Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 1332–1336, February 1995
Medical Sciences

Evidence that carcinogenesis involves an imbalance between epigenetic high-frequency initiation and suppression of promotion

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Communicated by Harry Rubin, University of California, Berkeley, CA, September 16, 1994

ABSTRACT **Evidence is presented in support of the hypothesis that cancer development depends on an imbalance between highly frequent epigenetic initiation and suppression of promotion of the initiated cells. When irradiated clonogenic mammary epithelial cells are transplanted and hormonally stimulated, they give rise to clonal glandular structures within which carcinomas may arise. In the current study, the cancer incidence in grafts of ≈13 7-Gy-irradiated clonogens per site indicated that at least 1 of ≈95 clonogens was radiogenically initiated. A similar initiation frequency had been seen in grafts of ≈5 methylnitrosourea (MNU)-treated clonogens. Such initiation is thus far more frequent than specific locus mutations. In sites grafted with larger cell inocula, cancer incidences per clonogen were suppressed inversely as the numbers of irradiated or MNU-treated clonogens per graft increased. Addition of unirradiated cells to small irradiated graft inocula also suppressed progression. Radiation and MNU thus produce quantitatively, and perhaps qualitatively, similar carcinogenesis-related sequelae in mammary clonogens.**

A key problem is whether initiation, the first step in carcinogenesis, involves a mutation of a specific gene or is epigenetic. Initiation is here defined as the first intracellular heritable event, induced or "spontaneous," that increases the probability that one or more of the affected cell's progeny will progress to cancer. Hence, the initiated cell is revealed by its future behavior (1).

If initiation is epigenetic, it could be common (2–4). If it is generally mutational, one would expect it to be rare and most carcinogens to be mutagens. Of 262 compounds for which there were adequate data in the U.S. National Toxicological Program screening, 162 (62%) were rodent carcinogens and 100 (38%) were not (5). Of the carcinogens, 90 (56%) were Ames *Salmonella* assay mutagens and 72 (44%) were not. Of the noncarcinogens, 25 (25%) were mutagens and 75 (75%) were not. Mutagenicity is thus neither necessary nor sufficient for carcinogenicity.

The most widely used experimental models for investigation of early events in carcinogenesis involve morphologic transformation of cultured cells. Transformation is here defined as a phenotypic change involving loss of sensitivity to contact inhibition, which enables cells to form randomly oriented multilayered colonies. Transformation may increase the probability of further neoplastic change, may be accompanied by anchorage-independent growth and tumorigenicity, and can be reversible (3, 4, 6, 7). The less common model type involves observations during cancer development in the sites of transplantation of known numbers of carcinogen-treated cells *in vivo*. Transformation models have commonly used embryonic or immortalized, often aneuploid, rodent fibroblasts (2–4). The transplantation models generally involve primary grafts and/or first subgrafts of epithelial cells and often epithelial cell cultures (8–13).

The rat mammary and thyroid transplantation models were developed for investigation of the early stages of carcinogenesis per cancer-susceptible cell *in vivo*. Normal growth, differentiation, and promotion to malignancy in both tissues is hormonally controlled (13) and surviving cancer precursor cell concentrations can be determined by transplantation assay (10). When transplanted and hormonally stimulated, epithelial clonogens from these glands can give rise to multicellular monoclonal structures within which cancers may arise (13). Transplantation is coupled with hormonal manipulation of the graft recipients designed to optimize promotion of initiated clonogens. This allows quantitative estimation of the cancer risk per clonogen *in vivo* and differentiates these two models from others.

We have observed cancer frequencies in grafts of irradiated cells as high as one cancer per 10–300 clonogens (14–18). High frequencies of induced preneoplastic changes occurred in tracheal pouch grafts and in cultured tracheal epithelia (8–11) as well as in a similar mouse mammary epithelial system (9, 12). Cell number-dependent suppression of progression was seen in the tracheal system (8, 11).

