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Protein synthesis inhibitors differentially superinduce c-*fos* and c-*jun* by three distinct mechanisms: lack of evidence for labile repressors

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Protein synthesis inhibitors strongly augment and prolong the usually transient induction of c-*fos* and c-*jun* by growth factors, phorbol esters etc., a phenomenon termed superinduction which is conventionally regarded as a secondary consequence of translational arrest. Our recent demonstration that some inhibitors can act positively as nuclear signalling agonists compromises this view and necessitates a re-evaluation of superinduction. First, we show that labile repressors, widely postulated to act negatively on diverse superinducible genes, are not involved in regulating c-*fos* and c-*jun*. Secondly, two components of c-*fos* and c-*jun* superinduction, namely the delay in shutting off transcription and stabilization of their mRNAs, arise from translational arrest and are common to all protein synthesis inhibitors. Thirdly, the recently described capacity to act positively as nuclear signalling agonists to stimulate pp33/pp15 phosphorylation is restricted to compounds such as anisomycin and cycloheximide; these, but not emetine or puromycin, will induce c-*fos*/c-*jun* on their own. Fourthly, the translational arrest-related components of superinduction are dissociable from the signalling agonist effects at sub-inhibitory concentrations of anisomycin, under which conditions a new type of c-*fos*/c-*jun* superinduction with 'spike' kinetics is observed. Finally, we show that in response to EGF plus anisomycin, the nuclear signalling responses are themselves augmented and prolonged in a manner that corresponds to c-*fos*/c-*jun* superinduction under these conditions.

Key words: mRNA stabilization/nuclear signalling/protein synthesis inhibitors/proto-oncogene expression/superinduction

Introduction

Polypeptide growth factors and phorbol esters initiate diverse intracellular signalling networks leading rapidly to the transcription of a subset of genes termed 'immediate early-response' (IE) genes (Bravo, 1990). These include members of the c-*fos* and c-*jun* families that comprise transcription factor AP1 (Greenberg and Ziff, 1984; Curran and Franza, 1988; Lamph *et al.*, 1988; Quantin and Breathnach, 1988; Ryseck *et al.*, 1988). The accumulation of IE gene transcripts is normally transient due first to the brief activation of the genes themselves and secondly to the extreme instability of their mRNA transcripts (Greenberg and Ziff, 1984; Greenberg *et al.*, 1986; Rahmsdorf *et al.*, 1987). The induction of IE genes does not require *de novo* synthesis of transcription factors since it is not blocked by inhibitors of protein synthesis. In fact, the presence of protein synthesis inhibitors along with an inducing growth factor leads to massive over-accumulation of IE transcripts, a phenomenon known as superinduction (Cochran *et al.*, 1983; Lau and Nathans, 1987). Furthermore, treatment of quiescent cells with certain protein synthesis inhibitors alone is sufficient to elicit IE gene expression (Greenberg *et al.*, 1986; Subramaniam *et al.*, 1989; Mahadevan and Edwards, 1991). Experimental evidence to explain induction and superinduction of IE genes by protein synthesis inhibitors, much of which comes from studies of c-*fos* regulation, has led to the four proposed contributory mechanisms discussed below.

First, it is a common observation that the normally labile IE gene transcripts become much more stable in the presence of protein synthesis inhibitors. Following stimulation, c-*fos* mRNA levels are maximal at ~30 min (Greenberg and Ziff, 1984; Wilson and Treisman, 1988) and the mRNA half-life is ~9 min (Rahmsdorf *et al.*, 1987). This extreme instability is due to the presence of multiple AU-rich destabilizing elements in its 3′ non-coding region (Shaw and Kamen, 1986; Fort *et al.*, 1987; Bonnieu *et al.*, 1989) and to additional sequences within the body of the c-*fos* gene (Shyu *et al.*, 1989, 1991; Kabnick and Housman, 1988). After cycloheximide treatment, c-*fos* mRNA half-life is extended to several hours (Fort *et al.*, 1987; Rahmsdorf *et al.*, 1987; Wilson and Treisman, 1988). At least three hypotheses have been proposed for the increased mRNA stability. First, degradation may involve labile mRNAases which disappear rapidly following inhibition of protein synthesis (Pontecorvi *et al.*, 1988). Secondly, some protein synthesis inhibitors cause RNAs to be trapped on polysomes, thus shielding them from cytoplasmic ribonucleases (Cochran *et al.*, 1983; see Discussion). Thirdly, the kinetics of mRNA turnover following addition and removal of CHM suggest that like histone and tubulin gene transcripts (Brawerman, 1989), ongoing translation is essential for c-*fos* mRNA degradation (Fort *et al*, 1987; Wilson and Treisman, 1988).

The second factor contributing to superinduction, the augmented transcription of the c-*fos* gene in the presence of protein synthesis inhibitors, has been assessed by nuclear run-on assays. After serum stimulation c-*fos* transcription reaches maximal levels by ~15 min and then is rapidly repressed, returning to basal levels within 1 h (Greenberg and Ziff, 1984; Greenberg *et al.*, 1986). The presence of anisomycin or cycloheximide enhances transcription rates and prolongs transcription for several hours (Greenberg *et al.*, 1986; Fort *et al.*, 1987) indicating that protein synthesis is necessary for shutting off transcription. The Fos protein itself is implicated in this transcriptional shut-off, possibly acting through the SRE (serum response element)

or AP1 sites upstream of the gene (Sassone-Corsi *et al.*, 1988; Lucibello *et al.*, 1989; Schöntal *et al.*, 1989; Rivera *et al.*, 1990). Though the mechanics of autorepression are not fully understood, it is conceivable that once c-*fos* is activated, inhibition of protein synthesis results in failure to produce the auto-repressing Fos protein, thus allowing its continued maintenance in the transcriptionally active mode (Chen and Allfrey, 1987; Chen *et al.*, 1990).

The third component of superinduction is described by a proposal referred to here as the 'labile repressor hypothesis'. This suggests that genes such as c-*fos* are kept inactive in quiescent cells by the continuous synthesis of labile repressors which rapidly disappear following inhibition of protein synthesis (for example, Wall *et al.*, 1986; Morello *et al.*, 1990) and is distinct from the post-induction repression mechanism discussed above. Subramaniam *et al.* (1989) showed that cycloheximide potentiates the serum responsiveness of reporter plasmids carrying a c-*fos* SRE in transient transfection assays and suggested that the putative labile repressor interacts with SRE−SRF (serum response factor) complexes. Though widely postulated in various superinducing systems, such as growth factor- and phorbol ester-inducible early response genes, various viral genes and NF-κB-regulated genes, labile repressors have not been definitively identified.

