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Hormonal Regulation of the *c-fms* Proto-Oncogene in Breast Cancer Cells is Mediated by a Composite Glucocorticoid Response Element

Maryann B. Flick,^{1*} Eva Sapi,¹ and Barry M. Kacinski^{1,2,3}

¹Department of Therapeutic Radiology, Yale University School of Medicine, 333 Cedar St, New Haven, Connecticut 06510-8040

²Department of Obstetrics and Gynecology, Yale University School of Medicine, 333 Cedar St, New Haven, Connecticut 06510-8040

³Department of Dermatology, Yale University School of Medicine, 333 Cedar St, New Haven, Connecticut 06510-8040

Abstract We have previously reported that glucocorticoids markedly increase and anti-glucocorticoids (such as RU-486) block *c-fms* RNA and protein expression in some breast cancer cell lines, but not in others, and that this increase is the consequence of increased transcription from the first, epithelial cell-specific promoter of the *c-fms* gene (encoding CSF-1R, macrophage colony-stimulating factor receptor). Employing DNaseI protection and electrophoretic mobility shift assays (EMSA), we now demonstrate that DNA-transcription factor protein complexes are formed on the *c-fms* first promoter at a composite regulatory element containing overlapping binding sites for AP-1 proteins, bHLH factors, and the glucocorticoid receptor (GR). Competition studies indicate that transcription factor proteins bind the AP-1 site and the GR element (GRE) and both GR and AP-1 proteins are involved in DNA-protein complex formation. The complexes differ in quantity and glucocorticoid inducibility in the different breast cancer cell lines studied depending on whether the promoter responds to glucocorticoid stimulation. Transient transfection of promoter/reporter gene constructs resulted in reduced basal transcription activity of this promoter and lack of glucocorticoid stimulation when the AP-1 site was mutated. We conclude that AP-1 proteins, GR and associated co-factors regulate transcription from the *c-fms* first promoter and that differences in recruitment of the various components are responsible for cell specific repression and activation of this gene in breast carcinoma cell lines. *J. Cell. Biochem.* 85: 10–23, 2002. © 2002 Wiley-Liss, Inc.

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Macrophage colony-stimulating factor receptor, CSF-1R, a cell surface tyrosine kinase, is transcribed from two distinct promoters of the proto-oncogene *c-fms* in a tissue specific manner [Visvader and Verma, 1989; Roberts et al., 1992; Sapi et al., 1995]. The activities of the CSF-1R/CSF-1 receptor/ligand pair are essential for trophoblastic implantation and placental differ-

entiation [Pampfer et al., 1992] and for normal mammary gland development [Pollard and Hennighausen, 1994]. In placental trophoblasts and mammary epithelial cells, the *c-fms* transcript initiates from the first promoter 26 kb upstream of exon 2 and includes a non-coding first exon [Roberts et al., 1988, 1992; Visvader and Verma, 1989; Sapi et al., 1995]. Expression of CSF-1R is observed in more than 80% of invasive breast carcinomas, and levels of expression of CSF-1 and CSF-1R have been shown to correlate with clinical stage and poor prognosis [Kacinski et al., 1991; Tang et al., 1992; Scholl et al., 1994]. In vitro studies have demonstrated that expression of CSF-1R by mammary epithelial cells confers both an invasive phenotype and a capability for anchorage independent growth, characteristics of malignant neoplasia

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*Correspondence to: Maryann B. Flick, Yale University, Department of Therapeutic Radiology, P.O. Box 208040, New Haven, CT 06520-8040.

E-mail: maryann.flick@yale.edu

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[Filderman et al., 1992; Sapi et al., 1996, 1998b]. Intracellular signal transduction from the activated receptor, acting through several autophosphorylated tyrosines in the tyrosine kinase domain proceeds down several independent pathways, one of which regulates a variety of phenotypes such as protease production and local invasiveness, while another regulates anchorage-independent growth and metastatic tumorigenicity [Sapi et al., 1996].

CSF-1 and CSF-1R are also essential for the survival, proliferation, and differentiation of monocytes [Stanley et al., 1983] in which the second promoter, immediately upstream of exon 2, drives transcription of *c-fms*. The activity and specificity of this macrophage-specific promoter is mediated by at least four different transcription factors: PU.1, AML1, C/EBP, and c-myc [Zhang et al., 1994a,b, 1996] and its activity can be increased by phorbol esters in the human myeloid leukemia cell line HL60 [Sapi et al., 1995].

Glucocorticoids are potent regulators of many cell functions including response to stress and anti-inflammatory activity. Glucocorticoid hormones exert their effects through the glucocorticoid receptor (GR), a transcription factor which interacts with DNA, steroid hormones, and other transcription factors [Yamamoto, 1985; Evans, 1988; Diamond et al., 1990; Pearce and Yamamoto, 1993; McNally et al., 2000]. GRs regulate gene transcription by several mechanisms: synergistic activity where transcription factors bound at other DNA sites cooperate with ligand-activated GR bound to a consensus glucocorticoid response element (GRE); interference with the actions of other transcription factors (e.g., AP-1 and NF- κ B) through protein-protein interactions, which does not require binding of GR to DNA; and either repression or stimulation via requisite interactions of GR with other transcription factors all bound at so-called composite GREs (cGREs) [Diamond et al., 1990; Pearce and Yamamoto, 1993; Pearce et al., 1998].

In normal mammary epithelium, glucocorticoids are required for normal lactogenic differentiation in the pre-partum period and for lactation post-partum [Topper and Freeman, 1980]. Our previous work and that of others has implicated a role for CSF-1 and its receptor, CSF-1R in these processes [Pollard and Hennighausen, 1994; Sapi et al., 1998a]. We have previously shown that the synthetic glucocorticoid dexamethasone (Dex) increases *c-fms*

transcript and protein levels in some breast carcinoma cell lines (SKBR3 and BT20), but not in others (MCF-7) [Sapi et al., 1995]. In short-term explant culture, 14 of 14 normal mammary epithelium specimens and 15 of 20 breast tumor specimens also expressed CSF-1R in response to Dex treatment [Sapi et al., 1998a; Kacinski et al., 2001]. Utilizing a *c-fms* first promoter-reporter gene construct, we were able to show that disruption of a GRE within the first promoter abolished stimulation by Dex in SKBR3 and BT20 breast carcinoma cell lines [Sapi et al., 1995].

Recent studies in our lab have shown that another non-steroid hormone is also able to stimulate *c-fms* transcription from the *c-fms* first promoter [Sapi et al., 1999]. All-*trans* retinoic acid, a potent factor in differentiation and development, stimulates transcription of the CSF-1R gene via RAR α receptors and stimulates other breast carcinoma cell phenotypes, such as anchorage-independent growth and invasion of basement membrane models [Sapi et al., 1999]. However, no hormones other than glucocorticoids and retinoids, or physiologic stimuli have been reported to modulate the first promoter. Moreover, the transcription factors that mediate this process, remain undefined.

