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Serine threonine kinases as signaling intermediates in v-Src-induced gene expression

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Serine Threonine Kinases as Signaling Intermediates in v-Src-Induced Gene Expression

by

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Abstract

Serine threonine kinases as signaling intermediates in v-Src-induced gene expression

by

Sajjad Aslam Qureshi

Advisor: Dr. David A. Foster

v-Src induces expression of two primary response genes, *TIS-10* and *Egr-1.* Depleting cells of protein kinase C (PKC) blocked v-Src-induced *TIS10* expression, but had no effect on v-Src-induced *Egr-1* gene expression. In addition, the induction of *TIS10* and *Egr-1* by v-Src could be distinguished pharmacologically. Therefore, the v-Src induces gene expression via at least two distinguishable pathways; one that is dependent upon PKC and one that is independent of PKC. A kinase-deficient c-Raf-1 that function as a dominant-negative mutant of c-Raf-1 inhibited the activation of *Egr-1* gene by v-Src. This inhibition was overcome by overexpression of wild type c-Raf-1. Consistent with the involvement of c-Raf-1 in *Egr-1* promoter activation, an activated Raf kinase also activated the *Egr-1* promoter. Thus, v-Src-induced signaling involves the activation of at least two serine/threonine kinases, PKC and c-Raf-1 that leads to the induction of two different primary response genes.

Preface

This thesis is presented in Six sections. The first section is general introduction to subject. Section two and three are presented as were published in two papers, section four is presented as a manuscript submitted for publication, section five is the summary of studies with a short discussion, and section six constitute material & methods section and reference section, and an appendix which though not related to the topic of thesis but did provide the basis for many useful conclusions made in this study.

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Section I

Introduction

Rous sarcoma virus transforms cells in culture and induces fibrosarcoma in vivo (Hanafusa, 1985). Rous sarcoma virus induced transformation is dependent upon tyrosine kinase activity of its gene product Src (Jove and Hanafusa, 1987). The cellular homologue of v-Src, c-Src, posses very low kinase activity relative to v-Src. Increased tyrosine kinase activity is responsible for the oncogenic activity of v-Src (Jove and Hanafusa, 1987).

The activation of protein kinase C (PKC) is an early event in many signaling pathways which lead to the induction of gene expression and cell division (Nishizuka, 1986). PKC is a Ca^{++}, phospholipid and diglyceride dependent protein kinase whose activity is increased in response to mitogens and growth factors (Nishizuka, 1986). Growth factors which activate PKC do so by activating phospholipid hydrolysis (Nishizuka, 1986; Barridge, 1987; Exton, 1990). Activation of PKC is transient in response to growth factors because diacylglycerol (DAG) which activates PKC is rapidly metabolized (Nishizuka, 1986). Tumor promoting phorbol esters like 12-O-tetradecanoylphorbol 13-acetate (TPA) also activate PKC by mimicking DAG. Since many oncogenes are altered forms of growth factors, growth factor receptors or proteins involved in propagating the signals induced by these growth factors, it is conceivable that the machinery involved in propagating signals induced by oncogenes is similar to growth factors (Drucker *et al.*, 1990).

PKC has been implicated in oncogene induced signals. Increased phospholipid hydrolysis has been reported in cells transformed by v-Src (Sugimoto *et al.,* 1984), v-ros (Macara *et al.,* 1984), v-abl (Fray *et al.,* 1984) and v-erb B (Kato *et al.,* 1987). We previously demonstrated that v-Src-induced expression of the transformation-related 9E3 gene (Sugano *et al.,* 1987) in chicken embryo fibroblasts requires PKC (Spangler *et al.,* 1989). However, in murine fibroblasts, the involvement of PKC in v-Src-induced signals is unclear. v-Src transformed mouse fibroblasts have been shown to have increased levels of DAG (Wolfman *et al.,* 1987). PIP_2 (Phosphatidyl inositol bisphosphate) hydrolysis leads to the generation of DAG, microinjection of an anti-PIP_2 antibody partially block mitogenesis induced by v-Src (Fukami *et al.,* 1988). PKC translocation, a marker for PKC activation, has been reported in v-Src transformed NIH 3T3 mouse fibroblasts (Halsey *et al.,* 1987). The activation of PKC rapidly leads to phosphorylation of a major PKC substrate (Rodriguez-Penna and Rozengurt, 1984), known as MARCKS (Stumpo, *et al.,* 1989). Increased phosphorylation of this protein is observed in v-Src transformed CEF (Sagara *et al.,* 1986, Spangler *et el.*, 1989). TNR9. 3T3 mouse fibroblasts, which do not respond to TPA dependent mitogenesis (Butler-Grall and Herschman, 1985) cannot be transformed by v-Src (Nori *et al.,* 1990). All these data are consistent with the involvement of PKC during v-Src dependent cellular transformation. With the exception of v-Src-induced PKC-dependent 9E3 induction, all these data were obtained from cells transformed by wild type v-Src, therefore, whether these effects are the primary effects of oncogene activation or a result of permanent

alterations induced by transformation have not been determined. In BALB/c 3T3 cells, activation of a temperature sensitive mutant of v-Src does not correlate with any increases in IP_3, a co-product of the PIP_2 hydrolysis (Gray and Macara,1989). v-Src-induced DNA synthesis, EGF receptor desensitization and v-Src induced synthesis of cyclooxygenase been reported to be independent of PKC (Wolfman and Macara, 1987; Han *et al.,* 1990a, 1990b). Furthermore v-Src activation does not cause any increase in phosphate content of MARCKS protein (Han *et al.,* 1990b).

Raf is a family of serine/threonine kinases (Rapp *et al.,* 1988). c-Raf-1 is a one of the members of this family which has been shown to have phosphorylated in v-Src transformed cells (Morrison *et al.,* 1989). Phosphorylation of Raf increase the kinase activity of Raf kinase (Morrison *et al.,* 1989). Whether v-Src-induced phosphorylation of Raf kinase has any functional consequence is not known. However, Raf is also phosphorylated by growth factors such as platelet derived growth factor and epidermal growth factor (Morrison *et al.,* 1989; App *et al.,* ,1991) and inhibiting the Raf-1 kinase by expressing an antisense transcript or a dominant negative mutant of c-Raf-1 blocks cellular proliferation induced by serum and TPA (Kolch *et al.,* 1991). This suggest that Raf-1 is an important component of growth propagating signals.

Other signaling intermediates have also been implicated in v-Src signaling. These include Ras (Smith *et al.,* 1986), S6 kinase and MAP_2 kinase (Vik *et al.,* 1990). Antibodies to Ras has been shown to block mitogenesis induced by v-Src (Smith *et al.,* 1987). v-Src has been shown to phosphorylate S6 kinase at tyrosine

(Vik *et al.,* 1990). v-Src activation leads to the phosphorylation of MAP_2 kinase (Weber *et al.,* 1984).

Thus it appears that transformation induced by v-Src involve activation of multiple pathways. To better understand the nature of signaling intermediates involved in transducing v-Src-induced signals we have looked at the involvement of PKC, cAMP-dependent kinase and c-Raf-1 kinase in v-Src induced gene expression.

Section II

v-Src Activates both Protein Kinase C-Dependent and Independent Signaling Pathways in Murine Fibroblasts

Introduction

Protein-tyrosine kinase (PTK) activity is frequently an early event in the transduction of intracellular signals leading to cell division (Drucker *et al.,* 1990). v-Src is a PTK whose uncontrolled PTK activity leads to cellular transformation (Jove and Hanafusa, 1987). The activation of protein kinase C (PKC) is also an early event in many signaling pathways which lead to cell division (Nishizuka, 1986). Diacylglycerol, which activates PKC, has been shown to be elevated in murine cells transformed by v-Src; however, increased phosphorylation of a PKC substrate was not detected (Wolfman and Macara, 1987). Thus, in murine fibroblasts, the involvement of PKC in v-Src-induced signals, has been unclear. The murine cell line TNR9 (Butler-Gralla *et al.,* 1981) which is resistant to the mitogenic effects of phorbol esters that activate PKC, is also resistant to transformation by v-Src suggesting a requirement for PKC for v-Src-induced transformation (Nori *et al.,* 1990). However, v-Src-induced DNA synthesis and v-Src-induced desensitization of the epidermal growth factor receptor have been reported to be independent of PKC (Han *et al.,* 1990; Gray & Macara, 1988). Because of data both implicating PKC and showing independence of PKC from different laboratories, the involvement of PKC in v-Src-induced signaling has

remained in question. However, it is not clear whether the different conclusions made regarding PKC involvement in v-Src-induced signals are due to the different intracellular contexts of different cell types or due to v-Src activating both PKC-dependent and independent signals in the same cell. In this report, we demonstrate that v-Src activates both PKC-dependent and independent signaling pathways in the same murine fibroblast cell type. Potential implications for induction of the transformed phenotype by v-Src are discussed.

Results

v-Src induces expression of the "primary-response" genes TIS10 and Egr-1: Cell division signals induce many changes in cellular metabolism including altered transcription patterns. Cellular responses leading to altered transcription patterns are initiated by induction of "primary response" genes (Herschman, 1989), which are activated in the absence of new protein synthesis. Recently, several primary response genes have been identified that are induced by a variety of stimuli (Almendral *et al.,* 1988; Lau and Nathans, 1987; Lim *et al.,* 1987). TIS10 (Lim *et al.,* 1987) and Egr-1 (Sukhatme *et al.,* 1987) are primary response genes first isolated as phorbol ester- or mitogen-inducible gene sequences. Egr-1 [also known as TIS-8 (Lim *et al.,* 1987), Zif/268 (Lau and Nathans, 1987; Christy *et al.,* 1988) Krox 24 (Almendral *et al.,* 1988), NGFI-A (Milbrandt, 1987) and CEF5 (Simmons *et al.,* 1989)] is a transcription factor (Lemaire *et al.,* 1990; Patwardhan *et al.,* 1991) with a "zinc finger" motif and a sequence-specific DNA

binding activity (Christy and Nathans, 1989; Cao *et al.,* 1990).

We examined the ability of v-Src to induce expression of TIS10 and Egr-1 in murine fibroblasts. We used BALB/c 3T3 cells infected with the LA90 temperature-sensitive strain of Rous sarcoma virus (LA90 cells). Shifting from the non-permissive (40°C) to the permissive (35°C) temperature rapidly activates the PTK activity of the v-Src gene product (Gray & Macara, 1988). Shifting LA90 cells from the non-permissive to the permissive temperature led to the induction of both TIS10 and Egr-1 as detected by northern gel analysis (Figure 1). Egr-1 expression peaked at about 1 h and TIS10 peaked at about 4 h. Parental BALB/c 3T3 cells subjected to the same temperature shift did not show increased levels of either TIS10 or Egr-1 mRNA (not shown). Pretreatment of cells with cycloheximide (20 μg/ml) for 30 min prior to temperature shift did not block v-Src-induced TIS10 or Egr-1 expression (not shown); thus v-Src induced expression of TIS10 and Egr-1 is independent of new protein synthesis.

Depleting cells of PKC activity blocks v-Src-induced TIS10 expression but has no effect upon v-Src-induced Egr-1 expression:

Both TIS10 and Egr-1 are induced by phorbol esters which activate PKC (Lim *et al.,* 1987; Sukhatme *et al.,* 1987). Therefore, we looked at the ability of v-Src to induce TIS10 and Egr-1 in cells depleted of PKC activity by prolonged exposure to phorbol ester (Rodriguez-Pena and Rozengurt, 1984). LA90 cells were treated with increasing concentrations of 12-O-tetradecanoylphorbol 13-acetate (TPA) for

26 h. This treatment reduced the level of PKC activity in LA90 cells, as measured by Ca++-, phospholipid-, and diacylglycerol-dependent histone phosphorylation, to background levels at 100 ng/ml (not shown). Depleting LA90 cells of PKC blocked more than 70% of v-Src-induced TIS10 gene expression; however, depleting cells of PKC activity had no effect upon v-Src-induced Egr-1 expression (Figure 2). Thus, v-Src-induced TIS10 and Egr-1 expression are differentially sensitive to PKC-depletion, suggesting that v-Src is activating different signaling pathways for induction of TIS10 and Egr-1.

