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A Transforming *ras* Gene Can Provide an Essential Function Ordinarily Supplied by an Endogenous *ras* Gene

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Microinjection of monoclonal antibody Y13-259, which reacts with all known mammalian and yeast *ras*-encoded proteins, has previously been shown to prevent NIH 3T3 cells from entering the S phase (L. S. Mulcahy, M. R. Smith, and D. W. Stacey, Nature [London] 313:241–243, 1985). We have now found several transformation-competent mutant v-*ras*^{H} genes whose protein products in transformed NIH 3T3 cells are not immunoprecipitated by this monoclonal antibody. These mutant proteins are, however, precipitated by a different anti-*ras* antibody. Each of these mutants lacks Met-72 of v-*ras*^{H}. In contrast to the result for cells transformed by wild-type v-*ras*^{H}, Y13-259 microinjection of NIH 3T3 cells transformed by these mutant *ras* genes did not prevent the cells from entering the S phase. These results imply that a transformation-competent *ras* gene can supply a normal essential function for NIH 3T3 cells. When the proteins encoded by the mutant *ras* genes were overproduced in *Escherichia coli*, several mutant proteins that lacked Met-72 failed to bind Y13-259 in a Western blot. However, a *ras* protein from a mutant lacking amino acids 72 to 82 did bind the antibody, but a *ras* protein from a mutant lacking amino acids 72 to 84 did not. These results suggest that Y13-259 may bind to a higher ordered structure that has been restored in the mutant lacking amino acids 72 to 82.

Monoclonal antibodies directed against *ras* proteins have been useful in elucidating the function of these proteins in vivo in addition to facilitating their identification and purification. One monoclonal anti-*ras* antibody (Y13-259) displays especially interesting properties in vitro and in vivo. It was initially developed by injecting rats with a tumor induced by Harvey murine sarcoma virus, which contains the v-*ras*^{H} transforming gene (2). This antibody binds in vitro to denatured v-*ras*^{H} protein in immunoprecipitation or Western blot assays (9). It also binds to other known *ras* proteins, such as those endogenous to yeast and mammals, as well as to highly transforming *ras* proteins that have been activated by amino acid substitution (1, 6, 7, 9, 11, 13–15).

Microinjection of monoclonal antibody Y13-259 has been used in vivo as a probe of normal *ras* function in mammalian cells (4, 8). Injection of this antibody into NIH 3T3 cells as well as into certain other fibroblastic cell lines prevents these cells from initiating S-phase DNA synthesis. These results suggest that one or more of the endogenous cellular *ras* genes serve(s) an essential function in the proliferative cycle of these cells, although the possibility that the antibody interferes with entry into the S phase by another mechanism has not been entirely ruled out.

It is not yet known whether the activation of *ras* proteins by amino acid substitution is associated with retention or loss of their normal functions. Since monoclonal antibody Y13-259 binds to activated *ras* proteins, these transforming proteins would not be expected to abrogate the defect induced by microinjection of the antibody into NIH 3T3 cells. However, this question could be addressed for the putative normal *ras* function identified by Y13-259 microinjection if a transformation-competent *ras* mutant that failed to bind Y13-259 were isolated.

The epitope to which this monoclonal antibody binds has not been identified. The capacity of Y13-259 to react with a protein denatured by heat and sodium dodecyl sulfate in an electroblot may imply that the antibody is binding to an epitope found in the primary structure of the protein; however, since a protein blotted in this way is still able to bind GTP (7), it must retain a considerable amount of secondary structure under these conditions. Using Western blot analysis of mutant *ras* proteins expressed in *Escherichia coli*, others have observed that a mutant that terminates prematurely after amino acid 89 still binds this antibody but that a mutant that terminates at amino acid 69 does not bind the antibody; this result led them to conclude that the epitope for Y13-259 must include at least some of the amino acids located between residues 70 and 89 (4a).

Using a series of in-frame deletion mutants, we recently noted (B. M. Willumsen, A. G. Papageorge, H. F. Kung, E. Bekesi, T. Robins, M. Johnsen, W. C. Vass, and D. R. Lowy, Mol. Cell. Biol., in press) that a transformation-competent v-*ras*^{H} mutant that substitutes three novel amino acids for Met-72 failed to bind monoclonal antibody Y13-259 in immunoprecipitation and electroblotting assays. The antibody also failed to immunoprecipitate the proteins encoded by several other closely related mutants. In this study we used NIH 3T3 cells morphologically transformed by these mutants to determine if the mutant genes can substitute functionally for the putative normal *ras* function that is disrupted by microinjection of Y13-259.

