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ras-induced c-*fos* expression and proliferation in living rat fibroblasts involves C-kinase activation and the serum response element pathway

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We have examined the early events involved in the proliferative activation of quiescent rat embryo fibroblasts by microinjection of oncogenic *ras* protein. Cells injected with *ras* show a transient expression of c-*fos* after 30–60 min visualized by immunofluorescence in the nucleus. This c-*fos* expression can be specifically suppressed by coinjection of a double-stranded oligonucleotide which corresponds to the serum response element (SRE) present in the c-*fos* promoter, implying that *ras* utilizes a pathway which activates the binding of serum response factor(s) (SRF) to SRE to induce c-*fos* transcription. Inhibition of this pathway also abolished *ras*-induced DNA synthesis indicating that the proliferative induction by *ras* requires expression of SRE-regulated genes. Both c-*fos* induction and DNA synthesis were prevented when *ras* oncoprotein was injected into quiescent cells together with either antibodies against calcium phospholipid-dependent protein kinase (C-kinase) or a synthetic peptide that specifically inhibits C-kinase. These data demonstrate the involvement of both functional C-kinase and the SRE pathway in the activation of quiescent cells by *ras* and suggest a potential relationship in their mechanism of action.

Key words: c-*fos*/C-kinase/*ras*/SRE

Introduction

The *ras* oncogenes have been associated with many types of human tumours and are thought to be at least partially responsible for the malignant phenotype (for a review, see Varmus, 1984). The mammalian *ras* family consists of three proto-oncogenes, H-*ras*, K-*ras*, and N-*ras*, each of which can acquire oncogenic properties by single nucleotide substitutions at either position 12, 13, 59 or 61 in their coding sequences (Tabin *et al.*, 1982) or by increased level of expression (Wigler *et al.*, 1984).

The *ras* genes code for proteins of 21–24 kd which bind guanine nucleotides, have GTPase activity and are associated with plasma membranes (for a review, see Barbacid, 1987). While little is known about the biological function of the *ras* proteins, these properties, together with their significant sequence homology with G proteins (Stryer *et al.*, 1986) suggest that *ras* proteins are regulatory proteins belonging to second messenger pathways. The introduction of oncogenic $p21^{ras}$ into quiescent fibroblasts induces both morphological transformation and DNA synthesis (Feramisco *et al.*, 1984). Moreover many different growth factors have been shown (by microinjection of an antibody which specifically blocks *ras* activity) to require functional $p21^{ras}$ for their activity (Mulcahy *et al.*, 1985). In an attempt to define the cellular signalling system affected by *ras*, different cells containing oncogenic *ras* genes have been analysed for changes in known second messenger molecules. In the yeast *Saccharomyces cerevisiae* (Toda and Wigler, 1985), the RAS proteins appear to be involved in the regulation of cAMP levels by coupling to the adenylate cyclase effector, but no such activity has yet been demonstrated in higher eukaryotic cells (Beckner *et al.*, 1985).

In mammalian cells, different studies utilizing either inducible expression of the *ras* genes in normal cell lines (Wakelam *et al.*, 1986) or constitutive expression of the *ras* genes in transfected cell lines (Fleischman *et al.*, 1985) have suggested that the *ras* protein could directly activate phospholipase C. The resulting production of diacylglycerol (DG) and inositol-1,4,5-triphosphate (IP_3), in turn, stimulates the Ca^{2+}-phospholipid-dependent protein kinase (C-kinase) and the release of Ca^{2+} from intracellular stores. Seuwen *et al.* (1988), however, failed to obtain evidence for interaction between *ras* proteins and phospholipase C.

Alternatively, since a reproducible increase in DG has been demonstrated in the absence of detectable changes in inositol phosphates in transformed cells containing activated *ras* (Lacal *et al.*, 1987a), the possibility remains that *ras* oncogenes specifically activate a source other than phosphoinositids for the generation of DG. Moreover, H-*ras* like the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Wasylyk *et al.*, 1987; Imler *et al.*, 1988) has been shown to activate the polyoma virus (Py) enhancer in both the myeloma cell line and NIH3T3 fibroblasts. These results, taken in combination with those of Morris *et al.* (1989), Berggren *et al.*, (1989) and Sassone-Corsi *et al.* (1989) are in keeping with a role for *ras* protein in signal transduction from outside the cell to a transcription factor in the nucleus through C-kinase.

It has been demonstrated (Mulcahy *et al.*, 1985; Stacey *et al.*, 1987) that introduction of *ras* oncoprotein into NIH3T3 cells or into rat pheochromocytoma PC12 cells (Sassone-Corsi *et al.*, 1989) activates c-*fos* expression. The proto-oncogene c-*fos*, which encodes a nuclear protein of 380 amino acids with DNA binding properties (Sambucetti and Curran, 1986), is expressed at relatively low levels in the majority of cell types, including fibroblasts. However, treatment of cells with mitogenic agents leads to a rapid increase of c-*fos* expression (Greenberg and Ziff, 1984; Kruiger *et al.*, 1984) that is necessary for cell proliferation (Riabowol *et al.*, 1988). The c-*fos* promoter contains a sequence element, termed serum response element (SRE) containing a 20 bp dyad symmetry element (DSE), that mediates the transcriptional response to serum (Treisman, 1985; Gilman *et al.*, 1986; Greenberg *et al.*, 1987; Gilman, 1988) through the binding of a protein called serum response factor (SRF) (Treisman, 1987; Prywes *et al.*, 1987; Norman

