the rosette stage, shortly after bolting, during flowering, and again at senescence.

16. We use the designation Dwf or Dwf::K$^R$Ti for the dwarf phenotype, *dwf*::K$^R$Ti for the disrupted allele, and *DWF* for the wild-type allele. We have not crossed the Dwf mutant described in this report to previously mapped dwarf mutants.
17. D. G. Jones, E. D. Gilbert, K. L. Grady, R. A. Jorgensen, *Mol. Gen. Genet.* **207**, 478 (1987); R. A. Jorgensen, C. Snyder, J. D. G. Jones, *ibid.*, p. 471.
18. M. D. Marks and K. A. Feldmann, unpublished data.
19. The transformation of genetically effective cells in the T1 plants occurs in such a way that only one or the other of the meiotic products is transformed. We have never observed siblings of any of the mutant phenotypes.
20. To assay for Kan$^R$ or Kan$^S$, we surface-sterilized seeds in pure Clorox (5.25% wt/vol sodium hypochlorite) for 8 min and rinsed three times with sterile water. After seeds were dry, they were sprinkled on agar solidified (0.8%) medium containing M&S salts and vitamins, 1% sucrose (*12*), and kanamycin-sulfate at 50 mg/liter (Sigma). Kanamycin was eliminated from the medium for nonselective conditions. The plates containing the seeds were transferred to the dark at 4°C for 48 hours. The plates were transferred to growth chamber conditions and observed after 10 to 12 days. (A) Tall and Dwf seedlings were identified on nonselective medium and transferred to kanamycin-containing medium. (B) Individual Kan$^R$ T3 seedlings (tall and Dwf) were identified and grown to maturity, and the selfed progeny, T4, were observed after growth on kanamycin.
21. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
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# Effect of Antisense c-*raf*-1 on Tumorigenicity and Radiation Sensitivity of a Human Squamous Carcinoma

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**Antisense RNA–mediated inhibition of gene expression was used to investigate the biological function of the c-*raf*-1 gene in a radiation-resistant human squamous carcinoma cell line, SQ-20B. S1 nuclease protection assays revealed that transfection of full-length *raf* complementary DNA in the antisense orientation (AS) leads to a specific reduction (greater than tenfold) of steady-state levels of the endogenous c-*raf*-1 sense (S) transcript in SQ-20B cells. In nude mice, the malignant potential of SQ-20B cells transfected with *raf* (S) was significantly increased relative to that of SQ-20B cells transfected with *raf* (AS). SQ-20B cells containing transfected *raf* (S) maintained a radiation-resistant phenotype as compared to those cells harboring the AS version, which appeared to have enhanced radiation sensitivity. These data indicate that the reduced expression of endogenous c-*raf*-1 is sufficient to modulate the tumorigenicity and the radiation-resistant phenotype of SQ-20B cells, thus implicating c-*raf*-1 in a pathway important to the genesis of this type of cancer.**

CELLULAR RADIATION SENSITIVITY is a complex function of genetic and environmental factors, not fully understood at the molecular level (*1, 2*). Only recently have applications of genetic engineering permitted probing of genomic events associated with radiation sensitivity or resistance (*3*). In the treatment of cancer, the sensitivity or resistance of tumor cells to ionizing radiation has substantial clinical consequences (*4, 5*). Using DNA-mediated gene transfer, we have observed the association of the *raf* oncogene in three radioresistant cancers of the head and neck (*6, 7*). Furthermore, Chang *et al.* have associated the expression of *raf* and *myc* oncogenes with radioresistant fibroblasts from a patient with the Li-Fraumeni syndrome (*8*). Others have reported correlations between *myc* expression and a more radioresistant phenotype of lung carcinomas (*9, 10*), as well as *ras* expression and radiation resistance in rodent cells (*11, 12*). An activated *raf* may also be present in oat cell lung carcinomas (*13*), some of which are radioresistant. More recently, Wasserman *et al.* have suggested that the sensitivity of squamous cell carcinomas to ionizing radiation may be attributable to altered poly(adenosine diphosphate–ribose) synthesis (*14*). Unequivocal evidence for oncogene modulation of radiation sensitivity has not been reported.

