Final Technical Report

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Project Title: Mutagenicity of Radon and Radon Daughters

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Objectives

The objective of this research was to investigate the dose-response relationship with regard to the lethal and mutagenic effects of exposure of cells to radon and its decay products. Dose-rate dependence was studied, as well as the nature of the DNA lesions. The effect of DNA repair on the lethal and mutagenic effects of exposure and on the character of the DNA lesions was investigated by comparing the response of L5178Y strains that differ in their ability to rejoin X radiation-induced DNA double strand breaks. The nature of radon/radon daughter-induced mutational lesions in human lymphoblasts was also investigated.

Summary of Significant Research Results

The major results of our research can be divided into four categories: (1) the construction of a radon generator/delivery system; (2) inter-laboratory comparisons of the cytotoxic and mutagenic effects of radon and radon daughters on CHO and L5178Y cells; (3) the cytotoxic and mutagenic effects of radon and radon daughters on L5178Y cells differing in DNA repair, including characterization of TK^{-/-} mutants; and (4) the cytotoxic and mutagenic effects of radon and radon daughters in human lymphoblast TK6 cells, including characterization of HPRT⁻ mutants as to deletion size and end-points and TK^{-/-} mutants as to their deletion size and growth rate. Most of the results of these experiments are described in the appended reprints and will be described here only briefly.

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1. **The Construction of the Radon Generator/Delivery System** (Bakale *et al.*, 1993). Radon emitted by 79 mCi (2.9 GBq) of $^{226}\text{RaCl}_2$ stored in an aluminum containment vessel was pumped through a delivery loop of copper tubing, controlled by three crossover ball valves. The radon was directed into a transfer syringe that is inserted into the delivery loop through a rubber septum. The measured amount of radon was then injected through a rubber septum into a sealed standard spinner flask containing 100 ml of cell culture medium. The residual radon present in the delivery loop was pumped into a charcoal trap. The spinner flasks containing radon were enclosed in zip-lock plastic bags, and placed on magnetic stirrers for the four or more hours required for establishment of radioactive equilibrium between radon and its short-lived daughters. After equilibrium was reached, a 1.5 ml aliquot of medium was withdrawn for determination of the radioactivity of the medium by gamma counting of the ^{214}Bi decay product. The cells were then added to the medium in the spinner flask and incubated with spinning at room temperature for the time necessary to deliver the desired dose. Dose rates of 30-40 cGy/hr from the medium were usually obtained by injection of 3 ml of radon-containing gas, but dose rates as high as 115 cGy/hr have been obtained by injection of more radon. Safety measures are described in the appended reprint.

Figure 1. Design of Radon Generator

Schematic diagram of the radon delivery system in which air from syringe S-1 is pumped into the radium-containment vessel via V-2 and radon-enriched air is concomitantly pumped to syringe S-2. Also shown is the charcoal trap that is used to collect residual radon in the loop comprised of ball valves B-1, B-2, and B-3, and the copper tubing.

The γ counting of ^{214}Bi was accomplished with a 5 x 5 cm NaI detector and multichannel analyzer. The area of the region corresponding to the 0.609 MeV gamma emission from ^{214}Bi was measured and converted to units of activity by means of the spectrum of a 0.5 μCi reference standard of $^{226}\text{RaCl}_2$. The activity of ^{214}Bi at any one time is a linear function of the initial activities of ^{222}Rn , ^{218}Po , ^{214}Pb and ^{214}Po . A multiple

linear regression calculation on the observed radioactivity of ^{214}Bi with time yielded the initial activities of those nuclides contributing to the dose. The dose rate contributed by the nuclides in the medium was calculated from the energies of the α and β particles emitted by the various radionuclides. The radon daughters also become associated with the cells and contribute to the dose. To measure the radioactivity associated with the cells, an aliquot of medium plus cells was removed at the end of the exposure. The cells in a measured aliquot of medium were pelleted and washed by centrifugation, taken up in fresh medium, and were counted using a hemocytometer. The α radioactivity of an aliquot with a known number of washed cells was determined by scintillation counting. The dose contributed by the α particles associated with the cells was calculated from the α cpm/cell as described below. Dosimetry details are discussed in an article by Jostes *et al.* (1991).

