The EMBO Journal vol.9 no.13 pp.4477 – 4484, 1990

# The SIF binding element confers *sis*/PDGF inducibility onto the c-*fos* promoter

**Belinda J.Wagner, Timothy E.Hayes, Carolyn J.Hoban and Brent H.Cochran**

MIT Center for Cancer Research and Department of Biology, 77 Massachusetts Ave E17-517, Cambridge, MA 02139, USA

Communicated by P.A.Sharp

**The c-*fos* proto-oncogene is rapidly and transiently induced by a variety of extracellular stimuli. We have previously shown that conditioned media from v-*sis* transformed NRK cells rapidly induces a DNA binding protein which binds to a conserved sequence upstream of the human c-*fos* gene. We now show that purified recombinant c-*sis*/PDGF can induce this binding activity which we have termed SIF, for *sis*-inducible factor. Oligonucleotides which bind to the SIF protein will confer *sis*/PDGF inducibility onto a truncated, unresponsive c-*fos* promoter. However, sequences lying between −100 and −57 of the c-*fos* gene are required for this induction. The *sis*-responsive element functions independently of a region of dyad symmetry previously identified as the serum responsive element (SRE). The time course of c-*fos* expression driven by the *sis*-responsive element is similar to that mediated by the SRE. Unlike the SRE, which can respond to signals generated by *sis*/PDGF, serum or phorbol esters, the SIF binding element mediates c-*fos* induction only in response to *sis*/PDGF. The SRE and SIF elements function in an additive manner to stimulate the transcription of the c-*fos* gene in response to *sis*/PDGF.**

*Key words:* c-*fos*/PDGF/*sis*/SIF/SRE

## Introduction

The c-*fos* proto-oncogene is regulated by a variety of extracellular stimuli in a variety of cell types (Verma and Sassone-Corsi, 1987). Its expression is regulated at the transcriptional level, and is typically induced in a transient fashion with maximal expression occurring only 30 min after stimulation (Greenberg and Ziff, 1984). By ~60 min after stimulation, c-*fos* transcription returns to basal levels. c-*fos* expression is capable of being induced by a number of different agents including growth factors, serum, cyclic AMP (cAMP) and calcium ionophore (Cochran *et al.*, 1984; Curran and Morgan, 1985; Fisch *et al.*, 1989b; Gilman, 1988; Sheng *et al.*, 1988).

In order to respond to such a diverse set of stimuli, the c-*fos* gene contains a number of regulatory elements. At −60 there is an element which has been implicated in regulation by cAMP, in basal level expression of the *fos* gene, and in responses to calcium (Gilman *et al.*, 1986; Fisch *et al.*, 1987, 1989a; Gilman, 1988; Sheng *et al.*, 1988). This element can bind to both the CREB factor and AP1 (Schonthal *et al.*, 1989). Another element (termed the serum response element) is a region of dyad symmetry which lies between −320 and −299 of the human gene. This element confers serum responsiveness on to the c-*fos* promoter (Treisman, 1985; Gilman *et al.*, 1986; Prywes and Roeder, 1986; Greenberg *et al.*, 1987). This element binds to a constitutive cellular factor (the serum response factor), which is sometimes complexed to a 62 kd protein termed 'ternary complex factor' (Treisman, 1986; Hayes *et al.*, 1987b; Shaw *et al.*, 1989b). The mechanisms by which the complexes formed over the serum response element (SRE) mediate the transcriptional activation of c-*fos* after serum stimulation are not understood. In addition, there are other elements which can induce c-*fos* in response to the v-*raf* kinase (Jamal and Ziff, 1990; Kaibuchi *et al.*, 1989).

Previously, we have described a DNA binding factor which binds to a conserved sequence at a position lying 346 bp upstream of the c-*fos* promoter (Hayes *et al.*, 1987a). Its DNA binding activity is induced when quiescent Balb/c-3T3 cells are treated with conditioned media from v-*sis* transformed NRK cells. Since this induction is independent of new protein synthesis, this factor is likely to be an intermediate in the pathway which leads from the PDGF receptor to the c-*fos* gene. In this report, we show that this factor is induced by pure recombinant c-*sis* and that its cognate DNA binding element is sufficient to confer *sis*/PDGF inducibility onto the c-*fos* promoter in the absence of the SRE.

## Results

Figure 1 shows the results of an experiment where quiescent Balb/c-3T3 cells were treated for 0.5 h with purified

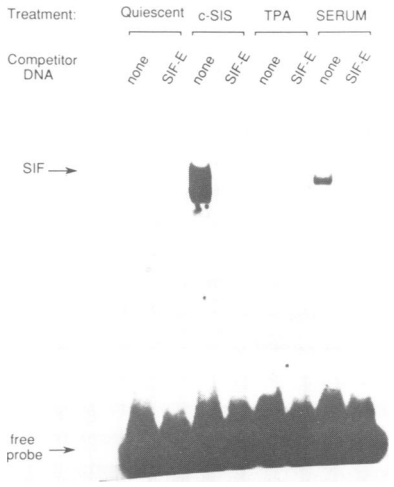


**Fig. 1.** Specificity of SIF induction. Balb/c-3T3 subclone A31 fibroblasts were grown to confluence in DME/10% calf serum and maintained in DME/5% platelet-poor plasma (PPP) for 48 h. Treatment with purified *sis* protein (25 ng/ml, Amgen), phorbol myristate acetate (50 ng/ml; TPA) or calf serum (20%) was in DME/5% PPP for 30 min. Nuclear extracts were prepared and gel shift assays were performed as described in Materials and methods. The sequence specific complex is indicated with an arrow.

