Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4794–4798, July 1986
Cell Biology

Inducible production of c-fos antisense RNA inhibits 3T3 cell proliferation

(protooncogenes/gene transfer/mouse mammary tumor virus/cell division/dexamethasone)

J. T. HOLT, T. VENKAT GOPAL, A. D. MOULTON, AND A. W. NIENHUIS

Clinical Hematology Branch, National Heart, Lung and Blood Institute, Bethesda, MD 20894

Communicated by Donald S. Fredrickson, March 11, 1986

ABSTRACT **Antisense RNA complementary to c-fos mRNA was produced in mouse 3T3 cells by gene transfer techniques. Transcriptional units were constructed consisting of a steroid-inducible mouse mammary tumor virus (MMTV) promoter, mouse or human 5′ c-*fos* gene fragments in either the sense (5′ to 3′) or antisense (3′ to 5′) orientation, and splice and poly(A) signals from the human β-globin gene. A gene that confers neomycin resistance was included in the vectors to allow isolation of stable transformants. Dexamethasone caused a marked induction of hybrid MMTV-fos-globin RNA. Induction of the hybrid transcript containing antisense c-fos RNA decreased colony formation following DNA transfer and inhibited the proliferation of cells into which the antisense transcriptional unit had been integrated. In contrast, colony formation and cell proliferation were not inhibited by induction of hybrid RNA containing c-fos RNA sequences in the sense orientation. These results indicate that the strategy of generating antisense RNA to inhibit gene expression may be useful in delineating the function of protooncogenes. The c-*fos* gene product appears to have a required role in normal cell division.**

Protooncogenes are thought to have regulatory roles in normal cellular proliferation and differentiation but to contribute to neoplastic transformation in their mutant forms (1–3). Protooncogene products may be grouped into four general categories: growth factors, growth factor receptors, membrane-associated GTP-binding proteins, and nuclear DNA-binding proteins. A specific function has been assigned to three protooncogene products; c-*sis* encodes one of the polypeptide chains of platelet-derived growth factor (4, 5), c-*fms* encodes the receptor for a hematopoietic regulator (6), and c-*erbB* encodes the receptor for epidermal growth factor (7). Precise functions for other protooncogenes are largely unknown but have been inferred from the pattern of tissue and cell expression and by observed variations in gene expression following mitogenic stimulation (2, 3, 8). The belief that these genes serve essential roles in cell growth regulation is further supported by their ability to transform cells in their mutated forms.

If protooncogenes have essential roles for normal cellular proliferation, then inhibition of their expression should alter cell growth. Specific antibodies may be used to block growth factor action (9–11), and microinjection of antibodies directed against the c-*ras* gene product has been shown to block the mitogenic response of 3T3 cells to serum (12). However, antibodies have limited value in analyzing the function of intracellular proteins. The use of "antisense" RNA has been proposed as a general method for inhibiting function of specific genes (13–15). RNA molecules containing sequences complementary to a portion or all of the RNA transcribed from a specific gene have been used to decrease the concentration of that gene's product. Inhibition apparently results from formation of RNA·RNA duplexes between the antisense RNA and RNA transcribed from the target gene (15). Such RNA·RNA duplexes have been shown to inhibit mRNA translation but the intranuclear location of duplexes suggests that RNA processing or transport might also be affected by antisense RNA (16). A large excess of antisense RNA compared to cellular mRNA may be necessary and sequences complementary to the 5′ end of the gene transcript may be most inhibitory of gene expression (13, 15). Transcriptional units that generate appropriate antisense RNAs have been introduced by DNA transfection and shown to inhibit expression of the cellular genes for thymidine kinase and actin (13, 14, 16), suggesting that protooncogene function might be studied by this approach.

The c-*fos* gene is the cellular homologue of the transforming gene of the Finkel–Biskis–Jinkins (FBJ) osteosarcoma virus and encodes a 55-kilodalton protein that is localized to the nucleus (17). c-fos mRNA is present in growing fibroblasts but disappears with quiescence (18). Induction of c-fos mRNA and protein to levels much higher than those present in dividing cells occurs within minutes of stimulation of growth-arrested 3T3 cells by platelet-derived growth factor and other growth factors (19, 20). Similar induction of c-fos mRNA has been observed in A431 cells on stimulation with epidermal growth factor, although the effect on cell growth is variable; some clones are inhibited, whereas others are stimulated (21). An association between cell differentiation and c-*fos* gene expression has also been inferred because of a rapid increase in c-fos mRNA on induction of HL60 promyelocytic leukemia cells to monocytic maturation (22–24). Increased production of c-fos mRNA causes F9 embryonal carcinoma cells to differentiate (25, 26) and is associated with neuronal differentiation of PC12 pheochromocytoma cells (27–29). These diverse results are difficult to reconcile with a single function for the c-*fos* gene and, indeed, a causal relationship between c-*fos* gene expression and altered cell behavior has usually not been demonstrated.

