Transcription Factor Interactions: Selectors of Positive or Negative Regulation from a Single DNA Element

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The mechanism by which a single factor evokes opposite regulatory effects from a specific DNA sequence is not well understood. In this study, a 25–base pair element that resides upstream of the mouse proliferin gene was examined; it conferred on linked promoters either positive or negative glucocorticoid regulation, depending upon physiological context. This sequence, denoted a "composite" glucocorticoid response element (GRE), was bound selectively in vitro both by the glucocorticoid receptor and by c-Jun and c-Fos, components of the phorbol ester-activated AP-1 transcription factor. Indeed, c-Jun and c-Fos served as selectors of hormone responsiveness: the composite GRE was inactive in the absence of c-Jun, whereas it conferred a positive glucocorticoid effect in the presence of c-Jun, and a negative glucocorticoid effect in the presence of c-Jun and relatively high levels of c-Fos. The receptor also interacted selectively with c-Jun in vitro. A general model for composite GRE action is proposed that invokes both DNA binding and protein-protein interactions by receptor and nonreceptor factors.

ON BINDING HORMONE, THE GLUCOCORTICOID RECEPTOR protein selectively regulates transcription by binding to specific DNA sequences, termed glucocorticoid response elements (GRE's), close to hormone-responsive promoters (*1, 2*). The structure and activities of the receptor have been extensively characterized (*3–5*). As with many transcriptional regulators, the receptor stimulates gene expression in some genetic and cellular contexts and represses expression in others (*6, 7*); indeed, a 150-amino-acid (aa) fragment encompassing the DNA binding domain of the 795-aa rat glucocorticoid receptor is sufficient for both positive and negative regulation (*7*). Such functional versatility expands the potential range of regulation by the receptor and raises interesting questions about the mechanisms by which a single gene product can both enhance and repress transcription.

An initial study implicated the specific DNA sequence recognized by the receptor as one of the determinants of positive or negative regulation. Thus, Sakai *et al.* (*6*) showed that a 34-bp sequence associated with the bovine prolactin gene is bound selectively by purified glucocorticoid receptor in vitro and confers in various cell types hormone-mediated repression on heterologous promoters to which it is fused. This so-called "negative glucocorticoid response element" (nGRE) differed substantially from the consensus sequence identified for positive GRE's (*6*) although as few as two site-directed alterations of the nGRE were sufficient to convert it to a positive GRE (*8*).

To explain the lack of receptor-mediated enhancement at nGRE's, it was suggested that the interaction of receptor with nGRE sequences might alter receptor conformation, thereby inhibiting its positive activity (*6*). Recent results indicate, however, that DNA sequence is not the sole determinant of positive or negative regulation, as an nGRE from the mouse proliferin gene (*9*) conferred hormone-dependent repression on a test promoter in certain mammalian cell types as expected, but activated expression upon hormone stimulation when introduced together with the receptor into cultured *Drosophila* cells (*10*). Thus, denoting a sequence element exclusively as an "nGRE" is an oversimplification, as the context of the cell can clearly affect the nature of the hormonal response.

In any case, the glucocorticoid receptor achieves negative regulation by counteracting transcriptional enhancement apparently conferred by nonreceptor stimulatory factors binding to the same segment of DNA that is recognized by the receptor. For example, when the prolactin nGRE fragment is fused to a test promoter, promoter activity is enhanced even in the absence of receptor, presumably through the action of a factor bound to the fragment (*6*). Characterization of the proliferin "nGRE" and of other nGRE's has revealed similar behavior, although different nonreceptor enhancement factors appear to act at the different nGRE's (*7, 11, 12*). Taken together, these findings suggest that the glucocorticoid receptor represses transcription by displacement or inactivation of nonreceptor stimulatory factors, that the DNA sequence of the element plays a role in determining positive or negative control, and that still other factors might also be required to preclude receptor-mediated enhancement at an nGRE.

To pursue further the determinants of enhancement or repression by the glucocorticoid receptor, we have begun to investigate the "nGRE" of proliferin (*9*). Mitogenic signals and phorbol esters such as TPA, which stimulate the activity of the transcription factor AP-1, activate expression of this gene in placenta and in various cultured cells; in the same cells, glucocorticoids repress proliferin gene transcription (*9*). Mordacq and Linzer (*9*) showed that a fragment from the proliferin regulatory region increases the basal activity of a test promoter and confers enhancement by TPA and repression by glucocorticoids in CHO, L, and 10T½ cells; indeed, this fragment encompasses a receptor binding site adjacent to a

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consensus AP-1 sequence (*9*).

AP-1 effects are mediated by protein dimers composed of products of the *jun* and *fos* gene families (*13*). The Fos-Jun heterodimer binds to AP-1 sites with much higher affinity than does the Jun homodimer, and Fos alone is unable to homodimerize or bind (*14–16*); in vitro, Fos-Jun heterodimers strongly enhance transcription and Jun homodimers enhance weakly (*17, 18*). In HeLa cells, AP-1 activity derives mainly from the c-Jun protein (*13, 17, 19*).

We now describe a minimal fragment of the proliferin regulatory region that is sufficient for receptor and AP-1 binding, for conferring positive regulation by TPA, and for specifying both negative and positive regulation by glucocorticoids. We then characterize receptor action at this element in the presence of various amounts of c-Jun and c-Fos. Finally, we test in vitro whether the receptor interacts with c-Jun or c-Fos.

Receptor binding site confers positive and negative regulation. Mordacq and Linzer (*9*) identified a site between −254 and −225 relative to the proliferin transcription initiation site that is selectively protected in deoxyribonuclease (DNase) I footprinting studies by a purified 168-aa fragment of the glucocorticoid receptor encompassing the DNA binding domain (*20*). We obtained similar results using either the full-length receptor or a 93-aa fragment that includes only the "zinc finger" DNA binding motif of the receptor (*20*). Our results were consistent with previous findings (*6*) that the full-length receptor binds to nGRE's with generally lower affinities than to consensus positive GRE's.

