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Attenuation of Serum Inducibility of Immediate Early Genes by Oncoproteins in Tyrosine Kinase Signaling Pathways

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Immediate early genes involved in controlling cell proliferation are rapidly and transiently induced following stimulation of susceptible cells with serum. To study how oncoproteins regulate immediate early genes, we examined serum inducibility of these genes in cells transformed by various oncoproteins. We found that induction of the immediate early gene, c-*fos*, by serum stimulation was markedly attenuated in four independent cell lines stably transformed by the v-Src tyrosine kinase. Cells chronically transformed by other oncoproteins implicated in tyrosine kinase signaling pathways, including v-Sis, v-Ras, and v-Raf, showed the same pattern of attenuation. In contrast, serum inducibility of c-*fos* was not attenuated in cells transformed by simian virus 40, which is thought to transform cells through a different pathway. Cell cycle analyses showed that proliferation of these transformed cell lines could be arrested effectively in 0.1% serum, demonstrating that the attenuation was not simply due to continuous cycling of transformed cells after serum deprivation. Moreover, serum inducibility of other immediate early genes, including c-*jun*, *junB*, *egr-1*, and *NGFI-B*, also was strikingly attenuated by these same oncoproteins. Nuclear run-on transcription assays established that this attenuation of serum inducibility occurred at the transcriptional level. Finally, flow cytometric analysis demonstrated that serum-starved v-Src-transformed cells were viable and able to progress into S phase of the cell cycle after serum stimulation, even though the induction of immediate early genes was greatly attenuated in these cells. Our results suggest that activation of immediate early genes is repressed by chronic stimulation of tyrosine kinase signaling pathways in transformed cells.

Growth factor receptors with tyrosine kinase activity convey signals from the plasma membrane to the nucleus, where transcriptional events that control cell proliferation are regulated (6, 13). Proteins encoded in many cellular proto-oncogenes, including c-Src, c-Sis, c-Ras, and c-Raf, are implicated in these signal transduction pathways involving tyrosine kinases. For example, c-Sis activates the tyrosine kinase activity of its cognate receptor, the platelet-derived growth factor (PDGF) receptor (25), which in turn phosphorylates and activates the c-Src tyrosine kinase (20, 35). In addition, c-Ras function is required for mitogenic stimulation by tyrosine kinases (43, 56), while the cytoplasmic c-Raf serine/threonine kinase is activated by treatment of cells with PDGF (41, 42). These proteins, therefore, appear to comprise a signaling network from receptor tyrosine kinases toward the interior of the cell. Constitutive activation of these signaling proteins, in the form of viral oncoproteins v-Src, v-Sis, v-Ras, and v-Raf, may transform cells by continuously stimulating mitogenic signal transduction pathways.

Ultimately, mitogenic signaling pathways converge on transcription factors that regulate cell proliferation. Several transcription factors are encoded in immediate early genes, including c-*fos* and c-*jun*, which are rapidly and transiently induced following stimulation of susceptible cells by growth factors in serum (9, 11, 22, 34, 44). Proteins encoded in the *fos* and *jun* families of genes have been shown to be required for cell cycle progression in fibroblasts (33). The molecular mechanism of c-*fos* induction by serum has been extensively studied and found to involve specific DNA-protein and protein-protein interactions. Major determinants of serum inducibility of c-*fos* are the serum response element (SRE), located in the promoter region, and serum response factor (SRF), which binds to the SRE (58, 59). Additional proteins, such as ternary complex factor p62 (53), Ets-related protein Elk-1 (27), and SRF accessory protein 1 (12), have been shown to form ternary complexes with the SRF-SRE binary complex. The mechanisms of serum induction of immediate early genes lacking an SRE, however, are less well understood.

Together, these findings suggest a model in which activation of receptor tyrosine kinases by growth factors stimulates expression of immediate early genes, which in turn promotes cell proliferation. Nevertheless, while many cellular components involved in growth control have been identified, the precise nature of mitogenic signaling networks is not yet fully defined. To better understand the signaling pathways in normal and transformed cells, we investigated the long-term effects of constitutive stimulation of these pathways by activated oncoproteins on the regulation of immediate early gene expression. Here we report that serum inducibility of the endogenous c-*fos* gene is markedly attenuated in cells chronically transformed by v-Src or other viral oncoproteins implicated in tyrosine kinase signaling pathways, including v-Sis, v-Ras, and v-Raf. This attenuation of serum inducibility occurs at the transcriptional level and is seen with other immediate early genes, including c-*jun*, *junB*, *egr-1*, and *NGFI-B*. These results suggest that a global regulatory mechanism governing activation of immediate early genes is perturbed in cells chronically transformed by oncoproteins in tyrosine kinase signaling pathways.

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MATERIALS AND METHODS

Cell lines. The normal rat fibroblast cell line 3Y1 and its v-Src-transformed counterpart SR-3Y1 have been described previously (31). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% iron-supplemented bovine calf serum (BCS). The following previously characterized derivatives of mouse NIH 3T3 fibroblasts were used: cells overexpressing normal chicken c-Src and cells transformed by v-Src or an activated c-Src mutant with a Y527-to-F527 substitution (29, 32); and cells transformed by v-Sis or v-Ras (19, 45). NIH 3T3 cells expressing high levels of v-Raf are highly transformed by the criteria of morphological alteration and growth in soft-agar suspension (47). The normal NIH 3T3 cell line and its derivatives were cultured in DMEM containing 5% BCS. Mouse BALB/c 3T3 cells and its derivatives transformed by v-Src or SV40 (5, 54) were maintained in DMEM containing 10% BCS. The Chinese hamster ovary cell line CHOr, which is a nontumorigenic flat revertant clone, and its v-Src-transformed counterpart (49) were cultured in minimum essential medium alpha containing 5% BCS.

