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Potentiation of growth factor activity by exogenous c-*myc* expression

(*ras* gene/anchorage-independent growth)

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ABSTRACT **The c-*myc* oncogene has been implicated in deregulation of cell growth in neoplastic cells and response to "competence-inducing" growth factors in normal cells. In the latter case, expression of c-*myc* has been shown to be associated with the transition from the G_0 to the G_1 phase of the cell cycle induced by platelet-derived growth factor (PDGF). In the work reported here, we have introduced the c-*myc* coding region, in a retroviral vector, into mouse and rat cells. We show that under conditions of anchorage-independent growth, constitutive c-*myc* expression increases the response of rodent cells to PDGF, as well as to other growth factors of both the competence-inducing and "progression" classes. These effects of the *myc* product are observed whether or not an exogenous *ras* gene has also been introduced into the same cells. Possible models for the influence of *myc* on growth responses are discussed.**

Cooperation between two or more oncogenes is widely assumed to be necessary for expression of a neoplastic phenotype by vertebrate cells (1–3). This phenotype is frequently identified with the capacity of cells to grow in an anchorage-independent manner, which has been found in some test systems to be correlated with ability to form tumors in animals (4). The fact that a single oncogene such as *ras* can confer both characteristics on some established cell lines has been attributed to prior activation in such lines of some additional gene(s) with growth-promoting activity (3). Because *ras* and oncogenes encoding nuclear proteins, such as *myc* or *p53*, can cooperate in transforming primary rodent embryo cells, it has been postulated that genes of the latter class might play a role in establishment of cell lines (3, 5). We report here that an established mouse embryo line, C3H/10T½ cells, can be converted to tumorigenicity by exogenous *ras* alone and that *myc* potentiates this conversion. However, anchorage-independent growth depends on *myc*, rather than *ras*, expression. The ability of cells receiving an exogenous copy of *myc* to grow in semisolid medium appears to be a consequence of enhanced sensitivity to growth factors exhibited by such cells. A similar increase in growth factor responsiveness was found in rat cells into which a *myc* vector was introduced.

The c-*myc* oncogene has been implicated in normal growth processes, particularly the transition from the G_0 to the G_1 phase of the cell cycle in serum-deprived cells exposed to "competence-inducing" growth factors (6). Because transcription of *myc* is stimulated by growth factors of the competence-inducing class, such as PDGF, it has been hypothesized that the *myc* product might abrogate the requirement for this type of growth factor in promoting cell division (7–9). We find, however, that constitutive expression of *myc* enhances anchorage-independent growth in response to growth factors of both competence-inducing and "progression" classes, as well as to transforming growth factor β (TGF_β).

MATERIALS AND METHODS

Cells were grown in minimal Eagle's medium (MEM) containing 10% fetal calf serum (GIBCO) at 37°C. C3H/10T½ clone 8 cells were from the American Type Culture Collection and were expanded no more than four cell passages. *ras*-transformed cells were derived by transfecting 100 ng of cloned Kirsten murine sarcoma virus (KiSV) DNA (courtesy of R. Ellis, Merck Sharp & Dohme) on 10^6 C3H/10T½ cells. The ras-2 cell line, derived from one transformed focus, was chosen as representative of *ras*-10T½ clones, as its characteristics were typical of the majority of other *ras*-transformed clones that we studied. These clones harbor a low copy number of the transfected K-*ras* gene (one copy in the case of ras-2 cells). Occasional *ras*-10T½ clones were found that displayed limited growth in agar; these clones all had high copy numbers of exogenous K-*ras*.