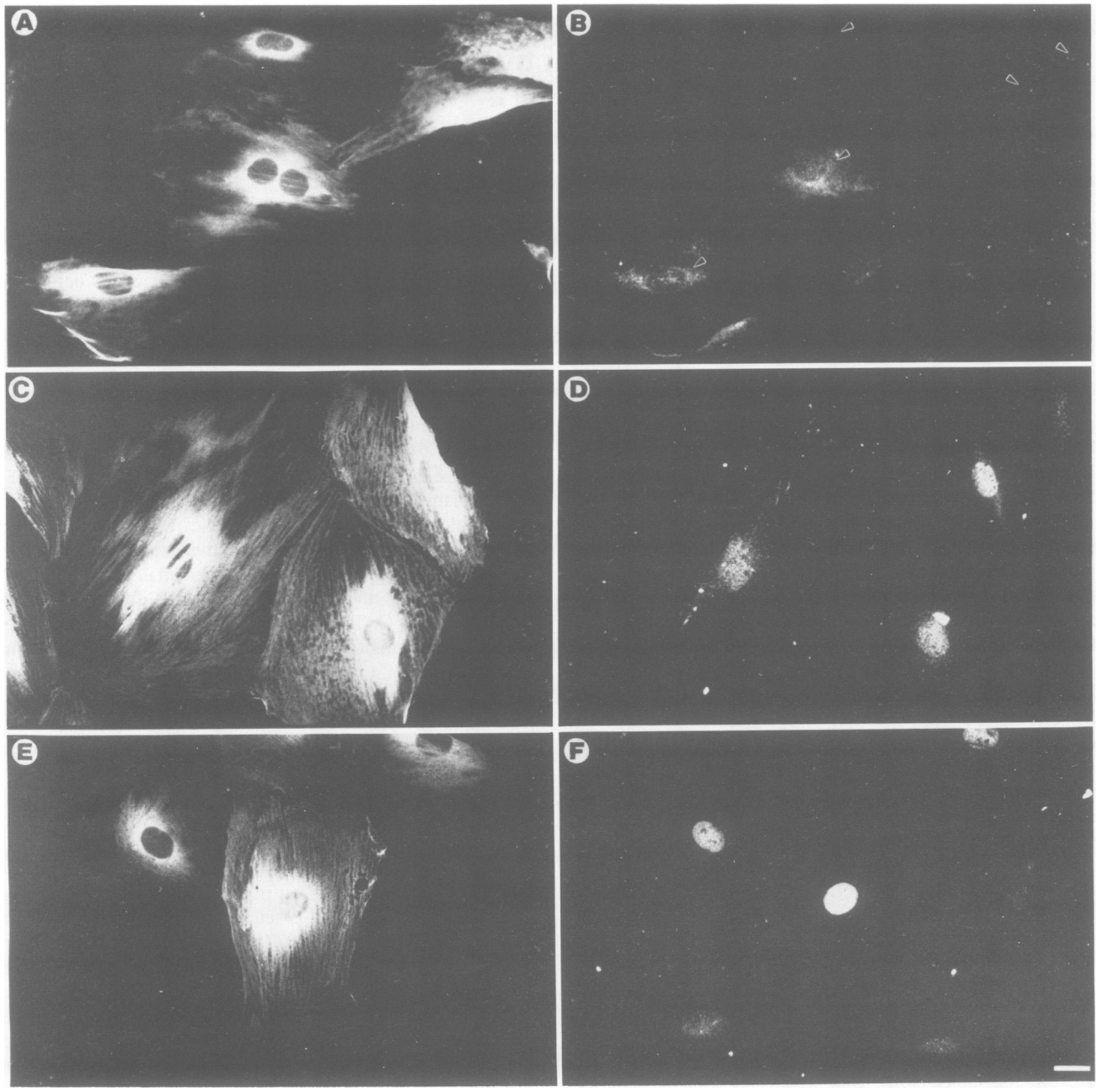

Fig. 1. Induction of c-*fos* in cells injected with *ras* oncoproteins. To investigate if *ras* induced c-*fos* expression, quiescent REF-52 cells plated on glass coverslips were microinjected with $\sim 2.5 \times 10^4$ molecules/cell of purified human *ras* oncoprotein. At various times afterwards, the cells were fixed and stained for c-*fos* protein and for the marker antibody. (**Panels A** and **B**) cells microinjected with control antibodies and non-oncogenic *ras* and stained for either the injected antibodies (**panel A**) or c-*fos* proteins (**panel B**) 60 min after injection. (**Panels C, D, E** and **F**) cells microinjected with control antibodies and *ras* oncoproteins and stained for either the injected antibodies (**panels C** and **E**) or c-*fos* proteins (**panels D** and **F**) 30 min (**panels C** and **D**) or 60 min (**panels E** and **F**) after injection. Bar = 10 μm. (However due to field limits of the camera two of the nuclei in panel D are off the field.).

et al., 1988) which is phosphorylated *in vivo* on serine residues, a phosphorylation required for its DNA binding activity (Prywes *et al.*, 1988).

In order to better define the early mediators of the effects of the *ras* oncoprotein and its role in cellular transformation, we have examined the consequences of elevating *ras* levels by microinjection of quiescent REF-52 fibroblasts with purified H-*ras* protein. In this study, we find that *ras*-induced c-*fos* expression and proliferation acts through a pathway utilizing SRE and C-kinase.

Results

Microinjection of human ras oncoprotein into REF-52 cells rapidly induces c-fos expression

We initially examined whether quiescent cells would respond to elevated levels of *ras* in the same way they respond to serum stimulation. One response to serum is the rapid and transient expression of c-*fos*. Quiescent cells injected with non-oncogenic *ras* (labelled with inert antibody (Figure 1A) showed little or no c-*fos* synthesis (Figure 1B) but, cells

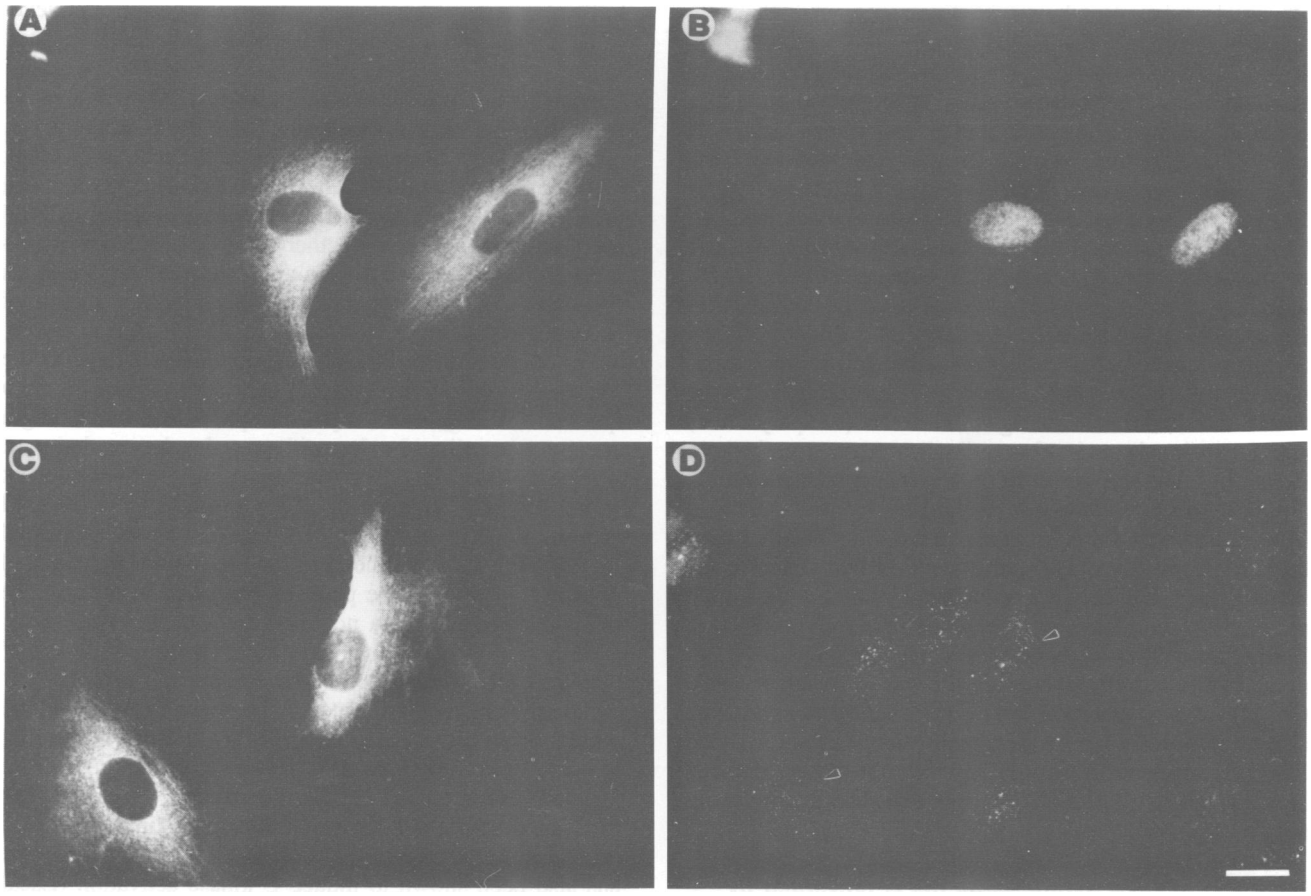

Fig. 2. Inhibition of c-*fos* expression in cells injected with *ras* oncoproteins and SRE oligonucleotides. In order to assess if c-*fos* induction by *ras* required the activation of SRE, quiescent REF-52 cells plated on glass coverslips were microinjected with purified human *ras* proteins alone as a control or with the double-stranded DNA oligonucleotides termed SRE or mutated SRE. After 1 h the cells were fixed and stained for c-*fos* proteins and for marker injected antibodies. (**Panels A** and **B**) cells microinjected with control antibodies, *ras* oncoproteins and the mutated SRE oligonucleotides; (**panels C** and **D**) cells injected with control antibodies, *ras* oncoproteins and the SRE oligonucleotide and stained for either the injected antibodies (**panels A** and **C**) or c-*fos* (**panels B** and **D**). Bar = 10 μm.

