MOLECULAR AND CELLULAR BIOLOGY, Mar. 1986, p. 870–877
0270-7306/86/030870-08$02.00/0

Vol. 6, No. 3

Differential Responsiveness of *myc*- and *ras*-Transfected Cells to Growth Factors: Selective Stimulation of *myc*-Transfected Cells by Epidermal Growth Factor

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Received 3 September 1985/Accepted 9 November 1985

To identify functional relationships between oncogenes and growth factors, we compared the effects of transfected *myc* and *ras* oncogenes on the responsiveness of Fischer rat 3T3 cells to three growth factors: epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor-beta (TGF-beta). Control cells did not grow in soft agar under any conditions. *ras*-Transfected cells grew in soft agar under all conditions tested and were insensitive to the stimulatory effects of exogenous growth factors. These cells secreted elevated levels of both EGF-like factors and TGF-beta, suggesting that the lack of responsiveness of these cells to exogenous growth factors arose from autocrine stimulation. *myc*-Transfected cells displayed conditional anchorage-independent growth: they formed numerous colonies in soft agar in the presence of EGF but relatively few colonies in the presence of PDGF or TGF-beta. Secretion of EGF-like factors and TGF-beta by these cells was not elevated above that of control cells. These results suggest a model for the mechanism of cooperation between *myc* and *ras* oncogenes in which *ras*-like genes induce growth factor production, while *myc*-like genes increase the responsiveness of cells to these factors.

A number of viral and cellular oncogenes have been identified that play a direct role in the genesis of virus-induced and spontaneous tumors. Introduction of such genes into cultured fibroblasts renders these cells tumorigenic and yields phenotypic alterations that correlate well with tumorigenicity (for reviews, see references 8 and 30). Many of these oncogenes act directly or indirectly on processes regulated by growth factors (GFs).

A role for GFs in the process of cellular transformation was first indicated by the finding that transformation of cells by viruses such as Kirsten and Moloney murine sarcoma viruses (which carry *ras* and *mos* oncogenes) causes secretion of GFs (13, 36). This suggested an autocrine model for transformation, in which malignant transformation results from inappropriate production of GFs by cells that bear functional receptors for these factors. Such cells would be stimulated to proliferate by their own secreted GFs (48). Direct support for this model has come from the recent finding that the *sis* oncogene encodes a GF that is structurally and functionally homologous to platelet-derived growth factor (PDGF, [14, 15, 20, 38, 52]). Transformation by v-*sis* is almost certainly caused by autocrine stimulation.

Other findings indicate that oncogenes can intervene in the GF response at the level of the GF receptors. Thus, the v-*erbB* oncogene is now known to be derived from the normal cellular gene that encodes the epidermal growth factor (EGF) receptor (16, 50). Introduction of the v-*erbB* oncogene into cells causes them to produce a truncated EGF receptor, which probably delivers mitogenic signals to the cell even in the absence of EGF. Similarly, the v-*fms* oncogene is probably derived from the gene for the macrophage colony-stimulating factor-1 receptor (46).

A further connection between GFs and oncogenes was established by the observation that expression of the normal progenitors of oncogenes (proto-oncogenes) is regulated by GFs. For example, PDGF stimulates accumulation of c-*myc* and c-*fos* transcripts in growth-arrested cells (11, 18, 26, 29, 35). Other work indicates that transforming growth factor-beta (TGF-beta) increases the number of cell surface EGF receptors, which are specified by the normal version of the *erbB* oncogene (4).

We wished to further probe these GF-oncogene relationships by determining whether certain oncogenes would alter the responsiveness of cells to exogenously supplied GFs. We examined the effects of the *ras* and *myc* oncogenes as representatives of two classes of genes that have distinct and complementary effects on the cellular phenotype (31, 44). Transformation of mammalian fibroblasts by *ras*-like oncogenes (*ras*, polyomavirus middle T, *src*) results in classical morphological transformation and anchorage-independent growth. In contrast, *myc*-like oncogenes (*myc*, *myb*, polyomavirus large T, and adenovirus E1a) only weakly promote morphological transformation and anchorage-independent growth but greatly facilitate establishment of continuous cell lines from primary cultures.

The different effects of *ras* and *myc* oncogenes on the cellular phenotype are paralleled by differences in the biochemical properties of the gene products. Products of the *ras* genes are located at the inner face of the plasma membrane and may be involved in transduction of extracellular signals (19, 23, 32, 49, 53). *myc*-Like genes specify nuclear proteins that may in turn regulate the expression of other genes (1, 27, 28). A final indication of functional differences between *myc*-like and *ras*-like genes is that these two types of genes act synergistically in induction of tumorigenicity. Under conditions in which neither of these types of genes, acting on its own, is sufficient to render early-passage fibroblasts tumorigenic, tumorigenicity is achieved on introduction into

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the cells of one oncogene from the *ras*-like group and one from the *myc*-like group (31, 44).