Finally, we have reported recently that anisomycin and cycloheximide independently stimulate the same nuclear signalling responses as EGF or TPA, namely the rapid phosphorylation of two chromatin-associated proteins pp33 and pp15 (Mahadevan and Edwards, 1991; Mahadevan *et al.*, 1991). pp33 exists in a second detergent-extractable complexed form, whereas pp15 is exclusively chromatin-associated and has been identified recently as histone H3. These responses are demonstrable using concentrations of anisomycin below the concentration that is necessary for inhibition of protein synthesis (Mahadevan and Edwards, 1991; Mahadevan *et al.*, 1991), suggesting that independent of its ability to block protein synthesis, anisomycin has a potent agonist-like action on intracellular signalling processes and can act positively to induce and superinduce proto-oncogenes. The observation that some protein synthesis inhibitors can act positively to activate signals is a departure from established thinking and compromises earlier explanations in which it has been sought to explain all the effects of these molecules solely through their ability to produce translational arrest.

In this light, we have re-evaluated the phenomenon of c-*fos*/c-*jun* superinduction by various protein synthesis inhibitors to establish the relative contributions of the various mechanisms postulated above. The effects of anisomycin, cycloheximide, puromycin and emetine, either alone or in combination with EGF, on the kinetics c-*fos* and c-*jun* transcription, mRNA stabilization and on the activation of the nuclear signalling responses described above have been assessed. In the sections that follow we use the term 'induction' to refer to gene activation in response to EGF or protein synthesis inhibitors used individually and 'superinduction' to that resulting from combined administration of EGF and inhibitor.

Results

For consistency with previous analyses, confluent, quiescent mouse C3H 10T1/2 fibroblasts have been used here. Protein synthesis inhibitors used were anisomycin, cycloheximide, puromycin and emetine, all of which produce virtually complete translational arrest (Mahadevan and Edwards, 1991; Figure 5D). EGF is used here because its nuclear signalling and proto-oncogene regulation characteristics are well established in these cells. The earliest nuclear signalling responses are the phosphorylation of two chromatin-associated proteins pp33 and pp15 (histone H3) detectable within 5 min, peaking at ~1 h and terminating at 2−3 h (Mahadevan *et al.*, 1988, 1989, 1990). These responses are not consequent upon the transcriptional activation of c-*fos*/c-*jun* and remain demonstrable in α-amanitin- or actinomycin D-treated cells. Transcription of c-*fos* and c-*jun* is maximal at 15 min after EGF stimulation and returns to basal levels within 1 h (see Figure 2).

Anisomycin, cycloheximide and puromycin differentially induce and superinduce c-fos and c-jun expression

The relative abilities of anisomycin, cycloheximide and puromycin to induce and superinduce c-*fos* and c-*jun* expression were assessed by Northern blotting (Figure 1A).

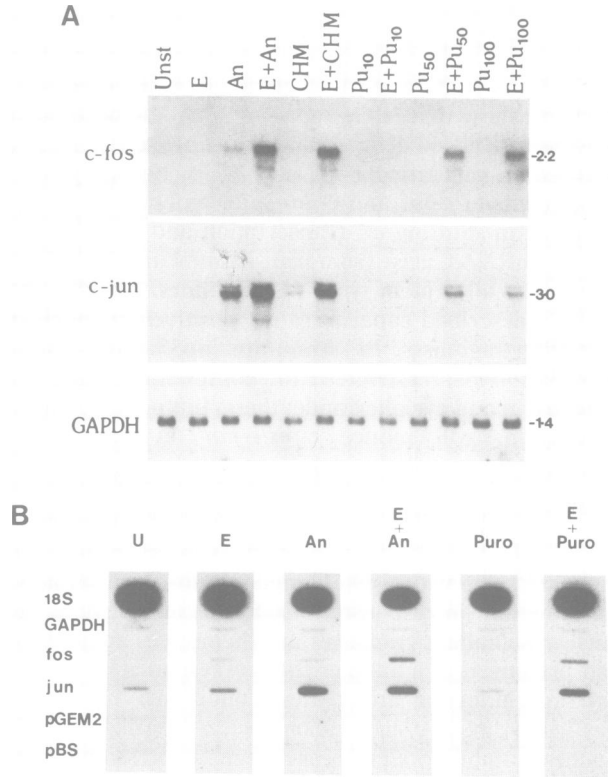

Fig. 1. Differential effects of protein synthesis inhibitors on c-*fos* and c-*jun* expression. **(A)** Northern blot analysis of cytoplasmic RNA from quiescent, confluent C3H10T1/2 cells and cells treated for 30 min with inhibitors alone or in combination with EGF. Replicate blots were hybridized with nick-translated c-*fos* and c-*jun* probes and equivalence of loading was confirmed by hybridization with the *GAPDH* probe. Unst, unstimulated cells; E, EGF 50 ng/ml; Anisomycin (An) and cycloheximide (CHM) were used at 10 μg/ml each and puromycin (Pu) was used at the indicated concentations in μg/ml (i.e. Pu_{10} 10 μg/ml). **(B)** Nuclear run-on transcription analysis. Nuclei were isolated from confluent, quiescent C3H10T1/2 fibroblasts and from cells treated with protein synthesis inhibitors alone or in combination with EGF (E) for 15 min. Anisomycin was used at 10 μg/ml and puromycin at 100 μg/ml. Nascent transcripts were allowed to elongate *in vitro* in the presence of [^{32}P]rUTP as described in Materials and methods and 2×10^6 c.p.m. of each probe was hybridized to nitrocellulose filters carrying the indicated DNAs. As controls, pGEM2 and pBluescriptII KS$^-$ (pBS) were included on the filters.

In combination with EGF, all three inhibitors superinduced c-*fos* and c-*jun* RNAs, however, even when used at high concentrations, puromycin produced relatively poor superinduction despite causing virtually complete inhibition of protein synthesis at 10 μg/ml (see Figure 5). The rank order of potency of superinduction by these inhibitors in combination with EGF is anisomycin > cycloheximide >> puromycin. However, when cells were treated with inhibitors in the absence of EGF, a different picture emerged (Figure 1A). Anisomycin produced stronger induction of c-*fos* and c-*jun* than EGF, whereas puromycin was without effect, although at very high concentrations (100 μg/ml) a weak effect was obtained. Cycloheximide, although less potent, also induced both genes. Although the induction of c-*fos* by cycloheximide is not clear in the experiment shown in Figure 1A, the effect on c-*jun* is evident. From our own repeated experiments and data from other laboratories, cycloheximide is clearly capable of inducing expression of c-*fos* and c-*jun* on its own, albeit with much lower potency than anisomycin. At these concentrations, anisomycin and cycloheximide are equally potent at inhibiting protein synthesis (see Figure 5); the difference in their ability to induce c-*fos* and c-*jun* may arise from the fact that anisomycin elicits much stronger nuclear signalling responses than cycloheximide (see below).

