The EMBO Journal vol.8 no.9 pp.2567 – 2574, 1989

Repression of c-*fos* transcription is mediated through p67^{SRF} bound to the SRE

Peter E.Shaw, Susanne Frasch and Alfred Nordheim

Zentrum für Molekulare Biologie Heidelberg (ZMBH), Universität Heidelberg, Im Neuenheimer Feld 282, 6900 Heidelberg, FRG

Communicated by P.Herrlich

Human c-*fos* expression is subject to tight transcriptional control. The gene is expressed at a high, constitutive level in some cell types and at a very low, but rapidly inducible level in many others. Induction of transcription by serum growth factors is mediated by the serum response element (SRE) to which at least two transcription factors, p67^{SRF} and p62, bind. In this paper it is demonstrated that the low basal level of transcription is mediated through p67^{SRF} bound to the SRE and that high, constitutive expression is observed when binding is prohibited. In this situation, an AP-1 consensus binding site adjacent to the SRE permits transactivation of the gene. Thus three levels of c-*fos* expression, induced, repressed and constitutive, appear to be determined by occupation of the SRE by p67^{SRF} and its interaction with other proteins.

Key words: c-*fos* expression/transcription/serum growth factors/serum response element

Introduction

The proto-oncogene c-*fos* encodes a 55–62 kd nuclear phosphoprotein (FOS) to which, on the basis of numerous recent results, a pivotal role in the transduction of extracellular and intracellular signals into changes in gene expression has been assigned (for a review see Cohen and Curran, 1988). FOS functions as a transcriptional regulator for a set of target genes and in that capacity it can either activate (Setoyama *et al.*, 1986; Chiu *et al.*, 1988; Kushtai *et al.*, 1988; Lech *et al.*, 1988; Schönthal *et al.*, 1988) or repress transcription (Distel *et al.*, 1987; Sassone-Corsi *et al.*, 1988a; Schönthal *et al.*, 1988, 1989; Wilson and Treisman, 1988). FOS has been shown to function in conjuction with Fos-associated proteins (FAPs) (Curran *et al.*, 1985), the best characterized being the sequence-specific DNA-binding protein JUN, with which it forms a heterodimeric complex (Chiu *et al.*, 1988; Rauscher *et al.*, 1988a; Sassone-Corsi *et al.*, 1988b).

The c-*fos* gene is subject to tight transcriptional control. In extra-embryonal cells, mid-gestation fetal liver and postnatal bone marrow cells c-*fos* is expressed at high constitutive levels (Cohen and Curran, 1988). In contrast, c-*fos* is expressed at a very low basal level in many other cell types, which can be induced rapidly and transiently by exposure to various extracellular stimuli (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Müller *et al.*, 1984; for a review see Cohen and Curran, 1988). Induction of human c-*fos* transcription by serum growth factors is mediated through the serum response element (SRE) (Treisman, 1985), which contains the dyad symmetry element (DSE), the binding site for the serum response factor (p67^{SRF}) (Treisman, 1986) and the binding site for p62 (Shaw *et al.*, 1989a).

The kinetics of c-*fos* induction and its subsequent inactivation, which requires ongoing protein synthesis (Greenberg *et al.*, 1986), suggest strongly that FOS represses its own expression. Indeed, the observation that C-terminal mutations of FOS block down-regulation of c-*fos* transcription implicates the protein in negative feedback regulation of its own promoter (Wilson and Treisman, 1988). Loss of c-*fos* repression is also caused by the expression of antisense FOS (and JUN) sequences (Schönthal *et al.*, 1989). As a result, FOS and JUN have been postulated to bring about repression in a cooperation that involves consensus AP-1 binding sites present within the c-*fos* promoter (Sassone-Corsi *et al.*, 1988a; Wilson and Treisman, 1988; Schönthal *et al.*, 1989). However, this has yet to be demonstrated by experiment.

We reported recently that the formation of a ternary complex between p67^{SRF}, the SRE and a novel protein, p62, is functionally involved in the induction of c-*fos* expression by serum (Shaw *et al.*, 1989a). Promoter mutations that specifically abolish formation of the ternary complex *in vitro* drastically reduce the serum responsiveness of the c-*fos* promoter *in vivo*.

The present analysis of further c-*fos* promoter mutants demonstrates that an important role in c-*fos* repression is played by the DSE–p67^{SRF} complex, a structure so far only implicated in promoter induction. We show that in HeLa cells, impairment of p67^{SRF} binding leads to a high level of uninducible c-*fos* transcription. This implies strongly that p67^{SRF} bound to the DSE permits repression prior to induction by serum ('poised' repression) of c-*fos* promoter activity. Additionally, the AP-1-like consensus site centred around position −295 allows *trans*-activation of c-*fos* expression when formation of the adjacent DSE–p67^{SRF} complex is prevented.

Results

Expression from c-fos SRE mutants unresponsive to serum stimulation correlates inversely with the affinity of p67^{SRF} for the DSE

In order to analyse the function of the c-*fos* SRE, numerous oligonucleotides have been synthesized that contain blocks of transversions at positions in and around the SRE. The sequences of three such pairs of mutant oligonucleotides are presented along with the wild-type probe in Figure 1a. It has been shown previously that none of these mutants binds p62 (Shaw *et al.*, 1989a). To demonstrate their affinities for p67^{SRF}, the mutant pairs were used as competitors against the unmutated sequence (wt) in a gel retardation experiment with purified p67^{SRF}. Each was included in the binding reactions at concentrations ranging from 5- to 1000-fold