injected with oncogenic *ras* (labelled with antibody, Figure 1C) showed detectable c-*fos* staining 30 min after injection (Figure 1D). This effect peaked by 60 min (Figure 1E and F) and the level of c-*fos* staining remained stable at least until 90 min (data not shown). At longer times, c-*fos* staining in the nucleus subsided, and completely disappeared from cells by 3−4 h post injection (data not shown). Between 60 and 120 min all the cells injected with oncogenic *ras* induced c-*fos* expression. The absence of c-*fos* staining in cells injected with non-oncogenic *ras* or with antibody alone (data not shown) demonstrated the specificity of the effects for oncogenic *ras*, effectively discounting that c-*fos* was induced by the microinjection technique (c-*fos* is a protein expressed during cell stress). Indeed, c-*fos* expression was induced by non-oncogenic *ras*, but required at least 8-fold higher concentration of protein. We observed that c-*fos* induced by *ras* injection localized in the nucleus was indistinguishable from that after serum stimulation, suggesting that in cells activated by *ras* microinjection, appropriate post-translational modifications of c-*fos* were also induced. A report by Mulcahy *et al.* (1985) showed that *ras* injection induced c-*fos* expression in NIH3T3 cells within 60 min. The effect we describe differs from the serum-induced expression of c-*fos* in REF-52 cells in its rapidity. As we have shown elsewhere (N.J.C.Lamb, A.Fernandez, N.Tourkine, P.Jeanteur and J.M.Blanchard, submitted) REF-52 cells rendered quiescent by 36 h of serum deprivation, start expressing c-*fos* 60 min after refeeding, an effect that does not peak, however, before 110−120 min after serum addition.

c-fos expression induced by ras is prevented by microinjection of SRE DNA oligonucleotides

There is extensive evidence that serum-induced c-*fos* expression at least partially involves the interaction of a specific protein, the SRF, at a defined sequence, the SRE on the c-*fos* promoter (Treisman *et al.*, 1985; Gilman *et al.*, 1986; Greenberg *et al.*, 1987). It has recently been shown (Gilman *et al.*, 1988; Lamb *et al.*, submitted) that serum-induced c-*fos* expression can be retarded or abolished completely by microinjecting an oligonucleotide identical to SRE, which competes with the *fos* promoter for the binding of SRF. To examine if *ras*-induced c-*fos* expression utilizes a mechanism involving SRF, we chose to microinject oncogenic *ras* protein with SRE oligonucleotides and stain for c-*fos* expression. As a control, mutated SRE oligonucleotide, was injected. In serum-stimulated cells only SRE oligonucleotide, not mutated SRE oligonucleotide, was efficient in abolishing serum-induced c-*fos* expression. We observed a similar result when *ras* was injected together with SRE oligonucleotide but not with the mutated SRE

oligonucleotide. As shown in Figure 2A and B, cells microinjected with *ras* in the presence of mutated SRE oligonucleotide (marked by antibody, Figure 2A) induced the expression of c-*fos* in the nucleus by 60 min (Figure 2B) as observed above without SRE oligonucleotide (Figure 1E and F), suggesting that the injection of mutated SRE oligonucleotide had little or no effect on the induction of c-*fos* by *ras*. (The microinjection solutions were adjusted to ensure identical concentrations of *ras* were injected in all cases.) In marked contrast, when SRE oligonucleotide, corresponding to the c-*fos* SRE, was included in the injection buffer with *ras* (cells marked by antibody in Figure 2C) we could no longer detect c-*fos* expression in the nucleus of injected cells 60 min post injection (Figure 2D). We have observed a similar result using two different (one polyclonal, one monoclonal) anti-c-*fos* antibodies (data not shown), suggesting that this result does not reflect induction of a different form of c-*fos* not recognized by our antibody. Moreover, when the cells were stained at times other than 60 min (from 30 to 210 min) we failed to detect c-*fos* staining, implying that the expression of c-*fos* was not simply retarded by the SRE oligonucleotide, but truly abolished. Furthermore other oligonucleotides not containing SRE had no such effects when included with *ras* (data not shown). Our previous observation that c-*fos* induction by serum can also be inhibited by SRE oligonucleotide, but not mutated SRE oligonucleotide, further supports the notion that *ras* induces cell proliferation through a pathway mimicking serum.

Induction of DNA synthesis by ras is prevented by microinjection of SRE DNA oligonucleotides

We chose to examine whether microinjection of SRE oligonucleotide had effects on the proliferative action of *ras*. Previous studies have detailed that injection of oncogenic *ras* was sufficient to induce quiescent cells to pass through DNA synthesis (Feramisco *et al.*, 1984). To examine this response, cells were injected with *ras* plus either SRE oligonucleotide or mutated SRE oligonucleotide and incubated for 24 h in the presence of 5-bromo-2-deoxyuridine (BdU) (an analogue of thymidine). Cells were fixed and stained for the incorporation of BdU with a monoclonal antibody directed against the analogue. Because cells induced with serum pass to S phase after 16–20 h (Lamb *et al.*, submitted), 24 h should be more than sufficient to monitor DNA synthesis. Cells injected with *ras* alone stained for injected antibody (Figure 3A) and for BdU (Figure 3B) implying that 80% have passed through S phase and synthesized DNA. When cells were labelled for 36 h after injection, although in different experiments the overall level of incorporation of BdU was observed to vary, 75–90% of injected cells were stained, which closely reflects the background level of incorporation in refed growing cells (data not shown). When mutated SRE oligonucleotide was included in the *ras* injected solution, we observed (Figure 3C) that the same proportion of injected cells incorporated BdU label (Figure 3D), confirming that mutated SRE oligonucleotide had little or no effect on the *ras* activation of cells to pass through DNA synthesis. In contrast, when SRE oligonucleotide is included in the injection solution most of the incorporation of BdU was missing from injected cells (Figure 3E and F). Indeed, usually only 0.1% of cells pass through DNA synthesis when SRE oligonucleotide is present in the injection buffer (a value which closely reflects the background level of BdU incorporation in serum starved cells) (unpublished observation). Blocking of the *ras* effect by SRE oligonucleotide was also observed using [^{3}H]thymidine and subsequent emulsion autoradiography to monitor DNA synthesis (data not shown). Therefore the inhibitory effect of SRE oligonucleotides reflects inhibition of *ras*-induced proliferation, not an alternative such as prevention of BdU entry into cells. Taken together these data imply that *ras*-induced proliferation involves a pathway in which c-*fos* is expressed. The pathway can be inhibited by introduction of SRE oligonucleotides.