The induction of Egr-1 expression by v-Src and phorbol ester is differentially sensitive to the protein kinase inhibitors H7 and sangivamycin: We have previously used protein kinase inhibitors to distinguish intracellular signaling pathways (Spangler *et al.,* 1989; Qureshi *et al.,* 1991, see appendix I). Induction of TIS10 by agonists of PKC was highly sensitive to H7 (Hidaka *et al.,* 1984) and sangivamycin (Loomis *et al.,* 1988), whereas induction of TIS10 by agonists of protein kinase A was relatively insensitive to these compounds (Spangler *et al.,* 1989). The dose responses of v-Src-induced TIS10 and Egr-1 expression to H7 and sangivamycin were compared with those for phorbol ester-induced TIS10 and Egr-1 (Figure 3). Whereas, the sensitivities of phorbol ester- and v-Src-induced TIS10 to both H7 and sangivamycin were indistinguishable (Figure 3a and 3b), the sensitivities of phorbol ester and v-Src-induced Egr-1 to these compounds were different (Figure 3c and 3d).

v-Src-induced Egr-1 was more sensitive to both H7 and sangivamycin than phorbol ester-induced Egr-1. The higher sensitivity of v-Src-induced Egr-1 to H7 and sangivamycin suggests the requirement of a protein kinase(s) not required for phorbol ester-induced Egr-1 or v-Src-induced TIS10. Since v-Src-induced Egr-1 is insensitive to depletion of PKC, the greater sensitivity to both H7 and sangivamycin is not likely due to inhibition of PKC. The in vivo specificities of both H7 and sangivamycin are unknown. Both H7 and sangivamycin have been reported to be effective in blocking PKC in vitro, however, this has not held true in vivo (Spangler *et al.,* 1989). Thus, it is not clear which protein kinase(s) is required for v-Src-induced Egr-1 that is not required for phorbol ester-induced Egr-1 or v-Src-induced TIS10. However, these data demonstrate that the intracellular signaling pathways activated by phorbol ester and v-Src leading to the induction of Egr-1 are different and that the induction of TIS10 by phorbol ester and v-Src are possibly the same. Taken together, the data further suggest that v-Src induces the expression of TIS10 and Egr-1 via different intracellular signaling pathways.

v-Src induces gene expression from the TPA response element (TRE): TPA-responsive genes contain an upstream non-coding consensus sequence which confers phorbol ester-responsiveness to heterologous promoters (Angel *et al.,* 1987). If v-Src is inducing TIS10 gene expression via PKC then v-Src might induce the expression of genes linked to the TRE. NIH 3T3 cells

stably transformed with wild type v-Src have been shown to have elevated levels of gene expression regulated by the TRE (Schonthal *et al.,* 1988). We wished to determine if TRE-regulated gene expression was activated in response to increased v-Src kinase activity. We therefore examined the ability of v-Src to induce expression of the surrogate gene, CAT, linked to the herpes simplex virus thymidine kinase promotor (HSV-TK) under the control of the TRE. p5xTRECAT contains 5 copies of the TRE, derived from the collagenase gene, upstream of the HSV-TK promotor (Angel *et al.,* 1987). p5xTRECAT was transfected into LA90 cells; 40 h after transfection, the cells were shifted from the non-permissive to the permissive temperature for v-Src for 6 h. As shown in Fig. 4a, CAT activity was enhanced greater than 9-fold in cells that were shifted to the permissive temperature for v-Src. The level of CAT activity induced by v-Src was comparable to that induced by TPA in LA90 cells maintained at the non-permissive temperature for v-Src (Fig. 4a). Under similar conditions there was no stimulation of gene expression from PBLCAT2 (Luckow and Schutz, 1987, a plasmid containing the HSV-TK promotor without the TRE (Fig 4b). The induction of CAT gene expression from the TRE by both v-Src and TPA was abolished in cells that were depleted of PKC by prolonged exposure to TPA (Fig. 4c). These data demonstrate that v-Src enhances gene expression from a promoter controlled by a PKC-responsive transcriptional control element and that the induction is dependent upon PKC activity.

v-Src activation leads to the phosphorylation of a major PKC substrate: Data presented above suggest that a PKC-mediated signaling pathway is used for v-Src-induced TIS10 gene expression. However, it could be argued that basal levels of PKC activity are required for v-Src-induced signaling and that PKC is not activated by v-Src. Activation of PKC in mouse fibroblasts leads to phosphorylation of a protein with molecular weight of 80 kilodalton (Rodriguez-Pena and Rozengurt, 1984) known as MARCKS protein (Stumpo *et al.,* 1989). Therefore, we investigated whether v-Src could induce phosphorylation of the 80 kilodalton MARCKS protein in LA90 cells. Consistent with the work by Macara and coworkers (Han *et al.,* 1990; Wolfman *et al.,* 1987), we could not detect phosphorylation of MARCKS protein in LA90 cells. However, we were unable to induce phosphorylation of MARCKS protein in LA90 cells with TPA at either the permissive or non-permissive temperature (data not shown); whereas, TPA-induced phosphorylation of MARCKS protein was readily detectable in the parental BALB/c 3T3 cell line (Qureshi *et al.,* 1991). We have found that the lack of inducibility of MARCKS phosphorylation in v-Src-transformed cells is due to the transcriptional down-regulation of this protein in LA90 cells rather than a lack of v-Src-induced PKC activity or high baseline levels of MARCKS phosphorylation (Joseph, *et al.,* manuscript submitted for publication). Han *et al.,* (1990) have suggested that LA90 cells are somewhat leaky for v-Src at the non-permissive temperature. This could be responsible for the inability to detect phosphorylation of MARCKS in LA90 cells. To circumvent this problem, we

employed a transient transfection assay using a plasmid carrying cloned ts LA90 DNA driven by the cytomegalovirus promoter (A. Maroney and J. Brugge, manuscript in preparation). Cloned LA90 DNA was transfected into NIH3T3 murine fibroblasts, which take up and express exogenous DNA very efficiently. We then examined MARCKS protein phosphorylation in these cells after activating v-Src by temperature shift. As shown in Figure 4, increased phosphorylation of MARCKS protein as detected by immunoprecipitation using an antisera raised against MARCKS protein (Rosen *et al.,* 1989), was detected by 15 min after activating v-Src by temperature shift. NIH 3T3 cells transfected with vector plasmid without v-Src did not show any change in the phosphorylation state of this protein upon temperature shift (not shown). Thus, activating the PTK activity of v-Src leads to the phosphorylation of a major PKC substrate in NIH-3T3 cells strongly suggesting that v-Src activates PKC.

Activation of v-Src PTK does not cause increases in the levels of intracellular cAMP: The induction of Egr-1 by v-Src is sensitive to protein kinase inhibitors suggest involvement of a protein kinase(s). Protein kinase A (PKA) is a serine/threonine kinase, increases in the intracellular cAMP levels lead to the activation of PKA (Gilman, 1987). We have previously shown that H-7 and sangivamycin blocks PKA-mediated gene expression in Balb/c 3T3 cells (Spangler *et al.,* 1989; Qureshi *et al.,* 1991a). To examine the involvement of PKA in v-Src-induced Egr-1 gene expression we looked at the levels of intracellular cAMP in

LA90 cells. As shown in table I, activation of the v-Src PTK did not cause any increases in cAMP contents in LA90 cells. Under similar conditions treatment of LA90 cells with forskolin (25μM), which increases cAMP levels by activating adenylate cyclase (Gilman, 1987), increased intracellular cAMP contents by 10 fold. These data ,therefore, shows that v-Src does not increase intracellular cAMP levels. Furthermore, agonist of PKA (forskolin and 8 Br-cAMP) (Qureshi *et al.,* ,1991a) do not induce expression of Egr-1 in LA90 cells (data not shown). Taken together these data suggest that PKA is not involved in v-Src-induced expression of Egr-1 in LA90 cells.

Discussion

In this paper we have presented data demonstrating that v-Src activates two primary response genes using two distinguishable signaling mechanisms in murine fibroblasts; one that is dependent on PKC and one that is independent of PKC. The induction of TIS10, a gene of unknown function, was dependent upon PKC for induction by v-Src, whereas the induction of Egr-1, a growth-factor responsive transcription factor (Sukhatme *et al.,* 1988; Lemaire *et al.,* 1990), by v-Src was independent of PKC. We also demonstrated that the activation of v-Src leads to the phosphorylation of the PKC substrate MARCKS and results in the activation of transcription from a PKC-responsive element. Taken together, these data further establish a role for PKC in v-Src induced signaling and demonstrate that v-Src is activating at least two distinguishable signaling pathways in murine

fibroblasts.

Whether the PKC-mediated signaling pathway leading to the induction of TIS10 or the signaling pathway leading to the induction of Egr-1 are required for v-Src for transformation remains to be determined. However, the TPA resistant cell line, TNR9 (Butler-Gralla and Herschman, 1981), is resistant to transformation by v-Src, suggesting that a PKC-mediated pathway is required for v-Src to transform (Nori *et al.,* 1990). In addition, since Egr-1 is a growth factor-responsive transcription factor, it is tempting to speculate a role for the signaling pathway leading to Egr-1 induction in v-Src-induced transformation. Thus, v-Src-induced transformation may require activation of both the PKC-dependent and PKC-independent signaling pathways demonstrated here.

Primary targets for activating the PKC-dependent and independent pathways identified here are not known. However, there are several candidate signaling intermediates that have been implicated as potential substrates or targets of v-Src. These include Ras (Smith *et al.,* 1986), Raf (Morrison *et al.,* 1988), phosphatidylinositol kinase (Kaplan *et al.,* 1987; Courtneidge and Heber, 1987) and GAP (Ellis *et al.,* 1990). In addition to these, we have preliminary data for the involvement of a G-protein of the heterotrimeric class (Alexandropoulos *et al.,* 1991a) and a type D phospholipase specific for phosphatidylcholine (Song *et al.,* 1991). Thus, it is not inconceivable that v-Src could activate six or more intracellular signaling pathways. The controlling promoter sequences of growth signal responsive genes contain multiple response elements that allow for subtle and complex control of gene expression (Mitchell and Tjian, 1989). It is therefore

likely that incoming signals that activate gene expression are also subtle and complex. One way to generate complexity of intracellular signals originating from a single source like v-Src is to act on several signal transducing substrates. Thus, v-Src may induce the transformed phenotype by activating a complex network of several intracellular signaling pathways.

Section III.

v-Src Activates Mitogen-Responsive Transcription Factor Egr-1 via Serum Response Elements

Introduction

Protein-tyrosine kinase activity has been implicated in events leading to cell division (Drucker *et al.,* 1989). Many growth factor receptors have protein-tyrosine kinase activity that is activated upon binding of ligand (Drucker *et al.,* 1989). v-Src is a protein-tyrosine kinase that transforms cells in culture and induces tumors in animals (Jove and Hanafusa, 1987). We previously demonstrated that v-Src induces expression of Egr-1 (Qureshi *et al.,* 1991b). Egr-1 is a transcription factor (Lamaire, *et al.,* 1990; Patwardhan *et al.,* 1991), that is induced in response to cell division signals in all mammalian cells tested thus far (Sukhatme *et al.,* 1987,1988; Almendral *et al.,* 1988;Christy *et al.,* 1988;Lim *et al.,* 1987;Seyfert *et al.,* 1989;Bartel *et al.,* 1989;Lau and Nathans, 1987;and Zerial *et al.,* 1989). Egr-1 is also induced in contexts other than cell growth (for review, see Sukhatme, V.P., 1990). Egr-1 is induced by signals causing differentiation of PC12 pheochromocytoma cells (Wisden, *et al.,* 1990;Milbrandt J., 1987) and in vivo in cells of the central nervous system (Wisden, *et al.,* 1990; Rusak, B., 1990). Thus, like c-Fos, Egr-1 is a nuclear signal transducer which couples early biochemical events to long-term changes in cellular phenotype through modulation of gene expression.

v-Src-induced expression of Egr-1 is independent of both protein kinase C and protein kinase A (Qureshi *et al.,* 1991b). Since v-Src also induces protein kinase C -dependent signals in both avian (Spangler *et al.,* 1989) and murine (Qureshi *et al.,* 1991b) fibroblasts, v-Src activates both protein kinase C-dependent and protein kinase C-independent intracellular signaling pathways. To characterize the protein kinase C- and protein kinase A-independent signaling pathway used by v-Src to induce Egr-1, we examined the mechanism by which v-Src increases Egr-1 MRNA levels in murine fibroblasts.

Results

v-Src-Induces Expression of Egr-1 at the Level of Transcription:

BALB/c 3T3 cells infected with the LA90 temperature-sensitive strain of Rous sarcoma virus were used to demonstrate v-Src-induced expression of Egr-1. Shift from the non-permissive (40° C) to the permissive temperature (35° C), which activates the PTK activity of v-Src (Gray and Macara, 1988), led to an increase of Egr-1 mRNA, as detected by "northern blot" analysis. (Qureshi *et al.,* 1991a). Cycloheximide treatment caused super-induction of Egr-1 by v-Src (Fig. 5), demonstrating that protein synthesis is not required for induction. Thus, Egr-1 is a "primary response" gene (Herschman, H., 1989) of v-Src-induced signaling.

To investigate whether v-Src-induced increases in Egr-1 mRNA were due to activation of transcription or increased mRNA stability, we examined the effect of v-Src on the initiation of Egr-1 transcription using the nuclear "run-on" assay.

As shown in Fig. 6, transcription of Egr-1 in isolated nuclei is increased in response to the PTK activity of v-Src. Transcription of the ß actin gene, although induced slightly, remained relatively constant over the time course studied in LA90 cells (Fig. 6). The level of induction of Egr-1 as detected by the run-on assay was comparable to the level seen using northern gel analysis. Thus, v-Src-induced increases in Egr-1 expression can largely be explained by increased levels of transcription.

v-Src Activates Transcription from the Egr-1 Promoter: To identify cis element targets for v-Src-induced Egr-1 expression, the Egr-1 promoter was attached to a surrogate gene encoding CAT to generate pEgr-1 P1.2 as described previously (Guis *et al.,* 1990;Tsai-M.,*et al.,* 1987). pEgr-1 P1.2 contains sequences between nucleotides -957 to +248 relative to the transcriptional start site for Egr-1 (Tsai-M., *et al.,* 1988) cloned into pCAT3M (Laimins *et al.,* 1984) upstream from the CAT gene. Pegr-1 P1.2 was co-transfected with the v-Src expression plasmid pMv-Src (Johnson *et al.,* 1985) into NIH 3T3 cells and CAT activity was determined. As shown in Fig. 7 , CAT activity was substantially increased in the presence of v-Src. Neither the v-Src expression vector pEVX (Kreigler *et al.,* 1984) alone, nor carrier DNA (PUC18) caused significant increases in CAT activity when co-transfected with pEgr-1 P1.2. Transfected cells maintained in either high (10%) or low (1%) serum showed similar levels of inducibility by v-Src indicating that the v-Src induced CAT activity from the Egr-1

promoter does not depend upon factors found in serum (Fig. 7). v-Src-dependent CAT activity peaked at 40 h post transfection; therefore, all subsequent experiments were performed 40 h post transfection. These data indicate that v-Src dependent activation of Egr-1 can be mediated by sequences present between -957 to +248 of the Egr-1 promoter.

