ANTICANCER RESEARCH *10:* 1543-1554 (1990)

Expression of the Normal H-*ras*1 Gene can Suppress the Transformed and Tumorigenic Phenotypes Induced by Mutant *ras* Genes

DEMETRIOS A. SPANDIDOS[1,2], MARGARET FRAME[1] and NEIL M. WILKIE[1,3]

[1]*The Beatson Institute for Cancer Research, Garscube Estate, Bearsden, Glasgow G61 1BD, Scotland, U.K.*
[2]*Institute of Biological Research and Biotechnology National,Hellenic Research Foundation,48 Vas. Constantinou Ave., Athens 11635, Greece*
[3]*Division of Hematology and Oncology, Department of Internal Medicine, Ohio State University, James Cancer Hospital, 300 West 10th Avenue, Colombus, Ohio 43210, USA*

Abstract. *The transformed phenotype of rat 208F cells transfected with the T24 H-ras1 oncogene is suppressed by simultaneous or subsequent transfection with the normal H-ras1 gene. The suppressed cells express both the normal and mutant forms of* ras *p21 but the normal form predominates. Rare transformed cells obtained after simultaneous transfection express mainly the T24 p21. Some suppressed cells induce tumours in nude mice after a long lag period and these tumour cell lines have much reduced expression of normal p21. The normal H-ras1 gene also suppresses the transformed phenotype induced by mutant N-ras, albeit less effectively. The tumorigenicity of the EJ bladder carcinoma cell line, which contains only the T24 mutant allele of H-ras1, is also suppressed following transfection with the normal H-ras1 gene. The results suggest that transforming alleles of* ras *genes do not behave in a fully dominant manner and that expression of the normal allele at elevated levels can lead to suppression of the transformed and tumorigenic phenotypes.*

The *ras* gene family encodes a set of gene products which are highly conserved in nature (for a review see ref. 1). In humans three members of the group have been identified, H-*ras*1 and K-*ras*2 (cellular homologues of the Harvey and Kirsten sarcoma viruses) and N-*ras* for which no transduced viral form has yet been identified. *Ras* genes encode guanosine nucleotide binding proteins of approximately 21,000 molecular weight (p21) which have weak intrinsic GTPase activity (2-4). The gene products localise to the inner side of the plasma membrane and show structural similarities to signal transducing G proteins (5, 6).

In a wide variety of experimental and spontaneous neoplasia, single point mutations in the protein coding sequences of *ras* have been identified which lead to 'activation' and the ability to malignantly transform NIH3T3 cells in DNA-mediated gene transfer experiments (for reviews see ref. 7 and 8). Since the recipient NIH3T3 cells contain and express normal *ras* alleles, such experiments have been interpreted as dominance of the activated gene in the determination of the transformed phenotype (for review see ref. 1 and 7). However, transfected cells often show higher levels of expression of the transfected *ras* gene than the resident alleles, either because of the integration of multiple copies or because of altered transcriptional regulation (9, 10). Moreover, many studies on tumor cell lines which express *ras* alleles with codon mutations fail to support a simple dominance model. Thus the EJ bladder carcinoma cell line contains the mutant T24 H-*ras*1 gene but not its normal allele (11), while the A1698 bladder carcinoma and the A2182 lung carcinoma cell lines contain only the mutant allele of K-*ras*2 (12). The SW480 lung carcinoma line contains both mutant and normal K-*ras*2 alleles but expresses only the mutant, while the CaLu1 lung carcinoma line expresses both normal and mutant alleles but the latter at much higher levels (13). In other cell lines, however, the situation is different. Human T-cell ALL lines express both mutant and normal N-*ras* p21 at equal levels (14) and the human fibrosarcoma HT1080 line also expresses both mutant and normal p21 at approximately equal levels (15).

The situation *in vivo* is not clear either. Most studies with human biopsy material are difficult to interpret because of problems in obtaining material uncontaminated by other cell types. The loss of one H-*ras*1 allele has been reported in 30% of primary breast carcinomas (16), while chemically induced murine thymic lymphoma contains only a mutant N-*ras* allele (17). In none of these situations is it known whether loss of an allele is due to an associated loss of an onco-suppressor gene on the same chromosome, or whether loss of the normal allele is itself important.

Thus, the simple dominance of mutant *ras* in tumorigenesis may be questionable. The ratio of expression of normal to mutant alleles may be important, or there may be a

Correspondence to: Prof. D.A. Spandidos, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Ave. Athens 11635, Greece.

Key Words: H-ras 1 gene, suppression of transformation.

0250-7005/90 $2.00+.40

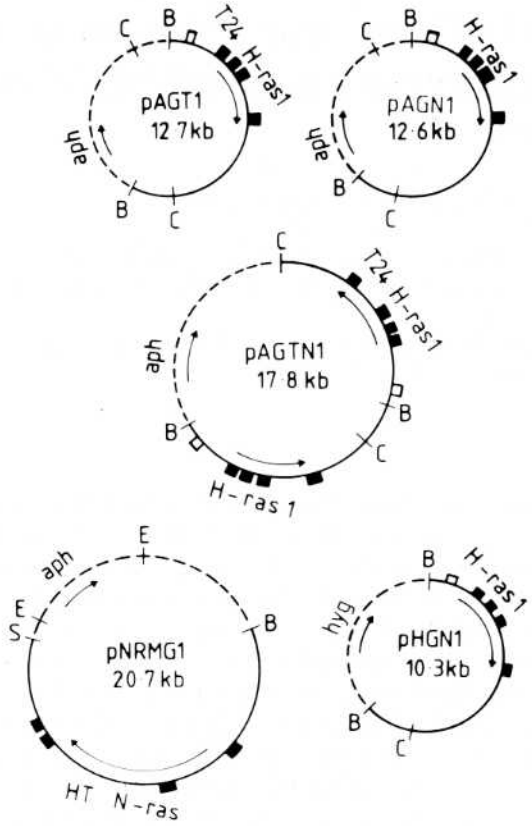


Figure 1. *Schematic representation of* aph *recombinant plasmids carrying* ras *genes. Plasmids pAGT1 and pAGN1 were constructed by inserting the BamHI 6.5 or 6.4 kb DNA fragment carrying the T24 mutant or the normal H-*ras*1 gene respectively into plasmid pAG60 as previously described (9). Both plasmids carry the H-*ras*1 gene in the same orientation as the aminoglycoside phosphotransferase* (aph) *gene of the pAG60 vector. The* aph *gene is under the trascriptional control of the 5' and 3' signals of the Herpes simplex virus thymidine kinase gene. Plasmid pAGTN1 was constructed as follows: the 6.5 kb BamHI DNA fragment carrying the T24 H-*ras*1 gene was inserted into the BamHI sites of plasmid pAG60 in the opposite orientation compared to the* aph *gene to obtain plasmid pAGT2. This plasmid was digested with ClaI and self-ligated to remove the 1.3 kb ClaI fragment containing one of the two BamHI site of plasmid pAG60 in the opposite orientation compared to the* aph *gene to obtain plasmid pAGT2. This plasmid was digested with ClaI and self-ligated to remove the 1.3 kb ClaI fragment containing one of the two BamHI sites and to obtain plasmid pCGT2. The 6.4 kb BamHI DNA fragment carrying the normal H-*ras*1 gene was then inserted into the single BamHI site of plasmid pCGT2 at the 5' end of the T24 H-*ras*1 gene to obtain plasmid pAGTN1. Plasmid pAGTN1 carries the normal H-*ras*1 gene in the same orientation to the T24 H-*ras*1 gene. Construction of plasmid pNRMG1 carrying the HT1080 N-*ras *gene has been desctribed previously (19). Briefly, a 2.9 kb EcoRI fragment carrying the* aph *gene under the 5' transcriptional control sequences of the MoMSV LTR and the 3' polyadenylation signal of the HSV-1* tk *gene was inserted into the single EcoRI site of plasmid pN*ras *HT1080 (20). Plasmid pHGN1 was constructed by inserting the 6.4 kb BamHI fragment containing the normal H-*ras*1 gene into the single BamHI site of plasmid pHMR272 carrying the gene conferring resistance against hygromycin B (21). The maps are not drawn to scale. Closed boxes represent the coding exons and open boxes the 5' non-coding exon of the T24 and normal H-*ras*1 genes. Arrows indicate the transcriptional orientation of the* ras, aph *and* hyg, *genes. B, BamHI; c, ClaI. Continuous line, human DNA; interrupted line, vector DNA.*

gene dosage effect. In the present study we provide evidence that, in rodent cells transformed with activated human *ras* genes, expression of the normal H-*ras*1 gene can suppress the transformed and tumorigenic phenotypes. A preliminary report on these studies has appeared previously (18).

Materials and Methods

Plasmids. Plasmids pAGN1, pAGTN1, pNRMG1 and pHGN1 are shown in Figure 1. Plasmids pAGT1 and pAGN1 have been previously described (9). The construction of plasmid pAGTN1, carrying both the mutant T24 and normal H-*ras*1 gene, and plasmids pNRMG1 and pHGN1, carrying the HT1080 N-*ras*1 in a hygromycin vector respectively, are described in the legend of Figure 1.

