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Analysis of the transforming potential of the human H-*ras* gene by random mutagenesis

(oncogenes/*in vitro* mutagenesis/gene transfer)

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ABSTRACT **Some tumor cells contain mutant *ras* genes that are capable of transforming NIH 3T3 cells. Those genes that have been analyzed arise from the wild-type, non-transforming *ras* genes by mutations producing single amino acid substitutions at position 12 or 61 of the encoded protein. We have performed random bisulfite-induced mutagenesis on the cloned wild-type human H-*ras* gene to find if mutations at other positions can activate the transforming potential of that gene. Most mutations are not activating, but mutations that specify single amino acid substitutions at position 12, 13, 59, or 63 of the encoded protein do activate the transforming potential of the H-*ras* gene. Some, but not all, mutant *ras* proteins show an altered electrophoretic mobility in NaDodSO$_4$/polyacrylamide gels.**

The *ras* genes are members of a highly conserved family of vertebrate genes first detected as the oncogenes of Harvey and Kirsten sarcoma viruses (1). They encode immunologically cross-reactive, guanine nucleotide binding proteins of ≈21,000 daltons (2). There are at least three human *ras* genes, H-, K-, and N-*ras*, which encode four distinct *ras* peptides of 188 or 189 amino acids, identical in sequence for the first 86 positions (3–5). Mutated *ras* genes have been detected in some human tumor cells by the ability of tumor cell DNA to induce tumorigenic transformation in NIH 3T3 cells upon DNA-mediated gene transfer. To date, many activating mutations of *ras* genes from human tumor cells have been analyzed and they all specify amino acid substitutions at position 12 or 61 of the encoded protein (3–11). To determine whether other mutations of the *ras* genes could also activate the transforming potential of the wild-type normal genes, we tested the transforming capacity of randomly mutagenized wild-type H-*ras* genes.

MATERIALS AND METHODS

Cells, Transfections, and Immunoprecipitations. NIH 3T3 cells were cultured and transfected as described (12). Morphologically altered foci were scored 18 days after DNA transfer. *Escherichia coli* strains were MM294 (r^-m^+, rec^+) for routine plasmid growths and BD1528 (ung^-) (the gift of D. Shortle) was used only for transfection of DNA after bisulfite mutagenesis. *E. coli* transfections were performed as described (13). Cell labeling and immunoprecipitations were performed as described (14).

Plasmid DNAs and Sequencing. The plasmid pT24 contained the transforming H-*ras* gene of T24 bladder carcinoma cells, encoding valine in position 12 (8, 15). The plasmid pP3 contained the wild-type human H-*ras* gene, encoding glycine in position 12, cloned initially from human placenta (8). The plasmid pTPT differs from pT24 only in containing the 242-base-pair (bp) *Mst* II/*Xba* I fragment of the wild-type H-*ras* gene contained in pP3. Its map is shown in Fig. 1. It therefore encodes glycine in position 12 and a normal H-*ras* protein. The plasmid EMS9 differs from pT24 in that it lacks any intron after the first coding exon. It was constructed by using the plasmids RC3 and RC6 containing cDNAs of the H-*ras* transcript (15). The protein it encodes also contains valine in position 12. The plasmid EMSH3 differs from EMS9 in that it contains the 34-bp *Hae* II/*Pvu* II fragment of the viral Harvey *ras* gene (16), cloned into the corresponding *Hae* II/*Pvu* II site of plasmid EMS9. It therefore encodes arginine in position 12. Clones of bisulfite-mutagenized pTPT were initially grown in ung^- BD1528 and then transfected into MM294 for large-scale growth. DNA restriction endonuclease fragments from the mutagenized regions were subcloned into the original pTPT plasmid and grown in MM294. Plasmid DNAs for DNA sequencing or transfection of animal cells was prepared by either of two methods. Large-scale individual plasmid DNAs were prepared from one-liter cultures of MM294 by the method of detergent lysis and CsCl/ethidium bromide banding (17). DNAs from ung^- *E. coli* containing mutagenized plasmids were made on 5-ml saturated cultures by previously published methods (18). DNA sequencing was performed according to Maxam and Gilbert (19) by using the strategy published previously (15).