2. Interlaboratory Comparisons

a. **CHO cells.** The results of these experiments have appeared in two articles: Jostes *et al.* (1991) and Schwartz *et al.*, (1992). Alpha radiation-induced cell killing was determined in four different laboratories using the same CHO cell line, C-18, exposed in suspension culture in three of the laboratories and attached to mylar film at Los Alamos National Laboratory. The alpha radiation sources were (1) ^{222}Rn and its short lived daughters at Pacific Northwest Laboratories (PNL) and at Case Western Reserve University (CWRU); (2) ^{212}Bi at the University of Chicago (UC); and (3) ^{238}Pu at Los Alamos National Laboratories (LANL). For calculation of dose from ^{222}Rn and its daughters and from ^{212}Bi , the following factors were considered: (1) the presence of different qualities and energies of nuclides in the medium; (2) the contribution to dose from radioactivity in the medium and from that associated with the cells; (3) the geometry of the cell and of the nucleus; (4) the intracellular localization of the radionuclides; (5) the attenuation of the α particles by the cytoplasm; (6) the radionuclide concentrations in the medium; and (7) the change in radioactivity associated with the cells with time; (8) the contribution to the dose from β -emitting progeny; and (9) the length of the exposure. Dose response curves were constructed plotting the colony forming ability of the cells against the α radiation dose. D_{0s} in Gy were 0.67, 0.66, 0.64, and 0.51, while n 's were 0.9, 0.9, 1.0, and 1.2 at UC, CWRU, PNL, and LANL respectively. Thus, the dose response parameters were surprisingly similar for α radiation at the four different laboratories. These parameters varied more widely for the dose response to X radiation, with the D_{0s} ranging from 1.15 to 1.64, and n varying from 1.8 to 3.3. The variability of dose-response characteristics with respect to low LET radiation in

contrast to the relatively invariable dose-response characteristics of high LET radiation has been observed previously (Thacker *et al.*, 1982; Iliakis, 1984).

b. **L5178Y Cells.** (Evans, *et al.*, 1993a). Following the completion of the CHO cell inter-laboratory comparison, a comparison of the cytotoxic and mutagenic effects of radon and radon progeny on L5178Y (LY) cells was undertaken at CWRU, UC, and PNL. The heterozygous $TK^{+/-}$ cell line LY-R16 was used for the investigation, with mutagenesis being studied at the autosomal *Tk1* locus. Dosimetry calculations were carried out as for the CHO cells, substituting values for the size of the LY cells and nuclei, and the time parameters of the association of radon daughters to the LY cells. The D_0 s obtained varied from laboratory to laboratory to a greater extent than for CHO cells, ranging from 0.2 to 0.4 Gy with the lower values being obtained when greater association of radon progeny with the cells occurred (PNL, and at UC with unchelated ^{212}Bi). We concluded that the variations were due to uncertainties in the measurement of the associated radioactivity, since this quantity was greater with LY cells than with CHO cells. The alpha radiation-induced mutant frequencies were quite similar at the three laboratories, particularly if plotted against survival rather than dose, with frequencies of 1480-2210 mutants per 10^6 surviving cells being observed at a survival level of 37%. It was of some interest that the D_0 values for radon were lower for LY cells (0.2 -0.4 Gy) than for CHO cells (0.6 Gy). The differences in D_0 would not be expected to be caused by differences in repair, since α particle-induced cytotoxic lesions do not appear to be repaired in either type of cell (see Evans, 1991, for review). Possibly the sensitivity of the LY cells is caused by their minimal cytoplasmic volume or by the occurrence of α -radiation-induced apoptosis in these murine lymphoblastoid cells (Macklis *et al.*, 1992).

