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ANTISENSE RNA TO THE C-FOS GENE: RESTORATION OF DENSITY-DEPENDENT GROWTH ARREST IN A TRANSFORMED CELL LINE

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Fibroblasts transformed by v-sis have elevated levels of c-fos relative to non-transformed controls. Transfection and integration of plasmids directing the synthesis of antisense RNA to the c-fos gene leads to restoration of density-dependent growth arrest in monolayer culture, but does not inhibit colony formation in soft agar.

The normal function of the proto-oncogene c-fos is not known in detail although considerable evidence suggests a role in cell cycle regulation (1-9). For example, agents such as PDGF that confer a state of "competence" on murine fibroblasts rapidly induce a large transient rise in c-fos expression (6-7) and, using antisense RNA-expressing plasmids, evidence has been presented that c-fos expression is indeed necessary for the progression of quiescent cells through the cell cycle (8). Further, it has been shown that elevated levels of c-fos expression in certain transfected cells causes extensive proliferation as growth foci in transformation assays (9).

These considerations may be relevant to the observations that numerous human and animal tumors exhibit autocrine stimulation based on the synthesis of PDGF-like molecules (10-17). Based on the induction pattern of the c-fos gene activity it has been proposed that c-fos and other PDGF inducible genes likely mediate the proliferative changes stimulated by the PDGF-like molecules which are produced by autocrine stimulated cells (18). As another example, human osteosarcoma cells in culture (U2-OS) also have been shown to exhibit

ABBREVIATIONS: v-sis, transforming gene of the simian sarcoma virus, SSV; PDGF, platelet-derived growth factor; neo, neomycin resistance bacterial gene; sof, antisense fos; MT, metallothionein gene; PBS, phosphate-buffered saline; DME, Dulbecco's modified Eagle's medium; TGFβ, transforming growth factor β; G-418, Geneticin, an aminoglycoside antibiotic; SSV-NIH, v-sis transformed NIH-3T3 cells, subclone; SSVn, pSV2neo transfected SSV-NIH cells; SSVnMT, pSV2neo/EV142 (28) co-transfected SSV-NIH cells; SSVnsof, pSV2neo/pSVsof co-transfected SSV-NIH cells.

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autocrine PDGF (α-chain homodimer) stimulation (17) and osteosarcoma is the principal tumor type induced in murine species by v-fos-bearing viruses (19).

A unifying hypothesis of one role of PDGF-like molecules in these systems is that they promote constitutively elevated levels of c-fos expression which in turn cooperates to promote a rapidly proliferating state. We have tested this hypothesis by using a model system based on SSV-NIH cells which are known to be stimulated in an autocrine fashion by a PDGF-like molecule, the beta chain homodimer of PDGF (16). The hypothesis that c-fos expression is a necessary and cooperating part of the complete v-sis transformed phenotype has been studied using antisense-fos-RNA expressing plasmids.

We have prepared plasmids directing the transcription of RNA complementary (antisense RNA) to a 1.7 Kb portion of the 5′ end of the c-fos gene. The major construct contains an SV40 viral promoter and is known as pSVsof (20-21). This plasmid has been used in co-transfection studies with pSV2neo to produce a series of stable G-418 resistant clones (22). Characterization studies which are being prepared for publication elsewhere (22) have shown that c-fos is elevated in SSV-NIH cells, that plasmid DNA can be stably inserted in these cells and that the levels of c-fos transcripts and c-fos protein then become markedly reduced. We now show that the decreased c-fos expression (22) is associated with marked phenotypic changes including restoration of density-dependent growth arrest.

MATERIALS AND METHODS

Cells. The NIH-3T3 cells used here are a clonal line obtained from Dr. R. Muller, EMBL, Heidelberg. The SSV-NIH cells were originally prepared by Dr. S. Aaronson (N.I.H.) and were provided by Dr. E. Adamson. These cells have been used for the preparation of a series of antisense fos RNA expressing cells (denoted by sof) by co-transfection with pSVsof and pSV2neo as described elsewhere (20-22). Six stable and G-418 resistant clones (SSVnsof, Cls. A,F,G,I,P and S) have been studied in detail. As controls, pSV2neo alone or pSV2neo or with a plasmid containing the MT promoter and irrelevant sequences derived from portions of pBR322 (20-21) have been used to develop additional clonal lines by co-transfection, called SSVn or SSVnMT, respectively.