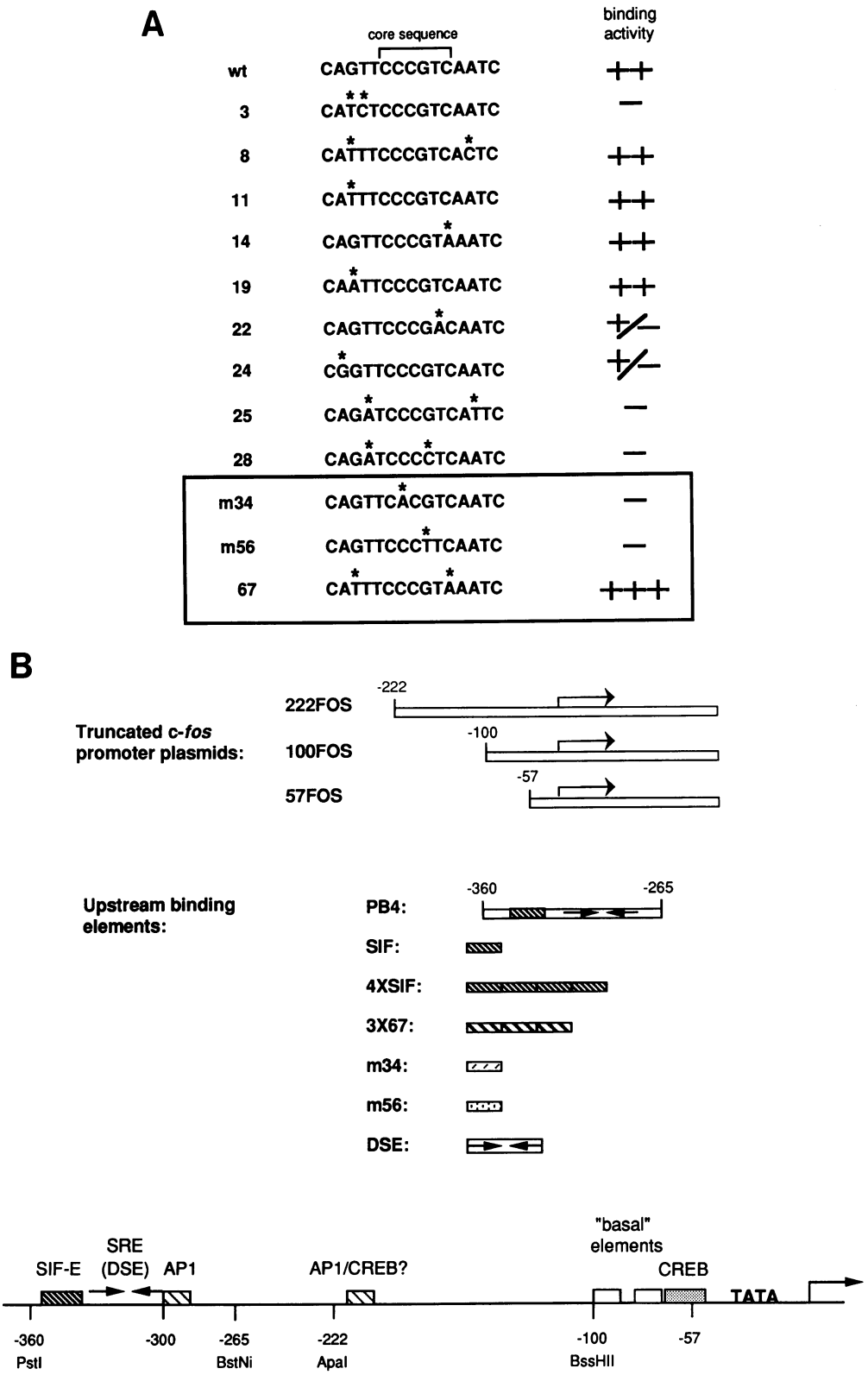


**Fig. 2.** (**A**) Mutational analysis of the SIF binding site. Point mutations in the SIF binding site were generated and $^{32}$P-body labelled DNA probes were made from each mutation as described in Materials and methods. Each probe was used to assay cellular extracts containing SIF proteins. Relative binding activities were judged as compared with the wild-type SIF element used as a probe. The asterisks above the sequences indicate deviations from the wild-type sequence. The mutations that are within the box were made into oligonucleotides which were used for further experiments in this study. (**B**) Plasmids used for transfection experiments. Oligonucleotides containing various wild-type and mutant SIF elements were cloned upstream of the human c-*fos* gene truncated at −222, −100 or −57 as described in Materials and methods and shown in the diagram. M34, m56, and 3X67 refer to the oligonucleotide sequences shown in panel A. The diagram below illustrates the c-*fos* promoter sequences between +1 and −400 bp. See text for explanation of sites in the promoter. Distances from the start site of transcription are indicated along with the restriction enzyme sites used for cloning. The copy number of each element cloned upstream of the constructs are indicated in the diagram.

recombinant c-*sis*, TPA, or 25% calf serum, and assayed for the induction of *sis*-inducible factor (SIF) DNA binding activity. From Figure 1, it can be seen that purified recombinant c-*sis* protein gives the best induction of the SIF binding factor (30-fold), whereas TPA and calf serum only weakly induce SIF−DNA binding (2- to 3-fold). Thus, SIF−DNA binding activity is specifically inducible by c-*sis* in Balb/c-3T3 cells.

To correlate SIF−DNA binding with the function of the SIF element, a series of point mutations in the SIF element were generated which have altered abilities to bind to SIF. Each of these mutant DNAs was assayed by band shift gel for their ability to bind SIF (data not shown). Figure 2A shows the sequence of these mutant SIF binding sites, and gives a relative indication of their affinity for SIF. M34 and M56 are point mutants which have no detectable SIF−DNA binding activity. M67 has enhanced DNA binding activity when used as a probe in a gel shift assay. This increase in binding is due to the combination of a slightly increased affinity and the elimination of a major non-specific DNA binding activity. Purified SIF preparations show a 2- to 3-fold increase in binding affinity for M67 relative to wild-type sequences (unpublished results).

To determine the function of the SIF element, wild-type and mutant SIF binding elements were positioned upstream of a human c-*fos* promoter truncated at −222, as diagrammed in Figure 2B. The 222FOS construct lacks the SIF element, the serum response element, and adjacent AP1 site, yet retains elements at −60 required for basal level expression. These DNAs were transfected into NIH 3T3 cells, along with an α-globin gene as a constitutively expressed internal control. After transfection, the cells were made quiescent by incubation in 0.5% calf serum for 48 h before being stimulated with purified c-*sis*, 20% calf serum, or the tumor promoter TPA. After 30 min, RNAs were isolated from the cells and assayed for expression of the human c-*fos* gene and the α-globin gene by an RNase protection assay. The results are shown in Figure 3.

As can be seen from Figure 3, when the 222FOS construct alone is transfected into cells and treated with these agents, there is no expression of the human c-*fos* mRNA. However, control α-globin mRNA is detected, indicating that the c-*fos* gene was successfully introduced into these cells. In addition, the human c-*fos* probe hybridizes to a small fragment of the endogenous mouse c-*fos* gene which can be visualized at the bottom of the gel. From the figure it can be seen that as expected, TPA, c-*sis*, and calf serum all induced the endogenous c-*fos* gene under these conditions.

When a single copy of the wild-type SIF element is positioned upstream of the −222 construct and introduced into NIH 3T3 cells, a different result is seen. A strong induction of the human c-*fos* gene is seen when these cells are treated with purified c-*sis*. However, only a weak induction is detected when cells transfected with this plasmid are treated either with the tumor promoter TPA or with 20% calf serum.

When oligonucleotides having sequences corresponding to the non-binding point mutants M34 and M56 are cloned upstream of the −222−*FOS* construct and assayed, induction of human c-*fos* by *sis*/PDGF is abolished. Since neither of these elements is capable of binding to the SIF protein *in vitro*, this indicates that the ability to bind the SIF protein is correlated with the ability of *sis*/PDGF to induce c-*fos*.