For typical serum stimulation experiments, cells were allowed to grow to confluence, the monolayers were washed twice with phosphate-buffered saline (PBS) and then maintained in medium containing 0.1% BCS for 48 h. Cells were stimulated with medium containing 20% fetal bovine serum (FBS) for the times indicated prior to harvesting. Exponentially growing cells were harvested when the monolayer reached 30 to 50% confluence.

Northern (RNA) blot analysis. Total RNAs were isolated by the guanidine isothiocyanate method as previously described (8). Five micrograms of total RNA was separated on 1.2% agarose–formaldehyde gels and electroblotted onto nylon membranes. Small aliquots of the same RNA samples were separated on parallel nondenaturing 1% agarose gels and stained with ethidium bromide to confirm the normalization and integrity of RNAs. Blotted membranes were hybridized at 65°C in 1 mM EDTA–0.5 M Na_2HPO_4 (pH 7.2)–7% sodium dodecyl sulfate (SDS) for 18 to 24 h with 3 $\times 10^6$ to 8 $\times 10^6$ cpm of the DNA probe per ml. DNA probes were labeled with [^{32}P]dCTP by nick translation to a specific activity of 3 $\times 10^8$ to 8 $\times 10^8$ cpm/μg. After hybridization, membranes were washed twice for 1 h in 1 mM EDTA–40 mM Na_2HPO_4 (pH 7.2)–5% SDS at 65°C and then twice for 1 h in 1 mM EDTA–40 mM Na_2HPO_4 (pH 7.2)–1% SDS at 65°C. Under these high-stringency conditions, only specific transcripts were detected. Equalization of RNA samples was further confirmed by sequential probing of blots for expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Each Northern blot analysis was repeated several times to ensure reproducibility.

DNA probes. Probes for hybridization of Northern blots were obtained by restriction endonuclease digestion of plasmids containing cDNAs for individual genes. Transcripts of c-*fos* were detected by a 790-bp *Bgl*II-*Nco*I fragment of v-*fos* cDNA (60). Transcripts of c-*jun* were detected by a DNA segment containing a 139-bp *Nhe*I-*Bss*HII fragment derived from the 5′ untranslated region of c-*jun* cDNA contiguous with a 929-bp *Hpa*I-*Eco*RI fragment derived from the 3′ untranslated region of c-*jun* cDNA (51). This probe contains sequences unique to c-*jun* and thus is specific for c-*jun* transcripts. Transcripts of *junB* were specifically detected by a 391-bp *Bam*HI fragment derived from the 5′ end of *junB* cDNA (50). A 1.1-kb *Eco*RI-*Bgl*II fragment of *egr-1* cDNA (57) was used in detecting *egr-1* transcripts. Transcripts of *NGFI-B* were analyzed by a 2.3-kb *Eco*RI-*Xho*I fragment of *NGFI-B* cDNA (24). A 1.1-kb *Pst*I fragment of GAPDH cDNA (14) was used in detecting GAPDH transcripts. In all cases, mRNA species of the expected sizes were detected on Northern blots. The same plasmids from which the probes for Northern analyses were derived were also used for making slot blots in nuclear run-on transcription assays.

Flow cytometric analysis. Monolayer cultures were trypsinized, and single-cell suspensions were prepared in ice-cold PBS. Cell samples containing 10^6 cells were centrifuged at 300 × *g* for 5 min at 4°C and then resuspended in 1 ml of ice-cold PBS. Cells were fixed by gradual addition of 2 ml of ice-cold absolute methanol with constant vortexing and then stored at 4°C. Before analysis by flow cytometry, methanol-fixed cells were centrifuged again at 300 × *g* for 5 min at 4°C, and the pellets were resuspended in 250 μl of propidium iodide solution (100 μg of propidium iodide per ml in PBS containing 0.1% Triton X-100 and 0.1 mM EDTA) for staining. Another 250 μl of solution containing bovine pancreatic RNase A (200 U/ml in PBS) was added to the cell suspension, and the mixture was incubated in the dark at room temperature for 30 min. Approximately 20,000 cells were analyzed from each sample on an EPICS C flow cytometer, and the average DNA content of cells (between 2N and 4N genome equivalents) was plotted as histograms. The percentages of cells within each phase of the cell cycle were determined by analysis with the computer program MULTICYCLE. Each cell cycle analysis was repeated at least twice, with similar results.

Nuclear run-on transcription assays. Nuclei were isolated from approximately 2 × 10^7 cells by lysing cells in hypotonic buffer containing 0.25% Nonidet P-40 and were then frozen at −80°C as described previously (22). Nuclear run-on transcription assays were performed by incubating thawed nuclei for 30 min at 30°C with 250 μCi of [^{32}P]UTP, and nascent transcripts were purified as previously described (39). In a given experiment, equivalent numbers of counts per minute of ^{32}P-labeled RNA transcripts were hybridized to slot blots containing 5 μg of denatured linearized plasmid DNAs with cDNA inserts, plasmid vector alone, or rat genomic DNA. Hybridization and washing conditions have been described elsewhere (10). Nuclear run-on assays were reproduced a minimum of three times, with identical results.

