The c-*fos* serum response element responds to protein kinase C–dependent and –independent signals but not to cyclic AMP

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Transcription of the c-*fos* proto-oncogene is rapidly induced by serum growth factors. A short c-*fos* DNA element, the serum response element (SRE), is required for this response to serum. However, serum activates a series of distinct intracellular signaling pathways, and it is not known to which of these pathways the SRE responds. To address this question, mutations have been introduced into the SRE of an otherwise intact c-*fos* promoter/enhancer. These mutations strongly reduce the binding of a nuclear factor to this site. Plasmids carrying either a wild-type or mutant c-*fos* SRE were transfected into fibroblasts and tested for their response to whole serum, purified recombinant c-*sis* protein, the protein kinase C activator phorbol myristate acetate, and activators of the cyclic AMP (cAMP) second messenger system. Assays were carried out under normal conditions and after chronic phorbol ester–treatment to deplete phorbol ester activatable protein kinase C activity from transfected cells. The results show that the SRE is necessary and sufficient for response to both protein kinase C–dependent and –independent intracellular signaling pathways but not for response to the cAMP pathway.

[*Key Words:* c-*fos*; inducible promoters; growth factors; protein kinase C; cyclic AMP; transient expression assay]

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Cellular growth and differentiation are largely coordinated by extracellular signals. Many such signals, in the form of polypeptide growth factors, act through high-affinity cell-surface receptors which, in turn, activate a variety of distinct signaling pathways within the target cell (for a review pertinent to fibroblast mitogens, see Rozengurt 1986). Some of these signals reach the nucleus where they lead to changes in the pattern of cellular gene expression. One of the earliest nuclear responses to such signals is transcriptional activation of the c-*fos* proto-oncogene (Greenberg et al. 1984; Kruijer et al. 1984; Muller et al. 1984). c-*fos* induction is a primary response to intracellular events triggered by growth factor–receptor interactions. It occurs within minutes and without a requirement for new protein synthesis (Greenberg et al. 1986). Induction of c-*fos* transcription can be triggered by several distinct second messenger systems, including protein kinase C, free calcium, cyclic nucleotides, and other uncharacterized pathways (for review, see Curran 1988). Whether these signals reach the c-*fos* gene through independent or convergent routes is not known.

One DNA sequence element in the c-*fos* gene plays a major role in the response of the gene to extracellular signals. Termed the serum response element (SRE), this 20-bp region of hyphenated dyad symmetry located 300 bp upstream of the c-*fos* cap site is both necessary and sufficient for the response of c-*fos* to whole serum in fibroblasts (Treisman 1985, 1986; Gilman et al. 1986; Greenberg et al. 1987). Deletion of the SRE abolishes induction by serum, whereas an oligonucleotide carrying a single copy of the sequence is sufficient to restore serum responsiveness to a deleted c-*fos* promoter. This sequence is a binding site for a protein factor found in nuclear extracts of a variety of cell types (Gilman et al. 1986; Prywes and Roeder 1986; Treisman 1986; Greenberg et al. 1987). Mutations in the SRE that affect the binding of this protein also affect the biological activity of the element (Greenberg et al. 1987; Treisman 1987). HeLa cell proteins that bind to the SRE have recently been purified (Prywes and Roeder 1987; Schroter et al. 1987; Treisman 1987). Such proteins are potentially recipients of incoming signals that activate c-*fos* transcription.

Although the SRE is critical for the response of the c-*fos* gene to serum, it is not yet clear to which intracellular signals it responds. Serum is a complex mixture of growth factors and hormones, each of which may activate multiple second messengers. To determine which intracellular signaling pathways use the SRE and which

use other sequence elements in the c-*fos* gene, point mutations have been introduced into the SRE and the resulting mutants have been tested for response to a set of purified growth factors and intracellular second messengers. The results suggest that in fibroblasts, at least three distinguishable intracellular signaling pathways reach the c-*fos* gene. Two pathways act through the SRE, whereas a third is completely independent of this element.

Results

Point mutations in the SRE abolish factor binding in vitro

The strategy used to evaluate the role of the SRE in the response of the c-*fos* gene to various stimuli was to inactivate the element in an otherwise intact mouse c-*fos* promoter–enhancer region and to test the resulting mutant for response to appropriate stimuli in mouse fibroblasts. Previous studies have identified critical nucleotides in the SRE required for binding of a nuclear protein that is presumed to mediate its activity (Gilman et al. 1986; Treisman 1986). These contact residues are two pairs of adjacent guanosines that are situated symmetrically within the SRE. Using site-directed mutagenesis, all four guanosines were mutated to cytosines (pm12 mutations; Fig. 1A), preserving the dyad symmetry of the element. Wild-type and mutant c-*fos* sequences from −356 to +109 were fused to the bacterial gene encoding chloramphenicol acetyltransferase (CAT). By using fusion genes, transcripts of the transfected gene could be distinguished easily from transcripts of the endogenous mouse c-*fos* gene in the transfected cultures.

The mutant SRE was first tested for its ability to bind the nuclear SRE-binding factor present in WEHI 231 cells (Gilman et al. 1986). Figure 2 shows a mobility-shift assay performed with a labeled c-*fos* DNA fragment (−356 to −274) carrying a wild-type SRE. Lane 2 shows the specific complex formed on this fragment in WEHI 231 nuclear extracts. When increasing amounts of an unlabeled purified c-*fos* restriction fragment (−356 to +109) also carrying a wild-type SRE were added to the binding reaction, binding to the wild-type probe was reduced as factor was diverted to the unlabeled competitor fragment (lanes 3–6). When a similar competitor fragment carrying the pm12 mutations in the SRE was added to the reaction, binding to the probe was unaffected at up to 100-fold molar excess of competitor (lanes 7–10). Thus, the pm12 mutant SRE binds this protein in vitro with at least two orders of magnitude lower affinity than does the wild-type site.

Effect of SRE point mutations on c-fos *induction in vivo*

c-*fos*–CAT fusion genes carrying either a wild-type or a pm12 mutant SRE were tested for their response to several types of stimuli. Plasmids carrying the fusion genes were transfected into BALB/c 3T3 cells, together with a plasmid carrying the human α-globin gene; the latter plasmid served as an internal control for transfection efficiency. After transfection, the cells were incubated in medium containing 0.5% calf serum for approximately 48 hr and then fed with either serum-free medium (NA in Figs. 3–6) or serum-free medium containing the following additions: 10% calf serum (CS); 40 ng/ml recombinant c-*sis* protein (SIS), a purified polypeptide growth factor that acts indistinguishably from authentic platelet-derived growth factor (PDGF); 50 ng/ml phorbol myristate acetate (PMA), an activator of protein kinase C (pkC); 0.5 mM isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, and 10 μM forskolin, an activator of adenylate cyclase (IF). After an additional 45-min incubation, the cells were harvested, and total cytoplasmic RNA was prepared.

