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Isolation of Recessive (Mediator$^-$) Revertants from NIH 3T3 Cells Transformed with a c-H-*ras* Oncogene

HISAFUMI YAMADA, TOSHIKO OMATA-YAMADA, NORIKO WAKABAYASHI-ITO,† STEPHEN G. CARTER,‡ AND PETER LENGYEL*

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511

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We have generated two serum- and anchorage-dependent revertants from NIH 3T3 cells transformed with multiple copies of the human c-H-*ras* oncogene. In both revertants, the c-H-*ras* oncogene was fully expressed. Fusion of either revertant with untransformed cells or of the two revertants with one another resulted in transformed progeny. These results indicated that the two revertants were recessive and in different complementation groups. We believe that in our two revertants some of the genes mediating the transforming activity of the c-H-*ras* oncogene are defective; we are attempting to identify these mediator genes.

We wish to identify genes that mediate the transforming activity of the c-H-*ras* oncogene (1). The approach chosen requires the isolation of recessive revertants from cells transformed with the oncogene (3, 9, 19). In the revertants sought, the oncogene is fully expressed, yet the transformed state is not manifested in consequence of a defect in a gene (mediator gene) required for mediating the transforming activity. We plan to retransform the revertants by transfection with DNA from normal cells and to identify the retransforming gene.

For this purpose, we have transfected (7) mouse NIH 3T3 cells with a plasmid carrying an activated human c-H-*ras* oncogene from the T24 bladder carcinoma cell line (13) and, as a selectable marker, the neomycin (G418) resistance gene (12). From the resulting G418-resistant clones, we picked one (FT9) carrying five to seven copies of the integrated *ras* oncogene. Revertants (serum dependent) were obtained from FT9 (serum independent) by incubation in serum-free medium with bromodeoxyuridine and irradiation (17). The procedure was repeated with the survivors except that the incubation with bromodeoxyuridine was in the presence of 1% fetal calf serum (FCS) to eliminate cells with a low serum requirement. After screening of 162 revertant clones, 2 with flat morphology (R116 and R260) were chosen for further studies. R116 cells were similar in size to NIH 3T3 cells; R260 cells were smaller (Fig. 1A). Both revertants were serum dependent: in the absence of serum, they formed only a few small colonies in conditions in which FT9 cells reached confluency (Fig. 2A). The efficiency of growth of the two revertants in 1% FCS was similar to that of NIH 3T3 cells and clearly lower than that of FT9 cells (Table 1). Furthermore, the anchorage dependence of growth of R116 cells was close to that of NIH 3T3 cells. R260 cells formed more colonies than did NIH 3T3 cells but still many fewer than did FT9 cells (Fig. 2B and Table 1). The generation of the revertants from FT9 cells was not accompanied by a large change in chromosome number. The number of chromosomes was 68 ± 6 in NIH 3T3 cells, 82 ± 5 in FT9 cells, 77 ± 5 in R116 cells, and 79 ± 8 in R260 cells.

We detected no difference in the *ras* oncogene integration pattern and in the mobility and level of *ras* mRNA and *ras* p21 protein among FT9 cells and the two revertants (Fig. 3). These findings make it unlikely that the reversion of R116 and R260 was a consequence of a defect in *ras* oncogene structure or expression. As a further test of this possibility, we introduced additional copies of the *ras* oncogene into the revertants: we transfected each with a plasmid carrying the c-H-*ras* oncogene linked to a hygromycin resistance gene (8). We characterized three hygromycin-resistant derivatives each from R116 and from R260. Every one of these six lines carried many more copies of the *ras* oncogene, as established by Southern blot analysis (Fig. 3A and C). Northern (RNA) blot analysis revealed that introduction of the additional *ras* oncogenes resulted in a strong increase in the *ras* mRNA level (Fig. 4). The intensity of the major *ras* mRNA band of the same mobility (approximately 1.2 kilobases) as in the revertants increased; in addition, high-molecular-weight *ras* mRNA band appeared. Western blot (immunoblot) analysis indicated that the six derivative lines with the extra copies of the *ras* oncogene expressed somewhat higher levels of the *ras* p21 protein than did the two revertants (Fig. 3B and D; compare lanes 4 to 6 with lanes 3). However, the level of p21 protein, as detected by Western blotting, did not increase in proportion with the gene copy number. It is conceivable that this level is regulated.