We examined the GF requirements of immortal Fischer rat 3T3 (FR3T3) fibroblasts into which we introduced activated *myc* or *ras* oncogenes. Three different GFs, EGF, PDGF, and TGF-beta, were chosen for these studies. EGF is a mitogenic GF that is one of the growth-promoting constituents of serum. EGF is structurally related to TGF-alpha, a factor that is often secreted by transformed cells. EGF and TGF-alpha both act by binding to the EGF receptor and have similar effects on cell physiology (41). PDGF is a second important serum mitogen and is of special interest because the viral oncogene v-*sis* specifies a protein closely related to PDGF. Pledger and Stiles have shown that resting BALB/c 3T3 cells can be induced to enter the cell cycle and synthesize DNA by sequential treatment with competence factors, like PDGF, and progression factors, like EGF (for a review, see reference 45). Thus EGF and PDGF have different effects on BALB/c 3T3 cells. TGF-beta has bifunctional properties: it either stimulates or inhibits growth, depending on the target cell and the presence of other GFs (40). All of the factors just described may play a role in tumorigenesis, because they are secreted by transformed cells and can cause phenotypic transformation of some cell lines (2, 9, 12, 24).

We found that cells transfected with activated *myc* or *ras* genes differed substantially in their GF requirements: *myc*-transfected cells had a specific requirement for EGF to exhibit anchorage-independent growth, while *ras*-transfected cells were unresponsive to exogenous GFs. These data support the idea that *myc* and *ras* oncogenes act via different mechanisms and indicate cooperativity between GFs and the *myc* oncogene.

MATERIALS AND METHODS

Cell culture. Cells were grown in Dulbecco-Vogt modified Eagle medium (DMEM) containing 10% calf serum and penicillin-streptomycin (50 μg/ml) in 5% CO_2–95% air at 37°C.

Growth factors. PDGF, purified from human platelets as described previously (40), was generously supplied by G. R. Grotendorst. EGF was purified from male mouse submaxillary glands (39, 40). For the experiment depicted in Fig. 2 only, receptor-grade EGF was obtained from Collaborative Research, Inc., Waltham, Mass. TGF-beta was purified from human platelets (6).

RNA assays. To prepare cytoplasmic RNA, cell monolayers on 100-mm-diameter culture dishes were washed three times with cold phosphate-buffered saline, scraped into cold phosphate-buffered saline, and concentrated by centrifugation. Cell pellets were suspended by vortexing in 400 μl of 50 mM Tris hydrochloride (pH 8.0)–100 mM NaCl–5 mM $MgCl_2$–0.5% (vol/vol) Nonidet P-40 and incubated at 0°C for 3 min. Nuclei were pelleted by centrifugation for 1 to 3 min in a microfuge. The supernatants were transferred to tubes containing 4 μl of 20% sodium dodecyl sulfate and extracted twice with phenol-chloroform (50% [vol/vol]) and once with water-saturated ether. RNA was recovered by ethanol precipitation and centrifugation. Similar results were obtained when total cell RNA was prepared by a different procedure.

RNA was assayed for the presence of mouse *myc* RNA (specified by pSVc-*myc*-1) using a RNase protection assay (33). The hybridization probe was produced by in vitro transcription of a plasmid (obtained from Michael Gilman, Whitehead Institute) which contains a 166-base-pair *XbaI-PstI* restriction fragment from pSVc-*myc*-1 that spans the junction between the first intron and the second exon of *myc* fused to an SP6 promoter. Transcription of this plasmid by SP6 RNA polymerase (Promega Biotech) in the presence of [α-^{32}P]UTP (New England Nuclear Corp., Boston, Mass.) yielded a body-labeled hybridization probe complementary to *myc* mRNA. A total of 2×10^5 cpm of probe was hybridized to 5 μg of RNA [in 80% formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–40 mM NaCl–5 mM EDTA at 42°C overnight], and single-stranded RNA was digested with RNases A (40 μg/ml) and T1 (2 μg/ml) in 10 mM Tris hydrochloride (pH 7.5)–5 mM EDTA–300 mM NaCl for 30 min at room temperature. Proteinase K was added to yield a final concentration of 70 μg/ml, sodium dodecyl sulfate was added to a concentration of 0.5% (wt/vol), and the samples were incubated for 15 min at 37°C. The samples were extracted once with phenol-chloroform, precipitated with ethanol, and analyzed by electrophoresis in 8% DNA sequencing gels. The probe fragment protected by hybridization to *myc* mRNA was identified by size and quantified by Cerenkov counting of the excised gel band. This fragment was not protected by endogenous rat *myc* sequences under our assay conditions.

