The basic region of AP-1 specifies glucocorticoid receptor activity at a composite response element

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Unrelated factors collaborate at composite response elements to confer novel patterns of transcriptional regulation. For example, AP-1 and glucocorticoid receptor bind and mutually affect their activities at a 25-bp composite element, plfG. We found that different members of the AP-1 factor family that behave similarly in the absence of receptor are strikingly distinct in its presence: They specify opposite (enhancement vs. repression) regulatory actions by the receptor. Four amino acids within the AP-1 DNA-binding domain were identified as crucial determinants of receptor regulatory activity at plfG. We conclude that interactions of factors from separate transcription factor families at composite response elements provide a mechanism by which a single factor can regulate both positively or negatively, and a potential resolution of the apparent functional redundancy within regulatory factor families.

[*Key Words:* AP-1; glucocorticoid receptor; transcriptional regulation; protein-protein interaction; DNA-binding domain]

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Many protein factors have been described that regulate the initiation of transcription by RNA polymerase II. Most of those characterized to date are sequence-specific DNA-binding factors, although it is likely that numerous non-DNA-binding regulators remain to be identified. In general, sequence-specific regulators are encoded by multigene families in which the family members bind to a cognate set of closely related DNA response elements using a particular protein structure motif, such as a zinc finger or basic-zipper domain (Evans 1988; Harrison 1991; Lamb and McKnight 1991; Macleod et al. 1992). The various family members commonly display qualitatively or even quantitatively similar activities at their cognate response element, suggesting either that the family members are redundant or that they may be functionally distinct under circumstances not yet defined.

Transcriptional regulatory regions commonly contain clustered or interdigitated binding sites for multiple factors from different families. Some of these consensus sequences, such as those recognized by the SP1, CTF, NF1 or AP-1 factor families (Friedman et al. 1989; Mitchell and Tjian 1989; Kerppola and Curran 1991a; Pascal and Tjian 1991), are found in many promoters; others, such as potential binding sites for nuclear receptors (Beato 1989, 1991), which transduce signaling by various small molecule ligands, reside at a more restricted subset of regulatory regions. The complex organization of these regions implies that they might serve as "junction points" at which regulatory networks defined by differ-

ent factor families may interact and communicate. Thus, protein-protein interactions between factors bound within a given regulatory region might produce novel regulatory consequences; DNA sequences at which such interactions occur have been denoted "composite" response elements, to distinguish them from "simple" elements at which a given factor functions alone (Yamamoto 1989; Diamond et al. 1990; Miner et al. 1991). Conceivably, factor interactions at composite elements might reduce or eliminate the apparent redundancy within factor families, as family members that are indistinguishable in the context of a simple response element might undergo quite distinct protein-protein interactions at composite elements (Miner and Yamamoto 1991).

Diamond et al. (1990) described a 25-bp composite response element from the regulatory region of proliferin, a gene that is transcriptionally activated by phorbol esters such as TPA and is repressed in several cell types by glucocorticoids (Mordacq and Linzer 1989). Consistent with these findings, the proliferin regulatory region contains a consensus binding sequence for AP-1 (which mediates TPA responsiveness) and a binding site for purified glucocorticoid receptor (GR). The plfG element, which corresponds precisely to the sequence footprinted by the receptor, is also bound specifically by purified AP-1 factor *in vitro* and can confer upon a heterologous promoter both TPA and glucocorticoid regulation (Diamond et al. 1990). Thus, although regulation from plfG alone is un-

likely to account for all of the regulatory events associated with the authentic proliferin promoter (Mordacq and Linzer 1989), it appears to be a relatively simple sequence at which to study the interaction of the receptor with AP-1.

AP-1 factors recognize specific DNA sequences through basic-zipper (bZIP) DNA-binding motifs (Kerppola and Curran 1991a). Each AP-1 family member carries a bZIP region that is closely homologous with either c-Jun or c-Fos, the two prototype representatives of the family (Kerppola and Curran 1991a); c-Jun-like proteins generally can homodimerize or heterodimerize across the AP-1 family, whereas c-Fos-like proteins heterodimerize with the Jun subclass but do not dimerize within the Fos subclass (Vogt and Bos 1990). The various AP-1 dimers all recognize, albeit with different affinities, the AP-1 consensus binding site and can stimulate, albeit with different efficiencies, initiation from nearby promoters (Abate et al. 1991; Kerppola and Curran 1991a).

Steroid receptors, a subset of the nuclear receptor superfamily (Evans 1988), enhance or repress transcription in a hormone-dependent, cell-type, and promoter-specific fashion. In the context of a consensus simple glucocorticoid response element (GRE), the GR functions independently of other regulators and enhances but does not repress transcription (Beato 1989; 1991). This suggests that simple response elements act solely to tether activation domains close to promoters (Ptashne 1988; Ptashne and Gann 1990). In contrast, regulation from composite hormone response elements requires the combined actions of a receptor together with one or more nonreceptor sequence-specific regulators (Miner and Yamamoto 1991; Miner et al. 1991). Such loci can specify either hormone-dependent enhancement or repression of transcription, and in the absence of the appropriate nonreceptor factor, fail altogether to confer hormonal regulation, despite the presence of a fully functional receptor.