Cells and gene transfer. The rat 208F cells (22) and their transfectants were grown in Ham's SF12 medium containing 15% FBS. Gene transfer was accompanied by using an electroporation technique (23) as described in the legend of Table I.

Anchorage dependence and tumorigenicity. Growth in semi-solid medium containing methocel was assayed by suspending a known number of cells in Ham's SF12 medium supplemented with 15% FBS and containing 0.9% methocel. Colonies in methocel containing semi-solid medium were counted using the technique of Bol *et al* (24) as follows. At day nine post-plating 1 ml of 1 mg INT (2-(p-iodophenyl)-2-(p-nitrophenyl)-5-phenyl tetrazolium chloride)/ml PBS was added to each plate and incubation continued overnight. The next day the colonies were counted. INT was purchased from Aldrich Chemical Co. It was kept as a solution of 1 mg/ml in PBS at 4°C and was sterilized by autoclaving. The staining of the colonies is based on the capacity of viable cells to reduce colourless tetrazolium salts to water-insoluble red formozan which precipitates inside the cells.

Tumorigenicity was tested by subcutaneous inoculation of $1x10^6$ cells into 1-month old nude mice (MFI-nu, Olac Ltd., England) and the animals were checked daily for the appearance of tumours.

Protein analysis. Metabolic labelling of cells with ^{35}S-methionine and immunoprecipitation of *ras* p21 with monoclonal antibody Y13-259 were carried out as previously described (25). Proteins were separated by one-dimensional SDS-polyacrylamide gel electrophoresis as previously described (25). The acrylamide concentration was 12.5% in the separating gel and 5% in the stacking gel.

For immunoblotting, cell extracts were resolved by discontinuous SDS-PAGE (26) using a 5% polyacrylamide stacking gel and 17% polyacrylamide resolving gel. Proteins were electroblotted onto nitrocellulose membranes (Schleicher and Schuell) as described (27). Membranes were blocked with 3% gelatin and then treated sequentially with Y13-259 anti-p21 rat monoclonal antibody, rabbit and anti-rat IgG and alkaline phosphatase-conjugated goat anti-rabbit IgG. Specific binding to p21 was then detected by treatment with BCIP and NBT as described by the manufacturers (BRL Laboratories).

Control experiments for both immunoprecipitation and immunoblotting showed that an excess of antibody was used in all cases.

End-labelling of oligonucleotide probes and hybridization. The antisense 20 mer oligonucleotides specific for the normal and T24 mutant H-*ras*1 genes (5'-CCCACACCGCCGGCGCCCAC-3' and 5'-CCCACACCGACGGCGCCCAC-3' respectively) were purchased from the MRC Institute of Virology, Glasgow. The oligonucleotides were end-labelled using γ-^{32}P-ATP (5000 Ci/mmol, Amersham, UK) and T4 polynucleotide kinase.

Agarose gel electrophoresis and direct-gel hybridization were carried out as follows. The DNA digests were electrophoresed in a 0.5 cm thick 0.8% agarose gel. The DNA was denatured *in situ* in 0.5M NaOH, 1.0M NaCl (2x20 min at room temperature) and neutralized in 0.5M Tris HCl

Table I. *Electroporation of 208F rat cells with* aph *recombinant plasmids.*

Donor DNA	*ras* gene	Voltage (kv/cm)	No. geneticin resistant colonies per 5×10^5 cells plated	
			Total No. AV±SD	Morphologically altered AV ± SD (%)
pAGT1	T24 H-*ras*1	2	623 ± 31	616 ± 31 (99)
pAGN1	H-*ras*1	2	589 ± 20	0
pAGTN1	T24 H-*ras*1 + H-*ras*1	2	474 ± 44	0
pAGT1	T24 H-*ras*1	4	479 ± 22	451 ± 34 (94)
pAGN1	H-*ras*1	4	473 ± 12	0
pAGTN1	T24 H-*ras*1 + H-*ras*1	4	423 ± 29	5.3 ± 2.5 (1.2)

Electroporation was carried out as previously described (23), using 1 μg of plasmid DNA per 5×10^6 cells. Following electroporation cells were incubated at 37° C in Ham's SF12 medium containing 15% FBS for 24h. Cells were then harvested and replated at a concentration of 1×10^5 cells per 5 ml medium per 25 cm^2 flask. The medium contained geneticin at 0.2 mg per ml and was replaced every 3-4 days. At day 10 after plating, geneticin resistant colonies were scored with the aid of an inverted microscope. To pick up colonies the top of the flask was removed with a heated scalpel. Colonies were picked up after they were isolated from the remaining colonies with a stainless steel ring and treated with trypsin. The data are derived from the results of three experiments each of which used 5 plates.

pH 7.5, 1.0M NaCl (2x20 min at room temperature). Gels were dried onto Whatman 3MM paper. The dried gel was wetted with distilled water to remove the Whatman 3MM paper and sealed in a plastic bag for hybridization. Hybridization was performed in 10 ml 5xSSPE buffer (1xSSPE = 10 mM sodium phosphate pH 7.0, 0.18M NaCl and 1mM EDTA) containing 0.3% sodium dodecylsulfate (SDS) and 10 μg/ml salmon sperm DNA at 55°C for 16h. Hybridized gels were washed as follows: 1. For the normal oligonucleotide antisense probe: twice at RT with 5xTATE (5.0M tetramethyl-ammonium chloride) containing 0.1% SDS and once at 60°C with the same buffer for 30 min. 2. For the T24 mutant oligonucleotide antisense probe: twice at RT with 5xTATE containing 0.1% SDS for 30 min each wash, once at 60°C for 30 min and once at 65°C for 30 min in the above buffer.

DNA dot hybridization analysis was performed as previously described (28) using synthetic oligonucleotides (29) and the hybridization and nitrocellulose washing conditions were as described above.

RNA dot hybridization analysis was performed using the method of White and Bancroft (30). Briefly, $5x10^6$ cells were pelleted by centrifugation, washed twice with PBS, resuspended in 45 μl of ice-cold 10mM Tris pH7.1, 1 mM EDTA and lysed by the addition of two 5 μl aliquots of 5% Nonidet P40. Nuclei were pelleted by centrifugation for 5 mm in eppendorf tubes and to the supernatant were added 30 ul of 20xSSC (1 x SSC = 0.15M NaCl, 0.015M trisodium citrate) and 20 μl of 37% (w/w) formaldehyde. This mixture was incubated at 60°C for 15 min and stored at −70°C. For analysis 20 μl of each sample were suitably diluted in 15xSSC, and 100 μl of each dilution were applied on a nitrocellulose sheet using a hybridot equipment. The nitrocellulose sheet was baked at 80°C for 90 min and prehybridized and hybridized as above. Filters were washed twice at RT for 30 min in 6xSSC, twice at 50°C for 30 min and once at 70°C for 15 min in 6xSSC.

Results

The transformed phenotype of 208F cells transfected by mutant and/or normal ras gene. The rat 208F cell is an established line with a normal morphology which grows in an anchorage-dependent manner and is non-tumorigenic in nude mice (8, 22). 208F cells were transfected by electroporation at two different voltage gradients with plasmid DNA containing the bacterial *aph* gene and either the normal or mutant T24 H-*ras*1 genes or with both *ras* genes together in the same molecule (see Figure 1 for schematic representation of the plasmid constructions). After transfection, clones expressing the *aph* gene were selected by growth in medium containing geneticin and the number and morphology of geneticin resistant colonies were determined.

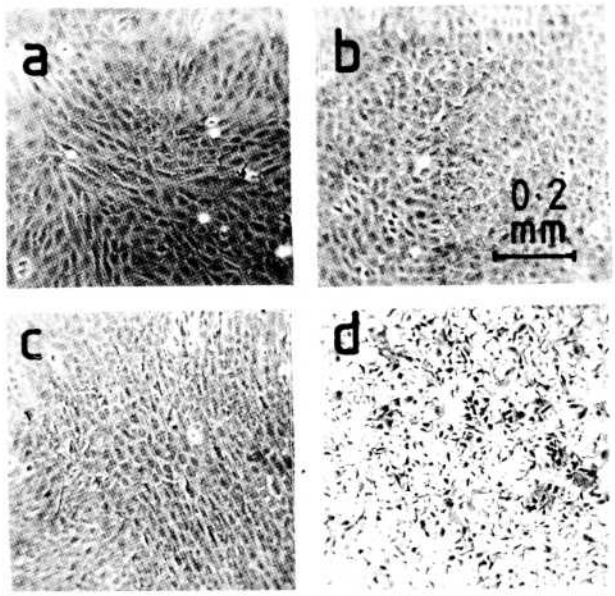


Figure 2. *Normal and transformed 208F rat cells with* aph *recombinants: a, 208F, b, RFAGTN1-1; c, RFAGN-1; d, RFAGT1-1.*

The results are summarized in Table I. As expected, transfection with the normal H-*ras*1 gene resulted in geneticin resistant colonies with a normal morphology, while transfection with the T24 gene resulted in 94-99% of colonies having a morphology characteristic of transformed cells (*eg.* see Figure 2). However, transfection with a plasmid containing both the normal and T24 genes produced geneticin resistant colonies with a normal morphological appearance. Using electroporation at 2kV/cm none of the colonies observed had a transformed appearance, while using 4kV/cm only about 1% of the resistant colonies appeared transformed.

