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Antisense RNA of Proto-Oncogene c-*fos* Blocks Renewed Growth of Quiescent 3T3 Cells

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Mouse 3T3 cells were transformed with an antisense c-*fos* gene fused to a mouse mammary tumor virus promoter. In transformants that integrated a large number of antisense c-*fos* sequences, the usual large increase in c-*fos* mRNA and protein following stimulation of quiescent cells by platelet-derived growth factor was blocked in the presence of dexamethasone. These cells subsequently also failed to show the stimulation of DNA synthesis normally induced by platelet-derived growth factor. Appropriate expression of c-*fos* appears to be a prerequisite for reentry of quiescent cells into the cell cycle.

Results of recent studies on expression of cellular oncogenes or proto-oncogenes in a variety of tumors have suggested that products encoded by these genes have pivotal roles for regulation of cell proliferation and cell differentiation (4, 22, 53). Some proto-oncogene products appear to work at the first step of concerted cell proliferation reactions, the transduction of an external cell growth signal. The c-*sis* proto-oncogene has been shown to encode the platelet-derived growth factor (PDGF) (15, 55), whereas the c-*erbB* and c-*erbA* genes are likely to encode the epidermal growth factor receptor (16) and the steroid hormone receptor (19, 20, 56), respectively. Except for these few examples, however, the physiological functions of most proto-oncogenes are not known (4).

Recently, results of a series of experiments (8, 9, 25, 26, 28, 35, 45) have shown that introduction of an antisense RNA complementary to a specific mRNA into cells can effectively create or mimic the null-mutant phenotype, which should be quite useful for investigating gene products of unknown physiological function. Although the exact molecular mechanism of antisense RNA inhibition of specific gene expression is not known, it has been assumed that the formation of a stable RNA-RNA hybrid in vivo results in inhibition of RNA transport, block of translation, or an increased rate of RNA turnover (25).

The proto-oncogene c-*fos*, which was originally identified as the normal cellular homolog of the transforming gene of FBJ-osteosarcoma virus (10, 17, 52), codes for a phosphoprotein of unknown function. The c-*fos* protein is found almost exclusively in the nucleus (12). Both protein and mRNA have very short half-lives (29, 38). Expression of c-*fos* is highly sensitive to mitogenic (21, 29, 38) or differentiation-inducing agents (11, 30, 37, 39). Transcription of c-*fos* genes is greatly increased within minutes of stimulation of mouse fibroblast 3T3 cells by serum or purified growth factors such as PDGF and fibroblast growth factor (21, 29, 38). This very rapid response of c-*fos* gene expression to mitogens precedes even that of c-*myc*, which has also been recognized as one of the early mitogen-responsive genes (27). These findings suggest that c-*fos* expression is a primary event in the complex response to mitogen, which in turn leads cells to proliferation, but direct proof is lacking. The possibility still remains that c-*fos* expression, although normally accompanying the response to mitogens, is not itself a prerequisite for the subsequent DNA replication and cell division.

The c-*fos* gene product was hypothesized to be important for the transition from quiescence to renewed cell growth because high levels of c-*fos* expression have been observed during this transition (21, 29, 38). In contrast, the extremely low levels of c-*fos* observed in continuously proliferating cells have suggested the hypothesis that c-*fos* does not play a role in steady-state growth (5, 38). A recent report, however, which appeared after our work described here was completed, has somewhat confused this simple picture of c-*fos* function. Holt et al. (24) recently reported antisense c-*fos* transformation of NIH 3T3 cells. Unexpectedly, the transformants described in that report showed an inhibition of growth in continuously proliferating cells. No experiments with quiescent cells were reported. These investigators did not demonstrate a change in c-*fos* mRNA or protein, nor a decreased response of the transformed cells to factors known to induce c-*fos* expression. These somewhat confusing results necessitate careful evaluation of each link in the chain of evidence implicating c-*fos* in the control of cell proliferation.

In this study, we used antisense RNA to elucidate the physiological function of c-*fos* gene products. In NIH 3T3 fibroblast cells transformed with multiple copies of antisense c-*fos* DNA fused to mouse mammary tumor virus (MMTV) promoter, antisense c-*fos* RNA was expressed, while the sense c-*fos* mRNA and protein levels were greatly decreased in the presence of dexamethasone. Although the transformants grew nearly normally in the exponential growth phase, a large fraction of cells synchronized in the quiescent state could not reenter S phase or, in other words, restart the cell cycle in the presence of dexamethasone. Our results show that c-*fos* proto-oncogene expression is a prerequisite for the transition from G_0 into renewed cell growth.

MATERIALS AND METHODS

Plasmid construction. pLTLcfosM.a was constructed from plasmids pc-fos(mouse)3 (52) and pLTL2 (58). pc-fos(mouse)3 plasmid contains approximately 7 kilobases of genomic mouse c-*fos* gene (52). pLTL2 contains two complete 5′ and 3′ long terminal repeats (LTRs) of MMTV and the herpes simplex virus thymidine kinase gene inserted between the two LTRs (58). A portion of the mouse genomic

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c-*fos* gene, a *Hinc*II 430-base-pair (bp) DNA fragment, consisting of 140 bp of the 5'-flanking region and a large part of the first exon (290 bp), including the initiation codon ATG, was excised from the pc-fos(mouse)3 plasmid. After *Bam*HI linkers were introduced at both ends, the DNA fragment was inserted into the *Bam*HI and *Bgl*II sites of the pLTL2 vector, from which the herpes simplex virus thymidine kinase gene had been removed. After bacterial transformation, individual clones were screened for the insert and its orientation relative to the MMTV transcription direction. In pLTLcfosM.a the orientation of the mouse c-*fos* gene is opposite to the MMTV transcription direction.

Cell culture and transfection. NIH 3T3 cells obtained from the American Type Culture Collection (Rockville, Md.) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) to confluence. Cells were trypsinized and replated into 24-well titer plates. On the following day, pLTLcfosM.a and pSV2-neo plasmids (1:1 ratio) were cotransfected into 5×10^6 NIH 3T3 cells by the $CaPO_4$ method (18). Selection in geneticin (G418, 200 μg/ml; GIBCO Laboratories, Grand Island, N.Y.)-containing medium was started 24 h later. After 2 weeks in the selection medium, positive clones were isolated.

DNA synthesis assays. (i) Autoradiography. A total of 10^4 cells was inoculated into each chamber of a 96-well culture plate in DMEM containing 10% FCS and grown to confluence at 37°C for 7 days. Cells were arrested in G_0 by removing the old medium and replacing it with DMEM containing 5% platelet-poor plasma (2). After 30 h this medium was replaced with 0.2 ml of fresh medium containing either 5% platelet-poor plasma plus purified PDGF (10, 50, or 250 U/ml), or FCS (5, 10, or 20%) and 5 μCi of [^{3}H]thymidine per ml, with or without 10^{-6} M dexamethasone. Twenty-four hours later the cells were rinsed, fixed in methanol, and processed for autoradiography and Giemsa staining. Photomicrographs were recorded on Plus-X film (Kodak) with an inverted microscope (Olympus) with ×20 objective, bright-field illumination.

