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Depletion of c-*myc* with Specific Antisense Sequences Reverses the Transformed Phenotype in *ras* Oncogene-Transformed NIH 3T3 Cells

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***ras* oncogene-transformed NIH 3T3 cells expressing glucocorticoid-inducible antisense c-*myc* cDNA transcripts at levels sufficient to deplete c-*myc* protein lost their transformed morphology and the ability to grow in soft agar; their ability to form tumors in nude mice was also impaired. These changes were dependent on the continuous expression of the antisense sequences. No major effects on plating efficiencies, growth rates in monolayer culture, or immortalization were observed in the revertant cells, indicating that the observed effects were not a toxic consequence of c-*myc* protein depletion. Transfection with the same vector expressing c-*myc* in the sense orientation or other control vectors had no effect on transformation. These results suggest that a certain minimum level of expression of c-*myc* is required for the maintenance of *ras* transformation in NIH 3T3 cells.**

The mechanism by which extranuclear oncoproteins such as *ras* and *src* transmit their signals for aberrant growth to the nucleus and mediate the cellular changes associated with transformation is one of the central questions in tumor cell biology. A related question is whether these changes are permanent or whether they are susceptible to reversion or modification through the manipulation of genes whose products participate in or interact with oncogene signaling pathways.

Oncoproteins appear to be components of pathways that are normally used to transmit mitogenic signals from the extracellular milieu to the nucleus (10, 19). In neoplasia, these products are altered qualitatively or quantitatively, thus leading to an inappropriate proliferative response (2, 26, 52, 56). Given the apparent intimate association between oncoproteins and otherwise normal mitogenic pathways, it seems reasonable to believe that oncogenic signals are transmitted along these same pathways.

We are interested in testing the hypothesis that maintenance of transformation by *ras* oncogenes involves nuclear proto-oncogenes whose expression might modulate some or all of the cellular features associated with transformation. That such genes might be the ultimate effectors of *ras* transformation seems reasonable in light of the fact that they themselves can function as transforming oncogenes in certain circumstances (30, 36, 42), that virtually all of them are proven or putative transcription factors (9, 21), and that their levels of expression are often increased by transformation and by normal growth stimuli (5, 15, 38–40, 44, 49, 54).

While transformation of mouse fibroblast cell lines by *ras* oncogenes increases the level of expression of c-*myc* only modestly if at all (7), an association between the *ras* and *myc* oncogenes has previously been suggested by experiments showing that their coexpression is necessary for the efficient transformation of primary rodent fibroblasts (25). The resistance of primary cells to *ras* transformation can also be overcome by the expression of certain other nuclear oncogenes such as simian virus 40 (SV40) large T and adenovirus E1a (55). In conjunction, these findings suggest a possible essential role for the c-*myc* proto-oncogene in the *ras* transformation pathway.

To determine whether maintenance of the transformed phenotype requires a minimum level of expression of c-*myc*, we expressed glucocorticoid-inducible c-*myc* antisense sequences in *ras* oncogene-transformed NIH 3T3 cells. We have obtained several clones which contain unrearranged vector sequences and express c-*myc* antisense transcripts, accompanied by lowered levels of c-*myc* protein, following exposure to dexamethasone (DM). In all cases tested, depletion of c-*myc* protein caused a loss of several properties typically associated with *ras* oncogene-mediated transformation. Our results suggest that the c-*myc* gene product may participate in or regulate the pathway(s) used by mutant *ras* genes to transform NIH 3T3 cells. Antisense-mediated inhibition of specific gene products may thus be a generally useful strategy for determining whether they are required for the maintenance of the transformed state.

MATERIALS AND METHODS

Plasmid constructions. In most cases, the starting plasmid was pMSG (Pharmacia, Piscataway, N.J.), which contains a murine mammary tumor virus (MMTV) promoter upstream of a polylinker cloning site. Downstream splice and polyadenylation sites are derived from the SV40 early region. A 2.25-kb *Bam*HI restriction fragment harboring an SV40-*Escherichia coli gpt* transcription unit was excised from the plasmid, which was then religated and subsequently designated pMS-G. A 2.8-kb dihydrofolate reductase (*dhfr*) tran-

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scription unit from plasmid pFR400 (43) was cloned into a unique *Eco*RI site of pMS-G to yield the construct pMS-Gdhfr. All proto-oncogene cDNAs except c-*myc* were blunt-end ligated into a unique *Sma*I site in the polylinker region of this plasmid located downstream of the MMTV promoter. In the case of c-*myc*, a 1.4-kb *Xho*I cDNA fragment (50) was cloned into the unique *Xho*I site in the pMS-Gdhfr polylinker. Other cDNAs used included those for murine c-*jun* (1.45-kb *Eco*RI-*Sca*I fragment) (40) and murine c-*myb* (0.83-kb *Eco*RI fragment) (4). Plasmids containing these cDNAs were gifts from R. Bravo and T. Bender, respectively. All cDNA orientations in the MMTV vector were confirmed by restriction enzyme mapping, and their expression was verified by Northern (RNA) blot or S1 nuclease protection analyses of RNAs extracted from DM-treated or untreated DT cells. Both sense and antisense cDNA orientations were obtained for all vectors. In the case of c-*myc*, these vectors are referred to as pMS-Gmyc-S-dhfr and pMS-Gmyc-AS-dhfr, respectively. Additional vectors were constructed in plasmid pSV_2dhfr, which has been previously described and which allows for the constitutive expression of desired sequences (33, 34). These constructs are referred to as pSV_2myc-S-dhfr or pSV_2myc-AS-dhfr. In these vectors, the c-*myc* cDNA insert was a 1.8-kb *Hin*dIII fragment. In subsequent experiments, we have exchanged the *dhfr* transcription unit in plasmid pMS-Gmyc-AS-dhfr for a neomycin transcription unit to allow for a more direct selection of clones with antisense sequences.

Cell culture and gene transfer. All cell lines were routinely cultured in Dulbecco's modified Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum or NuSerum (Collaborative Research), 200 mM L-glutamine, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml (D10 medium). Cell lines were maintained at 37°C in a 5% CO_2 atmosphere. As transfection recipients, we initially selected the DT cell line, derived from NIH 3T3 cells transformed by infection with v-K-*ras* (3). These cells display a highly transformed morphology and contain two copies of the v-K-*ras* genome, thus making spontaneous reversion of transformation a rare event. As our initial assay for an antisense-mediated effect was based on morphological reversion, a low frequency of spontaneous reversion was essential. Other NIH 3T3-derived transformed cell lines used included 5139, transformed by the EJ c-H-*ras* oncogene (five copies), and N/HL60, transformed by N-*ras* (46). Untransformed NIH 3T3 cells were obtained from D. Lowy.

All cell lines were cotransfected with the plasmid being tested and pSV_2neo at a 10:1 molar ratio, using the calcium phosphate coprecipitation protocol previously described (45, 46) or electroporation with a Bio-Rad Gene Pulser. Two days after transfection, the growth medium was replaced with fresh medium containing G418 (GIBCO, Grand Island, N.Y.) at a final absolute concentration of 0.5 mg/ml. The G418-containing growth medium was replaced with fresh medium every 3 to 4 days. After 7 to 10 days, G418-resistant colonies were screened for morphological reversion; flat colonies were picked into 24-well plates and expanded. When the transfected sequences were under the control of the MMTV promoter, DM was added to a final concentration of 1 μM and colonies were examined the next day for morphological change. Flat colonies were picked and subsequently recloned by limiting dilution in 96-well plates. After expansion, all clones were tested for the presence and expression of unrearranged, vector-associated c-*myc* sequences, for c-*myc* protein expression, and for several biological properties known to be hallmarks of *ras*-transformed fibroblasts. All the colonies reported here were derived from independent transfection events.