RESULTS

Serum inducibility of c-*fos* is attenuated in v-Src-transformed cells. To study the effects of activated oncoproteins on serum inducibility of immediate early genes, we examined the regulation of endogenous c-*fos* gene expression in cells chronically transformed by v-Src. These two components of mitogenic signaling pathways were selected for our initial studies because v-Src is a well-characterized tyrosine kinase associated with cell membranes (30) and because growth factor-mediated stimulation of c-*fos* expression is a paradigm for immediate early gene regulation (2, 26). Total RNAs were isolated from nontransformed or v-Src-transformed cells before or after serum stimulation, and the levels of c-*fos* transcripts were measured by Northern analysis. Figure 1 shows the kinetics of c-*fos* mRNA induction following serum stimulation of four independent cell lines derived from three different species. In all four nontransformed cell lines, c-*fos* mRNA was rapidly and transiently induced by serum as described previously (11, 22, 34, 44). By contrast, in v-Src-transformed cells, c-*fos* induction was significantly attenuated to various extents after serum stim-

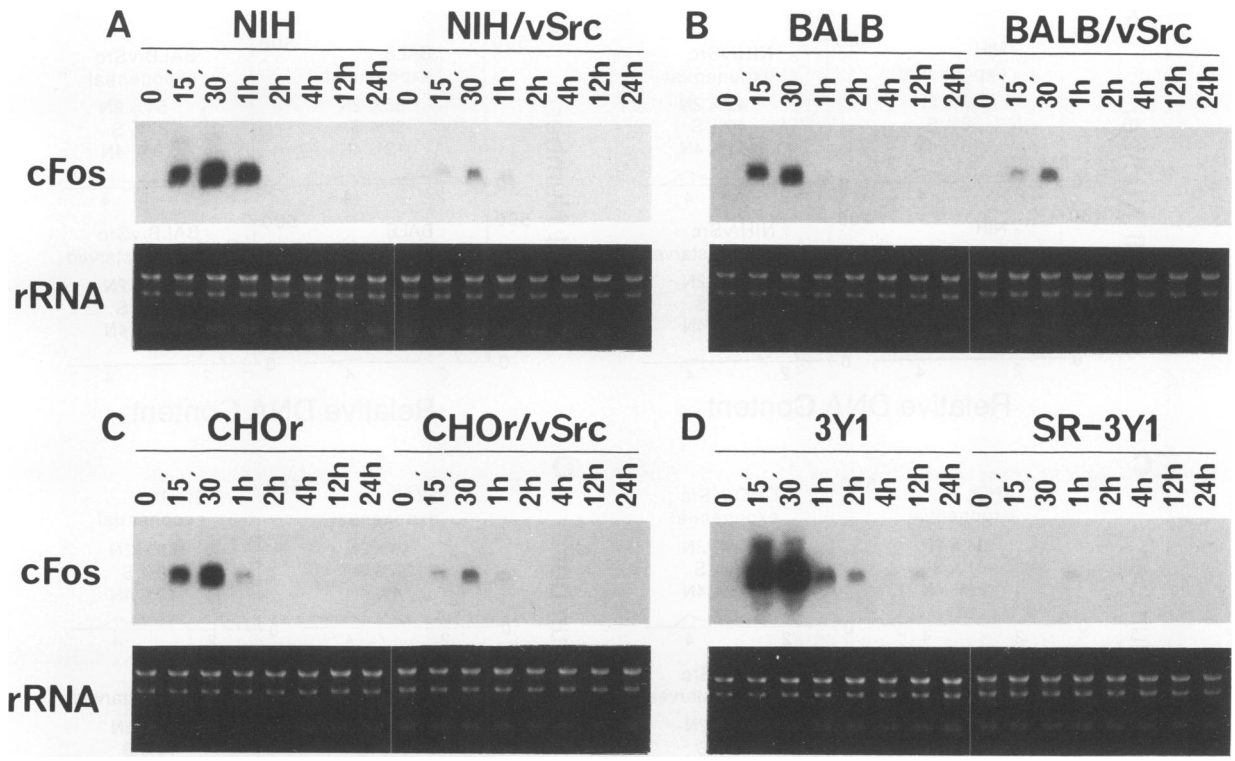

FIG. 1. Kinetics of c-*fos* mRNA induction following serum stimulation of cells transformed by v-Src. Eight different cell lines were analyzed: (A) mouse NIH 3T3 cells (NIH) and its v-Src-transformed counterpart (NIH/vSrc), (B) mouse BALB/c 3T3 cells (BALB) and its v-Src-transformed counterpart (BALB/vSrc), (C) nontransformed revertants of Chinese hamster ovary cells (CHOr) and its v-Src transformed counterpart (CHOr/vSrc), and (D) rat 3Y1 cells (3Y1) and its v-Src transformed counterpart (SR-3Y1). After reaching confluence, monolayer cultures were maintained in medium containing 0.1% BCS for 48 h (0 time point). Cells were then stimulated with medium supplemented with 20% FBS for 15 min (15') to 24 h, as indicated. Total RNAs (5 μg) were isolated and analyzed by Northern blotting for c-*fos* transcripts (see Materials and Methods). Small aliquots of the same RNA samples were analyzed by ethidium bromide staining of rRNAs in parallel nondenaturing gels to confirm the normalization and integrity of RNAs (bottom of each panel). Equalization of RNA samples was further confirmed by sequential probing for GAPDH transcripts (not shown; see Fig. 4).

ulation. Despite the attenuation, however, the kinetics of c-*fos* induction were similar in v-Src-transformed cells and their nontransformed counterparts. In addition, there was no detectable elevation of c-*fos* transcripts prior to serum stimulation in v-Src-transformed cells compared with their nontransformed counterparts (Fig. 1; compare 0 time points). Western immunoblot analysis using anti-Fos antibodies indicated that Fos protein was present at similarly low levels in both normal and transformed cells prior to serum stimulation; however, a consistent pattern of attenuation of Fos protein induction was observed in the transformed cells after serum stimulation (data not shown). These results demonstrate that serum inducibility of c-*fos* is attenuated in cells chronically transformed by v-Src and that this attenuation is not due to clonal variation.

