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# Characterization of the effect of protein phosphatase 2A upon v-Src-induced cell signalling

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# Characterization of the Effect of Protein Phosphatase 2A Upon v-Src-Induced Cell Signalling

by

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## ABSTRACT

**Characterization of the Effect of Protein Phosphatase 2A Upon v-Src-Induced Cell Signalling**

**by**

**Ruchika W. Gupta**

**Adviser: Professor David A. Foster**

Constitutive activity of the v-Src protein-tyrosine kinase results in cell transformation and initiates multiple intracellular signalling mechanisms. Okadaic acid (OA) is an inhibitor of the serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A). Addition of OA to v-Src-transformed BALB/c 3T3 cells reverted them to a flat morphology, increased fibronectin levels in the extra-cellular matrix, reduced saturation density, and inhibited the formation of colonies in soft agar. The ability of v-Src-transformed cells to proliferate in low serum was also inhibited by okadaic acid. These data indicate that OA can inhibit v-Src-induced cell transformation. v-Src can activate promoters under the control of 12-o-tetradecanoylphorbol-13-acetate (TPA) response elements (TREs) and serum response elements (SREs). The induction of SRE-mediated gene expression by v-Src requires Ras and Raf-1, while the induction of TRE-mediated gene expression by v-Src requires Ras but is independent of Raf-1. The induction of TRE-mediated gene expression by v-Src and v-HaRas increased in the presence of the catalytic subunit of PP2A. The induction

of SRE-mediated gene expression by v-Src and v-HaRas, however, was inhibited by PP2A. PP2A had no effect upon v-Raf-induced SRE-mediated gene expression. These findings implicate serine/threonine phosphatases in v-Src-induced cell signalling. They further suggest that PP2A differentially regulates two intracellular signals activated by v-Src and v-HaRas, and that the effect of PP2A upon v-Src- and v-HaRas-induced SRE-mediated gene expression is upstream from Raf-1 activation. Thus, serine/threonine phosphatases may play an important role in the transformation of cells by v-Src; their activity may either inhibit or potentiate v-Src-induced cell signalling depending upon the effect of dephosphorylation on the activity of the substrate protein.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| **BES** | N, N-bis(2-hydroxyethyl)-2-aminoethane-sulfonic acid |
| **CAT** | chloramphenicol acetyl transferase |
| **DMEM** | Dulbecco's modified Eagle's medium |
| **DMF** | dimethylformamide |
| **DMSO** | dimethylsulfoxide |
| **ERK** | extra-cellular-signal regulated protein kinase |
| **GDP** | guanosine diphosphate |
| **GTP** | guanosine triphosphate |
| **MAP kinase** | mitogen-activated protein kinase |
| **MEK** | MAP/ERK kinase |
| **OA** | okadaic acid |
| **PBS** | phosphate-buffered saline |
| **PKC** | protein kinase C |
| **PP1** | protein phosphatase 1 |
| **PP2A** | protein phosphatase 2A |
| **SRE** | serum response element |
| **TPA** | 12-o-tetradecanoylphorbol-13-acetate |
| **TRE** | TPA-response element |

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## INTRODUCTION

Many retroviral-encoded oncogenes that can transform cells and cause tumors in animals have subsequently been shown to be dominant, mutated forms of normal host genes (for review see Bishop, 1991; Cantley, et al., 1991). These oncogenes encode mutant proteins involved in the transmission of information through intracellular signalling cascades. Cellular homologues of oncoproteins include growth factors, growth factor receptors and other membrane-associated proteins, cytoplasmic proteins, and nuclear transcription factors. Since these proteins have such diverse roles in the cell, oncogenes must transform cells through many different mechanisms.

An example of a transforming protein homologous to a secreted growth factor is the product of the v-sis oncogene. v-sis encodes a mutant form of the B chain of platelet-derived growth factor (Johnsson et al., 1984). The transforming effect of v-Sis, however, may not be due simply to the over-stimulation of its cell-surface receptor (reviewed by Cross and Dexter, 1991). While overexpression of c-Sis can transform cells (Josephs et al., 1984), exogenous addition of even large quantities of platelet-derived growth factor does not have a transforming effect (Bejcek et al., 1989). A mutant v-Sis protein targeted to the endoplasmic reticulum and Golgi apparatus, however, retains transforming ability although it is no longer secreted in detectable amounts (Bejcek et al., 1989). Thus, the lack of transforming ability of the exogenously added growth factor may be due to receptor internalization and down-regulation, and the transforming ability of oncogenic forms of growth factor proteins may be due to premature association with receptors present on the internal membranes of the

endoplasmic reticulum and the Golgi apparatus (see Cross and Dexter, 1991).

Cell transformation may also arise due to mutations in genes encoding cell-surface growth factor receptors. Products of the v-erbB and v-fms oncogenes, for example, are mutant forms of receptor protein-tyrosine kinases that phosphorylate proteins on tyrosine residues. v-erbB and v-fms encode mutant forms of the epidermal growth factor receptor and the colony stimulating factor 1 receptor respectively, and may transform cells by delivering a continuous, ligand-independent mitogenic signal (see Hunter, 1991).

Proteins associated with the cytoplasmic face of the cell membrane may also become oncogenic upon mutation. These membrane-associated proteins include members of the Ras and Src families (see Cantley et al., 1991; Hunter, 1991). Proteins of the Ras family are guanine nucleotide binding proteins that are activated by the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP), and inactivated by the hydrolysis of GTP to GDP (Bourne et al., 1990; Gilman, 1987). The Ras family members are signal transducers of cell surface growth factor receptors, and oncogenic forms of the Ras family have sustained mutations that keep them in the active, GTP-bound state (Bourne et al., 1990; Gilman, 1987). Members of the Src family are protein-tyrosine kinases which phosphorylate proteins on tyrosine residues (see Cantley et al., 1991; Hunter, 1991). $pp56^{lck}$ is a member of the Src family of protein tyrosine-kinases, and binds tightly to the cytoplasmic tails of the CD4 and CD8 T cell receptors (Rudd et al., 1988; Veillette et al., 1988). $pp56^{lck}$ serves as the catalytic subunit for the CD4 and CD8 T cell receptor complexes, and its activity is stimulated upon binding of CD4 and CD8 to

their receptors (see Hunter, 1991). The activity of the remaining Src family of protein-tyrosine kinases may be regulated in a manner similar to that of $pp56^{lck}$.

Membrane-associated proteins activate signalling molecules present in the cytoplasm of cells. Cytoplasmic oncoproteins include serine/threonine kinases encoded by the v-mos and v-raf oncogenes, as well as the product of v-crk, a protein with Src-homology domains involved in protein-protein interaction, but no kinase domain (Mayer et al., 1989). In adults, c-Mos expression is restricted to germ cells, and transformation by v-Mos may be due to inappropriate expression of this protein in tissues which normally do not express c-Mos (see Hunter, 1991). The product of the v-raf oncogene has lost the amino-terminal regulatory sequences present in c-Raf, and transforms cells due to its constitutive kinase activity (Rapp et al., 1983). The Crk oncogene product can bind to protein-tyrosine kinases and other proteins through their phosphotyrosine residues (Mayer and Hanafusa, 1990; Matsuda et al., 1990). Association of these proteins with v-Crk may lead to inappropriate activation of their enzymatic activity (see Cantley et al., 1991). Thus, these cytoplasmic oncoproteins transform cells through diverse mechanisms.

When resting cells are stimulated with mitogens, membrane receptors activate cytoplasmic signalling molecules. Activation of these molecules leads to stimulation of nuclear transcription factors which initiate a cascade of events leading to cell division. These transcription factors include products of the c-myc, c-jun, and c-fos proto-oncogenes among others (see Hunter, 1991). Thus, mutations leading to constitutive activity of these transcription factors may cause continuous cell proliferation. Cell transformation may therefore result from the inappropriate expression or

activity of proteins at many points in the signalling cascade, from the cell surface to the nucleus.

Recently, work from several laboratories has converged to elucidate the mechanism of activation of one signalling pathway, known as the "Ras pathway", from the plasma membrane to the nucleus of the cell. Grb2, like Crk, is an adapter protein that consists of one SH2 domain flanked by two SH3 domains (Clark et al., 1992; Lowenstein et al., 1992; Rozakis-Adcock et al., 1992). SH2 and SH3 domains are Src homology regions present in many proteins that can bind to phosphotyrosine and proline-rich regions, respectively, in other proteins (see Pawson and Gish, 1992). In unstimulated cells, Grb2 is complexed with mSos1, the mammalian homologue of the Drosophila Son of Sevenless gene product, via its two SH3 domains (see Marx, 1993). Binding of epidermal growth factor to its receptor activates the tyrosine kinase activity of the receptor and causes it to autophosphorylate (see Hunter, 1991). The newly created phosphotyrosine sites on the receptor serve as binding sites for SH2-containing proteins (Hunter, 1991; Pawson and Gish, 1992; Marx, 1993). The Grb2-mSos1 complex binds to phosphotyrosines of the activated epidermal growth factor receptor via the SH2 domain of Grb2 (Egan et al., 1993; Li et al., 1993; Gale et al., 1993; Rozakis-Adcock et al., 1993; Simon et al., 1993; Olivier et al., 1993; Buday and Downward, 1993). This brings mSos1 to the plasma membrane, where Ras is located. The mSos1 protein, which is still attached to Grb2, then binds to Ras and stimulates its activity by causing it to exchange GDP for GTP (Simon et al., 1991; Bonfini et al., 1992). The activated Ras protein complexes with Raf-1 and stimulates its kinase activity (Moodie et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). Raf-1 can then activate the

mitogen-activated protein (MAP) kinase pathway, also known as the extra-cellular-signal-regulated protein kinase (ERK) pathway (Williams et al., 1993; Moodie et al., 1993; Macdonald et al., 1993). Raf-1 kinase activity results in a kinase cascade with Raf-1 phosphorylating a dual-specificity kinase termed MAP/ERK kinase (MEK) (Crews et al., 1991; Dent et al., 1992; Howe et al., 1992; Kyriakis et al., 1992; Tsuda et al., 1993). This activates MEK, causing it to phosphorylate MAP kinase on threonine and tyrosine residues (Crews et al., 1991). MAP kinase has been shown to phosphorylate c-Myc, a transcription factor found in the nucleus, and enhance its ability to stimulate gene expression (see Marx, 1993). MAP kinase may also stimulate the activity of other transcription factors (Marx, 1993). Thus, many of the major steps required in the "Ras pathway" to transmit signals from the cell surface to the nucleus have been elucidated.

The "Ras pathway" requires the activity of many protein kinases, and a large number of the oncogenes isolated so far encode kinases which phosphorylate other proteins on serine, threonine, and tyrosine residues. Phosphorylation of proteins on serine, threonine and tyrosine residues is one of the most common forms of post-translational modification in eukaryotic cells and is linked to the regulation of many cellular functions, including cell proliferation (for review see Hunter and Karin, 1992). The protein-tyrosine kinases fall into two categories: the transmembrane receptor family and the cytosolic non-receptor family. Rous sarcoma virus encodes a protein, $pp60^{v\text{-}src}$, which is a member of the latter family, and transforms cells in culture and induces tumors in animals (Jove and Hanafusa, 1987). The molecular events leading from the protein-tyrosine kinase activity of v-Src to cellular transformation are poorly understood; however it has been pointed out that transformation by v-Src leads to an

increase in the phosphorylation state of several proteins on serine and threonine residues as well as on tyrosine (Cooper and Hunter, 1981; Decker, 1981; Sefton et al., 1980). Due to the specificity of v-Src for tyrosine residues, the increase in serine/threonine phosphorylation must arise from either the activation of serine/threonine-specific protein kinases or the inhibition of phosphatases specific for serine and threonine residues. In agreement with this, the serine/threonine kinases Raf-1 and protein kinase C (PKC) have both been implicated in signals generated by v-Src (Qureshi et al., 1991a; 1992; Spangler et al., 1989). Furthermore, it has been demonstrated that v-Src can directly phosphorylate serine/threonine phosphatases 1 and 2A (PP1 and PP2A) on tyrosine and decrease their phosphatase activity *in vitro* (Chen et al., 1992; Johansen and Ingebritsen, 1986). v-Src may not, however, be able to inactivate all of the PP1 and PP2A present in cells *in vivo* since v-Src is localized near the plasma membrane while PP1 and PP2A are found in both the cytoplasm and the nucleus of the cell (for review see Cohen, 1989). Thus, the regulation of phosphatase activity in v-Src-transformed cells may be more complex than is implied by the *in vitro* data.