RNA was assayed by RNase protection using probes specific for c-*fos* and α-globin transcripts. The c-*fos* probe hybridizes to transcripts of the transfected c-*fos*–

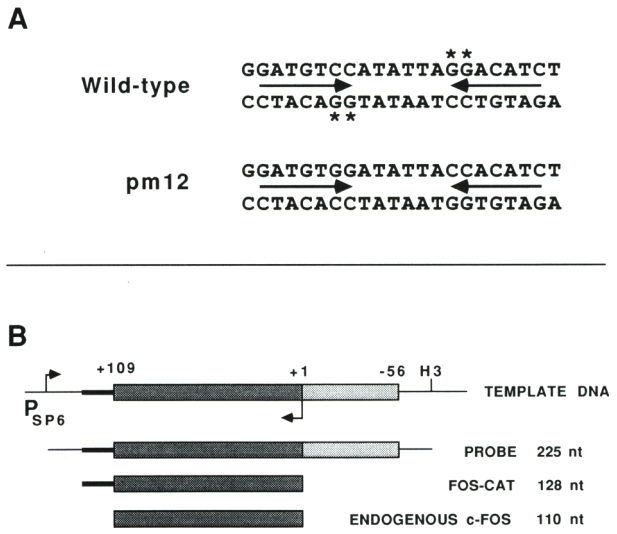

Figure 1. (*A*) Sequence of the c-*fos* SRE. The wild-type sequence shown corresponds to positions −315 to −294 in the mouse c-*fos* gene. The arrows indicate the limits of the dyad symmetry; the A-T base pairs on either side of the center of symmetry are not marked. The asterisks mark the guanosine residues that interfere with factor binding *in vitro*, when methylated by dimethylsulfate (Gilman et al. 1986; Treisman 1986). These are also the base pairs mutated in the pm12 mutant. The nucleotides shown also comprise the limits of the oligonucleotides used in these experiments. (For the complete sequences of the oligonucleotides used, see Materials and methods.) (*B*) c-*fos* hybridization probe used in the RNase protection assays. The top line represents the transcription template. The right-facing arrow indicates the start site for SP6 RNA polymerase. The left-facing arrow below the line indicates the initiation site for c-*fos* transcripts. (H3) The *Hin*dIII site used to linearize the template for transcription. The second line represents the RNA probe transcribed from this template, and the bottom two lines represent the fragments of the probe protected by transcripts of the transfected c-*fos*–CAT genes and the endogenous c-*fos* gene, respectively. The stippled boxes represent c-*fos* sequence: heavy stippling indicates transcribed sequence; light stippling is 5′-flanking sequence. The thick line represents linker sequences present in both the probe and the c-*fos*–CAT transcripts but not in endogenous c-*fos* mRNA.

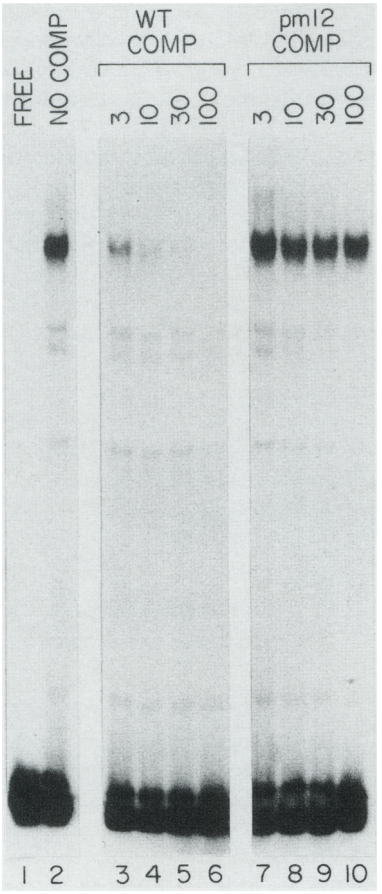

Figure 2. The pm12 mutations reduce factor binding in vitro. Mobility-shift assays were carried out using a 5′-end-labeled restriction fragment carrying mouse c-*fos* DNA from −356 to −274 (20,000 cpm, 0.4 ng) and crude nuclear extract from WEHI 231 cells (5 μg). (Lane *1*) No added extract; (lane *2*) standard binding reaction with no competitor restriction fragments added; (lanes *3–6*) reactions contained the indicated molar excess (3-fold, 10-fold, etc.) of a purified restriction fragment carry wild-type c-*fos* sequences from −356 to +109; (lanes *7–10*) reactions contained the indicated molar excess of a purified restriction fragment carrying c-*fos* sequences from −356 to +109 with the pm12 mutations.

CAT fusion genes and to endogenous c-*fos* mRNA, yielding two protected fragments in all assays (Fig. 1B). The largest fragment (128 nucleotides, T in Figs. 3–6) is protected by correctly initiated transcripts of the transfected c-*fos* gene. The next smaller fragment (110 nucleotides, E) is protected by correctly initiated transcripts of the endogenous c-*fos* gene of the host cells. This signal provides a control for the efficacy of induction for each dish of cells. The smallest fragment (95 nucleotides, marked α) derives from correctly initiated transcripts of the cotransfected α-globin gene. This signal provides a measure of the transfection efficiency for each dish. Because all dishes transfected with the same plasmids received aliquots of a single large transfection precipitate, transfection efficiencies were generally equivalent within that set of dishes, allowing direct comparison of the transfected c-*fos* signals within a panel in each figure. Between sets that received different plasmids, however, transfection efficiencies were not always equivalent, so direct comparisons of signals from the transfected c-*fos* gene in different panels may not be made.

Figure 3 shows the results of an experiment in which wild-type and mutant c-*fos* fusion genes were transfected into BALB/c 3T3 cells and induced by treatment with the agents listed above. The fusion gene carrying wild-type c-*fos* sequences was strongly induced by all stimuli (left panel), showing that it carries sequences sufficient for response to each activator. In contrast, the gene carrying the pm12 mutations in the SRE showed sharply reduced response to serum, c-*sis*, and PMA, although it still responded to activators of the cyclic AMP (cAMP) system (right panel, compare upper bands to control lane, NA). The endogenous c-*fos* genes in these