The construction of in-frame v-*ras*^{H} deletion mutants by the combination of two sequenced parts of v-*ras*^{H} through a *Bcl*I oligonucleotide linker of a Harvey murine sarcoma virus DNA-containing plasmid that can induce focal transformation of NIH 3T3 cells has been described previously

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TABLE 1. Biological activity and immunoreactivity of v-*ras*^{H} mutants[a]

Mutant	Structure[b]	Focus-forming activity[c]	% DNA synthesis after microinjection of:		Immunoprecipitation by:		Electroblotting with Y13-259[d]
			Y13-259	Y13-238	Y13-259	Y13-238	
1 (pBW600)[e]	1–189 (Wild type)	1.0			+	+	+
2 (pBW1160)[f]	1–189 (Wild type)	1.0	10	100	+	+	+
3 (pBW1303)[f]	1–63 SDQ 73–189	0.8	80	100[g]	−	+	
4 (pBW967)[e]	1–68 ADQ 73–189	0.1			−	+	−
5 (pBW968)[e]	1–68 ADQ 77–189	0.07	82	94	−	+	−
6 (pBW969)[e]	1–71 TDQ 73–189	0.1	85	103	−	+	−
7 (pBW1201)[f]	1–71 TDQ 77–189	0.3	85	114	−	+	−
8 (pBW1202)[f]	1–71 TDQ 83–189	None					+
9 (pBW1420)[f]	1–71 TDQ 85–189	None					−
10 (pBW1267)[f]	1–92 LIR 96–189	1.2	30	100[g]	+	+	+

[a] NIH 3T3 cells were grown in Dulbecco modified minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml). DNA transfection was carried out as previously described (16).

[b] The numbers indicate the v-*ras*^{H} amino acids in the front (first set of numbers) and tail (second set of numbers) ends. The letters indicate the three amino acids encoded by the oligonucleotide linker joining the front and tail ends. Amino acid abbreviations: S, serine; D, aspartic acid; Q, glutamine; A, alanine; T, threonine; L, leucine; I, isoleucine; and R, arginine.

[c] Relative number of foci induced as compared with the wild-type controls: pBW600 for *tk* vectors (250 to 400 foci per 0.2 μg of DNA); pBW1160 for *neo* vectors (700 to 1,000 foci per 0.2 μg of DNA).

[d] Electroblotted material was from bacterially synthesized *ras* proteins.

[e] *tk*-based vector.

[f] *neo*-based vector.

[g] Uninjected cells were used as controls for these clones.

(16). This linker results in the addition of three novel amino acids within the protein at the site of the deletion, as shown in Table 1 for each mutant. Two eucaryotic vectors and one procaryotic vector were used to express the mutant genes in NIH 3T3 cells and *E. coli*, respectively. One eucaryotic vector contains the Harvey murine sarcoma virus transforming region (the viral long terminal repeat plus v-*ras*^{H}) and a linked thymidine kinase (*tk*) gene (16). The other eucaryotic vector was developed by C. Jhappan, G. Vande Woude, and T. Robins (submitted for publication). In this vector, the v-*ras*^{H} gene is located upstream from the simian virus 40 and *neo*^{R} genes of pSV$_{neo}$ (12); these two genes are flanked by the Moloney murine leukemia virus long terminal repeat. The procaryotic vector, which places the *ras* mutants under the control of the lambda pL promoter, has been described previously (5). The *E. coli* cells that harbor the expression vector contain a temperature-sensitive allele of the lambda repressor gene, *c*I857.

The v-*ras*^{H} gene product is 189 amino acids in length. As part of a mutational analysis of the v-*ras*^{H} catalytic domain (Willumsen et al., in press), we noted that several transformation-competent in-frame deletion mutants (mutants 3 to 7 in Table 1) encoded proteins in NIH 3T3 cells that were not immunoprecipitated by monoclonal antibody Y13-259 (Fig. 1 and Table 1). The deletions were confined to a specific segment of the gene; each mutant lacked at least one codon from the segment encoding amino acid residues 64 to 76, and some lacked up to 10 of these amino acids. It may be significant that each of these mutant genes lacked Met-72, but we have not tested deletions of residues within codons 64 to 76 that have retained this Met-72 codon.

