High Frequency of Deletions at the Hypoxanthine-guanine Phosphoribosyltransferase Locus in an Ataxia-telangiectasia Lymphoblastoid Cell Line Irradiated with γ-Rays

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The molecular nature of γ-ray-induced mutations at the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus in an ataxia-telangiectasia (A-T) lymphoblastoid cell line was investigated. Twelve of 15 γ-ray-induced HPRT-deficient mutants showed deletions. Eight of them had lost the entire HPRT gene, one showed a 1.9-kb deletion, and three had deletions of about 40–150 base pairs. Of the eight mutants that lost the entire gene, five had also lost both DXS79 and DXS86, flanking markers of the HPRT locus. The spectrum of mutations induced by γ-irradiation in the A-T cells showed a high frequency of deletions in comparison with that in a control cell line, WIL2-NS. Sequence analysis of breakpoint junctions in four mutants revealed that three of them had junctions between short identical sequences at each breakpoint, leaving one copy at the junction. These results suggest that non-homologous end-joining is the major mechanism for deletion formation in A-T cells.

Key words: Ataxia-telangiectasia—Ionizing radiation—Non-homologous end-joining—Deletion junction

Ataxia-telangiectasia (A-T) is a human autosomal recessive disorder that is characterized by neurological deterioration, immunodeficiency, a high incidence of lymphoreticular malignancy and an increased sensitivity to ionizing radiation.1–2) Cells from A-T patients are hypersensitive to cell killing by ionizing radiation and radiomimetic agents.3–5) A-T cells also exhibit chromosomal instability. Increased spontaneous chromosomal abnormality is present in lymphocytes and fibroblasts from A-T patients, usually involving chromosomes 7 and 14.6, 7) Ionizing radiation markedly increases the number of chromosome breakages in A-T cells.8–11

The ATM protein kinase is activated in response to DNA damage, and phosphorylates proteins involved in general cellular functions, such as p53 and Chk2 proteins.13–15) Both p53 and Chk2 proteins are important factors of cell cycle regulation,16 which is in agreement with the deficiency in cell cycle checkpoints in A-T cells. The defective cell cycle checkpoint control is supposed to be the cause of radiation sensitivity of A-T.17) However, there have been many indications of the DNA repair defects in A-T cells.18–20) Premature chromosome condensation studies showed that A-T cells exhibit higher levels of chromosome damage than normal cells in the G1 phase.19, 20) The fact that radioresistant DNA replication does not conform to increased chromosomal aberrations in A-T cells also suggests the involvement of the ATM protein in the DNA repair process.21) Recently, the gene responsible for Nijmegen breakage syndrome, another heritable disease showing cellular phenotypes similar to A-T, was identified and designated as NBS1.22–24) The gene product was identified as a component of the Mre11-Rad50 DNA repair protein complex,22 and was shown to be phosphorylated by the ATM protein kinase in response to DNA damage.25–28) These results also suggest that the ATM protein is involved in DNA repair pathway.

Despite many indications of chromosome instability in A-T cells after irradiation, it has been reported that A-T fibroblasts are hypomutable or even immutable to ionizing radiation at the gene encoding hypoxanthine-guanine phosphoribosyltransferase (HPRT).29) Both limited increase in mutation frequencies induced by ionizing radiation and very poor growth of A-T fibroblasts might have made it difficult to obtain a sufficient number of mutants for detecting a statistically significant increase in mutation frequency in A-T fibroblasts. We overcame this difficulty by using lymphoblastoid cells from A-T patients, which

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are easier than fibroblasts to prepare in large numbers for mutation frequency analysis. We reported previously that an A-T lymphoblastoid cell line showed similar frequencies of 6-thioguanine (6TG)-resistant mutations to a control cell line at equivalent doses of γ-rays, but was still hypomutable when induced mutant frequencies were plotted against survival.\(^{30}\) In this study, to reveal the nature and the mechanism of DNA rearrangement in A-T, we carried out molecular dissection of γ-ray-induced mutations at the HPRT locus on X chromosome, which is hemizygous in male cells.

