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Differential activation of c-*fos* promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors

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The upstream regulatory region of the c-*fos* promoter contains two growth factor-regulated promoter elements: the serum response element, which binds a ternary complex comprising serum response factor (SRF) and a ternary complex factor (TCF); and the sis-inducible element (SIE) which binds STAT transcription factors. We used transient transfection of c-*fos* promoter mutants in NIH 3T3 cells to assess the contributions of these elements to activation by different extracellular stimuli. Colony-stimulating factor-1, platelet-derived growth factor and epidermal growth factor activate the c-*fos* promoter via cooperation of the SIE and the SRE; however, mutants that can bind SRF but not STATs or TCF remain inducible by whole serum. Activation by the SIE is context-dependent: interferons activate STAT DNA binding activity and transcription of SIE reporter genes, but not the c-*fos* promoter, which requires an additional ras-dependent signal. SRE activation by receptor tyrosine kinases requires TCF binding, and can be mediated by the TCF Elk-1. In contrast, SRE activation following activation of heterotrimeric G proteins by lysophosphatidic acid or aluminium fluoride ion requires SRF but is independent of TCF binding. These results suggest that heterotrimeric G proteins activate a signalling pathway distinct from those that activate the STATs and the TCFs, that controls SRF activity.

Keywords: c-*fos*/serum response element/serum response factor/sis-inducible element/STATs

Introduction

Many developmental and physiological processes are dependent on the activation of specific genes in response to extracellular signals. A number of signal-responsive promoter elements have now been identified, and in some cases signal transduction pathways can be traced from a cell surface receptor to activation of a particular transcription factor. However, the mechanisms that govern the differential activation of gene expression in a given cell type in response to extracellular signals are less well understood: specificity appears to arise from differences in the type, strength and duration of intracellular signals, and combinatorial interactions both between transcription factors and between signalling pathways (for review, see Hill and Treisman, 1995).

The c-*fos* promoter has been studied extensively as a model growth factor-regulated promoter. Its upstream region contains several regulatory sequences (see Figure 1), which include the serum response element (SRE; for review, see Treisman, 1990) and the sis-inducible element (SIE; Hayes *et al.*, 1987; Wagner *et al.*, 1990). At the SRE a ternary complex forms which contains serum response factor (SRF; Norman *et al.*, 1988) and an Ets domain protein, ternary complex factor (TCF), which can only bind the SRE via interaction with SRF (Shaw *et al.*, 1989a; Hipskind *et al.*, 1991; Dalton and Treisman, 1992). The TCFs, which bind an Ets motif adjoining the SRF binding site, are regulated by MAP kinase phosphorylation in response to extracellular signals (Gille *et al.*, 1992, 1995; Hill *et al.*, 1993; Janknecht *et al.*, 1993; Marais *et al.*, 1993; Kortenjann *et al.*, 1994; Janknecht and Nordheim, 1995; Price *et al.*, 1995; for review, see Treisman, 1994). The SIE binds cytokine- and growth factor-regulated transcription factors of the STAT family (Fu and Zhang, 1993; Sadowski *et al.*, 1993; Zhong *et al.*, 1994). Adjoining the SRE is a binding site for an uncharacterized AP1/ATF family member (Goding *et al.*, 1987; Piette and Yaniv, 1987; Shaw *et al.*, 1989b). Genomic footprinting studies of cultured cells indicate that the SRE and AP1/ATF sites are constitutively occupied *in vivo*, whereas SIE binding activity is regulated (Herrera *et al.*, 1989; Konig, 1991). Additional factors, some of which are also responsive to extracellular signalling pathways, bind to sequences in the vicinity of the TATA box (Sassone-Corsi *et al.*, 1988; Berkowitz *et al.*, 1989; Ginty *et al.*, 1994).

Our understanding of the mechanisms that regulate c-*fos* transcription is deficient in two important respects. First, only some of the different signalling pathways that target the promoter have been characterized. For example, although the linkage of the SRE to activity of the ras–raf–ERK pathway via TCF is well established, whole serum can also activate the SRE independently of TCF by an uncharacterized pathway (Graham and Gilman, 1991; Konig, 1991; Hill *et al.*, 1994; Johansen and Prywes, 1994) Second, the role of transcription factor interactions at the promoter in regulated promoter activity remains poorly understood. For example, the SRE appears less important for promoter activation by platelet-derived growth factor (PDGF) than by serum (Gilman, 1988), perhaps because PDGF, unlike serum, can also induce STAT activation. Moreover, although the SRE is sufficient for serum regulation of model reporter genes, in the context of the c-*fos* promoter its activity is dependent on cooperating promoter elements (Berkowitz *et al.*, 1989); indeed, studies with c-*fos* transgenes suggest that all the c-*fos* upstream elements are required for promoter activity

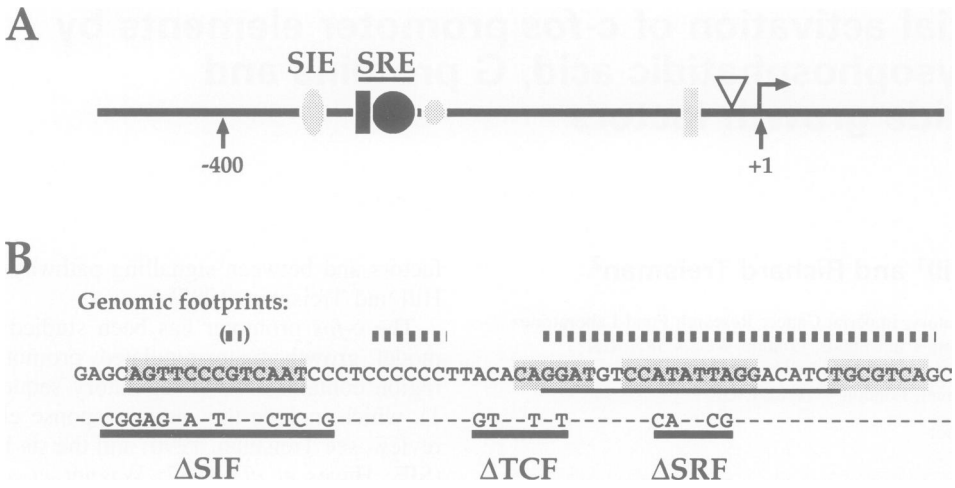

Fig. 1. The c-*fos* 5′ regulatory region. (**A**) Schematic representation of the c-*fos* promoter. The transcription initiation point, TATA box and CRE at –65 are indicated by an arrow, an inverted triangle and a grey rectangle respectively. Transcription factor binding sites in the upstream regulatory region are indicated by symbols as follows: SIE, grey ellipse, TCF binding site, black rectangle; SRF binding site, black circle; AP1/ATF site, grey circle. (**B**) Sequence of c-*fos* upstream regulatory region from –291 to –354 (according to Treisman, 1985). Oligonucleotide sequences that define the SRE (Treisman, 1986) and SIE (Wagner *et al.*, 1990) are underlined. The dotted line above the sequence indicates sequences protected from methylation *in vivo* (Herrera *et al.*, 1989); brackets denote sequence protected only after growth factor stimulation. The consensus binding sites for STAT factors, TCFs, SRF and AP1/ATF factors are shaded. Below the sequence the different promoter mutations are shown, with dashes indicating identity to the wild-type sequence; the ΔSIF, ΔTCF and ΔSRF mutations generate Gal4 operator, LexA half-site and MCM1 sites respectively. The different promoter mutants contain the underlined clusters of mutations either singly or in combination, i.e. ΔSIF/ΔTCF lacks both SIF and TCF sites but retains an intact SRF binding site.

in a bona fide chromatin context (Robertson *et al.*, 1995). Here we use transient c-*fos* transfections to investigate the roles of the SIE and SRE in transduction of different signals in fibroblasts. Our results demonstrate that the SRE and SIE can cooperate in promoter activation. Moreover, we show that lysophosphatidic acid (LPA) and aluminium fluoride ion (AlF_4^-), like serum, can activate the promoter via the SRE independently of TCF binding. We propose that these agents trigger an SRF-linked signalling pathway by activation of heterotrimeric G proteins.