In the case of the plfG composite element, the subunit composition of AP-1 determines the nature of the hormone response mediated by the GR (Diamond et al. 1990; Miner et al. 1991). That is, in cell contexts in which AP-1 is composed predominantly of c-Jun homodimers, glucocorticoids enhance transcription from plfG; whereas in cell contexts in which AP-1 is predominantly composed of c-Jun/c-Fos heterodimers, the receptor represses transcription. Furthermore, glucocorticoids have no effect on plfG-linked promoters in the absence of AP-1 or under conditions in which c-Jun homodimers and c-Jun/c-Fos heterodimers are functionally balanced (Diamond et al. 1990). The receptor can be chemically cross-linked to c-Jun and to c-Jun/c-Fos, but not to c-Fos alone (Diamond et al. 1990; Jonat et al. 1990). Point mutations in the receptor that preclude its DNA-binding are defective for composite regulation at plfG, whereas other mutations in the receptor DNA-binding domain that do not alter its DNA recognition have no effect on composite regulation (Diamond et al. 1990; J. Thomas, J.N. Miner and K.R. Yamamoto, un-

publ.). Together, these findings imply that ternary complexes of receptor and AP-1 at plfG determine the direction and magnitude of the subsequent hormone response (Diamond et al. 1990).

Thus, the plfG sequence represents a well-delineated composite element that mediates functional interactions of sequence-specific regulators from two well-characterized factor families. Why does the GR require AP-1 for activity at plfG while acting independently at simple GREs? How does the subunit composition of AP-1 determine the direction of glucocorticoid regulation at plfG? To begin to approach these questions, we have used protein chimeras of various AP-1 family members to define domains within specific subunits that might specify the regulatory fate of the receptor. Identification of such regulatory domains would be a first step in determining the mechanism by which selector activity is achieved. In addition, we have compared two members of the Fos subfamily to test the idea that factors whose activities seem redundant in the context of simple response elements might be distinguishable at composite response elements.

Results

AP-1 domains for composite regulation

To investigate composite regulation by the GR and AP-1, we monitored expression from a reporter construct, plfG3 chloramphenicol acetyltransferase (CAT), which contains three tandem plfG elements upstream of a fusion of the *Drosophila alcohol dehydrogenase* (*ADH*) TATA box to bacterial CAT-coding sequences (Diamond et al. 1990). The differential effect of c-Jun homodimers and c-Jun/c-Fos heterodimers on regulation by the receptor provided an approach to search for domains of these AP-1 subunits that might specify the direction of receptor activity at the plfG3 element. Cohen and Curran (1990) constructed chimeras between c-Jun and c-Fos and showed that the dimerization and specific DNA-binding activities map to the leucine zipper and basic domains of each subunit. We examined several of those constructs in mammalian expression vectors (Fig. 1), considering them as a series in which c-Fos is transformed progressively into c-Jun; thus, a carboxy-terminal swap (FFFJ) is followed by an amino- and carboxy-terminal swap (JFFJ) and then a basic region swap (JJFF). The chimeras were expressed in transient transfection assays from the Rous sarcoma virus (RSV) promoter in F9 cells (which lack endogenous AP-1 activity); chimera expression from the cytomegalovirus (CMV) promoter gave similar results.

As seen in Figure 2, cotransfection of each chimera together with intact c-Jun (Diamond et al. 1990; Yang-Yen et al. 1991) revealed that all chimeras stimulated transcription from the composite element in the absence of the receptor. Each chimera contained the c-Fos zipper and therefore could not homodimerize; as expected, transfection of a chimera without cotransfected c-Jun had little effect on reporter expression, confirming the absence of c-Jun activity in these F9 cells (data not

Figure 1. Chimeric constructs of c-Jun and c-Fos. Domains of c-Jun and c-Fos were exchanged as described (Cohen and Curran 1990); numbers below diagrams give positions of amino acid residues that define the borders of various domains. FFFJ (amino terminus + bZIP), c-Jun amino acids 317–334; c-Fos amino acids 1–199; JFFJ (bZIP), c-Jun amino acids 1–248 and 317–334; c-Fos amino acids 128–199; JJFJ (zipper), c-Jun amino acids 1–280 and 317–334; c-Fos amino acids 164–199. Intact c-Jun and c-Fos, and the chimera were cloned into mammalian expression vectors (RSV and CMV) for transfection experiments (see Fig. 2): RSV c-Jun, RSV JJFJ, RSV JFFJ, RSV FFFJ, RSV c-Fos; see Materials and methods.

shown). In this context, transfected c-Jun alone stimulated reporter expression only weakly, whereas cotransfection of a chimera markedly increased the extent of stimulation (Fig. 2); this likely reflects the strong dimerization affinity of the c-Jun and c-Fos zippers (O'Shea et al. 1992) and implies that we detect primarily heterodimers of c-Jun and the c-Fos zipper-containing chimera under our conditions.

When the receptor acted at plfG3 in conjunction with either c-Jun/c-Fos, c-Jun-FFFJ, or c-Jun/JFFJ heterodimers, transcription was repressed, suggesting that the amino- and carboxy-terminal regions of c-Jun are not sufficient as determinants of positive glucocorticoid regulation under these conditions. However, when the basic region of c-Jun replaced that of c-Fos (as in JJFJ), the receptor functioned as an activator. Thus, in the context of a c-Jun/c-Fos chimera heterodimerized with c-Jun, we conclude that the basic region of the chimeric subunit specifies the direction of receptor regulation from the composite element (Fig. 2).