To further characterize the transcriptional regulation of the *c-fms* gene in mammary epithelial cells, we carried out studies of the DNA-protein complexes formed in vitro between *c-fms* first promoter DNA probes and nuclear extracts from cell lines in which the CSF-1R promoter is or is not stimulated by glucocorticoids. Our results suggest that the first promoter of *c-fms* is regulated through interaction of GR and AP-1 proteins and associated co-regulators at a composite GR element (GRE).

MATERIALS AND METHODS

Cell Culture

Human breast carcinoma derived cell lines were obtained from the American Type Culture Collection (Bethesda, MD) and maintained at 37°C, 5% CO₂. SKBR3 was grown in McCoy's 5A plus 15% fetal bovine serum (FBS), and MCF7 in DMEM/F12 plus 10% calf serum, 10 μ g ml⁻¹ each of insulin and transferrin and 500 μ g ml⁻¹ bovine serum. Cells were incubated in serum-free DME/F12 medium for 48 h prior to addition

of 1 μ M dexamethasone (Dex, a synthetic glucocorticoid hormone, Sigma) directly to the medium. Hormone-treated cells were incubated for various times as indicated in the figure legends.

Preparation of Crude Nuclear Extracts (CNEs)

A modification of the method described by Schreiber et al. [1989] was used to prepare CNE from small numbers of cells. Nuclear proteins were extracted in one packed cell volume of cold buffer C (20 mM HEPES pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT and freshly added 0.4 mM PMSF; 2.5 μ g/ml aprotinin; 2.5 μ g/ml leupeptin; 1 mM sodium orthovanadate and 4 μ g/ml pepstatin). Protein concentrations of the supernatants (CNE) were measured by the BioRad Protein Assay and typically ranged from 5 to 10 mg/ml. Aliquots of the CNE were stored frozen at -70°C until further use.

Preparation of ^{32}P -Labeled DNA Probes

A portion of *c-fms* first promoter, 380 bp including exon 1, cloned into pBluescript at the BamHI and HindIII sites has been previously described [Sapi et al., 1995]. Double strand probes, labeled on only one strand, were prepared by first digesting with BamHI (leading to labeling of the sense strand) or HindIII (leading to labeling of the antisense strand). The resulting linearized plasmids were end-labeled by synthesizing complement to the 5' single strand overhangs resulting from the restriction digests utilizing the Klenow enzyme and ($\alpha^{32}\text{P}$)dATP (3,000 Ci/mmol, NEN Life Science Products) as described previously [Tabor et al., 1990]. Labeled products were digested with the second enzyme (HindIII or BamHI) and the resulting 380 bp probe was isolated by agarose gel electrophoresis and gel purification.

A shorter *c-fms* fragment (48 bp) was prepared by annealing two 45-mer complementary oligonucleotides (overlap of 42 nucleotides; see Fig. 1). Both ends were labeled with (^{32}P)dATP using Klenow DNA polymerase to fill in with complementary sequence. Labeled probe was purified by Sephadex G25 spin column (Boehringer). An AP-1 probe (non-*fms* sequence) was prepared by annealing the oligonucleotide cgcttgaTGAGTCAgccgaa (uppercase indicates consensus binding site) with its complement. The double strand probe (25 pmol) was labeled using 20 U T4 polynucleotide kinase, 25 pmol ATP and 150 μ Ci (γ - ^{32}P)ATP (6,000 Ci/mmol)

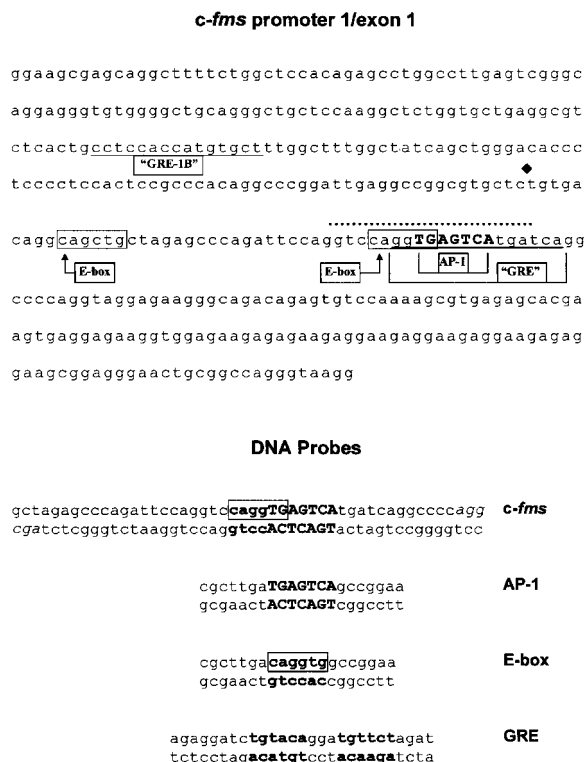


Fig. 1. Probes used for DNaseI protection and EMSA. DNaseI protection assays used the 378 bp double stranded sequence *c-fms* promoter/exon 1 (shown without complementary strand), which includes nucleotides -195 to 179 based on the start site (\blacklozenge) of the most abundant transcripts in breast carcinoma cell lines SKBR3 and BT20 [Sapi et al., 1995]. GRE-like sequences are underlined; cctccaccatgtgct is designated GRE-1B [Sapi et al., 1995] and ggtgagtcagatca is similar to negative GRE sequences. The AP-1 element is in bold uppercase letters. E-boxes are boxed. The DNaseI protected sequence is marked by a dotted overline. DNA probes used for EMSA are shown as double strands with recognition elements marked; E-box is bold and boxed and the AP-1 is in bold, uppercase letters. In the *c-fms* probe, nucleotides shown in italics are added by DNA polymerase after single strand oligonucleotides are annealed (see Materials and Methods). Flanking sequences for the AP-1 probe and the E-box probe are identical. The key elements of the consensus GRE (Santa Cruz Biotechnology, Inc.) are shown in boldface.

(Amersham) in 50 μ l [Cobianchi and Wilson, 1987]. After phenol extraction, the probe was purified by Sephadex G25 spin column (Boehringer Mannheim). A consensus GRE probe was purchased (Santa Cruz) and labeled using T4 kinase as described above.