Sodium Bisulfite Mutagenesis of Doubly Gapped, Circular pTPT Molecules. For mutagenesis of the *ras* gene coding regions, supercoiled pTPT DNA (20 μg in a 200-μl reaction volume) was digested to completion with pairs of restriction endonucleases. To mutagenize the region encoding amino acids 1–37, *Eco*RI and *Xba* I were used. To mutagenize the region encoding amino acids 38–110, *Xba* I and *Nco* I were used (see Fig. 1). At the end of the incubation, 20 μl of 0.25 M Na_2EDTA and 13 μl of 4.5 M NaOH were added to each reaction tube. After 5 min at room temperature, 1 ml of distilled water, 20 μl of 30% Ficoll 400, 0.02% bromophenol blue, and 40 mM EDTA were added, and the resulting solution was loaded into a 150 × 2 × 8 mm empty slot of a horizontal-wick 1% agarose gel at room temperature. After complete filling of the slot with distilled water, the gel was covered with SaranWrap and run at 80 V. The buffer system used was 40 mM Tris acetate, pH 7.9/4 mM sodium acetate/1 mM EDTA (TNE buffer). The location of the single strands was determined by ethidium bromide staining. Gel slices containing the fast- and slow-moving strands from the high molecular weight fragment of the above digested DNA were combined, respectively, with gel slices containing the slow and fast moving strands of the high molecular weight fragment of pTPT cleaved with *Bgl* II and *Sal* I, prepared in the same manner. The pairs of gel slices containing comple-

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Abbreviations: kbp, kilobase pair(s); bp, base pair(s).

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mentary DNA strands were electroeluted together in TNE buffer for 12 hr at 80 V. To the resulting solution (≈20 ml), 2 ml of 1 M Tris·HCl (pH 7.5), 2 ml of 5 M NaCl, and 0.2 ml of 0.25 M EDTA were added, and any insoluble material was removed by centrifugation in 50-ml Falcon tubes at 1800 × *g* for 15 min. Circular, partially single-stranded plasmid DNA was formed by immersion of the 50-ml Falcon tubes containing the above described solution in a 1-liter bath that was allowed to warm up to 76°C on a heating plate. After 10 min at this temperature, the plate was switched off and the solution was allowed to cool slowly to room temperature. After addition of 200 μg of tRNA and 2 vol of ethanol, the solution was kept for 12 hr at −80°C and then centrifuged at 25,000 rpm in siliconized polyallomer tubes for 30 min at 4°C in a SW 27 rotor. The plasmid DNA pellet was resuspended in 200 μl of 1.5 mM sodium citrate, pH 7.0/15 mM NaCl and treated with 3 M bisulfite for 10 min at 37°C as described by Shortle and Nathans (20). After ethanol precipitation, mutagenized and gapped DNA was dissolved in 50 μl of 10 mM Tris·HCl, pH 7.5/1 mM EDTA. ung⁻ *E. coli* was then transformed by the gapped and mutagenized DNA. We obtained from 200 to 2000 ampicillin-resistant colonies per μg of DNA. Ampicillin-resistant colonies were handled as described in the text. The extent of bisulfite mutagenesis was monitored both by the loss of restriction endonuclease sites and DNA sequence analysis of randomly selected mutagenized plasmids. By this method we detected no unmutagenized plasmids.

Oligonucleotide Screening of Mutants. Oligonucleotides were synthesized by the modified phosphate triester method (21) using a BIOSEARCH SAM DNA synthesizer and were purified by electrophoresis in a 20% acrylamide/8 M urea gel. Oligonucleotide screening of bacterial colonies containing the mutant plasmids was done as described by Wallace *et al.* (22).

RESULTS

Sodium bisulfite treatment of plasmid DNA containing a single-stranded gap will induce the chemical conversion of deoxycytidine into deoxyuridine residues preferentially in the single-stranded region (20, 23). The extent of this reaction can be controlled by time and bisulfite concentration. After treatment, introduction of the plasmid DNA into ung⁻ *E. coli*, lacking deoxyuridine N-glycosidase, results in gap repair and replication with the final result that some C·G pairs are converted to T·A pairs. We treated in this manner a clone of the human H-*ras* gene that encodes a normal *ras* protein in order to introduce random mutations in coding regions. Single-stranded gaps were introduced as shown in Fig. 1. Two regions were mutagenized separately: region I, 2.3 kilobase pairs (kbp) spanning *Eco*RI to *Xba* I; and region II, 509 bp spanning *Xba* I to *Nco* I. Both coding and noncoding strands were mutagenized. As a result, 25 codons in region I, encoding the first 37 amino acid positions, and 51 codons in region II, encoding amino acid positions 38–110, could be mutagenized, including the 12th but not the 61st codons.