Receptor/AP-1 complexes

The functional interaction between receptor and AP-1 at the plfG3 composite element suggested that the factors might interact physically. Receptor/AP-1 complexes had been implied from chemical cross-linking [dithio-bis(succinimidyl propionate) (DSP)] studies (Diamond et al. 1990; Yang-yen et al. 1990). We therefore sought conditions in which the putative complexes might be detect-

able even without cross-linking agents; such a finding would help to address concerns that weak complexes formed in vitro had been artifactually stabilized by cross-linking. We found that a receptor-specific monoclonal antibody (Gametchu and Harrison 1984) coimmunoprecipitated either in vitro translated c-Jun or c-Jun/c-Fos heterodimers from reticulocyte extracts under nondenaturing conditions (Fig. 3); in each case, AP-1 precipitation was receptor dependent. Consistent with the cross-linking results of Diamond et al. (1990), the receptor antibodies failed to precipitate c-Fos in the absence of either c-Jun or receptor. This implies that the receptor may interact only with dimerized forms of AP-1; whether the receptor directly contacts c-Fos in the receptor/c-Jun/c-Fos complex has not been tested.

Because both the receptor and AP-1 are DNA-binding proteins, it is conceivable that the apparent "factor interaction" inferred from the coimmunoprecipitation experiments actually represents nonspecific and independent binding of the two factor DNA fragments in our extracts (Lai and Herr 1992). Several lines of argument suggest that this is not likely to be the case. First, the efficiency of coprecipitation was unaffected by depletion of contaminating DNA with micrococcal nuclease or DNase I, or by the addition of high levels of nonspecific DNA. Second, the reactions were carried out with low

Figure 2. Role of the c-Jun and c-Fos basic regions in specifying receptor function. Chimeric c-Jun and c-Fos proteins were tested for their effects on plfG3 CAT expression, alone and together with receptor and hormone, in transfected F9 cells. The RSV chimeras (0.5 μ g), RSV c-Fos (0.5 μ g), and RSV c-Jun (0.5 μ g) were each cotransfected with plfG3 CAT (2.0 μ g) (Diamond et al. 1990), 6RGR expression plasmid (2.0 μ g) (Godowski et al. 1988), and β -galactosidase expression vector (0.2 μ g pLac82su) as an internal control (Jaynes and O'Farrell 1988). Each reaction also contained an additional 0.5 μ g of RSV c-Jun to provide a dimer partner for each construct listed. These amounts of plasmid are within the linear range of our assay conditions. Dexamethasone (0.1 μ M) was added where indicated (hatched bars) for 24 hr; (solid bars) no hormone added. Aliquots for each pair were taken from the same DNA precipitate. CAT activity was normalized to β -galactosidase activity and expressed as relative CAT activity. Each data point represents an average of four experiments.

Figure 3. Immunoprecipitation of the AP-1/GR complex. In vitro-transcribed and -translated [35 S]methionine-labeled c-Jun and/or c-Fos, and a labeled, truncated version of c-Jun (N187, denoted internal control, contains amino acids 1–187 and lacks the c-Jun bZIP region) were mixed together with unlabeled receptor for 30 min at 30°C. The reaction was diluted and protein A–Sepharose-bound receptor-specific mAb BUGR2 (Gametchu and Harrison 1984) was added for 30 min at 4°C with nutation to immunoprecipitate the receptor and associated proteins. Input: The labeled proteins in the extracts before immunoprecipitation are shown, using a sample size ~2% (lanes 1–5 from left) or ~4% (lane 6) of that used for immunoprecipitation. Immunoprecipitation: Labeled proteins coprecipitating with GR are shown. The efficiency of GR precipitation was equivalent in all lanes as determined by immunoblotting (data not shown).

concentrations of AP-1 and GR in the presence of a vast excess of other proteins; the potential artifact pointed out by Lai and Herr (1992) was observed only at very high concentrations of the interacting proteins. Third, the receptor and AP-1 are rapidly and covalently joined by a cross-linking reagent with a 12 Å arm length, indicating that the two species are separated by only atomic distances; moreover, Herrlich and colleagues (Jonat et al. 1990; König et al. 1992) have observed receptor/AP-1 complex formation using different short-range cross-linkers under different conditions, including in vivo. Fi-

nally, mutations that eliminate AP-1 DNA-binding activity have little or no effect on coprecipitation (see Fig. 5, below).

To begin to define a region of c-Jun that interacts with receptor, carboxy-terminal truncations of the 334-amino-acid c-Jun protein were prepared by restriction cleavage at various sites within the coding sequence before in vitro transcription and translation of the appropriate expression vector. The resultant c-Jun derivatives, labeled with [35 S]methionine, were then subject to coimmunoprecipitation studies in the presence of receptor and receptor specific antibodies (Fig. 4). In the experiment shown in Figure 4, our preparation of c-Jun truncation product N187 contained a small amount of intact c-Jun owing to incomplete restriction digestion of the original vector; this residual full-length product served as an internal positive control, as the results clearly show that the intact but not the truncated c-Jun (which lacks its bZIP region; see Fig. 1 for orientation) coprecipitated with receptor. Although these experiments do not distinguish a requirement for the dimerization or DNA-binding subregions of the bZIP domain, we can conclude from our results that c-Jun deletions that impinge on the bZIP region of c-Jun destroy the receptor/c-Jun interaction; in contrast, a deletion that affects only sequences downstream of this domain, such as N315, has no apparent effect on coprecipitation (Fig. 4).