The pZip-Neo vector (10) constructed by R. Mulligan and co-workers was supplied with an insert of mouse c-*myc* cDNA by K. Marcu (State University of New York, Stony Brook). The insert is an *Xho* I fragment encompassing the coding region in the second and third exons, provided with linkers and inserted into the *Bam*HI site of pZip-Neo (Zip-*myc* virus). Helper-free stocks of virus from the original vector (Zip virus) and of Zip-*myc* virus were produced in psi-2 cells transfected with the respective constructs (11). myc-5 cells are a pool of several hundred colonies resistant to the antibiotic G418 obtained by infection of C3H/10T½ cells with Zip-*myc* virus. These cells contain an average of 1 copy of *myc* DNA, and RNA blotting shows viral *myc* transcripts severalfold in excess of the cellular c-myc RNA. Results obtained with these cells were confirmed with another analogous pooled cell population, as well as with an individual *myc*-10T½ clone. A cell line derived by infection with Zip virus (lacking *myc*) was used as a control for transformed cell lines. Thus, in the work reported here, C3H/10T½ is in general represented by this control. A clone of *myc*+*ras*-transformed cells (myc-ras-2) was obtained by infection of ras-2 cells by Zip-*myc* virus and selection in G418-containing medium. Pooled clones of such cells obtained by G418 selection displayed growth properties similar to those of myc-ras-2. The control cell line for myc-ras-2 was a clone of ras-2 derived by infection with Zip virus and selection with G418. This clone and ras-2 itself were indistinguishable in all properties tested.

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Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; TGF_α and TGF_β, transforming growth factors α and β; FGF, fibroblast growth factor; KiSV, Kirsten murine sarcoma virus.

The F2408 rat cell line and its subclone Rat-1 cells were obtained from Brad Ozanne (University of Texas). F2408-zip and F2408-myc were obtained by infection with Zip virus and Zip-*myc* virus, respectively. Both cell types are pools of colonies obtained by G418 selection of infected cells.

Highly purified human PDGF was a generous gift from H. Antoniades (Harvard University) (12). Experiments were also performed with receptor grade PDGF from Seragen (Boston, MA) with equivalent results. Epidermal growth factor (EGF; receptor grade) was from Collaborative Research (Waltham, MA). TGF_β was a generous gift from M. Sporn (National Cancer Institute). Fibroblast growth factor (FGF) was from Bethesda Research Laboratories. G418 was supplied by GIBCO.

Agar growth assays were performed by plating 5×10^3 cells in 2 ml of MEM/10% fetal calf serum/0.25% agarose (SeaKem; FMC Bioproducts, Rockland, ME) on top of 2 ml of the same medium in 0.5% agarose, in 35-mm culture vessels. Colonies larger than 60 μm in diameter were scored under the microscope after 2 weeks and counted with the help of a grid. Conditioned media were prepared according to Kaplan and Ozanne (13).

RESULTS

The C3H/10T½ cell line was chosen for these studies because of the substantial background of data on its transformation *in vitro* (14–16). Since 10T½ is a permanent cell line, a role for exogenous oncogenes such as *myc* in immortalization *per se* is obviated in this system, and the effects of *myc* on cell growth can be studied.

The effects on cell morphology produced by introduction of *myc* and K-*ras* oncogenes under control of retroviral long terminal repeats are shown in Fig. 1. C3H/10T½ cells were morphologically transformed by *ras* but not by *myc*. *ras*-10T½ cells are more refractile and grow to a higher density in monolayer than *myc*-10T½ cells, which resemble parental 10T½ cells in their saturation density. Subconfluent *ras*-10T½ cells are flatter and maintain this appearance if prevented from reaching confluency. The ras-2 clone used in much of our work does not form colonies in soft agar, a characteristic noted by Morris in KiSV-transformed 10T½ cells (17). Interestingly, *ras*-10T½ cells can form tumors in nude mice, albeit with an extended latent period (Fig. 2). *myc*-10T½ cells do not form tumors.

Infection of *ras*-10T½ cells with a *myc*-containing retroviral vector results in cells that present a more transformed phenotype, with highly refractile cells able to pile up on the monolayer at confluence (Fig. 1). These cells can grow in agar, forming large colonies, and induce tumors rapidly in nude mice (Fig. 2). When such cells were plated in agar at increasing concentrations, a striking increase in the percentage of agar colonies was observed, in contrast to what was observed under the same conditions with cells into which a single oncogene had been introduced (Fig. 3). This suggested that the doubly transformed cells might specifically release a factor that stimulated their own growth at higher cell densities. Alternatively, the cells might be particularly responsive to a factor released from 10T½ cells, without the factor's production being dependent on *ras* or *myc* expression.