One, two, or three copies of a 25-bp oligonucleotide, plfG, corresponding to the receptor footprint sequence upstream of proliferin, were inserted upstream of a minimal promoter driving a chloramphenicol acetyltransferase (CAT) reporter gene (Fig. 1A). DNase footprinting assays confirmed that these sequences were bound selectively by the 440 to 525 receptor fragment and by the full-length receptor (Fig. 1B). The plfG3 insert substantially elevated basal CAT expression in CV-1 cells relative to the parental vector lacking the inserted element and conferred a modest stimulation of basal activity in HeLa cells (Fig. 2). This effect of plfG3 is independent of hormone (indeed, it is observed in the absence of the co-transfected receptor expression plasmid) and presumably reflects the activity of a nonreceptor enhancer factor. When dexamethasone was added to the cultures cotransfected with receptor and reporter plasmids, CV-1 and HeLa cells showed a striking difference; hormone treatment repressed CAT activity two- to threefold in CV-1 (Fig. 2A), whereas in HeLa cells, dexamethasone stimulated CAT activity three- to fourfold (Fig. 2B). Thus, plfG3, independent of either hormone or receptor, activates promoter function; moreover, the same element confers hormone-dependent positive or negative regulation depending on cell context. Similar effects were observed with the plfG CAT and plfG2 CAT constructs, although the magnitude of the regulatory effects was relatively modest.

Control of glucocorticoid response by Jun and Fos. Because the plfG element did not include the TPA-responsive AP-1 site identified by Mordacq and Linzer (*9*), the observed plfG3-mediated increase in basal promoter activity was unexpected. More surprisingly, treatment of the transfected HeLa cells with TPA produced a doubling of plfG3 CAT expression in the absence of hormone, and a 70-fold increase in dexamethasone-treated cultures (Fig. 3); TPA had no effect on transcription from the reporter construct lacking the plfG3 elements. Closer examination of plfG revealed two sequences within the receptor footprint region that resemble an AP-1 site, containing 4 and 6 bp, respectively, in common with the 7-bp consensus AP-1 element (Fig. 4A). Indeed, DNase footprinting with c-Jun and c-Fos revealed that AP-1 binds within the plfG element (Fig. 4B). Thus, in addition to the TPA response element identified within the proliferin regulatory region (*9*), we conclude that AP-1 also binds selectively within the plfG receptor-binding segment, and that AP-1 binding is likely responsible both for enhancing basal promoter activity and for conferring TPA responsiveness in the plfG3 CAT reporter construct. Moreover, the strong synergism of dexamethasone and TPA at plfG3 implies that the receptor and AP-1 simultaneously can occupy this element.

It seemed that the intracellular composition or concentration of AP-1 complexes might influence the receptor's conferring positive or negative regulation at plfG3. We therefore cotransfected HeLa cells with constant amounts of the plfG3 CAT and receptor expression vectors but with various amounts of c-*jun* or c-*fos* expression vectors. Increasing the amount of c-Jun produced progressive increases in CAT expression in the absence of hormone, and

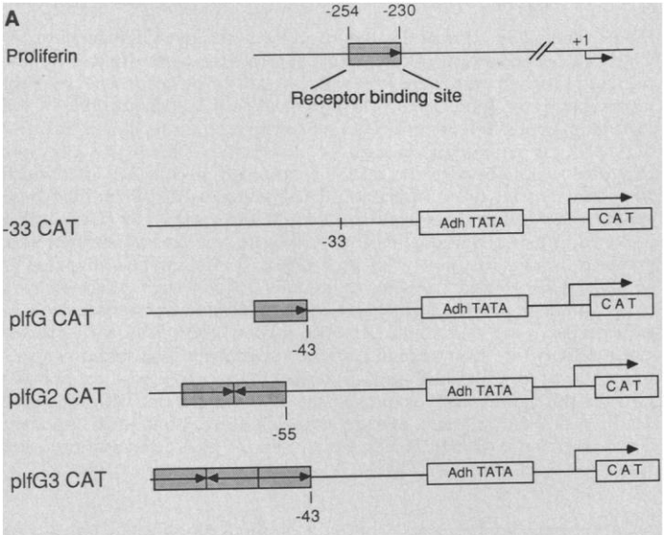


Fig. 1. CAT reporter genes containing one to three copies of a glucocorticoid receptor binding site from the mouse proliferin gene. (**A**) Diagrams of constructs. A 25-bp oligonucleotide corresponding to −254 to −230 relative to the proliferin gene transcription initiation site, and flanked by Pst I and Xba I sites, was synthesized and inserted as one, two, or three copies as shown upstream of the *Drosophila* distal Adh promoter TATA element. The vector contains *Drosophila* sequences between −33 and +53, relative to the alcohol dehydrogenase transcription initiation site fused to the bacterial chloramphenicol acetyltransferase (CAT) gene (*36*); this promoter is also functional in cultured animal cells (*37*). (**B**) DNase I footprint of full-length glucocorticoid receptor and the 440 to 525 receptor derivative (zinc finger fragment) on plfG2 DNA. A 260-bp fragment containing the plfG2 element was end-labeled with ^{32}P, incubated with proteins as described, and subjected to DNase I footprinting (*38*). Reactions in lanes 2 to 6 contained 100 ng of poly(dI · dC). (Lanes 1, 7, and 11) No protein; (lanes 2 and 6) 6 μl of cytoplasmic extracts from HeLa cells infected with wild-type vaccinia virus (3.25 μg/μl); (lanes 3, 4, and 5) these contain, respectively, 2, 4, and 6 μl of cytoplasmic extract from HeLa cells infected with a recombinant vaccinia virus producing full-length glucocorticoid receptor (3.25 μg/μl) (*22*); (lanes 8, 9, and 10) these contain, respectively, 300, 600, and 900 ng of purified glucocorticoid receptor derivative, 440–525 (*20*). Arrows on left margin indicate positions and orientation of plfG receptor binding sequences on the fragments.