Diamond et al. (1990) found that c-Fos coprecipitated with the receptor only in the presence of c-Jun, perhaps indicating that c-Fos is merely tethered to the c-Jun/receptor complex through its leucine zipper interaction with c-Jun. To examine this ternary complex further, we mixed various c-Fos derivatives together with either full-length c-Jun or a c-Jun bZIP deletion (N245) in the presence or absence of receptor, and monitored c-Fos coprecipitation with receptor-specific antibody (Fig. 5). As expected, intact c-Fos precipitated only in the presence of

Figure 4. c-Jun domains involved in receptor interaction. Input: [35 S]methionine-labeled c-Jun derivatives were prepared by cleaving T7 c-Jun (Turner and Tjian 1989) with particular restriction enzymes, generating specific linear templates for runoff transcription and translation, thus yielding a series of carboxy-terminal truncations of c-Jun (see Materials and methods). Each derivative contains the c-Jun amino terminus; numbers denote the last amino acid contained in the construct; the bZIP region of c-Jun lies between amino acids 250 and 315. Samples for this panel represent ~2%

of that used for immunoprecipitations. Reaction mixtures before precipitation demonstrate size and quantity of derivatives of c-Jun synthesized. HeLa cell extract (10 μ l) containing 20 ng of unlabeled GR was added as indicated. The restriction site used to produce N187 was not digested to completion and thereby allowed synthesis of a minor proportion of full-length c-Jun, which was efficiently precipitated by GR antibodies, in contrast to N187 itself. (Right) IP (immunoprecipitation): [35 S]methionine-labeled c-Jun and derivatives were mixed with unlabeled full-length receptor, cross-linked with DSP, precipitated with mAbBUGR2 (Gametchu and Harrison 1984) as described (see Materials and methods; Diamond et al. 1990), and loaded onto an SDS–polyacrylamide gel (under conditions that disrupt the cross-links) followed by fluorography and autoradiography. Similar results were obtained without the use of cross-linkers (data not shown). As noted above, the full length c-Jun in lane N187 precipitated efficiently with GR and functioned as an internal control for this reaction.

both receptor and c-Jun, and the c-Jun N245 derivative did not support c-Fos association with receptor. We examined two c-Fos derivatives with leucine zipper defects: c-Fos 1–172 is a carboxy-terminal truncation that lacks the bZIP region (see Fig. 1), and c-Fos Leu-Pro is a site-directed alteration that substitutes a critical leucine residue at position 172 with a proline (Turner and Tjian 1989). Neither of these mutants is competent to dimerize with c-Jun (Turner and Tjian 1989; F. Rauscher, pers. comm.), and we found that neither coprecipitated with receptor and c-Jun (Fig. 5). In contrast, the c-Fos derivative KCR-ICI, which contains 2 amino acid substitutions at amino acids 153 and 155 within the basic region of c-Fos that permit dimerization with c-Jun but abrogate DNA binding of the resultant complex (T. Curran and F. Rauscher, pers. comm.), remained competent to coprecipitate with receptor and c-Jun, albeit with somewhat reduced apparent efficiency (Fig. 5). Thus, the c-Fos leucine zipper, but not its DNA-binding activity, appears crucial for interaction with the c-Jun/receptor complex, supporting the notion that the c-Jun/receptor complex tethers c-Fos through its AP-1 dimer interface.

c-Fos and Fra1 action at plfG3

The functional connection between nuclear receptors and the AP-1 family extends beyond the GR and c-Jun/c-Fos; other members of these factor families appear to collaborate in regulatory interactions at other genes (Akberblom et al. 1988; Kourides and Gurr 1989; Jonat et al. 1990; Nicholson et al. 1990; Miner and Yamamoto 1991; E. Imai, J.N. Miner, K.R. Yamamoto, and D. Granner, in prep.; Pearce and Yamamoto, 1993). The c-Fos-related antigen Fra1 (Cohen and Curran 1988; Cohen et al. 1989) is immunologically related to c-Fos and is conserved particularly strongly within the bZIP region. Like c-Fos,

Fra1 enhances transcription by heterodimerization with a Jun subunit and subsequent binding to AP-1 sites; it cannot dimerize, bind DNA, or enhance transcription in the absence of Jun (Cohen and Curran 1988; Cohen et al. 1989). Transfection of a Fra1 expression vector alone into F9 cells had no effect on plfG3 CAT reporter expression, whereas cotransfection of Fra1, together with c-Jun, stimulated expression by eightfold over that observed with transfected c-Jun alone (Fig. 6). For comparison, cotransfection of c-Jun with c-Fos produced a 40-fold stimulation over c-Jun alone (Fig. 6). Whether this quantitative difference reflects distinctions in dimerization, DNA affinity, activation potency, or some combination is not known; in any case, it is clear that Fra1 and c-Fos are qualitatively similar at plfG3 in combination with c-Jun, and in the absence of the GR.