(ii) Flow cytometry. NIH 3T3 parental and clone NF2 cells were grown in dishes (diameter, 10 cm) in DMEM containing 10% FCS. When cells reached approximately 50% confluence, the medium was removed, and cells were incubated with DMEM containing 0.5% calf serum with or without 10^{-6} M dexamethasone. After 30 h of incubation in this serum-deprived condition, which synchronizes cells into a quiescent state, the cells were stimulated by fresh medium containing 10% FCS with or without dexamethasone and incubated for 24 h. Quiescent cells and serum-stimulated cells were both assayed for average DNA content by flow cytometry after trypsinization and staining of cells with propidium iodide. Fluorescence assays were carried out by a fluoresence-activated cell sorter analysis.

RNA analysis. Cells were lysed in 4 M guanidinium thiocyanate and total RNA was isolated (7). RNA was analyzed by the S1 nuclease mapping procedure described previously (3). End labeling was carried out by the method described by Maxam and Gilbert (34) with polynucleotide kinase. The uniformly ^{32}P-labeled DNA probe was prepared by using [α-^{32}P]dATP and [α-^{32}P]dCTP by the method of Ley et al. (32). The ^{32}P-labeled DNA probe was heat denatured, hybridized in 80% formamide to the various RNA samples at 56.5°C for 10 h, digested with S1 nuclease, and analyzed by electrophoresis on a 7 M urea–5% polyacrylamide gel as described previously (40). The ^{32}P-labeled DNA probe was always present in great excess over the specific RNA to be analyzed. All autoradiography was carried out with a fluorescent screen and preflashed x-ray film at −70°C. The amount of various RNAs detected was quantitated by scanning properly exposed autoradiograms of S1 nuclease analysis.

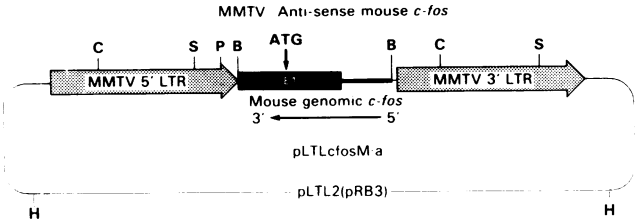


FIG. 1. Schematic representation of the pLTLcfosM.a antisense c-*fos* construct. The MMTV 5' and 3' LTRs and the direction of transcription are indicated by stippled arrows. The mouse genomic sequences are shown by black boxes. The wider black box is a part of the first exon; the narrower black box is the 5'-flanking sequence. The lines represent the parts of the original vector pLTL2 used for construction. The direction of transcription of the c-*fos* gene is indicated by the black horizontal arrow. The location of the translation initiation codon ATG of the c-*fos* gene is also indicated. Restriction sites: B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; S, *Sst*I; P, *Pvu*II.

Immunofluorescent staining. Cells were fixed with 3% formaldehyde in phosphate-buffered saline for 30 min. After permeabilization for 5 min in phosphate-buffered saline plus 1% Triton X-100, cells were incubated with affinity-purified rabbit antibody raised against peptides corresponding to a 26-amino-acid stretch of p55$^{c\text{-}fos}$ (residues 127 to 152) that was generously provided by Tom Curran (13). Antibody-labeled cells were made visible by using affinity-purified biotinylated donkey antibody against rabbit immunoglobulin G, and Texas Red-conjugated streptavidin (Amersham Corp., Arlington Heights, Ill.). Fluorescent cells were photographed on Tri-X pan film (Kodak) with epifluorescence equipment (Zeiss) and ×25 neofluar 0.8-NA objective lens.

RESULTS

Isolation of NIH 3T3 cells transformed with antisense c-*fos* plasmid. To counter the possibility that diminution of the c-*fos* level would prove to be lethal, and to more clearly elucidate the physiological role of the c-*fos* gene product, we prepared a construct to express antisense c-*fos* RNAs conditionally in the transformed cells. The chimeric recombinant pLTLcfosM.a, consisting of the MMTV LTR and a part of the mouse c-*fos* gene, is shown in Fig. 1. The promoter of the MMTV LTR is conditional. Transcription from the promoter can be controlled by a glucocorticoid hormone such as dexamethasone (44, 57). Part of the mouse c-*fos* gene, 140 bp of 5'-flanking region plus the first exon (290 bp) with the ATG initiation codon (52), was inserted in the opposite orientation to MMTV LTR transcription between the MMTV 5' LTR and the 3' LTR of vector pLTL2 (58). Transcription of antisense c-*fos* RNAs by this chimeric pLTLcfosM.a plasmid would be expected to initiate from the promoter of the MMTV 5' LTR; and the transcripts, containing the antisense mouse c-*fos* gene sequence, should be polyadenylated at the 3' LTR poly(A) site (58).

The antisense c-*fos* plasmid and a plasmid (pSV2-neo) (48) containing a bacterial neomycin resistance gene were cotransfected into mouse NIH 3T3 fibroblast cells. After selection in geneticin (G418)-containing medium for approximately 2 weeks, five clones were isolated. The transformation frequency was approximately 10^{-6}, which was 10-fold

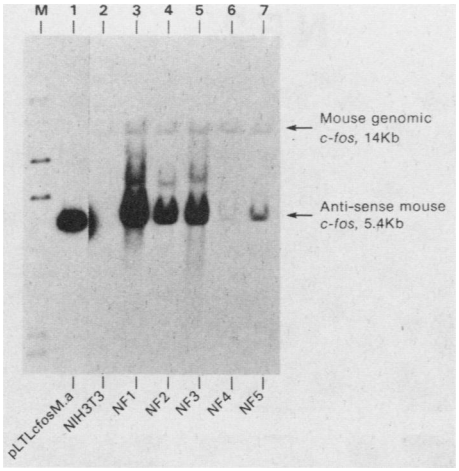


FIG. 2. Identification of antisense c-*fos* transformants. The result of Southern hybridization analysis for antisense c-*fos* DNA in various transformants is shown. A total of 20 μg of DNA, prepared from each clone as well as parental NIH 3T3 cells, was digested with *Hin*dIII, fractionated on a 0.7% agarose gel, and analyzed by the Southern hybridization procedure (47) with a genomic c-*fos* DNA fragment probe [*Hin*cII-*Hin*cII, 430 bp; prepared from the pc-fos(mouse)3 plasmid], which was the same fragment used for pLTLcfosM.a construction. Plasmid pLTLcfosM.a digested with *Hin*dIII was also included for analysis as a control (lane 1). Lane M; size marker; 5′-end ^{32}P-labeled λ phage *Hin*dIII digests.