In order to study in more detail the phenotypes of the cells obtained after transfection, individual geneticin resistant colonies were picked and established in liquid culture. Cells from representative colonies were then plated in semi-solid medium to determine anchorage dependence or inoculated sub-cutaneously into nude mice to determine the tumorigenic phenotype. The results are shown in Table II. As expected, the original 208F line was neither anchorage independent nor tumorigenic. A representative line obtained by transfection of the *aph* plasmid without any *ras* genes (line RFAG0-1) was

Table II. *Anchorage independence and tumorigenicity of rat 208F cells transfected with* aph *recombinants carrying* ras *genes.*

Cells	Donor DNA	*ras* genes	Anchorage independence (colonies/10^4 cells plated)[a] AV ± SD	Tumorigenicity (No. of mice with tumors /total No.)[b]
208F	–	–	0	0/5
RFAG60-1	pAG60	–	0	0/4
RFAGT1-1	pAGT1	T24 H-ras1	70 ± 13×10^2	10/10
RFAGN1-1	pGN1	H-ras1	0	0/8
RFAGTN1-1F	pAGTN1	T24 H-ras1 + H-ras1	11 ± 4.7	2/10*
RFAGTN1-2F	"	"	30 ± 9.5	3/8*
RFAGTN1-3F	"	"	7 ± 3.0	1/5*
RFAGTN1-4F	"	"	0	0/4
RFAGTN1-5F	"	"	10 ± 3.3	1/4*
RFAGTN1-6F	"	"	0	0/4
RFAGTN1-7F	"	"	16 ± 5.3	1/4*
RFAGTN1-8F	"	"	2.3 ± 1.9	0/4
RFAGTN1-15R	"	"	67 ± 9.4×10^2	5/5
RFAGTN1-16R	"	"	31 ± 11×10^2	3/3

a. Cells were plated in medium containing 0.9% methocel. The data are derived from the results of 3 experiments each of which used 2 plates at the appropriate cell dilution.

b. Tumorigenicity was tested by subcutaneous inoculation of 1×10^6 cells into 1-month old nude mice (MFI-nu, Olac Ltd., England). 1 cm diameter tumors appeared within 2 weeks with the RFAGT1-1, RFAGTN1-15R and RFAGTN1-16R cells and at between 1-3 months with the other tumorigenic cell lines (*). No tumors were obtained with the 208F, RFAG60-1 and RFAGN1-1, even after 3 months post inoculation.

also anchorage dependent and non-tumourigenic, as was line RFAGN1-1 obtained by transfection with a plasmid containing the normal H-*ras*1 gene. A representative morphologically-altered line obtained by transfection with the T24 H-*ras*1 oncogene (line RFAGT1-1) was anchorage independent and induced tumours in 10/10 nude mice.

The majority of colonies obtained after transfection with a plasmid containing both the normal and mutant T24 H-*ras*1 gene were flat and morphologically normal and were designated -F. Rare round, morphologically altered cells were designated -R. Eight -F cell lines were examined. Two were completely anchorage dependent and non-tumourigenic (-4F and -6F). The remainder showed a low but detectable degree of anchorage independence compared with 208F cells or RFAGN1, but much lower than transformed line RFAGT1-1. These lines showing some degree of anchorage indepedence also induced tumours in the minority of nude mice inoculated, but only after a long lag period of six to twelve weeks (in contrast to morphologically altered lines obtained with the T24 gene which normally induce tumours in 10-14 days). The two -R colonies tested showed a high degree of anchorage independence and induced tumours in all inoculated animals with a short lag period.

The results indicate that, while transfer of the T24 H-*ras*1 oncogene into 208F cells results in colonies with a marked transformed phenotype, simultaneous transfer of the normal gene with the mutant gene results in cells in which the transformed phenotype is strongly suppressed.

Table III. *Electroporation of 208F, RFAGT1-1, -2, -3 and RFNRMG1-1 cells with* hyg *recombinant plasmids.*

Recipient Cells (Exogenous *ras* gene)	Donor DNA (carrying *ras* gene)	No. of hygromycin resistant colonies / 5 ± 10^4 cells plated AV ± SD	
		Liquid medium (Morphologically altered) (%)	Semi-solid medium
208F	pHMR 272	35 ± 6.5 (0) (0)	0
208F	pHGN1 (H-ras1)	34 ± 7.0 (0) (0)	0
RFAGT1-1 (T24 H-ras1)	pHMR 272	45 ± 9.7 (42 ± 9.4) (93)	40 ± 9.4
RFAGT1-1 (T24 H-ras1)	pHGN1 (H-ras1)	46 ± 6.8 (3.7 ± 1.4) (8.0)	3.3 ± 2.0
RFAGT1-2 (T24 H-ras1)	pHMR 272	47 ± 8.9 (44 ± 8.0) (94)	43 ± 9.1
RFAGT1-2 (T24 H-ras1)	pHGN1 (H-ras1)	45 ± 8.4 (2.7 ± 1.6) (6.0)	2.2 ± 1.2
RFAGT1-3 (T24 H-ras1)	pHMR 272	47 ± 12 (45 ± 12) (96)	43 ± 11
RFAGT1-3 (T24 H-ras1)	pHGN1 (H-ras1)	43 ± 8.7 (2.5 ± 1.0) (5.8)	3.0 ± 1.4
RFNRMG1-1 (HT N-ras)	pHMR 272	38 ± 6.9 (36 ± 6.5) (95)	37 ± 6.6
RFNRMG1-1 (HT N-ras)	pHGN1 (H-ras1)	39 ± 6.7 (12 ± 3.3) (31)	12 ± 4.0

Electroporation was carried out as previously described (23) using 10 μg plasmid DNA per 5×10^6 cells at 2 kV/cm. Following electroporation, 5×10^4 cells were plated per 25 cm^2 flask in 5 mls of liquid medium or per 9 cm diameter bacteriological plate in 20 ml methocel containing semi-solid medium as described in Materials and Methods in the presence of 0.2 mg/ml hygromycin B (from Boehringer). The liquid medium was replaced every 3-4 days. At day 10 after plating, hygromycin resistant colonies were scored with the aid of an inverted microscope. Morphologically altered cells had a round morphology and grew in a disoriented fashion. Colonies in methocel containing semi-solid medium were counted using the technique of Bol *et al* (24) as described in The data are derived from the results of three experiments each of which used 2 plates.