When multiple copies of the M67 element are cloned upstream of the c-*fos* gene, again induction in response to c-*sis* treatment is seen. This element, though mutant, continues to bind to SIF *in vitro*. Thus, in the absence of the c-*fos* SRE, the SIF binding element specifically confers *sis*/PDGF responsiveness onto the c-*fos* promoter. This result is different from that which is seen for the SRE by itself, which can respond to both serum and TPA (Gilman, 1988, and Figure 5B; Siegfried and Ziff, 1989).

The results of the experiment (shown in Figure 3) were quantitated by densitometric analysis and normalized to the expression of the α-globin internal control. Induction by c-*sis* of the SIF/222 gene constructs is 4- to 8-fold higher than the *sis* induction of either 222FOS alone or with the non-binding mutant SIF elements. Multiple copies of the SIF element double the induction of c-*fos* relative to that given by a single copy. Serum or TPA result in no significant induction through the SIF element.

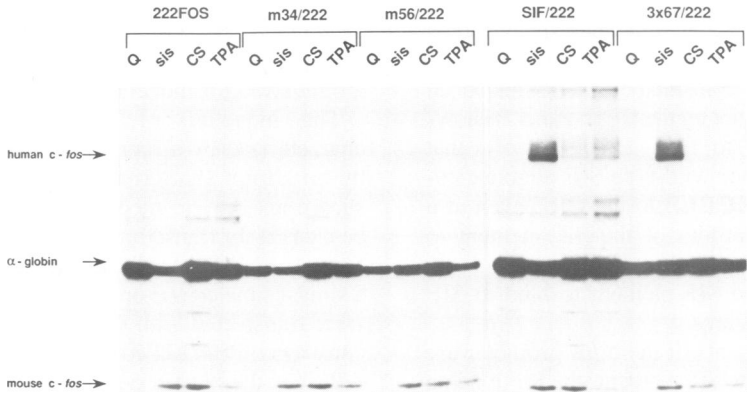


**Fig. 3.** The SIF binding element confers *sis* inducibility onto a truncated c-*fos* promoter. The indicated plasmids were transfected into NIH 3T3 cells, along with plasmid pSVA1, which expresses an α-globin gene as a control, as described in Materials and methods. After 48 h in 0.5% calf serum, the cells were treated with 25 ng/ml recombinant c-*sis* B-chain (Amgen), or 200 ng/ml TPA, or 20% calf serum for 30 min. RNA was isolated and used in RNase protection assays, as described in Materials and methods. The positions of the human c-*fos*, α-globin, and the mouse endogenous c-*fos* protected fragments are indicated by arrows.

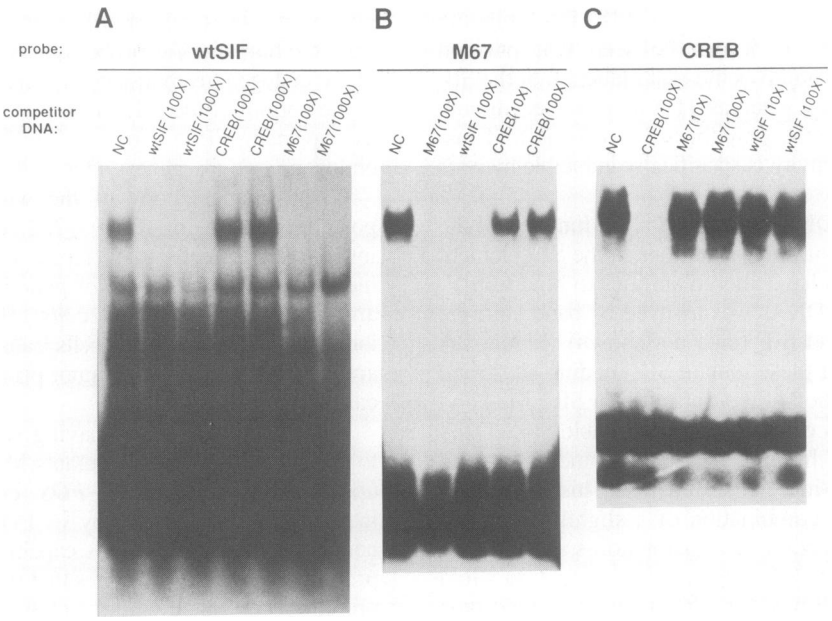


**Fig. 4.** The cAMP response element does not compete for binding to SIF and *vice versa*. Extracts of Balb/c-3T3 cells treated with v-*sis* conditioned media (Hayes *et al.*, 1987a) for 30 min were assayed for the SIF and CREB DNA binding proteins as described in Materials and methods. The oligonucleotide probes and competitor DNAs are indicated with the fold excess of competitor DNA indicated in parentheses. NC indicates that no competitor DNA was included in the binding reaction. (**A**) Binding to the wild-type SIF probe. (**B**) Binding to the high affinity M67 SIF probe. (**C**) Binding to the cAMP response element of the rat somatostatin gene.

The SIF recognition sequence overlaps a sequence which has homology to the cAMP response element [TGACGggA (6/8) on the non-coding strand] including a perfect match to the CGTCA core motif (Deutch *et al.*, 1988; Fink *et al.*, 1988). This site has a weak affinity for cAMP response element (CRE) binding proteins (Berkowitz *et al.*, 1989). Therefore, it seemed possible that the induction through the SIF element could be mediated by a CRE binding protein rather than by SIF. However, the M67 SIF element which binds SIF and mediates *sis*/PDGF induction of c-*fos* has a point mutation in the core CRE homology sequence (CGTaA), making it unlikely that this mutant element would bind CREB. To evaluate this possibility, the binding of SIF and CREB to the wild-type SIF element, M67, and an authentic CRE from the rat somatostatin gene (Yamamoto *et al.*, 1988) was assayed by band shift analysis as shown in Figure 4. Panel A shows that SIF binding to the wild-type SIF element is effectively competed by excess unlabelled self-competitor and M67, but not by the CRE. Panel B shows the same pattern for binding to M67. Panel C shows that neither the unlabelled M67 nor wild-type SIF oligonucleotides compete for binding of CREB to the CRE even at a 100-fold excess. Thus, the ability of the SIF element to confer *sis*/PDGF inducibility onto the c-*fos* promoter is correlated with the ability of the SIF element to bind to SIF and not to CRE binding proteins.