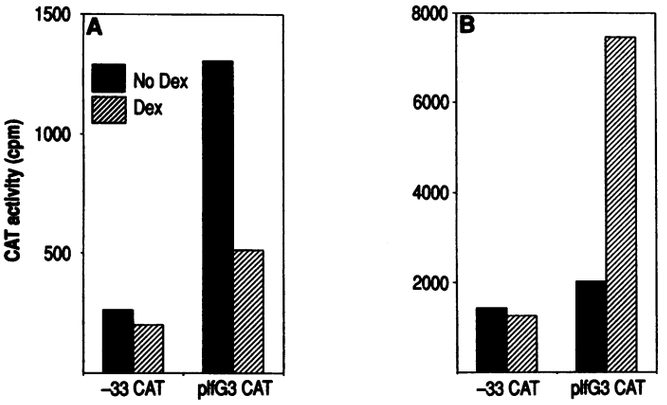


Fig. 2. The plfG3 element displays negative or positive GRE activity. (**A**) CV-1 cells. Subconfluent cultures of CV-1 cells were co-transfected with the calcium phosphate procedure (*39*) with 3 μg of rat glucocorticoid receptor expression vector (p6RGR) (*3*), 3 μg of either −33 CAT or plfG3 CAT reporter plasmids, and 50 ng of β-galactosidase expression plasmid (pLac 82 SU) (*40*); as an internal control of transfection efficiency. Cells were incubated overnight with the calcium phosphate precipitates, after which they were washed twice with phosphate-buffered saline (PBS), and fresh medium was added together with 100 nM dexamethasone (Dex) where indicated. After an additional 48 hours cells were harvested and extracts were prepared by four freeze-thaw cycles (−70°C, 37°C) and centrifugation at 15,000*g*. Heat-treated portions (5 minutes, 68°C) were subjected to a nonchromatographic CAT assay (*41*); a second portion was subjected to a β-galactosidase assay (*42*). CAT activities shown, normalized to β-galactosidase activity, are from a representative experiment. Transfection experiments shown here and in Figs. 3, 5, 6, and 8 were repeated three or more times. In this figure, average repression in CV-1 was 3.7-fold with a standard deviation of 0.61; in HeLa, average induction was 3.3-fold with a standard deviation of 0.65. (**B**) HeLa cells. Conditions for growth, transfection, and assay were as in (A).

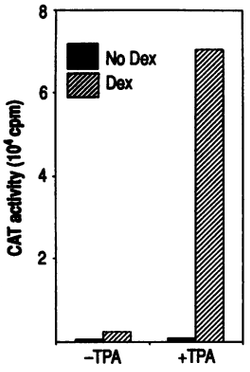
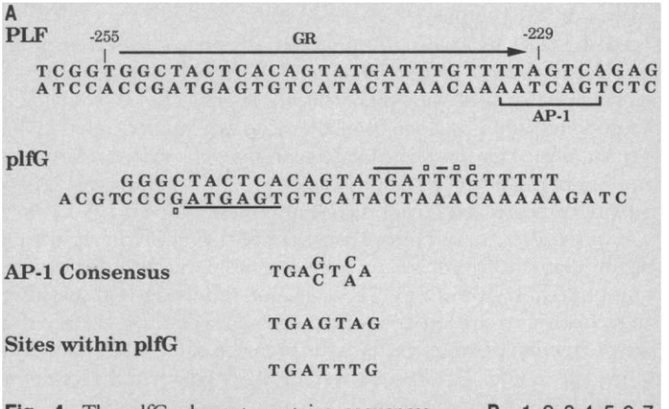


Fig. 3. Both TPA and dexamethasone modulate regulation in HeLa cells via the plfG3 element. HeLa cells were transfected as in Fig. 2 with 3 μg each of the plfG3 CAT and p6RGR DNA, and treated after transfection with 100 nM dexamethasone or TPA at 0.125 μg/ml for 48 hours as indicated. CAT and β-galactosidase were assayed as in Fig. 2.

dexamethasone addition resulted in a further three- to eightfold enhancement (Fig. 5A). In parallel transfections, increasing amounts of c-Fos stimulated CAT expression in the absence of hormone, but in contrast to the results with c-Jun, dexamethasone treatment had no effect on cultures transfected with 1 μg of c-*fos*, and repressed by about threefold the CAT expression in cultures transfected with 4 μg of c-*fos* (Fig. 5B). Thus, plfG3 conferred a positive glucocorticoid response in HeLa cells and in cells bearing transfected c-*jun* coding sequences. In contrast, the same element mediated negative glucocorticoid regulation in HeLa cells when c-Fos was elevated.

To examine further the c-Jun and c-Fos dependence of plfG3 activity, we performed a similar series of transfections in undifferentiated F9 embryonal carcinoma cells, which lack endogenous AP-1 activity (*14, 19, 21*). When receptor and plfG3 CAT were introduced into these cells in the absence of c-*jun* or c-*fos*, neither basal expression nor hormone-mediated enhancement was detected.