Cell Culture and Kinetics. For kinetic studies, freshly harvested cells using trypsin (ATV, Irvine Scientific Labs., Irvine, CA) 1:5 with PBS were seeded into wells of a 24-well plate (Flow Labs. Ins., VA) at 25 x $10^3/cm^2$ and grown in 1:0 ml of DME medium with high glucose (4.5 g./L), 10% (v/v) bovine fetal serum (Hyclone Labs. Inc., Utah), 10^4 IU/L. penicillin, 10^4 μg/L. streptomycin, and, for pSV2neo derived lines, 100 μg/ml. G-418 (Gibco Labs Inc., NY) and maintained at 37°C in 10% (v/v) CO_2. For proliferation measurements, four wells per time point were harvested and transferred to 10 mls of isoton (Fisher Chem Corp.) for counting using a Coulter Counter. All determinations were corrected for machine efficiency and non-cellular counts using a channel analyzer and expressed as cell density (cells/cm^2 of culture surface) $\pm$ SD_j (standard deviation). Average growth curves (Fig. 1) were obtained by combining the growth curves of individual clones according to the

expression $\bar{\rho} = \sum_{j}^{n} \rho_j \left(\frac{1/SD_j}{\Sigma 1/SD_j} \right)$ where ρ_j is the average cell density at t for clone j and SD_j is the corresponding standard deviation. The averaged standard deviations at t were obtained similarly with the SD_j in place of ρ_j which therefore reduces to the expression $\overline{SD} = n/\Sigma\ (1/SD_j)$. Doubling times, T, were determined graphically from the slopes of plots of ln ρ vs. t using the relation T = ln2/slope.

Clonogenic Assays in Soft Agar. Freshly harvested cells were suspended in 0.25 % (w/v) agarose (Sigma) in complete DME medium at 2.5 - 25 x 10^3 cells/ml and were layered (1.5 mls) into 35 mm plastic petri dishes previously layered with 0.5% agarose in the same medium and incubated as above. All plates were inspected prior to culture for inadvertent aggregates and all cells unused for the assay were returned to tissue culture to ensure viability. The dishes were fed every seven days and scored on day 14. Colony forming efficiency is expressed as the percent of seeded cells that grow to colonies $\geq$ 25-30 μM in diameter in 14 days.

Cytofluorimetry. Freshly harvested cells after various times in cultures seeded at 27 x 10^3 cells/cm^2 were washed twice in PBS, fixed by dropwise addition of 7 ml cold ethanol to 3 ml of continuously agitated cell suspensions to a final cell concentration of 0.2 - 4 x 10^6 cells/ml, washed three times with PBS, resuspended in 10 mls of PBS at 10-50 μg/ml propidium iodide and 10 μg/ml ribonuclease, incubated at 37°C for 10 min and analyzed using cytofluorograph systems 50-H (Ortho Diagnostic, Westwood, Mass.).

RESULTS

Transfection with control plasmids does not alter growth. The growth characteristics for the antisense RNA expressing cells and two control cell lines are summarized as average growth curves in Fig. 1A. Both control curves SSVn and SSVnMT are similar to each other. Further, as suggested by the average standard deviations (bars, Fig. 1A), the growth properties of each clone making up these curves are also similar. Since we have measured cell number, the doubling time for the cell population can be estimated reliably (Fig. 1, inset). The control lines yield a value of ca 11 hours comparable to that for the parent SSV-NIH cells and many other rapidly growing and transformed fibroblast lines (23). There is no evidence of a density-limited stage. The control cells grow to 450 x 10^3 cells/cm^2 or more, show extensive piling-up with focus formation (Fig. 2), deplete and acidify the medium and then rapidly deteriorate. Daily feeding with a 1/10 volume of fresh medium does not alter these observations. The features are typical of aggressively growing transformed cells. Thus, the results, which include growth curves determined at different passage numbers, suggest that the transfection and selection procedures used here lead to stable clonal lines with similar growth properties that are not significantly altered from the parent line.