The v-Src-Responsive Sequence in the Egr-1 Promoter Contains a Cluster of SREs: The promoter sequences present in pEgr-1 P1.2 contain several characterized cis transcriptional control elements (reviewed in Mitchell and Tjian, 1989) including six SREs with CArG boxes, four Sp-1 factor binding sites, 2 AP-1 binding sites, 2 cAMP response elements, a CAAT box, and a TATA box (Guis *et al.,* 1990;Tsai-M., *et al.,* 1988; Christy *et al.,* 1988). The position of these sites is shown in Fig. 4. To identify v-Src-responsive regions in pEgr-1 P1.2, we used a series of deletions in the Egr-1 promoter region contained within pEgr-1 P1.2. Salient features of these deletions have been described elsewhere (Guis *et al.,* 1990; Tsai, M., *et al.,* 1988) and are shown in Fig. 4. pΔEgr-1 P1.2 has a deletion between -550 and -50 of the Egr-1 promoter and retains both AP-1 sites, three of four Sp-1 sites and the TATA box. The -550/-50 deletion reduced CAT activity to basal levels in the presence of v-Src (Fig. 8). pE425, which contains most of the sequences deleted in p ΔEgr-1 P1.2 including all SREs (as shown by the location of the CArG boxes), but lacking both AP-1 sites and three of four SP-1 binding sites was more responsive to v-Src than the parental pEgr-1

P1.2. pΔE425 is a derivative of pE425 with a deletion from -250 to -50 in which the two SREs most proximal to the transcription start site have been deleted. The -250/-50 deletion responded to v-Src activation even more efficiently than pE425. Thus, the two proximal SREs were not essential for v-Src responsiveness. However, sequential deletion of the distal SREs began to eliminate v-Src responsiveness (compare pE395, pE359 and pE342 in Fig. 8). Deleting a cluster of four SREs distal to the promoter eliminated v-Src-responsiveness (note the reduced v-Src-responsiveness in plasmids pE359, pE342, and pE125). pE125, which is missing four distal SREs, but retains the two most proximal SREs did not respond to v-Src. These data strongly implicate the clustered SREs located between -425 and -250 of the Egr-1 promoter as the region responsive to v-Src.

v-Src Activates Gene Expression Controlled by a Single Egr-1 SRE Linked to a Heterologous Promoter: All constructs responsive to v-Src, retained an Sp-1 binding site and a CAAT box. Thus, although there was a strong correlation between decreased responsive to v-Src and the loss of SREs, it was still possible that v-Src-responsiveness was mediated through the Sp-1 site and/or the CAAT box. Therefore, constructs containing either one or two copies of the most distal Egr-1 SRE and a construct containing a mutation in the CArG box of this SRE were tested for v-Src-responsiveness. These constructs lacked both the Sp-1 site and the CAAT box. As shown in Fig. 9, a single SRE from the Egr-1 promoter was able to confer v-Src responsiveness to a heterologous promoter.

The induction was enhanced by the addition of a second identical SRE in tandem. A mutation to the CArG box of this SRE which reduces the binding of serum response factor (SRF) (Treisman, 1986), reduced CAT activity to basal levels. These data demonstrate that a target of v-Src-induced signaling is a SRE.

Discussion

In this report we have presented data characterizing the activation of Egr-1 expression by v-Src. We demonstrate that v-Src-induced expression of Egr-1 is independent of protein synthesis and can be explained by increased levels of Egr-1 transcription. Deletion analysis of Egr-1 promoter sequences showed that v-Src responsiveness was localized to a cluster of four CArG box-containing SREs located between -425 and -250 relative to the transcription start site. A single SRE from this region was sufficient to confer v-Src-responsiveness to a heterologous promoter. A mutation to the CArG box of this SRE abolished v-Src-responsiveness. Taken together, these data strongly implicate SREs as a target for the protein kinase C- and protein kinase A independent v-Src-induction of Egr-1.

v-Src has previously been shown to induce CAT gene expression regulated by the Fos gene promoter (Fuji, *et al.,* 1989). This induction could occur using only the dyad symmetry element of the Fos promoter which contains a CArG element (Fuji, *et al.,* 1989). However, v-Src does not induce endogenous Fos gene expression (Walham, *et al.,* 1990; Qureshi *et al.,* 1991d). The data

presented here show that v-Src activates gene expression from a CArG element of the Egr-1 promoter and induction correlates with the induction of endogenous Egr-1 expression (Qureshi *et al.,* 1991b). v-Src has also been shown to induce gene expression from phorbol ester-responsive elements (Schonthal *et al.,* 1988, and section II). Thus, v-Src-induced gene expression is likely to be very complex and involve the interaction of multiple activated signaling pathways.

The observation here that v-Src activates the distal SREs and not the proximal SREs suggests that activation of these two groups of SREs may be mediated by different factor(s). SRF, a factor binding to the SRE of the Fos promoter, has been characterized (Treisman, 1986, 1988;Prywes, *et al.,* 1988). Egr-1 SREs have been shown to have differential affinities for SRF (Christy and Nathans, 1989). The second SRE from the transcriptional start site competes weakly for the binding of SRF and the third SRE which contains only 5 A/T residues in the CArG box does not bind SRF at all (Christy and Nathans, 1989). The third SRE from the transcriptional start site retains some responsiveness to v-Src as suggested by the v-Src-induced CAT activity from deletion pE42 (see Fig. 4). This deletion mutant also contains an Sp-1 site and a CAAT box that could contribute to the full induction by v-Src seen with deletion mutants pE425 and pE395. However, it is likely that since the most distal SRE, which does bind SRF (Christy and Nathans, 1989), confers v-Src-responsiveness by itself (Fig .5), that SRF contributes to v-Src induced Egr-1 expression. In addition, a mutation to the CArG box which is known to affect SRF binding, eliminated responsiveness to v-Src. The intracellular components contributing to the activation of SRF are

unknown; however, SRF requires phosphorylation for induction of transcription (Prywes *et al.,* 1988), thus a protein kinase may be involved. Other factors responding to v-Src that could function synergistically with SRF to induce Egr-1 remain to be determined.

Although serum is an obligate requirement for most cells to grow in culture, transformed cells show a reduced requirement for serum. The mechanism by which oncogenes reduce the dependence of serum is not clear. One hypothesis is that oncogenes activate or propagate signals otherwise dependent upon factors present in serum (Drucker, *et al.,* 1989). Serum-responsiveness is often mediated by presence of an SRE in the promoter (Mitchell and Tjian, 1989); therefore, the ability of v-Src to activate a gene via an SRE provides a clue to understanding the mechanism leading to reduced serum requirements for transformed cells. An important signal induced by serum may be the induction of Egr-1 as nuclear signal transducer. If v-Src is inducing some of the same cell division signals induced by serum, such as the induction of Egr-1, then the induction of Egr-1 via a SRE may contribute to the uncontrolled cell division caused by v-Src.

Section III

An Inhibitory Mutant of c-Raf-1 Blocks v-Src-Induced Activation of the Egr-1 Promoter.

Results and Discussion

Protein-tyrosine kinase activity has been implicated in events leading to cell division (Drucker *et al.,* 1989). v-Src is a protein-tyrosine kinase that transforms cells in culture and induces tumors in animals (Jove and Hanafusa, 1987). We previously demonstrated that v-Src induces expression of the Egr-1 gene (Qureshi *et al.,* 1991b). Egr-1 [also known as TIS-8 (Lim *et al.,* 1987), Zif/268 (Lau and Nathans, 1987; Christy *et al.,* 1988) Krox 24 (Almendral *et al.,* 1988), NGFI-A (Milbrandt, 1987) and CEF5 (Simmons *et al.,* 1989)] is a transcription factor (Lemaire *et al.,* 1990) with a "zinc finger" motif and a sequence-specific DNA binding activity (Christy and Nathans, 1989; Cao *et al.,* 1990). Egr-1 is induced in response to mitogenic signals in all mammalian cells tested thus far (Sukhatme *et al.,* 1987; Sukhatme *et al.,* 1988; Almendral *et al.,* 1988; Christy *et al.,* 1988; Lim *et al.,* 1987; Seyfert *et al.,* 1989; Bartell *et al.,* 1989; Lau and Nathans, 1987; Zerial *et al.,* 1989). Thus, Egr-1 may be a nuclear signal transducer which couples early biochemical events to long-term changes in cellular phenotypic changes that accompany mitogenesis through modulation of gene expression.

v-Src-induced expression of Egr-1 is independent of both protein kinase C (PKC) and protein kinase A (PKA) (Qureshi *et al.,* 1991b, 1991c, and section II);

however v-Src induced Egr-1 expression was shown to be sensitive to protein kinase inhibitors (Qureshi *et al.,* 1991b). This suggested a requirement for a protein kinase(s) other than PKC or PKA. c-Raf-1 is a serine/threonine protein kinases that has been implicated in mitogenic responses (Rapp *et al.,* 1988). A dominant-negative mutant of c-Raf-1 was recently characterized that inhibits serum- and phorbol ester-induced DNA synthesis (Kolch *et al.,* 1991). In v-Src transformed cells, c-Raf-1 has been found to be phosphorylated on serine, threonine and tyrosine residues and to have increased kinase activity (Morrison *et al.,* 1988). Whether these events are important for mediating signals initiated by v-Src has not been established. To evaluate the role of c-Raf-1 in v-Src induced signaling we studied the effect of a dominant-negative mutant of c-Raf-1 kinase on v-Src induced expression of the mitogen-responsive transcription factor Egr-1.

We recently demonstrated that v-Src induces Egr-1 gene expression at the level of transcription and this induction is mediated by a cluster of serum response elements (SREs) found in the Egr-1 promoter (Qureshi *et al.,* 1991c). pEgr-1 P1.2 (Gius *et al.,* 1990) is a plasmid vector that contains Egr-1 promoter sequences between -957 and +248 of the Egr-1 gene (Tsai-M., *et al.,* 1988) attached to the reporter gene chloramphenicol acetyl transferase (CAT) (Fig. 7a). Co-transfection of pEgr-1 P1.2 along with a v-Src expression plasmid, pMv-Src (Johnson *et al.,* 1985), into NIH 3T3 cells led to a ten fold increase in CAT activity (Fig. 7b). Under these conditions neither pEVX, the parental vector used for the construction of pMv-Src (Kriegler *at al.,* 1984), nor carrier DNA had any effect on Egr-1 promoter-dependent CAT activity. Thus, expression of v-Src

results in increased gene expression under the control of the Egr-1 promoter.

In NIH 3T3 cells c-Raf-1 is the most highly expressed member of Raf gene family. Kolch *et al.,* (1991) recently demonstrated that a kinase-defective mutant of c-Raf-1 functions as a dominant-negative mutant of c-Raf-1 when overexpressed in cells expressing c-Raf-1. The lysine of the ATP binding site of the human c-Raf-1 was converted to tryptophan, resulting in a Raf protein lacking the ability to bind ATP and therefore lacking kinase activity (Kolch *et al.,* 1991). p3011 expresses the kinase-defective c-Raf-1 under control of the cytomegalovirus early promoter in plasmid pMNC (Fig. 10a). Co-transfection of p3011 along with Pmv-Src and pEgr-1 P1.2 resulted in reduced CAT activity induced by v-Src from the Egr-1 promoter. The effect was dose-dependent with a maximal effect observed at 2-3 ug of p3011 plasmid (Fig 10b). pMNC, the parental vector used for expression of the kinase-defective c-Raf-1, did not inhibit v-Src-induced increases in CAT activity (Fig. 10B). These data suggest that c-Raf-1 is a necessary component of the v-Src-induced signaling pathway leading to the induction of Egr-1 gene expression.

To determine if the effect of the kinase-defective c-Raf-1 mutant was specific to Raf-mediated signals, we added pRaf (see Fig. 10a), which expresses wild type c-Raf, to the transfection mixture to see if the effects of kinase-defective c-Raf-1 could be competed with wild type c-Raf-1. The inhibitory effect of kinase-defective c-Raf-1 on v-Src-induced Egr-1 promoter activation was overcome by the presence of an excess of wild type c-Raf-1 (Fig. 11). Thus, the effect of the kinase-defective c-Raf on v-Src-induced promoter activation was specific for

c-Raf-1 function.

If c-Raf-1 is required for v-Src-induced Egr-1 promoter activation then an activated Raf-1 kinase, by itself, might induce the Egr-1 promoter. p3611 is a molecular clone of murine sarcoma virus that expresses a Gag-Raf fusion protein with a constitutively activate kinase (Rapp *et al.,* 1984) (Fig. 12a). Co-transfection of p3611 along with pEgr-1 P1.2 resulted in 13 fold increase in CAT activity (Fig 12a). Under similar conditions pFSRAF (Jamal and Ziff, 1990), in which most of the coding region of Raf has been deleted, had no effect on gene expression regulated by the Egr-1 promoter. These data demonstrate that the Egr-1 promoter can be induced by an active Raf-1 kinase.

Intracellular signal transduction frequently involves protein phosphorylation (Hunter, 1987). The activation of cell surface receptors by growth factor is often followed by an increase in protein phosphorylation on serine, threonine and tyrosine residues. The precise control of this activity is important as many oncogenes are altered forms of protein kinases (Drucker *et al.,* 1989). We have shown previously that v-Src activates PKC-dependent (Spangler *et al.,* 1989; Qureshi *et al.,* 1991b) and PKC-independent (Qureshi *et al.,* 1991b; Qureshi *et al.,* 1991c) signaling pathways. The data reported here indicate that the PKC-independent signaling pathway activated by v-Src is dependent upon the c-Raf-1 kinase.

Increased phosphorylation on c-Raf-1 has been reported in response to mitogenic stimulation of quiescent cells (Morrison *et al.,* 1988; Morrison *et al.,* 1989; App *et al.,* 1991). Increased phosphorylation of c-Raf-1 is believed to be

responsible for activating the kinase activity of c-Raf-1 (Morrison *et al.,* 1988). This phosphorylation takes place on serine, threonine and tyrosine residues of c-Raf-1 (Morrison, *et al.,* 1988). A mechanism for activation of c-Raf-1 by v-Src has not been determined. However, it has been demonstrated that antibodies to H-Ras block transformation by v-Src but not by v-Raf (Smith *et al.,* 1986). Thus, by inference, it is possible that v-Src first activates Ras which then activates c-Raf-1. Tyrosine phosphorylation of the GTPase activating protein of Ras observed in response v-Src (Ellis *et al.,* 1990; Brott *et al.,* 1991) is consistent with Ras being a more proximal signaling component than c-Raf-1 in v-Src-induced Egr-1 expression. However, it has also been shown that v-Src induces tyrosine phosphorylation on c-Raf-1 (Morrison *et al.,* 1988). Thus, induction of c-Raf-1 by v-Src may involve direct activation through tyrosine phosphorylation as well as indirect activation through Ras and a putative serine/threonine protein kinase(s). A complex activating mechanism might contribute to the degree of activation and/or the substrate specificity of c-Raf-1.