We have reinforced the observation that *ras* requires the expression of SRE-regulated genes by injecting, into quiescent REF-52 cells, a plasmid pfos-LacZ containing a construct encoding β-galactosidase under the regulation of a region of the c-*fos* promoter including the SRE element (Lamb *et al.*, submitted). This plasmid is expressed efficiently in the presence of *ras*, whereas a control plasmid in which the dyad symmetry of the SRE region is disrupted does not. Neither is the plasmid expressed in the absence of co-injected *ras* (data not shown).

Changes in C-kinase activity modulate ras effects

Although a number of genes are thought to be under the regulation of SRF/SRE (Gilman *et al.*, 1986; Prywes and Roeder, 1986; Treisman *et al.*, 1986; Mohun *et al.*, 1987), detailed examination of c-*fos* induction via SRE has implicated a role for the calcium/phospholipid-dependent protein kinase in the modulation of SRF action. Since *ras* has also been shown to induce C-kinase activation (Lacal *et al.*, 1987b; Hsiao *et al.*, 1989; Lloyd *et al.*, 1989), we examined if *ras*-induced proliferation and c-*fos* induction involve C-kinase. We inhibited C-kinase activity *in vivo* either by injection of a monospecific antibody against C-kinase (anti-PKC kindly provided by K.P.Huang) (Huang *et al.*, 1986) or via a specific C-kinase inhibitory peptide (C-PKI). Our initial experiments (Figure 4) examined if either anti-PKC or C-PKI could modulate *ras*-induced c-*fos* expression. The inclusion of the inhibitory peptide C-PKI in the injection solution (Figure 4A) was clearly effective in preventing the induction of c-*fos* by *ras* (Figure 4B). If the same experiment was instead performed with serum starved REF-52 cells injected with *ras* and a non inhibitory peptide (which is the same as C-PKI except that it contains three amino acid deletions and is inactive as an inhibitor *in vitro*), c-*fos* induction occurred (Figure 4C and D), suggesting that *ras* activation involves C-kinase activation. As we observed with SRE oligonucleotide, incubating cells for longer or shorter periods, did not cause c-*fos* induction, nor have we observed c-*fos* expression with different anti-c-*fos* antisera (data not shown).

To ensure that the synthetic peptide utilized as a C-kinase inhibitor peptide is active in our cell system we performed an *in vitro* C-kinase assay with detergent-permeabilized REF-52 cells. Activity was monitored with autoradiograms of 2D gels, especially the region of the gel containing the 80 kd putative substrate for C-kinase. For Figure 4G, quiescent REF-52 cells were lysed and labelled with [^{32}P]-ATP for 10 min at 37°C. When phospholipids and calcium were added (Figure 4H) a protein of 80 kd containing numerous isoforms became heavily phosphorylated, as expected from a number of previous reports (Blackshear

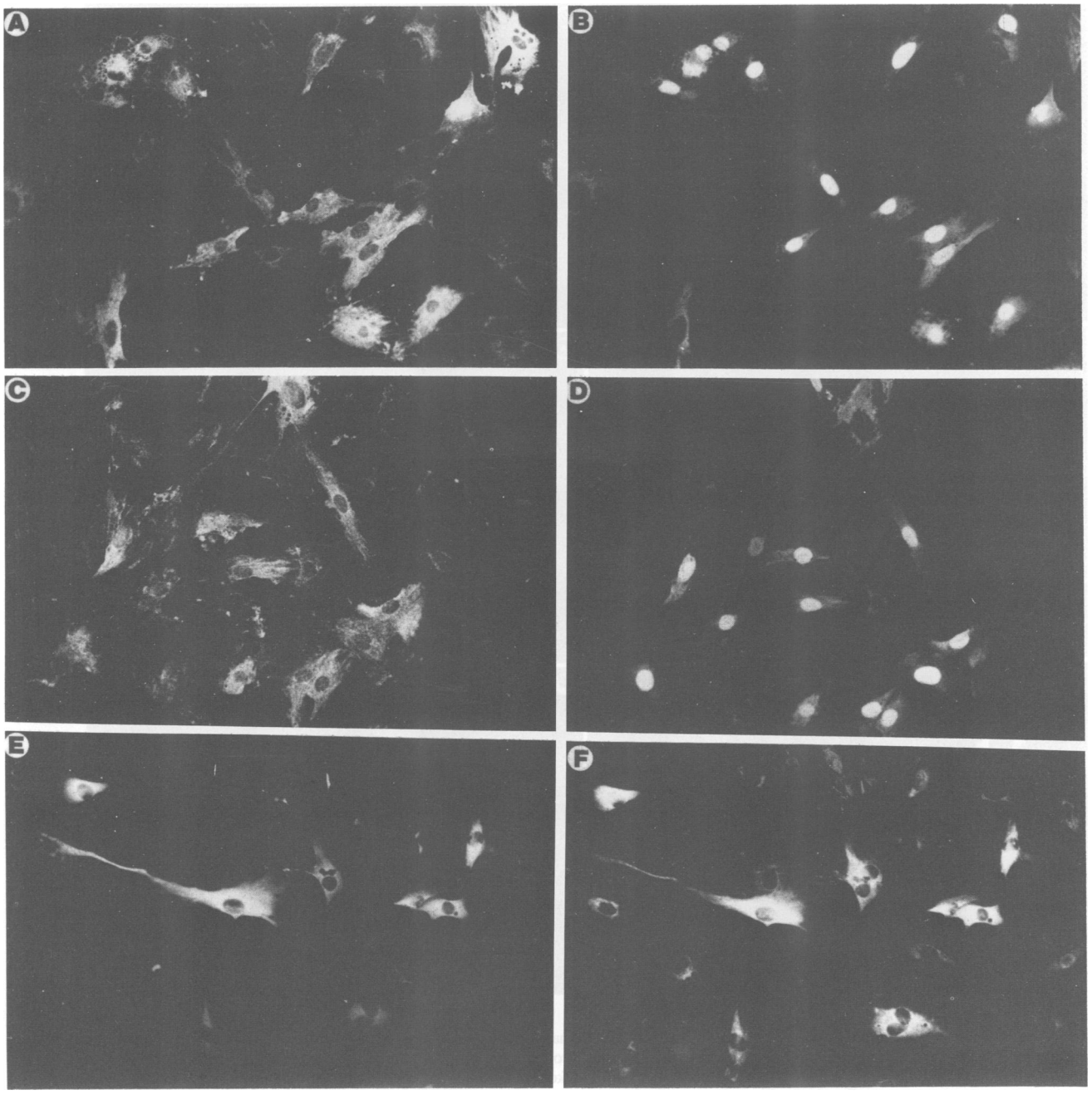

Fig. 3. Inhibition of SRE oligonucleotides of DNA synthesis induced by *ras*. Quiescent REF-52 cells plated on glass coverslips were microinjected with *ras* oncoproteins or with *ras* oncoproteins and double-stranded DNA oligonucleotides corresponding to SRE or mutated SRE. Thereafter the cells were labelled with BdU for 24 h. After this time, the cells were fixed and acid permeabilized and then incubated with mouse anti-BdU followed by incubation with fluorecein-anti-mouse antibodies and rhodamine-anti-rabbit antibodies. (**Panels A** and **B**) Cells injected with *ras* oncoproteins, marker antibodies (**panels C** and **D**) mutated SRE oligonucleotides or (**panels E** and **F**) SRE oligonucleotides and stained for either the injected antibodies (**panels A, C** and **E**) or BdU incorporation (**panels B, D** and **F**).

et al., 1986; Rodriguez-Pena and Rozengurt, 1986). When the same experiment was performed with the addition of C-PKI (Figure 4I), the overall level of 80 kd phosphorylation on all isoforms was markedly reduced, showing that the cellular C-kinase was inhibited by this pseudosubstrate inhibitory peptide. When non-inhibitory C-PKI was added to the reaction mixture, a similarly high level of 80 kd phosphorylation was observed, confirming our previous finding that non-C-PKI does not affect C-kinase activity (data not shown). Taken together these results suggest that C-PKI should be a suitable probe to use in living cells.