**MATERIALS AND METHODS**

**Cell culture and irradiation** The lymphoblastoid cell line GM2783 derived from a male A-T patient was obtained from the Human Genetic Mutation Cell Repository, Camden, NJ. The lymphoblastoid cell line WIL2-NS, which was derived from a male spherocytosis patient, was obtained from the Japanese Collection of Research Bioresources, Tokyo.

Cell cultures and mutagenesis experiments following γ-irradiation were performed as described previously.\(^{30}\) Briefly, about 10\(^8\) cells of GM2783 were irradiated with 2 Gy of γ-rays from a \(^{137}\)Cs source at a dose rate of about 4 Gy/min at room temperature, resulting in 4% cell survival. The average mutant fraction induced by 2 Gy γ-rays was 2.7×10\(^{-6}\) which constituted about a 4-fold increase over the background spontaneous mutant fraction of 0.7×10\(^{-6}\). Immediately after irradiation, the cells were split into 24 sib-cultures and incubated during the expression period to ensure the independent origin of mutants. Cells were then dispensed into microwells of 96-well microtiter plates in a concentration of 1000 cells/well to monitor the background spontaneous mutant fraction of 0.7×10\(^{-6}\).

**DNA sequencing** For direct sequencing of the PCR products, single-stranded templates were generated as described.\(^{34, 35}\) Briefly, the 5′-end of a primer (ex2pF (5′-CACCTAAATTCTCTGATAGACTAAGG-3′), ex3pF (5′-GTGGAAAGTTTAATGACTAAAGG-3′), int4U or int5U (Fig. 1) was phosphorylated using T4 polynucleotide kinase (TaKaRa) and ATP. PCR was performed using a phosphorylated primer and an unphosphorylated corresponding one (ex2pR′ (5′-GATACTAAGTAGTAGTGACC-3′), ex3pR (5′-GTATATATCTCCAAAGTGAC-3′), and int5L, respectively). Single-stranded DNA was generated from the double-stranded PCR product by treatment with λ exonuclease (Life Technologies, Rockville, MD).

Reactions for sequencing of the single-stranded PCR products and plasmid clones were performed using the ThermoSequenase II Kit (Amersham Pharmacia Biotech, Chalfont, Buckinghamshire, UK). FITC-labeled primers were used as the sequencing primers as follows; ex2pF for exon 2, ex3pF for exon 3, int4U for deletion breakpoint in intron 4 and 5, int5F for intron 5, and T7 and T3 primers for plasmid clones. The reaction products were analyzed on an automated laser fluorescent DNA sequencer (A.L.F. DNA Sequencer II, Amersham Pharmacia Biotech).

Nucleotides (n.t.) were numbered according to the published sequence of the human HPRT gene.\(^{36}\) Sequence data were analyzed using the sequence analysis software GENETYX (Software Development Co., Ltd., Tokyo).

**Statistical method** Mutation spectra were compared using 2×2 contingency tables. Significance levels were determined by Fisher’s exact probability test using the software StatView (SAS Institute, Inc., Cary, NC).
RESULTS