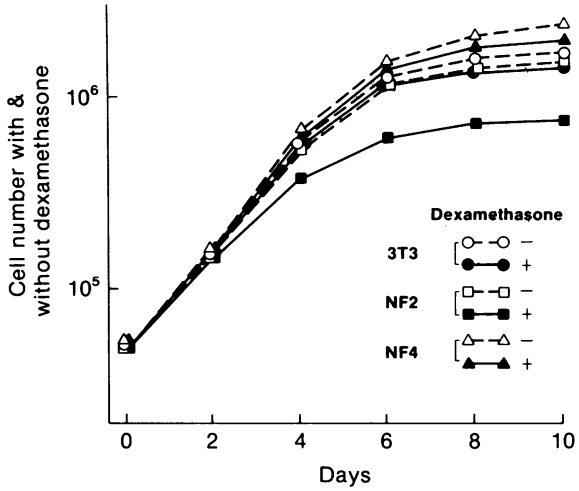


FIG. 3. Cell growth rate in antisense c-*fos*-transformed cells. Growth curves of unsynchronized cells are shown. A total of 5×10^4 cells were inoculated into dishes (diameter, 5 cm) with 5 ml of DMEM containing 10% FCS. Half of the dishes also included 10^{-6} M dexamethasone. Cells in duplicate sets were harvested, and the number of cells was counted after trypsinization. The difference between duplicate plates was negligible (<5%). Each point is the average of the duplicate plates. The growth curves (data not shown) for clone NF1 and NF5 were almost identical to those of the parental NIH 3T3 cells, and the curve for NF3 was similar to that for NF2, regardless of whether dexamethasone was present in the culture medium.

lower than the control transformation with pSV2-neo alone, suggesting some leakiness of the MMTV promoter (induction in the absence of glucocorticoid hormone) (33, 51) and that expression of antisense c-*fos* RNA may decrease the efficiency of transformation. The five isolated clones appeared to be morphologically identical to the parental NIH 3T3 cells.

Chromosomal DNAs prepared from individual clones were analyzed for the presence of antisense c-*fos* sequences by the Southern hybridization procedure (47). Employing the mouse c-*fos* DNA fragment used for pLTLcfosM.a construction as a probe, we confirmed that DNA from all five clones contain the expected 5.4-kilobase (kb) *Hin*dIII fragment derived from the transfected pLTLcfosM.a plasmid, in addition to a 14-kb DNA fragment derived from the endogenous mouse c-*fos* gene as shown in Fig. 2. Only the endogenous c-*fos* gene fragment is seen in the lane for parental NIH 3T3 cells (Fig. 2, lane 2). These results indicate that the transcription unit of pLTLcfosM.a remained intact in all five clones and also that the antisense c-*fos* sequences were integrated as tandem repeats of the original circular input DNA. Additional minor DNA fragments other than 5.4 and 14 kb in length, detected in some lanes, were probably generated by digestion at *Hin*dIII sites located in the chromosomal DNA adjacent to the integrated tandem repeats. Undigested DNA was also subjected to similar Southern hybridization analysis. It was found that the antisense c-*fos* DNA migrates together with high-molecular-weight mouse chromosomal DNA (data not shown). Using the endogenous c-*fos* 14-kb DNA fragment as an internal calibration, we estimated the number of MMTV antisense c-*fos* sequences integrated in individual clones. It varied from a single copy in clone NF4 (Fig. 2, lane 6) to three copies in clone NF5 (Fig. 2, lane 7) and 40 to 50 copies in clones NF1, NF2, and NF3 (Fig. 2, lanes 3, 4, and 5).

Cell growth rate in antisense c-*fos* transformants. Phenotypic alterations of five transformed clones were analyzed to investigate the role of c-*fos* in cell proliferation. First, we measured the growth rate of the transformed clones and the parental NIH 3T3 cells with or without dexamethasone in the culture medium. During the initial several days of log-phase growth, the different clones grew at nearly identical rates (Fig. 3). The doubling time of cell growth was unchanged in the presence of dexamethasone. When the growth of the cultures was alowed to continue to confluency, small but consistent differences in growth rates appeared. Clones NF2 (Fig. 3) and NF3 (data not shown), which had a large number of copies of the integrated antisense DNA as described above, grew more slowly and achieved lower final densities with dexamethasone than without the hormone. This decrease is not explained by an intrinsic effect of dexamethasone itself on cell growth, as can be seen by comparing the growth of transfected cells with that of the parental NIH 3T3 cells. Dexamethasone at 10^{-6} M had a very small retarding effect on NIH 3T3 cells, as seen by comparing growth curves with and without hormone (Fig. 3). This direct dexamethasone effect, however, would be expected to be approximately the same for the transformed and parental cells, whereas the observed effect was much larger in the NF2 and NF3 transformants. Thus, the growth rates of clones NF2 and NF3 appear to be influenced in the presence of dexamethasone and under conditions in which the cell is approaching a quiescent state. Transformants NF1 (data not shown), NF4 (Fig. 3), and NF5 (data not shown) grew at approximately the same rate as NIH 3T3 cells, regardless of the absence or presence of dexamethasone in the medium. In the case of NF4 and NF5, in which only one to three copies of antisense c-*fos* DNA were integrated, it is perhaps not surprising that the transformed cells behaved very much like the parental cells. The different behavior of NF1 from those of NF2 and NF3, however, was unexpected, because these