Unlike most other enzymes, the serine/threonine phosphatases show a broad and overlapping substrate specificity *in vitro*. They have therefore been classified based upon their sensitivity to inhibitors and requirement for divalent cations (for review see Cohen, 1989; Cohen and Cohen, 1989). The catalytic subunits of serine/threonine phosphatases have been classified into four major groups. Type 1 phosphatases can dephosphorylate the ß-subunit of phosphorylase kinase and are inhibited by the heat- and acid-stable proteins termed inhibitor 1 and inhibitor 2. The type 2 phosphatases are divided into three groups: PP2A, PP2B, and

PP2C. PP2A, like PP1, does not require divalent cations for activity, while PP2B and PP2C are dependent upon $Ca^{++}$/calmodulin and $Mg^{++}$, respectively (Cohen, 1989; Cohen and Cohen, 1989).

PP2A may play a significant role in the transformation of cells by v-Src. The regulatory and catalytic subunits of PP2A have been identified as p63 and p36 respectively, two cellular proteins known to associate with the polyoma middle and small T antigens, and the SV40 small T antigen (Pallas et al., 1990). The middle T antigen of polyoma virus interacts with c-Src (Courtneidge and Smith, 1983) and enhances its tyrosine kinase activity (Bolen et al., 1988; Courtneidge, 1985). In cells transformed with polyoma middle T, antisera against c-Src was found to immunoprecipitate p36, the catalytic subunit of PP2A (Pallas et al., 1990), suggesting that PP2A may play a role in the intracellular signals generated by c-Src.

It has previously been shown that OA, a potent inhibitor of PP1 and PP2A (Bialojan and Takai, 1988), can act as a tumor promoter (Suganuma et al., 1988). However, it has also been reported that OA reverts the transformation of cells by an activated Raf-1 (Sakai et al., 1989). v-Src activates both Raf-1-dependent and Raf-1-independent signalling pathways (Qureshi et al., 1991b; 1992; Williams et al., 1992; Kolch et al., 1991), and v-Src-induced transformation is inhibited by a dominant-negative Raf-1 mutant (Qureshi et al, 1993). Since OA has been reported to revert transformation induced by v-Raf (Sakai et al., 1989), we examined the effect of this phosphatase inhibitor upon v-Src-transformed BALB/c 3T3 fibroblasts. Furthermore, because it has been suggested that PP2A may play a role in c-Src-induced signalling (Pallas et al., 1990), we studied the effect of the overexpression of the catalytic subunit of PP2A upon Src-induced gene expression in transient transfection assays. We present data

showing that treatment with OA inhibits several transformation-related phenotypes induced by v-Src. We also demonstrate that the catalytic subunit of PP2A differentially regulates two signalling pathways activated by v-Src.

In summary, this thesis presents evidence demonstrating the following observations:

1. An OA-sensitive phosphatase may play a role in the transformation of cells by v-Src.
2. PP2A can potentiate the induction of TRE-mediated gene expression by v-Src and v-HaRas.
3. PP2A inhibits the induction of SRE-mediated gene expression by v-Src and v-HaRas, but has no effect upon v-Raf-induced SRE-mediated gene induction.

These data suggest a complex regulation of v-Src-initiated signalling cascades by serine/threonine phosphatases. Phosphatases may inhibit or potentiate signalling by v-Src depending upon the effect of dephosphorylation on the activity of the substrate protein.

## MATERIALS AND METHODS

### Cells and Culture Conditions:

SRD and LA90 transformed BALB/c 3T3 (kindly provided by J. Brugge) and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. LA90 cells are non-transformed at 39.5°C and transformed at 35°C (Gray and Macara, 1988; Qureshi et al., 1991a). Cell morphology was examined by phase contrast microscopy. The rate of cell proliferation was determined by seeding $3x10^5$ SRD cells per 35 mm dish in DMEM containing 20% or 1% calf serum and the appropriate drug. The next day (day 1), the medium was changed, and fresh drugs were added on days 1 and 3. Cell number was determined by detaching cells with trypsin and counting with an hemocytometer.

### Materials:

OA was obtained from Calbiochem and diluted in dimethylformamide (DMF) or dimethylsufoxide (DMSO). Fluoromount-G and fluorscein-labelled goat-anti-mouse IgM were from Southern Biotechnology. *N,N*-bis(2-hydroxyethyl)-2-aminoehtane-sulfonic acid (BES) was from Calbiochem. All other reagents were obtained from Sigma.

### Colony Formation in Soft Agar:

$1x10^3$ SRD cells were suspended in DMEM containing 0.35% Bacto agar and seeded onto basal agar containing 0.6% Bacto agar in DMEM. The basal and top agar both contained the indicated concentration of drug. Cells were incubated at 37°C and fed with 1 mL of fresh DMEM containing 10% calf serum and drugs at the appropriate concentration every third day. After 10 days, colonies were stained overnight with 1 mL of 40% p-

iodonitrotetrazolium violet, which stains viable cells, and visible colonies were counted the next day.

**Immunofluorescence Microscopy:**

LA90 cells were grown in Lab-Tek (Nunc) culture chambers as described previously (Sakai et al., 1989) with the following modifications. The cells were fixed in 3.7% paraformaldehyde for one hour and blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 15 minutes at room temperature to block non-specific binding sites. They were then incubated for 30 minutes at room temperature with monoclonal anti-cellular fibronectin (Sigma, diluted 1:200 in PBS), washed three times in PBS and incubated with fluorescein-labelled goat-anti-mouse IgM (Southern Biotechnology, diluted 1:100 in PBS) for 30 minutes at room temperature. After washing in cold PBS, the slides were mounted in fluoromount-G and immunofluorescence was examined under an inverted fluorescence light microscope.

**Protein Analysis:**

Phosphorylation and immunoprecipitation of the v-Src protein were performed as described previously by Spangler et al. Briefly, cells were starved of phosphate by incubating in phosphate-free DMEM supplemented with 0.1% dialyzed calf serum for 16 hours, and then labeled for 3 hours with 100 μCi/mL [$^{32}$P]orthophosphate (9000 Ci/mmol; 1 Ci=37 GBq) in phosphate-free DMEM. The cells were then lysed in Nonidet P-40 lysis buffer (0.2% Nonidet P-40/50 mM NaCl/10 mM Tris·HCl, pH 8.0/50 mM $NaH_2PO_4$/10 mM sodium pyrophosphate/ 50 mM NaF/1 mM $Na_3VO_4$/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride/ 1% aprotinin) and clarified by centrifugation in a microcentrifuge for 15 minutes. Supernatants were normalized for total protein by the Bradford assay. v-Src was

immunoprecipitated with monoclonal antibody 327, a generous gift of J. Brugge. Immunoprecipitates were separated on an 8% polyacrylamide/ 0.2% bisacrylamide gel. The gel was air dried overnight between two sheets of cellophane, and exposed to film (X-OMAT, Kodak).

**Plasmid Constructions:**

pPP2A was made by ligating Bam HI linkers to the blunt-ended, full length Eco RI fragment of the HL-14 PP2A catalytic subunit cDNA (a generous gift of Gary L. Johnson; Arino et al., 1988) and inserting the fragment into the single Bam HI cloning site of pZIPneoSV(X). The Eco RI fragment of HL-14 was blunt-ended with the Klenow fragment of DNA polymerase. pv-HaRas expresses the v-Ha-ras gene in pZIPneoSV(X) (Feig and Cooper, 1988). pMv-Src expresses v-src sequences under the control of the Moloney murine leukemia virus long terminal repeat in pEVX (Johnson et al., 1985). p3611 is a molecular clone of murine sarcoma virus 3611 and expresses a v-raf, a gag-raf fusion gene (Rapp et al., 1983). pHT25 expresses the v-mos gene of Moloney sarcoma virus (Blair et al., 1980). The construction of pE425 has been described previously (Gius et al, 1990), and it contains the region from -550 to the transcriptional start site of the Egr-1 promoter fused upstream of the chloramphenicol acetyl transferase (CAT) gene. p3xTRE contains three copies of the TPA-response element (TRE) of the collagenase gene promoter fused upstream of the CAT gene (Angel et al., 1987).

**Cell Transfection:**

NIH 3T3 cells were plated at $5x10^5$ cells/100-mm dish 16 hours prior to transfection. Transfections were performed using $CaPO_4$ as a co-precipitate as described previously (Chen and Okayama, 1987; Qureshi et al., 1991c). Transfection mixtures contained 5 μg of each experimental

DNA with salmon sperm DNA used to bring the total DNA concentration to 30 μg. Briefly, 500 μL of 2X BES-buffered saline was added to 500 μL of DNA solution, mixed gently, and allowed form a precipitate by incubating at room temperature for 30-45 minutes. The precipitate was then added dropwise while mixing to exponentially growing cells.

**CAT Assay:**

Transfected cultures were washed 16-20 hours post-transfection with isotonic buffer, and fresh growth medium containing 1% serum was added. After another 30 hours, cells were harvested after washing twice with cold isotonic buffer, resuspended in 125 μL of 250 mM Tris-HCl (pH 8.0), and lysed by five cycles of freeze-thaw (dry ice/methanol, 37°C) for 5 minutes each. Supernatants were collected after microcentrifuging for 5-10 minutes at 4°C. Supernatants were normalized for total protein by the Bradford assay. CAT activity in cell extracts was determined with the "flour diffusion assay" (Neuman et al., 1987).

## RESULTS

### OA Reverts v-Src-Transformed Cells to a Flat, Non-Transformed Morphology.

v-Src is the oncogenic form of c-Src and transforms cells in culture and induces tumors in animals (Jove and Hanafusa, 1987). To determine whether protein phosphatases might have any role in the transformation of cells by v-Src, we examined the effect of OA, an inhibitor of PP1 and PP2A (Bialojan and Takai, 1988; Suganuma et al., 1988), upon transformation-related phenotypes in BALB/c 3T3 fibroblasts expressing v-Src. Transformation of cells by oncogenes results in alterations in cellular morphology (Ossowski et al., 1973; Temin, 1960). We therefore observed the effect of OA upon the morphology of v-Src-transformed BALB/c 3T3 fibroblasts. BALB/c 3T3 fibroblasts transformed with the SRD strain of Rous sarcoma virus were incubated for 20 hours alone, with 20nM OA, or with 0.1% DMF, the solvent for OA. Untreated cells and cells treated with DMF showed a high degree of piling-up and loss of contact-inhibition, as well as a refractile, rounded morphology characteristic of transformed cells (Fig. 1A and 1B). Incubation with OA, however, resulted in the appearance of a flat, non-transformed morphology similar to that of the parental BALB/c 3T3 cell line (Fig. 1C and 1D). Removal of OA from the SRD cells resulted in the re-appearance of the transformed morphology within one day (data not shown). These data suggest that OA inhibits the morphological changes that accompany v-Src-induced transformation.

