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COMPARISON OF MUTATION INDUCTION BY EXTERNAL AND INTERNAL RADIATION SOURCES IN SYNCHRONIZED CHINESE HAMSTER OVARY (CHO) CELLS

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ABSTRACT

Synchronized CHO cells were either X irradiated or pulse-labeled with ^{3}H-TdR or ^{125}IUdR at 5 h (early S phase, ES), 8 h (mid S phase, MS) and 12 h (late S phase, LS) after mitotic selection. Radiation-induced hprt gene mutation frequencies were determined and mutants were isolated for further molecular analysis. Mutation frequencies of X irradiated ES cell populations increased linearly with dose, reaching 30 times the spontaneous level at 600 cGy. However, mutation frequencies of MS and LS cell populations increased at doses up to 200 cGy and then reached a plateau at doses up to 600 cGy. Although the same mutation frequencies were observed in ES and LS cell populations following X irradiation at 200 cGy, the cell survival

fractions were 0.6 and 0.98, respectively. Cells with DNA-incorporated ^{3}H-TdR had similar patterns of induced mutations. The results suggest that the most susceptible period for the induction of hprt mutations occurs while the gene is replicating in early S phase. Compared with X rays, DNA-incorporated ^{3}H-TdR and ^{125}IUdR produced D_0 doses with RBE values of 2.1 and 8.6, respectively, and hprt mutation frequencies with RBE values of 6.9 and >60, respectively. The structures of mutant hprt genes in radiation-induced mutant clones were analyzed by Southern hybridization. Seventeen to twenty-eight percent of hprt mutant clones had no detectable structural changes (point mutations). For X irradiation, 56% of mutant clones had complete hprt gene deletion, while for both internal radiation emitters (^{3}H-TdR and ^{125}IUdR), nearly 50% showed partial deletions at the hprt locus.

INTRODUCTION

Induction of 6-thioguanine resistance ($6TG^{r}$) mutations at the hypoxanthine-guanine-phosphoribosyl-transferase (hprt) gene locus has been widely studied with low- and high-LET radiation in the Chinese hamster ovary (CHO) cell system (1-7). The hprt gene product is one of the enzymes responsible for the salvage of preformed purine bases during normal nucleic acid turnover in mammalian cells (8). The Chinese hamster hprt gene is located at the distal end of the short arm of the X chromosome where DNA replication occurs in early S phase (9,10). High frequency X ray-induced hprt mutation has been reported at the G_1/S phase border of synchronized CHO cells (7,11). These studies suggest that DNA replication may influence the induction of mutations at the hprt locus.

The induction of hprt mutations with radioactive nucleic acid precursors (tritiated thymidine [^{3}H-TdR] and 5-[^{125}I]iodo-2'-deoxyuridine [^{125}IUdR]) has been studied in mammalian cells (12-15). Although beta rays from tritium are classified as low-LET particles, the ionizing radiation from ^{3}H-TdR incorporated in DNA may produce greater injury to cells than that from an equivalent amount of more generally distributed energy from internal (such as ^{3}HOH and ^{3}H-amino acid) (16) and external (such as X ray and γ ray) low-LET radiation sources.

Many studies have shown that the emission of a shower of low energy Auger electrons results in a highly localized biological effect similar to high-LET radiation (17-35). Nearly 30% of the electron tracks emitted from a single

decay of the Auger emitter ^{125}I incorporated into DNA as $^{125}IUdR$ interact as a direct hit with the atoms of DNA (36). The base most affected is the labeled base and in roughly 1% of all decays more than 100 eV is deposited here (37). Pomplun (37) calculated that ^{125}I incorporated in DNA produced 0.9 double strand breaks (DSB) per decay plus 1.9 additional single strand breaks (SSB) or a total of 3.7 SSB per decay. These findings are in agreement with those of LeMotte and Little (38).

In this paper, we discuss the mutation frequencies and molecular structural changes of the hprt locus in synchronized CHO cells induced by external (X ray) and internal (^{3}H-TdR and $^{125}IUdR$) radiation sources.