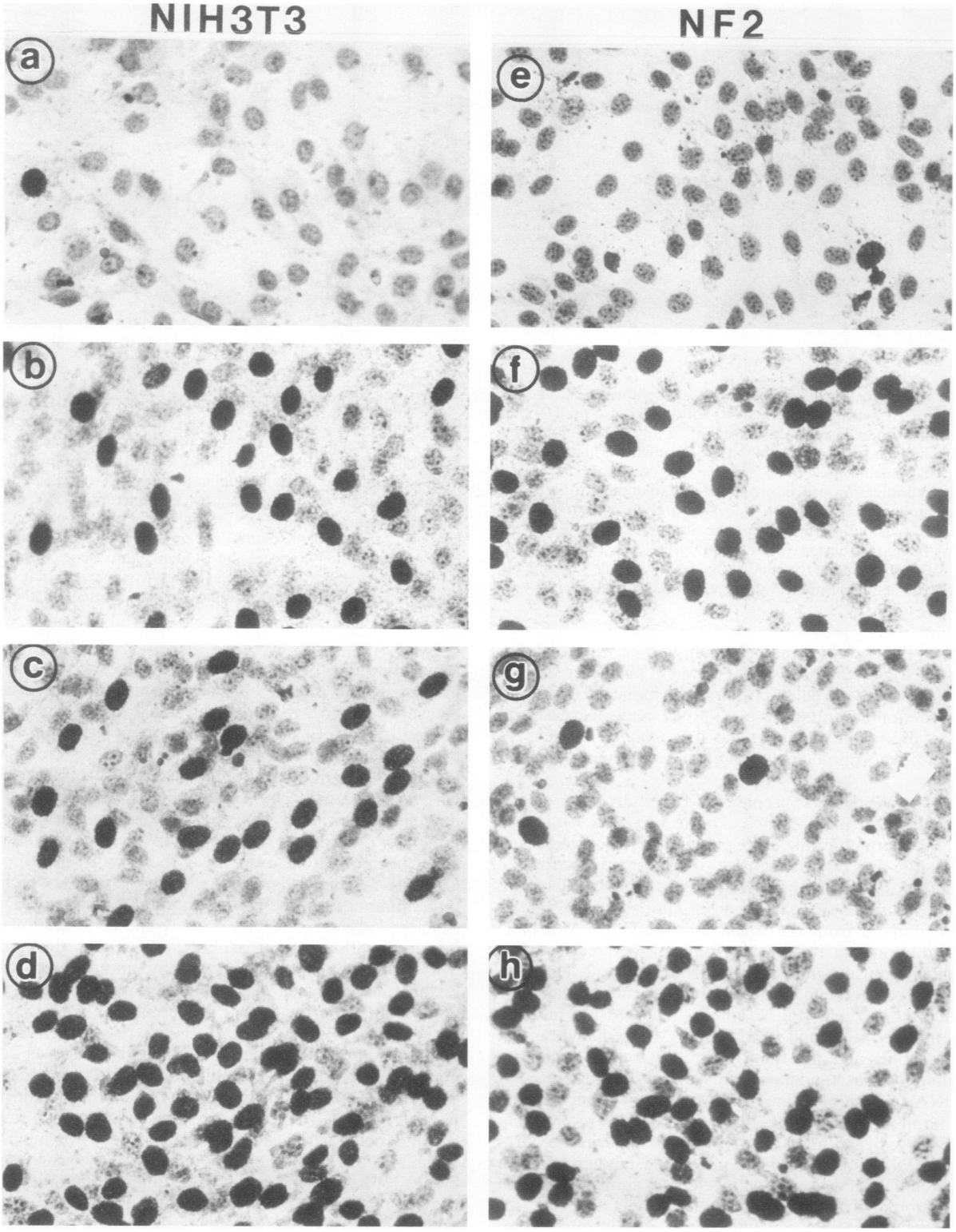
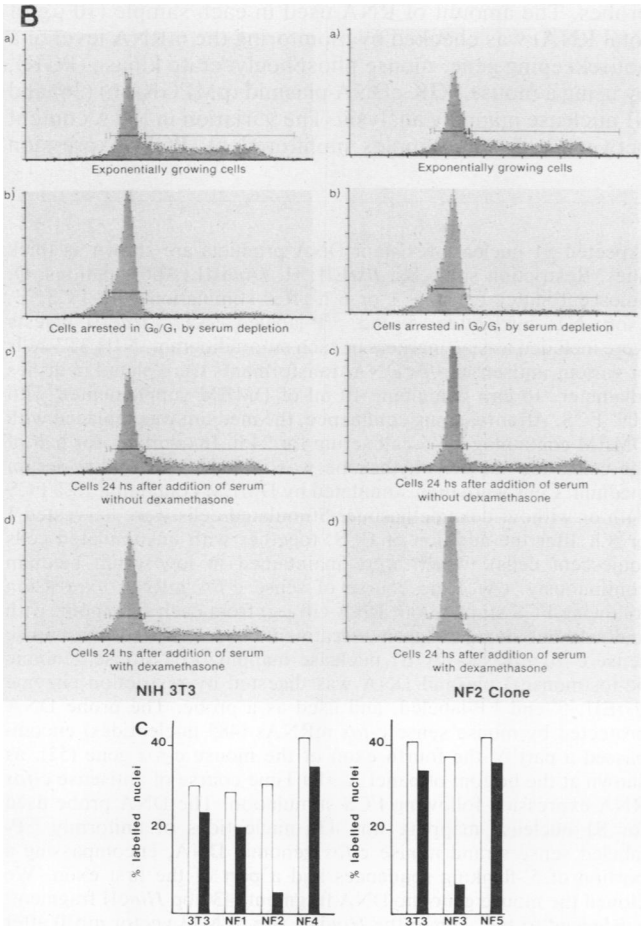


FIG. 4. DNA replication in antisense c-*fos*-transformed cells. (A) Autoradiography of [^{3}H]thymidine incorporated into NIH 3T3 and NF2 cells. (a to d) NIH 3T3 cells; (e to h) clone NF2. (a and e) Quiescent cells were treated with dexamethasone; (b and f) cells were stimulated by 50 U of PDGF per ml without dexamethasone; (c and g) cells were stimulated by 50 U of PDGF per ml with dexamethasone; (d and h) cells were stimulated by 250 U of PDGF per ml with dexamethasone. (B) Flow cytometric analysis of NIH 3T3 and NF2 cells. Exponentially growing, unsynchronized cells were examined as controls with (data not shown) and without (panels a) dexamethasone. Both NIH 3T3 and NF2 cells gave a bimodal DNA curve characteristic of exponentially growing cells, and the pattern was unaltered in the presence of dexamethasone. Serum-deprived cells showed a single peak with a diploid DNA content characteristic of G_0-G_1 cells (panels b). The flow cytometry analysis of the quiescent cells was unchanged in the presence of dexamethasone (data not shown). A large difference between NIH 3T3 and NF2 was observed when serum-stimulated cells incubated in the presence of dexamethasone were examined for reentry of the

three clones all apparently integrated a large number of copies of antisense c-*fos* DNA. This may be due to transcriptional inactivity of the site at which the antisense c-*fos* DNAs are integrated in NF1, or to a specific posttranscriptional modification such as aberrant RNA processing of antisense c-*fos* RNA transcribed in this clone (see below).

DNA replication in antisense c-*fos* transformants. We further explored this effect on cell growth by examining the transition from a nondividing quiescent state (G_0) into the normal cycle of growth and cell division. Following a period of serum starvation that has rendered them quiescent (41), NIH 3T3 cells can be induced to restart the cell cycle by exposure to fresh medium containing FCS or purified growth factors (2, 14, 23, 42, 50). DNA replication occurs in a large fraction of these cells within 24 h of exposure to PDGF supplemented with additional factors such as epidermal growth factor and somatomedins (2, 14, 23, 42, 46, 54). This sequence was interrupted in clones NF2 (Fig. 4A) and NF3 in the presence of dexamethasone. In the absence of dexamethasone, clone NF2 stimulated by PDGF (50 U/ml) incorporated [^{3}H]thymidine at a frequency similar to that of parental 3T3 cells (Fig. 4A; compare panel f with panel b). As expected, the parental NIH 3T3 cells were only slightly sensitive to dexamethasone (Fig. 4A; compare panel b with panel c). The transformant NF2 cells, however, showed a dramatic decrease in the number of cells undergoing DNA replication when dexamethasone was present (Fig. 4A, panel g). Thus, turning on expression of antisense c-*fos* RNA by dexamethasone in this clone appears to decrease greatly the reentry into the cell cycle from a quiescent state.



cells into S phase (panels d), whereas the difference was almost undetectable in the absence of dexamethasone (panels c). Vertical scale, number of cells; horizontal scale, DNA content. (C) Stimulation of DNA synthesis by PDGF in various transformants. Experiments similar to those described above for panel A, using 50 U of PDGF per ml for stimulation, were carried out with NIH 3T3, NF1, NF2, NF3, NF4, and NF5 cells. The results from two sets of experiments are shown separately. Labeled and unlabeled nuclei in several areas of each well were counted. A total of 700 to 1,000 cells per well were scored to arrive at the percentages shown. Open bar, without dexamethasone; solid bar, with dexamethasone.

