MOLECULAR AND CELLULAR BIOLOGY, July 1986, p. 2562–2570
0270-7306/86/072562-09$02.00/0

Vol. 6, No. 7

Oncogene Amplification during Tumorigenesis of Established Rat Fibroblasts Reversibly Transformed by Activated Human *ras* Oncogenes

EDWARD WINTER† AND MANUEL PERUCHO*

Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794

Received 27 January 1986/Accepted 31 March 1986

Normal rat fibroblasts of the established cell line Rat 4 were cotransformed with activated human *ras* oncogenes and with a cloned chicken thymidine kinase (*tk*) gene. Linkage between *tk* and *ras* genes allowed the isolation of oncogene deletion revertants and of cell clones showing varying degrees of malignant phenotype. Southern and Northern experiments in concert with tumorigenicity assays show that the malignant transformation of these cells by mutant *ras* oncogenes is a gradual but reversible process that depends on the relative abundance of oncogene sequences and their corresponding transcripts. We also show that moderate amplification of a c-K-*ras* oncogene in these cells results in a clear increase in their tumorigenicity and that the mutant gene present in low copy numbers in cultured cells undergoes amplification in the corresponding in vivo induced tumors.

A significant proportion of spontaneous and experimentally induced tumors contain *ras* genes altered by somatic mutations which result in the activation of their oncogenic potential in established rodent cell lines (for a review see reference 10). The mutations characterized to date result in single-amino acid substitutions at or near position 12 or 61 of the *ras* gene product (for a review see reference 36), but not all of these single-amino acid substitutions confer to *ras* genes the same transforming potency. Thus, rat fibroblasts transformed by the human c-H-*ras* gene containing different amino acids at codon 12 exhibit varying degrees of transformed morphology (28). Although mutant c-H-*ras* oncogenes are unable to achieve the oncogenic transformation of primary rodent embryo fibroblasts (11, 26), they can do so if they are supplied with strong transcriptional enhancers (30). It is also known that *ras* protooncogenes can transform NIH 3T3 cells when linked to a strong viral promoter-enhancer (2) or when incorporated in multiple copies in the host cell genome (23, 33). In addition, amplified *ras* genes have been found in animal (27) and human (14, 23, 34) tumors, and in some tumors the mutant *ras* allele is amplified relative to the normal (39). Therefore, quantitative as well as qualitative alterations in mammalian *ras* genes seem to be involved in the activation of their oncogenic potential.

We report here that the malignant transformation of established normal rat fibroblasts by mutant human *ras* oncogenes is reversible upon loss of the oncogene sequences and that moderate fluctuations in mutant *ras* gene amplification and expression have drastic consequences in the manifestation of their transformed phenotype. Moreover, we show that inoculation into syngenic animals of cell clones containing oncogene low copy number is accompanied by amplification of the mutant *ras* gene in the resulting tumors.

MATERIALS AND METHODS

Cell lines and DNA transfections. Rat 4 is an established normal cell line deficient in nuclear thymidine kinase, derived from the parental Fisher rat embryo fibroblast cell line Rat 1 (35). Calu 1 and T24 are cell lines derived from human lung and bladder carcinomas, respectively, and their origins have been previously reported (19). Cells were maintained as monolayer cultures in Dulbecco minimum essential medium (DMEM) supplemented with 10% bovine calf serum.

Rat 4 cells were transfected by the calcium-phosphate-DNA coprecipitate method (9) essentially as described before (19). Due to the toxicity of the calcium-phosphate-DNA coprecipitate in these cells, the conditions for optimal gene transfer were adjusted as follows: 10^6 cells were seeded in 10-cm culture dishes, and 1 day later 10 ml of fresh medium was added to the cultures 4 to 8 h before transfection. The calcium-phosphate precipitate contained 20 μg of tumor cell DNA per ml and, when indicated, 20 ng of a purified 3-kb kilobase *Hin*dIII fragment containing the chicken thymidine kinase (*tk*) gene (20). A 0.75-ml portion of the mixture was added per culture dish, and the precipitate was removed 6 to 10 h later. The cells from each culture dish were trypsinized and transferred into five dishes containing DMEM or hypoxanthine-aminopterin-thymidine (HAT) medium supplemented with 10% calf serum, and foci of transformed cells or HAT-resistant colonies showing a transformed morphology were scored 14 to 20 days later. tk^- revertants were isolated by plating increasing numbers of cells in medium containing 100 μg of bromodeoxyuridine (BUdR) or 1 μg of trifluorothymidine (TFT) per ml as previously described (22).

Anchorage dependence and tumorigenicity assays. Growth in soft agar was assayed by suspending a known number of cells in 0.3% agar in DMEM supplemented with 10% calf serum which was overlaid on a base consisting of 0.6% agar in the same medium. Colonies were scored by visual inspection and microscopic examination at 2, 4, and 6 weeks. One hundred cells were plated in tissue culture dishes without agar and colonies were scored at 2 weeks to obtain plating efficiencies. Relative soft-agar cloning efficiencies were determined by dividing the percentage of colonies observed in the soft-agar dishes by the plating efficiencies of the corresponding cell clones.

To test the tumorigenic properties in vivo of Rat 4 transformants, 10^6 and 5×10^6 cells in 0.25 ml of DMEM from

* Corresponding author.
† Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

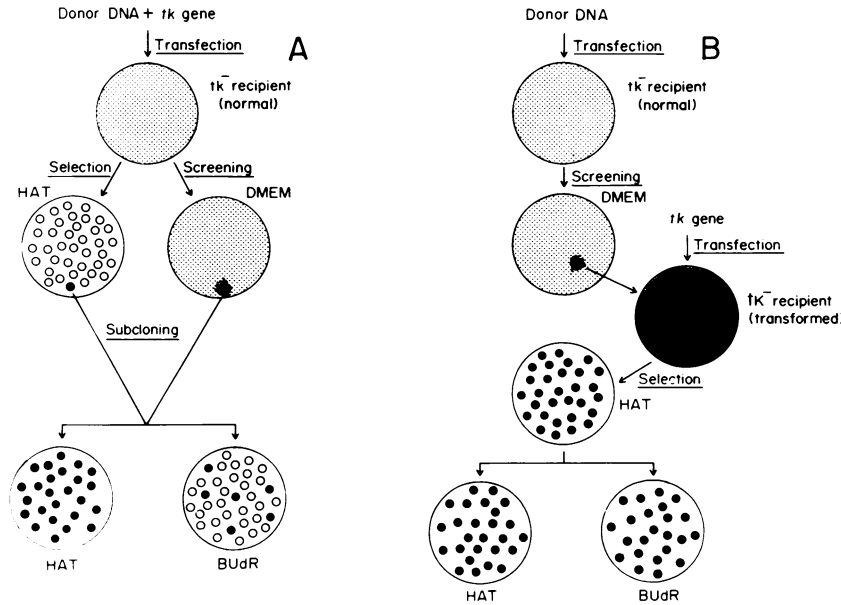

FIG. 1. Schematic representation of the cotransformation-reversion strategy. (A) Normal tk^- cells are transfected with a mixture of donor cell DNA containing a transforming gene and a cloned *tk* gene. Screening in neutral medium (DMEM) or selection in HAT medium allows the isolation of morphologically transformed tk^+ cells. After subcloning, the cells are plated in HAT medium or in medium containing BUdR, and the morphology of colonies is scored after 2 weeks in culture. (B) The cells are first transfected with the transforming gene, and foci of morphologically transformed tk^- cells are isolated by screening the neutral medium cultures and subcloned further. These cells are then used as recipients for transformation with the *tk* gene, and tk^+ colonies are isolated and plated in HAT or BUdR medium. Black and white circles represent colonies with transformed or normal morphology, respectively.

