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V-*sis* Induces Egr-1 Expression by a Pathway Mediated by c-Ha-Ras

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Abstract The early growth response gene, Egr-1, is up-regulated transiently by mitogens and many other stimuli in all cells tested. Using NIH3T3 cells conditionally expressing v-*sis* from a metallothionein promoter, we show that the addition of Zn^{2+} stimulates the production of PDGF-B (v-*sis*) and elicits the expression of Egr-1 in a dose-dependent and time-regulated manner. The signal is likely independent of protein kinase C, but depends on tyrosine kinase and other kinase activities and is mediated by c-Ha-Ras since the presence of dominant-negative mutants of Ras and Raf abrogates the induction of Egr-1 expression by Zn^{2+}. Transiently activated Ras expression in NIH3T3 cells also stimulates the transient expression of Egr-1, but cells that constitutively express Ras do not have elevated levels of Egr-1. Transient assays also demonstrated that Zn^{2+} or activated Ras expression stimulate the activity of a 950 bp Egr-1 promoter-reporter gene construct and this is abrogated in the presence of mutant Ras and Raf. The accumulated data show that Egr-1 gene expression is regulated by multiple mechanisms, as would be needed for putative roles in cell proliferation, in suppression of transformation and in differentiation.

Key words: inducible v-*sis* (PDGF-B), signal transduction, Egr-1 protein, mutant Ras

The early growth response gene, Egr-1 (zfp-6 in Standardized Genetic Nomenclature for Mice), also known as NGFIA [Milbrandt, 1987], Krox 24 [LeMaire et al., 1988], zif268 [Christy et al., 1988], and T1S-8 [Lim et al., 1987], encodes a protein with three adjacent zinc-finger motifs, structures that are present in many DNA-binding transcription factors. The Egr family of proteins consists of four members that all bind to the same DNA element: GCGGGGGCG or GCGTGGGCG [LeMaire et al., 1988; Christy and Nathans, 1989] because of the remarkable conservation of their "zinc finger" DNA binding domains. The Egr family is a highly evolutionarily conserved set of genes [Joseph et al., 1988] but it has proved difficult to define a precise role.

Immediate early genes, such as Egr-1, are induced within 5 min of the reception of the signal at the cell membrane. The signal transduction pathway for extracellular growth factors has been studied intensely in the last 3 years. Following the stimulation of the tyrosine kinase activity of the receptor, the invariant central component appears to be c-Ha-Ras [reviewed by McCormick, 1993]. From Ras, the signals are transmitted via Raf-1 kinase and MAP kinases to the nuclear transcription factor genes, such as the Jun/Fos family, that make up the complex, known as Activating Factor-1 (AP-1) [reviewed by Roberts, 1992]. Egr-1 is activated extremely rapidly by serum and is thought to contribute to the production of the mitogenic signal that stimulates cell proliferation. The target genes for Egr-1 activity that are responsible for growth stimulation are unknown, although many growth factor gene and oncogene promoters have specific binding sites (GC-rich elements or GCE) for Egr-1. For instance, Egr-1, c-*fos*, *jun* B, *jun* D, c-*ras*, c-*raf*, *int*2, PDGF-B, PDGF-A, and IGF-II have one or more GCE motifs [LeMaire et al., 1988; Christy and Nathans, 1989] in their 5′ promoter regions.

We have previously described functions for Egr-1 quite disparate from growth stimulation when its expression is constitutive. In differentiating cells, Egr-1 may play a role in the stabilization of differentiation [Darland et al., 1991]. Egr-1 is also involved in the suppression of transformed growth when over-expressed in a potentially transformed cell type [Huang et al., 1994]. Its duality may stem from the fact that

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this transcription factor can stimulate or inhibit the activity of the GCE to different degrees in different cells. For instance, Egr-1 stimulates the myosin heavy chain gene in cardiac cells [Gupta et al., 1991] and weakly activates a GCE-containing reporter gene in *Drosophila* SL2 cells [LeMaire et al., 1990]. In NIH3T3 cells, Egr-1 weakly inhibits the activity of the PDGF-A gene [Wang et al., 1992] and down regulates the activity of its own promoter [Cao et al., 1993]. In C1-D1 cells, Egr-1 down-regulates the adenosine deaminase gene probably by competing with Sp1 for overlapping binding sites [Ackerman et al., 1991]. Similarly, Egr-1 competes with activating Sp1 in order to down-regulate its own promoter in NIH3T3 cells [Cao et al., 1993]. Recently, several active domains and a repressive domain have been identified in Egr-1 protein [Gashler et al., 1993; Russo et al., 1993]. To understand the multifaceted roles of Egr-1, it is first necessary to discover how it is regulated and the components involved in its activation.

The experiments described here were designed to study the signal pathway for the transient regulation of Egr-1. The cell system that we use is the mouse fibroblast cell line, SN30-14, derived from NIH3T3 cells. The addition of heavy metal ions stimulates the induction of the oncogene v-*sis*, the homolog of PDGF-B. We hypothesize that the addition of heavy metal ions mimics the signal provided by the addition of PDGF-BB to cells. Although it is known that the PDGF and some transforming oncogenes send signals along a Ras-mediated pathway [Mulcahy et al., 1985; Smith et al., 1986], the mechanism has not been formally demonstrated for the v-*sis* oncogene. Studies by others have shown that the oncogenes v-*fps* and v-*src* stimulate the Egr-1 promoter via Ras and Raf-1 [Mulcahy et al., 1985; Smith et al., 1986]. We have used SN30-14 cells together with a specific antibody to Egr-1 and immunoblotting to characterize the pathway of induction for this transcription factor.

