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Causal Role for an Activated N-*ras* Oncogene in the Induction of Tumorigenicity Acquired by a Human Cell Line[1]

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ABSTRACT

***ras* oncogenes have been found in approximately 15% of the human tumors analyzed. However, a causal role for these genes in the tumorigenesis of human cells has yet to be shown. Tumorigenic late-passage PA-1 human teratocarcinoma cells (L-PA-1) contain an activated N-*ras* gene. In this report evidence is presented that nontumorigenic early passage revertant PA-1 cells (E-PA-1) contain only the germ-line protooncogene. Introduction by gene transfer of the activated L-PA-1 oncogene induces E-PA-1 cells to form tumors, suggesting that the activated N-*ras* oncogene has a causal role in the tumorigenesis of these cells.**

INTRODUCTION

Oncogenes in the *ras* family are forms of the germ-line protooncogenes with specific point mutations that when transfected onto NIH-3T3 murine fibroblasts induce foci of morphologically altered cells (1–13). The normal *ras* genes code for proteins of molecular weight of approximately 21,000; they have guanine nucleotide binding activity and are able to hydrolyze GTP (GTPase) (14). The oncogenic proteins with point mutations at amino acid positions 12 or 61 retain the ability to bind guanine nucleotides but have reduced GTPase activity (15–17). Approximately 15% of human tumors contain activated transfectable oncogenes. However, their role in the oncogenesis of human neoplasms is unclear. It has yet to be determined whether these genes have a causal role in tumor induction or are mutated simply as a consequence of their presence in tumor cells with increased mutation rates. Chemical carcinogenesis studies in rats have shown that specific oncogene activations can be detected following carcinogen treatment (18–21). However, it has not been demonstrated that these altered genes were responsible for tumor induction in these systems.

The present study addresses this issue using PA-1 human teratocarcinoma cells (22), a pseudodiploid cell line isolated by culturing the ascites fluid from a 12-year-old girl with an ovarian teratocarcinoma. PA-1 cells at early passages have reverted and do not form tumors in athymic nude mice, whereas late passage cells (>100) remain pseudodiploid but acquire the ability to form tumors in nude mice. In a previous report (23) it was shown that the presence of an activated oncogene correlated with the ability of PA-1 cells to form tumors in athymic nude mice. The tumorigenic PA-1 cells contain an oncogene that was activated by a point mutation at codon 12 of the *ras* p21 protein. In contrast, transfection of DNA isolated from E-PA-1 cells into NIH-3T3 murine fibroblasts failed to detect this activated N-*ras*, which was readily detected using DNA from L-PA-1 cells. In light of the extraordinary karyotypic stability of these cells, these data implied a role for the activated oncogene in the tumorigenic transformation of L-PA-1. In this report we demonstrate a causal role for the activated N-*ras* in the tumorigenicity of PA-1 cells. This was accomplished by introducing this oncogene into nontumorigenic, early passage cells and showing that the resulting cells exhibit a tumorigenic phenotype when injected into athymic nude mice. The nontumorigenic early passage cells used in this study retain some properties of a teratocarcinoma but represent revertants from the transformed phenotype.

MATERIALS AND METHODS

Cell Culture. PA-1 cells were grown in modified Eagle's medium with 10% fetal calf serum. The cells were subcultured weekly by treatment with 0.25% trypsin in phosphate-buffered saline with 2 mM EDTA. High-molecular-weight DNA was prepared from animal cells as previously described (24).

Molecular Cloning. We cloned the N-*ras* gene in DNA from passage 41 E-PA-1 cells to make certain that it did not contain an activating point mutation. The N-*ras* gene was contained on *Eco*RI fragments of 9 and 7 kilobases (25). Since the point mutation responsible for the activation of the N-*ras* L-PA-1 cells resides on the 9-kilobase 5′ *Eco*RI fragment, we cloned this fragment from DNA isolated from E-PA-1 cells at passage 41. DNA that had been digested to completion with *Eco*RI was electrophoresed on preparative agarose gels, and fractions containing 9 kilobase fragments that hybridized with a 5′-specific N-*ras* probe were isolated. The DNA in these fractions was ligated to purified *Eco*RI arms of the bacteriophage vector λ GT WES B (26), packaged, and screened by plaque hybridization to the N-*ras* "R" probe (9). The *Eco*RI fragments from eight independently isolated phage were ligated to the 3′ N-*ras* 7-kilobase fragment cloned from human placental DNA (a gift from M. Wigler) and tested for biological transforming activity by transfection in the NIH-3T3 focus formation assay.