each individual cell clone were injected subcutaneously into the backs of 4- to 6-week-old Fisher rats which were monitored thrice weekly for the appearance of tumors. After their appearance, tumors were periodically measured with a caliper to determine their growth rates. When the tumors reached a size of approximately 40 mm in diameter, the animals were sacrificed, and the tumors were dissected and frozen in liquid nitrogen until further use.

RNA and DNA extractions and hybridization analyses. RNA was prepared by sequential guanidine HCl-ethanol and phenol extractions, as previously described (38), from 8 to 10 plates of sparse cell cultures. Approximately 30 μg of total cellular RNA was electrophoresed through 1% formaldehyde-agarose gels (13) which were subsequently soaked for 15 min in water preheated to 65°C and for 1 h in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature. RNA was transferred to nitrocellulose filters as described before (19). The filters were subsequently prehybridized for 5 to 12 h at 42°C in 50% formamide–5× SSC–1× Denhardt solution–50 mM NaH_2PO_4 (pH 6.5) containing 50 μg of denatured salmon DNA per ml and hybridized at 42°C in the above solution containing 10% dextran sulfate and approximately 5×10^6 cpm of nick-translated DNA (1×10^8 to 2×10^8 cpm/μg) per ml. The filters were washed once in 5× SSC–0.1% sodium dodecyl sulfate at room temperature for 5 min and successively in 2× SSC–1× SSC and 0.5× SSC containing 0.1% sodium dodecyl sulfate at 65°C for 30 min each time. The filters were exposed to X-ray film for 12 to 42 h at −70°C.

DNA was prepared from cultured cells (three semiconfluent dishes) or from frozen tumors as previously described (17, 19) and digested with restriction endonucleases according to the manufacturer's specifications (New England Biolabs, Inc.). Southern blot hybridizations were carried out on approximately 8 μg of restriction endonuclease-digested DNA as previously described (19), with the exception that dextran sulfate (10%) was included in the hybridization solution. Quantitative densitometries were performed with a Joyce-Loebl densitometer as described previously (38), with different exposure times of the X-ray films to ensure operation in the linear response range of the films.

RESULTS

Reversible transformation of rat fibroblasts by mutant human *ras* oncogenes. Previous studies have shown that exogenous DNA sequences incorporated by cultured cells become ligated together in a large concatameric structure in which a transforming gene is genetically and physically linked to other cotransforming DNA sequences (21). This new genetic unit eventually integrates into the host cell genome (25). It was rationalized that cotransformation of appropriate cells with human *ras* oncogenes plus a selectable gene for which counterselection is available could simplify the isolation of oncogene revertants by partial or complete deletion of the foreign DNA sequences. Accordingly, Rat 4 tk^- cells were transformed with genomic or cloned activated *ras* oncogenes and a cloned chicken *tk* gene following an experimental protocol similar to that previously described (22) and that is represented in Fig. 1.

DNA from the human tumor cell lines Calu 1 and T24 which contain activated c-K-*ras* and c-H-*ras* oncogenes (8, 17) induced morphological transformation with efficiencies of 0.005 and 0.1 focus per μg, respectively. Foci which contained cells able to grow in HAT medium or HAT-selected colonies showing a transformed morphology were isolated and subcloned again, and cells from these tk^+ morphologically transformed clones were plated in HAT medium or in medium containing BUdR. Cells growing in HAT medium formed dense colonies showing a transformed

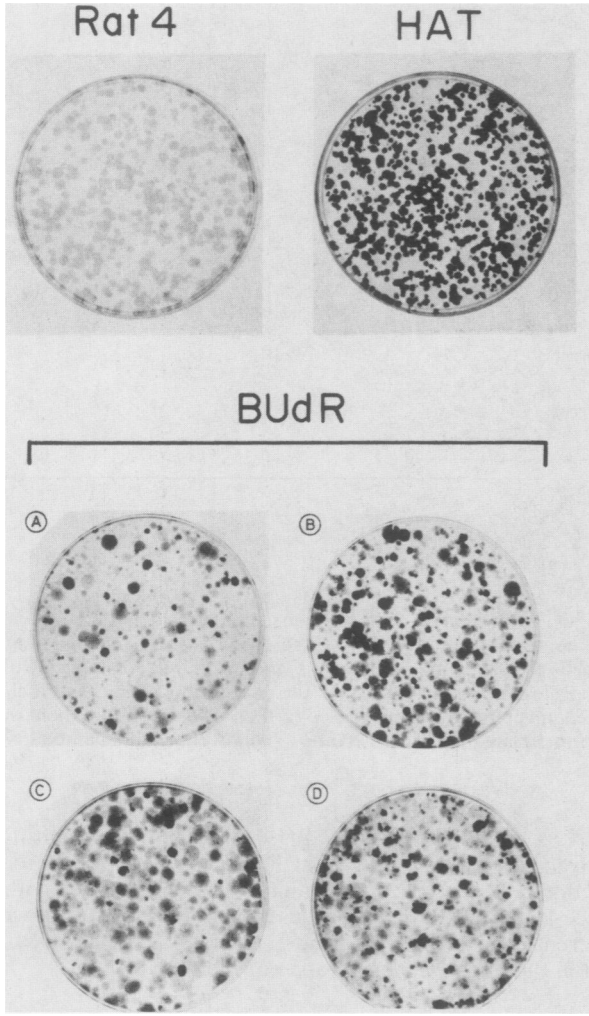

FIG. 2. Phenotypic reversion of Rat 4 c-H-*ras* oncogene transformants. Rat 4 cells were transformed with DNA of the bladder carcinoma cell line T24 and cotransformed with the chicken *tk* gene. Various tk^+ morphologically transformed cell clones were plated in BUdR medium (A through D). The number of cells from the different transformed clones (Table 1) were 2×10^4 (A), 15×10^4 (B), 15×10^3 (C), and 2×10^5 (D). Two hundred fifty and 500 cells from the parental Rat 4 cell line (R4) or from one tk^+ transformed clone (HAT) were also plated in neutral or in HAT medium, respectively. After 12 (R4 and HAT) or 15 (A to D) days, the culture dishes were stained with Giemsa.

morphology consisting of piled up, highly refractile cells. In contrast, selection in BUdR gave rise to morphologically normal colonies, although transformed colonies also developed with variable frequency depending on the different transformants (Fig. 2; Table 1).