DNaseI Protection Assay

The ^{32}P -labeled 380 bp DNA probe ($10-20 \times 10^3$ cpm, $\sim 3-10$ fmol) and protein (30 μ g CNE) were incubated on ice for 45 min in 50 μ l

containing 30 mM HEPES (pH 7.9), 10% glycerol, 50 mM KCl, 2% polyvinyl alcohol, 0.1 mM EDTA, 5 mM MgCl₂, 0.25 mM DTT, 0.25 mM PMSF plus 0.5 µg poly(dI-dC). Frozen stock of bovine pancreatic DNaseI (2.5 mg/ml in 50 mM Tris, pH 7.2, 10 mM Mg₂SO₄, 1 mM DTT, 50% glycerol, 3650 Kunitz U/mg, ICN) was diluted in 10 mM Tris, pH 7.5, 0.1 mM EDTA. DNA-protein mixtures were equilibrated to 10°C, 50 µl of 10 mM MgCl₂, 5 mM CaCl₂ was added, DNaseI (20 ng) was added and incubated at 10°C for 1 min. Control reactions (no protein) were digested with 5 ng DNaseI. The amounts of DNaseI required for optimum digestion had been predetermined. Reactions were stopped with 100 µl 0.2 M NaCl, 20 mM EDTA, 1% SDS, 0.25 mg/ml yeast tRNA and extracted with an equal volume phenol:chloroform (1:1). DNA was ethanol precipitated, resuspended in 60% formamide loading buffer and electrophoresed in an 8% denaturing acrylamide gel. Gels were autoradiographed (XAR film, Kodak) without drying. Maxam-Gilbert G and G + A DNA sequencing reactions were performed according to Pichersky [1996] on the same labeled DNA and run in adjacent lanes on the same gels to determine the sequence of DNaseI protected areas.

Electrophoretic Mobility Shift Assays (EMSA)

In a 10 µl volume, CNE (2–10 µg) was mixed with 0.1–0.3 pmol of ³²P-labeled DNA probe in the presence of 10 mM HEPES, pH 7.9, 10% glycerol, 50 mM KCl, 3 µg BSA, 6.25 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 1 µg poly(dI-dC). After a 10 min incubation at 25°C, DNA-protein complexes were separated from unbound DNA in non-denaturing, 6% acrylamide, 0.25 × TBE gels run at 4°C in low ionic strength buffer (0.25 × TBE). Wet gels were autoradiographed with intensifying screens at –70°C. For competition assays, CNEs were mixed on ice with unlabeled competitor at a 50-fold molar excess of subsequently added ³²P-labeled *c-fms* probe then incubated at 25°C and electrophoresed. For antibody competition assays, GR antibody NCL-GCR (Novocastra), *c-Jun/AP-1(D)X* (sc-44X, Santa Cruz) and *c-Fos (K-20)X* (sc-253X, Santa Cruz) TransCruz™ antibodies and anti-rat IgG (Sigma) were used. The GR antibody was prepared against the amino terminus, modulating domain. Antibody *c-Jun/AP1*, prepared against the highly conserved mouse *c-jun* DNA binding domain, is broadly reactive against human *c-jun*, *junB*, and *junD*. The

c-Fos antibody was prepared against the amino terminus and is crossreactive with *c-Fos*, *FosB*, *Fra-1*, and *Fra-2*. Anti-rat IgG was used as an irrelevant control. Antibody was added to 3–8 µg of CNE and incubated at 4°C for 1 hr. Labeled DNA in binding buffer was added and the mixture was incubated and electrophoresed as described above.

Reporter Gene Assays for Promoter Transactivation

Preparation of the reporter vector in which the 380 bp *c-fms* first promoter region is cloned upstream of the firefly luciferase reporter gene in the vector pGL2 (Promega) has been described previously [Sapi et al., 1995]. Complementary primers (5'-ccaggtccaggTGA-CGCGtgatcaggccccagg-3'; see Fig. 1), which incorporated a three nucleotide alteration (underlined) in the AP-1 site (uppercase) were designed according to the criteria in the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). Primers were synthesized and purified by the DNA Synthesis Lab, Department of Pathology, Yale University School of Medicine. The resulting construct was sequenced to verify the mutation.

Subconfluent SKBR3 or MCF-7 cells in six-well culture dishes were transiently transfected using LipofectAMINE 2000 Reagent™ (GIBCO-BRL) and 2 µg DNA/sample (1.5 µg of the firefly luciferase construct and 0.5 µg of pRL-SV40 Renilla luciferase control vector) following the manufacturer's transfection protocol. After incubation for 48 h, the cells were exposed to 1 µM Dex for various intervals. Cell lysates were prepared and the two separate luciferase activities were assayed using the Dual-Reporter Luciferase Assay System (Promega). Emitted light was measured with a Berthold luminometer using substrates for each luciferase according to the Dual-Reporter protocol. As a control for transfection efficiency, the Renilla luciferase activity was used to normalize the firefly luciferase data.

RESULTS

DNaseI Protection

Expression of the *c-fms* gene and its product, CSF-1R, is increased in SKBR3 and BT20, but not MCF-7 breast carcinoma cell lines after exposure to glucocorticoids such as dexamethasone [Sapi et al., 1995]. We have previously

shown that a 4 bp deletion, in a 15 bp glucocorticoid response element (designated GRE-1B) within 100 base pairs of the multiple transcription start sites, abolishes transcriptional stimulation of the first *c-fms* promoter by Dex [Sapi et al., 1995]. In order to analyze further the DNA-protein interactions, which are responsible for the cell line specific responses of the first promoter to glucocorticoids, we performed DNaseI protection assays with a 380 bp, 32 P-labeled double strand fragment of the 5' end of the *c-fms* gene which includes exon 1 and the immediate 5' flanking sequence including the GRE-1B (Fig. 1).

Because Dex stimulation of *c-fms* gene transcription can be detected on Northern blots as early as 1 h after treatment [Sapi et al., 1995], cells were treated for relatively short times. CNE of equal protein content from SKBR3 and MCF-7 cells, which were stimulated for 30 min with 1 μ M Dex, were mixed in a binding reaction with the DNA probe labeled on the sense strand. When compared to the control DNaseI digested

probe without added proteins (Fig. 2A, lane C), bound proteins protected a region of about 18 nucleotides in both cases (Fig. 2A, lanes 1 and 2). Extracts from the non-Dex-inducible line, MCF-7 (lane 2), appeared to provide less protection than extracts from SKBR3 (Fig. 2A, lane 1). The protected sequence was found to include a consensus AP-1 sequence (TGAGTCA), an E-box (CAGGTG, binding site for basic-Helix-Loop-Helix domain-containing transcription factors) overlapping the AP-1 site and a potential GRE (GGTGAGTCATGATCA) that overlaps both as shown in Figure 1. In other experiments employing extracts from both untreated and Dex treated cells and the same probe in which the antisense strand is labeled, the protected region may be somewhat less extensive, but still includes the AP-1 site (Fig. 2B). In addition, a DNaseI hypersensitive site (arrow, Fig. 2B) is evident just upstream of the AP-1 site suggesting that the bound proteins elicit a conformational change in the DNA which renders a previously insensitive bond