It has been recently discovered that a specific messenger RNA can be functionally inactivated by hybridization to complementary RNA or antisense RNA (*15, 16*). A number of investigators have now used antisense RNA to study the functional regulation of gene expression including cellular oncogenes. We have investigated the consequences of introduction of sense or antisense human c-*raf*-1 cDNA sequences on two major biological parameters in radiation-resistant human laryngeal squamous carcinoma cells (SQ-20B): tumor growth and radiation survival.

We have used a 3.05-kb Bam HI fragment of human c-*raf*-1 cDNA (*17*) that was isolated from an Okayama-Berg human fibroblast cDNA library (*18*) to construct various recombinant DNA molecules in the eukaryotic expression vector pADMLP. This vector contains an origin of replication and transcriptional initiation sequences of the adenovirus 2 major late promoter. Three different types of vector-insert plasmid DNA constructs were generated: (i) 3.05-kb Bam HI cDNA fragment in the "sense" orientation, downstream from the ad promoter, namely RAF (S); (ii) 3.05-kb Bam HI cDNA fragment in the reversed orientation, RAF (AS); and, (iii) 1.3-kb Bam HI–Bgl II cDNA fragment in the antisense orientation representing *raf* 3′ terminal sequences, 3′ RAF (AS). From SQ-20B, cells were transfected with these recombinant DNA molecules

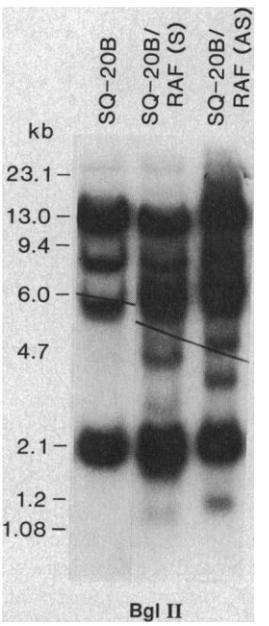


**Fig. 1.** Southern blot hybridization analysis of DNA isolated from various cell types. DNA samples (10 μg per lane) were digested to completion with Bgl II and hybridized to a $^{32}$P-labeled 1.09-kb Ava II fragment of c-*raf*-1 cDNA (*6*). DNA isolation, restriction endonuclease digestion, 0.8% agarose gel electrophoresis, Southern blotting onto nitrocellulose, and $^{32}$P-labeling of probes were all performed as described (*27*). Hybridization conditions and wash conditions have also been previously described (*6*).

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along with the pSV2neo selectable marker, which confers resistance to geneticin (G418 sulfate). Forty-eight hours after transfection, tumor cells were selected for their ability to grow in the medium supplemented with G418 (800 μg/ml). After 2 to 3 weeks in the selective medium, viable cell populations designated SQ20B/pADMLP (the control for vector sequences), SQ-20B/RAF (S), SQ-20B/RAF (AS), and SQ-20B/3′ RAF (AS) emerged from the vector-transfected cultures, whereas no SQ-20B cells survived in mock-transfected control plates.

Southern blot hybridization analysis with an Ava II cDNA fragment of c-*raf*-1 (1.09 kb) (*6*) confirmed the success of transfections of all of the *raf* cDNAs into the SQ-20B cells (*19*). Bgl II digestions of these DNAs distinguished SQ-20B RAF (S) transfectants (new 1850-bp Bgl II fragment) from SQ-20B/RAF (AS) transfectants (new 1200-bp Bgl II fragment) (Fig. 1). Densitometer scanning of the autoradiograph and a comparison with the endogenous [unamplified (*6*)] c-*raf*-1 sequence suggested that approximately three to five copies of the *raf* (S) cDNA and two copies of the *raf* (AS) cDNA were present per cell in the transfected RAF (S) and RAF (AS) cell populations, respectively.