The failure of monoclonal antibody Y13-259 to bind to the five mutants was not due to a lack of virally encoded protein. As noted above, each of these mutants was biologically active in that it induced focal transformation of the NIH 3T3 cells, although with variable efficiency. This result by itself suggested that they encoded a stable *ras* protein. Second, immunoprecipitation with a different anti-*ras* monoclonal antibody (Y13-238) demonstrated significant amounts of v-*ras*^{H}-encoded protein in the extracts from the transformed cells (Fig. 1 and Table 1).

Since these biologically active mutant proteins failed to react with monoclonal antibody Y13-259 in extracts made from transformed NIH 3T3 cells, we reasoned that these cells might not be blocked by microinjection with this monoclonal antibody. As noted earlier, normal NIH 3T3 cells were unable to initiate DNA synthesis following injection with monoclonal antibody Y13-259, but injection with a different anti-*ras* monoclonal antibody (Y13-238) did not have an inhibitory effect (8). Cells transformed by other oncogenes, including *ras*, have also been noted to be blocked by Y13-259 (4).

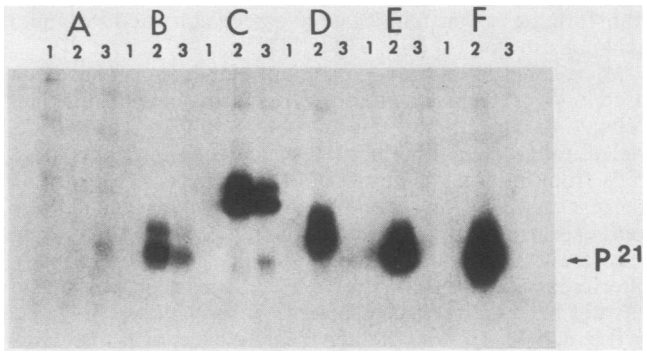

FIG. 1. Immunoprecipitation of NIH 3T3 cells transfected with *ras* mutants. Cultures were metabolically labeled overnight with [^{35}S]methionine (250 μCi/ml) in methionine-free medium. Extracts of whole cells were prepared and precipitated as previously described with p21 monoclonal antibody Y13-238 or Y13-259 (2, 10). Lanes: 1, no antibody; 2, Y13-238; 3, Y13-259. Panels: A, control NIH 3T3 cells; B, cells transformed by the wild-type v-*ras*^{H} gene; C, cells transformed by a previously described (17) v-*ras*^{H} duplication mutant (pBW976, whose *ras* structure is 1–172 PDQ 146–189); D, mutant 6; E, mutant 4; F, mutant 5. Overexposure of the gel (results not shown) revealed the precipitation of endogenous p21 protein in the extracts precipitated with Y13-259.