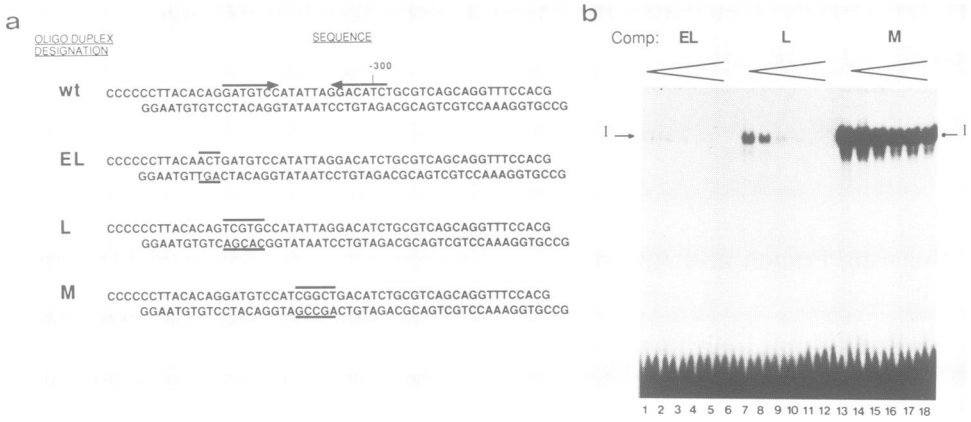

Fig. 1. Mutations within the SRE variously affect p67^{SRF} binding *in vitro*. (**a**) Synthetic oligonucleotide duplexes used. The dyad sequence element (DSE) is indicated by the converging arrows above the wild-type sequence. The duplex region from positions −328 to −279 spans the SRE with position −300 indicated above the sequence. (**b**) Competitor duplexes (Comp) were included in the binding reactions as shown at 5-, 10-, 50-, 100-, 500- and 1000-fold excess over the labelled probe (wt), increasing from left to right as indicated by the crescendo marks.

excess over the labelled wt probe (Figure 1b). Mutant EL competes very efficiently for p67^{SRF} against wt (Figure 1b, lanes 1−6), in fact as efficiently as wt itself (Shaw *et al.*, 1989a). Mutant L, in which the left arm of the palindrome is mutated, still competes well for p67^{SRF} binding (lanes 7−12), but 10-fold less efficiently than wt or EL (Shaw *et al.*, 1989a). Mutant M, in which the central region of the palindrome is mutated, fails to compete appreciably even at 1000-fold excess (lanes 13−18), demonstrating its complete inability to bind p67^{SRF}.

The effects of these mutations on c-*fos* transcription were subsequently tested *in vivo*. To analyse human c-*fos* promoter function in human cells, a c-*fos* promoter−SV40 gene fusion plasmid has been constructed (−EPf) such that expression from the transfected human c-*fos* promoter can be monitored by quantitative S1 analysis of SV40 early mRNA (Shaw *et al.*, 1989a). A number of linker insertion mutants and deletion mutants have subsequently been derived from this parent plasmid, one of which, ΔPS4, lacks sequences between positions −358 and −259 (Shaw *et al.*, 1989a).

Oligonucleotide pairs EL, L and M were each inserted into ΔPS4 and the resultant clones transfected, along with a suitable internal control, into HeLa cells. Analysis of RNA isolated from cells transfected with these plasmids yielded the result shown in Figure 2. The three promoter mutants ΔPS4EL, ΔPS4L and ΔPS4M are similar insofar as none of them is inducible by serum [ΔPS4wt is inducible similarly to −EPf (not shown; Shaw *et al.*, 1989a)]. However, the levels of uninducible expression from the three constructs differ dramatically. ΔPS4EL, which contains a DSE with unimpaired affinity for p67^{SRF}, is transcribed at very low levels (Figure 2, lanes 1 and 2), whereas ΔPS4L and ΔPS4M, both of which contain mutated binding sites for p67^{SRF}, give rise to higher constitutive levels of expression (lanes 3 and 4, 5 and 6). In fact, the relative levels of expression from these three constructs correlate inversely with the affinity of p67^{SRF} for the mutated SREs present in the promoters. Thus when p62 is prevented from interacting with the SRE and serum inducibility is lost as a consequence (Shaw *et al.*, 1989a), mutations that impair p67^{SRF} binding are seen to result in increased expression from the c-*fos* promoter. This result demonstrates that not only is p67^{SRF} necessary for induction of transcription by

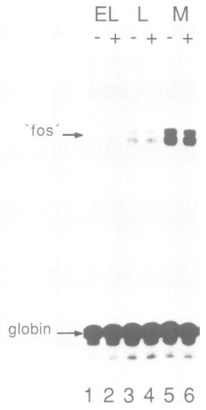

Fig. 2. Loss of p67^{SRF} binding leads to high constitutive c-*fos* expression *in vivo*. EL, L and M refer to mutants ΔPS4EL, ΔPS4L and ΔPS4M, − and + refer to RNA from starved and induced cells respectively. Ten micrograms of the test plasmids ('fos') were co-transfected with 5 μg of p9511 (globin) and 5 μg of plasmid carrier.

serum, poised repression of the promoter is also mediated through DSE-bound p67^{SRF}.

A cryptic enhancer element is activated by an insertion mutation of the human c-fos SRE

The mechanism that leads to the high level of c-*fos* expression observed when p67^{SRF} is unable to bind to the DSE is obscure. As an approach to its elucidation, we chose to test other c-*fos* promoter mutations *in vivo* in the same transient expression assay.

A number of linker insertion mutants of the human c-*fos* promoter have been prepared from −EPf, from which deletion mutants have subsequently been derived. Figure 3a presents the structures of several such −EPf derivatives that have been used as test plasmids in a second series of transient expression analyses. In these experiments, two plasmids were co-transfected along with the test plasmids, a rabbit β-globin gene with a 3′ synthetic enhancer element (see Materials and methods) that is uninducible by serum and a linker insertion mutant of −EPf (pR7) from which a distinctly longer mRNA is transcribed. These plasmids serve as controls both for the transfection and the subsequent serum induction. It should be noted, however, that pR7 contains a fully functional c-