The difference of effect between anisomycin and puromycin was examined further using nuclear run-on analyses. When used in conjunction with EGF, both inhibitors resulted in the superinduction of c-*fos* and c-*jun* at the transcriptional level to similar extents (Figure 1B). This demonstrates that the deficiency of c-*fos* and c-*jun* RNAs seen in Northern blotting analysis of puromycin superinduced cells (Figure 1A) is a post-transcriptional effect. However, the important observation here is that in agreement with the Northern blotting data, anisomycin on its own elicits c-*fos* and c-*jun* transcription, whereas puromycin (or emetine, see below) has no effect, despite producing complete translational arrest. Thus, translational arrest is in itself insufficient to initiate c-*fos* and c-*jun* transcription, showing that continuously synthesized labile molecules do not play a role in repressing these genes.

Anisomycin, puromycin and emetine delay transcriptional shut-off of c-fos and c-jun under superinducing conditions

Upon induction with EGF, c-*fos* and c-*jun* transcription is transient, being terminated by ~45 min after stimulation. The ability of anisomycin, puromycin and emetine to delay shut-off of c-*fos* and c-*jun* was assessed using nuclear run-on assays to monitor transcription at different times after stimulation (Figure 2). As reported previously, EGF-induced transcription of c-*fos* and c-*jun* was transient, peaking at 15 min and returning to basal levels by 1 h. Anisomycin alone or in combination with EGF strongly potentiated the transcriptional activation of both genes and sustained it for at least 1 h. Used on their own, neither puromycin nor emetine induced c-*fos* or c-*jun* transcription, but in combination with EGF both agents produced stronger c-*fos* and c-*jun* transcription after 30 min treatment compared to EGF alone. Taken together, these results suggest that following activation by an inducing stimulus, a delay in switching off transcription is a general feature of superinduction by all protein synthesis inhibitors.

RNA polymerase density across the c-fos gene during induction and superinduction

Apart from upstream regulatory sequences controlling transcriptional initiation, it has been reported recently that the c-*fos* gene is also regulated by a post-initiation block at an intragenic attenuation site (Fort *et al.*, 1987; Lamb *et al.*, 1990; Collart *et al.*, 1991; Mechti *et al.*, 1991). To produce mature cytoplasmic c-*fos* mRNA in response to inducing stimuli it is proposed that two events occur; (i) enhancement of transcriptional initiation mediated by the SRE and (ii) relief of the block to transcription elongation at a site close to the exon 1−intron1 boundary. The inability of puromycin on its own to induce c-*fos* transcription and mRNA accumulation (Figures 1 and 2) could therefore potentially result from continued operation of the attenuation mechanism in puromycin-treated cells. This was assessed by performing nuclear run-on analyses using probes corresponding to the 5′ and 3′ ends of the c-*fos* gene (Figure 2).

In contrast to previous studies with rat embryo fibroblasts (Lamb *et al.*, 1990), mouse Ltk$^-$ cells (Mechti *et al.*, 1991) and mouse macrophages (Collart *et al.*, 1991), we find that the c-*fos* attenuation mechanism is not clear in C3H 10T1/2 cells. Quiescent cells display background levels of hybridization of nascent transcripts to a 400 bp region of the 5′ end of the gene and a 1.1 kbp segment from the 3′ end (Figure 2). There was no enhancement of signal from the 5′ c-*fos* sub-fragment following treatment with puromycin alone, suggesting that it is unlikely that puromycin treatment had caused transcription initiation without lifting the block on elongation. Following induction by EGF or anisomycin

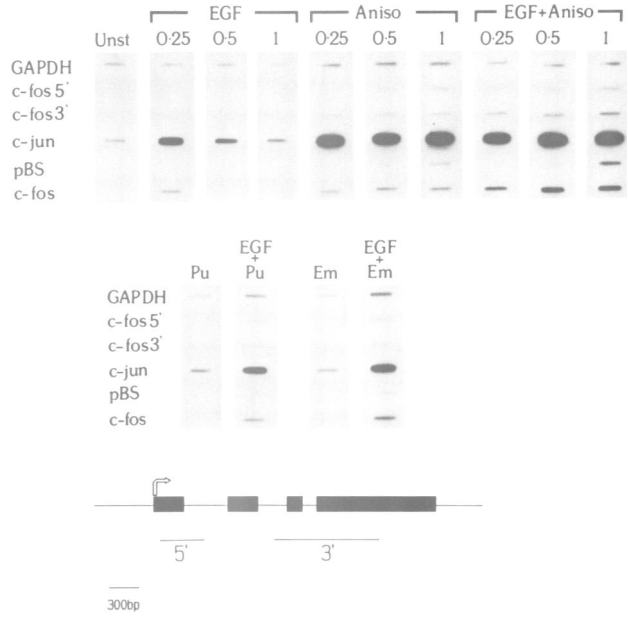

Fig. 2. Timecourse of c-*fos* and c-*jun* transcription following induction and superinduction and comparison of RNA polymerase densities in different parts of the c-*fos* gene. Nuclear run-on assays were carried out using nuclei from cells exposed to EGF, anisomycin, puromycin or emetine and combinations of EGF with the inhibitors for 15, 30 and 60 min. Nascent ^{32}P-labelled RNAs were hybridized to filters carrying the indicated cloned DNAs as described in the legend to Figure 1B. The line drawing at the bottom of the figure describes the origin of the sub-cloned regions of c-*fos*. Hybridization to the pBS plasmid seen at 1hour in the anisomycin treated cells may be due to ^{32}P-labelled transcript degradation arising from the cytotoxicity of this compound, thus causing increased non-specific hybridization.

and superinduction by combinations of EGF with anisomycin or puromycin, increased hybridization to both 5′ and 3′ c-*fos* sub-fragments was observed. The differences in signal intensities for c-*fos* 5′ and 3′-specific nascent RNAs reflect the representation of thymine residues in the target sub-fragments. On balance, this shows that following induction and superinduction, RNA polymerase density across the gene is relatively uniform. It is unclear why the attenuation mechanism observed in other cell types does not operate in the C3H 10T1/2 cells used here. The data show that the inability of puromycin on its own to induce c-*fos* is not attributable to a post-initiational block at the attenuation site.