The following observations indicate that the six lines retained the revertant characteristics despite their greatly

TABLE 1. Biological properties of revertant cells

Cell line	Morphology	Growth in:	
		1% serum[a]	Soft agar[b]
NIH 3T3	Flat	11.6	~0.1
FT9	Transformed	46.8	95.0
R116	Flat	11.0	~0.3
R260	Flat	11.6	4.5

[a] Expressed as 100 × number of cells grown in 1% FCS/number of cells grown in 10% FCS. A total of 5×10^4 cells were seeded on a 10-cm-diameter plate, incubated in 1 or 10% FCS for 6 days, and counted.

[b] Percentage of plated cells forming visible colonies. A total of 10^3 cells were seeded on a 3.5-cm-diameter soft agar plate in 10% FCS and cultured for 14 days. Colonies visible after staining with *p*-iodonitrotetrazolium violet were counted.

* Corresponding author.

† Present address: DNAX Research Institute, Palo Alto, CA 94304.

‡ Present address: Glaxo Research Laboratories, Research Triangle Park, NC 27709.

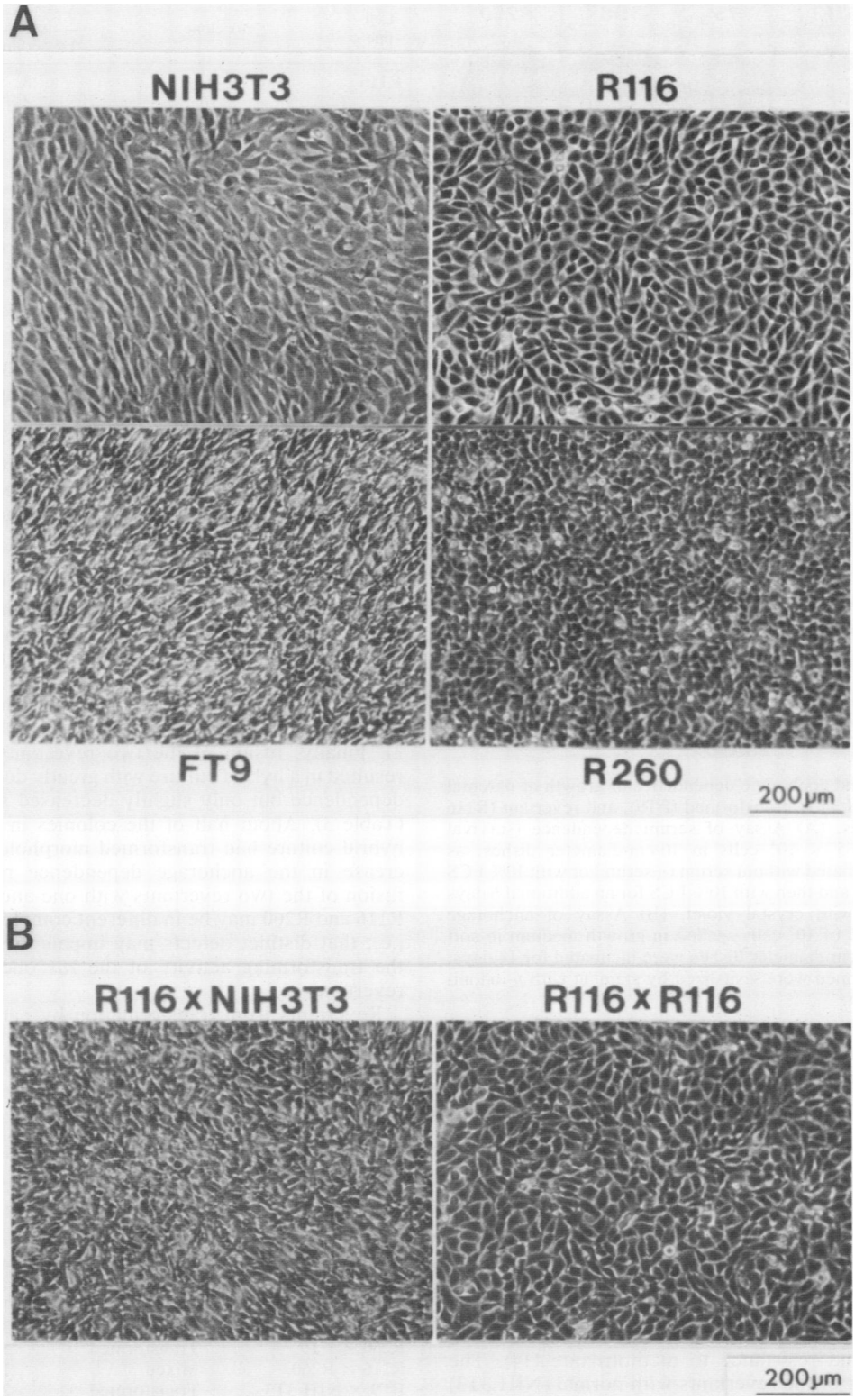

FIG. 1. Morphology of parental untransformed (NIH 3T3), transformed (FT9), and revertant (R116 and R260) cells (A) and of fused cells (R116 × NIH 3T3 and R116 × R116) (B). Cells were grown in monolayer cultures in growth medium (Dulbecco modified Eagle medium supplemented with 10% FCS) until they reached confluency.