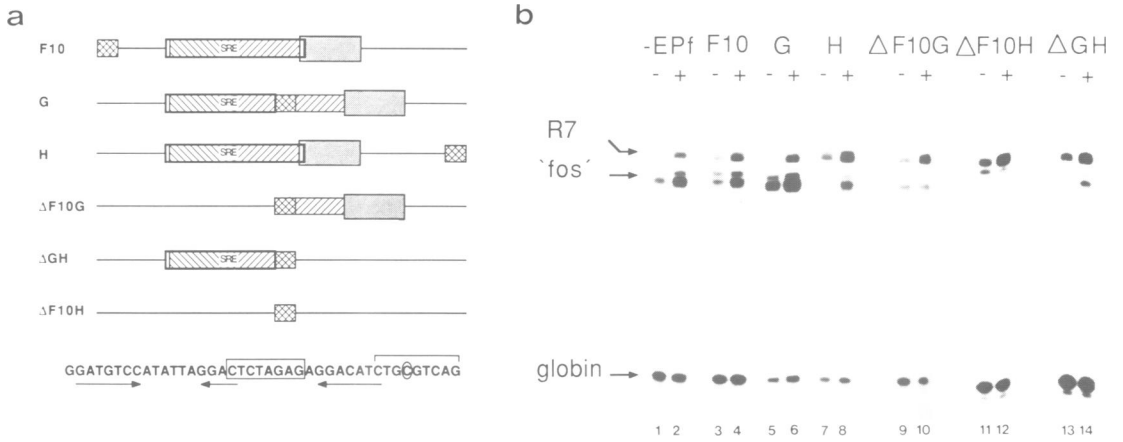

Fig. 3. Activation of a cryptic c-*fos* promoter element by a linker insertion into the SRE. (**a**) Diagram of the linker insertion and deletion clones used. Only c-*fos* promoter sequences between positions −354 and −278 are shown. The octameric *Xba*I linker is represented by the cross-hatched box, the DSE by the symmetrically hatched box within the SRE (bold rectangle) and the −295 element by the stippled box. The sequence of mutant pG across the linker insertion is given at the bottom of the diagram. (**b**) Ten micrograms of test plasmid ('fos') was transfected along with 10 μg of pR7 (R7) and 5 μg of core *Pvu*II 7½ (globin). Labelling corresponds to that of Figure 2.

fos promoter and thus can compete against p-EPf and its derivatives in these transfection experiments.

Insertion of a linker upstream of the SRE at position −354 (pF10) has no effect on expression or induction (3- to 5-fold) of the gene (Figure 3b, compare lanes 1 and 2 with 3 and 4) and insertion of the same linker downstream of the SRE at position −278 (pH) results in a slight but reproducible drop in the level of expression from the c-*fos* promoter (cf. lanes 1 and 2 with 7 and 8). Provisional evidence suggests that another *cis*-element bridges the site of this linker insertion (R.Hipskind, unpublished observation). However, insertion of a linker at a site within the DSE (pG: the sequence is given at the bottom of Figure 3a) leads to a marked increase in both basal level and serum-induced transcription from the c-*fos* promoter (cf. lanes 1 and 2 with 5 and 6).

Deletion of sequences between positions −354 and −307 (ΔF10G) removes most of the DSE (to which $p67^{SRF}$ binds) and abolishes serum responsiveness. However, a constitutive level of expression from the c-*fos* promoter is now seen (lanes 9 and 10). When the deletion is extended 3′ to position −278 (ΔF10H), transcription from the c-*fos* promoter detectable by this assay is abolished (lanes 11 and 12). Sequences between positions −306 and −278 must therefore harbour an element that confers the level of constitutive expression on the c-*fos* promoter observed when the DSE is removed.

When only the sequences between −302 and −278 are deleted (ΔGH), the promoter retains the ability to respond to serum stimulation (lanes 13 and 14). Thus although the ΔGH part of the DSE is replaced by linker sequences, $p67^{SRF}$ and p62 must still be able to bind to the mutated element.

Mutants ΔGH and ΔF10G thus distinguish two activities of the c-*fos* distal promoter element (Shaw *et al.*, 1989b), serum responsiveness mediated by the SRE (Treisman, 1985, 1986) and constitutive activation of expression in a manner similar to that described for enhancer elements. Immediately downstream of the DSE is a 9 bp sequence centred on position −295 that is closely related to the consensus binding site for AP-1 (Figure 3a). Indeed, it has been shown by *in vitro* footprinting experiments that the murine homologue to AP-1 (PEA 1) binds to this site in murine cell extracts that lack $p67^{SRF}$ (Piette and Yaniv, 1987). This element was therefore considered likely to be responsible for the constitutive activation seen with mutant ΔF10G. Furthermore, it suggested that the elevated level of expression seen with mutant pG in HeLa cells might be due to the combined function of the two elements distinguished above, a situation apparently not permissible in the unmutated promoter. For this reason we decided to examine the occupation of these two sites by factors present in HeLa nuclei.

Binding of affinity-purified AP-1 to the −295 element and $p67^{SRF}$ to the DSE appear to be mutually exclusive in vitro

$p67^{SRF}$ prepared from HeLa nuclei protects the distal element of the unmutated c-*fos* promoter from digestion by DNase I in the manner shown in Figure 4a (lanes 3 and 4). The region of protection extends beyond the 3′ boundary of the DSE to include the −295 element. This phenomenon is observed consistently regardless of the extent of purification of $p67^{SRF}$.

Affinity-purified AP-1 protects the −295 element 3′ of the DSE, the protection extending upstream into, but not through, the DSE (lanes 5 and 6). The two proteins thus protect overlapping sequences from DNase I digestion due to the proximity of their respective recognition sites. AP-1 is seen to protect a site immediately upstream of the DSE as well, which also shows partial homology to the AP-1 consensus binding site. However, this may reflect the excess of AP-1 that was used for this experiment rather than the presence of a functional AP-1 binding site at this position.

When both $p67^{SRF}$ and AP-1 are included in the binding reactions, the footprint seen over the DSE is the same as the one obtained with $p67^{SRF}$ alone even though AP-1 was present in excess of the amount required to give complete protection (lanes 7 and 8). Protection of the site lying 5′ of the DSE by AP-1 is still observed albeit slightly weaker. Thus under these conditions, $p67^{SRF}$ is bound to the DSE and appears to preclude binding of AP-1 to the site immediately 3′ of the DSE.

