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Malignant transformation of early passage rodent cells by a single mutated human oncogene

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*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 E1b 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

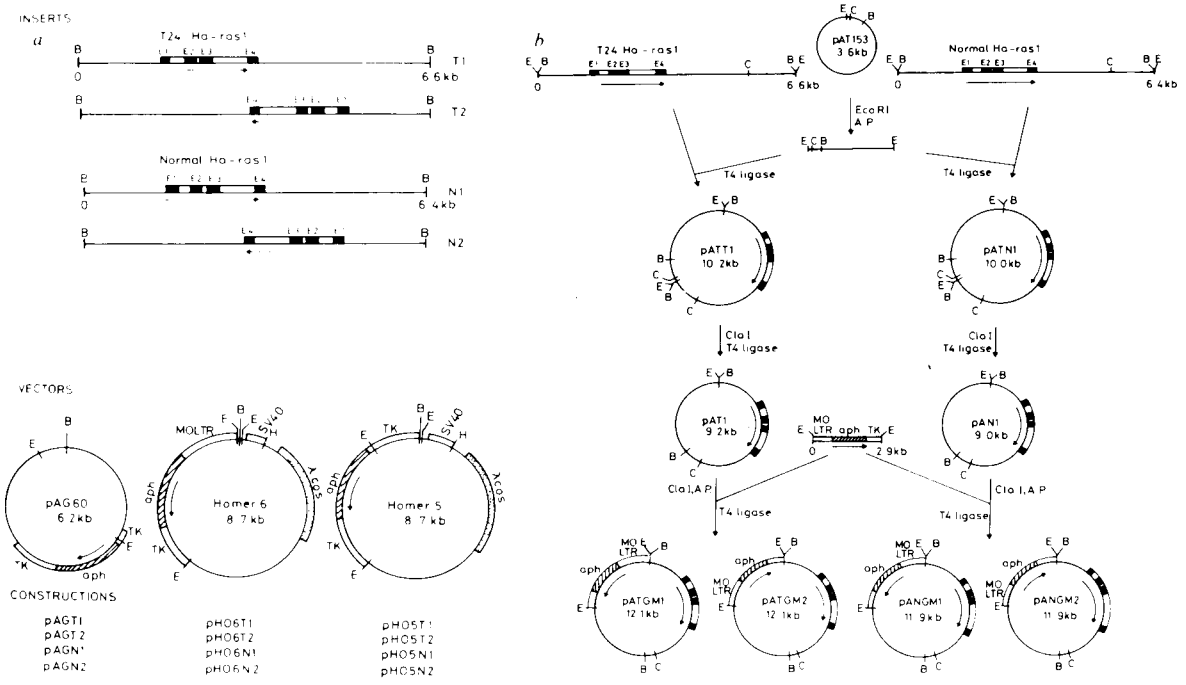

Fig. 1 *a*, Schematic representation of pAG60 (6.2 kb), Homer 6 (8.7 kb), Homer 5 (8.7 kb) and derivative plasmids carrying the human T24 or normal Ha-*ras*-1 gene. The *Bam*HI fragment containing the T24 Ha-*ras*-1 oncogene is 6.6 kb long[22], whereas the *Bam*HI fragment containing the normal human 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 pAT1 and pAN1 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.	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.