Measurement of GFs secreted by cell lines. Cells were seeded into three T-150 flasks in DMEM containing 10% calf serum. Three days after reaching confluence, the cultures were washed three times at half-hour intervals with serum-free DMEM and then placed in DMEM–2.5% plasma-derived serum (5). After 10 h, the conditioned medium was removed and centrifuged to remove cellular debris. The cells appeared healthy and remained attached throughout the collection period. Cell densities at this time were Neo-1, 7.7×10^6 cells per flask; Myc-1, 29×10^6 cells per flask; Ras-3, 39×10^6 cells per flask. The conditioned medium was dialyzed against 16 μl of 1 M acetic acid and lyophilized. Control medium containing 2.5% plasma-derived serum was treated identically. The dialysate was reconstituted at 0.5 ml per 10^7 cells, and fractions were lyophilized for assay on NRK-49F cells as previously described (39). The concentration of TGF-beta or EGF-like activity was determined by comparison of dilution curves of conditioned media with those of standards of human platelet TGF-beta or murine EGF. Assays for TGF-beta were carried out in the presence of 5 ng of EGF per ml; assays for EGF-like activity were carried out in the presence of 2 ng of TFG-beta per ml. The results (see Table 4) are the average of duplicate assays.

RESULTS

Cells transfected with *myc* genes or the polyomavirus large T gene have reduced serum requirements, suggesting an altered responsiveness to GFs (3, 7, 25, 34, 37, 51). Furthermore, Armelin and co-workers (3) found that *myc*-transfected BALB/c 3T3 cells showed increased responsiveness to EGF (the assays were performed with medium containing plasma, which lacks the PDGF found in serum). In these studies growth of cells in monolayer culture was measured. However, the growth parameter that correlates best with tumorigenicity of fibroblasts is growth under anchorage-independent conditions (10, 22). We therefore determined the effects of *myc* and *ras* oncogenes on growth factor responsiveness of cells grown in soft agar. We chose the FR3T3 cell line for these experiments because it was derived from Fischer rat embryo fibroblasts similar to those used in our previous experiments and because others have studied the effects of oncogenes on growth properties of these cells (37).

FR3T3 cells were transfected with an activated *myc*

TABLE 1. GF-dependent colony formation in soft agar of transfected FR3T3 cells[a]

GF	No. of the following colonies with >62-μm diam:		
	Neo-1	Myc-1	Ras-3
None	4	3	1,590
EGF (800 pM)	5	1,140	1,650
TGF-beta (70 pM)	2	140	1,820
PDGF (150 pM)	2	180	1,810

[a] A total of 4×10^3 cells were seeded in DMEM containing 10% calf serum and 0.3% agar in 35-mm-diameter culture dishes as described previously (39, 42). Colonies were scored after 7 days by counting a 5-cm^2 area of each plate with a Bausch and Lomb image analyzer.

oncogene (pSVc-*myc*-1, which contains the second and third exons of a translocated mouse c-*myc* gene under control of the simian virus 40 early promoter [31]) or *ras* oncogene (pEJ 6.6 [47]). These nonselected plasmids were transfected in 10-fold molar excess over pSV2-neo, which contains a G418 resistance gene. G418-resistant clones carrying pSV2-neo and the concomitantly introduced oncogene were selected and passaged as stable cell lines.

G418-resistant clones of *myc*-transfected cells exhibited a morphology that was distinct from that of *ras*-transfected cells and from that of control cell lines transfected only with pSV2-neo plasmid DNA. These *myc*-transfected cells had an epithelioid morphology, having well-defined cell margins and a polygonal shape. They were not well extended, even at low densities, and were smaller than control fibroblasts. Secondary rat embryo fibroblasts transfected with pSVc-*myc*-1 undergo similar morphological changes (H. Land and L. Parada, unpublished data).

The cell clone designated Myc-1 was chosen as a representative *myc*-transfected cell line. This cell line has the characteristic morphology of *myc*-transfected FR3T3 cells. The activity of the transfected mouse *myc* gene in Myc-1 cells was confirmed with an RNase protection assay (see above), which showed that *myc* mRNA is severalfold more abundant in Myc-1 cells than it is in serum-stimulated BALB/c 3T3 cells (D. F. Stern, unpublished data).

Ras-3 and Neo-1 cells were chosen as representative *ras*-transfected and control cell lines (having only a G418 resistance marker), respectively. Ras-3 cells display the refractile morphology typical of *ras* transformants. Neo-1 cells are morphologically similar to the parental FR3T3 cell line.

Ras-3, Myc-1, and Neo-1 cells differ in their requirements for anchorage-independent growth. We used Ras-3, Myc-1, and Neo-1 cells to determine whether the oncogenes would alter the response of the cells to exogenous GFs. Ras-3, Myc-1, and Neo-1 cells were seeded in soft agar in 10% fetal calf serum or in serum plus additional EGF, PDGF, or TGF-beta (Table 1). Neo-1 cells, which are representative of the parental FR3T3 cell line, did not form large colonies under any conditions. Ras-3 cells grew well under all conditions tested and were unaffected by GF additions. Myc-1 cells differed from Ras-3 and Neo-1 cells in displaying GF-dependent, anchorage-independent growth. Myc-1 cells formed few large colonies in 10% serum alone or serum supplemented with TGF-beta or with PDGF (Table 1). However, these cells could be induced to form large colonies in the presence of supplementary EGF. We tentatively concluded that the *myc* oncogene renders cells responsive to EGF, but not to PDGF or TGF-beta, in the assay for anchorage-independent growth.