Nucleic acid analyses. Southern blots were performed according to standard procedures, using 10 μg of *Xho*I-digested cellular DNA. Following electrophoresis in 1% agarose gels, DNAs were transferred to Nytran membranes (Schleicher & Schuell, Keene, N.H.). The probe was the same 1.4-kb c-*myc* *Xho*I cDNA fragment used in the construction of the antisense vectors. This was labeled with [α-^{32}P]dCTP by the random priming method (12). With Southern blot analysis, the endogenous murine c-*myc* gene is visualized as a single 5.0-kb band, whereas the 1.4-kb cDNA fragment is excised from unrearranged plasmid sequences.

The expression of c-*myc* antisense sequences was routinely monitored by S1 nuclease protection analysis (33, 34). In the case of pMS-Gmyc-AS-dhfr-transfected cells, 10 μg of total RNA from untreated or DM-treated cultures was hybridized with a 393-nucleotide (nt) long *Sst*I-*Xho*I restriction fragment from pMS-Gmyc-AS-dhfr. The fragment was end labeled at the *Xho*I site with polynucleotide kinase and [γ-^{32}P]ATP (specific activity, >7,000 Ci/mmol; Amersham, Arlington Heights, Ill.). In the case of pSV_2myc-AS-dhfr-transfected cell lines, the S1 probe consisted of a 670-nt-long *Nde*I-*Xho*I probe end labeled at the *Xho*I site (34). Conditions of hybridization were as previously described (34), and digestions were performed with 85 U of S1 nuclease (Sigma, St. Louis, Mo.). S1 nuclease digestion products were displayed on 2% agarose gels, which were subsequently dried and processed for autoradiography. RNAs were also examined by standard Northern blot analysis, using differences in the sizes of the endogenous and antisense messages to distinguish between the two.

Determination of c-*myc* protein levels. A 1.15-kb *Pst* fragment from the 3′ end of the murine c-*myc* cDNA was cloned into the pATH 23 expression vector (23) to generate a TrpE fusion protein containing the C-terminal 259 amino acids of c-*myc*. Up to 50% of the insoluble protein from indoleacrylic acid-induced *E. coli* harboring this plasmid consisted of the fusion product. Insoluble *E. coli* extracts were boiled in 1% sodium dodecyl sulfate (SDS) and resolved on 7.5% linear polyacrylamide-SDS gels. Fusion protein was visualized with fluorescamine (Sigma), excised, emulsified in complete Freund's adjuvant, and used for the generation of polyclonal rabbit antiserum. The generation of specific antibody was monitored by its ability to precipitate [^{35}S]methionine-labeled c-*myc* protein from in vitro translation reactions. Affinity purification of the antibody was performed as previously described (35, 47). To detect c-*myc* proteins in the various DT-derived cell lines, identical cultures were grown for 48 h in the absence or presence of 1 μM DM. At the end of this time, cultures were approximately 80% confluent. Cells were harvested by scraping and lysed in RIPA buffer containing protease inhibitors as described previously (47). Total cell extract (100 μg) was electrophoresed through a 7.5% linear SDS-polyacrylamide gel and transferred to a nitrocellulose filter by electroblotting. Blocking was performed overnight at room temperature in TBS buffer (0.15 M NaCl, 20 mM Tris-HCl, pH 7.6) containing a 5% suspension of nonfat dry milk. After extensive washing in TBS plus 0.5% Tween 20 (TBS-T), anti-c-*myc* antibody was added at a final dilution of 1:100 in TBS and incubation continued for an additional 4 to 6 h at room temperature with continuous rocking. The blot was washed extensively in TBS-T and then decorated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:100; Bethesda Research Laboratories). c-*myc* protein was visualized through the use of the

enhanced chemiluminescence kit (Amersham) as instructed by the supplier.

Growth in soft agar. A total of 10^2 to 10^3 cells were plated in 1 ml of 0.33% low-melting-point agarose (SeaPlaque; FMC) in Dulbecco's MEM containing 20% fetal bovine serum, glutamine, and antibiotics (D20 medium) over a 5-ml 0.9% Noble agar bottom layer prepared in the same medium. Plates were allowed to equilibrate at 4 to 20°C (no temperature-related differences in plating efficiency were observed) before being placed in a humidified 5% CO_2 incubator. Colonies were counted 10 to 14 days after the initial plating. In general, plates were fed (with D20–0.33% agarose) and observed at weekly intervals for as long as 17 to 28 days to take into account possible differences in growth rates. To assess cloning efficiencies in the presence of DM, cells were first grown for 24 to 48 h in culture medium containing 0.5 to 1 μM DM and were then plated under the conditions described above, with DM added to both agar layers at a final concentration of 0.5 to 1 μM. This concentration of DM did not affect the agar cloning efficiencies of DT or other control cells.

Determination of growth rate in monolayer culture. Growth rates were determined by plating 10^4 cells into 35-mm wells in the presence of 0.5 to 1 μM DM. One to three times a day, triplicate sets of plates were trypsinized and viable cell counts performed. Doubling times were calculated on the basis of growth curves obtained over at least six doublings (>5 days) during the exponential growth phase.

Cell cycle analysis. Nuclei were prepared as previously described (47) and stained with propidium iodide. DNA profiles were obtained from 10,000 to 20,000 nuclei, using a Coulter EPICS V flow cytometer (EPICS Division, Coulter Corp., Hialeah, Fla.). Data were analyzed by using the PARA I program to determine the percentage of cells in G_0/G_1, S, and G_2/M phases.

Tumorigenesis in nude mice. Six-week-old *nu/nu* mice were injected subcutaneously in both left and right upper dorsal regions with 10^5 cells, using an average of 10 animals per group. Animals were examined daily, and tumors were measured by a single observer using a calipers. Tumor volumes were calculated by the method of Morrison (31). Because interexperimental data were consistent, tumor volumes from multiple experiments were combined and plotted against time, and means and standard errors of each point were calculated. Statistical significance was calculated by Student's *t* test.

