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Molecular Characterization of X-ray-Induced Mutations at the HPRT Locus in Plateau-Phase Chinese Hamster Ovary Cells: The Effect of Dose, Dose Fractionation and Delayed Plating

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ABSTRACT: Plateau-phase CHO-K1 cells were irradiated to study the effects of dose, dose fractionation and delayed plating on the yield and type of mutations induced by 250 kVp X rays at the HPRT locus. DNA isolated from 89 mutant cell lines was examined by Southern blot analysis using a cDNA HPRT probe. Complete loss of the HPRT locus was the most frequently observed lesion, regardless of treatment protocol. Dose was the only parameter tested which appeared to significantly affect the fraction of full deletions induced by X rays. At 2 Gy, only 43% (9/21) showed a complete loss of HPRT sequences. The proportion of full deletion mutants observed in all other treatment groups (4 Gy, 2+2 Gy separated by 24 hr and 4 Gy with 24 hr delay in replating) combined was 66% (45/68). The specific fraction of full deletions was not altered by repair associated with recovery from sublethal or potentially lethal damage, suggesting that while DNA repair may mitigate the lethal effects of radiation damage, it acts with equal fidelity on all lesions. Evidence will also be discussed which suggests that the distribution of intragenic lesions is nonrandom.

1. INTRODUCTION

It is well known that cellular processes associated with repair of sublethal damage act on premutational lesions (Jostes and Painter, 1980; Rao and Hopwood, 1982; Thacker and Stretch, 1983). Several studies have also focused on the molecular structure of radiation-induced mutations and have found that the most common lesion is a large deletion of genetic information (Thacker, 1986; Vrieling et al., 1985). However, no systematic study has yet been reported in the literature determining how the molecular structure of radiation-induced mutations is affected by radiation dose or DNA repair. The present study was conducted to explore the effect of these variables on the yield and type of X-ray-induced mutations.

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2. MATERIALS AND METHODS

Chinese hamster ovary cells (CHO-K1) were routinely cultured in McCoy's 5a medium (GIBCO, Grand Island, New York) supplemented with 10% fetal calf serum and antibiotics. To reduce the background of spontaneous mutants, these cells were diluted to a low density (1000/75cm^2 flask) prior to initiation of this study. From this level, the population was expanded until sufficient numbers were available for storage by freezing in liquid nitrogen. Individual aliquots of these cells were thawed and cultured only long enough for an individual experiment. These cells had a low spontaneous background frequency of approximately 5 HPRT$^-$ mutant cells per 10^6 viable cells. Cells were irradiated in starved plateau phase as described previously (Nelson et al., 1984). Briefly, 10^5 cells were seeded into 25 cm^2 flasks and allowed to grow to confluence for 12 days without refeeding. Under these conditions, less than 0.1% of the cells are undergoing DNA replication (Nelson et al., 1984). Irradiated cells were subcultured into several flasks before any cell division occurred, to insure the independence of each mutant clone isolated. The flasks were maintained separately and only one mutant clone was picked from each population. Following subculture, the cells were maintained in exponential growth in nonselective medium for 9 days to permit degradation of pre-existing stocks of HPRT protein and mRNA in radiation-induced mutants. The cells were then reseeded at a density of $2x10^5$/100 mm dish in Ham's F12 medium formulated without hypoxanthine (GIBCO) and supplemented with 5% dialyzed fetal calf serum and 10 μM 6-thioguanine (TG; Sigma Chemical Co., St. Louis, MO). Individual TG^r clones were tryspsinized using cloning cylinders and propagated in McCoy's 5a medium.

X-irradiations were carried out using a Norelco MG-300 X-ray machine operating at 250 kVp and 10 mA (half-value layer 0.52 mm Cu). The dose rate was 1 Gy/min. The dose rate was monitored with a Farmer model 2502/3 air ionization chamber (Nuclear Enterprises, Beeham, Reeding, England). Cells were irradiated at 37°C in 25cm^2 flasks containing 5 ml of medium and 5%CO_2/95% air.

