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p21-*ras* Effector Domain Mutants Constructed by "Cassette" Mutagenesis

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A series of mutations encoding single-amino-acid substitutions within the v-*ras*^{H} effector domain were constructed, and the ability of the mutants to induce focal transformation of NIH 3T3 cells was studied. The mutations, which spanned codons 32 to 40, were made by a "cassette" mutagenesis technique that involved replacing this portion of the v-*ras*^{H} effector domain with a linker carrying two *Bsp*MI sites in opposite orientations. Since *Bsp*MI cleaves outside its recognition sequence, *Bsp*MI digestion of the plasmid completely removed the linker, creating a double-stranded gap whose missing *ras* sequences were reconstructed as an oligonucleotide cassette. Based upon the ability of the mutants to induce focal transformation of NIH 3T3 cells, a range of phenotypes from virtually full activity to none (null mutants) was seen. Three classes of codons were present in this segment: one which could not be altered, even conservatively, without a loss of function (codons 32 and 35); one which retained detectable biologic activity with conservative changes but which lost function with more drastic substitutions (codons 36 and 40); and one which retained function even with a nonconservative substitution (codon 39).

Mammalian *ras* genes encode 21-kilodalton protein products (p21) that localize to the inner aspect of the plasma membrane, bind guanine nucleotides GTP and GDP, and possess intrinsic GTPase activity (reviewed in references 2 and 9). p21 proteins are ubiquitously expressed and are thought to play an important role in the proliferation of normal cells (13). Although overexpression of normal p21 proteins can induce morphologic transformation of established rodent cells, *ras* genes that encode certain missense mutations induce these changes with much greater efficiency (15, 21). *ras* genes with such activating mutations have been identified as the cellular transforming genes of a wide variety of tumors in humans and animals and also as the viral oncogenes of several acute transforming retroviruses (reviewed in reference 2). The Harvey murine sarcoma virus oncogene v-*ras*^{H}, which was used in the experiments reported here, is one of the best-characterized mammalian *ras* transforming genes (19, 26). Compared with the normal c-*ras*^{H}, v-*ras*^{H} contains two missense mutations (encoding Arg-12 for Gly-12 and Thr-59 for Ala-59) that independently confer the high-transformation phenotype.

Mutational analysis of *ras* has elucidated some mechanistic aspects of p21 function. For normal p21, noncovalent binding of GTP places p21 in the biologically active state, while the GDP-bound form of p21 is inactive (4, 17, 23). The greater biologic activity of mutant p21 is believed to result from the mutant proteins being placed more or less constitutively in the active, GTP-bound form in vivo. In accord with this hypothesis, the intrinsic GTPase activity of most highly transforming p21 proteins, including p21$^{v\text{-}ras^H}$, is significantly lower than that of normal p21 proteins (6, 12, 20). Highly transforming forms of p21 are resistant to a widely expressed intracellular protein (GTPase-activating protein) that can markedly accelerate the intrinsic GTPase activity of normal p21 (23). Membrane association by p21 is required for the induction of cellular transformation even by v-*ras*^{H} (25). While adenylate cyclase appears to be a major target protein of *Saccharomyces cerevisiae* RAS (4), no effector protein of mammalian p21 has been identified, although mammalian p21 can stimulate yeast adenylate cyclase (22). However, mutational analysis of highly transforming *ras*^{H} genes, including v-*ras*^{H}, has identified an essential region of p21 that may interact with a mammalian p21 effector protein(s) (19, 26). This segment of p21 has been hypothesized to represent an effector region because the mutations in this region that are biologically inactive and fail to stimulate yeast adenylate cyclase retain the other characteristics of p21$^{v\text{-}ras^H}$, such as membrane association, guanine nucleotide binding, and low GTPase activity.