MATERIALS AND METHODS

Cell Culture

Chinese hamster ovary (CHO) cells were grown at 37°C in a humidified 95% CO_2 - 5% air atmosphere with McCoy 5a medium supplemented with 10% heat-inactivated bovine calf serum (56°C for 30 min), penicillin (50 units/ml), and streptomycin (50 μg/ml). To reduce the level of spontaneous hprt mutants, the cells were grown in HAT medium (2×10^{-4} M hypoxanthine, 2×10^{-7} M aminopterin, and 1.75×10^{-5} M thymidine) for 3 days. Synchronous cells (mitotic indices above 95%) were obtained by shaking loose the mitotic cells from asynchronous cultures as described previously (39). The mitotic cells ($1\text{-}3 \times 10^{8}$ cells) were pooled in an ice bath and frozen in dimethylsulfoxide (DMSO) freezing medium (40).[1] Prior to use, the cells were defrosted, seeded into plastic tissue culture flasks/tubes/petri dishes and returned to an incubator at 37°C.

X Irradiation

Synchronized cells were irradiated aerobically with a GE Maximar X ray generator operating at 190 kV and 15 mA with 1 mm/Al added filtration, yielding a dose rate of 80 cGy/min.

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[1]The progression of cells through the cell cycle is not affected by the freezing-defrosting procedure.

Incorporation of ^{3}H-TdR and ^{125}IUdR

Synchronized CHO cells were incubated at 37°C. At 1 to 2 h intervals, the cultures were pulse-labeled for 15 min with 3.7×10^{4} Bq/ml of ^{3}H-TdR (approximately 7.4×10^{11} Bq/mmol) or ^{125}IUdR (approximately 7.4×10^{13} Bq/mmol, Du Pont) to obtain labeling indices throughout the cell cycle. The cells were fixed with 100% methanol and Kodak nuclear emulsion NTB type 2 was applied to the dishes for autoradiography. The autoradiograms were stained with 0.1% crystal violet. Two hundred cells were scored from each dish to determine the pulse-labeling indices (41).

To investigate other biological endpoints (cytotoxicity and mutation, see below), various radioactive concentrations of ^{3}H-TdR or ^{125}IUdR were incorporated into early S (ES), mid S (MS), or late S (LS) cell populations for 15 to 30 min at 37°C. The labeled cells were washed twice with warm medium containing nonradioactive thymidine (final concentration 10^{-5} M). The cells were then stored at 2 to 4°C for 15 to 24 h to accumulate radiation doses from the decay of the DNA-incorporated ^{125}I or ^{3}H.

Cytotoxicity and Mutation Assays

Cytotoxicity was determined by the colony forming assay. The treated cells were seeded at low densities and incubated for 8 days to allow colony formation. Plating efficiencies were determined by a similar procedure. To determine mutation frequencies, each treated culture, containing at least 10^{6} surviving cells, was incubated with nonselective medium for 7 to 9 days to allow phenotypic expression. The cells (2×10^{5}) were then added to each of 20 P-100 petri dishes containing 5 μg/ml of 6-thioguanine and incubated for 10 days to select mutant clones. For further molecular analysis (see below), one mutant clone was isolated from one petri dish per treatment (to avoid sibling problems) and these clonal mutant cells were cultured in P-100 dishes containing 6-thioguanine medium until the cells reached the near confluence level.

Molecular Analysis

High molecular weight DNA was isolated from mutant clones by a standard method (42). Samples (10 μg) were digested (37°C, overnight) with 20 units of Pst-I (New England Biolabs), electrophoresed on 0.8% agarose gels, and transferred to Nytran membranes (pore size 0.2 μm, Schleicher and

Schuell) for ≥14 h according to the recommendations of the manufacturer. Membranes were hybridized with a PCR-amplified hprt probe labeled by a random primer labeling procedure (43). The PCR primers (5'- GGCTTCCTCC TCACACCGCT-3' and 5'-GGACTCCTCG TGTTTGCAGA-3') were used to amplify a 496 bp fragment from a Chinese hamster hprt cDNA. The Southern blot banding patterns were analyzed and assorted into three categories as reported previously (44): (i) full deletions: all hprt-specific bands are missing; (ii) alterations: partial deletions and rearrangements are seen; and (iii) no change: indistinguishable from control (including point mutations).