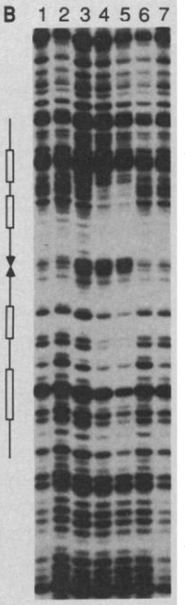


Fig. 4. The plfG element contains sequences related to AP-1 binding sites, and is specifically bound by AP-1 protein. (**A**) Nucleotide sequence of glucocorticoid receptor footprint region (underlying arrow) upstream of the proliferin promoter. The sequence of the plfG oligonucleotide itself is also shown, together with the AP-1 consensus sequence (*13*), and two related sequences within plfG. The AP-1–related sequence elements are marked on the plfG sequence; lines indicate consensus positions, small squares denote departures from consensus. (**B**) DNase I footprinting of AP-1 on plfG2. A plfG2-containing fragment, which also contains a head-to-head dimer of the PLF GR binding sequence, as in the diagram of (A), was end-labeled with ^{32}P for DNase I footprinting as in Fig. 1, except that the labeled fragments were mixed with HeLa cell nuclear extract containing high amounts of c-Jun and c-Fos (see below); similar results were obtained with purified c-Jun and c-Fos. Arrows on left margin indicate position and orientation of plfG elements; open rectangles denote positions of the putative AP-1 sites within plfG2. (Lanes 1 and 7) No protein; (lanes 2 and 6) 3.2 μg of control nuclear extracts (see below); (lanes 3, 4, and 5) these contain, respectively 0.25, 0.5, or 1.0 μl of AP-1–containing extract (3.2 μg/μl). Binding reactions were done in 10 percent glycerol, 2 percent polyvinyl alcohol, 25 mM tris-Cl, pH 7.9, 6.25 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT (dithiothreitol), 0.5 mM EDTA, 0.05 percent NP-40, with approximately 1 fmol of plfG2 probe. Reactions in lanes 2 to 6 contained 100 ng of poly (dI · dC). Reactions were incubated for 14 minutes at 0°C and 1 minute at room temperature before DNase I digestion; reactions in lanes 1 and 7 received 0.3 ng of DNase I; lanes 2, 5, and 6 received 7.5 ng; lane 3, 1.9 ng; lane 4, 3.8 ng. Samples were fractionated on an 8 M urea, 8 percent polyacrylamide gel. Overproduction of AP-1 was achieved by transfecting 150-mm dishes of subconfluent HeLa cells with 18 μg each of c-*fos* and c-*jun* expression vectors driven by a bacteriophage T7 promoter (*43, 44*). Four hours after removal of calcium phosphate precipitates, the cultures were infected with vaccinia virus encoding T7 polymerase (30 pfu per cell) (*45*) and were incubated for 24 hours. Cells were harvested by scraping into PBS, and nuclear extracts were prepared (*46*); control extracts were prepared in parallel from untransfected infected HeLa cells.

However, upon addition of relatively low levels of c-*jun* and c-*fos* together, basal promoter activity was strongly activated, and dexamethasone suppressed that activation by as much as 30-fold (Fig. 6A). Exogenous c-Fos expression in the absence of exogenous c-Jun yielded similar results (Fig. 6B). As observed in HeLa cells, cotransfection of c-*jun* into F9 cells in the absence of added c-*fos* reversed the direction of hormonal regulation: the Jun-stimulated basal expression was further enhanced upon dexamethasone addition (Fig. 6B).

These findings indicate that components of the AP-1 family are essential for each of the activities of the plfG3 element. That is, activation of basal expression is observed in F9 cells only in the

presence of exogenous c-Jun or c-Fos. The capacity for exogenous c-Fos alone to trigger plfG3-mediated enhancement in F9 cells implies a background level of an endogenous Jun-related protein that itself is insufficient to activate from plfG3 [or from other AP-1 sites (*14, 19, 21*)], but is functional upon heterodimerization with exogenously introduced c-Fos. The glucocorticoid receptor fails to regulate plfG3 CAT in F9 cells in the absence of added c-*jun* or c-*fos* (Fig. 6A) despite the fact that the receptor is fully competent under these same conditions to enhance transcription from the well-characterized tyrosine aminotransferase GRE (*22, 23*). This suggests that an interaction, direct or indirect, between receptor and c-Jun is required for hormonal regulation from plfG3, and that c-Jun alone (presumably as Jun homodimers) both activates the promoter and mediates glucocorticoid receptor-dependent enhancement, whereas Jun-Fos heterodimers strongly activate the promoter in a manner that is fully suppressed by the hormone-receptor complex.

Interactions of glucocorticoid receptor with AP-1 and DNA. To examine the possibility that the glucocorticoid receptor might associate with components of the AP-1 complex, we tested whether the proteins in solution could be chemically cross-linked. For these experiments, c-Fos and c-Jun were synthesized in reticulocyte lysates in the presence of [^{35}S]methionine, and glucocorticoid receptor was produced in HeLa cells infected with a recombinant vaccinia virus expressing receptor coding sequences (*22*). Cytoplasmic extracts from the infected cells were incubated with hormone, mixed with labeled c-Jun, c-Fos, or both, and the mixtures were treated with the cross-linking agent dithio-bis (succinimidyl proprionate) (DSP). The reactions were immunoprecipitated with receptor-specific monoclonal antibody (*24*), the cross-links were reversed, and the labeled proteins were subjected to SDS gel electrophoresis.

Under these conditions c-Jun was coprecipitated, whereas c-Fos alone was not (Fig. 7, lanes 2 and 4); however, labeled c-Fos was detected in complexes with the receptor when c-Jun was also present in the reaction (Fig. 7, lane 6). Control reactions with cytoplasmic extracts from HeLa cells infected with wild-type vaccinia virus yielded no detectable coprecipitated c-Jun or c-Fos (Fig. 7, lanes 1, 3, and 5); similarly, labeled proteins were not precipitated by control antiserum. Thus, the receptor appears to form specific complexes with c-Jun, perhaps as Jun homodimers, and with Jun-Fos heterodimers. No complexes of receptor and AP-1 were observed in the absence of DSP, implying that the interactions are transient under these conditions.

Binding of receptor to the plfG sequence is selective but weak relative to its association with consensus GRE's (Fig. 1B). Together with results indicating that c-Jun interacts with receptor in vitro and is required for plfG activity in vivo, it seemed conceivable that AP-1 (and c-Jun in particular) might passively "tether" the receptor at plfG sequences, and that DNA binding by the receptor itself might therefore be wholly dispensable. To test this idea, we cotransfected into HeLa cells the plfG3 CAT reporter, c-*fos*, and receptor derivatives bearing point mutations in the zinc finger region that selectively abrogate DNA binding (*4*). We found no hormonal regulation under these conditions (Fig. 8), consistent with the idea that DNA binding by the receptor at plfG is essential. Similarly, NLxC, a chimeric receptor lacking entirely the zinc finger region and instead containing the DNA binding domain of the bacterial lexA repressor (*6*), also failed to modulate plfG3 CAT activity, whereas it strongly enhanced expression from a lex operator-linked promoter (Fig. 8). In addition, the receptor zinc finger mutants failed to activate transcription from plfG3 CAT in HeLa cells when transfected alone or with c-*jun*. We infer from these results that receptor action at the plfG3 element requires receptor binding both to c-Jun and to a specific DNA sequence.

