MOLECULAR AND CELLULAR BIOLOGY, July 1987, p. 2649–2652
0270-7306/87/072649-04$02.00/0

Vol. 7, No. 6

# Modulation of Transforming Growth Factor Type β Action by Activated *ras* and c-*myc*

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Received 18 December 1986/Accepted 21 April 1987

**Transfection of C3H/10T½ cells with a c-*myc* gene resulted in the acquisition of responsiveness to transforming growth factor type β. Cells transfected with an activated H-*ras* gene or an H-*ras* and c-*myc* gene, however, exhibited a transformed morphology and spontaneous soft-agar growth, a phenotype induced reversibly by transforming growth factor type β in responsive fibroblasts.**

Transforming growth factors (TGF) are proteins which have been operationally defined by their ability to support the anchorage-independent growth of anchorage-dependent mesenchymal cells in soft agar (5, 11, 16, 20). Two general classes of TGF have been well characterized: TGF type α (TGFα) and TGFβ. TGFα is produced by various transformed cells as well as by embryonic and placental tissues (23). TGFβ, however, while being a growth inhibitor for most cell types (13), is a highly ubiquitous molecule which is capable of promoting the anchorage-independent growth, in the absence of TGFα or epidermal growth factor, of many mesenchymal cells (12a, 22).

C3H/10T½ (10T½) mouse cells form very few colonies in soft agar in response to TGFβ (12, 18). We reasoned that the nonresponsiveness of 10T½ cells to TGFβ might be overcome by transfection with activated protooncogenes. The results indicate that the *myc* gene may modulate the responsiveness of cells to TGFβ, while transfection with the activated H-*ras* gene induces a morphological and soft-agar growth phenotype similar to that observed following the addition of TGFβ to responsive mesenchymal cell lines.

Cultures of 10T½ cells were treated with a calcium phosphate precipitate containing pRSVneo (2) and the appropriate oncogene(s) (pEJ6.6 or pSVc-*myc* or both) (6). Following protooncogene transfection, 13 to 15 clones were isolated by limiting dilution in the presence of the antibiotic G-418 (3). Two clones representative of the predominant phenotype for each group were selected for further study.

Before an extensive biological characterization of the consequence of activated protooncogene transfection in mouse 10T½ cells could begin, it was necessary to confirm the presence and expression of the transfected genes. High-molecular weight DNA was prepared and subjected to restriction endonuclease treatment and Southern hybridization (9). Digestion with *Sac*I or *Bam*HI resulted in discrete bands associated with the *ras* or *myc* gene transfectants, respectively (data not shown). Northern analysis of the transfectants demonstrated expression of the respective transfected gene(s) (Fig. 1). The *myc* and *ras* plus *myc* gene transfectants expressed high levels of *myc* mRNA encoded by the transfected vector (Fig. 1A). It was possible to distinguish endogenous *myc* expression seen in the control and *ras*-transfected clones from expression of the transfected gene owing to the shorter *myc* transcript produced in the *myc* and *ras* plus *myc* cultures; the vector used contains only *myc* exons 2 and 3 (6). Interestingly, the *ras* clone C10 expressed approximately fivefold higher amounts of endogenous *myc* mRNA than did the control clone H6. The significance of this is unknown; however, flow cytometry indicates that it was not due simply to an increased growth fraction of cells in the *ras* culture (data not shown). When the transfectants were analyzed for *ras* expression, there was no detectable *ras* mRNA in the control (21) or *myc* transfectants, while both the *ras* and *ras* plus *myc* cultures showed varying levels of *ras* mRNA (Fig. 1B).

We wished to determine whether transfection with an activated *ras* or *myc* gene or both results in any morphological alteration. Both the control transfectants and the *myc* gene transfectants appeared morphologically similar (Fig. 2). The *myc* cultures, however, grew to a somewhat higher final saturation density (approximately threefold) and were slightly more cuboidal. However, the selected clones that were transfected with either an activated *ras* or the *ras* plus *myc* genes showed a distinct transformed morphology, similar to that described in previous reports (1, 4, 6, 10).