3. Cytotoxic and Mutagenic Effects of Radon and Radon Daughters in LY Strains Differing in DNA Repair. (Evans, *et al.*, 1993b).

Three different LY strains were used in this study. The X radiation-sensitive strain LY-S1 is deficient in the repair of X radiation-induced DNA double-strand breaks (Evans *et al.*, 1987; Wlodek and Hittelman, 1987), while the UV-sensitive strains LY-R16 and LY-R83 are presumed to be deficient in the excision of UV-induced dimers as is their parental strain LY-R (Hagan *et al.*, 1988; Szumiel *et al.*, 1988). Strains LY-S1 and LY-R16 are heterozygous at the *Tk1* locus, while strain LY-R83 is hemizygous at this locus (Table I) (Evans *et al.*, 1986).

The three strains responded similarly to the cytotoxic effects of radon and radon daughters, giving D_{0s} of 0.30-0.31 Gy. After exposure to X radiation, in contrast, the D_0 for LY-S1 is 0.7 Gy and for the two LY-R strains is 1.3 Gy (Table II) (Evans *et al.*, 1986). These results indicate that while strain LY-S1 is deficient in the repair of damage induced by X radiation, none of the strains repair the cytotoxic damage induced by α radiation. Mutant frequencies induced by α radiation were higher per unit dose than were frequencies induced by X radiation, but at equitoxic doses the mutant frequencies at the *Tk1* locus were similar for α and X radiation. A very low mutant frequency was induced by α radiation in the $TK^{+/-}$ hemizygous strain LY-R83, indicating that multilocus lesions were induced by the α radiation. (Table II)

TABLE I
Properties of L5178Y Lines

	LY-R16	LY-R83	LY-S1
Relative sensitivity to X radiation	Resistant	Resistant	Sensitive
Relative sensitivity to UV radiation	Sensitive	Sensitive	Resistant
Repair defect	Dimer excision ^a	Dimer excision ^a	DSB repair ^b
<i>tk</i> genotype	<i>tk</i> ^{+/-}	<i>tk</i> ^{+/-}	<i>tk</i> ^{+/-}
Presumptive <i>gk</i> genotype	<i>gk</i> ^{+/+}	<i>gk</i> ^{+/-}	<i>gk</i> ^{+/+}

TABLE II
Comparison of the Mutagenicity and Lethality of α -Particle and X Radiation for Three Cell Lines

	LY-R16	LY-R83 ^a	LY-S1
D_0 (Gy)			
α particles	0.31	0.31	0.30
X radiation	1.3	1.3	0.70
Mutant frequency ^b /Gy			
α particles	4.9	0.36	2.7
X radiation	1.3	0.05	2.0
Mutant frequency ^b at 37% survival			
α particles	1.7	0.16	1.0
X radiation	2.7	0.05	1.0

Mutant frequency is expressed as the number of $TK^{-/-}$ mutants per 1×10^3 surviving cells.

The nature of the $TK^{-/-}$ mutants was determined by Southern blotting procedures and by measuring galactokinase activity in $TK^{-/-}$ mutants. The *Gk* gene that codes for galactokinase is located within 200 kb of the *Tk1* gene in Chinese hamster cells (Peterson and McBride, 1980). The entire active *Tk1* allele was lost by 81% of the $TK^{-/-}$ mutants of strain LY-R16. Approximately 40% of the $TK^{-/-}$ mutants of strains LY-R16 and LY-S1 exhibited inactivation of galactokinase, indicating that the mutational lesion inactivating the *tk* gene extends to the neighboring *gk* gene. The percentage of $TK^{-/-}$ mutants with inactivation of galactokinase after treatment with X radiation, UV radiation, and radon is shown in Figure 2. As can be seen, this percentage is high in the X radiation-sensitive strain LY-S1 after X

radiation, in the UV-sensitive strain LY-R16 after UV radiation, and in both strains after α radiation, an agent known to induce irreparable cytotoxic lesions (Evans, 1991). We have suggested that the inhibition of DNA replication at the site of unrepaired lesions in each case provides a greater opportunity for the induction of large deletions.