To determine whether the c-*fos* gene product is essential in growing fibroblasts, we introduced constructs that allowed production of c-fos antisense RNA in stably transformed cells. Because of the distinct possibility that inhibition of c-*fos* gene expression would be lethal to cells, the production of antisense transcripts was regulated by a steroid-inducible mouse mammary tumor virus (MMTV) promoter. Our results suggest that c-*fos* gene expression is required for normal cell proliferation.

EXPERIMENTAL METHODS

Plasmid Constructions. Vectors capable of generating RNA molecules containing sequences complementary to portions

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Abbreviations: MMTV, mouse mammary tumor virus; CAT, chloramphenicol acetyltransferase; bp, base pair(s); ^{S}, sense; ^{AS}, antisense.

of the c-*fos* gene were constructed by standard recombinant DNA techniques. The details are provided in Fig. 1 and its legend. The structure of these recombinants was verified by restriction enzyme digestion of isolated DNA. Three fragments were used to construct pMMTVCat: a linker-containing *Sal* I–*Hin*dIII MMTV promoter fragment, a *Hin*dIII–*Sal* I fragment containing coding sequences for chloramphenicol acetyltransferase (CAT) derived from pRSVCat (35), and *Sal* I-linearized pUC-9.

Cell Culture and Nucleic Acid Analysis. Mouse Swiss 3T3 cells were grown in improved minimal essential medium with 10% fetal calf serum. A clone of 3T3 cells (D1) that grew well in 1 μM dexamethasone was isolated by limiting dilution. For transfection, cells were exposed to calcium phosphate-precipitated DNA for 4 hr followed by glycerol shock (36). The following day, the cells were divided into two aliquots and grown subsequently in 1 mg of the antibiotic G418 per ml; dexamethasone (1 μM) was added to one aliquot. Stable transformants obtained after transfection with DNA of either pHfos^{AS} (antisense) or pHfos^{S} (sense) were selected from plates grown without dexamethasone and expanded under continued G418 selection. DNA and RNA were isolated as described (34, 37). S1 nuclease analysis, RNA transfer blot analysis, and Southern blot analysis of DNA were performed by using standard methodology (37–40). Growth curves were performed by plating 2×10^5 cells in each of several 6-cm Petri dishes. At various time intervals, cells were removed from duplicate plates by trypsinization and washed twice in Hepes-buffered saline (pH 7.4). Cell counts were determined and protein determinations were performed on cell pellets. Duplicate samples were harvested at each time point.

RESULTS

Inducible Production of Hybrid Hfos^{S} and Hfos^{AS} RNA in 3T3 Cells. The presence of the transcriptional units producing the MMTV-fos-globin hybrid RNAs was inferred by isolation of colonies that were resistant to G418 and confirmed by