To study this region in greater detail, we have now made numerous single-amino-acid-substitution mutations in the region spanning codons 32 to 40 of v-*ras*^{H} by a modified version of "cassette" mutagenesis, in which oligonucleotides that contain the desired mutations are used to directly reconstruct a DNA segment (24). Conservative amino acid substitutions were introduced for most mutants, since previous studies had indicated that nonconservative amino acid changes in this region often resulted in null mutants (19). Our results indicate that a range of phenotypes can be induced by various mutations in the effector region and that not all missense mutations in codons 36 and 40 produce identical phenotypes.

Cassette mutagenesis. The strategy for constructing the single-codon changes in the p21 effector domain is shown schematically in Fig. 1. The technique exploits the fact that *Bsp*MI cleaves outside its recognition sequence, in contrast to most commonly used restriction endonucleases. Unlike the standard cassette mutagenesis protocol (24), which results in the obligatory presence of nonauthentic sequences, *Bsp*MI digestion of an appropriately designed linker removes all linker sequences, creating a gap whose ends are composed entirely of authentic sequences. The self-complementary dodecameric *Bsp*MI linker (5'GCAGGTACCTGC3') is designed to form a duplex consisting of two *Bsp*MI sites which are oriented in opposite directions so that *Bsp*MI cleavage of DNA that contains the linker results in excision

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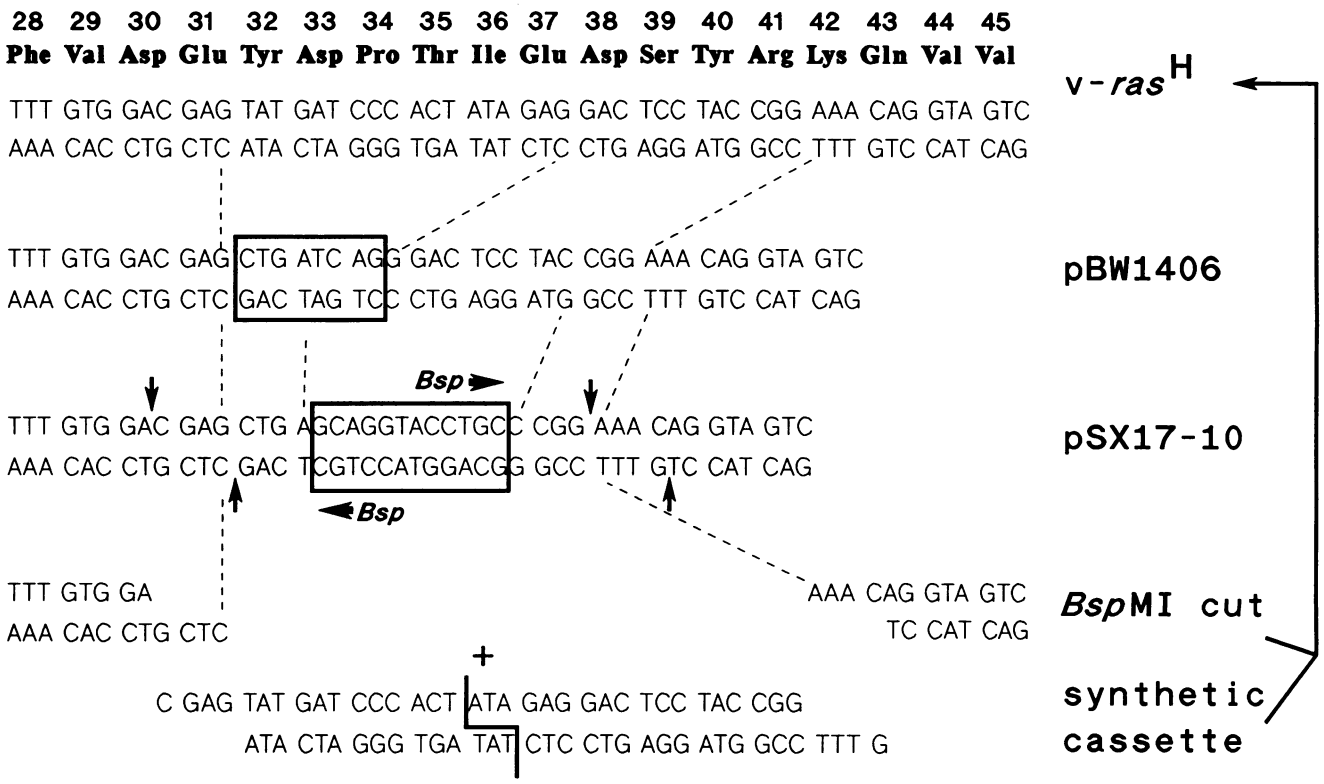

