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The normal human H-*ras*1 gene can act as an onco-suppressor

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Summary The altered morphology and tumorigenic phenotypes of rat 208F fibroblasts transformed with the human T24 H-*ras*1 oncogene is suppressed by transfection with the human normal H-*ras*1 gene. In the suppressed cells, both the normal and mutant T24 *ras* gene products are expressed although the normal p21 is expressed at a higher level. Rare transformants or tumours derived from suppressed cells possess reduced expression of normal *ras* p21. Our findings suggest that transforming *ras* alleles do not behave in a dominant manner and that elevated expression of the normal allele could cause suppression of the morphologically transformed and tumorigenic phenotypes.

Evidence exists for three classes of genes directly involved in tumorigenesis (for a review see Spandidos, 1986). Firstly, oncogenes, which act dominantly at the cellular level, can convert normal cells to transformed or malignant cells. Originally identified by transduction with retroviruses (for a review see Bishop, 1987) and subsequently by DNA-mediated gene transfer (for a review see Spandidos, 1988), this is the best studied group and around forty such genes have been directly identified and isolated. The first human cellular oncogene was cloned in 1982 from T24 bladder carcinoma cells and shown to be homologous to the viral H-*ras* oncogene (Reddy *et al.*, 1982; Tabin *et al.*, 1982; Taparowski *et al.*, 1982). A second class of gene is associated with predisposition to cancer in patients with recessively inherited disorders such as ataxia telangiectasia, Bloom's syndrome and Fanconi's anaemia. Patients with these disorders have defects in DNA repair which indirectly result in increased incidence of malignancy (for a review see Knudson, 1986). So far only one such gene has been identified and cloned, the human excision repair gene ERCC-1 (Van Duin *et al.*, 1986).

The third class of gene comprises a diverse group which share the property that their expression inhibits the cancer phenotype. Their existence was first deduced from studies of cancers associated with heritable single gene traits which are genetically dominant but which behave recessively at the cellular level (Knudson, 1986). Their presence has been particularly well documented in *Drosophilas melanogaster* in which at least 25 recessive genes have been implicated in developmental tumours (for a review see Gateff, 1982). Cell hybrid studies, in which the tumorigenic phenotype of malignant cells is suppressed by fusion with normal cells, has also been used as evidence for suppressor genes (Stanbridge, 1986). Various names have been given for this third class of cancer gene, e.g. anti-oncogenes (Knudson, 1983), tumour-suppressor genes (Stanbridge, 1986) ortho-genes or emero-genes (Todaro, 1986). However, to avoid limiting the term to the suppression of tumourigenicity (other cancer phenotypes such as immortalisation, morphological parameters and metastatic potential are also suppressible), we have suggested the term onco-suppressor gene (Spandidos & Anderson, 1988).

In this report we provide evidence that expression of the normal proto-oncogene product of the H-*ras*1 gene can suppress the transformed and tumorigenic phenotypes of cells transformed with mutant oncogenic *ras* genes. H-*ras*1 can therefore act as either an onco-suppressor gene (this report) or as an oncogene inducing immortalised or tumorigenic phenotypes, depending on the context (Spandidos & Wilkie, 1984).

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Results

Gene transfer experiments

In order to study the effect of introducing different *ras* genes into cells we constructed plasmids containing selectable markers (either the bacterial *aph* gene which confers resistance to geneticin or the bacterial *hyg* gene which confers resistance to hygromycin, or both) and the normal H-*ras*1 proto-oncogene, the mutant T24 H-*ras*1 oncogene and the mutant HT1080 N-*ras* in various combinations. In some constructions normal H-*ras*1 was placed under transcriptional regulation by strong transcriptional enhancers. The constructions are shown schematically in Figure 1.

Initially we studied the phenotypes of rat 208F cells. This is an immortal cell with normal morphology, anchorage-dependent growth and is non-tumourigenic in nude mice. These parameters were determined for the geneticin-resistant colonies obtained by transfection of 208F cells with plasmid DNA containing the mutant T24 H-*ras*1 oncogene, the normal H-*ras*1 gene or both *ras* genes in the same plasmid (Table I). As expected the T24 H-*ras*1 gene induced morphological alterations, anchorage independent growth and tumorigenicity, while normal H-*ras*1 had no apparent effect. However, transfection with plasmid DNA containing both the proto-oncogene and the T24 H-*ras*1 gene resulted in colonies the major proportion of which were morphologically normal, anchorage-dependent and non-tumorigenic. Simultaneous transfer of the normal gene with the T24 H-*ras*1 gene apparently led to suppression of the cancer phenotype normally induced by the T24 gene.