Gapped plasmids were treated so that on average 1 deoxycytidine residue per 30 was altered. After transformation of ung⁻ *E. coli*, several hundred colonies of ampicillin-resistant *E. coli* were individually picked and grouped into pools of 10 colonies each. Plasmid DNA was prepared from each pool of 10 and tested for the ability to induce the typical foci of morphologically altered NIH 3T3 murine fibroblasts that we associate with mutated *ras* genes that have transforming capacity. Plasmid DNAs were then individually tested from pools that were positive. The vast majority of plasmids was incapable of transforming NIH 3T3 cells (see below), from which we conclude that most mutations do not activate the transforming potential of the *ras* genes.

Four of 40 pools of plasmids mutagenized in region I were positive. From these pools, five mutants were found that could efficiently transform NIH 3T3 cells. These five were analyzed further. First, the 242-bp *Mst* II/*Xba* I fragment containing the entire first coding exon was purified from mutants and ligated with the 2.0-kbp *Eco*RI/*Mst* II and 8.5-kbp *Xba* I/*Eco*RI fragments of the H-*ras* gene contained on pTPT (see *Materials and Methods*) to create a chimeric gene. These chimeric genes were also active in the NIH 3T3 transformation assay (see Table 1), indicating that the activating mutation was contained on the 242-bp *Mst* II/*Xba* I fragment. The coding sequences of the mutagenized region were then sequenced. All five mutants encoded proteins with single amino acid substitutions at either position 12 or 13, with serine or aspartic acid replacing glycine.

A similar protocol was followed for mutants of region II. Twenty pools of 10 colonies each were screened and 2 were positive. From these, two individual plasmid clones with transforming activity were identified. The 509-bp *Xba* I/*Nco* I fragment from these was ligated with the 2.3-kb *Eco*RI/*Xba* I and 7.9-kbp *Nco* I/*Eco*RI fragments of the H-*ras* gene contained on pTPT to create chimeric genes. The activity of the resulting chimeras was tested in transfection assays (see Table 1). Finally, the coding sequences contained on the 509-bp *Xba* I/*Nco* I fragment were sequenced. The first mutant with transforming activity we analyzed encoded a protein with a single amino acid substitution: lysine for glutamic acid at position 63. The second mutant encoded a protein with four amino acid substitutions: lysine for glutamic acid at position 49, threonine for alanine at position 59, lysine for glutamic acid at position 62, and histidine for arginine at position 73.

Since the substitution of threonine for alanine in position 59 is also found in the *ras* genes of the Harvey and Kirsten sarcoma viruses, we were interested in determining if this mutation alone sufficed to activate the transforming potential of *ras* genes. To this end, we synthesized an oligonucleotide, T-G-G-C-C-G-G-T-G-G-T-A-T-C-C, which is complementary to the sequence of the mutant encoding a threonine substitution, and used this as a probe to isolate threonine substitution mutants among a larger population of plasmids mutagenized in region II. Eight were isolated. Including the previous threonine mutant, at least four of nine mutants were unique, as judged by the pattern of loss of restriction endonuclease sites in the mutagenized region. Seven of the eight new mutants had transforming activity when tested on NIH 3T3 cells. One of these was sequenced, and it too contained multiple amino acid substitutions: arginine for lysine at position 42, asparagine for aspartic acid at position 47, and methionine for valine at position 81, in addition to threonine for alanine at position 59. However, since seven of eight randomly picked threonine substitution mutants efficiently transformed NIH 3T3 cells and since most mutations do not activate the *ras* gene, we conclude that it is highly probable that substitution of threonine for alanine in position 59 is sufficient to activate the transforming potential of the *ras* genes.

We measured the transforming capacity of our chimeric genes in NIH 3T3 focus assays. For comparison, we utilized the pT24 plasmid specifying valine for glycine in position 12. We also included in this survey an H-*ras* gene we constructed specifying another single amino acid substitution at position 12: arginine for glycine. This was made by recombining *in vitro* the wild-type human H-*ras* gene with the Harvey sarcoma virus *ras* gene that encodes arginine at position 12 (see *Materials and Methods*). The efficiency of transformation of the various mutants varied considerably. The differences we observed between mutants were reproducibly obtained in independent experiments but may not be biologically significant since we have not excluded the existence of additional mutations within introns.