One possible explanation for the results shown in Fig. 1 is that v-Src-transformed cells, being less reliant on serum for their growth, continue to proliferate after serum starvation and are therefore less responsive to the inductive effects of added serum. We tested this idea by examining the cell cycle characteristics of the various cell lines (Fig. 2). The cell cycle profiles of v-Src-transformed NIH 3T3 and BALB/c 3T3 cells were almost identical to those of their normal counterparts after serum starvation (Fig. 2A and B). Although the percentages of cycling cells appeared slightly higher in v-Src-transformed CHO revertants and 3Y1 cells after serum starvation than in their nontransformed counterparts, there was no consistent correlation with the extent of attenuation observed in the Northern analyses (compare Fig. 1C and D and Fig. 2C and D). This cell cycle analysis demonstrates that the majority of v-Src-transformed cells, like their nontransformed counterparts, can be arrested effectively at a 2N DNA content by serum starvation under the stringent conditions used here (0.1% serum). In addition, attenuation of c-*fos* induction was observed after serum stimulation of v-Src-transformed cells that were growing exponentially in complete medium containing 5% serum (data not shown), indicating that this attenuation is not dependent on serum starvation.

Attenuation of c-*fos* mRNA induction by serum in cells transformed by various oncoproteins. To examine the effects of different Src proteins on c-*fos* induction by serum, two additional NIH 3T3 cell lines were analyzed. The first line overexpresses high levels of normal chicken c-Src, a tightly regulated tyrosine kinase, and is only partially transformed (29). The second line is highly transformed by a c-Src mutant harboring a substitution of Tyr-527 to Phe (F527), which results in a constitutively activated kinase (32). Northern blot analysis (Fig. 3A) shows the kinetics of induction of c-*fos* transcripts following serum stimulation of these cell lines. Similar to the case with v-Src, the F527 c-Src mutant or overexpressed normal c-Src also attenuated c-*fos* induction without changing the kinetics. The degree of attenuation by the various Src proteins was correlated with the overall

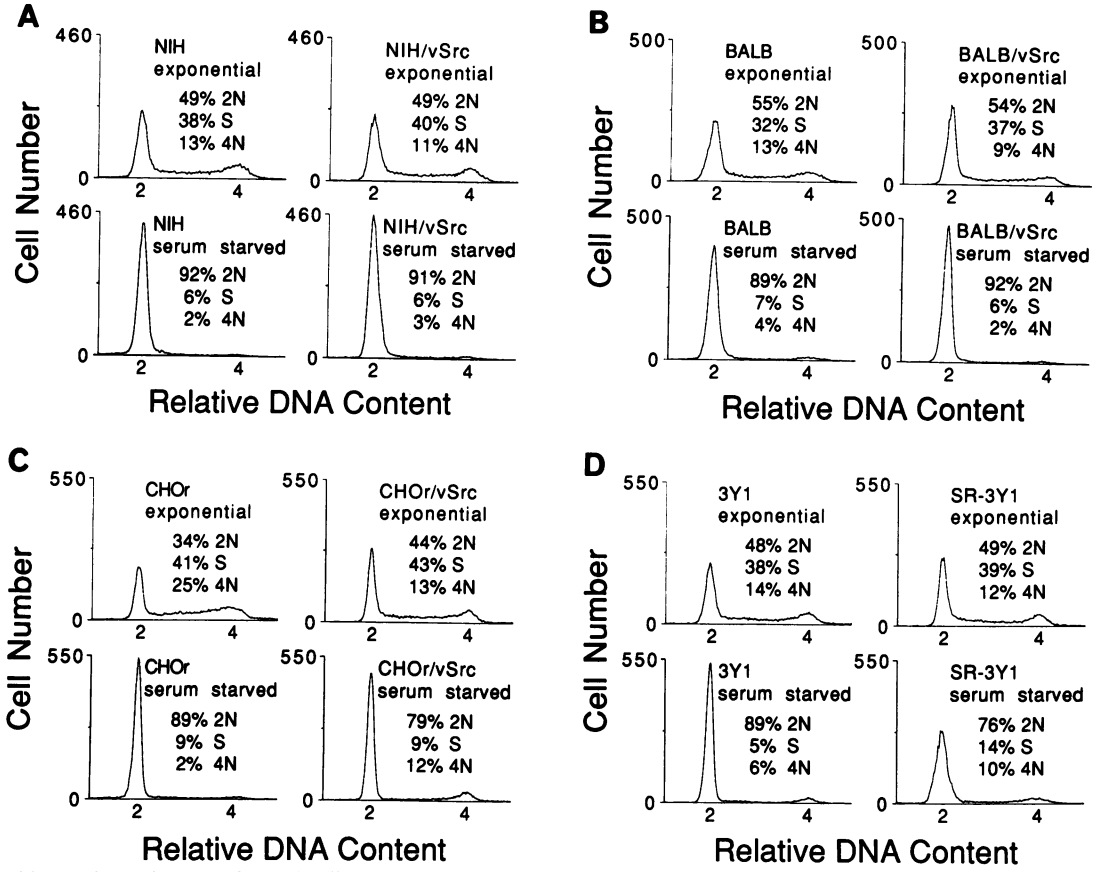

FIG. 2. Evidence that v-Src-transformed cells can be arrested by serum starvation. All cell lines shown in Fig. 1 were analyzed by flow cytometry to determine their cell cycle profiles under conditions of exponential growth or serum starvation as described in Materials and Methods. The average DNA content of cells (between 2N and 4N genome equivalents) was determined by fluorescence-activated cell sorting analysis and plotted as histograms. The percentages of cells in each phase of the cell cycle are given in the inset of each plot.

levels of protein phosphotyrosine in these cells, as determined by Western blot analysis with antiphosphotyrosine antibodies (62). Furthermore, cell cycle analysis confirmed that these cells could also be arrested effectively by serum starvation (not shown).