The reversion of BUdR-selected clones to a normal morphology was not due to an effect of this drug because similar results were obtained with the nucleotide analog TFT as selection for the tk^- phenotype (Table 1). In addition, sequential experiments were performed in which Rat 4 cells were first transformed with the T24 c-H-*ras* oncogene and then used in another transfection experiment to generate tk^+ clones with the chicken *tk* gene (Fig. 1B). Several morphologically transformed tk^+ colonies were subjected to selection in BUdR as before. In this case, no linkage was observed between the two phenotypes: all BUdR-selected tk^- revertants were still morphologically transformed (Table 1) (data not shown).

To have more objective criteria for the characterization of the cellular phenotype, individual colonies were isolated and expanded into mass culture. Three classes of cells were chosen: HAT-selected cells, all morphologically transformed; BUdR-selected cells which maintained a transformed morphology; and BUdR-selected cells exhibiting a morphologically normal phenotype. Cells from these three classes were analyzed for their cloning efficiency in soft agar, for their tumorigenicity in vivo, and for the presence of human *ras* oncogene sequences in their genome (Table 1). A direct correlation was found between the morphological appearance of these cells and their malignant phenotype: HAT- and BUdR-selected, morphologically transformed cells contained oncogene sequences, showed high cloning efficiencies in soft agar, and were highly tumorigenic. In contrast, tk^- flat revertants which had lost detectable oncogene sequences were not able to grow in soft agar and, like the parental Rat 4 cells, were nontumorigenic in our experimental conditions.

Similar results were obtained with viral *ras* oncogenes (v-H-*ras*), with cloned *ras* oncogenes including the T24 c-H-*ras* oncogene and c-K-*ras*/c-H-*ras* chimeric oncogenes (40), and also with another tk^- normal rat fibroblast cell line (Rat 2) as recipient (data not shown). We conclude from these experiments that deletion of *ras* oncogenes in transformed rodent fibroblasts results in their reversion to a normal phenotype.

1HT1 and its descendants have variable transformed phenotypes. We used this experimental strategy to analyze in greater detail the behavior of a Rat 4 tk^+ transformed clone (hereafter referred to as 1HT1) induced by genomic DNA from the c-K-*ras* oncogene-containing cell line Calu 1. When cells from this clone were plated in BUdR medium, approximately 2% survived. The majority of these cells exhibited a normal morphology. Southern blot hybridization analyses revealed that they were deletion revertants, and they were not able to survive when plated back in HAT medium. However, a smaller proportion (about 5% of the BUdR-selected tk^- clones) exhibited varying degrees of transformed morphology. When these clones were expanded into mass culture, the majority of them contained cells that were able to survive in HAT medium with efficiencies that correlated with the morphological appearance of the original clones. Thus, 1 to 5% of the cells from the most transformed clones (T clones), 0.1 to 1% of the cells showing an intermediate morphology (NT clones), and 0.001 to 0.1% of the cells exhibiting a more normal morphology (N clones) survived and gave rise to fully transformed clones when plated in HAT medium.

Southern and Northern hybridization experiments (data not shown) revealed that, while these clones still contained *tk* gene sequences, they showed no detectable or very low levels of *tk* gene transcripts when maintained in BUdR medium. In contrast, 1HT1 cells grown in HAT medium expressed *tk*-specific transcripts. These results suggest that the phenotypic reversion of these tk^- clones was due to repression of the *tk* gene expression. In addition to these general classes of cells, we occasionally observed highly transformed clones which never gave rise to cells with ability to survive in HAT medium, suggesting that they had lost the *tk* but not the oncogene sequences.

In contrast with 1HT1 and all cells derived from this clone which displayed a stable transformed morphology when maintained in HAT medium, the morphological appearance

TABLE 1. Phenotypic linkage of *tk* and *ras* genes in Rat 4 transformants

Donor[a]	Medium	Morphology (%)[b]			Gene[c]		Anchorage[d]	Tumorigenicity[e]
		T	I	N	*ras*	*tk*		
T24 (4)	HAT (354)	98.8	1.2	0	6/6	3/3	4/4	2/2
	BUdR (1,636)	29.7	16.0	54.3	0/8	0/4 (N)	0/4 (N)	0/4 (N)
					5/5	0/3 (T)	4/4 (T)	2/2 (T)
	TFT (282)	42.5	5.6	51.7	0/2	ND (N)	ND	ND
					2/2	ND (T)	ND	ND
Calu 1 (2)	HAT (469)	92.7	7.3	0	6/6	4/4	2/2	2/2
	BUdR (1,336)	4.0	3.0	97.0	0/12	0/12 (N)	0/6 (N)	0/4 (N)
					5/5	3/5 (T)	3/3 (T)	3/3 (T)
	TFT (235)	10.6	12.7	76.5	2/2	ND	ND	ND
					0/3	ND	ND	ND
T24[f]	HAT (166)	99.2	0.8	0	ND	ND	ND	ND
	BUdR (277)	99.6	0.4	0	ND	ND	2/2	ND
Rat 4	BUdR						0/2	0/2

[a] Rat 4 transformants induced by DNA from the human tumor cell lines T24 or Calu 1 were analyzed for linkage of their transformed morphology with the *tk* gene as described in the text. The numbers in parentheses indicate the number of independently derived transformed clones analyzed.

[b] Morphology of cells growing in the indicated culture media. The values represent the average percentage of colonies showing a transformed (T), intermediate (I), or normal (N) morphology. The numbers in parentheses represent the total number of colonies analyzed. The cloning efficiencies in BUdR medium (and the percentage of colonies showing a normal morphology) of the different clones was as follows: T24 transformants (four clones)—0.13 (35), 0.15 (22), 0.78 (53), and 1.5 (60); Calu 1 transformants (two clones)—0.02 (93) and 1.8 (94).

[c] Presence of c-K-*ras* or c-H-*ras* oncogenes (*ras*) or chicken thymidine kinase (*tk*) sequences in isolated clones as analyzed by Southern blot hybridization. The probes used were the 0.8-kb *Pst*I fragment of the human c-H-*ras* oncogene cloned in pBR322 (8), the 0.9-kb *Eco*RI-*Hin*dIII fragment of the human c-K-*ras* oncogene cloned in plasmid pLRH0.9 (17), and the purified 3.0-kb *Hin*dIII fragment of the chicken *tk* gene (20). The values represent the number of positive cases/total number analyzed. The morphology of the different BUdR- selected clones is indicated in parentheses. ND, Not determined.

