Reprinted from Nature, Vol. 310, No. 5977, pp. 469-475, 9 August 1984
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Malignant transformation of early passage rodent cells by a single mutated human oncogene

Demetrios A. Spandidos*† & Neil M. Wilkie*

* Beatson Institute for Cancer Research, Garscube Estate, Bearsden, Glasgow G61 1BD, UK
† Hellenic Anticancer Institute, Athens, Greece

*When linked to transcriptional enhancers, the mutant Ha-*ras*-1 gene from the T24 bladder carcinoma cell line induces the complete malignant transformation of early passage cells, while the normal Ha-*ras*-1 proto-oncogene only induces immortalization. Therefore, mutated Ha-*ras*-1 does not require a cooperating gene to trigger malignant conversion and* ras *genes may be involved in the process of tumorigenesis at an earlier stage than previously suspected.*

CARCINOGENESIS *in vivo* has been shown to be a multi-step process. Evidence for this comes from epidemiological studies[1], experimental chemical carcinogenesis in animal model systems[2] and recent studies on virally induced tumours in chickens[3,4]. The process has broadly been subdivided into two steps, an initiating step and a completing step[5]. Initiation is thought to be an absolute requirement involving mutational events in as yet unknown genes, while completion involves subsequent alterations that result in the tumorigenic state. In attempts to develop experimental systems suitable for *in vitro* testing of this model, much recent work has focused on the tumorigenic conversion of cells in culture[6-8]. In such systems two steps can again be distinguished: a first step in which cells are immortalized (rescue from senescence) but have few phenotypic characteristics of malignant cells, and a second step (completing step) in which cells acquire phenotypes characteristic of malignant cells (for example, reduced serum requirement, anchorage independence and ability to induce tumours in experimental animals).

Initial attempts to identify genes acting dominantly at the cellular level, which could affect one of these steps, concentrated on viral genes. As a result of these experiments several viral genes have been identified which are thought to effect rescue from senescence but not completion. These include the polyoma large T antigen and the adenovirus Ela genes[9,10]. Genes which exhibit the ability to cause completion to a malignant phenotype include the adenovirus Elb and polyoma virus middle T genes[11,12]. Another set of viral genes (termed v-*onc* genes) involved in the malignant conversion of cells has been identified in the acutely transforming, highly oncogenic retroviruses. Few of these have been rigorously tested in quantitative assays for rescue from senescence or completion, but it is clear that at least some cases, retroviral oncogenes can trigger complete malignant conversion of primary cells (for reviews see refs 13, 14). In these cases, it seems likely that both mutation of amino acid coding sequences and transcriptional activation contribute to the highly transforming phenotype. The retroviral oncogenes are closely related to and are assumed to be derived from normal cellular genes (termed proto-oncogenes). Alterations in these genes are associated with *in vivo* carcinogenesis. The alterations so far identified include amplification, rearrangements such as transposition or chromosomal translocation and mutation (for review see ref. 15).

DNA-mediated gene transfer techniques are among the most useful ways of detecting altered cellular proto-oncogenes with transforming ability in tumour cells and cell lines[6-8]. Most of these experiments have used NIH 3T3 cells as recipients, and most experiments have detected mutated cellular homologues of the oncogenes contained in Harvey and Kirsten murine sarcoma viruses (*ras* genes)[7,8]. As NIH 3T3 cells are considered to be an abnormal cell line, already rescued from senescence and predisposed to completion, it has been suggested that *ras* oncogenes are second-step transforming genes or 'progressogenes'[16,17]. Several recent studies support this proposal[18-20]. They suggest that the Ha-*ras*-1 oncogene from the human T24 bladder carcinoma cell line, which has a substitution at amino acid position 12, could cause the malignant conversion of early passage rodent cells only if they are immortalized by chemical carcinogens, or when a second gene mediating rescue from senescence is co-transferred.

Here we show that these requirements can be eliminated. The T24 oncogene alone can rescue low passage rodent cells from senescence and, if transferred to recipient cells in recombinant DNA constructions containing transcriptional enhancers, can also directly trigger low passage cells to convert to a fully tumorigenic phenotype. Moreover, the normal *ras* proto-oncogene can also rescue low passage rodent cells from senescence when introduced into recipient cells in high expression vectors.

Transformation

In order to introduce normal and mutant *ras* genes into low passage rodent cells, the cloned oncogenes were linked to a dominant selectable marker. The marker used was the aminoglycoside phosphotransferase gene (*aph*) from bacterial transposon Tn*5*, which confers on eukaryote cells resistance to the antibiotic geneticin (G418). *Bam*HI fragments containing either the activated or the normal Ha-*ras*-1 gene were introduced into the *Bam*HI site of three different bacterial plasmids containing the *aph* gene (Fig. 1). Plasmids pAG60 and Homer 5 contain an *aph* gene under the transcriptional control of the herpes simplex virus thymidine kinase gene (HSV *tk*) while in Homer 6 the 5' *tk* control sequences have been replaced by those of Moloney murine sarcoma virus (MoMSV) which contain a transcriptional enhancer. Both Homer 5 and Homer 6 include a fragment containing the simian virus 40 (SV40) origin of replication, which also contains an enhancer. Thus, in the pAG series, no known transcriptional enhancers are present, in the pHO5 series one enhancer (from SV40) is present, while in the pHO6 series two enhancers (from SV40 and MoMSV) flank the inserted fragments. We also constructed plasmids pANGM1, pANGM2, pATGM1 and pATGM2 containing the normal or the T24 Ha-*ras*-1 genes and the *aph* gene with a single MoMSV enhancer, by inserting a 2.9-kilobase (kb) *Eco*RI fragment containing the enhancer and the *aph* gene into plasmids pAT1 and pAN1 (Fig. 1*b*).