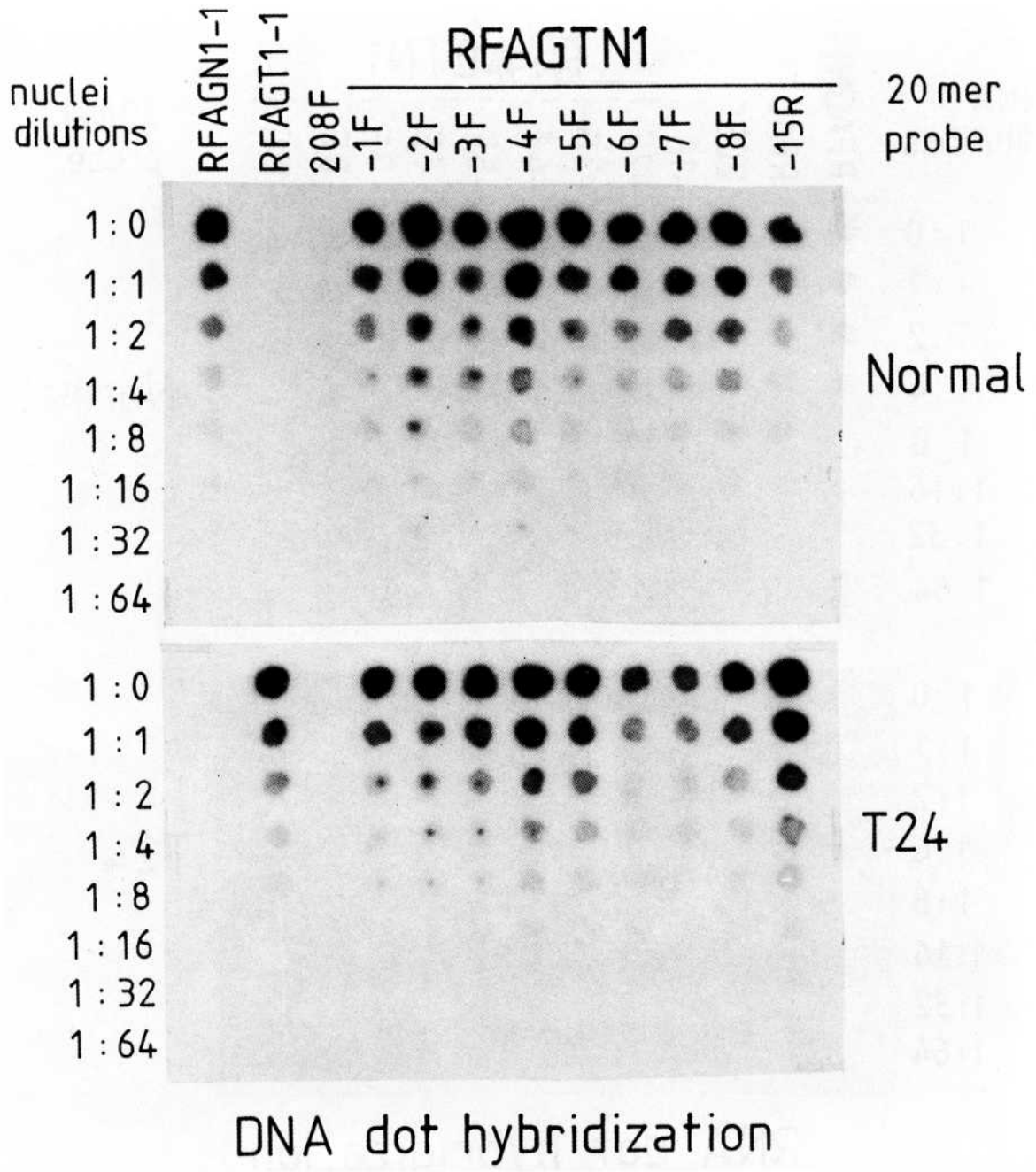


Figure 3. *DNA dot hybridization analysis of transfected rat 208F cells using the oligonucleotide probes for the normal and mutant T24 H-*ras*1 gene.*

It was not necessary to transfer the normal and mutant genes simultaneously to obtain suppression. Introduction of the normal H-*ras*1 gene into cells previously transformed with mutant *ras* genes also resulted in suppression. Independent, moprhologically altered cell lines were established from geneticin resistant colonies obtained by transfection with plasmids containing the T24 gene (lines RFAGT1-1, -2 and -3) or a mutant N-*ras* gene from HT1080 cells (line RFNRMG1-1). The cell lines were transfected with plasmids containing the *hyg* gene conferring resistance to hygromycin, with or without the normal H-*ras*1 gene (see Figure 1 for constructions). 24 hours after transfection the cultures were trypsinised and plated directly into liquid culture or semi-solid medium containing hygromycin.

The results are given in Table III. In each case, introduction of the normal gene into the cells resulted in suppression of the transformed phenotype as judged by the morphology of cells in liquid culture, or anchorage independent growth in methocel. In this experiment the proportion of drug resistant colonies obtained by transfection with the normal gene which still showed a transformed morphology (6-8%) was higher than in the simultaneous transfer experiment shown in Table I, although 2kV/cm was used. In the case of cells transformed originally with the mutant N-*ras* gene, suppression was also

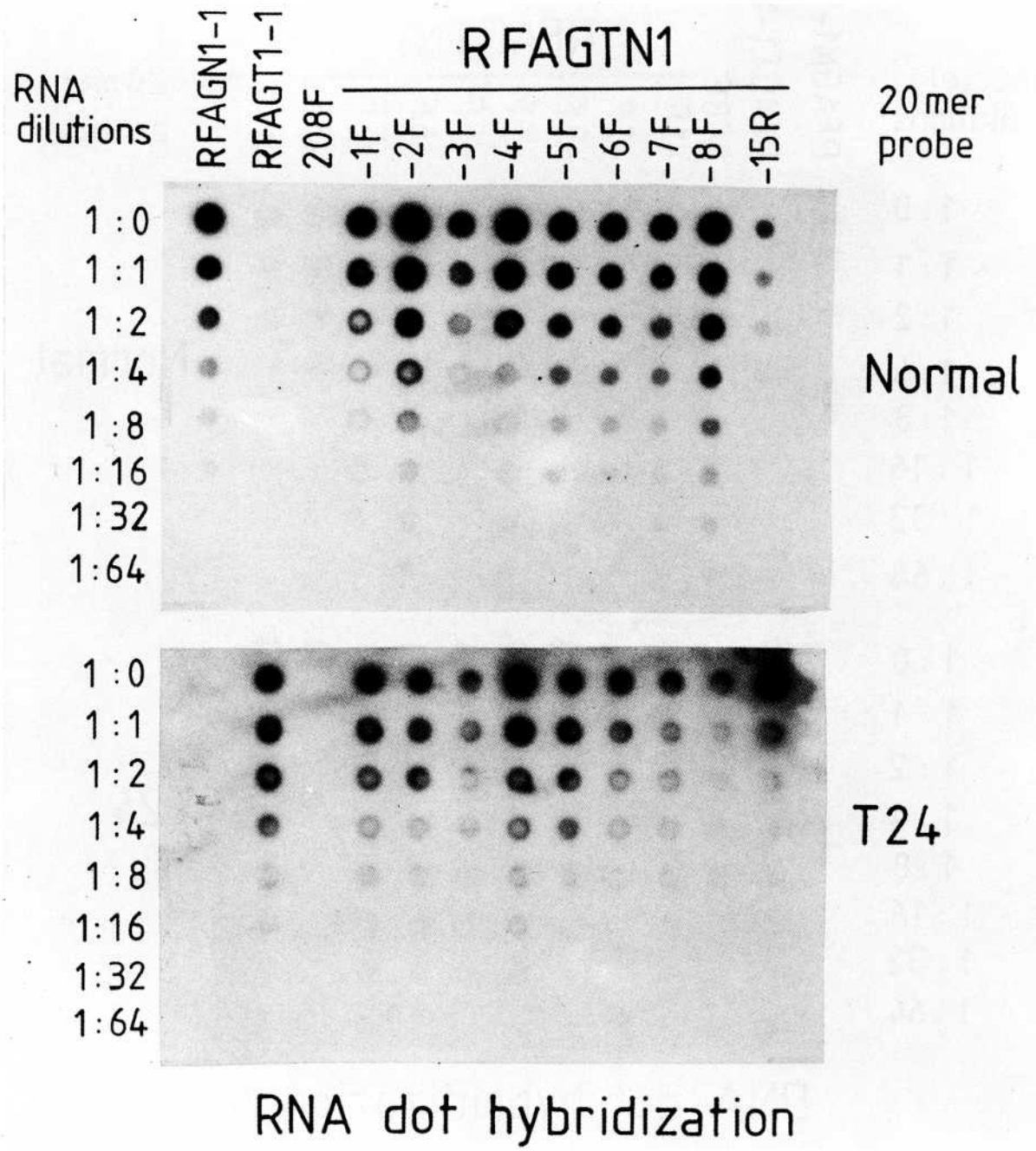


Figure 4. *RNA dot hybridization analysis of transfected rat 208F cells using the oligonucleotide probes for the normal and mutant T24 H-*ras*1 gene.*

obtained but at a noticeably lower level (31%). This indicates that whatever the mechanism, suppression can be obtained across members of the *ras* gene family, albeit apparently less effectively.

Expression of normal and mutant ras genes in transfected cells. To determine whether there was a correlation between the levels of human *ras* gene expression and the transformed and tumorigenic phenotypes, the DNA and RNA contents and p21 expression of individual cell lines were analysed.

DNA content was detected by dot-blot hybridization using synthetic oligonucleotide probes specific for the human normal or mutant H-*ras*1 alleles (Figure 3). As expected, the probes did not detect any DNA in 208F cells, while in cells transfected with either the normal or T24 H-*ras*1 genes only the appropriate DNA was detected. Regardless of morphology, anhcorage independence or tumorigenicity, cell lines obtained after simultaneous transfer of both the normal and T24 H-*ras*1 genes contained both types of DNA. This was confirmed by Southern blot analysis after cleavage of cell DNA with BamH1 which generates different sized fragments from the two genes (data not shown).