To determine if other elements of the c-*fos* promoter are necessary for the activity of the SIF element, the SIF binding elements and the SRE were cloned upstream of a c-*fos* construct deleted at −100 and −57, transfected into NIH 3T3 cells along with an α-globin control plasmid, and stimulated with either c-*sis*, calf serum, or TPA. From Figure 5A, it can be seen that when the SIF element is positioned at −100, it is still able to confer *sis*/PDGF responsiveness onto the c-*fos* promoter. Interestingly, the SRE at this position responds better to calf serum than TPA or c-*sis*/PDGF. This result is in contrast to the nearly equal inductions given by these three treatments when the SRE is at position −222 (Figure 5B) and similar to that seen when the SRE is placed upstream of a β-globin reporter gene (Siegfried and Ziff, 1989). Thus, there must be a sequence between −222 and −100 which is required to cooperate with the SRE to give the maximal response to TPA and PDGF. From Figure 5B, it can be seen that the SIF element positioned at −57 in four copies confers no induction upon the c-*fos* gene. The same result is seen with a single copy of the SIF element at this position (data not shown). However, at the same position (which is just upstream of the TATA box), the SRE by itself is responsive to calf serum, although only weakly responsive to c-*sis* treatment. Neither element confers TPA responsiveness at this position. Thus, sequences between −100 and −57 of the c-*fos* gene are required for induction of c-*fos* through the SIF element.

The induction of c-*fos* by extracellular agents is characterized by a rapid increase in transcription followed by an abrupt transcriptional shut-off. The SRE has been shown not only to mediate induction by serum but also to be a target for transcriptional repression (Konig *et al.*, 1989; Shaw *et al.*, 1989a). To determine if the SIF element confers a similar time course onto the c-*fos* promoter, NIH 3T3 cells were transfected with the SIF/222, SRE/222 and PB4/222 constructs. PB4/222 contains sequences between −360 and −260 of the human c-*fos* promoter thus spanning the SIF element, the SRE and the adjacent AP1 site. After transfection, the cells were made quiescent by incubation in 0.5% calf serum for 48 h before being stimulated by purified c-*sis* for 30, 60, 120 or 240 min. RNAs were isolated from the cells and assayed for expression of the human c-*fos* gene and the α-globin gene by an RNase protection assay. The results are shown in Figure 6. As can be seen from the

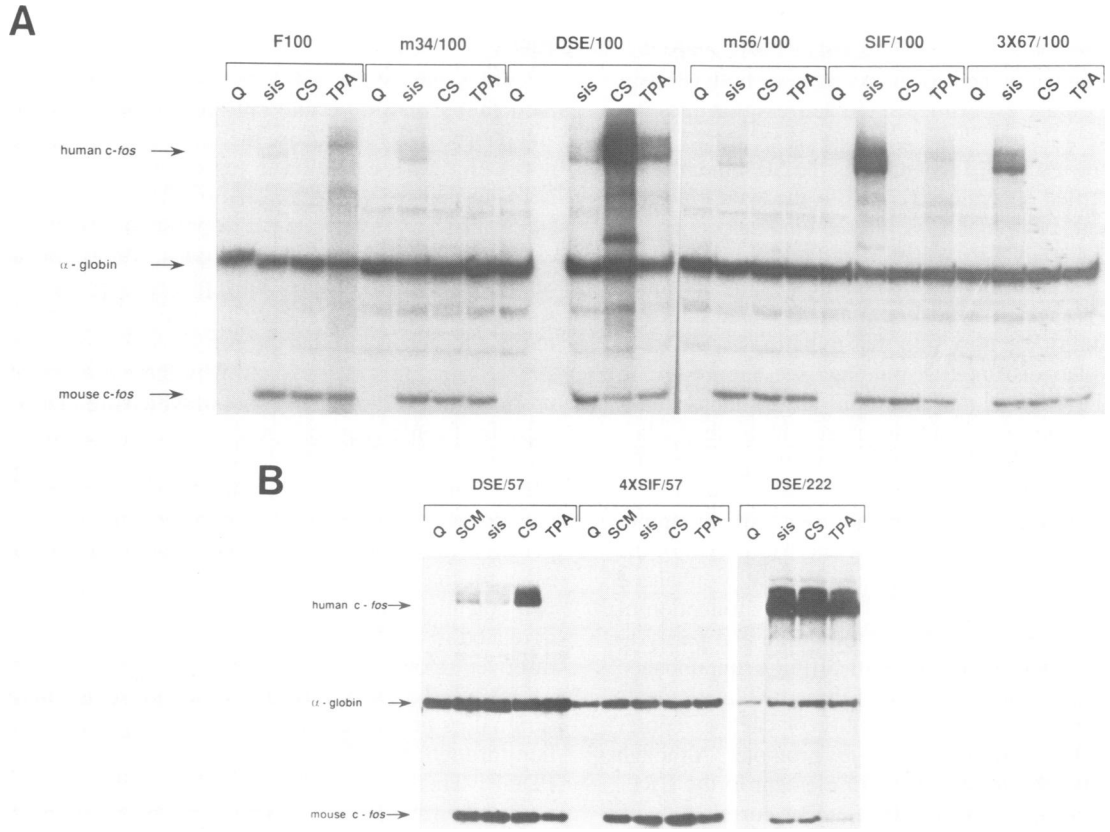


**Fig. 5.** Effect of positioning the SIF element and DSE at −100 or −57 of the human c-*fos* gene. The indicated plasmids (see Figure 2B and Materials and methods) were transfected into NIH 3T3 cells, treated with v-*sis* conditioned medium (SCM), c-*sis*, 20% calf serum, or TPA for 30 min, and analyzed by RNase protection assay as described in Figure 3. (**A**) SIF element and DSE (SRE) at −100 of c-*fos*. (**B**) SIF element and DSE at −57 of c-*fos*. The response of the DSE at −222 is shown for comparison.

figure, the time course of c-*fos* expression mediated by the SIF element in the absence of the SRE is essentially the same as for the SRE itself. Since the kinetics of expression are nearly equivalent for all three constructs, there may be a common mechanism for repressing both the SIF element and the SRE.

Since the SIF element requires sequences between −100 and −57 to mediate efficient induction, we investigated the possibility that the SRE might also serve as a cooperating element. The relative inductions of the SIF/222, SRE/222 and PB4/222 constructs were compared after transfection into NIH 3T3 cells and treatment of quiescent cells with purified c-*sis* for 45 min. In this experiment, the presence of a single copy of the wild-type SIF element confers a 5-fold induction of the c-*fos* gene. A single copy of the SRE confers a 16-fold induction. The presence of both elements confers a 23-fold induction indicating that the two elements act in an additive manner to mediate c-*fos* induction by purified *sis*/PDGF.

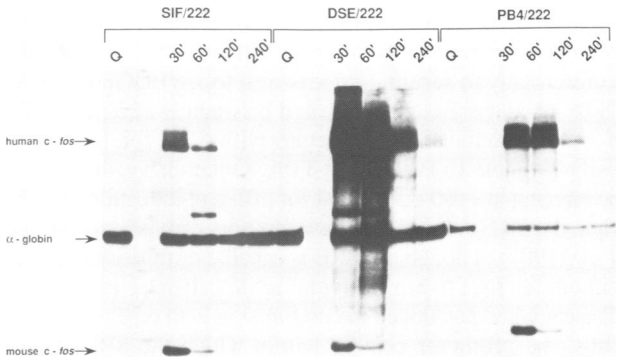


**Fig. 6.** Time course of induction of c-*fos* mediated by the SIF binding element. The indicated plasmids were transfected into NIH 3T3 cells, placed into media containing 0.5% calf serum for 40−48 h, and treated with 25 ng/ml of c-*sis* for 30, 60, 120 or 240 min. Total RNA was isolated and assayed for c-*fos* and α-globin expression as detailed in Materials and methods.