Sof cells appear density arrested. In contrast to the control cells, SSVnsof cells show a number of differences (Fig. 1A). Upon seeding at low density, the cells exhibit a lag phase characterized by a decrease in cell numbers. Following recovery the cells enter a typical log-phase of growth

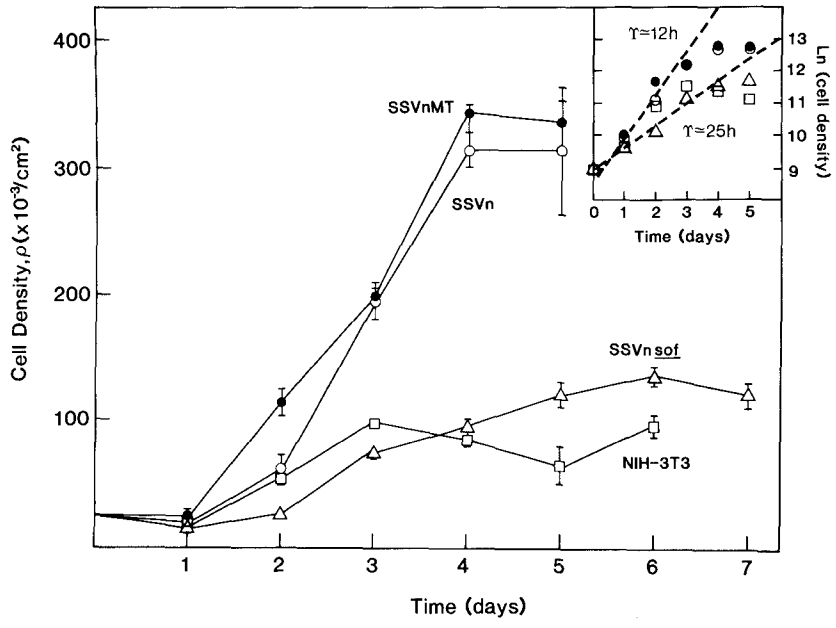

Figure 1. Kinetics of proliferation of transfected SSV-NIH cells. The growth curves for each SSV-NIH line are averages of multiple clones. Controls, ●—● SSVnMT, clones 1,2 & 3; ○—○ SSVn, clones 1,2,3,5 & 6. Antisense, △—△ SSVnsof, clones A,G & I. □—□ NIH-3T3 fibroblasts. Inset, Natural log plot of same data with limiting slope of log phase growth indicated by the straight line. T, doubling time.

(Fig. 1, inset) similar in duration to control cells, but with an extended doubling time of 25 hours. Further, after 3 days in culture the growth rate decreases further and cell numbers become relatively fixed in the 100 - 150 x $10^3/cm^2$ range, values only slightly greater than NIH-3T3 cells (Fig. 1A). Several observations argue that this phase is not due to depletion of the medium, toxic secretion, toxicity associated with antisense RNA expression or other untoward effects: (1) the plateau is established at a time when control cells growing in identical medium attain average densities 3.5 times that of the SSVnsof clones, (2) replicate experiments fed daily for a week by addition of 1/10 volume of fresh medium obtained a maximum density of 16% above that of unfed cells, (3) cells harvested during the plateau phase and recultured at low (25 x $10^3/cm^2$) cell densities again regrow to the same limiting density. Thus the antisense expressing cells taken from the plateau stage are perfectly capable of division and proliferation once reduced in density.

Further cytofluorimetric analyses of cells in early plateau stage (day 3) shows an average (three SSVnsof clones) ratio of 3.37 ± 0.42 for the proportion of quiescent ($G_0 + G_1$) cells to cycling ($S + G_2 + M$) cells compared to 1.70 ± 0.06 for SSV-NIH cells of the same culture age or 2.14 ± 0.47 for the same SSVnsof clones in log phase (day 2). The ratio is 10 to 1 in late