Regulation by composite GRE's. Our experiments define a new class of glucocorticoid response element at which hormonal regulation depends not only on DNA binding by the receptor protein, but also on nonreceptor factors with which the receptor interacts. We denote these as "composite GRE's" to distinguish them from previously characterized "simple GRE's," which are defined by three types of experiments: First, fusion of simple GRE's to test promoters has no effect on basal promoter activity in the absence of

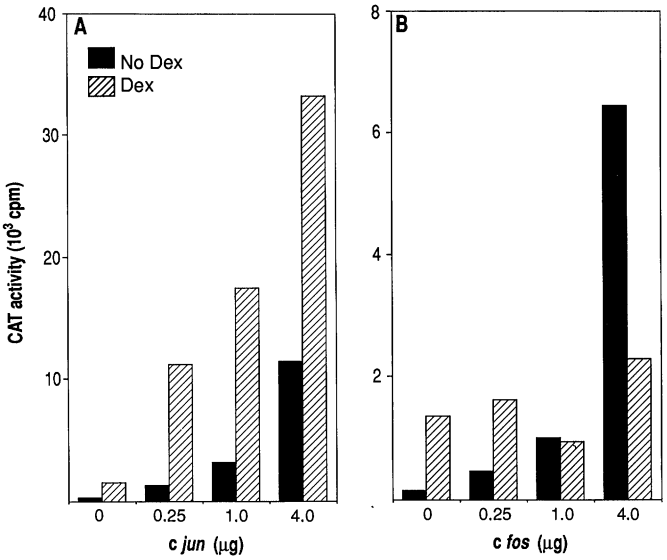


Fig. 5. Exogenous c-Jun or c-Fos have differential effects on plfG3 action in HeLa cells. (**A**) HeLa cell cultures were transfected with 2 μg of plfG3 CAT reporter, 2 μg of p6RGR receptor expression vector, 50 ng of pLac82 SU internal control plasmid, and either 0, 0.25, 1.0, or 4.0 μg of pRSV c-*jun* (*47*) expression vector. Extracts were prepared and assayed as in Fig. 2, except that a mock reaction containing no protein was included in the CAT assay to establish a baseline activity which was subtracted from each value. (**B**) HeLa cells were transfected and assayed as above, except that pRSV c-*fos* (*47*) replaced pRSV c-*jun*.

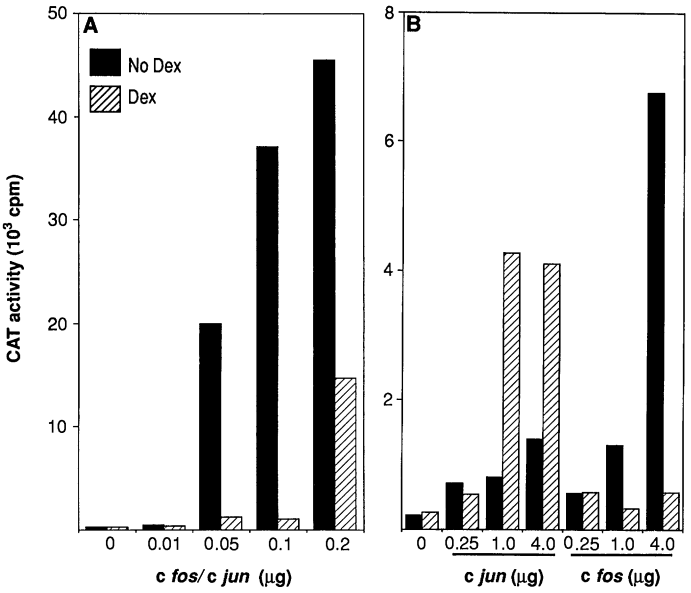


Fig. 6. Effects of exogenous c-Jun and c-Fos on plfG3 composite GRE activity in F9 embryonal carcinoma cells. (**A**) Co-transfection of c-*jun* and c-*fos*. Subconfluent monolayers of F9 cells were cotransfected as in Fig. 2 with increasing amounts of pRSV c-*fos* and c-*jun* expression vectors together with 2 μg of plfG3 CAT, 2 μg of p6RGR receptor expression vector, and 50 ng of pLac82 SU control plasmid. Extracts were prepared and enzymes assayed as in Fig. 2. (**B**) Separate transfections of c-*jun* and c-*fos*. Experiment was carried out as in (A), except the pRSV c-*jun* and c-*fos* expression plasmids were introduced separately.

