Cellular and molecular studies of mutation induction by low energy heavy ions*

Tom K Hei[1], Li X Zhu[1], David J Chen[2] and Charles A Waldren[3]
([1] *Center for Radiological Research, College of Physicians and Surgeons, Columbia University, New York 10032;*
[2] *The Genetics Group, Los Alamos National Laboratory, Los Alamos, NM 87545;*
[3] *Department of Radiological Health Sciences, Colorado State University, Fort Collins, CO 80523*)

Abstract Mutation induction by low energy heavy ions was scored at the hypoxanthine guanine phosphoribosyl transferase (HGPRT) locus using both normal human fibroblasts and the human-hamster hybrid A_L cells. In addition, the mutation yield at a non-essential chromosome was also examined by using the S1 marker gene locating on human chromosome 11 in A_L cells. Mutagenicity induced by low energy heavy ions was dose and LET dependent. The induced mutant fractions at the S1 locus were consistently higher than those for HGPRT. Using a mutation system that can detect multilocus changes, it can be shown by either Southern blotting or multiplex PCR techniques that radiation can induce chromosomal deletions in the millions of basepairs.

Keywords Mutation, Molecular spectrum, Southern blot, PCR, Multilocus deletions

1 Introduction

The biological effects of ionizing radiation on cells in culture have been well documented. Low energy heavy ions with energy 10~200 keV/μm have been shown to induce cell death, chromosomal aberrations, gene and chromosomal mutations and malignant transformation.[1~3] In general, the efficiency of these DNA damaging processes depends, among other factors, on the total dose, dose rate, culture conditions and the linear energy transfer (LET) of the heavy ions.[4,5] It is thought that the high relative biological effectiveness (RBE) of these heavy ions are due to the induction of poorly-repaired or irrepairable DNA lesions.[6]

In vitro assay systems such as oncogenic transformation and mutagenesis have been used to examine the biological effectiveness of these low energy heavy ions.[7~9] Mutation induction has been examined at several genetic loci using a variety of human and rodent cell lines.[2,10~12]. These low energy ions are more efficient per unit absorbed dose at mutant induction than low LET radiation such as X- or γ-rays. Although the use of gene markers, such as HGPRT that locates on essential, monosomic chromosomes, provide an accurate and convenient measure for mutagenic agents that produce mainly gene mutations, chromosomal mutations, such as large deletions and non-disjunctional process, may be underestimated as has been shown for ionizing radiation and certain chemicals.[13~14] The frequency of induced mutants has been found to be much higher when the heterozygous thymidine kinase gene, as opposed to the hemizygous one, is the target gene for radon induced mutagenesis in the mouse lymphoblast cell line.[15] The rationale is that many of the lesions induced by the low energy heavy ions are intergenic lesions that are poorly recovered when the target gene is located in the vaccinity of other essential genes.

In the present study, the cellular and molecular characterization of mutagenesis induced by low energy heavy ions and γ-rays were examined using both normal human skin fibroblasts and the human-hamster hybrid A_L cells. The A_L cells contain a single copy of chromosome 11 as its only human chromosome that encodes a series of human cell surface antigens.[16] This assay can detect sensitively both intra-

*This work was supported by the National Cancer Institute grants CA-49062, CA-56392, and by the National Institute of Environmental Health Sciences grant ES 05801 of the U.S. National Institute of Health.
Address all correspondence to Dr. Tom K. Hei
Manuscript received date: 1997-01-20

genic and multilocus mutations since virtually the entire human chromosome serves as a target for mutation.[17] Since only a small portion of the human chromosome 11 is essential for viability of the hybrid cell, even large chromosomal deletions involving millions of base pairs are not lethal. Southern blotting and polymerase chain reaction (PCR) techniques were used to determine the molecular nature of the mutations induced by γ-rays and various low energy heavy ions.