Dose Determination

Radiation doses (cGy) were calculated from the DNA-incorporated ^{3}H-TdR or ^{125}IUdR activity per cell as described by Kassis and co-workers (26,31,32,34,35). Basically, the cumulative mean lethal dose (D) to the cell nucleus during these *in vitro* experiments is given by $D = E/V_N$, where V_N is the nuclear volume of CHO cells and E, the total amount of energy deposited in the nucleus. E is given by $E = (N_{37})\varepsilon$, N_{37} being the cumulated number of decays of the radionuclide (during the 15 to 24 h 4°C and the subsequent 8 day 37°C incubation periods) in the nucleus at 37% survival, and ε the average energy deposited in the nucleus (for ^{125}I, ε = 10.8 keV; for ^{3}H, ε = 4.3 keV) per decay in the nucleus.

RESULTS

The progression of pooled, previously frozen, mitotic cells through the cell cycle was followed by obtaining pulse-labeling indices (Fig. 1). The synchronized cells entered S phase 4 h after mitosis at 37°C, with nearly 95% of cells in S phase during mid S phase (8-10 h after mitosis). Labeling indices rapidly declined when cells entered G_2 phase.

With X irradiation, the early S phase (5 h) cell population showed the highest sensitivity to induced cell lethality (Table I) and the late S phase population the greatest radioresistance. Hprt mutation frequencies increased linearly with dose up to 600 cGy in early S phase cells (Fig. 2A). The relationship of cell survival and mutation frequencies of early S phase cells was nearly linear to the 5% survival level (Fig. 2B). X irradiation induced 1.3×10^{-7} mutants/cGy and the mutation frequencies increased linearly as a

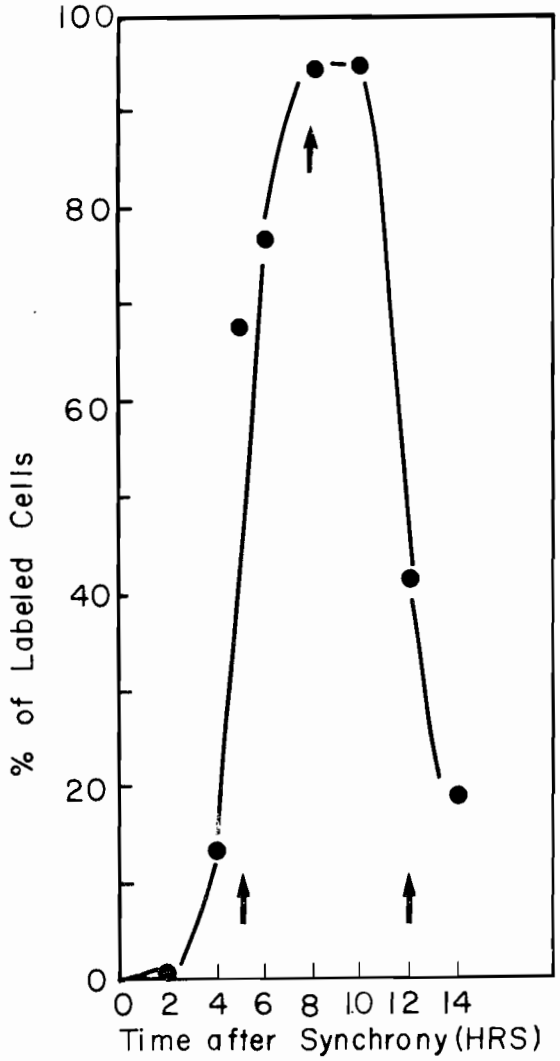

FIG. 1. Pulse-labeling indices of synchronized CHO cells after mitosis. Arrows indicate time when cell population was studied.

function of dose. Although mid and late S phase cells were more X ray resistant than early S phase cells (Table I), X ray-induced mutation frequencies increased at the same rate as in early S phase cells up to 200 cGy, with no increase between 200 cGy and 600 cGy (Fig. 2A). With mid S phase (Fig. 2A)

and late S phase (Fig. 2B) cell populations, X ray-induced hprt mutations increased only in the shoulder region of the cell survival curve (up to 200 cGy).