[d] Ability to grow in soft agar of clones selected in the indicated medium. Number of positive samples (giving colonies of more than 20 cells after 2 weeks in culture)/total number analyzed.

[e] Tumorigenicity in vivo of clones selected in the indicated medium. Number of positive samples (inducing detectable tumors 4 weeks after subcutaneous injection of 5×10^6 cells in 4- to 6-week-old Fisher rats)/total number analyzed.

[f] Cells from two independent morphologically transformed Rat 4 tk^- clones induced by T24 DNA were used as recipients in transfection experiments, using the chicken *tk* gene and Rat 4 carrier DNA. One tk^+ colony of transformed cells selected in HAT medium was subcloned from each original transformant and analyzed as before.

of those transformed cells which survived in BUdR medium was unstable. Thus foci of T cells appeared in cultures of N cells; conversely, colonies of N cells appeared in cultures of T cells (Fig. 3). In most cases the changes in cellular morphology appeared to be gradual, involving intermediate NT cells.

A BUdR-selected clonal derivative of 1HT1 (1BT1), exhibiting a transformed morphology, was plated at low cell

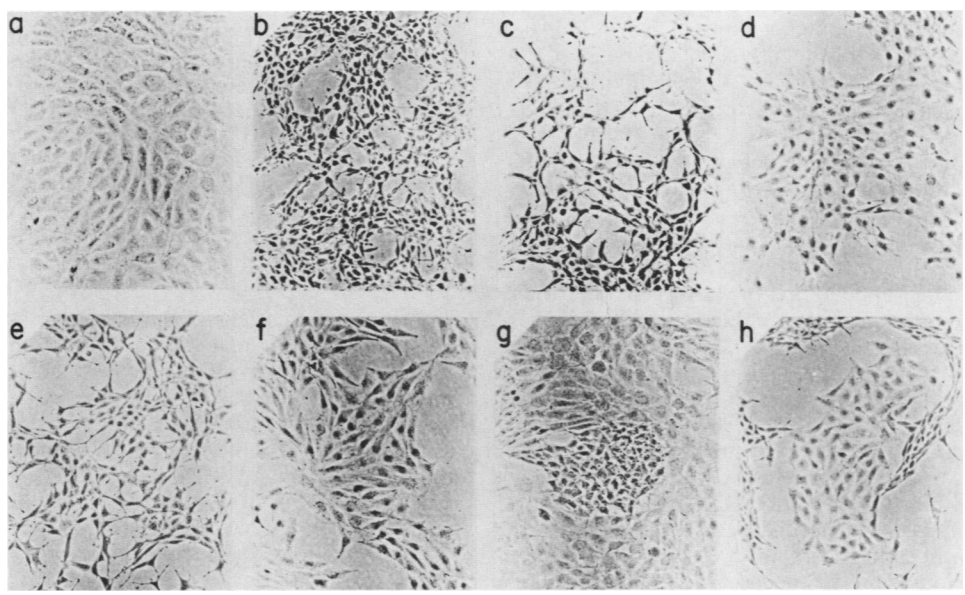

FIG. 3. Morphological instability of Rat 4 c-K-*ras* oncogene transformants. Cells from the indicated clones were photographed in the culture dishes with a Wild MPS45 camera, using a Leitz Diavert phase-contrast microscope (×100 magnification). (a) Rat 4; (b) 1HT1; (c) T1; (d) NT1; (e) NT2; (f) N1; (g) a focus of transformed cells in a culture of N1; (h) a colony of N cells in a culture of T1.

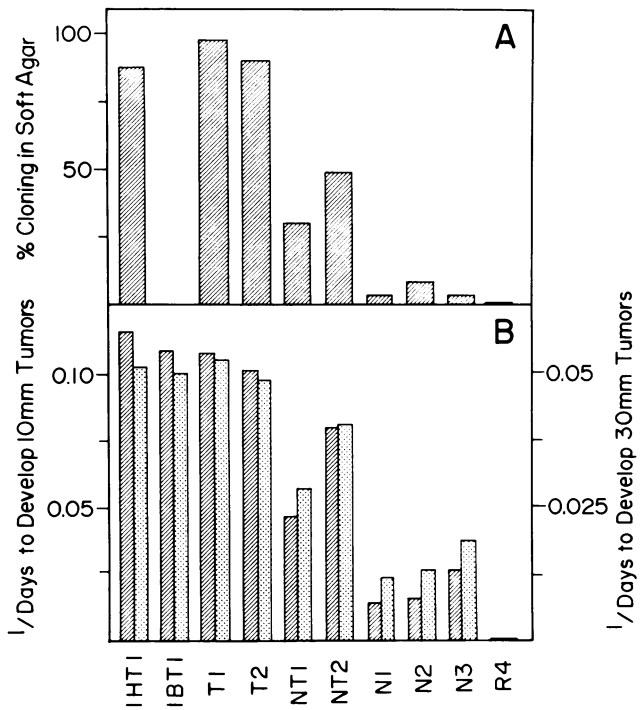

FIG. 4. In vitro and in vivo tumorigenic properties of Rat 4 c-K-*ras* oncogene transformants. Soft-agar cloning efficiencies (A) and ability to induce tumors in Fisher rats (B) of 1HT1 and its descendants. (A) Inocula of 10^2, 10^3, and 10^4 of the indicated cells were analyzed for ability to grow in soft agar. The number of colonies containing more than 20 cells was scored at 2 weeks post-inoculation and divided by the plating efficiency of each inoculum in standard growth medium. (B) Totals of 10^6 and 5×10^6 cells were injected subcutaneously into Fisher rats. The values are expressed as the reciprocal of the average time required for tumors of 10 (left bars) or 30 (right bars) mm to develop.