2 Materials and methods

2.1 Cell culture

Primary, diploid human skin fibroblasts were initiated by enzymatic digestion of foreskin preparations according to the method described by Noyes *et al.*[18] Cells were maintained in α-Minimum Essential Medium (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 25 μg/ml gentamycin. Primary explants were subcultured and frozen down in liquid nitrogen at the second passage. Exponentially growing cells from passages 3$\sim$ 6 were used in the present studies. The A_L cells, developed by Puck *et al*, contain a single copy of human chromosome 11 in addition to the standard set of hamster chromosomes.[16] Cell surface antigenic markers such as S1 and S2 have been regionally mapped on chromosome 11. Normal rabbit serum was used as a source of complement and specific monoclonal antibody against the S1 antigenic marker was produced as described in Ref.[19]. Cells were maintained in Ham's F-12 medium supplemented with 8% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), 2X normal Glycine (2×10^4 mol/L) and 25 μg/ml gentamycin.

2.2 Irradiation

Charged particles of defined LET ranging from 10 keV/μm protons to 150 keV/μm ^{3}He ions were accelerated at the 4 MV van de Graff accelerator at the Radiological Research Accelerator Facility of Columbia University. The particle beam was directed vertically through the mylar bottom of specially constructed stainless-steel irradiation dishes. The mylar foil had an average thickness of 6μm onto which cells were grown as monolayers. Details of the physical arrangement of the track segment irradiation had been published previously.[2,5] For low LET control radiation, γ-rays from a ^{136}Cs irradiator with an absorbed dose rate of 118 cGy/min were used.

2.3 Cytotoxicity

To determine the cytotoxicity of the various charged particles, exponentially growing HSF and A_L cells were trypsinized and replated onto mylar dishes at a density of 1×10^5 cells/dish as described previously.[2,13] Cells were irradiated 48 h after plating with graded doses of 10 keV/μm protons, 20 or 40 keV/μm deuterons, and 80, 120 or 150 keV/μm helium-4 ions. Representative cultures were also irradiated with graded doses of γ-rays. After irradiation, cultures were immediately trypsinized, counted with an electronic cell counter (Coulter Electronic) and replated in 100 mm diameter tissue culture dishes at a density such that 50$\sim$60 cells would survived after the radiation treatment and form colonies. Cultures were incubated for 10$\sim$12 days, fixed with formaldehyde, stained with Giemsa and the number of colonies counted.

2.4 Mutation assay

Following irradiation, the cultures were trypsinized and replated for mutagenesis assay at both the S1 and the HGPRT loci as described previously[2,12,13,17,19]. For the S1 marker gene, briefly, $2\times$ 10^5 cells per 10 cm diameter dish were challenged with 1.5% complement together with 0.3% antiserum. Cultures were incubated for 8 $\sim$10 d at which time they were fixed, stained and scored for surviving colonies. Controls included identical sets of dishes, with appropriate numbers of cells, containing antibody alone, complement alone or neither agent. The induced mutant fractions, expressed as the number of surviving colonies per 10^5 survivors, remained fairly constant for several weeks after irradiation. To assay for the induction of HGPRT$^-$ mutants, irradiated HSF and A_L cells were plated in completed medium containing 40 μmol/L 6-thioguanine at a density of 2×10^5 cells/dish. After incubation for 9$\sim$10 d, all dishes were fixed and stained. S1$^-$ and HGPRT$^-$ mutants were plucked, expanded in culture and retested in media containing antiserum plus complement or HAT respectively to verify their true mutant nature before DNA analysis.

2.5 Molecular analysis of mutation spectrum

Molecular spectra of the induced mutants were analyzed using either standard Southern blotting or multiplex polymerase chain reaction techniques.[20,21] Briefly, 15μg of DNA prepared from the various HGPRT$^-$ mutants were digested by Pst I restriction endonuclease (Biolab. Beverly, MA). The DNAs were then separated by electrophoresis and blotted onto Hybond membranes (Amersham, Arlington Hts, IL.). The blots were pre-hybridized, then hybridized to the ^{32}P-labelled cDNA probe, pHPT12 was used for the A_L cells and pPR1 for the HSF cells. The filters were then washed and exposed to X-ray film at -80°C for 4 d in the presence of intensifying screens. For multiplex PCR analysis of the human chromosome markers, primers were synthesized for the 4 chromosome 11 genes with known locations relative to the M1T1 gene which codes for the S1 locus (Wilm's tumor, PTH, RAS and APO-Al). With EcoR1, 1μg of mutant DNA was digested and used as a template for the 35 cycle reaction using a Perkin-Elmer thermocycler as described previously.[22] The entire reaction mixture (20 μl) consisted of 0.2μl of each of the primers, 0.2μl of Stoffel fragment enzyme (Perkin-Elmer, S. Plainfield, N.J.) together with the buffer and dNTP mixture. The reaction products were then run on a standard minigel, stained with ethidium bromide, and examined under an UV trans-illuminator.