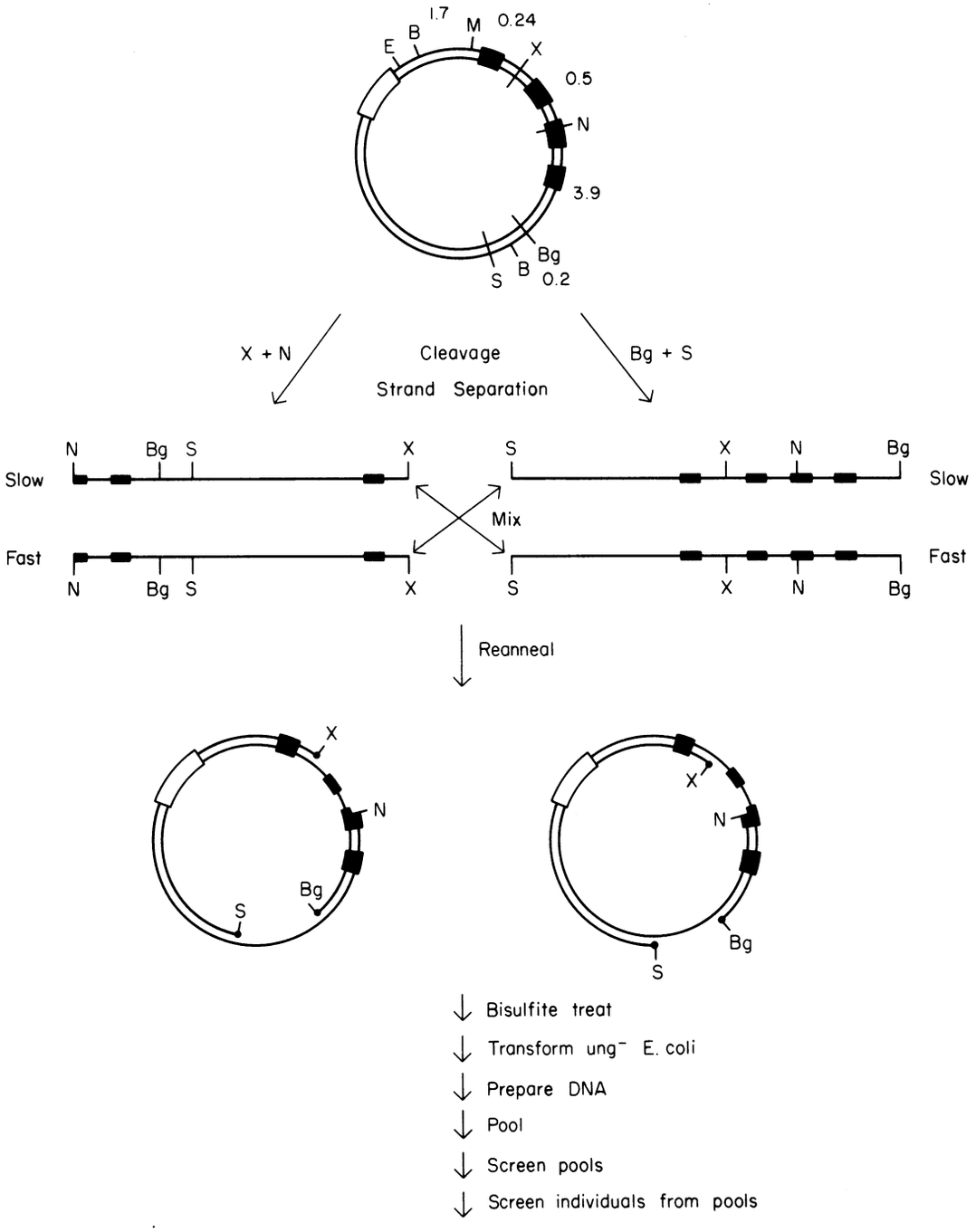

FIG. 1. The scheme for H-*ras* mutagenesis. Shown at the top is the structure of the plasmid pTPT containing a nontransforming human H-*ras* gene as a 6.5-kbp *Bam*HI insert in pBR322. Restriction endonuclease sites shown are *Eco*RI, E; *Bam*HI, B; *Mst* II, M; *Xba* I, X; *Nco* I, N; *Bgl* II, Bg; and *Sal* I, S. The restriction map is complete except for *Mst* II. There is one *Mst* II site only in the 2.0-kbp *Eco*RI/*Xba* I fragment, but there are multiple sites in the 4.6-kbp *Xba* I/*Bam*HI fragment. The above restriction map is not to scale, but distances between restriction endonuclease sites are indicated in kbp. Filled boxes indicate coding exons. The empty box indicates the pBR322 β-lactamase gene. Shown below is the scheme for creating a plasmid with a single-strand gap exposing either strand of region II.

It was of interest to observe the transforming capacity of genes with double mutations. To this end, we recombined genes with mutations in region II with the gene encoding valine at position 12. The resulting double mutants were tested in NIH 3T3 cells (see Table 1). Mutants encoding valine at position 12 and threonine at position 59 had an efficiency of transformation that was intermediate between either parental mutant. By contrast, the mutant gene encoding valine at position 12 and lysine at position 63 had a lower transformation efficiency than either parental mutant gene, suggestive that there may be interference between these two mutations.

Mutant *ras* proteins can have altered electrophoretic mobility. The H-*ras* protein of T24 bladder carcinoma cells, containing a valine substitution at position 12, runs slower than wild-type protein in $NaDodSO_4$/polyacrylamide gels (6), as does the K-*ras* protein of Calu-1 lung carcinoma cells (24), which contains a cysteine substitution at position 12 (4, 5). On the other hand, the N-*ras* protein of SK-N-SH neuroblastoma cells, which contains a lysine