### OA Increases Fibronectin Levels in v-Src-Transformed Cells.

Fibronectin is an extracellular matrix protein that plays a central role in cell adhesion (see Ruoslahti, 1988 for review). Both transformed cells and normal cells can secrete fibronectin into the surrounding growth medium,

but the level of fibronectin in the extracellular matrix of transformed cells is often low with respect to that in normal cells (Hayman et al., 1981; Ruoslahti, 1988). Fibronectin secreted by transformed cells can be deposited into the extracellular matrix if added to a culture of normal cells (Hayman et al., 1981; Ruoslahti, 1988). This suggests that the decreased level of fibronectin in the extracellular matrix of transformed cells is due to a failure by transformed cells to efficiently bind fibronectin (Hayman et al., 1981; Ruoslahti, 1988). Since OA reverted the transformed morphology of v-Src-transformed cells, we examined the effect of OA upon the level of fibronectin in the extracellular matrix of these cells. To avoid artifacts arising from variability between different cell lines in the secretion of the extracellular matrix, we used BALB/c 3T3 cells transformed with LA90, a temperature-sensitive strain of Rous sarcoma virus (described by Gray and Macara, 1988, and Qureshi et al., 1991a). LA90 transformed BALB/c 3T3 cells were grown at either the permissive (35$^{o}$C) or non-permissive (39.5$^{o}$C) temperature for v-Src. LA90 transformed cells cultured at the permissive temperature exhibit less fibronectin in their extracellular matrix than do those grown at the non-permissive temperature (Fig. 2A and 2B). Incubation with OA, however, resulted in an increase in the level of fibronectin in the extracellular matrix of LA90 cells maintained at the permissive temperature (Fig. 2B and 2C). These data further suggest that OA inhibits transformation-related phenotypes induced by v-Src.

**OA Inhibits the Ability of v-Src-Transformed Cells to Reach High Saturation Densities and to Grow Under Low-Serum Conditions.**

Transformed cells often overcome the density-dependent growth arrest that is characteristic of normal cells and grow to high saturation densities (Holley and Kiernan, 1968). The effect of OA upon the saturation density of

v-Src-transformed cells was therefore examined. SRD cells were cultured in growth medium supplemented with 20% serum either alone, or in the presence of either DMF or OA. Untreated and DMF treated SRD cells grew rapidly and reached a high cell density that was maintained for two days (Fig. 3A). In contrast, SRD cells incubated with OA had a slower rate of proliferation and reached a lower saturation density that was similar to that observed for the parental BALB/c 3T3 cells. Furthermore, both the parental BALB/c 3T3 cells and SRD cells incubated with OA declined in number after reaching maximal cell density on the third day. Thus, the presence of OA caused SRD cells to behave like the non-transformed parental BALB/c 3T3 cells with regard to growth rate and saturation density.

Transformed cells also often have a reduced requirement for serum growth factors (Dulbecco, 1970). We therefore examined the effect of OA upon the growth of v-Src-transformed cells under low serum conditions. SRD cells were cultured in growth medium supplemented with 1% serum either alone, with 0.1% DMF or with 20nM OA. Untreated and DMF treated SRD cells grew rapidly and reached a high cell density by the third day (Fig. 3B). In contrast, the parental BALB/c 3T3 cells were completely growth-arrested under these conditions. SRD cells incubated with OA were still able to proliferate under low serum conditions, but with a slower growth rate and lower saturation density than SRD cells or SRD cells incubated with DMF. This decrease in the growth rate and saturation density can not be explained by an inability of the cells to proliferate in the presence of OA since cells could be grown in the presence of OA for at least 10 days (data not shown). Furthermore, removal of OA from the growth medium resulted in the reappearance of the transformed morphology (data not shown).

Thus, OA inhibits the ability of v-Src-transformed cells to overcome density-dependent growth arrest and inhibits their ability to grow under low serum conditions.

**OA Inhibits Anchorage-Independent Growth by v-Src-Transformed Cells.**

Perhaps the most stringent transformation-related phenotype in culture is the ability to grow in suspension. Normal fibroblasts must attach to a solid matrix in order to proliferate. In contrast, transformed fibroblasts can proliferate in suspension without attachment to a solid surface (Macpherson and Montagnier, 1964). We therefore examined the effect of OA upon the ability of v-Src-transformed cells to grow in semi-solid medium. SRD cells were suspended in soft agar alone, with DMF, or with OA. There was no difference between the number of colonies formed by the untreated cells and cells incubated with DMF (Fig. 4). Treatment with OA, however, led to a concentration-dependent decrease in colony formation by SRD cells (Fig. 4). Thus, OA also inhibits anchorage-independent growth induced by v-Src-transformed cells.

**OA Does Not Increase the Level of Phosphorylation of v-Src.**

It is possible that the observations made so far upon the effect of OA in v-Src-transformed cells are due to the inhibition of the activity of an OA-sensitive phosphatase towards the v-Src protein. If this is the case, then treatment with OA should result in an increase in the level of phosphorylation of v-Src. We therefore examined the effect of OA upon the phosphorylation of v-Src in LA90-transformed BALB/c 3T3 cells. Shifting these cells from the non-permissive to the permissive temperature for v-Src-activation led to a 2-fold increase in the total phosphorylation of the v-Src protein (Fig. 5). Treatment with OA did not increase the level of

phosphorylation of v-Src with respect to cells treated with solvent alone at the same temperature (Fig. 5). Thus the effect of OA upon transformation-related phenotypes in v-Src-transformed cells is not due to the inhibition of serine/threonine phosphatase activity towards the v-Src protein.

**PP2A Does Not Stimulate SRE- or TRE-Mediated Gene Expression.**

The inhibition of transformation-related phenotypes by OA in v-Src-transformed cells implicates a serine/threonine phosphatase in v-Src-induced cell transformation (Gupta et al., 1993). PP2A, which is inhibited by OA (Bialojan and Takai, 1988; Suganuma et al., 1988), has also been implicated in Src-induced signalling in polyoma middle T-transformed cells (Pallas et al., 1990). We therefore wished to examine the effect of PP2A upon v-Src-induced cell signalling. To express PP2A in NIH 3T3 cells, we ligated the cDNA for the catalytic subunit of PP2A (Arino et al., 1988) into the single Bam HI cloning site of pZIPneoSV(X) (Cepko et al., 1984) to generate pPP2A (Fig. 6A). pPP2A expresses the catalytic subunit of PP2A under the transcriptional control of the Moloney murine leukemia virus long terminal repeat. We have previously characterized two different signalling mechanisms induced by v-Src which lead to gene expression; v-Src activates SREs of the Egr-1 promoter (Qureshi et al., 1991c) and TREs isolated from the collagenase gene promoter (Qureshi et al., 1992) in transient transfection assays (Fig. 6B). We therefore studied the effect of pPP2A upon SRE- and TRE-mediated gene activation. To determine whether overexpression of the catalytic subunit of PP2A alone might have any effect upon the induction of SRE- and TRE-mediated gene expression, NIH 3T3 cells were transfected with pPP2A and either p3xTRE or pE425. p3xTRE contains three TRE elements of the collagenase gene promoter fused upstream of the chloramphenicol acetyl transferase (CAT) gene

(Angel et al., 1987). pE425 contains Egr-1 promoter sequences from -550 to the transcriptional start site cloned upstream of the CAT gene (Gius et al., 1990; Qureshi et al., 1991c). This region contains four SREs responsible for the transactivation of the Egr-1 promoter by v-Src (Qureshi et al., 1991c). Neither pPP2A nor pZIPneoSV(X), the parental vector for pPP2A, induced gene expression from either p3xTRE or pE425 (Fig. 6, C and D). Transfection with either pv-HaRas, which expresses the v-HaRas protein from pZIPneoSV(X), or pMv-Src, which expresses v-Src from the pEVX expression vector, does stimulate SRE- and TRE-mediated gene expression (Fig. 6, C and D). Thus, overexpression of PP2A alone in transient transfection assays does not induce SRE- or TRE-mediated gene expression in NIH 3T3 cells.

**v-Src-Induced TRE-Mediated Gene Expression is Potentiated by PP2A.**

TRE-mediated gene expression is induced by v-Src (Qureshi et al., 1992). Transfection of NIH 3T3 cells with pMv-Src, which expresses v-Src, increased gene expression from p3xTRE as compared to transfection with pEVX, the parental vector for pMv-Src (Fig. 6C). Although overexpression of PP2A alone did not induce TRE-mediated gene expression (Fig. 6C), we wished to determine whether it might affect v-Src-induced TRE-mediated gene expression. The effect of PP2A upon the induction of p3xTRE by v-Src was therefore examined. In cells transfected with pMv-Src, co-transfection with pPP2A leads to a two-fold increase in transcriptional activation of p3xTRE with respect to cells transfected with pZIPneoSV(X), the parental vector for pPP2A (Fig. 7). This suggests that while the catalytic subunit of PP2A can not stimulate TRE-mediated gene expression on its own, it can potentiate v-Src-induced TRE-mediated gene expression.

**PP2A Potentiates v-HaRas-Induced TRE-Mediated Gene Expression.**

The activation of TRE-mediated gene expression by v-Src has been shown to be mediated by HaRas (Qureshi et al., 1992; Fig. 6B). We therefore examined the effect of PP2A upon the activation of p3xTRE by v-HaRas. Transfection with pv-HaRas, which encodes the v-HaRas protein, leads to increased gene expression from p3xTRE (Fig. 6C). In NIH 3T3 cells stimulated with pv-HaRas, co-transfection with pPP2A results in more than a 2.5-fold increase in gene induction from p3xTRE as compared to cells co-transfected with the parental pZIPneoSV(X) (Fig. 8). Thus, the induction of TRE-mediated gene expression by v-HaRas is also potentiated by the catalytic subunit of PP2A. It has previously been demonstrated that phorbol-dibutyrate-induced TRE-mediated gene expression is inhibited by a dominant-negative mutant of HaRas, suggesting that HaRas functions downstream of protein kinase C in the activation of TRE-mediated gene expression (Alexandropoulos et al., 1993). Since PP2A potentiated HaRas stimulated TRE-mediated gene expression, the effect of PP2A upon TRE-mediated gene expression is likely to be downstream of protein kinase C in NIH 3T3 cells.

**v-Src-Induced SRE-Mediated Gene Expression is Inhibited by PP2A.**

Transactivation of the Egr-1 promoter by v-Src has been shown to be mediated through a different signalling pathway than that used to transactivate TRE-mediated gene expression (Qureshi et al., 1992; Fig. 6B). We therefore examined the effect of PP2A upon the induction of the Egr-1 promoter by v-Src. v-Src induces gene expression from pE425 (Fig. 6D). pE425, contains four SREs that are responsible for the transactivation of the Egr-1 promoter by v-Src (Qureshi et al., 1991c). Co-transfection of pMv-Src with pPP2A leads to almost 60% inhibition of the induction of

pE425, as compared to co-transfection with pZIPneoSV(X), the parental vector for pPP2A (Fig. 9). Thus, the catalytic subunit of PP2A inhibits the induction of SRE-mediated gene expression by v-Src.

**PP2A Inhibits Induction of SRE-Mediated Gene Expression by v-HaRas.**

It has previously been demonstrated that HaRas, a guanine nucleotide binding protein, is a signalling intermediary in the induction of SRE-mediated gene expression by v-Src (Qureshi et al., 1992; Fig. 6B). Transfection with pv-HaRas, which expresses v-HaRas, increases gene expression from pE425 (Fig. 6D). The effect of PP2A upon the induction of SRE-mediated gene expression by v-HaRas was therefore examined. SRE-mediated gene expression from pE425 is induced by pv-HaRas (Fig. 6D). In NIH 3T3 cells stimulated with pv-HaRas, co-transfection with pPP2A results in an approximately 60% decrease in gene expression from pE425 as compared to cells co-transfected with the parental pZIPneoSV(X) (Fig. 10). Thus, PP2A also inhibits the induction of SRE-mediated gene expression by v-HaRas, which functions downstream of v-Src in the activation of SRE-mediated gene induction (Qureshi et al., 1992).