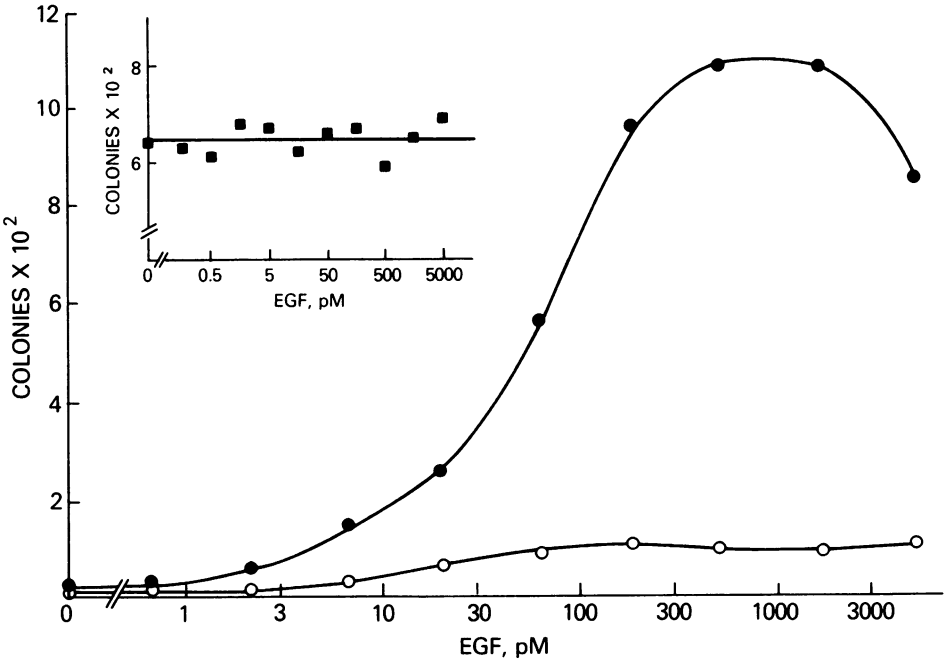

FIG. 1. EGF-dependent growth of transfected FR3T3 cells in soft agar. A total of 4.2×10^3 cells were seeded in DMEM containing 10% calf serum and 0.3% agar in 35-mm-diameter culture dishes and incubated at 37°C in the presence of various concentrations of murine EGF (39, 42). Colonies formed by Neo-1 and Myc-1 cells were counted after 7 days. (Inset) colonies formed by EJ cells were counted after only 5 days to reduce the incidence of colony overlap resulting from the rapid growth of Ras-3 cells in soft agar. The data represent the average number of colonies that were >62 μm in diameter counted per 5-cm^2 area in duplicate plates. Anchorage-independent growth of Neo-1 (○), Myc-1 (●), and Ras-3 (■) cells is shown.

TABLE 2. Effects of transfected *myc* gene on EGF-induced growth of FR3T3 cells in soft agar

DNA transfected and transfection no.[a]	Amt of pSVc-*myc*-1 RNA relative to Myc-1[b]	No. of colonies with >62-μm diameter for the following additions[c]:	
		No EGF	EGF (0.8 nM)
pSV2-neo–pSVc-*myc*-1			
11	Not detectable	2	2
12	0.17	1 ± 1.0	50 ± 11
13	Not determined	3 ± 0.3	4 ± 3
14	3.3	2 ± 2.0	120 ± 14
15	0.32	19 ± 6.1	130 ± 6
pSV2-neo			
1		10	8
2		2	10
3		2	3
4		2	4
5		3	2

[a] Five 100-mm-diameter cultures of FR3T3 cells were each transfected with pSV2-neo alone or pSV2-neo plus a 10-fold molar excess of pSVc-*myc*-1 by coprecipitation with calcium phosphate in the presence of sheared herring sperm DNA carrier. Each culture was split to yield five daughter cultures, and G418-resistant colonies were then pooled.

[b] Cytoplasmic RNA was isolated from the transfected pools, and sequences encoded by pSVc-*myc*-1 were quantified with a RNase protection assay (see text). The amount of radioactivity in the protected probe fragment is expressed as a fraction relative to the amount protected by an equivalent amount of RNA from Myc-1 cells analyzed in parallel.

[c] Pooled G418-resistant colonies were assayed for anchorage-independent growth as described in the legend to Fig. 1. For transfections 11 to 15, the average number of colonies for no EGF added was 5.4 ± 6.8, and for those colonies to which 0.8 nM EGF was added the average was 61 ± 55. For transfections 1 to 5, the average number of colonies for no EGF added was 3.8 ± 3.1, and for those colonies to which 0.8 nM EGF was added the average was 5.4 ± 3.1.