DNA from mutant and unirradiated cells was analyzed for mutations at the HPRT locus by the method of Southern (1975). High-molecular-weight DNA was isolated from exponentially growing cells by lysis in buffer containing 50 mM Tris, 10 mM EDTA, 100 mM NaCl, 0.5% sodium laurel sarcosine (pH 8). Following digestion with proteinase K (100 μg/ml; Sigma) at 50°C overnight, lysates were extracted with phenol-chloroform (Maniatis et al., 1982) and treated with RNase A (50 μg/ml; Sigma) at 37°C for 60 min. After extraction with phenolchloroform, the purified DNA was exhaustively dialyzed against TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at 4°C. 20 μg of DNA from each of these samples were digested with restriction endonucleases under conditions specified by the enzyme supplier. Digested DNA was subjected to electrophoresis through 0.8% agarose gels overnight in TBE buffer (Maniatis et

al., 1982). Following alkaline denaturation and neutralization, the DNA was transferred to GeneScreen Plus hybridization membrane (Dupont-NEN, Wilmington, DE) as recommended by the membrane supplier. Membranes were air dried and stored at 4°C. Prior to hybridization, the membranes were pretreated at 65°C for at least 1 hr with 20-30 ml of 1 M NaCl, 1% SDS, 10% dextran sulfate, 1 X Denhardt's reagent (Maniatis et al., 1982). Following addition of 2-3 mg of denatured salmon sperm DNA and 50-100 ng of labeled probe DNA, hybridization was carried out overnight at 65°C. The membranes were washed with 2 X SSC at room temperature (two washes of 5 min each), 1% SDS, 2 X SSC at 65°C (two washes of 30 min each), and 0.1 X SSC at room temperature (two washes of 30 min each). Autoradiography was carried out at -80°C using Kodak X-omat R film and intensifying screens.

Blots were probed with hamster HPRT cDNA contained in the 1.3 kbp Msp I fragment of the plasmid pHPT12 (Konecki et al., 1982). pHPT12 was provided by J.C. Fuscoe (Lawrence Livermore National Laboratory) and was isolated from E. coli HB101 by alkaline/SDS lysis and banding on CsCl gradients as described by Maniatis et al. (1982). Probe DNA was labelled with ^{32}P using a random primer oligolabeling reaction kit (Pharmacia, Piscataway, NJ). Unincorporated radionuclides were removed by NACS-52 column chromatography (Bethesda Research Labs, Gaithersburg, MD).

3. RESULTS AND DISCUSSION

Starved plateau-phase CHO-K1 cells were employed in this study to allow investigation of cellular responses in an environment where DNA replication was not occurring. Several treatment protocols required holding the cells for 24 hr prior to subculture. If DNA replication and cell division occurred during this time, interpretation of results would be much more complicated. In the present study CHO-K1 cells were irradiated with graded doses of X rays or a 4 Gy dose split into two equal fractions separated by 24 hr or were replated 24 hr following a single 4 Gy dose. The results of these experiments are shown in Table 1. Cells irradiated with 4 Gy and replated immediately showed three times as many induced mutants as those treated with 2 Gy (120 vs 38 TG^r mutants/10^6 viable cells). Relative to immediate replating, delaying replating of cells irradiated with 4 Gy by 24 hrs resulted in no significant change in the mutation frequency (120 vs 113), despite a modest increase in cell survival (from 25% to 43% survival). Delivering 4 Gy in two equal fractions separated by 24 hr also caused an increase in survival (to 37%) as well as a reduction in the mutation frequency to approximately two-thirds of that observed with an acute dose of 4 Gy (79 vs 120 TG^r mutants/10^6 surviving cells). The results of the delayed plating and split dose experiments suggest that plateau-phase cells are capable of repairing a modest amount of sublethal and potentially lethal damage. However, as found by Thacker and Stretch (1983), delayed plating does not significantly reduce the number of potentially mutagenic lesions induced by a given

dose of X rays. Several studies (Thacker, 1979; Jostes and Painter, 1980; Nakamura and Okada, 1981; Radner et al., 1982; Ueno et al., 1982; Thacker and Stretch, 1983) have found that low dose rate or dose fractionation decreases the induced mutation frequency at the HPRT locus. The results presented here are consistent with these studies.