3 Results and conclusions

In Figs.1,2 survival data were analyzed using the linear quadratic model and curves represent the best fit to the data by using the method of maximum likelihood. For γ-rays and charged particles of lower LET, the survival curves show an initial shoulder, while the curves approximate to an exponential function of dose at higher LET's. The efficiency for cell killing reaches a plateau at 80 keV/μm ^{3}He ions. The RBE for cell lethality at the 10% survival level ranged from 1.6 for 10 keV/μm protons to 5.3 for 150 keV/μm ^{3}He ions.

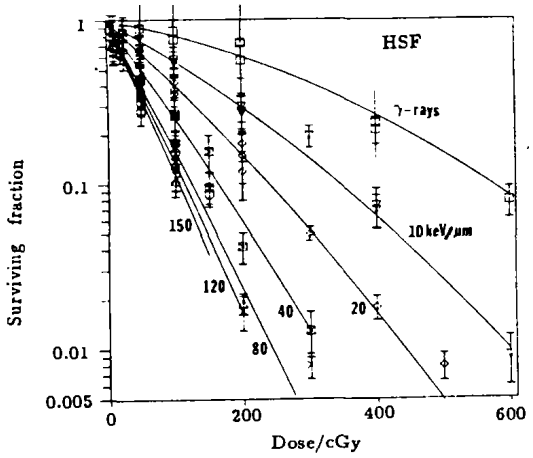

Fig.1 Surviving fractions of human skin fibroblasts either with graded doses of γ-rays or various low energy heavy ions. Bars represent 95% confidence intervals

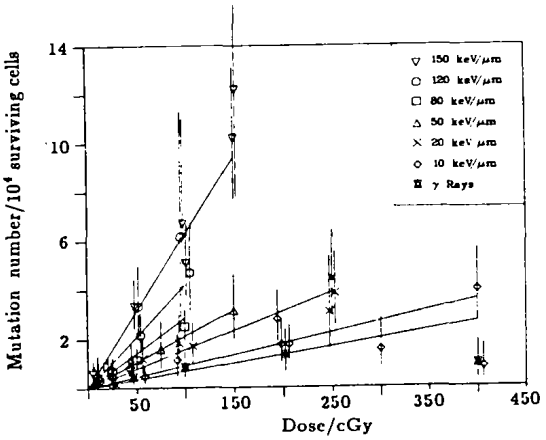

Fig.2 Mutation induction at the HGPRT locus in human fibroblasts by graded doses of either γ-rays or various low energy heavy ions

It can be seen from Fig.2 that mutation frequencies have a direct correlation to LET over the dose ranges examined. The RBE for mutagenesis, based on the slopes of the dose response curves, ranged from 1.3 for 10 keV/μm protons to 9.4 for 150 keV/μm ^{3}He ions. When 6-thioguanine-resistant mutants were plucked out, expanded in cultures and subsequently cultured in medium containing aminopterin and hypoxanthine, none of the clones survived, in-

dicating that they were true mutants.

Wild-type A_L cells express a set of human surface antigen markers such as S1, S2, S3 and lactic dehydrogenase that have been regionally mapped on chromosome 11.[17,19] A gene at 11p13 (M1C1) encodes the S1 antigen. In the presence of complement and specific antiserum against the S1 antigen, wild type A_L cells are lysed, while mutated cells having lost the marker survive to form colonies as shown in Fig.3. The maximum expression of loss of surface antigen markers normally occured after 7~14 d, depending on radiation dose[13,19]. The background mutant fraction at the S1 locus is 73~121 mutants per 10^5 survivors.