The inhibitory effect of dexamethasone on exit from the G_0 phase was also demonstrated in a different way. By using fluorescent cell sorting of cells stained with propidium iodide, we measured the proportion of cells in each phase of the cell cycle after stimulating quiescent cultures with fresh medium containing FCS. Figure 4B shows the cell sorter trace for NIH 3T3 cells and clone NF2. As expected from results of the [^{3}H]thymidine uptake experiment, a large decrease in the proportion of NF2 cells in G_2, M, and S phases was observed in the presence of dexamethasone (30 to 8%), whereas NIH 3T3 parental cells were essentially unaffected (39 to 34%).

We carried out an extended series of similar experiments on all of our antisense c-*fos*-transformed clones. We measured the proportion of cells which began DNA synthesis following stimulation of quiescent cultures with fresh medium containing PDGF or FCS at various concentrations. The results of typical experiments are presented in Fig. 4C. At this level of PDGF (50 U/ml), approximately 30 to 40% of the control cells initiated DNA synthesis. The clones NF1, NF2, NF3, and NF5 responded similarly to the control cells in the absence of dexamethasone. When dexamethasone was present, a slightly lower level of stimulation was found with control NIH 3T3 cells and with clones NF1 and NF5. Stimulation of clones NF2 and NF3 fell dramatically in the presence of dexamethasone in these experiments. Clone NF4 showed a slight increase in stimulation relative to that of the parental clone. The pattern of response shown in Fig. 4C by each of the six cell lines was consistently found in all of our experiments at intermediate levels of stimulation (50 U of PDGF per ml or 10% FCS). The responses were somewhat modified both at extremely low (10 U of PDGF per ml) and at near saturating (250 U of PDGF per ml or 20% FCS) levels. At very low levels of stimulation, the proportion of stimulated cells fell to near zero, and the different cell lines became indistinguishable. At near saturating levels of PDGF or FCS, the inhibitory effects were overridden, and 80 to 90% of the cells of all clones were stimulated to begin DNA synthesis even in the presence of dexamethasone (Fig. 4A, panels d and h). In the intermediate range of stimulus levels, however, whether with PDGF or FCS, each of the transfected clones consistently showed its characteristic response: slight enhancement of stimulation in clone NF4, stimulation only slightly lower than that of NIH 3T3 cells in clones NF1 and NF5, and severe inhibition (four- to sixfold) in clones NF2 and NF3.

In the experiment shown in Fig. 4C, clone NF4 differed from the control even in the absence of dexamethasone. In other experiments without dexamethasone at different levels of PDGF or with FCS, we also observed slight differences between control cells and clones NF2 and NF3. Again, we must consider the possibility that the MMTV promoter is slightly leaky, allowing a low level of transcription in the absence of inducer (33, 51). These differences between