MATERIALS AND METHODS

Cells and Culture

SN30-14 cells were derived from NIH3T3 cells as a G418-resistant clone stably expressing v-*sis* from the expression construct pMT*sis*, driven by the heavy metal-inducible mouse metallothionein promoter [Mercola et al., 1992]. This cell line and the control line, N9, were selected by cotransfection with pSV2neo to allow selection for the integrated DNA. Culture medium consists of DMEM containing 5% bovine serum with antibiotics and 100 μg/ml G418 as a maintenance dose. To induce the expression of v-*sis* product (a close homolog of platelet-derived growth factor-B (PDGF-B), 50 μM Zn acetate or up to 0.5 μM Cd nitrate is added to the medium. The characteristics of the cell line have been described by Mercola et al. [1992].

For treatment of cells with inhibitors (Sigma Chemical Co., St. Louis, MO), stock solutions of genistein, 20 mM in DMSO; H7, 30 mM in water; tetradecanoyl phorbol ester (TPA), 0.025 mM in DMSO were made. Working concentrations were 100 μM, 30 μM, and 0.2 μM, respectively.

Antibodies

Two rabbit polyclonal antibodies were used. For immunoperoxidase studies, an affinity-purified antiserum raised to the full length protein was employed. Egr-1 protein was prepared from a nuclear extract of Baculovirus-infected insect cells [Ragona et al., 1991] and gel-purified from preparative SDS-PAGE. Egr-1 from gel bands was electroeluted, concentrated, and mixed with Freund's complete adjuvant for immunizing a rabbit. Antibodies were purified by chromatography on Agarose-protein A (Sigma, St. Louis, MO). A second polyclonal antiserum was prepared similarly but was raised to the bacterially synthesized fusion protein in pGEX-2T (Pharmacia, Alameda, CA). The glutathione-S-transferase-ΔEgr-1 (aminoterminal half) residues 27 to 318 were purified as described by the manufacturer on a glutathione affinity column for the preparation of the immunogen. Both antisera recognize an Mr 80 kDa protein after immunoprecipitation or immunoblotting assays. Both antisera inhibit the binding of Egr-1 protein to its specific DNA sequence in gel shift assays [Huang and Adamson, 1993, and unpublished data]. Ras antibody (v-H-Ras, 259) was purchased from Santa Cruz Biotech, Inc., Santa Cruz, CA.

Plasmids and Promoter Plasmid Constructs

The two mutated c-Ha-Ras-encoding plasmids were obtained from C. Der. They code for activated p21, containing leucine in codon 61 and mutant p21, containing asparagine in codon 17 inserted into the vectors pZIP-vRasH [Feig and Cooper, 1988]. The Asn17 plasmid acts as a dominant negative mutation in the presence of

normal p21 protein. Raf301 was kindly donated by Drs. U. Rapp and M. Karin. For β-galactosidase expression used as a control in transient assays, pLKLac containing the β-galactosidase sequences driven by the β-actin promoter was obtained from R. Oshima of this Institute.

Promoter Constructs

Plasmid pEgr-1B950 was constructed by partial digestion of 268CAT AMC, which contains 2.8 kb of Egr-1 5′ flanking region (kindly provided by D. Nathans) with BamHI. It contains 950 bp 5′ Egr-1 flanking DNA ligated 5′ to the chloramophenicol acetyltransferase (CAT) gene as a reporter. Standard DNA manipulation techniques were used in plasmid constructions according to Sambrook et al. [1989].

Transfection and CAT Assays

SN30-14 cells (2×10^5) were seeded into 60 mm tissue culture dishes 15–20 h prior to transfection. Eight micrograms of DNA, consisting of 2 μg of LKLac as control for transfection efficiency, pUC8 as carrier DNA to make up total 8 μg DNA, 0.5 μg of CAT reporter vector, and different amounts of expression vector DNA were cotransfected by calcium phosphate precipitation. About 8–10 h after transfection, cells were treated with 2 ml of 10% of glycerol in culture medium for 15 min and allowed to recover 30 to 34 h. Cells were rinsed twice with ice-cold Hepes-buffered saline (HBS), scraped from plates in HBS buffer and pelleted for 2 min at 2,000 rpm at 4°C in an Eppendorf microcentrifuge. Cell pellets were resuspended in 150 μl 0.25M Tris-HCl (pH 7.3) and lysed by 3 cycles of freezing and thawing. The cell lysates were then collected by spinning (15,000 rpm) for 5 min at 4°C. Lysates (15 μl) were subjected to β-galactosidase assays according to Pfahl et al. [1990]. Chloramphenicol acetyltransferase (CAT) assays were performed by thin-layer chromatography (TLC) with the lysate volumes normalized by β-galactosidase activity essentially as described in Gorman et al. [1983]. The intensity of signals was imaged and analyzed in an Ambis Radioanalytical Imaging System.

Immunoperoxidase

Cells were cultured on plastic 8-well slides (Costar) and fixed in methanol (10 min at −20°C) after different times of exposure to 50 μM Zn^{2+}. Affinity-purified anti-Egr-1 at 4 μg/ml was incubated overnight at 4°C with the fixed cells. The ABC peroxidase method of staining was employed as described by the manufacturers (ABC, Vector Laboratories, Burlingame, CA). The slides were reacted with the substrate H_2O_2 in conjunction with diaminobenzidine which stains brown and with 0.1% aqueous Fast Green for cytoplasmic counterstaining.