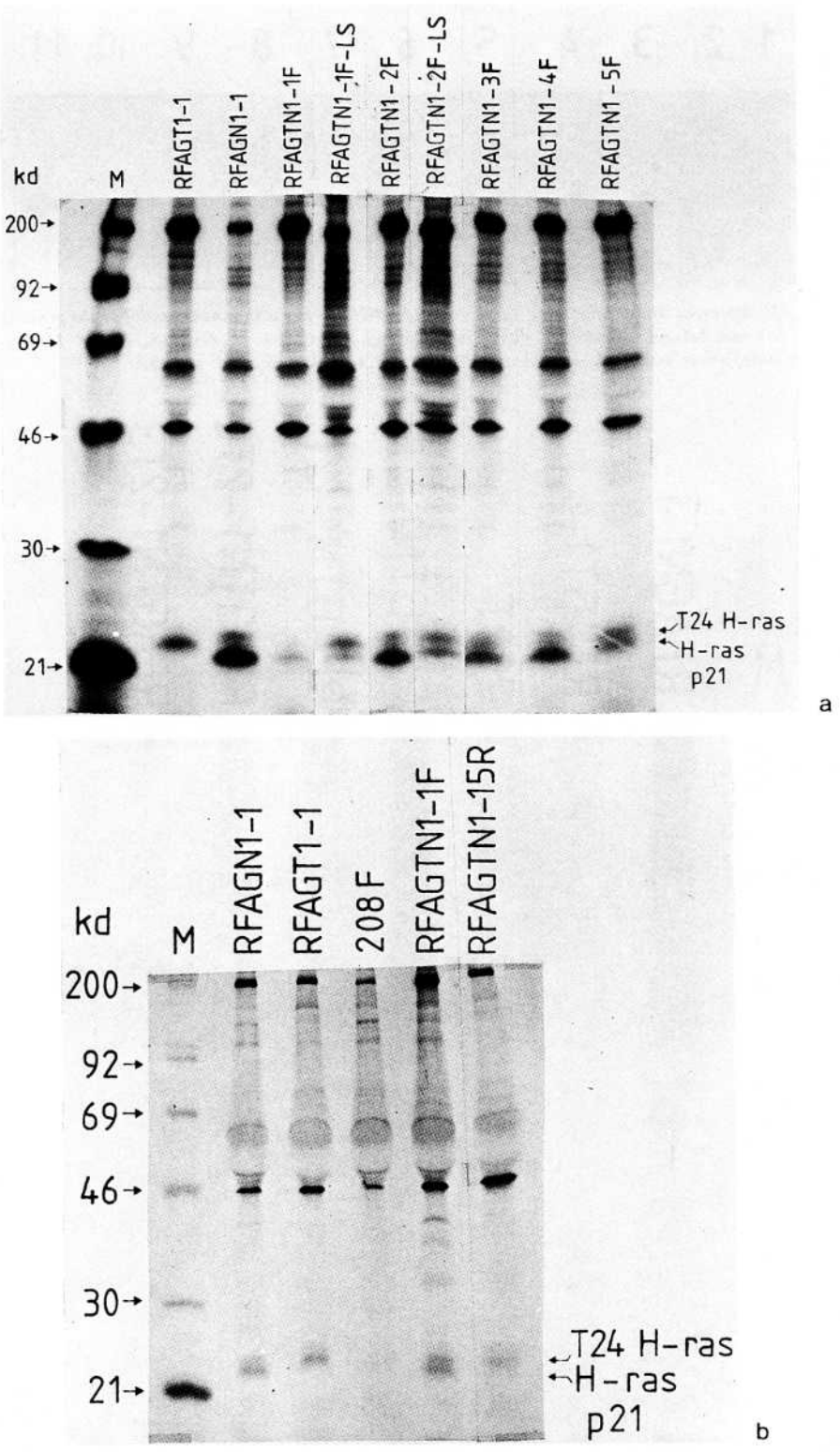


Figure 5. *a. Analysis of p21* ras *oncogene products in 208F cells transfected with plasmids pAGN1 (RFAGN1-1 cells), pAGT1 (RFAGT1-1 cells) and pAGTN1 (RFAGTN1-1F to -5F cells). RFAGTN1-1F-LS and RFAGTN1-2F-LS represent the RFAGTN1-1F and RFAGTN1-2F cells respectively grown for 1 month in medium containing 2% serum. [^{35}S] methionine-labelled cell extracts were immunoprecipitated with anti-p21 monoclonal antibody Y13-259 and analyzed by electrophoresis on 12.5% SDS-polyacrylamide gels as described previously (25). b. Analysis of p21* ras *oncogene products as above in clones RFAGN1-1, RFAGT1-1, RFAGTN1-1F and RFAGTN1-15R.*

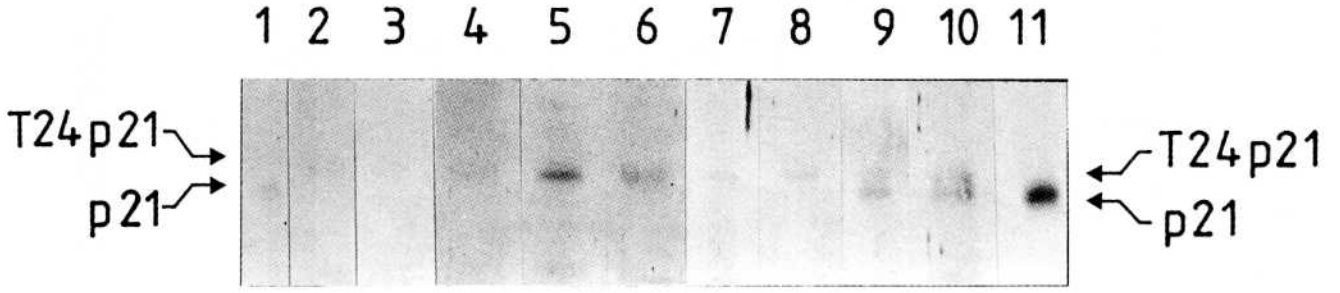


Figure 6. *Immunoblots of p21 expressed in cell lines after transfection of rat 208F cells with normal and T24* ras *genes. Lane 1; cells transfected with normal* ras *only, RFAGN1-1. Lanes 2-8; cells transfected with mutant T24* ras, *RFAGT1-1, -4, -6, -7, -8, -10 and -11. Lanes 9-11; flat morphologically normal cells obtained after transfection with both normal and T24* ras, *RFAGTN1-11F, -13F and -2F.*