Table I
Cytotoxicity and induced mutation frequency as a function of X-ray dose.

Dose	Replating[a]	Surviving Fraction[b]	Induced Mutation Frequency (TG^r Colonies/10^6 Viable Cells)[c]
1 Gy	Immediate	0.82 ± 0.11	19 ± 14
2 Gy	Immediate	0.75 ± 0.15	38 ± 14
3 Gy	Immediate	0.44 ± 0.16	80 ± 46
4 Gy	Immediate	0.25 ± 0.05	120 ± 52
4 Gy	24 hr delay	0.43 ± 0.07	113 ± 20
4 Gy	Split	0.42 ± 0.11	78 ± 20

a: Plateau-phase CHO-K1 cells were irradiated and plated immediately, given 4 Gy and held at confluence without refeeding for 24 hr, or given two 2 Gy doses separated by 24 hr.
b: Values represent the mean and standard deviation of at least 3 experiments.
c: Values represent the mean and standard deviation of at least 5 experiments.

High-molecular weight DNA was isolated from unirradiated CHO-K1 cells and digested and subjected to Southern blot analysis following restriction enzyme treatment. The results are shown in Figure 1. Molecular weight standards are noted on the left side of each panel. Band sizes of approximately 17, 13, and 6.7 kbp were observed in Hind III digestions (Panel A, lane 12). With Pst I band sizes of 10, 9, 6.7, 5, 3.4, 2.9, and 0.9 kbp were observed (Panel B, lane 12). Fuscoe et al. (1983) determined that the 6.7 kbp Hind III and 5 and 2.9 kbp Pst I bands were caused by an unlinked pseudogene which hybridizes strongly to HPRT probes. DNA from unirradiated V79 cells showed band sizes and migration patterns identical to CHO-K1 cells when either Pst I or Hind III was employed (Lane 1 in Figures 1A and 1B).

To determine the affect of dose, dose fractionation, and delayed plating on the molecular structure of radiation-induced mutations at the HPRT locus, a total of 89 TG^r mutants were isolated from four different treatment protocols: (i) 2 Gy of X rays, with immediate replating; (ii) 4 Gy, with immediate replating; (iii) 4 Gy, split into two equal fractions separated by 24 hr, followed by immediate subculture; and (iv) 4 Gy, followed by 24 hr delay before subculture. On the basis of their Southern blot banding pattern, we sorted the observed patterns into three categories: (i) full deletions--all HPRT-specific bands were missing (viz., 4PG in lane 6 of Figure 1A

and lane 5 of Figure 1B); (ii) alterations--modified banding patterns indicating the presence of partial deletions, insertions, rearrangements, or modifications of one or more restriction enzyme cutting sites within the HPRT locus (viz., 4DA in Figure 1A, lane 5); (iii) no change--the banding pattern was indistinguishable from untreated CHO-K1 DNA.