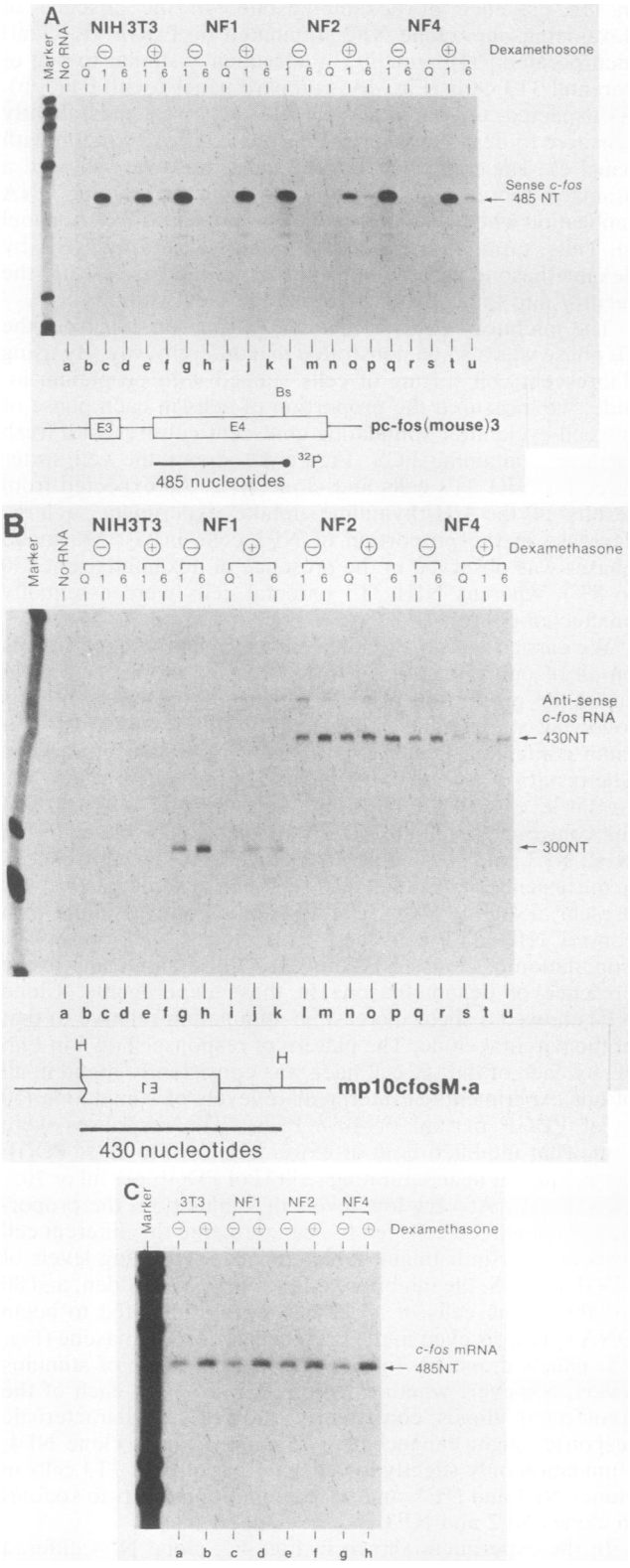


FIG. 5. Sense and antisense c-*fos* RNA expression in NIH 3T3 and various transformants. Total RNA was analyzed by S1 nuclease mapping (3). In the lower part of panels A and B the probe used (either 5′-end ^{32}P-labeled or uniformly ^{32}P-labeled) and the expected S1 nuclease-resistant DNA products are shown. Open boxes represent exons; thinner lines indicate either 5′- and 3′-flanking regions and introns or plasmid sequences. Location and size of expected S1 nuclease-resistant DNA products are shown as thick lines. Restriction sites: Bs, *Bst*EII; H, *Hin*dIII. Abbreviations: Q, quiescent state; 1 and 6, 1 or 6 h after stimulation with FCS; E, exon; NT, nucleotide. 5′-end, ^{32}P-labeled φX174 *Hae*III digests were included as size markers in each autoradiogram. NIH 3T3 cells or various antisense c-*fos* DNA transformants were plated in dishes (diameter, 10 cm) containing 10 ml of DMEM supplemented with 10% FCS. After reaching confluence, the medium was replaced with DMEM containing 0.5% calf serum for 24 h. In addition, for half of the cells, 10^{-6} M dexamethasone was included in this low-serum medium. Cells were then stimulated by DMEM containing 10% FCS with or without dexamethasone. Stimulated cells were harvested 1 or 6 h after the addition of FCS, together with unstimulated cells (quiescent cells), which were maintained in low-serum medium continuously. (A) Time course of sense c-*fos* mRNA expression following FCS stimulation. RNA (10 μg) from each time point with and without dexamethasone treatment was analyzed for mouse sense c-*fos* mRNAs by S1 nuclease mapping (3). Mouse genomic pc-fos(mouse)3 plasmid DNA was digested by restriction enzyme *Bst*EII, 5′-end ^{32}P-labeled, and used as a probe. The probe DNA protected by mouse sense c-*fos* mRNAs (485 nucleotides) encompassed a part of the fourth exon of the mouse c-*fos* gene (52), as shown at the bottom of panel A. (B) Time course of antisense c-*fos* RNA expression following FCS stimulation. The DNA probe used for S1 nuclease mapping was 430 nucleotides of uniformly ^{32}P-labeled sense strand mouse c-*fos* genomic DNA, encompassing a portion of 5′-flanking sequences and a part of the first exon. We cloned the mouse genomic DNA fragment (430-bp *Hin*cII fragment; see legend to Fig. 1) into the *Hin*dIII site of M13 vector mp10 after the *Hin*cII sites were converted into *Hin*dIII sites by linker ligation. The orientation of c-*fos* transcription in the selected clone mp10cfosM.a was the same as the DNA sequencing direction of this M13 clone. (C) Steady-state level of c-*fos* mRNA level in continuously proliferating NIH 3T3 cells and various transformants. NIH 3T3 cells and various transformants were harvested when they were exponentially growing and 50% confluent. Total RNA (20 μg; twice as much as that used for the experiment shown in panel A) was used for detection of sense c-*fos* mRNAs, as described above, except the autoradiographic exposure time was 10 times longer than that for the experiment shown in panel A.

clones in the absence of dexamethasone, however, are well within the range of clonal variation found in normal cell populations (1, 43). Thus, these differences may reflect preexisting slight differences between individual cells in the original NIH 3T3 parental culture.

Expression of sense and antisense c-*fos* RNAs. Because clones NF2 and NF3 exhibited a dramatic decrease in DNA synthesis in the presence of dexamethasone, we next asked whether this resulted from a decrease in the endogenous, translatable, sense c-*fos* mRNA level. We chose three clones, NF1, NF2 (high-copy-number clones), and NF4 (single-copy clone), for further study. The transformed clones, together with parental NIH 3T3 cells, were synchronized into the quiescent state; and then total RNAs were isolated at various times after the addition of 10% FCS, which gave a stimulation of DNA synthesis similar to that of 50 U of PDGF per ml, as was used for the [^{3}H]thymidine incorporation experiments, and resulted in the maximum phenotypic change in the presence of dexamethasone. The level of the sense c-*fos* mRNAs and antisense c-*fos* RNAs were analyzed by S1 nuclease mapping (3) by using specific probes. The amount of RNA used in each sample (10 μg of total RNA) was checked by monitoring the mRNA level of a housekeeping gene, mouse phosphoglycerate kinase (PGK), by using a mouse PGK cDNA plasmid (pMPGK-5b) (36) and S1 nuclease mapping analysis. The variation in RNA content between different samples monitored by PGK expression

was negligible (data not shown). The level of sense c-*fos* mRNA in NIH 3T3 parental cells in the quiescent state in the absence (Fig. 5A, lane b) or presence (data not shown) of dexamethasone was very low, as reported previously (21, 29, 38). At 1 h after stimulation of these quiescent cells with FCS, the level dramatically increased (approximately 100-fold; Fig. 5A, lanes c and e) and by 6 h had returned to a low level, similar to that at quiescent state (Fig. 5A, lanes d and f). Although the level of sense c-*fos* mRNAs 1 h after stimulation in the presence of dexamethasone slightly decreased (10%) from that in the absence of hormone, the general pattern of sense c-*fos* mRNA kinetics in NIH 3T3 cells was unchanged whether or not the dexamethasone was included in the culture medium. Similar results were obtained with clones NF1 and NF4, except that the peak levels of sense c-*fos* mRNA (1 h after stimulation) were slightly higher (approximately 30 to 40%) than those of parental cells, regardless of the absence or presence of dexamethasone in the culture medium (Fig. 5A, lanes g, j, q, and t). When, however, the level of sense c-*fos* mRNA in clone NF2 was analyzed, a large effect of dexamethasone was observed. In the absence of dexamethasone, clone NF2 contained a slightly (30%) higher level of sense c-*fos* mRNAs 1 h after stimulation of quiescent cells with FCS (Fig. 5A, lane l) in comparison with NIH 3T3 cells (Fig. 5A, lane c). In the presence of dexamethasone the level at the same time point dropped by approximately sixfold (Fig. 5A, lane o). In the quiescent state, or 6 h after stimulation, no significant difference in the sense c-*fos* mRNA levels between NIH 3T3 and NF2 cells (both very low) was observed, regardless of whether dexamethasone was present. A similar experiment, but with RNAs prepared at more closely spaced time points from synchronized NIH 3T3 and NF2 cells, was carried out to exclude the possibility that in clone NF2 the c-*fos* mRNA level in the presence of dexamethasone was not lower than in the parental cells, but rather that the peak of the expression had been shifted to earlier or later time points. Our results show that the time course of c-*fos* expression after stimulation in NF2 cells is identical to that in NIH 3T3 cells, but the peak level (at 45 min in both cell types) in NF2 is simply much lower than that in parental cells in the presence of dexamethasone (data not shown). The steady-state level of sense c-*fos* mRNAs prepared from unsynchronized and exponentially growing cells was low but almost identical among NIH 3T3 and NF2 cells as well as the other two clones examined in the absence and presence of dexamethasone (Fig. 5C).

The simplest interpretation of our results is that the blockage of DNA synthesis observed in synchronized NF2 cells in the presence of dexamethasone is a consequence of the observed decrease in the sense c-*fos* mRNA level, and that this decrease is due to increased transcription of antisense c-*fos* RNA under control of the MMTV promoter. We next investigated whether these findings could be correlated with steady-state levels of antisense RNA present in the transformed clones.