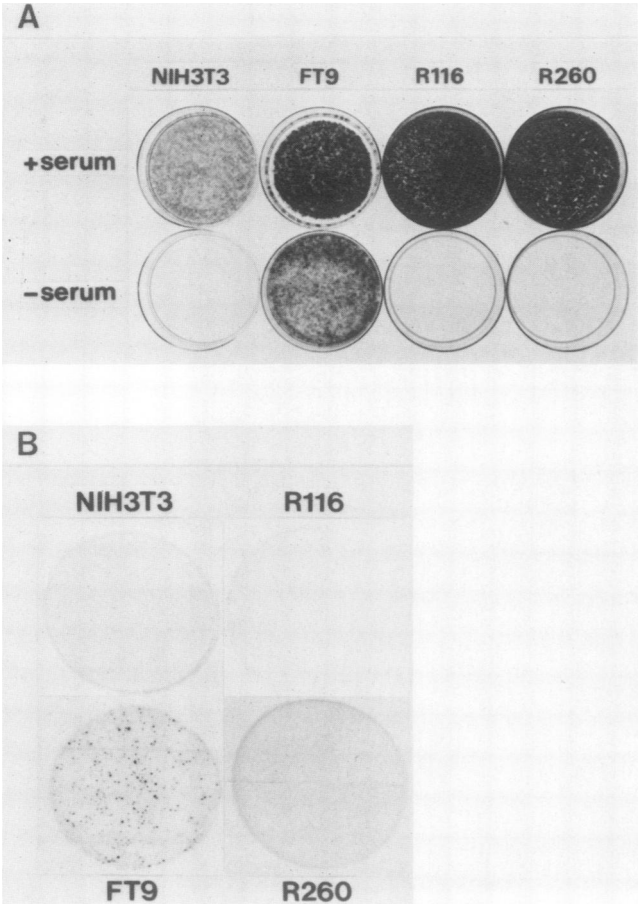

FIG. 2. Serum and anchorage dependence of growth of parental untransformed (NIH 3T3), transformed (FT9), and revertant (R116 and R260) cell lines. (A) Assay of serum dependence (survival assay). A total of 5×10^4 cells in 10-cm-diameter dishes, as indicated, were incubated without serum (−serum) or with 10% FCS (+serum) for 6 days and then with 10% FCS for an additional 6 days and were stained with crystal violet. (B) Assay of anchorage dependence. A total of 10^3 cells seeded in growth medium in soft agar (0.33%) in 3.5-cm-diameter dishes were incubated for 14 days, and the colonies formed were visualized by staining with *p*-iodonitrotetrazolium violet.

increased *ras* gene copy number: the efficiency of growth of the six lines in low (1%) FCS was not increased above that of R116 or R260 (Table 2). The anchorage dependence of the three R116 derivatives remained essentially complete. Although the anchorage dependence of the R260 derivatives diminished, it still remained significantly below that of FT9 (Table 2).