substitution in position 61 (3), runs more rapidly than expected (25). To explore whether this is a general property, we examined the electrophoretic mobility of our mutant proteins in $NaDodSO_4$/polyacrylamide gels (see Fig. 2). Like the mutant H-*ras* proteins containing valine (lane b) or cysteine in position 12, the

Table 1. Amino acid changes that activate the transforming potential of the normal H-*ras* gene

Plasmid	Amino acid at positions in the H-*ras*-encoded p21				Relative potency in focus induction
	12	13	59	63	
pP3 (negative control)	Gly	Gly	Ala	Glu	<0.0001
pTPT (negative control)	Gly	Gly	Ala	Glu	<0.0001
pT24 (positive control)	*Val*	Gly	Ala	Glu	1.0
EMSH3	*Arg*	Gly	Ala	Glu	0.6*
BSS176†	*Asp*	Gly	Ala	Glu	0.5
BSS194	*Ser*	Gly	Ala	Glu	0.4
BSS180	Gly	*Asp*	Ala	Glu	0.2
BSC454	Gly	*Asp*	Ala	Glu	0.2
BSS197	Gly	*Ser*	Ala	Glu	0.001
TPO24†	Gly	Gly	Ala	*Lys*	0.1
TTO24†	*Val*	Gly	Ala	*Lys*	0.05
TPO87‡	Gly	Gly	*Thr*	Glu	0.2
TTO87‡	*Val*	Gly	*Thr*	Glu	0.8
THR09§	Gly	Gly	*Thr*	Glu	0.6

Plasmids pP3, pT24, pTPT, and EMSH3 are described in *Materials and Methods*. All others derive from pTPT and contain only point mutations. With the exception of EMSH3 (see footnote *), all transforming potencies are relative to that of pT24 in a standard focus-induction assay. At least two assays were performed on each plasmid. The encoded amino acids that differ from the wild type are shown in italics.

*The potency of EMSH3 is relative to that of the corresponding cloned intronless gene (EMS9) encoding valine in position 12. The potency of EMS9 relative to that of pT24 was about 0.05.

†An additional silent mutation (G-to-A transition) was present in the third position of the codon corresponding to valine in position 7 (BSS176) and glutamic acid in position 76 (TPO24 and TTO24).

‡Additional mutations leading to amino acid change were present in positions 49 (lysine instead of glutamic acid), 62 (lysine instead of glutamic acid), and 73 (histidine instead of arginine).