Section IV

Summary and Discussion

Activation of the v-Src tyrosine kinase activity leads to the induction of two immediate early response genes TIS-10 and Egr-1. Egr-1 encodes for a transcription factor (Lamair *et al.,* 1989; Patwardhan *et al.,* 1991), whereas TIS-10 has homology to the prostaglandin synthase gene (Herschman *et al.,* 1991).

Involvement of Serine threonine kinases namely PKC, c-Raf-1 and PKA was investigated during v-Src-induced expression of Egr-1 and TIS-10. Depleting cells of PKC by prolonged exposure to 12-O-tetradecanoylphorbol 13-acetate (TPA), blocked v-Src-induced TIS10 expression, but had no effect on v-Src-induced Egr-1 gene expression. In addition, the induction of TIS10 and Egr-1 by v-Src could be distinguished using protein kinase inhibitors. Therefore, the induction of TIS-10 is dependent upon PKC activity whereas Egr-1 is induced via a PKC-independent process. Consistent with the use of a PKC-mediated signaling pathway by v-Src, activating the kinase activity of v-Src led to increased phosphorylation of a major PKC substrate. Furthermore, v-Src activates transcription from a PKC-responsive transcriptional element in a PKC-dependent manner. These data demonstrate that one of the early events of v-Src transformation is activation of PKC.

To understand the PKC-independent signalling pathway activated by v-Src leading to the induction of Egr-1, the Egr-1 promoter was examined in transient

transfection assays for v-Src. Deletion analysis of the Egr-1 promoter revealed that a region between -425 and -250 of the Egr-1 gene is sufficient for v-Src dependent activation of the Egr-1 promoter. This region contains many cis-control elements, of which a CArG containing box was found to be sufficient for v-Src responsiveness.

Serum response elements are activated after binding of serum response factor. The activation of serum response factor requires phosphorylation. Casein kinase II (serine/threonine kinase) have been shown to induce this phosphorylation in vitro (Manack, *et al.,* 1990). Whether v-Src activates this kinase is not known. However, sangivamycin which blocked v-Src-induced Egr-1 expression also blocks casein kinase II in vitro (Loomes and Bell, 1987). Since activation of v-Src PTK in LA90 cells does cause any increases in the cAMP, an activator of PKA, levels in LA90 cell, PKA does not appear to be involved in the v-Src-induced signaling.

v-Src induced Egr-1 expression is independent of both PKC and PKA but is still sensitive to protein kinase inhibitors. This suggests the involvement of a protein kinase. v-Src phosphorylates Raf kinase (Morrison *et al.,* 1988). Phosphorylation of Raf kinase increases its kinase activity. Involvement of c-Raf-1 kinase in v-Src-induced signaling was examined using a dominant-negative mutant of c-Raf-1. Cotransfection of a c-Raf-1 inhibitor plasmid along with Egr-1 CAT plasmid inhibited the v-Src induciblity. This effect can be overcome by using an excess of c-Raf(WT) plasmid. Consistent with the involvement of c-Raf-1 during Egr-1 promoter activation we have found that an activated Raf also increases

CAT expression from the Egr-1 promoter. These data, therefore, suggest that v-Src-induced Egr-1 expression is mediated by c-Raf-1 kinase.

The involvement of PKC, and c-RAf-1 kinase suggest that v-Src induced transformation involves complex pathways and may involve more serine threonine kinases. PKC depletion only inhibits 70% of the v-Src-induced TIS 10 gene expression whereas protein kinase inhibitors completely blocks v-Src-induced TIS10 gene expression is indicative of involvement of another protein kinase(s). Indeed v-Src phosphorylate and possibly activate two other serine threonine kinases, S6 kinase (Vik *et al.*, 1989) and MAP_2 kinase (Weber *et al.*, 1984).

Although the exact mechanism leading to the activation of serum response elements of the Egr-1 gene by v-Src has not been determined. Observation that the v-Src activates only few of the many Egr-1 serum response elements suggest broader implications. One such implication would be the lack of induction of all serum response element containing genes. Lack of Fos gene expression in response to v-Src PTK activity is one such example (Walhem, *et al.*, 1989, Qureshi *et al.*, 1991d). Since coordinate regulation of gene expression is necessary, selective induction of growth responsive gene may be a way to induce transformation.

Contribution of TIS 10 and Egr-1 gene expression to transformation has not been determined. But Egr-1 is induced in response to at least two other transforming oncogenes v-raf (Rim *et al.*, submitted for pulication) and v-Fps (Alexandropoulos *et al.*, submitted for publication) suggest it have some role in transformation. Egr-1 is a transcription factor and its binding site is found in

genes usually involved in growth control (Ras, Abl, PDFG a chain, etc.) (Christy and Nathan, 1989; Sukhatme, 1990). Whether any of these genes are also induced in LA90 cells by v-Src has not been determined.

In summary, activating the v-Src PTK activity leads to the induction of two early response genes and this induction is mediated by PKC and c-Raf-1 kinase.

Materials and methods

Cells and cell culture conditions:

BALB/c-3T3 and NIH 3T3 cells (obtained from the American Type Culture Collection) and LA90-transformed BALB/c 3T3 cells (provided by J. Brugge) were maintained in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum. Confluent cell cultures were made quiescent by maintaining cells for three days in 1% newborn calf serum.

Materials: H7 was obtained from Seikagaku of America, Ltd., St. Petersburg, FL. Sangivamycin was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment of the National Cancer Institute (NSC-6546). TPA, phorbol dibutyrate (PDB) and cycloheximide, and chloramphenicol were obtained from Sigma. [^{14}C]-butyryl-co-enzyme A, [^{14}C]-chloramphenicol, [^{32}P]-orthophosphate, [^{32}P]-dCTP and [^{32}P]-UTP were obtained from New England Nuclear.

RNA analysis: RNA was extracted as described by Hatch and Bonner (1987) with modifications described previously (Qureshi *et al.,* 1991a). Electrophoretic transfer of RNA to nylon membranes and immobilization on filters by UV cross-linking was as described by Church and Gilbert, (1984). Filters were probed with DNA, radiolabeled by the random primed DNA labelling technique (Feinberg & Vogelstein, 198) using a kit (US Biochemical). Hybridizations and washings were performed as described (Davis *et al.,* 1986).

PKC assay: The total PKC activity present in cultured cells was determined after partial purification of cellular extracts as described by Ballester and Rosen (1985). Briefly, cells were washed twice with cold isotonic buffer, collected in 5 ml of homogenization buffer (20 mM Tris [pH 7.5], 5mM NaCl, 1 mM EDTA, 5mM MgCl2, 2mM DTT, 200 μM PMSF, 20 ug/ml Aprotinin and 500 ng/ml Leupeptin) containing 1% Triton X-100, and disrupted with 30 strokes in a Dounce homogenizer. After centrifugation at 100,000g for 1 h, the supernatant was loaded onto a DEAE-Cellulose column (Sigma) equilibrated with buffer A [20 mM Hepes buffer (pH 7.5), 5 mM EGTA, 2mM dithiothreitol]. The column was washed with 10 ml of buffer A and eluted with 1.5 ml of Buffer A containing 100 mM NaCl. Under these conditions, PKC activity eluted in the first 1 ml of eluent. 100 μl of this fraction was assayed for PKC activity using histone type III-S as a substrate with or without Ca^{++}, phosphatidylserine and diacylglycerol as described by Wolfman, et al. (1987).

Cell transfection for MARCKS analysis and protein analysis:

NIH 3T3 cells were plated at 5x10 cells per 100 mm dish 16 hours prior to transfection. Transfections were performed using $CaPO_4$ as a coprecipitate as described by Chen and Okayama (1987). Each transfection mixture contained 20 μg of DNA. ts-LA90 DNA was cloned by polymerase chain reaction amplification of DNA from RSV ts-LA90 virus transformed BALB/c 3T3 fibroblasts (Anna Maroney and Joan Brugge, unpublished results). 20 μg of ts-LA90 DNA was transfected into NIH 3T3 cells. Phosphorylation of the 80 kilodalton PKC substrate was characterized as described previously (Spangler *et al.,* 1989) using antibody to the MARCKS protein (Rosen *et al.,* 1989).

Nuclear "Run-On" Assay: Run-on assays (Greenberg and Ziff, 1984) were performed as described by Lineal et al. (1985). Radiolabeled RNA ($2x10^7$ cpm) was hybridized for 48 h to DNA containing Egr-1, murine ß actin (obtained from American Type Culture Collection) and pUC-18 gene sequences that were immobilized on Nylon membrane as described by Marzluff and Huang, (1984). Nylon membranes were washed and exposed to X-Ray film for 2 days with intensifying screens.

Plasmid Constructions: The construction of pEgr-1 P1.2, pΔEgr-1 P1.2, pE425, pΔE425, pE395, pE359 pE342, and pE125 was described previously (Guis *et al.,* 1990). pTK35CAT-SRE1 and pTK35CAT-SRE2 contain

one and two copies respectively of the most distal Egr-1 SRE cloned into the XbaI site of pTK35CAT. pTK35CAT contains the minimal promoter of the thymidine kinase gene of herpes simplex virus and was kindly provided by S. Jamal and E. Ziff (NYU School of Medicine). pTK35CAT-SREm contains a single copy of the most distal Egr-1 SRE where the CArG box has been changed from CCATATAAGG to GGATATAACC as described by Gius *et al.*, (1990). p3011 expresses mutant c-Raf-1 gene under the control of cytomegalovirus in plasmid pMNC and has been described (Kolch *et al.*, 1991). p3611 is a molecular clone of murine sarcoma virus and expresses a gag-raf protein (Rapp, *et al.*, 1984). pFSRAF was constructed from p3611 by deleting most of the raf coding region and therefore does not express any functional protein (Jamal and Ziff, 1990)

Cell Transfection for CAT assay: NIH 3T3 cells were plated at $5x10^5$ cells per 100 mm dish 16 hours prior to transfection. Transfections were performed using $CaPO_4$ as a coprecipitate as described by Chen and Okayama, (1987). Each transfection mixture contained 20 μg DNA (4 μg pMv-Src or pEVX, 4 μg of reporter chloramphenicol acetyl transferase (CAT) plasmid, carrier DNA- salmon sperm DNA and other DNA if mentioned).

CAT Assay: Transfected cultures were washed 16-20 h post transfection and fresh media containing 1% serum was added. After another 40 h cells were harvested after washing twice with cold isotonic buffer, resuspended in 200 μl of

250 mM Tris-HCl (pH 7.8), and lysed by 5 cycles of freeze-thaw (dry-ice methanol/ 70°C) for 5 min each. Supernatants were collected after microcentrifuging for 5 minutes at 40°C. CAT activity in cell extracts, using equal amounts of protein (Bradford , 1976), was determined either by the procedure of Gorman et al. (1982) using thin layer chromatography or by using the "fluor diffusion assay" (Neuman *et al.*, 1987).

cAMP assay: cAMP levels in LA90 cells were determined as described (Qureshi *et al.*, 1991a).

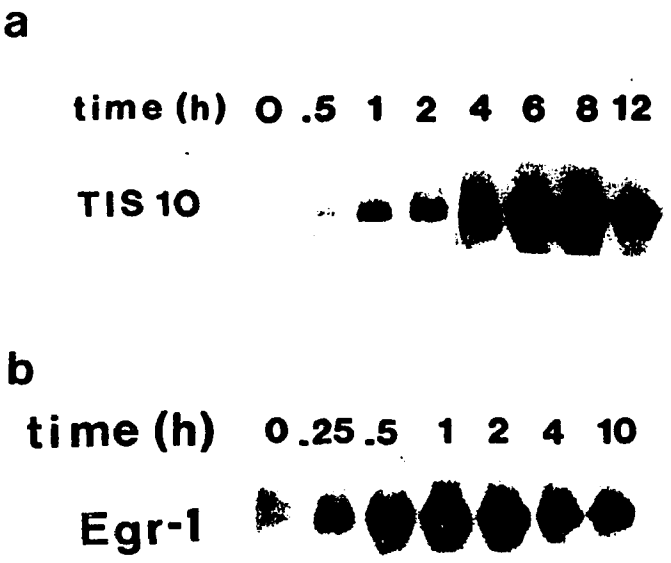

Figure 1. **Induction of TIS10 and Egr-1 expression by v-Src.** Quiescent LA90 cells were shifted from the non-permissive (40°C) to the permissive (35° C) temperature for v-Src. RNA was harvested at the times indicated, electrophoresed, and hybridized to radiolabeled TIS10 DNA as described in Materials and Methods. (a) TIS10. (b) Egr-1.

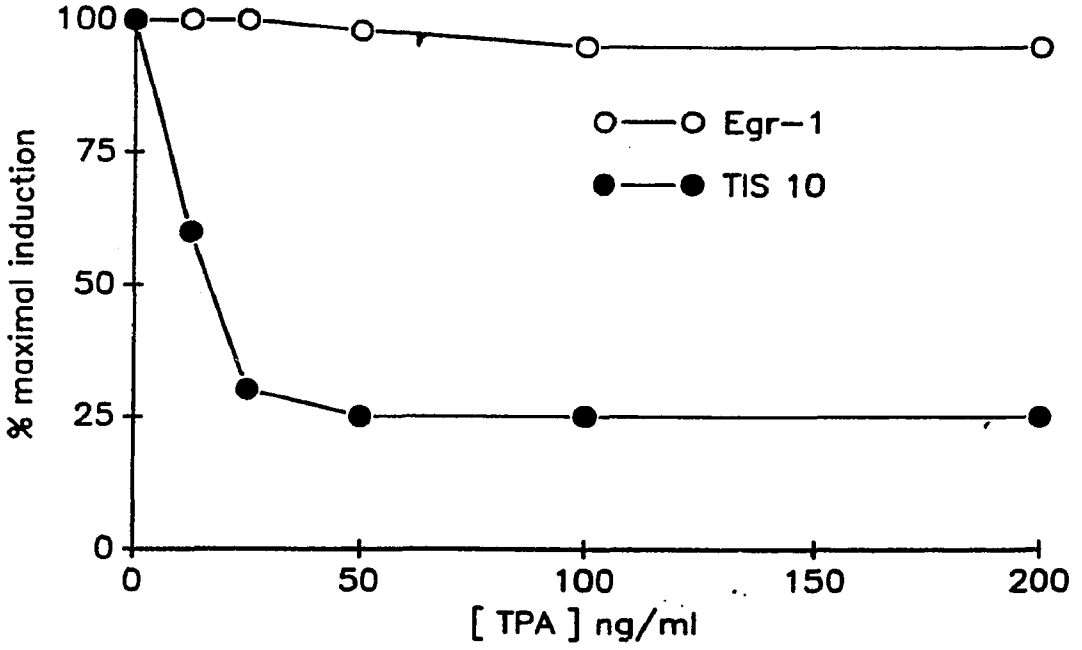

Figure 2. The effect of depleting cells of PKC on v-Src-induced TIS10 and Egr-1 expression. Increasing concentrations of TPA (ng/ml) were added to LA90 cell cultures for 26 h prior to either temperature shift. TIS10 and Egr-1 RNA levels were measured as in Figure 1 after two h induction by temperature shift.