Immunoblotting

Cells (100 mm dish), grown in log phase to near-confluence, were harvested and directly lysed by SDS-containing lysis buffer. For serum stimulation, cells were cultured in 0.5% serum for 24–48 h, then 20% serum was added for 1 h before harvesting. Equal amounts of protein were applied to sodium dodecyl sulfate (SDS)-polyacrylamide gel (7.5%) electrophoresis under reducing conditions. After transferring the proteins to the PVDF membrane (Immobilon, Millipore), the Egr-1 protein was detected by anti-Egr-1 antiserum and the ECL system (Amersham Corporation). At least two experiments were performed and the signals were then quantified by Scanning Densitometry (Ultroscan XL, LKB). Several exposures of each blot were obtained and the averages of at least two measurements are shown.

RESULTS

V-*sis* Transiently Stimulates Egr-1 Protein Expression

The cell model used here is based on NIH3T3 fibroblasts that produce v-*sis*, a homolog of the growth factor PDGF-B, when induced by the application of heavy metal ions to the culture medium. The induction of v-*sis* provides a simple and reproducible mitogenic signal, whereby the cells can respond during logarithmic growth or in quiescence. The SN30-14 cell line was established and characterized as a reversibly transformed cell line by the stable integration of a v-*sis* expression vector driven by a mouse minimal metallothionein promoter, mMT-I. This neomycin resistant cell line exhibits normal growth characteristics in culture medium but grows with a transformed phenotype when chronically provided with Zn or Cd [Mercola et al., 1992]. In the work described here, however, we are utilizing the early events after metal addition when the v-*sis* vector is strongly induced and the production of growth factor is high from 4 h onwards [Grover-Bardwick et al., in press]. In

the presence of heavy metals the production and secretion of v-*sis* contributes primarily to stimulating the proliferation of SN30-14 cells. This is supported by the fact that the antisense sis vector (pMTsas) can completely abrogate the effect [Huang et al., 1994].

The time course of the appearance of Egr-1 protein was first demonstrated by immunoperoxidase staining of monolayers of SN30-14 cells stimulated with 50 μM Zn^{2+}. A control NIH3T3 G418-resistant clone, N9, was similarly tested for Egr-1 expression. Figure 1 shows that Egr-1 protein was found in the nucleus and was strongly induced in SN30-14 cells with highest expression at 4 h, decreasing in level by 7 h after Zn addition to cultures. There was a small induction by metal addition on the N9 clone or NIH3T3 cells but this was substantially weaker (see Fig. 2B) and was likely caused by the stimulation of the metal responsive element present in the Egr-1 promoter. The polyclonal antibody used here was raised to the whole Egr-1 protein and is known to recognize a polypeptide that migrates with an Mr of 80 kDa in SDS-PAGE [Ragona et al., 1991]. This protein has been identified as the product of the mouse Egr-1 gene by tests on serum stimulated fibroblasts [Mercola et al., 1993] and by the recognition of a baculovirus-transformed Sf9 insect cell line [Ragona et al., 1991], as well as recognition of the protein produced by the in vitro transcription and translation of an Egr-1 vector [Huang and Adamson, 1993].

The Egr-1 protein was also assayed using a second antibody that was raised in a rabbit to a fusion protein between the glutathione-S-transferase gene product and the amino-terminal half of the Egr-1 protein, a polypeptide encoded by amino acids 27 to 318 in the Egr-1 protein. This antibody is more efficient in immunoblotting assays than the antibody above and was used for the Western blots shown in Figures 2 to 7. In Figure 2 we show that the stimulation of Egr-1 protein in SN30-14 cells after 4 h of exposure to Zn was equal to that in 1 h serum- or PDGF-stimulated cells (the time of maximum effect in