The efficacy of the transcription of transfected cDNA sequences in SQ-20B cells was tested with RNA probes specific for hybridization with *raf* (S) or *raf* (AS) transcript. A 479-bp Pst I–Hind III fragment from the 5′ end of the *raf* cDNA was subcloned either in 5′ → 3′ or 3′ → 5′ orientation downstream from the SP6 promoter of the in vitro transcription vector, pGEM-1 (Promega Biotech). For detection of the antisense RNA, the vector containing the insert in the 3′ → 5′ orientation was linearized with Eco RI, and an antisense transcript (556 bp) was generated with SP6 RNA polymerase. For detection of the sense RNA, the vector containing an insert in the 5′ → 3′ orientation was linearized with Pvu II, and a sense transcript (211 bp) was generated with SP6 RNA polymerase. Specificity and sensitivity were tested by solution hybridization with complementary and noncomplementary sense RNA probes as well as unrelated transcripts. In a nuclease protection assay, only the complementary sense RNA probe (119 bp) was identified (Fig. 2B). No protected fragments could be visualized with unrelated transcripts. The total cellular RNA was isolated from SQ-20B/RAF (AS) cells at passage four, 3 months after transfection. Antisense 5′ *raf* RNA probe detected a distinct endogenous sense transcript in SQ-20B cells as evidenced by the protected 479-bp hybrid, whereas the expression was inhibited to less than one-tenth in SQ-20B cells transfected with full-length *raf* in the reversed orientation, RAF (AS) (Fig. 2A). Similar inhibition of sense RNA in SQ-20B/RAF (AS) cells was noted when a shorter probe (280 bp) was used (*19*). Antisense *raf* transcripts were not identifiable with the $^{32}$P-labeled sense probe in SQ-20B/RAF (AS) cells. This observation may reflect the unstable nature of this transcript, as has been observed in murine cells transfected with an antisense *myc*-producing plasmid DNA (*20, 21*). However, in order to confirm our ability to express genes from the pADMLP vector in SQ-20B cells, pADMLP containing the bacterial chloramphenicol acetyltransferase (CAT)