FIG. 1. Scheme of cassette mutagenesis of the region containing codons 32 to 42 of v-*ras*^{H}. The top lines show the DNA sequence of codons 28 to 45 of v-*ras*^{H} and the encoded amino acids. In plasmid pBW1406, whose origin was described previously (26), codons 32 to 37 have been deleted, and a *Bcl*I linker has been inserted (boxed area). Plasmid pSX17-10, which lacks codons 32 to 40, contains two *Bsp*MI sites in opposite orientations. It was derived from pBW1406 as described in the text. Each horizontal arrow indicates the side of the recognition sequence on which *Bsp*MI cleaves. The vertical arrows indicate the *Bsp*MI cleavage sites. Following *Bsp*MI digestion, the deleted sequences were reconstructed with a synthetic cassette composed of four single-stranded oligonucleotides that can contain any designated mutation; the wild-type sequence is shown here. The "+" indicates that the oligonucleotide below was ligated to the gap created by *Bsp*MI digestion.

of the linker as well as a few flanking residues. The middle of the linker also forms a *Kpn*I site (GGTACC), which is useful in identifying molecules that contain the linker. The linker can be used for virtually any DNA segment, provided there are no other *Bsp*MI sites in the DNA. The only *Bsp*MI site present in most commonly used bacterial cloning vectors is located in the polylinker of PUC plasmids, between the *Pst*I and *Sal*I sites, which can be readily deleted.

Standard recombinant DNA techniques were used throughout these studies (10). The starting plasmid was pBW1406, which contains a v-*ras*^{H} gene with a *Bcl*I linker in the sequences encoding the effector domain (26) (Fig. 1). In this clone, the v-*ras*^{H} gene is embedded in a murine retroviral vector that contains a selectable marker, the neomycin resistance gene (*neo*^{r}) (7). Since this 9.3-kilobase vector contained several *Bsp*MI and *Kpn*I sites that would compromise the mutagenesis strategy, the 843-base-pair *Sac*II-*Xho*I fragment containing v-*ras*^{H} was transferred to a pSV2gpt derivative that contained an irrelevant sequence that provided single *Sac*II and *Xho*I sites (data not shown). The resulting 4.4-kilobase plasmid, pSXras, has neither *Bsp*MI nor *Kpn*I sites. To position the *Bsp*MI linker within the coding region for the effector domain, we linearized pSXras with *Bcl*I and then digested it with the "slow" form of exonuclease *Bal* 31 (International Biotechnologies, Inc.) under conditions designed to remove 2 to 16 base pairs from either end. After phenol extraction and ethanol precipitation, the plasmid ends were made flush with the Klenow form of DNA polymerase.

To ensure that single copies of the *Bsp*MI linker were introduced, we ligated the unphosphorylated form of the *Bsp*MI linker and recircularized the plasmids by the linker-tailing method (8). After introduction into *Escherichia coli*, modified plasmids that contained the linker were readily identified by the presence of the introduced *Kpn*I site. The presence of the *Bsp*MI sites had to be inferred, since the enzyme displayed negligible activity toward the crude DNA preparations. The sequences of several pSXras derivatives were determined by dideoxy sequencing (16) after subcloning of the appropriate fragment into mp18. Two plasmids that contained the desired 5′ and 3′ insertion points of the dimer were recombined by means of the *Kpn*I site within the linker and a unique *Eco*RI site located elsewhere in the plasmids. After confirmation of the sequence of the recombinant, pSX17-10 (Fig. 1), the plasmid was purified by equilibrium banding in CsCl.