TABLE I
Radiosensitivities of Synchronized CHO Cells to X Ray, ^{3}H-TdR, and ^{125}IUdR

Radiation Source	Time after Mitosis (h)	N[a]	D_0 (cGy)	RBE[b]
X ray	5 (ES)	3	170	1.0
	8 (MS)	2	180	1.0
	12 (LS)	4	190	1.0
^{3}H-TdR	5 (ES)	1	20	8.5
	8 (MS)	~1	88	2.1
	12 (LS)	1	65	2.9
^{125}IUdR	5 (ES)	1	0.6-2.0	85-288
	8 (MS)	1	5.6	32.1
	12 (LS)	1	35.8 (1.6)[c]	5.3 (11.8)[c]

[a] extrapolation number
[b] RBE = D_0 (^{3}H-TdR or ^{125}IUdR)/D_0 (X ray)
[c] Parentheses show D_0 dose and RBE from sensitive cell populations

The cell survival curves of CHO cells pulse-labeled with ^{3}H-TdR at 5, 8, or 12 h after mitosis had no shoulder. There were no clear cell cycle effects on cellular lethality in MS and LS cell populations (Table I). Early S phase cell populations had mutation frequencies that increased linearly with dose to nearly 100 cGy of ^{3}H-TdR (9×10^{-7} mutants/cGy, RBE = 6.9), while mid and late S phase cell populations demonstrated increased mutation frequencies at doses from 5 to 10 cGy, with no further increase between 10 and 270 cGy (Fig. 3). With ^{125}IUdR incorporation, there were no dose-response or cell cycle effects when the mutation frequencies were calculated on the total cell populations. Only early S phase cell populations showed slightly higher mutations frequencies (Fig. 4).

In order to study the molecular structural changes at the hprt locus, genomic DNA isolated from independent mutant clones was examined by Southern hybridization analysis. Fifty-eight percent of spontaneously arising