To confirm that C-kinase activation following *ras* injection was essential to c-*fos* induction, we also injected a polyclonal antibody directed against all three isoforms of C-kinase (Figure 4E and F). As shown in Figure 4D, cells injected with *ras* and anti-C-kinase antibody failed to induce c-*fos* (Figure 4F). Therefore blocking C-kinase activity either with an inhibitory peptide or with specific antibodies abrogated *ras*-induced c-*fos* expression.

Inhibition of C-kinase could also modulate *ras*-induced DNA synthesis. In an experiment similar to that described in Figure 3, quiescent cells were injected with *ras* in the

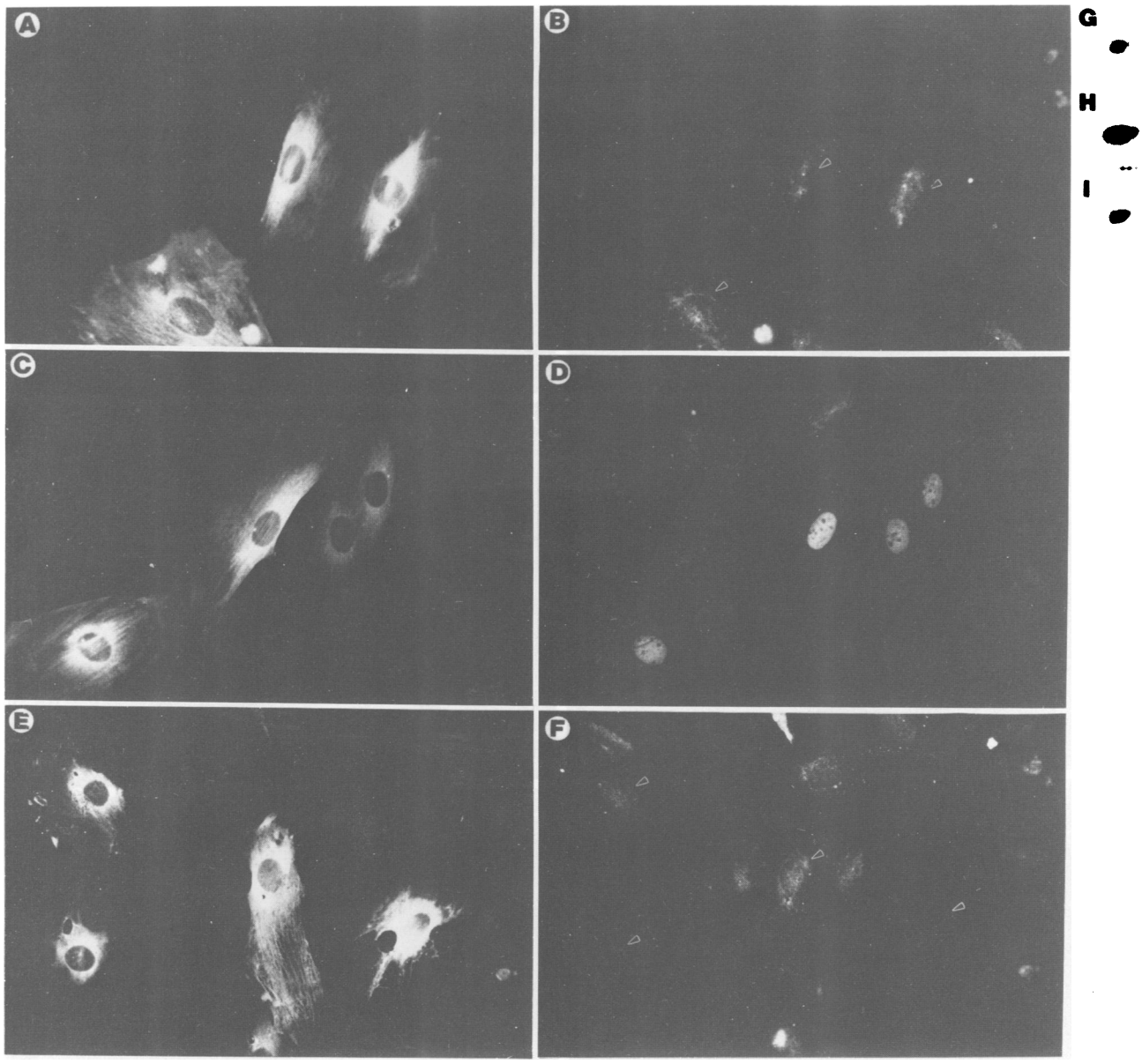

Fig. 4. Inhibition of *ras*-induced c-*fos* by injection of C-PKI or anti-C-kinase antibodies. To investigate the role of C-kinase in c-*fos* induction by *ras*, quiescent REF-52 cells were microinjected with the oncogenic *ras* protein and marker antibodies alone (mouse Ig) either with non C-PKI, C-PKI or anti-C-kinase antibody. A control experiment was performed with marker antibodies alone. After 1 h, the cells were fixed and stained for c-*fos* proteins and marker antibodies. (**Panels A, C** and **E**) staining for the marker antibodies. (**Panels B, D** and **F**) staining for c-*fos* proteins. (**Panels A** and **B**) cells injected with *ras* protein, C-PKI and marker antibodies. (**Panels C** and **D**) cells injected with *ras* protein, non-C-PKI and marker antibodies. (**Panels E** and **F**) cells injected with *ras* proteins, anti-C-kinase antibodies and marker antibodies. Activity of C-PKI was examined using a C-kinase assay with permeabilized REF-52 cells. Each pellet of cells obtained from 35 mm dishes was incubated with 50 mM MES; 1 mM $MgCl_2$; 1 mg/ml BSA; 0.5% Trixon X-100 (**panel G**); in **panel H** with, in addition, 2 mM $CaCl_2$; 800 nM phosphatidylserine; 80 nM dioleine and in **panel I** with, in addition, 2 mM $CaCl_2$; 800 nM phosphatidylserine; 80 nM dioleine and 0.5 μM C-PKI. The labelled proteins were analysed by 2D gel electrophoresis. Panels G, H and I are the regions of the autoradiograms covering the area of migration of the 80 kd phosphoprotein.

presence of non-C-PKI, C-PKI or anti-C-kinase antibody. Cells were again incubated in the presence of BdU for 24 h before fixation and staining for the incorporation of label. As shown in Figure 5, cells injected with *ras* and non-C-PKI (Figure 5A) incorporated BdU in 70–80% of injected cells (Figure 5B), whereas cells injected with *ras* and C-PKI (Figure 5C and D) or anti-C-kinase antibody (Figure 5E and F) show little or no incorporation of BdU (Figure 5D and F respectively) above background. Again these results, like those following c-*fos* induction suggest that *ras*-induced proliferation involves a pathway utilizing c-*fos*, SRE and C-kinase.