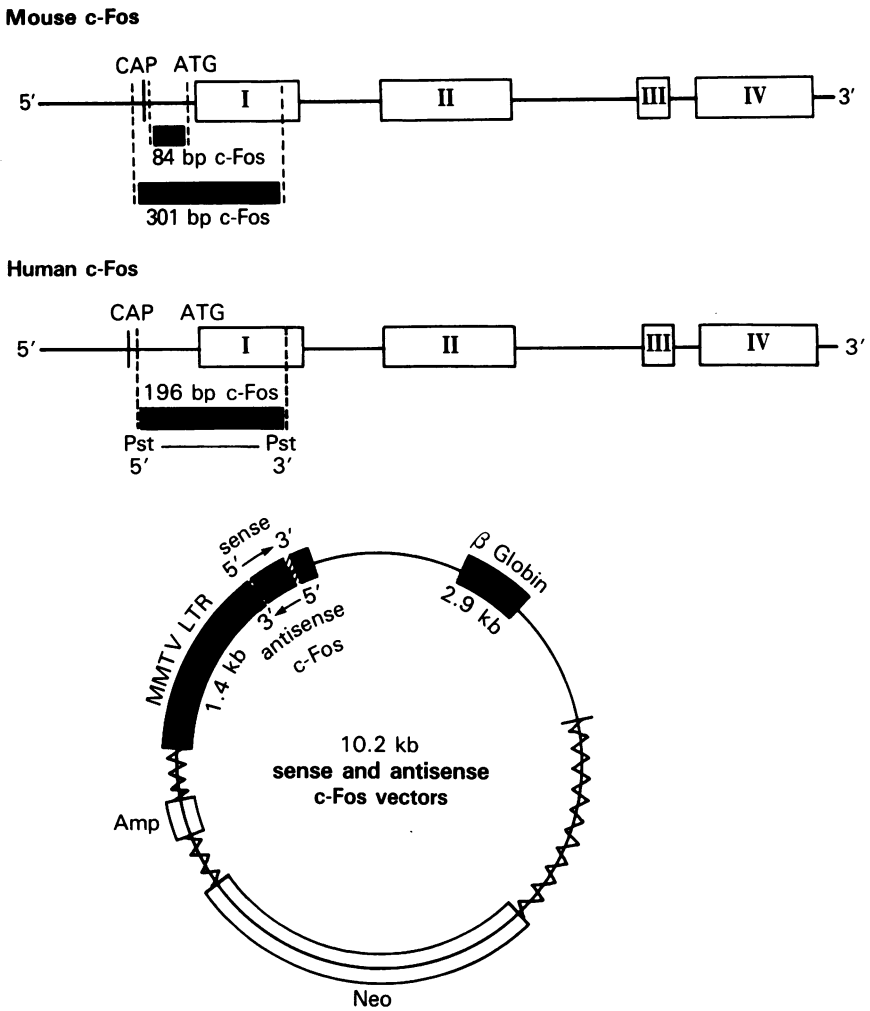

FIG. 1. Structure of human and mouse c-*fos* genes and of vectors containing fragments of these genes in the sense or antisense orientation. The open boxes in the gene maps represent exons with flanking and intervening sequences denoted by lines. DNA fragments isolated from c-*fos* (30) and cloned into the expression vectors are marked by black boxes flanked by dotted lines to delineate their precise position. Closed boxes in the vector map represent promoter and exon sequences as described. Arrows demonstrate the orientation of the c-*fos* fragments and are listed as 5′ to 3′ (sense) or 3′ to 5′ (antisense). Other components of the vector include a 1.4-kilobase (kb) *Pst* I fragment from plasmid p8-29 (31), containing the MMTV long terminal repeat (LTR) inserted as a linker-containing *Eco*RI (5′) to *Hin*dIII (3′) fragment, the human β-globin gene from the intragenic *Bam*HI site to the 3′ flanking *Bgl* II site, and the neo transcription unit. This transcriptional unit contains the simian virus 40 (SV40) early promoter, neomycin phosphotransferase coding sequences, and the SV40 t splice and poly(A) signals isolated as a *Pvu* II–*Bam*HI fragment from pSV2neo (32) and inserted as a linker-containing *Sal* I fragment. The large intron of the globin gene is shown as a line. The vector is a derivative of pBR322, designated pLTN1 (33, 34). Plasmids are defined as follows: pHfos^{AS} and pHfos^{S} are the MMTV-fos-globin transcriptional units containing the 196-base-pair (bp) *Pst* I human c-*fos* fragment in antisense (3′ to 5′) and sense (5′ to 3′) orientations, respectively. pM(84)fos^{AS} and pM(84)fos^{S} contain the 84-bp *Pst* I mouse c-*fos* fragment, and pM(301)fos^{AS} and pM(301)fos^{S} contain the 301-bp *Pvu* II–*Hin*cII mouse c-*fos* fragment in antisense and sense orientations, respectively. Amp, ampicillin; Neo, neomycin.