By using our standard S1 nuclease protection assay with the 5′-end ^{32}P-labeled probe (as used for sense mRNA detection in Fig. 5A and C), antisense c-*fos* RNA was undetectable. This is consistent with results of previous investigations in which the antisense approach was used and in which strong specific phenotypic effects were observed but steady-state antisense RNA levels were extremely low (9, 28). When we increased the sensitivity of our assay system by using a uniformly ^{32}P-labeled probe and long exposure times for autoradiography, we detected S1 nuclease-resistant DNA products of the size expected from protection by antisense c-*fos* RNA (Fig. 5B). However, the quantity detected was quite small, of the order of 1% of the level of sense c-*fos* RNA measured in the same cells. Because even the observed decrease in steady-state c-*fos* mRNA induced by antisense RNA transcription was two orders of magnitude greater than the measured steady-state antisense RNA level, we conclude that steady-state levels of antisense RNA do not correlate well with the observed effects of this antisense RNA. Antisense thymidine kinase gene transcripts have been shown to be very unstable, and the hybrid sense-antisense duplex RNA is also thought to be very short-lived (25). Thus, the vast majority of the transcribed antisense c-*fos* RNA is quickly degraded, leaving for us to detect only that very small fraction which somehow escapes the normal degradative pathway. The size of this small fraction does not appear to reflect accurately differences in transcription rate of antisense RNA between different clones or different transcription rates induced by dexamethasone (compare Fig. 5B, NF4 with NF2, or NF2 plus or minus dexamethasone). Nevertheless, this measurement may be of some relevance to future investigations of the mechanism of antisense RNA action. Particularly interesting in this regard is the finding that in clone NF1, which failed to show significant phenotypic effects in spite of a large number of integrated copies of antisense DNA, the expected S1 nuclease-protected DNA fragment was not observed but was replaced by a shorter product of about 300 nucleotides (Fig. 5B, lanes g to k). Because the probe used for S1 nuclease analysis was a uniformly ^{32}P-labeled DNA, we cannot locate precisely the position of the 300-nucleotide

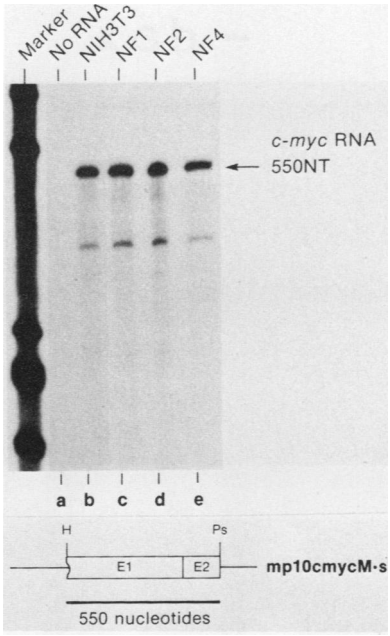


FIG. 6. Detection of c-*myc* mRNA in NIH 3T3 and various transformants. Total RNA (10 μg) from NIH 3T3 or various clones stimulated with 10% FCS for 1 h with or without dexamethasone was analyzed for c-*myc* mRNA by S1 nuclease mapping, as described in the legend to Fig. 5. The DNA probe used was 550 nucleotides (NT) of the uniformly ^{32}P-labeled antisense strand of mouse c-*myc* cDNA. We cloned the cDNA fragment of pMcmyc54 plasmid (49) (550-bp *Hin*dIII-*Pst*I fragment), encompassing a part of the first and second exons (E1 and E2), into the *Hin*dIII and *Pst*I sites of M13 vector mp11. Restriction sites: H, *Hin*dIII; Ps, *Pst*I.

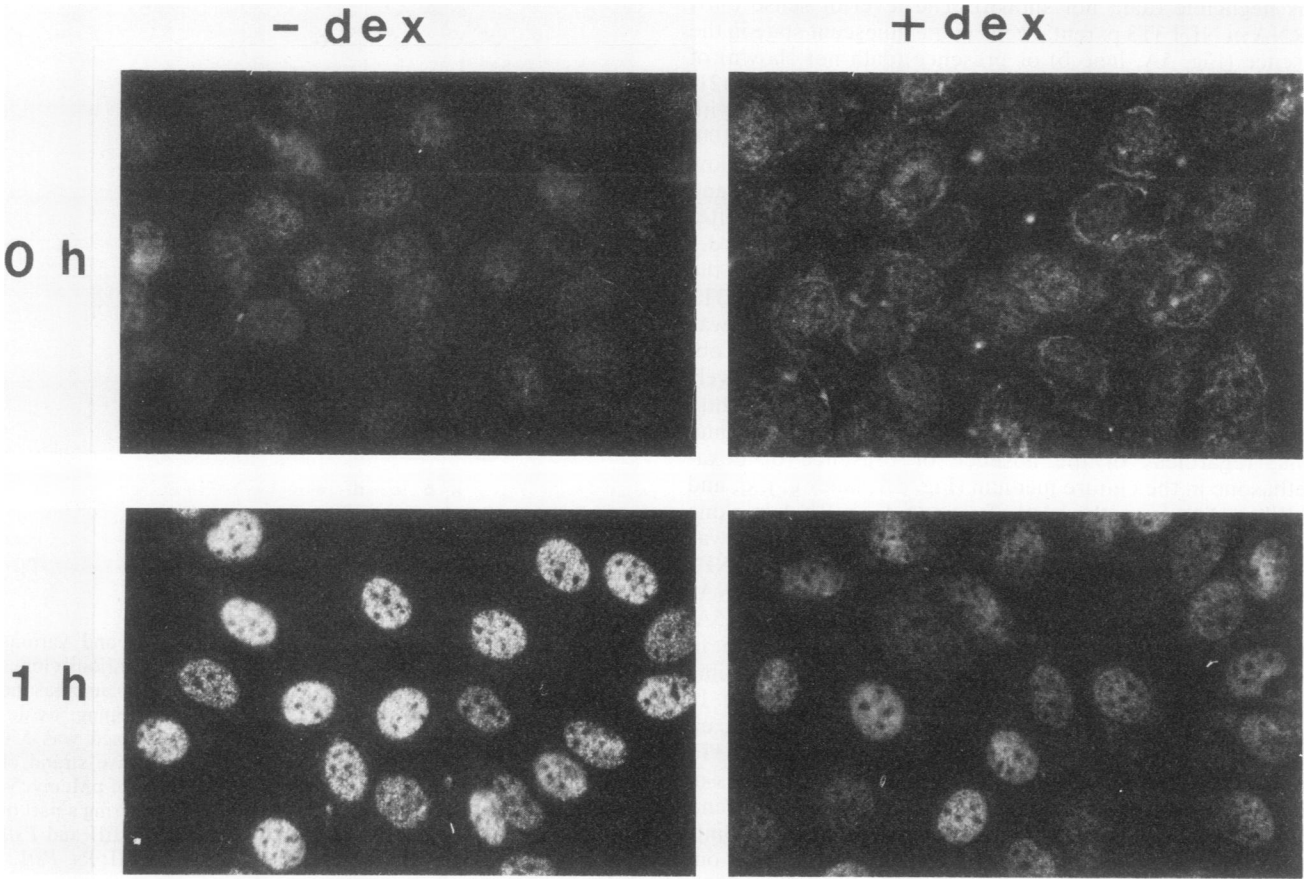


FIG. 7. Detection of the c-*fos* protein by immunochemical fluorescent labeling. Quiescent cultures of clone NF2 were exposed to fresh medium containing 10% FCS with and without 10^{-6} M dexamethasone (dex), as described in the legend to Fig. 4. Cells were fixed either immediately (0 h) or 1 h after stimulation. All photographs were recorded and printed with identical exposure times to enable comparison of fluorescence intensities among different samples. See the text for a description of the technique.

protected DNA fragment along the 430-nucleotide probe DNA (Fig. 5B). This unexpected S1 nuclease signal may possibly be generated by antisense c-*fos* RNAs transcribed from the MMTV promoter but spliced aberrantly.