Figure 2. Effect of DNA Repair Capacity on the Induction of Multilocus Lesions

Unrepaired DNA lesions and the induction of multilocus lesions. The inactivation of galactokinase in $TK^{-/-}$ mutants has been assumed to be an indication of the extension of the mutational lesion from the *tk* gene to the neighboring *gk* gene. Line LY-S1 is sensitive to X radiation and is unable to repair X-radiation-induced DNA double-strand breaks. Line LY-R16 is sensitive to UV radiation and is assumed to be deficient in the repair of UV-radiation-induced pyrimidine dimers as has been shown for its parental line, LY-R... Neither line appears to be able to repair α -particle radiation-induced lesions. The percentages of spontaneous and radon-induced mutants showing GK loss did not differ significantly between LY-R16 and LY-S1 cells ($P = 0.40$ and 0.46 , respectively). However, the percentage of X-radiation-induced mutants showing GK loss is significantly greater for LY-S1 than LY-R16 cells ($P = 0.0005$ according to Fisher's exact test). In the case of UV radiation, the percentage of mutants losing GK was higher for LY-R16 $TK^{-/-}$ mutants than for LY-S1 mutants ($P = 0.056$) according to Fisher's exact test. The numbers at the top of the columns show the number of mutants analyzed in each case. (□) Spontaneous, (▨) X-radiation-induced, (▩) UV-radiation-induced, (■) radon-induced.

4. Cytotoxic and Mutagenic Effects of Radon and Radon Daughters in Human Lymphoblastoid Cells, TK6. (Bao *et al.*, 1995; Chaudhry *et al.*, 1996)

a. Comparison of the cytotoxicity of X radiation and radon for TK6 cells.

We used human cells for this research because of the sequence data available for *HPRT* and other genes. We carried out our initial studies with *HPRT*⁻ mutants, since we wished to isolate a number of mutants with partial deletions (almost all radiation-induced $TK^{-/-}$ mutants have full deletions of the *TK* gene). The TK6 cells were obtained from Dr. Wm. Thilly of Massachusetts Institute of Technology, and were grown in RPMI-1640 medium plus 10% defined calf serum (HyClone). Colony formation was measured in soft agar medium (0.25% BBL agar in growth medium). The dose response to X radiation gave a D_0 of 0.62 Gy, a value similar to that obtained previously for this cell line by Liber *et al.* (1983). The D_0 for radon was 0.31 Gy (Table III).

b. Comparison of the mutagenicity of X radiation and radon in TK6 cells.

The mutagenicity of X radiation and of radon and radon daughters was measured at the *HPRT* and *TK* loci. Although the mutant frequencies induced by radon as a function of dose were higher than the frequencies induced by X radiation at both loci, at the D_{37} or D_0 doses, the frequencies induced by radon were somewhat lower than induced by X radiation (Table III).

Table III Mutant Frequencies Induced by Radon vs. X radiation in TK6 Cells

Agent	D_0 or D_{37}	Mutant Frequency ($\times 10^6$)			
		<i>HPRT</i> Locus		<i>TK</i> Locus	
		per Gy	at D_0 or D_{37}	per Gy	at D_0 or D_{37}
X Radiation	0.61	8.1	5.0	52	32
Radon	0.29	13.2	4.5	76	22

c. The nature of mutations induced by X radiation and radon in TK6 cells (Bao *et al.*, 1995; Chaudhry *et al.*, 1996)