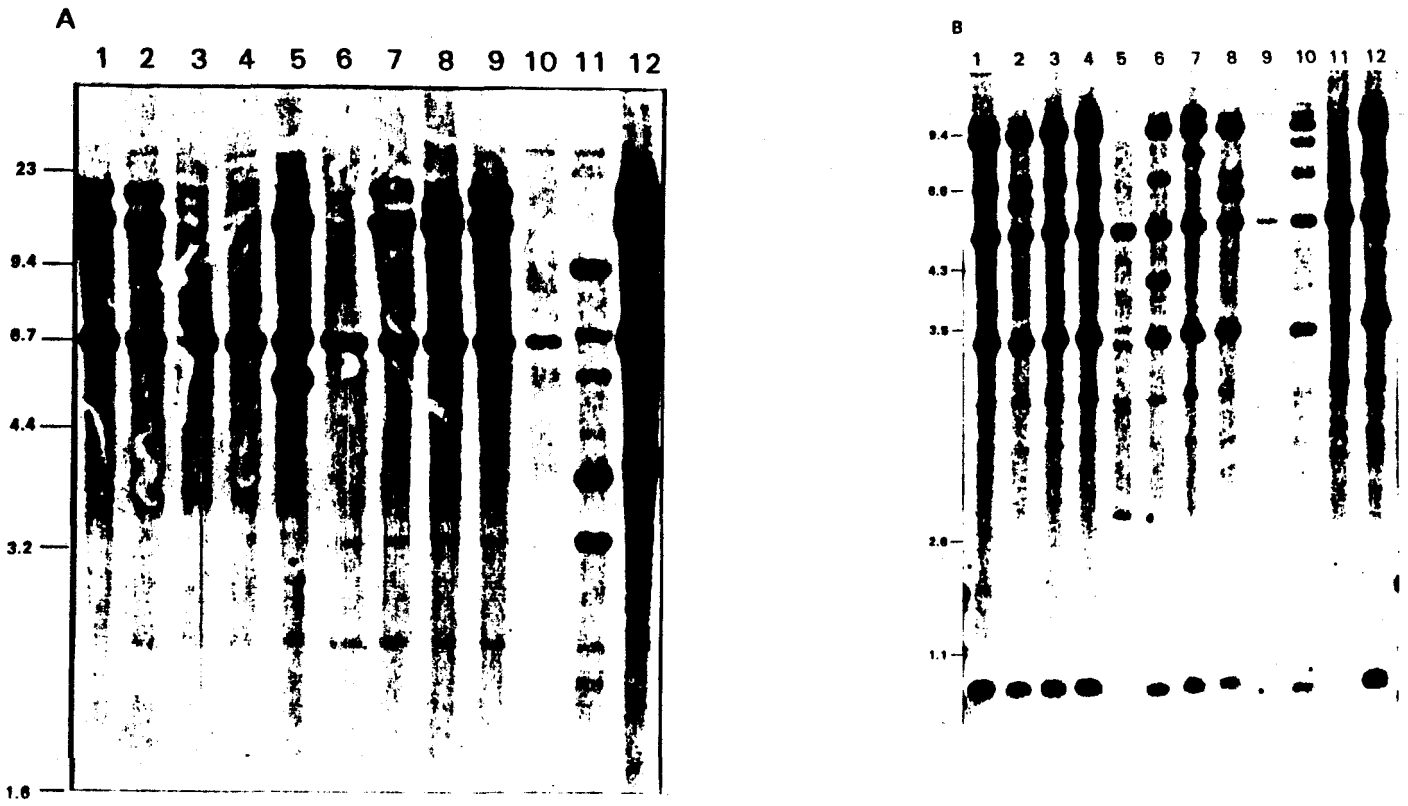

Figure 1. Southern blot of selected mutant cell lines. Panel A: Hind III digestion. Lane 1: V79, Lanes 2-11: 2SH, 4DD, 4DB, 4DA, 4PG, 4PC, 2SF, 4SF, 4SE, 13A, Lane 12: CHO-K1. Panel B: Pst I digestion. Lane 1: V79, Lanes 2-11: 2SF, 2SB, 4DA, 4PG, 4PC, 4SF, 4SE, 13A, Lane 12: CHO-K1.