A similar DNase I footprinting analysis was carried out with the equivalent fragment of promoter mutant pG (Figure 4b) in which the DSE is separated from the −295

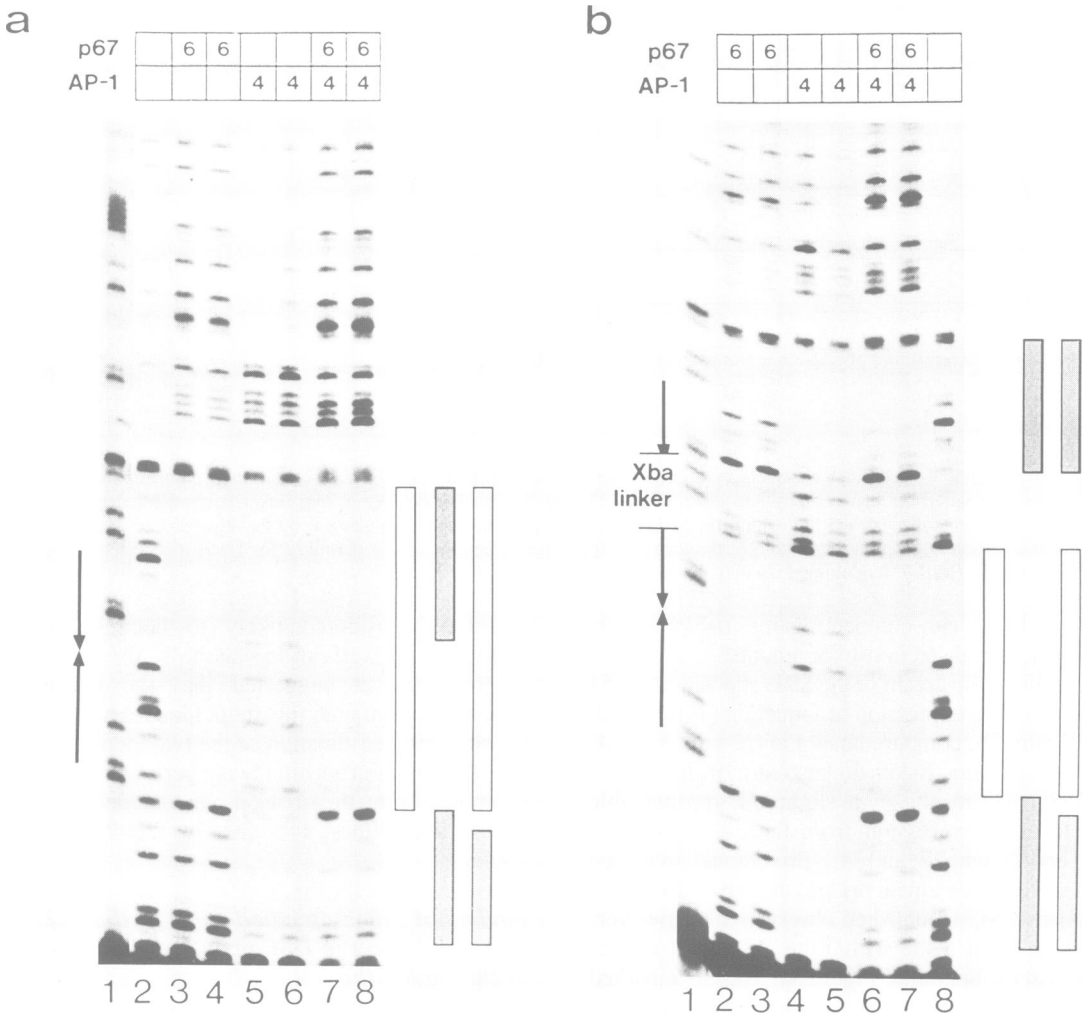

Fig. 4. Comparison of $p67^{SRF}$ and AP-1 binding to the SRE in the unmutated promoter and mutant pG by DNase I footprinting. The DNA probes used for the analysis of the coding strands presented here are described in Materials and methods. Arrows to the left of the gel panels show the extent of the DSE in the wild-type sequence (**a**) and in mutant G, which contains a linker insertion (**b**). Lane 1 of each panel contains a partial sequence of each probe (G reaction) reading towards the cap site up the gel. To the right of the gel panels, open rectangles indicate the extent of protection due to $p67^{SRF}$ and shaded rectangles indicate protection by AP-1, whereby weaker shading represents weaker protection. **Lane 2**, panel a and **lane 8**, panel b show DNase I reactions performed in the absence of added protein.

element by 11 bp. In this case, the same amount of $p67^{SRF}$ protects the DSE as before except that the protection does not reach into the −295 element. AP-1 alone protects the same two sites as before with the similar exception that the 3′ half of the DSE remains unprotected. Thus the linker insertion does not impair the binding of either factor but serves to separate the two binding sites from each other.

When $p67^{SRF}$ and AP-1 are both included in the binding reactions exactly as before, two distinct footprints are observed over the DSE and the −295 element. Thus in the case of promoter mutant pG, $p67^{SRF}$ does not interfere with AP-1 binding to the −295 element as it appears to do in the unmutated c-*fos* promoter. Instead, the two proteins can bind together.

It should be noted that affinity-purified ATF (Lee *et al.*, 1987a) also binds to the AP-1 consensus sequence 3′ of the DSE to produce a footprint indistinguishable from the one seen for AP-1 (result not shown) and CREB (Montminy and Bilezikjian, 1987) is reported to do so too (P.Herrlich, personal communication). In the footprinting experiments shown here, AP-1 serves only as an example for a number of *trans*-activating factors that have the potential to recognize this site in the c-*fos* promoter.

The result of the footprinting experiment shown in Figure 4a indicates that $p67^{SRF}$ binds to the DSE more readily than AP-1 to the −295 element. However, this experiment was performed necessarily with fractions enriched for $p67^{SRF}$ and AP-1. Therefore, in order to assess more realistically which factor preferentially occupies its binding site, gel retardation experiment was performed in which increasing amounts of DNA were titrated against a fixed amount of an unfractionated HeLa nuclear extract.