ras microinjection induces phosphorylation of the C-kinase 80 kd specific substrate

To confirm that *ras* microinjection induced C-kinase activation, we performed metabolic labelling of serum refed or *ras* injected cells with $[^{32}P]H_3PO_4$ and examined if we could discern a change in phosphorylation of the 80 kd putative substrate for C-kinase (Rodriguez-Pena and Rozengurt, 1986). A typical autoradiogram from 2D electrophoresis showing the phosphorylation status of the 80 kd substrate from 1000 serum starved REF-52 cells labelled for 2 h with 0.3 mCi $[^{32}P]H_3PO_4$ is shown in Figure 6A. A similar pattern is obtained if the cells were injected with

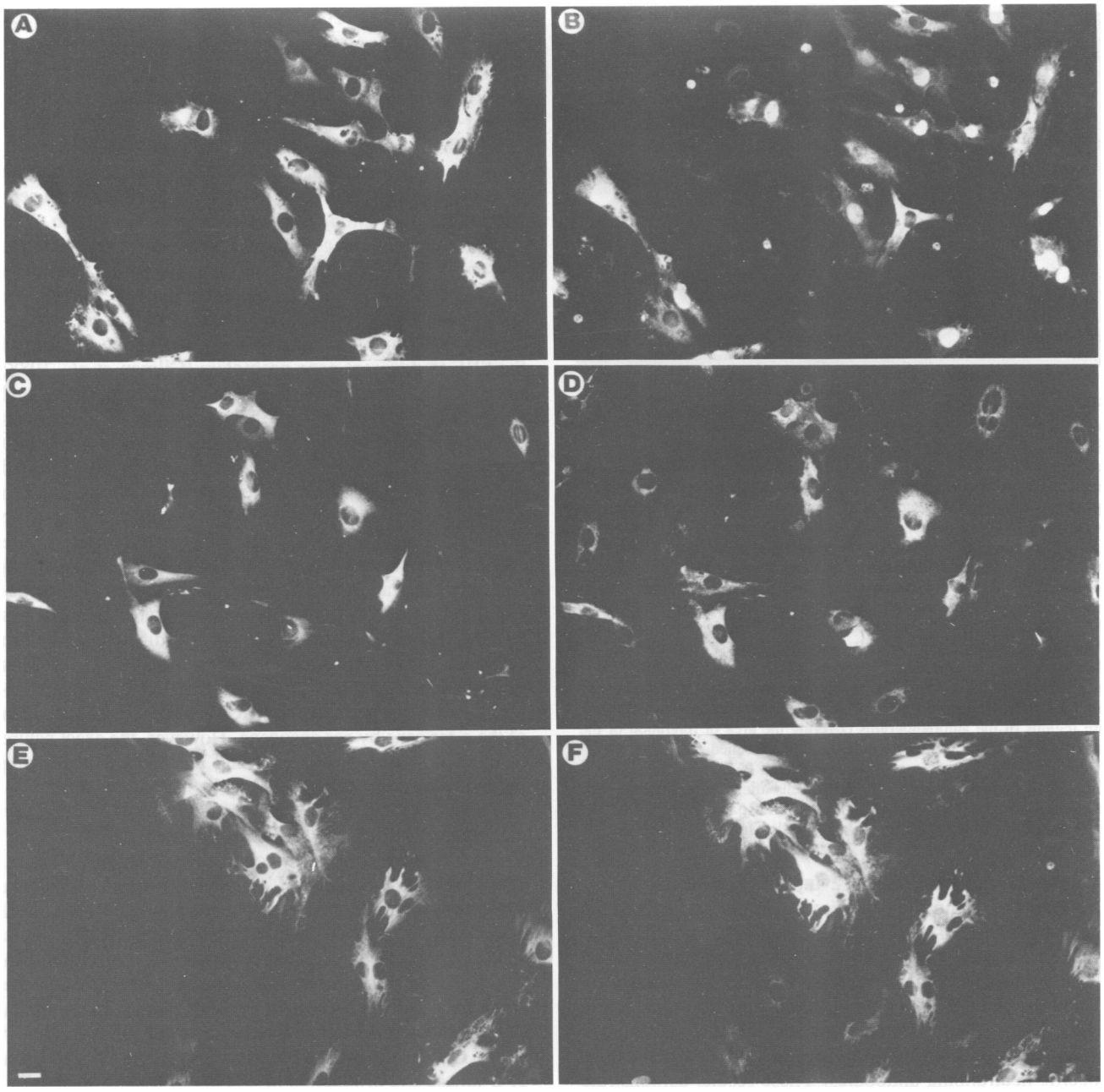

Fig. 5. BdU incorporation into REF-52 cells after microinjection of the *ras* proteins or the *ras* protein and C-PKI. To investigate the role of C-kinase in the proliferative mechanism induced by *ras* oncoprotein, quiescent REF-52 cells were microinjected with the oncogenic *ras* protein with either C-PKI or anti-C-kinase antibodies. Thereafter the cells were labelled with BdU for 24 h. After this time, the cells were fixed and acid extracted and then incubated with BdU followed by incubation with fluorescein-anti-mouse antibodies and rhodamine-anti-rabbit antibodies. (**Panels A** and **B**), cells injected with control antibodies, *ras* oncoproteins and non-C-PKI; (**panels C** and **D**), cells injected with control antibodies, *ras* oncoproteins and C-PKI; (**panels E** and **F**), cells injected with control antibodies, *ras* oncoproteins and anti-C-kinase antibodies and stained for injected antibodies; (**panels A, C** and **E**) or DNA synthesis (**panels B, D** and **F**). Bar = 10 μm.

buffer alone (data not shown). After 1 h of TPA treatment, to induce specific activation of the C-kinase, and pulse labelling from 30 to 60 min with 0.3 mCi $[^{32}P]H_3PO_4$, we observed a large increase in 80 kd phosphorylation (Figure 6B). Analysis of a similar region of a 2D gel of 1000 REF-52 cells 90 min after refeeding and labelled for the period 45−90 min with 0.3 mCi $[^{32}P]H_3PO_4$ shows the level of phosphorylation was increased 8- to 10-fold over that of the level of quiescent cells (Figure 6C). These results agree with a previous report showing augmentation of C-kinase activity after serum stimulation (Rozengurt, 1986). The levels of 80 kd phosphorylation as well as the overall isoform distribution of the 80 kd protein from serum refed and *ras* injected cells were similar. See Figure 6D showing the same region of a 2D gel of 230 serum quiescent REF-52 cells injected with activated *ras* protein. Moreover, the overall level of label incorporation is very similar even though fewer cells were analysed (230 versus 1000), implying that *ras* may even activate C-kinase more potently than serum.