§Additional amino acid changes were present in positions 42 (arginine instead of lysine), 47 (asparagine instead of aspartic acid), and 81 (methionine instead of valine). The change in position 42 was the result of an A-to-G transition; all the other mutations were G-to-A transitions.

mutant protein containing arginine in this position (lane k) migrates more slowly than wild-type protein (lanes c, h, and m). The mutant protein encoded by Harvey sarcoma viral *ras* containing both arginine in position 12 and threonine in position 59 migrates even more slowly (lane l, lower band; the upper band corresponds to the form phosphorylated on threonine at residue 59). A very minor decrease in electrophoretic mobility is seen also for proteins containing serine in position 12 or 13 (lanes d and e) and possibly for proteins containing aspartic acid in position 13 (lane g), whereas no change is observed for proteins containing aspartic acid in position 12 (lane f). Mutant *ras* proteins with alterations in this region thus either have the same or a decreased electrophoretic mobility compared to wild type. On the other hand, like the N-*ras* protein SK-N-SH, mutant proteins containing a substitution in region II (lysine 63, lane i, or a quadruple mutant including threonine 59, lane j) have an increased electrophoretic mobility.

Both the Harvey and Kirsten sarcoma viral *ras* proteins have a threonine residue at position 59 in place of the alanine residue encoded by wild-type *ras* genes (16, 26). This residue is the site of phosphorylation of the viral proteins (27). In contrast, the wild-type H-*ras* protein is phosphorylated only at very low levels at one or more serine residues (2). We have found that all of the mutant *ras* proteins containing threonine at position 59 are phosphorylated efficiently *in vivo* (data not shown). It is highly probable that this modification occurs at threonine 59 and results from autophosphorylation using GTP as substrate, as has been shown for the viral *ras* proteins (2, 27, 28). We conclude that transforming mutations affecting residue 12 are not required and that threonine 59 alone suffices for efficient phosphorylation of the H-*ras* protein.

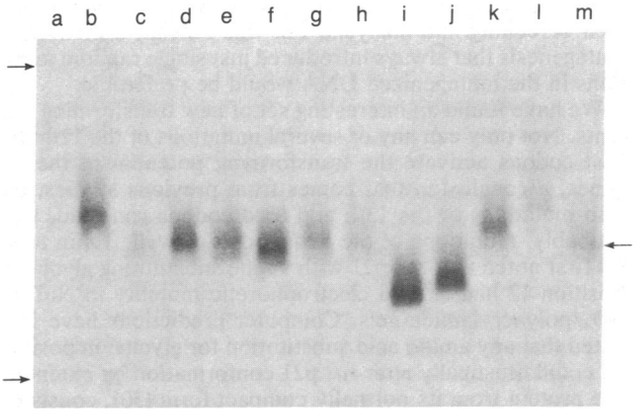

FIG. 2. NIH 3T3 cells transformed with the indicated H-*ras* genes were labeled with [^{35}S]methionine for 16 hr at 37°C and then incubated for 1 hr in complete medium, to permit post-translational processing of labeled polypeptides (14). Cell extracts were immunoprecipitated with anti-*ras* monoclonal antibody Y13-238 (14). Immunoprecipitates (14) were electrophoresed through a 4% polyacrylamide stacking gel and a 30-cm-long 12% polyacrylamide resolving gel as described (32), and radioactive bands were visualized by fluorography. For ease of comparison, samples were approximately normalized for amount of precipitated H-*ras* protein. Lane a, immunoprecipitate from NIH 3T3 cells. Lanes b–k and m, immunoprecipitates from NIH 3T3 cells transfected with human H-*ras* containing the indicated amino acid substitutions. Lanes: b, valine at position 12; c, wild type; d, serine at position 12; e, serine at position 13; f, aspartic acid at position 12; g, aspartic acid at position 13; h, wild type; i, lysine at position 63; j, lysine at position 49, threonine at position 59, lysine at position 62, and histidine at position 73; k, arginine at position 12; m, wild type. Lane l, immunoprecipitate from NIH 3T3 cells transfected with the Harvey sarcoma viral *ras* gene. The arrow on right indicates the position of wild-type human H-*ras* protein. Arrows on left indicate positions of ^{14}C-labeled molecular weight markers (Bethesda Research Laboratories): β-lactoglobulin (M_r 18,400) and α-chymotrypsinogen (M_r 25,700).