The relationship of transformation of cultured embryonic or immortal fibroblasts to oncogenic initiation of normal epithelial clonogens *in vivo* is not clear. Despite the uncertainties, *in vitro* models have yielded valuable information on carcinogen-induced early cellular events. Evidence that irradiation altered virtually every cell was presented 3 decades ago (18), and essentially all 3-methylcholanthrene-treated immortal fibroblasts were shown to give rise to transformed cells that were tumorigenic (2). After replating at a variety of densities, irradiated C3H10T½ mouse fibroblasts formed the same frequencies of transformed foci per unit dish surface irrespective of initial or total final cell numbers or dish sizes (19, 20). Kennedy and associates (4, 20) postulated a generalized epigenetic effect followed by a rare confluent cell-dependent transformation event. Transformation is 10–1000 times as frequent as specific locus mutations in the general literature (21) and in parallel determinations (22, 23). High mutation frequencies in tumors may occur in a time-dependent but not in a proliferation-dependent fashion (24), and minisatellite DNA in irradiated transformed cells is less stable in growing tumors than in cultures (25). NIH 3T3 fibroblast sublines have been selected that did not yield transformants and either supported or inhibited growth of transformed cells (7, 26). In this light, Rubin (3, 7), Holliday (27), and Kennedy (4, 28) have reassessed the data and concluded that epigenetic processes play a major role in carcinogenesis.

In the current experiment, the neoplastic initiation frequency per 7-Gy γ-irradiated hormonally promoted mammary clonogen was $\approx 10^{-2}$ and is very similar to the previously

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Abbreviation: MNU, methylnitrosourea.

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observed frequency of initiation by methylnitrosourea (MNU) (29). Furthermore, the efficiency of promotion of initiated mammary clonogens to overt cancer was reduced in inverse relationship to the total mammary cell numbers transplanted per site. We postulate that highly frequent oncogenic initiation is epigenetic and that an epigenetic physiologic process(es) within the mammary glands *in situ* normally suppresses promotion of initiated cells. Carcinomas arise when initiated cells or their progeny escape such inhibition by further epigenetic and/or genetic change.

MATERIALS AND METHODS

Rats and Routine Maintenance. Virgin female F344 Fischer rats (Harlan–Sprague–Dawley) 7–8 weeks old at the time of mammary cell harvest and transplantation were used. They were fed and housed under veterinary supervision as described (17). Surgery was done under ether anesthesia. Rats were killed by ether or carbon dioxide overdose followed by severance of the cervical spine and/or opening of the pleural cavity.

Experimental Chronology. On day −15—i.e., 15 days before transplantation—the cephalolateral mammary gland of each recipient's inguinal fat pad was cannulated (30) and infused with 0.3 ml of 70% ethanol to eradicate the mammary cells *in situ*. On day −5, the recipients were adrenalectomized to produce glucocorticoid deficiency, which blocks differentiation for lactation. They were also implanted intrasplenically with a pituitary gland adjacent to an estrone capsule to induce Pr^+ (hyperprolactinemia) to stimulate mammary growth without hyperestrinism (31). The rats were maintained thereafter as described (31).

On day −5, mammary epithelial organoids were prepared from glands of untreated donors by enzymatic digestion and differential filtration and were placed in culture (32). On day −1, the organoid cultures were sham exposed or irradiated with 7-Gy ^{137}Cs γ-rays in a 4π chamber at 8.1 Gy/min and returned to the incubator for 24 hr for repair of radiation damage (32).

On day 0, irradiated and sham-irradiated cultures were enzymatically monodispersed, and the concentrations of morphologically intact cells were determined. Clonogenic fractions of cell suspensions were taken as 2.48×10^{-3} sham-irradiated cells and 0.67×10^{-3} irradiated cells as determined by transplantation assay (32). Serial dilutions containing the desired numbers of surviving irradiated or sham-irradiated cells, or mixtures of both, per 120-μl graft volume were then prepared for transplantation (10, 32). Recipient rats were grafted at one site each in the interscapular white fat pad and the cephalolateral ends of each inguinal pad. Groups 1–5 received 13.3–1080 surviving irradiated clonogens (1.99×10^4–1.61×10^6 total cells), groups 6–8 received 13.3–1080 sham-irradiated clonogens (5.36×10^3–4.35×10^5 total cells), and groups I–III received 40–360 irradiated clonogens admixed with 4.35×10^5 sham-irradiated cells (≈1080 unirradiated clonogens) in two adjacent inoculations of 60 μl each per site (Table 1). The experiment was set up in 13 runs during 7 weeks.