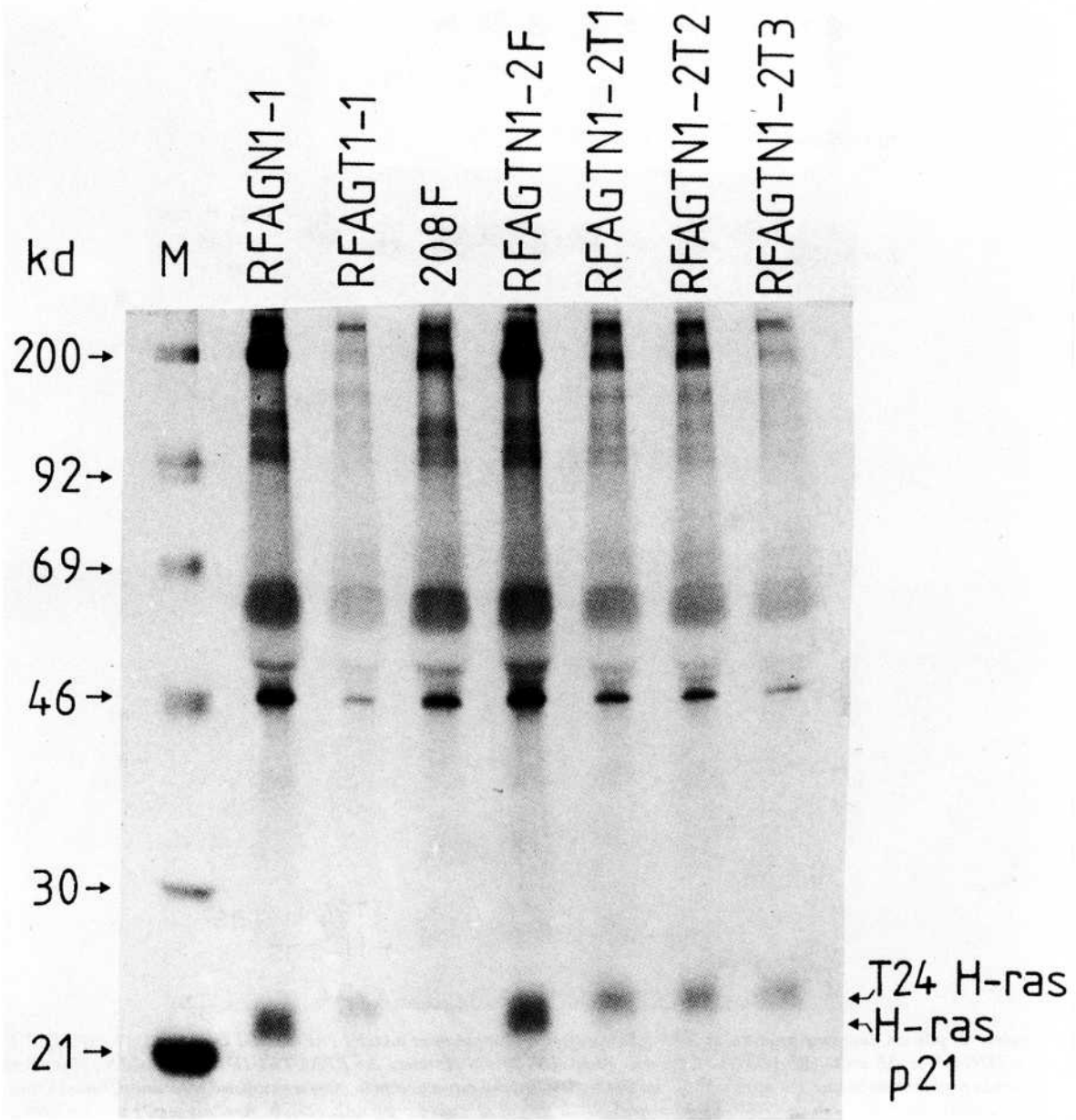
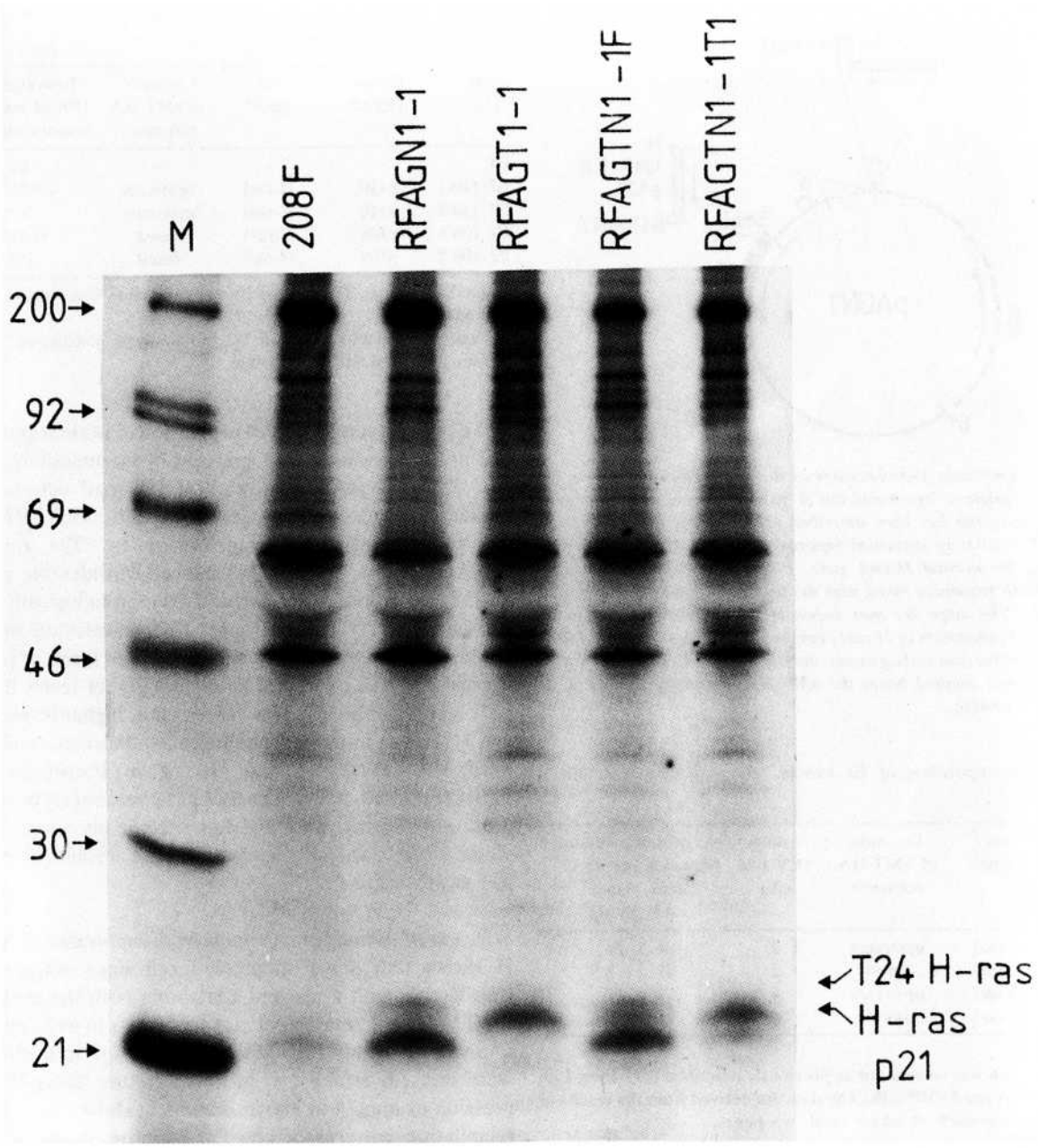


Figure 7. *a. Analysis of p21* ras *oncogene products in 208F cells transfected with plasmids pAGN1 (RFAGN1-1 cells), pAGT1 (RFAGT1-1 cells), and pAGTN1 (RFAGTN1-2F cells). RFAGTN1-2T1, RFAGTN1-2T2 and RFAGTN1-2T3 cells were derived by* in vitro *culture of the tumors from different nude mice induced with the RFAGTN1-2F cells.*



b. Analysis of ras *p21 in cells transfected with plasmid pAGTN1 (RFAGTN1-1F cells) and the RFAGTN1-1T1 (tumor derived) cells after inoculation of RFAGTN1-1 cells into nude mice. [^{35}S] methionine-labelled cell extracts were analyzed as in Figure 5.*

RNA dot blot hybridization was also performed using the same two oligonucleotide probes (Figure 4). Once again the RNA detected was specific for the gene used to transfect the cells, and cells transfected by both the normal and mutant gene contained both types of RNA. Within the -F series of morphologically normal lines, there was some variation in the relative amounts of both the normal and mutant RNA from line to line. In the case of the round transformed line -15R there was noticeably less normal RNA than in the -F series, although the mutant RNA was expressed at a similar level to some -F lines.

Expression of *ras* p21 was examined by immunoprecipitation using the pan-*ras* monoclonal antibody Y13-259 (25) followed by SDS-PAGE electrophoresis which separates the products of the two genes (31). Figure 5 shows that while no p21 was detected in 208F cells using this antibody, cells transfected with only the normal or the mutant genes expressed only the related gene products. Of the five representative flat cell lines obtained after simultaneous transfer of the normal and mutant genes (-1F to 5F), Figure 5a shows that, while there was some variability in the level of p21 from one line to another, all the lines expressed both the normal and

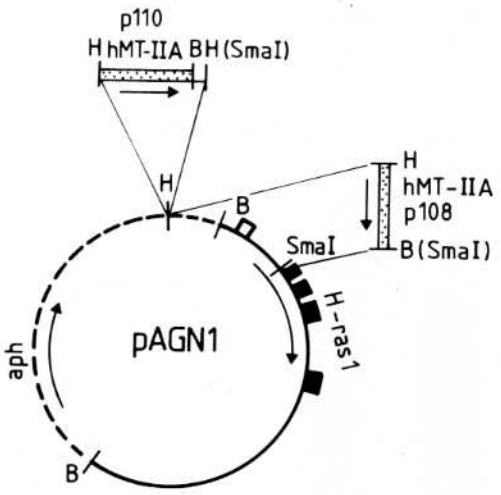


Figure 8. *Recombinant plasmids carrying the human metallothionein IIA (hMT-IIA) regulatory region and the H-ras1 gene. Construction of plasmids p110 and p108 has been described (32). Briefly, p110 carries the hMT-11A 5' regulatory sequences (approximately 0.8 kb of DNA) (33), upstream of the normal H-ras1 gene. Plasmid p108 carries the same metallothionein sequences fused near the first coding exon of the normal H-ras1 gene. The maps are mot drawn to scale. Arrows represent the transcriptional orientation of H-ras1,* aph *and hMT-IIA sequences. Closed boxes represent the four coding exons, open box the non-coding exon of the H-ras1 gene and stippled boxes the hMT-IIA regulatory sequences. B, BamHI; H, HindIII.*

Table IV. *Electroporation of EJ human cells with* aph *recombinant plasmids.*

Donor DNA	*ras* gene	Location of hMT-IIA enhancer	Voltage (KV per cm)	No geneticin resistant colonies per 1×10^5 cells plated[b] AV ± SD
p110	H-*ras*1	upstream	2	84 ± 10
p108	H-*ras*1	fused	2	85 ± 7.5
p110	H-*ras*1	upstream	4	72 ± 11
p108	H-*ras*1	fused	4	71 ± 6.6

[a] See Figure 7.

[b] Electroporation was carried out as previously described (23) using 1 ug of plasmid DNA per 5×10^6 cells. The data are derived from the results of three experiments each of which used two plates.

mutant gene products. In each case there was more normal p21 expressed than T24 p21. The -15R round tranformed line, which expresses much less normal RNA than the -F series, expressed mainly T24 p21, and no normal gene product could be detected (Figure 5b).

Comparing the results of Figure 5 with Table 2, the only apparent correlation is that suppression of the transformed phenotype is observed in cells which express mainly the normal p21. There seems no apparent correlation between the absolute or relative levels of normal or mutant p21 and the intermediate phenotypes of the suppressed cells. Cells expressing only or mainly p21 are completely transformed and tumorigenic.

Table V. *Tumorigenicity of EJ human cells transfected with* aph *recombinants carrying* ras *genes.*

Cells	Donor DNA[a]	*ras* gene[a]	Location of hMT-IIA enhancer[a]	Tumorigenicity (No of mice with tumors/total no)[b]
EJ	–	–	–	8/8
EJ 110-1	p110	H-ras1	upstream	6/10*
EJ 110-2	p110	H-ras1	upstream	1/5*
EJ 108-1	p108	H-ras1	fused	1/10*
EJ 108-2	p108	H-ras1	fused	0/5

a. See Figure 7. b. Tumorigenicity was assayed as described in Materials and Methods.