The wild-type *ras* sequence was reconstructed in *Bsp*MI-digested pSX17-10 with short oligonucleotides corresponding to the deleted portion of the *ras* gene (Fig. 1). For mutations, two oligonucleotides with appropriate substitutions in the relevant codon were used with two wild-type oligonucleotides. In either case, a mixture containing 1 μg of each oligonucleotide was phosphorylated with T4 polynucleotide kinase. After ethanol precipitation, the mixture of phosphorylated oligonucleotides was dissolved in Tris-EDTA and ligated to 0.2 μg of the 4.4-kilobase *Bsp*MI-digested pSX17-10 fragment at 15°C overnight in a final volume of 10 liters. After transformation into HB101, plas-

TABLE 1. Biologic activity of v-*ras* mutants[a]

ras Structure and substitution	Focus formation	Growth in soft agar
Wild type		
c-*ras*	0.05	+
v-*ras*	1.00	+++++
32-Tyr mutant (Phe)	0.00	−
35-Thr mutant (Ser)	0.00	−
36-Ile mutants		
Val	0.38	++
Leu	0.31	++
Met	0.05	ND
Ala	0.00	−
39-Ser mutant (Cys)	0.95	++++
40-Tyr mutants		
Phe	0.47	+++
Ile	0.00	−
Val	0.00	−
Gly	0.00	−
Ser	0.00	−
Arg	0.00	−

[a] Values for focus formation represent the relative efficiency of focus formation on NIH 3T3 cells as compared with that of the wild type, which yielded 1,000 to 5,000 foci per 0.3 μg of DNA in different assays. ND, Not done. The NIH 3T3 cells and DNA transfection procedure have been previously described (25). Cells were grown in Dulbecco modified minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin, 100 U/ml, and streptomycin, 100 μg/ml). G418 (0.5 mg/ml) was added when resistance to this antibiotic was used to select mutants.

mids from individual colonies which had been restreaked to ensure that only single plasmid types were present were prepared and subjected to DNA sequence analysis. Wild-type and mutant v-*ras*^{H} genes were then excised from the pSX17-10 derivative with *Sac*II and *Xho*I and transferred to the retroviral vector used to construct pBW1406.

Effector domain mutants. The above-described technique was used to construct a series of single-codon mutations in the effector-coding region of v-*ras*^{H} spanning codons 32 to 40 and to reconstruct wild-type v-*ras*^{H} (Table 1). Several mutations were introduced into codons 36 and 40. Only one mutation was introduced into codons 32, 35, and 39. Most of the codon-36 and codon-40 mutations were generated with pairs of mixed oligonucleotides; resulting clones were then selected on the basis of their sequences. The remainder of the mutations were constructed with oligonucleotides that would encode specific amino acid substitutions. Following transfer of mutant v-*ras*^{H} genes to the retroviral vector that carried the selectable marker (*neo*^{r}) (7, 26), the Phe-32 and Thr-35 mutants were verified by resequencing of their effector domain regions.

The biologic activity of the mutants was assessed by testing their ability to induce focal transformation of NIH 3T3 cells (Table 1). Despite the conservative nature of the mutations introduced into codons 32 (tyrosine to phenylalanine) and 35 (threonine to serine), both of these mutants were transformation defective (td) (a mutant whose transforming activity is more than three orders of magnitude lower than that of the wild type will score as td in this assay). By contrast, at codon 39 the substitution of cysteine for the normal serine resulted in a mutant whose transforming activity approached that of the wild type. When the codon-