Results

c-fos promoter mutants

We used RNase protection to investigate the roles of the SIE and SRE promoter elements in the context of the entire c-*fos* gene. Mutations were introduced into the c-*fos* promoter designed to prevent SRF, TCF and SIF binding, either singly or in combination, as shown in Figure 1. At the SRE, SRF binding was prevented by conversion of the SRF binding site to a consensus site for the yeast SRF-related factor MCM1; this mutation, ΔSRF, prevents binding of both SRF and TCF, since TCF binding requires DNA-bound SRF. An alternative SRE mutation, ΔTCF, converts the SRE Ets motif to a LexA half-site, which prevents TCF binding while leaving SRF binding unaffected. The ΔSRF and ΔTCF mutants can be bound efficiently *in vivo* by altered specificity mutants of SRF and the TCF Elk-1 (Hill *et al.*, 1993, 1994). At the SIE, the ΔSIF mutation destroys the SIE consensus, creating a GAL4 operator. To analyse the promoter mutants, we used transient transfection assays in T56 cells, an NIH 3T3 derivative cell line that expresses the human colony-stimulating factor-1 (CSF-1) receptor (Roussel *et al.*, 1990). Apart from their ability to respond to CSF-1, these cells behave essentially indistinguishably from NIH 3T3 cells in our assays.

Cooperation of the SIE and SRE in transcriptional activation

We first used the promoter mutants to compare c-*fos* transcriptional activation induced by whole serum, the polypeptide growth factors CSF-1, PDGF and epidermal growth factor (EGF) and phorbol myristate acetate (TPA). The results of several experiments are summarized in Figure 2 (for representative results, see Figures 6, 7 and 8). We observed that the endogenous c-*fos* gene appeared more responsive to serum stimulation than the transfected gene when compared with the other inducers (data not shown; see Figures 6–8 below); it is possible that this reflects a limiting amount of an intracellular factor necessary for transducing serum-induced signals in the cells. However, the kinetics of induction of the transfected and endogenous genes were very similar (data not shown; see Figure 7). The relative efficiency with which the wild-type promoter was activated by the different stimuli was extremely repoducible, ranging from 25% (EGF) to 71% (CSF-1) of that obtained with whole serum (see Figure 2 legend).

Mutation of the TCF binding site significantly impaired the response to TPA, but had only a small effect on serum inducibility (Figure 2A, compare lanes 1 and 2 with 6 and 7). In contrast, mutation of the SRF binding site, which prevents binding of both SRF and TCF, impaired the response to both serum and TPA (Figure 2A, compare lanes 6 and 7 with 11 and 12). The effect of the ΔTCF mutation on the response to CSF-1, EGF and PDGF was comparable with the effect on the TPA response (Figure 2A, compare lanes 2–5 with 7–10). These responses were also impaired by the ΔSRF mutation, but the effect of this mutation was reproducibly less than that of the ΔTCF

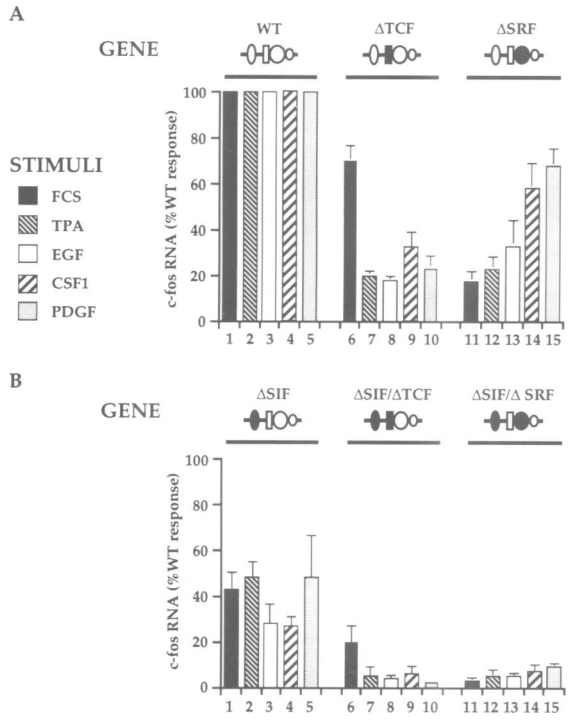

Fig. 2. Transcriptional activation of c-*fos* promoter mutants by serum and polypeptide growth factors. In this and subsequent figures, the c-*fos* regulatory region is shown schematically with symbols indicating (from left to right) the SIE, TCF, SRF and ATF/AP1 sites; open and filled symbols denote intact and mutated sites respectively. (**A**) Effects on regulated transcriptional activation of mutations in the TCF and SRF binding sites. Cells were transfected with the intact c-*fos* gene (lanes 1–5) or mutants ΔTCF (lanes 6–10) or ΔSRF (lanes 11–15), together with a reference plasmid containing the human α-globin gene. Following maintenance of the cells in medium containing 0.5% FCS for 36 h, total cell RNA was prepared following a 30 min treatment with 15% serum (lanes 1, 6 and 11), 50 ng/ml TPA (lanes 2, 7 and 12) 50 ng/ml EGF (lanes 3, 8 and 13), 107 ng (200 U)/ml CSF-1 (lanes 4, 9 and 14) or 25 ng/ml PDGF (lanes 5, 10 and 15) as indicated. (**B**) Contribution of the SIE to regulation of c-*fos* transcription. Cells were transfected with mutants ΔSIF (lanes 1–5), ΔSIF/ΔTCF (lanes 6–10) or ΔSIF/ΔSRF (lanes 11–15) together with a reference plasmid containing the human α-globin gene. Inductions performed as above, were as follows: FCS (lanes 1, 6 and 11); TPA (lanes 2, 7 and 12); EGF (lanes 3, 8 and 13); CSF-1 (lanes 4, 9 and 14); PDGF (lanes 5, 10 and 15). Human c-*fos* and reference α-globin transcripts were detected by RNase protection and data were quantitated using the PhosphorImager. The c-*fos* RNA signal, normalized to that of the reference transcript, is expressed as a percentage of the level of RNA produced by the wild-type c-*fos* promoter in response to the same stimulus. The data are averages from up to 11 independent experiments, with standard deviations indicated as error bars. The relative responses of the wild-type gene to the various stimuli were as follows, means and standard deviations in abitrary units: FCS, 100; TPA, 51 ± 5; CSF-1, 71 ± 10; EGF, 25 ± 5; PDGF-BB, 46 ± 11.

mutation (Figure 2A, compare lanes 8–10 with 13–15). This may reflect the ability of novel factors to bind the AP1/ATF site when SRF binding is prevented (Fisch *et al.*, 1989; Shaw *et al.*, 1989b; see Discussion).

We next considered the role of the SIE. Mutation of this site resulted in a promoter, ΔSIF, that was 2-fold less responsive to serum, TPA and PDGF but 4-fold impaired in its response to EGF and CSF-1 (Figure 2, compare panels A and B, lanes 1–5), suggesting that the SIE contributes to promoter activation. To test this more rigorously, we assessed the effects of combining the ΔSIF mutation with SRE mutations that block binding of either TCF only or both SRF and TCF. A promoter mutant lacking both the SIE and the TCF binding site, ΔSIF/ΔTCF, exhibited only a residual response to TPA and the polypeptide growth factors, but remained significantly serum inducible (Figure 2B, lanes 6–10). To determine whether this serum inducibility requires SRF binding, we tested a second double mutant, ΔSIF/ΔSRF, which can bind neither SRF nor TCF. In contrast to the ΔSIF/ΔTCF mutant, this gene exhibited only a residual response to serum, TPA and the polypeptide growth factors (Figure 2B, compare lanes 6–10 with 11–15).

These data demonstrate that the SIE and the SRE cooperate in activation of the promoter in response to growth factor signals. However, the relative contribution of the two sites varies according to the stimulus. EGF and CSF-1 induction is affected equally by mutations that prevent TCF or STAT factor binding, while TPA and PDGF induction appears more sensitive to SRE mutations than to SIE mutations. Only serum can activate a promoter mutant that retains the SRF site, but lacks both the TCF binding site and the SIE. Taken together, these data suggest that serum activates a signalling pathway distinct from those activated by polypeptide growth factors and TPA, which targets SRF. In the following sections, we will present experiments to examine the roles of the SIE and the SRF/TCF ternary complex in more detail.

Induction of STAT DNA binding activity

The involvement of the SIE region in growth factor induction suggests that transcription factors of the STAT family might be involved. To analyse STAT induction, extracts prepared from cells treated with various agents were analysed by gel mobility-shift assay using as a probe a high affinity, relaxed-specificity variant of the c-*fos* SIE (the M67 sequence; Wagner *et al.*, 1990). As a control for induction of STAT DNA binding activity, we treated the cells with interferon (IFN)-α or -γ.