## Discussion

Previously, we have shown that there is a DNA binding factor which is inducible in quiescent Balb/c-3T3 cells by conditioned medium from NRK cells transformed with the v-*sis* oncogene (Hayes *et al.*, 1987a). This conditioned medium contains PDGF-like growth factor activity (Deuel *et al.*, 1983; Owen *et al.*, 1984), and when added to quiescent Balb/c-3T3 cells rapidly activates a new DNA binding activity which binds to a site ~346 bp upstream of the c-*fos* oncogene. This activity is induced in the presence inhibitors of protein synthesis and is therefore a good candidate for being an intermediate in the signal transduction pathway between PDGF receptor and the nucleus. In this report, we have demonstrated that purified recombinant c-*sis* B-chain homodimer is able to induce SIF−DNA binding. Because BB homodimeric PDGF is able to bind to both

PDGF receptors ($\alpha$ and $\beta$), both of which are present on these cells, it cannot be determined whether the receptor that generates this signal is the $\alpha$ or $\beta$ receptor, or both (Matsui *et al.*, 1989). Future experiments will address this question more directly.

By generating a series of mutations in the SIF binding site, we have identified sequences related to the wild-type SIF binding element, but which have altered binding properties. In our previous analysis, we had identified by methylation interference, a series of five guanines in the core sequence 5′-CCCGTC-3′, which, when methylated, would interfere with SIF binding. We now find that mutations adjacent to this site can also disrupt SIF binding (see Figure 2). In addition, we have found that mutations at guanine residues which were previously shown to interfere with binding when methylated, such as the C to A transversion represented by mutant M14 can be tolerated. However, other mutations in the core sequences can abolish the ability of SIF to bind to the element as demonstrated by M34 and M56. One of the mutant sequences, M67, in fact shows slightly enhanced binding to SIF, which is partially due to the elimination of binding of a non-specific factor to the probe and partly to a slight enhancement of the overall affinity (unpublished observations). The ability of each of these mutant elements to bind SIF correlates with the ability of each to confer *sis*/PDGF responsiveness on to the c-*fos* promoter. Although there is a weak homology of the SIF element to the CRE, the induction of c-*fos* via the SIF element correlates with its ability to bind SIF and not CREB.

Previous work has identified several distinct response elements upstream of c-*fos*. One is a region of dyad symmetry that centers between −319 and −299 of the human gene, and which will confer serum inducibility onto a c-*fos* promoter truncated at −222. This element can confer serum-, PDGF-, and TPA-inducibility onto the c-*fos* promoter at this position (Figure 5B). However, when this element is moved to position −100 or −57, it responds preferentially to serum, and less well to *sis*/PDGF or TPA. Thus, a sequence element located between −222 and −100 of c-*fos* is required to cooperate with SRE for maximum responsiveness to PDGF and TPA. Others have shown that the element at −60 is required for efficient induction of c-*fos* when the SRE is located at the more upstream position (Fisch *et al.*, 1987). Though the SRE is capable of functioning even without a TATA-box (Rivera *et al.*, 1990), it is more efficient when positioned in combination with other elements. Thus, the promoter context within which the SRE lies as well as the presence or absence of other elements within the promoter determines the response of the SRE to a given stimulus.

The SIF binding recognition element is capable of conferring PDGF inducibility on to the c-*fos* gene in a manner that is independent of the SRE. That is, constructs which have been deleted for the SRE, but which contain the SIF element, are able to respond specifically to *sis*/PDGF, but only very weakly to serum or phorbol ester. Deletion of the sequences between −100 and −57 of the c-*fos* gene eliminates the responsiveness of these constructs to *sis*/PDGF. Thus, SIF as well as SRE appears to function in conjunction with other basal level elements of the c-*fos* promoter. The −60 CRE element of the c-*fos* promoter is a likely candidate for a cooperating element since this element has been shown to have the ability to modify the activity of other upstream elements (Webster and Kedes, 1990).

Unlike the SRE, SIF specifically confers *sis*/PDGF inducibility onto c-*fos* and only responds weakly to serum and phorbol esters. Because the *sis*-responsive element does not confer c-*fos* induction in response to phorbol esters, it is likely that the activation of protein kinase C by PDGF is not sufficient to activate the response mediated by the SIF element (Nishizuka, 1984; Williams, 1989). Because the SRE does respond to TPA when placed upstream of −222, it is a reasonable hypothesis that unlike SIF, the SRE can respond to signals initiated through protein kinase C (Gilman, 1988). Thus, this may explain why there are two elements upstream of the c-*fos* gene, both of which are independently capable of conferring *sis*/PDGF responsiveness onto the c-*fos* promoter. That is, the SIF element may respond to a different PDGF-generated second message than does the SRE. There may be other extracellular factors as yet undiscovered which generate the same second signal as does PDGF to induce SIF, but that do not activate *fos* through the SRE. CSF-1 is a potential candidate for such a factor since its receptor is related to the PDGF-$\beta$ receptor, yet fails to stimulate PI turnover (Downing *et al.*, 1989).

One puzzling aspect of the results presented here is that serum does not induce SIF binding activity or c-*fos* transcription through the SIF element, even though serum contains PDGF. There are several possible explanations for this observation. (i) It may be that the concentration of PDGF in serum is high enough to induce proliferation, but not high enough to induce SIF−DNA binding. Bovine calf serum contains $<1$ ng/ml of PDGF (Bowen-Pope *et al.*, 1989) so that 20% serum would then contain amounts of PDGF 100-fold lower than the doses used for stimulation. (ii) Alternatively, it could be the isoform of PDGF that is in serum that is critical for SIF induction. PDGF is a dimer and can be encoded by two separate genes. Thus, there are at least three forms of PDGF; AA homodimer, BB homodimer, and AB heterodimer. In addition, the PDGF-A chain can be alternatively spliced. Different forms of the molecule could be generating different types of second signals (Bowen-Pope *et al.*, 1989; Hart *et al.*, 1988). (iii) A third possibility is that there may be factors in serum which inhibit the signal transduction pathway required for *sis* activation. Dose−response and mixing experiments argue against explanations (i) and (iii), and therefore we currently favor the explanation that only certain isoforms of PDGF activate SIF (unpublished results).

Complete understanding of the mechanism of c-*fos* transcriptional activation through the SIF element will require a determination of the mechanisms by which the SIF protein is activated after PDGF stimulation. As of yet, we have not been able to activate SIF−DNA binding activity *in vitro* as can be done with NF$\kappa$B (Baeuerle and Baltimore, 1988). Further characterization of the SIF protein and the modifications which may be made to it before and after PDGF treatment will be the next step in this analysis.