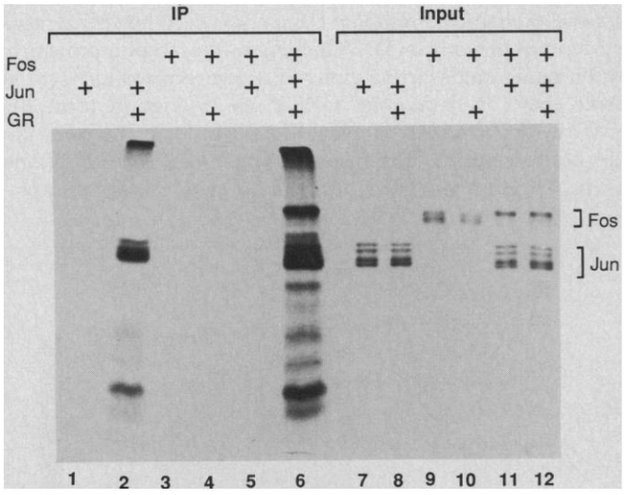


Fig. 7. Glucocorticoid receptor interacts selectively with c-Jun and AP-1 in vitro. [^{35}S]methionine-labeled c-Fos and c-Jun proteins were produced by in vitro transcription (T7 and SP6 RNA polymerase, respectively; Promega) and in vitro translation in reticulocyte lysates (Promega). The labeled products were mixed with cytoplasmic extracts of HeLa cells infected either with wild-type vaccinia virus or with a recombinant vaccinia virus expressing the rat glucocorticoid receptor (*22*). The labeled Jun and Fos products in these reaction mixes are shown in lanes 7 to 12. The reaction mixtures were then subjected to chemical cross-linking with DSP and the cross-linked products were immunoprecipitated with a receptor-specific monoclonal antibody (*48*); the immunoprecipitates were washed extensively before cross-links were reversed in 4 percent β-mercaptoethanol and the products were subjected to electrophoresis, fluorography, and autoradiography. Labeled Jun and Fos immunoprecipitated with the receptor are shown in lanes 1 to 6. In each lane, the presence of a given protein in the initial reaction is denoted by plus signs. Bands at the stacking gel interface in lanes 2 and 6 are due to inefficient reversal of cross-links.

hormone or receptor (*25*). Second, all mutations that affect the activity of simple GRE's in vivo also affect receptor binding in vitro (*26*). Finally, "domain swaps," in which the zinc finger DNA binding region of the receptor is replaced by the DNA binding domains of heterologous proteins, yield chimeric receptors that recognize the corresponding heterologous target sequences as fully functional GRE's (*3*). Thus, all simple GRE's confer enhancement in a manner that depends solely on "tethering" the receptor to DNA. In contrast, all of the described "nGRE's" appear to depend upon both receptor and nonreceptor factors, and certain positive steroid responses similarly have been shown to be mediated by composite elements (*27*); we show here, for example, that hormone-dependent enhancement from plfG3 requires c-Jun.

Because simple GRE's depend only upon a single factor, their analysis is relatively straightforward, but their regulatory diversity is limited. In contrast, composite GRE's employ protein-protein interactions between the receptor and other factors to achieve combinatorial regulation. In fact, hormonal control of the intact proliferin promoter likely involves components in addition to those implicated with the synthetic plfG3-minimal promoter constructs characterized in this study (*28*). Because of their regulatory versatility, it seems likely that composite GRE's will prove to be the prevalent mode for regulation by glucocorticoids.

Without altering cell type, regulatory element, promoter, receptor, or hormonal ligand, we have shown that transcription from plfG3 can be activated, repressed, or unaffected by hormone, and that c-Jun and c-Fos serve as "selectors" for these shifts of regulatory activity. Thus, the positive glucocorticoid response conferred by plfG3 in HeLa cells was switched to a negative response in the presence of increased c-Fos. Similarly, elevated expression of c-Jun in CV-1 cells reversed the repression normally mediated by plfG3 in that line, producing a stimulation of transcription in response to hormone. Therefore, the ratio of functional Jun and Fos, not their absolute amounts, is the key determinant; in this way, cell-specific action of plfG3 is defined by the relative activities of two non-cell-specific factors.

On the basis of our results, we propose a simple model to account for selector activity (Fig. 9). According to this speculative scheme, the receptor would function at plfG3 only by interacting both with a specific DNA sequence and with bound c-Jun; hence, plfG3 would be inactive in F9 cells, which lack AP-1 activity (Fig. 9A). The model envisions further that the "transcriptional enhancement domains" of receptor and c-Jun would remain functional in a receptor-Jun-plfG3 complex (Fig. 9B); thus, plfG3 would be a strong positive GRE in this state. In contrast, receptor association with a Jun-Fos heterodimer at plfG3 would produce a DNA-protein complex in which Jun-Fos and receptor co-occupy the element but are inactive (Fig. 9C); alternatively, the receptor interaction with bound Jun-Fos at plfG3 might produce a conformational change that destabilizes DNA binding by both receptor and Jun-Fos. Preliminary results are consistent with co-occupancy of the DNA (*29*), although in either case, plfG3 would be a strong negative GRE under these conditions.

It remains formally possible that c-Jun and c-Fos might affect plfG3 indirectly, perhaps by inducing the expression of other factors that bind at plfG3 and interact with receptor. We have shown, however, that the plfG element contains AP-1–like consensus sequences, that AP-1 binds specifically within the element, that the receptor can interact selectively with c-Jun, and that regulation by the receptor requires its DNA binding domain. Thus, the simplest interpretation of our experiments is that c-Jun and c-Fos act directly at plfG3, and that receptor-AP-1 complexes form on the element. As higher order oligomers are well known among molecules that interact via coiled coils (*30*), the protein structure motif that governs dimerization of AP-1 components, heterotrimerization of factors, resulting in protein complexes with distinct activities, provides a feasible mechanism for combinatorial control. We suggest that

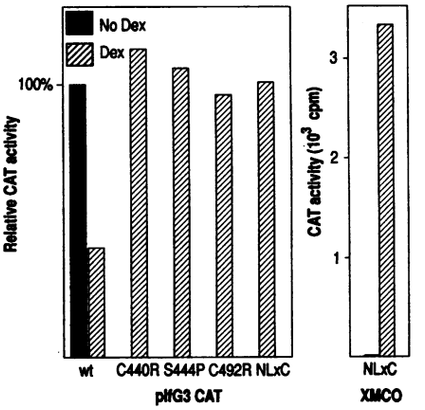


Fig. 8. Role of the receptor DNA binding domain in composite GRE activity. HeLa cells were transfected as in Fig. 2 with 2 μg of plfG3 CAT, 4 μg of pRSV c-*fos*, 50 ng of pLac82 SU, and 2 μg of expression plasmids encoding various receptor derivatives: wt, wild type; C440R, S444P, C492R, three receptor derivatives with point mutations in the zinc finger region, each of which abrogates DNA binding (*4*); NLxC, a chimeric receptor in which the zinc finger region has been substituted by the DNA binding motif of the bacterial LexA repressor (*3*). To demonstrate that NLxC is functional, a separate control transfection was carried out in which NLxC was co-transfected with a CAT reporter plasmid linked to the *lex* operator sequence (*3, 49*). Extracts were prepared and enzymes were assayed as in Fig. 2; for ease of comparison, CAT activities in the absence of hormone were normalized to the maximum activity obtained for cells transfected with the wild-type receptor. Repression by wild-type receptor is somewhat weaker in this case due to relatively low levels of receptor expression from the particular expression vector [pVARO (*50*)] used for this experiment.