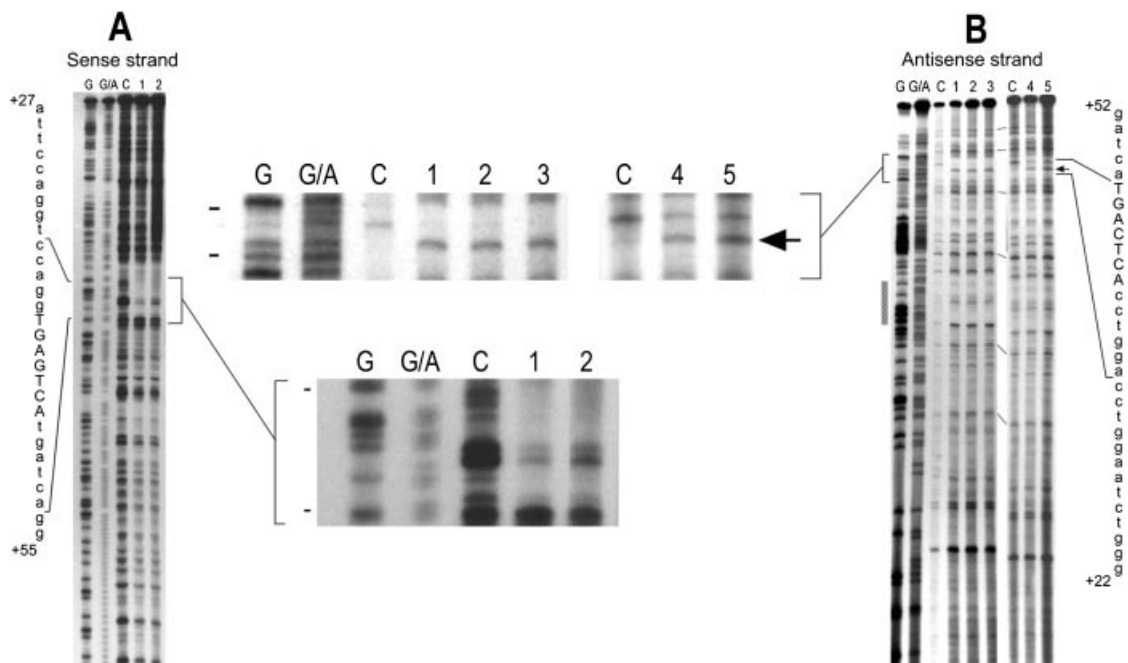


Fig. 2. Nuclear proteins from two breast carcinoma cell lines protect a small region of the *c-fms* first promoter from DNaseI digestion. Thirty micrograms of CNE prepared from SKBR3 or MCF-7 cells were incubated with labeled DNA probe followed by limited digestion with DNaseI as described in Materials and Methods. Digestion products were separated on denaturing polyacrylamide gels and compared with digests of DNA that had not been mixed with protein. Maxam-Gilbert sequencing reactions (G and G/A) of the same oligonucleotide were electrophoresed simultaneously to identify the sequence of the

protected region (shown by brackets). The AP-1 site is in upper case letters. **A:** Labeled sense strand (see Materials and Methods). **Lane C:** control (no CNE); **lane 1,** SKBR3, 30 min Dex; **lane 2,** MCF-7, 30 min Dex. **B:** Labeled antisense strand. **Lanes C:** control (no CNE); **lanes 1–3,** SKBR3, 0, 20, and 90 min treatment with Dex; **lanes 4 and 5,** MCF-7, 0, and 30 min treatment with Dex. The DNaseI hypersensitive site is marked by an arrow. The position of the GRE-1B is identified by the stippled bar. The two separate gels are aligned with reference marks.

now sensitive to DNaseI cleavage. This hypersensitivity appears equivalent in both cell lines since the digestion product at this site looks equally intense in both cases (Fig. 2B, arrow). However, protection of the region flanking this site is less extensive with MCF-7 extracts as seen by the still visible cleavage product above the hypersensitive site in Figure 2B, lanes 4 and 5 (MCF-7) vs. its absence in lanes 1–3 (SKBR3) confirming the result seen in Figure 2A. Treatment with Dex did not result in any obvious change in DNaseI protection in either SKBR3 or MCF-7. Surprisingly, the region corresponding to the glucocorticoid response element (GRE-1B), which is required for Dex induction of the *c-fms* gene [Sapi et al., 1995] was not observed to be protected by bound protein under the conditions used in this assay (Fig. 2B, stippled bar).

Gel Shift Assays With a *c-fms* Sequence Probe

DNA–protein binding was further examined using more sensitive EMSA. When a 48 base pair ³²P-labeled DNA probe (Fig. 1) based on the sequence identified by DNaseI protection was incubated with CNE from SKBR3 cells, two principle, shifted bands were observed and labeled A and B (Fig. 3A, lanes 1–2). There is a significant increase in both bands after 30 min Dex treatment. By contrast, Dex treatment of MCF-7 had little if any effect on the intensity of any bands (Fig. 3A, lanes 3–4). However, the migration of bands A and B is identical for both cell lines suggesting that both cells form identical complexes. For equivalent amounts of protein, MCF-7 extracts on average gave significantly less intense bands.

Oligonucleotide Competition Studies

An oligonucleotide competition assay was employed to determine which of the overlapping sequence elements in the 48 bp probe might be responsible for the observed protein binding. Unlabeled double stranded competitors, AP-1, E-box, and GRE consensus sequences (Fig. 1) were chosen based on the transcription factor binding motifs found in the DNaseI protected region. The sequence flanking the AP-1 and E-box motifs is different from flanking sequence in the *fms* probe and was purposely matched in these two probes in order to rule out competition by any sequence other than the elements themselves. The GRE is a commercial preparation of the simple hormone response element, an imperfect palindrome. The relative competition