Recombinant DNAs were introduced into early passage rodent cells using the calcium phosphate precipitation technique first described by Graham and Van der Eb[21]. The ability of cells to take up and stably express the exogenous DNA was determined by measuring the number of geneticin-resistant colonies obtained with each cell type and donor DNA. The ability of donor DNA to rescue cells from senescence, regardless of mor-

phology, was determined by isolating geneticin-resistant colonies and passaging the cells *in vitro*. In the growth conditions used here, individual hamster clones and Wistar rat cells senesced after approximately 30 doublings. (Cloned cells capable of *in vitro* passage for >100 doublings were considered rescued from senescence.) Second-step transformation (completion) was determined by the ability of the cells to grow in semi-solid media (anchorage independence).

Table 1 shows the results obtained on the introduction of 100 ng of recombinant DNA into passage 3 Chinese hamster lung cells (CHL(F3)). Geneticin-resistant colonies were obtained with all recombinant DNAs tested, but at different frequencies. As might be expected, plasmids containing known transcriptional enhancers produced more colonies than the pAG series which did not contain enhancers. Microscopic examination of the geneticin-resistant colonies revealed a most striking result. Colonies obtained after transfection with plasmids containing the normal Ha-*ras*-1 gene or the T24 Ha-*ras*-1 gene with no known enhancers appeared normal, with flat morphology, and exhibited contact inhibition. However, >90% of the colonies obtained with plasmids containing the T24 Ha-*ras*-1 gene and either or both the SV40 and MoMSV enhancers contained highly refractile, pebble-shaped cells which grew in a disoriented manner and were not contact-inhibited (Fig. 2*A*). Table 1 also shows the results when transfected cells were simultaneously selected for both geneticin-resistance and anchorage independence. Again, doubly selected colonies were obtained only after transfection with DNA containing the T24 gene and at least one enhancer, at about the same frequency as the morphologically distinct geneticin-resistant colonies obtained in liquid medium. This suggests that the morphological changes are characteristic of the second or completing transformation step, and it was confirmed by showing that cells from morphologically altered colonies plated with a high efficiency in semi-solid medium.

These results were not confined to hamster cells. Table 1 also shows the transformation of early passage rat cells from rat embryo and muscle and skin from 2-week-old Wistar rats. The results obtained were very similar to those observed with CHL(F3) cells. Geneticin-resistant colonies were obtained with all recombinant DNAs and all types of recipient cells and again the presence of transcriptional enhancers increased the