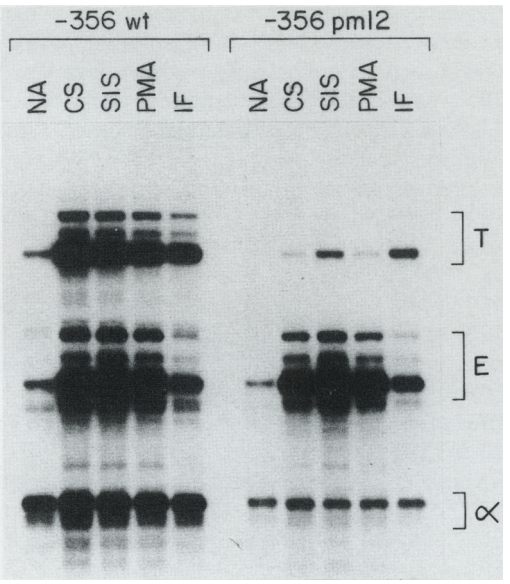

Figure 3. The pm12 mutations reduce the response of transfected c-*fos*–CAT fusion genes to whole serum, c-*sis*, and PMA, but not to IBMX and forskolin. BALB/c 3T3 cells were transfected with the indicated plasmids, Δ-356 wt and Δ-356 pm12, as described in Materials and methods. Following 48 hr of incubation in medium containing 0.5% calf serum, cells were exposed for 45 min to the agents shown at the top of each lane. RNA was isolated and assayed by RNase protection as described in the text. (NA) No addition, cells treated with serum-free medium; (CS) cells treated with medium containing 10% calf serum; (SIS) cells treated with 40 ng/ml c-*sis* protein in serum-free medium; (PMA) cells treated with 50 ng/ml (~80 nM) PMA; (IF) cells treated with 0.5 mM IBMX and 10 μM forskolin in serum-free medium. (T) The position of c-*fos* probe fragments protected by transcripts of the transfected genes; (E) fragments of the c-*fos* probe protected by endogenous c-*fos* transcripts; (α) fragments protected by transcripts of the α-globin control plasmid. Note that the relative intensities of the α-globin bands show that the dishes that received −356 pm12 DNA were transfected with lower efficiency than the dishes that received the wild-type plasmid. This difference in transfection efficiency accounts for much of the absolute difference in signal intensity for the transfected genes.

cells were induced normally, showing that the reduction in response of the transfected genes was due to the introduced mutations, not to the absence of an inducing signal. The residual response of the pm12 mutant to c-*sis* is reproducibly seen, although the magnitude is highly variable. That the pm12 mutations do affect c-*sis* response may be seen by comparing the c-*sis* signal to that obtained with IBMX and forskolin. Induction by c-*sis* is stronger than IBMX and forskolin, with the wild-type gene, whereas induction by c-*sis* is weaker with the mutant. This experiment, together with the binding assays shown in Figure 2, confirms that an intact protein-binding site is required for the serum response (Greenberg et al. 1987; Treisman 1987). Furthermore, it shows that the SRE is also required for c-*fos* induction by PMA, presumably through activation of pkC and for full response to c-*sis* protein. Third, this experiment suggests that c-*fos* induction via the cAMP second messenger system does not require an intact SRE and that sequences sufficient for this response lie elsewhere in the gene.

The previous experiment showed that c-*fos* activation by serum, c-*sis*, and PMA requires the SRE. PMA is thought to act largely through activation of pkC, but serum growth factors act through other pathways besides pkC. To determine whether the SRE was required for response to pkC-independent signals, transfected c-*fos* genes were induced in cells depleted of pkC activity. Cultures of cells were transfected with either the wild-type or the pm12 c-*fos*–CAT fusion genes. Immediately following transfection, cells were treated for 48 hr with medium containing 0.5% calf serum and 100 ng/ml phorbol dibutyrate (PDBu). Control cultures received the solvent dimethylsulfoxide (DMSO). Chronic treatment with active phorbol esters results in down-regulation of pkC and the disappearance of assayable pkC activity in extracts of the treated cells (Collins and Rozengurt 1984; Rodriguez-Pena and Rozengurt 1984). Therefore, this protocol establishes a temporary state in which cells are deficient in phorbol ester–activatable pkC, and any residual growth factor–induced response is presumably due to signaling pathways independent of such enzymes. Although endogenous c-*fos* expression in control cultures was induced by all stimuli (Fig. 4, left panel), the PDBu-treated cultures were refractory to induction by PMA (Fig. 4, middle and right panels), suggesting that these cells lacked PMA-activatable pkC. Induction by serum and c-*sis* was partially reduced, suggesting a partial reliance on pkC. Induction by IBMX and forskolin was largely unaffected, suggesting that this pathway is pkC independent. Under these conditions, the transfected wild-type fusion gene still responded to serum and c-*sis* (middle panel), though not as strongly as in the control cells. Therefore, this gene must carry sequences sufficient for response to pkC-independent signals. Mutations in the SRE abolished response to serum and c-*sis* in the pkC-deficient cells (Fig. 4, right panel). Induction by IBMX and forskolin was again unaffected by the SRE mutations. Thus, this experiment establishes that in addition to the pkC pathway, the SRE is required for response to one or more pkC-independent pathways triggered by serum and c-*sis*.

An SRE oligonucleotide confers responsiveness to both pkC-dependent and -independent signaling pathways

Short oligonucleotides carrying the SRE restore serum inducibility to deleted c-*fos* and heterologous promoters (Treisman 1986, 1987; Greenberg et al. 1987; Mohun et al. 1987). To determine whether the SRE alone is sufficient for response to different signaling pathways, synthetic oligonucleotides encoding 22 bp of wild-type SRE sequence or the corresponding sequence carrying the pm12 mutations (see Fig. 1A) were inserted just upstream of a deleted c-*fos*–CAT fusion gene. The deleted gene carries c-*fos* sequences through −151 and includes several basal promoter elements (Gilman et al. 1986). Figure 5 shows that the parental −151 construct responds weakly, if at all, to serum, c-*sis*, and PMA, although it does respond to IBMX and forskolin (left panel). Insertion of a single copy of the wild-type SRE oligonucleotide immediately upstream restores response

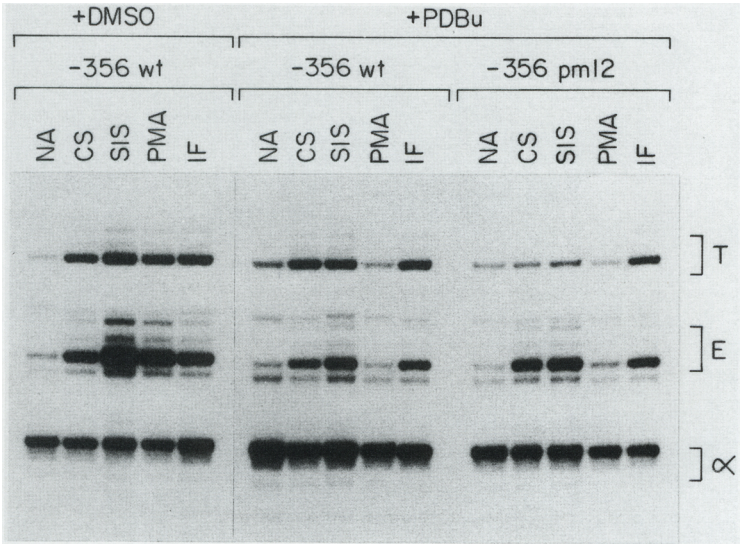

Figure 4. The pm12 mutations reduce the response of transfected c-*fos*–CAT fusion genes to pkC-independent signals. BALB/c 3T3 cells were transfected and treated for 48 hr with medium containing 0.5% calf serum and 0.1% DMSO (*left* panel) or medium containing 0.5% calf serum and 100 ng/ml PDBu (*middle* and *right* panels). (NA) no addition; (CS) 10% calf serum; (SIS) 40 ng/ml c-*sis* protein; (PMA) 50 ng/ml PMA; (IF) 0.5 mM IBMX and 10 μM forskolin. (For details, see Materials and methods and the legend to Fig. 3).