* Tumors appeared between 1-3 months post inoculation. All other tumors appeared within a month.

The steady state levels of expression of normal and mutant p21 in cell lines were also analysed by immunoblotting using Y13-259 antibody, rabbit anti-rat IgG and detection with alkaline phosphatase-conjugated goat anti-rabbit IgG (Figure 6). Cells malignantly transformed by T24 *ras* alone (RFAGT1-1, -4, -10 and -11) showed considerable variation in p21 expression, as expected. Two morphologically normal cell lines (RFAGTN1-11F and -13F) transfected with both the normal and the T24 *ras* expressed both forms of p21. The normal p21 was expressed at slightly higher levels than T24 p21. Significantly, T24 was expressed at higher levels in -11F and -13F than in the morphologically abnormal, tumorigenic lines RFAGT1-1, -4 and -10 of in transformed lines RFAGT1-2 and -9, in which T24 p21 could barely be detected (data not shown). Thus the tumorigenic phenotype in individual clones does not correlate with the absolute level of T24 p21 expressed.

Selection of tumorigenic variants from suppressed cells. Table II shows that some suppressed cell lines obtained after transfection with a plasmid containing both the normal and the T24 H-*ras*1 genes gave rise to tumors in nude mice after a long lag period. Individual tumors from individual mice were excised, established in liquid culture and p21 *ras* expression examined as above. Figure 7a shows the immunoprecipitation patterns of one flat suppressed line (-2F) and three independent tumour lines derived from it (-2T1, -2T2 and -2T3). It is clear that while the parent -2F expresses more normal p21 than T24 p21, the three tumor lines express predominantly mutant p21. It is also notable that the tumor lines did not express the mutant gene product at detectably higher levels that the flat parent cell line. Figure 7b shows a similar experiment with parent line -1F and tumor line -1T1 with similar results. Apparently acquisition of the tumorigenic phenotype is correlated with suppression of normal p21 expression.

Similar results were obtained by growth of flat suppressed cell lines in low serum. After one month of culture of -1F and -2F cells in 2% serum (splitting the cells 1/8 every three days), the cultures showed a mixed phenotype with some cells

remaining flat and others showing a round transformed morphology. Figure 5a shows that, compared to the parent-F lines, the low serum cells had reduced levels of normal p21.

*Transfection of EJ bladder carcinoma cells with normal H-*ras *1.* The human EJ bladder carcinoma cell line contains and expresses only the T24 mutant form of the H-*ras*1 oncogene. It was of interest to determine whether the tumorigenic phenotype of this line could be suppressed by transfection with the normal gene. *Aph* plasmids were constructed containing the normal gene with its own promoter, the normal gene with a 5′ insertion of the human metallothionine IIA promoter or the normal gene with its own promoter (see Figure 8). The human metallothionine promoter contains a strong enhancer element (38, 33).

EJ cells were transfected with these plasmids and geneticin resistant colonies selected. The number of colonies obtained are shown in Table IV. In this case there are no morphological criteria to assist assessement of the transformed phenotype. Representative colonies were picked, established in liquid culture and the tumorigenic phenotypes determined by inoculation into nude mice. Table V shows that transfection with the normal H-*ras*1 gene under the control of its own promoter led to little or no suppression of the tumorigenic phenotype. There was gene suppression when the metallothionine promoter was inserted 5′ to normal H-*ras*1 but when the gene was placed under the direct control of the metallothionine promoter a marked reduction in the proportion of mice developing tumours was observed and those tumours which arise did so after a delayed lag period.

The experiment strongly suggests that the tumorigenic phenotype of the EJ cell line can be suppressed by expression of the normal H-*ras*1 gene but that in this case the level of gene expression must be elevated using a strong promoter.

Discussion

In a wide variety of tumours cellular *ras* genes are activated to highly transforming forms by point mutation. Detection of such activated forms by DNA-mediated gene transfer into 'normal' cells has led to the concept that activated *ras* behaves dominantly in determining the transformed or tumorigenic phenotype. In contrast, the results presented here provide evidence that in rat 208F and human EJ cells dominance can at best be partial and the level of expression of a normal H-*ras*1 gene contributes to the phenotype.

We have shown that transfer of the normal gene into recipient cells, either simultaneously with or subsequent to transfection with mutant *ras,* suppresses the transformed phenotype. Cells transfected with both the normal and mutant genes contain and express both the normal and mutant RNAs and p21 proteins. Analysis of p21 expression shows that in flat suppressed cells normal p21 is expressed at higher levels than T24 p21, while in a rare round cell which expresses a transformed phenotype the T24 p21 form predominates. Tumour cell lines derived directly from suppressed parent cells show reduced levels of normal p21 and a predominance of T24 p21. Importantly, there is no apparent correlation between the absolute levels of mutant gene expression and the type of transformed phenotype observed. These results strongly imply that the expression of normal H-*ras*1 p21 suppresses the transformed phenotype induced by mutant *ras.*

The experimental situation described above is quite different from cell-hybrid experiments in which the tumorigenic phenotype of tumour cell lines is suppressed by fusion to normal fibroblasts or selection of flat revertants of transformants obtained after transformation with viral oncogenes. In some such experiments there is unambiguous evidence that suppression is achieved without detectable alteration in the level or ratio of normal to mutant p21 (34, 35). In these cases suppression is due to other genetic or epigenetic events perhaps involving specific 'suppressor genes'. We do not suggest that our results throw doubt on the existence of such genes but that in different circumstances suppression can be achieved by different mechanisms. For example, in the studies of Geiser *et al* (35) the tumorigenicity of the EJ cell line was suppressed by fusion to normal fibroblasts. Non-tumorigenic hybrids expressed mainly T24 p21 and the normal p21 was barely detectable. However, in the present study the tumorigenicity of the EJ cells is suppressed by transfection with the normal gene, but only when the *ras* promoter is replaced by the very strong human metallothionine promoter, suggesting the importance of the level of normal p21 in achieving suppression. It seems likely that as yet unknown genetic or epigenetic factors may determine the most readily observed route of suppression.

Recently, Paterson *et al* (15) analysed two non-tumorigenic revertants of the human HT1080 cell line. This line contains two normal alleles of N-*ras* and two mutant alleles. The revertants had lost one mutant allele and underexpressed mutant p21 by about 50%. Since they failed to induce reversion of the HT1080 line by transfection with normal N-*ras* or inoculation of the normal N-*ras* p21, the authors concluded that the absolute level of mutant p21 determines the tumorigenic phenotype and that the ratio of normal/mutant p21 was not important. The reasons for the apparent discrepancy between this study and ours are not obvious, although the experimental conditions are quite different. The conclusion reached by Paterson *et al* cannot be general since the EJ line is suppressed by transfer of the normal H-*ras*1 gene. It is possible that each tumour cell line has different properties and this is underlined by the fact that the HT1080 line expresses normal and mutant N-*ras* p21 at approximately equal levels while the EJ cells express only a mutant allele of H-*ras*1 and that at highly elevated levels.

The mechanism(s) of the suppression described in the present study remains to be determined. There are several possibilities for the level at which suppression might operate. We do not favour the idea that transfection of the normal gene leads to transcriptional interference with the mutant gene, since suppression can be obtained subsequent to establishment of tumorigenic cells by T24 *ras* and also because in the tumorigenic 15R line there are reduced levels of normal

ras rather than elevated levels of mutant *ras*. We cannot exclude an element of translational efficiency or competition, since suppressed cells (which are the most common cells derived from simultaneous transfer) all express more normal than mutant p21 although they were transfected with stoichiometric amounts of each gene. The most likely explanation derives from the studies on p21 expression. Suppressed cells usually express much more normal p21 than T24 p21, whereas rare transformed cells derived directly following double transfection or from selection in nude mice or growth in low serum express more T24 p21 than normal p21. Since the transformed phenotype does not correlate with the absolute level of T24 p21 expression (Figure 6), we suggest that normal p21 can compete with mutant p21 for other specific cellular proteins, sites or substrates.

Ras p21 becomes anchored at the inner side of the cell membrane through lipid modification (36-38) but there is no evidence that the attachment sites are specific. One important function of p21 is in transmitting the proliferative signal triggered by tyrosine kinase growth factor receptors (39, 40), possibly subsequent to altered lipid metabolism (41). Conceivably, competition could be for a receptor protein or metabolites activated during signal transduction. Mutational analyses have identified an effector domain within p21, thought to be the site of interaction with an effector protein during signal transmission (42), which could be a strong candidate for competition.