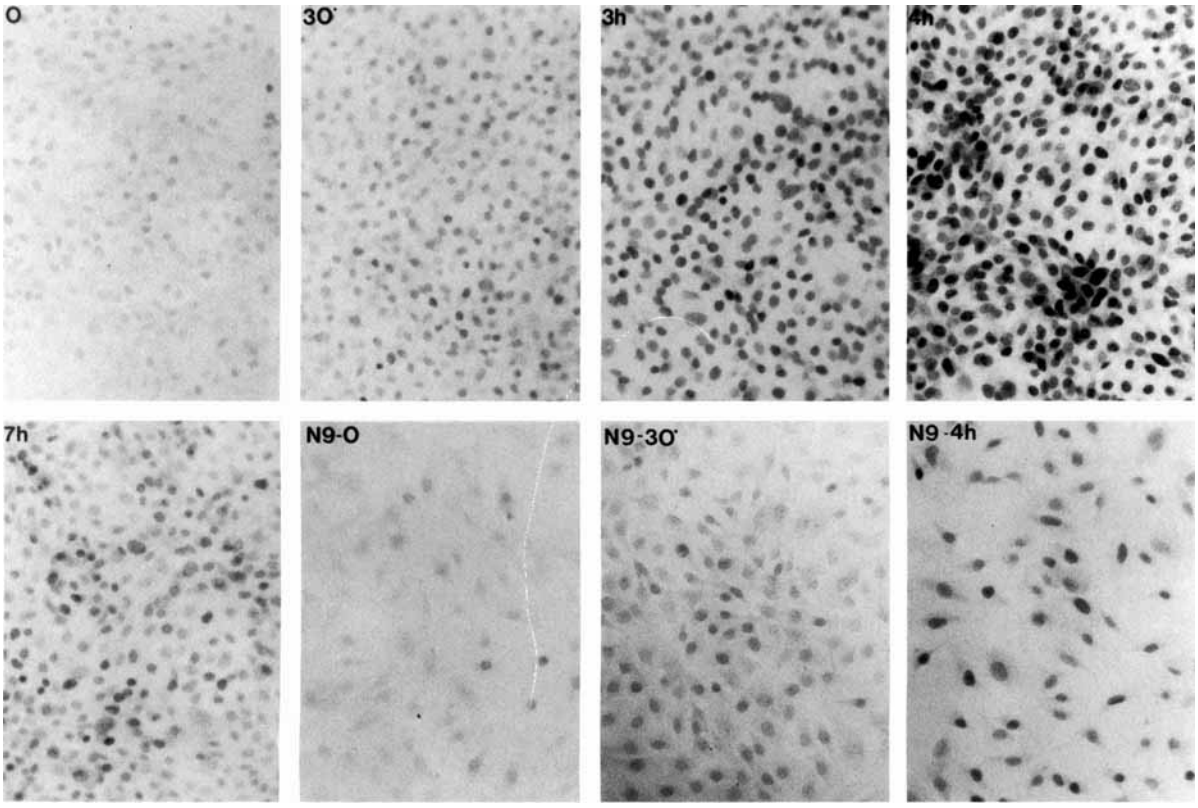


Fig. 1. Immunoperoxidase staining of Egr-1 protein in SN30-14 cells stimulated with 50 μM Zn^{2+}. Affinity-purified rabbit anti-Egr-1 (4 μg/ml) was incubated for 4 h at room temperature with cells previously fixed 5 min in methanol at 4°C after various times of stimulation. Control cells (**N9**) with no v-*sis* expression were similarly treated. Detection was by the avidin-biotin-peroxidase method and diaminobenzidine. The Egr-1 protein is seen largely in the nucleus, very strongly induced in the SN30-14 cells, and is maximally activated after 4 h of Zn stimulation.

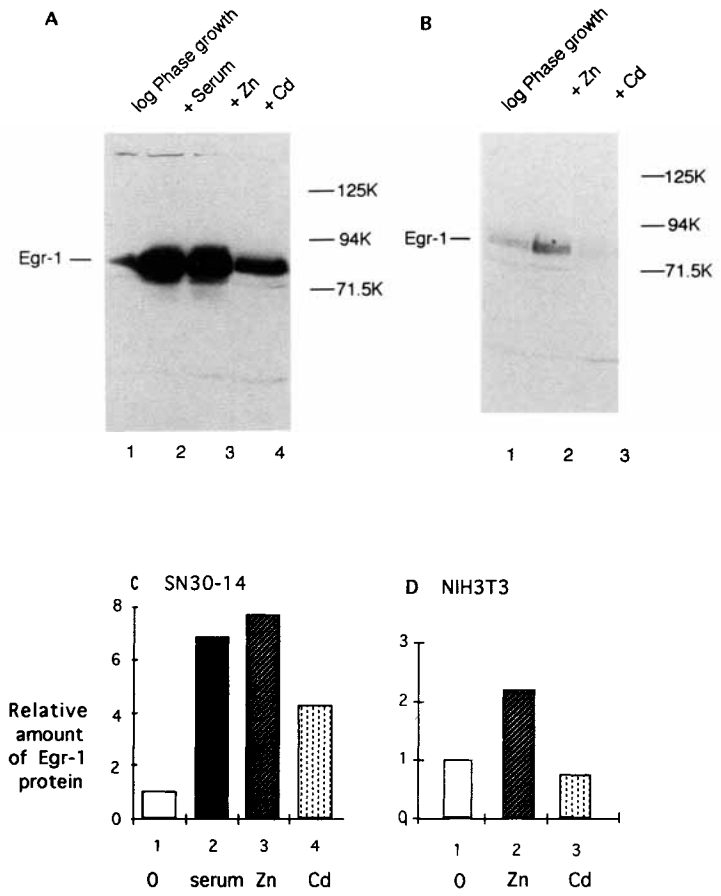


Fig. 2. Immunoblotting to detect Egr-1 protein. **A:** SN30-14 cells were stimulated with serum or heavy metals to induce immediate early growth response gene Egr-1. Heavy metals induced Egr-1 protein because the presence of inducible MT-v-*sis* produced v-*sis* maximally at 4 h. **B:** A similar analysis of NIH3T3 cells. **C** and **D** are the quantitated results of A and B, respectively. The value of each band was determined by scanning densitometry as described in Materials and Methods and expressed as the ratio over the value obtained from log phase growth.

the case of serum). The effect of Cd was always weaker in these studies, perhaps because the toxic dose level is close to the maximum stimulation level. The results of 2 similar experiments was averaged for the quantification shown in panels C and D of Figure 2. Note that Egr-1 protein is evident even in cycling cells (Fig. 2A, lane 1) and is expressed heterogeneously in the population (Fig. 1). We have not tested whether the level of staining corresponds with the stage of the cell cycle. The stimulation of the immediate early genes in response to metals is slower in SN30-14 cells than in growth factor-stimulated cells and this could indicate either that more time is needed for the induction of the v-*sis* gene or that the mediators in the signal pathway may be distinct.

The stimulation of Egr-1 expression by the addition of heavy metals to the medium of SN30-14 cells was dose-dependent as shown by immunoblotting (Fig. 3A) and the quantification of the results of two repeated Western blots (Fig. 3B). The induction of Egr-1 was transient, however, and 4 h exposure to Zn or Cd was maximal for the elicitation of Egr-1 protein expression. After this time the expression declined and returned to base line levels by 48 h (Fig. 4). When the heavy metal inducer was removed, the Egr-1 and v-sis levels dropped rapidly (data not shown). Therefore, we concluded that Egr-1 expression was transiently up-regulated by v-sis.