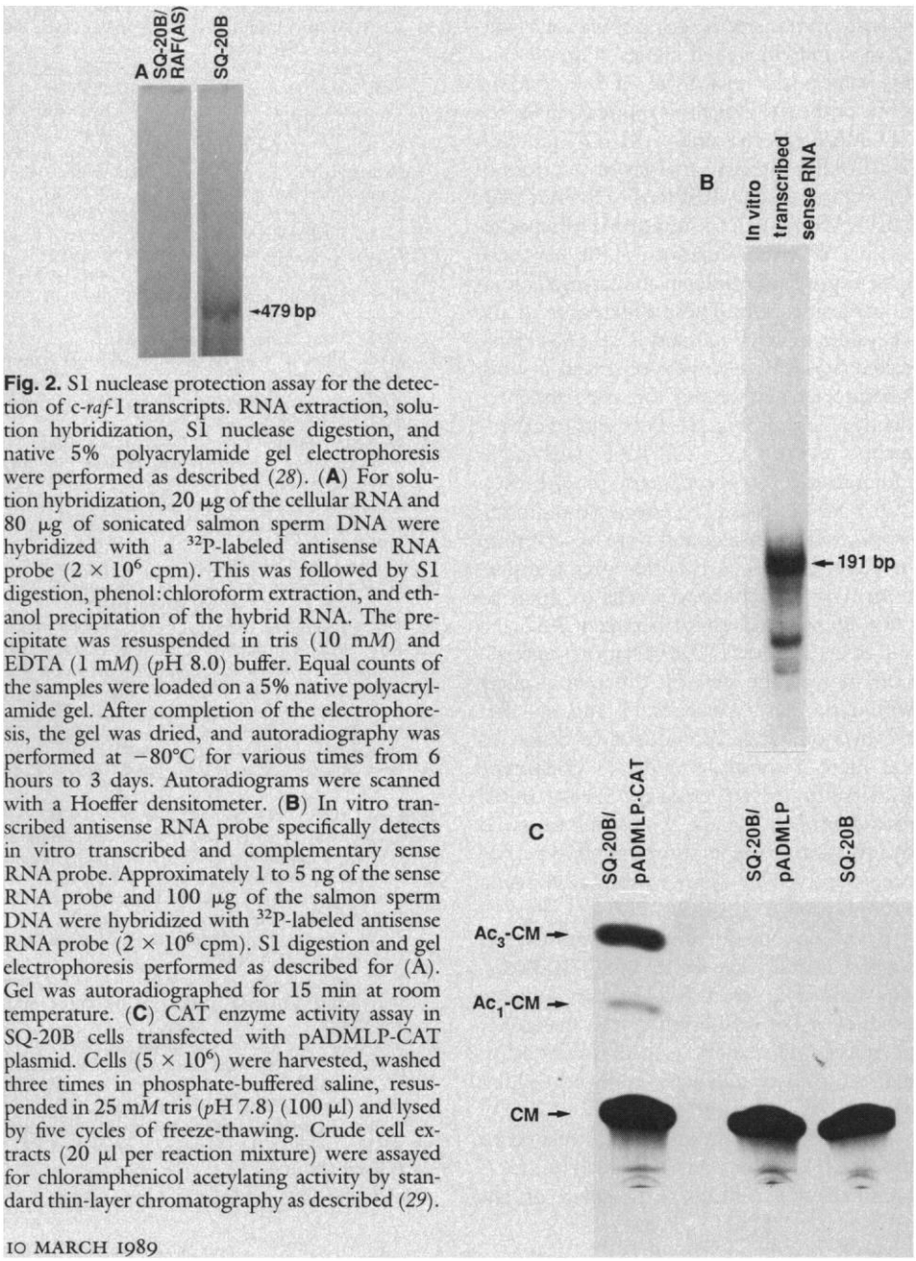


**Fig. 2.** S1 nuclease protection assay for the detection of c-*raf*-1 transcripts. RNA extraction, solution hybridization, S1 nuclease digestion, and native 5% polyacrylamide gel electrophoresis were performed as described (*28*). (**A**) For solution hybridization, 20 μg of the cellular RNA and 80 μg of sonicated salmon sperm DNA were hybridized with a $^{32}$P-labeled antisense RNA probe (2 × 10$^6$ cpm). This was followed by S1 digestion, phenol:chloroform extraction, and ethanol precipitation of the hybrid RNA. The precipitate was resuspended in tris (10 m*M*) and EDTA (1 m*M*) (*p*H 8.0) buffer. Equal counts of the samples were loaded on a 5% native polyacrylamide gel. After completion of the electrophoresis, the gel was dried, and autoradiography was performed at −80°C for various times from 6 hours to 3 days. Autoradiograms were scanned with a Hoeffer densitometer. (**B**) In vitro transcribed antisense RNA probe specifically detects in vitro transcribed and complementary sense RNA probe. Approximately 1 to 5 ng of the sense RNA probe and 100 μg of the salmon sperm DNA were hybridized with $^{32}$P-labeled antisense RNA probe (2 × 10$^6$ cpm). S1 digestion and gel electrophoresis performed as described for (A). Gel was autoradiographed for 15 min at room temperature. (**C**) CAT enzyme activity assay in SQ-20B cells transfected with pADMLP-CAT plasmid. Cells (5 × 10$^6$) were harvested, washed three times in phosphate-buffered saline, resuspended in 25 m*M* tris (*p*H 7.8) (100 μl) and lysed by five cycles of freeze-thawing. Crude cell extracts (20 μl per reaction mixture) were assayed for chloramphenicol acetylating activity by standard thin-layer chromatography as described (*29*).

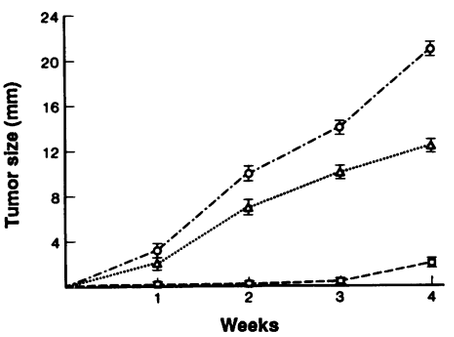


**Fig. 3.** Tumorigenic potential of SQ-20B/RAF (S and AS) transfectants. BALB/c *nu/nu* mice were maintained in the animal research resources facility of Georgetown University. Cells (5 × 10$^6$) of SQ-20B transfectants containing either the sense (S) or antisense (AS) oriented c-*raf*-1 cDNA downstream from the adenovirus 2 major late promoter (pADMLP), were injected subcutaneously between the scapulae into groups of seven to ten males. Sizes of the tumors were determined at weekly intervals. Values are means ± SEM. ○, SQ-20B/RAF (S); △, SQ-20B/pADMLP or SQ-20B/3′ RAF (AS); □, SQ-20B/RAF (AS).

gene was transfected into SQ-20B cells, and the CAT enzyme activity was tested in the cell extracts 48 hours after transfection. Acetylated forms of the chloramphenicol were observed (Fig. 2C).