Stabilization of c-fos and c-jun mRNAs following superinduction with anisomycin, cycloheximide or puromycin

A further component of superinduction, the stabilization of c-*fos* and c-*jun* transcripts in the presence of protein synthesis inhibitors, was evaluated by analysis of rates of mRNA degradation in superinduced cells. Following superinduction by EGF plus inhibitors for 1 h, further transcription was arrested by the addition of DRB (5,6-dichlororibofuranosyl benzimidazole) and mRNA decay was then followed over a 4 h period by Northern blotting analysis (Figure 3A). Rates of decay obtained by densitometry of the Northern blots are displayed in Figure 3B. Treatment with anisomycin, cycloheximide or puromycin resulted in mRNA stabilization, although the extent depended upon the inhibitor used. With any particular inhibitor, c-*fos* transcripts disappeared with similar kinetics to those of c-*jun* suggesting that in translationally-arrested cells they are likely to be degraded by the same enzymic route. In the puromycin-treated cells, mRNA loss is observed immediately after DRB addition, and c-*fos* and c-*jun* transcripts have similar half-lives of ~40 min. In cycloheximide- or anisomycin-treated cells, c-*fos* and c-*jun* RNA levels remained stable for ~1 h after DRB

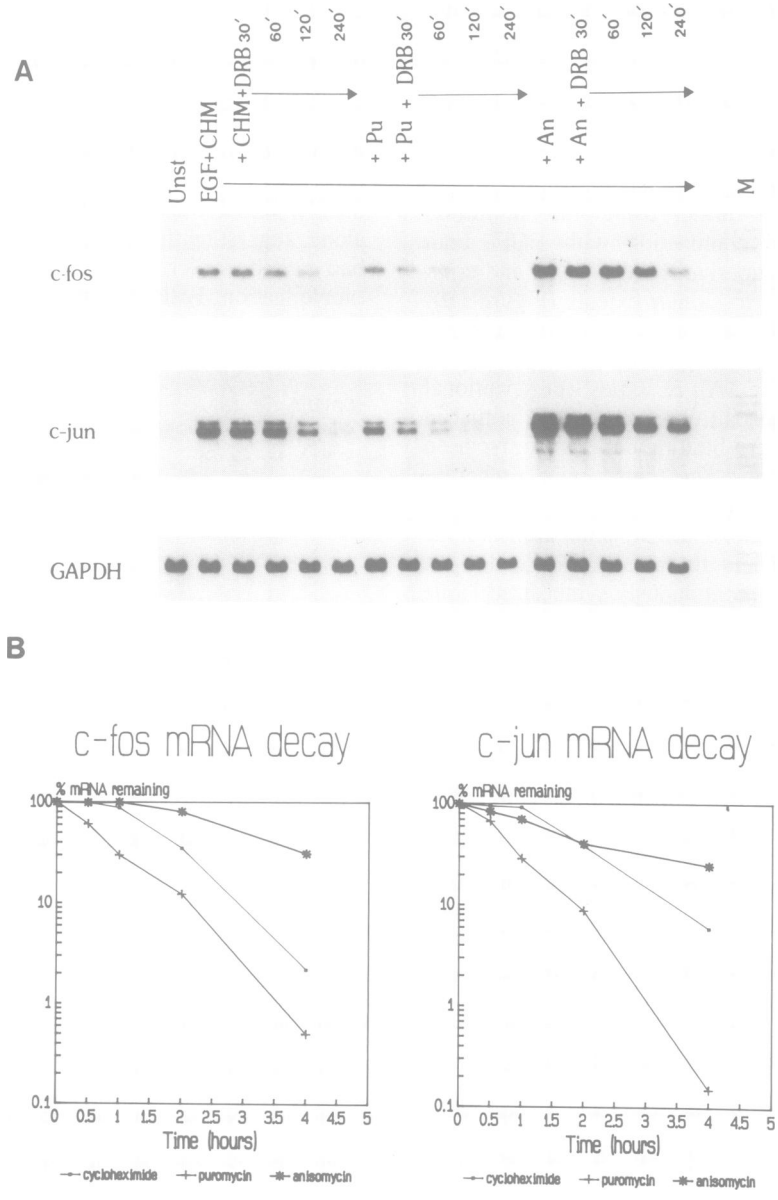

Fig. 3. Stabilities of c-*fos* and c-*jun* mRNAs under superinducing conditions. **(A)** Analysis of c-*fos* and c-*jun* RNA levels following inhibition of transcription in cells exposed to superinducing conditions. Cells were treated with EGF (50 ng/ml) and either anisomycin (10 μg/ml), cycloheximide (10 μg/ml) or puromycin (100 μg/ml) for 1 h and further transcription was arrested by addition of 25 μg/ml DRB. Cytoplasmic RNA was isolated at the indicated times. **(B)** Shows the results of densitometry of the data in (A), in which time 0 refers to the time of addition of DRB.

addition, but subsequent decay was faster in cycloheximide-treated cells than in cells exposed to anisomycin ($t_{1/2}$ ~1 h versus 2 h). Thus, when administered together with EGF, the differential effects of anisomycin and puromycin on the superinduced levels of c-*fos* and c-*jun* RNAs seen in Figures 1 and 3 can be in part attributed to the lower stability of the RNAs in puromycin-treated cells. There may be a simple mechanistic explanation for this. Both anisomycin and cycloheximide cause polysome stabilization whereas puromycin causes polysome disaggregation (Vázquez, 1979); the freezing of mRNAs on polysomes by the former compounds may afford some protection from cytoplasmic nucleases.

Sub-inhibitory levels of anisomycin induce c-fos and c-jun RNAs without prolonging transcript stability

The results above, in agreement with the literature (Wilson and Treisman, 1988) suggest that the stabilization of message is associated with translational arrest and is common to all protein synthesis inhibitors. However, we have shown recently that it is possible to produce superinduction of c-*fos* and c-*jun* and to stimulate signalling responses by phosphorylation of two proteins pp33 and pp15, using anisomycin at concentrations below that necessary for translational arrest (Mahadevan and Edwards, 1991). pp33 exists in two forms, complexed and chromatin-associated, which are resolved by a sequential extraction procedure, whereas pp15 is exclusively chromatin-associated and has been recently identified as histone H3 (Mahadevan *et al.*, 1991). This indicated that anisomycin possessed an intrinsic ability to interact with intracellular signalling pathways and could act positively to activate nuclear signalling and proto-oncogene induction without producing translational arrest (Mahadevan and Edwards, 1991). From the data presented above, we predicted that at these sub-inhibitory concentrations, there should be no message stabilization and consequently no prolonged persistence of c-*fos*/c-*jun* transcripts at these low concentrations. This was assessed by Northern blotting analysis (Figure 4). Using 50 ng/ml anisomycin, c-*fos* and c-*jun* transcript levels after 30 min of treatment correspond to ~25–50% of those found in cells treated with 10 μg/ml anisomycin (Mahadevan and Edwards, 1991). However, after prolonged treatment, dramatic differences in RNA levels are apparent (Figure 4A). At the inhibitory concentration of anisomycin (10 μg/ml), high levels of induced and superinduced c-*fos* and c-*jun* RNAs persist for at least 6 h, whereas at the sub-inhibitory level (50 ng/ml) c-*fos* transcripts were not evident and c-*jun* RNA levels were similar to those observed in cells stimulated with EGF alone. Decay rates for c-*fos* and c-*jun* transcripts at sub-inhibitory anisomycin concentrations were determined by transcription inhibition with DRB after 30 min of treatment with anisomycin either alone or in combination with EGF. Under these conditions both c-*fos* and c-*jun* showed much reduced $t_{1/2}$ values (~15 min and 30 min, respectively) relative to cells treated with inhibitory concentrations of anisomycin ($t_{1/2}$ values ~2 h, see Figure 3). Thus, at sub-inhibitory concentrations, anisomycin activates expression of c-*fos* and c-*jun* with spike kinetics similar to that of EGF itself and without the prolonged persistence of message associated with translational arrest.