To determine whether activated protooncogene transfection can make a TGFβ nonresponsive cell line (10T½) responsive to TGFβ, transfected 10T½ cell clones were seeded in soft agar in the presence or absence of 10 ng of TGFβ per ml and the number of colonies larger than 50 μm were determined after 7 days of growth. Few colonies spontaneously grew in either the control or *myc* gene-transfected groups (Fig. 3). As previously reported for NIH/3T3 cells (6, 14), transfection of 10T½ cells with an activated H-*ras* gene resulted in spontaneous colony formation. This action of *ras* could be potentiated approximately three- to sixfold by cotransfection with *myc* (Fig. 4). The addition of TGFβ to the control, *ras*, or *ras* plus *myc* gene transfectants had little additional colony-stimulating effect. However, the addition of 10 ng of TGFβ per ml to *myc* gene-transfected clones resulted in a 20-fold or greater stimulation of colony formation in soft agar. These results were consistent over a wide range of initial cell inocula (2,500 to 75,000 cells per ml) and TGFβ concentrations (0.1 to 10 ng/ml) (Fig. 3 and 4 and data not shown). The ability of *myc* gene transfection to increase the responsiveness of 10T½ cells to TGFβ is not owing simply to a generalized sensitivity to growth factors, since the addition of epidermal growth factor, insulin, epidermal

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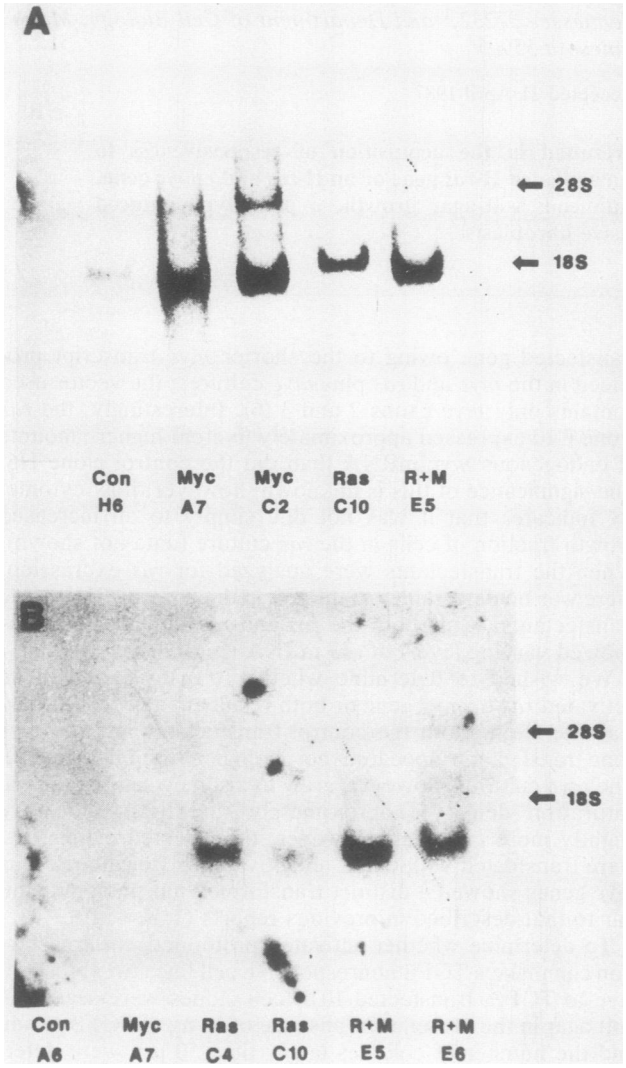


FIG. 1. Northern hybridization of transfected 10T½ clones. Total poly(A)$^+$ mRNA was prepared from cultures growing in 5% fetal bovine serum-supplemented McCoy 5A medium (approximately 60% confluent). Equivalent quantities of RNA (1.5 μg) were electrophoresed in formaldehyde-agarose gels (2.2 M formaldehyde), blotted to nitrocellulose, and hybridized at 43°C. Washes were performed at 43°C in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]). (A) The probe was the 4.8-kilobase *Bam*HI-*Xba*I fragment containing *myc* exons 2 and 3 from pSVc-*myc* (6). (B) The labeled probe was the transforming 3.0-kilobase *Sac*I fragment from pEJ6.6 (15). Con, Clones transfected with pRSVneo alone; Ras or Myc, clones transfected with the *ras* or *myc* gene, respectively; R+M clones transfected with both *ras* and *myc*.