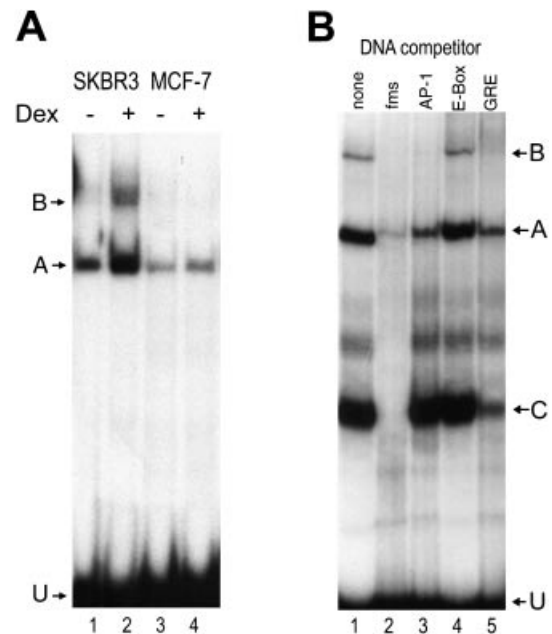


Fig. 3. Nuclear proteins that bind the composite recognition element of the *c-fms* first promoter also recognize consensus sequences for GR and AP-1 recognition elements. **Panel A:** Six micrograms of CNE from SKBR3 (lanes 1 & 2) and MCF-7 (lanes 3 & 4) cells, untreated or treated with 1 μ M Dex for 30 min, were incubated with labeled *fms* DNA probe and electrophoresed as described in Materials and Methods. **Panel B:** CNE from Dex treated (30 min) SKBR3 cells (6 μ g), was mixed with a 50-fold molar excess (over labeled *fms* probe) of unlabeled *fms* probe, AP-1, E-box or GRE DNA consensus sequence probe on ice. Labeled *fms* probe was added, and reactions were incubated at 25°C for 10 min prior to electrophoresis. Bands A and B represent specific DNA–protein complexes, U is unbound, labeled DNA probe.

for protein binding can be seen in Figure 3B for each of the two principal bands seen in Figure 3A formed by extract from Dex treated SKBR3 cells as well as a third band (labeled C), which we found in about half of our extract preparations regardless of cell type. Band C was not responsive to Dex treatment and was considered an artifact. The unlabeled probe itself and a consensus GRE were efficient competitors of all three bands. The consensus AP-1 sequence only competed bands A and B, whereas the consensus E-box had no detectable effect under the conditions used for this experiment. The same relative competition was seen with MCF-7 extract (not shown).

Gel Shift Assays With GRE and AP-1 Consensus Sequence Probes

Because the competition assay suggests that the same proteins that bind the *c-fms* first promoter sequence also bind GRE and AP-1

consensus elements, EMSA was performed with labeled GRE and AP-1 consensus sequence probes (see Fig. 1) to further characterize the binding proteins present in the two cell lines and compare binding to recognized consensus sequences with that of the *c-fms* composite sequence. As seen in Figure 4A (lanes 1–4), when the GRE consensus sequence probe was labeled and mixed with CNE from SKBR3 cells, the gel shift pattern of DNA–protein complexes formed was similar to that observed using labeled *c-fms* sequence, although no increase in complex formation is apparent with increasing time of Dex treatment. Three complexes are sensitive to pre-incubation of the CNE with an antibody to GR (Fig. 4A, lane 4) and the relative migration of two of them (bands g2 and g3) is similar to that of the GR antibody-sensitive bands A and B generated with the *fms* probe (Fig. 3). Although MCF-7 cells are known to be positive for GR and sensitive to Dex treatment at other gene promoters [Sun et al., 1983;

Tsai et al., 2000], there is significantly less binding to the consensus GRE when compared to SKBR3 (Fig. 4A, lanes 5–7). In all MCF-7 samples, there is a noticeable absence of the g3 band complex, while the formation of g1 and g2 remains sensitive to pretreatment with GR antibody (lane 8).

In order to compare the AP-1 binding proteins in the two cell lines, antibody competition gel shifts were performed using the labeled consensus AP-1 probe. CNE from SKBR3 cells forms two complexes with the consensus AP-1 probe, which are not noticeably changed after 15 min Dex treatment (Fig. 4B, lanes 1 and 2). Only the faster migrating complex (g) is sensitive to pre-incubation with GR antibody (Fig. 4B, lane 3). The slower complex (j) is sensitive to the Jun-family antibody (Fig. 4B, lane 4) in contrast to the slowest migrating complex formed with the *c-fms* sequence. This suggests that these complexes may not be identical either in identity of components or in the way the components are assembled on the DNA. In striking contrast to SKBR3, twice as much CNE from untreated MCF-7 fails to produce the j band and forms very little band g complex (Fig. 4B, lane 5). Treatment of MCF-7 with Dex had no effect on AP-1 binding proteins (not shown).

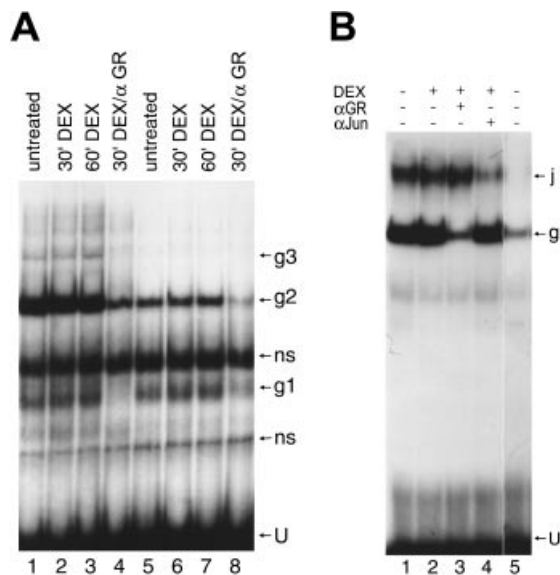


Fig. 4. Complex formation on simple GRE and consensus AP-1 elements. **Panel A:** CNE from Dex stimulated SKBR3 (3 μ g) and MCF-7 (5 μ g) cells were incubated with labeled GRE DNA probe and electrophoresed as described in Materials and Methods. CNE samples in lanes 4 and 8 were preincubated with GR antibody at 4°C for 1 h prior to incubation with labeled probe. Bands g1, g2, and g3 are specific DNA–protein complexes, ns are non-specific, U is unbound probe, g1, g2, and g3 are sensitive to GR antibody. **Panel B:** CNE (3 μ g) from Dex treated SKBR3 (lanes 1–4) and MCF-7 (lane 5) cells were incubated with labeled AP-1 DNA probe and electrophoresed as described in Materials and Methods. Bands j and g are specific DNA–protein complexes, U is unbound probe, g is sensitive to GR antibody, j is sensitive to the pan-Jun antibody.

Antibody Competition Assays With *c-fms* Sequence Probe

To help identify the proteins bound to the *c-fms* DNA probe, antibodies to AP-1 and GR were pre-incubated with the CNE before addition of the labeled DNA probe (Fig. 5). Although the Jun-family antibody pre-incubated with CNE from SKBR3 did not affect complex formation or cause a supershift (Fig. 5A, lane 2), the presence of the Fos-family antibody results in a new, faster migrating band contrary to typical supershift results, wherein, the antibody further retards the migration (Fig. 5A, lane 4). The faster migrating band is possibly a complex related to the band B complex, but lacking the Fos-family protein component. In a separate experiment, compared to no antibody (Fig. 5A, lane 5) and the irrelevant control antibody (Fig. 5A, lane 7), interaction of CNE with the GR antibody effectively eliminates the complex at band B and significantly diminishes the band A complex (Fig. 5A, lane 6). This result is consistent with the results of the GRE consensus oligonucleotide competition assay and suggests that GR is part of both complexes.