**v-Raf-Induced SRE-Mediated Gene Expression is Unaffected by PP2A.**

A dominant-negative Raf-1 mutant can block the induction of SRE-mediated gene induction by both v-Src and v-HaRas (Qureshi et al., 1991c, 1992; Alexandropoulos et al., 1992), suggesting that the serine/threonine kinase Raf-1 functions downstream of both v-Src and v-HaRas in the SRE-mediated activation of the Egr-1 promoter (Fig. 6B). v-Raf is a mutant form of c-Raf-1 with constitutive kinase activity (Rapp et al., 1983). We therefore examined the effect of PP2A upon the induction of the Egr-1 promoter by v-Raf. Co-transfection of p3611, which encodes v-Raf, with

pPP2A had no effect upon gene expression from pE425 as compared to co-transfection with the parental pZIPneoSV(X) (Fig. 11). Thus, the catalytic subunit of PP2A has no effect upon the induction of SRE-mediated gene expression by v-Raf. Since Raf-1 functions downstream of v-Src and v-HaRas in SRE-mediated gene induction (Qureshi et al., 1992), the inhibitory effect of PP2A upon v-Src- and v-HaRas-stimulated SRE-mediated gene induction must be upstream or independent of Raf-1 activation.

**v-Mos-Induced SRE-Mediated Gene Expression is Increased by PP2A.** It is possible that v-Raf-induced SRE-mediated gene expression is unaffected by PP2A because overexpression of serine/threonine kinases in transient transfection assays might non-specifically counteract the action of a serine/threonine phosphatase towards its substrates. We therefore wished to determine what effect, if any, PP2A might have upon the induction of SRE-mediated gene expression by another serine/threonine kinase. v-Mos, a serine/threonine kinase, has previously been shown to induce the Egr-1 promoter (Alexandropoulos et al., 1992). In cells stimulated by v-Mos, co-transfection with PP2A leads to a 2.5-fold increase in gene expression from pE425 with respect to cells transfected with the parental pZIPneoSV(X) (Fig. 12). Thus, PP2A potentiates SRE-mediated gene expression by v-Mos. This implies that the lack of effect of PP2A upon v-Raf-induced SRE-mediated gene expression is not due to an inability of the phosphatase to function efficiently in cells transfected with activated serine/threonine kinases.

## DISCUSSION

Constitutive activity of the v-Src protein-tyrosine kinase results in cell transformation (Jove and Hanafusa, 1987) and initiates multiple intracellular signalling mechanisms, including the activation of kinases that phosphorylate proteins on serine and threonine residues (for review see Cantley et al., 1991; Foster, 1993). Progress has been made in the identification of intermediates required for signalling by v-Src. These intermediates include guanine nucleotide binding proteins, transcription factors such as Fos and Jun, as well as serine/threonine kinases (for review see Cantley et al., 1991). Two serine/threonine kinases, protein kinase C and Raf-1, have been shown to be required for v-Src-induced intracellular signals (Qureshi et al., 1991a; 1992; Spangler et al., 1989). Since v-Src activates serine/threonine kinases, serine/threonine phosphatases may also play an important role in signalling by tyrosine kinases. In general, however, much less is known about the role of protein phosphatases as compared to protein kinases in signalling by v-Src. The serine/threonine phosphatases, in particular, have a broad and overlapping substrate specificity, making them difficult to study (for review see Cohen, 1989; Karin and Smeal, 1992). Until recently, phosphatases were considered to be passive partners of kinases that simply returned signalling pathways to their original or equilibrium state. It is becoming evident, however, that phosphatases play a more complex role, and the regulation of their activity may be an important mechanism in cell signalling. We have found that OA, an inhibitor of PP1 and PP2A (Suganuma et al., 1988), reverts v-Src-transformed fibroblasts to a flat morphology, increases the level of fibronectin in the extra-cellular matrix, inhibits their ability to overcome density-dependent growth arrest and to grow in low serum, and

blocks colony formation in soft agar (Gupta et al., 1993). Taken together, these data suggest that OA inhibits v-Src-induced cell transformation. We also show that overexpression of PP2A in transient transfection assays can regulate v-Src-induced gene activity in both a positive and negative manner. The induction of TRE-mediated gene activity by v-Src was potentiated by the catalytic subunit of PP2A, while the induction of SRE-mediated gene activity by v-Src was inhibited by PP2A. A cartoon describing the effect of PP2A upon v-Src-induced SRE- and TRE-mediated gene expression is presented in figure 13. Thus, the regulation of phosphatase activity may be significant to v-Src-induced cell signalling.

OA inhibits PP2A much more potently than PP1 *in vitro* (Bialojan and Takai, 1988; Suganuma et al., 1988). The *in vitro* $IC_{50}$ value of OA for PP2A is between 0.2-1 nM, while that for PP1 is much higher, between 20-500 nM (Ishihara et al., 1989; Bialojan and Takai, 1988; Suganuma et al., 1988; Cohen, 1989). Because the optimal concentration of OA for the inhibition of v-Src-induced cell transformation was between 10-20 nM (see Fig. 4), the observed effects of OA upon v-Src-induced transformation-related phenotypes may be due to the inhibition of PP2A; 20nM OA may be sufficient to inhibit most if not all of the PP2A present in the cell, but is unlikely to inhibit all PP1 activity in the cell due to the higher $IC_{50}$ value of OA for PP1 (Ishihara et al., 1989; Bialojan and Takai, 1988; Suganuma et al., 1988; Cohen, 1989). Our data implicated an OA-sensitive phosphatase in v-Src-induced cell transformation (Gupta et al., 1993). It is not known whether the inhibitory effect of OA upon v-Src-induced cell transformation is due to the inhibition by OA of just one or both OA-sensitive phosphatases, although the concentration of OA used in the experiments is consistent with a role for PP2A.

Because we implicated an OA-sensitive phosphatase in v-Src-induced cell transformation, and since it has been suggested that PP2A may play a role in the signals generated by c-Src in polyoma middle-T-transformed cells (Pallas et al., 1990), we examined the effect of PP2A upon Src-induced gene expression. v-Src has been previously shown to increase gene expression by activating promoters under the control of TREs (Qureshi et al., 1992). Transactivation by v-Src of TRE-mediated gene expression was previously found to be dependent upon Ras activation as determined by a dominant-negative mutant of HaRas (Qureshi et al., 1992) which preferentially binds GDP and inhibits Ras activity (Feig and Cooper, 1988). The Ras protein is a member of a family of guanine nucleotide binding proteins with GTPase activity, and is active when bound to GTP and inactive when bound to GDP (Gilman, 1987; Bourne et al., 1990). We have shown that transactivation of TRE-mediated gene expression by v-Src or v-HaRas is potentiated by the catalytic subunit of PP2A. This is consistent with the inhibitory effect of OA upon the transformation of cells by v-Src, which suggests that a phosphatase is required for v-Src-induced cell transformation.

The transcription factor AP-1, which binds to TRE (Angel et al., 1987; Lee et al., 1987), consists either of Jun homodimers or of Fos/Jun heterodimers (Bohmann et al., 1987; Rauscher et al., 1988; reviewed in Lewin, 1991). It has previously been demonstrated that c-Jun is phosphorylated on five major sites (see review by Hunter and Karin, 1992). Two of these sites are serine residues that are phosphorylated in response to mitogen treatment and are located in the amino-terminal regulatory region of the protein. The remaining three sites consist of one threonine residue and two serine residues that lie in the carboxy-terminal DNA-

binding domain of c-Jun. Dephosphorylation of the sites in the DNA binding domain with PP2A is correlated with increased DNA binding activity of AP-1 (Papavassiliou et al., 1992; reviewed in Hunter and Karin, 1992). Treatment of cells with the phorbol ester TPA, which activates protein kinase C, decreases phosphorylation of these sites which regulate DNA binding (Boyle et al., 1988). The expression of several oncogenes, including v-src, v-Ha-ras, and v-sis also leads to dephosphorylation of these inhibitory phosphorylation sites (Hunter and Karin, 1992). Protein kinase C and these oncogenes must therefore activate a serine/threonine phosphatase to stimulate AP-1 DNA-binding activity. Serum induced c-Jun expression, which has previously been shown to be autoregulated by AP-1 itself (Angel, 1988), is potentiated by PP2A but not by PP1 (Alberts et al., 1993). Thus the potentiation by PP2A of TRE-mediated gene expression induced by v-Src and v-HaRas from the collagenase gene promoter may be due to the dephosphorylation of the negative-regulatory phosphorylation sites in the DNA-binding domain of c-Jun.

v-Src can also activate promoters under the control of SREs (Qureshi et al., 1991c). Transactivation by v-Src of SRE-mediated gene expression was shown to require Ras activation (Qureshi et al., 1992). We have demonstrated that the induction of SRE-mediated gene expression by both v-Src and v-HaRas is inhibited by the catalytic subunit of PP2A. Since TRE-mediated gene expression by v-Src and v-HaRas was potentiated by PP2A, PP2A differentially regulates two intracellular pathways activated by these oncogenes.

Both Raf-1-dependent and Raf-1-independent pathways have been identified in signals initiated by Src and Ras (Kolch et al., 1991; Qureshi et al., 1991b; 1992; Williams et al., 1992; Dickinson et al., 1992). Induction of

TRE-mediated gene expression by v-Src and v-HaRas is independent of Raf-1 activation, while SRE-mediated gene expression by v-Src and v-HaRas is mediated by Raf-1 (Qureshi et al., 1992). We have shown that PP2A has no effect upon v-Raf-induced SRE-mediated gene expression. This lack of effect of PP2A towards v-Raf-induced SRE-mediated gene induction is not a general, non-specific effect of the overexpression of a serine/threonine kinase since PP2A potentiated SRE-mediated gene expression by v-Mos, another serine/threonine kinase. Thus, the inhibitory effect of PP2A upon SRE-mediated gene expression by v-Src and v-HaRas must be upstream from Raf-1-activation.

Raf-1 contains a conserved kinase domain in the carboxy-terminal half of the protein, and two highly conserved regulatory regions termed CR1 and CR2 in the amino-terminal half of the protein (Heidecker et al., 1992). It has recently been shown that Ras interacts directly with the amino-terminal CR1 domain of Raf-1 and activates the kinase activity of the carboxy-terminal catalytic domain in a GTP-dependent manner (Moodie et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). In most cells, activation of Raf-1 is accompanied by multi-site serine/threonine phosphorylation of the Raf-1 polypeptide in a protein kinase C-independent manner (reviewed in Heidecker et al., 1992). Treatment of the activated Raf-1 polypeptide *in vitro* with the serine/threonine phosphatase PP1 inactivates the kinase activity of Raf-1 (Kovacina, et al., 1990). Since Ras does not have kinase activity, the stimulation of Raf-1 kinase activity by Ras must be accompanied by phosphorylation of Raf-1 by a serine/threonine kinase. It is therefore possible that activation of Raf-1 by v-Src or v-HaRas in SRE-mediated gene expression requires not only the physical association and activation

of Raf-1 by Ras, but also phosphorylation of Raf-1 by a serine/threonine kinase. The inhibitory effect of PP2A upon SRE-mediated gene expression by v-Src and v-HaRas might be due to the dephosphorylation of Raf-1 at sites which must be phosphorylated for Raf-1-kinase activity. v-Raf, which is missing most of the regulatory amino-terminal domain, has constitutive kinase activity (Rapp et al., 1983), and may no longer have a requirement for serine/threonine phosphorylation to stimulate its kinase activity. This is consistent with the lack of effect of PP2A upon v-Raf-induced SRE-mediated gene expression.

The role of phosphatases in signal transduction by tyrosine kinases is poorly understood (see Cohen, 1992). In particular, little is known about the role serine/threonine phosphatases in v-Src-initiated signalling cascades. We have implicated a serine/threonine phosphatase(s) in v-Src-induced cell transformation (Gupta et al., 1993). We have also shown that the serine/threonine phosphatase PP2A can differentially regulate signalling mechanisms activated by v-Src. The control of PP2A activity is therefore likely to be an important mechanism in v-Src-initiated cell signalling. PP2A is a heterotrimer that exists in multiple forms (Cohen, 1989). The catalytic subunit of PP2A may exist freely, or in a core enzyme complex when bound to the regulatory A subunit (Cohen, 1989). The core enzyme can bind to one of several regulatory B subunits (Kamibayashi et al., 1991; Cohen, 1989). Interaction of the catalytic subunit with the regulatory A subunit suppresses activity towards some proteins without affecting activity towards other proteins (Usui et al., 1988; Cohen, 1989). Addition of the regulatory B subunit to the core enzyme can further inhibit the activity of PP2A towards other substrates (Usui et al., 1988; Imaoka et al., 1983; Cohen, 1989). v-Src may regulate phosphatase activity by

directly inactivating the catalytic subunit (Chen et al., 1992) or by influencing the binding of the catalytic subunit to different regulatory subunits. Elucidation of the mechanisms through which v-Src regulates PP2A activity should result in a clearer understanding of the role of this phosphatase in v-Src-induced cell transformation.