Discussion

Using the microinjection technique, we have examined the early mediators of the proliferative activation induced by *ras*

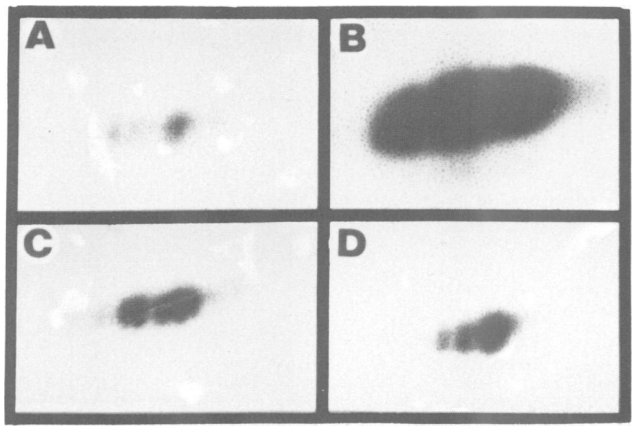

Fig. 6. Phosphorylation of the 80 kd specific substrate of C-kinase is induced by serum, TPA and *ras* oncoprotein. Changes in 80 kd and SRF phosphorylation in quiescent cells were investigated following either incubation with DMEM containing 6% fetal calf serum or treatment with 25 nM PMA or *ras* oncoprotein injection. Essentially, quiescent REF-52 cells plated on 35 mm plastic dishes were treated with either 8% fetal calf serum or 25 nM PMA (to activate C-kinase). Quiescent cells plated on small glass coverslips, were microinjected with $\sim 2.5 \times 10^4$ molecules/cell of purified human *ras* oncoprotein (each cell on the coverslip was injected). At various times after treatment or injection, the cells were metabolically labelled with 0.25 mCi of $[^{32}P]H_3PO_4$ in phosphate-free DMEM for 30 or 45 min. After the labelling period, the cells were briefly washed in PBS and the chips were transferred to Eppendorf tubes containing $\sim 5 \times 10^6$ unlabelled cells (as carrier proteins) in lysis/sample buffer and analysed by 2D electrophoresis as described in Materials and methods. Shown are regions of the autoradiograms covering the area of migration of the 80 kd protein from: (**panel A**) quiescent cells labelled from 0 to 120 min; (**panel B**) quiescent cells treated with PMA and labelled from 30 to 60 min; (**panel C**) quiescent cells after addition of 8% fetal calf serum and labelled from 45 to 90 min and (**panel D**) quiescent cells injected with *ras* oncoprotein and labelled from 60 to 105 min.

oncoprotein in quiescent cells. Introduction of human *ras* oncoprotein into normally quiescent fibroblasts induces the rapid expression of c-*fos* and stimulates DNA synthesis and cell proliferation. The induction of both c-*fos* expression and DNA synthesis by *ras* is prevented by the coinjection of a double-stranded DNA oligonucleotide corresponding to the SRE. Both effects of *ras* injection are also blocked if C-kinase activity is inhibited by an inhibitor peptide or specific neutralizing antibodies. These results demonstrate the involvement of both C-kinase and SRE promoter in *ras*-induced proliferation. In addition to its mitogenic effect, *ras* microinjection is known to induce marked morphological changes within a few hours following injection (Feramisco *et al.*, 1984). Interestingly, such morphological modifications were not prevented by the coinjection of SRE oligonucleotides or by inhibition of C-kinase. This suggests that *ras* has at least two separate effects during oncogenesis. One leading to C-kinase activation as demonstrated by these experiments, the other C-kinase independent pathway leading to modification of the cell shape. The mediator of this second pathway remains to be elucidated.

ras mimics the mitogenic effect of serum

Upon stimulation by serum growth factors, REF-52 cells made quiescent by serum deprivation induce the rapid and transient transcription of the proto-oncogene c-*fos*, DNA synthesis and mitosis. Similarly, the injection of *ras* oncoprotein into quiescent NIH3T3 cells induces c-*fos* expression (Mulcahy *et al.*, 1985) and into REF-52 cells stimulates DNA synthesis and cell proliferation (Feramisco *et al.*, 1984), suggesting that *ras* can mimic the mitogenic effects of serum. At this point it is important to notice that 100% of *ras*-injected cells induced c-*fos* expression whereas 75–90% of them go on to DNA synthesis. This is probably because some serum-arrested cells do not re-enter the division cell cycle. The same result is obtained for refed cells reinforcing the similarities between *ras* and serum. The serum inducibility of c-*fos* transcription correlates with the presence of a SRE, located 300 bp upstream of the c-*fos* mRNA start site. The induction of c-*fos* by serum can be specifically inhibited through microinjection of SRE oligonucleotides which presumably compete for the binding of the SRF to different gene promoters (Gilman *et al.*, 1988; Lamb *et al.*, submitted). The similarity between *ras*- and serum-induced effects is further supported by the results obtained after coinjection of SRE and *ras* oncoprotein into quiescent cells. Indeed, the addition of SRE to microinjected *ras* oncoprotein effectively prevents the proliferative function of *ras*. Furthermore, we show that this inhibition is specific for SRE since injection of control oligonucleotides, where SRE had been mutated at key sites, is ineffective in inhibiting cell cycle activation by oncogenic *ras*. Recently, Sassone-Corsi *et al.* (1989) have observed via transfection of *ras* mutants that *ras*-induced PC12 neuronal differentiation also activated c-*fos* transcription through the DSE. Results presented here suggest that an interaction between SRE-binding proteins and the c-*fos* SRE is required for induction of the c-*fos* gene by *ras* oncogene. However, the exact nature of the SRE-binding protein(s) and the stimuli modulating its/their activation remain to be elucidated.

Activation of C-kinase by ras oncogene

Various reports using transfection (Wolfman and Macara, 1987) or scrape-loading of cell (Morris *et al.*, 1989) in coordination with C-kinase chronic down-regulation (Lacal *et al.*, 1987a,b) have implied that *ras* proliferation is dependent on functional C-kinase. Indeed, it has been shown that a H-*ras* responsive transcription element from the polyoma virus is identical to the sequence element which also mediates promoter activation by phorbol ester (Imler *et al.*, 1988). However, these reports supposed an activation of C-kinase and production of DG in *ras*-transformed cells. None of the experiments using transformed cell lines satisfactorily address the question of whether these effects were mediated directly by *ras*. Moreover, the chronic treatment of cells with phorbol ester, used to down-regulate C-kinase, does not completely eliminate C-kinase activity from cells and may have other effects as well. In an attempt to obviate some of these problems, we have used the microinjection technique utilizing a specific C-kinase inhibitor peptide. The results we obtained are in keeping with the results described above. Indeed, the specific inhibition of C-kinase by either inhibitory peptide or specific neutralizing antibodies abolish both c-*fos* expression and DNA synthesis induced by *ras* implying that C-kinase activity is required for these effects. Moreover, through metabolic labelling of injected cells, analysis of the changes in phosphoprotein after *ras* injection reveals the increased phosphorylation of the 80 kd C-kinase specific substrate as well as other proteins including a group of proteins of 67–72 kd that comigrate with proteins which react immunologically with antibodies to the mammalian

SRF (C.Gauther, N.J.C.Lamb and A.Fernandez, manuscript in preparation). Analysis of the 80 kd phosphoproteins by Cleveland mapping confirms that the phosphoprotein in *ras* injected cells is the same protein that is phosphorylated in response to PMA (data not shown).