Proteins encoded in the cellular proto-oncogenes c-*sis*, c-*ras*, and c-*raf* have been implicated in the same mitogenic signal transduction pathways as the Src protein; therefore, we examined the effects of their oncogenic forms on c-*fos* induction by serum. As shown in Fig. 3B, serum inducibility of c-*fos* mRNA was attenuated in NIH 3T3 cell lines transformed by all three oncoproteins, albeit to different extents. The extent of attenuation did not correlate with the degree of transformation because the v-Raf cell line is highly transformed (47). Like v-Src-transformed cells, those transformed by v-Sis, v-Ras, or v-Raf exhibited no detectable elevation of c-*fos* transcripts prior to serum stimulation. Flow cytometric analysis demonstrated that these transformed cell lines could be arrested by serum starvation to a degree comparable to that seen in Fig. 2 (data not shown). These results are consistent with the notion that Src, Sis, Ras, and Raf proteins are involved in the same or related signaling pathways (6, 13).

Because all of the cell lines exhibiting attenuated c-*fos* induction are at least partially transformed, it was important to determine whether there is an obligatory correlation between attenuation of c-*fos* induction and cell transformation per se. For this purpose, we analyzed a well-characterized BALB/c 3T3 cell line (WTB1a) transformed by wild-type simian virus 40 (SV40), which is believed to transform cells through a mechanism different from that of v-Src (21, 38). As shown in Fig. 3C, cells transformed by SV40 exhibited no attenuation of c-*fos* induction. These results are consistent with the idea that SV40 transforms cells through a different signaling pathway and establish that attenuation of c-*fos* induction by serum is a direct consequence of the specific oncoproteins used to transform cells.

Attenuation of serum inducibility of immediate early genes occurs at the transcriptional level. In addition to c-*fos*, many other proven or putative transcription factors also belong to the immediate early gene family. Some of these, including c-*jun* and *junB*, have known oncogenic forms (36, 50), whereas others, such as *egr-1/NGFI-A* (7) and *NGFI-B/nur77* (16), do not. To determine whether attenuation of serum inducibility also extends to these immediate early genes, Northern blot analyses were performed by using probes specific for the individual transcripts. As shown in Fig. 4, serum inducibility of c-*jun*, *junB*, *egr-1*, and *NGFI-B* transcripts was attenuated in v-Src-transformed SR-3Y1 cells. By contrast, constitutively elevated expression of GAPDH in these transformed cells compared with their normal counterparts indicated that the attenuation was not due to a general reduction in the overall levels of gene expression. In addition, NIH 3T3 cells stably transformed by