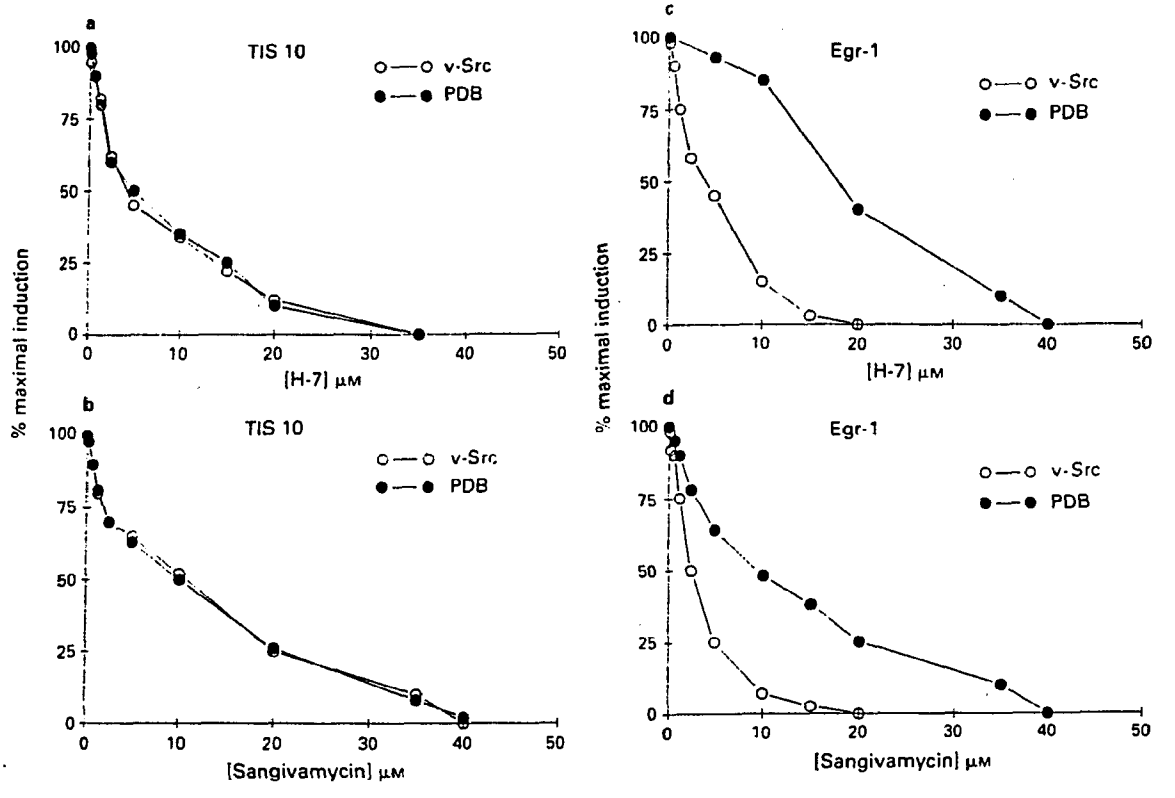

Figure 3. The sensitivity of v-Src- and phorbol ester-induced TIS10 and Egr-1 expression to the protein kinase inhibitors H7 and sangivamycin. LA90 cell cultures were either subjected to temperature shift to activate v-Src or were treated with PDB (100 ng/ml). H7 and Sangivamycin at the concentrations given (μM) were added 10 minutes prior to stimulus. Northern analysis was performed and densitometric analysis of autoradiographs is presented. TIS10; (a) H7, (b) sangivamycin. Egr-1; (c) H7, (d) sangivamycin.

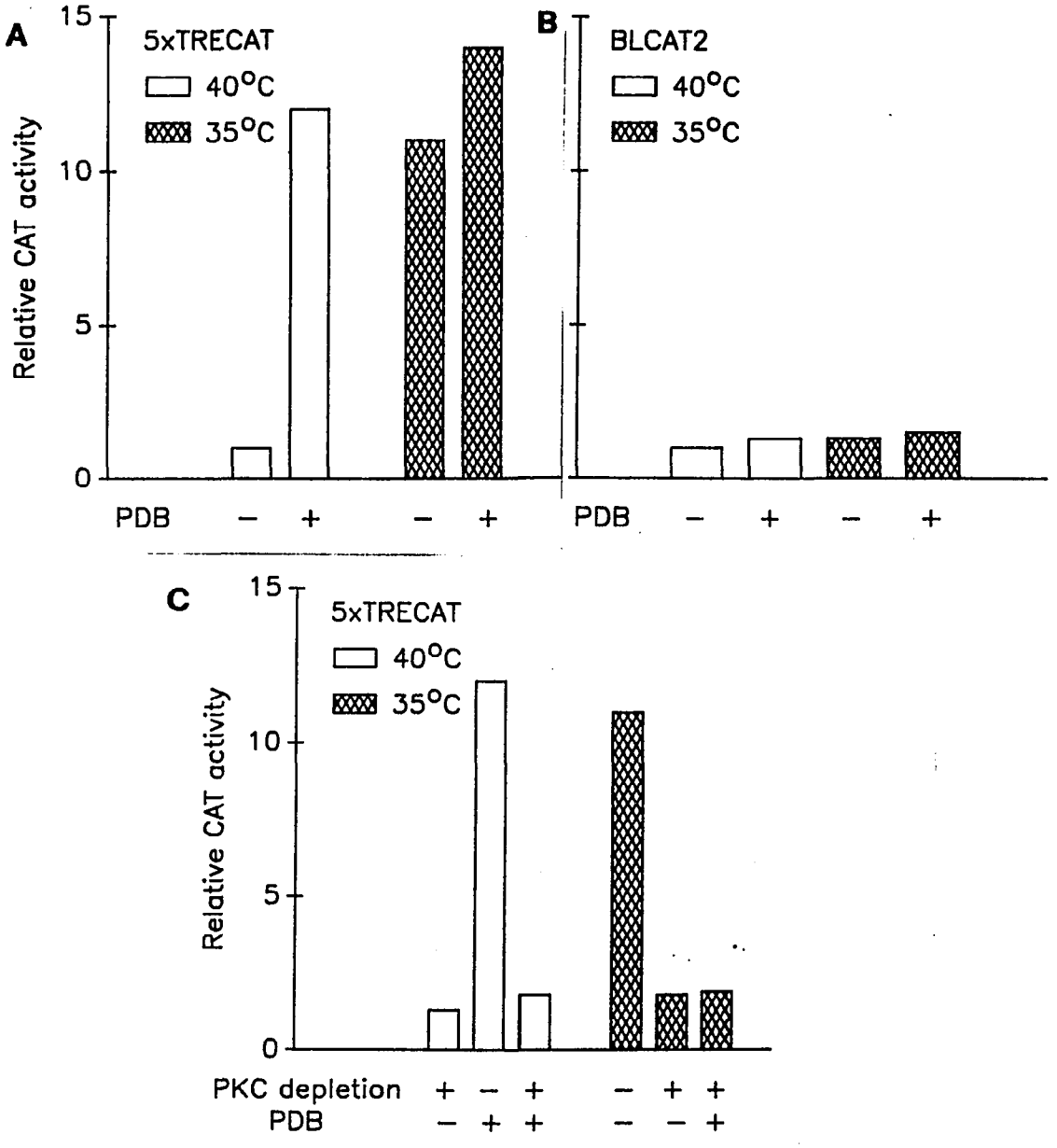

Figure 4. Induction of gene expression regulated by the TRE.
Cells were transfected with either p5xTRECAT or pBLCAT2. a. CAT activity was assayed in LA90 cells transfected with p5xTRECAT. 36 h after transfection, the cells were induced either by shifting from the non-permissive to the permissive temperature for 6 h or by addition of PDB (100 ng/ml) for 6 h. b. CAT activity was assayed in LA90 cells transfected with pBLCAT2 as in a. c. The ability of PDB and v-Src to induce gene expression from p5xTRECAT in LA90 cells depleted of PKC was tested using conditions established in Fig. 3 (100 nm TPA, 26 h).

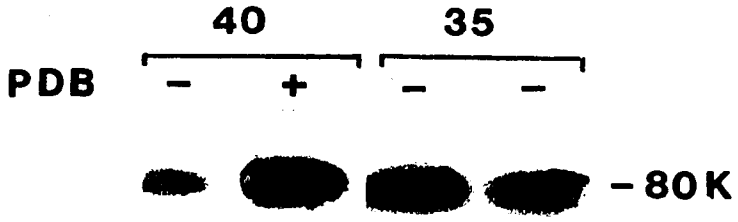

Figure 5. **Phosphorylation of the PKC substrate MARCKS**
Molecularly cloned LA90 DNA, expressed under the control of cytomegalovirus promoter was transfected into NIH 3T3 cells as described in Materials and Methods. 16 h after transfection, the cells were washed and fed with medium containing 1% serum. 40 h later, cells were induced either with PDB (100 ng/ml) or temperature shift. Phosphorylation of the 80 kilodalton MARCKS protein was examined 15 min after the addition of PDB and at 15 and 30 min after shifting to the permissive temperature for v-Src. PDB-induced (lane 2), and v-Src-induced (lanes and 4; 15 and 0 min time points, respectively) were immunoprecipitated with antisera raised against MARCKS protein (Rosen *et al.,* 1989) and electrophoresed as described previously (Spangler *et al.,* 1989).

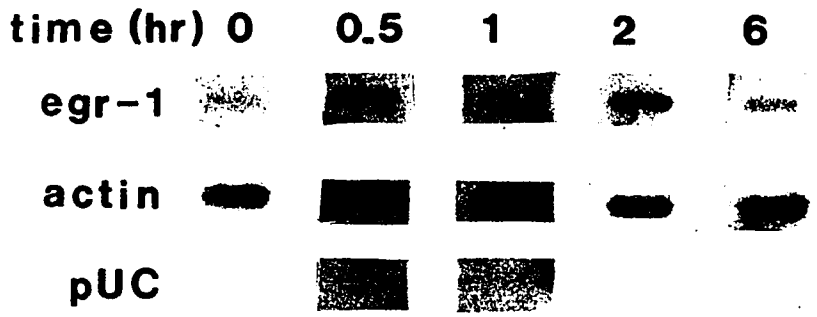

Figure 6. v-Src-induced Egr-1 expression is at the transcriptional level. "Run-on" transcription in isolated nuclei was examined in LA90 cells at the times indicated (hours) after shifting from the non-permissive to the permissive temperature. Transcription of Egr-1 is compared with transcription of ß actin. pUC-18 vector DNA was used as a negative control.

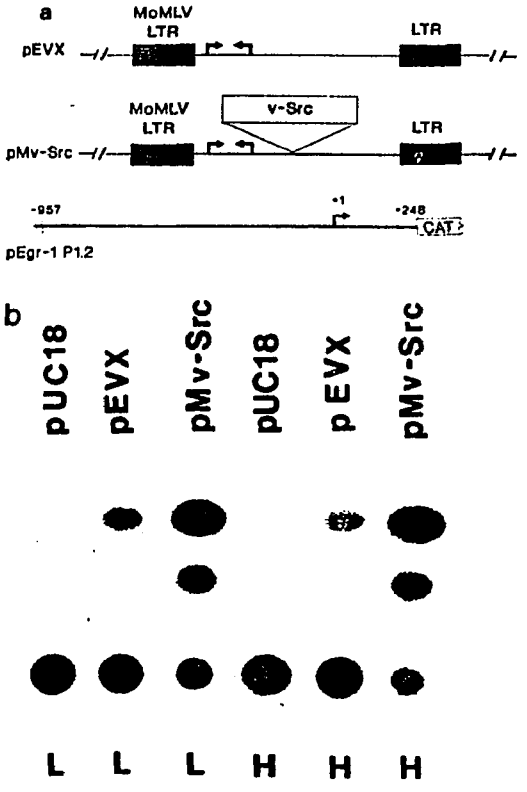

Figure 7. Activation of the Egr-1 promoter by v-Src.
pEgr-1 P1.2 contains the CAT gene under control of the Egr-1 promoter (Guis *et al.,* 1990). CAT activity was assayed in extracts from NIH 3T3 cells that had been transfected with pEgr-1 P1.2 and either pMv-Src, a v-Src expression vector (Johnson *et al.,* 1985; pEVX (Kreigler *et al.,* 1984), the vector used to construct pMv-Src, or pUC18. Transfections were performed using the calcium phosphate co-precipitation technique as described previously (Chen and Okayama, 1987). 24 h later the cells were washed and cultured in either 10% serum (H) or 0.5% serum (L) and CAT activity was assayed from extracts prepared 16 h later. a) Diagram of constructs used. Bent arrows represent splice donor and acceptor sites. b) CAT assays were performed as described by Gorman *et al.,* (1982).