Table II shows the results of experiments in which 208F cells were first transformed by transfection with *aph* plasmids containing either the T24 H-*ras*1 or HT1080 N-*ras* oncogenes, cells from individual geneticin-resistant colonies grown and subsequently transfected with *hyg*-containing plasmids carrying the normal H-*ras*1 gene. Most of the geneticin-resistant colonies obtained in the first round of transfection with either oncogene were morphologically altered and grew in an anchorage-independent manner (data not shown). However the majority of the *hyg*-resistant colonies obtained by transfection with the plasmid containing the normal H-*ras*1 gene were morphologically normal and failed to grow in semi-solid medium. Thus, the normal H-*ras*1 gene can act as an onco-suppressor in cells previously transformed with *ras* oncogenes. Furthermore the suppression can be obtained in cells transformed with an N-*ras* oncogene, suggesting a pan-*ras* effect.

In order to determine whether suppression might also be obtained in human tumour cell lines, we investigated the effect of normal H-*ras*1 on human T24 bladder carcinoma cells. The T24 cell line is tumorigenic in nude mice and contains and expresses only a mutant oncogenic form of H-

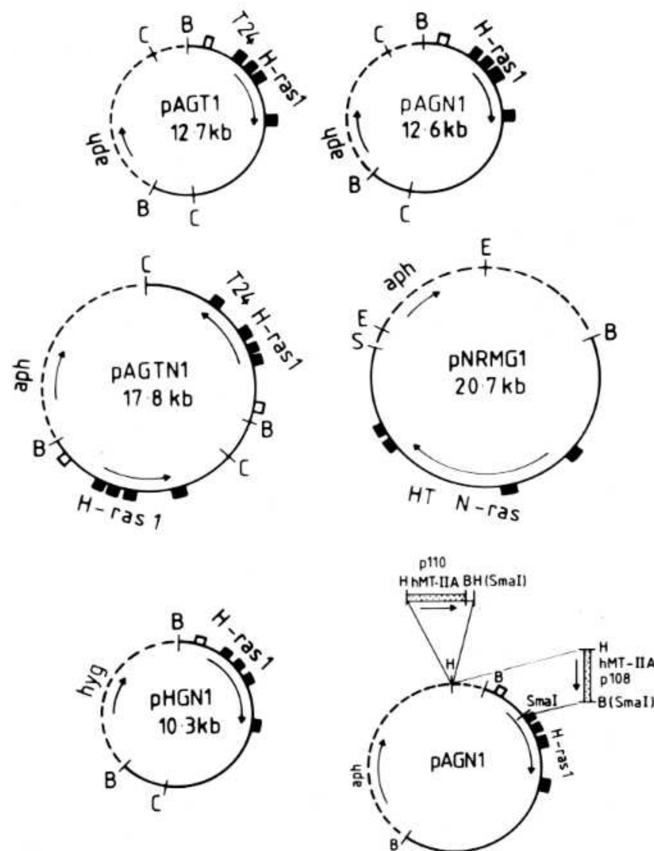

Figure 1 Recombinant plasmids carrying *ras* genes. Plasmids pAGT1 and pAGN1 were constructed by inserting the BamH1 6.5 or 6.4 kb DNA fragment carrying the T24 mutant or the normal H-*ras*1 gene respectively into plasmid pAG60 as previously described (Spandidos & Wilkie, 1984). Plasmid pAGTN1 was constructed as follows: the 6.5 kb BamH1 DNA fragment carrying the T24 H-*ras*1 gene was inserted into the BamH1 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. Construction of plasmid pNRMG1 carrying the HT1080 N-*ras* gene has been described previously (Spandidos, 1985). 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 hygromyan B (Bernard *et al.*, 1985). Construction of plasmids p110 and p108 carrying the human metallothionein IIA (hMT- A) regulatory region has been described (Lang & Spandidos, 1986). Plasmid p110 carries the hM-IIA 5′ regulatory sequences (~0.8 kb of DNA) upstream of the normal H-*ras*1 gene whereas plasmid p108 carries the same metallothionein sequences fused near the first coding exon of the normal H-*ras*1 gene. Plasmids pAGT1, pAGN1, pAGTN1, pNRMG1, p110 and p108 also carry the aminoglycoside phosphotransferase (*aph*) gene as a selectable marker. The maps are not drawn to scale. Closed boxes represent the coding exons, open boxes the 5′ non-coding exon of the T24 and normal H-*ras*1 genes and stipled boxes the hMT- A regulatory sequences. Arrows indicate the transcriptional orientation of the *ras*, *aph*, *hyg* and hMT- A sequences. Continuous line human DNA; interrupted line, vector DNA. B, BamHI, C, ClaI, H, Hind III.