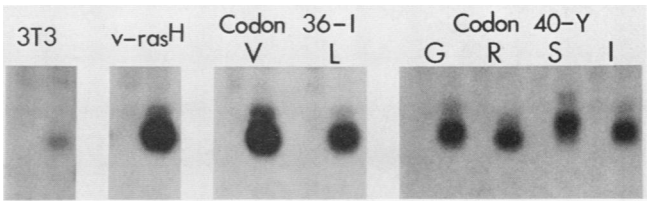

FIG. 2. Immunoprecipitation of mutant p21 proteins from NIH 3T3 cells. Cells transfected with mutant DNAs were selected by geneticin resistance and metabolically labeled with [^{35}S]methionine (250 μCi/ml) as previously described (14). Extracts were immunoprepitated with p21 antibody Y13-238 (5) or no antibody. 3T3, NIH 3T3 control cells transfected with *neo*^{r}; v-ras^H, wild type. For the mutants, the single-letter designations refer to the mutant amino acids that are encoded (I, isoleucine; V, valine; L, leucine; Y, tyrosine; G, glycine; R, arginine; S, serine). Each extract was run in paired lanes; the right lane of each pair contained the p21 antibody, and the left lane contained no antibody. The band seen in the NIH 3T3 cells represents endogenous p21. The p21 proteins seen in the other lanes contain a doublet characteristic of p21$^{v\text{-}ras^H}$.

36 and codon-40 mutants were tested, some retained transforming activity, while others were defective. At both codons, the more drastic amino acid changes led to the loss of biologic activity. The Ile-36 mutants carrying the three most conservative substitutions (leucine, valine, and methionine) were still able to transform the cells but with an efficiency that was significantly lower than that of the wild type; they induced fewer foci, and these foci developed later and grew more slowly than did those induced by the wild type. The less conservative Ala-36 mutant was td, as reported previously (19). In the Tyr-40 mutant, only the most conservative substitution (phenylalanine) allowed biologic activity to be retained; as was true of the transformation-competent Leu-36 and Val-36 mutants, its activity was lower than that of v-*ras*^{H} but higher than that of c-*ras*^{H}. Each of the other Tyr-40 mutants tested lacked biologic activity.

To verify that the mutants encoded stable p21 proteins, we used the presence of *neo*^{r} in the vector to select NIH 3T3 transfectants by growing the cells in geneticin. Geneticin-resistant mass cultures were then metabolically labeled with [^{35}S]methionine, and extracts were immunoprecipitated with a p21-specific monoclonal antibody (5, 14). For each mutant, whether it was biologically active or td, a typical p21$^{v\text{-}ras^H}$ doublet was immunoprecipitated from the cells (representative results are shown in Fig. 2). Metabolic labeling with ^{32}P confirmed that the upper band of the doublet represented the phosphorylated form (data not shown) resulting from autophosphorylation of Thr-59 (reviewed in reference 18).

We also assessed the capacity of most mutants to form colonies in soft agar as an additional test of their biologic activity (Table 1 and Fig. 3). To study this property independently of their ability to induce focal transformation, we selected mass cultures of NIH 3T3 cells transfected with the mutants by using geneticin. As demonstrated previously for other v-*ras*^{H} mutants (26), only those mutants that had been shown to form foci in monolayer cultures grew in agar, and the relative efficiency of the mutants in forming colonies in agar correlated with their ability to induce foci in monolayers.

Significance of the p21 effector domain. In this report, we have studied mutations of a small segment, spanning amino acids 32 to 40, of a highly transforming *ras* gene. This segment, which is conserved among virtually all *ras* genes, has been previously identified as a region of p21 that