HPRT⁻ mutations: Independent spontaneous HPRT⁻ mutants have been compared with mutants induced by X vs. α radiation as to the type of alteration in the HPRT DNA using PCR methods. In this analysis each independent mutant colony was recloned and expanded in culture to enable isolation of DNA and RNA. HPRT cDNA was produced from the total cellular RNA using reverse transcriptase and specific primers, and amplified by the PCR. DNA from each exon, from selected introns, and regions of interest flanking the *HPRT* gene were also amplified by the PCR. Alterations were confirmed by Southern and Northern analysis of genomic DNA. We have analyzed 84 independent mutants: 24 spontaneous, 31 mutants induced by X radiation, and 29 induced by radon. Deletions were the most common alteration in the recovered mutants. Thus, we found that 63% of the spontaneous mutants, 81% of the X radiation-induced mutants and 86% of the radon-induced mutants had deletions in the HPRT DNA. Deletion of the entire *HPRT* gene occurred in 21% of the spontaneous mutants, 55% of the mutants induced by X radiation and 55% of the mutants induced by α radiation. The size of these large deletions was investigated by determining whether or not linked DNA sequences, for which we had obtained or synthesized the appropriate probes, were present or absent. A greater percentage of X-radiation-induced

mutants (35%) than radon-induced mutants (12%) harbored deletions extending more than 800 kb from the *HPRT* gene. The breakpoints of partial deletions appeared to be randomly distributed throughout the *HPRT* gene, with breakpoints appearing in most of the exons. For the deletions extending from exon 9 into the 3' flanking regions, the breakpoints in the flanking regions were located at different sites, as shown by restriction fragments of different sizes generated by *Ban*II and *Pst*I digestion. Mutants showing no DNA alterations, as measured by Southern blotting or PCR analysis, were classified as presumptive base substitutions. These small mutations accounted for 31% of the spontaneous mutants, 12% of the X radiation-induced mutants and 16% of the radon-induced mutants. We conclude that the predominant type of mutation induced by both X radiation and α radiation at the *HPRT* locus is a large deletion encompassing the *HPRT* gene and large stretches of neighboring DNA sequences. No evidence for clustering of breakpoints was observed.

TK^{-/-} mutations. A large majority (82%) of the radon-induced mutants and 74% of the X radiation-induced mutants, but only 36% of the spontaneous mutants lost the entire active *TK* allele. Although 21% of the X radiation-induced mutants harbored lesions extending into the *col*1A1 gene in a neighboring chromosomal band and approximately 31 Mb distant from the *TK* gene, only 5% of the radon-induced mutants lost heterozygosity at this distant locus. Most (77%) of the radon-induced mutants harbored lesions that involved the *TK* allele alone or extended from the *TK* locus to only one of the neighboring markers. (Figure 3, 4).

Figure 3. Extent of Lesions in Chromosome 17 Induced by Radon and X Radiation

The horizontal lines show the extent of the loss of heterozygosity for the various types of mutant. The sequences have been ordered by deletion analysis, but the distances between S4, tk, S77, and S24 are unknown to our knowledge. On the basis of chromosomal bands, the *col*1A1 gene is estimated to be approximately 30 Mb from the *tk* gene. The numbers in parenthesis show the number of mutants with the respective lesion.