Decrease of c-*fos* expression does not affect c-*myc* expression. Although expression of both proto-oncogenes c-*fos* and c-*myc* are mitogen responsive (21, 29, 38), the activation of c-*fos* gene transcription (5 min after stimulation) precedes that of c-*myc* (60 min after stimulation), suggesting the possible coupled expression of these two genes (21). We therefore asked whether the decrease in c-*fos* expression would block the subsequent c-*myc* expression. S1 nuclease mapping analysis of c-*myc* mRNAs for various cells 1 h after FCS stimulation in the presence of dexamethasone is shown in Fig. 6. Clone NF2, in which the c-*fos* mRNA level dramatically decreased in the presence of dexamethasone, synthesized the same amount of c-*myc* RNA as the parental NIH 3T3 cells or other transformants (Fig. 6, lane d). We conclude that c-*fos* and c-*myc* expression is independently regulated rather than tightly coupled.

Nuclear c-*fos* protein content is diminished by antisense c-*fos* RNA expression. Previously, it has been shown that stimulation of quiescent cells by PDGF gives rise to a rapid transient increase in nuclear c-*fos* protein level (29, 38). We have shown that expression of antisense c-*fos* RNA blocks the rise in the c-*fos* mRNA level which normally precedes this increase in protein and would be expected to have a strong effect on the protein levels as well. Figure 7 shows that expression of the antisense RNA induced by dexamethasone does indeed diminish dramatically the nuclear accumulation of the c-*fos* protein in clone NF2. In contrast, dexamethasone had almost no effect on nuclear accumulation of the c-*fos* protein in clones NF1 and NF4 (data not shown), which is consistent with the nearly normal sense c-*fos* RNA levels in these clones.

DISCUSSION

Specificity of effects of antisense RNA expression. In the presence of dexamethasone, quiescent cells of clones NF2 and NF3 showed a greatly diminished level of DNA replication following PDGF stimulation. The presence of dexamethasone also prevented the large increase in c-*fos* RNA and protein that would normally occur with PDGF treatment, but it had no effect on the level of either PGK or c-*myc* RNA. Thus, the inhibition seems to be a specific effect on the target gene, which is a consequence of the blockage in expression of the c-*fos* gene caused by sense-antisense RNA duplex formation.

One possible nonspecific effect of antisense RNA expression should be considered. Double-stranded RNA is known to be an inducer of interferon, which is an inhibitor of protein synthesis (31). Because the stimulation of DNA replication caused by PDGF can be blocked by protein synthesis

inhibitors (6), it might be argued that the observed effects are mediated by interferon induction in response to the c-*fos* sense-antisense RNA duplex formation rather than by the decreased c-*fos* sense mRNA levels per se. There are several reasons, however, for rejecting this argument. First, the inhibition of DNA replication observed in the presence of dexamethasone was shown to be completely reversed by treating with high levels of PDGF. Second, the measured c-*fos* RNA level was lower when dexamethasone was present, whereas inhibition of protein synthesis is known to greatly enhance the induction of c-*fos* RNA by PDGF (29, 38). Finally, sense-antisense RNA duplex formation has been shown not to induce detectable levels of interferon in earlier applications of this technique (26).

Phenotype variation in antisense c-*fos*-transformed cells. Stimulation of DNA replication by PDGF was not affected by dexamethasone in the antisense c-*fos*-transformed clones NF1, NF4, and NF5, nor was the PDGF-induced rise in c-*fos* RNA and protein diminished. The antisense RNA appeared to be processed aberrantly in clone NF1, which may explain the lack of phenotypic alteration in this clone. Alternatively, the result may simply be due to inactivity of the chromosomal site at which the antisense c-*fos* DNA was integrated. The transcription rate from the MMTV promoter has been reported to be strongly affected by the integration site (51). Variations in the phenotype of antisense RNA-transformed cells have also been noted in previous studies using this technique (28). This variability makes it necessary to determine by direct measurement, as we have here, that the expected decrease in expression of the target gene actually occurs. Another means of demonstrating the specificity of the phenotype changes, which we have not used here, would be to carry out a rescue experiment by transfecting the antisense transformants with a sense RNA construct. If the transformants revert to the wild type on supertransfection with the sense RNA construct, this would also confirm, albeit indirectly, that the original altered phenotype was the result of lowered expression of the target gene.

Role of c-*fos*. Results of previous studies have demonstrated the dramatic rise in both c-*fos* mRNA and c-*fos* protein levels that occurs within minutes of stimulation of quiescent fibroblasts by FCS or PDGF (21, 29, 38). We have shown here that this large increase in c-*fos* mRNA level is a necessary feature of the transition from quiescence through the G_1 phase, subsequent DNA replication, and on to renewed growth. The increase in the nuclear c-*fos* protein level following stimulation in our antisense c-*fos* transformants, while much smaller than that in control cells, was still detectable yet insufficient to support the normal progression to replicative DNA synthesis. The requirement for such a large quantity of c-*fos* protein may indicate that it plays a structural role in the events leading up to DNA replication.

Cells that are continuously proliferating have much lower levels of c-*fos* mRNA and protein than PDGF-stimulated quiescent cells (38) and, presumably, also have lower requirements for c-*fos* (5, 38). This presumption is supported by our observation of nearly normal steady-state growth rates of clones NF2 and NF3 in the presence of dexamethasone; i.e., steady-state growth is normal under conditions in which enough antisense c-*fos* RNA is being expressed to inhibit the transition from quiescence to renewed growth. Holt et al. (24), however, report a significant decrease in the steady-state growth rate after transfection of NIH 3T3 cells with antisense c-*fos* DNA, although they did not address the issue of reentry into growth of quiescent cells. Unfortunately, that report did not include a measurement of actual levels of c-*fos* expression in the transformed cells, which leaves open the possibility that some nonspecific effect on growth rate was being observed. Alternatively, the discrepancy between our results with continuously proliferating cells and those of Holt et al. (24) may be due simply to a higher level of antisense c-*fos* RNA expression in their transformed cells. As measured by hybridization to a β-globin gene fragment, Holt et al. (24) reported very high expression of the chimeric globin antisense c-*fos* RNA used for their transformations. Higher expression, however, may not account completely for the discrepancy, because Holt et al. reported that they found no correlation between measured levels of their chimeric antisense RNA and inhibition of growth. Thus, the presently available data are not sufficient to define clearly the role of c-*fos* in continuously proliferating cells.

In summary, we have demonstrated that the large increase in c-*fos* that follows growth factor stimulation of quiescent cells is indeed a requirement for, not merely an accompaniment of, renewed growth. Our results also suggest that the later increase in c-*myc* is not dependent on the large increase in c-*fos*. Finally, in spite of some variability in phenotypic effects, these experiments demonstrate that the technique of antisense transformation is a useful tool for studying cellular oncogene function.

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