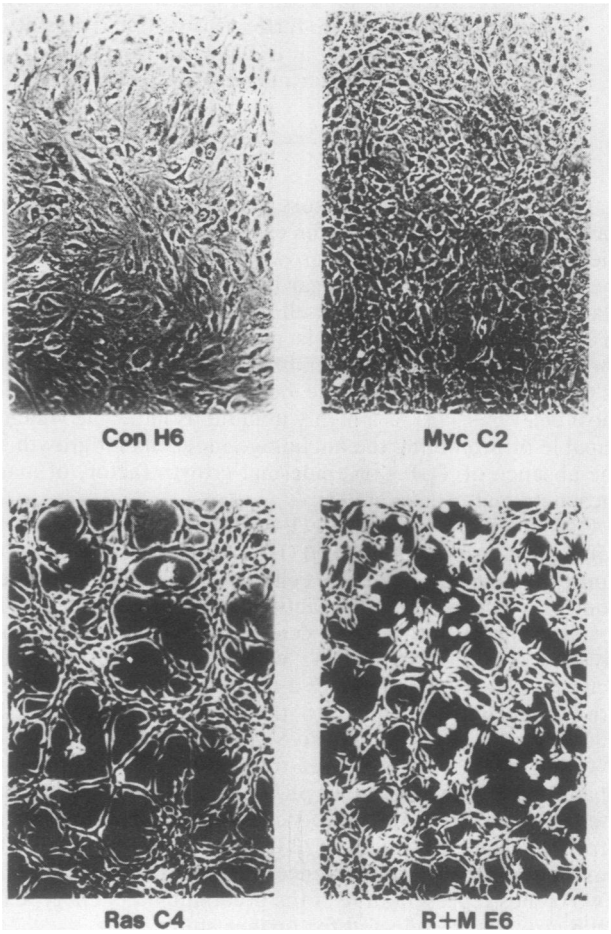


FIG. 2. Morphology of transfected clones. Cultures were plated at 5 × $10^3$ cells per $cm^2$ in 60-mm culture dishes in McCoy 5A medium supplemented with 5% fetal bovine serum and 250 μg of G418 per ml. After 6 days of growth, the plates were fixed with 10% buffered Formalin and photographed with a phase-contrast microscope. For abbreviations, see legend to Fig. 1.

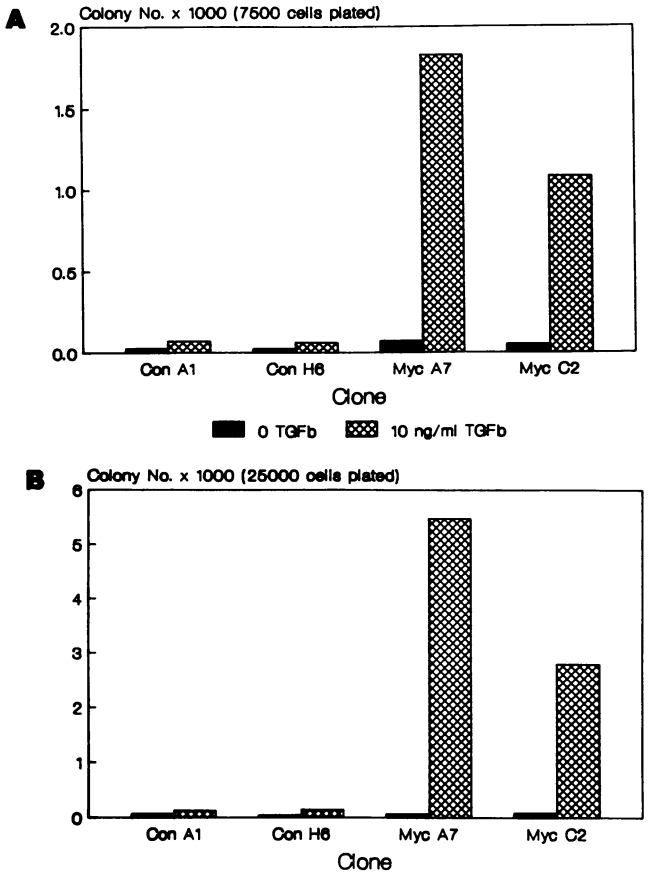


FIG. 3. *Myc* gene modulation of responsiveness to TGFβ. Control (Con) and *myc* (Myc) transfectants were plated in soft agar at either 7,500 or 25,000 cells per ml (A and B, respectively) with or without 10 ng of TGFβ per ml. After 7 days of growth, the number of colonies larger than 50 μm was determined. The data represent the means of three separate experiments, each done in duplicate. Replicate plates for each experiment did not vary more than 15% from the mean.