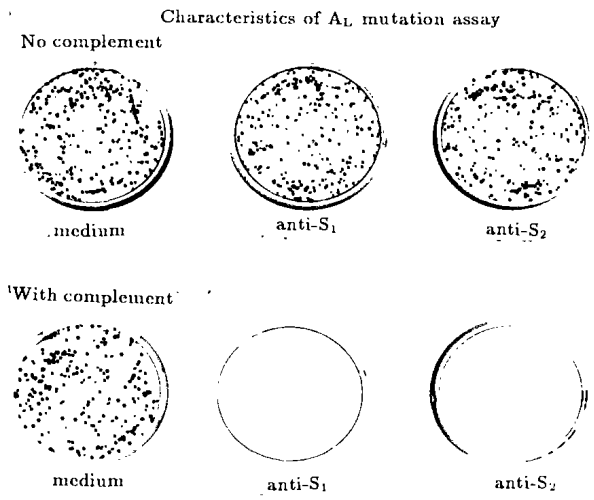

Fig.3 Characterization of the A_L cell using the complement-antibody cell lysate assay. 300 cells from exponentially growing cultures were plated in completed medium containing either 1.5% or no complement followed by addition of 0.3% of either anti-S1 or anti-S2 antiserum

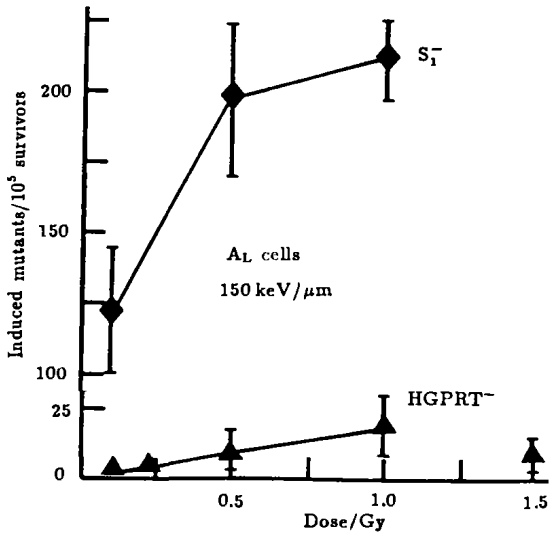

Fig.4 Induced mutant fractions at the S1 and HGPRT loci in A_L cells irradiated with graded dose of 150 keV/μm helium ions. Data are pooled from 4 experiments. Bars represent ± S.E. of means

The mutant fractions induced by graded doses of 150 keV/μm ^{3}He ions at the S1 and HGPRT loci of the A_L cells are shown in Fig.4. At a dose of 50 cGy of charged particles, the mutant yield at the S1 locus was ~20 fold higher than at the corresponding HGPRT. The single copy of human chromosome 11 in the A_L cells codes for genes that are not essential for the survival of the cells. As such, mutational damages in this chromosome are less likely to be lost through cell damages. A higher mutation frequency is expected, especially at lower radiation doses as compared to conventional mutational assay system such as the HGPRT. Similar results have also been obtained with the heterozygous tk^+/tk^- locus in both the L5178 mouse lymphoblast[23] and the TK6 human lymphoblastoid cell lines.[24] In radon irradiated L5178 cells, the mutation frequency at the heterozygous tk locus has been shown to be 50 fold higher than the corresponding tk hemizygous cell line.[10]

Analysis of molecular changes in the DNA of mutant cells is essential for an understanding of the mechanisms of mutagenesis. It is apparent from a variety of studies that the majority of radiation induced mutation result from gene and chromosomal deletions.[8,10-12,22,25] Southern blotting techniques can be used to investigate the kinds of lesions that underlie

mutants obtained at either the HGPRT or S1 locus. Fig.5 shows a representative Southern blot analysis of human fibroblasts DNA from 10 HGPRT$^-$ mutants induced by 80 keV/μm ^{3}He ions. After Pst I restriction digestion, DNA from normal HSF was hybridized to 6 fragments with the full length human HGPRT cDNA probe, pPR1 ranging in size from 1.25 to 7.6 kB (lane 1). Of the 10 mutants examined, 6 showed total deletions of the HGPRT sequence (lanes 2, 3, 5, 9, 10 and 11). One showed partial deletion (lane 8), and 3 had no apparent changes at the HGPRT gene (lanes 4, 6 and 7). Among the 30 to 50 individual mutants isolated from either γ-rays or ^{3}He irradiated cultures, there was a significant difference in their deletion spectrum and those of the spontaneous origin (see Fig.6).