Anisomycin, cycloheximide and puromycin differentially elicit mitogen-regulated intracellular signals

The final constituent of superinduction analysed here extends our recent demonstration, discussed above, that some protein

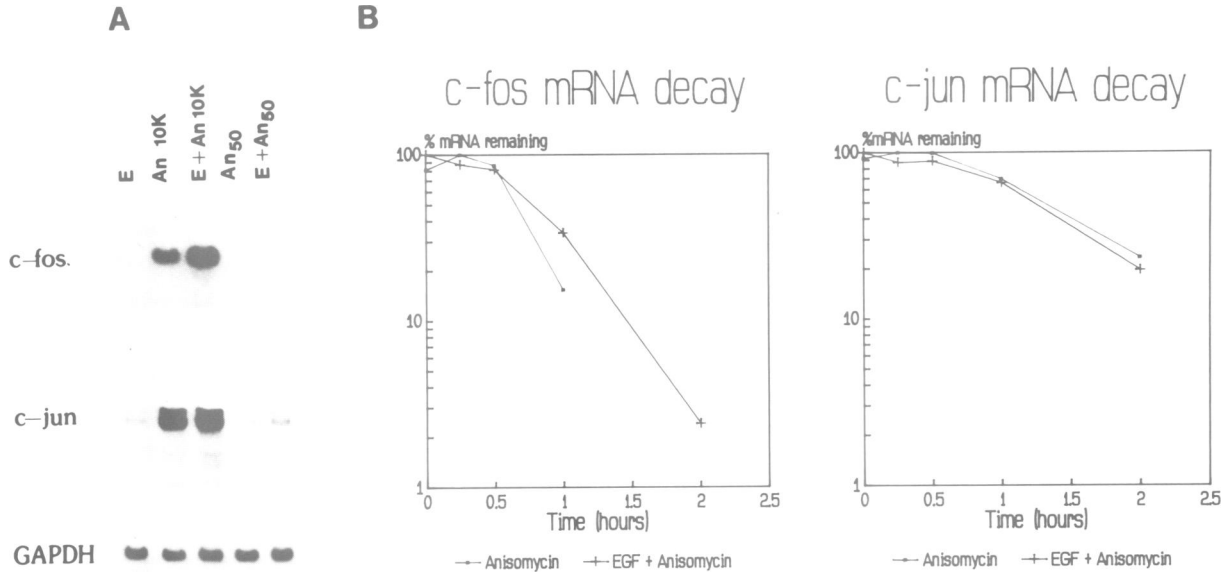

Fig. 4. Sub-inhibitory concentrations of anisomycin induce and superinduce c-*fos* and c-*jun* RNAs but do not cause mRNA stabilization. **(A)** Northern blot analysis of RNA isolated from cells after 6 h stimulation with EGF (50 ng/ml), anisomycin at 10 μg/ml (An10K) or 50 ng/ml (An50) and combinations of EGF and anisomycin at both concentrations. The lower concentration of anisomycin strongly induces c-*fos* and c-*jun* expression (see Mahadevan and Edwards, 1991), but the induced transcripts do not persist to 6 h. **(B)** Kinetics of decay of c-*fos* and c-*jun* mRNAs in cells stimulated with sub-inhibitory concentrations of anisomycin. DRB (25 μg/ml) was added to cells 30 min after stimulation with anisomycin (50 ng/ml) either alone or in combination with EGF (50 ng/ml) and c-*fos* and c-*jun* RNA levels were monitored by Northern blot analysis of RNA isolated at the indicated times after DRB addition. The results show densitometry of the blot data, normalized to equivalent signals for *GAPDH*.

synthesis inhibitors elicit early nuclear signals identical to EGF or TPA (Mahadevan *et al.*, 1988, 1991; Mahadevan and Edwards, 1991). To assess the extent of their signalling influence, the relative abilities of anisomycin, cycloheximide and puromycin to stimulate intracellular signals were compared directly with those of EGF and TPA (Figure 5). Only TPA activated protein kinase C, as shown by phosphorylation of the 80 kDa substrate (Figure 5A). Anisomycin and cycloheximide were both strong inducers of pp33 and histone H3 phosphorylation (Figure 5, panels B and C), whereas puromycin was extremely weak. As reported previously, pp33 and histone H3 phosphorylation were stimulated by EGF and TPA (Mahadevan *et al.*, 1989, 1990, 1991). Anisomycin produces the highest levels of pp33 and histone H3 phosphorylation, the response being stronger than that seen with EGF, TPA or cycloheximide. At the concentrations used here (10 μg/ml for each inhibitor), virtually complete inhibition of [^{35}S]methionine incorporation into protein was obtained after 5 min of treatment (Figure 5D). Thus, although they each produce virtually complete inhibition of protein synthesis, anisomycin, cycloheximide and puromycin differ in their relative abilities to stimulate pp33 and histone H3 (pp15) phosphorylation, showing that the signalling is not a consequence of translational arrest. This is in accord with our recent demonstration that anisomycin will stimulate intracellular signals at concentrations below that necessary for inhibition of protein synthesis.

Time-course analysis was performed using anisomycin and puromycin to determine the duration of the signalling responses under conditions of induction and superinduction. Two timepoints, 1 and 3 h after stimulation, were chosen because EGF-stimulated pp33 phosphorylation is maximal within 1 h and is almost undetectable at 3 h (Figure 6; Mahadevan *et al.*, 1988). Anisomycin strongly stimulated phosphorylation of complexed and chromatin-associated pp33, the response being sustained and clearly demonstrable at both 1 and 3 h. Puromycin was a weak inducer of these responses. Notably, the combination of EGF with anisomycin but not puromycin produces stronger phosphorylation than either agent individually and the response was clearly detectable at 3 h, at which time the response to EGF alone would have been terminated. Thus, anisomycin prolongs the normally transient signalling responses. The relative extent and duration of histone H3 (pp15) phosphorylation under these conditions was similar to that of pp33. Thus, the induction and superinduction of proto-oncogene expression elicited by anisomycin is paralleled by the augmented and prolonged stimulation of the nuclear signalling responses.