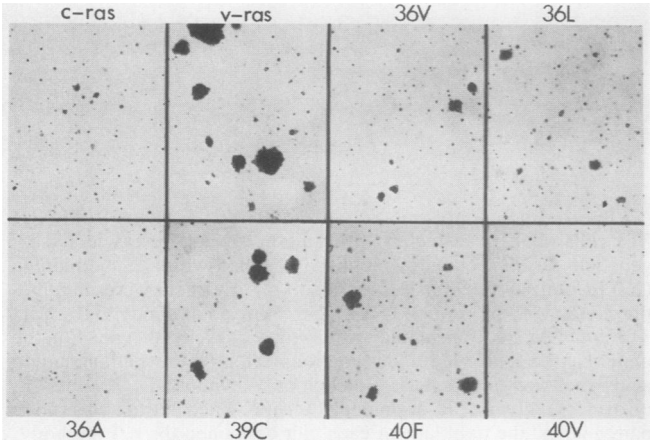

FIG. 3. Growth in soft agar of NIH 3T3 cells transfected with v-*ras*^{H} mutants. Transfected cells were selected with geneticin, and trypsin-dispersed cells from the mass culture were suspended in 0.35% agar and grown in regular growth medium and serum. Shown are representative fields of colonies 18 days after placement in agar. c-ras, Proto-oncogene; v-ras, wild-type viral oncogene. The mutants are listed according to the mutant codons (V, valine; L, leucine; A, alanine; C, cysteine; F, phenylalanine). The Ala-36 and Val-40 mutants did not form colonies; similar results were obtained with a negative control geniticin-resistant line transfected with *neo*^{r} and with the other td mutants listed in Table 1.

probably interacts with the p21 target (19, 26), since td mutations in this region encode stable proteins that retain the known biochemical functions of biologically active p21 protein and fail to stimulate *S. cerevisiae* adenylate cyclase, which probably represents the major RAS target protein in *S. cerevisiae* (4). Further support for this segment representing an effector domain has been recently obtained in *S. cerevisiae*; an *S. cerevisiae* RAS mutant with a lesion in this region was used to map a suppressor mutation to adenylate cyclase (11). It is still possible, however, that mutations in this region affect biological activity via putative conformational changes in other parts of the protein.

Previous results have shown that nonconservative single-amino-acid substitutions in some of the amino acids encoded by codons 32 to 40 result in mutants that are td (19). In the current study, we have determined the effect of making single-amino-acid substitutions that are more conservative. Mutants with various degrees of biologic activity have been identified.

The results demonstrate that there are three functional classes of amino acids within this segment. Some amino acids cannot be altered, even conservatively, without a virtual loss of function. Even the most conservative substitutions at Tyr-32 (phenylalanine) and Thr-35 (serine) abolished transforming activity in our NIH 3T3 cell transformation assay. The Ser-42 mutation in *S. cerevisiae* RAS (analogous to Ser-35 in v-*ras*^{H}) has been reported to possess very low biologic activity in *S. cerevisiae* (11). In other codons, minor amino acid changes were permitted, although with some loss of function. Ile-36 and Tyr-40 mutants with more conservative substitutions (Val-36, Leu-36, Met-36, and Phe-40) retained transforming activity, although their efficiency was reduced. Less conservative mutations at these codons abolished activity. Some amino acids within the segment, such as Ser-39, may be less critical. Ser-39 appeared to tolerate the divergent substitution of Ala-39 without major loss of function, despite the amino acids upstream and downstream having stringent requirements to retain function (19). Consistent with this observation, we found that the Cys-39 mutation had almost no effect on transformation.

The availability of mammalian p21 effector domain mutants with a hierarchy of biologic activities may be useful for the identification and assessment of candidate effector proteins of p21, such as the recently described GTPase-activating protein, which appears to interact with the p21 effector domain (1, 3).