DISCUSSION

Transforming genes have been found in RNA tumor viruses and in tumor cells. In general, they derive from normal cellular genes by a variety of complex processes: translocation, transduction, deletion, fusion, and point mutation (29). The clearest examples are the transforming *ras* genes, which differ from normal *ras* genes by a single point mutation resulting in a single amino acid substitution of the encoded protein (3–11).

In our present study we have utilized random bisulfite mutagenesis, a generally applicable procedure, for finding point mutations that can activate the transforming potential of a normal gene. We have not conducted an exhaustive study. We have not, for example, mutagenized the 3′ one-third of the H-*ras* gene, and we have probably not assessed all of the possible bisulfite-inducible mutations even in the regions we did mutagenize. There are intrinsic limits to the bisulfite mutagenesis: not all positions can be mutated and of those that can, only a circumscribed set of nucleotide changes can result. Moreover, recent work from our laboratory (unpublished) indicates that not all cytosine residues in a single-stranded region are equally likely to be modified by bisulfite treatment. Nevertheless, the rate-limiting step in endeavors

of this kind is not the number and variety of mutants but their screening and analysis. For this reason, a method of mutagenesis that always introduced just single random mutations in the mutagenized DNA would be preferable.

We have found an interesting set of new transforming mutants. Not only can any of several mutations of the 12th and 61st codons activate the transforming potential of the *ras* genes, a conclusion that comes from previous studies, but also mutations of the 13th and 63rd codons can, and, very probably, mutations of the 59th codon as well. Tabin *et al.* (6) first noted that *ras* p21 with valine substituting glycine in position 12 had altered electrophoretic mobility in NaDodSO_4/polyacrylamide gels. Computer predictions have indicated that any amino acid substitution for glycine in position 12 could drastically alter *ras* p21 conformation by extending the protein from its normally compact form (30), consistent with the observed shifts in electrophoretic mobility of mutant p21s. Our data indicate that some, but not all, amino acid substitutions at positions 12 and 13 that have drastic effects on *ras* p21 function can significantly alter protein mobility in NaDodSO_4/polyacrylamide gels. Our data also indicate that amino acid substitutions in the region about positions 59–63 also greatly alter p21 mobility. These observations are consistent with the idea that the amino acid substitutions that activate *ras* p21 do so by disrupting protein structure, thereby disrupting some critical protein function.

We argue that the region about positions 59–63 neighbors the GTP binding site of the *ras* proteins, since when threonine is encoded in position 59, it is a site for autophosphorylation by GTP (27). Others have argued that region about amino acids 12 and 13 may neighbor the *ras* GTP binding site, since this region has amino acid homology to known nucleotide binding proteins (31). If indeed activating mutations occur near the guanine nucleotide binding site, this binding must be relevant to the physiologic function of the *ras* protein. For example, there may be two conformational isomers of *ras* p21, induced upon binding of GTP and/or GDP, in which only one of the isomers can stimulate cell growth. The high binding affinity of both nucleotides for *ras* p21 is in accord with their role as conformational effectors rather than substrates in the usual sense (2, 27, 28). If this line of thinking is correct, the activating mutations might lead to altered conformational properties of the p21 nucleotide complexes either by disrupting conformational changes induced by interaction with guanine nucleotides or by disrupting the interaction between p21 and another protein that might in turn regulate guanine nucleotide binding.

Although amino acid substitutions at positions 13, 59, and 63 appear to activate the transforming potential of the *ras* protein as assayed on NIH 3T3 cells, to date all mutations of *ras* genes from human tumor cells that have been analyzed specify amino acid substitutions at position 12 or 61 (3–11). This discrepancy may have a biological basis. As a partial explanation, not all mutations may be equally effective at inducing tumorigenic transformation, and our data are consistent with this. For example, the mutant encoding serine in place of glycine at position 13 is particularly weakly transforming. A second problem is raised by the observation that the *ras* genes contained on Harvey and Kirsten sarcoma viruses each have at least two activating mutations. The Harvey sarcoma virus *ras* gene encodes arginine at position 12 and the Kirsten sarcoma virus *ras* gene encodes serine at this position, while both encode threonine at position 59 (16, 26). Yet, the present study indicates that any one of these mutations is sufficient to strongly activate *ras*. We can offer no simple explanation for this at present.