Treatment of cells with IFN-α or -γ generated readily detectable SIE binding activity. IFN-α induced three complexes (Figure 3, lane 3, complexes A–C); in contrast IFN-γ induced only complex C (Figure 3, lanes 2 and 3). CSF-1 and PDGF also induced complexes A–C, although complex formation by PDGF was inefficient (Figure 3A, lanes 6 and 7). However, complex induction by EGF, serum and TPA was virtually undetectable, even though the SIE contributes to transcriptional activation by each of these stimuli (Figure 3A, lanes 4, 5 and 8). Complexes A and C reacted virtually quantitatively with antisera specific for STAT3 and STAT1-α respectively, while complex B reacted with both antisera; it is likely that unreactive complex C material contains STAT1-β, which does not react with the antiserum (Figure 3A, lanes 9–16). Formation of each complex was competed by a 400-fold excess of either the authentic or M67 SIEs, but not by a mutated SIE derivative (Figure 3B, lanes 1–12). These findings are in agreement with previous studies (Wagner *et al.*, 1990; Ruff-Jamison *et al.*, 1993; Sadowski *et al.*, 1993; Silvennoinen *et al.*, 1993; Zhong *et al.*, 1994).

Transcriptional activation by the SIE is context dependent

Taken together with the transient transfection data, the results above suggest that the ability of stimuli to activate

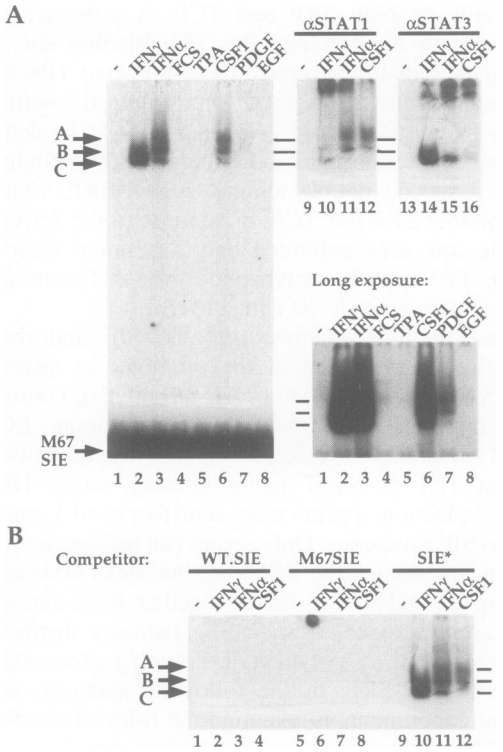

Fig. 3. Induction of SIE binding activity by different stimuli. (**A**) IFNs and polypeptide growth factors induce DNA binding by STAT1 and STAT3. SIE binding proteins were detected by gel mobility-shift analysis using the relaxed-specificity high-affinity M67 SIE probe (Wagner *et al.*, 1990). Extracts were prepared from cells maintained in medium containing 0.5% serum for 36 h and then stimulated for 30 min by the following agents: no stimulus (lanes 1, 9 and 13); 1000 U/ml IFN-γ (lanes 2, 10 and 14); 1000 U/ml IFN-α (lanes 3, 11 and 15); 15% FCS (lane 4); 50 ng/ml TPA (lane 5); 107 ng (200 U)/ml CSF-1 (lanes 6, 12 and 16); 25 ng/ml PDGF (lane 7); 50 ng/ml EGF (lane 8). The binding reactions contained 1.0 μl STAT1 antibody (lanes 9–12) or 1.0 μl STAT3 antibody (lanes 13–16). (**B**) Binding competition analysis of SIE binding proteins. Binding reactions were performed with extracts from unstimulated cells (lanes 1, 5 and 9) or from cells treated with IFN-γ (lanes 2, 6 and 10), IFN-α (lanes 3, 7 and 11) or CSF-1 (lanes 4, 8 and 12) as above. Reactions contained a 400-fold excess of unlabelled SIE.WT (lanes 1–4), M67 SIE (lanes 5–8) or SIE* (lanes 9–12).

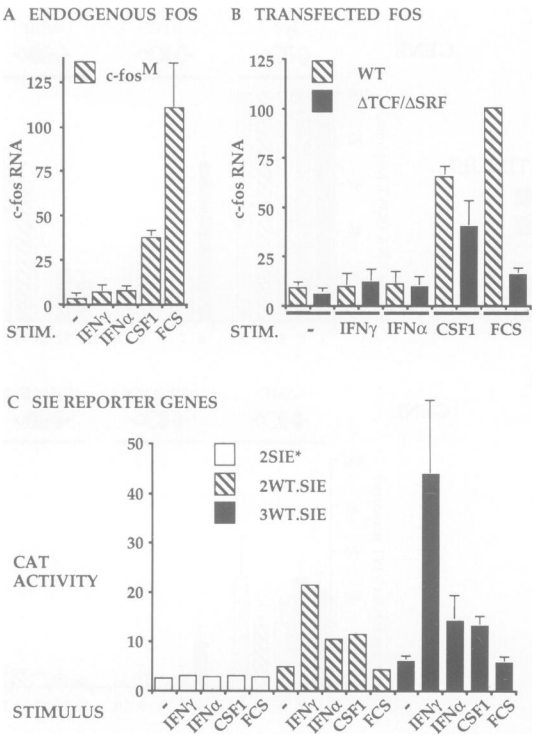

Fig. 4. Context-dependent transcriptional activation by the SIE. Cells were transfected with the intact c-*fos* gene or the ΔTCF/ΔSRF mutant which retains only the SIE, together with the α-globin reference plasmid. Following maintenance of the cells in medium containing 0.5% FCS for 36 h, total cell RNA was prepared either directly or following a 30 min treatment with 1000 U/ml IFN-γ, 1000 U/ml IFN-α, 107 ng (200 U)/ml CSF-1 or 15% FCS. (**A**) Activation of the endogenous mouse c-*fos* gene. Mouse c-*fos* transcripts were detected by RNase protection, quantitated using the PhosphorImager, and expressed in arbitrary units. Data presented are the average of triplicate experiments; bars indicate estimated standard deviation. (**B**) Activation of human c-*fos* derivatives. Human c-*fos* transcripts were detected by RNase protection, quantitated relative to α-globin reference transcript using the PhosphorImager, and expressed in arbitrary units, taking the serum-stimulated level of the wild-type gene as 100. Results presented are the average of two independent experiments; bars indicate estimated standard deviation. CSF-1-induced transcript levels were within 10% of the means established in Figure 2. (**C**) Transcriptional activation by SIE-controlled reporter genes. Cells were transfected with TK–CAT reporter plasmids containing two copies of SIE*, or two or three copies of WT.SIE. Following maintenance of the cells in medium containing 0.5% FCS for 36 h, cells were stimulated with serum, IFNs or CSF-1 as above and extracts were prepared 8 h later and analysed for CAT activity, expressed as percent acetylation. Results are from a representative experiment, with 3WT.SIE transfected in duplicate; bars indicate estimated standard deviation. The vector pBLCAT2 itself is responsive to none of these stimuli (data not shown).

STAT binding correlates only poorly with the contribution of the SIE to transcriptional activation. To investigate this further, we examined c-*fos* induction in cells stimulated with interferons. Both IFN-α and IFN-γ induce STAT activation, and the STAT complexes induced by IFN-α are indistinguishable from those induced by CSF-1, which efficiently activates c-*fos* transcription.

In contrast to CSF-1 and serum, IFNs induced transcription of the endogenous c-*fos* gene very inefficiently (Figure 4A). A similar result was obtained with the intact transfected c-*fos* gene: IFN induction was <2-fold, in contrast to CSF1 and serum (Figure 4B, hatched bars). These results suggest that induction of STAT DNA binding activity is insufficient for activation of the c-*fos* promoter. To exclude the possibility that this arises because of the presence of the intact SRE, we also tested IFN induction of a mutant that lacks a functional SRE, ΔTCF/ΔSRF. Again, IFN induction was inefficient while CSF-1 induced transcription relatively efficiently (Figure 4B, solid bars). Thus, in the context of the c-*fos* promoter, transcriptional activation by the SIE appears to be induced only by polypeptide growth factors.