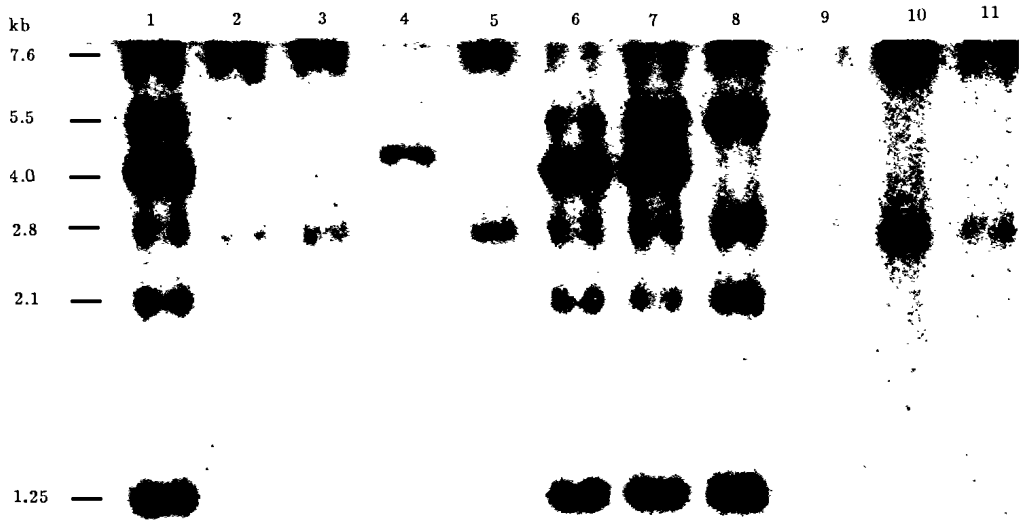

Fig.5 Southern blot analysis of human fibroblasts DNA from control (lane 1) and ten HGPRT$^-$ mutants induced by 80 keV/μm ^{3}He ions. The bands that correspond to 7.6 and 2.8 kb are from autosomal pseudogenes

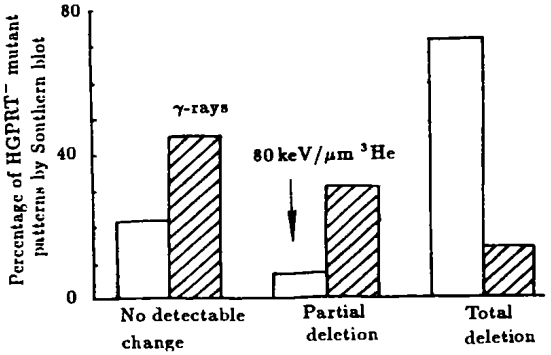

Fig.6 Deletion spectrum based on Southern analysis of human fibroblasts DNA from HGPRT$^-$ mutants induced by either γ-rays or 80 keV/μm ^{3}He ions

Using cDNA probes mapped to specific regions of human chromosome 11, one can delineate precisely the presence or absence of particular DNA segments. As a result, the size of DNA lesions from various S1$^-$ mutants can be calculated. Previous studies have shown that at doses of γ-rays that resulted in comparable survival to that of ^{3}He ions exposure, the percentage of S1$^-$ mutants with deletions larger than 3 megabase pairs is significantly higher in the charged particle group.[12] The availability of primer sequences for several human chromosome 11 marker genes has allowed one to use the multiplex PCR techniques to evaluate the deletion pattern from mutants using small quantities of DNA. Fig.7 shows a representative gel of PCR products using S1$^-$ mutant DNA as template and primers synthesized for three marker

genes located either on the long arm (APO) or short arm (RAS, PTH) of the human chromosome 11. The presence or absence of the corresponding PCR products indicates whether particular segments of DNA are present or not.