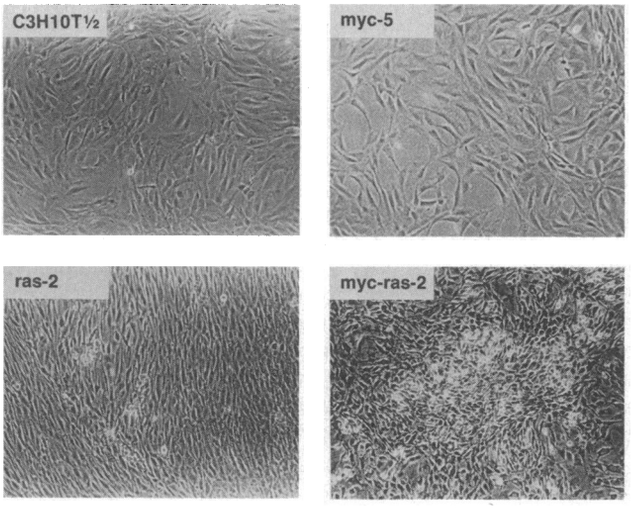

FIG. 1. Photomicrograph of normal C3H/10T½ cells and 10T½ cells after introduction of *myc*, *ras*, or both oncogenes. (×60.)

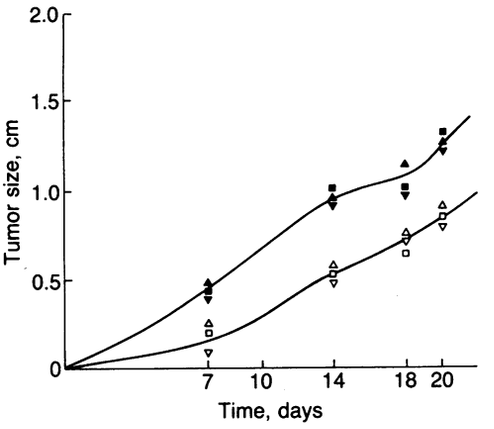

FIG. 2. Tumorigenicity of *ras*- and *ras*+*myc*-transformed C3H/10T½ cells. BALB/c *nu/nu* mice, 4–6 weeks old, were injected subcutaneously with 10^6 cells in 0.25 ml of phosphate-buffered saline. The size of tumors was monitored at the indicated times and the average diameters of resulting tumors are shown. Open symbols, *ras*-10T½ clones; closed symbols, *myc* transformants of the same *ras*-10T½ clones. The *myc*-transformed 10T½ cells were not tumorigenic.

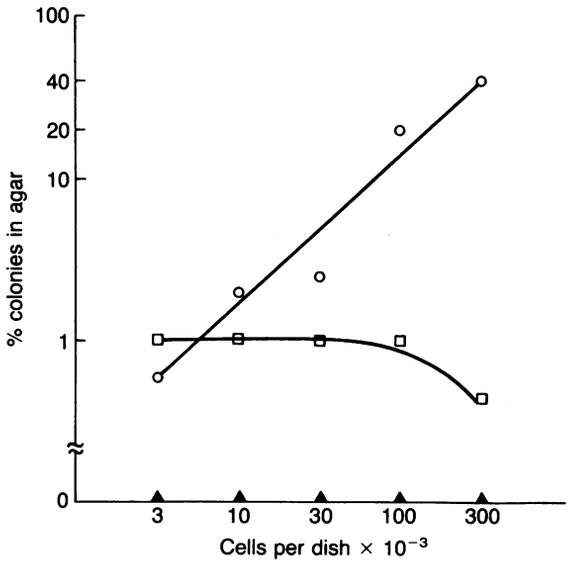

FIG. 3. Effect of plating density on growth of transformed C3H/10T½ cells in agar. The indicated cell numbers were seeded in 2 ml of MEM containing 10% fetal calf serum and 0.25% agarose over a 5-ml 0.5% agarose layer with the same medium in 60-mm Petri dishes. Colonies larger than 60 μm in diameter were counted after 14 days of incubation. The percentage of the total cells plated that formed colonies is shown. ▲, ras-2 cells; □, myc-5 cells; ○, myc-ras-2 cells. This is a representative experiment out of four that were performed.