However, we found that Fra1 differed strikingly from c-Fos in its functional effect on receptor action at the composite element. That is, in marked contrast to the strong repression by the receptor in the presence of c-Jun/c-Fos, c-Jun/Fra1 dimers specified enhancement by the receptor (Fig. 6). Thus, Fra1 has the dimerization, DNA-binding, and transcriptional activation properties of c-Fos at the composite element, but interacts with receptor with opposite consequences.

We constructed a c-Fos/Fra1 chimera to test whether, like c-Jun, the basic region of Fra1 might play a determining role in the regulation of receptor function. As shown in Figure 7, we found that the FRFF chimera, which contains a precise replacement of only the basic region of c-Fos (amino acids 128–164) with that of Fra1 (amino acids 98–133), specified receptor-mediated activation, whereas bona fide c-Fos specified repression. Sequence comparisons reveal that FRFF differs from c-Fos at only 4 amino acid residues and that all four differences represent conservative changes that may reside on a sol-

Figure 5. Domains involved in c-Fos coprecipitation with the GR/c-Jun complex. Input: c-Fos and c-Jun proteins were [35 S]methionine-labeled during in vitro protein synthesis as in Fig. 4. c-Fos mutant Leu-Pro contains a substitution of a Pro for a Leu at amino acid 172 within the leucine zipper of c-Fos (see Fig. 1) that disrupts dimerization and DNA binding (Turner and Tjian 1989); c-Fos 1–172 is a carboxy-terminal truncation that truncates the zipper of c-Fos from amino acid 172 (T. Curran, pers. comm.); mutations KCR-ICI (amino acids 153–155) within the c-Fos basic region abrogate DNA-binding but not dimerization (T. Curran and F. Rauscher, pers. comm.). Samples for this panel represent ~2% of that used for immunoprecipitations. IP (immunoprecipitation): [35 S]Methionine-labeled c-Jun and c-Fos were incubated at 30°C for 30 min; an aliquot was set aside for the input gel, and a cell extract containing or lacking full-length GR was added, incubated, cross-linked, and immunoprecipitated as in Fig. 4.

Figure 6. Comparison of c-Fos and Fra1 interactions with receptor at plfG. RSV c-Jun (0.5 μ g), c-Fos-related antigen (RSV Fra1; 0.5 μ g), and RSV c-Fos (0.5 μ g) were cotransfected with plfG3 CAT (2.0 μ g), 6RGR receptor expression plasmid (2.0 μ g), and β -galactosidase expression vector pLac82su (0.2 μ g) as an internal control (Jaynes and O'Farrell 1988). Each reaction also contained an additional 0.5 μ g of RSV c-Jun to provide a dimer partner for each construct listed. Dexamethasone (0.1 μ M) was added where indicated (hatched bars) for 24 hr; (solid bars) no hormone added. Aliquots for each pair were taken from the same DNA precipitate.

vent-accessible surface of the AP-1 DNA-binding domain (see below) (Fig. 8).

Discussion

Complex regulatory regions containing consensus binding sequences for multiple factor families are commonly associated with cellular and viral promoters and provide potential junction points for combinatorial interactions between regulatory networks. The plfG element, for example, represents only a portion of the regulatory region that controls proliferin expression (Mordacq and Linzer 1989). Similarly, a composite element termed the glucocorticoid response unit (GRU) (Imai et al. 1990) resides within the regulatory region of the phosphoenolpyruvate carboxykinase gene. As with plfG, the GRU is bound specifically by the GR but in addition, contains binding sites for at least two nonreceptor factors that are also required for GRU-mediated glucocorticoid regulation (Imai et al. 1990). Moreover, the GRU interacts with factors bound elsewhere within the PEPCK regulatory region, and together, these various interactions produce the overall pattern of regulation of this promoter (Imai et al. 1990; E. Imai, J.N. Miner, K.R. Yamamoto, and D. Granner, in prep.). Numerous additional putative composite response elements, at which members of the nuclear receptor family, for example, appear to interact functionally with nonreceptor regulators, have been described by other investigators; in some of these cases, the receptor is thought to function even without specific DNA binding (Akerblom et al. 1988; Kourides and Gurr 1989; Lichtler et al. 1989; Mordacq and Linzer 1989; Morrison et al. 1989; Gaub et al. 1990; Jonat et al. 1990; Nicholson et al. 1990; Owen et al. 1990; Schüle et al. 1990a,b; Weisz and Rosales 1990; Yang-yen et al. 1990;

Zhang et al. 1991; König et al. 1992). These findings support the notion that complex regulatory regions in general correspond to combinatorial junction points, but in no case are the interactions or their mechanistic consequences understood in detail.