Data Collection and Analysis. Recipient rats were inspected frequently until death; after the first tumors appeared, inspections were daily. Most cancers were found when recipients were killed when moribund or found dead. If a tumor in a gland *in situ* or a graft site grew to 1 cm diameter in a healthy rat, it was surgically removed, and the animal was returned to the experiment. Particular attention was paid at autopsy to the graft sites, mammary glands, intrasplenic pituitary and estrone capsule implants, and the endocrine organs. Only histopathologically confirmed cancers ≥4 mm in diameter in cell grafts were included in the data analyses. Grafts in rats with improperly functioning estrone capsules were censored from day 0; those lost to autolysis or cannibalism were censored from the last day of observation. Cancer incidences per site were calculated according to an adaptation (17) of the Kaplan–Meier procedure (33). Group comparisons were by the Wilcoxon and logarithm-rank methods (34, 35) and tests of significance between final cancer incidences were performed in accord with the hypotheses under test: between irradiated clonogen groups 1–5 and sham-irradiated groups 6–8 and between irradiated clonogen groups 2–4 and mixed cell graft groups I–III that had received the same irradiated clonogen numbers. Only comparisons that yielded $P \leq 0.05$ are noted in Table 1.

RESULTS

Ninety-one mammary cancers, predominantly adeno- and/or comedocarcinomas, were randomly distributed among 676

Table 1. Effect of numbers of irradiated mammary clonogens (groups 1–5), unirradiated clonogens (groups 6–8), or mixtures of both (groups I–III) grafted on final mammary cancer incidences

Group	γ-rays, Gy	Clonogens per graft site	Total sites scored	Total cancers scored	Final cancer incidence*	Cancer incidence per 10^4 clonogens†	Clonogens per cancer
1	7	13.3	124	9	0.152 (0.049)	114 (37)	87
2	7	40	137	13	0.298 (0.081)	75 (20)	134
3	7	120	140	16	0.341 (0.102)	28 (9)	352
4	7	360	141	19	0.380 (0.125)	11 (3)	948
5	7	1080	134	34	0.837 (0.083)	8 (1)	1290
6	0	13.3	91	1	0.012 (0.012)	9 (9)	1109
7	0	120	95	7	0.187 (0.066)	16 (6)	641
8	0	1080	105	8	0.115 (0.039)	1 (0)	9434
I	7 + 0‡	40 + Epi‡	99	2	0.022 (0.015)	6 (4)	1667
II	7 + 0‡	120 + Epi‡	108	9	0.323 (0.590)	27 (11)	370
III	7 + 0‡	360 + Epi‡	94	7	0.269 (0.113)	7 (3)	1429

*Final cancer incidences (fractions of sites with cancer) were corrected for intercurrent graft site loss (33) as described (17). Standard errors are in parentheses. Significant differences in irradiated vs. unirradiated cell groups: group 1 vs. 6, $P < 0.003$; group 5 vs. 8, $P < 0.000$; groups 1, 3, and 5 vs. 6, 7, and 8, $P < 0.000$. Significant differences in irradiated cell graft inocula with vs. without added unirradiated cells: group 2 vs. I, $P < 0.000$.

†Final cancer incidence per 10^4 clonogens grafted per site. Standard errors are in parentheses. Significant differences in irradiated vs. unirradiated cell groups: group 1 vs. 6, $P < 0.003$; group 5 vs. 8, $P < 0.000$; groups 1, 3, and 5 vs. 6, 7, and 8, $P < 0.000$. Significant differences in irradiated cell graft inocula with vs. without added unirradiated cells: group 2 vs. I, $P < 0.000$. Data for groups I–III are expressed in terms of irradiated clonogen numbers only.

‡+ 0 and + Epi indicate that 4.4×10^5 unirradiated mammary epithelial cells including 1080 clonogens were added to the indicated numbers of 7-Gy-irradiated clonogens per inoculum.

sites grafted with ≈219,409 total irradiated clonogens in groups 1–5; 16 similar carcinomas were scored among 291 sites grafted with ≈126,010 sham-irradiated clonogens in groups 6–8; and 18 carcinomas were scored among 301 sites grafted with ≈50,760 irradiated clonogens mixed with ≈325,080 total unirradiated clonogens in groups I–III (Table 1). The glandular tissues in the grafts did not occupy all the contiguous fat pad spaces at the times of autopsy.