Interpretation of the result of this experiment is complicated by the interaction of a third protein with the DNA probe. As seen in Figure 5, under conditions of limiting wt probe (see Figure 1a for sequence of probe), only complex II is detected. This complex has been shown to be a ternary complex formed by p62, $p67^{SRF}$ and the SRE (Shaw *et al.*, 1989a). $p67^{SRF}$ bound to the DSE is an absolute requirement for the formation of this complex. Only when excess probe is included in the binding reaction are complexes observed that correspond to the interaction of

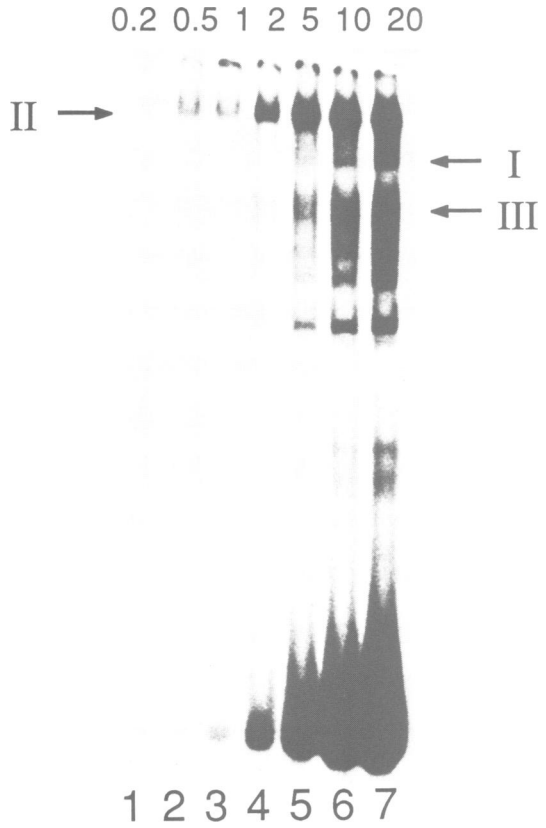

Fig. 5. Gel retardation analysis of HeLa SRE-binding proteins with limiting amounts of DNA probe. A fixed amount of HeLa nuclear extract (~25 μg protein) was incubated with increasing amounts of labelled oligonucleotide duplex wt (Figure 1a). Numbers above the lanes refer to counts per minute ($\times 10^{-3}$) of probe included in each binding reaction. Complexes I, II and III are described in the text.

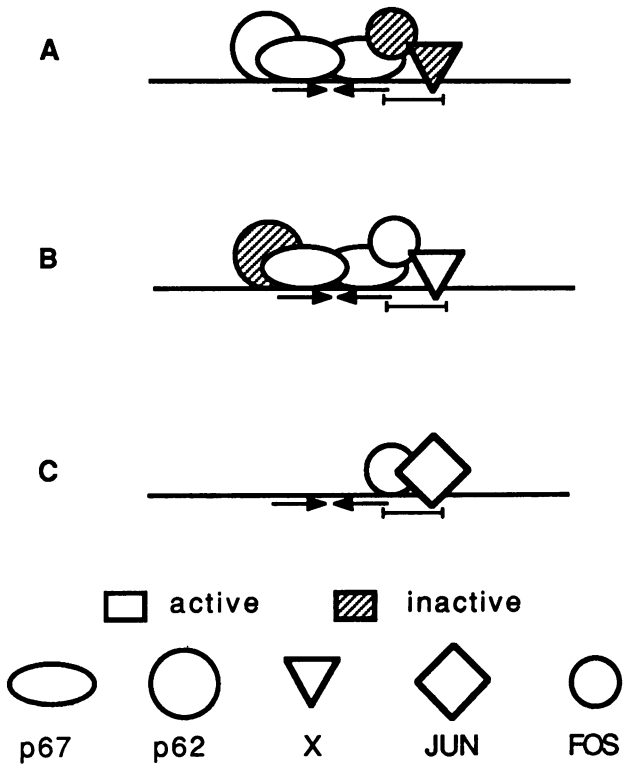

Fig. 6. Schematic representation of transcription factors binding to the SRE as proposed for the levels of expression observed. (**A**) corresponds to inducible expression, (**B**) repression and (**C**) uninducible, constitutive expression, as described in Discussion.

p67^{SRF} alone with the DSE (complex I) and a factor with the −295 element (complex III), as confirmed by methylation interference analysis (result not shown). No complex is detected that corresponds to p67^{SRF} and AP-1 bound to the probe together. Thus in HeLa nuclear extracts, the DSE is occupied by p67^{SRF}, albeit in association with p62, in preference to the AP-1 consensus site being occupied by AP-1 or a related factor.

From the results of the footprinting and gel retardation experiments described above, we infer that the elevated levels of basal and serum-induced expression from promoter mutant pG compared with −EPf (Figure 3b, lanes 5 and 6, 1 and 2) result from the combined activities of p67^{SRF} and p62 bound to the SRE, which confer serum inducible expression, and an activator protein (possibly AP-1) bound to the AP-1 consensus site, which confers an elevated level of constitutive expression.

Discussion

In a recent paper, it was shown that serum induction mediated by the SRE depends on the interaction of a second protein, p62, with DSE-bound p67^{SRF} (Shaw *et al.*, 1989a). Here an attempt has been made to ask what function, if any, does p67^{SRF} have besides that of a transcriptional inducer in association with p62. On the basis of our previously published work, the experiments described above and those in the accompanying paper (König *et al.*, 1989), it can now be proposed that three levels of c-*fos* expression (induced, repressed and constitutive) are determined by occupation of the SRE by p67^{SRF} and its interaction with other proteins, as presented schematically in Figure 6.

Poised repression

In vivo competition experiments, in which the c-*fos* promoter attached to a reporter gene was co-transfected with specific promoter fragments demonstrated that, prior to induction by serum, the c-*fos* promoter is repressed by a diffusible factor (Sassone-Corsi and Verma, 1987). This state of repression may be referred to as 'poised' repression and is distinct from the down-regulation that follows induction, for example, by serum (Treisman, 1985; Wilson and Treisman, 1988) as during the latter, the promoter is refractory to induction (Morgan *et al.*, 1987). Taken together, the results of the experiments presented in Figures 1 and 2 demonstrate that when p62 is prevented from interacting with the SRE, the level of expression from the human c-*fos* promoter in HeLa cells correlates inversely with the affinity of p67^{SRF} for the DSE. This result implies very strongly that poised repression of the human c-*fos* promoter is mediated by DSE-bound p67^{SRF}. Leung and Miyamoto (1989) have recently published results that are also consistent with this assertion.

Constitutive expression

Further *in vivo* experiments were carried out to identify the promoter element(s) responsible for the high constitutive expression in the absence of p67^{SRF} binding. A linker insertion mutant of the human c-*fos* promoter (pG) was found to have an elevated level of both uninduced and serum-induced expression. Three explanations for this phenomenon

were considered plausible: a duplication of the functional SRE, the loss of a repressor function or the recruitment of an activator.

