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Neoplastic transformation of immortalized human epidermal keratinocytes by ionizing radiation

(radiation carcinogenesis/adenovirus type 12/simian virus 40/p21/malignant transformation)

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ABSTRACT **Efforts to investigate the progression of events that cause human cells to become neoplastic in response to ionizing radiation have been aided by the development of tissue culture systems of epithelial cells. In the present study, nontumorigenic human epidermal keratinocytes immortalized by adenovirus type 12 and simian virus 40 have been transformed by exposure to x-ray irradiation. Such transformants showed morphological alterations, formed colonies in soft agar, and induced carcinomas when transplanted into nude mice, whereas primary human epidermal keratinocytes exposed to radiation in this manner failed to show any evidence of transformation. These findings demonstrate the malignant transformation of human primary epithelial cells in culture by the combined action of a DNA tumor virus and radiation, indicating a multistep process for radiation-induced neoplastic conversion. This *in vitro* system may be useful as a tool for dissecting the process of radiation-induced neoplastic transformation of human epithelial cells and for detecting previously unreported human oncogenes.**

It is now well-accepted that cancer develops in a multistep fashion and that environmental exposures, particularly to physical, chemical, and biological agents, are major etiological factors (1, 2). While the majority of studies of transformation have relied on the use of rodent cells in culture, experimental models to define the role of carcinogenic agents in the development of cancers must be established by using human cells. Unlike rodent cells, normal human cells in culture do not undergo spontaneous transformation and have generally proved to be resistant to neoplastic transformation by carcinogens. Previous attempts to induce neoplastic transformation of human cells have mostly been with fibroblastic cells, which are relatively easy to culture. While the use of tumor viruses (3, 4), x-ray (5, 6), and chemical carcinogens (7, 8) have led to the development of established, biologically abnormal lines of fibroblasts, neoplastic transformation has proven very difficult to achieve. Since most human cancers are of epithelial origin, it is important to obtain a better understanding of this particular cell type. Until recently, our inability to grow epithelial cells in culture has made it difficult to define the process of neoplastic transformation of human epithelial cells. To our knowledge, there is no report so far in which radiation-induced neoplastic transformation of human epithelial cell cultures has been described.

We have recently developed an *in vitro* multistep model suitable for the study of human epithelial cell carcinogenesis (9, 10). Upon infection with adenovirus type 12 and simian virus 40 (Ad12–SV40) hybrid virus, primary human epidermal keratinocytes acquire an indefinite lifespan in culture but do not undergo malignant conversion. Subsequent addition of Kirsten murine sarcoma virus (Ki-MSV), which contains the Ki-*ras* oncogene or chemical carcinogens *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or 4-nitroquinoline-1-oxide, induces striking morphological alterations and the acquisition of neoplastic properties. The availability of a human epithelial cell line that can undergo neoplastic conversion in response to a *ras* oncogene or chemical carcinogens has led us to inquire whether this system may be useful in detecting x-ray irradiation as a carcinogenic agent for human epithelial cells.

MATERIALS AND METHODS

Cell Cultures and Media. The human epidermal keratinocyte line, designated RHEK-1 (F-4582), was used at passage 23 for these transformation studies. This cell line was established from primary foreskin epidermal keratinocytes after infection with the Ad12–SV40 hybrid virus (9). This cell line did not produce virus, had a "flat" epithelial morphology, and expressed a number of markers associated with epithelial cells. It also contained the SV40 tumor antigen but was not tumorigenic in nude mice. Growth and maintenance medium for these cells consisted of Dulbecco's minimal essential medium with 10% fetal bovine serum, hydrocortisone (5 μg/ml), and gentamicin (10 μg/ml).