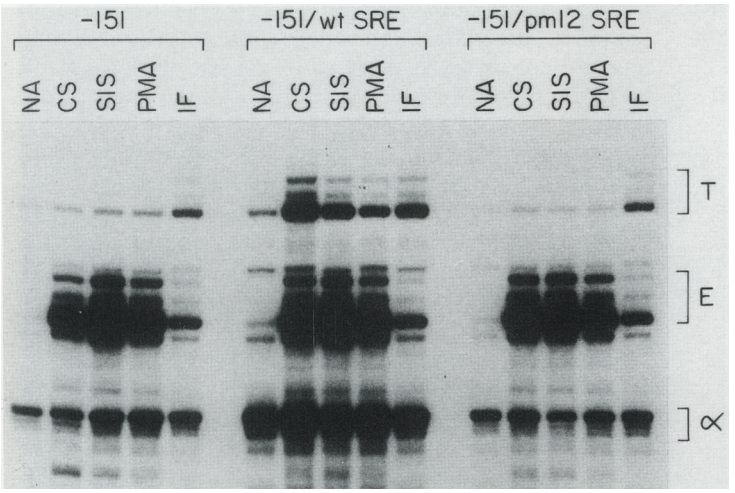

Figure 5. A wild-type but not a pm12 SRE oligonucleotide restores response to serum, c-*sis*, and PMA. BALB/c 3T3 cells were transfected with a c-*fos*–CAT fusion gene carrying c-*fos* sequences through −151 (*left* panel), the same construct carrying a single copy of a wild-type SRE oligonucleotide (*middle* panel), or the same construct carrying a pm12 SRE oligonucleotide (*right* panel). (For the sequences of these oligonucleotides, see the legend to Figure 1 and Materials and methods.) (NA) No addition; (CS) 10% calf serum; (SIS) 40 ng/ml c-*sis* protein; (PMA) 50 ng/ml PMA; (IF) 0.5 mM IBMX and 10 μM forskolin.

to serum, c-*sis*, and PMA with little effect on induction of IBMX and forskolin (middle panel). Insertion of a mutant SRE oligonucleotide in the same position has no effect on any response (right panel). Therefore, a single copy of the SRE is sufficient for response to serum, c-*sis*, and PMA, at least in the presence of the downstream basal elements. A single copy of the mutant SRE is inactive. Further, these data show that sequences sufficient for response to the putative cAMP pathway lie downstream of −151.

The same constructs carrying the wild-type and pm12 SRE oligonucleotides were transfected into cells, and the cells were treated for 48 hr with PDBu, as described previously. Figure 6 shows that genes carrying a mutant SRE oligonucleotide responded only to IBMX and forskolin (left panel). Genes with a wild-type oligonucleotide were also induced by serum and c-*sis* (right panel). Thus, the SRE is also sufficient for response to growth factor–activated pkC-independent pathways operating in these cells. Therefore, the SRE is necessary and sufficient for the response of c-*fos* to at least two distinct intracellular signaling pathways, one dependent on pkC, and one or more pkC-independent pathways. The SRE does not appear to play a direct role in the response to cyclic nucleotide–mediated signals.

Discussion

The c-*fos* proto-oncogene is a highly inducible gene that is sensitively coupled to external signals. Transcription of c-*fos* is activated in response to signals that are diverse in both their chemical structures and biological activities. Previous work has shown that a single sequence element, the SRE, is essential for the response of c-*fos* to whole serum (Treisman 1985, 1986; Gilman et al. 1986; Greenberg et al. 1987). But serum is a complex mixture of biologically active agents, with cooperating and antagonistic activities on cells. Furthermore, constituents of serum activate several distinct intracellular signaling pathways. The goal of the studies described here was to determine which of these pathways act on the c-*fos* gene through the SRE and which require other sequences. The results show that the c-*fos* SRE is the target of at least two intracellular signaling pathways, whereas a third pathway induces c-*fos* expression independently of the SRE.

Fresh serum and several purified growth factors stimulate inositol phospholipid turnover in sensitive cells (for review, see Michell and Putney 1987). One product of this reaction is diacylglycerol, which activates a family of closely related protein kinases known as pkC (Nishizuka 1986). This intracellular signaling pathway appears to communicate with the c-*fos* gene primarily through

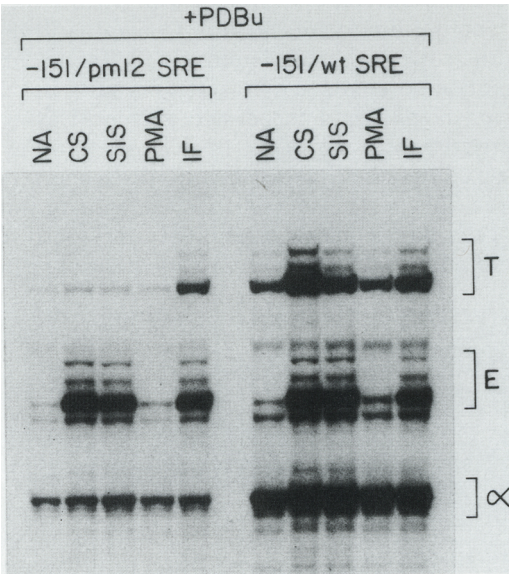

Figure 6. A wild-type but not a pm12 SRE oligonucleotide restores response to pkC-independent signals. BALB/c 3T3 cells were transfected with c-*fos*–CAT fusion genes carrying c-*fos* sequences through −151 plus a pm12 SRE oligonucleotide (*left* panel) or a wild-type SRE oligonucleotide (*right* panel). Cells were treated with PDBu, as described in the legend to Figure 4 and in Materials and methods. (NA) No addition; (CS) 10% calf serum; (SIS) 40 ng/ml c-*sis* protein; (PMA) 50 ng/ml PMA; (IF) 0.5 mM IBMX and 10 μM forskolin.

the SRE. Point mutations in the SRE almost completely eliminate c-*fos* induction by the potent pkC activator PMA. The small remaining induction seen with a mutant SRE may be due to PMA-induced modification of other cellular transcription factors. A growing group of sequence elements have been implicated in regulation of gene expression by activators of pkC. These include sequences in the SV40 enhancer (Imbra and Karin 1986; Angel et al. 1987; Chiu et al. 1987; Imagawa et al. 1987; Lee et al. 1987a), the HIV LTR (Kaufman et al. 1987; Nabel and Baltimore 1987; Siekevitz et al. 1987; Tong-Starksen et al. 1987), and several cellular genes (Comb et al. 1986; Elsholtz et al. 1986; Angel et al. 1987; Imagawa et al. 1987; Lee et al. 1987a). Related sequences are found in the c-*fos* gene. In particular, the c-*fos* gene carries a consensus binding site for the PMA-inducible transcription factor AP-1 (Angel et al. 1987; Lee et al. 1987a, 1987b), and Piette and Yaniv (1987) have shown that this site binds a cellular factor. But the data shown here and by others (Fisch et al. 1987; Stumpo et al. 1988) suggest that this site is not important for c-*fos* induction by PMA.