*ras*1, the normal allele being deleted. The T24 cells were transfected with *aph* plasmids containing the normal H-*ras*1 gene under the control of its own promoter, with the human metallothionine promoter inserted 5′ to the gene or with the endogenous *ras* promoter replaced by the metallothionine promoter (see Figure 1). The metallothionine promoter contains a strong enhancer for transcription. Individual geneticin-resistant colonies were picked, cell lines established and tested for tumorigenicity in nude mice. We found little effect of the normal gene under the regulation of its own promoter on the tumorigenicity of T24 cells. However, when the metallothionine promoter was used, a marked suppression of the tumourigenic phenotype was observed, especially when the endogenous promoter was replaced with the metallothionine promoter (data not shown). The experiment shows that normal H-*ras*1 can behave as an onco-suppressor in human cancer cells and further suggests that in this case the level of proto-oncogene expression is crucial for the effect.

Expression of ras *p21 protein in cells*

We measured the level of mutant and normal H-*ras*1 expression in the cells transfected by the various plasmid constructions and selected for drug resistance. Proteins were labelled with ^{35}S-methionine, extracted under native conditions, immunoprecipitated using the anti-*ras* monoclonal antibody Y13-259 and subjected to electrophoretic separation as described (Furth *et al.*, 1982; Srivastava *et al.*, 1985). Figure 2 shows that while no p21 was detected in 208F cells under these conditions, cells transfected with either only the normal or only the mutant gene expressed only the related gene products. Several representative suppressed cell lines obtained after simultaneous transfer of the normal and the mutant genes were also analysed. While there is some variability in the level of p21 from one line to another, all the suppressed cells (flat transfectants RFAGTN1-1F, 3F and -5F) expressed both gene products and in each case there was more normal p21 than mutant p21. Moreover, in the phenotypically round transfectant RFAGTN1-15R the mutant p21 was expressed predominantly. Expression was also measured using RNA hybridisation to labelled ologonucleotide probes specific for the normal and mutant genes with similar results (data not shown).

Analysis of tumorigenic variants

While testing the tumorigenicity of suppressed 208F cells

Table I Phenotypic properties of 208F rat fibroblast cells electroporated with recombinant plasmids carrying *ras* genes.

Cells	*Donor DNA*	ras *genes*	*Cell morphology*	*Anchorage independence (colonies/10⁴ cells plated)*[a] *AV±s.d.*	*Tumorigenicity (no. of mice with tumours/total no.)*[b]
208F	—	—	flat	0	0/5
RFAG60-1	pAG60	—	flat	0	0/4
RFAGT1-1	pAGT1	T24 H-*ras*1	round	$70 \pm 13 \times 10^2$	10/10
RFAGN1-1	pAGN1	H-*ras*1	flat	0	0/8
RFAGTN1-1F	pAGTN1	T24 H-*ras*1 + H-*ras*1	flat	11 ± 4.7	2/10*
RFAGTN1-2F	pAGTN1	T24 H-*ras*1 + H-*ras*1	flat	30 ± 9.5	3/8*
RFAGTN1-3F	pAGTN1	T24 H-*ras*1 + H-*ras*1	flat	7.0 ± 3.0	1/5*
RFAGTN1-4F	pAGTN1	T24 H-*ras*1 + H-*ras*1	flat	0	0/4
RFAGTN1-5F	pAGTN1	T24 H-*ras*1 + H-*ras*1	flat	10 ± 3.3	1/4
RFAGTN1-15R	pAGTN1	T24 H-*ras*1	round	$67 \pm 9.4 \times 10^2$	5/5

[a]Cells were plated in semi-solid Ham's SF12 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 s.c. inoculation of 1×10^6 cells into 1-month old nude mice. One centimetre diameter tumours appeared within 2 weeks with the RFAGT1-1 and RFAGTN1-15R cells and between 1–3 months with the other tumourigenic cell lines (*). No tumours were obtained with the 208F, RFAG60 and RFAGN1-1 at 3 months post inoculation.

Table II Electroporation of 208, RFAGT1-1 and RFNRNG1-1 cells with *hyg* recombinant plasmids.

