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

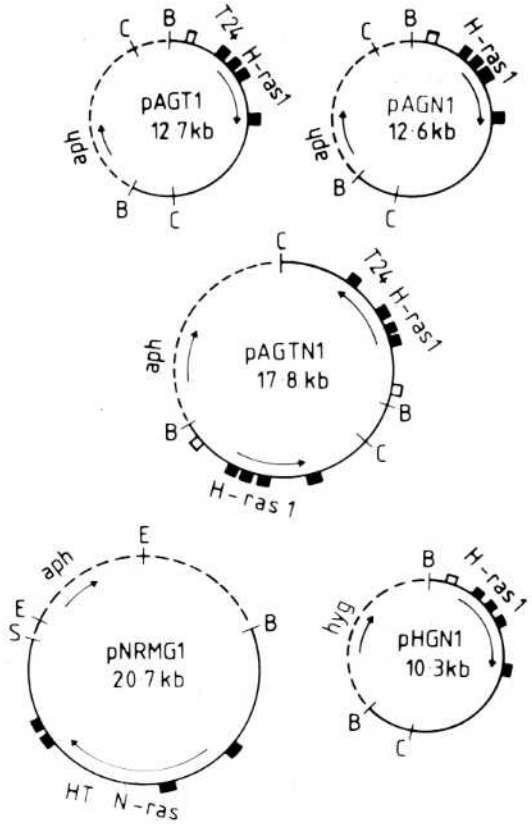

Figure 1. *Schematic representation of* aph *recombinant plasmids carrying* ras *genes. Plasmids pAGT1 and pAGN1 were constructed by inserting the BamHI 6.5 or 6.4 kb DNA fragment carrying the T24 mutant or the normal H-*ras*1 gene respectively into plasmid pAG60 as previously described (9). Both plasmids 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.

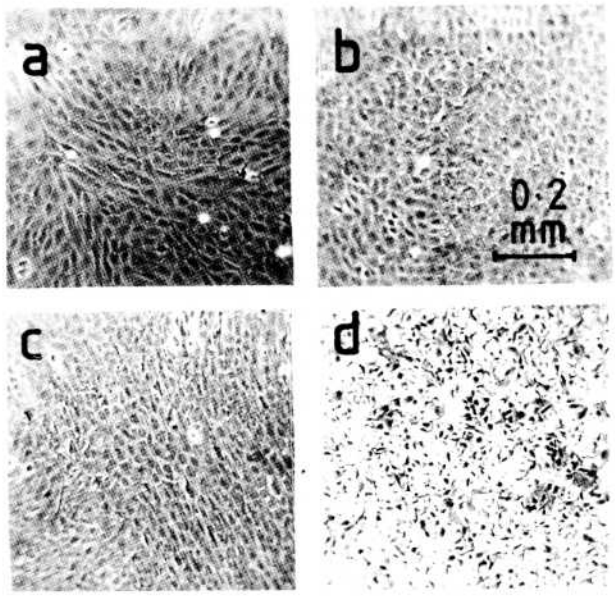

Figure 2. *Normal and transformed 208F rat cells with* aph *recombinants: a, 208F, b, RFAGTN1-1; c, RFAGN-1; d, RFAGT1-1.*

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.

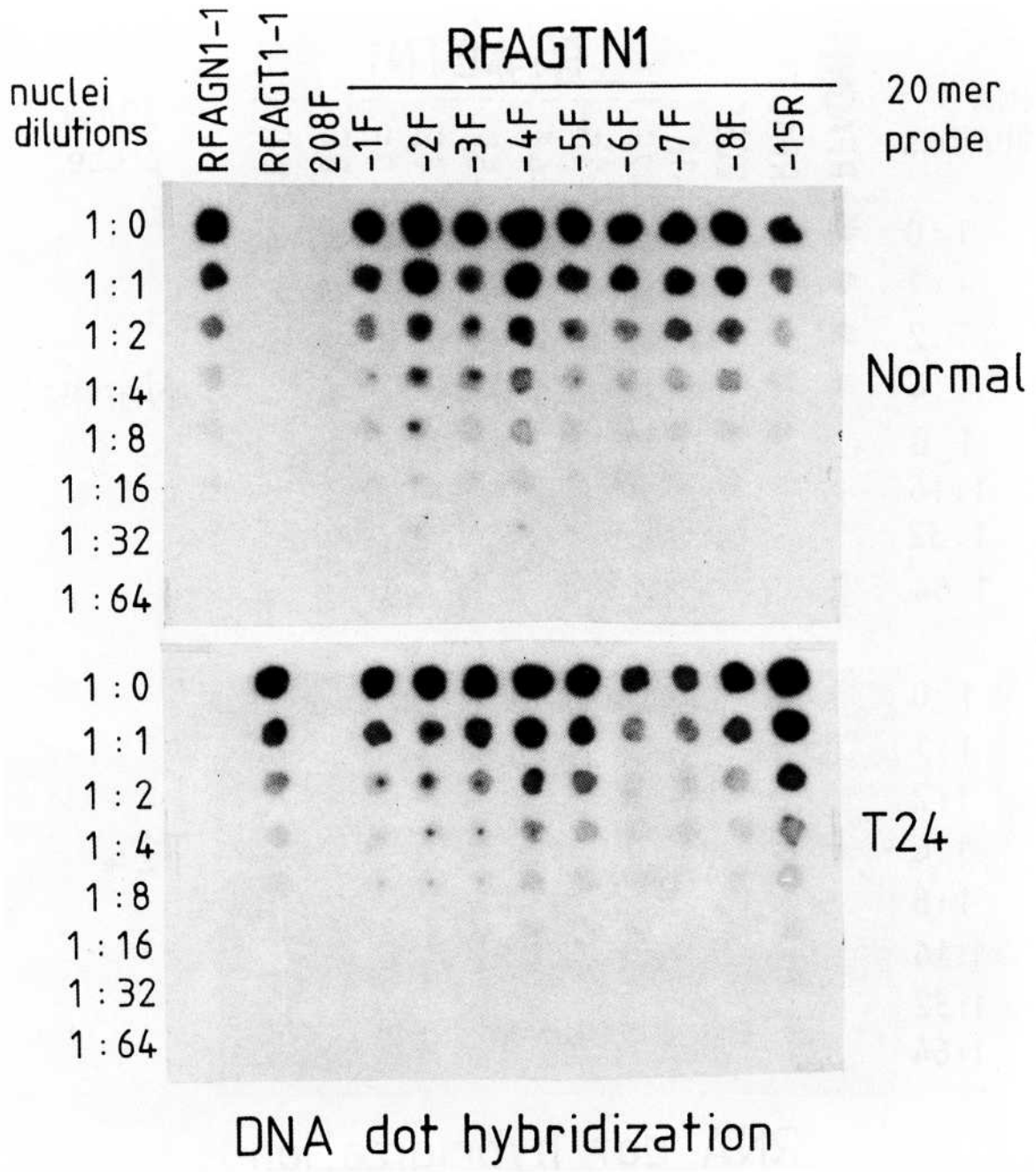

Figure 3. *DNA dot hybridization analysis of transfected rat 208F cells using the oligonucleotide probes for the normal and mutant T24 H-*ras*1 gene.*

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