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1. Ellis, R. W., Defeo, D., Shih, T. Y., Gonda, M. A., Young, H. A., Tsuchida, N., Lowy, D. R. & Scolnick, E. M. (1981) *Nature (London)* **292,** 506–511.
2. Papageorge, A., Lowy, D. & Scolnick, E. (1982) *J. Virol.* **44,** 509–519.
3. Taparowsky, E., Shimizu, K., Goldfarb, M. & Wigler, M. (1983) *Cell* **34,** 581–586.
4. Shimizu, K., Birnbaum, D., Ruley, M., Fasano, O., Suard, Y., Edlund, L., Taparowsky, E., Goldfarb, M. & Wigler, M. (1983) *Nature (London)* **304,** 497–500.
5. McGrath, J. P., Capon, D. J., Smith, D. H., Chen, E. Y., Seeburg, P. H., Goeddel, D. V. & Levinson, A. D. (1983) *Nature (London)* **304,** 501–506.
6. Tabin, C., Bradley, S., Bargmann, C., Weinberg, R., Papageroge, A., Scolnick, E., Dhar, R., Lowy, D. & Chang, E. (1982) *Nature (London)* **300,** 143–148.
7. Reddy, E., Reynolds, R., Santos, E. & Barbacid, M. (1982) *Nature (London)* **300,** 149–152.
8. Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M. & Wigler, M. (1982) *Nature (London)* **300,** 762–765.
9. Santos, E., Reddy, E. P., Pulciani, S., Feldman, R. J. & Barbacid, M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5253–5257.
10. Sukumar, S., Notario, V., Martin-Zanca, D. & Barbacid, M. (1983) *Nature (London)* **306,** 658–661.
11. Yuasa, Y., Srivastava, S., Dunn, C., Rhim, J., Ready, E. & Aaronson, S. (1983) *Nature (London)* **303,** 775–779.
12. Perucho, M., Goldfarb, M., Shimizu, K., Lama, C., Fogh, J. & Wigler, M. (1981) *Cell* **27,** 467–476.
13. Dagert, M. & Ehrlich, S. D. (1979) *Gene* **6,** 23–28.
14. Furth, M. E., Davis, L. J., Fleurdelys, B. & Scolnick, E. M. (1982) *J. Virol.* **43,** 294–304.
15. Fasano, O., Taparowsky, E., Fiddes, J., Wigler, M. & Goldfarb, M. (1983) *J. Mol. Appl. Genet.* **2,** 173–180.
16. Dahr, R., Ellis, R. W., Shih, T. Y., Oroszlan, S., Shapiro, B., Maizel, J., Lowy, D. & Scolnick, E. (1982) *Science* **217,** 934–937.
17. Tanaka, T. & Weisblum, B. (1975) *J. Bacteriol.* **121,** 354–362.
18. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7,** 1513–1518.
19. Maxam, A. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–580.
20. Shortle, D. & Nathans, D. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2170–2174.
21. Efimov, V. A., Reverdatto, S. V. & Chakhmakhcheva, O. G. (1982) *Nucleic Acids Res.* **10,** 6675–6694.
22. Wallace, R. B., Schold, M., Johnson, M. J., Dembek, P. & Itakura, K. (1981) *Nucleic Acids Res.* **9,** 3647–3656.
23. Giza, P. E., Schmit, D. M. & Murr, B. L. (1981) *Gene* **15,** 331–342.
24. Der, C. & Cooper, G. (1983) *Cell* **32,** 201–208.
25. Shimizu, K., Goldfarb, M., Suard, Y., Perucho, M., Li, Y., Kamata, T., Feramisco, J., Stavnezer, E., Fogh, J. & Wigler, M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2112–2116.
26. Tsuchida, N., Ryder, T. & Ohtsubo, E. (1982) *Science* **217,** 937–939.
27. Shih, T., Stokes, P., Smythes, G., Dhar, R. & Oroszlan, S. (1982) *J. Biol. Chem.* **257,** 11767–11773.
28. Shih, T. Y., Papageorge, A. G., Stokes, P. E., Weeks, M. O. & Scolnick, E. M. (1980) *Nature (London)* **287,** 686–691.
29. Cooper, G. (1982) *Science* **217,** 801–806.
30. Pincus, M. R., van Renswoude, J., Harford, J. B., Chang, E. H., Carty, R. P. & Klausner, R. D. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5253–5257.
31. Wierenga, R. & Hol, W. (1983) *Nature (London)* **302,** 842–844.
32. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.