Transformation Assay. One-day-old cultures of the RHEK-1 cells (plated at 5×10^5 cells per 80-cm^2 flask) were irradiated with graded doses of x-rays (0, 2, 4, 6, and 8 Gy). Irradiations were performed with an ATC-4 4-MeV linear accelerator at a dose rate of 2.3 Gy·min^{-1} at a distance of 80 cm. After irradiation, the cultures were allowed to grow to confluence with a change of medium every 3 days, were subsequently passaged by trypsin treatment, and were irradiated again with similar doses. Cultures were subcultured every 7–10 days and observed biweekly for changes in morphology or growth pattern.

Colony Formation in Soft Agar. Cell suspension (1×10^5 cells per ml) in 5 ml of 0.35% Noble agar was overlaid in a 60-mm dish containing a 0.6% agar base. Viable colonies were scored at 21 days.

Tumorigenicity in Nude Mice. Adult NIH/Swiss athymic nude mice were inoculated subcutaneously with 1×10^7 and 1×10^6 freshly trypsin-treated cells to determine tumorigenicity.

Analysis for *ras* p21 Production. [^{35}S]Methionine-labeled cell extracts from untransformed and transformed cells were immunoprecipitated by using anti-p21 monoclonal antibody Y13-259 (11) and analyzed by SDS/PAGE as described (9).

RESULTS

Transformation of RHEK-1 Cells by Ionizing Radiation. After exposure of primary human epidermal keratinocytes to

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Abbreviations: Ad12–SV40, adenovirus type 12 and simian virus 40 hybrid virus; Ki-MSV, Kirsten murine sarcoma virus.

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various graded fractions of radiation, no morphological differences between irradiated and control unirradiated cultures could be seen. Neither control nor irradiated cultures were able to grow serially beyond two or three subcultures. The cells underwent progressive deterioration and were lost. In contrast, the RHEK-1 line irradiated twice at either 2 or 4 Gy demonstrated morphological alterations of cells and an abnormal pattern of growth by the third subculture (6–7 weeks after irradiation). Similar changes were not observed in the unirradiated and twice 6- and 8-Gy-treated RHEK-1 cells. Changes were first observed in the cells irradiated with two doses of 4 Gy; however, morphological alterations in the cells irradiated with two doses of 2 Gy became more pronounced after further subcultivation. These morphological changes were similar to those observed after exposure to chemical carcinogens (10)—namely, the transformed cells began to pile up in focal areas, formed small projections, and released round cells from the foci (Fig. 1 *A* and *B*). These foci grew in chains or as islets that stained heavily with Giemsa. In contrast, the cellular morphology in the cells irradiated with