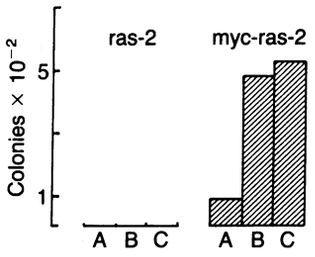

FIG. 4. Effect of conditioned medium from normal and transformed C3H/10T½ cells on agar growth of ras-2 and myc-ras-2 cells. Cells (5×10^3) were plated in 35-mm Petri dishes. Cells were plated in 0.25% agarose in fresh medium or a 1:1 mixture of fresh medium and conditioned medium (from an overnight culture) from normal 10T½ or *ras*+*myc*-transformed 10T½. Bars A, fresh medium; bars B, fresh plus conditioned medium from normal C3H/10T½; bars C, fresh plus conditioned medium from myc-ras-2 cells. Colonies were scored after 14 days.

To test these hypotheses, *ras*-10T½ cells and the same cells infected by the *myc* viral vector were seeded in agar in the presence of medium conditioned by 10T½ cells or by 10T½ transformed by both oncogenes. Agar growth of *ras*-10T½ cells was not appreciably affected by the conditioned media. In contrast, it was found that both conditioned media were capable of specifically stimulating the growth of the myc-ras-2 cells in agar (Fig. 4). The stimulation by medium from myc-ras-2 cells themselves initially suggested that TGF_α production associated with *ras* transformation (18, 19) might be playing a role. On the other hand, since medium from 10T½ cells was active, release of a factor operative in this assay did not appear to be dependent on expression of exogenous *ras* (or *myc*).

A growth factor that has been reported to be produced from various normal cells in culture is TGF_β (20, 21). Accordingly, we decided to test TGF_β, as well as certain other factors, for their effects on the growth of 10T½ and oncogene-expressing 10T½ cells. The results are shown in Fig. 5. It can be seen that *myc*-10T½ cells (myc-5) responded, in terms of agar growth, to added TGF_β. A positive response was also exhibited by myc-ras-2 cells. On the other hand, normal 10T½ and *ras*-10T½ cells did not grow better in agar in the presence of the exogenous factor. When EGF was used, it was again observed that *myc* expression was needed for a positive growth effect—with or without exogenous *ras* expression. TGF_β acts synergistically with EGF in stimulating anchorage-independent growth in some systems (19). An inhibitory effect of this combination of growth factors for *myc*-transformed cells has also been reported by Roberts *et al.* (22). We found that for *myc*- or *myc*+*ras*-transformed 10T½, combination of TGF_β with EGF resulted in a small additional increase in agar cloning efficiency (not shown). Addition of PDGF produced the strongest response for both cell types (Fig. 5). FGF was also stimulatory, but less so than PDGF.

Taken as a whole, these data indicate that in C3H/10T½ cells constitutive *myc* expression can increase sensitivity to several different growth factors. It is noteworthy that the factors in question do not belong exclusively to the competence-inducing or progression classes defined by Pledger *et al.* (23). Since competence induction involves a transient expression of *myc* (6), deregulation of *myc* might have been expected to act primarily to mitigate a requirement for competence-inducing factors such as PDGF or FGF. Armelin *et al.* (7) reported a partial compensation for PDGF requirement in attached cultures of BALB/c 3T3 cells, and Stern *et al.* (9) recently described a selective response of *myc*-transformed rat 3T3 cells to EGF, a progression factor, for growth in agar. We do not see a preference for progression factors in the *myc*-induced growth factor response in 10T½ cells, under our assay conditions.

To determine whether the sensitization toward growth factors induced by constitutive *myc* expression was in some way peculiar to C3H/10T½ cells, the effect of long terminal repeat-linked *myc* expression was studied in clone F2408 rat fibroblasts. Kaplan and Ozanne have described an enhanced response to growth factors correlated with increased susceptibility to transformation in several subclones of this line (13). We compared the original F2408 clone, transformed by the *myc* vector, or by vector alone, with the Rat-1 subclone. As can be seen in Table 1, exogenous *myc* expression greatly increased the sensitivity of F2408 to either PDGF or EGF for growth in agar. Kaplan and Ozanne (13) described a marked difference in susceptibility to transformation by KiSV for F2408 vs. Rat-1 cells, paralleling the difference in agar growth potential between the parent clone and the subclone. We found a similar correlation for F2408 and its *myc*-transformed derivative (Table 1).