# FIGURES

## Figure Legends

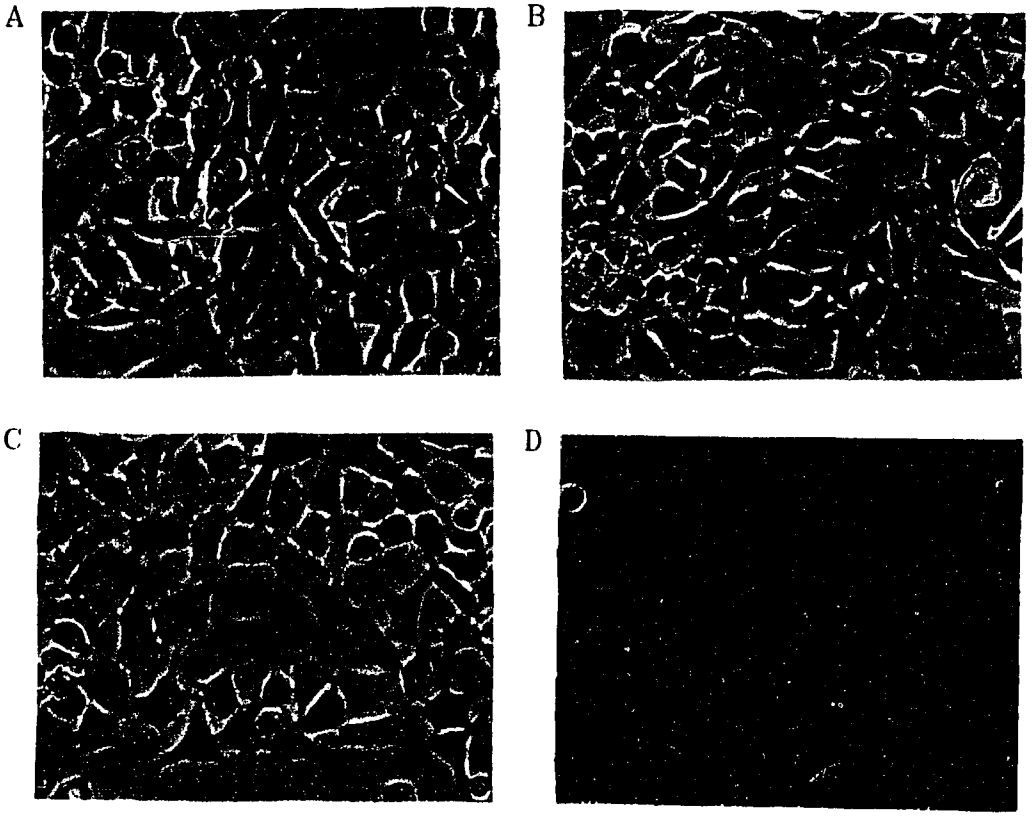



**Fig. 1. OA reverts v-Src-transformed cells to a flat, non-transformed morphology.**

SRD (A-C) and BALB(D) cells were cultured alone (A and D), with 0.1% DMF (B) or with 20 nM OA in 0.1% DMF (C). After 20 hours, the cells were photographed under a phase contrast microscope (200x magnification).

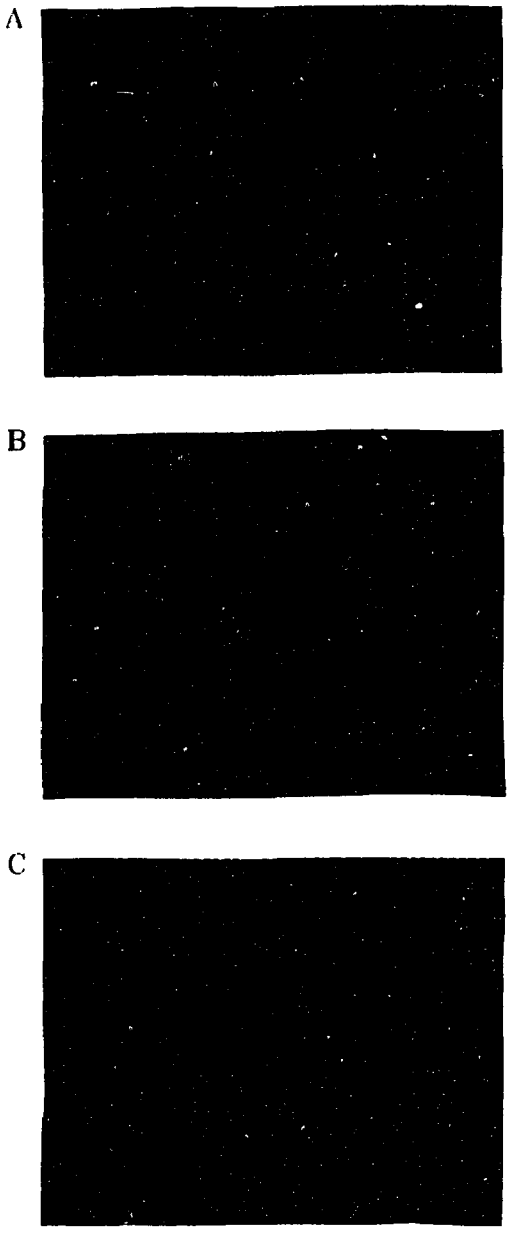



**Fig. 2. OA increases the level of fibronectin in v-Src-transformed cells.**

LA90 cells were incubated for 2 days at 39.5°C (A) or 35°C (B and C) in the absence (A and B) or presence (C) of 20 nM OA. Fibronectin was detected as described under "Materials and Methods" and cells were examined at 400x magnification with a fluorescence microscope.

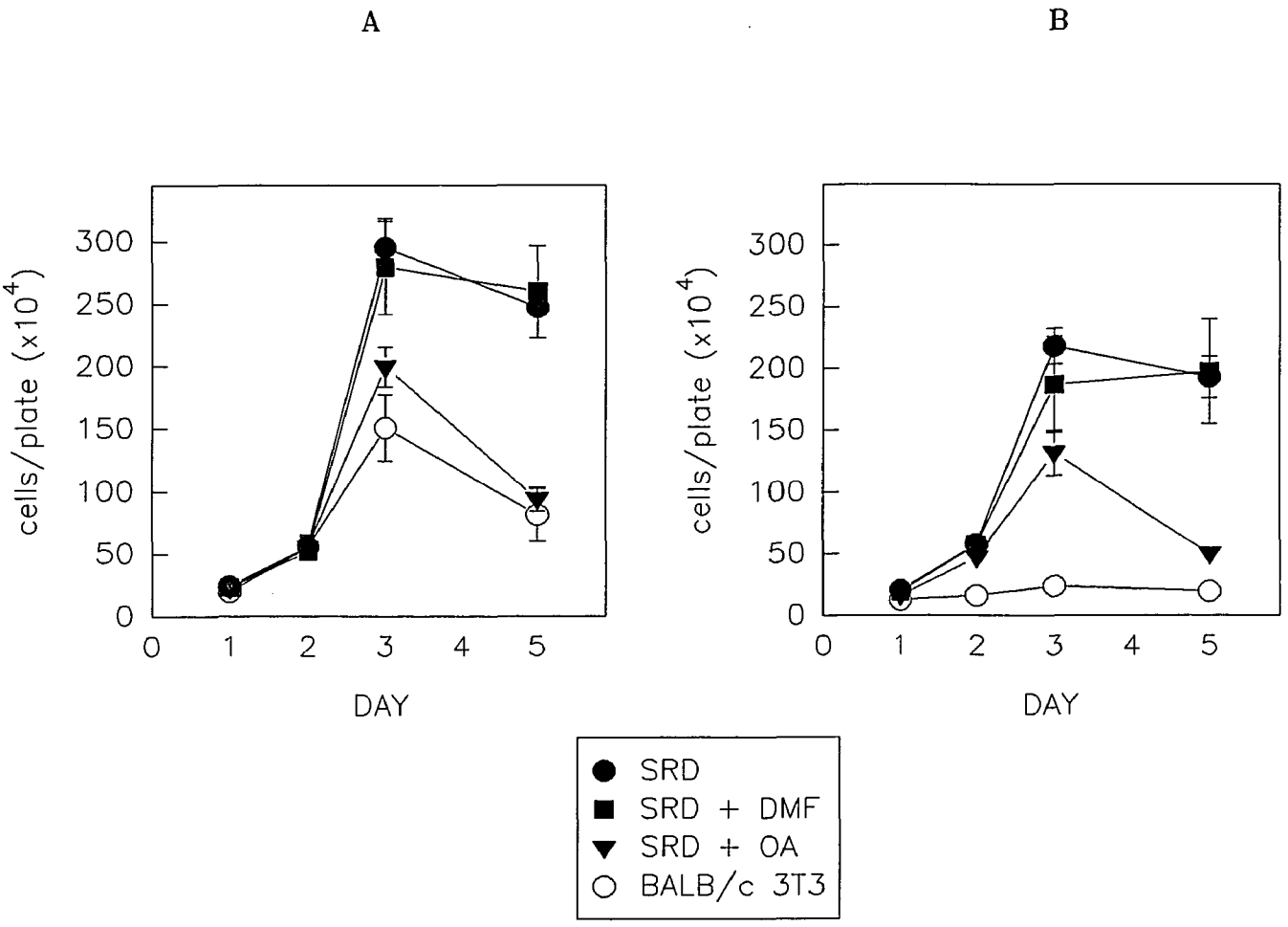



**Fig. 3. OA inhibits the ability of v-Src-transformed cells to reach high saturation densities and to grow in low serum.**

$3x10^5$ SRD cells (filled symbols) or BALB/c 3T3 (open circle) cells were seeded per 35mm tissue culture dish and incubated with DMEM supplemented with 20% (A) or 1% (B) calf serum. Cells were either untreated (circles), or incubated with 0.1% DMF (squares) or 20 nM OA (triangles). On the indicated days, cells were detached with trypsin and counted with a hemocytometer. Values are the means of triplicate samples for SRD cells, and of duplicate samples for BALB/c 3T3 cells.