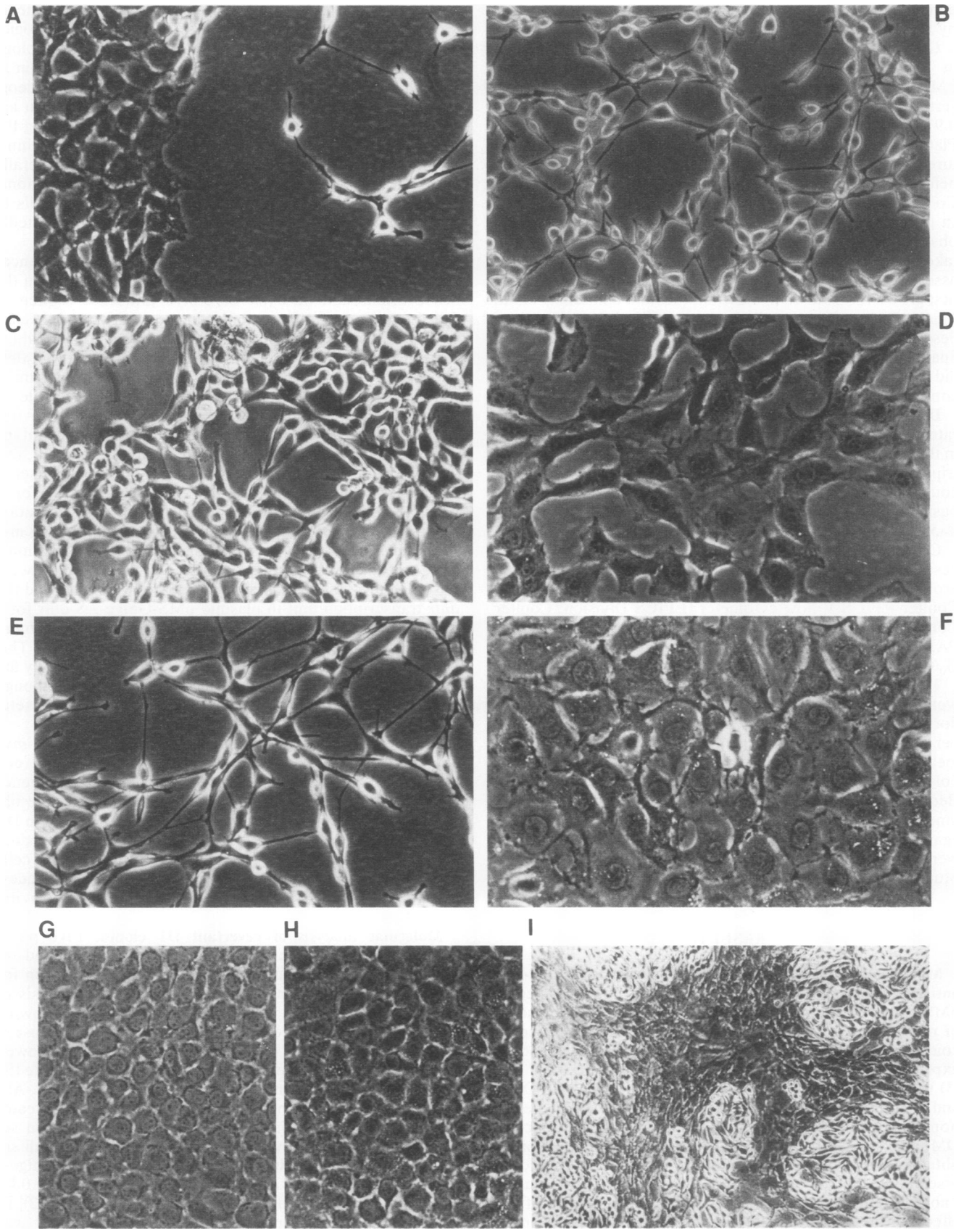
RESULTS

Morphological reversion of DT cells transfected with c-*myc* antisense expression vectors. We first constructed the vector pMS-Gmyc-AS-dhfr, which allowed conditional expression of murine c-*myc* antisense transcripts from the glucocorticoid-responsive MMTV promoter. In several independent experiments, the v-K-*ras*-transformed NIH 3T3 DT cell line (3) was transfected with 10 μg of supercoiled plasmid DNA and 1 μg of supercoiled pSV_2neo DNA, followed by selection in the aminoglycoside antibiotic G418. In the absence of DM, approximately 10 to 15% of the G418-resistant colonies obtained possessed a flattened or revertant morphology, resembling that of untransformed NIH 3T3 cells (Fig. 1; Table 1). This presumably reflects some combination of the effects of the presence of serum glucocorticoids with constitutive expression of c-*myc* antisense transcripts due to positional effects on the MMTV promoter. Within 24 h of the addition of 0.1 to 1 μM DM, however, up to 80% (mean, 55%) of the G418-resistant clones had flat morphologies (Table 1; Fig. 1). The effect of DM on colony morphology was readily reversible and was generally complete within 18 to 24 h of removal of the inducer. Using time lapse videography, we detected noticeable morphologic changes in antisense clones within as little as 30 to 60 min following the addition of DM. Many cells became completely flat within 6 to 8 h, and the entire reversion process was generally complete within 16 to 18 h after addition of DM. Additional cycles of addition and removal of DM caused the cells to flatten and regain the transformed morphology repeatedly (unpublished observations).

DT cells transfected with pMS-Gdhfr vector sequences alone or the same vector containing c-*myc* sequences in the sense orientation or c-*jun* or c-*myb* antisense sequences failed to give rise to flat colonies in the presence or absence of DM (Table 1). We also performed transfections in which antisense sequences were expressed from the pSV_2dhfr expression vector (33, 34). The results obtained were similar to those seen with pMS-Gdhfr expression vectors (Table 1). However, many of the flat cell lines generated with this plasmid eventually reverted to a transformed phenotype after weeks or months in culture. In all cases examined, this was associated with the concurrent loss of c-*myc* antisense sequences (not shown). As an additional control for a possible role for double-stranded RNA in transformation reversion, two different pSV_2dhfr plasmids, containing murine c-*myb* sequences in sense and antisense orientations, were cotransfected into DT cells. No flat colonies were observed (Table 1). In later experiments, we exchanged the *dhfr* transcription unit in plasmid pMS-Gmyc-AS-dhfr for a *neo* transcription unit from pSV_2neo to allow for a more direct selection of clones with antisense sequences. This caused no difference in our ability to derive conditionally flat DT revertants (Table 1; unpublished observations), although it did facilitate selection and indicated that the *dhfr* cassette played no role in morphologic reversion.

In other experiments, we determined the effect of c-*myc* antisense expression on the morphology of NIH 3T3 cell lines transformed by mutant c-H-*ras* and N-*ras* oncogenes (46). In both cases, morphologically flat colonies were obtained with frequencies comparable to those observed in DT cells (Table 1). Thus, c-*myc* antisense sequences appeared able to cause morphologic reversion in NIH 3T3 cells transformed by all three common classes of *ras* oncogenes, irrespective of whether *ras* is driven by cellular or retroviral promoters.

Molecular analysis of revertant DT clones. Five pMS-Gmyc-AS-dhfr-transfected DT cell clones that showed revertant morphologies after DM treatment were chosen for further characterization. All were selected on the basis of striking and reversible changes in morphologies following DM addition. A sixth clone (46B2) was chosen because it was constitutively flat in the absence of DM and showed little morphological change following its addition. Unlike the clones generated by transfection with plasmid pSV_2myc-AS-dhfr, however, this clone demonstrated considerably greater in vitro stability, tending to retain transfected plasmid sequences over several months in culture. DNAs from all six clones were examined by Southern blotting following digestion with *Xho*I. This revealed the presence of a 5.0-kb endogenous c-*myc* band in all cell lines examined (Fig. 2). In each of the transfectants, an additional 1.4-kb band was observed, indicating the presence of unrearranged exogenous antisense c-*myc* sequences. This band was not ob-


A
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I

TABLE 1. Morphologic reversion of *ras*-transformed cells by c-*myc* antisense expression plasmids

