beam was directed into a Zeiss Standard microscope via a sidearm tube and focused onto the specimen with a Plan 100× objective. Each cell to be killed was irradiated a number of times until there was visible bubbling. Animals were examined by Nomarski microscopy on the day after the laser treatment to confirm the loss of the ablated cells.
10. M. Chalfie, J. N. Thomson, J. E. Sulston, *Science* **221**, 61 (1983).
11. The procedures used for laser microsurgery on embryos were described in J. E. Sulston, E. Shierenberg, J. G. White, J. N. Thomson, *Dev. Biol.* **100**, 64 (1983). Both of the ALM-BDU precursors divide 430 minutes after the first cleavage of the embryo, approximately the time when the animal begins to move within the egg. These movements make identification and ablation of the BDU cells difficult. To avoid this difficulty, the ALM-BDU precursor on the right side was killed before it divided. The egg was then reoriented so that the left side was uppermost and observed with Nomarski optics. When the ALM-BDU precursor on the left had divided, the posterior daughter (BDUL) was killed. The resulting animals were touch-sensitive upon hatching, an indication that the ALML had formed appropriate synapses in the nerve ring.
12. M. Chalfie *et al.*, *J. Neurosci.* **5**, 956 (1985).
13. M. Chalfie, H. R. Horvitz, J. E. Sulston, *Cell* **24**, 59 (1981); J. E. Sulston, personal communication.
14. Five animals (four adults and one L4 stage larva) in which the BDU cells had been killed at hatching were fixed and embedded for electron microscopy as described in M. Chalfie and J. N. Thomson, *J. Cell Biol.* **82**, 278 (1979). Serial sections (106 to 132) covering the region of the nerve ring from the excretory pore anterior to the branches of the ALM cells (a distance of approximately 10 μm) were examined in a JEOL 1200 microscope. [AVM normally branches 1 to 2 μm anterior to the excretory pore (6).] The AVM and ALM touch cells were identified by their characteristic large-diameter microtubules.
15. J. J. M. Chun, M. J. Nakamura, C. J. Shatz, *Nature (London)* **325**, 617 (1987).
16. J. G. White, E. Southgate, J. N. Thomson, S. Brenner, *Cold Spring Harbor Symp. Quant. Biol.* **48**, 633 (1983).
17. The mutant strain CB3241 [*dr-1 (e1745)*] was used in these experiments. Mutant animals appear to become intrinsically starved in appearance when shifted overnight from 15° to 25°C; under these conditions many neuronal processes are easily seen (E. Hedgecock, personal communication).
18. We thank J. E. Sulston who initiated our thinking on this project, M. Finney for providing us with the *unc-86(n848)* mutant, C. Masuoka and D. Hall for assisting in the electronic microscopy, and E. Berghoz for art work. Supported by a grant from the Muscular Dystrophy Association, a U.S. Public Health Service grant (GM 30997 to M.C.), and an NIH postdoctoral fellowship (W.W.W.).

23 July 1987; accepted 31 December 1987

The *ras* Oncogenes Increase the Intrinsic Resistance of NIH 3T3 Cells to Ionizing Radiation

MARSHALL D. SKLAR

Identification of genes that function to protect cells from radiation damage is an essential step in understanding the molecular mechanisms by which mammalian cells cope with ionizing radiation. The intrinsic radiation resistance (D_0) of NIH 3T3 cells was markedly and significantly increased by transformation with *ras* oncogenes activated by missense mutations. This radiobiologic activity appeared to be a specific consequence of the *ras* mutations rather than of transformation, since revertant cells that contained functional *ras* genes (but were no longer phenotypically transformed) retained their increased D_0's.

CELLS HAVE DEVELOPED MULTIPLE biochemical mechanisms to protect the integrity of their DNA from damage by ionizing radiation, to which they are constantly exposed. While these molecular mechanisms are not well understood in mammalian cells, identification of genes involved in conferring radiation resistance should prove a useful step in understanding the fundamental nature of the radiation response. Increasing environmental exposure and extensive medical uses of radiation in diagnosis and cancer therapy make understanding these processes of considerable medical importance.