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LITERATURE CITED

1. **Adari, H., D. R. Lowy, B. M. Willumsen, C. J. Der, and F. McCormick.** 1988. Guanosine triphosphatase activating protein (GAP) interacts with the p21 *ras* effector binding domain. Science **240**:518–521.
2. **Barbacid, M.** 1987. ras genes. Annu. Rev. Biochem. **56**:779–827.
3. **Cales, C., J. F. Hancock, C. J. Marshall, and A. Hall.** 1988. The cytoplasmic protein GAP is implicated as the target for regulation by the *ras* gene product. Nature (London) **332**:548–551.
4. **Field, J., D. Broek, R. Kataoka, and M. Wigler.** 1987. Guanine nucleotide activation of, and competition between, RAS proteins from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7**:2128–2133.
5. **Furth, M. E., L. J. Davis, B. Fleurdelys, and E. M. Scolnick.** 1982. Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and the cellular *ras* gene family. J. Virol. **43**:294–304.
6. **Gibbs, J. B., I. S. Sigal, M. Poe, and E. M. Scolnick.** 1984. Intrinsic GTPase activity distinguishes normal and oncogenic ras p21 molecules. Proc. Natl. Acad. Sci. USA **81**:5704–5708.
7. **Jhappan, C., G. F. Vande Woude, and T. S. Robins.** 1986. Transduction of host cellular sequences by a retroviral shuttle vector. J. Virol. **60**:750–753.
8. **Lathe, R., M. Kieny, S. Skory, and J. P. Lecocq.** 1984. Linker tailing: unphosphorylated linker oligonucleotides for joining DNA termini. DNA **3**:173–182.
9. **Lowy, D. R., and B. M. Willumsen.** 1986. The ras gene family. Cancer Surv. **5**:275–289.
10. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Marshall, M. S., J. B. Gibbs, E. M. Scolnick, and I. S. Sigal.** 1988. An adenylate cyclase from *Saccharomyces cerevisiae* that is stimulated by *RAS* proteins with effector mutations. Mol. Cell. Biol. **8**:52–61.
12. **McGrath, J. P., D. J. Capon, D. V. Goeddel, and A. D. Levinson.** 1984. Comparative biochemical properties of normal and activated human ras p21 protein. Nature (London) **310**:644–649.
13. **Mulcahy, L. S., M. R. Smith, and D. W. Stacey.** 1985. Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature (London) **313**:241–243.
14. **Papageorge, A. G., D. R. Lowy, and E. M. Scolnick.** 1982. Comparative biochemical properties of p21 *ras* molecules coded for by viral and cellular *ras* genes. J. Virol. **44**:509–519.
15. **Reddy, E. P., R. K. Reynolds, E. Santos, and M. Barbacid.** 1982. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature (London) **300**:149–152.
16. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
17. **Satoh, T., S. Nakamura, and Y. Kaziro.** 1987. Induction of neurite formation in PC12 cells by microinjection of proto-oncogenic Ha-*ras* protein preincubated with guanosine-5′-*O*-(3-thiotriphosphate). Mol. Cell. Biol. **7**:4553–4556.
18. **Shih, T. Y., and M. O. Weeks.** 1984. Oncogenes and cancer: p21 *ras* genes. Cancer Invest. **2**:109–123.
19. **Sigal, I. S., J. B. Gibbs, J. S. D'Alonzo, and E. M. Scolnick.** 1986. Identification of effector residues and a neutralizing epi-

tope of Ha-ras-encoded p21. Proc. Natl. Acad. Sci. USA **83:** 4725–4729.

20. **Sweet, R. W., S. Yoroyama, T. Kamata, J. R. Feramisco, M. Rosenberg, and N. Gross.** 1984. The product of ras is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature (London) **311:**273–275.
21. **Tabin, C. J., S. M. Bradley, C. I. Bargmann, R. A. Weinberg, A. G. Papageorge, E. M. Scolnick, R. Dhar, D. R. Lowy, and E. H. Chang.** 1982. Mechanism of action of a human oncogene. Nature (London) **300:**143–149.
22. **Toda, T., I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broaek, S. Cameron, J. Broach, K. Matsumoto, and M. Wigler.** 1985. In yeast *RAS* proteins are controlling elements of adenylate cyclase. Cell **40:**27–36.
23. **Trahey, M., and F. McCormick.** 1987. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science **238:**542–545.
24. **Wells, J. A., M. Vasser, and D. B. Powers.** 1985. Cassette mutagenesis: an efficient method for generation of multiple mutations at defined sites. Gene **34:**315–323.
25. **Willumsen, B. M., A. Christensen, N. L. Hubbert, A. G. Papageorge, and D. R. Lowy.** 1984. The p21 ras C-terminus is required for transformation and membrane association. Nature (London) **310:**583–586.
26. **Willumsen, B. M., A. G. Papageorge, H.-F. Kung, E. Bekesi, T. Robins, M. Johnsen, W. C. Vass, and D. R. Lowy.** 1986. Mutational analysis of a *ras* catalytic domain. Mol. Cell. Biol. **6:** 2646–2654.