The failure of IFNs to induce c-*fos* transcription efficiently could reflect either their inability to induce other signals required for promoter activation, or their inability to induce transcriptionally active STAT complexes. We used a reporter gene assay to test whether the induced STAT complexes could activate transcription. Single or multiple SIE oligonucleotides were inserted 5′ to a thymidine kinase–chloramphenicol acetyltranferase (CAT) reporter gene, and transcriptional activation in response to IFNs and CSF-1 compared following transfection of T56 cells. Single copies of both the wild-type and

M67 SIE sequences were unable to activate reporter genes in response to any stimulus (data not shown); however, reporters containing two or three wild-type SIEs were weakly activated by both CSF-1 and IFN-α, and more strongly activated by IFN-γ (Figure 4C). The mutated SIE derivative, which cannot bind STATs, was inactive (Figure 4C). Thus, both IFNs and CSF-1, but not serum, induce STAT complexes that are transcriptionally competent when bound at multiple sites close to the TATA box. However, an additional, CSF-1-specific signal is required for transcriptional activation by the single SIE in the context of the c-*fos* promoter. These results are consistent with the idea that STATs contribute to c-*fos* induction by CSF-1 and PDGF, but do not explain the role of this sequence in regulation of the gene by serum or TPA (see Discussion).

SIE-dependent activation of the c-fos promoter requires ras

To investigate the nature of additional signals necessary for c-*fos* induction via the SIE, we compared the ability of polypeptide growth factors and IFNs to activate the ras–raf–ERK pathway. As assessed by immunoblotting, CSF-1 activated ERK2 as efficiently as whole serum, while IFN treatment had little if any effect (Figure 5A, compare lanes 1–7 with 8–13). Thus, IFNs fail to induce efficiently at least one of the known signalling pathways associated with c-*fos* transcriptional activation.

We next investigated whether ras, whose regulated activity potentiates ERK activation, is required for activation of the SIE in the context of the c-*fos* promoter. Cells were co-transfected with c-*fos* genes and a plasmid that produces a dominant interfering ras mutant, rasN17 (Feig and Cooper, 1988). The rasN17 protein inhibited activation of the intact c-*fos* gene by serum, TPA, CSF and PDGF; in addition, rasN17 inhibited transcriptional activation in response to treatment with LPA, an inducer which we shall discuss in more detail below (Figure 5B compare lanes 1–6 and 7–12). We next examined the ras-dependence of the c-*fos* ΔTCF/ΔSRF mutant, which lacks a functional SRE. Although the transcriptional regulation of this gene by polypeptide growth factors is mediated by the SIE, induction was also inhibited in the presence of rasN17 (Figure 5B, compare lanes 13–16 and 17–20).

The results above show that activation by the SIE in the context of the c-*fos* promoter requires both activation of STAT factor DNA binding activity and an additional ras-dependent signalling pathway, which must target the SIE and/or other elements in the c-*fos* promoter. To investigate this further, we tested whether activation of our SIE reporter gene is also sensitive to inhibition by rasN17. Cells were transfected with the CAT reporter containing three wild-type SIEs, either with or without the rasN17 expression plasmid. rasN17 expression did not significantly affect induction by IFN-α or IFN-γ, but did inhibit induction by CSF-1 (Figure 5C). In a control experiment, rasN17 also strongly inhibited induction of a FOS–CAT reporter gene by CSF-1 (data not shown). Thus SIE activation by CSF-1, but not IFNs, involves a ras-dependent step (see Discussion).

The Elk-1 TCF can mediate SRE activation by CSF-1

We next examined the involvement of the SRF/TCF ternary complex in the c-*fos* induction. The experiments

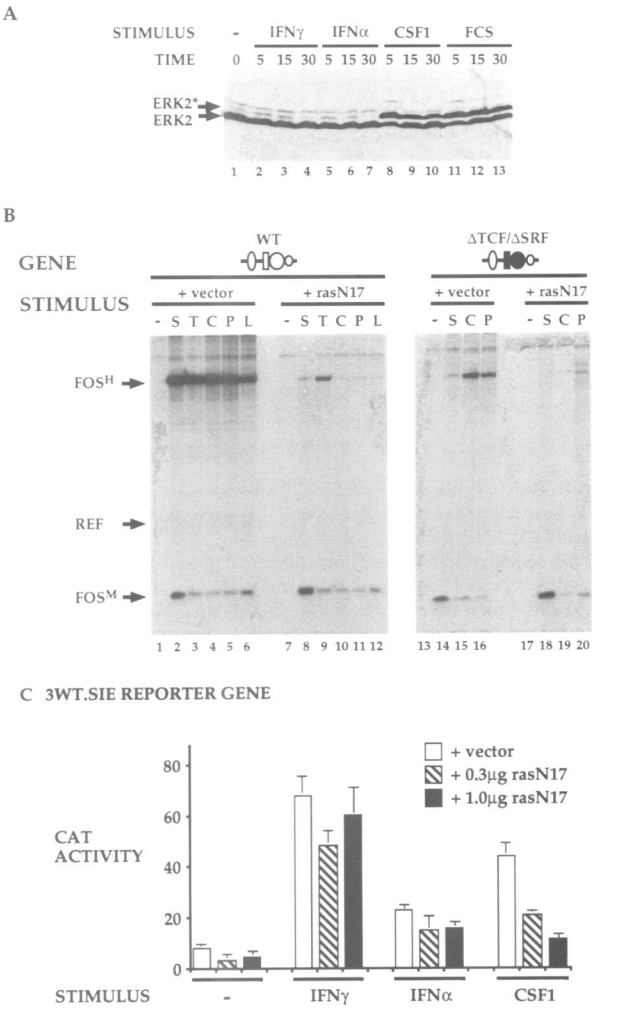

Fig. 5. Differential action of IFNs and CSF-1 in NIH 3T3 clone T56 cells. (**A**) IFN-γ and IFN-α do not activate ERKs. Cells were maintained in medium containing 0.5% FCS for 36 h and total cell lysates were prepared either directly (lane 1) or following stimulation with 1000 U/ml IFN-γ (lanes 2–4), 1000 U/ml IFN-α (lanes 5–7), 107 ng (200 U)/ml CSF-1 (lanes 8–10) or 15% FCS (lanes 11–13) for the times indicated above each lane. Activated ERK2 was detected by immunoblotting as described in Materials and methods. The band above ERK2* is ERK1, which is shifted in lanes 8 and 11. (**B**) Induction by both the intact c-*fos* promoter and the SIE requires ras. Cells were transfected with either the wild-type c-*fos* gene (lanes 1–12) or the ΔTCF/ΔSRF mutant, which retains the SIE (lanes 13–20), together with the α-globin reference plasmid. Transfections also included 10 μg of either the β-globin expression plasmid RSVβ128 (lanes 1–6 and 13–16) or its derivative RSVrasN17 (lanes 7–12 and 17–20). Following maintenance of the cells in medium containing 0.5% FCS for 36 h, total cell RNA was prepared either directly (lanes 1, 7, 13 and 17) or following a 30 min treatment with 15% FCS (lanes 2, 8, 14 and 18), 50 ng/ml TPA (lanes 3 and 9), 107 ng (200 U)/ml CSF-1 (lanes 4, 10, 15 and 19), 25 ng/ml PDGF (lanes 5, 11, 16 and 20) or 10 μM LPA (lanes 6 and 12; LPA will be discussed in detail below). Human c-*fos* (FOS^H), reference α-globin (REF) and endogenous mouse c-*fos* (FOS^M) transcripts were detected by RNase protection, as described in the legend to Figure 2. Note that transcripts from the reference plasmid are slightly reduced by the rasN17 co-transfection. (**C**) IFN induction of SIE reporter gene does not require ras. Cells were transfected with a reporter plasmid containing three copies of the WT.SIE, together with either β-globin expression plasmid RSVβ128 (open bars), 0.3 μg RSVrasN17 (hatched bars) or 1.0 μg RSVrasN17 (solid bars); DNA was kept constant with RSVβ128. Following maintenance of the cells in medium containing 0.5% FCS for 36 h, cells were stimulated with IFNs or CSF-1 as described in Figure 4 legend; extracts were prepared 8 h later and analysed for CAT activity, expressed as percent acetylation. Results are from a representative experiment performed in duplicate; bars indicate estimated standard deviation.