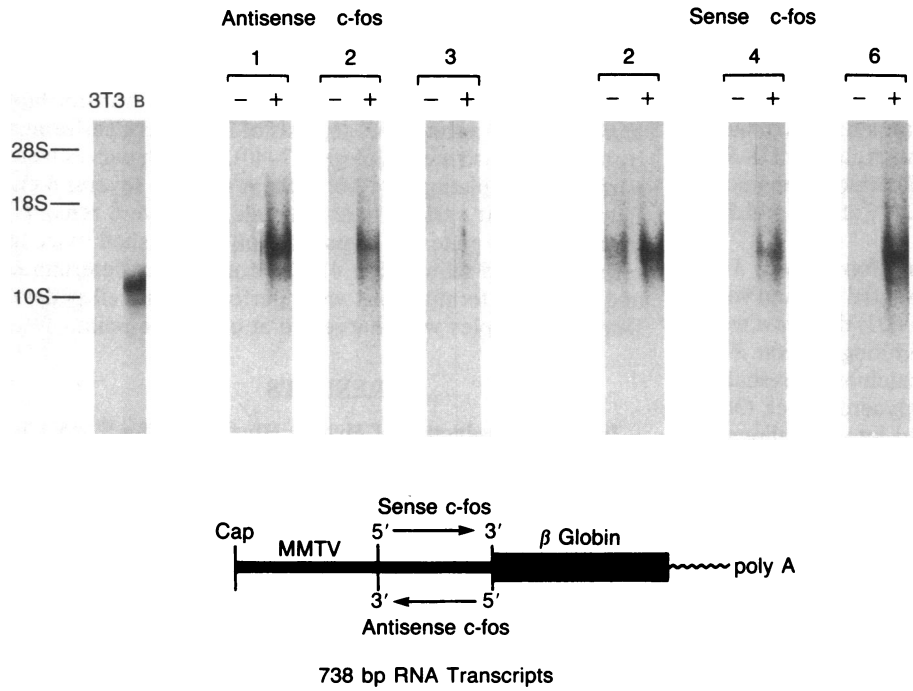

FIG. 2. Induction of hybrid Hfos^{S} or Hfos^{AS} RNAs by dexamethasone in stably transformed 3T3 cell clones. Ten micrograms of RNA was analyzed by the RNA transfer blot technique and probed with a 750-bp *Eco*RI–*Pst* I fragment from the 3′ end of the human β-globin gene radiolabeled in an M13 clone (37). Locations of the 28S and 18S rRNA markers and the 10S globin mRNA are indicated. Lanes labeled 3T3 and B contain RNA from 3T3 cells (10 μg) and human bone marrow (2 μg), respectively. RNA samples obtained from cells transformed with pHfos^{AS} are designated as antisense c-fos and those obtained from cells transformed with pHfos^{S} as sense c-fos. Lanes designated + contain RNA from cells incubated for 4 hr in 1 μM dexamethasone, whereas those designated − contain RNA from cells of that clone cultured without dexamethasone. The drawing below illustrates the structure of the Hfos^{S} and Hfos^{AS} hybrid RNA transcripts. The arrows demonstrate the orientation of the c-*fos* fragment and the solid box indicates the portion of the transcript that hybridizes with the β-globin gene probe fragment.

Southern blot analysis of DNA from cells of clones transfected with pHfos^{S} (designated S2, S4, and S6) or pHfos^{AS} (designated AS1, AS2, and AS3). RNA transfer blot analysis demonstrated a hybrid RNA of predicted size in cells of each clone and, furthermore, this hybrid RNA was shown to be highly inducible with dexamethasone (Fig. 2). An S1 nuclease analysis of RNA from uninduced and induced cells of each clone is shown in Fig. 3. A probe specific for the 3′ end of the hybrid RNA was utilized. This analysis verified the dexamethasone inducibility of the hybrid RNA in each clone and demonstrated that 3′ processing had occurred correctly on the globin portion of the transcriptional unit. Furthermore, this experiment provided a quantitative estimate of the number of RNA molecules in each clone. Based on densitometric analysis and the assumption that bone marrow and 3T3 cells contain roughly equal amounts of total RNA, we obtained the rough estimates of hybrid RNA copies per cell presented in Table 1. The β-globin mRNA copy number for the control, a patient with β-thalassemia, was ≈5000 per cell. Note that 2 μg of bone marrow RNA but 20 μg of 3T3 cell RNA were used in these analyses. This experiment was done a second time using several dilutions of bone marrow RNA from a patient with sickle cell anemia as a control with independently isolated samples from the 3T3 cell clones. Results identical to those shown in Table 1 were obtained. There are several major conclusions from these experiments: (*i*) there was an at least 5- to 600-fold increase in hybrid RNA concentration on induction, (*ii*) the hybrid RNA copy number in induced cells is relatively high, ranging from 250 to 3000 copies per cell, and (*iii*) the amount of hybrid RNA in uninduced cell clones transfected with pHfos^{AS} was low, being undetectable in one (S1) and barely detectable in another (S2), whereas there was readily detectable hybrid RNA in cells in each of the clones derived by transfection with pHfos^{S}.