Efforts to identify genes affecting radiation response have focused on genes coding for DNA repair enzymes (*1*) because the lethal effects of ionizing radiation in the clinically relevant dose range appear to be due primarily to DNA damage. Genes coding for repair of ultraviolet-induced DNA damage have been isolated and identified (*2*). Our working hypothesis was that variations in expression or structure of genes that directly or indirectly regulate repair and other vital cellular processes may also significantly affect radiation response. Certain oncogenes might affect response to cancer therapeutic agents such as ionizing radiation, since abnormalities of *ras* and *myc* oncogenes have been associated with poor cancer prognosis, and radioresistant cell lines (*3*) and their normal cellular homologs appear to play fundamental roles in regulation of other aspects of cellular growth and proliferation (*4*). Furthermore, a cell line transformed by *ras* was recently shown to have a D_0 greater than that of the parental cell line, although a causative role for the exogenous *ras* gene could not be established (*5*). In the present work, we assessed the role of *ras* genes, the oncogene family most commonly associated with human tumors (*4*), in inducing resistance to ionizing radiation.

To minimize the inherent difficulties of distinguishing the effects of specific genes among different cell lines (*6*), we evaluated the effect of each oncogene by first adding it to the same NIH 3T3 subline by transfection, then determining the radiation survival curve of the transfected cell lines by clonogenic survival curve assays. NIH 3T3 cells were selected because they can be readily transfected and transformed by *ras* and several other oncogenes (*4*) and because they have a radiation survival curve similar to that of many human tumor cell lines (*6*).

An NIH 3T3 cell line, a Kirsten murine sarcoma virus–transformed NIH 3T3 cell line (DT) containing two copies of the virus gene (*7*), and two transformation-revertant cell lines derived from DT (*7*) were obtained from R. Bassin (NIH). The NIH 3T3 cells used for all transfections were obtained from D. Lowy (NIH) as was NN 192, an NIH 3T3 cell line transformed by transfection with an overproducing rat c–H-*ras* protooncogene that had been transformationally activated by linkage with a retroviral long terminal repeat (LTR) [constructed as in (*8*)]. NIH 3T3 cells were transfected by the calcium phosphate precipitation technique (*9, 10*) with either genomic DNA containing human c–H-*ras* [EJ bladder cancer (*11*)] or N-*ras* [Hodgkin's disease (*10*) and HL60 leukemia (*12*)] or cloned oncogenes, including c–H-*ras* (pUCEJ6.6) (*13*), v–H-*ras* (*8*), and v-*fms* (*14*). Transformed cells had previously been cloned and tested for the ability to grow in soft agar and for the presence of the transfected gene by DNA hybridization analysis (*9, 10*). Level of expression was determined by dot-blot analysis (*10*). Detailed descriptions of the transformed cell lines have been presented (*9*). With the exception of the *ras* protooncogene in NN 192, all *ras* genes were activated by missense mutations at codon 12 or 61 (*9–14*).

The effect of *ras* genes bearing missense mutations on radiation survival is shown in Fig. 1 and Table 1. All cell lines transformed with *ras* genes that had been activated by a missense mutation showed a large increase in intrinsic radiation resistance (D_0; the slope of the single dose radiation survival curve as compared to untransformed NIH 3T3). This change was significant at $P < 0.005$ by t test (Table 1).

There were no significant differences among *ras* genes in their effect on D_0 regardless of the type of *ras* gene (H, K, or N; viral or cellular), the site of activating mutation,

Department of Radiation Oncology, University of Michigan Medical School, Ann Arbor, MI 48109.

the means of introducing the *ras* gene (infection or transfection), or whether the *ras* gene was introduced as genomic DNA or as a gene molecularly cloned in a plasmid. The D_0 did not increase with the number or level of expression of *ras* gene copies in a cell, since the HSV 3A cell line, which contains 20 to 50 copies of v–H-*ras* (*9*) and comparably elevated messenger RNA levels, had a D_0 similar to those of cell lines containing two copies [DT (*7*)] or two to ten copies (*9, 10*).

These experiments demonstrate that: (i) the presence of missense mutation–activated *ras* was sufficient to significantly alter the radiation survival curve of NIH 3T3 cells in a characteristic fashion; (ii) non-*ras* human genes that might be present in the secondary and tertiary transformants from HL60 and the other human tumors were not responsible for increasing the D_0 of NIH 3T3, since cloned *ras* genes had the same effect on D_0; and (iii) specific sequences in the transfecting plasmids or viral LTR sequences were not necessary for increased D_0, since genomic DNA containing missense mutation–activated *ras* genes also conferred the same effect. In addition, the shoulder of the survival curves (represented by *n*, the extrapolation number) was noticeably less in cells transformed by mutation-activated *ras* than in untransformed NIH 3T3 cells. While this difference is statistically significant ($P < 0.01$), its biological significance in these cells is uncertain. It will be necessary to determine whether *ras* genes affect repair of sublethal DNA damage as suggested by changes in *n* (*15*).