Cell line	Transfected plasmid[a]		Flat/total G418-resistant colonies[b]	
	Vector	Insert	−DM	+DM
DT (v-K-*ras*)	pSV_2neo	None	0/>1,000	0/>1,000
DT	pMS-Gdhfr[c]	None	0/85	0/85
	pMS-Gdhfr[c]	c-*myc*AS	92/798	354/798
	pMS-Gdhfr[c]	c-*myc*S	0/>300	0/>300
	pMS-Gdhfr[c]	c-*jun*AS	0/797	0/797
	pSV_2dhfr[d]	c-*myc*AS	40/562	
	pSV_2dhfr[d]	c-*jun*AS	0/112	
	pSV_2dhfr[d]	c-*myb*AS	0/190	
	pSV_2dhfr[d]	c-*myb*AS + c-*myb*S	0/90	
5139 (c-H-*ras*)	pSV_2neo	None	0/>1,000	
	pSV_2dhfr	c-*myc*AS	65/96	
N/HL60 (N-*ras*)	pSV_2neo	None	0/1,000	0/>1,000
	pMS-Gneo[c]	c-*myc*AS	12/186	56/186

[a] All cells were cotransfected with 1 μg of pSV_2neo DNA and the indicated proto-oncogene expression plasmid at a 10-fold molar excess; 48 h after transfection, cells resistant to G418 were selected in the continuous presence of G418. AS, antisense; S, sense.

[b] Colony morphologies were evaluated 7 to 10 days after G418 addition. For cells transfected with glucocorticoid-inducible vectors only, fresh culture medium containing 1 μM DM was then added to plates for 16 to 24 h, at which time colony morphology was determined.

[c] Inducible MMTV promoter.

[d] Constitutive SV40 promoter.

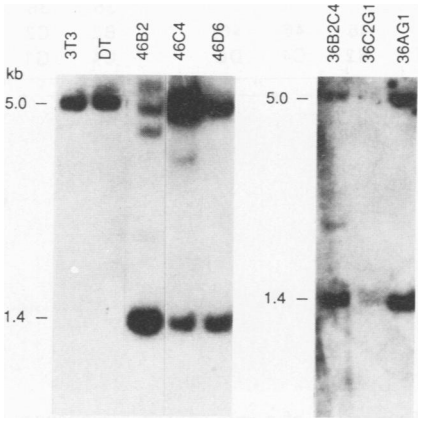

FIG. 2. Southern blot analysis of DT clones. DNAs from untransformed NIH 3T3 cells, v-K-*ras*-transformed DT cells, or individual DT clones transfected with the glucocorticoid-inducible pMS-Gmyc-AS-dhfr vector were digested with *Xho*I, electrophoresed, blotted, and hybridized with a random-primed ^{32}P-labeled murine c-*myc* cDNA probe as described in Materials and Methods. An endogenous c-*myc* band of 5.0 kb is seen in all lanes. The incorporation of unrearranged antisense vector sequences is indicated by the presence of a 1.4-kb c-*myc* fragment. Additional bands in some lanes indicate the presence of rearranged c-*myc* sequences as well as clonal uniqueness.

served in control NIH 3T3 or DT cells. Digestion of the DNAs with other restriction enzymes revealed the presence of unique plasmid junctional fragments in each case, thus confirming that each cell line arose from an independent transfection event (not shown).

The expression of c-*myc* antisense sequences in the absence or presence of DM was assessed by an S1 nuclease protection assay. As expected, no c-*myc* antisense transcripts were detected in untransfected cells (Fig. 3). In five of the six antisense cell lines, a low level of antisense transcription was detected in the absence of DM. The 46B2 cell line was the only exception, demonstrating a significantly higher basal level of antisense transcript, which may account for its constitutively flat morphology. DM induced significant increases of antisense transcripts in all clones, including 46B2. In other studies, we have shown that maximal expression of these transcripts occurs within 1 to 2 h of the addition of DM and that an additional 6 to 8 h is required for them to reach basal levels after its removal (not shown). To measure the relative concentrations of antisense to endogenous sense transcripts, Northern blot analyses were performed on selected RNAs under conditions that allowed for resolution of the two types of transcripts. Depending on the cell line, each of the antisense clones expressed from 3 to 100 times more antisense than sense c-*myc* message following the addition of DM (not shown). Thus, we conclude that all clones examined contained unrearranged and highly expressible c-*myc* antisense sequences.

Antisense-mediated reductions in c-*myc* protein levels in antisense clones. To determine whether c-*myc* antisense transcript expression led to a reduction in c-*myc* protein levels, control DT cells and individual antisense cell lines were cultured separately in the presence of 1 μM DM for 48 h. Identical plates of cells were cultured in parallel but without DM. Cells were harvested and lysed, and 100 μg of each lysate was subjected to SDS-polyacrylamide gel electrophoresis followed by Western immunoblot transfer to nitrocellulose. The filter was reacted with the anti-mouse c-*myc* antibody described in Materials and Methods, and c-*myc* protein was detected by chemiluminescence. As shown in Fig. 4, treatment of pMS-G-dhfr-transfected DT cells with DM had no demonstrable effect on c-*myc* protein levels. On the other hand, each of the antisense clones showed 3- to 10-fold reductions in the levels of c-*myc* protein following addition of DM. Consistent with its constitutively

FIG. 1. Reversion of transformed morphology in monolayer culture. DT cells cotransfected with the plasmid being tested and pSV_2neo were selected for resistance to G418. After 7 to 10 days, G418-resistant colonies were screened for morphological reversion. DM was added to a final concentration of 1 μM, and colonies were examined the next day for morphological change. Photographs were taken at a magnification of ×250 (B to F) or ×100 (A and G to I) on an inverted Leitz photomicroscope under phase contrast. (a) Adjacent transformed and flat G418-resistant DT cell colonies cotransfected with antisense c-*myc* and pSV_2neo plasmids following exposure to 1 μM DM for 16 h. (b) DT cells in the presence of 1 μM DM (DM does not affect the morphology of DT cells). (c) 46D6 (inducible *myc* antisense) cell line, no DM. (d) 46D6 cell line 18 h after addition of 0.1 μM DM. Refractile cells are dividing (confirmed by time lapse videomicrography). (e) 36AG1 (inducible *myc* antisense) cell line, no DM. (f) 36AG1 cell line 18 h after addition of 0.1 μM DM (g) 46B2 cell line (inducible *myc* antisense but constitutively flat), no DM. (h) 46B2 cell line 18 h after addition of 0.1 μM DM. (i) 46B2 cell line transfected with v-*myc*; retransformed colonies on a flat background.