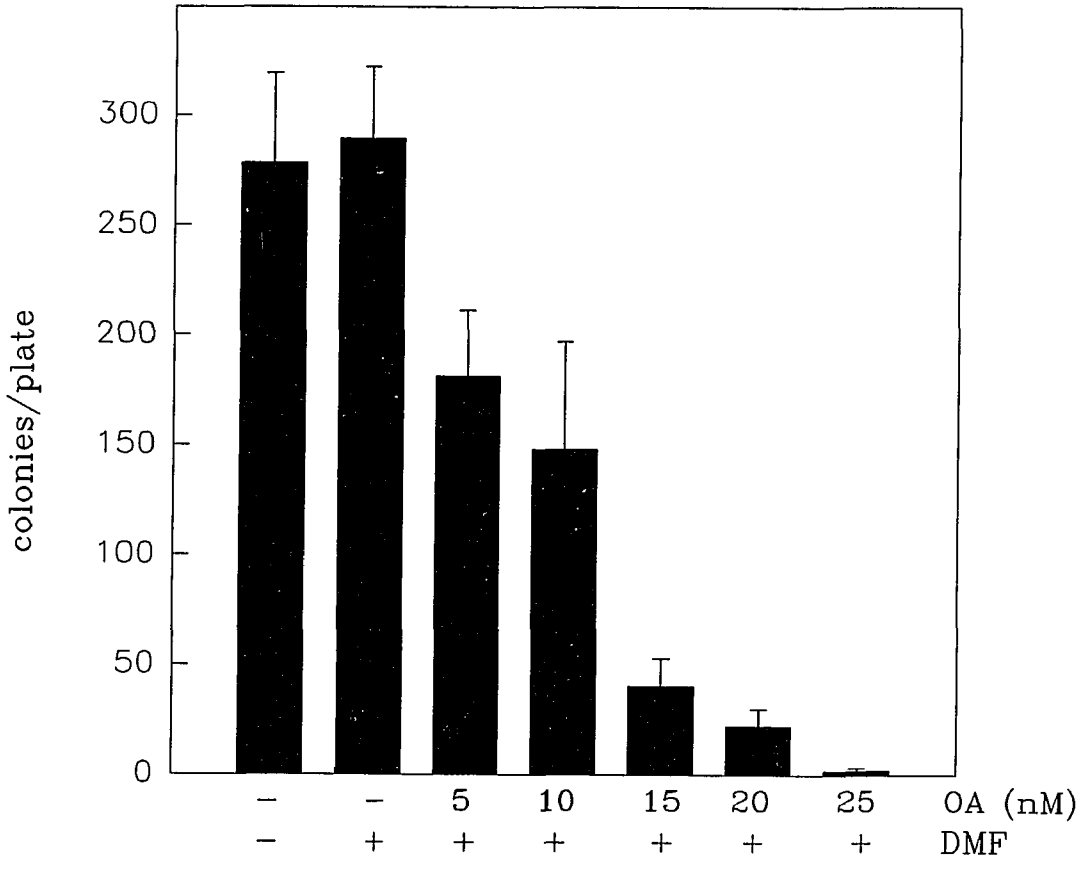



**Fig. 4. OA inhibits anchorage-independent growth by v-Src-transformed cells.**

$1x10^3$ SRD cells were suspended in Bacto-agar as described under "Materials and Methods" with the indicated concentration of OA and/or 0.1% DMF. Colonies were counted after 11 days. Values are the mean of at least 4 independent samples.

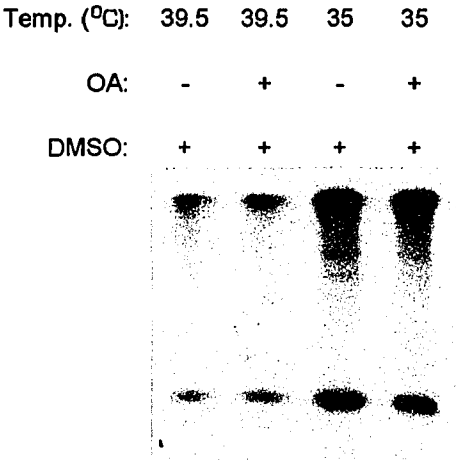



**Fig. 5. OA does not increase the level of phosphorylation of v-Src.** LA90 cells were incubated at 39.5°C (lanes 1 and 2) or 35°C (lanes 3 and 4) with 10 nM OA (lanes 2 and 4) or 0.1 % DMSO (lanes 1 and 3), the solvent for OA. v-Src was immunoprecipitated from cell lysates with monoclonal 327 as described under "Materials and Methods".

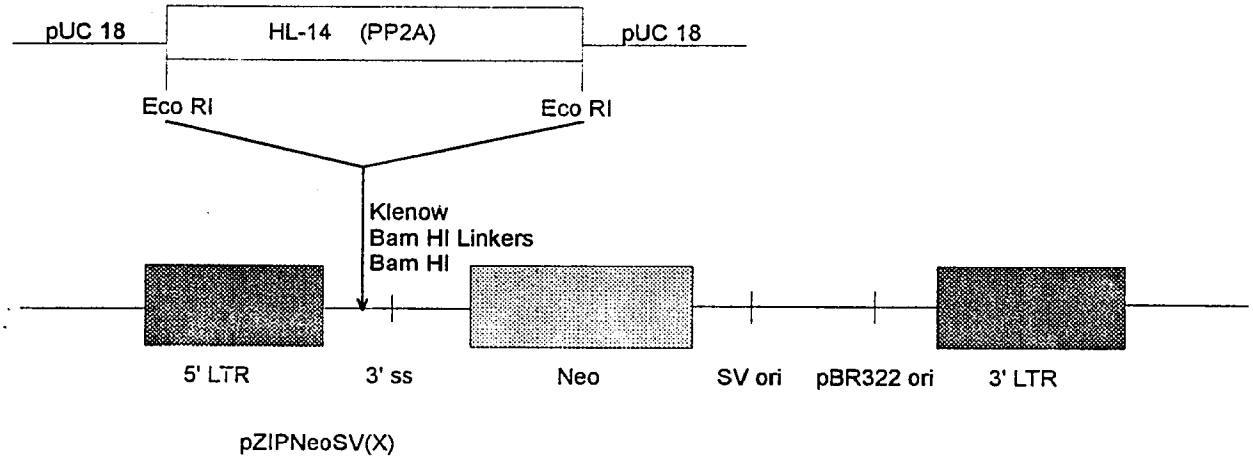



**Fig. 6A. pPP2A expresses the catalytic subunit of PP2A.**

pPP2A was made as described under "Materials and Methods" by inserting the cDNA from HL-14, which encodes the catalytic subunit of human liver PP2A, into the eukaryotic expression vector pZIPneoSV(X). HL-14 was the generous gift of Gary L. Johnson (Arino et al., 1988). Orientation of the resultant clone was determined by restriction enzyme mapping. LTR= Moloney murine leukemia virus long terminal repeat. 3' ss= 3' splice acceptor sequence. Neo= DNA sequences derived from the transposon Tn5 which confer resistance to G418 in mammalian cells and kanamycin resistance in E. coli. SV ori= SV40 origin of replication. pBR322 ori= pBR322 origin of replication. pUC18= DNA sequences from the pUC18 plasmid which contains the HL-14 PP2A insert.

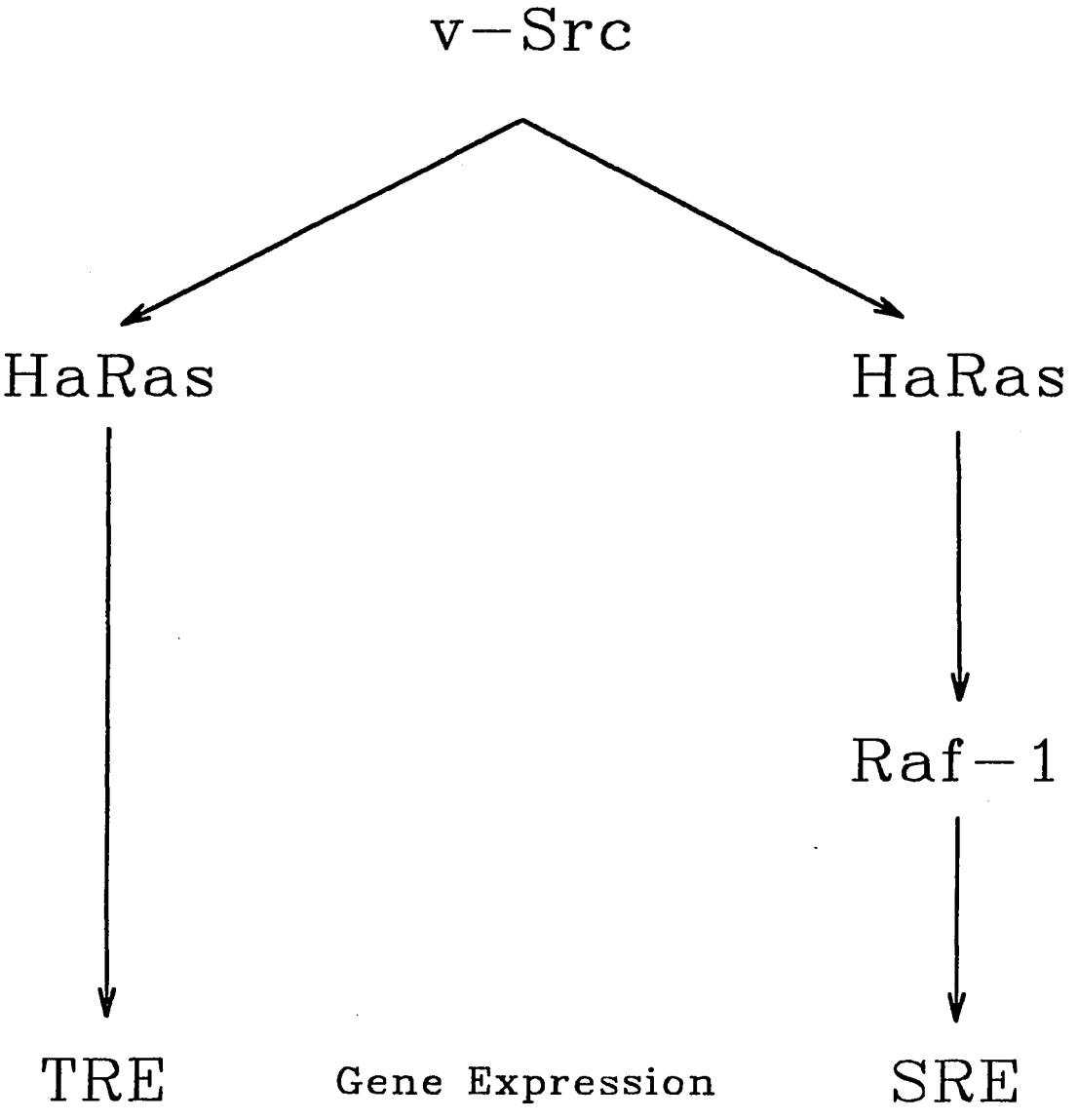
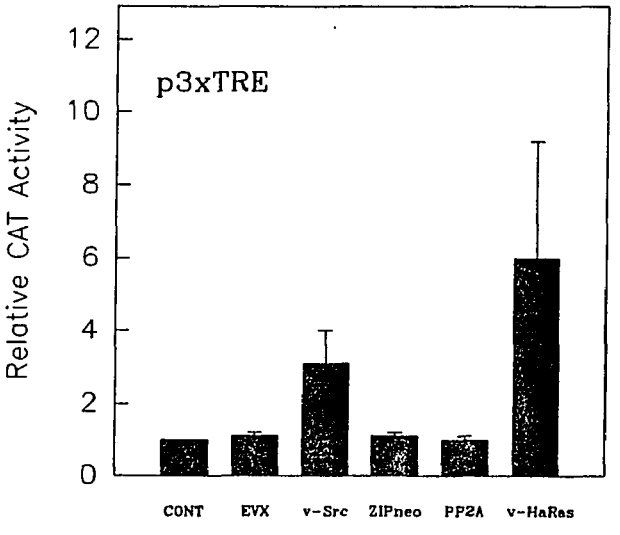



**Fig. 6B. v-Src induces SRE- and TRE-mediated gene expression through distinguishable intracellular signalling mechanisms.**

Model of v-Src-induced gene expression mediated by SREs of the Egr-1 gene promoter and TREs of the collagenase gene promoter.

C




D

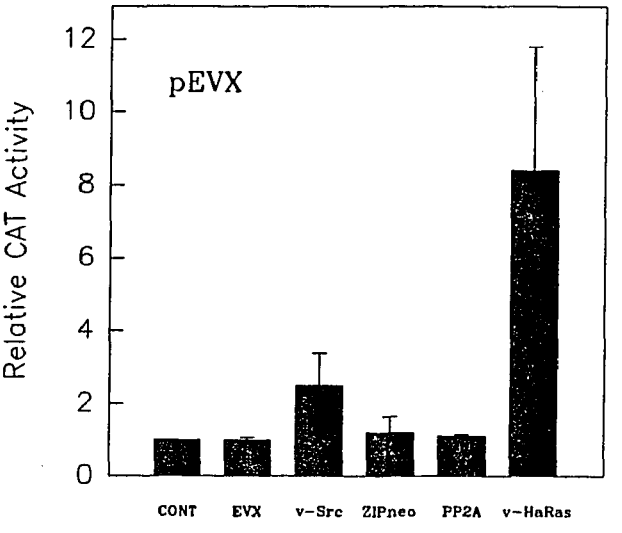



**Fig. 6 C&D. PP2A does not stimulate SRE- or TRE-mediated gene expression.**

NIH 3T3 cells were transfected with p3xTRE (C) or pE425 (D), and either pEVX, pMv-Src, pZIPneoSV(X), pPP2A, or pv-HaRas as indicated in the figure. Data are the mean of at least 3 independent experiments. Data were normalized to the CAT activity generated in the presence of salmon sperm DNA, which was assigned a value of 1.