Transfection. DNA-mediated transfection was performed by the method of Graham and Vander Eb (27) as previously described (13). Monolayers of NIH-3T3 cells were carried at confluency for 14–21 days after transfection in preselected calf serum in DMEM[3] and observed for the appearance of foci of morphologically altered cells.

Protoplast Fusion. Protoplast fusion (28) is a useful gene transfer method to introduce cloned genes into certain cells that, like PA-1 human teratocarcinoma cells, do not survive the standard transfection protocol. Human recipient cells received oncogene plasmid constructions by protoplast fusion, and the resulting cell lines were analyzed for tumor formation in athymic nude mice. By linking the genes of interest to the plasmid pSV2-neo (29), which is selectable for resistance to the neomycin analogue G-418, oncogenes could be introduced and selected in a single step. The resulting drug-resistant cells were used as a pool or as individual colony derived clonal lines. Plasmids that were to be introduced into cells were freshly transformed into the *Escherichia coli* DH-1 prior to each experiment. A bacterial colony was picked 2 days before the actual fusion experiment, and an overnight culture was prepared in L-broth with the appropriate antibiotic. We added 0.2 ml of the overnight culture to 50 ml of L-broth with appropriate antibiotic in 250-ml flasks and grew this culture to A600 = 0.5, at which time chloramphenicol was added to a concentration of 0.2 mg/ml. This culture was then incubated for an additional 14–18 h. The PEG (BDH

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[3] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; PEG, polyethylene glycol 1500.

Chemical Co.) was prepared the day before the fusion experiment: 50 ml of molten PEG was mixed with 50 ml of DMEM and filtered immediately through a Nalgene 0.45-μm sterilizing unit. This 50% PEG was kept overnight in a tissue culture incubator in 50-ml sterile plastic tubes.

The protoplasts were prepared as follows: each 50-ml bacterial culture was centrifuged at 4000 rpm in a Sorvall RT-6000 centrifuge for 10 min. The medium was decanted and the bacteria resuspended on ice in 2.5 ml of a buffer containing 20% sucrose and 50 mM Tris-HCl, pH 8.0. This suspension was incubated on ice with 0.5 ml of 5 mg/ml lysozyme (freshly prepared) in 0.25 M Tris-HCl, pH 8.0, for 5 min, at which point 1 ml of 0.25 M EDTA, pH 8.0, was added. After 5 min, 1 ml of 50 mM Tris-HCl, pH 8.0, was added and this mixture incubated at 37°C until the bacteria were converted to protoplasts (usually 15 min) as judged by phase contrast microscopy. The protoplasts were then diluted very slowly and gently with 20 ml serum-free DMEM containing 10% sucrose and 10 mM MgCl and kept at 37°C for 15 min.

Recipient cells for protoplast fusion were seeded at 300,000/60-mm dish 14–18 h before the experiment. The cells were washed once with 5 ml of prewarmed serum-free DMEM; 6.25 ml of the protoplast suspension was then added to each 60-mm dish and centrifuged in a Sorvall RT-6000 centrifuge at 2000 rpm for 12 min at room temperature. At the inside edge of the tissue culture dish nearest the center of rotation, few cells or protoplasts remained after centrifugation. Therefore, all subsequent additions were performed at this edge. The medium was removed by aspiration and 2.5 ml of the 50% PEG was added. After 2 min the 50% PEG was removed and the cells washed five times with prewarmed serum-free DMEM. Complete DMEM was then added to the cells, which were then returned to the incubator. After 24 h the cells from one 60-mm dish were trypsinized and transferred to a T-75 flask. After an additional 24 h the selection medium was added. The culture medium was changed twice weekly until large colonies were apparent. (No cells remained in control cultures receiving no plasmid.) The G-418-resistant colonies were then subcultured, expanded, and inoculated into animals.