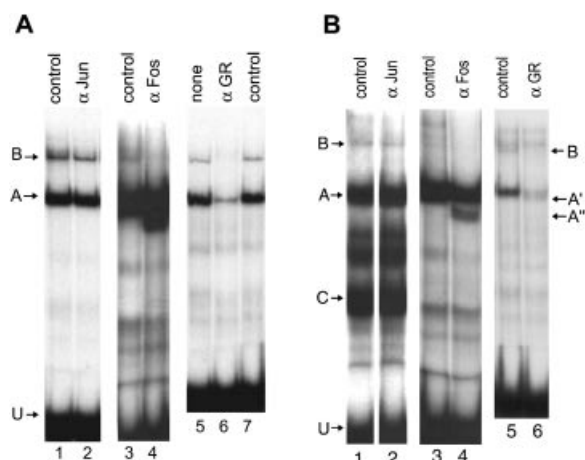


Fig. 5. Supershift analyses indicates GR and AP-1 family proteins in complexes bound to *c-fms* first promoter sequence. CNE from SKBR3 (6 μ g, **Panel A**) and MCF-7 (6 μ g, **Panel B**) were pre-incubated with pan-Jun antibody, pan-Fos antibody, GR antibody, anti-rat IgG (control) or buffer only (none) at 4°C for 1 h. Samples were incubated with labeled *fms* DNA probe and electrophoresed as described in Materials and Methods. A, A', A'', B, and C are specific DNA-protein complexes, U is unbound labeled DNA probe.

A similar experiment using CNE from MCF-7 cells is shown in Figure 5B. Compared to the irrelevant antibody (Fig. 5B, lane 1), addition of the Jun-family antibody again has no significant effect on bands A or B (Fig. 5B, lane 2). The Fos-family antibody effectively blocks formation of the complex seen as band B, as well as the minor bands above, but not band A (Fig. 5B, lanes 3 and 4). Adding GR antibody to the binding mix resulted in the elimination of band B and a much-reduced band A (Fig. 5B, lanes 5 and 6). In the presence of the GR antibody, with the formation of complex A inhibited, it is possible to see the doublet nature of band A (designated in the figure as A and A') with MCF-7 extracts, which was not seen with SKBR3 extracts under any conditions and is usually hidden by the intensity of band A. Formation of band C was blocked only by incubation with GR antibody in extracts in which it was present, from both cell lines (not shown).

Wild-Type and Mutant Promoter Activity in Reporter Gene Assays

Two promoter-reporter gene constructs were used to demonstrate the role of the AP-1 site in glucocorticoid regulation of the *c-fms* first promoter. The first, E1P-B, has been described previously [Sapi et al., 1995] and consists of the 380-bp promoter region (as shown in Fig. 1)

cloned upstream of the luciferase reporter gene in the vector pGL2. This construct was then employed to create the second promoter-reporter construct, E1P-BMAP in which the AP-1 site is altered, changing the consensus TGAGTCA to TGACGCG. Each was transiently transfected into SKBR3 and MCF-7 cells, which were then treated with 1 μ M Dex for various times. Promoter activity was determined by assaying cell lysates for luciferase activity.

In agreement with previous results [Sapi et al., 1995], the promoter region included in E1P-B has a low basal level of activity and can be stimulated *in vitro* by treatment of SKBR3 cells with the synthetic glucocorticoid dexamethasone. Stimulation reaches a peak of approximately eight-fold after 3 h (Fig. 6). In contrast, the promoter region having an altered AP-1 site has a very low basal level of activity *in vitro*, comparable to the promoterless control vector, which had an activity of 100–200 relative units (not shown); and as seen in Figure 6,

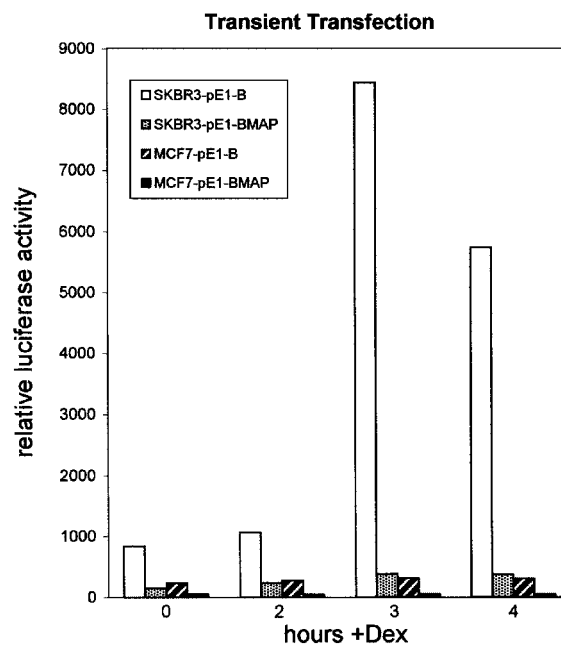


Fig. 6. Alteration of the AP-1/GRE site in the *c-fms* first promoter blocks transcriptional ability and Dex inducibility. The pGL2 luciferase reporter gene vector carrying *c-fms* first promoter constructs with either an intact AP-1 element (E1-B) or altered AP-1 element (E1-BMAP) were transiently transfected into SKBR3 and MCF-7 cells, which were then treated with Dex for various times as described in Materials and Methods. Lysates were prepared and assayed for luciferase activity. To control for variable efficiency of transfection, the activity of each sample was normalized to Renilla luciferase activity from a co-transfected vector. Error bars represent the standard error of triplicate samples for each time point.

Dex has no effect on the promoter activity in the absence of an intact AP-1 element. In MCF-7 cells, both the normal sequence promoter and the altered AP-1 sequence have similar low basal activity which is not stimulated by Dex.

DISCUSSION

Expression of CSF-1R, product of the proto-oncogene *c-fms*, in breast and other cancers has been shown to correlate with advanced stage and poor prognosis in patients and is able to confer phenotypes necessary both for local invasion and distant spread [Kacinski et al., 1991; Filderman et al., 1992; Tang et al., 1992; Sapi et al., 1996]. Since glucocorticoids have been shown to dramatically increase the expression of CSF-1R in breast carcinoma cells, levels of expression of the transcription factors and cofactors that stimulate the first, epithelial cell specific, *c-fms* promoter could be prognostically significant and these factors themselves are possible therapeutic targets. The mechanisms responsible for the regulation of the first promoter might also underlie the striking tissue specificity of promoter utilization observed in cells of epithelial vs. those of hematopoietic origin. In order to elucidate these mechanisms, we have studied protein-DNA interactions employing DNaseI protection and EMSA. Our results demonstrate that the promoter region contains a composite transcription factor binding element that binds both GR and AP-1 protein complexes. Comparing nuclear extracts from a breast carcinoma cell line that can be induced to express *c-fms* by glucocorticoids with one which does not respond, we found a significant difference in proteins that bind this sequence element. Mutation of the AP-1 site of the cGRE significantly reduces the basal activity of this promoter in cells not exposed to glucocorticoids, and renders the promoter unresponsive to glucocorticoid stimulation when assayed in a transfected luciferase reporter gene.