If the differences in response to EGF among Neo-1, Ras-3, and Myc-1 cells were significant, then they should be evident over a wide range of EGF concentrations. Consequently, we measured anchorage-independent growth of these cells in response to EGF concentrations ranging between 10 and 4,000 pM (Fig. 1). Myc-1 cells grew best at a concentration of 500 to 1,700 pM but were stimulated by EGF concentrations as low as 10 pM. In contrast, Neo-1 cells failed to grow even at high EGF concentrations, and growth of Ras-3 cells was completely insensitive to the effects of EGF. Thus, the conditional growth of Myc-1 cells, the unconditional growth of Ras-3 cells, and the lack of growth of Neo-1 cells were all maintained when EGF concentrations were varied over nearly three orders of magnitude.

The apparent preference of Myc-1 cells for EGF over PDGF or TGF-beta was evident over a range of concentrations of these factors (A. B. Roberts, unpublished data), demonstrating that this preference was not an artifact arising from the particular concentrations of GFs chosen. We conclude that the *myc* oncogene is able to cooperate with exogenous EGF to a much greater extent than with either PDGF or TGF-beta to induce anchorage-independent growth of Myc-1 cells.

Although these results suggest that there is cooperativeness between *myc* gene expression and EGF in supporting anchorage-independent growth of cells, this conclusion was based on the properties of only a single cell line. To determine whether the properties of the Myc-1 cells were typical of FR3T3 cells transfected with pSVc-*myc*-1, we examined the growth of a large number of clones transfected with the active *myc* oncogene. Five parallel cultures of FR3T3 cells were each transfected with either pSV2-neo alone or pSV2-neo and pSVc-*myc*-1. G418-resistant colonies that had taken up DNA were selected, pooled, and assayed for their ability to grow in soft agar in the absence or presence of EGF (Table 2). Cells derived from the five transfections with pSV2-neo alone yielded few large colonies, even in the presence of EGF. In contrast, three of five cultures transfected with the *myc* oncogene yielded many large EGF-dependent colonies. To determine whether the transfected *myc* gene was being expressed in these cultures, *myc* RNA was measured by a nuclease protection assay that does not detect RNA specified by the endogenous rat *myc* gene (Table 2). Of the four cultures in which RNA levels were measured, the only one which did not contain detectable mouse *myc* RNA was culture number 11, which did not show EGF-dependent growth. The remaining cultures tested (numbers 12, 14, and 15) contained mouse *myc* RNA and grew in soft agar only in the presence of EGF (Table 2). Taken together, these data confirm that expression of pSVc-*myc*-1 permits EGF-dependent, anchorage-independent growth of FR3T3 cells.

Myc-1 cells are not selectively sensitive to EGF when grown in monolayer culture. To determine whether the selective responsiveness of Myc-1 cells to EGF, as well as the differential response of Neo-1, Myc-1, and Ras-3 cells to EGF, could be measured under different culture conditions, we examined the growth of the cells in monolayer culture. To measure the effects of exogenous GFs on cells in monolayer culture, it was first necessary to establish culture conditions under which GFs were limiting. Neo-1 and Myc-1 cells grew poorly in 2% calf serum, indicating that components of serum, presumably GFs, were limiting.

To compare the growth of Myc-1, Ras-3, and Neo-1 cells grown in 2% serum supplemented with GFs, we measured cell number 3 or 4 days after plating, while the cells were still in the logarithmic phase of growth (Table 3). Ras-3 cells were insensitive to the effects of exogenous EGF and PDGF in this assay. This result echoed the insensitivity of these cells to exogenous GFs observed in the soft agar assay. The selective responsiveness of Myc-1 cells to EGF that was observed in soft agar assays was not seen in monolayer

TABLE 3. Growth of transfected FR3T3 cells in monolayer culture[a]

GF	Percentage of cell no. in 2% calf serum for the following cell lines:		
	Neo-1	Myc-1	Ras-3
None	100 ± 6.4	100 ± 5.3	100 ± 3.7
EGF (800 pM)	160 ± 1.8	110 ± 7.3	98 ± 2.2
TGF-beta (40 pM)	66 ± 2.1	40 ± 0.4	55 ± 2.6
PDGF (230 pM)	150 ± 8.7	195 ± 8.9	100 ± 2.3
EGF-TGF-beta	110 ± 5	61 ± 1.8	35 ± 2.8
EGF-PDGF	210 ± 11	180 ± 4.1	95 ± 6.5
TGF-beta-PDGF	98 ± 2	89 ± 2.9	52 ± 1.1

[a] Cells were seeded in 16-mm-diameter multiwell culture dishes in DMEM–2% calf serum at 1×10^4 cells per well (Neo-1 and Myc-1 cells) or 0.5×10^4 cells per well (Ras-3 cells). Growth factors were added 4 to 8 h later. Cells were counted while they were in the exponential phase of growth, which was either 3 days (Neo-1, Myc-1) or 4 days (Ras-3) later. At this time the control cells (no added GFs) had reached the following densities: Neo-1, 4.7×10^4 cells (2.3 cell doublings); Myc-1, 1.6×10^5 cells (4.4 cell doublings); Ras-3, 1.8×10 cells (5.3 cell doublings). The numbers used were averaged from four parallel wells and normalized so that the number of cells after growth in 2% serum only was 100%.

culture, in which PDGF was slightly more effective than EGF at promoting cell growth (Table 3).