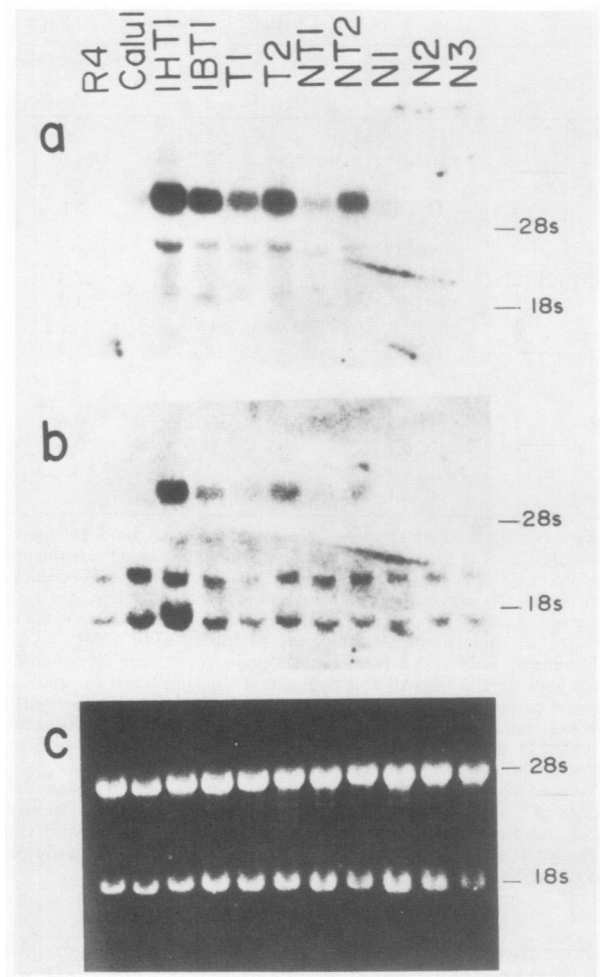

FIG. 5. Expression levels of the c-K-*ras* oncogene in Rat 4 transformants. Northern blot hybridization analysis of human c-K-*ras* (a) and rat α-tubulin (b) mRNA levels in 1HT1 and derived cultured cell clones. Total cellular RNAs (30 μg each) were electrophoresed through a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pLR2.4 (17), which is the 2.4-kb *Eco*RI fragment containing the fourth alternative coding exon and 3′-end untranslated sequences of the c-K-*ras* gene cloned into pBR322 (a). Subsequently, the filters were rehybridized with ^{32}P-labeled pILαT1 (12), a plasmid containing α-tubulin cDNA sequences (b). Calu 1 represents RNA extracted from the human Calu 1 tumor cell line. (c) One-tenth of each RNA sample was electrophoresed through a separate formaldehyde-agarose gel and stained with ethidium bromide to ensure that equivalent amounts of RNA were analyzed in each case.

density, and colonies of cells of varying morphologies ranging from T (T1 and T2) to NT (NT1 and NT2) to N (N1, N2, and N3) were isolated, subcloned again, and grown in mass culture. These cells were subsequently analyzed by all of the criteria described below after 25 to 30 cell doublings to minimize the heterogeneity which eventually became apparent after prolonged growth.

The differences in the oncogenic potential of 1HT1 and its descendants correlates with the c-K-*ras* oncogene expression levels. The oncogenic potential in vitro and in vivo of 1HT1 and derived clones was analyzed by soft-agar and tumorigenicity assays. A clear correlation was found between the microscopic appearance of these cells and their ability to grow in soft agar and to induce tumors in Fisher rats (Fig. 4). While 1HT1 and T clones had high cloning efficiencies in soft agar and induced tumors in about 1 week, the NT clones exhibited intermediate cloning efficiencies and induced tumors in 2 to 4 weeks. The N clones grew poorly in soft agar and took about 2 months to induce detectable tumors. However, upon appearance of a palpable tumor the growth rates were indistinguishable (Fig. 4B). At the same time, inspection of the soft-agar dishes at 2, 4, and 6 weeks revealed no differences in the relative cloning efficiencies, suggesting that cell division was necessary for the transition of an N cell to a T cell.

The human c-K-*ras* mRNA levels in these cells were determined by Northern hybridization experiments (Fig. 5). The expression pattern of the human c-K-*ras* oncogene was the same in 1HT1 and its derived clones and in Calu 1 human cells (two major and distinct RNA species of approximately 5.0 and 3.0 kb), indicating that the c-K-*ras* gene present in the rat transformants conserved all of the sequences necessary for its apparently correct expression. However, the highly transformed cells (1HT1, 1BT1, T1, and T2) accumulated high levels of *ras*-specific mRNA than the slightly transformed cells (N1, N2, and N3). Cells with an intermediate morphology (NT1 and NT2) expressed intermediate levels (Fig. 5a). As an internal control, the filter was rehybridized with a radioactive probe complementary to rat α-tubulin mRNA, revealing only minor differences in α-tubulin mRNA accumulation (Fig. 5b). The relative levels of