Discussion

The transient induction of immediate early-response genes is a widely utilized control system to orchestrate precise and regulated availability of specific proteins including transcription factors such as c-*Fos* and c-*Jun*, overproduction of which lead to transformation *in vitro* (Miller *et al.*, 1984; Castellazzi *et al.*, 1990) and tumourigenesis *in vivo* (Rüther *et al.*, 1989; Schuh *et al.*, 1990). In general, the strategies adopted to ensure transient expression are rapid transcriptional shut-off following induction (Greenberg and Ziff, 1984; Treisman, 1985; Greenberg *et al.*, 1986; Lamph *et al.*, 1988) and extreme instability of the gene products (Fort *et al.*, 1987; Shyu *et al.*, 1989, 1991). These processes depend upon ongoing translation in at least two ways. The present consensus (Greenberg *et al.*, 1986; Fort *et al.*, 1987; Wilson and Treisman, 1988; Laird-Offringa *et al.*, 1990) is that all protein synthesis inhibitors (i) cause a failure of transcriptional shut-off after activation, most probably by blocking the synthesis of a repressing, or auto-repressing protein and (ii) prolong mRNA half-lives primarily due to the coupling of the degradation of a particular mRNA to its own translation. Auto-repression and translation–degradation coupling are obvious ways to ensure the discrete availability of a particular transcription factor. However, some protein synthesis inhibitors such as anisomycin and cycloheximide have a unique additional property unrelated to translational arrest: they act as nuclear signalling agonists, inducing phosphorylation of nuclear proteins that are normally the targets of growth factor-regulated signal transduction pathways and can therefore act positively to

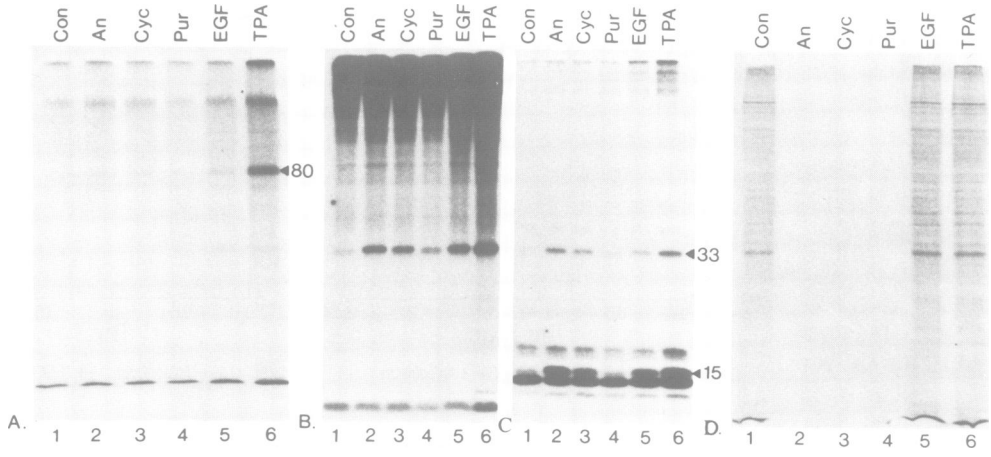

Fig. 5. Protein synthesis inhibitors differentially stimulate intracellular signals. Quiescent C3H 10T1/2 cells were stimulated for 30 min with 10 μg/ml anisomycin (lane 2), 10 μg/ml cycloheximide (lane 3), 10 μg/ml puromycin (lane 4), 100 ng/ml EGF (lane 5) and 100 nM TPA (lane 6). Lane 1 is an unstimulated control. For analysis of protein phosphorylation (A, B and C), lysates were prepared from ^{32}P-labelled cells and subjected to sequential extraction to produce a Triton–DOC fraction containing the 80 kDa protein kinase C substrate **(A)**, a pellet containing complexed pp33 **(B)** and a chromatin-derived fraction containing pp33 and pp15 **(C)**. For analysis of protein synthesis **(D)** [^{35}S]methionine was added to the cells 5 min after addition of each agent and cells were harvested after 25 min. Thus, the analysis of protein phosphorylation is directly comparable with that of protein synthesis.

induce IE gene expression (Mahadevan and Edwards, 1991). These findings, summarized in Table I, somewhat alter the accepted view of superinduction.

Anisomycin as a signalling agonist or antagonist

Used on their own, only anisomycin and cycloheximide are capable of eliciting pp33/histone H3 phosphorylation and c-*fos*/c-*jun* transcription, with anisomycin being more potent. This cannot be the consequence of translational arrest, first because neither puromycin nor emetine produce these effects and secondly because these responses remain demonstrable at sub-inhibitory concentrations of anisomycin. This invalidates the 'labile represor hypothesis' for c-*fos* and c-*jun* (discussed below) and raises an interesting question of signalling, namely, where exactly does anisomycin intervene to produce the activation of nuclear signalling pathways?

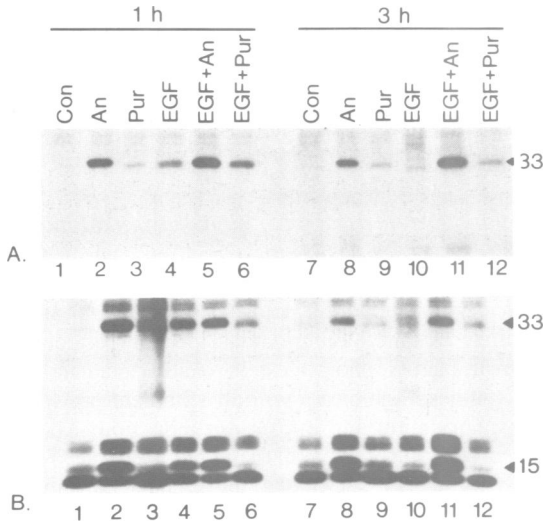

Fig. 6. Intracellular signalling by anisomycin and puromycin at 1 and 3 h. Intracellular signalling (A and B): Quiescent C3H 10T1/2 cells were stimulated for 1 h (lanes 1–6) or 3 h (lanes 7–12) with 10 μg/ml anisomycin (lanes 2 and 8), 10 μg/ml puromycin (lanes 3 and 9), 100 ng/ml EGF (lanes 4 and 10), 100 ng/ml EGF + 10 μg/ml anisomycin (lanes 5 and 11) and 100 ng/ml EGF + 10 μg/ml puromycin (lanes 6 and 12). Cells were harvested and fractions containing complexed pp33 **(A)** and chromatin-associated pp33 and pp15 **(B)** were prepared as described in the legend to Figure 1. Protein synthesis inhibitors, when present were added 10 min prior to addition of EGF (lanes 1 and 7, unstimulated controls).