Since radiation resistance was induced by genes introduced by infection (the v–K-*ras* gene in the DT cell line) as well as by transfection, DNA perturbations caused by the transfection process seemed unlikely to be the cause of the *ras* effect. As expected, there was no significant difference ($P > 0.2$) among survival curves of NIH 3T3 cells, NIH 3T3 cells transfected with pSV2*neo*, and human genomic DNA not containing transforming *ras* genes (*16*).

Malignant transformation involves many cellular changes. Thus, it was necessary to rule out the possibility that the increased radiation resistance was a nonspecific consequence of transformation. We did this by determining the survival curves of NIH 3T3 cells transformed with an unrelated oncogene, v-*fms*, and of a cell line transformed with a c–H-*ras* protooncogene that had been transformationally activated by linkage with a Moloney retrovirus LTR. NIH 3T3 cells transformed by these genes had survival curves very different from those of missense mutation–activated *ras* transformants (Fig. 2 and Table 1). The D_0's of these cell lines ranged from 90 to 110 cGy, meaning that they had markedly less intrinsic resistance to ionizing radiation than NIH 3T3 and far less than cells transformed with the activated *ras* genes. As only a few cell lines trans-

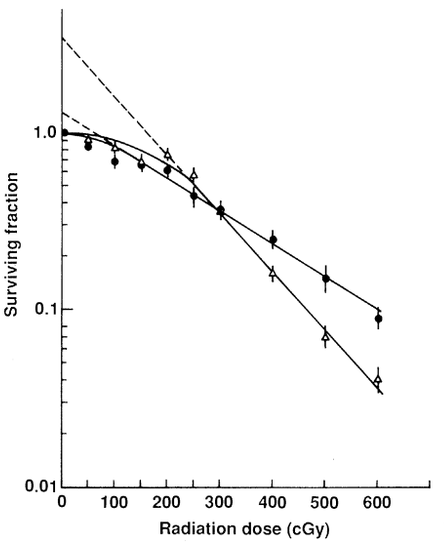

Fig. 1. Clonogenic radiation survival curves for NIH 3T3 cells and NIH 3T3 cells transformed by missense mutation–activated *ras* genes. Cell lines were maintained in exponential growth in Dulbecco's modification of Eagle's minimal essential medium with high glucose and 10% fetal bovine serum, 2 m*M* glutamine, and sodium pyruvate (D10 medium). Single cell suspensions were irradiated in isotonic phosphate-buffered saline (PBS; *p*H 7.4) under euoxic conditions in 15-ml Corning centrifuge tubes with a Theratron 80 ^{60}Co unit; a lateral field in a specially constructed Lucite block holder was used. Water was kept in the bottom of the holder to eliminate the air gap at the conical end of the tube. Dose calculations were confirmed by thermoluminescent dosimetry. Immediately after irradiation, cells were plated in triplicate at 100 to 10,000 cells per 60-mm dish in D10. After 8 to 10 days, the plates were fixed and stained in 70% methanol with 1% methylene blue; colonies containing >50 cells were scored. Survival curves were plotted as the log of the surviving fraction of cells versus the radiation dose. Experimental points are plotted as the mean ± SE (where greater than the symbol size) of 12 (NIH 3T3) or 23 (*ras*) separate experiments. The closed circle represents data pooled from survival curves of all the different *ras* oncogenes tested. Statistical significance was determined by *t* test. There were no statistically significant differences among the D_0's among any of the *ras* missense mutation-transformed cell lines ($P > 0.2$).

Table 1. Radiation survival of NIH 3T3 cells transformed by oncogenes. D_0 and *n* were calculated by linear regression of the means of triplicate experimental points taken from 3 to 23 separate experiments. All numbers represent the mean ± SE. Data were obtained from one or more cell lines derived by independent transfections, as shown. There were no significant differences ($P \geq 0.2$) among the cell lines pooled. To rule out the possibility that non-*ras* sequences were responsible for the effects, cell lines transformed with either cloned *ras* genes or genomic DNA from *ras*-containing human tumor cells (*10–14*). Tertiary transfectants were assayed.

NIH 3T3 cells		D_0 (cGy)	Extrapolation number (*n*)
Number of cell lines	Transformed with		
2	—	141 ± 12	3.4 ± 0.2
3	c–H-*ras* (genomic DNA)	238 ± 8	1.4 ± 0.2
2	c–H-*ras* (cloned DNA)	220 ± 12	1.4 ± 0.2
1	v–H-*ras* (cloned DNA)	205 ± 13	1.3 ± 0.3
2	N-*ras* (genomic DNA)	203 ± 14	1.1 ± 0.1
1	v–K-*ras* (DT cell line)*	220 ± 8	1.3 ± 0.1
2	v–K-*ras* (transformation revertants of DT)	219 ± 10	2.2 ± 0.4
1	c–H-*ras* protooncogene†	101 ± 4	4.3 ± 0.5
2	v-*fms* (cloned DNA)	105 ± 6	2.2 ± 0.2

*Cells transformed by infection with Kirsten sarcoma virus (*7*). †Transformationally activated by linkage to a Moloney virus LTR (*8*).