Analysis of the HPRT gene in mutants isolated from γ-irradiated cells  We isolated 15 independent 6TG-resistant mutant clones from independent sib-cultures of GM2783 cells after irradiation with 2 Gy of γ-rays. The structure of the HPRT gene in the mutant clones was examined by multiplex PCR of the HPRT exons. As the HPRT gene is hemizygous in the male cells, gross changes in the gene are easily identified by PCR analysis. Fig. 2 shows examples of the electrophoresis pattern of amplified bands in GM2783 mutants. Mutant ATG103 showed all the bands of the expected sizes, indicating that there was no large sequence alteration in the HPRT gene in this mutant. Although there was an extra band between the bands of exon 3 and 4 in mutant ATG103 in this experiment, as seen in the figure, this faint band was not reproduced in a separate independent experiment (data not shown), indicating that this was a false band. Mutant ATG111, on the contrary, showed no bands, suggesting deletion of the entire HPRT gene. Mutant ATG03 did not show the band for exon 5, but retained all the other bands with expected sizes, indicating loss of exon 5. The absence of the exon 5 in this mutant was confirmed by individual PCR for exon 5 (data not shown). The banding pattern of ATG106 in Fig. 2B suggested that exon 2 was lost in this mutant. However, the individual PCR for exon 2 revealed a smaller band in this mutant (Fig. 2C). Because the size of this smaller PCR product of exon 2 was approximately the same as the size of the exon 6 product, both products co-migrated and appeared as if a single band in Fig. 2B. Mutant ATG21 lost the band for exon 3 and acquired a new band that was slightly smaller than the normal band for exon 3, although all the other bands were intact (Fig. 2A). Mutant ATG22 also lost the normal band for exon 3 and gained a new band (Fig. 2A). Bands smaller than the normal exon 3 were observed by individual PCR for exon 3 in ATG21 and ATG22 (Fig. 2D), indicating that these smaller bands are really derived from the region of exon 3. Sequence analysis revealed that these smaller PCR products found in ATG21, ATG22 and ATG106 were caused by small deletions in the amplified regions (see below). Altogether, of 15 GM2783 mutant clones examined, 12 clones were caused by deletions, and eight of them had lost all the HPRT exons (Fig. 3). We ruled out the possibility that the absence of amplified bands was caused by some error, such as degradation of the DNA samples, by amplifying exon 3 of the adenine phosphoribosyltrans-
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ferase gene (data not shown). Three mutant clones that did not show any changes in the PCR band pattern presumably have small mutations such as a base substitution or a frameshift.

We reported previously the results of similar PCR analysis of γ-ray-induced 6TG-resistant mutants of a cell line, WIL2-NS. The number of 6TG-resistant mutants of WIL2-NS cells induced by 3 Gy of γ-rays was increased, and we analyzed them by multiplex PCR. As shown in Fig. 3, 20 of 47 mutants examined had deletions, and 11 of them had deletions of all the HPRT exons.

Analysis of the extent of deletions by using flanking marker loci To examine further the extent of deletions in the mutants that had lost one or both ends of the HPRT gene, we analyzed two DNA markers closely linked to the HPRT gene by PCR. The proximal marker, DXS79, is located 400 kb upstream of the HPRT gene, and the distal marker, DXS86, is located 800 kb downstream. As shown in Fig. 3, of eight mutants with deletions of the entire HPRT gene from GM2783 cells, two retained both loci, one had lost the DXS79 locus and retained the DXS86 locus, and five had lost both loci. Thus, 33% (5/15) of HPRT-deficient mutants of GM2783 had large deletions encompassing DXS79 and DXS86, indicating that the size of the deletions were larger than 1.2 Mb. Of 47 γ-ray-induced mutants of WIL2-NS cells, three mutant clones had lost either DXS79 or DXS86 loci, and other mutant clones retained both loci. No mutant clone that lost both DXS loci was identified among WIL2-NS mutant clones.

The results of the analyses on the HPRT gene and the DXS loci are summarized in Table I. Statistical analysis revealed that the proportion of deletion mutations induced by γ-rays in A-T cells was significantly increased compared to that in control cells (P=0.0169). Although the increase of the proportion of deletions of the entire HPRT gene in A-T cells was not quite significant (P=0.0509), loss of either DXS marker was significantly increased in A-T cells (P=0.0044).

Sequence analysis of deletion breakpoint junctions To identify the changes in the smaller PCR products found in mutants ATG21, ATG22 and ATG106, these PCR fragments were analyzed by direct sequencing. Mutant ATG21 had a 46-bp deletion in exon 3, and upstream and downstream donor sequences shared a homology of dinucleotides GG at the junction (Fig. 4A). Similarly mutant ATG22 had an 85-bp deletion with an overlapping three-nucleotide repeat (TTT) at the junction (Fig. 4B).