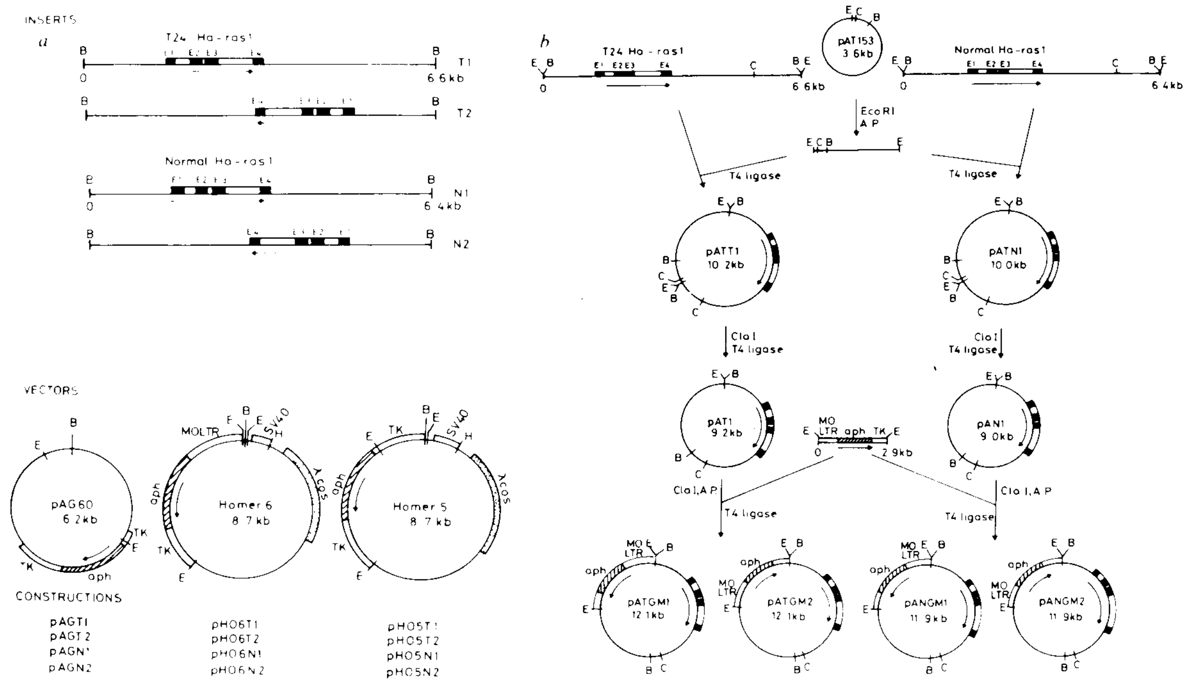

Fig. 1 ***a***, Schematic representation of pAG60 (6.2 kb), Homer 6 (8.7 kb), Homer 5 (8.7 kb) and derivative plasmids carrying the human T24 or normal Ha-*ras*-1 gene. The *Bam*HI fragment containing the T24 Ha-*ras*-1 oncogene is 6.6 kb long[22], whereas the *Bam*HI fragment containing the normal human Has-*ras*-1 oncogene from fetal liver is 6.4 kb[30]. Plasmid pAG60[31] contains the bacterial Tn*5*-encoded aminoglycoside phosphotransferase (*aph*) gene under the transcriptional control of the 5′ and 3′ signals of the herpes simplex virus thymidine kinase gene (HSV-1 *tk*). Plasmids pAGT1 and pAGT2 were obtained by insertion of a 6.6-kb *Bam*HI fragment containing the human T24 bladder carcinoma Ha-*ras*-1 oncogene into the *Bam*HI site of pAG60, and plasmids pAGN1 and pAGN2 by insertion of the 6.4-kb *Bam*HI fragment containing the human normal Ha-*ras*-1 oncogene. Plasmids pAGT1 and pAGN1 contain the T24 and the normal Ha-*ras*-1 genes in the same orientation as the *aph* gene and plasmids pAGT2 and pAGN2 in the opposite orientation. Homer 6 was derived from the cosmid vector Homer 5 by Jonathan Wolf. Homer 6 contains the *aph* gene under the 5′ transcriptional control of the Moloney murine sarcoma virus long terminal repeat (LTR) promoter and enhancer regions[32] and the 3′ polyadenylation signal derived from the HSV-1 *tk* gene. The MoMSV LTR-containing sequences consist of a 530-base pair (bp) *Eco*RI–*Xba*I fragment of cloned integrated MoMSV carrying 230 bp of mink sequences upstream of the remaining 300 bp of viral LTR sequences[33]. The *Xba*I site in the MoMSV LTR was converted to *Bam*HI using molecular linkers. Homer 6 also contains the 430-bp *Hpa*II–*Hin*dIII fragment of SV40 spanning the origin of replication and carrying the 'enhancer' region and the packaging signal of phage λ (λ cos). Plasmids pHO6T1 and pHO6T2 were obtained by inserting the 6.6-kb T24 Ha-*ras*-1 oncogene fragment into the *Bam*HI site of Homer 6 and plasmids pHO6N1 and pHO6N2 by inserting the 6.4-kb normal Ha-*ras*-1 gene into the same site. Plasmids pHO6T2 and pHO6N2 contain the T24 and the normal Ha-*ras*-1 genes in the same orientation as the *aph* gene and plasmids pHO6T1 and pHO6N1 in the opposite orientation. Homer 5 differs from Homer 6 in that it contains the *aph* gene under the 5′ transcriptional control of the HSV-1 *tk* gene instead of the MoMSV LTR. ***b***, The construction of recombinant plasmids containing the normal or mutant Ha-*ras*-1 genes, the *aph* gene and a single MoMSV LTR enhancer. *Eco*RI fragments containing the T24 Ha-*ras*-1 gene (6.6 kb) or the normal Ha-*ras*-1 gene (6.4 kb) were cleaved from plasmids pHO6T1 or pHO6N1 (see *a*) and isolated from low melting agarose gels. The fragments were ligated to phosphatase *Eco*RI-cleaved pAT153 and plasmids pATI and pANI isolated. *Cla*I digestion followed by ligation removed a 1.0-kb fragment containing adjacent *Eco*RI and *Bam*HI sites producing plasmids pAT1 and pAN1 which contain single *Eco*RI sites. Ligation of the 2.9-kb *Eco*RI fragment containing the *aph* gene linked to the MoMSV LTR fragment (isolated from pHO6T1, see *a*) into the *Eco*RI sites of pAT1 and pAN1 produced plasmids pATGM1, pATGM2, pANGM1 and pANGM2. The maps are not drawn to scale; arrows represent the transcriptional orientation of the Ha-*ras*-1 and *aph* genes. AP, alkaline phosphatase; B, *Bam*HI; c, *Cla*I; E, *Eco*RI.

frequency of geneticin-resistant colonies. However, the frequency was at least 10 times lower than that obtained with CHL(F3) cells (the results shown for rat cells in Table 1 were obtained with 1 μg of recombinant DNA), confirmed by dose response tests (data not shown). The frequencies obtained following transfection of low passage lung and kidney cells from 2-week-old rats were even lower. Most importantly, morphologically altered cells and anchorage-independent colonies were obtained only after transfection with DNA containing the T24 gene and enhancers. Typical morphological appearances of normal cells and their transformed counterparts are shown in Fig. 2*B*. Transformation with pHO6N1 resulted in a significant number of geneticin-resistant colonies in liquid medium, but these were morphologically normal. Simultaneous selection for geneticin resistance and growth in Methocel failed to yield any anchorage-independent colonies. Transfection with pAGT1 resulted in a very low number of geneticin-resistant colonies and no anchorage-independent cells. When cultures were transfected with pAGT1 or pHO6T1 and plated in Methocel in the absence of geneticin, numerous small anchorage-independent colonies appeared which rapidly senesced (200 colonies per 10^6 cells for pAGT1 and ⩾500 per 10^6 cells plated for pHO6T1). Transfection of 2×10^5 rat cells in liquid culture with 20 μg of plasmid pT24 (ref. 22), which contains the T24 oncogene without a biochemically selectable marker, followed by selection in low serum, resulted in the transient appearance of morphologically altered cells which subsequently senesced.

In summary, in both the hamster and rat cells the presence of the mutant T24 *ras* gene and transcriptional enhancers correlates with our ability to isolate directly morphologically altered, anchorage-independent cells with almost the same frequency as geneticin-resistant cells.

Phenotypic properties of cloned cells

Individual geneticin-resistant colonies of Chinese hamster and rat cells were picked and propagated *in vitro*. The phenotypes of individual colonies were analysed by determining which cells were rescued from senescence, which showed striking morphological changes and grew in semi-solid medium, and which induced tumours in nude mice (Table 2).