Figure 4. Spectra of Mutational Lesions Induced by Radon and X Radiation

The bars show the percentage of mutants with (1) no apparent loss of heterozygosity; (2) the percentage of mutants losing heterozygosity at the *TK* locus and the *TK* locus plus one or both of the closest markers, D17S77 and/or D17S4; (3) the percentage of mutants with LOH involving the D17S24 sequence but not the *col1A1* locus; and (4) the percentage of mutants losing heterozygosity at all five loci tested, including *col1A1*, 30 Mb distant from the *TK* gene. The number of mutants analyzed was 19 induced by X radiation, 39 induced by radon and 20 spontaneous mutants. Since the mutants to be analyzed were picked approximately equally among the normally and slowly growing colonies, the number of each type of colony analyzed could influence the calculation of the percentage of mutants with extensive LOH. However, if the percentage of mutants with extensive LOH is calculated based on the slowly or very slowly growing population analyzed, the percentage of radon-induced mutants with extensive LOH is still less than in the case of mutants induced by X radiation (2/16 or 12.5% for radon and 4/7 or 57% for X radiation). The statistical difference in each class of mutants was determined using the Fisher exact test. For mutants showing no LOH, the percentage of radon-induced mutants differed from the percentage of spontaneous mutants ($P = 0.005$). In the case of radon-induced compared to X-radiation-induced mutants that exhibited no LOH, $P = 0.07$. For mutants showing LOH at the *TK* gene and *TK* plus D17S4, D17S77 or both, the percentage of radon induced mutants was significantly higher than for X-radiation-induced or spontaneous mutants ($P = 0.03$ and 0.008 , respectively). The percentage of radon-induced mutants exhibiting LOH at all tested markers differed significantly from the percentage of spontaneous mutants with this genotype ($P = 0.014$). In the case of the percentage of radon-induced compared to X-radiation-induced mutants with this genotype, $P = 0.083$. The difference between the percentage of mutants showing LOH extending from the *TK* gene to D17S24 but not to *col1A1* was not significant for any combination of spontaneous compared to X-radiation-induced compared to radon-induced mutants.

These results are in agreement with the analysis of radon and X-radiation-induced lesions inactivating the *HPRT* gene of TK6 cells, described above, in which a lower percentage of radon- than X radiation-induced mutants showed lesions extending to markers 800 or more kB from the *HPRT* gene on the X chromosome. It is likely that the densely ionizing tracks of the α particles create more closely-spaced DNA double-strand breaks (dsb) than the sparsely ionizing tracks produced by X radiation. It is possible that these closely neighboring dsb tend to rejoin more frequently than do distant dsb.

d. **The sequence of a deletion end-point.** We have analyzed an X radiation-induced mutant with a partial deletion in the *HPRT* gene, with one end-point within the gene and the other in the 3' flanking region. In this study, the region in which the deletion breakpoint occurred was first narrowed down to about 200 base pairs by running a series of

PCR reactions that were not completed if a primer site was deleted. The DNA was then subjected to restriction enzyme digestion, using a "5-cutting" enzyme that hydrolyzed a sequence just outside of the last PCR priming site before the breakpoint and that produced fragments of approximately 1000 bp with overlapping sequences. The fragments were separated by agarose gel electrophoresis and the fragments containing the breakpoints identified by hybridization to the appropriate radioactively-labeled probe. The fragment of interest was circularized by ligation and amplified with two flanking oligonucleotide primers on opposite strands to produce linear double-stranded DNA for sequencing. Comparison of the altered sequence with the known sequence defined three of the four deletion endpoints (the end point of the deleted sequence within the *HPRT* gene was determined by the known *HPRT* sequence) The sequence of the fourth breakpoint (the deleted endpoint in the flanking region) was determined by similar methodology using DNA from wild-type cells. The deletion endpoint consisted of a repeated sequence of three base pairs, one of which was in the deleted region (lower case letters):

ACTTGA / ggtca.....ttatga / TGTCA

The sequence is similar to those identified by Lutze *et al.* (1990) for the deletions induced by X radiation and radon in the extra-nuclear plasmid, pHAZE. The occurrence of short repeated sequences at deletion junctions is typical of the rejoining of dsb with overlapping ends by non-homologous recombination (Pfeiffer and Vielmetter, 1988).

e. **Growth rate of radon-induced TK^{-/-} mutants.** It was found that the percentage of slowly growing TK^{-/-} mutants was two times greater after radon than X radiation exposure, regardless of the extent of the lesion in the *TK* gene (Figure 5, Table IV).

Figure 5. Time of Mutant Colony Appearance

Mutant frequency of TK6 cells measured at various times after plating. We have termed the mutant colonies appearing at 4 and 5 weeks after plating "very slow-growth-rate" mutants. TFT was present in the selective medium at a concentration of 2 µg/ml and was added to the same plates after 2 weeks of incubation to give an additional 2 µg/ml.