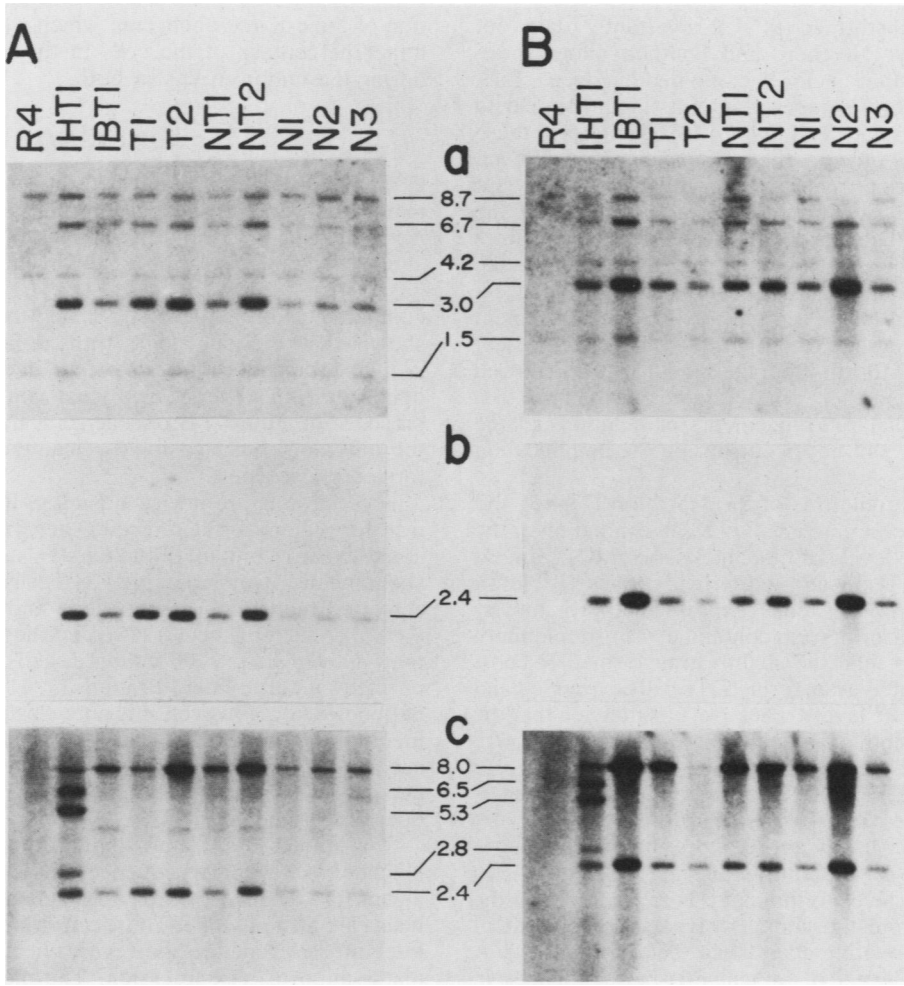

FIG. 6. Amplification of the c-K-*ras* oncogene in Rat 4 transformants. Southern blot hybridization analysis of endogenous rat c-K-*ras*, human c-K-*ras*, and chicken *tk* sequences in DNA from 1HT1 and derived cultured cell clones (A) and in their in vivo induced tumors (B). DNA was extracted from the indicated cell clones, and approximately 8 μg of each sample was digested with *Eco*RI, electrophoresed through a 1% agarose gel, and transferred to nitrocellulose. The filters were hybridized with the 0.6-kb *Stu*I-*Eco*RI fragment of HiHi3, a plasmid containing the coding sequences of the rat v-K-*ras* oncogene derived from Kirsten murine sarcoma virus (5) (panels a), and subsequently rehybridized with pLR2.4 (panels b) and with the 3-kb *Hin*dIII chicken *tk* gene fragment (panels c), radioactively labeled by nick translation.

c-K-*ras* mRNA were also determined in isolated T-cell populations which arose in N-cell cultures and conversely in isolated N-cell populations which arose in T-cell cultures (data not shown), revealing again a tight coupling between the abundance of c-K-*ras* mRNA in these cells and their malignant phenotype.

The differences in the malignant phenotype of 1HT1 and its descendants correlates with the c-K-*ras* oncogene dosage. The relative copy number of the c-K-*ras* oncogene present in these cells was determined by Southern blot hybridization experiments, using as probe v-K-*ras* gene sequences (Fig. 6A). Upon digestion with *Eco*RI, Rat 4 DNA revealed three distinct bands of 8.7, 4.2, and 1.5 kb, corresponding to the endogenous rat c-K-*ras* gene. The intensities of these bands were similar in all rat transformants, confirming that similar amounts of DNA were analyzed in each sample. In addition, two other bands of 6.7 and 3.0 kb were observed in the transformant DNA which represented cross-hybridization to the integrated human c-K-*ras* sequences. However, the relative intensities of these bands were not identical. The highly transformed T cells showed relatively higher levels of human c-K-*ras* sequences than did the NT cells, which in turn showed higher levels than the N cells (Fig. 6A, a). The same filter, rehybridized with a human c-K-*ras*-specific probe, again showed clear although moderate differences in the intensities of the specific human c-K-*ras* 2.4-kb *Eco*RI fragment (Fig. 6A, b). The filter was subsequently hybridized with chicken *tk*-specific sequences (Fig. 6A, c). While 1HT1 contained four different DNA fragments which hybridized to the *tk* probe, cells grown in BUdR contained only one of an identical size in each case. The relative intensity of this band was essentially coincident with that of the human c-K-*ras* band, indicating a tight linkage between the chicken *tk* and the human c-K-*ras* genes.

The differences observed in the tumorigenic properties and in the relative levels of oncogene transcripts and copy number between these cell clones could be explained if the more normal (N and NT) clones were heterogeneous populations with variable proportions of cells containing oncogene sequences. However, cloning experiments performed with cells from N1 and N2 cultures at passages identical to those used for the experiments described before revealed that the majority of these cells still harbored *tk* and oncogene sequences, as shown by their ability to generate

morphologically transformed tk^+ re-revertants (data not shown). Comparative Northern and Southern blot experiments also revealed that N clones contained oncogene RNA levels (Fig. 5) and copy number (data not shown) similar to those of the human tumor cell line Calu 1. Therefore, these differences were not due to the presence of a significant percentage of deletion revertants in these cultures, but rather to differences in the degree of amplification of the oncogene, with the N clones containing oncogene sequences equivalent to a single or a few copies per genome. At the same time, the human bands corresponding to different regions of the c-K-*ras* locus, from the 6.7-kb *Eco*RI fragment containing the first coding exon to the 2.4-kb *Eco*RI fragment containing the last fourth coding exon (1, 17), showed parallel fluctuations in their relative intensities (Fig. 6A, a and b), indicating that rearrangements or deletions in the oncogene sequences did not occur during the amplification events.

Quantitative densitometries of the Northern (Fig. 5) and Southern (Fig. 6A) experiments allowed an estimation of the relative steady-state levels of human c-K-*ras* mRNA and of its copy number in 1HT1 and its derived clones (Table 2). The highly transformed T cells expressed approximately 10-fold more *ras* transcripts and contained 5- to 10-fold more oncogene sequences than the slightly transformed N cells, while the intermediately transformed NT cells expressed and contained intermediate levels. These results indicate that the changes in expression of the human c-K-*ras* gene were primarily due to changes in its amplification.

The c-K-*ras* oncogene of 1HT1 and its descendants undergoes amplification in vivo. It was of interest to study the oncogene expression levels and its copy number in the in vivo induced tumors. In contrast to the cells grown in culture, the relative levels of human c-K-*ras* mRNAs in the tumors dissected from the animals revealed no consistent differences in their relative abundance (data not shown). A comparison of the levels of human c-K-*ras* sequences in DNA extracted from tumors with the DNA extracted from the corresponding cultured cells prior to inoculation revealed only minor differences in the cases of 1HT1, 1BT1, T1, T2, and NT2. However, the c-K-*ras* sequences had become clearly amplified in the tumors induced by the more normal N cells (Fig. 6B). These results indicate that the induction of tumors in vivo required the previous amplification of the c-K-*ras* oncogene, which was either present in a minor percentage of the cells in the inoculum or selected during the tumor growth or both.

TABLE 2. Quantitative analysis of human c-K-*ras* mRNA and DNA levels in 1HT1 and derived cultured cell clones[a]

Clone	mRNA			DNA			mRNA/DNA
	ras	Tubulin	*ras*/ tubulin	h-*ras*	r-*ras*	h/r *ras*	
1HT1	3.04	2.10	1.44	2.66	2.0	1.33	1.08
1BT1	1.0	1.0	1.0	1.0	1.0	1.0	1.0
T1	0.71	0.50	1.42	2.80	1.65	1.69	0.84
T2	1.14	0.76	1.50	3.15	1.75	1.80	0.83
NT1	0.22	0.58	0.38	0.90	1.05	0.85	0.44
NT2	0.67	0.71	0.94	2.80	1.90	1.47	0.63
N1	0.15	0.81	0.19	0.22	0.90	0.24	0.79
N2	0.11	0.93	0.11	0.33	1.45	0.22	0.50
N3	0.09	0.68	0.13	0.39	1.40	0.26	0.50

[a] In each case the values represent the average of two separate experiments which were quantitated by integrating the areas under peaks generated by densitometric tracings of autoradiograms. Representative experiments are shown in Fig. 6 and 7. The values were normalized relative to the areas of the 1BT1 clone, which was given an arbitrary value of 1.0. *ras*, Human c-K-*ras* RNA sequences; h-*ras* and r-*ras*, human and rat c-K-*ras* DNA sequences, respectively.