Animal Inoculations. Athymic nude mice, 3–6 weeks old, were inoculated s.c. with 1,000,000 cells in 0.1 ml Hanks' balanced salt solution at an intrascapular location. Mice were treated with X-rays at a dose of 500 rads 24 h prior to injection and checked weekly for tumor growth and general health. Mice were sacrificed when their tumors reached 1–2 cm by cervical dislocation and the tumors excised under sterile conditions for the establishment of cell lines and the extraction of DNA.

RESULTS

Previous transfection studies (23) showed that DNA from nontumorigenic E-PA-1 cells does not contain the activated N-*ras* oncogene that was found in DNA from L-PA-1 cells. At that time we correlated the presence of this oncogene with tumorigenicity. It was possible that the activated oncogene was present in the E-PA-1 cells but sequestered by some mechanism (such as methylation) that masked its biological activity in transfection assays. Therefore, the N-*ras* locus from DNA isolated from passage 41 E-PA-1 cells was molecularly cloned. The gene is contained on *Eco*RI fragments of 9 and 7 kilobases (24). Since the point mutation responsible for the activation of N-*ras* in L-PA-1 cells resides on the 9-kilobase 5′ *Eco*RI fragment, this fragment was cloned from DNA isolated from E-PA-1 cells at passage 41. The 9-kilobase 5′ N-*ras* *Eco*RI fragments from eight independently isolated phage were ligated to the 3′ N-*ras* 7-kilobase fragment cloned from human placental DNA (a gift from M. Wigler) and tested for biological transforming activity in the NIH-3T3 focus formation assay. None of the eight early passage genes induced foci on transfection, whereas genes constructed utilizing the equivalent *Eco*RI fragment cloned from L-PA-1 DNA produced numerous foci. Therefore, the N-*ras* genes in E-PA-1 cells did not contain an activating mutation within the 5′ portion of the gene.

Table 1 *Tumorigenicity of plasmid bearing PA-1 clone 1 cells*

PA-1 cells with the pSV2-neo N-*ras* plasmid construction were inoculated s.c. into 3–6-week-old athymic nude mice at 1,000,000 cells/animal. Mice were observed for tumor growth. Experiment 1 was performed using cell lines derived from individual G-418-resistant colonies. Experiments 2–4 were performed using pools of 20-100 G-418-resistant colonies.

	pSV2-neo N-*ras* oncogene		pSV2-neo	
Exp.	Fractions with tumors	Latent period (weeks)	Fractions with tumors	Latent period (weeks)
1	1/3 2/3 2/3 0/3 0/3 0/3	12 13 21 22 25	0/3 0/3	>40
2	5/8	15 18 19 21 22	NT[a]	
3	1/3	7	0/3	>40
4	2/3	8 13	0/3	>40

[a] NT, not tested.