Recently, Trahey and McCormack (43) have described a cytoplasmic activity (GAP) which accelerates the hydrolysis of GTP bound to normal but not mutant N-*ras*. The biologically active form of p21 is assumed to be GTP bound, by analogy with G proteins (5), leading to the suggestion that mutant *ras* is highly transforming because the p21 cannot be hydrolysed efficiently to the inactive GDP bound form. Unless GAP is itself an effector protein activated by p21 interactions, we suggest it is an unlikely candidate for competition; competition with normal p21 would have the effect of leaving mutant p21 in the active GTP-bound form. Whatever the mechanism, the fact that normal H-*ras* can suppress the phenotype induced by mutant N-*ras* suggests that the putative target can interact with both proteins.

Acknowledgements

We should like to thank Lesley Holmes for technical assistance and Dr John Wyke for critical reading of the manuscript. The Beatson Institute is supported by the Cancer Research Campaign of Great Britain.

References

1 Barbacid M: *ras* genes. Ann Rev Biochem *56:* 779-827, 1987.
2 Gibbs JB, Sigal IS, Poe M and Scolnick EM: Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. Proc Natl Acad Sci USA *81:* 5704-5708, 1984.
3 McGrath JP, Capon DJ, Goeddel DV and Levinson AD: Comparative biochemical properties of normal and activated human *ras* p21 protein. Nature *310:* 644-649.
4 Sweet RW, Yokoyama S, Kamata T, Feramisco JR, Rosenberg M and Gross M: The product of *ras* is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature *311:* 273-275, 1984.
5 Gilman AG: G proteins: Transducers of receptor-generated signals. Ann Rev Biochem *56:* 615-649, 1987.
6 McCormick F, Clark BFC, La Cour TFM, Kjeldgaard M, Norskov-Lauritson L and Nyborg J: A model for the tertiary structure of p21, the product of the *ras* oncogene. Science *230:* 78-82, 1985.
7 Marshall CJ: Human oncogenes. *In:* Weiss RA, Teich NM, Varmus HE and Cofin J (eds), RNA Tumor Viruses. 2nd ed. Cold Spring Harbor Lab. New York, USA. Vol 2, 1985, pp 486-558.
8 Spandidos DA and Anderson MLM: A study of mechanisms of carcinogenesis by gene transfer of oncogenes into mammalian cells. Mutation Res *185:* 271-291, 1987.
9 Spandidos DA and Wilkie NM: Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature *310:* 469-475, 1984.
10 Pulciani S, Santos E, Long LK, Sorrentino V and Barbacid M: *ras* gene amplification and malignant transformation. Mol Cell Biol *5:* 2836-2841, 1985.
11 Feinberg AP, Vogelstein B, Droller MJ, Baylin SB and Nelkin BD: Mutation affecting the 12th amino acid of the c-Ha-*ras* oncogene product occurs infrequently in human cancer. Science *220:* 1175-1177, 1983.
12 Santos E, Martin-Zanca D, Reddy EP, Pierotti MA, Della Porta G and Barbacid M: Malignant activation of a k-*ras* oncogene in lung carcinoma but not in normal tissues of the same patient. Science *223:* 661-664, 1984.
13 Capon DJ, Chen EY, Levinson AD, Seeburg Ph and Goeddel DV: Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. Nature *309:* 33-37, 1983.
14 Shen WP, Aldrich TH, Venta-Perez G, Franza BR Jr and Furth ME: Expression of normal and mutant ras proteins in human acute leukemia. Oncogene *1:* 157-165, 1987.
15 Paterson H, Reeves B, Brown, R Hall A, Furth M, Bos J, Jones P and Marshall C: Activated N-ras controls the transformed phenotype of HT1080 human fibrosarcoma cells. Cell *51:* 803-812, 1987.
16 Theillet C, Liderau C, Liderau R, Escot C, Hutzell P, Brunet M, Gest J, Schlom J and Callahan R: Loss of a c-H-ras1 allele in aggressive human primary breast carcinomas. Cancer Res *46:* 4776-4781.
17 Guerrero I, Villasante A, Corses V and Pellicer A: Loss of the normal N-*ras* allele in a mouse thymic lymphoma induced by a chemical carcinogen. Proc Natl Acad Sci USA *82:* 7810-7814, 1985.
18 Spandidos DA and Wilkie NM: The normal human H-ras1 gene can act as oncosuppressor. Br J Cancer *58:* suppl ix: 67-71, 1988.
19 Spandidos DA: Mechanism of carcinogenesis: the role of oncogenes, transcriptional enhancers and growth factors. Anticancer Res *5:* 485-498, 1985.
20 Brown R, Marshall CJ, Pennie SG and Hall A: Mechanism of activation of an N-*ras* gene in the human fibrosarcoma cell line HT1080. EMBO J *3:* 1321-1326, 1984.
21 Bernard HV, Krammer G and Rowekamp WG: Construction of a fusion gene that confers resistance against hygromycin B to mammalian cells in culture. Exp Cell Res *158:* 237-243, 1985.
22 Quade K: Transformation of mammalian cells by avian myelocytomatosis virus and avian erythroblastosis virus. Virology *98:* 461-465, 1979.
23 Spandidos DA: Electric field mediated-gene transfer (electroporation) into mouse Friend and human K562 erythroleukemic cells. Gene Analysis Techn *40:* 50-56, 1987.
24 Bol S, Van den Engh G and Visser J: A technique for staining hemopoietic colonies in agar cultures. Exp. Hemat *5:* 551-553, 1977.
25 Furth ME, Davis LJ, Fleurdelys B and Scolnick EM: Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular ras gene family. J Virol *43:* 294-304, 1982.
26 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *277:* 680-685, 1970.
27 Tombin H, Staehlin T and Gordon J: Electrophoretic transfer of proteins polyacrylamide gels to nitrocellulose sheets: Proceedures and some applications. Proc Natl Acad Sci USA *76:* 4350-4354, 1979.
28 Spandidos DA, Harrison PR and Paul: Transfer and expression of Herpes simplex virus thymidine kinase and human globin genes in mammalian cells studied by spot hybridization assays. Biosci Rep *1:* 911-920, 1981.
29 Verlaan-de Vries M, Bogaard ME, van den Elst H, Van Boom JH, van der Eb AJ and Bos JL: A dot-blot screening procedure for mutated ras oncogene using synthetic oligodeoxynucleotides. Gene *50:* 313-320, 1986.
30 White B and Bancroft F: Cytoplasmic dot hybridization; simple analysis of relative mRNA levels in multiple small cell on tissue sample. J Biol Chem *257:* 8562-8572, 1982.
31 Srivastava SK, Yuasa Y, Reynold SH and Anderson SA: Effects of two major activity lesions on the structure and conformation of human *ras* oncogene products. Proc Natl Acad Sci USA *82:* 38-42, 1985.
32 Lang JC and Spandidos DA: The structure and function of eukaryotic enhancer elements and their role in oncogenesis. Anticancer Res *6:* 437-450, 1986.
33 Haslinger A and Karin M: Upstream promoter element of the human metallothionein-IIA gene can act like an enhancer element. Proc Natl Acad Sci USA *82:* 8572-8576, 1975.
34 Noda M, Selinger Z, Scolnick EM and Bassin RH: Flat revertants isolated from Kirsten sarcoma virus-transformed cells are resistant to the actions of specific oncogenes. Proc Natl Acad Sci USA *80:* 5602-5606, 1983.
35 Geiser AG, Fer CJ, Marshall CJ and Stanbridge EJ: Suppression of tumorigenicity with continued expression of the c-Ha-*ras* oncogene in EJ bladder carcinoma-human fibroblast hybrid cells. Proc Natl Acad Sci USA *83:* 5209-5213, 1986.
36 Willington MC, Pastan I, Shih TY and Scolnick EM: Localization of the src gene product of the Harvey strain of MSV to plasma membranes of transformed cells by electron microscopic immunocytochemistry. Cell *19:* 1005-1014, 1986.
37 Willumsen BM, Christensen A, Hubbert NL, Papageorge AG and Lowy DR: The p21 *ras* C-terminus is required for transformation and membrane association. Nature *310:* 583-586, 1984.
38 Willumsen BM, Norris K, Papageorge AG, Hubbert NL and Lowy DR: Harvey murine sarcoma virus p21 *ras* protein: biological and biochemical significance of the cysteine nearest the carboxy terminus. EMBO J *3:* 2581-2585, 1984.
39 Mulcahy LS, Smith MR and Stacey DW: Requirement for ras proto-oncogene function during serum-stimulated growth of NIH3T3 cells. Nature *313:* 241-243, 1985.
40 Smith MR, DeGudicibus SJ and Stacy DW: Requirement for c-ras proteins during viral oncogene transformation. Nature *320:* 540-543, 1986.
41 Yu CL, Tsai MH and Stacey DW: Cellular ras activity and phospholipid metabolism. Cell *52:* 63-71, 1988.
42 Sigal IS, Gibbs, JB, D' Alonzo JS, and Scolnick EM: Identification of effector residues and a neutralizing epitope of Ha-*ras*-encoded p21. Proc Natl Acad Sci USA *83:* 4725-4729, 1986.
43 Trahey M and McCormick F: A cytoplasmic protein stimulates normal N-*ras* p21 GTPase, but does not affect oncogenic mutants. Science *238:* 542-545, 1987.

Received September 10, 1990
Accepted October 24, 1990