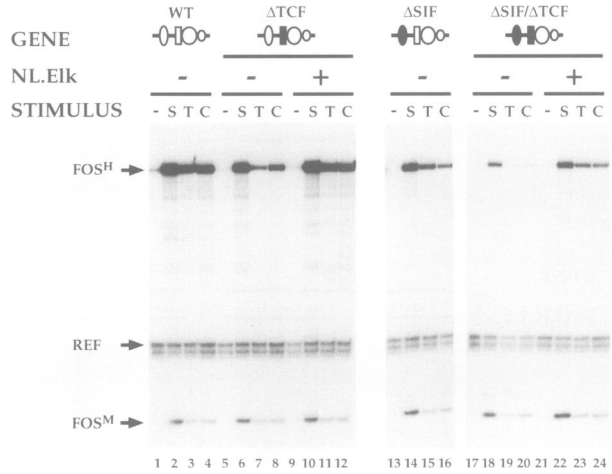

Fig. 6. The TCF Elk-1 can confer CSF-1 responsiveness on the SRE. Cells were transfected with the intact c-*fos* gene (lanes 1–4), or mutants ΔTCF (lanes 5–12), ΔSIF (lanes 13–16) or ΔSIF/ΔTCF (lanes 17–24), together with the α-globin reference plasmid. In addition, transfections included 2 μg of either the β-globin expression plasmid MLVβ128 (lanes 1–8 and 13–20) or its derivative MLV.NLElk, which expresses an altered binding specificity Elk-1 derivative that can bind the ΔTCF mutation (lanes 9–12 and 21–24; Hill *et al.*, 1994). Following maintenance of the cells in medium containing 0.5% FCS for 36 h, total cell RNA was prepared either directly (lanes 1, 5, 9, 13, 17 and 21) or following a 30 min treatment with 15% FCS (lanes 2, 6, 10, 14, 18 and 22), 50 ng/ml TPA (lanes 3, 7, 11, 15, 19 and 23) or 107 ng (200 U)/ml CSF-1 (lanes 4, 8, 12, 16, 20 and 24). Human c-*fos*, reference α-globin and endogenous mouse c-*fos* transcripts were detected by RNase protection as described in the legend to Figure 2; for quantitation see Table I.

summarized in Figure 2 demonstrate that, in addition to activation of the SIE, signals elicited by polypeptide growth factors also activate the SRE, and that this activation requires an intact binding site for TCF. To investigate more directly the role of TCF in this transcriptional response, we used an altered-specificity derivative of the TCF Elk-1. This protein, NL.Elk, can form a ternary complex with SRF at the ΔTCF mutant SRE, and its expression can restore TPA regulation to the ΔTCF gene (Hill *et al.*, 1993, 1994).

To test whether the TCF Elk-1 can mediate the response to polypeptide growth factors, cells were transfected with wild-type or mutant c-*fos* genes together with an expression plasmid that produces the NL.Elk protein. A representative experiment is shown in Figure 6, and the data are summarized in Table I. The ΔTCF mutant gene is impaired in its response to TPA and CSF-1 (Figure 6, compare lanes 3 and 4 with 7 and 8). Expression of NL.Elk significantly restored both these responses, but had little effect on regulation by serum (Figure 6, compare lanes 5–8 with 9–12; see Hill *et al.*, 1994). We next used c-*fos* promoter mutants that lack the SIE to test whether transcriptional activation by NL.Elk also requires factors bound to the SIE. As summarized in Figure 2 and Table I, CSF-1 and TPA inducibility of the ΔSIF gene, which lacks the SIE, is reduced to background levels by additional mutation of the TCF binding site (Figure 6, compare lanes 15 and 16 with 19 and 20); this activity was substantially restored by expression of NL.Elk (Figure 6, compare lanes 19 and 20 with 23 and 24). Again, expression of NL.Elk had relatively little effect on the response of these genes to serum (Figure 6, compare lanes 14, 18 and 22; see Table I). Taken together, these data show that the effects of the ΔTCF mutation can indeed be explained by its effects on TCF binding, and that the TCF Elk-1 can mediate the TCF-dependent response of the c-*fos* promoter to TPA and CSF1.

Table I. Reactivation of c-*fos* promoter mutants by TCF and SRF altered-specificity mutants

Gene	Activator[a]	Induced RNA level[b]		
		Serum	TPA	CSF-1
Wild-type		100[c]	100[c]	100[c]
ΔTCF[d]	none	71 (3)	21 (4)	34 (9)
	NL.Elk	65 (19)	59 (17)	74 (11)
ΔSIF[d]	none	44 (7)	42 (1)	28 (4)
ΔSIF/ΔTCF[d]	none	23 (7)	6 (5)	7 (3)
	NL.Elk	32 (9)	26 (7)	20 (6)
ΔSRF[d]	none	17 (5)	23 (3)	57 (13)
	SRF-M2	49 (14)	77 (17)	107 (9)
ΔTCF/ΔSRF	none	5	10	31
	SRF-M2	9	12	48

[a]Cells were transfected with c-*fos* promoter mutants as described in Materials and methods together with 2 μg MLVplink vector or 2 μg NL.Elk or SRF-M2 expression plasmids.
[b]RNase protection analysis was used to detect c-*fos* and α-globin RNA and data were quantitated using the PhosphorImager. The c-*fos* RNA signal, normalized to that of the reference transcript, is expressed as a percentage of the level of RNA produced by the wild-type c-*fos* promoter in response to the same stimulus. The data are averages from independent experiments: for ΔTCF ± NL.Elk, five experiments; for ΔSIF and ΔSIF/ΔTCF ± NL.Elk, four experiments; for ΔSRF ± SRF-M2, three experiments; for ΔTCF/ΔSRF ± SRF-M2, one experiment. Standard deviations are shown in brackets.
[c]The relative responses of the wild-type gene to the various stimuli were as follows, in abitrary units: serum, 100; TPA, 51 ± 5; CSF-1, 71 ± 10.
[d]The small differences between these data and those in Figure 2 arise because the data presented here use only a subset of the 11 experiments used to compile Figure 2.

An altered-specificity SRF mediates only TCF-dependent c-fos transcription

We previously developed an altered binding specificity derivative of SRF which can bind the ΔSRF mutant SRE used in this study. This protein, SRF-M2, can restore serum regulation to model reporter genes, but only in the context of the ternary complex with TCF (Hill *et al.*, 1993), because the sequence changes in its DNA binding domain prevent it acting independently of TCF (Hill *et al.*, 1994). We used the promoter mutants to test whether other regulatory elements in the c-*fos* promoter could cooperate with SRF-M2 to mediate TCF-independent serum regulation. The results, summarized in Table I, indicated that this is not the case. Co-expression of SRF-M2 restored regulated transcription of the ΔSRF mutant in response to TPA and CSF-1, but not serum, almost to wild-type levels; however, this transcriptional regulation was almost entirely dependent on TCF binding, because it was prevented by mutation of the TCF binding site (Table I). These results suggest that TCF-independent signalling to the promoter requires the intact SRF DNA binding domain, in agreement with our previous findings using model promoters (Hill *et al.*, 1994).

TCF is not required for c-fos induction by LPA

The analysis of c-*fos* promoter mutants summarized in Figures 2 and 6 shows that promoters that lack both intact

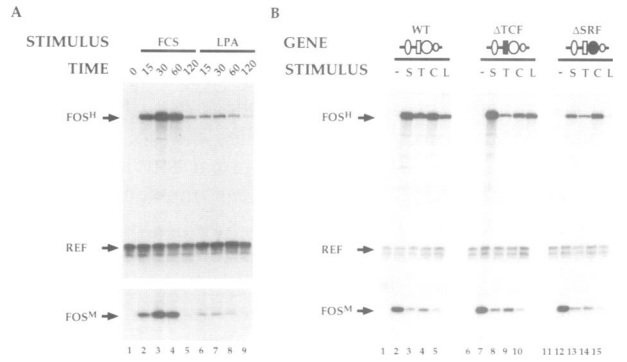

Fig. 7. Activation of the c-*fos* promoter by LPA. (**A**) Kinetics of c-*fos* transcriptional activation in response to serum and LPA. Cells were transfected with the intact c-*fos* gene together with the α-globin reference plasmid. Following maintenance of the cells in medium containing 0.5% FCS for 36 h, total cell RNA was prepared either directly (lane 1) or following treatment with either 15% FCS (lanes 2–5) or 10 μM LPA (lanes 6–9) for the times (in minutes) indicated above the figure. Human c-*fos*, reference α-globin and endogenous mouse c-*fos* transcripts were detected by RNase protection as described in the legend to Figure 2. A longer exposure of the protected products generated by the endogenous mouse c-*fos* gene is shown. Note the similarity between the kinetics of accumulation of RNA from the endogenous and transfected gene. (**B**) Induction by LPA can occur independently of TCF. Cells were transfected with the intact c-*fos* gene (lanes 1–5), the ΔTCF mutant (lanes 6–10) or the ΔSRF mutant (lanes 11–15), together with the α-globin reference plasmid. Following maintenance of the cells in medium containing 0.5% FCS for 36 h, total cell RNA was prepared either directly (lanes 1, 6 and 11) or following a 30 min treatment with 15% FCS (lanes 2, 7 and 12), 50 ng/ml TPA (lanes 3, 8 and 13), 107 ng (200 U)/ml CSF-1 (lanes 4, 9 and 14) or 10 μM LPA (lanes 5, 10 and 15). Human c-*fos*, reference α-globin and endogenous mouse c-*fos* transcripts were detected by RNase protection as described in the legend to Figure 2. The average response to LPA after 30 min was 17% (± 5) of that to serum; the responses of the mutants to LPA stimulation (expressed as a percentage of the level of RNA produced by the wild-type c-*fos* promoter in response to LPA) were as follows: ΔTCF mutant, 119 ± 14; ΔSRF mutant 18 ± 5.