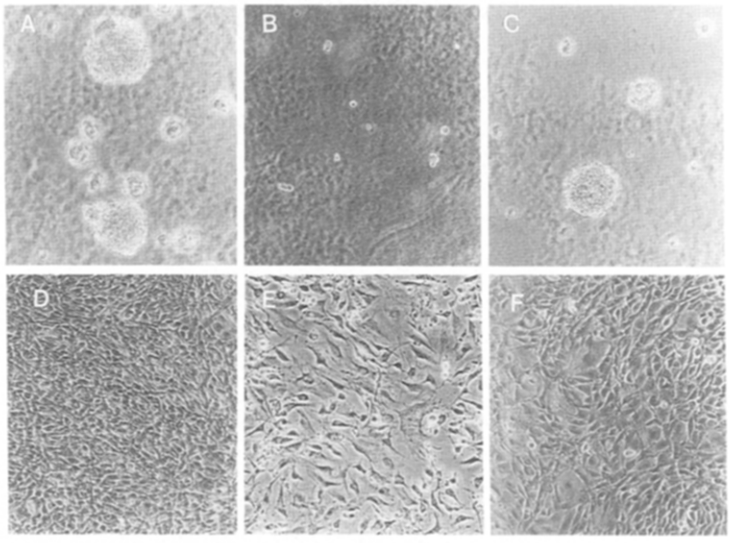

Figure 2. Examples of phase contrast micrographs of SSV-derived clones in soft agar, day = 7 (top) and in monolayer culture, day = 5 (bottom). Control cells (here SSVnMT3); B & E, NIH-3T3 cells; C & F, antisense RNA expressing cells (here SSVnsof G).

plateau stage SSVnsof cells. Finally, these cells show distinctive morphological changes from control cells (Fig. 2). They appear larger and flatter than the control cells and form uniform monolayers at/or near maximum cell densities. In contrast, the refractile spindle-shaped morphology of the control cells is apparent even at low density (Fig. 2) and these form interlacing layers at high densities. However, these distinctions are confined to culture on adhesive plastic.

Sof cells remain transformed. Freshly harvested SSVnsof cells are clonogenic (Fig. 2) in soft agar with high efficiency. The average colony-forming efficiency after two weeks of culture in soft agar is 3% (three clonal lines) compared to an average of 7 % (five clonal lines) for the control lines. The observation argues that the underlying transforming principle associated with v-sis transformation remains intact in SSVnsof cells. This is supported by northern transfer analysis of polyadenylated RNA isolated from 6 SSVnsof clonal lines (22) which confirms that the complete v-sis gene is actively transcribed in these lines. It appears therefore that fos antisense RNA expression has led to a preferential alteration of growth properties consistent with a restoration of density-dependent growth control without altering the underlying transformed nature of these cells.

DISCUSSION

The studies described here provide evidence that v-sis-transformed cells which constitutively express elevated c-fos products (22) show a marked

decrease in growth following transfection with plasmids directing the expression of antisense RNA to the c-fos gene. These cells exhibit a normal log-phase, but with an extended doubling time and reproducibly exhibit a marked slowing of growth at ca 100-150 x 10^3 cells/cm^2, characteristics similar to NIH-3T3 cells. Morphologically these cells have much less tendency to pile-up. Cytofluorimetric analyses confirms that even early plateau stage cells are predominantly in G_0/G_1 stages of the cell cycle and strongly supports the conclusion that the plateau stage is largely due to density arrest. Cells derived from apparently density-arrested cultures readily regrow to a similar density and a similar value is obtained when cells are grown in fresh or supplemented medium.

Our results implicate a density-dependent phenomenon in the growth regulation of pSVsof transfected cells. When taken together with the biochemical evidence of markedly reduced c-fos expression in these cells (22), the results emphasize the possibility that exit from the cell cycle may involve an interplay of signals derived from cell-cell contacts and a negative regulatory control mechanism for the c-fos gene. This view is therefore entirely complementary to the previous conclusion that the rapid rise in c-fos activity promoted by addition of PDGF to quiescent cells may be a prerequisite for re-entry of these cells into the cell cycle (8,27). In terms of the transformed phenotype, the results suggest that constitutively elevated c-fos activity may serve to overcome negative regulatory signals associated with cell-cell interactions, thereby bypassing the G_0 state and promoting a continuously cycling population of cells. In this way, the c-fos gene may cooperate with v-sis to bring about the complete transformed phenotype of v-sis transformed cells.

The basis of colony growth in SSVnsof cells is of interest and raises the possibility of the involvement of TGF-β or a similar factor. TGF-β is produced by numerous transformed cells (24) including SSV-NIH cells (25). For several normal and transformed epithelial cells and certain mesenchymal cells (24,26) TGF-β is a potent inhibitor of monolayer growth but in several cases, including some of the same epithelial and mesenchymal cell lines, TGF-β stimulates colony formation in soft agar (24). Further, elevated expression of both c-sis and c-fos may be important for the stimulatory action of TGF-β (18). It is conceivable, therefore, that antisense RNA, by lowering c-fos expression restores TGF-β regulated growth in monolayer culture. It will be of interest to determine the level of c-fos expression in the SSVnsof clones under conditions of anchorage independent growth.

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