Involvement of Protein Kinases on the Pathway of v-*sis* Stimulation of Egr-1

The signal pathway that stimulates the Egr-1 gene is likely to involve the PDGF-receptors and their tyrosine kinase activities. This idea is supported by finding that the induction of Egr-1 by serum or Zn^{2+} is largely, but not completely, inhibited by the tyrosine kinase inhibitor, genis-

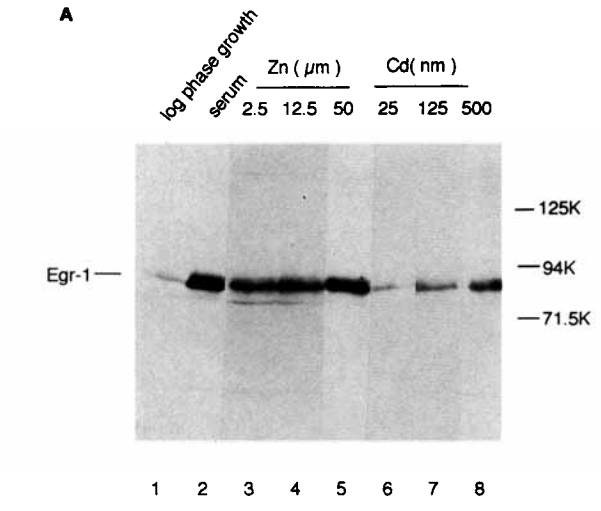


Fig. 3. Dose-dependent stimulation of Egr-1 protein expression by v-*sis*. **A:** SN30-14 cells were treated with different concentrations of either Zn^{2+} or Cd^{2+} for 4 h. For a positive control, cells were also stimulated with serum. Equal amounts of proteins were subjected to immunoblotting. **B:** Graphic analysis of A.

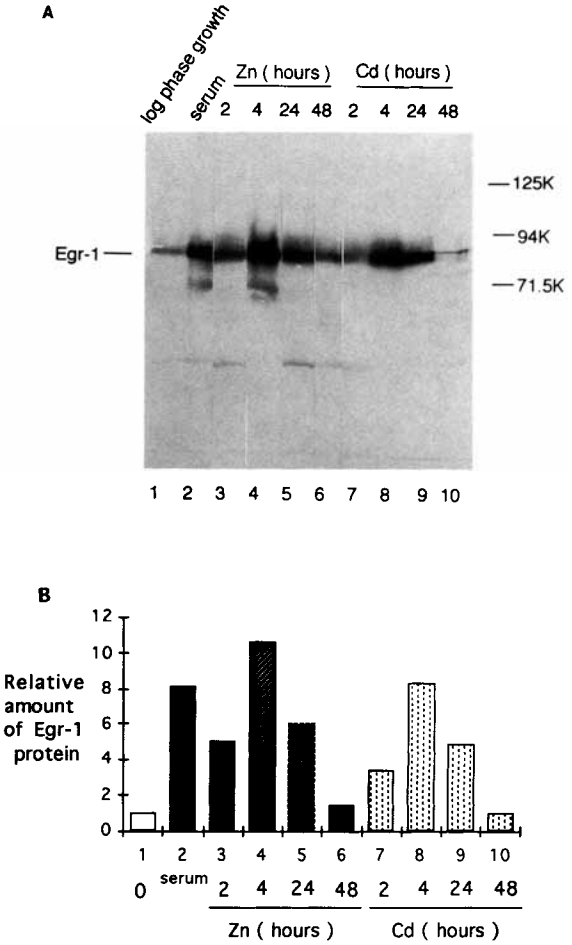


Fig. 4. Transient induction of Egr-1 protein by v-*sis*. **A:** SN30-14 cells were incubated with either 50 μM Zn^{2+} or 500 nM Cd^{2+} for the times indicated. Western blotting was carried out to reveal Egr-1 protein. **B:** Average of at least two experiments obtained from A by scanning densitometry.

tein, added simultaneously with Zn^{2+}. A concentration of 100 μM was used because this is usually sufficient to inhibit tyrosine kinases (Fig. 5A).

We also tested if protein kinase C (PKC) is involved by first exposing the cells to high levels of the phorbol ester, TPA, for 48 h to deplete PKC. The cells were still able to make a full response to serum and to heavy metals, as shown by the induction of Egr-1 in Figure 5B, indicating that PKC was not involved (but see Discussion). This response was not the result of incomplete removal of PKC because TPA itself could no longer elicit a response, while a rapid response was observed in untreated cells (data not shown). In contrast, H7, an inhibitor of PKC, blocked all induction of Egr-1 by serum and v-sis. Previous research has indicated, however, that H7 is not a specific inhibitor of PKC in vivo [Qureshi et al., 1991b].

c-Ha-Ras Is Involved in the Induction of Egr-1 by v-*sis*

In order to determine if v-sis stimulates Egr-1 expression via c-Ha-Ras, we used a SN30-14-derived clone that stably expresses mutated Ras-Asn17. The Ras protein expressed by this vector is altered by the replacement of Ser by Asn at codon 17 [Feig and Cooper, 1988; Cai et al., 1990]. This mutant protein acts as a powerful dominant negative mutation, abrogating the activity of normal Ras in cells that express both. Cells were stimulated with serum or Zn and the levels of Egr-1 were measured by immunoblot-