DISCUSSION

We have used the established rat fibroblast cell line Rat 4 in our studies of the oncogenic properties of activated human *ras* oncogenes. The cotransformation-reversion approach allows the rapid isolation of oncogene deletion revertants based on its linkage with the *tk* gene for which a counterselection is available. Using this strategy, we have shown that the oncogenic transformation of established normal rodent fibroblasts by activated human cellular *ras* oncogenes is a reversible process and that the continuous presence of mutant *ras* oncogenes is necessary not only for the initiation, but also for the maintenance of their transformed phenotype.

These results are not surprising in light of the reversible transformation of rodent established cell lines by a temperature-sensitive mutant of the Kirsten sarcoma virus (29), the spontaneous reversion of NIH 3T3 cells transformed with an activated human N-*ras* oncogene and cotransformed with the *dhfr* selectable vector (15), and the transient transformation and reversion of cultured cells microinjected with purified mutant c-H-*ras* proteins (7, 31) or with *ras*-specific antibodies (6). However, it is noteworthy that the deletion of incorporated mutant *ras* genes in stably transformed cells is accompanied by a rapid reversion to a phenotype indistinguishable by the criteria analyzed from the parental, nontransformed cells.

Our studies also show that, although deletion of the chicken *tk* exogenous sequences is the most frequent mechanism for the reversion to a tk^- phenotype, other mechanisms are also possible. Thus, cell clones that still contain an integrated *tk* gene are, however, phenotypically tk^- due to inactivation of its expression. The mechanism responsible for the reversible repression of the *tk* gene in these cells is not clear. By parallelism with similar observations using the herpesvirus *tk* gene, it could be due to changes in chromatin conformation (4, 32) or to alterations in the methylation patterns (3, 18). However, it is noteworthy that, while the parental 1HT1 clone contains several independently integrated copies of the *tk* gene, the BUdR-selected clones which still are able to survive in HAT medium retain only one (Fig. 6, c). The *tk* gene that remains in these tk^- clones has lost sequences 5′ of the *Eco*RI site located at the promoter region of the gene (data not shown). It is conceivable that the levels of gene expression could be abnormally low or aberrant in these cells due to the loss of essential sequences at its promoter region and that this could be the reason for their phenotypic instability. Growth in HAT medium could select those cells containing *tk* genes amplified above a critical level required for expressing sufficient *tk* activity to allow the cells to survive in HAT medium (24). This is supported by the different reversion frequency of N and T clones to the tk^+ phenotype and the tight linkage observed between the *tk* and the oncogene sequences which results in their coamplification.

Whatever processes led to the reversible inactivation of the *tk* gene in 1HT1 cells, its linkage with the *ras* oncogene allowed us to identify those cells which had undergone fluctuations in the degree of amplification of the oncogene sequences because this resulted in the manifestation of different cellular phenotypes. Therefore, our studies show that the malignant transformation of rodent fibroblasts by an

activated human c-K-*ras* oncogene is a quantitative process that depends on its expression levels, which in turn correlates with its degree of amplification.

Amplification of DNA sequences introduced into cultured cells by the calcium-phosphate coprecipitation method occurs very often, probably due to the instability of the exogenous DNA sequences in the transformed cells (24, 37). Thus, cultured rodent cells stably transformed with cellular *ras* oncogenes usually contain amplified oncogene sequences (16, 19, 36). It is not clear whether this is a prerequisite for the induction of distinguishable foci of morphologically transformed cells or if this is a later phenomenon selected during the propagation in culture. However, our observations suggest that amplification of the incorporated oncogene sequences may be necessary to achieve detectable morphological transformation at least with those oncogenes that do not contain mutations conferring to them strong oncogenic potential or with those cells which appear more resistant to transformation.

Perhaps the most striking result of our experiments is that a relatively small increase (<10-fold) in the gene dosage of the incorporated mutant c-K-*ras* gene that is readily detected in the NIH 3T3 transfection assay (19) and is representative of a mutation (cysteine at codon 12) that shows a clear transforming potency in the Rat 1 assay (28) results in a drastic difference in the malignant phenotype of the transformed cells. Thus, while cells containing one or a few copies of the mutant gene exhibit a very low cloning efficiency in soft agar and are weakly tumorigenic, subclones of cells derived from them that have amplified a few fold the mutant gene have clearly increased their ability to grow in soft agar and their tumorigenic potential in vivo. In support of this is the finding that propagation in vivo of cell clones having low oncogene dosage is accompanied by amplification of the gene. These observations also suggest that the expression of a minimum level of mutant *ras* gene products may be critical in tumorigenesis, and that one way this can be accomplished is by gene amplification. In this context it is noteworthy that previous studies have shown that mutant *ras* genes are preferentially expressed in several human tumor cell lines (1, 39) and that this is accompanied, in at least one case (that of the PR371 lung adenocarcinoma), by differential amplification of the mutant allele (39).

ACKNOWLEDGMENTS

We thank Concepcion Lama and Concepcion Almoguera for their excellent technical assistance and E. Scolnick and S. Farmer for their gifts of pHiHi3 and pILαT1 plasmids.

This work was supported by Public Health Service grants CA33021 and CA38579 awarded by the National Cancer Institute.