All of the geneticin-resistant colonies derived from vector plasmids pAG60 or Homer 6 (four out of four individual colonies tested in each case) senesced and were unable to form cell lines (Table 2). To test for tumorigenicity, 10–20 geneticin-resistant colonies from pAG60 or Homer 6 transfections were pooled to obtain sufficient cells, and inoculated into nude mice. No tumours were obtained. However, 7 out of 10 colonies tested from transfections on hamster cells with pAGT1, which contains the T24 gene but no known transcriptional enhancers, were rescued from senescence. These cells showed no striking morphological changes, were not anchorage independent and did not produce tumours in nude mice. In contrast, all of the tested geneticin-resistant colonies derived from pHO5T1, pATGM1, pHO6T1 and pHO6T2, in which the T24 gene is adjacent to either one or two enhancers, were morphologically altered, anchorage independent and produced tumours in nude mice. The tumours grew rapidly (the animals became moribund within 10 days) and on necropsy, metastatic infiltrations of other organs were observed in some cases. These results were repeated with Wistar rat cells transformed with pHO6T1 and pHO6T2 and strikingly, the anchorage-independent cells induced rapidly growing progressive tumours in nude mice (Table 2) and 3-week-old Wistar rats (data not shown). Very few geneticin-resistant colonies were obtained with pAGT1 and rat cells (see Table 1). Two such colonies were picked but failed to grow.

Table 1 Transfection of Chinese hamster and rat cells with *aph* recombinant plasmids

Recipient cells	Donor DNA	No. geneticin-resistant colonies per flask (liquid medium): Total No. av. ± s.d.	No. geneticin-resistant colonies per flask (liquid medium): Morphologically transformed av. ± s.d. (%)	No. geneticin-resistant colonies per plate (semi-solid medium) av. ± s.d.
CHL(F3)|	pAG60	3.3 ± 2.0	0	0
	pAGT1	9.0 ± 4.3	0	0
	pAGN1	7.3 ± 3.8	0	0
	Homer 6	45 ± 12	0	0
	pHO6T1	99 ± 13	92 ± 10 (95)	79 ± 17
	pHO6T2	89 ± 9.4	84 ± 8.3 (94)	71 ± 17
	pHO6N1	80 ± 13	0	0
	pHO6N2	83 ± 12	0	0
	Homer 5	38 ± 12	0	0
	pHO5T1	28 ± 7.3	25 ± 6.5 (89)	25 ± 6.8
	pATGM1	73 ± 8.0	69 ± 7.0 (94)	65 ± 16
	pATGM2	62 ± 17	59 ± 16 (95)	56 ± 16
	pANGM1	60 ± 12	0	0
	pANGM2	67 ± 14	0	0
	Salmon	0	0	0
Rat embryo†	pAG60	2.5 ± 1.3	0	0
	pAGT1	1.0 ± 0.8	0	0
	Homer 6	17 ± 4.7	0	0
Rat embryo	pHO6T1	25 ± 5.2	23 ± 3.6 (9)	15 ± 2.9
	pHO6T2	21 ± 4.9	20 ± 4.2 (95)	14 ± 5.9
	pHO6N1	15 ± 4.5	0	0
	Rat embryo	0	0	0
Rat muscle†	pAG60	3.3 ± 2.1	0	0
	pAGT1	4.3 ± 3.0	0	0
	Homer 6	46 ± 8.3	0	0
	pHO6T1	95 ± 11	90 ± 6.7 (95)	76 ± 9.2
	pHO6T2	54 ± 7.9	81 ± 7.5 (96)	54 ± 6.1
	pHO6N1	69 ± 11	0	0
	Rat embryo	0	0	0
Rat skin†	pAG60	3.3 ± 2.2	0	0
	pAGT1	3.8 ± 2.4	0	0
	Homer 6	40 ± 15	0	0
	pHO6T1	88 ± 7.0	83 ± 9.0 (94)	68 ± 13
	pHO6T2	63 ± 14	59 ± 13 (94)	63 ± 20
	pHO6N1	70 ± 9.3	0	0
	Rat embryo	0	0	0

* Transfection of third-passage Chinese hamster lung cells CHL(F3) was carried out as follows: 100 ng of each superhelical plasmid DNA mixed with 10 μg salmon sperm DNA as carrier was co-precipitated with calcium phosphate in a volume of 0.5 ml[21] amd added to 1×10^5 recipient cells in 5 ml medium per 25 cm^2 flask. 24 h later the medium was changed with 5 ml non-selective medium (SF12; Flow) containing 15% Hyclone serum (Sterile Systems Inc.) and incubation was continued at 37 °C for 24 h. The medium was then changed to selective medium containing 15% Hyclone serum and 200 μg ml^{-1} geneticin (Gibco). Duplicate 25-cm^2 flasks were trypsinized, the cells counted and 1×10^5 cells plated per 9-cm bacteriological plate in 20 ml, Methocel medium (SF12 containing 0.9% Methocel (Fluka), 30% Hyclone serum and 200 μg ml^{-1} geneticin). The medium of liquid cultures was changed every 2–3 days for up to 10 days, when colonies were examined and counted using an inverted microscope. Foci consisting of predominantly highly refractile, pebble-shaped cells which contained occasional giant nuclei, and which grew in a disoriented manner were classified as morphologically transformed. Colonies were picked using cloning rings and trypsin after removing the top of the flask with a heated scalpel. Colonies in Methocel-containing plates were counted 10 days post-plating and picked with Pasteur pipettes. The data are derived from the results of four to six flasks or plates per donor DNA from two to three experiments.

† Transfection of second-passage rat (Wistar) cells was carried out as follows: 1 μg of each superhelical plasmid DNA mixed with 10 μg rat embryo DNA as carrier in 0.5 ml calcium phosphate DNA co-precipitate were added per 1×10^5 recipient cells (passage 2 at 1:4 split ratio) in 5 ml medium per 25-cm^2 flask. Selection of geneticin-resistant colonies in liquid or semi-solid medium was carried out as described above. The results are derived from four flasks per donor DNA.

None of the geneticin-resistant colonies obtained from hamster or rat cells after transfection with recombinants containing the normal Ha-*ras*-1 gene were anchorage independent or able to produce tumours in experimental animals. However, a high proportion of the hamster colonies produced by pHO6N1 (9 out of 10) and pANGM1 (4 out of 4), where the normal gene is flanked by enhancers, were rescued from senescence and grew into established cell lines. The same was true for rat muscle or skin cells transformed with pHO6N1. Four out of four muscle and three out of four skin cell foci grew into established lines. In no case did we observe any obvious 'crisis' during the immortalization process. Geneticin-resistant colonies derived from transfections with pAGN1 or pAGN2, which contain no known enhancers, were very difficult to propagate. Only two colonies grew *in vitro* and both senesced after about 20 doublings.