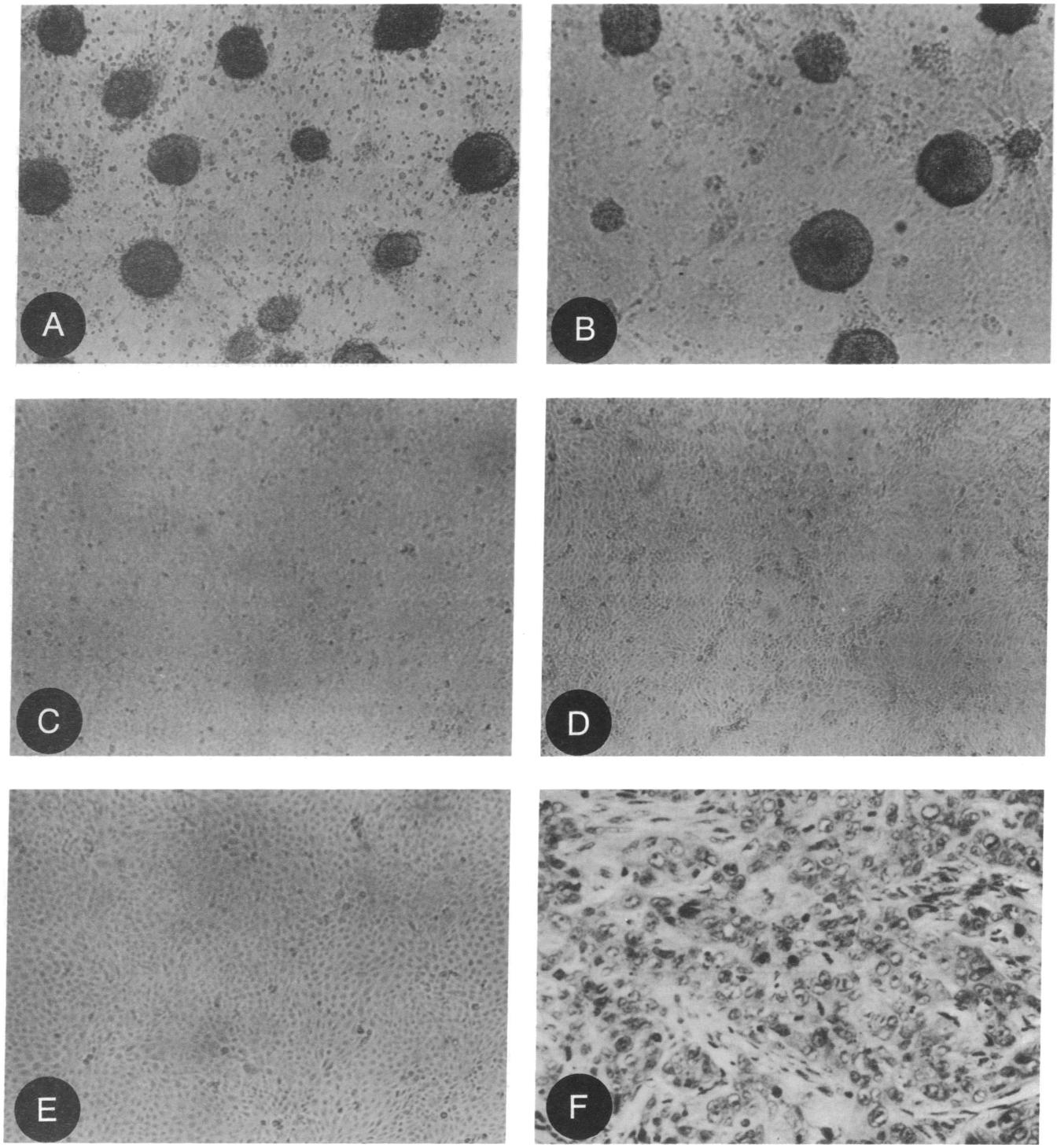

FIG. 1. Human epidermal keratinocytes (RHEK-1) irradiated with x-rays twice, followed by subculture three times in nutrient medium. (*A*) Four grays (2 Gy twice). (*B*) Eight grays (4 Gy twice). (*C*) Twelve grays (6 Gy twice). (*D*) Sixteen grays (8 Gy twice). (*E*) Unirradiated. (*F*) *In vivo* tumor induced by RHEK-1 cells treated with 4-Gy (2 Gy twice) x-ray; moderately differentiated squamous carcinoma.

Table 1. Biological properties of RHEK-1 human epidermal cell line transformed by radiation

Total dose	Saturation density, cells per $cm^2 \times 10^{-5}$	Soft agar colony formation, %	Nude mice with tumors	
			10^7 cells	10^6 cells
None	2.1	<0.01	0/4	0/4
4 Gy (2 Gy twice)	4.5	0.56	2/4*	ND
4 Gy (2 Gy twice) SA	5.2	0.63	4/4*	4/4
8 Gy (4 Gy twice)	4.6	0.22	2/4	ND
8 Gy (4 Gy twice) SA	4.8	0.30	3/4	ND
12 Gy (6 Gy twice)	2.5	0.02	0/0	ND
16 Gy (8 Gy twice)	2.7	<0.01	0/4	ND

SA, lines derived from soft agar colonies; ND, not done. Saturation density was measured as the maximum number of cells obtained after initial plating with 3×10^3 cells per cm^2 and then incubating at 36°C with growth medium changed every 3 days.
*Tumors were reestablished in tissue culture and confirmed as human; their resemblance in the cells of origin was determined by karyological analysis.