These data also show that serum and c-*sis* transmit signals to the c-*fos* gene that are independent of phorbol ester–activatable pkC, as shown previously by others (Stumpo and Blackshear 1986; Blackshear et al. 1987; Bravo et al. 1987) and show further that these signals act through the SRE. The nature of these signals is unclear. pkC is now known to comprise a large family of enzymes, some of which may not be depleted by phorbol ester treatment (Coussens et al. 1986; Knopf et al. 1986; Makowske et al. 1986; Ono et al. 1986; Parker et al. 1986; Housey et al. 1987). Therefore, involvement of some form of pkC in c-*fos* induction after chronic phorbol ester treatment cannot be ruled out formally. Another candidate signal that could operate under these conditions is the increase in cytoplasmic calcium levels that is also a consequence of inositol phospholipid turnover. Indeed, in many cell types, agents that act to increase intracellular calcium levels induce c-*fos* expression efficiently (Bravo et al. 1985, 1987; Milbrandt 1986; Moore et al. 1986; Morgan and Curran 1986). However, in the cells used here, calcium ionophores fail to induce c-*fos* expression significantly (data not shown), an observation also reported for another clone of BALB/c 3T3 cells (Ran et al. 1986). Therefore, the identity of the growth factor–induced signaling pathways that act on c-*fos* under these conditions is not known.

Another intracellular signal that induces c-*fos* expression in some cells is accumulation of intracellular cAMP. Agents that increase intracellular cAMP are reported to induce transient c-*fos* expression in the PC-12 pheochromocytoma cell line (Greenberg et al. 1985; Kruijer et al. 1985) but not in two fibroblast lines (Ran et al. 1986; Bravo et al. 1987). In macrophages, cAMP induces c-*fos* but with markedly different kinetics (Bravo et al. 1987). In the fibroblast line used in the present experiments, induction by cAMP is rapid and transient, although relatively weak and with slightly slower kinetics than induction by serum (L.A. Berkowitz and M.Z. Gilman, unpubl.). It has not yet been directly shown that treatment of these cells with IBMX and forskolin significantly augments cellular cAMP levels. However, this pathway has sequence requirements distinct from the other two pathways examined. It is independent of the SRE and requires, at least in part, c-*fos* sequences between −151 and +109. It has been noted that the c-*fos* gene contains sequences located at −290 and −65 with similarity to cAMP regulatory elements in other genes (Comb et al. 1986; Montminy et al. 1986). The c-*fos* gene also contains several matches to the consensus binding site for transcription factor AP-2 (Imagawa et al. 1987; Mitchell et al. 1987), and polymerized binding sites for this factor are cAMP responsive in vivo (Imagawa et al. 1987).

A reproducible observation in these experiments was the slight induction of the pm12 mutants with c-*sis* (see Figs. 3 and 4). The variable nature of the residual induction obtained with the mutant suggests that this construct may be responding to a signaling pathway that is variably active in these cells, depending on the precise growth conditions (Ran et al. 1986; Rollins et al. 1987). This observation raises the possibility that other sequences in the c-*fos* gene are capable of specifically responding to c-*sis* or PDGF. One candidate is a sequence 25 bp upstream of the SRE that binds a cellular factor specifically induced by conditioned medium from v-*sis*-transformed cells (Hayes et al. 1987). However, induction by c-*sis* in both the presence and absence of pkC is strongly affected by SRE mutations (Figs. 3 and 4), suggesting a major role for the SRE, and induction by c-*sis* also occurs in the absence of this upstream sequence (Figs. 5 and 6). A second possible explanation for induction of the pm12 mutant by c-*sis* is that this induction is due to effects on intracellular cAMP concentrations elicited by PDGF (Rozengurt et al. 1983). Arguing against this model is the observation that c-*fos* constructs containing sequences through −151 and lacking an SRE fail to respond to c-*sis*, although they do respond to IBMX and forskolin (Figs. 5 and 6). Additional mutagenesis studies will be necessary to distinguish between these alternatives.

How are the distinct signals that act through the SRE coupled to c-*fos* transcription? One possibility is that these pathways converge, each acting through the same SRE-binding protein. An alternate possibility is that each pathway communicates with a unique or dedicated SRE-binding protein. Only one SRE-binding activity is detected in nuclear extracts using the mobility-shift assay, but the presence of this activity in extracts does not correlate perfectly with the transcriptional activity of the c-*fos* gene. For example, the activity is present in many extracts of cells not transcribing c-*fos* (Gilman et al. 1986; Prywes and Roeder 1986; Treisman 1986; Fisch et al. 1987; Greenberg et al. 1987; Stumpo et al. 1988). It is reported absent in cells in which c-*fos* transcription has been induced with PMA or calcium ionophore (Prywes and Roeder 1986). Nevertheless, the results presented here suggest that cellular proteins with the binding specificity of this nuclear factor are indeed re-

quired for response to PMA and to pkC-independent signals. Therefore, if these pathways all act through this single factor, its activity does not appear to be strictly regulated through control of its DNA-binding activity, in contrast to factors that bind to other PMA-inducible elements (Sen and Baltimore 1986; Angel et al. 1987; Franza et al. 1987; Dinter et al. 1987; Nabel and Baltimore 1987; Wu et al. 1988). Alternatively, there may be additional SRE-binding proteins not detected by the mobility-shift assay. With the recent purification of HeLa cell proteins that bind to the SRE (Prywes and Roeder 1987; Schroter et al. 1987; Treisman 1987) direct data bearing on the roles cellular proteins play in c-*fos* induction may soon be available.

Materials and methods

Materials

PMA, PDBu, forskolin, and IBMX were obtained from Sigma. PMA stocks were made up at 100 μg/ml (162 μM) in DMSO. Forskolin stocks were 10 mM in ethanol. IBMX stocks were 0.5 M in DMSO. Final concentrations of DMSO and ethanol applied to cells were no more than 0.1% (vol/vol). At these concentrations, the solvents had no effect on c-*fos* expression. c-*sis* protein, B-chain homodimers of PDGF, was purchased from Amgen Biologicals.