In the DNaseI protection assays, only one relatively short segment of the *c-fms* first promoter was found to be protected. In a cell line (SKBR3) in which glucocorticoids stimulate *c-fms* transcription the protection by equal amounts of input protein was somewhat greater than that observed for another cell line (MCF-7) where *c-fms* transcription is not stimulated by glucocorticoids. A hypersensitive site within

the protected region suggests the bound protein(s) elicits a conformational change in the promoter DNA in agreement with other studies demonstrating that bound transcription factors such as GR and AP-1, are capable of bending DNA [Petz et al., 1997]. The observation that there is less protection by an equal amount of MCF-7 protein compared to that from SKBR3 cells, but no difference in appearance of the hypersensitive site suggests that there must be some difference in the proteins bound to this site in the two cell lines.

A computer search for transcription factor consensus binding sites within the protected sequence revealed several overlapping elements; an E-box (basic Helix-Loop-Helix recognition element), an AP-1 element (binding site for Jun- and Fos-family proteins) and a sequence with close homology to so-called negative GREs. Since the first report that positive and negative regulation from a single GRE is dependent on the relative proportions of AP-1 constituent proteins present in the cell [Diamond et al., 1990], there have been numerous reports of GR interaction with other transcription factors. The interaction of GR with AP-1 proteins has been variously reported to either repress or stimulate a wide variety of genes [Shemshedini et al., 1991; Teurich and Angel, 1995; Sikora et al., 1996; Pearce et al., 1998]. A recent review explains the three major modes by which glucocorticoids are believed to regulate gene transcription: (1) activation via binding of GR to positive GREs; (2) repression via binding of GR to nGREs; and (3) transcriptional interference via protein-protein interaction of GR with other transcription factors, for example, AP-1 complexes and NF- κ B [Karin, 1998].

Competition assays where consensus sequences for the three elements in the protected *c-fms* sequence were added to see whether they can compete with the promoter sequence for transcription factor binding, suggest that the two major complexes formed by extracts from SKBR3 are also able to bind an AP-1 element whose flanking sequence differs from that which flanks the AP-1 element in the *c-fms* promoter. The same was true of a competing consensus GRE. These results are consistent with our interpretation that the protected *fms* promoter sequence is a cGRE. Compared to the simple GRE, cGREs have only a short stretch of homology to the consensus GRE sequence, but

also include a consensus or near consensus AP-1 site which overlaps the GRE sequence [Diamond et al., 1990; Pearce et al., 1998]. cGREs provide a means for regulatory diversity of a promoter by employing protein–protein interactions between receptor and other factors that may vary from one cell type to another. Evidence suggests that both GR and AP-1 bind to the element *in vitro* and that regulation is not due simply to competition between GR and AP-1 complexes for binding [Diamond et al., 1990]. Rather, stimulation or repression of the promoter that includes a cGRE is determined by the type of non-receptor factors in the bound complex [Pearce and Yamamoto, 1993]. Typically, when the composition of the AP-1 is a c-Jun-c-Jun homodimer, GR activates transcription and when it is a heterodimer of c-Jun and c-Fos, the receptor represses transcription [Diamond et al., 1990; Miner and Yamamoto, 1991; Pearce et al., 1998]. Teurich and Angel [1995] concluded that negative or positive modulation of expression of AP-1 dependent genes depends on the subunits of the AP-1 dimer bound at the AP-1 site and does not require the presence of a GRE in the promoter since GR can interact with bound AP-1 complexes by protein–protein interaction. Even before Dex treatment, which activates GR, there is binding to the *c-fms* probe, an observation, which suggests that *c-fms* is negatively regulated by bound proteins at the composite element in the absence of an inducer such as glucocorticoid.

Transcriptional activation by GR is likely a dynamic event involving the cycling of proteins on and off the complex accompanied by changes in local DNA conformation [McNally et al., 2000]. Steroid receptors are thought to regulate rates of transcription through protein–protein interactions with other transcription factors and through the recruitment of a number of coactivators or corepressors that associate with the transcription apparatus [Glass and Rosenfeld, 2000; McNally et al., 2000]. Such cofactors may have histone/protein acetyltransferase activity, which overcomes chromatin-mediated repression or deacetylase activity, which has the opposite effect.

While the DNaseI protection assays suggested a difference in the protein complexes bound to the composite element in the *c-fms* promoter, the mobility shift assays revealed marked differences between the two cell lines. Nuclear extracts from MCF-7 showed less

binding to the *c-fms* sequence particularly the formation of a complex seen as band B; and while Dex treatment of SKBR3 cells produces an increase in DNA–protein complexes with time, the same was not observed for MCF-7 cells. EMSA using GRE and AP-1 consensus sequence probes helped to elucidate the differences between SKBR3 and MCF-7 cells. Most apparent in MCF-7 was an absence, or significant reduction in formation, of bands with the GRE and the AP-1 sequence probes, notably bands sensitive to both the pan-Jun and the GR antibodies suggesting significant differences in the relative proportion of transcription factors between the two cell lines. Expression of c-Fos and/or c-jun results in inhibition or stimulation of nuclear receptor-induced transcription, dependent not only on the promoter and the receptor, but also on cell type [Shemshedini et al., 1991]. Significant differences in AP-1 activity between SKBR3 and MCF-7 cells have been reported elsewhere. AP-1 activity was found to be lower in MCF-7 than in SKBR3 [Chen et al., 1996; Smith et al., 1997] and transient expression of c-jun increased AP-1 transactivating activity seven-fold in SKBR3, but only two-fold MCF-7 [Smith et al., 1997]. A profound difference in the assembly of factors on the *c-fms* first promoter could, therefore, result in stimulation in one case and repression in the other.