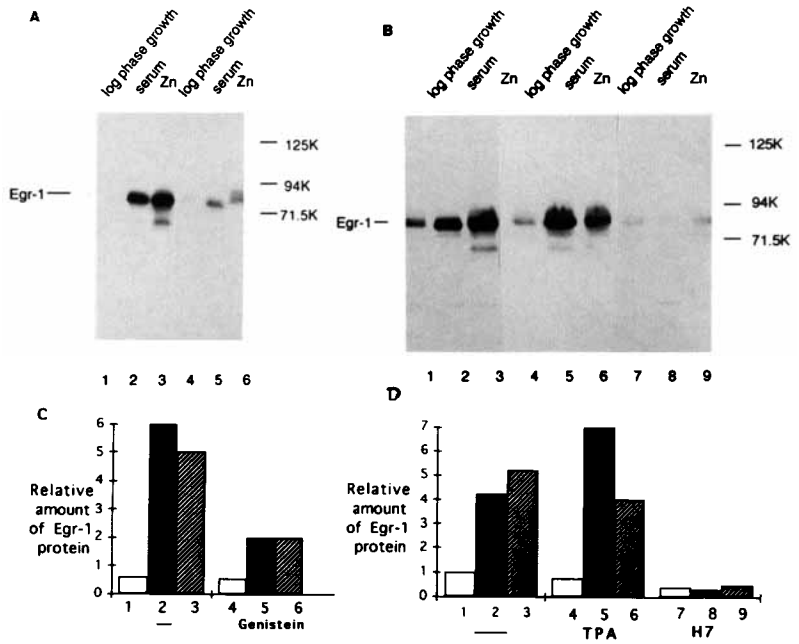


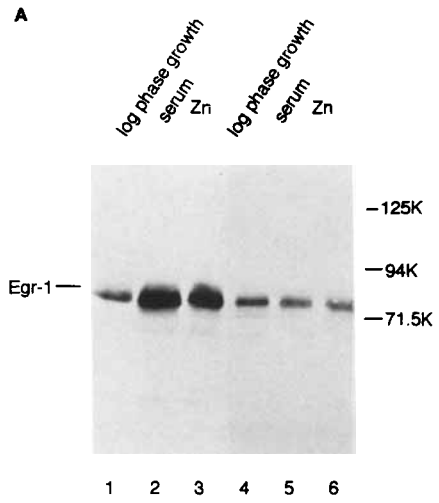
Fig. 5. The stimulation of Egr-1 is mediated by tyrosine kinase activity. **A:** Immunoblotting was used to detect Egr-1 in SN30-14 cells in the presence of various inhibitors. One hundred micromolar genistein, a protein kinase inhibitor, largely inhibited the effect of Zn^{2+} or serum in the induction of Egr-1. **B:** Egr-1 induced by metal or serum in cells depleted of PKC activity by 48 h treatment with 0.2 μM TPA suggests that PKC is not involved in Egr-1 induction; however, 30 μM H7, a putative inhibitor of PKC, is able to inhibit the induction completely. **C:** Quantitation of the Egr-1 induced in A. **D:** Quantitation of the average results of duplicate assays shown in B.

ting. The stimulation of Egr-1 protein by v-*sis* was completely inhibited by the presence of mutant Ras-Asn17 (Fig. 6). As evidence for the expression of Ras-Asn17 in this cell line, we noted its inability to grow in soft agar, compared with the rapid growth of large colonies by cells transfected with Ras61 (data not shown). Constitutive expression of Ras61 in SN30-14 cells after transfection and selection was demonstrated by immunoblotting (Fig. 7B, lane 1) and did not elevate the steady state expression of Egr-1 (Fig. 7A, lane 1) which is a short-lived, tightly regulated protein. Transient expression of Ras61 in NIH3T3 cells, however, did induce transient Egr-1 expression (data not shown). Neither control (Fig. 7A, lane 3) nor mutant Ras-expressing (lane 2) cells affected constitutive Egr-1 expression. These data are consistent with other data that suggest that Ras is a component of the intracellular signaling mechanism for the transient induction of Egr-1 gene expression by v-*sis*. We therefore concluded that the induction of Egr-1 expression by v-*sis* follows an identical pathway to normal PDGF-B stimulation of fibroblasts, at least as far as the intermediary c-Ha-Ras.

Up-Regulation of the Egr-1 Promoter by v-*sis* Is Mediated by c-Ha-Ras

In order to clearly define the signal pathway, we examined whether v-*sis* or Ras can independently activate the Egr-1 promoter. To observe the effect of v-sis on the Egr-1 promoter, we transfected an Egr-1 promoter containing 950 base pairs gene sequence ligated upstream of the choline acetyl transferase reporter gene (CAT) sequences into SN30-14 cells. The activity of the promoter was measured by the level of CAT activity in cells harvested 40 h after transfection. As shown in Figure 8, the addition of Zn^{2+} to the culture medium to turn on the v-*sis* gene significantly enhanced the Egr-1 promoter-driven CAT activity.

C-Raf-1 is a serine/threonine protein kinase that has been implicated in signal transduction downstream of Ras. Recently, Kolch et al. [1993] demonstrated that the kinase-defective mutant, c-Raf301, in which lysine in the ATP binding site was converted into tryptophan, functions as a dominant negative mutant of c-Raf-1. As shown in Figure 8, the presence of Ras17 or Raf301 in Zn-stimulated SN30-14 cells nearly abolished