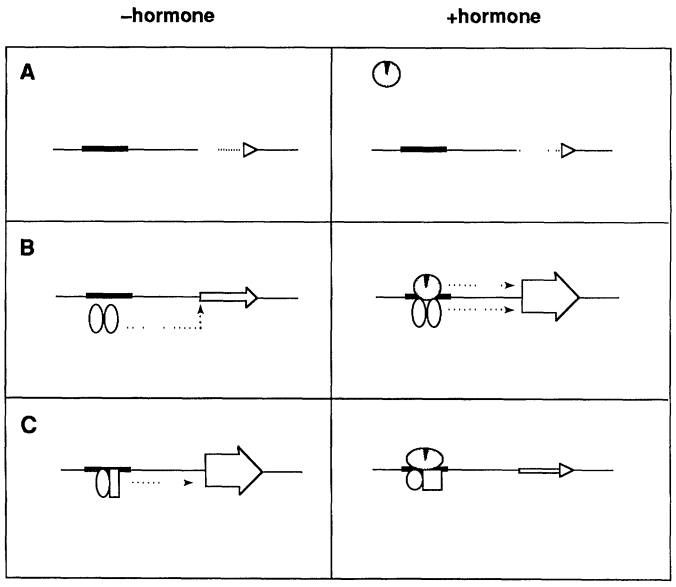


Fig. 9. A model for composite GRE activity by the plfG3 element. In each panel, the solid line depicts DNA surrounding the promoter (different sized arrows represent relative levels of transcriptional activity) and plfG sequence element (thickened line). Stippled forms with solid sector depict the receptor-hormone complex; open ovals represent c-Jun; open rectangles, c-Fos; dotted arrows between factors and promoter indicate enhancement activity. Shown are three conditions in the absence and presence of hormone: (**A**) In the absence of AP-1 activity, the hormone-receptor complex fails to interact functionally with the plfG element. (**B**) The c-Jun homodimer alone binds weakly to plfG and activates the promoter; the hormone-receptor complex interacts both with the plfG sequence and with c-Jun, producing a stable complex that strongly enhances promoter function. (**C**) The Jun-Fos heterodimer strongly enhances promoter function; the hormone-receptor complex interacts both with plfG and with Jun-Fos producing a complex with altered conformation that is not functional for enhancement; an alternative possibility is that the structural alteration leads to release of all components from the DNA.

selector functions of the types inferred here might operate at composite GREs in vivo to modulate the extent and the direction of glucocorticoid responsiveness under different physiologic conditions. In fact, certain single copy genes, such as phosphoenolpyruvate carboxykinase (PEPCK), are glucocorticoid-stimulated in some tissues and repressed in others (*31*).

The selector function of Jun and Fos provides a simple mechanism for communication between distinct signal transduction networks. In this regard, it is intriguing that AP-1 itself represents a family of transcription factors that may derive functional diversity from alternate pairwise association of different Jun- and Fos-related components (*16, 17*). In fact, we have not determined whether c-Jun and c-Fos are the precise AP-1 components that serve as selectors at plfG3 in vivo. Interestingly, glucocorticoid-mediated repression of the glycoprotein hormone α-subunit and PEPCK genes appears to involve receptor-dependent inhibition of CREB (*11, 31*), a cAMP signal transducer that is c-Jun–related (*32*). Thus, different composite GRE's may employ a range of AP-1 family members as selectors.

General features of negative regulation. One view of negative transcriptional regulation is that repressor proteins exclude positive factors by competitive binding to common or overlapping sites (*2, 33*). Such a scheme requires that repressors bind tightly and efficiently to their target sites. The receptor, however, binds to plfG with relatively low affinity in vitro. Moreover, the receptor is produced at limiting concentrations, and therefore occupies inefficiently even strong consensus GRE elements (*34*); similarly, other regulatory factors are expressed at limiting levels (*35*). Therefore, the competition model cannot readily explain repression by the receptor or by many other factors.

It is instructive to consider that even low fractional occupancy of a single regulator at sites near an otherwise inactive promoter can suffice to strongly activate transcription. In contrast, efficient repression by steric hindrance requires that the repressor efficiently occupy binding sites at all potentially activatable promoters. As the amount of regulatory proteins produced is generally limiting, negative regulation in particular must occur by other mechanisms. In our model for the plfG3 composite GRE, we suggest a scheme whereby limiting amounts of receptor repress efficiently and specifically despite inability to occupy fully even cognate GRE sequences. The key feature is that the receptor interacts at the composite GRE not merely with a DNA sequence but also with the bound Jun-Fos complex. Thus, the receptor associates only with activated promoters. The finding that the receptor interacts both with DNA and with Jun-Fos implies that the composite GRE may facilitate or stabilize the protein-protein interaction, and provides a mechanism for selectivity that explains why glucocorticoids do not regulate all promoters that utilize AP-1. Moreover, our studies of the plfG3 composite GRE underscore the regulatory versatility of such elements.