Plasmids and mutagenesis

Most of the mouse c-*fos*–CAT fusion plasmids used in these studies were described previously (Gilman et al. 1986). For mutagenesis, a *Hin*dIII–*Eco*RI fragment from the Δ-356 c-*fos*–CAT fusion was transferred to M13 mp11. Mutagenesis was carried out with the two-primer method described by Zoller and Smith (1984) and was done in two steps. In the first step, the 3′ pair of G-C base pairs was mutagenized using the oligonucleotide 5′-CGCAGATGTGGTAATATGGACATCCTG-3′. The resulting mutant was mutagenized further using the oligonucleotide 5′-TAATATCCACATCCTGTGTAAAGGAGG-3′. Mutant phage were identified by plaque hybridization using the mutagenic oligonucleotides, plaque purified, and sequenced. Mutant c-*fos*-CAT fragments were recloned into plasmid vectors.

SRE oligonucleotides (5′-GTCGGATGTCCATATTAGGACATCT-3′ and 5′-GACAGATGTCCTAATATGGACATCC-3′ for wild-type; 5′-GTCGGATGTGGATATTACCACATCT-3′ and 5′-GACAGATCTGGTAATATCCACATCC-3′ for pm12) were phosphorylated, annealed, ligated briefly, and treated with the Klenow fragment of DNA polymerase to blunt overhanging ends. These mixtures of tandemly repeated oligonucleotides were cloned into the *Hin*cII site of pUC119 and screened by DNA sequence analysis. Restriction fragments carrying the cloned oligonucleotides were recovered as *Hin*dIII–*Sal*I fragments and inserted between the *Hin*dIII and *Sal*I sites immediately upstream of the c-*fos* promoter sequences in c-*fos*–CAT Δ-151 (Gilman et al. 1986).

All plasmid constructions were verified by DNA sequence analysis. Plasmids for transfections were purified twice in CsCl/EtBr gradients. DNA concentrations were determined by UV absorbance and confirmed by agarose gel electrophoresis.

Mobility-shift assay

The assay procedures, extracts, and probes were described in detail in Gilman et al. (1986). Competitor fragments were prepared by digestion of the Δ-356wt and Δ-356pm12 plasmids with *Hin*dIII and *Bam*HI, and purification by polyacrylamide gel electrophoresis of the 484-bp fragment carrying c-*fos* sequences −356 to +109. The concentrations of the purified fragments were estimated by comparison to standards on polyacrylamide gels.

Transfections

BALB/c 3T3 cells were carried in Dulbecco's modified essential medium (DMEM) containing 10% calf serum and antibiotics. Cells were split 1 : 6 to 1 : 12 for 12–24 hr prior to transfection, so that cultures were approximately 20–30% confluent at transfection. Cells were fed with 9 ml warm fresh medium 1 hr before transfection. Cells were transfected using a calcium phosphate coprecipitation procedure. c-*fos*–CAT DNA (52 μg) and πSVHPα2 (Treisman et al. 1983) DNA (10.4 μg) were diluted to 2.6 ml of 10 mM Tris (pH 7.5), 1 mM EDTA, and 250 mM $CaCl_2$. This solution was added with gentle vortexing to 2.6 ml of 50 mM HEPES (pH 7.12), 280 mM NaCl, and 1.5 mM Na_2HPO_4. The precipitate was allowed to stand for 2 min and then distributed in 1-ml aliquots onto five dishes. Each plate of cells received 10 μg of c-*fos*–CAT DNA and 2 μg of the internal control plasmid πSVHPα2. All plates receiving the same constructions received aliquots of the same large precipitate, minimizing plate-to-plate variability among these dishes. Cells were incubated in the precipitates for 16–18 hr at 37°C. The monolayers were washed three times with warm phosphate-buffered saline (PBS) and refed with 10 ml of DMEM containing 0.5% calf serum. Incubation continued for 48 hr at 37°C. The medium was removed, the cells were washed once with warm PBS, and inducers were added in 3-ml serum-free DMEM. The control cells were handled identically and incubated in 3-ml serum-free DMEM alone. After 45 min at 37°C, the medium was removed, and cells were placed on ice and flooded with ice-cold PBS. The monolayers were washed a total of three times with ice-cold PBS prior to RNA extraction.

pkC down-regulation experiments were carried out identically, except that after transfection, cells received DMEM containing 0.5% calf serum and 100 ng/ml PDBu. Control cultures received the same medium with an equal amount of DMSO (0.1%).

RNA analysis

Total cytoplasmic RNA was prepared essentially as described previously (Gilman et al. 1988) with two modifications. The cytoplasmic extract was treated with 125 μg/ml proteinase K for 15 min at 37°C prior to organic extraction. Second, the precipitated cytoplasmic RNA was treated with DNase I as follows. The RNA pellets were redissolved in 50 μl RNase-free TE buffer. Following the addition of 50 μl of a solution containing 10 mM Tris (pH 8), 1 mM EDTA, 20 mM $MgCl_2$, 2 mM dithiothreitol, 500 U/ml RNasin (Promega), and 200 U/ml RNase-free DNase I (Worthington DPRF), the RNA was incubated for 15 min at 37°C. DNase digestion was stopped by the addition of 25 μl of a solution containing 50 mM EDTA, 1% (wt/vol) SDS, and 1.5 M sodium acetate. RNA was finally recovered by extraction with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and chloroform/isoamyl alcohol (24 : 1), followed by ethanol precipitation.

RNase protection assays were carried out as outlined in Melton et al. (1984), with a number of modifications. Probes were prepared with either SP6 or T3 RNA polymerases using 100 μCi α-[^{32}P]CTP and were gel purified before use. Hybridizations contained 10 μg total cytoplasmic RNA, 500,000 dpm c-*fos* probe, and 200,000 dpm of α-globin probe in a total volume of 30 μl. Hybridization mixtures were incubated for 5

min at 85°C and then overnight (8–16 hr) at 45°C. RNase digestions were carried out for 60 min at room temperature and contained only RNase T1 (2 μg/ml). Electrophoresis was on 8% (wt/vol) polyacrylamide, 50% (wt/vol) urea sequencing gels.

The c-*fos* probe was prepared from a plasmid carrying c-*fos* sequences from −56 to +109 [nucleotides 79–243 in Van Beveren et al. (1983)]. The plasmid was prepared by inserting a *Hin*dIII–*Bam*HI fragment from c-*fos*–CAT fusion plasmid Δ-56 (Gilman et al. 1986) into *Hin*dIII–*Bam*HI–digested pSP65. Plasmid DNA was linearized with *Hin*dIII and transcribed with SP6 RNA polymerase (New England Biolabs) to yield a 225-nucleotide probe containing c-*fos* sequences found in transcripts of both the endogenous c-*fos* gene and the transfected genes and flanking linker sequences present only in the transfected gene transcripts (see Fig. 1B). The α-globin probe was prepared from a plasmid consisting of a 110 bp *Bss*HII fragment of the human α-globin gene (−15 to +95) cloned into the *Hin*dIII site of pBSM13+ (Stratagene). The probe was prepared by transcription of *Xba*I-digested template with T3 RNA polymerase (Stratagene).