c-fos Antisense RNA Inhibits Transformation and Growth of 3T3 Cells. Addition of dexamethasone to cells transfected with pHfos^{AS} and pM(84)fos^{AS} during G418 selection resulted in a 90–96% reduction in the number of G418-resistant transformants, respectively, whereas with pM(301)fos^{AS}

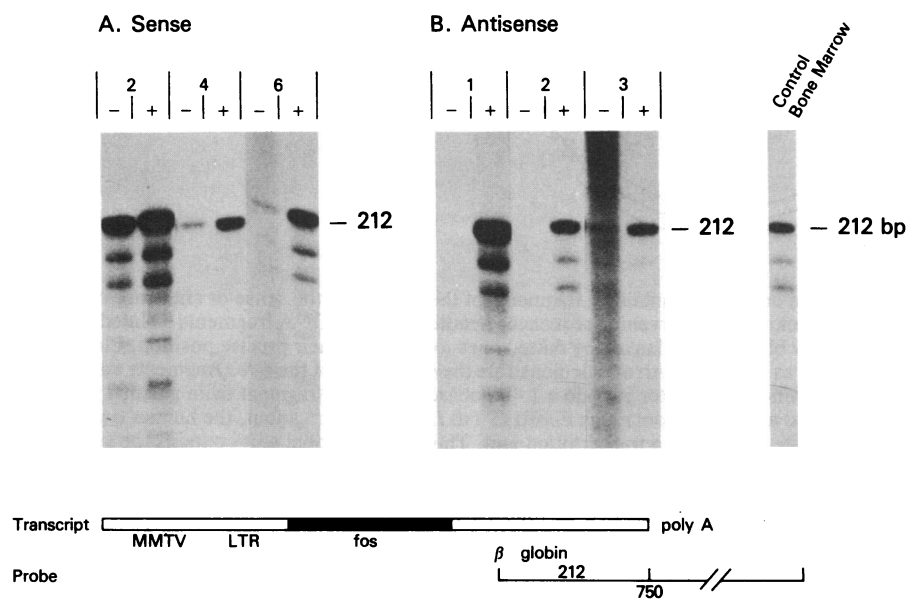

FIG. 3. Induction of hybrid Hfos^{S} and Hfos^{AS} RNAs in stably transformed 3T3 cell clones as demonstrated by S1 nuclease analysis. The identity of the individual clones is as designated in the legend to Fig. 2. The symbols − and + refer to RNA obtained from control cells and those incubated in 1 μM dexamethasone, respectively. Two micrograms of bone marrow RNA was employed as a positive control. All lanes were exposed for 8 hr. The drawing illustrates the structure of the RNA transcript and the 212 bp of the probe protected by correctly cleaved and poly(A) RNA. The smaller protected fragments observed on analysis of authentic globin mRNA from bone marrow cells and the hybrid RNAs reflect nicking by S1 nuclease at (A+T)-rich portions of the duplex (37).

Table 1. Copy number of hybrid RNAs from sense and antisense human c-fos transformants

Transformant	Copies per cell		Fold induction
	Control	Dex*	
$Hfos^{AS1}$	5	3000	600
$Hfos^{AS2}$	5	300	60
$Hfos^{AS3}$	20	250	15
$Hfos^{S2}$	600	3000	5
$Hfos^{S4}$	30	250	8
$Hfos^{S6}$	20	1000	50

The copy number was estimated by using densitometry of S1 nuclease autoradiographs and comparison to the known copy number of a bone marrow RNA sample obtained from a patient with β-thalassemia.
*1 μM dexamethasone was added to the medium 4 hr before cell lysis.