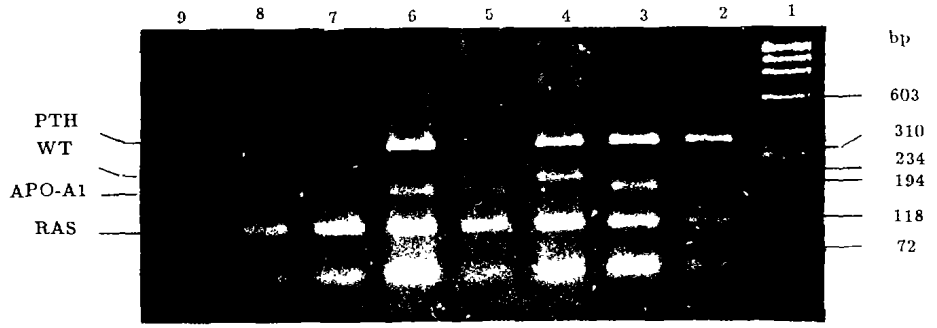

Fig.7 Gel electrophoresis of PCR products using DNA from $S1^-$ mutants as templates and primers synthesized for PTH (parathyroid hormone), WT (Wilm's Tumor), RAS and APO-A1 (apolipoprotein). Hae III digested ϕX174 DNA was used as size markers (lane 1). Lanes 2 through 6 show mutation patterns of mutants induced by a 10 cGy dose of 90 keV/μm ^{3}He ions. Lanes 7 through 9 show mutants induced by a 1 Gy dose of the charged particles. The size of the corresponding PCR products in basepair are shown in the far right

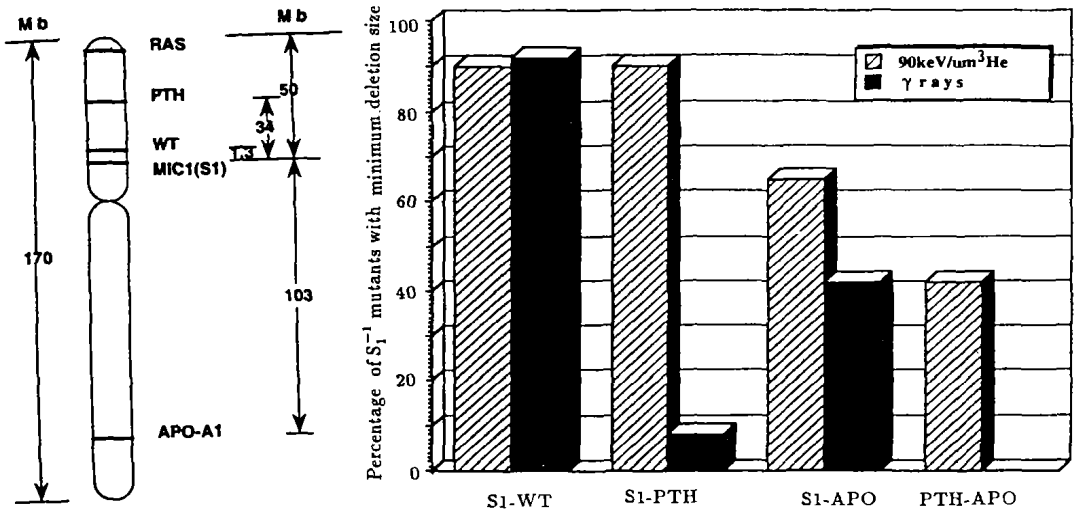

Fig.8 Molecular spectrum of $S1^-$ mutants induced by equitoxic doses of either γ-rays or 90 keV/μm ^{3}He ions as determined by multiplex PCR using synthesized primers for the various human chromosome 11 marker genes. The distance between the various genes is shown in megabase (left panel).

The application of the PCR techniques to amplify short regions of the genome have been shown to be a powerful method in mutation analysis. DNA deletion screening using multiplex PCR proves to be a much faster method and can also eliminate the need for radioactive

probes that are often required in conventional Southern analysis.