Recipient cells (exogenous ras *gene)*	*Donor DNA (carrying* ras *gene)*	*No. of hygromycin resistant colonies/ 5×10⁴ cells plated AV+s.d.* — *Liquid medium (morphologically altered (%))*	*Semi-solid medium*
208F	pHMR272	35 ± 6.5 (0) (0)	0
208F	pHGN1 (H-*ras*1)	34 ± 7.0 (0) (0)	0
RFAGT1-1 (T24 H-*ras*1)	pHMR272	45 ± 9.7 (42 ± 9.4) (93)	40 ± 9.4
RFAGT1-1 (T24 H-*ras*1)	pHGN1 (H-*ras*1)	46 ± 6.8 (3.7 ± 1.4) (8.0)	3.3 ± 2.0
RFNRMG1-1 (HT H-*ras*1)	pHMR272	38 ± 6.9 (36 ± 6.5) (95)	37 ± 6.6
RFNRMG1-1 (HT H-*ras*1)	pHGN1 (H-*ras*1)	39 ± 6.7 (12 ± 3.3) (31)	12 ± 4.0

Electroporation was carried out as previously described (Spandidos, 1987). The concentration of DNA was 10 μg plasmid DNA per 5×10^6 cells electroplated at 2 KV/cm. Following electroporation 5×10^4 cells were plated per 25 cm² flask in 5 ml of liquid medium or per 9 cm diameter bacteriological plate in 20 ml methocel containing semi-solid medium in the presence of 0.2 mg ml⁻¹ hygromycin B (from Boehringer). The liquid medium was replaced every 3–4 days. Colonies were scored at day 10 post plating with the aid of an inverted microscope. Morphologically altered cells had a round morphology and grew in a disorientated fashion. Colonies in methocel containing semi-solid medium were counted using the technique of Bol *et al.* (1977) as follows: At day 9 post plating 1 ml of 1 mg ml⁻¹ PBS of INT (2-(P-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride) (Aldrich Chemical Co.) was added to each plate and incubation continued overnight. The next day the red-stained colonies were counted. The data are derived from the results of three experiments each of which used 2 plates. HT=HT1080 cells.

obtained by simultaneous transfer of the normal and mutant H-*ras*1 genes, we observed that in a minor proportion of the cell lines tested, tumours arose in nude mice after a very long lag period. The parent lines giving rise to these late tumours were otherwise morphologically normal and anchorage-dependent. Individual tumours from individual mice were excised and established in liquid culture. These cells gave rise to tumours with short latent periods with 100% incidence. Figure 3 shows the p21 immunoprecipitation patterns obtained with one suppressed parent line (−2F) and three independent tumour lines derived from it (−2T1, −2T2 and −2T3). While the parent line expresses more normal than mutant p21, the three tumour lines express predominantly the mutant T24 form. In addition it is notable that the tumour lines express similar amounts of T24 p21 to the suppressed parent line. Similar results were obtained with other parent and derived tumour cell lines and with tumorigenic cells obtained by growing suppressed cell lines in low serum conditions (data not shown).

These results show that the suppressed phenotype obtained by expression of the normal H-*ras*1 p21 is not stable. Furthermore they strongly suggest that the tumorigenic phenotype may be determined by the ratio of expression of normal to mutant p21.

Discussion

We have shown that the normal H-*ras*1 gene can behave as an onco-suppressor when transferred into recipient cells either simultaneously with or subsequent to transfection with mutant *ras* genes. Analysis of p21 expression shows that suppressed cells express more normal than mutant p21 while tumorigenic variants show reduced levels of normal p21 and a predominance of mutant p21. The results strongly imply that expression of normal H-*ras*1 can suppress the transformed and tumorigenic phenotypes induced by mutant *ras*

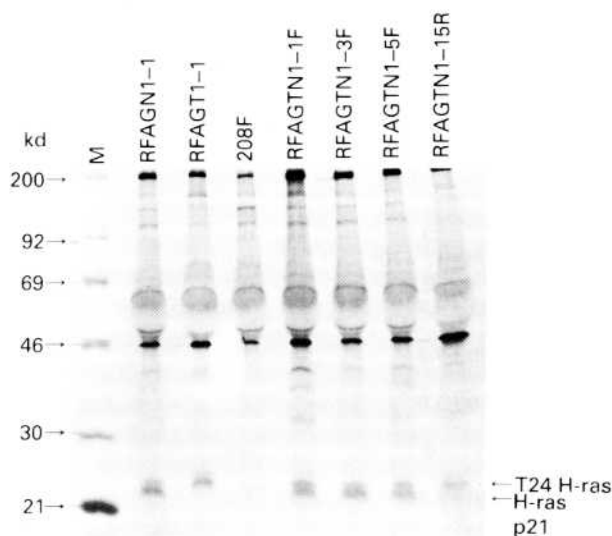

Figure 2 Analysis of p21 *ras* gene products in 208F cells transfected with plasmids pAGN1 (RFAGN1-1 cells), pAGTN (RFAGT1-1 cells) and pAGTN1 (RFAGTN1-1F, -3F, -5F and -15R). [^{35}S] methionine-labelled cell extracts were immunoprecipitated with anti-p21 monoclonal antibody Y13-259 and analyzed by electrophoresis on 12.5% SDS-polyacrylamide gels as described previously (Furth *et al.*, 1982).