LITERATURE CITED

1. **Capon, D. J., P. H. Seeburg, J. P. McGrath, J. S. Hayflick, V. Edman, A. D. Levinson, and D. V. Goeddel.** 1983. Activation of Ki-ras-2 gene in human colon and lung carcinomas by two different point mutations. Nature (London) **304**:507–512.
2. **Chang, E. H., M. E. Furth, E. Scolnick, and D. R. Lowy.** 1982. Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature (London) **297**:479–484.
3. **Christy, B., and G. Scangos.** 1982. Expression of transferred thymidine genes is controlled by methylation. Proc. Natl. Acad. Sci. USA **79**:6299–6303.
4. **Davies, R. L., S. Fuhrer-Krusi, and R. S. Kucherlapati.** 1982. Modulation of transfected gene expression mediated by changes in chromatin structure. Cell **31**:521–529.
5. **Ellis, R. W., D. DeFeo, T. Y. Shih, M. A. Gonda, H. A. Young, N. Tsuchida, D. R. Lowy, and E. M. Scolnick.** 1981. The p21 *src* genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature (London) **292**:506–510.
6. **Feramisco, J. R., R. Clark, G. Wong, N. Arnheim, R. Melley, and F. McCormick.** 1985. Transient reversion of *ras* oncogene-induced cell transformation by antibodies specific for amino acid 12 of *ras* protein. Nature (London) **314**:639–642.
7. **Feramisco, J. R., T. Kamata, M. Gross, M. Rosenberg, and R. W. Sweet.** 1984. Microinjection of the oncogene form of the human H-*ras* (T24) protein results in rapid proliferation of quiescent cells. Cell **38**:109–117.
8. **Goldfard, M., K. Shimizu, M. Perucho, and M. Wigler.** 1982. Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells. Nature (London) **296**:404–409.
9. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
10. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Cellular oncogenes and multistep carcinogenesis. Science **222**:771–778.
11. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) **304**:596–602.
12. **Lemischka, I. R., S. Farmer, V. R. Racaniello, and P. A. Sharp.** 1981. Nucleotide sequence and evolution of a mammalian alpha-tubulin messenger RNA. J. Mol. Biol. **151**:101–120.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. A laboratory manual, p. 202–203. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
14. **McCoy, M., J. J. Toole, J. M. Cunningham, E. H. Chang, D. R. Lowy, and R. A. Weinberg.** 1983. Characterization of a human colon/lung cacinoma oncogene. Nature (London) **302**:79–81.
15. **Murray, M. J., J. M. Cunningham, L. F. Parada, F. Dautry, P. Lebowitz, and R. A. Weinberg.** 1983. The HL-60 transforming sequence: a *ras* oncogene coexisting with altered *myc* genes in hematopoietic tumors. Cell **33**:749–757.
16. **Murray, M. J., B. Z. Shilo, C. Shih, D. Cowing, H. W. Hsu, and R. A. Weinberg.** 1981. Three different human tumor cell lines contain different oncogenes. Cell **25**:355–361.
17. **Nakano, H., F. Yamamoto, C. Neville, D. Evans, T. Mizuno, and M. Perucho.** 1984. Isolation of transforming sequences of two human lung carcinomas: structural and functional analysis of the activated *c-K-ras* oncogenes. Proc. Natl. Acad. Sci. USA **81**:71–75.
18. **Ostrander, M., S. Vogel, and S. Silverstein.** 1982. Phenotypic switching in cells transformed with the herpes simplex virus thymidine kinase gene. Mol. Cell. Biol. **2**:708–714.
19. **Perucho, M., M. Goldfard, K. Shimizu, C. Lama, J. Fogh, and M. Wigler.** 1981. Human-tumor-derived cell lines contain common and different transforming genes. Cell **27**:467–476.
20. **Perucho, M., D. Hanahan, L. Lipsich, and M. Wigler.** 1980. Isolation of the chicken thymidine kinase gene by plasmid rescue. Nature (London) **285**:207–210.
21. **Perucho, M., D. Hanahan, and M. Wigler.** 1980. Genetic and physical linkage of exogenous sequences in transformed cells. Cell **22**:309–317.
22. **Perucho, M., and M. Wigler.** 1981. Linkage and expression of foreign DNA in cultured animal cells. Cold Spring Harbor Symp. Quant. Biol. **45**:829–838.
23. **Pulciani, S., E. Santos, L. Long, V. Sorrentino, and M. Barbacid.** 1985. *ras* gene amplification and malignant transformation. Mol. Cell. Biol. **5**:2836–2841.
24. **Roberts, J. M., and R. Axel.** 1982. Gene amplification and gene correction in somatic cells. Cell **29**:109–119.
25. **Robins, D. M., S. Ripley, A. S. Henderson, and R. Axel.** 1981. Transforming DNA integrates into the host chromosome. Cell **23**:29–39.
26. **Ruley, H. E.** 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (London) **304**:602–606.
27. **Schwab, M., K. Alitalo, H. E. Varmus, J. M. Bishop, and D.**

George. 1983. A cellular oncogene (c-Ki-ras) is amplified, overexpressed and located within karyotipic abnormalities in mouse adrenocortical tumor cells. Nature (London) **303:** 497–501.

28. **Seeburg, P. H., W. W. Colby, D. J. Capon, D. V. Goeddel, and A. D. Levinson.** 1984. Biological properties of human c-H-ras-1 genes mutated at codon 12. Nature (London) **312:**71–75.
29. **Shih, T. Y., M. O. Weeks, H. A. Young, and E. M. Scolnick.** 1980. p21 kirsten murine sarcoma virus is thermolabile in a viral mutant temperature sensitive for the maintenance of transformation. J. Virol. **31:**546–556.
30. **Spandidos, D. A., and N. M. Wilkie.** 1984. Malignant transformation of early passage rodent cells by a single human oncogene. Nature (London) **310:**469–475.
31. **Stacey, D. W., and H. F. Kung.** 1984. Transformation of NIH 3T3 cells by microinjection of Ha-ras p21 protein. Nature (London) **310:**503–511.
32. **Sweet, R. W., M. V. Chao, and R. Axel.** 1982. The structure of the thymidine kinase gene promoter: nuclease hypersensitivity correlates with expression. Cell **31:**347–353.
33. **Taparowsky, E., Y. Suard, O. Fasano, K. Shimizu, M. Goldfarb, and M. Wigler.** 1982. Activation of the T-24 bladder carcinoma transforming gene is linked to a single amino acid change. Nature (London) **300:**762–765.
34. **Taya, Y., K. Hosogai, S. Hirahasi, Y. Shimosato, R. Tsuchiya, N. Tsuchida, M. Fushimi, T. Sekiya, and S. Nishimura.** 1984. A novel combination of K-*ras* and myc amplification accompanied by point mutational activation of K-*ras* in a human lung cancer. EMBO J. **3:**2943–2946.
35. **Topp, W.** 1981. Normal rat cell lines deficient in nuclear thymidine kinase. Virology **113:**408–411.
36. **Varmus, H.** 1984. The molecular genetics of cellular oncogenes. Annu. Rev. Genet. **18:**553–612.
37. **Wigler, M., M. Perucho, D. Kurtz, S. Dana, A. Pellicer, R. Axel, and S. Silverstein.** 1980. Transformation of mammalian cells with an amplifiable dominant-acting gene. Proc. Natl. Acad. Sci. USA **77:**3567–3570.
38. **Winter, E., C. Palatnik, D. Williams, J. R. E. Wells, L. S. Coles, and J. S. Gordon.** 1985. Changes in the H-1 histone complement during myogenesis. II. Regulation by differential coupling of H-1 variant mRNA. J. Cell Biol. **101:**175–181.
39. **Winter, E., F. Yamamoto, C. Almoguera, and M. Perucho.** 1985. A method to detect and characterize point mutations in transcribed genes: amplification and overexpression of the mutant c-Ki-*ras* allele in human tumor cells. Proc. Natl. Acad. Sci. USA **82:**7555–7559.
40. **Yamamoto, F., and M. Perucho.** 1984. Activation of a human c-K-*ras* oncogene. Nucleic Acids Res. **12:**8873–8885.