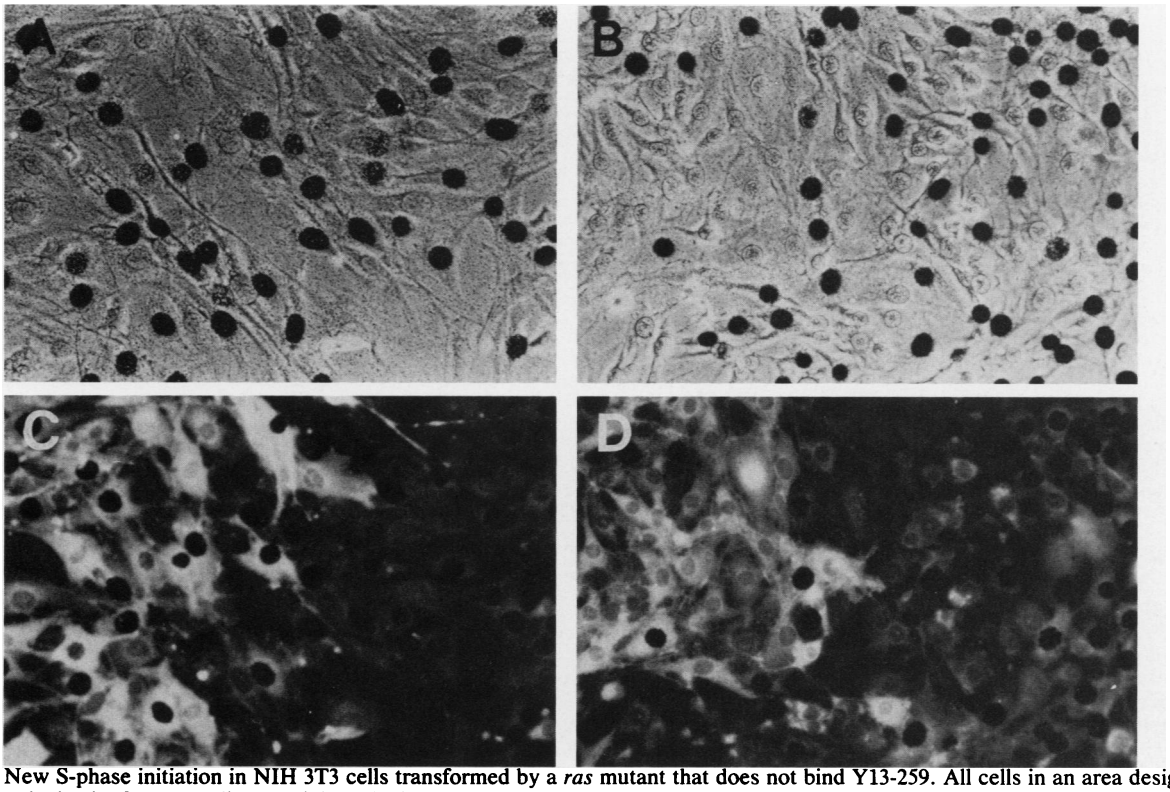

FIG. 2. New S-phase initiation in NIH 3T3 cells transformed by a *ras* mutant that does not bind Y13-259. All cells in an area designated by marks on the back of a cover slip were injected with monoclonal antibody Y13-259 labeled with fluorescein (8). After 18 h, the cells were pulsed with [^{3}H]thymidine, fixed, and autoradiographed. Injected cells were identified by fluorescent-antibody staining, first with rabbit anti-rat immunoglobulin and then with rhodamine-conjugated goat anti-rabbit immunoglobulins. The proportion of thymidine-labeled injected cells was compared with the proportion of thymidine-labeled uninjected cells. Panels A and C, which are identical fields, are cells with mutant 7, which does not bind Y13-259. Photomicrographs A and B were taken with visible light, and photomicrographs C and D were taken with fluorescent light to indicate which cells had been injected. Many fluorescent cells took up thymidine. Panels B and D are identical fields of cells transformed by the wild-type gene (clone 2), photographed as were panels A and C. The vast majority of the injected cells did not take up thymidine.

As expected, when cells transformed either by the wild-type viral gene (mutant 2) or by a deletion mutant encoding a *ras* protein that did bind Y13-259 (mutant 10) were injected with this antibody, they reverted to the morphologic appearance of untransformed cells, and their DNA synthesis was markedly impaired (Table 1 and Fig. 2). By contrast, cells transformed by mutants whose proteins were not immunoprecipitated by this antibody (mutants 3 and 5 to 7) retained the morphologic appearance of transformed cells. In addition, they continued to undergo DNA synthesis characteristic of the S phase at a frequency only marginally lower than that in uninjected cells or in cells injected with the anti-*ras* antibody Y13-238, which does not ordinarily inhibit DNA synthesis. The results imply that these mutant *ras* genes can bypass the block introduced by microinjection with Y13-259.

The capacity of the transformation-competent mutants to provide the function that is impaired by microinjection of monoclonal antibody Y13-259 into NIH 3T3 cells suggests that the block in wild-type cells results from the anti-*ras* activity of the antibody rather than from a nonspecific effect. Since normal *ras* gene expression is required for the cells to enter the S phase, our results indicate that an activated *ras* gene can provide this normal function. This is the first evidence in mammalian cells that an activated *ras* gene can supply a normal cellular function. This result is consistent with the hypotheses that the acquisition of the high transformation phenotype may not necessarily be associated with a loss of the ability to carry out at least some normal *ras* functions and that *ras*-induced transformation may represent an exaggerated normal *ras* function. Our results are analogous to the previously demonstrated capacity of *ras* proteins with activating mutations to partially complement the defect induced in *Saccharomyces cerevisiae* by the disruption of the two yeast *ras* genes (1, 3).