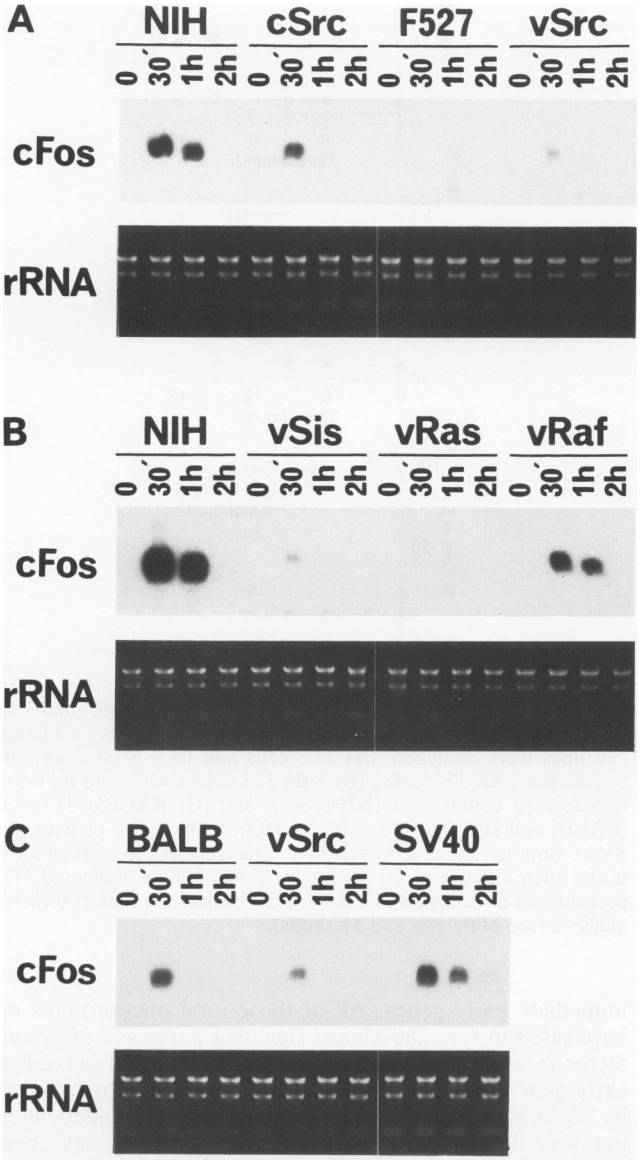

FIG. 3. Attenuation of c-*fos* mRNA induction by serum in cells transformed by various oncoproteins. Serum-starved cells (0 time point) were stimulated with medium containing 20% FBS for 30 min (30′), 1 h, or 2 h. Total RNAs (5 μg) were analyzed by Northern blotting, using a c-*fos*-specific probe as described for Fig. 1. Parallel gels were stained with ethidium bromide to confirm RNA normalization. (A) Comparison of the effects of different Src proteins on c-*fos* induction by serum. Four cell lines were used: NIH 3T3 cells (NIH), NIH 3T3 cells overexpressing normal c-Src (cSrc), NIH 3T3 cells transformed by an activated c-Src mutant with a Y527-to-F527 substitution (F527), and NIH 3T3 cells transformed by v-Src (vSrc). (B) Comparison of the effects of different oncoproteins on c-*fos* induction by serum. The cell lines analyzed were NIH 3T3 cells and NIH 3T3 cells transformed by v-Sis, v-Ras, or v-Raf. The film was overexposed to show the slight c-*fos* induction in v-Sis-transformed cells. (C) Comparison of the effects of SV40 transformation on c-*fos* induction by serum. The cell lines used were BALB/c 3T3 cells (BALB) and BALB/c 3T3 cells transformed by v-Src or wild-type SV40.

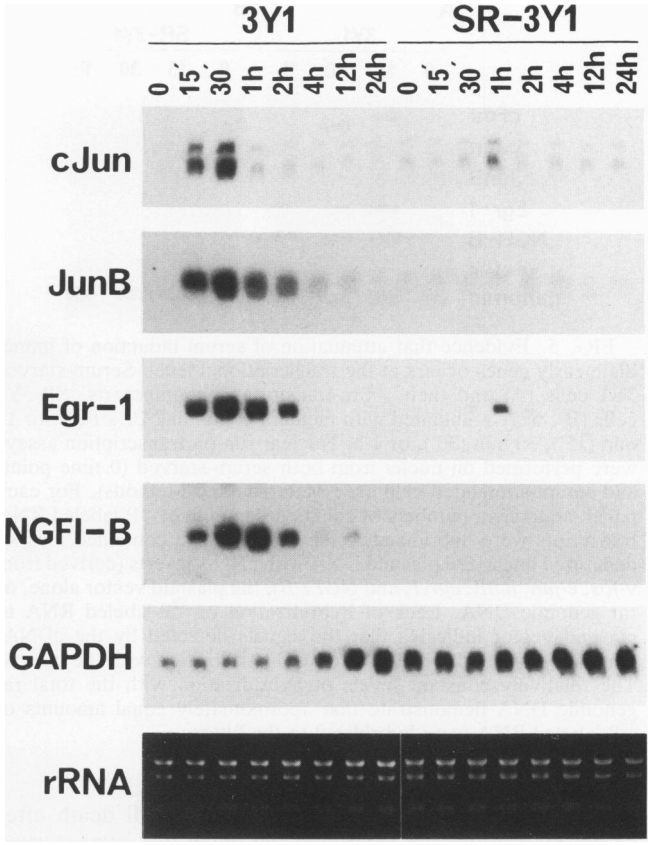

FIG. 4. Attenuation of serum inducibility of c-*jun*, *junB*, *egr-1*, and *NGFI-B* by v-Src transformation. 3Y1 cells and their v-Src-transformed counterparts, SR-3Y1 cells, were used in serum stimulation experiments (see the legend to Fig. 1). Total RNAs (5 μg) were analyzed by Northern blotting, using probes specific for c-*jun*, *junB*, *egr-1/NGFI-A*, or *NGFI-B/nur77* (see Materials and Methods). Normalization of RNA samples was confirmed by probing Northern blots for GAPDH mRNA and by ethidium bromide staining. Note that levels of GAPDH expression are constitutively elevated in cells transformed by v-Src compared with normal cells.

v-Sis, v-Ras, or v-Raf also showed the same pattern of attenuation (data not shown). These results demonstrate that attenuation of serum inducibility is not restricted to c-*fos* but is a general property of other immediate early genes.

Nuclear run-on assays were performed to determine whether inducibility of these immediate early genes is attenuated at the transcriptional or posttranscriptional level. Figure 5 shows that the transcription rates of all of the immediate early genes were elevated following serum stimulation of normal 3Y1 cells. In striking contrast, there were no detectable increases in the transcription rates of these genes when v-Src-transformed SR-3Y1 cells were stimulated with serum. As a control, hybridization of run-on transcripts to total rat genomic DNA showed that approximately equal amounts of ^{32}P-labeled RNA were hybridized to the filters (Fig. 5). These results indicate that the attenuation of serum inducibility of immediate early genes occurs at the transcriptional level.