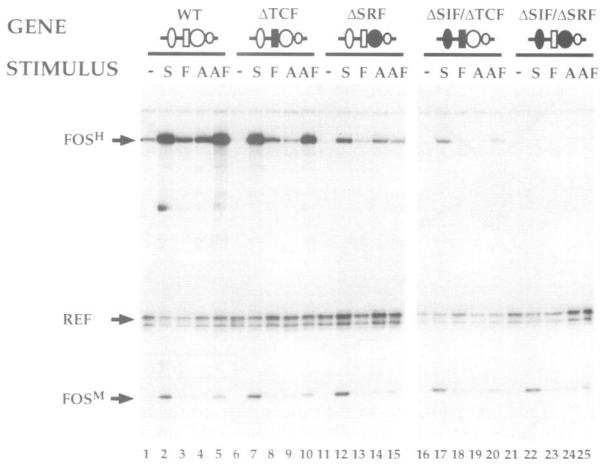

Fig. 8. Activation of the c-*fos* promoter by aluminium fluoride ion. Cells were transfected with the intact c-*fos* gene (lanes 1–5) or mutants ΔTCF (lanes 6–10), ΔSRF (lanes 11–15), ΔSIF/ΔTCF (lanes 16–20) or ΔSIF/ΔSRF (lanes 21–25), together with the α-globin reference plasmid. Following maintenance of the cells in medium containing 0.5% FCS for 36 h, total cell RNA was prepared either directly (lanes 1, 6, 11, 16 and 21) or following a 30 min treatment with 5% FCS (lanes 2, 7, 12, 17 and 22), 10 mM NaF (lanes 3, 8, 13, 18 and 23), 100 μM $AlCl_3$ (lanes 4, 9, 14, 19 and 24) or AlF_4^- (NaF and $AlCl_3$ together; lanes 5, 10, 15, 20 and 25). Human c-*fos*, reference α-globin and endogenous mouse c-*fos* transcripts were detected by RNase protection as described in the legend to Figure 2.

SIE and TCF binding sites retain the ability to respond to serum, albeit weakly, but not the other stimuli tested, and that this response is blocked by mutations that prevent SRF binding. Thus, serum must activate a signalling pathway distinct from those activated by the other stimuli. However, our further investigation of TCF-independent activation of the SRE was hampered by the fact that serum is a complex mixture of growth factors and mitogens. Since the c-*fos* promoter mutants provide a way of ascertaining whether a particular agent can promote TCF-independent SRE activation, we used them to evaluate the ability of defined compounds to induce such signals. We were particularly interested to investigate c-*fos* regulation by the phospholipid LPA, a mitogen that acts through a heterotrimeric G protein-coupled receptor which is present in high concentrations in serum (Jalink *et al.*, 1994; Moolenaar, 1995; see Discussion).

LPA induced transcription of the intact c-*fos* gene, albeit relatively weakly (peak ~17% of serum-induced level; Figure 7A, compare lanes 1–5 and 6–9). We next tested the effect of c-*fos* promoter mutations on LPA-induced transcription. We compared the response of promoter mutants to LPA with their response either to agents that require TCF binding for maximal activation, such as TPA and CSF-1, or to those which require only SRF, such as serum. The responses to serum and LPA were essentially unaffected by mutation of the TCF binding site (Figure 7B, compare lanes 2 and 5 with 7 and 10); however, both were significantly impaired by mutation of the SRF binding site (Figure 7B, compare lanes 2 and 5 with 12 and 15). In contrast, the responses to TPA and CSF-1 were equally impaired by mutation of either the TCF or SRF binding sites (Figure 7B, compare lanes 3 and 4 with 8, 9, 13 and 14). These results show that LPA, unlike TPA or CSF-1, can signal to the SRE independently of TCF binding.

c-fos activation by heterotrimeric G proteins does not require TCF and SIF

Although the properties of LPA are consistent with its action via a heterotrimeric G protein-coupled serpentine receptor, the LPA receptor has not been characterized molecularly (Jalink *et al.*, 1994; Moolenaar, 1995). To confirm that TCF-independent signalling to the c-*fos* promoter can indeed occur as a result of G protein activation, we stimulated cells with AlF_4^-, which binds to GDP-bound G protein α subunits in a manner that mimics the γ phosphate of GTP, leading to subunit dissociation and G protein activation (Bigay *et al.*, 1985, 1987). Cells were transfected with the intact c-*fos* gene or the promoter mutants, and stimulated either with serum or by AlF_4^-, produced *in situ* by the combination of aluminium chloride and sodium fluoride, which were also each tested alone. The results are shown in Figure 8. The intact c-*fos* promoter was induced efficiently by AlF_4^- (Figure 8, compare lanes 2 and 5); aluminium chloride or sodium fluoride also led to a variable weak induction (Figure 8, lanes 3 and 4). As with whole serum and LPA, mutation of the TCF binding site had little effect upon induction by AlF_4^- (Figure 8, compare lanes 2 and 5, and 7 and 10); the ΔTCF mutation did, however, impair c-*fos* induction by aluminium chloride or sodium fluoride alone (Figure 8,

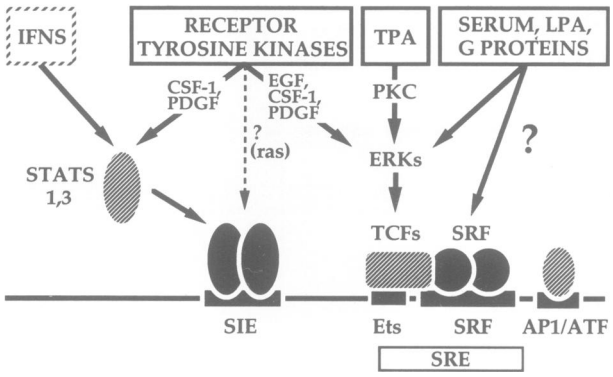

Fig. 9. Signalling pathways to the c-*fos* upstream regulatory region in NIH 3T3 clone T56 cells. Stimuli are boxed, with the hatched box indicating that IFNs do not induce c-*fos* transcriptional activation. Signalling pathways are shown by arrows. The dotted arrow indicates that STAT factors are subject to additional growth factor-specific *ras*-dependent regulation. Signalling pathways to other c-*fos* promoter elements that are activated by these stimuli are omitted. The SIE also contributes to c-*fos* activation by serum, TPA and EGF, which do not induce STAT DNA binding activity detectably in these cells; for discussion see text.

lanes 8 and 9). In contrast to the ΔTCF mutant, the ΔSRF mutant was significantly impaired in its response to both serum and AlF_4^- (Figure 8, compare lanes 2 and 5 with 12 and 15); in this case the combination of aluminium chloride and sodium fluoride was no more effective than either agent alone (Figure 8, lanes 13–15). Finally, we tested ΔSIF/ΔTCF, the mutant gene which can bind SRF but neither TCF nor STATs. In addition to its response to serum, this gene remains inducible by AlF_4^- (Figure 8, compare lanes 16 and 20); this response requires an intact SRF binding site as it is abolished in the ΔSIF/ΔSRF mutant (Figure 8, compare lanes 16–20 with 21–25). Taken together, these results suggest that serum, LPA and heterotrimeric G proteins activate a signalling pathway that potentiates the activity of SRF independently of TCF.