growth factor plus insulin, platelet-derived growth factor, interleukin 1, or phorbol 12-myristate 13-acetate did not stimulate colony formation (data not shown). Moreover, injection of the *ras* plus *myc* transfectants into nude mice resulted in tumor formation, while mice injected with control cells or cells transfected with either H-*ras* or c-*myc* alone did not develop tumors (data not shown). Thus, transfection of $10T\frac{1}{2}$ cells with a *myc* gene construct conferred TGFβ responsiveness, while an activated H-*ras* gene induced a phenotype similar to that produced by TGFβ stimulation of responsive cells.

We have previously shown that TGFβ stimulation of responsive (AKR-2B) cells results in the accumulation of c-*myc* mRNA (8). However, when quiescent $10T\frac{1}{2}$ cells were treated with TGFβ, there was no change in the levels of c-*myc* mRNA (data not shown). Thus, the lesion in $10T\frac{1}{2}$ cells is the inability to induce *myc*, and presumably the additional pathways dependent on c-*myc* activation, following stimulation with TGFβ. This is not, however, due to an inherent defect in the expression of the *myc* gene or to complete nonresponsiveness to TGFβ; $10T\frac{1}{2}$ cells rapidly growing in the presence of fetal bovine serum expressed *myc* mRNA, and both β and τ cytoplasmic actin genes were induced following TGFβ stimulation of quiescent cultures (Fig. 1A; 7 and data not shown).

In contrast to our findings for *myc* gene modulation of TGFβ responsiveness, recent reports suggest that *myc* gene transfection can result in alternative responses; both colony stimulation and inhibition was observed, depending upon the combinations of growth factors used (17–19). Our results differ in that only TGFβ (0.1 to 10 ng/ml)-supplemented medium stimulated growth in soft agar. The reasons for these apparent differences is growth factor responses of cells transfected with the *myc* gene are presently unknown.

We thank A. S. Goustin, R. M. Lyons, and W. R. Wharton for helpful discussions; Bruce Howard for pRSVneo; and Robert Weinberg for pEJ6.6 and pSVc-*myc*. Mary Aakre, Muriel Cunningham, and Jennifer Pietenpol provided excellent technical assistance, and Rebecca Koransky typed the manuscript.

This work was supported by Public Health Service grants CA 42572 to H.L.M. and CA 42836 to E.B.L. from the National Cancer Institute.

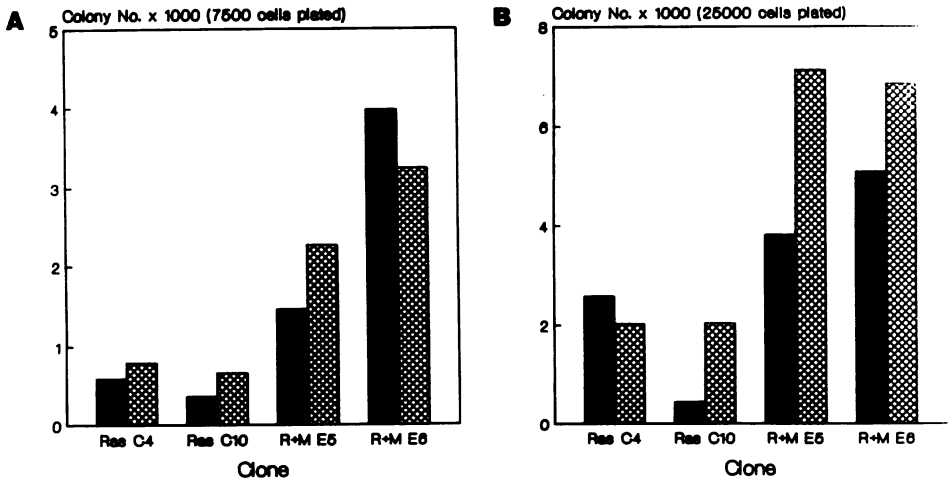


FIG. 4. TGFβ effect on *ras* gene-stimulated colony formation. Clones transfected with *ras* (Ras) and *ras* plus *myc* (R+M) were plated in soft agar as described in the legend to Fig. 3. The data represent the means of three separate experiments, each done in duplicate. Replicate plates for each experiment varied less than 10% from the mean. ■ and ▩, 0 and 10 ng of TGFβ per ml, respectively.

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