The SQ-20B cell line was established from a biopsy of a laryngeal carcinoma that had progressed in the face of a full course of radiation therapy. We next investigated the malignant potential of SQ-20B/RAF (S, AS, or 3′AS) transfected cell populations by their inoculation into seven to ten male nude mice (*nu/nu*). Representative results from one such experiment using cells at passage two and approximately 40 days after transfection are shown in Fig. 3. SQ-20B cells transfected with the control vector pADMLP and the SQ-20B/3′ RAF (AS) cells produced distinct tumors at the site of inoculation in all mice within 2 weeks. In contrast, SQ-20B/RAF (AS) cells showed no evidence of tumor growth during an observation period of up to 4 weeks. However, small nodules that eventually grew into tumors appeared in these mice at 5 weeks after inoculation. SQ-20B/RAF (S) cells showed an increased growth rate of the tumor as compared to control cells, SQ-20B/pADMLP (Fig. 3). No metastases were observed in any of the animals (*19*). The modulation of tumorigenicity by altering c-*raf*-1 expression in SQ-20B cells provides important insight into its role in this cancer.

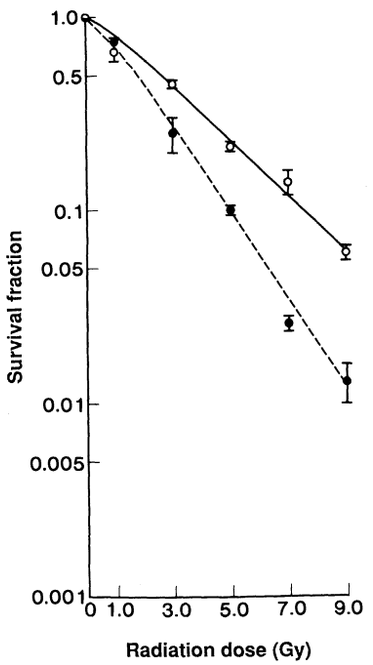


**Fig. 4.** Clonogenic radiation survival curves for RAF (S) (○) and RAF (AS) (●) cDNA transfected SQ-20B cell populations. Cells were maintained in exponential growth in complete Dulbecco's minimal essential medium in the presence of geneticin (800 μg/ml). Geneticin was deleted for the radiation survival experiments. Cells at passage two (posttransfection) were plated in 25-cm$^2$ flasks (Co-Star) and were allowed to attach for 6 hours before irradiation. Irradiations were performed with a Theratron 80 cobalt-60 unit at 80 cm source-to-target distance with the source under the flasks and 1/2-cm bolus material to assure dose buildup. Dosimetry was confirmed with an ionization chamber. After irradiation, the cells were incubated for 8 to 14 days for colony formation. The colonies were fixed and stained with 1% methylene blue, and colonies containing 50 cells or more were scored. Survival curves were fit to a single-hit, multitarget model ($S = 1 - (1 - e^{-D/D_0})^n$ (*30*), and experimental points are plotted ± SEM.

Radiation survival studies were performed on the transfected cell populations. In our experience, both NIH 3T3 and human tumor cell populations have within them cells that exhibit sufficient heterogeneity of radiation survival responses to confound subsequent interpretations of oncogene effects (*19, 22*). We therefore elected to focus our studies on radiation survival responses in transfected cell populations rather than individual cell clones (Fig. 4). The $D_0$ values (the reciprocal of the terminal slope of the survival curve) were 3.10 Gy for SQ-20B/RAF (S) and 1.91 Gy for SQ-20B/RAF (AS). The analysis of the ratio of $D_0$ values of SQ-20B/RAF (S) and SQ-20B/RAF (AS) (1.6) supports our hypothesis that down-modulation of the *raf* oncogene expression results in an increased radiation sensitization. These differences in the $D_0$ values of RAF (S) and RAF (AS) transfected SQ-20B cells were observed in both laboratories performing the assay independently [(*23*) and Fig. 4]. Both sets of experiments revealed SQ-20B/RAF (AS) cells radiosensitive as compared to the SQ-20B/RAF (S) cells. The effects on radiosensitivity were transient and were observed up to passage four (3 months after transfection). The SQ-20B control cells are intermediate in radiosensitivity between RAF (S) and RAF (AS) cells. The radiation sensitization showed the greatest differential effect within the early passages (2 and 4) after transfection, with subsequent reversion to the more resistant, "parental" phenotype with continued cell passage. Similar initial modulation of the gene expression, followed by reversion to the wild-type phenotype, has been observed by other investigators using antisense RNA constructs (*24*).