The deletion in mutant ATG106 removed a 148 bp sequence from intron 2 including T of the invariant GT at the splice donor site, resulting in a sequence not similar to

![Fig. 2. PCR analysis of the HPRT gene in 6TG-resistant mutants of GM2783 cells induced by γ-rays. (A) HPRT exons 3, 4, 5 and 9 were amplified in four separate fragments using multiplex PCR. Although the bands of exon 5 in mutant clones ATG21, ATG22 and ATG103 are very faint in this figure, another experiment using multiplex PCR confirmed the presence of these bands. The absence of the band of exon 5 in ATG03 was confirmed by PCR of exon 5 (data not shown). (B) HPRT exons 2, 6, 7 and 8 were amplified in three DNA fragments by multiplex PCR. ATG106 had lost the band corresponding to the normal size of exon 2, and showed a thick band corresponding to the size of exon 6. (C) PCR of exon 2 revealed a smaller band in ATG106. (D) PCR of exon 3 showed smaller bands in ATG21 and ATG22. The exons contained within the individual bands (A and B) and the size of the amplified products of exon 2 (C) and exon 3 (D) are given to the right of each panel. “WT” is wild-type DNA used as positive control to show the normal size of each band.]
the consensus splice donor site (Fig. 4C). Therefore, this deletion may cause the aberrant splicing in the HPRT mRNA. There was no overlap between the upstream and downstream donor sequences of this deletion. However, if we assume a loss of a nucleotide T, there would be an overlapping three-nucleotide repeat (AAG) at the junction (Fig. 4C), suggesting multiple changes at this junction.

Mutant ATG03 lost exon 5 but retained both exon 4 and exon 6. To identify the deletion junction in this mutant, the region from exon 4 to exon 6 was amplified by long PCR with ex4pF and ex6pR as primers. The resulting 5.5 kb amplified fragment, which is about 2 kb shorter than the fragment from wild-type cells, was cloned into pBluescript II SK(−). Restriction analysis revealed that this fragment retained the HindIII site at n.t. 30 559 in intron 4 and the EcoRI site at n.t. 34 301 in intron 5, but the digestion with both HindIII and EcoRI generated a 1.8 kb fragment (Fig. 1). Sequencing of the HindIII-EcoRI fragment from three clones revealed a deletion of about 1.9 kb sequence with a direct repeat (TA) at the junction and a base substitution from C to A at n.t. 30 046, i.e., 4 bp downstream of the junction (Fig. 4D). The junction sequence was confirmed by direct sequencing of the PCR product using primers int4U and int5L, both of which reside outside of the deleted region (Fig. 1). Direct sequencing of PCR products of wild-type GM2783 cells and four other mutant clones using primers surrounding the 3’ breakpoint (int5U, int5F and int5L; Fig. 1) showed sequences identical to the published sequence. This result indicated that the base substitution from C to A at n.t. 33 046 found in ATG03 was really a mutation in this mutant clone, instead of a polymorphism in GM2783 cells.

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Table I. Mutation Spectra in γ-Ray-induced Mutants of GM2783 and WIL2-NS

| Cell line GM2783 | WIL2-NS |
|------------------|---------|
| No. of mutants analyzed | 15 | 47 |
| No gross change | 3 | 27 |
| Deletions | 12 | 20 |
| P = 0.0169 a) | |
| Deletions of the entire gene | 8 | 11 |
| P = 0.0509 a) | |
| Deletions of the entire gene and at least one flanking marker | 6 | 3 |
| P = 0.0044 a) | |

a) The P values were calculated using Fisher’s exact probability test between the two mutant collections of GM2783 and WIL2-NS.
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We have examined the molecular nature of $\gamma$-ray-induced mutations at the HPRT locus in GM2783 A-T lymphoblastoid cells, as there are few studies on mutations at the endogenous genes in A-T cells. The results showed that 80% (12/15) of mutations induced by $\gamma$-rays were deletions, and that approximately half (8/15) of the mutants had lost the entire region of the gene. Multiplex PCR analysis of the HPRT gene in $\gamma$-ray-induced mutants of WIL2-NS cells revealed that 43% (20/47) of mutants had deletions, and 11 of the mutant clones (23%) had lost all the HPRT exons.

Since other deletion mutant clones had lost the entire region of the HPRT gene, it was impossible to identify the breakpoint junction in these mutant clones.