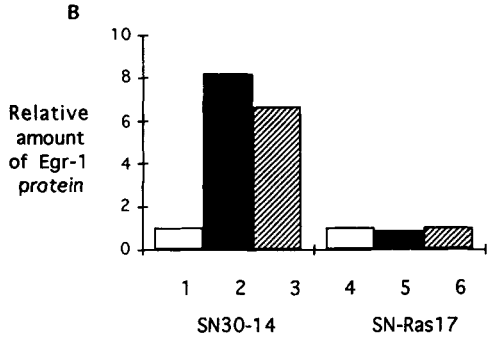


Fig. 6. Inhibition of v-sis-mediated Egr-1 protein induction by dominant-negative mutant Ras17. **A:** SN30-14 cells were co-transfected with pRas17 and pHygro expression vectors and selected with hygromycin B for 3 weeks. The pooled transfected colonies were processed for Western blotting to analyze Egr-1 protein levels from SN30-14 cells induced by serum or zinc in the absence or in the presence of Ras17 expression plasmids. **B:** The graph indicates the relative Egr-1 protein level determined by scanning densitometry.

the activity of the Egr-1 promoter. In the case of Raf301, the failure to activate the Egr-1 promoter could be explained by its ability to bind and complex with Ras-GTP to prevent its activity. This study confirms that c-Ras is an intermediary in the stimulation of Egr-1 expression by v-sis.

To demonstrate that c-Ha-Ras induces the activity of the Egr-1 gene promoter even in the absence of v-sis, we transfected activated Ras 61 into NIH3T3 cells together with the Egr-1 promoter-CAT reporter gene. Figure 9 shows that Ras induced CAT activity proportionally to the level of Ras vector used, while mutant Ras17 was unable to elicit this response.

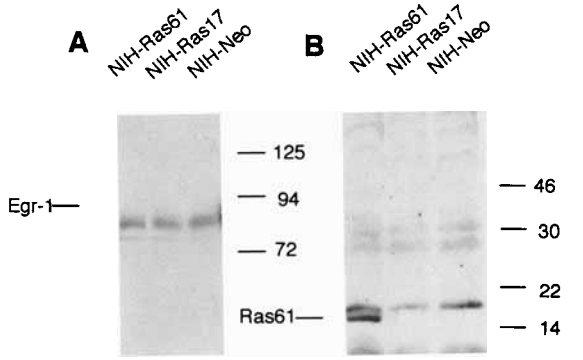


Fig. 7. Constitutive Ras expression does not alter steady-state Egr-1 expression. SN30-14 cells constitutively expressing activated Ras (Ras61) or mutant Ras (Ras17) were compared with control (neo) cells for expression of Egr-1 levels (**A**). No differences were found, although activated Ras (**B**) was strongly expressed in the appropriate cell line. (The antibody to Ras does not detect mutant Ras.)

DISCUSSION

The signal transduction pathway is a topic of considerable interest, but the information is still rather limited as to how a growth factor passes the signal to nuclear transducers. We have shown here that the mitogenic signal, v-*sis*, induces the transcriptional activity of the Egr-1 gene and the appearance of Egr-1 protein by a pathway that involves tyrosine kinases and c-Ha-Ras. The hypothesis that the v-*sis* signal pathway in SN30-14 cells is the same as the PDGF-B pathway in these cells is supported by the evidence that c-*ras* is a common mediator in the activation of the Egr-1 gene. Although v-*sis* is also a transforming oncogene through its constitutive expression in SN30-14 cells, we used metal-induction over a short 4 h period to stimulate the transient induction of the Egr-1 gene. In this 4 h period, the production of v-sis (PDGF-B) increases rapidly, but is not yet maximal until after 10–24 h (data not shown). Activation of the PDGF-R kinases are presumed to be necessary components in the signal pathway from v-sis that was blocked by genistein. A small portion of the signal that was not blocked by genistein was likely due to the stimulation of Egr-1 gene by heavy metals along a different pathway.

Protein kinase C (PKC) does not appear to be involved in the signal pathway from v-sis to Egr-1, since depletion of PKC activity by prolonged TPA treatment did not prevent the induction of Egr-1 by Zn/v-sis. However, total abrogation of the response of the Egr-1 gene by the

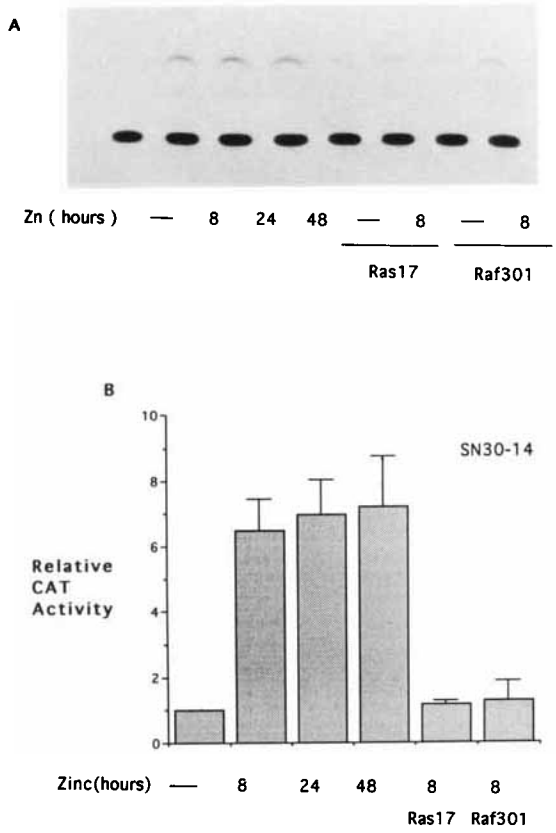


Fig. 8. Activation of Egr-1 promoter by v-sis. **A:** SN30-14 cells were transiently cotransfected with the Egr-1 promoter pEgr-1B950 and either mutants Ras17 or Raf301 expression plasmids. After transfection, the cells were treated with 50 μM Zn^{2+} for the times indicated. CAT assays were applied as described in the Materials and Methods. **B:** The results of three experiments were quantified by the Ambis system, averaged, and plotted.