Final carcinoma incidences progressively increased with the numbers of irradiated clonogens grafted per site (groups 1–5 in Fig. 1*A* and Table 1). The data were well fit by the equation $y = -1.1525 + 0.3323x$, where $y = \log_{10}$(cancer incidence per site) and $x = \log_{10}$(clonogens per site) (correlation coefficient, $r^2 = 0.8973$; test of regression fit, $P = 0.014$; data plot not shown). Lower final incidences of cancer per site developed in control groups 6–8 that were grafted with unirradiated clonogens, and there was no clear grafted unirradiated clonogen dose–carcinoma incidence response (Fig. 1*B*; Table 1).

The >80-fold difference in mean clonogens per site between groups 1 and 5 resulted in but a 5- to 6-fold increase in final cancer incidence per site (Table 1; Fig. 1*A*), reflecting a decline in final cancer incidence per grafted clonogen as the number of irradiated clonogens per site increased (Table 1). The inverse relationship between the cancer incidence per clonogen and the number of irradiated clonogens grafted per site in groups 1–5 is well fit by a log/log regression (Fig. 2).

Carcinoma incidences in group I that had received ≈40 irradiated clonogens to which unirradiated cells had been added were significantly less per site and per irradiated clonogen than those in group 2 grafted with 40 irradiated clonogens without added unirradiated cells (Table 1). However, addition of the same unirradiated cell numbers of inocula of 120 and 360 irradiated clonogens in groups II and III had no effect on cancer incidences per site or per irradiated clonogen when compared with groups 3 and 4 (Table 1).

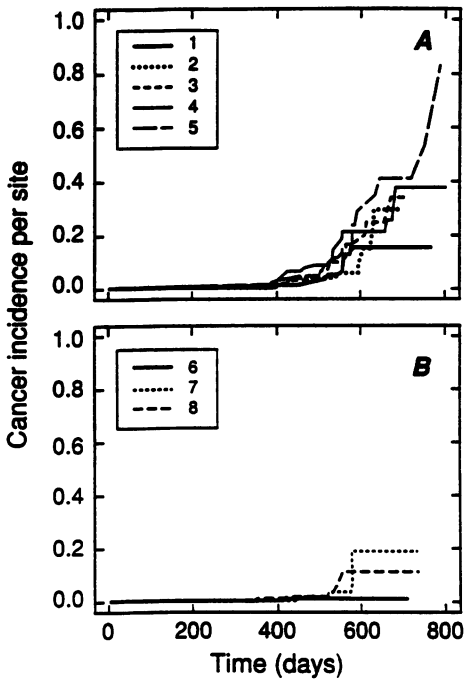

FIG. 1. Effect of irradiation and of grafted clonogen numbers on cumulative cancer incidences as fractions of total sites corrected for intercurrent loss (17, 33). (*A*) Groups 1–5, cancer incidences in sites grafted with averages of 13.3–1080 7-Gy-irradiated mammary clonogens each. (*B*) Groups 6–8, cancer incidences in sites grafted with averages of 13.3, 120, and 1080 unirradiated clonogens each. See also Table 1.

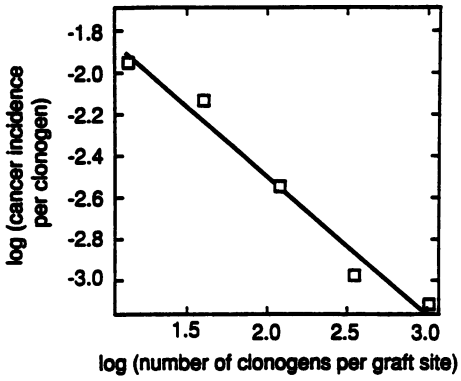

FIG. 2. Log/log plot of final cancer incidences per 7-Gy-irradiated mammary clonogen against mean numbers of surviving mammary clonogens grafted per site. □, Observed data points. Line is a computer fit of the equation $y = -(1.1525 + 0.6677x)$, where $y = \log_{10}$(cancer incidence per clonogen) and $x = \log_{10}$(number of clonogens per graft site). $r^2 = 0.9724$; probability of regression, $P = 0.002$.