Table 2. Transfection frequency of 3T3 cells with c-fos sense and antisense vectors

Vector	Colonies per 10 μg of transfected DNA		% of control
	Control	Dex*	
$pM(84)fos^{S}$	444	352	80
$pM(84)fos^{AS}$	96	4	4
$pM(301)fos^{S}$	360	412	112
$pM(301)fos^{AS}$	356	224	63
$pHfos^{S}$	160	160	100
$pHfos^{AS}$	160	16	10

*1 μM dexamethasone was added 24 hr after the DNA transfer procedure.

there was only a 37% reduction in colony number (Table 2). The corresponding transformations with the control plasmids having the *fos* fragments in the sense orientation gave essentially equal numbers of colonies in the presence or absence of dexamethasone. Comparable results were obtained in three separate experiments with $pHfos^{S}$ and $pHfos^{AS}$. To further control for any nonspecific effect of dexamethasone in this assay, several of the transfections were performed with the D1 cell clone. These cells grow very well at low density in 1 μM dexamethasone. The MMTV promoter was shown to be steroid responsive in D1 cells using pMMTVCat; the activity of the CAT enzyme was increased 20-fold when transfections were done in the presence of 1 μM dexamethasone (data not shown). Suppression of colony formation of D1 cells in the presence of dexamethasone upon transfection with the antisense vectors but not with the sense vectors was comparable to the results obtained with the parental 3T3 cells.

Dexamethasone was shown to inhibit the growth of three clones containing antisense hybrid c-fos RNA (AS1, AS2, and AS3) in three separate experiments; representative results are displayed in Fig. 4. The cell doubling time in the absence of dexamethasone was estimated as ≈24 hr, whereas it was increased by dexamethasne to 48 hr for clone AS1 and to >72 hr for clones AS2 and AS3. In contrast, the cell doubling time for clones S1, S2, and S3 were roughly 24 hr in the presence or absence of dexamethasone. There is no obvious correlation between the concentration of antisense c-fos RNA and extent of growth inhibition. Six additional clones, transformed with $pHfos^{AS}$, were also shown to be growth inhibited by dexamethasone. Thus, the presence of the composite transcript containing antisense fos mRNA sequences consistently inhibited cell growth in all clones that were isolated. Removal of dexamethasone allows cells to recover from this inhibition, demonstrating that this phenomenon is reversible (data not shown).

DISCUSSION

Recombinant vectors have been created that generated hybrid mRNA transcripts containing c-fos sequences in the sense or antisense orientation. The hybrid transcripts, generated under control of a steroid-inducible promoter, were formed in stably transformed mouse 3T3 cells. Inhibition of cell growth by transcripts containing c-fos mRNA sequences in the antisense orientation was shown in a colony-forming assay and by comparing the growth rate of cells stably transformed with the appropriate vectors in the presence or absence of dexamethasone. These results demonstrate that generation of antisense RNA is a useful approach for studying protooncogene function and, furthermore, establish a role for the c-*fos* gene product in the growth of mouse fibroblasts.

Use of an inducible promoter for generation of antisense RNA is essential for analysis of genes whose products are necessary for cell growth. Isolation of stable transformants would clearly not be possible if antisense RNA were constitutively produced and significantly inhibited cell division. In our experiments suppression of colony formation in the presence of dexamethasone can be attributed to the generation of c-fos antisense sequences upon introduction of the vector into 3T3 cells. In addition to the steroid-inducible MMTV promoter, inducible promoters of the interferon (41), heat shock protein (42, 43), and metallothionein genes (44) might also be suitable, depending on the type of cells to be studied. The MMTV promoter was used in our experiments because its inducibility in 3T3 cells has been well established (45).