Deletion of sequences immediately upstream from the linker (−354 to −307) to produce ΔF10G resulted in the loss of serum inducibility. Thus there is no functional duplication of the SRE. However, an uninducible level of expression from ΔF10G was observed that was lost upon extending the deletion further downstream to position −278, implying that sequences between −306 and −278 can function to activate transcription. If, on the other hand, these sequences are deleted (ΔGH), serum inducibility is not impaired. Thus we concluded that sequences between −306 and −278 harbour a cryptic *trans*-activator binding site and that the third explanation for the behaviour of promoter mutant pG is applicable.

Because the sequence around position −295 has been previously shown to bind PEA1, the murine analogue of AP-1 (Piette and Yaniv, 1987), *in vitro*, it was considered to be a potential activator binding site, especially as the linker insertion in pG displaces it from immediately adjacent to the DSE in the unmutated promoter by one helical turn. Footprint and gel retardation experiments with fractions enriched for $p67^{SRF}$ and AP-1 indicate that AP-1 is unable to bind readily to the −295 element in the unmutated promoter if the DSE is occupied by $p67^{SRF}$. However, both proteins are able to occupy their respective sites in promoter mutant pG.

Studies of stably integrated *fos−CAT* fusion genes in HeLa cell lines led to the conclusion that if the SRE is destroyed by point mutations, the −295 element can mediate induction of the promoter by tetradecanoylphorbolacetate and epidermal growth factor as efficiently as the SRE itself (Fisch *et al.*, 1989). This again points to the −295 site being a cryptic promoter element.

It should be emphasized that other transcriptional activators have been reported to bind to the −295 site in the c-*fos* promoter, and in our hands, affinity-purified ATF binds to the site comparably to AP-1 (result not shown). Such observations may result from the degree of cross-contamination observed with affinity-purified fractions of related proteins (Hai *et al.*, 1988), which is presumably a general limitation of the method. Nevertheless, although we conclude that these *in vitro* experiments support the notion that a *trans*-activator is recruited to the c-*fos* promoter in mutant pG but is unable to bind to the unmutated promoter, we do not ascribe this activity specifically to AP-1.

It is interesting to note that recent findings show the most active form of AP-1 to consist of a heterodimer between JUN and FOS, the products of the c-*jun* and c-*fos* proto-oncogenes respectively (Halazonetis *et al.*, 1988; Nakabeppu *et al.*, 1988; Rauscher *et al.*, 1988b). Therefore in the absence of $p67^{SRF}$, FOS may function as part of a positive feedback loop to *trans*-activate its own transcription. High levels of c-*fos* transcription have been observed in prenatal parietal endoderm (Mason *et al.*, 1985) as well as in extra-embryonal tissues, e.g. placenta, amnion and yolk sac (Müller *et al.*, 1983). It is conceivable that these cells express a *trans*-activator similar to the one present in HeLa cells, but fail to express $p67^{SRF}$. Alternatively, a shift in the relative affinities of the two factors for their respective binding sites may suffice to switch from high, uninducible to inducible (and repressible) c-*fos* expression. Experiments to distinguish between these possibilities are underway.

Repression by FOS mediated through $p67^{SRF}$

Does the −295 element serve as a promoter element in the presence of DSE-bound $p67^{SRF}$? Both FOS and JUN have been implicated in the down-regulation of c-*fos* expression following induction of the gene (Sassone-Corsi *et al.*, 1988a; Schönthal *et al.*, 1989). The proteins are known to activate transcription when bound to sites closely related to the −295 element (Chiu *et al.*, 1988; Schönthal *et al.*, 1988) and to be involved in repression of adipocyte-specific gene expression through similar *cis*-elements (Distel *et al.*, 1987). In the accompanying paper, König *et al.* (1989) present compelling, corroborative evidence that the SRE is the promoter element through which repression of c-*fos* expression is mediated. The effect observed can be modulated by co-expression of anti-sense FOS sequences or over-expression of FOS, demonstrating the involvement of FOS in its own repression. Removal of the −295 element adjacent to the DSE results in a 3-fold increase in c-*fos* expression but, interestingly, this can be entirely counteracted by over-expression of FOS.

Based on this observation, we propose that poised repression and down-regulation of c-*fos* expression are achieved by the interaction of a second component with $p67^{SRF}$ bound to the DSE. The interaction may be stabilized by sequences downstream of the DSE, i.e. the −295 element, in a manner similar to that proposed for p62 and sequences upstream of the DSE during induction (Shaw *et al.*, 1989a,b). Thus the high level of expression from pG seen in starved cells (Figure 4, lane 5) may reflect impared repression of this promoter mutant. Although the results of König *et al.* imply that a FOS−JUN heterodimer is this additional component, it might also consist of a FOS−FOS homodimer or a FOS−X heterodimer whereby X might represent ATF or a hitherto unidentified FOS-associated protein (Franza *et al.*, 1987).

Two further observations support this model. Firstly, two murine genes, *Krox*20 and 24 that are co-regulated with c-*fos* also contain SREs with the adjacent sequence CGTCA, similar to the c-*fos* promoter configuration (Wilson and Treisman, 1988; Chavrier *et al.*, 1989). Secondly, *in vivo* footprinting experiments reveal that the DSE and the −295 element can be occupied simultaneously, which is consistent with the formation of a multicomponent complex over the SRE (Herrera *et al.*, 1989). At present there is no evidence to suggest how the repressed status of the c-*fos* promoter progresses from down-regulated to poised, thus permitting induction to recur (Morgan *et al.*, 1987).

Our experiments and those described by König *et al.* differ in an important respect: the choice of cells. In NIH3T3 cells, p-EPf and pG behave similarly, as do ΔF10G and ΔF10H (result not shown). On the other hand, repression in HeLa cells is not improved by the −295 element as in NIH3T3 cells. Taken together, these observations suggest that HeLa cells express the factor(s) involved in *trans*-activation and repression at a higher level than NIH3T3 cells.

In several important aspects, the regulation mediated by the c-*fos* SRE parallels the control mechanisms regulating yeast cell type-specific gene expression (Bender and Spraque, 1987; Goutte and Johnson, 1988; Keleher *et al.*, 1988; for a review see Nasmyth and Shore, 1987). Firstly, both systems employ sequence-specific DNA binding proteins ($p67^{SRF}$ and PRTF) that have extended amino acid homology (Norman *et al.*, 1988; Passmore *et al.*, 1988) and related DNA-binding specificities (Hayes *et al.*, 1988).