DISCUSSION

These results support the hypothesis that initiation is a frequent epigenetic process within the clonogenic mammary target cell population *in vivo*. Furthermore, the observed cell number-dependent inhibition of promotion may be an important physiologic cancer control mechanism.

The greatest cancer frequency observed, uncorrected for spontaneous malignancies, was one carcinoma per ≈87 clonogens in sites grafted with ≈13 irradiated clonogens each. Final cancer incidences per irradiated clonogen decreased as the numbers of clonogens per site were increased with the larger grafted irradiated cell numbers as well as in sites transplanted with 40 irradiated clonogens to which sham-irradiated cells were added. Perhaps because promotion was suppressed by the relatively large numbers of cells already present in the 120 and 360 irradiated clonogen inocula, addition of unirradiated mammary cells to these larger inocula did not further suppress cancer incidence.

The study of MNU-induced cancer in mammary clonogen grafts was performed with WF strain rats (29), whose mammary glands contain clonogen concentrations comparable to those of F344 rats (36). About 27% of F344 rat mammary clonogens survived 7-Gy γ-rays in culture (32). Similarly, ≈34% of WF rat mammary clonogens survived exposure *in situ* to MNU (29). Comparable cumulative cancer incidences occurred per site over time, and similar final cancer incidences per clonogen occurred in grafts of MNU-exposed and of irradiated clonogens (Fig. 3).

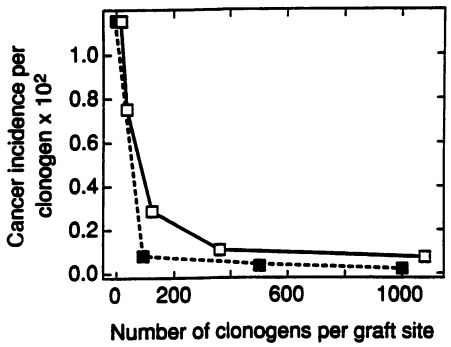

FIG. 3. Comparison of final cancer incidences per mammary clonogen against numbers of 7-Gy-irradiated clonogens (□) and MNU-treated clonogens (■) grafted per site. MNU data are from ref. 29.

Within treatment groups, all irradiated and all MNU-exposed clonogens presumably had equal probabilities of initiation, and the cancers arose within glandular structures composed of their progeny. Were promotion completely effective, the cancer frequencies per grafted exposed clonogen, corrected for control frequencies, would equal the frequencies of radiogenic or chemically induced initiation per clonogen. In practice, only one cancer can be diagnosed per graft site with confidence although there were likely to have been sites with more than one initiated clonogen. Furthermore, the hormonal promotion may not have caused every initiated clonogen or its progeny to progress to overt cancer. These sources of error tend to reduce the observed carcinoma frequencies below the "true" frequency of initiated cells. Hence, the highest observed cancer incidence per irradiated clonogen (114×10^{-4}; group 1) minus its respective sham-irradiated control incidence (9×10^{-4}; group 6) is a conservative estimate of the radiogenic initiation frequency. Accordingly, 7-Gy γ-rays initiated at least 1 of $\approx$95 clonogens exposed. About the same fraction of MNU-treated clonogens gave rise to cancer in grafts of five clonogens per site (Fig. 3).

Three points deserve further consideration: (*i*) if initiation could occur in epithelial cells other than clonogens, the high frequencies calculated above would be overestimates; (*ii*) assuming initiation is as frequent as calculated, how can the relatively low cancer frequencies in hormonally promoted irradiated mammary glands *in situ* be explained; and (*iii*) what are the possible mechanisms of epigenetic initiation and of suppression of promotion of initiated cells by other epithelial cells.