Malignant conversion

FAGT1-2 and FHO6N1-1 are immortalized Chinese hamster cell lines obtained by transfection of CHL(F3) cells with recombinants pAGT1 and pHO6N1, respectively. These cells have a near normal morphology (see Fig. 2*A*), are contact-inhibited, anchorage dependent and are not tumorigenic (see Table 2). However, they have a $\sim 10^{-7}$ frequency of spontaneous transformation to anchorage independence, detected by plating in semisolid media, and these achorage-independent cells are tumorigenic (data not shown).

Table 3 shows the ability of various recombinants to convert these two lines to an anchorage-independent phenotype. As expected from the previous results, pHO6T1 can readily transform FAGT1-2 and FHO6N1-1 cells to anchorage independence. However, the frequencies obtained (7.6×10^{-3} to 1.7×10^{-2}) are much higher than that obtained with primary cells ($\sim 8\times10^{-5}$, Table 1). Transfection with R15[23], a clone which contains the adenovirus-2 Ela gene, also produces anchorage-independent colonies with both cell lines, although at a lower frequency. The R15 clone had no detectable effect on the anchorage independence of CHL(F3) cells (data not shown). Plasmid pHO6N1, containing the normal gene in the presence of enhancers, also transformed both cell lines, as did pAGT1, containing the T24 gene with no enhancers. Thus, immortalized cells could be transformed to anchorage independence using the same donor plasmid as in the original immortalization step. Because no anchorage-independent cells could be obtained by transfection of 2×10^5 CHL(F3) cells with up to 20 μg of these donor DNAs, we suggest that this represents a qualitative change in immortalized cells. That is, the immortalization process results in qualitative changes to the cells which renders them more senstive to further transformation even with the same gene construction. Presumably, conversion to anchorage independence with the same gene construction reflects a quantitative increase in the expression of that gene in the already immortalized cells. No further transformation of FAGT1-2 cells was obtained using pAGN1, which contained the normal gene with no enhancers, suggesting that conversion to anchorage independence requires either the mutation of the T24 gene or enhancer expression of the normal Ha-*ras*-1 gene. As Homer 6 produced no measurable increase in the frequency of anchorage-indepen-