In the figures, the major protected bands correspond to RNase T1 cleavage of the probes at the guanosine residues nearest the hybrid boundaries. Therefore, they do not indicate the precise 5′-end of the transcripts. Weak bands running above the major cleavage products represent incomplete RNase T1 digestion products that are cleaved at the next guanosine residue either upstream or downstream of the hybrid.

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References

Angel, P., M. Imagawa, R. Chiu, B. Stein, R.J. Imbra, H.J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. 1987. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. *Cell* **49:** 729–739.

Blackshear, P.J., D.J. Stumpo, J.-K. Huang, R.A. Nemenoff, and D.H. Spach. 1987. Protein kinase C-dependent and -independent pathways of proto-oncogene induction in human astrocytoma cells. *J. Biol. Chem.* **262:** 7774–7781.

Bravo, R., J. Burckhardt, T. Curran, and R. Muller. 1985. Stimulation and inhibition of growth by EGF in different A431 cell clones is accompanied by the rapid induction of c-*fos* and c-*myc* proto-oncogenes. *EMBO J.* **4:** 1193–1197.

Bravo, R., M. Neuberg, J. Burckhardt, J. Almendral, R. Wallich, and R. Muller. 1987. Involvement of common and cell type-specific pathways of c-*fos* gene control: Stable induction by cAMP in macrophages. *Cell* **48:** 251–260.

Chiu, R., M. Imagawa, R.J. Imbra, J.R. Bockoven, and M. Karin. 1987. Multiple *cis*- and *trans*-acting elements mediate the transcriptional response to phorbol esters. *Nature* **329:** 648–651.

Collins, M.K.L. and E. Rozengurt. 1984. Homologous and heterologous mitogenic desensitization of Swiss 3T3 cells to phorbol esters and vasopressin: Role of receptor and post-receptor steps. *J. Cell. Physiol.* **118:** 133–142.

Comb, M.C., N.C. Birnberg, A. Seasholtz, E. Herbert, and H.M. Goodman. 1986. A cyclic AMP- and phorbol ester-inducible DNA element. *Nature* **323:** 353–356.

Coussens, L., P.J. Parker, L. Rhee, T.L. Yang-Feng, E. Chen, M.D. Waterfield, U. Francke, and A. Ullrich. 1986. Multiple distinct forms of bovine and human protein kinase C suggest diversity in cellular signalling pathways. *Science* **233:** 859–866.

Curran, T. 1988. *fos*. In *Oncogene handbook* (ed. E.P. Reddy, A.M. Skalka, and T. Curran), Elsevier, Amsterdam (in press).

Dinter, H., R. Chiu, M. Imagawa, M. Karin, and K.A. Jones. 1987. *In vitro* activation of the HIV-1 enhancer in extracts from cells treated with a phorbol ester tumor promoter. *EMBO J.* **6:** 4067–4071.

Elsholtz, H.P., H.J. Mangalam, E. Potter, V.R. Albert, S. Supowit, R.M. Evans, and M.G. Rosenfeld. 1986. Two different *cis*-active elements transfer the transcriptional effects of both EGF and phorbol esters. *Science* **234:** 1552–1557.

Fisch, T.M., R. Prywes, and R.G. Roeder. 1987. c-*fos* sequences necessary for basal expression and induction by epidermal growth factor, 12-*O*-tetradecanoyl phorbol-13-acetate and the calcium ionophore. *Mol. Cell. Biol.* **7:** 3490–3502.

Franza, B.R., S.F. Josephs, M.Z. Gilman, W. Ryan, and B. Clarkson. 1987. Characterization of cellular proteins recognizing the HIV enhancer using a microscale DNA-affinity precipitation assay. *Nature* **330:** 391–395.

Gilman, M.Z., R.N. Wilson, and R.A. Weinberg. 1986. Multiple protein-binding sites in the 5′-flanking region regulate c-*fos* expression. *Mol. Cell. Biol.* **6:** 4305–4316.

Greenberg, M.E. and E.B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. *Nature* **311:** 433–438.

Greenberg, M.E., L.A. Greene, and E.B. Ziff. 1985. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC-12 cells. *J. Biol. Chem.* **260:** 14101–14110.

Greenberg, M.E., A.L. Hermanowski, and E.B. Ziff. 1986. Effect of protein synthesis inhibitors on growth factor activation of c-*fos*, c-*myc*, and actin gene expression. *Mol. Cell. Biol.* **6:** 1050–1057.

Greenberg, M.E., Z. Siegfried, and E.B. Ziff. 1987. Mutation of the c-*fos* dyad symmetry element inhibits serum inducibility of transcription *in vivo* and the nuclear regulatory factor binding *in vitro*. *Mol. Cell. Biol.* **7:** 1217–1225.

Hayes, T.E., A.M. Kitchen, and B.H. Cochran. 1987. Inducible binding of a factor to the c-*fos* regulatory region. *Proc. Natl. Acad. Sci.* **84:** 1272–1276.

Housey, G.M., C.A. O'Brian, M.D. Johnson, P. Kirschmeier, and I.B. Weinstein. 1987. Isolation of cDNA clones encoding protein kinase C: Evidence for a protein kinase C-related gene family. *Proc. Natl. Acad. Sci.* **84:** 1065–1069.

Imagawa, M., R. Chiu, and M. Karin. 1987. Transcription factor AP-2 mediates induction by two different signal transduction pathways: Protein kinase C and cAMP. *Cell* **51:** 251–260.

Imbra, R.J. and M. Karin. 1986. Phorbol ester induces the transcriptional stimulatory activity of the SV40 enhancer. *Nature* **323:** 555–558.

Kaufman, J.D., G. Valandra, G. Rodriquez, G. Breshar, C. Giri, and M.A. Norcross. 1987. Phorbol ester enhances human immunodificiency virus-promoter gene expression and acts on a repeated 10-base-pair functional enhancer element. *Mol. Cell. Biol.* **7:** 3759–3766.

Knopf, J.L., M.-H. Lee, L.A. Sultzman, R.W. Kriz, C.R. Loomis, R.M. Hewick, and R.M. Bell. 1986. Cloning and expression of multiple protein kinase C cDNAs. *Cell* **46:** 491–502.

Kruijer, W., D. Schubert, and I.M. Verma. 1985 Induction of the proto-oncogene *fos* by nerve growth factor. *Proc. Natl. Acad. Sci.* **82:** 7330–7334.

Kruijer, W., J.A. Cooper, T. Hunter, and I.M. Verma. 1984. PDGF induces rapid but transient expression of the c-*fos* gene. *Nature* **312:** 711–716.

Lee, W., P. Mitchell, and R. Tjian. 1987a. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* **49:** 741–752.