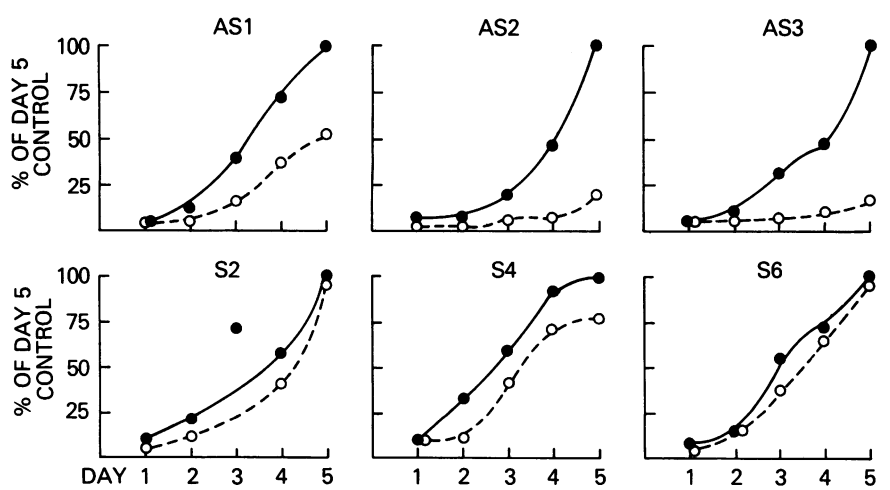

FIG. 4. Effect of dexamethasone on the growth of cells containing $Hfos^{AS}$ (*Upper*) or $Hfos^{S}$ (*Lower*) hybrid RNAs. Three individual clones of each type were analyzed; these were designated as indicated in Figs. 2 and 3. All values were normalized, with 100% representing the protein value of the control plates on day 5. The "100%" protein values for the individual clones are as follows: AS1 = 385 μg, AS2 = 194 μg, AS3 = 176 μg, S2 = 148 μg, S4 = 263 μg, and S6 = 338 μg. One hundred micrograms of protein is the amount derived from ≈2 × 10^6 cells. Circles represent the mean of duplicate values: ●, control; ○, 1 μM dexamethasone.

Several factors may determine whether the function of a gene can be inhibited by antisense RNA. Pertinent to our results is the very high ratio of antisense RNA to the endogenous c-fos mRNA. Growth inhibition was observed in cells that contain 250–3000 copies of the hybrid RNA containing the antisense c-fos sequences (Table 1), whereas the amount of c-fos mRNA in growing 3T3 cells has been estimated as <5 copies per cell (18). Other studies had generally shown that antisense sequences complementary to the 5′ end of mRNA molecules are most effective in inhibiting gene function, and thus we selected three fragments from the 5′ end of the mouse or human c-*fos* gene in making our constructs. Consistent with the results of others (14, 16), we found variable inhibition of colony formation by the different vectors. At present it is impossible to predict with certainty which antisense vectors will be effective, and therefore empirical testing of several is necessary. Inhibition of mouse c-*fos* gene function was observed with an antisense construction containing a fragment of the human c-*fos* gene. The human c-*fos* fragment has >80% homology with the corresponding portion of the mouse c-*fos* gene. The calculated T_m (46) of the mouse–human RNA·RNA duplex in physiological saline is 75°C. Our data suggest that complete homology is not required for effective inhibition of gene function by antisense RNA sequences.

An association between the c-*fos* gene product and cellular proliferation has been inferred because of its rapid induction by growth factors, but the gene transcript and c-fos protein are both present in very low levels in normally proliferating fibroblasts. Attempts to define correlation between c-fos protein concentration and the rate of cell growth seemed impractical. Nonsynchronized fibroblasts appear to contain fairly uniform amounts of the c-fos protein, suggesting that its concentration does not vary markedly during the cell cycle (20). The use of vectors capable of generating antisense c-fos mRNA sequences provided a direct test of the role of this gene product in cell growth. Indeed, our results appear to indicate that the amount or concentration of the c-fos protein may be directly related to the rate of cell division.

We thank Drs. J. Majors and H. Varmus for providing us p8-29 plasmid through Drs. N. Copeland and N. Jenkins. We also thank Drs. B. Howard and C. Gorman for plasmid pRSVCat. We are grateful to Anne Baur and Patricia Turner for expert technical assistance and Rhonda Mays who skillfully prepared the manuscript.