Serum-starved v-Src-transformed cells begin cycling in response to serum stimulation. Previous studies indicated that Rat-1 fibroblasts expressing high levels of c-Myc protein are more prone to cell death (or apoptosis) upon serum deprivation (15). To determine whether attenuation of serum induc-

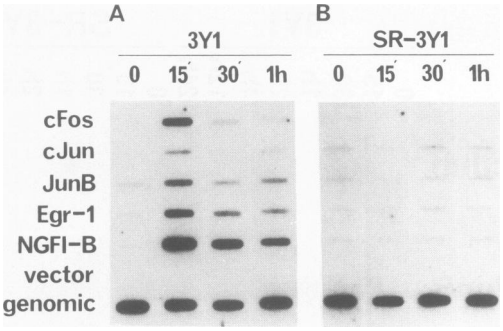

FIG. 5. Evidence that attenuation of serum induction of immediate early genes occurs at the transcriptional level. Serum-starved 3Y1 cells (A) and their v-Src-transformed counterparts, SR-3Y1 cells (B), were stimulated with medium containing 20% FBS for 15 min (15'), 30 min (30'), or 1 h. Nuclear run-on transcription assays were performed on nuclei from both serum-starved (0 time point) and serum-stimulated cells (see Materials and Methods). For each panel, equivalent numbers of counts per minute of ^{32}P-labeled RNA transcripts were hybridized to the filters, which contained 5 μg of denatured linearized plasmid DNA with cDNA inserts (derived from v-*fos*, c-*jun*, *junB*, *egr-1*, and *NGFI-B*), the plasmid vector alone, or rat genomic DNA. Lack of hybridization of ^{32}P-labeled RNA to plasmid vector indicates that the signals detected by the cDNA-containing plasmids are the result of hybridization with the inserts. The relatively constant levels of hybridization with the total rat genomic DNA demonstrate that approximately equal amounts of ^{32}P-labeled RNA were hybridized to the filters.

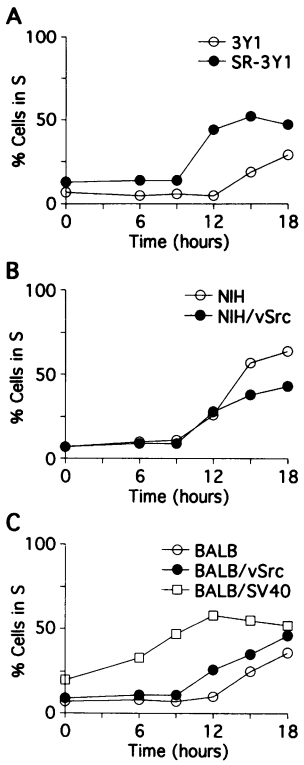

FIG. 6. Evidence that serum-deprived v-Src-transformed cells are viable and responsive to serum growth factors. Seven different cell lines were analyzed: (A) 3Y1 cells and its v-Src-transformed counterpart, SR-3Y1 cells, (B) NIH 3T3 cells (NIH) and its v-Src-transformed counterpart (NIH/vSrc), and (C) BALB/c 3T3 cells (BALB) and BALB/c 3T3 cells transformed by v-Src or wild-type SV40. Both normal and transformed cells were serum starved for 48 h and then stimulated by serum for 6 to 18 h, as indicated. The percentages of cells in S phase were determined by flow cytometric analysis (see Materials and Methods).

ibility of immediate early genes is due to cell death after serum starvation, we examined the entry of serum-starved normal and transformed cells into S phase after serum stimulation. As shown in Fig. 6, v-Src-transformed cells progressed into S phase with either faster kinetics (in the case of 3Y1 and BALB/c 3T3 cells) or the same kinetics (in the case of NIH 3T3 cells) as did their normal counterparts. Consistent with these cell cycle analyses, serum-starved SR-3Y1 cells remained greater than 90% viable by the trypan blue exclusion assay and showed normal doubling times by growth curve analysis after replating of the cells into complete culture medium at low cell densities (data not shown). These results demonstrate that serum-starved v-Src-transformed cells are viable and responsive to serum stimulation and that the attenuation of immediate early gene induction is not the result of cell death. In addition, this attenuation is not correlated with the rate of entry into S phase, since the SV40-transformed cells enter S with the most rapid kinetics (Fig. 6C) yet retain a normal immediate early gene response (Fig. 3C).

DISCUSSION

Mitogenic signal transduction initiated by activation of growth factor receptors at the plasma membrane ultimately leads to the induction of immediate early gene expression in the nucleus (26). This induction of immediate early genes is thought to be essential for stimulation of cell proliferation by growth factors. Oncoproteins may usurp normal restraints on cell proliferation by chronic stimulation of the mitogenic signaling pathways used by growth factor receptors. These considerations prompted us to study how oncoproteins affect regulation of immediate early genes in response to growth factors in serum. Our results demonstrate that the oncoproteins v-Src, v-Sis, v-Ras, and v-Raf attenuate serum inducibility of the endogenous c-*fos* gene and several other immediate early genes. All of these viral oncoproteins are implicated in tyrosine kinase signaling pathways of growth factor receptors. In contrast, this attenuation of immediate early gene induction was not observed in cells transformed by SV40, which may transform cells through an independent pathway. Results similar to those reported here have been obtained by Bravo and coworkers, who found that serum inducibility of c-*fos*, *fosB*, *Krox24*, *N10*, and *junB* expression was attenuated in NIH 3T3 cells transformed by v-Src or v-Ras (3). In addition, earlier studies reported that v-Ras inhibits induction of c-*fos* expression by PDGF in rodent fibroblasts (63). While the physiological significance of these findings remains to be determined, the strikingly consistent pattern of our results suggests that a fundamental regulatory mechanism of mitogenic signaling pathways is perturbed by activated oncoproteins.