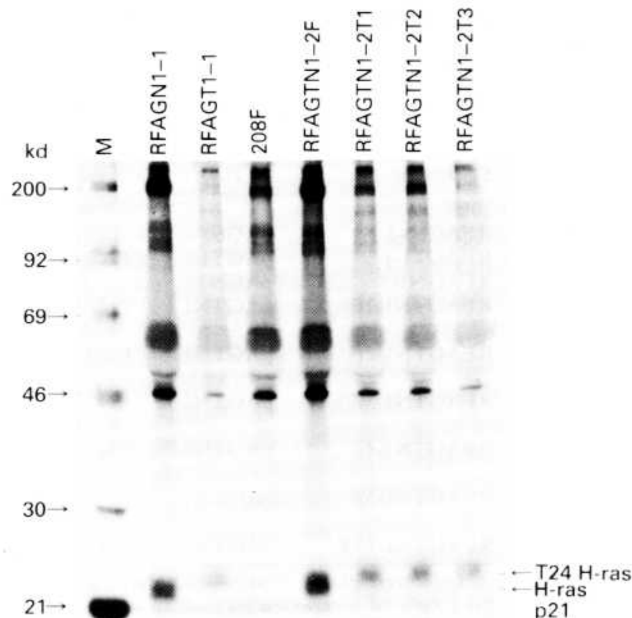

Figure 3 Analysis of p21 *ras* gene products in 208F cells transfected with plasmids pAGN1 (RFAGN1-1 cells), pAGT1 (RFAGT1-1 cells) and pAGTN1 (RFAGTN1-2F cells). RFAGTN1-2T1, RFAGTN1-2T3 cells were derived by *in vitro* culture of the tumours from different nude mice induced with the RFAGTN1-2F cells. [^{35}S] methionine-labelled cell extracts were analyzed as in **Figure 2**.

genes and that this correlates with the ratio of expression of normal to mutant protein.

The original DNA mediated transfer experiments which led to the detection and isolation of the mutant *ras* genes associated with tumours led to the concept that they behave dominantly in determining the transformed and tumorigenic phenotypes of cells. The results reported here suggest that at least in rat 208F cells and human T24 cancer cells, cellular dominance can at best be partial.

Results from the study of spontaneous cancer and established cancer cell lines are conflicting. Cancer cell lines often either contain and express only mutant alleles of *ras* genes, suppress expression of the normal allele or increase expression of the mutant allele (Feinberg *et al.*, 1983; Santos *et al.*, 1983; Capon *et al.*, 1983). On the other hand human T-cell ALL cell lines and the human HT1080 fibrosarcoma cell line express both mutant and normal forms of N-*ras* at equal levels (Shen *et al.*, 1987; Paterson *et al.*, 1987). In the latter case the cells are triploid for the mutant allele, and non-tumorigenic variants lose one copy of the chromosome containing the mutant allele. However, in this case the parent cell line is not suppressed by transfection with the normal N-*ras* gene. The reason for such differences in results is not yet obvious, but it seems possible that each tumour cell line has different properties which result in differences in the most frequently observed mechanism of suppression.

The molecular mechanism(s) behind the onco-suppression described in the present study remain(s) to be determined. We suggest that the most likely explanation is competition by the normal H-*ras*1 gene product with the mutant gene product for cellular proteins or sites which interact with p21. One important function of p21 may be in transmission of the proliferative signal triggered by the interaction of growth factors with tyrosine kinase related receptors (Mulcahey *et al.*, 1985; Smith *et al.*, 1986). Since it is likely that the activity of p21 is regulated by binding to guanosine nucleotides, the recently described GAP protein, which accelerates the hydrolysis of bound GTP to GDP, might be one possible target for such competition (Trahey & McCormick, 1987). *Ras* p21 most probably interacts with other proteins or metabolites which may be equally plausible targets.

Our results suggest that *ras* may be able to act as either an oncogene or an onco-suppressor gene, depending on the cellular context. The mechanism by which *ras* can suppress the transformed and tumorigenic phenotypes of cells may be important in the analysis of gene products which modulate onco-suppressor gene activity of cells *in vitro* and *in vivo*.

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