Taken together, our data demonstrate that activation of PKC is one of the important events integral in the proliferative effect of *ras* and it will be of interest to determine how this activation takes place. Indeed, the increased cellular level of DG with (Fleischman *et al.*, 1985; Wakelam *et al.*, 1986; Berggren *et al.*, 1989) or without (Lacal *et al.*, 1987a,b; Seuwen *et al.*, 1988) changes in the other phosphoinositide metabolites observed in *ras*-transformed cells leads to the question: how does *ras* activate C-kinase? It has been suggested that *ras* protein may function in regulating the activity of phospholipase C. There remains, however, no direct evidence for this hypothesis. Another point of interest to elucidate is the post-translational modification on SRF involved in its activation. In this respect Prywes *et al.* (1988) have observed an *in vitro* phosphorylation of SRF occurring on serine residues necessary to its DNA binding activity but the sequence of these phosphorylation sites as well as the protein kinase involved are still unknown. Since our data point at the role of both C-kinase activation and SRF−SRE interaction in *ras*-induced mitogenesis, it is tempting to speculate that SRF could be activated by a C-kinase dependent phosphorylation. We are currently investigating this possibility following growth stimulation in *ras* injected cells.

Materials and methods

Cell culture

Rat embryo fibroblast (REF-52) (McClure *et al.*, 1982) cells were cultured in DMEM supplemented with 8% fetal calf serum in a humidified atmosphere containing 5% CO_2, 95% air on 35 mm plastic dishes, glass coverslips or 2 mm glass chips, as described elsewhere (Lamb *et al.*, 1988). Cells were plated 2−3 days before use for microinjection.

Purification of oncogenic ras protein

Oncogenic *val-12* K-*ras* protein was purified from an *Escherichia coli* expression vector as described by Feramisco *et al.* (1984). Prior to use the protein samples were diluted into an injection buffer (50 mM HEPES pH 7.3, 1 mg/ml $MgSO_4$, 25 mM NaCl).

Microinjection and metabolic labelling

Quiescent REF-52 cells plated on glass chips were microinjected with the oncogenic H-*ras* protein (2.5×10^4 molecules/cell) (the same number of cells were microinjected in each case) (Lamb *et al.*, 1988). For labelling, the chips were transferred to humidified chambers at varying times after injection, washed once in phosphate-free DMEM before incubation for 30 min periods in 5 μl of phosphate-free DMEM containing 0.25−0.5 mCi [^{32}P]H_3PO_4. After the end of the labelling period, the cells were washed with PBS and collected with 15 μl of lysis buffer [5% (w/v) β-mercaptoethanol, 1% (w/v) NP-40)] and 15 μl of sample buffer [40 mM Tris−Cl pH6.8, 5 mM dithiothreitol, 1% (w/v) SDS, 7.5% (w/v) glycerol and 1% (w/v) bromophenol blue]. After boiling, samples were lyophilized and resuspended in 10 μl of 1 mg/ml DNase and 1 mg/ml RNase for 5 min at 37°C to digest nucleic acids. Samples were then diluted with IEF sample buffer [9.5 M urea, 2% (w/v) NP-40, 50% (w/v) β-mercaptoethanol, 2% (w/v) ampholines] such that the final concentration of SDS was <0.1% (w/v) before analysis by 2D gel electrophoresis.

For immunofluorescence experiments, REF-52 cells were plated on glass coverslips and microinjected with 1 mg/ml of a solution of *ras* oncoprotein. The *ras* solution contained marker antibodies (mouse Ig 0.5 mg/ml) and either with double-stranded DNA oligonucleotides corresponding to SRE (5′-AGGATGTCCATATTAGGACATCTGC-3′), mutated SRE (5′-AGGATGTCCATATTAACTATTGATG-3′ (1 mg/ml in HEPES, 100 mM), C-PKI (Arg^{19}-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val^{31}) (House and Kemp, 1987) (10^{-2} in HEPES 100 mM), non-inhibitory peptide or anti-C-kinase antibodies (Huang *et al.*, 1986).

Drug treatment and metabolic labelling

REF-52 cells on 2 mm chips were made quiescent by removal of serum for 36 h, incubated in phosphate-free DMEM with 0.25 mCi [^{32}P]H_3PO_4 (Amersham) and with phorbol 12-myristate, 13-acetate (PMA) (20 nM). After the appropriate incubation period, the cells were prepared as described above for analysis by 2D gel electrophoresis.

Immunofluorescence

At different times after microinjection cells were fixed for 5 min in 3.7% formalin (in PBS) followed by a 30 second extraction in −20°C acetone and rehydration in PBS containing 0.1% BSA. Injected antibodies and c-*fos* staining were visualized by a 60 min incubation with affinity-purified fluorescein-conjugated goat anti-mouse antibody diluted 1:100 (Cappel-Organon Technika) and a rabbit anti-*fos* antibody (Verrier *et al.*, 1986) diluted 1:100 for 60 min. After a brief wash in PBS, the cells were incubated with biotinylated anti-rabbit diluted 1:100 (Amersham) for 60 min and with streptavidine-Texas-red diluted 1:500 for 30 min. Cells were washed in PBS, mounted in 0.25% (w/v) Airvol-205 in PBS and examined on a Zeiss Axiophot using 63× (1.3NA), 40× or 16× planopochromat lens. Fluorescent images were recorded on Kodak Tri-X pan film and processed as described before (Lamb *et al.*, 1988).

In situ phosphorylation by calcium/phospholipid dependent protein kinase in permeabilized cells

Quiescent REF-52 cells grown on 35 mm dishes were washed twice with PBS. Each dish was scraped into 1 ml of PBS and collected by centrifugation at 15 000 *g*. Each pellet was incubated with 60 μl of 50 mM MES; 1 mM $MgCl_2$; 1 mg/ml BSA; 0.5% Triton X-100; and with or without 800 nM phosphatidylserine; 80 nM dioleine and 5×10^{-5} M of C-kinase inhibitor peptide. The reaction was started by addition of [γ^{32}P]ATP (75 μCi/assay) for 30 min and terminated by the addition of 15 μl of lysis buffer and 15 μl of boiling sample buffer. The samples were immediately heated at 100°C for 1 min prior to analysis by 2D electrophoresis.

Two-dimensional gel electrophoresis

Samples were analysed by 2D electrophoresis using the method of O'Farrell (1975). First dimension electrofocussing gels contained 9.5 M urea, 2% (w/v) ampholines, 2% (w/v) NP-40 and 5% (w/v) DTT. The ampholine mixture used contained 60% (w/v) ampholine pH 3−10, 40% (w/v) ampholine pH 4−6. Second dimension gels contained 12.5% (w/v) acrylamide and 0.1% (w/v) bis-acrylamide. The gels were stained to visualize the mol. wt markers and after drying, the phosphoproteins visualized by autoradiography on Kodak XAR-5 film at −70°C, using Cronex Lightening plus intensifying screens.

BdU labelling

REF-52 cells after coinjection of purified *ras* oncoprotein with or without C-kinase inhibitor peptide, anti-C-kinase antibodies, SRE or mutated SRE oligonucleotides were incubated at 37°C for 24 h with a thymidine analogue, BdU (Amersham), diluted 1:500 in DMEM. At the end of the labelling period, the cells were fixed by formaldehyde (3% in PBS) for 5 min and acetone (−20°C) extracted for 1 min. To allow access to BdU the cells were treated with 4 N HCl for 10 min and then rehydrated by extensive washing with water and PBS. The cells were incubated with mouse anti-BdU (Amersham) for 1 h at 37°C, washed with PBS, incubated with fluorescein-anti-mouse antibody (Cappel) diluted 1:50 plus rhodamine-anti-rabbit antibody (Cappel) diluted 1:50 for 1 h at 37°C. The cells were analysed on a Zeiss microscope using a 16× lens as described elsewhere.

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