Table 2 summarizes the data collected on all the TG^r cell lines. Fifty-four of the 89 mutants lacked any detectable HPRT-specific sequences and therefore were caused by deletions of the entire locus. This was the most frequently observed lesion, regardless of the treatment regime employed. Loss of or a shift in size of individual bands was the second most common pattern noted. Twenty-five mutants were found to have altered banding patterns. Ten of the 89 mutants showed no change in the band migration pattern. Although 89% (79/89) of the observed mutations were caused by a detectable loss or alteration of HPRT coding sequences, the proportion of full deletions was dependent on how the radiation dose was administered. For cells irradiated with 4 Gy and replated immediately, 69% (20/29) showed full deletion of the HPRT gene, while 24% (7/29) had altered banding patterns. In cell populations irradiated with two 2 Gy doses separated by 24 hr, 62% (16/26) of the mutants had full deletions. This shift in the mutation spectrum, toward a smaller fraction of full deletions, was not statistically significant ($p = 0.84$; Chi-squared test; Bevington, 1969). The number of clones selected for molecular characterization under these exposure conditions was too small to resolve effects of repair on the spectrum of induced mutations, which, if present, are much less pronounced than the effect on the mutation frequency described above. Similarly, we did not detect statistically significant changes in the mutation spectrum due to holding cells in a nonproliferative state for 24 hr after irradiation ($p = 0.61$). For a dose of 2 Gy, only 43% (9/21) showed full deletions while the proportion of mutants having altered banding patterns rose to 38% (8/21).

Table 2.
Summary of Mutation Type as Determined by Southern Blot Analysis[a]

	Mutation Type[b]			
Dose Schedule	Full	Alteration	No Change	Total
2.0 Gy	9 (43%)	8 (38%)	4 (19%)	21
4.0 Gy	20 (69%)	7 (24%)	2 (7%)	29
4.0 Gy (24 hr delay)	9 (70%)	2 (15%)	2 (15%)	13
4.0 Gy (Split)	16 (62%)	8 (31%)	2 (7%)	26
Total	54 (61%)	25 (28%)	10 (11%)	89

a: Each DNA sample was digested independently with at least two restriction enzymes.
b: Full- no HPRT coding sequences detected
Alteration- loss of and/or change in size of one or more bands
No change- no apparent change in band size or migration pattern

The difference between the fraction of mutant cell lines showing full deletions isolated following 2 Gy irradiation (9/21) and the same fraction in all 4 Gy treatment groups combined

(45/68) was statistically significant at the p = 0.056 level and indicates that exposure level affected the mutation spectrum. The principal reason for this difference was the higher fraction of alteration-type events observed in the cell population irradiated with 2 Gy (8/21). The cells used in this study exhibited a low background mutation frequency; therefore we believe that contamination by alteration-type mutations arising spontaneously in the 2 Gy population was unlikely. This data indicates that the dose effect trend is towards more large scale deletions at higher doses.

The hamster HPRT gene is composed of 9 exons ranging in size from 18 bp to 601 bp, spanning approximately 40 kbp (B.J.F.R., in preparation). Exons 2, 3 and 4 are located within the 10, 3.4, and 6.7 kbp Pst I bands, respectively. Similarly, the 9 and 0.9 kbp Pst I fragments contain exons 6-8 and 9, respectively. In Hind III digestions, exons 2-4 are contained in the 17 kbp band while exons 6-9 are located within the 13 kbp band. Bands containing exons 1 and 5 were not detected in the present study and has not been seen by Rossiter (1987).

Using this information, we were able to determine the approximate location of the deletion breakpoints in 14 of the 25 mutants which showed altered banding patterns. Substantial portions of the 3' half of the gene appeared to be missing in 11 of these mutants. Only four of the mutants showed any alteration in the 5' region (one mutant appeared to be caused by a deletion of all but the 3' end of the locus). These results indicate a nonrandom distribution of deletion endpoints, suggesting that the 3' region of the hamster HPRT gene is abnormally sensitive to radiation-induced events creating deletion-type mutations. Models of mutation induction and transformation may need to be revised to account for deletions of large segments of DNA in specific regions of the mammalian genome.

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