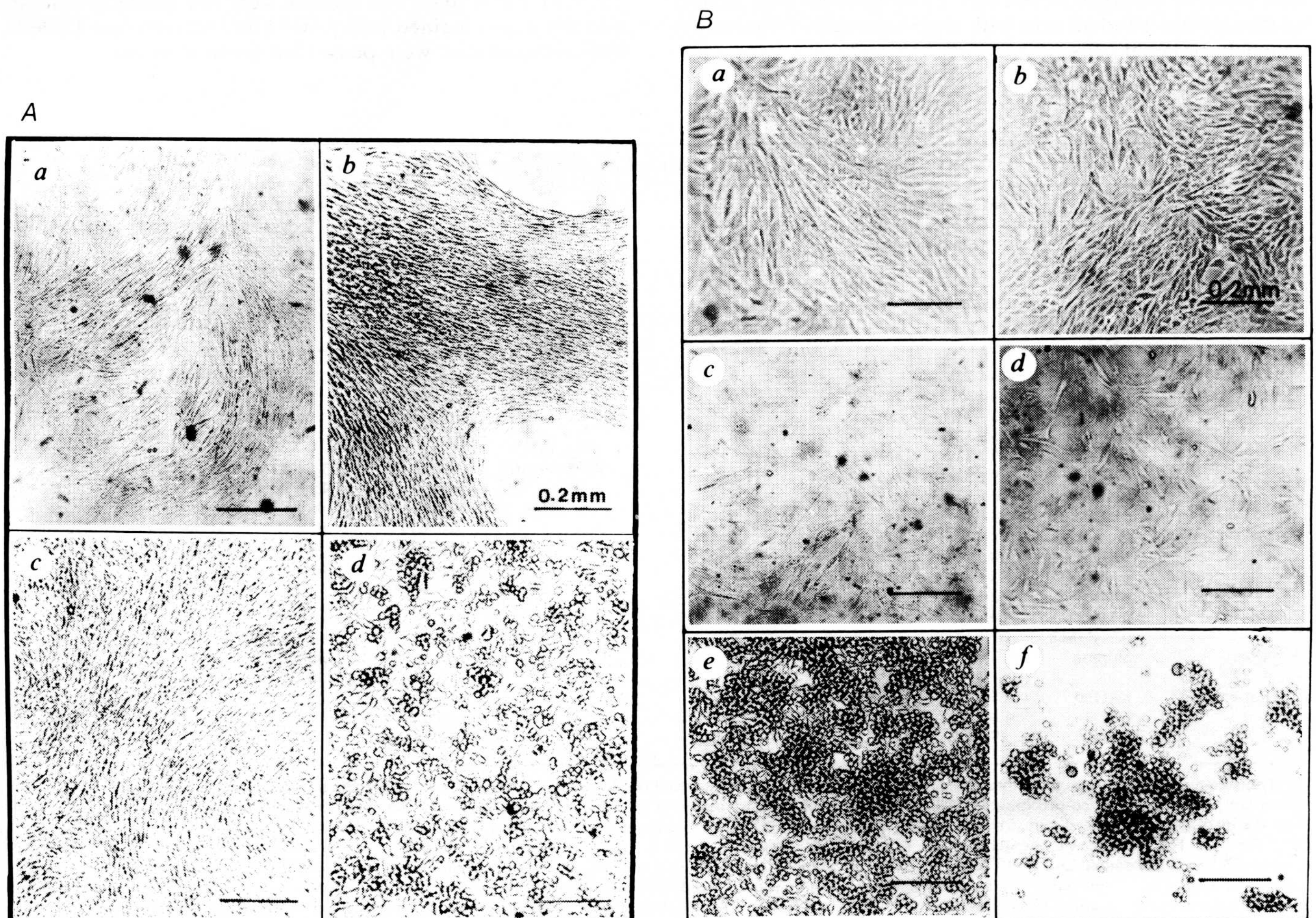

Fig. 2 *A*, Normal and transformed Chinese hamster lung cells: *a*, CHL(F3); *b*, FAGT-2; *c*, FHO6N1-1; *d*, FHO6T1-1 cells. *B*, Normal and transformed Wistar rat cells; *a*, second passage muscle cells; *b*, second passage skin cells; *c*, muscle cells transformed with pHO6N1; *d*, skin cells transformed with pHO6N1; *e*, muscle cells transformed with pHO6T1; *f*, skin cells transformed with pHO6T1.

Table 2 Phenotypes of geneticin-resistant hamster and rat cells

Cells	No. of clones	Transfected with	Rescued from senescence	Morphologically transformed	Anchorage independent	Tumorigenicity
Chinese hamster						
CHL(F3)	5	—	0/5	0/5	0/5	0/1*
FAG	4	pAG60	0/4	0/4	0/4	0/1*
FHO6	4	Homer 6	0/4	0/4	0/4	0/1*
FAGN1	2	pAGN1	0/2	0/2	0/2	0/1*
FAGT1	10	pAGT1	7/10	0/10	0/10	0/2
FHO6N1	10	pHO6N1	9/10	0/10	0/10	0/2
FHO6T1	4	pHO6T1	4/4	4/4	4/4	2/2
FHO6T2	4	pHO6T2	4/4	4/4	4/4	2/2
FHO5T1	4	pHO5T1	4/4	4/4	4/4	2/2
FATGM1	4	pATGM1	4/4	4/4	4/4	2/2
FATGM2	4	pATGM2	4/4	4/4	4/4	2/2
Rat						
Muscle	4	—	0/4	0/4	0/4	0/1*
Skin	4	—	0/4	0/4	0/4	0/1*
RMHO6	4	Homer 6	0/4	0/4	0/4	0/1*
RSKHO6	4	Homer 6	0/4	0/4	0/4	0/1*
RMHO6N1	4	pHO6N1	4/4	0/4	0/4	0/2
RSKHO6N1	4	pHO6N1	3/4	0/4	0/4	0/2
RMHO6T1	4	pHO5T1	4/4	4/4	4/4	2/2
RSKHO6T1	4	pHO6T1	4/4	4/4	4/4	2/2
RMHO6T2	4	pHO6T2	4/4	4/4	4/4	1/1
RSKHO6T2	4	pHO6T2	4/4	4/4	4/4	1/1

Individual colonies from the experiments shown in Table 1 were picked as described in Table 1 legend and propagated in SF12 liquid medium containing 15% Hyclone serum and 200 $\mu g\ ml^{-1}$ geneticin. Cells which grew for more than 100 cell doublings were classified as rescued from senescence. The classification of morphologically altered cells is described in Table 1 and Fig. 2 legends and anchorage independence was classified by a plating efficiency of >20% in the semi-solid medium described in Table 1. Tumorigenicity was tested by inoculating 1×10^6 cells subcutaneously into four or five 1-month-old nude mice. With tumorigenic cells lines, experimentally induced tumours appeared within 1 week in all inoculated animals, whereas no tumours were detected up to 2 months after inoculation of non-tumorigenic cells.

* In these cases insufficient numbers of cells from individual colonies were obtained for the tumorigenicity tests because the cells senesced. To obtain sufficient numbers the cells from 10–20 colonies were pooled and propagated for a limited number of passages before testing.

Table 3 Transformation of immortalized Chinese hamster lung cells to anchorage independence

Recipient cells	Immortalized with	Donor DNA	Colonies per plate per μg DNA per 10^4 cells* (av. ± s.d.)
FAGT1-2	pAGT1	pHO6T1	139 ± 30
FAGT1-2	pAGT1	R15	22 ± 11
FAGT1-2	pAGT1	pHO6N1	80 ± 19
FAGT1-2	pAGT1	pAGT1	9 ± 6.2
FAGT1-2	pAGT1	pAGN1	0
FAGT1-2	pAGT1	Homer 6	0
FAGT1-2	pAGT1	Salmon	0
FHO6N1-1	pHO6N1	pHO6T1	92 ± 16
FHO6N1-1	pHO6N1	R15	13 ± 8.5
FHO6N1-1	pHO6N1	pHO6N1	50 ± 16
FHO6N1-1	pHO6N1	pAGT1	16 ± 6.5
FRO6N1-1	pHO6N1	Homer 6	0
FRO6N1-1	pHO6N1	Salmon	0

FAGT1-2 and FHO6N1-1 are cells rescued from senescence after transfection of CHL(F3) with plasmids pAGT1 and pHO6N1, respectively (see also Table 2).

* 1 μg of each superhelical plasmid DNA mixed with 10 μg salmon sperm DNA as carrier in 0.5 ml calcium phosphate DNA co-precipitate were added per 1×10^5 recipient cells in 5 ml medium per 25-cm^2 flask. 24 h later the medium was changed with 5 ml non-selective medium and incubation continued at 37 °C for 24 h. Cells were then trypsinized, counted and plated at 10-fold dilutions in 20 ml Methocel-containing medium per 9-cm plate in the presence of 200 $\mu g\ ml^{-1}$ geneticin. Colonies were counted 8 days post-plating. The data are derived from the results of six plates per donor DNA from two experiments.

dent colonies, we assume that the enhancers in pHO6N1 act in a *cis* rather than a *trans* manner, probably at the level of transcription.