Several of the immediate early genes encoding transcription factors have been shown to be important in regulating normal cell proliferation and differentiation. For example, previous studies demonstrated that the Fos and Jun protein families are required for cell cycle progression and for entry into S phase in normal fibroblasts (33). Therefore, it is notable that serum-starved, v-Src-transformed cells progressed into S phase after serum stimulation (Fig. 6), even though the induction of several immediate early genes, including c-*fos* and c-*jun*, was greatly attenuated. It is

important to emphasize, however, that our findings do not rule out an essential role for Fos and Jun family proteins in Src transformation. Functions essential for cell cycle progression may be regulated at the level of Fos and Jun proteins, perhaps by posttranslational modification, independently of transcriptional regulation. In this case, it is possible that low levels of immediate early gene expression are sufficient for cell cycle progression in both normal and transformed cells. Alternatively, transformed cells may have reduced requirements for immediate early gene expression during cell cycle progression compared with normal cells.

Earlier studies showed activation of the c-*fos* promoter by v-Src in transient cotransfection experiments and increased c-*fos* expression after transient activation of the tyrosine kinase activity of temperature-sensitive v-Src mutants (18, 23, 28, 55). However, contrasting results also have been reported indicating a lack of increased c-*fos* expression in response to v-Src (61). Our results demonstrate that in four different cell lines stably transformed by v-Src, there were no detectable c-*fos* transcripts during serum starvation (Fig. 1). We have also examined the steady-state levels of c-*fos* transcripts in these four cell lines under conditions of exponential growth in complete medium containing 5 or 10% serum. Northern blot analysis indicated that there was no consistent elevation in basal levels of c-*fos* mRNA among the various v-Src-transformed cells compared with their nontransformed counterparts (62). Therefore, elevation of endogenous c-*fos* expression is not a general property of cells chronically transformed by v-Src. This observation stands in apparent contrast to reports using transient v-Src expression in cotransfection assays with c-*fos* promoter constructs or transient activation of v-Src mutants by temperature shift. One plausible explanation for this discrepancy is that the intracellular environment of chronically transformed cells differs in critical ways from that of cells which only transiently express active v-Src. Alternatively, it is possible that elevated c-*fos* expression is required for initiation of transformation (mimicked by transient v-Src expression) but not for maintenance of transformation. In contrast to the results with c-*fos*, expression of the endogenous *junB* gene is constitutively elevated in exponentially growing cells stably transformed by v-Src (1).

While it may be argued that the attenuation of serum inducibility of immediate early genes reported here is due to an inability of transformed cells to enter a quiescent state after serum starvation, this explanation is not supported by all of the available data. Our flow cytometric analysis demonstrated that the majority of transformed cells are arrested by serum starvation in G_1 phase with a 2N DNA content before initiation of DNA synthesis (Fig. 2). Nevertheless, we cannot exclude the possibility that transformed cells are arrested at a point in G_1 phase distinct from that of normal cells. Consistent with this possibility, we found that serum-starved SR-3Y1 and BALB/v-Src cells progressed faster into S phase after serum stimulation than did their normal counterparts (Fig. 6). Earlier studies showed that induction of DNA synthesis in quiescent normal BALB/c 3T3 cells can be resolved into distinct phases with different requirements for PDGF, epidermal growth factor, and supraphysiological concentrations of insulin (37, 46, 48). Using this classical system, we determined that serum-starved, v-Src-transformed BALB/c 3T3 cells require only epidermal growth factor and insulin to enter S phase, suggesting that these cells are blocked in early G_1 at a point closer to S than are quiescent normal cells (62). In contrast, we determined that SV40-transformed BALB/c 3T3 cells require only insulin to enter S after serum starvation, suggesting that they are blocked in late G_1 at a point even closer to S than are the v-Src transformed cells. Together with our finding that serum inducibility of c-*fos* occurs normally in SV40-transformed cells (Fig. 3C), these results provide compelling evidence that c-*fos* induction does not require a quiescent state unique to normal cells. Consistent with this conclusion, other studies have shown that arrest of cells at any particular point in the cell cycle is not a requirement for c-*fos* induction by serum because this induction can occur equally well throughout the cell cycle (4).

In the case of c-*fos*, the SRE and regulatory proteins that bind to it have been shown to have important roles in serum inducibility of this gene (53, 58, 59). We have determined that attenuation of serum inducibility of immediate early genes occurs at the transcriptional level (Fig. 5), suggesting that these transcriptional components may be involved. However, it is important to note that some immediate early genes, such as *junB* (1, 50), lack a discernible SRE in their promoter regions, and thus the mechanisms of how these genes are induced by serum remain to be determined. In addition, it has been shown that overexpressed c-Fos protein negatively regulates its own gene's promoter soon after induction of c-*fos* transcripts by serum stimulation (40, 52). It appears unlikely that the attenuation reported here is due to such an autoregulatory mechanism, however, because there was no detectable elevation of c-*fos* or other immediate early gene expression in the transformed cells prior to serum stimulation. Instead, our results point to the existence of a global regulatory mechanism, shared by all of the immediate early genes examined, which is perturbed by various oncoproteins. This global regulator is a potential target for both activation of immediate early genes in normal cells and their attenuation in chronically transformed cells in response to serum growth factors. In this regard, it is of interest that a seven-nucleotide motif present in the 3′ untranslated region of a large set of immediate early genes, including all those studied here, has been implicated in growth factor induction of these genes (17). Because attenuation of serum inducibility involves oncoproteins that chronically stimulate tyrosine kinase signaling pathways, this attenuation could reflect a negative regulatory mechanism acting on signaling pathways normally stimulated by growth factor receptors. We are further characterizing the molecular mechanisms of this attenuation, which may have important implications for understanding signal transduction pathways controlling cell proliferation in response to growth factors and oncoproteins.

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