To further study the EGF responsiveness of Neo-1 and Myc-1 cells in monolayer culture, we measured the EGF dose dependence of the growth rate of these cell lines. Although Myc-1 cells grew more rapidly than Neo-1 cells, these two cell lines had nearly identical dose responses to EGF (Fig. 2). Therefore, the transfected *myc* oncogene did not render Myc-1 cells more responsive to EGF when they were growing in monolayer culture.

EGF and TGF-beta receptors in transfected cells. The increased responsiveness of Myc-1 cells to EGF in the soft agar assay suggests that they might display a greater number of EGF receptors than Neo-1 cells. We therefore measured the number and affinity of EGF receptors in binding experiments using radiolabeled EGF (Fig. 3A, Table 4). Neo-1 cells had the highest level of EGF binding. Myc-1 cells had 40% the number of EGF receptors found on Neo-1 cells, and receptors were barely detectable on Ras-3 cells. Scatchard analysis of the data showed that the differences in binding were due principally to changes in receptor number rather than receptor affinity (Fig. 3B). These results indicate that the high responsiveness of Myc-1 cells to EGF does not result from receptor amplification.

More subtle differences among the cell lines were found in binding experiments with TGF-beta. Myc-1 and Ras-3 cells displayed 44 and 22%, respectively, the number of receptors found on Neo-1 cells (Table 4). The modest difference in EGF receptor number between Myc-1 and Neo-1 cells and the differences between the number of TGF-beta receptors in the three cell lines may be due in part to differences in cell surface area.

Ras-3 cells secrete GFs. Secretion of GFs by transformed cells is generally accompanied by the loss of cell surface receptors for these factors (2, 9, 13). Because Ras-3 and Myc-1 cells had fewer receptors for EGF and TGF-beta than Neo-1 cells, we determined whether these cells were secreting GFs. Medium conditioned by Ras-3, Myc-1, or Neo-1 cells were assayed for EGF-like activity (stimulation of anchorage-independent growth of NRK-49F cells in the presence of TGF-beta) and TGF-beta activity (stimulation of anchorage-independent growth of NRK-49F cells in the presence of EGF). Neo-1 cells produced little or no EGF-like activity and some TGF-beta (Table 4). Myc-1 cells secreted little EGF-like activity; secretion of TGF-beta was not significantly higher than that found in Neo-1 cells. In contrast, Ras-3 cells secreted elevated amounts of both EGF-like factors and TGF-beta. These results provide an explanation for the reduced numbers of free EGF receptors on the surface of Ras-3 cells and show that Ras-3 cells produce substantially higher levels of GFs than do the other two cell lines.

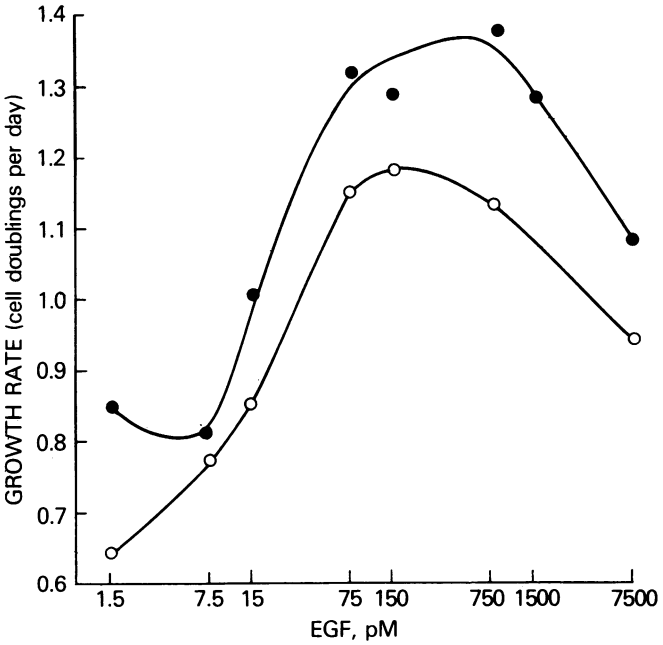

FIG. 2. EGF dependence of growth rate of Neo-1 and Myc-1 cells in monolayer culture. A total of 5×10^3 cells were seeded into 16-mm-diameter wells of 24-well culture dishes in DMEM containing 2% calf serum. The following day EGF was added as a 100× stock solution. Cell numbers were determined with a Coulter counter on the subsequent 3 days, during which period cell growth was in the exponential phase. Numbers derived from duplicate wells were averaged for each data point. The growth rate (k) was calculated by using linear regression to fit the line $\log_{10} N_t = 0.301\ kt + \log_{10} N_0$, where N_0 is the initial cell number, and N_t is the cell number on day t. Growth of Neo-1 (○) and Myc-1 (●) cells as a function of EGF concentration is shown.