6 and 8 Gy and in the unirradiated RHEK-1 epithelial cells remained unchanged. They continued to grow as nonoverlapping round to polygonal adherent cells that were flat and cobblestone-like in appearance (Fig. 1 *C–E*).

Characteristics of Radiation-Transformed RHEK-1 Cell Lines. The radiation-transformed cells were further characterized by quantitative differences in growth properties, such as saturation density and soft agar colony-forming efficiency associated with the neoplastic phenotype. The saturation densities of the radiation transformants were 2–3 times higher than that of the unirradiated RHEK-1 cells. Moreover, the radiation transformants grew in soft agar with colony-forming efficiencies of 0.2–0.6%, whereas the unirradiated and twice 6- and 8-Gy-irradiated cells did not grow in soft agar (Table 1).

Evidence for the human origin of all the cell lines was obtained by isoenzyme analysis and cell membrane species-specific immunofluorescence. The chromosomal findings are summarized in Table 2. A detailed description of the banding patterns will be reported elsewhere. All the cell lines are aneuploid human male (XY) derivatives with chromosome counts in the near-diploid range. Unirradiated and twice 2- and 4-Gy-transformed cells had five marker chromosomes and were quite alike, the only minor variances being observed in the normal and marker chromosome distributions. Abnormality of chromosome 5 was observed in some metaphases of karyotyping in each of these cell lines. The twice 4-Gy-transformed cell line had a significantly higher population of tetraploid cells than any of the other cell lines. The twice 6- and 8-Gy-irradiated, untransformed cells both had five marker chromosomes and a new marker chromosome that involved a deletion in the q arm of chromosome 11. This marker was more prominent in the twice 8-Gy-irradiated cells. Random alterations in chromosomes 1 and 9 were found in both of these cells. Chromosome 5 was altered in the metaphases selected for karyotyping in the twice 8-Gy-irradiated cells.

Tumorigenicity of Radiation-Transformed Cells. When athymic nude mice were inoculated subcutaneously with 10^7 radiation-transformed cells, the animals developed tumors within 3–4 weeks. The transformed lines derived from soft agar colonies were highly tumorigenic; all the mice inoculated with as few as 10^6 twice 2-Gy-irradiated transformed cells developed progressively growing tumors within 4 weeks (Table 1). Such tumors were diagnosed as squamous cell carcinomas (Fig. 1*F*) and the cells were typically arranged in sheets, with individual cells often containing keratohyalin granules or prekeratin. Cultures established from these tumors resembled the radiation-treated cells, were confirmed as human, and resembled the cells of origin by karyological analysis. In contrast, subcutaneous inoculation of 10^7 unirradiated RHEK-1 cells into nude mice produced usually regressing cystic nodules containing epidermal cells (Table 1).

Analysis of p21 Proteins in the Radiation-Transformed Cells. Since RHEK-1 cells can be transformed by Ki-MSV infection and become tumorigenic (9), we analyzed the *ras* oncogene p21 product in the radiation-transformed as well as in the Ki-MSV-transformed RHEK-1 cells by using antibody to p21 and SDS/PAGE. In contrast to the findings in the Ki-MSV-transformed cells, neither altered mobility nor increased expression of p21 was observed in the radiation-transformed RHEK-1 cells (Fig. 2). Moreover, the DNAs from these radiation-altered cells failed to induce detectable transformed foci upon transfection of NIH 3T3 cells. These findings indicate that the activation of *ras* oncogenes is not involved in the radiation-induced human epithelial cell lines analyzed.