Table IV. Growth Rate of Radon-Induced TK^{-/-} Mutants with Various-Sized Lesions

	Percentage mutants in each growth rate class			Total mutants
	Normal	Slow	Very slow	
A. Radon-induced mutants				
No LOH	43	43	14	7
LOH at TK + D17S4 or D17S77 or both	45	20	35	20
LOH at TK extending to D17S24, but not to col1A1	0	17	83	6
LOH at col1A1, D17S4, TK, D17S77, D17S24	0	50	50	2
B. X-radiation-induced mutants				
No LOH	100	0		5
LOH at TK + D17S4 or D17S77 or both	100	0		6
LOH at TK extending to D17S24, but not to col1A1	33	67		3
LOH at col1A1, D17S4, TK, D17S77, D17S24	0	100		5
C. Spontaneous				
No LOH	88	12		8
LOH at TK + D17S4 or D17S77 or both	100	0		2
LOH at TK and D17S24, but not at col1A1	50 ^a	50 ^a		2
LOH at col1A1, D17S4, TK, D17S77, D17S24	0	100		3

Notes. Normal-, slow- and very slow-growth-rate mutants are those that formed colonies at 2, 3 and 4–5 weeks after plating in soft agar medium, respectively. The few very slow-growing X-radiation-induced mutants were not analyzed.

^aLoss of heterozygosity in both of these mutants was not contiguous.

The slow growth rate of these mutants appeared to be caused by an extended G1 period. About 30 generations following the exposure to α radiation, the growth rate of approximately half of these slow-growing mutants had increased to the rate of the normal TK6 parental cells. In order to determine if this change in growth rate resulted from the induction of genomic instability, the characteristics of these mutants, as well as of mutants not exhibiting a change in growth rate, were investigated. Characteristics investigated included plating efficiency, the mutant frequency at the *HPRT* locus, the frequency of unstable aberrations per cell, and the response to a second X radiation dose of 1.5 Gy in terms of survival, level of apoptosis, p53 expression, and cell cycle distribution. In comparison to the characteristics of the parental TK6 cells, one of these mutants showed alterations in four of these characteristics, one showed alterations in three, seven showed alterations in one characteristic, and one (a spontaneous TK^{-/-} mutant) showed no alterations. Following the second irradiation, all of the mutants tested showed an increase in p53 expression similar to that of the parental TK6 cells (Table V). The mutants that maintained a slow growth rate showed fewer alterations in the tested characteristics than did the mutants exhibiting a growth rate increase (Table VI).

Our results show that these putative unstable mutants exhibit a variety of individual phenotypes. If these mutants are indeed unstable, the results indicate that there are multiple targets for the induction of genomic instability, the characteristics of the eventual unstable sub-clones depending on the nature of the initial target.