## Materials and methods

### *Construction and sequencing of point mutants*

The oligo 3′-CTTAACATCTGTT**CTAACTGCCCTTGAC**GTG-5′ (BHC-008) was synthesized as follows: the first 13 nucleotides were coupled as usual, the next 15 (in bold type) were added as mixtures which contained 90% of the wild-type nucleotide phosphoramidites and 10% of a solution

of all four deoxynucleotide phosphoramidites, the last three nucleotides were coupled as usual. A second oligo 3′-CAGATCTTAAG (BHC-007) was synthesized to serve as a primer. Both oligos were kinased and annealed as follows: 20 pmol BHC-008 and 5 pmol BHC-007 were added to a reaction mix containing 50 mM Tris−HCl pH 7.5, 10 mM $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine, 1 mM EDTA, 1 mM ATP and 20 U polynucleotide kinase (NEB). The reaction was incubated at 37°C for 20 min. An additional 20 U of enzyme was added and the reaction incubated 37°C for 20 min. The reaction was heated to 65°C for 15 min and allowed to cool slowly to 0°C. NaCl and dNTPs were added to final concentration of 50 mM and 80 nM respectively. Klenow fragment (5 U) was added and the reaction was incubated at 0°C for 2 h. The extended product was then ligated into *Sma*I digested M13 mp19 using standard protocols. Competent JM101 cells were transformed with one half of the ligation reaction and light blue or clear plaques picked at random, and the DNA sequence of the inserts determined.

### *DNA binding assays*

Confluent, quiescent 150 mm tissue culture plates of Balb/c-3T3 cells were rinsed with phosphate buffered saline at 0°C and then scraped into the same buffer. Nuclear extracts were prepared according to Dignam *et al.* (1983). Briefly, harvested cells were resuspended in 5 vol of hypotonic buffer, incubated 10 min on ice and centrifuged. Swollen cells were resuspended in 2 vol of hypotonic buffer, dounced and pelleted nuclei were incubated in high salt buffer for 30 min with stirring on ice. Material released from the nuclei was dialyzed for 4 h and stored at −80°C. DNA binding assays were done as in Hayes *et al.* (1987a). Briefly, 8 μg of nuclear extract was incubated with 1 or 2 fmol of radiolabeled probe (see below for sequence), 1 μg poly(dIdC):poly(dIdC) and unlabeled competitor oligonucleotide (if included) in 20 μl of 10 mM HEPES, pH 7.9/50 mM NaCl/1 mM EDTA/10% glycerol for 30 min at 30°C. Binding reactions were electrophoresed on a 4% polyacrylamide gel (80:1) with 2.5% glycerol in 6.7 mM Tris, pH 7.9/3.3 mM NaOAc/1 mM EDTA, fixed, dried and exposed to X-ray film.

Probes for the analysis of the SIF element point mutations were made as follows. Template DNA from each point mutant was annealed with forward sequencing primer (NEB) and extended with Klenow fragment (5 U) in the presence of [α-$^{32}$P]dATP (>800 Ci/mmol). Appropriate restriction enzyme (20 U) was added to the reaction and incubated at 37°C for 1 h. The reaction was run on a 5% PAGE gel and the labeled probe fragment visualized by autoradiography. Probe fragment was electrophoresed on to DEAE paper and eluted. Probe was ethanol precipitated in the presence of 2 μg poly(dIdC):poly(dIdC) (Pharmacia) carrier. After pelleting the samples were rinsed in 70% ethanol, dried briefly, resuspended in TE and used immediately.

### *Oligonucleotides and plasmids*

222FOS contains the entire *fos* transcription unit and 222 bp of promoter sequence. The plasmid p-c-fos-1 (Curran *et al.*, 1983) was digested completely with *Bam*HI and partially with *Apa*I. The 4.5 kb fragment was excised from a 1% agarose gel, blunted with T4 DNA polymerase and ligated into *Sma*I digested pUC18 to make plasmid fos-Apa-Bam. Fos-Apa-Bam was digested with *Eco*RI and *Xba*I and ligated into pGEM7Z(+) to make 222FOS.

SIF/222 contains one copy of the SIF binding sequence in direct orientation relative to *fos* transcription. Oligonucleotides 5′-AGCTTCAGTTCCCGTCAATCA and 5′-AGCTTGATTGACGGGAACTGA were annealed, blunted with Klenow fragment and ligated into *Sma*I digested pUC18. White colonies were picked at random and sequenced to determine insert orientation. Plasmid 346−312 was digested with *Kpn*I and *Bam*HI, the 35 bp fragment was excised from a 1% agarose gel and ligated into pGEM7Z(+) to make 3127Z. Fos-Apa-Bam was digested with *Kpn*I and *Xba*I, the 4.5 kb fragment was excised from a 1% agarose gel and ligated into 3127Z to make SIF222.

3×67/222 contains three copies of point mutant 67 (see Figure 2A), positive for SIF binding activity, in direct−opposite−direct orientation relative to the c-*fos* promoter. Oligonucleotides 5′-AGCTTCATTTCCGTAAATCCCTA and 5′-AGCTTAGGGATTTACGGGAAATGA were treated with kinase, annealed and ligated. The ligation mix was cloned into the *Hin*dIII site of pGEM7Z(+). White colonies were picked at random and sequenced to determine insert copy number and orientation. The 4.5 kb *Eco*RI−*Xba*I fragment from fos-Apa-Bam was cloned into plasmid MA to make 3×67/222.

m34/222 and m56/222 were constructed by annealing and blunting with Klenow fragment oligonucleotides corresponding to the M13 insert sequences 34 and 56 (see Figure 2). Both sequences are negative for SIF binding activity. 222FOS was digested with *Hin*dIII and blunted with Klenow fragment. The blunted oligonucleotides were ligated into 222FOS and colonies positive for insert were sequenced to determine orientation. Both constructs contain single copies of oligonucleotide insert in direct orientation relative to the *fos* promoter.

PB4/222 contains a genomic *Pst*I−*Bst*NI fragment of the human c-*fos* promoter which encompasses sequences from −365 to −265 (see Figure 2B). It was constructed by complete digestion of p-c-*fos*-human-1 with *Pst*I and *Bst*NI, isolation of the 96 bp fragment and subcloning between the *Pst*I and *Sma*I sites of pGEM1 (Promega Biotech) to make plasmid PB4. PB4 was digested with *Kpn*I and *Hin*dIII and the fragment containing the 96 bp insert was isolated and subcloned into pGEM7Z(+) to make plasmid 4K1. The 4.5 kb *Eco*RI−*Xba*I fragment from fos-Apa-Bam was cloned into plasmid 4K1 to make PB4/222.