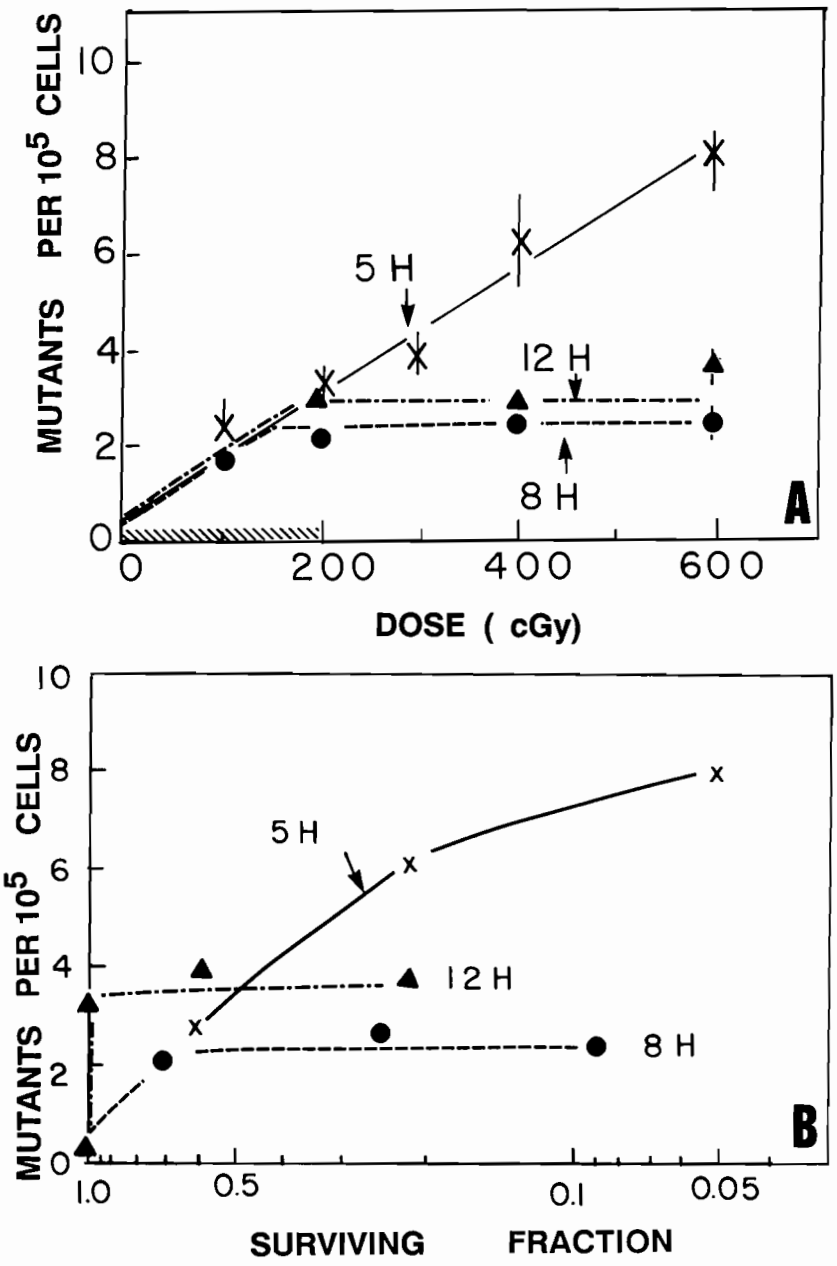

FIG. 2. A. Mutation frequencies at hprt locus in synchronized CHO cells following X irradiation. Early S (ES): X; Mid S (MS): solid circles; Late S (LS): solid triangles. Spontaneous background frequencies ([0.27 ± 0.12] X 10^{-5}) shown as shaded area. B. Relationship of cell survival and X ray-induced mutation frequencies at hprt locus in synchronized CHO cells. Early S : X; Mid S : solid circles; Late S : solid triangles.

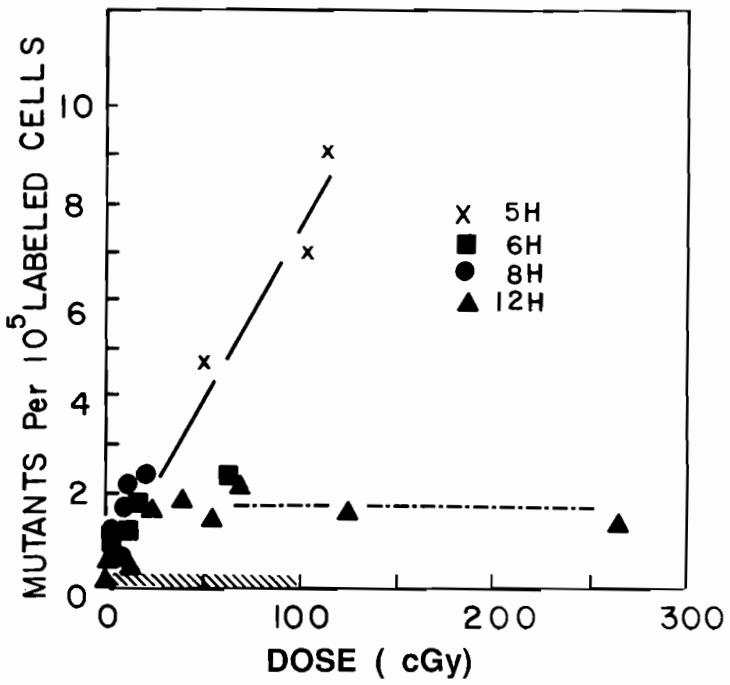

FIG. 3. Mutation frequencies at hprt locus in synchronized CHO cell populations following ^{3}H-TdR pulse-labeling. Early S (ES): X, solid squares; Mid S (MS): solid circles; Late S (LS): solid triangles. Spontaneous background frequencies ([0.50 ± 0.12] X 10^{-5}) shown as shaded area.

mutants had no detectable structural changes, while 56% of 600 cGy X ray-induced mutant clones had total gene deletions and 27% partial gene deletions. In contrast, half the mutant clones obtained after ^{3}H-TdR or ^{125}IUdR incorporation had partial gene deletions. Seventeen to twenty-eight percent of mutant clones were point mutations after both external and internal radiation (Table II).