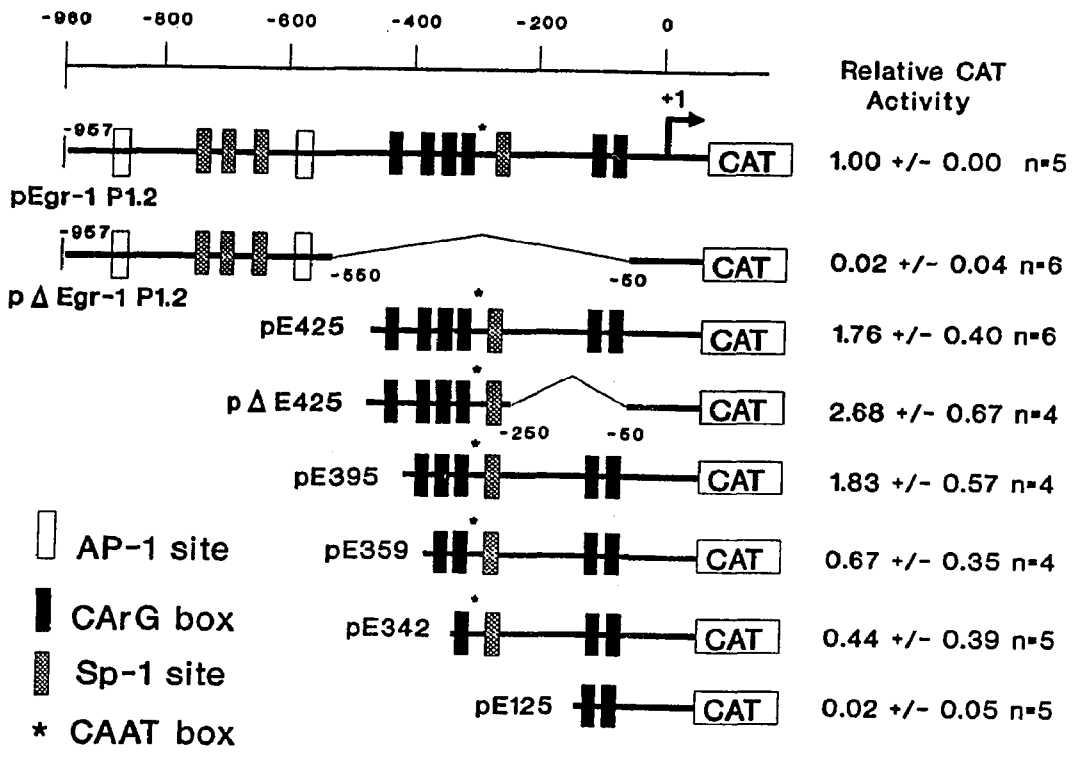

Figure 8. Deletion analysis of the Egr-1 promoter.
NIH 3T3 cells were transfected with pEgr-1 P1.2 and derivatives containing various Egr-1 promoter deletions (Guis *et al.,* 1990) along with pMv-Src. CAT activity was determined using the "fluor diffusion" assay as described by Neuman *et al.,* (1987). The location of transcriptional control elements in the Egr-1 promoter are shown in the diagram of parental pEgr-1 P1.2 plasmid from which the deletion mutants were constructed. *"Relative CAT activity"* is the amount of CAT activity generated by the constructs containing deletions in the Egr-1 promoter normalized to CAT activity generated by pEgr-1 P1.2 in the absence of v-Src. Basal CAT activity generated by pEgr-1 P1.2 which contains entire Egr-1 promoter. The data presented are from the experiments in the presence of v-Src. Basal CAT activity generated in the absence of v-Src by pEgr-1 P1.2 was equivalent to the CAT activity observed for pΔEgr-1 P1.2 and pE125. Similar levels of basal activity were observed for all Egr-1 promoter deletion CAT constructs in the absence of v-Src. Data are the mean of at least four independent experiments.

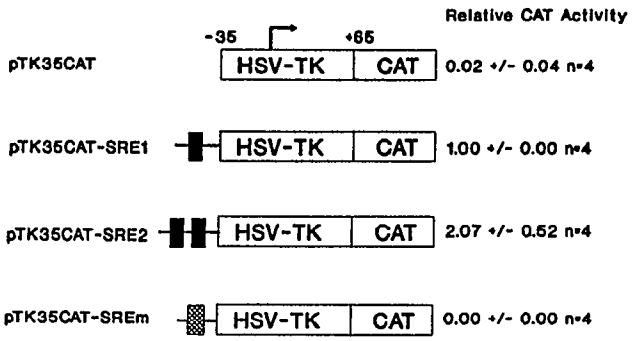

Figure 9. v-Src induces gene expression from a heterologous promoter containing a single SRE from the Egr-1 promoter. Oligonucleotide sequences representing 40 base pairs of the most distal SRE including the CArG box were cloned upstream of the herpes virus thymidine kinase minimal promoter. pTK35CAT-SRE1 contains a single copy of the most distal SRE, pTK35CAT-SRE2 contains two copies in tandem, and pTK35CAT-SREm contains a mutation in the CArG box. CAT activity generated by these plasmids was assayed as in Fig. 7. CAT activity was normalized to that generated by pTK35CAT-SRE1 in the presence of v-Src as in Fig. 7. Basal CAT activity generated in the absence of v-Src from these constructs was equivalent to that seen with pTK35CAT in the presence of v-Src. Data are the mean of four independent experiments.

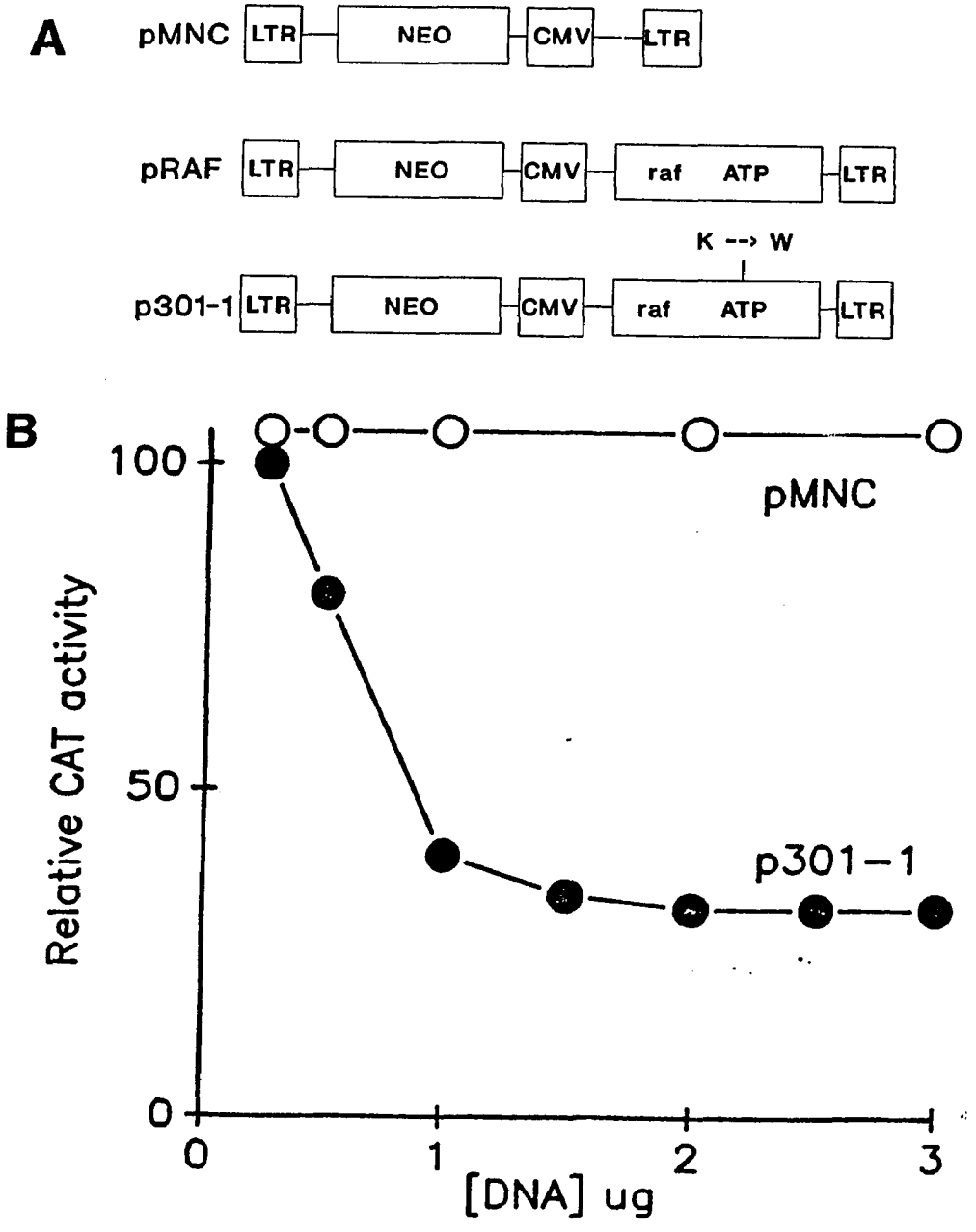

Figure 10. A kinase-defective mutant of c-Raf-1 blocks v-Src-induced Egr-1 promoter activation.

A. Schematic of vectors used. B. Effect of increasing amounts of p3011, which expresses the kinase-defective c-Raf-1 mutant, and pMNC, the parental vector of p3011, on v-Src-induced promoter activation. Transfections were performed as described in Fig. 7 except that the transfection mixture also contained either p3011 or pMNC as indicated.

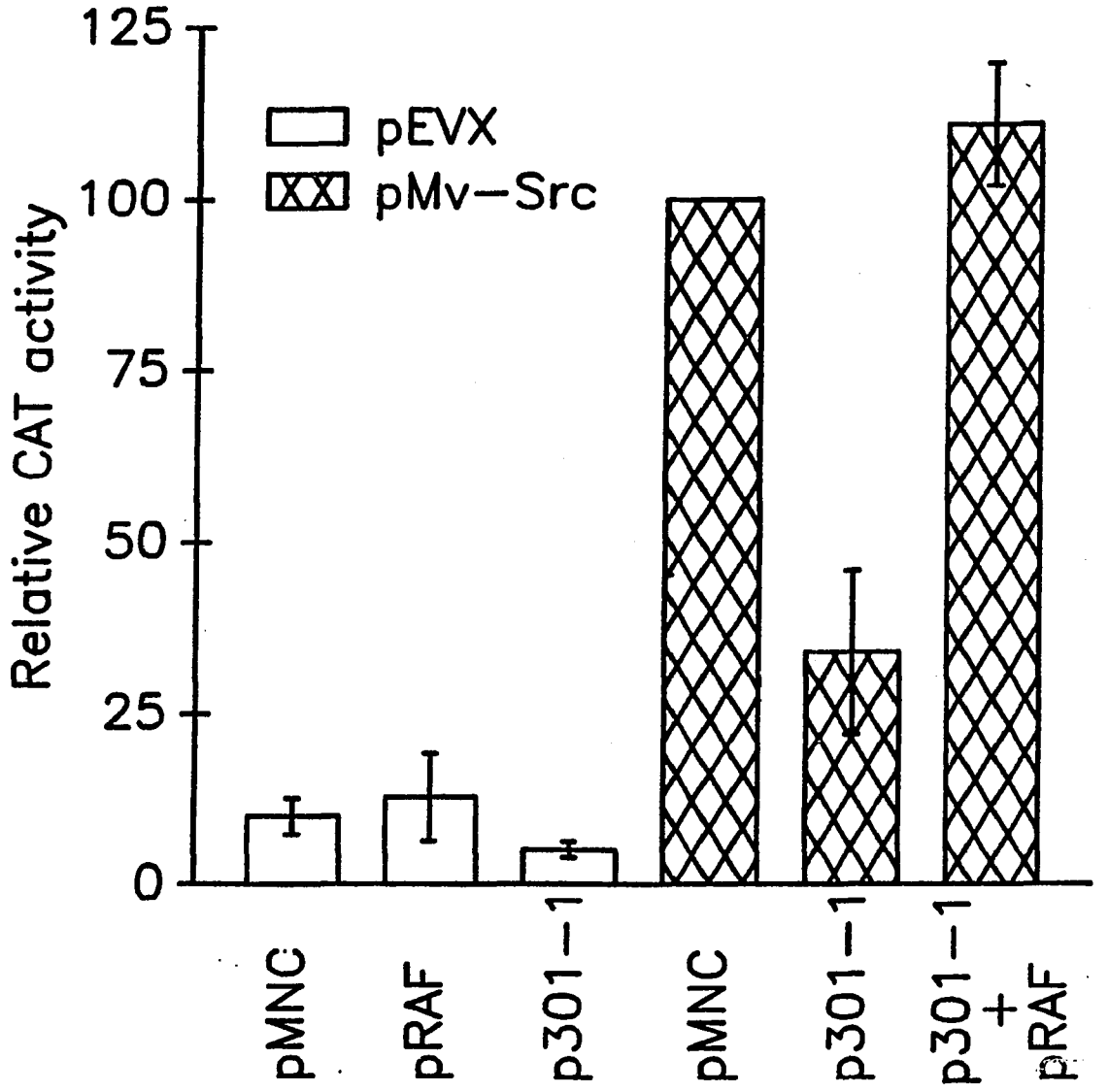

Figure 11. The inhibitory effect of the kinase-defective c-Raf-1 mutant is overcome by wild type c-Raf-1.
NIH 3T3 cells were transfected with pEgr-1 P1.2 in the presence or absence of pMv-Src as indicated. p3011, expressing the kinase-defective c-Raf-1, and pRaf, expressing wild type c-Raf-1 were included (2 μg p3011; 5 μg pRaf) in the transfection mixture where indicated. CAT activity was determined as in Fig. 8.