The human *fos* promoter contains a unique *Bss*HII site at −100 relative to the start of transcription. Plasmids designated as /100 were constructed by digesting their /222 parent plasmid with *Asp*718 and *Bss*HII and blunting with Klenow fragment. The digests were electrophoresed on a 1% agarose gel, the 7.4 kb fragment containing 3 kb of vector backbone, 100 bp of c-*fos* promoter sequence and the *fos* transcription unit was excised and ligated intramolecularly to make the /100 plasmids.

57FOS was constructed from pF57 (Sheng *et al.*, 1988) which contains 57 bp of *fos* promoter sequence and the *fos* transcription unit. pF57 was digested with *Eco*RI and *Hin*dIII. The 4.4 kb fragment was excised from a 1% agarose gel and blunted with Klenow fragment. The *fos* insert was ligated into pGEM7Z(+) which had been digested with *Eco*RI and blunted with Klenow fragment.

SRE/57 (also DSE/57), containing the SRE (Treisman, 1986) upstream of 57FOS in the opposite orientation relative to transcription, was constructed in three steps. The oligonucleotides 5′-CTAGAGGATGTCCATATTAGGACATCTG and 5′-GATCCAGATGTCCTAATATGGACATCCT were annealed and cloned into the *Sma*I site of pUC18. White colonies were picked at random and sequenced to determine insert orientation. Plasmid 310/371 was digested with *Kpn*I and *Bam*HI, the 45 bp fragment was excised from a 1% agarose gel and cloned into pGEM7Z(+) to make KB7Z. The 4.3 kb *Kpn*I−*Xba*I fragment from 57FOS was cloned into KB7Z to make SRE/57.

4×SIF/57 contains four copies of the SIF binding site upstream of 57FOS and whose orientations are direct−opposite−direct−direct. Oligonucleotides 5′-AGCTTCAGTTCCCGTCAATCA and 5′-AGCTTGATTGACGGGAACTGA were kinased, annealed and ligated. The ligation reaction was run on a 5% PAGE gel and stained with ethidium bromide. Fragments containing between three and six tandem copies of the oligonucleotide sequence were electroeluted onto DEAE paper, isolated and ligated into the *Hin*dIII site of 222FOS. Colonies positive for oligonucleotide insert were picked and sequenced. The *Asp*718−*Xba*I fragment containing the −222 *fos* sequence was removed and replaced with the *Asp*718−*Xba*I fragment from 57FOS.

AHFOS plasmid used to make labeled RNA probes for RNase protection assays was constructed by digesting 222FOS with *Hin*cII and *Xba*I. The *fos* sequence has a unique *Hin*cII site 4 bp 5′ to the end of the first exon. The 3.5 kb fragment containing the vector backbone, 222 bp of the c-*fos* promoter sequence and the first exon was excised from a 1% agarosed gel, blunted with Klenow fragment and ligated intramolecularly to make AHFOS.

The CREB oligonucleotide was made from the sequence of the CREB site of the rat somatostatin gene (Yamamoto *et al.*, 1988). The sequence of this oligonucleotide pair is 5′-GATCCGGCGCCTCCTTGGCTGACGTCAGAGAT-3′ and 5′-CTAGATCTCTGACGTCAGCCAAGGAGGGGCCG-3′.

### *Transfection and pulsing of NIH 3T3 cells*

NIH 3T3 cells were passaged at $10^6$ cells per 10 cm plate. Transfection was performed 24−28 h after passage. Plasmid DNA (20 μg per plate) and pSVA1 control DNA (1 μg per plate) in 1 ml 0.25 M $CaCl_2$ was added dropwise to 1 ml 2 × HEBS (50 mM HEPES pH 7.05, 280 mM NaCl, 1.5 mM $Na_2HPO_4$) to form a precipitate. Plates were rinsed twice with DME. Precipitate was added to plate and cells incubated for 20 min at 37°C in 5% $CO_2$. DME containing 10% calf serum and 50 μM chloroquine was added to 10 ml. Cells were incubated 4 h at 37°C in 5% $CO_2$. Cells were rinsed twice in DME and then incubated 48 h in DME containing 0.5% calf serum. Cells were pulsed for 30 min in 5 ml of DME + 0.5% calf serum containing 25 ng/ml c-*sis* (Amgen), 200 ng/ml TPA (Sigma), v-*sis* conditioned media, or 5 ml of DME + 20% calf serum. v-*sis* conditioned media was prepared as previously described (Hayes *et al.*, 1987a). Cells were then rinsed three times in PBS and scraped into 3.5 ml 5 M guanidine isothiocyanate buffer (5 M guanidine isothiocyanate, 25 mM Na citrate pH 7.0, 0.1 M β-mercaptoethanol).

***RNA isolation and RNase protection assays***

Total RNA was isolated by centrifugation through a 2 ml 5.7 M CsCl cushion for 18 h at 20°C in SW50.1 rotor at 40 000 r.p.m. Pellet was resuspended in PK buffer (100 mM Tris−HCl pH 7.5, 12.5 mM EDTA, 150 mM NaCl, 1% SDS) containing 100 μg/ml proteinase K (Boehringer Mannheim) and incubated at 37°C for 1 h. Samples were extracted three times with phenol:$CHCl_3$ (1:1) and ethanol precipitated at −20°C. After pelleting, samples were rinsed with 70% ethanol, dried briefly and resuspended in $H_2O$ and stored at −70°C until use.

*Fos* riboprobe template was prepared using *Bss*HII or *Eco*RI digested AHfos plasmid and T7 RNA polymerase. α-globin riboprobe template was prepared using *Bam*HI digested SP6-α-globin plasmid (Sheng *et al.*, 1988) and SP6 RNA polymerase. Reaction was performed using 10 U of appropriate enzyme (Promega) in 40 mM Tris−HCl pH 7.5, 7.5 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 1000 U/ml RNasin (Promega), 0.5 mM each ATP, GTP, CTP, 20 mCi/ml [$\alpha$-$^{32}$P]UTP (>800 Ci/mmol), 6.25 μM cold UTP and 0.5 μg of linearized template. Reaction was incubated at 37°C for 30 min. 10 U of RQ1-DNase (Promega) was added and reaction incubated at 37°C for 15 min. 50 μl of TE was added and reaction was passed through a G-50 spin column (Boehringer Mannheim). Sample was extracted three times with phenol:$CHCl_3$ (1:1) and ethanol precipitated with 20 μg of carrier tRNA (Boehringer Mannheim). After pelleting the labeled probe was rinsed with 70% ethanol, dried briefly and resuspended in 100 μl hybridization buffer (80% formamide, 40 mM PIPES pH 6.7, 0.4 M NaCl, 1 mM EDTA).