The hypothesis that cancer commonly arises from nonclonogenic cells will be considered with data from two experiments. In group 3 (Table 1), the cells were harvested for grafting from 7-Gy-irradiated 5-day cultures; culturing favors clonogen accumulation (32). The inocula was composed of $\approx$120 irradiated clonogens among a total of $\approx 1.8 \times 10^5$ morphologically intact cells. One cancer developed per $\approx$350 clonogens or per $\approx 5.3 \times 10^5$ total cells. In a previous experiment (17), the cells were irradiated with 5 Gy *in situ* in donor rats 24 hr before harvest and their clonogenic fractions were lower; inocula were composed of $\approx 4.0 \times 10^6$ morphologically intact cells including $\approx$120 irradiated clonogens per site. One cancer developed per 245 clonogens or per 8.2×10^6 total inoculated cells. When expressed per total inoculated cells, there was a 15-fold difference between cancer incidences. In contrast, there was a 1.4-fold difference in incidences expressed per clonogen. Thus, the agreement between experiments is much better if the data are expressed as cancers per clonogen.

Cultures of carcinogen-treated tracheal cells (8) and mammary cells (12) gave rise to altered foci, and the latter gave rise to ductal dysplasias when grafted (9). With time, increasing numbers of foci grew when subcultured and many grew in agarose. After 4- to 6-Gy x-radiation, foci occurred at frequencies of 1 per 37–45 putative mucosal stem cells (37–39). In these systems, cancers developed when 10^5–10^6 cells were transplanted only after many culture passages. Although focus-forming capacity in culture is a common carcinogen-induced effect, its relationship to initiation and cancer development *in vivo* is unclear.

In the current studies, cancer incidences per clonogen significantly decreased as the clonogen numbers per site increased. For example, the incidence per site grafted with 1080 irradiated clonogens was 5- to 6-fold greater than in sites grafted with 13 irradiated clonogens; when expressed per clonogen, the cancer incidence in sites grafted with 13 irradiated clonogens was 15-fold greater than in sites grafted with 1080 irradiated clonogens. This effect is unrelated to attainment of limiting cell population densities as seen in cell cultures (19, 20, 26), in that the grafted mammary tissues did not fill the available fat pads. As irradiated clonogens had been at equal risk of radiogenic initiation, differences among groups in final cancer incidences per clonogen are related to postirradiation events. This bears on the second discussion point, the high estimated initiation frequency and the low observed cancer incidences in glands *in situ*—i.e., three to four cancers per irradiated hormonally promoted rat (ref. 31; unpublished data). About 16,000 clonogens can be recovered in suspension from all the mammary glands of an untreated rat (ref. 40; unpublished data). Assuming that 20% of the clonogens are lost during preparation and that 27% survive 7 Gy, there would be $\approx$5400 total surviving clonogens per irradiated rat. In the current experiments, 1 of $\approx$1290 surviving irradiated clonogens gave rise to cancer in sites grafted with $\approx$1080 clonogens. Hence, a bit more than four radiation-related cancers might be expected per hormonally promoted 7-Gy-irradiated rat, in reasonable agreement with observation.

Suppression of promotion of radiation-initiated thyroid cells (14, 15) has been related in part to inhibition of secretion of thyrotropin (16, 41), the primary thyroid cell promoter. Other reports of cell interactions include higher cancer frequencies among grafts of dispersed preneoplastic mouse mammary cells than in grafts of such cells in small pieces of tissue (42) and more "severe" ductal dysplasias among grafts of 10^4 than of 10^5 dispersed carcinogen-treated mouse mammary cells (9). A mucosa-free tracheal surface was essential to establishment of cancer cells injected into rat tracheal pouches; normal cells admixed with the malignant or preneoplastic cells prevented the latter from growing (43). Normal epithelial cell-conditioned medium inhibited growth of carcinogen-altered tracheal cells (44). The latter was mediated at least in part through active transforming growth factor type β from the differentiating normal cells (45).

Cell interactions may impinge on the malignant process at one or more stages and in several ways: (*i*) by gap junctional exchange of molecules (46), (*ii*) by paracrine growth factor release and uptake (45, 47), (*iii*) by cell number-dependent effects on secretion of specific hormones (16, 41), and (*iv*) by cell number-dependent local competition for space and nutrition. Gap junctional communication could not occur widely in grafts until new glandular tissue developed. As the glandular tissue in the graft sites did not fill the available fat pads at autopsy, it is unlikely that competition for space and nutrition played a role in the current results. Growth and differentiation of mammary glands is mediated by the hypothalamic–pituitary–ovarian axis and placenta; we are not aware of hormonal feedback from the mammary epithelium to the endocrine system. We conclude that the suppression of promotion in this experiment was likely mediated by one or more paracrine growth factor(s).