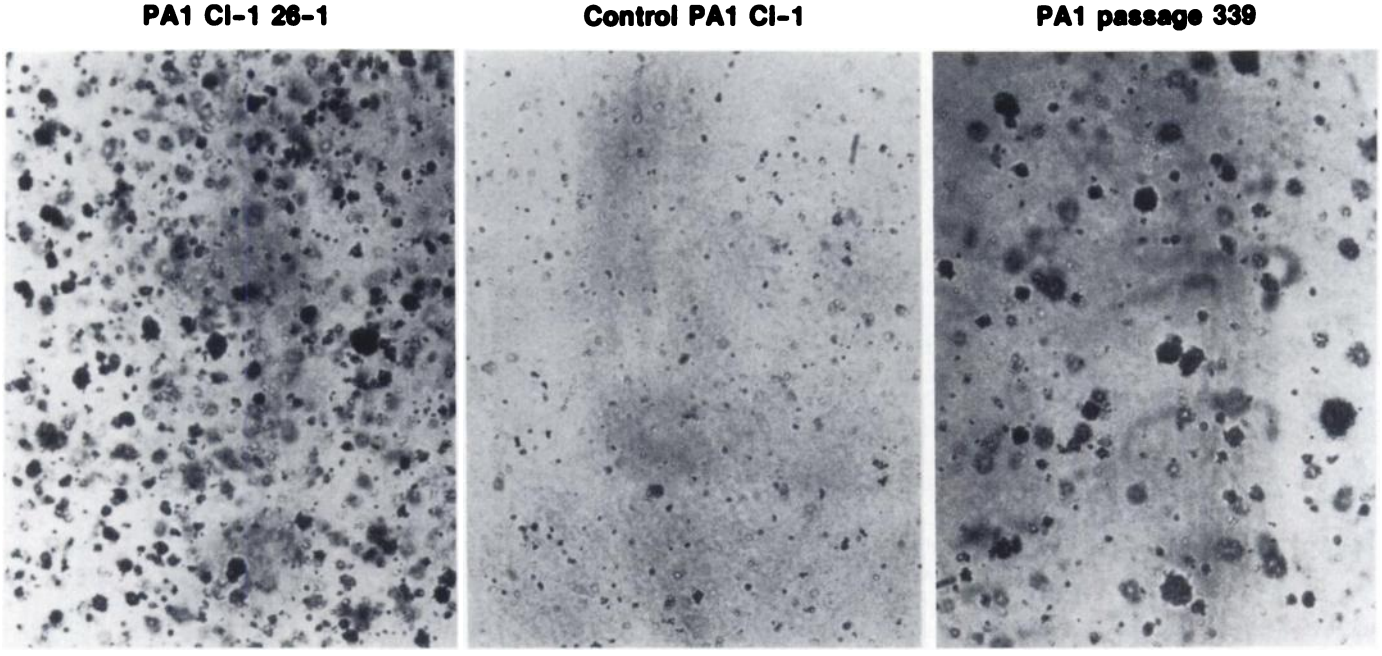

Fig. 1. Anchorage-independent growth of PA-1 cells. Sixty-mm tissue culture plates were layered with 2 ml of 0.6% agarose in DMEM with 10% fetal bovine serum. The indicated PA-1 cells were suspended in 4 ml of DMEM with 10% fetal bovine serum with 0.3% agarose. The plates were photographed after 14 days.

In order to determine whether this activated N-*ras* oncogene had a causal role in the tumorigenesis of these cells, gene transfer studies were undertaken. The goal of this study was to determine whether a nontumorigenic E-PA-1 cell line could form tumors in athymic nude mice after the addition of the activated oncogene. For these experiments a nontumorigenic clone of E-PA-1 cells (clone 1) which was isolated at passage 63 was used. The cloned cells were carried an additional 50 passages before these experiments during which time they remained nontumorigenic (Table 1).

The activated N-*ras* was cloned onto the drug-selectable plasmid pSV2-neo (26). Introduction of this plasmid into recipient cells, followed by selection with the neomycin analogue G-418, should select for a population of cells carrying both the selected plasmid sequences and the N-*ras* oncogene. Since PA-1 cells do not survive the standard calcium phosphate transfection procedure (27), protoplast fusion (28) was employed to introduce the construct into the recipient cells. In an initial experiment six G-418-resistant colonies were isolated and expanded into cell lines. Three of these six cell lines also were able to exhibit anchorage-independent growth (Fig. 1) and form tumors in athymic nude mice (Table 1). We presume that the three colony-derived cell lines which do not form tumors in nude mice may have alterations in the oncogene which inactivate its transforming potential. Two cell lines derived from transfection of the pSV2-neo vector alone did not exhibit anchorage-independent growth or form tumors in nude mice.

Further, in multiple independent experiments, clone 1 E-PA-1 cells into which the activated N-*ras* was introduced acquired the ability to form tumors in nude mice. Since we had found that as few as 100 L-PA-1 cells in a mixture with 1,000,000 nontumorigenic E-PA-1 cells could form tumors, the remaining three experiments were performed using pools of G-418-resistant colonies. These populations were able to form tumors when injected into the nude mice (Table 1). Thus either method (individual colonies or pools of colonies) appears capable of giving rise to tumorigenic PA-1 cells, and the presence of nontumorigenic cells apparently does not inhibit tumor formation by the transformed cells. Introduction of the pSV2-neo vector alone or a pSV2-neo construct containing the normal human N-*ras* protooncogene into clone 1 cells did not result in tumor formation in athymic nude mice when pools of G-418-resistant colonies were employed.