Combining the above results, we suggest that the transforming potential of the Ha-*ras*-1 gene is modulated at the level of transcription as well as mutation. In the absence of enhancers the mutant T24 gene can rescue cells from senescence, but cannot transform them to a tumorigenic phenotype. In the presence of enhancers acting in *cis*, the gene directly triggers events which result in a tumorigenic phenotype. A similar effect is observed with the normal Ha-*ras*-1. In the absence of enhancers, no rescue from senescence or complete transformation is obtained. In the presence of enhancers the normal gene is capable of rescuing cells from senescence. Apparently, both transcriptional activation and mutation are required to trigger tumorigenic conversion.

Expression of human Ha-*ras*-1

Selection of geneticin-resistant colonies after transfection with recombinant DNA ensures that all selected cells contain donor DNA. This was confirmed by Southern blot analysis (data not shown) which showed the presence of one to five copies of donor DNA, in some cases as full-length copies, in other cases individually integrated into host or carrier DNA.

The level of Ha-*ras*-1-specific transcripts in transformed cells was analysed by Northern blot and dot-blot analyses, using the 6.6-kb *Bam*HI fragment of the cloned T24 gene as a hybridization probe. The expected 1.2-kb Ha-*ras*-1-specific RNA was detected in most transformed cells. Much higher levels of the 1.2-kb RNA were detected in cells transformed with pHO6T1, containing two enhancers, than in two cell lines obtained with pAGT1, which contains no known enhancers. Moreover, when the pHO6T1 and pHO6T2 transformed cells were passaged in nude mice, the resultant tumour cells also contained high levels of the *ras* transcript (data not shown).

The results were confirmed by dot-hybridization tests (Fig. 3). Cells transformed with recombinants containing the T24 gene and no known enhancers (FAGT1-1 and FAGT1-2) contained relatively low amounts of Ha-*ras*-1-specific RNA (even

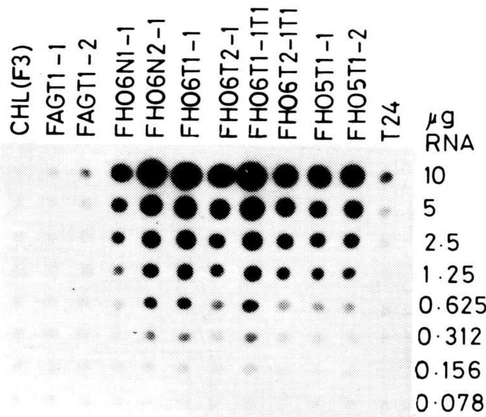

Fig. 3 An autoradiograph showing RNA spot-hybridization analysis of Ha-*ras*-1 transcripts present in recipient CHL(F3) and transformed with pAGT1 (FAGT1-1, FAGT1-2), pHO6N1 (FHO6N1-1) pHO6N2 (FHO6N2-1), pHO6T1 (FHO6T1-1), pHO6T2 (FHO6T2-1), pHO5T1 (FHO5T1-1, FHO5T1-2), tumours induced in nude mice by FHO6T1-1 (FHO6T1-1T1) or FHO6T2-1 (FHOT2-1T1) and the T24 human bladder carcinoma cell lines. Total cell RNA extracted from cells as previously described[34] and twofold serial dilutions of 10 µg down to 0.078 µg were spotted onto nitrocellulose[35]. The ^{32}P-labelled 6.6-kb *Bam*HI fragment carrying the T24 oncogene was used as a probe.

lower than the human T24 bladder carcinoma cell line). In contrast, much higher levels of Ha-*ras*-1-specific RNA were found in cells transformed with recombinants which contained either the normal or mutant T24 genes plus known enhancers (FHO6N1-1, FHO6N2-1, FHO5T1-1, FHO5T1-2, FHO6T1-1 and FHO6T2-1). From Fig. 3 it can be estimated that these cells contained 20–60 times as much RNA as FAGT1-1 or FAGT1-2. Again, the high transcription level was maintained in tumour cells produced by passage in nude mice (FHO6T1-1T1 and FHO6T2-2T1). Similar results were obtained with transformed rat cells (data not shown).

Karyotypic analysis

Morphologically altered geneticin-resistant hamster and rat colonies were picked and the karyotypes analysed after 15–20 cell doublings. All of the transformed lines were subsequently shown to have a tumorigenic phenotype. As expected, the hamster CHL(F3) cells and the normal Wistar rat skin and muscle cells had a normal modal chromosome number (22 and 42, respectively). Three out of four tumorigenic hamster cells, isolated after transfection with pHO6T1 or pHO6T2, contained a near normal modal distribution, while the other was almost tetraploid. In contrast the transformed rat skin and muscle cells exhibited marked aneuploidy. On further passage the proportion of cells with a near normal number of chromosomes decreased and eventually most cells became heteroploid. These results suggest that the transformed cells were chromosomally abnormal. When, to investigate this in more detail, the banding patterns of the transformed hamster cells were determined, in each case a distinctive karyotype different from CHL(F3) cells was obtained and the presence of marker chromosomes noted (D.A.S., M. Freshney, R. Balfour and N.M.W., unpublished results).

Discussion

We have demonstrated here the potential for a single mutated human oncogene, the Ha-*ras*-1 from the T24 bladder carcinoma line, to trigger both step 1 (rescue from senescence) and step 2 (tumorigenic conversion) transformation of early passage rodent cells. Furthermore, we show that the 'normal' Ha-*ras*-1 proto-oncogene can rescue early passage cells from senescence. This transforming ability of the proto-oncogene, and the ability of the T24 gene to cause tumorigenic conversion, depends on the presence in the vector DNA of transcription enhancers. We suggest that the increased transforming potential of the two genes results from increased expression of the p21 proteins. Strong support for this suggestion comes from the experiments of Table 3, which show that the enhancer effect is in *cis* and not in *trans*, and from those of Fig. 3, which show increased levels of Ha-*ras*-1 transcripts in cells transformed with enhancer-containing DNA.