Secondly, both p67^{SRF} and PRTF interact specifically with other proteins (p62 and α1 or α2 respectively) when bound to the DNA. Thirdly, p67^{SRF} and PRTF display the common functional characteristic of mediating both gene activation (i.e. induction of c-*fos* and activation of α-specific genes respectively) and repression (i.e. c-*fos* and **a**-specific genes respectively). By analogy, repression of c-*fos* expression mediated by SRE-bound p67^{SRF} would depend on the interaction of p67^{SRF} with FOS in some form or other, thus mirroring the interaction of PRTF with α2 that leads to the repression of **a**-specific genes.

Materials and methods

Cells, extracts and chromatography

Adherent HeLa cells were grown in DMEM supplemented with 10% fetal calf serum. HeLa cells used to prepare extracts were grown as spinner cultures in Joklik's medium supplemented with 5% newborn calf serum. Nuclear salt extracts were prepared as described (Dignam *et al.*, 1983) with slight modifications (Wildeman *et al.*, 1984). Protein concentrations of the nuclear extracts were in the range of 10–15 mg/ml.

The p67^{SRF} fraction used for the DNase I footprinting experiments was enriched for p67^{SRF} by wheatgerm lectin (Sigma) affinity chromatography (H.Schröter and A.Nordheim, in preparation) with suitable modifications. Protein from 15 ml of extract was bound to the resin (0.5 ml packed volume) by gentle shaking in buffer 'A100' on ice for 30 min, after which the resin was pelleted by centrifugation at 1000 r.p.m. for 5 min. The supernatant was removed, the resin resuspended in 1 ml A100 and transferred to a small column. After the resin had settled, the buffer was drained off, the column washed with 5 ml A100 and eluted with three 0.5 ml fractions of A100 containing *N*-acetyl glucosamine.

Affinity-purified p67^{SRF} was prepared as described (Schröter *et al.*, 1987), affinity-purified AP-1 was a gift from D.Bohmann and affinity-purified ATF was a gift from T.Hai.

DNA binding studies

Gel retardation assays were performed as described previously (Schröter *et al.*, 1987). DNase I footprinting experiments were carried out essentially as described (Lee *et al.*, 1987b) except that binding reactions were performed in 32 μl of 15 mM Hepes pH 7.9, 15% glycerol, 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM spermidine, 0.4 mM dithiothreitol, 2% polyvinyl alcohol, and that 32 μl of 2 mM $MgCl_2$, 9 mM $CaCl_2$ were added prior to digestion with DNase I. *N*-acetyl glucosamine (50 mM) was also present in the binding reactions that contained p67^{SRF}. DNA probes used were the *Pst*I–*Hin*dIII fragments of p-EPf and pG from positions −359 to +72 cloned into pUC19, excised with *Eco*RI and *Hin*dIII, 5′-labelled with polynucleotide kinase (Pharmacia) and recut with *Xho*I.

DNA transfer, serum stimulation, RNA preparation and analysis

HeLa cells were transfected by DNA–calcium phosphate co-precipitation (Weber *et al.*, 1984), starved and stimulated with serum (Shaw *et al.*, 1989a) and RNA was isolated and analysed (Shaw *et al.*, 1985) as described. The probe used for S1 analysis of RNA transcribed from p-EPf and all its derivatives was the relevant strand of a 570 bp *Ava*II fragment of p-EPf that spans the cap site, labelled at its 5′ end. S1 analysis of rabbit β-globin RNA was performed in parallel as described previously (Shaw *et al.*, 1985).

Construction of plasmids

p-EPf, ΔPS4, ΔPS4EL and ΔPS4L have been described previously (Shaw *et al.*, 1989a). ΔPS4M was constructed in an analogous fashion to ΔPS4EL and ΔPS4L. Linker insertion mutants each contain a single octameric *Xba*I linker inserted into restriction sites within the human c-*fos* promoter: pF10, *Fnu*DII (−354); pG, *Hga*I (−302) and pH, *Hae*III (−278). Deletion mutants were derived by combining complementary halves of the relevant linker insertion mutants. Sequences were verified by the chain termination method (Sanger *et al.*, 1977; Seedorf *et al.*, 1985).

The rabbit β-globin constructs used as transfection controls have been described elsewhere: p9511 (de Villiers *et al.*, 1982) and core *Pvu*II 7½ (Schirm *et al.*, 1987).

Acknowledgements

We wish to thank Klaus Meese for growing vast quantities of HeLa cells and Hennrik Schröter for providing affinity-purified p67^{SRF} prepared from some of them. We thank Dirk Bohmann for the gift of AP-1 and Tsonwin Hai for ATF. We are grateful to Peter Herrlich and colleagues for making data available prior to publication and for discussions on the manuscript, to Laura Runkel for critically reading the manuscript and to C.Hynde and B.Ferry for sound encouragement. This research was supported by the Deutsche Forschungsgemeinschaft (SFB 229) and the Fonds der Chemischen Industrie.