The specific nature of epigenetic initiation is unclear. Several processes that alter gene function but not DNA base sequences are induced by ionizing radiation (4, 28). These include increased transcription of genes (48) including induction of "SOS"-like DNA repair (49), altered DNA methylation patterns that influence transcription (27, 50, 51), and somatic recombination (25, 52). Although none of these may be involved in initiation, they illustrate that radiation and perhaps other agents induce nonmutational processes in high frequency that may persist for several cell generations. One or more such processes may render the irradiated clonogen and its progeny more susceptible to oncogenic promotion and hence constitute initiation. Alternative hypotheses, either (*i*) that mutation of any one of, say, $\approx 10^3$ genes may serve as initiation, or (*ii*) that there is a gene(s) that is orders of magnitude more sensitive to oncogenic mutation than has yet been described, seem far less probable. Finally, it is unclear how large cell numbers suppressed promotion. In analogy to the tracheal system (47), we speculate that such suppression is

mediated through a paracrine factor(s) such as transforming growth factor type β.

We are indebted to Mr. Fernando Quintana for statistical analyses and to Ms. Peggy Ziebarth for manuscript preparation. This work was supported by National Cancer Institute Grants R37-CA13881 and RO1-CA44387 and by National Cancer Institute Grant P30-CA14520 to the University of Wisconsin Comprehensive Cancer Center for partial salary support.