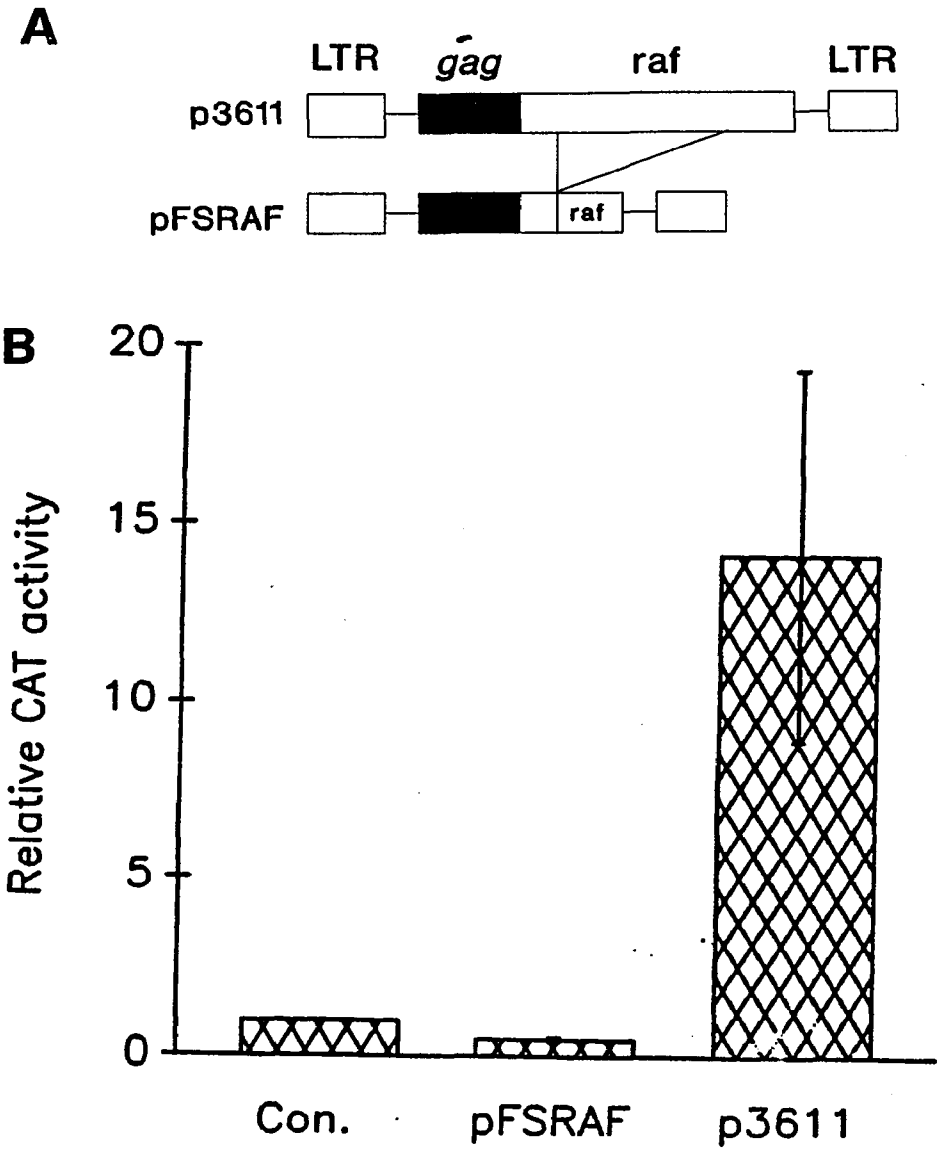

Figure 12. Activation of Egr-1 promoter by v-Raf
NIH 3T3 cells were co-transfected as described in Fig. 7 with 4 μg of p3611 or pFSRAF and 5μg of pEgr-1 P1.2 CAT. p3611 is a molecular clone of murine sarcoma virus 3611, which expresses a Gag-Raf fusion protein (Rapp *et al.*, 1984). pFSRAF was derived from p3611 by deleting most of the RAF coding sequences from p3611 (Jamal and Ziff, 1990) and does not express a functional c-Raf-1 protein. A. Schematic of vectors used. B. CAT activity as determined as in Fig. 8.

Table I

Relative levels of cAMP in LA90 cells

	40° C	35° C
v-Src	1.00	1.00 +/- 0.04
Forskolin	10.00 +/- 1.20	10.00 +/- 1.30

LA 90 cells were maintained at 40° C as described in figure 1. LA 90 cells were stimulated for 15 min either by activating the v-Src PTK by shifting cells to 35° C, or by addition of Forskolin (25 μM), after which cells were harvested and lysed by boiling in 5mM EDTA (pH 8.) as described previously (Qureshi *et al.,* 1991). cAMP levels in the supernatants were determined using a radioimmune assay kit (Amersham) as per vendor's instruction as described (Qureshi *et al.,* 1991). "Relative cAMP levels" were determined by giving the amount of cAMP in LA90 cells kept at 40° C value of 1 and is mean of two independent experiments performed in duplicate.

Appendix I

Cholera toxin induces expression of the immediate-early response gene JE via a cyclic AMP-independent signaling pathway.

Qureshi, S.A., Alexandropoulos, K., Joseph, C.K., Spangler, R. & Foster, D.A. (1991). *Mol. Cell. Biol.* **11**, 102-107.

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Cholera Toxin Induces Expression of the Immediate-Early Response Gene JE via a Cyclic AMP-Independent Signaling Pathway

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Cholera toxin (CT) activates expression of two immediate-early response genes (JE and TIS10) in quiescent BALB/c 3T3 cells. Increases in cyclic AMP (cAMP) levels in response to CT are likely responsible for the induction of TIS10 gene expression, since treatment with 8-Br-cAMP and increasing the intracellular levels of cAMP by treatment with forskolin induce TIS10 gene expression. In contrast, neither forskolin nor 8-Br-cAMP induces JE gene expression. 3-Isobutyl-1-methylxanthine, which stabilizes intracellular cAMP, potentiates CT-induced TIS10 gene expression but has no effect on CT-induced JE gene expression. Thus, induction of JE by CT is independent of the cAMP produced in response to CT. Induction of JE by CT does not require protein kinase C (PKC), since depleting cells of PKC activity has no effect on the induction of JE by CT. CT-induced expression of JE can be distinguished from CT-induced TIS10 gene expression by using protein kinase inhibitors and inhibitors of arachidonic acid metabolism, further suggesting distinct signaling pathways for CT-induced JE and TIS10 gene expression. Thus, induction of JE gene expression by CT results from the activation of an intracellular signaling pathway that is independent of cAMP production. This pathway is independent of PKC activity and uniquely sensitive to inhibitors of protein kinases and arachidonic acid metabolism.

Guanine-nucleotide-binding proteins (G proteins) transduce signals from cell surface receptors to effector molecules within the cell (10, 23, 33). The activation of a G protein results in the replacement of GDP with GTP and the dissociation of a heterotrimeric complex ($\alpha\beta\gamma$) into α and $\beta\gamma$ subunits. Dissociation activates the signal-transducing ability of G proteins (10, 23, 27, 33). Cholera toxin (CT) is a bacterial enterotoxin that modifies a subset of G proteins by ADP-ribosylation of the α subunit (10, 33). This modification reduces the intrinsic GTPase activity of the α subunit and may also promote subunit dissociation (10, 33). The GTPase activity of the α subunit down-regulates the effect of the G protein α subunit on effector molecules. The effector molecule for the α subunit of G_s is adenylate cyclase, which converts ATP into the second messenger cyclic AMP (cAMP). By catalyzing the ADP-ribosylation of G_s, CT increases production of cAMP by promoting subunit dissociation and blocking GTPase activity. Increasing the level of intracellular cAMP has many effects on the modulation of gene expression (7, 36). Most effects of cAMP are mediated by the cAMP-dependent protein kinase A (PKA) (10). In fibroblasts, most effects of CT are thought to be mediated by G_s, adenylate cyclase, cAMP, and PKA. However, recent reports suggest that CT may induce cAMP-independent events via G_s (3, 19, 21, 25, 26, 37). Other studies have suggested that CT may ADP-ribosylate G proteins other than G_s (20, 32, 35). In this report, we present data demonstrating that CT induces expression of the immediate-early response gene JE (5) through an intracellular signaling pathway that does not involve cAMP production.

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MATERIALS AND METHODS

Cells and cell culture conditions. BALB/c 3T3 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% newborn-calf serum. Confluent cell cultures were made quiescent by being maintained for 4 days without feeding.

Materials. H7 was obtained from Seikagaku of America, Ltd., St. Petersburg, Fla. Sangivamycin was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment of the National Cancer Institute (NSC-65346). 8-Br-cAMP, CT, purified A and B subunits of CT, forskolin, indomethacin, nordihydroguaiaretic acid (NDGA), phorbol dibutyrate, and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma. For reconstitution of CT holotoxin, purified A and B subunits were mixed in equimolar ratios and stored at 4°C for at least 24 h prior to being used. Forskolin and IBMX were dissolved in ethanol. When added to cell culture, the solvent constituted less than 0.5% of the total volume. Solvent alone was used in control experiments.

RNA analysis. RNA was extracted as described by Hatch and Bonner (12), with modifications. Briefly, cells were washed with cold isotonic Tris-saline and resuspended in isotonic loading buffer (2% bentonite, 24% sucrose, and 0.1% bromophenol blue in 1× phosphate-buffered saline [137 mM NaCl, 2.7 mM $CaCl_2$, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4]), freeze-thawed (−70 and 4°C), dissolved with hot formaldehyde-formamide-sodium dodecyl sulfate (SDS), and microcentrifuged. For RNA analysis, supernatants were loaded directly onto 1.0% formaldehyde–SDS–agarose gels and fractionated as described previously (12). RNA levels were compared by examination of ethidium bromide staining in both gels and transfer filters. RNA was electrophoretically transferred to nylon membranes and immobilized on filters by UV cross-linking as described by Church and Gilbert (4).

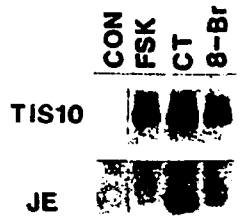

FIG. 1. Effects of CT and activators of PKA on JE and TIS10 gene expression. CT at 2 μg/ml, forskolin (FSK) at 25 μM, and 8-Br-cAMP (8-Br) at 1 mM were added to quiescent BALB/c 3T3 cell cultures, and mRNA levels were examined 90 min later. RNA was prepared for Northern (RNA) gel analysis as described in the text, and filters were hybridized to JE and TIS10 probes sequentially as described in the text. CON, Control.

Filters were probed sequentially with JE and TIS10 DNA radiolabeled by the random-primed-DNA labeling technique (9) with a kit from U.S. Biochemical. Hybridization and washings were performed as described previously (6), with the final wash at 65°C for 1 h. Filters were dried and exposed to X-ray film (X-AR5; Kodak) with an intensifying screen at −70°C for 1 to 2 days. For quantification of signals, autoradiograms were scanned with a laser densitometer. For multiple probing, filters were stripped of probe by being washed in stripping buffer (5 mM Tris hydrochloride [pH 8.0] 0.2 mM EDTA, 0.05% PP_i, 0.1× Denhardt reagent) for 2 h at 65°C.

cAMP assay. For cAMP determination, cells were harvested as for RNA analysis, resuspended in 4 mM EDTA (pH 8.0) and lysed by placing the tubes in boiling water for 3 min. After centrifugation at 4°C for 15 min, supernatants were assayed for cAMP levels by a radioimmune assay in accordance with the vendor's instructions (Amersham). Samples were normalized by assaying for total protein by the method of Bradford (2) with a protein assay kit (Bio-Rad).

Protein analysis. Phosphorylation of the 80-kDa PKC substrate was characterized as described previously by Spangler et al. (31).

RESULTS

Induction of JE gene expression by CT is independent of cAMP production. CT has been shown to induce a variety of responses by ADP-ribosylating G proteins (10). ADP-ribosylation of the adenylate cyclase-stimulatory G protein, G_s, leads to elevated levels of cAMP. cAMP activates PKA by promoting dissociation of catalytic and regulatory subunits of PKA (8). We found that CT induced expression of the immediate-early response gene JE (5) in quiescent murine fibroblasts. However, forskolin, which activates adenylate cyclase, and 8-Br-cAMP, a slowly hydrolyzed analog of cAMP, did not induce JE gene expression (Fig. 1). In contrast, the immediate-early response gene TIS10 (17) was induced by CT, forskolin, and 8-Br-cAMP.

The above data suggest that CT-induced TIS10 gene expression involves a cAMP-dependent signaling pathway, whereas CT-induced JE gene expression involves a cAMP-independent pathway. We therefore compared the dose-response curves for induction of cAMP production and gene expression by both CT and forskolin. The levels of CT and forskolin required to induce cAMP production correlated with the levels of CT and forskolin required to induce TIS10 gene expression; maximal induction of TIS10 by both CT and forskolin was observed when similar levels of cAMP were reached (Fig. 2a and b). Therefore, it is likely that induction of TIS10 by CT is the result of cAMP production. The induction of TIS10 gene expression by 8-Br-cAMP (Fig. 1) supports this hypothesis. The induction of JE gene expression by CT did not correlate with cAMP production (Fig. 2c). Forskolin, which did not induce JE gene expression (Fig. 1), generated the same levels of cAMP induced by 2 μg of CT per ml (Fig. 2a and c). This level of CT strongly induced JE gene expression. In addition, JE gene expression was not induced by 8-Br-cAMP (Fig. 1). Thus, the induction of JE by CT is apparently independent of the effect of CT on adenylate cyclase.

The induction of JE by CT could be the result of a contaminant in commercially obtained preparations of CT or the result of nonspecific effects of CT on BALB/c 3T3 cells. We therefore looked at the abilities of purified A and B subunits of CT and CT reconstituted from purified A and B subunits to induce JE gene expression. In Fig. 2d, it is shown that CT reconstituted from purified A and B subunits also induces JE. Purified A or B subunit alone did not induce JE gene expression at concentrations that produced induction when CT was reconstituted. The catalytic A subunit did, however, weakly induce JE at higher concentrations. In addition, we have found that CT obtained from several different lots and different sources all induce JE gene expression with the same dose responses (not shown). These data demonstrate that CT-induced JE gene expression is not the result of a contaminant in commercially obtained CT preparations. The data also suggest that CT-induced JE gene expression is due to the catalytic action of CT, since the B subunit, which facilitates passage of the catalytic A subunit into the cell, had no effect upon JE gene expression. Thus, the induction of JE by CT is not a nonspecific effect of CT on BALB/c 3T3 cells and is likely the result of ADP-ribosylation of an intracellular substrate.