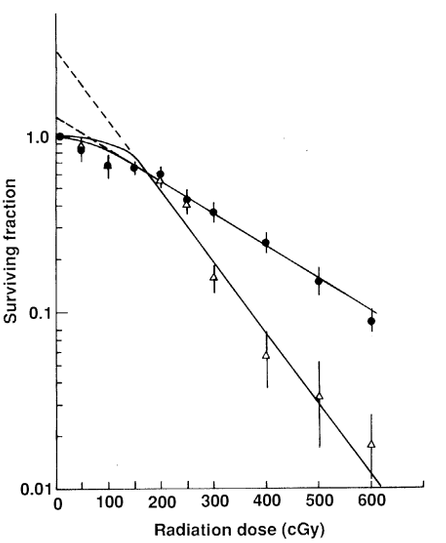

Fig. 2. Clonogenic radiation survival curves for NIH 3T3 cells transformed by missense mutation *ras* genes (●) and by a composite of two cell lines transformed by v-*fms* and one cell line transformed by a retrovirus LTR-activated c–H-*ras* protooncogene (△). The D_0's of cell lines transformed by v-*fms* and by the LTR-activated H-*ras* protooncogene were similar to each other ($P > 0.2$) and differed significantly ($P < 0.001$) from the D_0's of the *ras* transformants.

formed by v-*fms* and the H-*ras* protooncogene gene have been examined thus far, no firm conclusions can yet be drawn as to the ability of these genes to affect radiation response in a gene-specific way. Nevertheless, this strikingly different radiation resistance of cells transformed with these genes indicates that the effect of *ras* oncogenes was not just an incidental consequence of transformation.

Survival curves of two cell lines (KR-F2 and KR-C11) that were transformation revertants of DT were analyzed to determine whether the effect of *ras* oncogenes required the transformed phenotype. They were no longer phenotypically transformed, but still contained the active *ras* gene and still produced the mutant protein (*7*). The composite results of the radiation survival curves on these cells are shown in Table 1. The Bassin NIH 3T3 line and the DT transformant (v–K-*ras*) behaved radiobiologically like their Lowy NIH 3T3 and *ras*-transformed counterparts. However, the revertants still showed the increased intrinsic resistance of the other missense mutation *ras* transformants. Thus, the presence of the mutated *ras* protein appears to be sufficient to increase intrinsic resistance, whether or not the cell is morphologically transformed.

The magnitude of these differences in D_0 caused by single genes is remarkable. The total reported range of intrinsic resistance for mammalian cells irradiated under similar (euoxic, exponential growth) conditions that do not, like ataxia telangiectasia cells, have serious DNA repair defects (*17*) is about 80 to 250 cGy (*6*). Thus, adding single genes to NIH 3T3 can cause increases or decreases in D_0 that encompass a substantial fraction of the total known range of mammalian cell D_0's.

These results indicate that genes whose protein products are in and around the cell membrane can have major effects on a predominately nuclear process, specifically the ionizing radiation response of mammalian cells. These cell membrane–associated proteins cannot have a direct DNA repair function, nor are they in the right place to scavenge free radicals in the nucleus, another mechanism known to affect radiation response. Their effect must be indirect, perhaps causing the induction or repression of genes coding for specific nuclear molecules involved in free radical scavenging or DNA repair, or altering DNA or chromosomal conformation in a way that affects access of repair enzymes to damaged DNA.

Another possible indirect effect might be through cell cycle modification since radiation resistance varies considerably in different stages of the cell cycle (*18*). Growth curves and flow cytometry data for cell cycle analysis were obtained from the following cell lines, which were used in Table 1: both NIH 3T3 cell lines, four *ras*-transformed lines [representing c–H-*ras* (genomic; EJ), v–H-*ras*, N-*ras* (genomic; HL60), and v–K-*ras* (Kirsten virus–transformed)], two lines transformed by *fms*, and one line transformed by the c–H-*ras* protooncogene. No correlation between D_0 and any cell cycle parameter was observed (*19*). The mean cell cycle length (15 to 16 hours), phase distribution, and absolute time in radioresistant or radiosensitive cell cycle phases (S and G_2/M, respectively) was similar for NIH 3T3 and for cells transformed by either *ras* or *fms*. For example, the v–H-*ras*–transformed Lowy NIH 3T3 cell line (S, 9%, 1.6 hours; G_2/M, 27%, 5.6 hours) differs little from the Lowy NIH 3T3 (S, 10%, 1.6 hours; G_2/M, 30%, 5.4 hours), although its D_0 is ~50% higher (205 cGy as compared to 141 cGy). Furthermore, no *ras*-transformed cell line ($D_0 \sim 230$ cGy) had a higher S:G_2/M ratio than the v-*fms*–transformed cells ($D_0 \sim 105$).