DNA from the clone 1 N-*ras* transformants prior to injection into mice, as well as DNA from tumors formed by these cells was analyzed by Southern blotting to determine the state of the newly acquired N-*ras* sequences. The N-*ras* from L-PA-1 cells, which was cloned from a secondary NIH-3T3 focus, was truncated during transfection 5′ to the gene. This produced a 5′ N-*ras Eco*RI fragment of 7-kilobase pair rather than the 9-kilobase pair fragment found in normal human cells and PA-1 cells themselves. This alteration is unrelated to the oncogene activation which was determined by DNA sequence analysis to be due to an amino acid 12-point mutation. This size difference allows the analysis of the oncogene introduced by gene transfer in presence of the endogenous N-*ras* protooncogene sequence. Because we observed (Fig. 2) approximately equal hybridization to the endogenous N-*ras* and the exogenous N-*ras* (introduced by gene transfer) we concluded that approximately single copy levels of the N-*ras* gene were introduced by gene transfer in the G-418-selected PA-1 cells prior to injection into athymic nude mice. No change in copy number was observed in the tumors formed after injection into athymic nude mice, indicating that gene amplification of the N-*ras* oncogene sequences is not required for tumor formation by PA-1 clone 1 cells. This was true whether the tumors were derived from individual G-418-resistant colonies (Fig. 2, *lanes 4* and *5*) or pools of colonies (Fig. 2, *lane 3*). Chromosomal banding analyses of the cells before and after the pSV2-neo N-*ras* construct were indistinguishable (data not shown). Therefore, the induction of the tumorigenic phenotype of PA-1 cells was consistent with its being the result of the addition of a single copy of an activated oncogene.