DISCUSSION

We present data in this report indicating that the c-*myc* oncogene enhances the ability of mouse and rat cells to grow in semisolid medium by affecting responsiveness to growth factors. It has been postulated that *myc* contributes to transformation of normal cells by causing immortalization

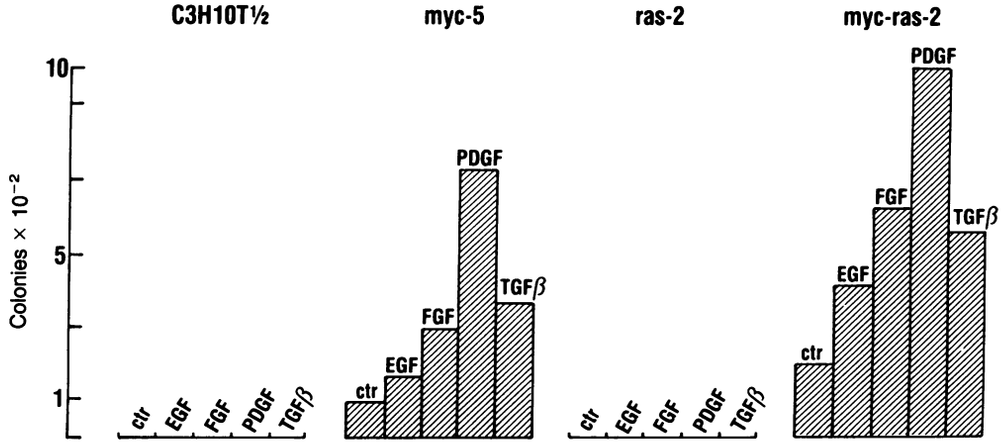

FIG. 5. Effects of purified growth factors on agar colony formation by 10T½ and transformed derivatives. Cells were plated in agarose medium and growth factors were added just before addition of agarose to the cell suspension. All growth factors were at 10 ng/ml except TGF_β, which was at 2.5 ng/ml. ctr, Control with no added factors.

Table 1. Effect of growth factors on normal and *myc*-transformed rat fibroblasts and focus-forming titers of KiSV on same cells

Cell type	% colonies in agar			KiSV titer, foci/ml
	Control	EGF (10 ng/ml)	PDGF (5 ng/ml)	
F2408-Zip	6	20	19	$<10^3$
F2408-*myc*	16	53	35	5×10^6
Rat-1	36	32	30	2×10^7

To determine the sensitivity to growth factors, cells were plated in agarose as described in the legend to Fig. 5, except that 10% calf serum was used instead of 10% fetal calf serum. Focus formation was determined by infection of 10^5 cells in 6-cm plates with 10-fold dilutions of KiSV (amphotropic virus NS292 pseudotype). Cells were fed weekly and foci were counted after 11 days by staining monolayers with Giemsa solution.

(3). Our results show that *myc* significantly alters the growth characteristics of cell lines that are already immortal. The *ras* oncogenes can produce alterations in cellular morphology of embryonic fibroblasts, often causing cells to "round up" and even detach from a monolayer. These effects are more pronounced in *ras* transformation of established cell lines. Such changes, which are suggestive of decreased requirement for substratum attachment, are not readily produced by *myc*. It has therefore been tempting to associate *ras* with the property of progressive anchorage-independent growth in primary rodent embryo cells transformed by a combination of *ras* and *myc* oncogenes. Our results indicate that *myc* can contribute directly to anchorage independence; indeed in some systems unscheduled *myc* expression can be a primary determinant of growth in semisolid medium.