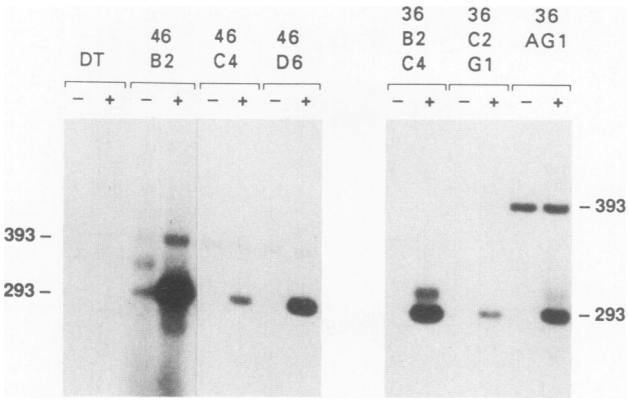

FIG. 3. Expression of c-*myc* antisense sequences in individual DT clones. Each of the indicated cell lines or clones was left untreated (−) or was treated with 1 μM DM for 16 h (+). Ten micrograms of total RNA was hybridized with the ^{32}P-end-labeled 393-nt-long *Sac*I-*Xho*I probe and digested with S1 nuclease, and the products were resolved on a 2% agarose gel. The expression of c-*myc* antisense transcripts is indicated by the presence of a 293-nt-long protected fragment. To measure the relative concentrations of antisense to endogenous sense transcripts, Northern blot analyses were performed on selected RNAs under conditions that allowed for resolution of the two types of transcripts. The 46D6 cell line, for example, contained approximately 10-fold more antisense than sense transcripts (not shown).

flat morphology and high level of antisense transcript expression, the 46B2 cell line showed a low level of c-*myc* protein even in the absence of DM. The addition of DM to this cell line produced an additional modest (approximately twofold) decrease of c-*myc* protein levels.

Loss of ability of antisense clones to grow in soft agar. Each of the c-*myc* antisense cell lines described above was examined for its ability to grow in soft agar. As controls, we included DT cells and four DT-derived clonal cell lines that had been transfected with the pMS-Gmyc-S-dhfr (c-*myc* sense) vector. As seen in Table 2, control clones demonstrated plating efficiencies of approximately 25 to 50%. This was not significantly altered by DM, with the ratios of cloning efficiencies in its presence and absence being >0.85.

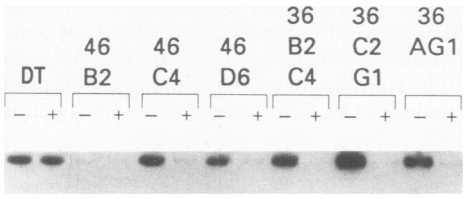

FIG. 4. Reduction in c-*myc* protein levels by the conditional expression of c-*myc* antisense transcripts. Exponentially growing cells from each of the indicated clones were grown in either the presence (+) or absence (−) of 1 μM DM for 48 h prior to harvest. Cells were lysed, and 100 μg of total protein per well was electrophoresed through a 7.5% SDS-polyacrylamide gel and electroblotted to a nitrocellulose filter. Following incubation with the anti-c-*myc* polyclonal rabbit antibody described in Materials and Methods, the filter was decorated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G, and the protein was detected with an enhanced chemiluminescence system (Amersham).

TABLE 2. Growth of DT, NIH 3T3, and transfected clones in agar[a]

Cell line	Colonies/1,000 cells		Ratio of plating efficiencies, +DM/−DM
	−DM	+DM	
NIH 3T3	0	0	
DT	543 ± 44[b]	493 ± 40[b]	0.91
Individual sense c-*myc*			
INDSB	322	274	0.85
INDSD	250	240	0.96
INDSH	520	377	0.73
INDSJ	285	178	0.62
Individual antisense c-*myc*			
36B2C4	175	4	0.023
36C1AG1	157	18	0.11
36C2G1	175	25	0.14
46D6	329	20	0.061
46C4	85	16	0.19
46B2[c]	8	2	0.20

[a] A total of 10^3 cells were suspended in 0.33% agarose in Dulbecco's MEM with glutamine and 20% fetal calf serum and plated onto a gelled bottom layer consisting of 0.9% agarose in the same medium. When DM was used, it was added to both layers at a final concentration of 1 μM. Colonies were counted after 10 to 14 days of growth. Values are means of at least three to six experiments for each cell line. All transfected cell lines but one (INDSH) showed significantly lower plating efficiencies in the presence of DM, irrespective of the orientation of the *myc* inserts. The reason for this is not clear. There is no significant difference between plating efficiencies or ratios of DT cells and control inducible sense *myc* cell lines in the absence or presence of DM ($P > 0.06$ to $P > 0.1$). DM treatment significantly decreased plating efficiency only in cell lines expressing antisense sequences.

[b] Mean and standard error.

[c] Constitutively flat in the absence of dexamethasone.

In the absence of DM, four of the six antisense cell lines showed cloning efficiencies (16 to 33%) that did not significantly differ from those of control DT cells expressing the inducible sense c-*myc* transcripts ($P > 0.2$). The 46C4 clone had a somewhat reduced cloning efficiency (9%), but the difference from the antisense cell lines was not statistically significant ($P > 0.1$). The 46B2 clone had a significantly lower agarose cloning efficiency (0.8%; $P < 0.01$), which was compatible with its high constitutive level of antisense c-*myc* transcript expression and constitutive morphologic reversion.

Following the addition of DM, all of the antisense clones showed dramatic reductions in their cloning efficiencies; the relative cloning efficiencies in the presence of DM (compared with cloning efficiency in its absence) were 0.023 to 0.20. Thus, the ability of the antisense clones to grow in soft agar correlated well with their expression of c-*myc* antisense transcripts and with the depletion of c-*myc* protein.

The data presented in Table 2 are even more striking when the relative sizes of the antisense colonies which did develop in agar in the presence of DM are taken into account. While DM did not significantly affect the sizes of control DT cell agar colonies, it dramatically affected the sizes of colonies of the antisense cell lines (Fig. 5). The c-*myc* antisense colonies that formed in the presence of DM had diameters that were, on average, only 1/5 to 1/20 the diameters of colonies that developed in the absence of DM. Assuming spherical colonies, this represented 1/125 to 1/8,000 the number of cells per colony in grown in the presence of DM than for colonies grown in its absence. Thus, DM not only dramatically

reduced the absolute cloning efficiency of the antisense cell lines but also inhibited the rates of growth of the colonies which did form in agarose to a much greater degree than it did in monolayer culture (see below).

To further confirm that the reversion phenomenon was a specific consequence of c-*myc* depletion, we cotransfected constitutively flat 46B2 cells with an MC29 v-*myc* expression vector (1) and the selectable marker pSV2*his* (17). Histidinol-resistant clones were pooled and tested for colony-forming ability in soft agar. These clones appeared transformed (Fig. 1I) and repeatedly demonstrated a significantly greater agar cloning efficiency than did 46B2 cells transfected with the pSV2*his* vector alone (not shown). Similar results were obtained when the inducible c-*myc* sense vector was used in place of v-*myc*. We interpret these results to indicate that increased levels of expression of c-*myc* sense sequences can override the antisense effect and thus restore the transformed phenotype.

TABLE 3. Growth rates and cell cycle analyses of control and *myc* antisense DT cell lines[a]

Cell line	DM	Mean doubling time (h) ± SEM[b]	% of cells in:		
			G_0/G_1	S	G_2/M
Controls					
DT	−	12.8 ± 1.3	53	25	22
	+	14.1 ± 1.3	45	25	30
DT/pMS-G (vector only)	−	13.2 ± 1.1		ND[c]	
	+	13.9 ± 1.2		ND	
INDSB (sense c-*myc*)	−	12.4 ± 1.4	44	28	28
	+	12.0 ± 1.4	48	29	23
Antisense c-*myc*					
46B2	−	20.2 ± 1.5	47	23	30
	+	23.1 ± 1.3	41	19	40
46D6	−	18.8 ± 1.7	55	20	25
	+	19.1 ± 1.9	49	19	32
36AG1	−	17.8 ± 1.4	49	25	24
	+	19.5 ± 1.5	42	22	34

[a] Growth rates and cell cycle distribution were calculated as described in Materials and Methods. When DM was present, it was added to a final concentration of 1 μM without any further additions over the course of the experiment. Cell cycle distribution determinations were performed following 24 to 48 h of exposure to 1 μM DM.
[b] From two to four experiments.
[c] ND, not determined.