Lee, W., A. Haslinger, M. Karin, and R. Tjian. 1987b. Two factors that bind and activate the human metallothionien-IIA gene *in vitro* also interact with the SV40 promoter and enhancer region. *Nature* **325:** 368–372.

Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12:** 7035–7056.

Michell, R.H. and J.W. Putney. 1987. *Inositol lipids in cellular signaling*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Milbrandt, J. 1986. Nerve growth factor rapidly induces c-*fos* mRNA in PC-12 rat pheochromocytoma cells. *Proc. Natl. Acad. Sci.* **83:** 4789–4793.

Mitchell, P.J., C. Wang, and R. Tjian. 1987. Positive and negative regulation of transcription in vitro: Enhancer-binding protein AP-2 is inhibited by SV40 T antigen. *Cell* **50:** 847–861.

Mohun, T., N. Garrett, and R. Treisman. 1987. *Xenopus* cytoskeletal actin and human c-*fos* gene promoters share a conserved protein-binding site. *EMBO J.* **6:** 667–673.

Montminy, M.R., K.A. Sevarino, J.A. Wagner, G. Mandel, and R.H. Goodman. 1986. Identification of a cAMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci.* **83:** 6682–6686.

Moore, J.P., J.A. Todd, T.R. Hesketh, and J.C. Metcalfe. 1986. c-*fos* and c-*myc* activation, ionic signals, and DNA synthesis in thymocytes. *J. Biol. Chem.* **261:**8158–8162.

Morgan, J.I. and T. Curran. 1986. Role of ion flux in the control of c-*fos* expression. *Nature* **322:** 552–555.

Muller R., R. Bravo, J. Burckhardt, and T. Curran. 1984. Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. *Nature* **312:** 716–720.

Nabel, G. and D. Baltimore. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* **326:** 711–713.

Nishizuka, Y. 1986. Studies and perspectives of protein kinase C. *Science* **233:** 305–312.

Ono, Y., T. Kurokawa, T. Fujii, K. Kawahara, K. Igarashi, U. Kikkawa, K. Ogita, and Y. Nishizuka. 1986. Two types of complementary DNAs of rat brain protein kinase C. *FEBS Lett.* **206:** 347–352.

Parker, P.J., L. Coussens, N. Totty, L. Rhee, S. Young, E. Chen, S. Stabel, M.D. Waterfield, and A. Ullrich. 1986. The complete primary structure of protein kinase C–The major phorbol ester receptor. *Science* **233:** 853–858.

Piette J. and M. Yaniv. 1987. Two different factors bind to the α-domain of the polyoma virus enhancer, one of which also interacts with the SV40 and c-*fos* enhancers. *EMBO J.* **6:** 1331–1337.

Prywes, R. and R.G. Roeder. 1986. Inducible binding of a factor to the c-*fos* enhancer. *Cell* **47:** 777–784.

———. 1987. Purification of the c-*fos* enhancer-binding protein. *Mol. Cell. Biol.* **7:** 3482–3489.

Ran, W., M. Dean, R.A. Levine, C. Henkle, and J. Campisi. 1986. Induction of c-*fos* and c-*myc* mRNA by epidermal growth factor or calcium ionophore is cAMP dependent. *Proc. Natl. Acad. Sci.* **83:** 8216-8220.

Rodriguez-Pena, A. and E. Rozengurt. 1984. Disappearance of Ca-sensitive, phospholipid-dependent protein kinase activity in phorbol ester-treated 3T3 cells. *Biochem. Biophys. Res. Commun.* **120:** 1053–1059.

Rollins, B.J., E.D. Morrison, and C.D. Stiles. 1987. A cell-cycle constraint on the regulation of gene expression by platelet-derived growth factor. *Science* **238:** 1269–1271.

Rozengurt, E. 1986. Early signals in the mitogenic response. *Science* **234:** 161–166.

Rozengurt, E., P. Stroobant, M.D. Waterfield, T.F. Dueul, and M. Keehan. 1983. Platelet-derived growth factor elicits cyclic AMP accumulation in swiss 3T3 cells: Role of prostaglandin production. *Cell* **34:** 265–272.

Schroter, H., P.E. Shaw, and A. Nordheim. 1987. Purification of intercalator-released p67, a polypeptide that interacts specifically with the c-*fos* serum response element. *Nucleic Acids Res.* **15:** 10145–10158.

Sen, R. and D. Baltimore. 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-βB by a posttranslational mechanism. *Cell* **47:** 921–928.

Siekevitz, M., S.F. Josephs, M. Dukovitch, N. Peffer, F. Wong-Staal, and W.C. Greene. 1987. Activation of the HIV-1 LTR by T cell mitogens and the *trans*-activator protein of HTLV-1. *Science* **238:** 1575–1578.

Stumpo, D.J. and P.J. Blackshear. 1986. Insulin and growth factor effects on c-*fos* expression in normal and protein kinase C-deficient 3T3-L1 fibroblasts and adipocytes. *Proc. Natl. Acad. Sci.* **83:** 9453–9457.

Stumpo, D.J., T.N. Stewart, M.Z. Gilman, and P.J. Blackshear. 1988. Identification of c-*fos* sequences involved in induction by insulin and phorbol esters. *J. Biol. Chem.* **263:** 1611–1614.

Tong-Starksen, S.E., P.A. Luciw, and B.M. Peterlin. 1987. Human immunodeficiency virus long terminal repeat responds to T-cell activation signals. *Proc. Natl. Acad. Sci.* **84:** 6845–6849.

Treisman, R. 1985. Transient accumulation of c-*fos* RNA following serum stimulation requires a conserved 5′ element and c-*fos* 3′ sequences. *Cell* **42:** 889–902.

———. 1986. Identification of a protein-binding site that mediates transcriptional response of the c-*fos* gene to serum factors. *Cell* **46:** 567–574.

———. 1987. Identification and purification of a polypeptide that binds to the c-*fos* serum response element. *EMBO J.* **6:** 2711–2717.

Treisman, R., M.R. Green, and T. Maniatis. 1983. *cis* and *trans* activation of globin gene transcription in transient assays. *Proc. Natl. Acad. Sci.* **80:** 7428–7432.

Van Beveren, C., F. van Straaten, T. Curran, R. Muller, and I. Verma. 1983. Analysis of FBJ-MuSV provirus and c-*fos* (mouse) gene reveals that viral and cellular *fos* gene products have different carboxy termini. *Cell* **32:** 1241–1255.

Wu, F., J. Garcia, R. Mitsuyasu, and R. Gaynor. 1988. Alterations in binding characteristics of the human immunodeficiency virus enhancer factor. *J. Virol.* **62:** 218–225.

Zoller, M.J. and M. Smith. 1984. Oligonucleotide-directed mutagenesis: A simple method using two oligonucleotide primers and a single-stranded DNA template. *DNA* **3:** 479–488.

The c-fos serum response element responds to protein kinase C-dependent and -independent signals but not to cyclic AMP.

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