DISCUSSION

The hprt gene of CHO cells is functionally hemizygous in pseudodiploid cells, since one X chromosome is inactivated in female cells during embryonic development (45). Farrell and Worton (9) localized the

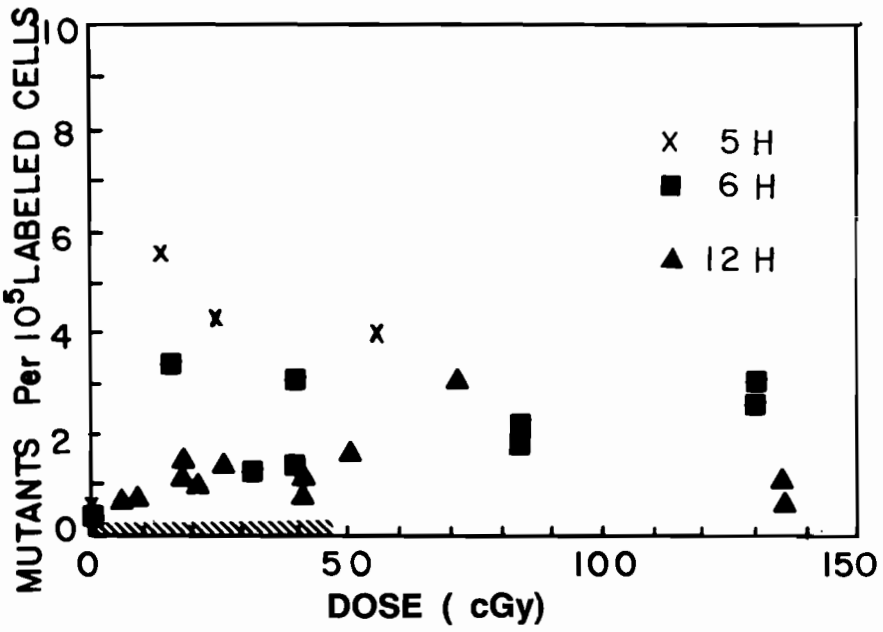

FIG. 4. Mutation frequencies at hprt locus in synchronized CHO cell populations following ^{125}IUdR pulse-labeling. Early S (ES): X, closed squares; Late S (LS): solid triangles. Spontaneous background frequencies ($[0.60 \pm 0.13] \times 10^{-5}$) shown as shaded area.

hprt gene to the distal end of the short arm on the Chinese hamster X chromosome. DNA replication in these cells occurs intensively at the short and long arm of the X chromosome during early and late S phase, respectively (10). At early, mid, and late S phase, the survival fractions of X irradiated CHO cells showed the same age responses as demonstrated previously (39,46-48) (Table I).

It has been reported that in many mutation systems in eukaryotic cells, plots of radiation-induced mutation frequencies (mutants/survivors) against the logarithm of the surviving fraction often give a straight line with low- and high-LET radiation (7,29,49-51). In this study, only early S phase cell populations showed a linear increase in the induction of hprt mutants following X irradiation to 600 cGy and cell survival levels of about 5% (Fig. 2B). This suggests that a close relationship between cell killing and

TABLE II
Molecular Analysis of the HPRT Mutant Clones

Radiation Source	Number of Clones	Structural changes at hprt locus: Total Deletion	Partial Deletion	Point Mutation
Control	29	4 (14%)	8 (28%)	17 (58%)
X ray (600 cGy)	48	27 (56%)	13 (27%)	8 (17%)
^{3}H-TdR (1-265 cGy)	36	9 (26%)	16 (46%)	11 (28%)
^{125}IUdR (7-137 cGy)	57	15 (26%)	31 (54%)	11 (22%)

induction of hprt mutants in early S phase cells exists. On the other hand, late S phase cells were the most radioresistant (Fig. 2A) (39,46-48). When hprt mutants were induced with nonlethal doses (up to 200 cGy), however, the mutation frequency level remained constant (Fig. 2A).

The same effects of X ray-induced hprt mutational patterns through the cell cycle have been reported in synchronous CHO cells by others (7,11). This phenomenon may be related to the order of gene replication in the cell cycle. Housekeeping genes are located in the euchromatin area and tend to replicate in early S phase, whereas tissue-specific genes (transcriptionally inactive genes located in heterochromatin) tend to replicate in late S phase (52). The hprt locus might be highly susceptible to radiation during the replication period when cells escape from lethal radiation damage. Once the hprt gene has finished replicating, however, it may become more resistant to radiation-induced mutation while other essential genes may be more sensitive to damage in mid and late S phase.