1. Shilo, B.-Z. & Weinberg, R. A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6789–6792.
2. Bishop, J. M. (1985) *Cell* **42**, 23–38.
3. Muller, R. & Verma, I. M. (1984) *Curr. Top. Microbiol. Immunol.* **112**, 73–116.
4. Waterfield, M. D., Scrace, G. J., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.-H., Huang, J. S. & Deuel, T. F. (1983) *Nature (London)* **304**, 35–39.
5. Doolittle, R. F., Hunkapiller, M. W., Hood, L. E., DeVare, S. G., Robbins, K. C., Aaronson, S. A. & Antoniades, H. N. (1983) *Science* **221**, 275–277.
6. Sherr, C. J., Rettenmier, C. W., Sacca, R., Roussel, M. F., Look, A. T. & Stanley, E. R. (1985) *Cell* **41**, 665–676.
7. Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. & Waterfield, M. D. (1984) *Nature (London)* **307**, 521–527.
8. Muller, R., Slamon, D. J., Tremblay, J. M., Cline, M. J. & Verma, I. M. (1982) *Nature (London)* **299**, 640–644.
9. Huang, J. S., Huang, S. S. & Deuel, T. F. (1984) *Cell* **39**, 79–87.
10. Johnsson, A., Betsholtz, C., Heldin, C.-H. & Westermark, B. (1985) *Nature (London)* **317**, 438–440.
11. Leal, F., Williams, L. T., Robbins, K. C. & Aaronson, S. A. (1985) *Science* **230**, 327–330.
12. Mulcahy, L. S., Smith, M. R. & Stacey, D. W. (1985) *Nature (London)* **313**, 241–243.
13. Izant, J. G. & Weintraub, H. (1984) *Cell* **36**, 1007–1015.
14. Izant, J. G. & Weintraub, H. (1985) *Science* **229**, 345–352.
15. Melton, D. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 144–148.
16. Kim, S. K. & Wold, B. J. (1985) *Cell* **42**, 129–138.
17. Curran, T., Miller, A. D., Zokas, L. & Verma, I. M. (1984) *Cell* **36**, 259–268.
18. Kruijer, W., Cooper, J. A., Hunter, T. & Verma, I. M. (1984) *Nature (London)* **312**, 711–716.
19. Greenberg, M. E. & Ziff, E. B. (1984) *Nature (London)* **311**, 433–438.
20. Muller, R., Bravo, R., Burkhardt, J. & Curran, T. (1984) *Nature (London)* **312**, 716–720.
21. Bravo, R., Burckhardt, J., Curran, T. & Muller, R. (1985) *EMBO J.* **4**, 1193–1197.
22. Gonda, T. J. & Metcalf, D. (1984) *Nature (London)* **310**, 249–251.
23. Mitchell, R. L., Zokas, L., Schreiber, R. D. & Verma, I. M. (1985) *Cell* **40**, 209–217.
24. Muller, R., Muller, D. & Guilbert, L. (1984) *EMBO J.* **3**, 1887–1890.
25. Muller, R. & Wagner, E. F. (1984) *Nature (London)* **311**, 438–442.
26. Ruther, U., Wagner, E. F. & Muller, R. (1985) *EMBO J.* **4**, 1775–1781.
27. Curran, T. & Morgan, J. I. (1985) *Science* **229**, 1265–1268.
28. Kruijer, W., Shubert, D. & Verma, I. M. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7330–7334.
29. Greenberg, M. E., Greene, L. A. & Ziff, E. B. (1985) *J. Biol. Chem.* **260**, 14101–14110.
30. Van Straaten, F., Muller, R., Curran, T., Van Beveren, C. & Verma, I. M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3183–3187.
31. Majors, J. E. & Varmus, H. E. (1981) *Nature (London)* **289**, 253–258.
32. Southern, P. J. & Berg, P. (1982) *J. Mol. Appl. Genet.* **1**, 327–341.
33. Anagnou, N. P., Karlsson, S., Moulton, A. D., Keller, G. & Nienhuis, A. W. (1986) *EMBO J.* **5**, 121–126.
34. Humphries, R. K., Ley, T. J., Turner, P., Moulton, A. D. & Nienhuis, A. W. (1982) *Cell* **30**, 173–183.
35. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
36. Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S. & Axel, R. (1979) *Cell* **16**, 777–785.
37. Ley, T. J., Anagnou, N. P., Pepe, G. & Nienhuis, A. W. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4775–4779.
38. Humphries, R. K., Ley, T. J., Anagnou, N. P., Baur, A. W. & Nienhuis, A. W. (1984) *Blood* **64**, 23–32.
39. Alwine, J. C., Kemp, D. J. & Stark, G. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5350–5354.
40. Southern, S. M. (1975) *J. Mol. Biol.* **98**, 503–517.
41. Goodbourn, S., Zinn, K. & Maniatis, T. (1985) *Cell* **41**, 509–520.
42. Topol, J., Ruden, D. M. & Parker, C. S. (1985) *Cell* **42**, 527–537.
43. Wu, C. (1984) *Nature (London)* **309**, 229–234.
44. Mayo, K. E., Warren, R. & Palmiter, R. D. (1982) *Cell* **29**, 99–108.
45. Armelin, H. A., Armelin, M. C. S., Kelly, K., Stewart, T., Leder, P., Cochran, B. H. & Stiles, C. D. (1984) *Nature (London)* **310**, 655–660.
46. Davis, R. W., Botstein, D. & Roth, J. R. (1980) in *Advanced Bacterial Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 622–623.