Cell growth and cell cycle parameters. Certain minimum levels of c-*myc* protein are required to maintain cells in a proliferative state (18, 20, 34, 57). This finding led us to ask whether the reduced agar cloning efficiencies of various DM-treated antisense cell lines might not simply be due to a generalized effect of lowered c-*myc* protein levels on DT cell growth rates. As proliferative arrest should result in the accumulation of cells with a 2n DNA (G_0/G_1) content, we compared cell cycle parameters of several cell lines in monolayer culture in the presence or absence of DM. Cell nuclei were stained with propidium iodide, and the fractions of cells in different phases of the cell cycle were determined

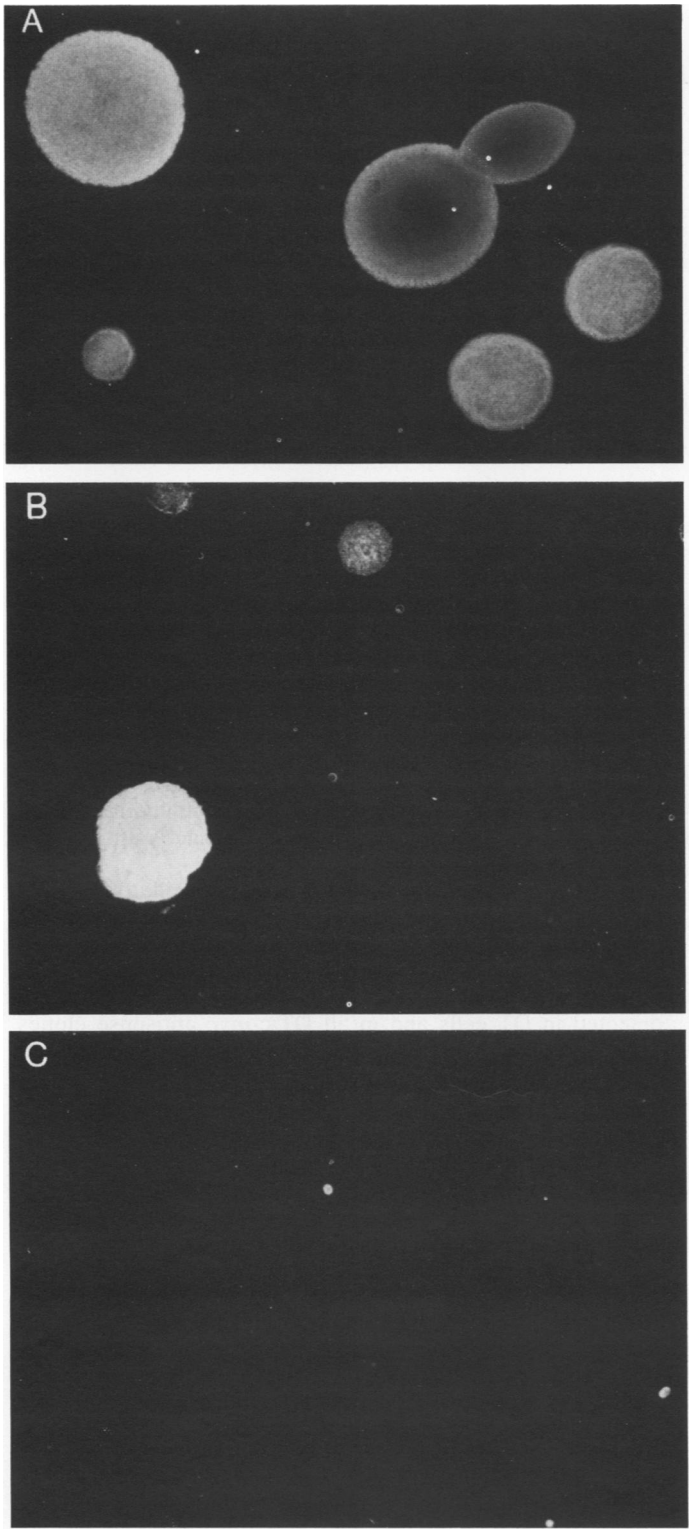

FIG. 5. Growth in soft agar. A total of 10^2 to 10^3 cells grown for 24 h in the presence or absence of 1 μM DM were plated in 0.33% low-melting-point agarose in Dulbecco's MEM containing 20% fetal bovine serum, glutamine, and antibiotics over a 0.9% Noble agar bottom layer prepared in the same medium. Colonies were counted and photographed (magnification, ×25) on a Leitz inverted photomicroscope 10 to 14 days after the initial plating. (A) DT cells control, 1 μM DM; (B) 36AG1 cells, no DM; (C) 36AG1 cells, 1 μM DM. There is a significant decrease in colony size as well as plating efficiency in the presence of DM in cells expressing c-*myc* antisense.

by fluorescence-activated cell sorting. As seen in Table 3, essentially no differences were noted between control DT cells and antisense clones in the presence of DM.

We also determined the doubling times in monolayer culture of several of the clones in the presence and absence of DM. DM had only a minimal effect on the doubling times of DT cells or of DT cell clones transfected with either pMS-G-dhfr or the inducible c-*myc* sense plasmid. In contrast, modest effects on growth rates (20 to 50% inhibition) were seen with each of the antisense clones examined. This effect was seen in the absence of DM and was only minimally enhanced by its addition. This slowed growth rate did not reflect any loss of proliferative capacity; antisense c-*myc* cell lines cultured in the presence of DM readily proliferated in the flat state for many weeks, undergoing at least 20 to 60 doublings. Thus, we conclude that the minimal effect of DM on the growth rates of antisense clones in monolayer culture was insufficient to account for the much more dramatic effects on growth in soft agar.

We observed a greater effect of c-*myc* depletion on growth in monolayer culture when antisense c-*myc* cell lines were cultured in low serum concentrations (0.5 or 1% fetal calf serum). Whereas the plating efficiencies of control DT cells were minimally affected by either DM or low serum concentration, the addition of DM to the antisense cell lines cultured in low serum concentrations caused a 70 to 90% reduction in plating efficiencies (data not shown). Furthermore, the colonies that did arise in the presence of DM were smaller and had far fewer cells, indicating a significant inhibition of cell growth. These results are qualitatively similar to those we previously reported for Friend murine erythroleukemia cells expressing high constitutive levels of c-*myc* antisense transcripts (34).