It is conceivable that anisomycin resembles endogenous intracellular signalling molecules on the pathway to c-*fos* and c-*jun* activation and functions simply as a nuclear signalling agonist. From data on protein kinase C-mediated 80 kDa protein phosphorylation (Figure 5) and from unpublished data, it seems clear that anisomycin does not intervene in the PI pathway, nor activate protein kinase C. This also implies that anisomycin probably does not act at points upstream of protein kinase C activation, such as by activating receptors linked to PI turnover etc. Excluding the PI pathway, other potential points of intervention currently under examination are serine/threonine kinases such as c-*raf* and the *ras*/GAP system. It is worth noting that even at sub-inhibitory concentrations, EGF and anisomycin are strongly synergistic in their activation of pp33/histone H3 phosphorylation and c-*fos*/c-*jun* induction. A possible explanation may be that anisomycin resembles and functions as the antagonist of a molecule involved in one of the several negative feedback loops that ensure transience of signalling and gene induction.

It is known that anisomycin and cycloheximide bind to different sites on the 60S ribosomal subunit, producing translational arrest and polysome stabilization. That these are their only known targets (Barbacid and Vazquez, 1975; Vazquez, 1979) may be because the analysis thus far has been restricted to their effects on polysomes and protein synthesis. It is possible, though somewhat unlikely, that this 60S subunit binding may activate some as yet unknown ribosome-associated intracellular signalling system. This may be dissociable from translational arrest because binding to a small number of ribosomes, insufficient for observable translational inhibition, may be sufficient to activate a signal that is amplified to lead to nuclear signalling and gene activation. Any such mechanism must be highly specific, because anisomycin and cycloheximide are the only inhibitors tested to have these signalling properties. The

Table I. Summary of protein synthesis inhibitors and their effects on c-*fos* and c-*jun* expression and signalling-related phosphorylation

Inhibitor	Action	Effects on c-*fos* and c-*jun* expression		Effects on pp33 and pp15 phosphorylation
		alone (induction)	+EGF (superinduction)	
Anisomycin	Blocks peptide bond formation, binds to 60S subunit polysome stabilizer	++++	++++	++++
Cycloheximide	Inhibits initiation, elongation and termination, acts on 60S subunit polysome stabilizer	+	+++	++
Puromycin	Analogue of 3′ end of aminoacyl tRNA, interacts with A site on 60S subunit polysome destabilizer	–	+++	–
Emetine	Blocks translocation, acts on 40S subunit, stabilizes polysomes	–	+++	ND

–, no stimulation; +, stimulation (number of + indicate magnitude of the effect); ND, not determined.

possibility that the 'freezing' of polysomes by these agents may be the origin of the signal was eliminated because emetine, which causes polysome stabilization but acts on the 40S subunit is completely inactive in gene induction.

The labile repressor hypothesis

Data presented here show that c-*fos*/c-*jun* superinduction cannot be explained in terms labile repressors. The existence of rapidly degraded nuclear repressors of transcription is frequently invoked as a possible explanation of superinduction (for example, Wall *et al.*, 1986; Morello *et al.*, 1990). Transient transfection studies of c-*fos* regulation demonstrated that unstimulated mouse fibroblasts contain repressors that can be titrated out by the presence of high levels of SRE sequences (Sassone-Corsi and Verma, 1987), but did not assess the stability of the proteins involved. More recent transfection studies showed that a functional SRE could confer cycloheximide-inducibility to a reporter gene (Subramaniam *et al.*, 1989), which led to the proposal that a labile protein may interact with SRE−SRF to repress c-*fos* transcription. If this were the case, inhibition of protein synthesis by any translational inhibitor should result in c-*fos* induction, which was not seen in our experiments. Other explanations of the experiments of Subramaniam *et al.* (1989) are possible. For instance, by acting as a signalling agonist, cycloheximide may stimulate transcription directly from the reporter plasmids. Alternatively, by acting as an inducer of IE genes and as a strong stabilizer of their transcripts, cycloheximide could cause accumulation of significant amounts of IE mRNAs, which upon wash-out of the inhibitor are translated to yield products that transactivate the SRE-driven reporter gene.

Translational arrest results in delayed transcriptional shut-off and increased transcript stability

All four translational inhibitors, when used together with EGF, produce a delay in shutting off transcription and an increase in message stability. These phenomena are not discussed at length here, as they are well described in the literature. The nuclear run-on assays (Figures 1B and 2) demonstrate that superinduction is characterized by elevated, persistent c-*fos* and c-*jun* transcription, consistent with a general failure of post-induction repression. This is likely to be a direct consequence of translational arrest suppressing synthesis of a repressing protein, which in the case of c-*fos* may be the auto-repressing Fos protein itself (Sassone-Corsi *et al.*, 1988; Lucibello *et al.*, 1989; Schöntal *et al.*, 1989; Rivera *et al.*, 1990).

Stabilization of the short-lived c-*fos* and c-*jun* mRNAs following inhibition of protein synthesis arises in two possible ways (Wilson and Treisman, 1988; Shyu *et al.*, 1989, 1991). First, normal RNA turnover may depend upon the ongoing translation of the mRNA itself, as shown for c-*fos* (Wilson and Treisman, 1988) and c-myc (Wisdom and Lee, 1991). Alternatively, it could reflect the operation of labile nucleases that are rapidly lost from the cell following translational inhibition (Harford *et al.*, 1990). The lower level of stabilization of c-*fos* and c-*jun* RNAs observed in cells treated with puromycin compared to cycloheximide or anisomycin (Figure 3) argues against a labile nuclease being the rate limiting factor. The difference may reflect the increased accessibility of mRNAs to nucleases when polysomes are disassembled.

Sub-inhibitory levels of anisomycin elicit nuclear signalling and transcriptional activation without mRNA stabilization: spike superinduction

Data presented here extend our recent demonstration that anisomycin can induce gene expression and signalling at very low doses (25 ng/ml or ~100 nM). Under these conditions cellular protein synthesis is not inhibited (Mahadevan and Edwards, 1991). In addition, Figure 4 demonstrates that at these non-inhibitory concentrations, gene activation is transient and the induced message not stabilized, confirming earlier indications that the prolonged transcription and mRNA stabilization are the consequence of translational arrest. In these circumstances we predominantly obtain the positively-acting inducing component of anisomycin that is independent of its effectiveness as an inhibitor of protein synthesis. This gives rise to a new type of superinduction where the mRNAs are over-produced but not stabilized, giving rise to a transient excess of message, which we propose to refer to as 'spike' superinduction.