In the present work, we have analyzed composite regulation specified by two well-characterized regulators at a discrete 25-bp element. Our immunoprecipitation experiments both with and without cross-linking suggest that the receptor interacts closely and probably directly with c-Jun and that c-Jun/c-Fos, but not c-Fos alone, also

Figure 7. A surface of AP-1 that specifies the direction of receptor regulation at plfG. RSV FRFF was constructed by inserting the basic region of Fra1 (see Materials and methods) into the comparable location in a parental c-Fos plasmid that had been modified by the introduction of *Cla*I and *Mlu*I sites at the borders of the basic region (Cohen and Curran 1990). The chimera was cloned into an RSV-driven mammalian expression vector (Godowski et al. 1988) and tested for activity by cotransfection (0.5 μ g) into F9 cells together with plfG3:CAT (2.0 μ g) (Diamond et al. 1990), GR expression plasmid 6RGR (2.0 μ g) (Godowski et al. 1988), and β -galactosidase expression vector pLac82su as an internal control (0.2 μ g) (Jaynes and O'Farrell 1988). Each reaction also contained an additional 0.5 μ g of RSV c-Jun to provide a dimer partner for each construct listed. After measuring CAT activity, normalizing to β -galactosidase activity, and averaging three experiments, the experimental values for the experiment lacking dexamethasone were divided into those for the experiment with dexamethasone to calculate fold activation, and vice versa, to calculate fold repression. c-Jun, Fra1, and c-Fos controls are also shown. Note that in these experiments, Fra1 activated with c-Jun relatively strongly and therefore synergized proportionally weaker with GR than was commonly observed, e.g., in Fig. 6.

Figure 8. Helical representation of the basic regions of c-Fos and Fra1. Of the 4 amino acid residues that appear to serve as determinants of regulation by the receptor at plfG, 3 reside on one surface of the basic region α -helix, as indicated by hatch marks between the c-Fos and Fra1 sequences and extending to their putative locations on the helix. By alignment of this region with the crystal structure of the basic region helix of another bZIP protein, GCN4 (Ellenberger et al. 1992), we infer that the residues that contact DNA are positioned below the helix and that the upper surface residues, including all of those that differ between c-Fos and Fra1, are solvent accessible. The fourth difference, R-K, is also shown on the sequence but is outside of the helical region and therefore could not be assigned.

associates with the receptor. Thus, either c-Fos assumes an altered conformation in c-Jun/c-Fos heterodimers that facilitates its interaction with receptor, or it is tethered to the receptor/c-Jun complex through its zipper-mediated dimerization with c-Jun. This second scheme implies that the basic region of c-Fos (or Fra1) might transduce information, presumably through c-Jun and/or the plfG DNA sequence, that specifies the direction of receptor regulation.

Remarkably, c-Fos/Fra1 chimeric constructs implicate 4 amino acid residues in the basic regions of these factors as crucial determinants of glucocorticoid regulation at plfG. Molecular, genetic and structural analyses of the basic regions in bZIP factors indicate that these segments are α -helical and that they make sequence-specific DNA contacts (Landschulz et al. 1988; Rauscher et al. 1988; Vinson et al. 1989; Abate et al. 1990; O'Neil et al. 1990; Patel et al. 1990; Weiss et al. 1990). By alignment with the determined crystal structure of another bZIP protein (Ellenberger et al. 1992), we can infer that the 4 critical residues are clustered near the end of this DNA recognition helix, and that they are solvent accessible, positioned on the helical surface away from the DNA. In principle, such a region could interact with other regulatory factors, such as GR, that are bound contiguously on the DNA.

One interpretation of these results is that the 4 amino acid residues in the c-Fos-type patch of the basic helix might comprise a signal (when bound together with c-Jun at plfG) for the receptor to repress transcription. Conversely, the 4 residues in the Fra1-like patch might signal the receptor to confer enhancement.

By what mechanisms might the subunit composition of a nonreceptor factor act as a determinant of receptor activity at a composite element? It is intriguing that c-Jun homodimers and c-Jun/c-Fos heterodimers bend DNA in opposite directions when they bind in vitro to a

consensus AP-1 site (Kerppola and Curran 1991b). AP-1-induced DNA bending has not been examined at plfG, but it is conceivable that different DNA conformations might elicit distinct regulatory consequences from the receptor. By this view, c-Jun/Fra1 heterodimers should bend DNA in a manner similar to c-Jun homodimers and opposite that induced by c-Jun/c-Fos heterodimers.

Perhaps a simpler notion is that a regulatory surface on AP-1, such as the putative 4 amino-acid patch on the backside of the Fra1 or c-Fos DNA recognition helix, may contact the receptor, and likely together with other determinants, specify receptor activity via protein-protein interactions. It might be useful in this regard to carry out pairwise studies on the effect of each component in the ternary complex (receptor, AP-1, and plfG) on the formation, stability or conformation of each binary combination, namely, receptor-plfG, receptor/AP-1, and AP-1/plfG. Whether receptor and AP-1 can associate simultaneously with each other and with DNA has been controversial (Diamond et al. 1990; Jonat et al. 1990; Schüle et al. 1990a; Yang-yen et al. 1990; Miner and Yamamoto 1991; Miner et al. 1991), but recent studies in vivo and in vitro support the notion that these ternary complexes are competent to form (König et al. 1992; J.N. Miner and S.T. Jeng, unpubl.). Our present studies have employed only relatively crude protein fractions in the absence of added plfG DNA. Notably, we observe only a very low efficiency of coimmunoprecipitation of receptor and AP-1 (<1% of the labeled AP-1 is precipitated) under our in vitro conditions, despite evidence that the interactions are biologically significant in vivo. We predict that these interactions may occur in vivo only at specific DNA sites (Miner and Yamamoto 1991; Miner et al. 1991; Zacharchuk and Ashwell 1992) and that more efficient receptor/AP-1 contacts in vitro may be detected upon the addition of plfG DNA.