Since ^{3}H-TdR and ^{125}IUdR are incorporated into nuclear DNA, they pass into daughter nuclei after cell division. In addition, G-C-rich DNA (euchromatin) is synthesized in early S phase and low G-C DNA (heterochromatin) in late S phase (52). The same mutation frequencies in early S phase CHO cells with ^{3}H-TdR incorporated into DNA required nearly a sixfold lower radiation dose than with X ray (Fig. 2B and 3). This RBE value

correlates well with that of 5.9 reported in a previous study in which ^{3}H-TdR incorporation was compared to chronic gamma ray induction of hprt mutations in mouse L5178Y lymphoblastoid cells (13). When ^{3}HOH and ^{3}H-amino acids were incorporated into cells, the radioactivity was homogeneously distributed throughout the cells and RBE values of 1 to 3 were reported for cell killing, chromosomal aberrations and mutations (16). Since ^{3}H-TdR is incorporated into thymine-rich DNA, these areas received more radiation than expected, and the RBE values observed were higher.

It has been reported that ^{125}IUdR incorporated into the DNA of cells generates highly localized, low energy, Auger electrons resulting in extremely high RBE values for cell lethality, mutations and malignant transformations (7,15,17-35,38). In the present studies, the RBE (at D_0 doses) of ^{3}H-TdR and ^{125}IUdR treated cells were extremely high in ES phase (85-288) as compared to MS and LS phase cells (Table I). These results and those published earlier (29), in which an RBE value of 49 for ^{125}IUdR-induced hprt mutations in human lymphoblast cells had been reported, suggest that very low doses of internal emitters (especially ^{125}IUdR) are efficient inducers of mutations in the absence of cell killing. While significantly high rates of hprt mutations were induced at low DNA-incorporated ^{125}IUdR levels, we were unable to measure a dose response in the present studies (Fig. 4). It is possible that the decay of DNA-incorporated ^{125}IUdR and the subsequent shower of Auger electrons could have led to nonrepairable DNA damage and death of the irradiated cells. In such a scenario, the surviving cells are those in which (a) the decay of ^{125}I occurred within nonlethal genes, or (b) DNA damage had been repaired.

As determined by Southern hybridization analysis, 58% of the spontaneous hprt mutants were shown to have undetectable changes (Table II). These findings are in agreement with results from other cell systems (14,15,53). Fifty-six percent of 600 cGy X ray-induced mutant clones were total gene deletions (Table II). This value was higher than that in 100 cGy X ray-induced TK-6 cells, where 30% of mutant clones were reported as total gene deletions (14,15). However, other studies with CHO, V79, and TK-6 cells indicated over 50% of X ray-induced mutant clones were total hprt gene deletions (12,44,53,54).

Similar molecular structural changes were observed after DNA incorporation of both internal emitters (^{3}H-TdR and ^{125}IUdR) (Table II). Forty-six and fifty-four percent of the mutant clones were partial gene deletions after exposure to ^{3}H-TdR and ^{125}IUdR, respectively. Twenty-six percent of the

mutant clones were missing a total gene with both of these radionuclides. It is possible that ^{3}H-TdR and ^{125}IUdR incorporated within, or in close proximity to, the hprt gene damage local regions. In the presence of high radioactive concentrations within the hprt gene, total deletions might result. In the present study, however, nearly half the molecular structural changes appeared as partial gene deletions. Whaley and Little (14,15) reported that the molecular structural changes were dependent on the dose of ^{125}IUdR incorporated. Currently, mutant clones are being analyzed to determine the relationship between DNA replication patterns and molecular structural changes of the hprt gene in different phases of the cell cycle with X, ^{3}H-TdR, and ^{125}IUdR irradiation.

In conclusion, our data suggest that the most susceptible period for the induction of hprt mutation occurs while the gene is replicating in early S phase. Compared with X rays, the RBE values for ^{125}I incorporated into DNA for cell killing and induction of hprt mutations were much higher than those of tritium. With these internal emitters, approximately 50% of the mutant clones exhibited partial gene deletions and 25% were missing a total gene.

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