Based on our present observations, we suggest a "dual" role for *raf* in SQ-20B cells. The first is a direct role of the *raf* gene product in the exhibition of the malignant phenotype. The second is in all likelihood an indirect role of *raf* with multiple cellular responses to radiation damage.

Since the biochemical cascade related to the function of *raf* has not been fully established, it is difficult to speculate on the mechanism by which transcripts from human c-*raf* cDNA (S or AS) might influence the radiation response of tumor cells. Based on our limited understanding of protooncogenes, we would not expect the *raf* gene product to be a repair protein, but rather to modulate the repair cascade by indirect mechanisms or to alter the cell cycle distribution (a consequence of which may be alterations in radiosensitivity) (*25, 26*). Such modifications of the radiation response of tumor cells may be nevertheless significant, and may hold important clinical implications. Our studies demonstrate a close relation among *raf* expression, tumor growth rate, and response to ionizing radiation.

## REFERENCES AND NOTES


1. T. Thacker, *Int. J. Rad. Biol.* **50**, 1 (1986).
2. P. G. Debenham, M. B. Webb, N. J. Jones, R. Cox, *J. Cell Sci.* **6**, 177 (1987).
3. M. MacInnes, J. Bingham, L. Thompson, G. Strniste, *Mol. Cell. Biol.* **4**, 1152 (1984).
4. R. R. Weichselbaum *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2684 (1986).
5. L. J. Peters, L. E. Hopwood, H. R. Withers, H. D. Suit, *Cancer Treatment Symposia* **1**, 67 (1984).
6. U. Kasid, A. Pfeifer, R. R. Weichselbaum, A. Dritschilo, G. E. Mark, *Science* **237**, 1039 (1987).
7. U. Kasid, A. Pfeifer, R. R. Weichselbaum, A. Dritschilo, G. E. Mark, presented at the Third Annual Meeting on Oncogenes, p. 325 (1987).
8. E. H. Chang *et al.*, *Science* **237**, 1036 (1987).
9. C. D. Little, M. M. Nau, D. N. Carney, A. F. Gazdar, J. D. Minna, *Nature* **306**, 194 (1983).
10. D. N. Carney *et al.*, *Cancer Res.* **43**, 2806 (1983).
11. T. J. Fitzgerald *et al.*, *Am. J. Clin. Oncol.* **8**, 517 (1985).
12. M. D. Sklar, *Science* **239**, 645 (1988).
13. G. E. Mark *et al.*, *Adv. Biochem. Psychopharmacol.* **44**, 45 (1988).
14. K. Wasserman *et al.*, *Biochem. Biophys. Res. Commun.* **154**, 1041 (1988).
15. H. Weintraub *et al.*, *Trends Genet.* **1**, 22 (1985).
16. P. J. Green *et al.*, *Annu. Rev. Biochem.* **55**, 569 (1986).
17. T. Bonner *et al.*, *Nucleic Acids Res.* **14**, 1009 (1986).
18. H. Okayama and P. Berg, *Mol. Cell. Biol.* **3**, 280 (1983).
19. U. Kasid, unpublished data.
20. H. M. Lachman, G. Cheng, A. I. Skoultchi, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6480 (1986).
21. A. E. Griep and H. Westphal, *ibid.* **85**, 6806 (1988).
22. R. R. Weichselbaum, M. A. Beckett, W. Dahlberg, A. Dritschilo, *Int. J. Rad. Onc. Biol. Phys.* **14**, 907 (1988).
23. R. R. Weichselbaum, unpublished data.
24. J. B. Bolen, S. Amini, V. DeSeau, S. Reddy, D. Shalloway, *J. Virol.* **61**, 1079 (1987).
25. W. K. Sinclair and R. Morton, *Radiat. Res.* **29**, 450 (1966).
26. J. B. Little, *N. Engl. J. Med.* **278**, 308 (1968).
27. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
28. F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Wiley Interscience, New York, 1987).
29. C. M. Gorman, L. F. Moffat, B. H. Howard, *Mol. Cell. Biol.* **2**, 1044 (1982).
30. J. R. Andrews, in *The Radiology of Human Cancer Radiotherapy* (University Park Press, Baltimore, MD, 1978).


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