Diamond et al. (1990) demonstrated that the zinc finger region of the GR, which encompasses its DNA-binding domain, is necessary for its functional interaction with AP-1 at plfG. Analogously, we have found that the bZIP regions of c-Jun and c-Fos, which define the AP-1 DNA-binding domain, are essential in vitro for the AP-1/receptor interaction and in vivo for composite regulation. Moreover, a portion of the Fra1 DNA recognition helix that does not itself contact the DNA is necessary for enhancement by the receptor. We propose that amino acid residues within or close to the DNA-binding motifs of these factors also participate in protein-protein interactions. The zinc finger motif within the receptor is highly multifunctional, specifying not only DNA recognition, but also receptor dimerization (Luisi et al. 1991), nuclear localization (Picard and Yamamoto 1987), and transcriptional regulation (Miesfeld et al. 1987, 1988). Similarly, DNA-binding motifs from other transcriptional regulators appear to interact with proteins. For example, the herpesvirus activator VP16 contacts Oct-1 in a region that is probably on the backside of a homeo domain involved in Oct-1 DNA binding (W. Herr, pers. comm.). Clearly, the DNA-binding motifs are the most strongly conserved portions of the sequence-specific reg-

ulators and transcription factors, serving as the defining feature of many factor families. We suggest that strong evolutionary pressure to conserve these DNA recognition structures may have yielded conserved surfaces that have been exploited for other intermolecular interactions, such as contacts between different factor families that produce combinatorial regulation.

In conclusion, we report two novel and likely general findings about factor interactions at composite response elements. First, composite elements can be viewed as junction points that integrate multiple regulatory inputs and provide combinatorial versatility, allowing a single factor to confer several distinct regulatory effects even within a single cell. In the case of plfG, we have described a simple scheme by which glucocorticoid regulation can be continuously "tuned" from positive through zero to negative, a sort of molecular rheostat in which the composition of AP-1, reflecting in part the presence or absence of a putative enhancement surface on certain family members, serves as a key determinant. The frequent incidence of AP-1 sites within promoters and regulatory regions seems to suggest that the AP-1 factor family may commonly act at such junction points. It will be interesting in future studies to determine whether the same putative surfaces defined here are employed for interactions of AP-1 with other regulators and eventually to define the precise molecular mechanisms by which these surfaces specify composite regulation. Secondly, our studies show that members of a given factor family that appear similar or indistinguishable at simple response elements may differ dramatically at composite elements, where protein-protein interactions assume crucial roles. Indeed, the plfG element has also been shown to distinguish different steroid receptors that bind to the GRE consensus sequence (Pearce and Yamamoto 1993). Thus, this scheme for composite regulation provides a rationale for the existence of large families of closely related factors that seem at first sight to be redundant; it also provides a testable mechanism by which multiple regulatory consequences can be generated by a modest complement of regulators.

Materials and methods

Plasmid construction

The c-Jun/c-Fos chimeric constructs were excised from their original *in vitro* transcription-translation vectors (Cohen and Curran 1990) with *EcoRI*. The ends were changed to *HindIII* with *EcoRI*-*HindIII* adapters (New England Biolabs) and cloned into the *HindIII* site of an expression vector containing the RSV enhancer-promoter region and the SV40 transcription termination and polyadenylation signals (p6R) (Godowski et al. 1988). RSV Fra1 was prepared by excising an *EcoRI* fragment containing the Fra1-coding region from CMV Fra1 (Cohen and Curran 1988; Cohen et al. 1989) and inserting it into the *EcoRI* site of Bluescript SK; re-excision with *XbaI* and *SalI* allowed directional cloning into the p6R *XbaI* and *SalI* sites.

The c-Fos/Fra 1 chimera FRFF was constructed by cleaving the parental vector SP6 FJFF (Cohen and Curran 1990) with *ClaI* and *MluI* to remove the c-Jun basic region. The basic region of Fra 1 was amplified by polymerase chain reaction (PCR)

from wild-type Fra1 using primers that added *ClaI* and *MluI* ends onto the amplified fragment (primers: GGFRClal = GG-GGGATCGATGAGCAGATCAGCCCGGAG; GGRFMLU 1 REV = CCCCCACGCGTCAATTCCTTTCTTCGGTT). Ligation of these two fragments produced SP6 FRFF, which was used as a source for a 700-bp *BglII*-*NcoI* fragment encompassing the inserted Fra1 basic region. This fragment was cloned into the *BglII* and *NcoI* sites in RSV Fos, generating RSV FRFF. In addition to the 4 amino acid differences between c-Fos and Fra1, RSV FRFF has three alterations introduced in the course of constructing SP6 FJFF: an Asp to Arg change at the *MluI* site corresponding to amino acid 281 of c-Fos and amino acid 133 of Fra1 (Fra1 and c-Fos are identical at this position); a Lys (c-Fos 128) or Pro (Fra1 98) to Ile change; and a Val (c-Fos 129) or Cys (Fra1 99) change to Asp. In extensive analyses *in vitro* and *in vivo*, these changes did not affect dimerization, DNA binding (Cohen and Curran 1990), or interactions with GR (Figs. 1 and 2; J.N. Miner, unpubl.).