Discussion

We used c-*fos* promoter mutants and transient transfection assays to investigate the roles of different transcriptional regulatory elements in the response of the promoter to different signalling pathways. Our results are summarized in Figure 9. We find that two regulatory sites, the SIE and the SRE, cooperate in reception of growth factor-induced signals at the promoter. Some stimuli, such as CSF-1 and PDGF, both activate STAT factors and potentiate SRE activity via TCF; however, the SIE also contributes to activation by stimuli such as TPA, serum and EGF that do not detectably activate the STATs in our cells. Serum activates two pathways to the SRE: one, involving the ERKs, acts on the TCF component of the SRF/TCF ternary complex, while the other acts on SRF itself and can function independently of TCF binding. LPA, a mitogen present at high concentrations in serum, and AlF_4^-, a global activator of heterotrimeric G proteins, can also activate SRF independently of TCF, suggesting that this pathway may be specific to receptors acting through heterotrimeric G proteins.

Signalling at the SIE

While the regulation of SIE binding activity in response to growth factor stimuli has been characterized in some detail, surprisingly little work has been done to elucidate the role of the SIE in regulation of the c-*fos* promoter. Growth factor-regulated activation of transcription by the SIE was reported previously (Wagner *et al.*, 1990; Fu and Zhang, 1993); however, these studies did not assess the contribution of the SIE to transcriptional activation of the c-*fos* promoter itself. In the transient transfection assay, we found that the SIE contributed substantially to c-*fos* promoter activation by those polypeptide growth factors which induce STAT binding activity; these stimuli also activated an SIE-controlled reporter gene. Surprisingly, we found that the SIE also contributed to transcriptional induction by serum, TPA and EGF, none of which induced detectable STAT DNA binding activity (see below). When combined with mutations that block binding of TCF, SIE mutations reduced activation of the c-*fos* promoter in response to TPA and the growth factors to background levels. Our results are consistent with previous studies which have demonstrated that SRE mutations affect the PDGF response less than the serum response (Gilman, 1988; C.Alexandre and M.Gilman, personal communication).

The finding that the SIE contributes to c-*fos* transcriptional induction by EGF, serum and TPA cautions against a simple correlation of STAT activity and transcription, as these agents do not significantly activate STAT DNA binding activity. The result with EGF is puzzling since, in A431 cells, which overexpress the EGF receptor, EGF activates STAT DNA binding (Herrera *et al.*, 1989; Sadowski and Gilman, 1993), while in COS cells STAT1 overexpression can potentiate the EGF-stimulated activity of an SIE reporter gene (Fu and Zhang, 1993). EGF may induce STAT binding in our cells but at a level below that detectable in the gel mobility-shift assay. However, this mechanism is unlikely in the cases of serum and TPA, since these agents neither activate a simple SIE-controlled reporter gene, nor affect genomic footprints at the SIE *in vivo* (Herrera *et al.*, 1989; V.Bardwell and R.Treisman, unpublished data). Instead, the contribution of the SIE to activation by serum and TPA may reflect the binding of a positively acting but non-regulated factor distinct from the STATs: indeed, other factors have been observed to interact with the authentic c-*fos* SIE in gel mobility-shift assays (Sadowski *et al.*, 1993). Both transfection assays and studies with mice containing c-*fos–lacZ* transgenes demonstrate a role for the SIE in response to stimuli not associated with STAT activation (Berkowitz *et al.*, 1989; Robertson *et al.*, 1995). In fibroblasts from these mice, the SIE appears absolutely required for response of the transgene to both TPA and serum, while the SIE is also required for kainate-induced c-*fos* expression in certain parts of the brain (Robertson *et al.*, 1995). In this study, the dependence of transcription of the SIE was more striking than in our experiments, perhaps owing to differences in template copy number or chromatin structure in the two assay systems. We are currently using oligonucleotide microinjection to evaluate the contributions of SIE binding factors to induction of the chromosomal c-*fos* and other immediate-early genes.

Our data demonstrate that transcriptional activation by the SIE is dependent on promoter context: IFN-α induces STAT1 and STAT3 binding activity more effectively than CSF-1 or PDGF in our cells, yet fails to activate c-*fos*

transcription efficiently. In contrast, a simple SIE-controlled reporter gene could be activated by both CSF-1 and IFNs, and the comparatively strong induction of this reporter by IFN-γ, which induces STAT1 activity, provides evidence that STAT1 can bind to and activate the authentic c-*fos* SIE efficiently *in vivo*. Our results suggest that CSF-1 must generate a signal, distinct from those activated by IFNs, which is required for c-*fos* promoter activation. This signal probably involves the ras–raf–ERK pathway: SIE-dependent c-*fos* promoter activation is inhibited by the interfering ras mutant rasN17, and CSF-1, but not IFNs, induces ERK activation.

ras-dependent signals could affect STAT activity in either or both of two ways. First, ras-dependent activation of other c-*fos* promoter elements, such as the AP1/ATF site or –65 CRE element (see Ginty *et al.*, 1994), may be required for activation by STAT complexes remote from the TATA box, such as the SIE, even though these complexes are competent to activate transcription from TATA-proximal sites such as those in the reporter gene. Second, the signal may target the STAT complexes themselves. Recent reports suggest that serine phosphorylations modulate transcriptional activation by STAT3 (Lutticken *et al.*, 1995), and regulate STAT3 DNA binding activity (Zhang *et al.*, 1995). ras-dependent modifications cannot be essential for activation of transcription by STAT1 or STAT3 complexes, since reporter gene activation by IFNs is insensitive to rasN17. It is likely that they potentiate their activity, however, since CSF-1 induction of the SIE reporter is inhibited by rasN17. Further experiments will be necessary to elucidate the contribution of secondary signalling pathways to STAT-mediated activation of IFN- and cytokine-regulated promoters.

Signalling at the SRE

Our data show that TPA and the polypeptide growth factors CSF-1, EGF and PDGF activate the SRE via TCF; the role of SRF thus appears to be predominantly that of a recruitment factor for TCF. However, only mutations that block both SRF and TCF binding substantially impair the response of the whole gene to serum, LPA or AlF_4^- stimulation, indicating that these agents can signal to the SRE via an SRF-linked signal pathway. All these agents also activate TCF-linked signalling pathways (Hill *et al.*, 1993, 1995; M.A.Price and R.Treisman, unpublished data). Curiously, we found that mutations which block both SRF and TCF binding apparently have a smaller effect on induction by polypeptide growth factors than mutations that block TCF binding alone. This may reflect the fact that when SRF binding is prevented, the neighbouring AP1/ATF site becomes accessible to factors that cannot bind when SRF is present (Shaw *et al.*, 1989b; V.Bardwell and R.Treisman, unpublished observation); indeed, others have reported that the AP1/ATF site appears growth factor responsive in this situation (Fisch *et al.*, 1989).

In the case of CSF-1, the defective c-*fos* activation caused by mutation of the TCF binding site can be restored by expression of an appropriate altered-specificity mutant of the TCF Elk-1. We and others have previously used a similar approach to show that Elk-1 can also mediate activation of the promoter by oncogenic raf kinase, EGF and TPA (Hill *et al.*, 1994; Kortenjann *et al.*, 1994; Gille *et al.*, 1995). In addition, our altered-specificity derivative of Elk-1 can also mediate serum-induced signals to the promoter in conjunction with an SRF derivative which is deficient in mediating the SRF-linked signalling pathway. Taken together, the results provide further evidence that the TCF site in the c-*fos* SRE is a major target for signalling to the promoter in response to activation of the ras–raf–ERK pathway, whether by receptor tyrosine kinases, TPA or whole serum.

To investigate the role of SRF in SRE function, we previously developed an altered-specificity derivative of SRF, SRF-M2, which binds the mutated SRE present in the ΔSRF mutant described here. Our data show that SRF-M2 requires TCF to restore serum-regulated activation to an SRE containing the ΔSRF mutation, both in the context of an SRE-controlled reporter gene (Hill *et al.*, 1993), and the c-*fos* promoter itself. However, analysis of the c-*fos* promoter mutants shows that TCF binding is apparently not required for serum-induced promoter activation. We suggest that this apparent paradox can be explained by previous studies, both by ourselves and others, demonstrating that SRF can confer serum-regulated transcriptional activation independently of TCF, but only when bound to DNA via its own, intact DNA binding domain, which is mutated in SRF-M2 (Hill *et al.*, 1994; Johansen and Prywes, 1994). We have attempted to adapt the assay used in the latter work to the c-*fos* promoter itself but without success, probably owing to the inefficiency and relatively small range of the assay (8–10% wild-type SRE activity restored; 10- to 20-fold induction of c-*fos* RNA compared with 50- to 100-fold induction of reporter gene activity; Hill *et al.*, 1994). Further work will clarify the role of the SRF DNA binding domain in transcriptional regulation.