The failure of the different mutants with a deletion of Met-72 to bind Y13-259 in the immunoprecipitation assay (mutants 3 to 7) suggested that the epitope recognized by this antibody might include sequences that had been deleted from the mutants. To examine this question in a cleaner background, we placed mutants 3 to 7 in a procaryotic expression vector to overproduce the mutant proteins. When the bacterially synthesized *ras* proteins were denatured and electroblotted onto nitrocellulose, they did not bind Y13-259, in agreement with the results of the immunoprecipitation assay of the mutant proteins extracted from NIH 3T3 cells (Fig. 3 and Table 1). We also tested the binding of this antibody to two mutants (mutants 8 and 9) that were similar to mutants 6 and 7, which failed to bind Y13-259, except that additional amino acids had been deleted in mutants 8 and 9. Unlike mutants 6 and 7, mutant 8 was positive in the electroblotting assay (Fig. 2 and Table 1 [identical results were obtained independently by M.J. with crude extracts and by H.-F.K. with purified protein]). How-

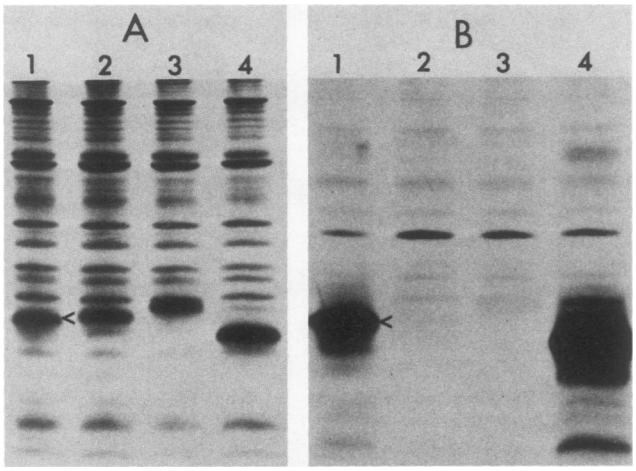

FIG. 3. Electroblot of monoclonal antibody Y13-259 binding to *ras* proteins synthesized in *E. coli*. Bacteria containing the procaryotic *ras* expression plasmids were grown at 32°C in minimal medium supplemented with 19 amino acids (no methionine) and glucose. At about 10^8 cells per ml, *ras* protein synthesis was induced by incubation at 42°C for 1 h. Cells were pulse-labeled with [^{35}S]methionine during this period to facilitate monitoring of the extent of protein transfer onto nitrocellulose paper at a later step. Crude extracts were loaded onto 12.5% sodium dodecyl sulfate-polyacrylamide gels and subjected to electrophoresis. The gels were then electroblotted onto nitrocellulose filters, which were incubated overnight at 4°C with monoclonal antibody Y13-259, washed, and then incubated with peroxidase-conjugated goat anti-rat immunoglobulin (Dakopatts) at 25°C for 3 h. The blot was photographed after color had developed. An autoradiogram was also made to verify that the *ras* protein had been transferred to the nitrocellulose (panel A). Binding to Y13-259 was monitored with immunoperoxidase (panel B). Lanes: 1, wild-type v-*ras*^{H}; 2, mutant 4; 3, mutant 6; 4, mutant 8. The arrowhead in each panel indicates the location of the wild-type *ras* protein.

ever, the deletion of two more amino acids (mutant 9) once again resulted in a protein that failed to bind Y13-259.

These antibody-binding results can be explained by either of two mutually exclusive possibilities. The lack of Y13-259 binding to mutants 3 to 7 could be due either to the deletion of all or part of the amino acid sequences that form the epitope recognized by the antibody or to a masking of the epitope by a change in *ras* structure brought about by these deletions. The latter possibility would imply that the epitope is formed by amino acids other than those deleted in mutants 3 to 7, whereas the former would suggest that the epitope includes sequences around amino acid 72. The transformation-competent nature of mutants 3 to 7 suggests that the mutant proteins do not have a grossly altered structure, arguing against the possibility of the deletions masking an epitope located outside this region. The deletion in mutant 8, which binds Y13-259, does not restore a wild-type amino acid sequence. If the epitope includes the amino acid 72 region, the positive binding obtained with mutant 8 would imply that the antibody recognizes a secondary structure that was fortuitously restored by this deletion. However, the positive binding of mutant 8 is also compatible with the possibility that the epitope lies outside this region. Additional studies will be required to determine which possibility is correct.

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