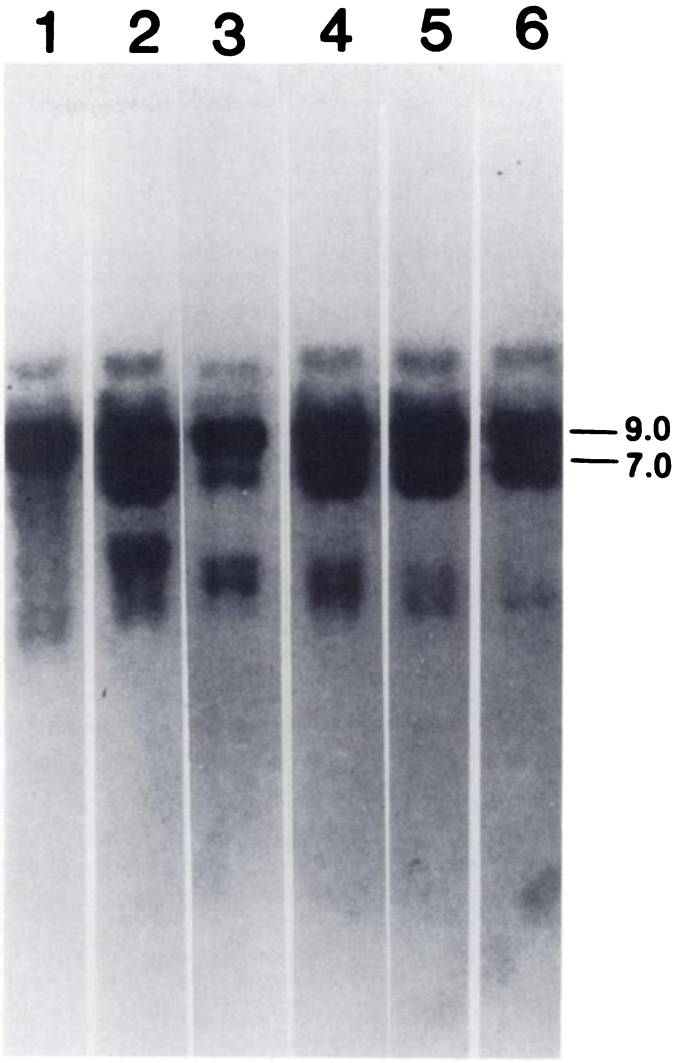

Fig. 2. Southern blot analysis: PA-1 genomic DNA samples (10 μg) were digested with the restriction enzyme *Eco*RI and electrophoresed on a 0.8% agarose gel and blotted and hybridized as previously described (26) with the N-*ras* 9. *Lane 1*, passage 120 PA-1; *lane 2*, tumor 32b forms, a pooled population of clone 1 PA-1 cells containing the pSV2-neo N-*ras* plasmid; *lane 3*, pooled population which gave rise to tumor 32b; *lanes 4* and *5*, tumors derived from a cell line, 26-1, established from a single G-418 resistant colony; *lane 6*, cell line 26-1.

DISCUSSION

PA-1 cells were shown to be tumorigenic in athymic nude mice when they contained an activated N-*ras* oncogene and nontumorigenic when it was absent. In order to prove that the activated N-*ras* oncogene caused the tumorigenicity, the oncogene was introduced into a nontumorigenic E-PA-1 cell clone: the resulting cells were tumorigenic. Introduction by gene transfer of the nonmutated protooncogene did not induce tumorigenesis. These data suggest that the activated N-*ras* fulfills Koch's postulates for a causal role in the acquisition of the

tumorigenic phenotype by PA-1 teratocarcinoma cells. It was curious that of the animals which received potentially tumorigenic populations of cells some did not develop tumors. It remains a possibility that the activated N-*ras* initiates a series of steps one or more of which may be rate limiting in tumorigenic process. The long latent period for some of the tumors may also indicate that another event initiated by the activated N-*ras* is required for tumorigenesis.

It remains to be determined how activated *ras* oncogenes induce the ability to form tumors. The activated oncogenes may simply increase the growth rate of these cells or they may alter some other factor in the cell that affects the growth in the animal host. Since the growth rates of PA-1 clone cells with gene transfer of the oncogene or protooncogene are similar, we suggest the latter holds true.