Reduced tumorigenic capacity of antisense clones. The conditional nature of most of the antisense clones complicated evaluation of their tumorigenic potential, since maintenance of adequate levels of expression of antisense transcripts is difficult to achieve in vivo. Unfortunately, we found that perhaps the most reliable method for achieving high continuous levels of DM, implantation of continuous-release DM subcutaneous pellets, was toxic to the nude mice used in our studies. We therefore took advantage of the 46B2 cell line, which displayed a revertant phenotype in the absence of DM (Fig. 1; Table 3). When cells from this clone were inoculated into nude mice, the tumors that formed did so at a significantly slower rate ($P < 0.05$) than did those arising from either DT cells or DT cells transfected with vectors containing c-*myc* in the sense orientation (Fig. 6). In other experiments, we also inoculated nude mice with several constitutively flat clones derived from DT cells expressing c-*myc* antisense transcripts under the control of the SV40 early promoter. Tumor growth in these animals was also significantly delayed (not shown). However, when the tumors that did eventually arise were examined, we found that plasmid sequences could no longer be detected by Southern blotting (unpublished observations). Thus, as was the case with prolonged in vitro propagation, these constitutively flat clones eventually reacquired a transformed phenotype that correlated with the loss of c-*myc* antisense vector sequences.

Continued presence and expression of v-K-*ras* in antisense cell lines. The presence of two copies of the v-K-*ras* genome in DT cells (3), the conditional nature of reversion in most of the antisense clones examined, and the biological instability of pSV_2myc-AS transfectants argued strongly against the possibility that reversion of these clones was due to loss of the v-K-*ras* genome. This consideration was of somewhat greater concern in the case of clone 46B2, whose phenotype was independent of the presence of glucocorticoid hormone. Each of the antisense clones, including 46B2, was therefore infected with nontransforming Moloney murine leukemia virus. In each case, we were able to rescue focus-forming virus from supernatants collected several days after infection (not shown). No focus-forming virus could be rescued from NIH 3T3 cells, however. Thus, the nontransformed morphology and biologic behavior of 46B2 cells could not be explained by loss or inactivation of the v-K-*ras* oncogenes.

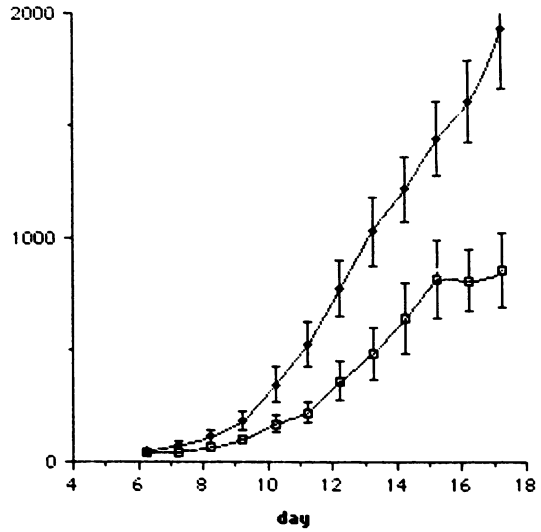

FIG. 6. In vivo growth of cell line 46B2. Nude mice were injected subcutaneously in the left and right upper dorsal regions with 10^5 cells per site. Tumor volumes were calculated as described previously (34) and are presented as the average tumor volume ± standard error for each group of mice. Symbols: ⊡, 46B2 (constitutively flat antisense c-*myc*); ◆, INDSB (sense c-*myc*).

We also examined the levels of K-*ras*-specific transcripts in each of the cell lines grown in the absence and in the presence of DM (Fig. 7). Control NIH 3T3 cells contained very low levels of endogenous c-K-*ras* transcript. On the other hand, significant levels of K-*ras*-specific mRNA were detected in DT cells and in all DT/c-*myc* antisense clones examined. The levels of K-*ras* transcripts were not altered by growth in the presence of DM.

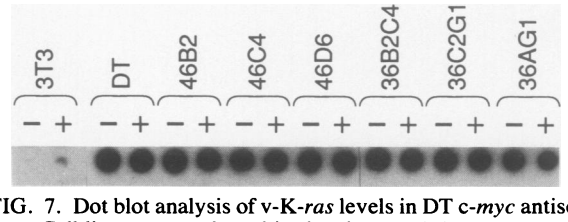

FIG. 7. Dot blot analysis of v-K-*ras* levels in DT c-*myc* antisense clones. Cell lines were cultured in the absence (−) or presence (+) or 1 μM DM for 24 h; 10 μg of total RNA from each sample was then dot blotted onto nitrocellulose and hybridized with a random-primed ^{32}P-labeled K-*ras*-specific probe (pSW-11; ATCC 4107). No differences were observed between DM-treated and untreated cultures.

DISCUSSION

The mechanism(s) by which oncogenes transform cells is thought to involve the sequential transfer of aberrant growth signals from the periphery to the nucleus. The products of most oncogenes and proto-oncogenes appear to be involved in regulating various aspects of normal cellular function, including proliferation and differentiation. Thus, it seems likely that peripherally located transforming oncoproteins utilize the same signaling pathways as do their normal counterparts.

Nuclear oncoproteins such as the c-*myc*, c-*fos*, and c-*jun* proteins have been shown in many instances to be important for exponential growth, for the transition into exponential growth from quiescence, and for differentiation (20, 26, 33, 34, 37–40, 57). Many of these proteins encode proven or putative transcription factors whose levels are often elevated following transformation by other oncogenes (14, 44, 49, 54). Indeed, many of these so-called nuclear proto-oncogenes are themselves transforming when aberrantly expressed (30, 36, 42). Thus, it seems reasonable to suspect that these genes may serve as the ultimate effectors of both normal and oncogenic proliferative signals.

Direct evidence to support such a role for c-*myc* has been obtained by several groups. Heikkila et al. (18) have shown that a c-*myc* antisense oligodeoxynucleotide prevented the entry of mitogen-activated T lymphocytes into S phase from G_1 but did not inhibit their entry from G_0 into G_1. Holt et al. (20) and Yokoyama and Imamato (58) showed that the suppression of endogenous c-*myc* by antisense sequences resulted in an inhibition of proliferation and induced spontaneous myeloid or monocytic differentiation. In the Friend murine erythroleukemia cell line, the constitutive expression of c-*myc* antisense transcripts resulted in the acceleration and enhancement of dimethyl sulfoxide-induced differentiation and in the increased suppression of proliferation under reduced-serum conditions (34).

In this work, we have demonstrated that a specific nuclear proto-oncogene (c-*myc*) is important for *ras* oncogene-mediated transformation of NIH 3T3 cells. Previous work has shown that c-*myc* or other *myc*-like genes may cooperate with *ras* oncogenes in the transformation of primary cells in vitro (25, 28). However, since the target cells presumably were already expressing the endogenous c-*myc* gene, it was not apparent from such studies whether their successful transformation by *ras* genes was the result of quantitative or qualitative changes in c-*myc* expression. In addition, since several genes of viral origin (e.g., SV40 large T and adenovirus E1a) can functionally substitute for c-*myc* (55), it is also not apparent that the c-*myc* gene product, when expressed at normal levels, is specifically required for *ras* transformation. Consistent with this notion is the observation that *ras* oncogenes alone can, in fact, transform primary cells when the influence of surrounding cells is eliminated (24) or when the expression of *ras* is enhanced (48).