These results are consistent with recent reports which show that the T24 gene readily transforms already immortalized Chinese hamster cells[24] and rat cells[18], as well as the standard NIH 3T3 cell line[7,8]. However, our data depart from recent claims that the T24 gene will only cause tumorigenic conversion of primary cells in combination with either the adenovirus Ela gene or the avian v-*myc* gene[18,19]. While we agree that in the absence of enhancers the T24 gene may require transformation with other oncogenes to induce focus formation and tumorigenicity, our results clearly show that the T24 gene when linked to enhancers is able to trigger tumorigenic conversion on its own. We suggest that these differences reflect the levels of expression of the mutant T24 gene: at a low level of expression the presence of an additional gene is required; at high levels of expression the T24 gene alone suffices. Our recent data suggest that similar results can be obtained for the mutant N-*ras* gene[25] cloned from the HT1080 human fibrosarcoma cell line (unpublished results). It is therefore important to distinguish between quantitative and qualitative effects in attempting to define the effect of oncogenes in *in vitro* transformation systems.

Our inability to induce morphologically altered foci in liquid culture by transfection of early passage cells with the T24 gene in the absence of enhancers is consistent with the data of Ruley[19] and Newbold and Overall[20] with early passage rat and Syrian hamster cells. However, like Newbold and Overall[20] and Land *et al*[18], we did observe anchorage-independent colonies which senesced, in the absence of geneticin selection. We believe that these apparently discrepant results can be explained by the transient expression and release of transforming growth factors (TGFs) in cultures transfected with *ras* genes (I. B. Pragnell, N.M.W. and D.A.S., unpublished results). These TGFs efficiently induce anchorage-independent phenotypes in already immortalized cells and we see no reason that they should not do the same in early passage cells, albeit at a lower frequency. In the presence of geneticin, these indirectly stimulated cells would not grow, and only cells containing activated *ras* genes stably expressing at high levels would form colonies.

While it is clear that the T24 gene in the absence of enhancers can immortalize CHL(F3) cells, the only two rat colonies we tested after transfection with this gene, rapidly senesced. Further experiments on a larger scale are required to determine if there is a qualitative difference between CHL(F3) cells and rat cells in this respect, apart from the quantitative differences in transformation frequencies observed between the two systems. Nonetheless, transfer of the normal Ha-*ras*-1 proto-oncogene in the presence of enhancers leads to immortalization of both CHL(F3) cells and Wistar rat cells. All eight rat cell geneticin-resistant colonies obtained with vectors pAG60 or Homer 6 and tested independently, senesced and failed to form established cell lines. After transfer of pHO6N1, 9 out of 10 CHL(F3) colonies and 7 out of 8 rat cell colonies grew into established cell lines.

It is of considerable interest that CHL(F3) cells immortalized with either the T24 gene without enhancers or the normal Ha-*ras*-1 gene in the presence of enhancers, can be converted to anchorage-independent cells with the same gene construction as used in the immortalization step (see Table 3). We interpret this to mean that unknown qualitative changes accumulate in immortalized cells which render them very sensitive to further tumorigenic conversion, as anchorage-independent conversion of primary cells cannot be achieved even with large amounts of

the same donor DNA. Interestingly, the adenovirus Ela gene behaves as a second-step conversion gene in immortalized Chinese hamster cells (Table 3), although it is usually considered to be an 'immortalization' gene[18]. Similarly, in our hands Ha-*ras*-1 can behave as an immortalization gene, although it is usually considered to be a late step or conversion gene[18–20]. These results are consistent with those of Blair *et al.*[26] and Chang *et al.*[27], who showed the proto-oncogenes c-*mos* and Ha-*ras*-1 to have second-step transformation activity in NIH 3T3 fibroblasts when activated by transcriptional enhancers. Thus, a proto-oncogene and the equivalent activated oncogene can have both step 1 and step 2 transforming potential, depending on the assay system chosen.

It is of interest to attempt to relate our findings to *in vivo* carcinogenesis. Although there are few detailed reports on the expression of *ras* genes in pre-malignant lesions *in vivo*, we note that chemically induced benign papillomas of mouse skin contain a cellular Ha-*ras* gene which is active in transfection assays and gives rise to elevated transcript levels[28]. Moreover, pre-malignant polyps of the human colon as well as adenocarcinomas have greatly elevated levels of *ras*-related transcripts compared with normal colonic mucosa[29]. In the latter case it is not known whether transcription is from a mutant, activated gene, but the experiments provide support for the notion that *ras* genes may participate in early stages of the *in vivo* carcinogenic process, perhaps even in the establishment phase.

The mechanisms by which *ras* gene products effect transformation *in vitro* and *in vivo* are not understood. We emphasize that although our results suggest a quantitative aspect to *in vitro* transformation by the *ras* oncogene, they are completely consistent with multi-step models of carcinogenesis[5,6]. Several events are required to reveal the most active transforming potential of Ha-*ras*-1 oncogenes. Although activated Ha-*ras*-1 can trigger the complete tumorigenic conversion of very early passage rodent cells *in vitro*, it seems likely that if similar mutational and transcriptional activation occurs *in vivo*, as yet unknown changes in other cellular genes may be necessary before the fully malignant cell emerges.

We thank Mary Freshney and Rosemary Balfour for technical assistance, Drs John Paul and James Neil for critical reading of the manuscript, M. Barbacid for plasmids pT24C3 and pbc-N1, Nigel Stow for clone R15, Jonathan Wolf and Peter Rigby for the Homer 5 and Homer 6 vectors, and A. C. Garapin for pAG60. We gratefully acknowledge the continuing support of the Cancer Research Campaign of Great Britain

Note added in proof: The introduction of plasmid pHO6T1 into primary cultures of adult Syrian hamster muscle cells resulted in the appearance of geneticin-resistant, anchorage-independent cells, which induced progressively growing tumours in nude mice.

Received 27 January; accepted 22 May 1984.

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