30 μg aliquots of RNA samples were precipitated in ethanol just prior to use. Pellets were resuspended in 30 μl hybridization buffer containing 200 000 c.p.m. of each probe, heated to 80°C for 5 min then incubated overnight at 55°C. 0.3 ml RNase digestion buffer (10 mM Tris−HCl pH 7.5, 5 mM EDTA, 300 mM NaCl) containing 40 μg/ml RNase A (Boehringer Mannheim) and 2 μg/ml RNase T1 (Boehringer Mannheim) and reaction incubated at 37°C for 1 h. Reaction was terminated by addition of 20 μl 10% SDS and 50 μg proteinase K (Boehringer Mannheim) and incubation at 37°C for 15 min. Samples were extracted once with phenol:$CHCl_3$ (1:1) and double-stranded product was precipitated by the addition of 20 μg tRNA and 1 ml ethanol. The pellet was rinsed once with 70% ethanol, dried briefly and resuspended in 6 μl loading buffer (80% formamide, 1 × TBE, 0.1% bromphenol blue, 0.1% xylene cyanol). Samples were denatured at 85−90°C for 3 min and loaded onto a 6% PAGE gel (1.5 mm). Samples were electrophoresed at 250−300 V. Gels were rinsed in water to remove urea and dried onto 3 mm Whatman paper. Pre-flashed XAR film was exposed to gels for autoradiography. Results were quantified on an LKB densitometer and expression from transfected c-*fos* plasmid was normalized to α-globin control expression.

## Acknowledgements

We thank Karen Traite for help in preparing this manuscript and M.E.Greenberg for his gift of the pF57, pSVA1 and SP6α-globin plasmids. This work was supported in part by NIH grant PO1 CA42063. B.H.C. is a scholar of the Rita Allen Foundation and the Searle Scholars Program/Chicago Community Trust and holds the Pfizer Career Development Professorship.

## References

Baeuerle,P.A. and Baltimore,D. (1988) *Cell*, **53**, 211−217.
Berkowitz,L.A., Riabowol,K.T. and Gilman,M.Z. (1989) *Mol. Cell. Biol.*, **9**, 4272−4281.
Bowen-Pope,D.F., Hart,C.E. and Seifert,R.A. (1989) *J. Biol. Chem.*, **264**, 2502−2508.
Cochran,B.H., Zullo,J., Verma,I.M. and Stiles,C.D. (1984) *Science*, **226**, 1080−1082.
Curran,T. and Morgan,J.I. (1985) *Science*, **229**, 1265−1268.
Curran,T., MacConnell,W.P., van Straaten,F. and Verma,I.M. (1983) *Mol. Cell. Biol.*, **3**, 914−921.
Deuel,T.F., Huang,J.S., Huang,S.S., Stroobant,P. and Waterfield,M.D. (1983) *Science*, **221**, 1348−1350.
Deutch,P.J., Hoeffler,J.P., Jameson,J.L., Lin,J.C. and Habener,J.F. (1988) *J. Biol. Chem.*, **263**, 18466−18472.
Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475−1489.
Downing,J.R., Margolis,B.L., Zilberstein,A., Ashmun,R.A., Ullrich,A., Sherr,C.J. and Schlessinger,J. (1989) *EMBO J.*, **8**, 3345−3350.
Fink,J.S., Verhave,M., Kasper,S., Tsukada,T., Mandel,G. and Goodman,R.H. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6662−6666.
Fisch,T.M., Prywes,R. and Roeder,R.G. (1987) *Mol. Cell. Biol.*, **7**, 3490−3502.
Fisch,T.M., Prywes,R., Simon,M.C. and Roeder,R.G. (1989) *Genes Dev.*, **3**, 198−211.
Gilman,M.Z. (1988) *Genes Dev.*, **2**, 394−402.
Gilman,M.Z., Wilson,R.N. and Weinberg,R.A. (1986) *Mol. Cell. Biol.*, **6**, 4305−4316.
Greenberg,M.E. and Ziff,E.B. (1984) *Nature*, **311**, 433−442.
Greenberg,M.E., Siegfried,Z. and Ziff,E.B. (1987) *Mol. Cell. Biol.*, **7**, 1217−1225.
Hart,C.E., Forstrom,J.W., Kelly,J.D., Seifert,R.A., Smith,R.A., Ross,R., Murray,M.J. and Bowen-Pope,D.F. (1988) *Science*, **240**, 1529−1531.
Hayes,T.E., Kitchen,A.M. and Cochran,B.H. (1987a) *Proc. Natl. Acad. Sci. USA*, **84**, 1272−1276.
Hayes,T.E., Kitchen,A.M. and Cochran,B.H. (1987b) *J. Cell. Physiol.*, Suppl. 5, 63−68.
Jamal,S. and Ziff,E. (1990) *Nature*, **344**, 463−466.
Kaibuchi,K., Fukumoto,Y., Oku,N., Hori,Y., Yamamoto,T., Toyoshima,K. and Takai,Y. (1989) *J. Biol. Chem.*, **264**, 20855−20858.
Konig,H., Ponta,H., Rahmsdorf,H. and Herrlich,P. (1989) *EMBO J.*, **8**, 2559−2566.
Matsui,T., Heidaran,M., Miki,T., Popescu,N., La-Rochelle,W., Kraus,M., Pierce,J. and Aaronson,S. (1989) *Science*, **243**, 800−804.
Nishizuka,Y. (1984) *Nature*, **308**, 693−698.
Owen,A.J., Pantazis,P. and Antoniades,H.N. (1984) *Science*, **225**, 54−56.
Prywes,R. and Roeder,R.G. (1986) *Cell*, **47**, 777−784.
Rivera,V.M., Sheng,M. and Greenberg,M.E. (1990) *Genes Dev.*, **4**, 255−268.
Schonthal,A., Buscher,M., Angel,P., Rahmsdorf,H.J., Ponta,H., Hattori,K., Chui,R., Karin,M. and Herrlich,P. (1989) *Oncogene*, **4**, 629−636.
Shaw,P.E., Frasch,S. and Nordheim,A. (1989a) *EMBO J.*, **8**, 2567−2574.
Shaw,P.E., Schroter,H. and Nordheim,A. (1989b) *Cell*, **56**, 563−572.
Sheng,M., Dougan,S.T., McFadden,G. and Greenberg,M.E. (1988) *Mol. Cell. Biol.*, **8**, 2787−2796.
Siegfried,Z. and Ziff,E.B. (1989) *Oncogene*, **4**, 3−11.
Treisman,R. (1985) *Cell*, **46**, 889−902.
Treisman,R. (1986) *Cell*, **46**, 567−574.
Verma,I.M. and Sassone-Corsi,P. (1987) *Cell*, **51**, 513−514.
Webster,K.A. and Kedes,L. (1990) *Mol. Cell. Biol.*, **10**, 2402−2406.
Williams,L.T. (1989) *Science*, **243**, 1564−1570.
Yamamoto,K.K., Gonzalez,G.A., Biggs,W.H. and Montminy,M.R. (1988) *Nature*, **334**, 494−498.

*Received on August 3, 1990; revised on October 15, 1990*