The difference in deletion spectra between mutants induced by equitoxic doses of γ-rays and 90 keV/μm ^{3}He ions is shown in Fig.8 where the percent of S1$^-$ mutants with specific deletion size based on multiplex PCR analysis is given. While γ-rays and the charged particles are equally proficient in inducing mutants with relatively small deletions (WT to S1:1.3 Mb), ^{3}He ions, at an equitoxic dose to that of γ-rays, induced significantly a larger number of mutants with deletions $\geq$34 Mb (S1-PTH, PTH-APO). Furthermore, the deletion spectrum appeared to be dose dependent as was shown previously.[22] As the dose of radiation increased, the percentage of mutants with relatively larger deletions also increased.

Results of the present study demonstrate that low energy heavy ions are mutagenic in cultured mammalian cells. Both the mutagenic potential and the molecular spectra of gene and chromosomal deletions are dose and energy dependent. While there is no unique mutation attributable to a specific type of radiation, there is indication that the distribution of deletion spectrum is dependent on radiation quality. Furthermore, the size of the radiation-induced lesions is governed by the chromosomal location in which the target gene resides. The possibility of hypersensitive site(s) in the target gene remains unclear. Recent study suggests that the 3' end of the HGPRT gene may be abnormally sensitive to X-ray induced damage.[26]

Acknowledgement

The authors would like to thank Dr. G Randers-Pehrson and Mr. S. Marino of the Radiological Research Accelerator Facility of Columbia University for performing the dosimetry and irradiation using the track segment mode.

References

1 Hall E J , Hei T K. Radiat Protect Dos, 1985; 13:149
2 Hei T K, Chen D J, Brenner D J *et al.* Carcinogenesis, 1988; 9:1233
3 Geard C R. Radiation Res, 1985; 104S:112
4 Yang T C, Craise L M, Mei M T *et al.* Radiat Res, 1985; 104:S177
5 Hei T K, Komatsu K, Hall E J *et al.* Carcinogenesis, 1988; 9:747
6 Goodhead D T. Int J Radiat Biol, 1989; 56:558
7 Tsuboi K, Yang T C, Chen D. Radiat Res, 1992; 129:171
8 Kronenberg A, Little J B. Mutat Res, 1989; 211:215
9 Hall E J, Hei T K. Environ Helth Persp, 1990; 88:149
10 Evans H H. Advances in Mut Res, 1991; 3:28
11 Thacker J. Mutation Res, 1986; 49:1011
12 Hei T K, Zhu L X, Vannais D *et al.* Molecular analysis of mutagenesis by high LET radiation. Advances in Space Res. (in press).
13 Hei T K, Hall E J, Waldren C A. Radiat Res, 1988; 115:281
14 Hsie A W, Xu W, Yu Y *et al.* Terato Carcino Mut, 1990; 10:115
15 Evans H H, Mencl J, Hui T E *et al.* Radiat Res, 1993; 136:57
16 Puck T T, Wuchier P, Jones C *et al.* Proc Natl Acad Sci, 1971; 68:3102
17 Waldren C A, Jones C C, Puck T T. Proc Natl Acad Sci, 1979; 76:1358
18 Noyes E. Tissue Culture Association Manual, 1979; 5:1173
19 Waldren C A, Correall L, Sognier M A *et al.* Proc Natl Acad Sci, 1986; 83:4839
20 Southern E M. J Mol Biol, 1975; 98:503
21 Mullis K B, Faloona F A. Methods in Enzymology, 1987; 155:335
22 Hei T K, Zhu L X, Waldren C A. Molecular mechanisms of mutagenesis by radiation of different qualities. NATO ASI series H (in press)
23 Evans H H, Mencl J, Horng M F. Proc Natl Acad Sci, 1986; 83:4379
24 Liber H L, Yandell D W, Little J B. Mutation Res, 1989; 216:9
25 Jostes R F, Morgan T L, Gies R A *et al.* J Cell Biochem, 1990; S14A:55
26 Morgan T, Fleck E W, Poston K A *et al.* Mutation Res, 1990; 232:171