Correlations between nuclear signalling, induction and superinduction of c-fos/c-jun

The correlations between pp33/histone H3 phosphorylation and c-*fos*/c-*jun* induction and superinduction are now compelling. Despite having distinct primary effects in the cell, growth factors, phorbol esters, okadaic acid and certain protein synthesis inhibitors will all stimulate pp33/histone H3 phosphorylation and c-*fos*/c-*jun* induction. There is a distinct order of potency of the inhibitors (see Table I), namely anisomycin > cycloheximide >> puromycin, in their effects on c-*fos* and c-*jun* expression, which correlates well with their effects on pp33/histone H3 phosphorylation. The difference between anisomycin and cycloheximide on c-*fos*/c-*jun* induction and superinduction may arise from the fact that anisomycin is a much stronger nuclear signalling agonist. At sub-inhibitory concentrations of anisomycin, pp33/histone H3 phosphorylation and c-*fos*/c-*jun* induction remain demonstrable. Finally, we have shown here that under conditions of superinduction, pp33/histone H3 phosphorylation is augmented and prolonged in a manner that parallels the effects on proto-oncogene induction. By prolonging the normally transient nuclear signals (Figure 6), anisomycin may make an additional contribution to the prolonged transcriptional activation of c-*fos* and c-*jun* under superinducing conditions. Thus, despite our extensive attempts, we are unable to dissociate pp33/histone H3 phosphorylation from c-*fos*/c-*jun* expression in their relative intensities and duration at present. This makes for a pressing case that the two are mechanistically linked in some way, but this remains to be proven.

In conclusion, our data demonstrate that depending on the nature of the protein synthesis inhibitor, superinduction may be regarded as the cumulative result of intervention at either two or three different points in the process of signal delivery, transcriptional activation and shut-off and mRNA degradation that normally orchestrates transient proto-oncogene induction.

Materials and methods

Cell culture

Mouse C3H 10T1/2 fibroblasts were used in all experiments. Cells were grown in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12 1:1) in the presence of 10% vol/vol fetal calf serum (FCS). For experimental

purposes, cultures were rendered quiescent by growth to confluence and the medium was then replaced with DMEM/F12 supplemented with 0.1% vol/vol fetal calf serum (Gibco/BRL) and 1 μg/ml insulin and 5 μg/ml transferrin (Gibco/BRL). Cells were incubated overnight before receiving the additions specified in each experiment.

Additions

Epidermal growth factor (EGF; Boehringer Mannheim) was used at a final concentration of 50ng/ml. The protein synthesis inhibitors anisomycin, cycloheximide, puromycin and emetine were purchased from Sigma and solutions (5 mg/ml Anisomycin, 10 mg/ml all other reagents, prepared in H_2O) were prepared freshly on the day of use. DRB (5,6, Dichlorobenzimidazole riboside, Sigma) was used at 25 μg/ml from a stock solution at 20 mg/ml in dimethylsulphoxide.

Northern blot studies and gene probes

Northern blot studies were carried out using cytoplasmic RNA as described previously (Edwards and Denhardt, 1985; Edwards *et al.*, 1985). The gene probes used were as follows. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was a 1 kbp fragment of murine cDNA cloned into pBluescript KS^- (Stratagene). The c-*fos* probe was either a full-length human c-*fos* cDNA (pG7hfos) provided by Dr Lynn Matrisian, Vanderbilt University, Nashville, TN., or a *BglII*–*SalI* fragment of v-*fos* DNA previously described (Edwards *et al.*, 1987). Human c-*fos* clones corresponding to the 5′ and 3′ ends of the genes were a *NaeI* fragment (nucleotides 42–434) and a *SphI*–*NaeI* fragment (1524–2624) sub-cloned from a genomic clone provided by Dr R.Johnston, University of Calgary. Mouse c-*jun* (pAH119, Ryseck *et al.*, 1988) was generously provided by Dr R.Bravo.

Nuclear run-on assays

Nuclear run-on transcription studies used a modification of the protocol of Greenberg and Ziff (1984). Briefly, following incubation of nuclei for 25 min in reaction buffer (Greenberg and Ziff, 1984), unlabelled UTP was added to a final concentration of 40 μM for 2 min. Nuclei were lysed in 1 ml 4 M guanidinium isothiocyanate and nascent ^{32}P-labelled RNAs were isolated using the hot phenol extraction procedure (Sambrook *et al.*, 1989). The aqueous phase from this extraction was re-extracted with Tris-saturated phenol–chloroform (1:1) at room temperature and RNA was precipitated by addition of sodium acetate pH 5.5 to a final concentration of 0.3 M and ethanol to 70%. The precipitated nucleic acids were collected by centrifugation and resupended in 0.1 ml 10 mM Tris–HCl pH7.6, 5 mM $MgCl_2$, 50 mM NaCl. Residual DNA was removed by addition of 1 unit RQ1 DNase (Promega) and incubation at 37°C for 30 min. Samples were extracted once with phenol–chloroform (1:1) and contaminating nucleotides were removed by spun-column chromatography using Sephadex G50. Nitrocellulose filters carrying slot-blotted plasmid DNAs were hybridized with equivalent amounts (generally in the range $2-5 \times 10^6$ c.p.m.) of nascent RNA probe using the conditions of Greenberg and Ziff (1984), which were likewise used for post-hybridization washes. Autoradiography was performed with Kodak XAR5 film and a single Dupont Cronex intensifying screen.

[^{32}P]phosphate labelling and sequential extraction of C3H 10T1/2 cells

Quiescent C3H 10T1/2 cells were labelled for 3 h in phosphate-free medium containing 0.5 mCi/ml [^{32}P]phosphate (NEN), stimulated as appropriate and lysates prepared in Triton–DOC buffer (20 mM HEPES pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 100 mM sodium chloride, 50 mM sodium fluoride, 5 mM EDTA, 100 μM sodium molybdate) with protease inhibitors exactly as described (Mahadevan and Bell, 1990; Mahadevan *et al.*, 1990). After removal of insoluble material by a 15 min spin in a microfuge, aliquots of Triton–DOC supernatant were either ultracentrifuged (100 000 *g* for 30 min) to produce a pellet containing complexed pp33 or TCA-precipitated to analyse 80 kDa protein kinase C substrate phosphorylation. The microfuge pellet was extracted with 9 M urea as described previously to remove cytoskeletal proteins and the resultant pellet extracted with 0.3 M HCl to isolate a fraction containing chromatin-associated pp33 and histones. 80 kDa protein phosphorylation was analysed on 12.5% and complexed and chromatin-associated protein fractions on 15% SDS–polyacrylamide gels.

[^{35}S]methionine labelling of C3H 10T1/2 cells

Quiescent cells were set up and stimulated exactly as for the phosphate labelling studies described above. 5 min after stimulation 50 μCi/ml [^{35}S]methionine (Amersham) was added and after 25 min cells were harvested in Triton–DOC buffer. Aliquots of lysate were TCA-precipitated and labelled proteins analysed on 10% SDS–polyacrylamide gels. As cells were harvested 30 min after stimulation, these experiments are directly comparable to the associated signalling experiments (Figure 5).

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