To determine whether the revertants were dominant or recessive, they were fused with transformed and normal cells. This procedure required the introduction of further selectable markers. The markers chosen were resistance to hygromycin (8) and resistance to methotrexate (14). The fusion (6) of either of the revertants with normal (NIH 3T3) cells resulted in hybrids with transformed morphology. This result is compared with the nontransformed morphology of the control hybrid obtained by fusing R116 with itself (Fig. 1B). The R116 × NIH 3T3 hybrid was much less serum dependent and much less anchorage dependent than either

TABLE 2. Effects of transfection of further copies of the *ras* oncogene on revertants[a]

Cell line	*ras* clone	Growth in:	
		1% serum	Soft agar
R116	1-7	7.8	~0.1
	1-10	7.4	~0.1
	2-6	12.9	~0.1
R260	1-2	3.6	33.2
	1-5	4.2	35.6
	2-4	5.5	30.8

[a] R116 and R260 were transfected (7) with a plasmid containing the *ras* oncogene linked to a hygromycin resistance gene (8) and selected in the presence of 500 μg of hygromycin per ml. All clones were morphologically flat. For details, see footnotes to Table 1.

the control R116 × R116 hybrid or NIH 3T3 cells (Table 3). From 13 colonies picked from the population of the R116 × NIH 3T3 hybrid, 8 resulted in cultures with transformed morphology. The basis of this lack of uniformity is unclear. Zarbl et al. (19) also reported that from the hybrid cells obtained by fusing normal cells with recessive revertants from transformation with the *fos* oncogene, only 50% exhibited a transformed phenotype. The R260 × NIH 3T3 hybrid was also less serum dependent and less anchorage dependent than either the control R260 × R260 hybrid or NIH 3T3 cells (Table 3). These results indicate that both R116 and R260 were recessive revertants. This conclusion was tested and verified by fusing each of the revertants to the transformed FT9 cells: the hybrids obtained retained the transformed morphology of FT9 cells (not shown) and also the ability to grow efficiently in 1% FCS and in soft agar (Table 3). Finally, fusion of the two revertants with each other resulted in a hybrid culture with greatly decreased anchorage dependence but only slightly decreased serum dependence (Table 3). About half of the colonies in the R116 × R260 hybrid culture had transformed morphology. The large decrease in the anchorage dependence resulting from the fusion of the two revertants with one another suggests that R116 and R260 may be in different complementation groups, i.e., that distinct defects may impair the manifestations of the transforming activity of the *ras* oncogene in the two revertants.

Revertants from transformation by particular oncogenes (including *ras*) were reported to be resistant to retransformation by some of the other oncogenes (9, 11, 16, 19). This

TABLE 3. Characteristics of hybrid cells[a]

Cell line	Morphology	Growth in:	
		1% serum	Soft agar
R116 × R116	Flat	7.2	0.5
R116 × NIH 3T3	Transformed	41.2	55.7
R116 × FT9	Transformed	42.6	54.0
R260 × R260	Flat	14.5	16.8
R260 × NIH 3T3	Transformed	33.6	72.5
R260 × FT9	Transformed	38.3	81.2
R116 × R260	Mixed	18.4	86.5
FT9 × NIH 3T3	Transformed	28.3	75.0
FT9 × FT9	Transformed	42.9	92.3

[a] Cell lines carrying a hygromycin resistance gene (8) or a methotrexate-resistant dihydrofolate reductase gene (14) were fused with polyethylene glycol (6) and selected in the presence of hygromycin (500 μg/ml) and methotrexate (500 nM). For details, see footnotes to Table 1.