DISCUSSION

The initial aim of these experiments was to identify physiological connections between GFs and oncogenes. We found that a transfected *myc* oncogene renders cells sensitive to the effects of EGF under anchorage-independent conditions. These effects did not reflect an increased responsiveness to all mitogens because Myc-1 cells showed a much greater response to one mitogenic GF, EGF, than to another mitogenic GF, PDGF.

Although either PDGF or TGF-beta acting alone on Myc-1 cells induced only 15% of the number of colonies in soft agar induced by treatment of the cells with EGF (Table 1), we have shown elsewhere that the combined action of PDGF and TGF-beta on the cells induces colony formation equivalent to that induced by EGF alone (40). This suggests that GF sensitivity of *myc*-transfected cells is not confined to only EGF. However, results of further studies comparing colony formation of Myc-1 cells induced by either EGF alone or by the combination of PDGF and TGF-beta suggest that these two sets of GFs act via different cellular pathways; they show differential sensitivity to inhibition by retinoic acid (43). Expression of the *myc* gene is essential for each of these pathways because Neo-1 cells do not respond to either EGF or the combination of PDGF and TGF-beta in soft agar.

Monolayer versus suspension culture. Results of this study indicate that there are qualitative differences between cellular growth control in monolayer and suspension culture. The requirements of Myc-1 cells for optimal anchorage-independent growth differ substantially from the conditions that favor growth in monolayer culture. EGF was far more effective than PDGF in inducing anchorage-independent growth (Table 1), but these two factors had similar effects on Myc-1 cells grown in monolayer culture (Table 3). Furthermore, in the presence of PDGF, TGF-beta stimulated anchorage-independent growth (40) of Myc-1 cells but inhibited growth in monolayer culture (Table 3). These results indicate that measurement of anchorage-independent growth is not merely a more rigorous assay for parameters measured in monolayer culture but instead measures a phenotype that is mechanistically distinct. Although the enhanced responsiveness of Myc-1 cells to EGF was not observed when the cells