REFERENCES

1. Shih, C., Shelo, B. Z., Goldfarb, M. P., Dannenberg, A., and Weinberg, R. A. Passage of phenotypes of chemically transformed cells via transfection of DNA and chromatin. Proc. Natl. Acad. Sci. USA, *76:* 5714–5718, 1979.
2. Chang, E., Furth, M., Scolnick, E., and Lowy, D. Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature (Lond.), *297:* 479–483, 1982.
3. Parada, L. F., Tabin, C. J., Shih, C., and Weinberg, R. A. Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene. Nature (Lond.), *297:* 474–478, 1982.
4. Santos, E., Tronick, S. R., Aaronson, S. A., Pulciani, S., and Barbacid, M. T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. Nature (Lond.), *298:* 343, 1982.
5. Der, C. J., Krontris, T. G., and Cooper, G. M. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. Proc. Natl. Acad. Sci. USA, *79:* 3637–3640, 1982.
6. Der, C. J., and Cooper, G. M. Altered gene products are associated with activation of cellular ABSk genes in human lung and colon carcinomas. Cell, *32:* 201–208, 1983.
7. Goldfarb, M. P., Shimizu, K., Perucho, M., and Wigler, M. H. Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells. Nature (Lond.), *296:* 405–409, 1982.
8. Hall, A., Marshall, C. J., Spurr, N. K., and Weiss, R. A. Identification of transforming gene in two human sarcoma cell lines as a new member of the ras gene family located on chromosome 1. Nature (Lond.), *303:* 396, 1983.
9. Shimizu, K., Goldfarb, M., Perucho, M., and Wigler, M. Isolation and preliminary characterization of the transforming gene of a human neuroblastoma cell line. Proc. Natl. Acad. Sci. USA, *80:* 383–387, 1983.
10. Reddy, E. P., Reynolds, R. K., Santos, E., and Barbacid, M. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature (Lond.), *300:* 149–152, 1982.
11. Tabin, C. J., Bradley, S., Bargmann, C., Weinberg, R., Papageorge, A., Scolnick, E., Dhar, R., Lowy, D., and Chang, E. Mechanism of activation of a human oncogene. Nature (Lond.), *300:* 143–149, 1982.
12. Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M., and Wigler, M. Activation of the T24 bladder carcinoma transforming gene is linked to a single amino-acid change. Nature (Lond.), *300:* 762–768, 1982.
13. Cooper, C. S., Blair, D. G., Oskarsson, M. K., Tainsky, M. A., Eader, L. A., and Vande Woude, G. F. Characterization of human transforming genes from chemically-transformed, teratocarcinoma, and pancreatic carcinoma cell lines. Cancer Res., *44:* 1–10, 1984.
14. Papageorge, A., Lowy, D., and Scolnick, E. Comparative biochemical properties of p21 ras molecules coded for by viral and cellular ras genes. J. Virol., *44:* 509–519, 1982.
15. Sweet, R. W., Yokoyama, S., Kamata, T., Feramisco, J., Rosenberg, M., and Gross, M. The product of *ras* is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature (Lond.), *311:* 273–275, 1984.
16. McGrath, J., Capon, D., Goeddel, D., and Levinson, A. Comparative biochemical properties of normal and activated human ras p21 protein. Nature (Lond.), *310:* 644–649, 1984.
17. Gibbs, J., Sigal, I., Poe, M., and Scolnick, E. Intrinsic GTPase activity distinguishes the normal and oncogenic *ras* p21 molecules. Proc. Natl. Acad. Sci. USA, *81:* 5704–5709, 1984.
18. Sukamar, S., Notario, S. V., Martin-Zanca, D., and Barbacid, M. Induction of mammary carcinomas in rats by nitroso-methylurea involves malignant activation of *H-ras-1* locus by single point mutations. Nature (Lond.), *306:* 658–661, 1983.
19. Balmain, A., and Pragnell, I. B. Mouse skin carcinomas induced *in vivo* by chemical carcinogenes have a transforming Harvey-*ras* oncogene. Nature (Lond.), *303:* 72–74, 1983.
20. Eva, A., and Aaronson, S. A. Frequent activation of c-*kis* as a transforming gene in fibrosarcomas induced by methycholanthrene. Science, (Wash. DC), *220:* 955–956, 1983.
21. Guerrero, I., Calzada, P., Mayer, A., and Pellicer, A. A molecular approach to leukemogenesis: mouse lymphomas contain an activated C-*ras* oncogene. Proc. Natl. Acad. Sci. USA, *81:* 202–205, 1984.
22. Zeuthen, J., Norgaard, J. D. R., Avner, P., Fellows, M., Wartiovaara, J., Vaheri, A., Rosen, J., and Giovannela, B. C. Characterization of a human ovarian teratocarcinoma-derived cell line. Int. J. Cancer, *25:* 19–32, 1980.
23. Tainsky, M. A., Cooper, C. S., Giovanella, B. C., and Vande Woude, G. F. An activated rasN gene: detected in late but not early passage human PA-1 teratocarcinoma cells. Science (Wash. DC), *225:* 643–645, 1984.
24. Tainsky, M. A. Analysis of the virogenes related to the rhesus monkey endogenous type C retrovirus in monkeys and apes. J. Virol., *37:* 922–930, 1981.
25. Taparowsky, E., Shimza, K., Goldfarb, M., and Wigler, M. Structure and activation of the human N-*ras* gene. Cell, *34:* 581–586, 1983.
26. Tilghman, S. M., Tiemeier, D. C., Polsky, F., Edgell, M., Seidman, J. G., Leder, A., Enquist, I. W., Norman, B., and Lee, P. Cloning specific segments of the mammalian genome: bacteriophage lambda containing mouse globin and surrounding gene sequences. Proc. Natl. Acad. Sci. USA, *74:* 4406–4410, 1977.
27. Graham, F. L., and Vander Eb, A. J. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology *52:* 456, 1973.
28. Yoakum, G. H. Protoplast-fusion: a method to transfect human cells for gene isolation, oncogene testing and construction of specialized cell lines. Bio. Techniques, 24–29, 1984.
29. Berg, P., and Southern, P. J. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. Mol. Appl. Genet., *1:* 327–341, 1982.