Differential effect of the phosphodiesterase inhibitor IBMX on CT-induced TIS10 and JE gene expression. If CT-induced gene expression is a response to elevated cAMP levels, then inhibition of the phosphodiesterase activity which converts cAMP to AMP might enhance CT-induced gene expression by preventing the turnover of cAMP produced in response to CT. Conversely, if CT-induced gene expression is independent of cAMP, the inhibiting phosphodiesterase should have no effect. We examined the effect of the phosphodiesterase inhibitor IBMX on CT-induced JE and TIS10 gene expression (Fig. 3). As predicted, IBMX enhanced CT-induced TIS10, whose expression correlated with cAMP production, and had no effect on CT-induced JE, whose expression did not correlate with cAMP production. These data further suggest that induction of JE gene expression by CT is via a cAMP-independent signal transduction pathway.

Protein kinase inhibitors distinguish the CT-activated signaling pathways for induction of JE and TIS10. Signal transduction pathways frequently involve protein kinases or protein kinase cascades (8, 24). We have previously used protein kinase inhibitors with different K_is for a variety of protein kinases to distinguish signal transduction pathways (31). Of the protein kinase inhibitors we examined, H7 (13) and sangivamycin (18) have been the most useful for distinguishing signaling pathways. We previously reported that induction of TIS10 gene expression by agonists of PKC and PKA is differentially sensitive to H7 and sangivamycin (31).

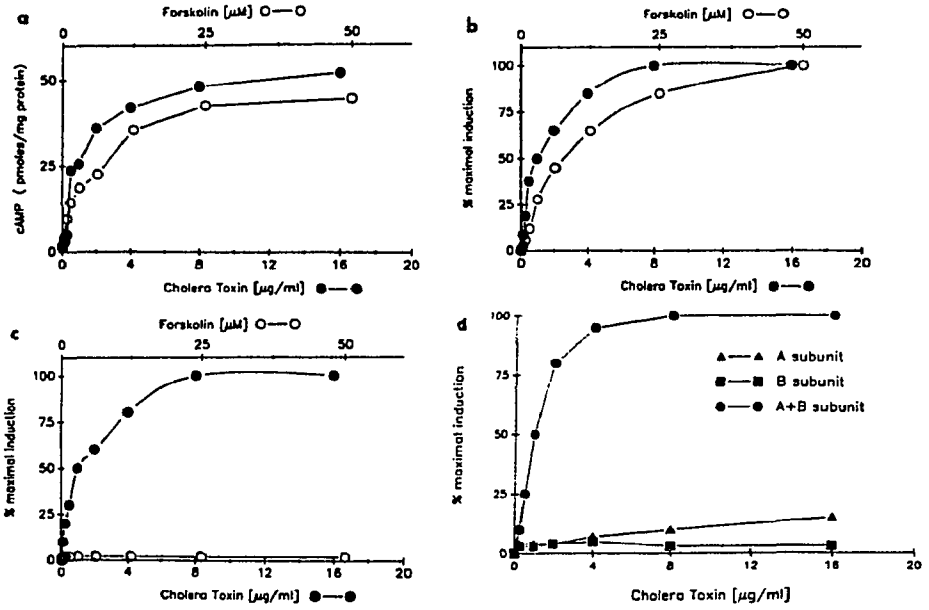

FIG. 2. Induction of TIS10, JE, and cAMP levels by CT. (a) CT and forskolin were added to quiescent BALB/c 3T3 cell cultures in the presence of 0.5 mM IBMX, and cAMP levels were determined 30 min later. Values represent the means of two independent experiments performed in duplicate (independent values did not vary by more than 20%). (b) CT and forskolin were added as described for panel a, and RNA was prepared for Northern gel analysis as described in the legend to Fig. 1. Densitometric analysis of autoradiographs was performed after probing with TIS10 DNA. (c) Filters used for panel b were stripped and reprobed with JE DNA and scanned as in panel b. (d) Purified CT A and B subunits and CT reconstituted from purified A and B subunits were added at the indicated concentrations, and JE gene expression was determined as for panel c.

Induction of TIS10 gene expression by agonists of PKA was relatively insensitive to H7 and sangivamycin, whereas induction of TIS10 by PKC agonists was highly sensitive to these two compounds. While the differential effects of these compounds on PKC- and PKA-mediated events do not necessarily imply direct effects of H7 and sangivamycin on PKC or PKA in vivo (31), they do imply that different signal transduction pathways are being used.

The sensitivity of CT-induced expression of JE and TIS10 to H7 and sangivamycin is shown in Fig. 4. CT-induced JE gene expression is far more sensitive to both H7 and sangivamycin than CT-induced TIS10 gene expression. This observation suggests a dependence on different protein kinases for CT to induce expression of these two genes. The pattern of inhibition observed for induction of TIS10 by CT was identical to that observed for induction of TIS10 by other agonists of PKA (31), further establishing that CT is inducing TIS10 gene expression by activating G_s, which in turn activates adenylate cyclase, leading to elevated levels of cAMP and subsequent activation of PKA. The greater

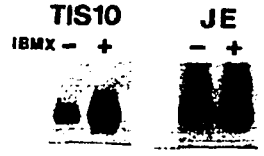

FIG. 3. Effect of phosphodiesterase inhibitor IBMX on CT-induced JE and TIS10 gene expression. CT (2 μg/ml) was added to cell cultures in the absence (–) and presence (+) of 0.5 mM IBMX, and RNA was prepared for Northern gel analysis and probed sequentially with JE and TIS10 as for Fig. 1.

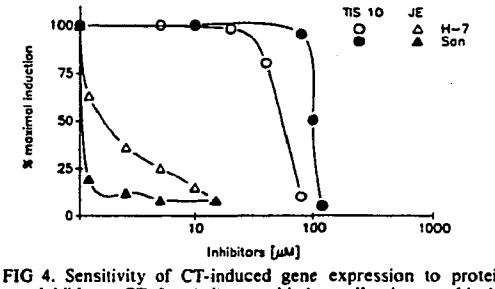

FIG 4. Sensitivity of CT-induced gene expression to protein kinase inhibitors. CT (2 μg/ml) was added to cell cultures with the protein kinase inhibitors H7 and sangivamycin (San) at the indicated concentrations. Inhibitors were added 10 min prior to addition of CT. Northern analysis was performed as for Fig. 1. Densitometric analysis of the autoradiographs is presented.

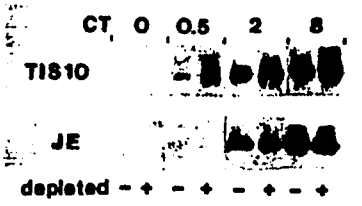

FIG. 5. Effect of PKC depletion on CT-induced expression of JE and TIS10. CT at the indicated concentrations (in micrograms per milliliter) was added to quiescent BALB/c 3T3 cells that were either depleted (+) or not depleted (−) of PKC activity by prolonged exposure to phorbol ester. To deplete cells of PKC activity, 12-*O*-tetradecanoylphorbol-13-acetate (50 ng/ml) was added to quiescent cultures for 26 h prior to the addition of CT. Under these conditions, neither gene expression nor phosphorylation of the 80-kDa PKC substrate (Fig. 6) could be induced with phorbol dibutyrate (not shown).

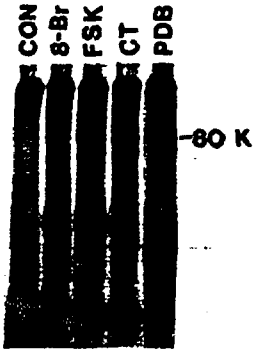

FIG. 6. Effect of CT on phosphorylation of the 80-kDa PKC substrate. CT at a concentration that induced JE gene expression (2 μg/ml) was added to quiescent BALB/c 3T3 cultures, and phosphorylation of the 80-kDa PKC substrate was examined 15 min later as described previously (31). Phorbol dibutyrate (PDB) was added to 50 ng/ml. 8-Br-cAMP (8-Br) and forskolin (FSK) were used at the concentrations indicated in the legend to Fig. 1. CON, Control.

sensitivity to H7 and sangivamycin observed for CT-induced JE expression suggests a more complex pathway that is dependent upon additional protein kinases or protein kinases different from those required for the induction of TIS10.

CT-induced JE gene expression does not require PKC activity. The profile for inhibition of CT-induced JE gene expression by H7 and sangivamycin seen in Fig. 4 is similar (although not identical) to the pattern of inhibition seen for PKC-mediated signal transduction pathways (31). We therefore examined whether depleting cells of PKC by prolonged treatment with phorbol ester (24, 26, 28, 29, 31) affected CT-induced JE gene expression. Prolonged treatment with phorbol ester had no effect on the induction of JE (Fig. 5). In contrast, depletion of PKC activity potentiated CT-induced TIS10 gene expression (Fig. 5), suggesting cross talk between PKC and cAMP-mediated induction of TIS10 gene expression.

Activation of PKC leads to the rapid phosphorylation of an 80-kDa substrate (1, 34). Treatment with the PKC agonist phorbol dibutyrate leads to increased phosphorylation of an 80-kDa protein (Fig. 6). However, CT at levels that stimulated JE gene expression did not increase phosphorylation of this PKC substrate. Thus it is unlikely that CT is using a PKC-mediated signal transduction pathway to induce JE gene expression.

Inhibition of arachidonic acid metabolism distinguishes CT-induced JE gene expression from CT-induced TIS10 gene expression. Phospholipase A2 activity has recently been implicated in G-protein-mediated signal transduction (14–16). Phospholipase A2 generates arachidonic acid from phospholipids (22). Arachidonic acid is converted into a variety of biologically active molecules, including prostaglandins, thromboxanes, and leukotrienes, through cyclooxygenase and lipoxygenase activity. We examined the effects of inhibitors of both cyclooxygenase and lipoxygenase activities on CT-induced JE and TIS10 gene expression. NDGA, which blocks lipoxygenase activity, inhibited CT-induced JE gene expression (Fig. 7). In contrast, NDGA potentiated CT-induced TIS10 gene expression (Fig. 7). The cyclooxygenase inhibitor indomethacin had a less dramatic effect opposite to that of NDGA. Neither indomethacin nor NDGA was able to completely inhibit CT-induced expression of either JE or TIS10 at concentrations of up to 200 μM (not shown). Thus, arachidonic acid metabolism is unlikely to be a vital component in CT-induced expression of either JE or TIS10. However, these data further demonstrate that CT is activating a distinct signal transduction pathway to induce expression of JE.

DISCUSSION

Data presented in this paper demonstrate that CT-induced expression of the immediate-early response gene JE is via a signaling pathway that is distinct from CT-induced signaling pathways mediated by cAMP. CT, forskolin, and cAMP analogs all induce TIS10 gene expression; however, of these compounds, only CT induced JE gene expression. Consistent with these observations, the phosphodiesterase inhibitor IBMX potentiated CT-induced TIS10 gene expression but had no effect upon CT-induced JE gene expression. Depleting cells of PKC activity had no effect on CT-induced JE gene expression, whereas this treatment potentiated CT-induced TIS10 gene expression, demonstrating that CT-induced JE gene expression is independent of PKC. CT-induced JE and TIS10 gene expression is differentially sensitive to protein kinase inhibitors and to inhibitors of arachidonic acid metabolism. Thus, CT-induced expression of JE is via a signal transduction pathway which is indepen-

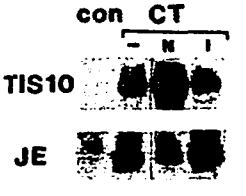

FIG. 7. Sensitivity of CT-induced gene expression to NDGA and indomethacin. Induction of gene expression by CT (2 μg/ml) alone (−) and by CT in the presence of 40 μM NDGA (N) or 20 μM indomethacin (I) is shown. con, Control.

dent of both cAMP production and PKC activity and which is uniquely sensitive to inhibitors of protein kinases and arachidonic acid metabolism.

The differential induction of JE and TIS10 by CT could be explained by the effect of CT on G_s (the best-characterized substrate for CT), since several cAMP-independent responses to CT have been characterized previously (3, 19, 21, 25, 26, 30, 37). It is also possible that induction of JE could be mediated by the $\beta\gamma$ subunit of G_s or another G protein ADP-ribosylated by CT. The $\beta\gamma$ subunits of G proteins have recently been demonstrated to transduce signals independent of those transduced by the α subunit. The $\beta\gamma$ subunit of transducin (a CT substrate) has been reported to induce phospholipase A2 activity (14). It has also been reported that K^+ channels can be opened by $\beta\gamma$ subunits, again acting through phospholipase A2 (15, 16). Thus, induction of JE by CT could result from the action of the $\beta\gamma$ subunit of G_s or another CT-sensitive G protein. We have not been able to achieve complete inhibition of CT-induced JE gene expression by using inhibitors of arachidonic metabolism, suggesting that activation of phospholipase A2 is not required for CT to induce JE gene expression. However, arachidonic acid metabolism may both positively and negatively regulate cAMP-dependent and cAMP-independent signaling pathways induced by CT (Fig. 7).

The most likely explanation for the different signaling pathways induced by CT is that CT is activating more than one G protein in fibroblasts. Lapetina and coworkers have recently reported a second substrate (in addition to G_s) for CT in platelets (20). This substrate is likely a G protein. Such a G protein would be a candidate for induction of JE gene expression. In addition, recent cloning of genes encoding G protein α subunits with polymerase chain reaction technology has revealed several additional G-protein α-subunit genes with consensus ADP-ribosylation sites for CT (32, 35). Any of these G proteins might be a candidate for a G protein involved in the induction of JE. Such a G protein might also be involved in the induction of JE by platelet-derived growth factor in BALB/c 3T3 cells, since this induction has been shown to be independent of PKC (11), as demonstrated here for CT-induced JE expression.

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