NIH 3T3 cells, although immortalized, contain unrearranged c-*myc* genetic loci which are subject to apparently normal regulatory controls. Thus, the gene is down regulated following serum deprivation or density arrest, is induced rapidly and independently of protein synthesis in growth factor-stimulated cells, and is superinducible in the presence of protein synthesis inhibitors (6, 15, 22). Other transcriptional, posttranscriptional, and translational controls seem to be intact as well. These cells, therefore, appeared to represent an ideal model system in which to test the hypothesis that single-hit transformation by *ras* oncogenes requires a minimum level of expression of a normal c-*myc* gene. The results presented here suggest this to be the case. The reversibility of the effects which we have observed also indicates that c-*myc* expression is required both for the maintenance and establishment of the *ras*-transformed state. Whether c-*myc* expression is also required to maintain transformation by other extranuclear oncogene products is currently under investigation. The finding that antisense c-*jun* expression did not affect the phenotype of DT cells suggests that the requirement for c-*myc* expression is relatively specific. However, the statement that the c-*jun* protein is not required must be made cautiously, since it is possible that insufficient depletion of c-*jun* protein was achieved. Nevertheless, our negative results are intriguing and merit further investigation, particularly in view of evidence relating *ras* transformation to the expression of the c-*fos* nuclear proto-oncogene (27, 49).

Several studies have shown that c-*myc* and c-*fos* levels are actually lower in v-K-*ras*-transformed cells than in untransformed cells (8, 29, 59). Furthermore, in these cells the de novo induction of these transcripts by platelet-derived growth factor, but not necessarily other growth factors, is markedly attenuated (29, 59). Similar results have been obtained in PC12 pheochromocytoma cells expressing an activated N-*ras* oncogene, in which it has been shown that nerve growth factor fails to induce either c-*myc* or c-*fos* transcripts (16). Taken together, these results indicate that *ras* oncogenes can attenuate the c-*myc* response to a selected subset of mitogens. Whether this is related to the well-known ectopic production of various growth factors by *ras*-transformed cell lines is not known (11, 32). A similar down regulation of endogenous c-*myc* has been observed in primary rat fibroblasts which express only an activated *ras* oncogene but are not otherwise transformed (11a). Thus, the widely reported cooperative role of exogenous c-*myc* in *ras* transformation may be to overcome this *ras*-mediated down regulation by restoring levels of c-*myc* expression adequate to support the transformed phenotype. The differences in susceptibilities of primary versus established cell lines to single-hit transformation by *ras* oncogenes may thus reflect differences in the degree to which c-*myc* is suppressed by *ras* in different cells. In the case of DT cells, antisense suppression of c-*myc* as described here might deplete c-*myc* levels to a degree comparable to those in primary fibroblasts.

In monolayer culture, the antisense c-*myc* cell lines grew only slightly slower (20 to 50% longer doubling times) than control clones, even when c-*myc* protein levels were substantially reduced by the addition of DM (Fig. 3). This suggests that the growth of *ras*-transformed NIH 3T3 fibroblasts in monolayer culture is relatively independent of c-*myc* levels, at least until the serum levels are drastically lowered. This view is consistent with our previous observations that Friend murine erythroleukemia cell lines constitutively expressing high levels of c-*myc* antisense transcripts are only growth inhibited at very low serum concentrations (34). Such an explanation would also be in keeping with the known property of *ras*-transformed cells to be relatively serum independent (51), a condition which normally lowers c-*myc* levels (22). Thus, the depletion of c-*myc* protein exerted a much more profound effect on the transformed phenotype of DT cells than it did upon their growth properties.

Consistent with this idea, the behavior of c-*myc* antisense clones in soft agar, as measured by both plating efficiency and colony size, was very dependent on c-*myc* levels. In every case examined, DM inhibited plating efficiency to a

much greater degree than could be explained by the differences in growth rates in monolayer culture (Table 3). Moreover, those colonies which did form in soft agar were significantly smaller than control DT colonies, even when cultured for longer periods of time (up to 3 weeks) to compensate for the observed differences in monolayer culture growth rates. Thus, the qualitative and quantitative differences in soft agar cloning cannot be explained by the relatively minor effects on DT cell growth that occur in monolayer culture. More likely, they reflect alterations in the features of *ras*-transformed cells that typically allow for the establishment of anchorage-independent growth.

The unstable nature of the revertant phenotype in DT cells which constitutively express high levels of c-*myc* antisense transcript has complicated a systematic evaluation of the long-term effects of c-*myc* reduction on DT cells. In retrospect, such instability might have been anticipated, given the growth advantage of transformed cells over flat revertants. Nevertheless, several of these cell lines retained flat morphology after at least 3 months in continuous culture. Furthermore, even with a relatively slow growth rate, the 46B2 inducible c-*myc* antisense clone has maintained its revertant morphology for over 6 months of continuous culture without changes in its growth characteristics or morphology. The reduction in c-*myc* protein levels in this clone has thus far not seemingly affected its immortalized nature. In addition, a subclone derived from the inducible 46D6 cell line, 46D6AD2, also retains a flat revertant morphology in the absence of DM. While not characterized in detail, several other similar clones have also been kept in culture for several weeks to months without obvious loss of proliferative capacity. This suggests that the putative immortalizing role of *myc* is distinguishable from the transformation maintenance role identified in this study. Thus, as originally suggested by Franza et al. (13), nuclear oncogenes such as c-*myc*, SV40 T antigen, and adenovirus E1a antigen may provide functions other than immortalizing ones which allow them to cooperate with *ras* genes in the transformation of primary fibroblasts.

The following points argue against the possibility that the biologic findings could have been caused by diminished *ras* expression as a consequence of *myc* depletion: (i) there was no change in the K-*ras* mRNA levels when the cells were grown in DM (Fig. 7); (ii) *ras* proteins have long half-lives (20 to 42 h) (53), while induction of the antisense c-*myc* sequences caused morphological changes within the hour and complete phenotypic reversion within 8 to 16 h; (iii) even low levels of mutant *ras* oncoprotein are sufficient to maintain the transformed phenotype; and (iv) similar reversion effects were observed with cells transformed by *ras* genes expressed under the control of both viral and cellular promoters (Table 1), which suggested that a specific effect on the retroviral portions of v-K-*ras* was improbable.

The mechanism by which oncogenes induce neoplastic transformation is thought to involve the sequential passage of signals from the periphery of the cell to the nucleus over otherwise normal growth regulatory pathways. The interruption of such pathways might therefore be expected to exert profound effects on the transformed phenotype. Although we have been able to achieve reversion of NIH 3T3 transformation, our results should not be interpreted to indicate that the c-*myc* gene product is necessarily a primary component of the *ras* transformation pathway. It is conceivable that the function of c-*myc* is to supplement this pathway by providing additional cellular functions that produce the fully transformed state.

c-*myc* may not be the only nuclear proto-oncogene involved in mediation of the *ras*-transformed phenotype. Indeed, recent evidence suggesting a role of c-*fos* has been presented by several groups. Stacey et al. (49) first demonstrated that the microinjection of c-*ras* protein into NIH 3T3 cells induced the expression of c-*fos*, whereas others have demonstrated that the transcriptional stimulation of several genes by *ras* requires AP-1 sites (41). Finally, Ledwith et al. (27) have recently demonstrated the partial reversion of c-H-*ras*-transformed NIH 3T3 cells by the conditional expression of c-*fos* antisense transcripts. These findings, coupled with our own, suggest that multiple cooperating nuclear effectors might be involved in signal transduction by oncoproteins located outside the nucleus.

The work presented here suggests that conditional antisense expression may be a general means of classifying oncogene products on the basis of the physiologic substrates with which they interact. In turn, this may reveal heretofore unappreciated relationships among structurally unrelated oncoproteins by identifying final common nuclear effectors of their action.

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