1. Foulds, L. (1969) *Neoplastic Development* (Academic, New York), Vol. 1, pp. 44–45.
2. Mondal, S. & Heidelberger, C. (1970) *Proc. Natl. Acad. Sci. USA* **65,** 219–225.
3. Rubin, H. (1980) *J. Natl. Cancer Inst.* **64,** 995–1000.
4. Kennedy, A. R. (1985) in *Carcinogenesis*, eds. Barrett, J. C. & Tennant, R. W. (Raven, New York), Vol. 9, pp. 355–364.
5. Ashby, J. & Tennant, R. W. (1991) *Mutat. Res.* **257,** 229–306.
6. Stanbridge, E. J., Der, C. J., Doerson, C.-J., Nishimi, R. Y., Peehl, D. M., Weissman, B. E. & Wilkinson, J. E. (1982) *Science* **215,** 252–259.
7. Rubin, H. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1039–1043.
8. Terzaghi, M. & Nettesheim, P. (1979) *Cancer Res.* **39,** 4003–4010.
9. Ethier, S. P. & Ullrich, R. L. (1982) *Cancer Res.* **42,** 1753–1760.
10. Clifton, K. H. & Gould, M. N. (1985) in *Cell Clones: Manual of Mammalian Cell Techniques*, eds. Potten, C. S. & Hendry, J. H. (Churchill Livingstone, Edinburgh), pp. 128–138.
11. Terzaghi-Howe, M. (1987) *Carcinogenesis* **8,** 145–150.
12. Adams, L. M., Ethier, S. P. & Ullrich, R. L. (1987) *Cancer Res.* **47,** 4425–4431.
13. Clifton, K. H. (1990) in *Scientific Issues in Quantitative Cancer Risk Assessment*, ed. Moolgavkar, S. M. (Birkhauser, Boston), pp. 1–21.
14. Mulcahy, R. T., Gould, M. N. & Clifton, K. H. (1984) *Int. J. Radiat. Biol.* **45,** 419–426.
15. Watanabe, H., Tanner, M. A., Domann, F. E., Gould, M. N. & Clifton, K. H. (1988) *Carcinogenesis* **9,** 1329–1335.
16. Domann, F. E., Freitas, M. A., Gould, M. N. & Clifton, K. H. (1994) *Radiat. Res.* **137,** 330–337.
17. Clifton, K. H., Tanner, M. A. & Gould, M. N. (1986) *Cancer Res.* **46,** 2390–2395.
18. Sinclair, W. K. (1964) *Radiat. Res.* **21,** 584–611.
19. Kennedy, A. R., Fox, M., Murphy, G. & Little, J. B. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7262–7266.
20. Kennedy, A. R. & Little, J. B. (1984) *Radiat. Res.* **99,** 228–248.
21. Parodi, S. & Brambilla, G. (1977) *Mutat. Res.* **47,** 53–74.
22. Barrett, J. C. & Ts'o, P. O. P. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3297–3301.
23. Landolph, J. R. & Heidelberger, C. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 930–934.
24. Strauss, B. S. (1992) *Cancer Res.* **52,** 249–253.
25. Paquette, B. & Little, J. B. (1994) *Cancer Res.* **54,** 3173–3178.
26. Yao, A. & Rubin, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7486–7490.
27. Holliday, R. (1991) *Mutat. Res.* **250,** 351–363.
28. Kennedy, A. R. (1991) *Environ. Health Perspect.* **93,** 199–203.
29. Zhang, R., Haag, J. D. & Gould, M. N. (1991) *Cell Growth Differ.* **2,** 1–6.
30. Wang, B., Kennan, W. S., Yasukawa-Barnes, J., Lindstrom, M. J. & Gould, M. N. (1991) *Cancer Res.* **51,** 2642–2648.
31. Clifton, K. H., Yasukawa-Barnes, J., Tanner, M. A. & Haning, R. V., Jr. (1985) *J. Natl. Cancer Inst.* **75,** 167–175.
32. Kamiya, K., Kim, N. D., Gould, M. N. & Clifton, K. H. (1991) *Int. J. Radiat. Biol.* **59,** 1207–1216.
33. Kaplan, E. L. & Meier, P. (1958) *J. Am. Stat. Assoc.* **54,** 457–481.
34. Cox, D. R. & Oakes, D. (1984) *Analysis of Survival Data* (Chapman & Hall, London).
35. Kalbfleish, J. D. & Prentice, R. L. (1980) *The Statistical Analysis of Failure Time Data* (Wiley, New York).
36. Kamiya, K., Gould, M. N. & Clifton, K. H. (1991) *Proc. Soc. Exp. Biol. Med.* **196,** 284–292.
37. Terzaghi-Howe, M. (1989) *Radiat. Res.* **120,** 352–363.
38. Ford, J. R. & Terzaghi-Howe, M. (1992) *Exp. Cell Res.* **198,** 69–77.
39. Ford, J. R. & Terzaghi-Howe, M. (1992) *Am. J. Physiol.* **263,** L568–L574.
40. Shimada, Y., Yasukawa-Barnes, J., Kim, R. Y., Gould, M. N. & Clifton, K. H. (1994) *Radiat. Res.* **137,** 118–123.
41. Domann, F. E., Mitchen, J. M. & Clifton, K. H. (1990) *Endocrinology* **127,** 2673–2678.
42. Medina, D., Shepherd, F. & Gropp, T. (1978) *J. Natl. Cancer Inst.* **60,** 1121–1126.
43. Terzaghi-Howe, M. (1987) *Carcinogenesis* **8,** 145–150.
44. Terzaghi-Howe, M. & McKeown, C. (1986) *Cancer Res.* **46,** 917–921.
45. Terzaghi-Howe, M. (1989) *Carcinogenesis* **10,** 973–980.
46. Trosko, J. E. & Chang, C. C. (1989) *Toxicol. Lett.* **49,** 283–295.
47. Terzaghi-Howe, M. (1990) *Radiat. Res.* **121,** 242–247.
48. Boothman, D. A., Meyers, M., Fukunaga, N. & Lee, S. W. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7200–7204.
49. Wolff, S., Afzal, V., Wiencke, J. K., Olivieri, G. & Michaeli, A. (1988) *Int. J. Radiat. Biol.* **53,** 39–48.
50. Hardwick, J. P., Schlenker, R. A. & Huberman, E. (1989) *Cancer Res.* **49,** 2668–2673.
51. Feinberg, A. P., Gehrke, C. W., Kuo, K. C. & Ehrlich, M. (1988) *Cancer Res.* **48,** 1159–1161.
52. Benjamin, M. B. & Little, J. B. (1992) *Mol. Cell. Biol.* **12,** 2730–2738.