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46. Cell monolayers were scraped into PBS, centrifuged, and lysed in buffer A [10 mM Hepes 7.6, 15 mM KCl, 2 mM MgCl2, 1 mM DTT (dithiothreitol), 0.1 mM EDTA, 1 mM PMSF, and 0.2 percent NP-40]. Nuclei were isolated by centrifugation, resuspended in buffer B (10 percent glycerol, 50 mM Hepes 7.6, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF) and lysed by addition of ammonium sulfate to 0.3 M at 4°C. The lysate was centrifuged at 400,000*g*, the supernatant was brought to 1.5 M ammonium sulfate, and the sample was centrifuged at 90,000*g* to collect precipitated protein. The pellet was resuspended in buffer B and stored at −70°C.
47. Provided by V. Baichwal and R. Tjian.
48. Reaction mixes containing reticulocyte lysates and cell extracts were incubated at 30°C for 30 minutes, diluted one-third with 100 mM triethanolamine, pH 8.0, 2 mM EDTA, 5 percent glycerol, and cross-linked with 5 mM DSP for 15 minutes at 25°C (*30*); cross-linking was quenched by the addition of lysine to 20 mM. Samples were diluted tenfold in RIPA buffer (1 percent sodium deoxycholate, 1 percent Triton X-100, 0.2 percent sodium dodecyl sulfate, 150 mM NaCl, 50 mM tris-HCl, pH 7.4) plus 5 percent fetal calf serum, and cleared by the addition of swollen, washed protein A–Sepharose (100 mg/ml) to 0.03 percent, at 4°C for 30 minutes. The Sepharose was removed and antibody to the receptor (*24*) was added for 1 hour at 0°C, protein A–Sepharose was added to 0.02 percent and incubated for 2 hours, at 4°C with rotation. The Sepharose beads were washed extensively in RIPA buffer and 2 M urea. Bound protein was eluted in SDS sample buffer containing 4 percent β-mercaptoethanol to reverse cross-links, and subjected to electrophoresis, fluorography, and autoradiography.
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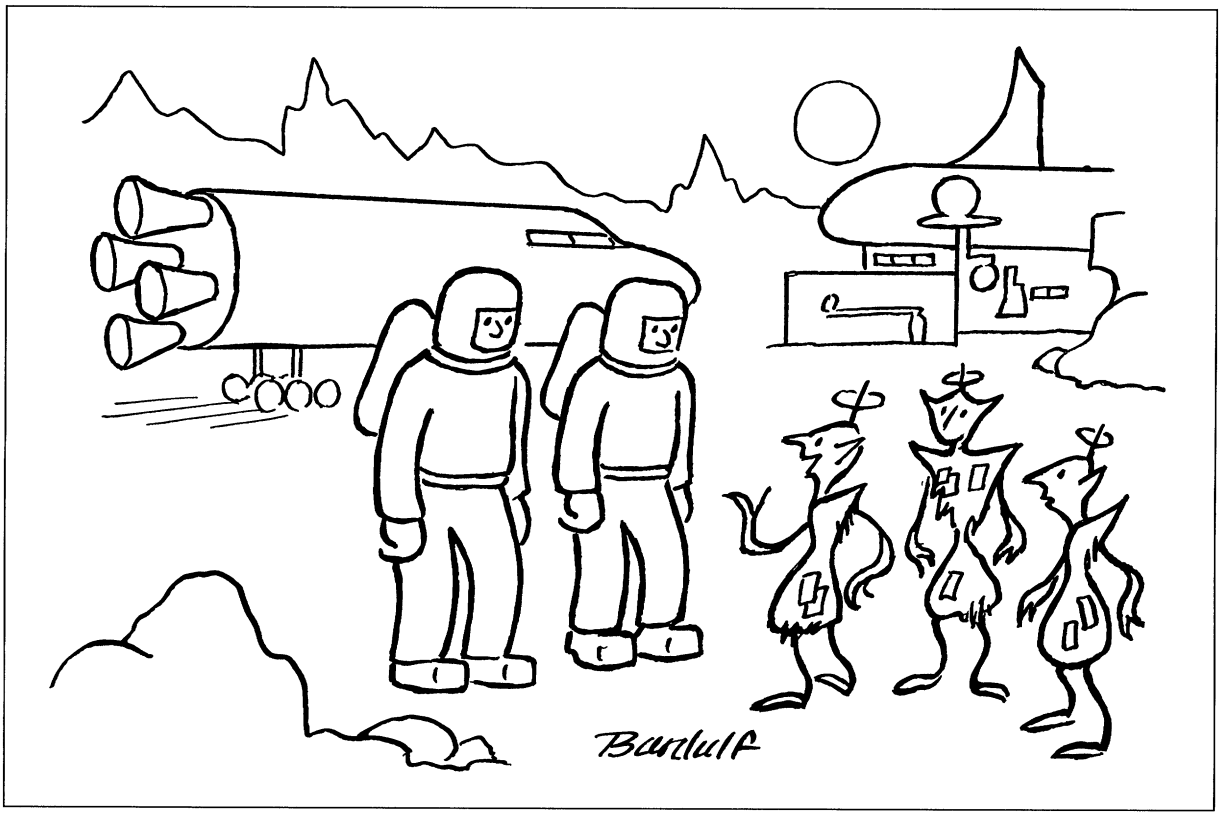
51. We thank D. Linzer and J. Mordacq for providing materials and information prior to publication; V. Baichwal, T. Curran, E. Fodor, N. Hay, D. Hruby, B. Moss, F. Rauscher, H. Stunnenberg, R. Treisman, and R. Tjian for plasmid and vaccinia virus clones; K. Perkins for initial binding assays with AP-1 and for providing AP-1 protein; R. Turner for plasmids and for AP-1 protein; C. Abate and T. Curran for c-Jun and c-Fos proteins; M. Jacobson, M. Privalsky, and J. Thomas for discussion and assistance with experiments; R. Grosschedl, J. Jaynes, A. Johnson, J. Mordacq, D. Linzer, P. O'Farrell, B. Polisky, and S. Weinrich for critically reviewing the manuscript; and B. Maler and K. Mulherin for preparation of the figures and manuscript, respectively. Supported by grants from NIH and NSF, an ACS Postdoctoral Fellowship (J.N.M.), a California Division ACS Junior Fellowship J-47-88 (S.K.Y.), and a Howard Hughes Medical Student Research Training Fellowship (M.I.D.).

19 June 1990; accepted 17 August 1990



"...furthermore, thirty-four percent of our population control one percent of the wealth which is the same as in your country – if I'm not mistaken."