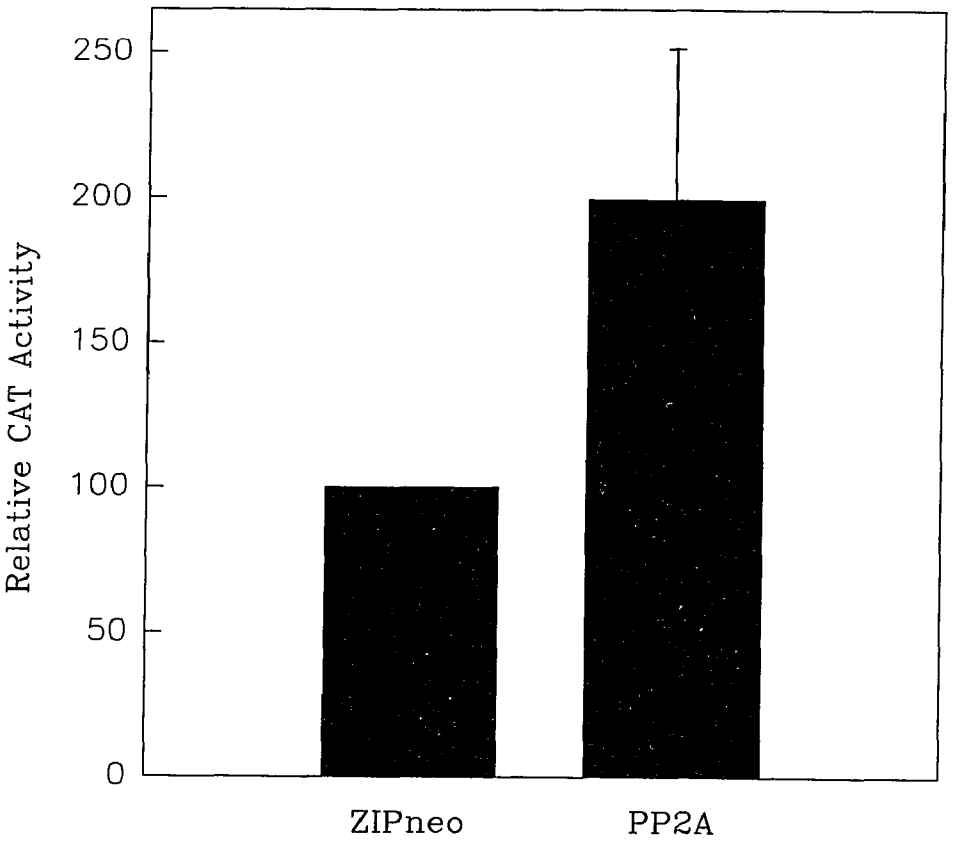



**Fig. 7. v-Src-induced TRE-mediated gene expression is potentiated by PP2A.**

NIH 3T3 cells were co-transfected with p3xTRE, pMv-Src, which expresses v-Src, and either pPP2A which expresses PP2A or pZIPneoSV(X), the parental vector for pPP2A. CAT activity was assayed with the "flour diffusion assay" (Neuman et al., 1987). The data are the mean of 5 independent experiments. Values were normalized to the CAT activity generated in the presence of pMv-Src plus pZIPneoSV(X), which was assigned a value of 100.

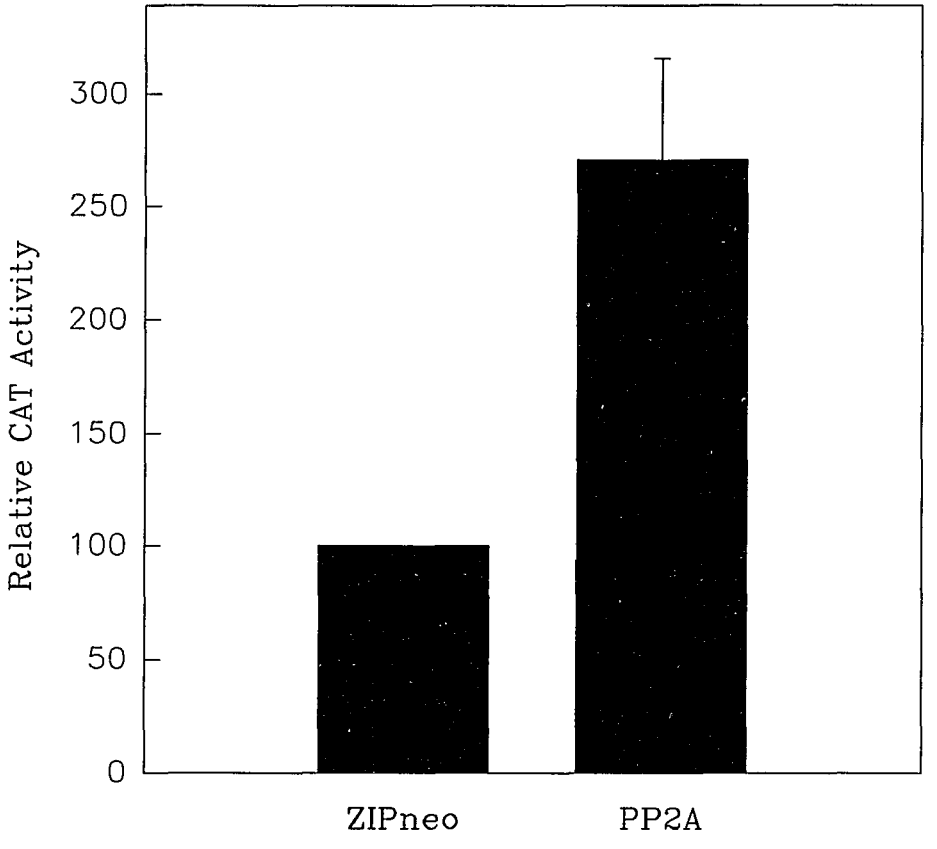



**Fig. 8. PP2A potentiates v-HaRas-induced TRE-mediated gene expression.**

(A) NIH 3T3 cells were co-transfected with p3xTRE, pv-HaRas, and either pPP2A which expresses PP2A, or pZIPneoSV(X), the parental vector for pPP2A. Data are the mean of 4 independent experiments. Values were normalized to the CAT activity generated in the presence of pv-HaRas plus pZIPneoSV(X), which was arbitrarily assigned a value of 100.

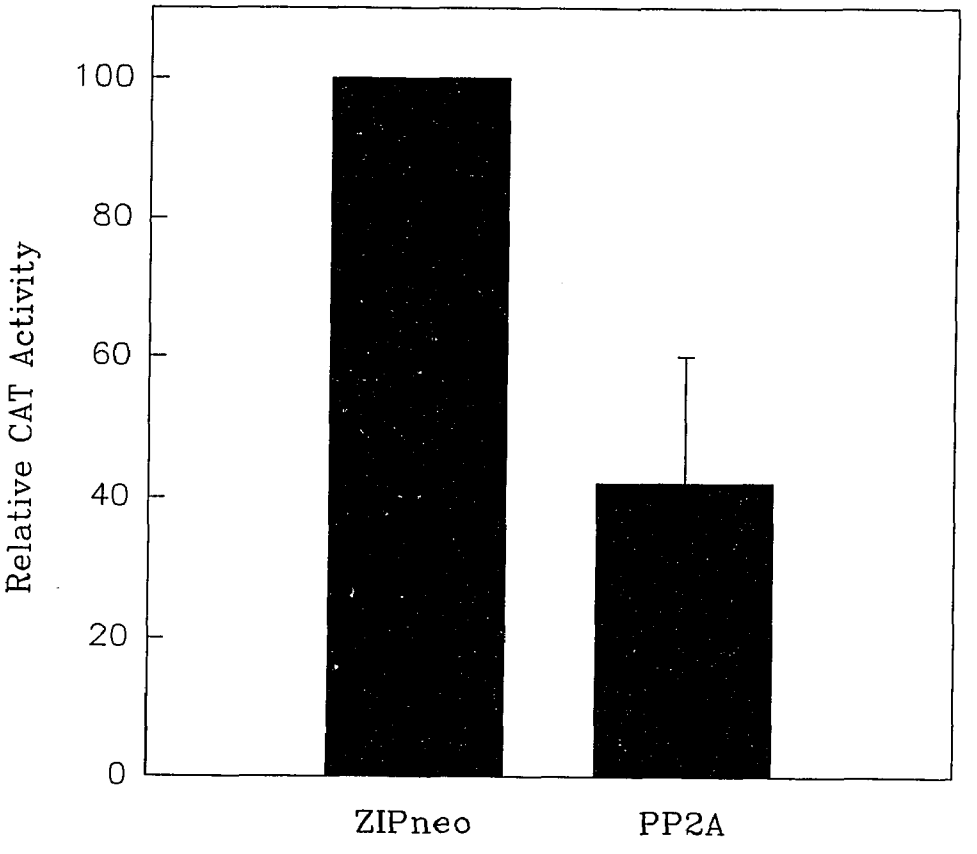



**Fig. 9. v-Src-induced SRE-mediated gene expression is inhibited by PP2A.**

NIH 3T3 cells were co-transfected with pE425, pMv-Src, and either pPP2A or pZIPneoSV(X), the parental vector for pPP2A as indicated in the figure. Salmon sperm DNA was used to normalize the total DNA to 30 μg. Data are the mean of 3 independent experiments. Values were normalized to the CAT activity obtained in the presence of pMv-Src plus pZIPneoSV(X), which was given a value of 100.

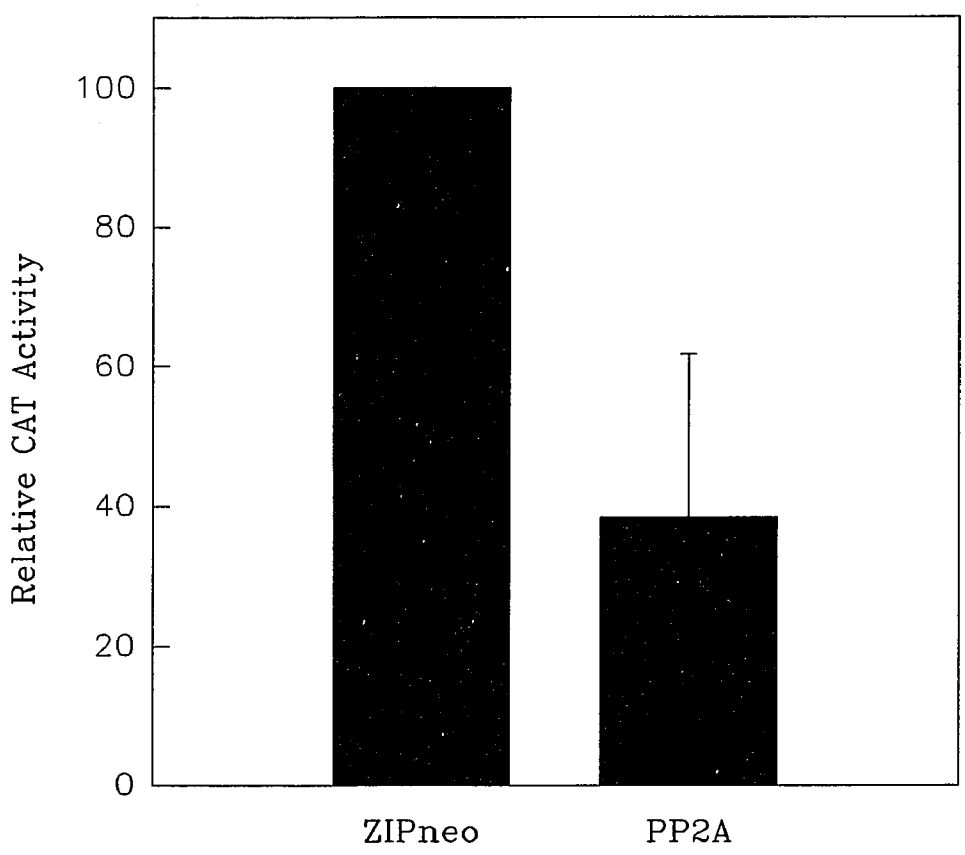



**Fig. 10. PP2A inhibits induction of SRE-mediated gene expression by v-HaRas.**

NIH 3T3 cells were cotransfected with pE425, pv-HaRas, and either pPP2A or pZIPneoSV(X), the parental vector for pPP2A. Data are the mean of 4 independent experiments. Values were normalized to the CAT activity generated in the presence of pv-HaRas plus pZIPneoSV(X), which was randomly assigned a value of 100.