DISCUSSION

Our results appear to represent induction of human epithelial cancer cells in culture by the concerted action of a DNA tumor virus and x-ray radiation. At least two and possibly

Table 2. Chromosomal characteristics of x-ray-irradiated and unirradiated cells

Irradiation	Passage	% metaphases by chromosome number		Marker chromosomes					
		43–47	80+	M1	M2	M3	M4	M5	M6
None	30	89	11	+	+	+	+	+	–
2 Gy twice	7*	80	20	+	+	+	+	+	–
4 Gy twice	7*	56	44	+	+	+	+	+	–
6 Gy twice	7*	91	9	+	+	+	+	+	+
8 Gy twice	7*	96	4	+	+	+	+	+	+

M1, iso(11q); M2, 6q+; M3, t(10q;11p); M4, del(18)(q12:); M5, t(8q;9q); M6, del(11)(q14:).
*Passage number after x-ray irradiation.

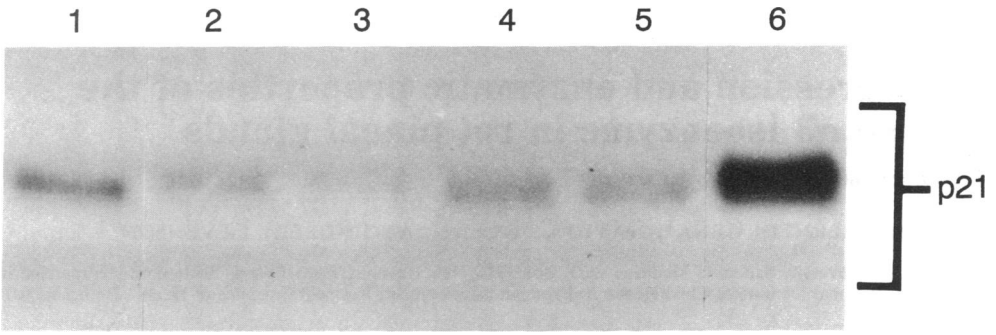

FIG. 2. Analysis of *ras* oncogene p21 product in RHEK-1 cells irradiated with x-rays. [^{35}S]Methionine-labeled cell extracts from unirradiated RHEK-1 cells (lane 1), 4-Gy (2 Gy twice)-irradiated RHEK-1 cells (lane 2), 8-Gy (4 Gy twice)-irradiated RHEK-1 cells (lane 3), 12-Gy (6 Gy twice)-irradiated RHEK-1 cells (lane 4), 16-Gy (8 Gy twice)-irradiated RHEK-1 cells (lane 5), or Ki-MSV-transformed RHEK-1 cells (lane 6) were immunoprecipitated with anti-p21 monoclonal antibody Y13-259 (11) and analyzed by SDS/PAGE as described (9).

more alterations in cell growth properties seem to be required. The measurable event was the acquisition of apparently unlimited growth potential as a result of Ad12–SV40 infection (9). Treatment of nontumorigenic early-passage Ad12–SV40 immortalized epithelial cells with radiation resulted in further changes in their growth properties. Morphological alterations as well as the abilities to grow in soft agar and to form rapidly growing squamous cell carcinomas in athymic nude mice appeared to be concomitantly acquired properties of the radiation-transformed cells.

The significance of the combined action of Ad12–SV40 virus and radiation in the induction of neoplastic human epithelial cells is emphasized by the inability of ionizing radiation to induce continued proliferation of primary epithelial cells under our assay conditions. Thus, x-ray irradiation is similar to chemical carcinogens or the Ki-*ras* oncogene in their ability to complement Ad12–SV40 virus in fully transforming human epidermal cells. Unlike the rapid transformation of RHEK-1 cells observed after Ki-MSV infection (9), the alterations in the growth pattern after radiation treatment were similar to those observed after exposure to chemical carcinogen (10). These changes were delayed in their appearance and required several subcultures for visualization. These findings suggest that multiple cell divisions are required for fixation and expression of the transformed phenotype in response to radiation. It is possible that more than one genetic lesion may be required as well. Cooperating cellular or viral oncogenes have been shown to induce neoplastic transformation of embryonic rodent fibroblasts (12–16). In addition, the combined action of γ-irradiation and Ha-*ras* oncogene has been demonstrated to produce neoplastic transformation of human fibroblasts (17). Our ability to obtain neoplastic transformation as a result of x-ray treatment of Ad12–SV40-altered human epidermal cells provides additional support for a multistep process of neoplastic conversion.