The presence of exogenous *ras* as well as *myc* in C3H/10T½ cells induces a stronger ability to grow in soft agar than when *myc* alone is present. This could be due to an autocrine mechanism (24, 25) being operative for these cells (Figs. 3 and 4). *ras*-transformed cells are known to produce TGF_α (18, 19), which is related to EGF. *ras*+*myc*-transformed 10T½ produce relatively low levels of TGF_α, as monitored by reduction in EGF-binding capacity and by the ability of medium from these cells to stimulate the agar growth of NRK cells, in the presence of TGF_β (unpublished data; ref. 19). The *myc* gene product might therefore mediate sensitivity to low levels of self-generated TGF_α, as well as to TGF_β, in doubly transformed cells. Stern *et al.* have also proposed this type of collaboration between *myc* and *ras* (9). Another example of cooperation between two classes of oncogenes by an autocrine growth mechanism has been observed in an avian system, where hemopoietic cells transformed by *myc* or *myb* are rendered independent of exogenous growth factor by superinfection with viruses containing *src*-related oncogenes (26). Such a mechanism has also been invoked (27) to explain the described enhancement of *ras* transformation by tumor promoters such as phorbol esters (16, 27), which induce expression of *myc*.

Other laboratories have reported data consistent with an effect of constitutive *myc* expression on responsiveness to growth factors in serum (7–9, 22, 28–31). Vennström (29) has found that a v-*myc* gene of avian origin, tested in mouse 3T3 cells, produces effects on sensitivity to PDGF, FGF, and EGF similar to those that we have observed. Stern *et al.* (9) have reported a more limited effect of exogenous *myc* expression on anchorage-independent growth of rat 3T3 cells: sensitivity primarily to EGF, rather than to PDGF, was enhanced. However, these workers did find an increased response to PDGF induced by *myc* in monolayer cultures. More cell types and culture conditions will have to be tested to identify definitively the common features of *myc*-induced growth responses. When tested, no substantial effect of constitutive *myc* expression on receptor levels for PDGF and EGF was observed (refs. 9 and 29; unpublished data). Since the *myc* product appears to be confined to the cell nucleus and may act at the level of transcription, it might exert its ultimate effect at various points in the pathways of growth factor–receptor interaction and intracellular signalling.

Three models can be considered for the sensitization to growth factors caused by *myc* expression in these experiments. (*i*) Expression of *myc* primes the cell for response to progression factors such as EGF. This model is an extrapolation of published data on induction of *myc* transcription by PDGF (6, 32, 33). It does not explain the increased sensitivity to PDGF itself in *myc*-10T½ cells and *myc*-F2408 cells. (*ii*) *myc* is one of several competence genes, but its product is rate limiting. Constitutive expression of *myc* allows a stronger response when other competence genes are induced by PDGF. Data consistent with this type of model have been published (7, 34). By itself this model does not explain the potentiation of other growth factors by *myc* in the absence of PDGF. (*iii*) The *myc* product acts as a transducing switch or filter for signals from a number of different growth factor receptors. Higher levels of the product amplify the action of these signals in driving the cell toward mitosis. This model, which we favor, raises the interesting question whether products of other oncogenes related to *myc*, such as N-*myc* or *myb*, will be found to interact with growth factors in an analogous fashion.

Alteration or deprivation of substrate attachment has been shown to affect the requirement of various cell types for growth factors in culture (21, 22, 35). This consideration, as well as the limited diffusibility of polypeptide growth factors in agar, is built into the use of semisolid media to test for cellular "transformation." Our experiments suggest that *myc*, and possibly other oncogenes of this type, may critically influence a positive test in such a system by quantitative effects on responsiveness to a spectrum of different growth factors. Cooperative transformation with other oncogenes, such as *ras*, could reflect these effects.

We thank Michael Sporn for supplying us with purified TGF_β and Harry Antoniades for PDGF. We are indebted to Ken Marcu for the Zip-*myc* virus construct and to Jim DiSanto and Michèle Souyri in this laboratory for deriving Zip-*myc* producer clones in psi-2 cells. Peter Besmer provided helpful consultations in the course of this work. This research was supported by Grants CA-08748, CA-41518, CA-16599, and CA-09022 from the National Cancer Institute and CD-195 from the American Cancer Society.

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