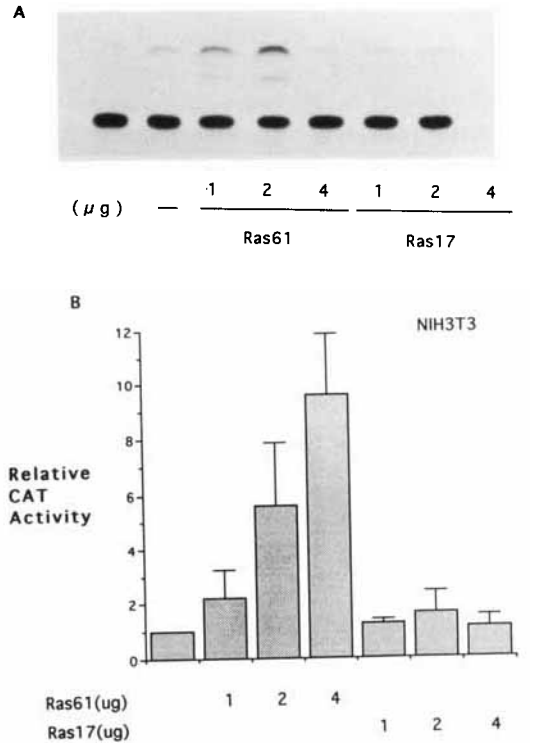


Fig. 9. c-Ras induces the Egr-1 promoter. **A:** Transient transfection CAT assays were used with different amounts of Ras 61 vector cotransfected with the Egr-1 promoter-CAT construct into NIH3T3 cells. Dose dependent Egr-1 promoter activation was abrogated by the presence of low levels of mutant Ras17. **B:** Quantification of the averages of at least two experiments.

PKC inhibitor, H7, in SN30-14 cells apparently contradicted this conclusion. A similar finding was described for Egr-1 expression induced by v-src in 3T3 cells [Qureshi et al., 1991b]. We suspect that H7 inhibits another kinase on the pathway and/or that a PKC isoenzyme, such as zeta, may be involved in signal transduction since this form is not affected by TPA treatment.

So far the accumulated data suggest that multiple signal transduction pathways may mediate the effects of v-sis activation [Mercola, 1992], and components could include c-ras, c-src, c-raf-1, phospholipase-C-γ (PLCγ), phosphoinositol-3-kinase (PI-3-K), c-fyn, and so on. By using the dominant negative mutants of c-Ras and c-Raf-1, we investigated possible factors involved in v-sis-mediated Egr-1 expression. The results indicate that Ras and Raf-1 participate in the pathway. The involvement of Ras was confirmed by finding that an Egr-1 promoter construct is induced in cells carrying transiently expressed Ras. (Figs. 8, 9). Consistent with our results, Qureshi et al. [1991a,b,c, 1992] demonstrated that v-Src induces Egr-1 mRNA expression through a pathway involving c-Raf-1. A similar mechanism also operates in v-fps-mediated Egr-1 gene activation. Thus, several different activating mechanisms may operate in each circumstance. v-*sis*-induced Egr-1 expression may provide a good system for further investigation of these signal transduction mechanisms.

The roles of Egr-1 are not well defined but are clearly complex. On the one hand, Egr-1 is rapidly induced by growth factors resulting in cell proliferation. On the other hand, its expression is associated with differentiation in teratocarcinoma cells [Sukhatme et al., 1988; Darland et al., 1991] and in HL60 cells [Nguyen et al., 1993]. The production of Egr-1 varies in a biphasic manner in mitogen-stimulated cells, since

Egr-1 expression rises for 1 h and falls to base values within 3 h. The same is true but, with a longer time frame, the v-sis expressing cells used here, whether or not zinc is withdrawn. However, when Zn^{2+} remains in the culture medium, the cells start to accelerate in proliferation rate and eventually achieve a morphology characteristic of transformed cells. At this stage, the cells grow in soft agar and are tumorigenic in nude mice, but at this time the level of Egr-1 protein has fallen to low levels (Fig. 4). We have shown that the over-expression of Egr-1 by the stable integration of an expression vector, pCMV-Egr-1, into the SN30-14 line suppresses the tendency of these cells to grow in transformed foci and soft agar colonies [Huang et al., 1994]. Clearly then, Egr-1 can inhibit transformed cell proliferation, although the mechanism of this effect is unknown. This diverse range of activities illustrates the multi-faceted nature of the Egr-1 transcription factor, a characteristic which probably underlies a requirement for complex regulation of the gene.

The results described here may appear to be in conflict with our observation that Egr-1 can suppress sis-mediated transformation [Huang et al., 1994]. However, here we show that oncogenic challenge stimulates a mitogenic signal pathway leading to transient expression of Egr-1, whereas constitutive over-expression of Egr-1 is needed for suppression of transformation. p53 tumor suppressor gene also becomes transiently expressed by many stimuli and can both stimulate and suppress growth when over-expressed [Lowe and Ruley, 1993]. Several possible explanations may be suggested for the transient versus constitutive expression effects of Egr-1. Long-term v-sis expression stimulates the formation of hyperphosphorylated forms of Egr-1 in the later stages of induction by Zn. Therefore, modified Egr-1 may be associated with transformed growth in SN30-14 cells. It is also possible that the elevated early production of Egr-1 is necessary for hyperphosphorylation in later stages. Further studies are in progress to study this intriguing phenomenon. The SN30-14 cell line will be a useful resource for the examination of the roles of Egr-1 in normal and transformed cells.

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