Transfection

Partially confluent (50%) monolayers of F9 embryonal carcinoma cells were grown at 8% CO₂ in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Cultures in 60-mm dishes were transfected by the calcium phosphate procedure (Graham and van der Eb 1973; Diamond et al. 1990). One hour before cells were transfected, fresh medium with Pen-Strep (penicillin G at 100 U/ml and streptomycin SO₄ at 100 µg/ml) was added. Calcium phosphate-DNA precipitate was prepared in 125 mM CaCl₂, HEBS (pH 6.93) [25 mM HEPES, 0.75 mM Na₂HPO₄/7H₂O, 140 mM NaCl]. DNA transfected: 2 µg of plfG3 CAT reporter plasmid (Diamond et al. 1990), 2 µg of 6RGR GR expression vector (Godowski et al. 1988), 0.2 µg of β-galactosidase expression plasmid (pLac82su) (Jaynes and O'Farrell 1988) as an internal control of transfection efficiency, and indicated amounts of RSV c-Jun, RSV c-Fos, RSV Fra1, or chimera. These amounts of plasmid are within the linear range of our assay conditions. Cells were incubated for 15 hr with precipitate, shocked by the addition of 2 ml of 15% glycerol in 37°C DMEM for 2 min, washed twice in 37°C phosphate buffered saline (PBS), and refed with fresh medium, together with 100 nM dexamethasone, where indicated.

After 24 hr, cells were washed twice and scraped from the plates in 25°C PBS, resuspended in 120 µl of 250 mM Tris-HCl (pH 7.8), and lysed by four cycles of freeze/thaw (−70°C/37°C); cell debris was pelleted by centrifugation at 15,000g. CAT activity was assayed with a 4-hr nonchromatographic procedure using ¹⁴C-labeled acetyl coenzyme A and extraction of labeled acetylated chloramphenicol with ethylacetate (Sleigh 1986). Scintillation fluor was added to the ethylacetate supernatant and monitored for radioactivity. β-Galactosidase activity was monitored as described (Stuart et al. 1984). Briefly, 2 µl from the unheated CAT extract was mixed with 8 µl of 5× β-gal assay buffer [750 mM Tris (pH 7.5), 50 mM MgCl₂, 125 mM NaCl, 50 mM β-mercaptoethanol], 2 µl of 30 mM 4-methylumbelliferyl in dimethyl sulfoxide, 1 µl of 40 mg/ml BSA in a 40-µl reaction. After 15 min at 37°C, the reaction was stopped by the addition of 1 ml of 250 mM glycine (pH 10.65). Reactants were assayed in a Hoefer DNA fluorometer (TKO 100) excited at 365 nm and read at 445 nm. The CAT values presented were all normalized to β-galactosidase levels.

Immunoprecipitation

Labeled c-Jun and c-Fos were prepared by *in vitro* protein synthesis in rabbit reticulocyte lysates (Promega) in the presence of

[³⁵S]methionine (New England Nuclear; 1190 Ci/mmol). Labeled carboxy-terminal deletion derivatives of c-Jun were produced by cleaving the c-Jun in vitro transcription-translation vector T7 c-Jun (Turner and Tjian 1989) at various restriction sites: *HpaI* = N315; *BstXI* = N245; *PstI* = N222; *NarI* = N187; *SacII* = N146; (numbers following the N denote the last amino acid in the construct). These derivatives were subjected to run-off transcription and translation to produce a range of [³⁵S]methionine-labeled, truncated c-Jun polypeptides. Intact c-Fos (SP6 c-Fos) and mutant derivatives were also prepared with this reticulocyte translation system, using vectors in which they were originally cloned (Turner and Tjian 1989; T. Curran, pers. comm.). All cDNA used for these experiments were from rat. Labeled protein levels for each experiment were equalized by TCA precipitation and quantitation after SDS-PAGE. Generally, the T7 c-Jun vector expressed c-Jun at higher levels and was routinely diluted ~10-fold; the others required only minor adjustments.

The [³⁵S]methionine-labeled c-Jun and/or c-Fos proteins were mixed with crude extracts from HeLa cells infected with either wild-type vaccinia virus or with a GR expressing recombinant virus (Schmid et al. 1989) for 30 min at 30°C. The reactions were diluted 10-fold in HEGNO50 + 0.1% Triton X-100 [10 mM HEPES (pH 8.0) 1 mM EDTA, 10% glycerol, 50 mM NaCl] and precleared by the addition of preswollen, prewashed protein A-Sepharose (100 mg/ml) to 0.03% for 30 min at 4°C. After removal of the Sepharose, receptor-specific monoclonal antibody (BUGR2) (Gametchu and Harrison 1984) and another aliquot of fresh protein A-Sepharose were added and the slurry was incubated at 4°C for 1 hr with gentle nutation. The beads were centrifuged and washed quickly four times in 20 volumes of HEGNO50 + 0.1% Triton X-100 and one time in fresh tubes in HEGNO50 without Triton X-100. Proteins were eluted from the beads with sample buffer and analyzed on SDS-polyacrylamide gels.

Cross-linking experiments were done as described (Diamond et al. 1990). Briefly, reaction mixtures were prepared as described above, diluted in triethanolamine containing buffer, cross-linked with DSP (Pierce), and quenched with 20 mM lysine. Samples were then immunoprecipitated under stringent conditions as described (Diamond et al. 1990); the precipitated proteins were analyzed in SDS-polyacrylamide gels under strongly reducing conditions to reverse DSP cross-links, and subjected to autoradiography.

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