These findings are, at this time, limited to NIH 3T3 cells irradiated in vitro under specific conditions. Nevertheless, *ras* genes are ubiquitous in mammalian cells (*4*), suggesting a potentially broader applicability of these findings. For example, if human tumors are similarly affected by activated *ras* and perhaps other oncogenes, then their presence or aberrant expression may help predict whether a patient's tumor will respond to ionizing radiation. Greater understanding of the mechanism by which these genes induce radiation resistance may eventually permit us to minimize tumor cell resistance to ionizing radiation and thus improve our ability to treat cancer.

REFERENCES AND NOTES

1. J. Rubin *et al.*, *Nature (London)* **306**, 206 (1983); M. MacInnes, J. Bingham, L. Thompson, G. Strniste, *Mol. Cell. Biol.* **4**, 1152 (1984).
2. A. Westerveld *et al.*, *Nature (London)* **310**, 425 (1984).
3. R. Seeger *et al.*, *N. Engl. J. Med.* **313**, 1111 (1985); M. Viola, F. Fromowitz, S. Oravez, S. Deb, J. Schlom, *J. Exp. Med.* **161**, 1213 (1985); M. Viola *et al.*, *N. Engl. J. Med.* **314**, 133 (1985); D. Carney, J. Mitchell, T. Kinsella, *Cancer Res.* **43**, 2806 (1983); C. Little, M. Nau, D. Carney, A. Gazdar, J. Minna, *Nature (London)* **306**, 1984 (1983).
4. R. Weinberg, *Science* **230**, 770 (1985).
5. T. Fitzgerald *et al.*, *J. Am. J. Clin. Oncol.* **8**, 517 (1985).
6. A. Russo, J. Mitchell, T. Kinsella, G. Morstyn, E. Glatstein, *Semin. Oncol.* **12**, 332 (1985); R. Weichselbaum *et al.*, *Eur. J. Cancer* **12**, 47 (1976); R. Weichselbaum *et al.*, *Radiology* **139**, 485 (1981).
7. R. Bassin *et al.*, in *Oncogenes and Viral Genes*, G. Vande Woude, A. Levine, W. Topp, J. Watson, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1984), p. 463.
8. D. DeFeo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3328 (1981); E. Chang, M. Furth, E. Scolnick, D. Lowy, *Nature (London)* **297**, 479 (1982).
9. M. Sklar, *Cancer Res.*, in press.
10. M. Sklar and G. Kitchingman, *Int. J. Rad. Ther. Biol. Phys.* **11**, 49 (1985).
11. M. Murray *et al.*, *Cell* **33**, 749 (1983).
12. L. Parada, C. Tabin, C. Shih, R. Weinberg, *Nature (London)* **297**, 474 (1982).
13. C. Tabin *et al.*, *ibid.* **300**, 143 (1982); E. Taparowsky, K. Shimizu, M. Goldfarb, M. Wigler, *Cell* **34**, 581 (1983).
14. L. Donner, L. Fedele, C. Garon, S. Anderson, C. Sherr, *J. Virol.* **41**, 489 (1982).
15. M. Elkind and H. Sutton, *Nature (London)* **184**, 1293 (1959).
16. M. Sklar, unpublished data.
17. A. Taylor *et al.*, *Nature (London)* **258**, 427 (1975).
18. T. Terasima and L. J. Tolmach, *Science* **140**, 490 (1963).
19. M. Sklar and A. Flint, in preparation.

20. We thank D. Lowy and R. Bassin for providing NIH 3T3 cells and the NN 192 and DT cell lines, respectively; C. Sherr and E. Chang for providing cloned v-*fms* and v–H-*ras*, respectively; A. Lichter and J. Mitchell for helpful discussions; and M. C. Holden, S. McQuiston, and V. Terry for technical assistance. Supported by research grant CA 41166 from the National Cancer Institute.

17 September 1987; accepted 30 November 1987