Characteristics of Mutants that Changed from Slow to Normal Growth Rate

A Mutant Number	B Radon Dose (Gy)	C Extent of LOH	D Weeks to 1 st Cols.	E Weeks to 1 st Cols (later test)	F Later Doubling Time	G Aberra's. per cell	H HPRT Mutant Freq.	I % Plating Eff.	J %	Response to 1.5 Gy X irradiation			
										% apop. (16 h)	Ratio irradi./control		G ₂ M
											GI	S	
13	0.74	0	3	2 & 3	17	<0.04	<0.3	(25)	(8.6)	2.4	1.0	0.5	(4.0)
17	0.82	4-TK-77	3	2	15	0.04	<0.3	60	15.7	(5.0)	0.9	0.6	2.8
24	0.82	TK	4	2	16	0.04	(7.0)	44	13.1	(6.5)	1.3	0.6	2.1
29	0.00	0	5	2	16	<0.04	<0.3	68	15.1	2.8	(0.6)	0.5	(3.9)
30	0.74	4-TK-77	5	2 & 3	17	0.08	<0.3	(22)	12.6	3.4	1.3	0.5	2.3
38	0.00	0	3	2	16	(0.2)	2.1	44	16.9	3.2	1.3	0.4	1.8
39	0.32	4-TK	3	2 & 3	18	(0.1)	(10.1)	(26)	(8.1)	2.3	1.4	0.3	2.2
40	0.66	0	3	2	15	<0.04	(22.4)	42	14.9	2.5	1.3	0.4	2.2
41	0.75	0	3	2	17	<0.04	<0.3	32	14.3	2.8	(0.7)	0.6	(4.5)
42	1.13	TK	3	2	16	<0.04	<0.3	49	16.8	(7.1)	1.3	0.5	1.3
44	0.75	4-TK-77	4	4	44 → 32	<0.04	<0.3	64	16.6	ND	ND	ND	ND
TK6	0.00	0	2	2	14	0.04	(6.8)	73	19.9	2.9	1.4	0.5	1.8

Column Notes:

A. Mutant # 44 changed its doubling time from 43 h to 32 h during the measurement of its doubling time at least 30 generations after radon treatment.

C. Loss of heterozygosity; Fig. 1 shows a map of chromosome 17 with the relevant sequences.

D. Initial colonies were formed from cells plated 48 hours after radon treatment.

E. At least 30 generations elapsed between radon treatment and the second estimation of colony growth and doubling time.

E. For mutant #'s 13, 30, and 39, a substantial number of colonies appeared at two weeks and a substantial additional number at three weeks.

G. Aberrations include chromatid and chromosome aberrations, dicentric, rings, and tetraploid and aneuploid cells.

K. Apoptosis was determined 16 h after 1.5 Gy by flow cytometry, following propidium iodide staining.

L, M & N. Cell cycle distributions were determined 16 h after administration of 1.5 Gy X radiation.

L, M & N. Cell cycle distribution is indicated as the % of cells in the indicated phase divided by the % of unirradiated cells in that phase.

Characteristics of Mutants Maintaining Slow Growth Rate

A	B	C	D	E	F	G	H	I	J	K	L	M
Mutant Number	Radon Dose (Gy)	Extent of LOH	Weeks to 1 st Colonies	Weeks to 1 st Colonies (retest)	Later Doubling Time	Aberrations per cell	HPRT Mutant Frequency	% Plating Efficiency	% apoptotic cells	% cells in G1	% cells in S	% cells in G2
14	0.74	4-TK-77-24	3	3 & 4	32	0	31	49	1.2	40	53	9
16	0.82	4-TK-77	3	3	30	0	<0.3	44	1.3	44	46	9
25	0.82	4-TK-77	4	4	42	0	<0.3	24	1.4	52	43	6
28	0.74	4-TK-77-24	4	4	36	0	<0.3	14	2	44	50	6
44	0.75	4-TK-77	4	4	44 → 32	0	<0.3	20	3	44	48	8
48	0	0	4	3	30	0.16	0.3	54	1	52	43	5
TK6	0	0	2	2	14	0	1	58	1.3	30	56	14

Column Notes:

- Mutant #44 changed its doubling time from 43 h to 32 h during the measurement of its doubling time at least 30 generations after radon treatment.
- Loss of Heterozygosity; Fig. 1 shows a map of chromosome 17 with the relevant sequences.
- Initial colonies were formed from cells plated 48 hours after radon treatment.
- At least 30 generations elapsed between radon treatment and the second estimation of colony growth and doubling time.
- For mutant #14, a substantial number of colonies appeared at 3 weeks and a substantial additional number at 4 weeks.
- Aberrations in Mutant #48 were all dicentric.
- Apoptosis was determined in unirradiated cells by flow cytometry, following propidium iodide staining.
- K, L & M. The cell cycle distributions were determined in unirradiated cells by flow cytometry.

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