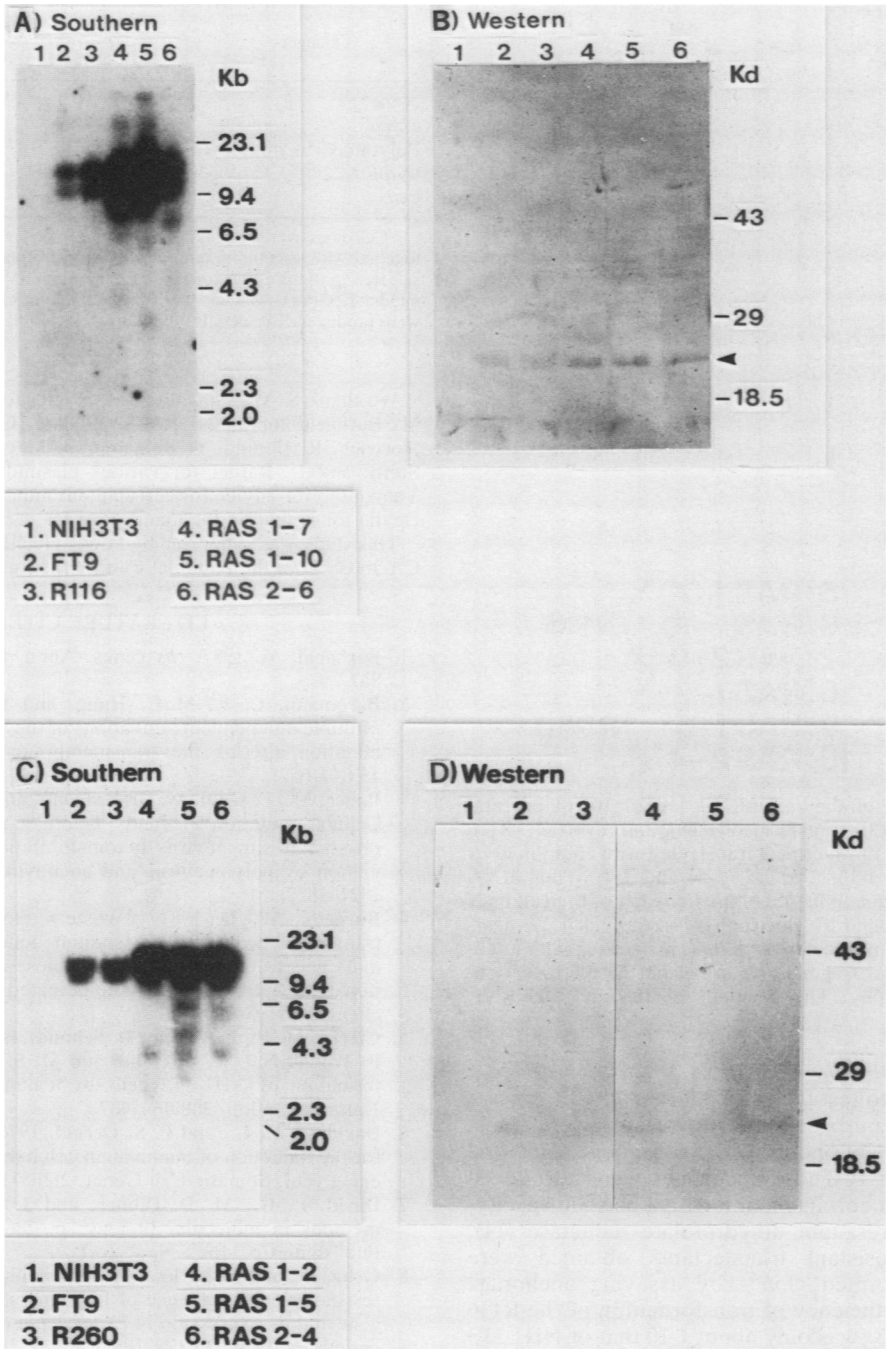

FIG. 3. Comparisons of the *ras* gene content and p21 protein content of parental untransformed (NIH 3T3), transformed (FT9), and revertant (R116 and R260) cell lines and of revertants transfected with additional *ras* DNA (*ras* clones 1-7, 1-10, 2-6, 1-2, 1-5, and 2-4). (A and C) Assay of H-*ras* gene content by Southern blot analysis of DNA (10). DNA samples (30 μg) from each of the cell lines indicated were digested with *Hin*dIII, fractionated by electrophoresis through 1% agarose gels, transferred to nitrocellulose filters, hybridized with a ^{32}P-labeled 2.8-kilobase *Sac*I fragment from plasmid EJ 6.6, and visualized by autoradiography. The positions of marker DNA (sizes indicated in kilobases [kb]) are shown. (B and D) Assay of the p21 protein content of cell extracts by Western blot analysis (4) Total cell extracts containing 10 μg of protein from each of the cell lines indicated were fractionated by electrophoresis through 12.5% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose filters, treated with an antibody to p21 (Triton Biosciences Inc.) and peroxidase-conjugated rabbit anti-sheep immunoglobulin G, and visualized by staining with 4-chloro-1-naphthol. The positions of marker proteins (sizes indicated in kilodaltons [kD]) are shown. The *ras* 1-7, 1-10, and 2-6 cell lines were derived from R116, and the *ras* 1-2, 1-5 and 2-4 cell lines were derived from R260 by the introduction of further copies of the *ras* oncogene by transfection with a c-H-*ras* oncogene linked to a hygromycin resistance gene and selection in the presence of hygromycin (500 μg/ml).