The availability of c-*fos* promoter mutants that retain the ability to respond to serum but which are refractory to stimulation by TPA or polypeptide growth factors provided a way to identify purified growth factors and mitogens that can activate SRF independently of TCF. Two considerations led us to speculate that these agents might act through serpentine receptors. First, the abundant serum mitogen LPA has properties consistent with its action through such a receptor (Moolenaar, 1995). Second, in *Saccharomyces cerevisiae*, many of the genes regulated by the SRF-related protein MCM1 are controlled by mating pheromone via a signalling pathway activated by a serpentine receptor. However, although the pheromone pathway involves a putative SRF homologue, it does not involve a TCF homologue, suggesting that it might represent an evolutionarily ancient counterpart of the TCF-independent signalling pathway (Yuan *et al.*, 1993; for review, see Schultz *et al.*, 1995). Consistent with these speculations, we found that LPA can indeed activate the SRE independently of TCF. While this work was in progress, others reported that LPA can activate SRE-controlled reporter genes (Perkins *et al.*, 1994), although in this case the contribution of TCF to transcriptional activation was not assessed. Consistent with the notion that an SRF-linked signalling pathway can be activated by heterotrimeric G proteins, we found that AlF_4^-, which activates these proteins intracellularly, also activates the SRE independently of TCF. LPA activates multiple signalling pathways, including activation of the ras–raf–ERK pathway, phospholipase C, tyrosine kinases and the small GTPase Rho, a member of the Ras superfamily of

small GTPases (Jalink *et al.*, 1994; Moolenaar, 1995). In a related study, we have shown that members of the Rho family of small GTPases can activate SRF independently of TCF, and that both serum- and LPA-induced signalling to SRF is dependent on RhoA (Hill *et al.*, 1995). It is intriguing to note that the mammalian homologue of STE20, a kinase involved in the pheromone pathway, is regulated directly by the Rho family members CDC42hs and rac1 (Manser *et al.*, 1994). It will be interesting to compare the mammalian SRF-linked signalling pathway with the yeast pheromone signalling pathway.

Materials and methods

Cell culture

NIH 3T3 cells expressing the human CSF-1 receptor (Clone T56; Roussel *et al.*, 1990), a generous gift from Martine Roussel (St Jude Children's Hospital, Memphis, TN), were maintained in DME/10% fetal calf serum (FCS) containing 400 μg/ml G418. Similar results, except for those obtained with CSF-1, were obtained using NIH 3T3 cells. Cells were stimulated as follows: human PDGF-BB (Calbiochem) 25 ng/ml, mouse EGF (Calbiochem), 50 ng/ml; human CSF-1 (Genetics Institute, Cambridge, MA), 107 ng (200 U)/ml; LPA [Sigma; 2.4 mM stock in phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA)], 10 μM; $AlCl_3$, 100 μM; NaF (stock solution stored in plastic), 10 mM; AlF_4^- (10 mM NaF/100 μM $AlCl_3$); mouse IFN-α (recombinant human A/D), 1000 U/ml; mouse recombinant IFN-γ, 1000 U/ml; TPA (Sigma), 50 ng/ml.

Transfections and analysis of gene expression

Cells were transfected using the DEAE–dextran technique as previously described (Hill *et al.*, 1993, 1994). Except where indicated in the figure legends, 14 μg DNA was transfected per 9 cm dish, which included 10 μg human c-*fos* plasmid and 4 μg of the plasmid pSVKα, which expresses the human α1-globin gene under control of the SV40 early promoter (Treisman *et al.*, 1983). After 36–40 h, cells were then stimulated as above, generally for 30 min. Total cell RNA was prepared and c-*fos* and reference α-globin transcripts analysed by RNase protection (Hill *et al.*, 1994; Treisman, 1985). Protected fragments were generated as follows: human c-*fos*, 287 nucleotides (mRNA cap to *Hin*cII, exon 1); human α-globin, 95 nucleotides (α-globin initiation codon to 3′ end exon 1); mouse c-*fos*, ~60 nucleotides (from partially protected exon 1). Human c-*fos* RNA was quantified relative to the α-globin reference RNA using the PhosphorImager. CAT assays were performed exactly as described (Hill *et al.*, 1993, 1994); 4 μg TK–CAT reporter plasmid was used with 1 μg MLVlacZ reference plasmid.

Plasmids

All DNA manipulations were carried out by standard techniques and the plasmid structures were verified by sequencing. The c-*fos* promoter mutants are derivatives of pF711, which contains the entire human c-*fos* gene including 711 bp of 5′ flanking sequences (Treisman, 1985): sequences of the mutations are given in Figure 1. The mutants are named according to which transcription factor binding sites are mutated, i.e. ΔSIF/ΔTCF contains both the ΔSIF and ΔTCF mutations. The following plasmids were as described previously: MLVSRF-M2, MLV128β, MLVNL.Elk (Hill *et al.*, 1993); pF711ΔTCF (Hill *et al.*, 1994); human α-globin reference plasmid pSVKα (Treisman *et al.*, 1983).

RSVplink was derived from MLVplink (Dalton and Treisman, 1992) by replacement of the murine leukaemia virus (MLV) enhancer and globin promoter and 5′ untranslated sequences with the Rous sarcoma virus (RSV) long terminal repeat (R.Treisman, unpublished). RSVrasN17 and RSVβ are derivatives of RSVplink that express the human Ha-rasN17 mutant and human β-globin gene respectively.

For c-*fos* RNA probe preparation, a derivative of pSP6Fos 5′ (Treisman, 1985), which contains sequences from the *Eco*RI linker at −711 to the exon I *Hin*cII site, was linearized by *Bss*HII; for α-globin probe, SP6α132 was linearized by *Bam*HI (Treisman, 1985).

TK–CAT reporter plasmids controlled by two or three copies of the wild-type human c-*fos* SIE (SIE.WT) in the same sense as in the c-*fos* promoter, or two copies of the mutant SIE oligonucleotide (SIE*) in reverse orientation were constructed by inserting SIE oligonucleotides into the *Xba*I site of pBLCAT2Bam$^-$ (Treisman *et al.*, 1992).

SIE oligonucleotides

The following oligonucleotide pairs were annealled before use: SIE*, ctagCAGTACGCCTTAATC/ctagGATTAAGGCGTACTG; WT.SIE, ctagCAGTTCCCGTCAATC/ctagGATTGACGGGAACTG; M67 SIE, ctagCATTTCCCGTAAATC/ctagGATTTACGGGAAATG.

Underlining indicates divergence from the sequence of the human c-*fos* SIE; lower case indicates linker sequence.

Extract preparation and analysis

Whole cell extract preparation and gel mobility-shift analysis were done using a modification of previous methods (Marais *et al.*, 1993; Sadowski and Gilman, 1993). Extract protein (15 μg) in 10 μl 20 mM HEPES pH 7.9, 2.6 mM EDTA, 5.2 mM EGTA, 200 mM KCl, 0.2% Triton X-100, 1 mM dithiothreitol (DTT), 0.2% NP-40 and 10% glycerol with protease and phosphatase inhibitors as described (Marais *et al.*, 1993) was incubated alone or with 1 μl of antibody or competitor oligonucleotide at room temperature for 20 min. Non-specific competitor poly(dI–dC)·poly(dI–dC) (1 μg) and M67 SIE probe (20 fmol) were added in 10 μl of 4 mM HEPES, 2% glycerol, 0.2 mM DTT with protease inhibitors, and the reaction continued for 20 min. Complexes were resolved in a 5% 40:1 acrylamide:bis-acrylamide 0.5× Tris–borate–EDTA gel containing 2.5% glycerol. M67 SIE probe was generated by end repair of annealled M67 oligonucleotides with radiolabelled dNTPs. The anti-STAT1 antibody recognizes the C-terminal 38 amino acids of STAT1α; the anti-STAT3 antibody recognizes residues 688–727 of STAT3.

Assessment of ERK2 activation was carried out by lysis of cells directly into SDS–PAGE loading buffer, sonication and fractionation on a 15% 200:1 acrylamide:bis-acrylamide gel followed by immunoblotting and detection using pan-ERK antiserum (Transduction Laboratories No. E17120).

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