References

Bender,A. and Sprague,G.,Jr (1987) *Cell*, **50**, 681–691.
Chavrier,P., Janssen-Timmen,U., Mattei,M.-G., Zerial,M., Bravo,R. and Charnay,P. (1989) *Mol. Cell. Biol.*, **9**, 787–797.
Chiu,R., Boyle,W.J., Meek,J., Smeal,T., Hunter,T. and Karin,M. (1988) *Cell*, **54**, 541–552.
Cohen,D.R. and Curran,T. (1988) *CRC Crit. Rev. Biochem.*, in press.
Curran,T., Van Beveren,C., Ling,N. and Verma,I.M. (1985) *Mol. Cell. Biol.*, **5**, 167–172.
de Villiers,J., Olson,L., Tyndall,C. and Schaffner,W. (1982) *Nucleic Acids Res.*, **10**, 7965–7976.
Dignam,J.C., Lebowitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475–1489.
Distel,R.J., Ro,H.-S., Rosen,B.S., Groves,D.L. and Spiegelman,B.M. (1987) *Cell*, **49**, 835–844.
Fisch,T.M, Prywes,R. and Roeder,R.G. (1989) *Mol. Cell. Biol.*, **9**, 1327–1331.
Franza,B.R.,Jr, Sambucetti,L.C., Cohen,D.R. and Curran,T. (1987) *Oncogene*, **1**, 213–221.
Goutte,C. and Johnson,A.D. (1988) *Cell*, **52**, 875–882.
Greenberg,M.E. and Ziff,E.B. (1984) *Nature*, **311**, 433–438.
Greenberg,M.E., Hermanowski,A.L. and Ziff,E.B. (1986) *Mol. Cell. Biol.*, **6**, 1050–1057.
Hai,T., Liu,F., Allegretto,A., Karin,M. and Green,M.R. (1988) *Genes Dev.*, **2**, 1216–1226.
Halazonetis,T.D., Georgopoulos,K., Greenberg,M.E. and Leder,P. (1988) *Cell*, **55**, 917–924.
Hayes,T.E., Sengupta,P. and Cochran,B.H. (1988) *Genes Dev.*, **2**, 1713–1722.
Herrera,R.E., Shaw,P.E. and Nordheim,A. (1989) *Nature*, **340**, 68–70.
Keleher,C.A., Goutte,C. and Johnson,A.D. (1988) *Cell*, **53**, 927–936.
König,H., Ponta,H., Rahmsdorf,U., Büscher,M., Schönthal,A., Rahmsdorf,H. and Herrlich,P. (1989) *EMBO J.*, 2559–2566.
Kruijer,W., Cooper,J.A., Hunter,T. and Verma,I. (1984) *Nature*, **312**, 711–716.
Kushtai,G., Barzilay,J., Feldman,M. and Eisenbach,L. (1988) *Oncogene*, **2**, 119–127.
Lech,K., Anderson,K. and Brent,R. (1988) *Cell*, **52**, 179–182.
Lee,K.A.W., Hai,T.Y., SivaRamanan,L., Thimmappaya,B., Hurst,H.C., Jones,N.C. and Green,M.R. (1987a) *Proc. Natl. Acad. Sci. USA*, **84**, 8355–8359.
Lee,W., Mitchell,P. and Tjian,R. (1987b) *Cell*, **49**, 741–752.
Leung,S. and Miyamoto,N.G. (1989) *Nucleic Acids Res.*, **17**, 1177–1195.
Mason,I., Murphy,D. and Hogan,B.L.M. (1985) *Differentiation*, **30**, 76–81.
Montminy,M.R. and Bilezikjian,L.M. (1987) *Nature*, **328**, 175–178.
Morgan,J.I., Cohen,D.R., Hempstead,J.L. and Curran,T. (1987) *Science*, **237**, 192–197.
Müller,R., Verma,I.M. and Andamson,E.D. (1983) *EMBO J.*, **2**, 679–684.
Müller,R., Bravo,R., Burckhardt,J. and Curran,T. (1984) *Nature*, **312**, 716–720.
Nakabeppu,Y., Ryder,K. and Nathans,D. (1988) *Cell*, **55**, 907–915.
Nasmyth,K. and Shore,D. (1987) *Science*, **237**, 1162–1170.
Norman,C., Runswick,M., Pollock,R. and Treisman,R. (1988) *Cell*, **55**, 989–1003.
Passmore,S., Maine,G.T., Elble,R., Christ,C. and Tye,B.-K. (1988) *J. Mol. Biol.*, **204**, 593–606.
Piette,J. and Yaniv,M. (1987) *EMBO J.*, **6**, 1331–1337.
Rauscher,F.J.,III, Cohen,D.R., Curran,T., Bos,T.J., Vogt,P.K., Bohmann,D., Tjian,R. and Franza,B.R.,Jr, (1988a) *Science*, **240**, 1010–1016.
Rauscher,F.J.,III, Voulalas,P.J., Franza,B.R.,Jr and Curran,T. (1988b) *Genes Dev.*, **2**, 1687–1699.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Sassone-Corsi,P. and Verma,I.M. (1987) *Nature*, **326**, 507–510.
Sassone-Corsi,P., Sisson,J.C. and Verma,I.M. (1988a) *Nature*, **334**, 314–319.

Sassone-Corsi,P., Lamph,W.W., Kamps,M. and Verma,I.M. (1988b) *Cell*, **54**, 553–560.
Schirm,S., Jiricny,J. and Schaffner,W. (1987) *Genes Dev.*, **1**, 65–74.
Schönthal,A., Herrlich,P., Rahmsdorf,H.J. and Ponta,H. (1988) *Cell*, **54**, 325–334.
Schönthal,A., Büscher,M., Angel,P., Rahmsdorf,H.J., Ponta,H., Hattori,K., Chiu,R., Karin,M. and Herrlich,P. (1989) *Oncogene*, **4**, 629–636.
Schröter,H., Shaw,P.E. and Nordheim,A. (1987) *Nucleic Acids Res.*, **15**, 10145–10158.
Setoyama,C., Frunzio,R., Liau,G., Mudryj,M. and de Crombrugge,B. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3213–3217.
Seedorf,K., Krämmer,G., Dürst,M., Suhai,S. and Röwekamp,W. (1985) *Virology*, **145**, 181–185.
Shaw,P.E., Bohmann,D. and Sergeant,A. (1985) *EMBO J.*, **4**, 3247–3252.
Shaw,P.E., Schröter,H. and Nordheim,A. (1989a) *Cell*, **56**, 563–572.
Shaw,P.E., Hipskind,R.A., Schröter,H. and Nordheim,A. (1989b) In *Nucleic Acids and Molecular Biology*, **3**, Springer-Verlag, Heidelberg, pp. 120–132.
Treisman,R. (1985) *Cell*, **42**, 889–902.
Treisman,R. (1986) *Cell*, **46**, 567–574.
Weber,F., de Villiers,J. and Schaffner,W. (1984) *Cell*, **36**, 983–992.
Wildeman,A., Sassone-Corsi,P., Grundström,T., Zenke,M. and Chambon,P. (1984) *EMBO J.*, **3**, 3129–3133.
Wilson,T. and Treisman,R. (1988) *EMBO J.*, **7**, 4193–4202.

Received on April 7, 1989; revised on June 1, 1989