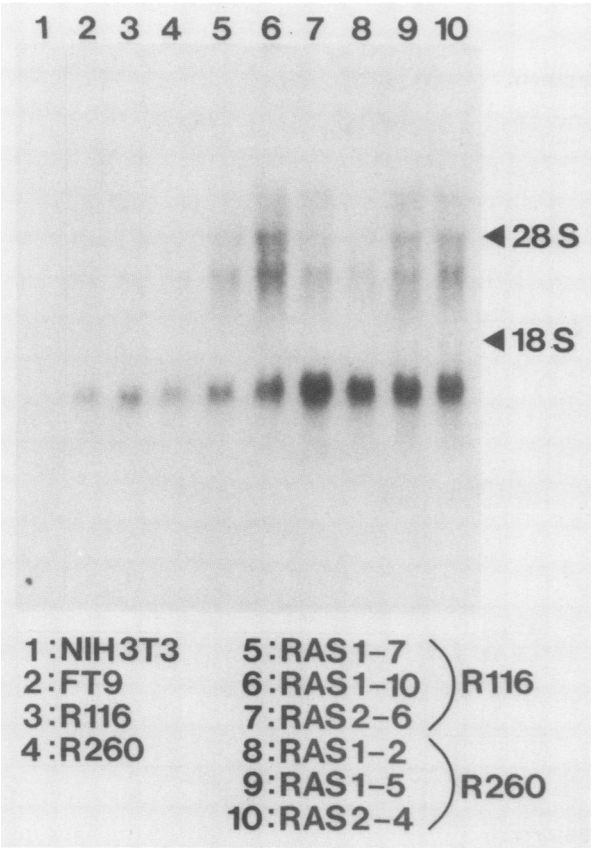

FIG. 4. Comparison of the *ras* mRNA levels (10) of parental untransformed (NIH 3T3), transformed (FT9), and revertant (R116 and R260) cell lines and of revertants transfected with additional *ras* DNA (*ras* clones 1-7, 1-10, 2-6, 1-2, 1-5, and 2-4). Total RNA samples (25 μg) from subconfluent cultures of each of the cell lines indicated were fractionated by electrophoresis through 1.2% agarose gels, transferred to nitrocellulose filters, hybridized with a ^{32}P-labeled 2.8-kilobase *Sac*I fragment from plasmid EJ 6.6, and visualized by autoradiography. The positions of 18S and 28S RNA markers are indicated.

finding prompted us to test the retransformability of the two revertants. For this purpose, these revertants were transfected with four types of oncogenes: c-*sis*, c-*neu*, v-*abl*, and polyomavirus middle T. These oncogenes were introduced into cells by unlinked cotransfection with a plasmid specifying a methotrexate-resistant dihydrofolate reductase (14). The methotrexate-resistant transfectants obtained were screened for transformed clones by assaying anchorage independence. The efficiency of transformation of the R116 revertant by v-*abl* (18) was only about 1/10 that of NIH 3T3 cells, whereas the c-*sis* (5), c-*neu* (2), and polyomavirus middle-T oncogenes (15) transformed R116 and NIH 3T3 cells with similar efficiencies. c-*sis* transformed R260 with a fourfold higher efficiency than it transformed NIH 3T3, whereas c-*neu* transformed R260 with an about twofold higher efficiency than it transformed NIH 3T3 cells (Table 4). These results also indicate that the two revertants differ in characteristics.

We are attempting to identify the defective mediator genes by using an approach involving the transfection of DNA from normal cells into the revertants and the identification among the transfected cells of those that became transformed.

TABLE 4. Retransformation of the revertants by various oncogenes[a]

Oncogene	Growth in soft agar		
	NIH 3T3	R116	R260
None	0.1	0.7	1.0
c-*neu*	38.2	47.9	68.7
Polyomavirus middle T	72.3	58.7	
v-*abl*	54.4	5.7	
c-*sis*	19.0	15.0	73.5

[a] Cell lines were transfected with the oncogenes indicated (2, 5, 15, 18) together with a methotrexate-resistant dihydrofolate reductase gene (14) and selected in the presence of methotrexate (500 nM). When the polyomavirus middle-T or the v-*abl* gene was transfected into R260, only very few colonies were produced. For details, see footnote *a*, Table 1.

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