**DISCUSSION**

We also found that six of 15 GM2783 mutants (40%) had lost at least one of the loci linked closely to the HPRT locus, and in particular, five of them (33%) had lost both marker loci, covering about 1.2 Mb. In contrast, only three of 47 WIL2-NS mutants had lost either one of the flanking marker loci, and we did not identify any mutant clones that had lost both marker loci in the mutant collection examined. Statistical analysis showed that the proportion of deletions in GM2783 was significantly increased ($P=0.0169$), and therefore, we concluded that GM2783 cells showed much higher proportions of deletions at the HPRT locus than WIL2-NS cells. Although the proportion of deletions of the entire HPRT gene was not significantly increased ($P=0.0509$), the proportion of mutations that had lost one of the flanking loci in addition to all the HPRT exons was significantly increased in A-T cells ($P=0.0044$). In particular, large deletions eliminating...
sequences of at least 1.2 Mb were induced by ionizing radiation at an extremely high frequency in GM2783 cells ($P=0.0005$). The dose of $\gamma$-rays irradiated was different between the two cell lines, that is, GM2783 cells were exposed to 2 Gy whereas WIL2-NS cells were exposed to 3 Gy. Yamada et al. reported that the proportion of deletions of the entire HPRT gene was dependent on radiation dose. Therefore, it is probable that we underestimated the difference in the proportion of deletions of the entire gene between GM2783 and WIL2-NS cells. Our data show that $\gamma$-rays induce deletions at a higher frequency in GM2783 cells, implying a high frequency of concomitant deletions of genes adjacent to the HPRT gene. This may have resulted in the substantial reduction of the number of mutant clones recovered after irradiation, because of the co-elimination of a nearby putative gene that is essential for proliferation in culture.

In addition to large deletions, small deletions (46–148 bp) were also found with A-T mutants. Since such small deletions were not detected among 47 WIL2-NS mutants, a high frequency of small deletions (3/15) is also a distinctive characteristic of mutations induced by $\gamma$-irradiation in A-T cells ($P=0.0361$). Another sequence characteristic found in A-T mutants was a point mutation close to the small deletion in two of four mutants sequenced (Fig. 4).

There was little homology between the breakpoint sequences in $\gamma$-ray-induced A-T mutants, except short direct repeats. The presence of short direct repeats at breakpoint junctions has been observed in many cases of deletion mutations arising spontaneously or induced by ionizing radiation. The mechanism of rejoining DNA ends with little or no sequence homology is called illegitimate recombination or non-homologous end-joining. Our results show that non-homologous end-joining is the predominant mechanism for deletion formation induced by ionizing radiation in A-T cells, as is the case in normal cells.

Dar et al. introduced bleomycin-treated linear plasmid DNA into A-T cells, and recovered the plasmid DNA from the cells followed by the selection of mutants. They concluded that deletions in the plasmid DNA recovered from A-T cells did not involve direct repeats. However, ten of 27 deletions from A-T cells were loss of one or two bases, in which direct repeats are not expected. Furthermore, careful inspection of the sequence data of deletion junctions revealed direct repeats in some deletions that the authors classified as no direct repeat. Therefore, further analysis should be done to evaluate the involvement of direct repeats in deletion formation in A-T cells.

Human cells contain a DNA repair complex consisting of hMre11, hRad50 and Nbs1 proteins, which is homologous to the Mre11-Rad50-Xrs2 complex in Saccharomyces cerevisiae. It seems very likely that the human complex is operating in the recognition and repair of double-strand breaks in DNA, like its yeast counterpart. The complex shows an exonuclease activity that is important for processing the ends of DNA in the non-homologous end-joining pathway. Recently, it has been shown that the ATM protein phosphorylates and activates the Nbs1 protein in the response to DNA damage. Therefore, it is possible that the activity of the hMre11-hRad50-Nbs1 complex in A-T cells for processing of double-strand breaks is affected by the alteration of ATM kinase. This would result in incomplete processing of DNA ends. Most of the DNA ends will not be rejoined soon, and will be subjected to ligation with a sequence located at a remote site, resulting in a large deletion. In some cases, an incompletely processed end will be immediately mis-rejoined with the nearby end, resulting in the formation of a small deletion, often associated with a base change. Our results suggest that the ATM protein is an important component for processing double-strand breaks in DNA after irradiation, and support the notion that A-T cells have defects not only in cell cycle checkpoints, but also in DNA repair.

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