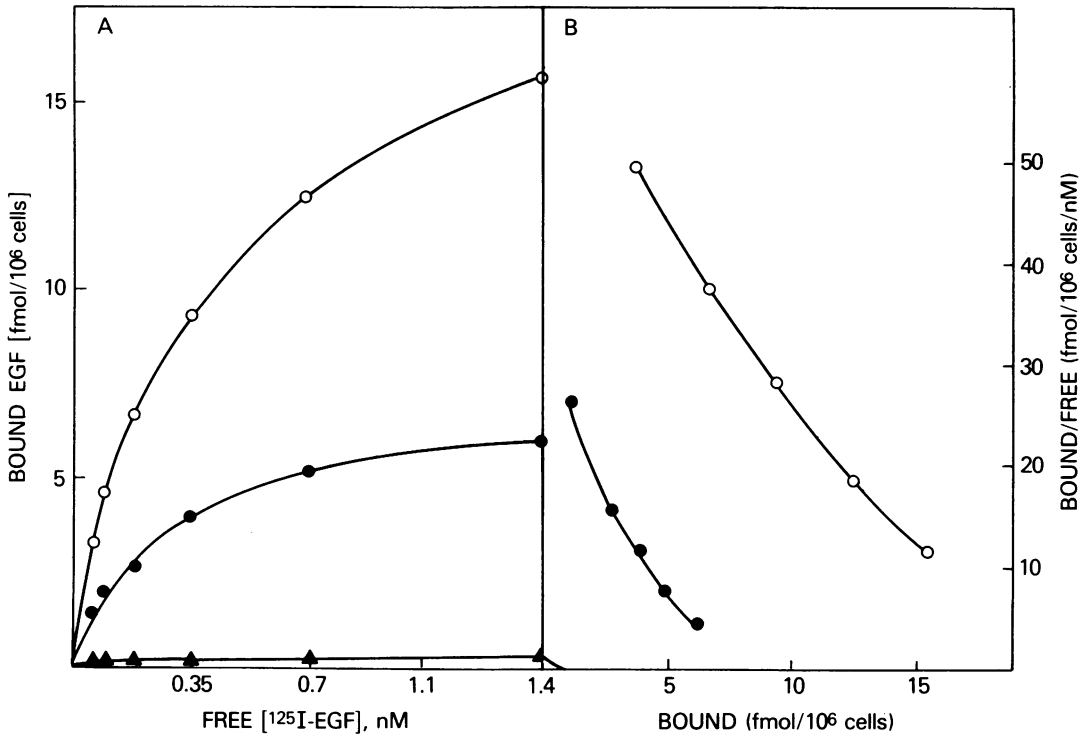

FIG. 3. Binding of ^{125}I-labeled EGF to transfected FR3T3 cells. Cells were seeded in 1 ml of DMEM containing 10% calf serum at a density of 10^5 cells per 16-mm diameter well in 24-well culture dishes. Binding of ^{125}-labeled EGF was measured after a 2-h incubation period at room temperature as described previously (4). Nonspecific binding measured in the presence of unlabeled EGF at a concentration of 1 μg/ml was subtracted from all values. Cell counts were determined at the end of the incubation period; results were determined in triplicate. (A) Binding to Neo-1 (○), Myc-1 (●), or Ras-3 (▲) cells is shown. (B) Scatchard plots of the data shown in panel A. The average calculated K_d was 0.27 nM. Calculated receptor numbers are shown in Table 4.

were grown in monolayer culture, the tight correlation of anchorage-independent growth and tumorigenicity of fibroblasts suggests that this specific response of Myc-1 cells is relevant to the mechanism of transformation by *myc* oncogenes.

Competence and progression. The increased responsiveness of Myc-1 cells to GFs may have implications for the interaction between competence and progression factors in stimulation of DNA synthesis. Sequential or concurrent addition of competence factors (such as PDGF) and progression factors (such as EGF) to quiescent BALB/c 3T3 cells causes them to leave the G_0 phase and move into the S phase (45). Because progression factors have no effect if they are added before competence factors, one effect of competence factors may be to render cells responsive to progression factors. The effects of competence factors may be mediated primarily through regulation of *myc* mRNA accumulation because the abundance of *myc* mRNA is dramatically increased after the addition of PDGF (26), and induction of expression of a transfected *myc* gene or microinjection of *myc* protein into cells is sufficient to induce competence in a substantial fraction of treated cells (3, 21). These results imply that increased sensitivity to EGF may be one of many consequences of increased *myc* expression in competent cells. This would explain the greater responsiveness of Myc-1 cells to EGF than to PDGF which is observed in agar culture. If this model is correct, then other progression factors, but not other competence factors, should stimulate anchorage-independent growth of Myc-1 cells.

TABLE 4. GF binding and secretion by transfected FR3T3 cells

Cell line	GF binding (no. molecules/cell) in:		GF secretion (pmol/10^7 cells) in[c]:	
	EGF[b]	TGF-beta[c]	EGF-like	TGF-beta
Neo-1	10,300 (100)	27,000 (100)	<18 (1.0)	270 (1.0)
Myc-1	4,200 (41)	13,000 (48)	<28 (1.6)	370 (1.4)
Ras-3	360 (3)	10,000 (37)	600 (33)	1,200 (4.4)

[a] Numbers in parenthesis are the number of molecules secreted relative to that of Neo-1 cells. Experimental procedures are described in the text.

[b] Numbers in parentheses are percent binding relative to that of Neo-1. Experimental procedures are described in the legend to Fig. 3.

[c] Numbers in parentheses are percent binding relative to that of Neo-1. Cells were seeded as described in the legend to Fig. 3, and binding was determined after a 2-h incubation period, as described previously (17). Nonspecific binding, measured in the presence of 500 ng of unlabeled TGF-beta per ml, was subtracted from all values.

Cooperation between *myc* and *ras*. Our findings support the idea that *ras* and *myc* oncogenes have different effects on cells and suggest a model for the mechanism of cooperation between *myc* and *ras* oncogenes in transformation of cells. It is well established that transformation of cells by *ras* oncogenes causes them to secrete GFs (36). In consonance with this, we found that Ras-3 cells secrete elevated levels of EGF-like factors and TGF-beta (Table 4). Secretion of abundant mitogenic GFs by Ras-3 cells may account for the unresponsiveness of these cells to the stimulatory effects of exogenous factors and implies a major role for autocrine stimulation in transformation of these cells.

In contrast, secretion of EGF-like factors and TGF-beta by *myc*-transfected cells was not significantly elevated over the background level observed in control cells. These results

suggest that autocrine stimulation is much less important for transformation by *myc* than it is for *ras*. We found, instead, that *myc* renders cells more sensitive to the effects of GFs.

Because transformation by *ras* oncogenes causes cells to secrete GFs and *myc* sensitizes cells to GFs, cooperation between *myc* and *ras* may result, in part, from GF production induced by *ras* coupled with increased responsiveness to these factors induced by *myc*. In more general terms, oncogenes like *myc* may amplify the effects of autocrine circuits induced by *ras*-like oncogenes.

ACKNOWLEDGMENTS

We thank Lalage Wakefield for determination of TGF-beta binding, Joseph M. Smith for assistance with the EGF binding assays, Michael Gilman for help with the *myc* RNA assays, and Tish Heffernan for technical assistance.

This work was supported by Public Health Service grants CA 07285-02 (D.F.S.) and CA 39964-01 (D.F.S. and R.A.W.) from the National Institutes of Health.

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