While the activation of cellular *ras* oncogenes has been demonstrated in rodent tumor induced by ionizing radiation (18–20), the activation of unique non-*ras* oncogenes has been shown in malignant radiogenic transformed rodent cells (21). The reproducible neoplastic transformation of the RHEK-1 human epithelial cell line by x-ray irradiation suggests that cellular oncogenes may be activated as part of the process. Our evidence further indicates that *ras* oncogenes, which have been commonly implicated in radiation-induced animal tumors (18–20) and spontaneous human tumors (22), were not activated in the transformation. Thus, this system may be useful in efforts to detect and characterize other cellular genes that can contribute to the neoplastic phenotype of human epithelial cells.

The carcinogenic action of ionizing radiation in humans has been well recognized from epidemiological data. The human epithelial cell system described here may be a useful *in vitro* tool for evaluating risk and dissecting the process of radiation-induced malignant transformation in human epithelial cells, the cell type from which carcinomas are derived.

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1. Farber, E. (1984) *Cancer Res.* **44,** 4217–4223.
2. Klein, G. & Klein, E. (1985) *Nature (London)* **315,** 190–195.
3. Girard, A. J., Jensen, F. C. & Koprowksi, H. (1965) *J. Cell. Physiol.* **65,** 69–84.
4. Aaronson, S. A. & Todaro, G. J. (1970) *Nature (London)* **225,** 458–459.
5. Borek, C. (1980) *Nature (London)* **283,** 776–778.
6. Namba, M., Nishitani, K., Hyodoh, F., Fukushima, F. & Kimoto, T. (1985) *Int. J. Cancer* **35,** 275–280.
7. Milo, G. & DiPaolo, J. (1978) *Nature (London)* **275,** 130–132.
8. Rhim, J. S., Huebner, P., Arnstein, P. & Kopelovich, L. (1980) *Int. J. Cancer* **26,** 565–569.
9. Rhim, J. S., Jay, G., Arnstein, P., Price, F. M., Sanford, K. & Aaronson, S. A. (1985) *Science* **227,** 1250–1252.
10. Rhim, J. S., Fujita, J., Arnstein, P. & Aaronson, S. A. (1985) *Science* **232,** 385–388.
11. Furth, M. E., Davis, L. J., Fleurdelys, B. & Scolnick, E. M. (1982) *J. Virol.* **43,** 294–304.
12. van der Eb, A. J., van Ormondt, H., Schrier, P. I., Lupker, J. H., Jochemsen, H., van den Elsen, P. J., DeLeys, R. J., Maat, J., van Beveren, C. P., Dijkema, R. & deWaard, A. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **44,** 383–399.
13. Houweling, A., van den Elsen, P. & van der Eb, A. (1980) *Virology* **105,** 537–550.
14. Treisman, R., Novak, U., Favoloro, J. & Kamen, R. (1981) *Nature (London)* **292,** 595–600.
15. Ruley, H. E. (1983) *Nature (London)* **304,** 602–606.
16. Land, H., Parada, L. F. & Weinberg, R. A. (1983) *Nature (London)* **304,** 596–602.
17. Namba, M., Nishitani, K., Fukushima, F., Kimoto, T. & Nose, K. (1986) *Int. J. Cancer* **37,** 419–423.
18. Guerrero, I., Villasante, A., Corces, V. & Pellicer, A. (1984) *Science* **225,** 1159–1162.
19. Guerrero, I., Calzada, P., Mayer, A. & Pellicer, A. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 202–205.
20. Sawey, M. J., Hood, A. T., Burns, F. J. & Garte, S. J. (1987) *Mol. Cell. Biol.* **7,** 932–935.
21. Borek, C., Ong, A. & Mason, H. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 794–798.
22. Weinberg, R. A. (1982) *Adv. Cancer Res.* **36,** 49–163.