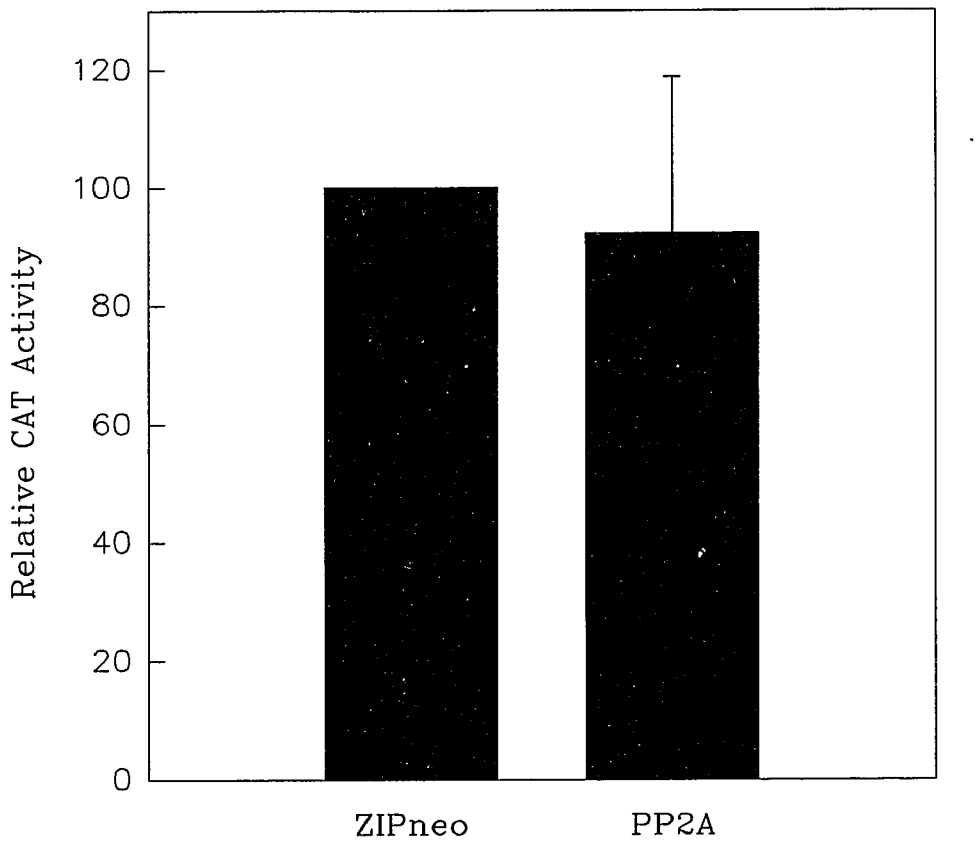



**Fig. 11. v-Raf-induced SRE-mediated gene expression is unaffected by PP2A.**

NIH 3T3 cells were co-transfected with pE425, p3611 and either pPP2A or pZIPneoSV(X). p3611 is a molecular clone of murine sarcoma virus 3611, and expresses v-Raf, a Gag-Raf fusion protein (Rapp et al., 1983). Data are the mean of 3 independent experiments. CAT activity was normalized to the value obtained for p3611 plus pZIPneoSV(X), which was assigned a value of 100.

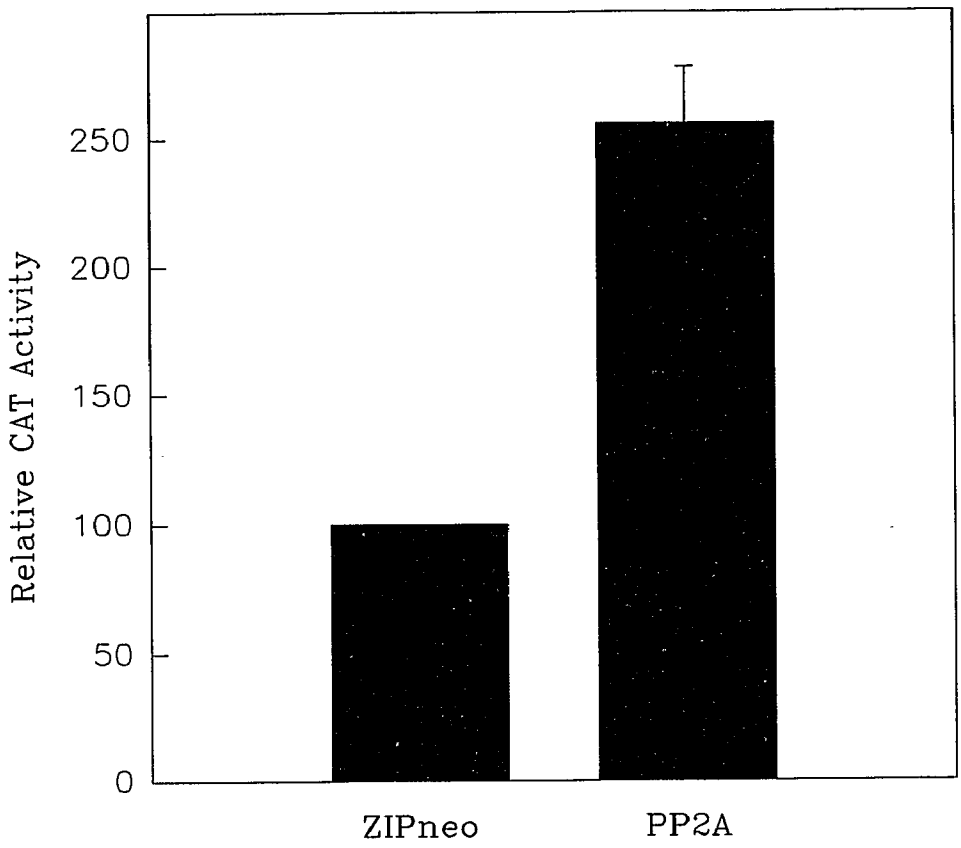



**Fig. 12. v-Mos-induced SRE-mediated gene expression is increased by PP2A.**

NIH 3T3 cells were co-transfected with pE425, pHT25, which expresses v-Mos, and either pPP2A or pZIPneoSV(X) as indicated in the figure. Salmon sperm DNA was used to normalize the total DNA to 30 μg. Data are the mean of 3 independent experiments. Values were normalized to the CAT activity observed with pHT25 plus pZIPneoSV(X), which was given a value of 100.

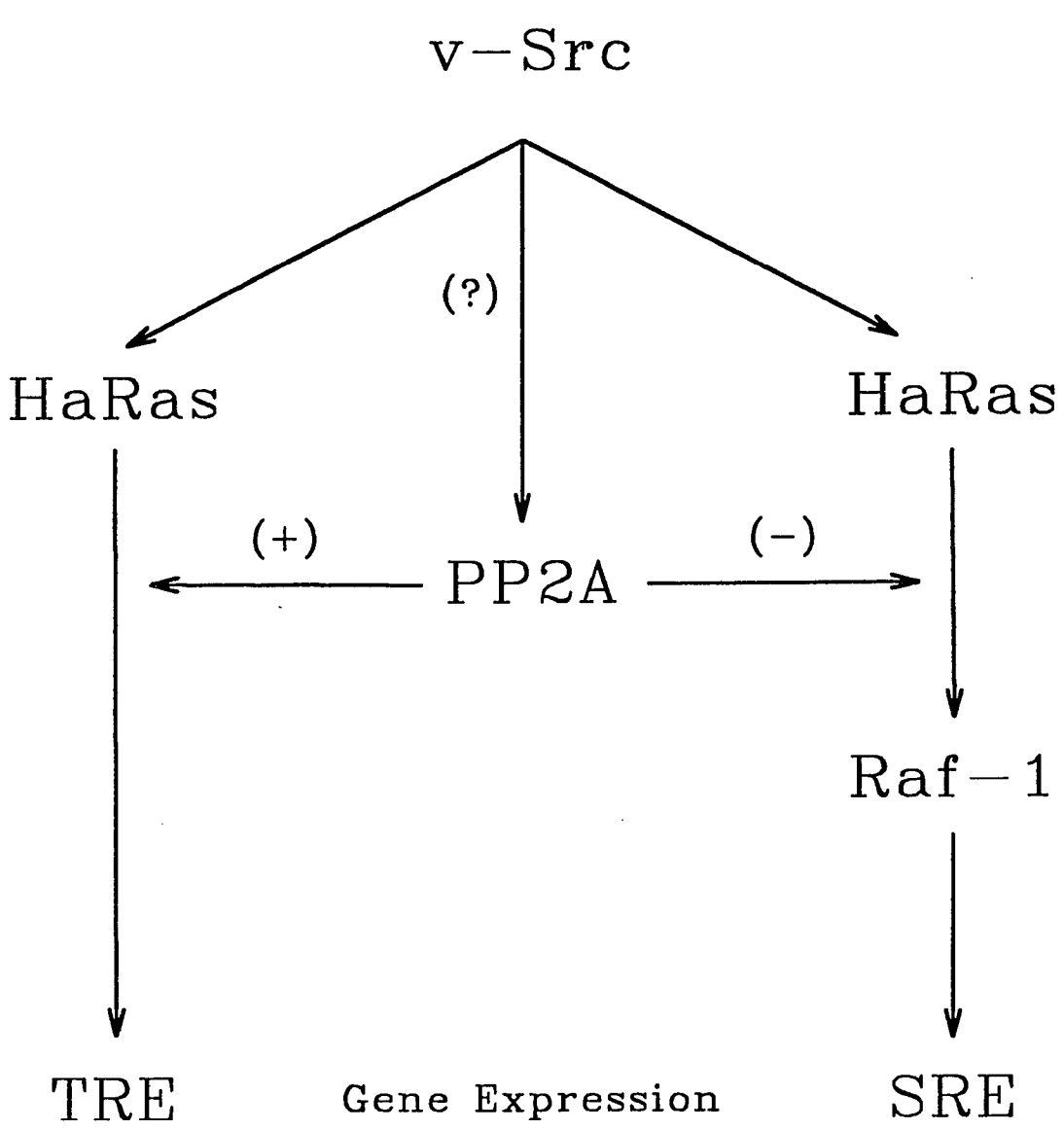



**Fig. 13. Model for the Effect of PP2A Upon the Induction of SRE- and TRE-Mediated Gene Expression by v-Src.**

v-Src activates SRE- and TRE-mediated gene expression via distinguishable intracellular signals. The induction of SRE-mediated gene expression is inhibited by PP2A, whereas the induction of TRE-mediated gene expression is potentiated by PP2A. The mechanisms through which v-Src activates HaRas, Raf-1 and PP2A are not known. Thus, the arrows in the figure represent a dependence for signal transduction in transient transfection expreiments; they do not imply a direct interaction or a linear pathway.

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