Experiments in which antibodies to GR, Jun-family proteins, and Fos-family proteins were added to the binding reactions provide further evidence for the presence of GR in at least two specific promoter/protein complexes. Rather than supershifting, the complexes the GR antibody inhibited complex formation as might be expected if the epitope recognized by the antibody is part of a domain involved in DNA binding or protein complex formation. Incubation with a Fos-family antibody resulted in a somewhat surprising reverse shift, a faster migrating band and the elimination of the slower migrating (larger) complexes suggesting that a Fos protein is critical for formation of a multi-protein complex. Fos itself has also been implicated as the preferential target of GR inhibition of AP-1 activity [Kerppola et al., 1993]. Studies with antibodies to known transcription factors are useful when either supershifting or inhibition of DNA–protein complexes is observed, a negative result is unfortunately indeterminate since there are

many reasons why such experiments fail. For this reason, we interpret our failure to observe any Jun-family proteins in this complex as inconclusive, particularly since we find evidence of a Fos-family protein in these complexes, and at least to date, AP-1 binding complexes, which include Fos also include a Jun-family protein to form a heterodimeric DNA binding complex.

A recent study of the levels of expression of AP-1 family proteins in breast tumors, normal breast tissue, human breast cancer cell lines (T47D and MDA-MB231), and a transformed human breast epithelial cell line, HBL100 demonstrates that levels of the various Jun-family and Fos-family proteins may play a significant role in the pathogenesis of breast cancer [Bamberger et al., 1999]. While these investigators found a uniform expression of *c-jun*, *junB*, *c-fos*, and *fra2* among tumors, expression of *JunD* and *fosB* varied strongly. Expression of *fosB* correlated significantly with positive steroid hormone receptor status and a more differentiated tumor phenotype, while *fra-1* showed a significant negative correlation with *fosB* expression. They concluded that certain members of AP-1 family proteins such as *fosB* and *fra-1*, might be involved in the pathogenesis of these tumors. Others have reported a correlation of endocrine unresponsiveness (a well known factor in breast cancer) with Fos overexpression [Gee et al., 1995].

Such results suggest that we might expect a correlation between levels of certain AP-1 proteins (particularly a Fos-family protein) with *c-fms* expression as well. Since the various Jun-family proteins are of nearly identical size and the Fos-family proteins are also similar to one another, co-migrating complexes may not necessarily contain the same proteins. Both SKBR3 and MCF-7 have similar levels of expression of *c-fos* and *JunB* by Northern blot analysis (not shown). However, Western blots (not shown) for Fos-family, Jun-family, and GR proteins revealed differences in levels of GR and Jun proteins in the two cell lines. SKBR3 has at least two-fold more GR than MCF-7. In contrast, MCF-7 has at least twice as much Jun protein as SKBR3, which makes it difficult to explain the gel shift result showing less DNA binding to an AP-1 consensus probe with extracts from MCF-7 compared to SKBR3. In addition, we checked the level of the steroid receptor coactivator SRC-1 in both cell lines by

Western blot and found substantially more in SKBR3 than in MCF-7 (not shown). Further studies are needed to identify the components of these complexes which may include accessory factors such as the steroid receptor cofactors (SRC) as well as various forms of the AP-1 family proteins.

The Dex-induced increase we observed in one GR-containing complex in SKBR3, but not MCF-7 is most likely due to differences in GR cofactors required for efficient binding. Western blot analysis indicates that within 15 min (not shown) after Dex addition GR has translocated to the nucleus in both cell lines although SKBR3 appears to have 2–3 times as much nuclear GR as MCF-7. A Western blot performed with an antibody against the steroid receptor coactivator SRC-1 shows it is present at significantly lower levels in MCF-7 than in SKBR3 cells. Additional experiments will be needed to clearly establish the role, if any, that SRC-1 plays in the regulation of glucocorticoid-induced *c-fms* expression and to ascertain whether any additional coactivators and/or corepressors are components of the *c-fms* first promoter DNA–protein complexes.

We were quite surprised to find no binding to a sequence, which was shown by deletion to be required for promoter activity [Sapi et al., 1995]. This observation might be the consequence of the artificiality of the *in vitro* conditions employed for DNaseI protection where a relatively small, naked DNA fragment is employed while the promoter DNA in the expression vector may be assembled into a more chromatin-like structure. Located approximately 100 bp upstream of the protected sequence, we may have a case where the nucleosome context facilitates an interaction of this upstream imperfect GRE and the AP-1 site, which cannot occur on a naked DNA probe *in vitro*. Others have reported that interacting AP-1 and GRE elements may be spaced as much as 210 bp apart [Pearce et al., 1998]. In the MMTV promoter, Chavez and Beato [1997] have demonstrated that functional synergism between GR and nuclear factor I is compromised by removal of nucleosomes. DNaseI protection and gel shift assays can only suggest which of the known DNA binding proteins are present in the complexes and do not provide information on the dynamic aspects of chromatin function involving rapidly changing DNA–protein and protein–protein interactions. Thus, neither assay is truly representative of

endogenous genes in normal chromosomes. Transient transfection of mutated AP-1 promoter/luciferase reporter gene constructs demonstrated that the AP-1 element of the promoter is involved in glucocorticoid induction of the *c-fms* gene in SKBR3 cells. Since the AP-1 site is integral to the composite element this might suggest that any disruption of the cGRE will affect the regulation of the *c-fms* first promoter. In our previous study, disrupting the GRE 113 bp upstream from the AP-1/cGRE was also found to abolish promoter activity in transient transfection assays [Sapi et al., 1995]. We would conclude then that both regions of the first promoter are required for promoter activity and it is probable that they cooperate in some manner to regulate its activity. In agreement with our previous study, MCF-7 transiently transfected with the normal promoter/reporter gene constructs showed no *c-fms* first promoter activity and likewise, no Dex induction of first promoter activity in agreement with Northern blot analyses of Dex treated cultured cells [Sapi et al., 1995]. At that time, we proposed that this was due to some difference in recruitment of transcription factors to the promoter. Experiments described here show that this hypothesis was correct and that the observed hormone regulation involves both GR and AP-1 proteins.

The ability to hormonally induce *c-fms* from the first promoter seems to require a well regulated set of factors as might be expected for developmental and tissue specific regulation. Expression and activation of transcription factors involved in the regulation of *c-fms* is especially important in the study of tumor cells. Dissecting the molecular mechanisms underlying the expression of genes, which contribute to the malignant cell phenotype may yield information, which is applicable to cancer diagnosis, assessment of prognosis, and new therapies. Our results are consistent with a simple model in which GR interacts with an AP-1 protein complex, but do not preclude more complicated regulation involving differing components of the AP-1 complex in different cells and/or the presence of specific coactivators or corepressors. Further studies of specific AP-1 proteins as well as the various steroid receptor coactivators required for induction of transcription by activated receptors will be needed to fully understand regulation of the *c-fms* first promoter by glucocorticoids and retinoids and their antagonists. Besides gaining a better un-

derstanding of the dynamics of the regulation of *c-fms* gene expression, this knowledge can be applied to devise novel hormonal therapies for breast cancer directed against glucocorticoid action and thus independent of tumor cell estrogen and/or progesterone receptor status.

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