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

Akira Tachibana,[1,3] Kouichi Tatsumi,[2] Ikuko Furuno-Fukushi[2] and Masao S. Sasaki[1]

[1]*Radiation Biology Center, Kyoto University, Yoshida-Konoecho, Sakyo-ku, Kyoto 606-8501 and* [2]*Division of Biology and Oncology, National Institute of Radiological Science, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555*

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]

The gene responsible for A-T, designated as *ATM*,[9] encodes a protein kinase that may play a central role in the regulation of a number of cellular processes.[10–12] A-T cells are unable to arrest at both the G1/S and G2/M boundaries after irradiation, indicating a deficiency in G1/S and G2/M cell cycle checkpoint control. A-T cells also show radioresistant DNA synthesis that is thought to be a manifestation of a defective S phase cell cycle checkpoint. 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] 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

[3] To whom correspondence should be addressed.
E-mail: atachibana@house.rbc.kyoto-u.ac.jp

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 the presence of 5 μg/ml 6TG on the eighth day following irradiation. Positive wells were scored after 10–12 days of incubation at 37°C. One 6TG-resistant colony was isolated from each sib-culture and expanded for DNA extraction.

Polymerase chain reaction (PCR) The reaction protocol for amplifying exons of the *HPRT* gene is as described.[31, 32] Briefly, exon 1 was amplified in a separate reaction, since a clear band of exon 1 was not always obtained in a multiplex PCR mixture, presumably because of its high GC content. Two sets of primers for exon 1 were used to make the results certain. Other exons were examined using multiplex PCR. Primers were designed to amplify each exon including part of the flanking introns. To make the amplified bands discernible on agarose gel, the seven pairs of primers were separated into two groups. One group contained primers for exons 3, 4, 5 and 9, and the other for exons 2, 6 and 7/8. Exons 7 and 8 were amplified as a single PCR fragment as they are located very close to each other. The results were confirmed by performing multiplex PCR at least twice for each mutant clone. For the exons which gave very faint bands in multiplex PCR, reactions to amplify the individual exons were also performed to establish the presence or absence of the bands.

The reaction product (5 μl) was subjected to electrophoresis on a 1.8% agarose gel and stained with ethidium bromide. A 123 bp DNA ladder (Life Technologies, Inc., Gaithersburg, MD) was used as molecular weight marker. All PCR reactions were performed with control reactions that do not contain template DNA to ensure no contamination of DNA in the reaction components.

The region from exon 4 to exon 6 was amplified using a LA-PCR Kit (TaKaRa Shuzo Co., Kyoto) following the manufacturer's protocol with ex4pF (5′-GCTATGGATATTAGCTAGCTAAC-3′) and ex6pR (5′-CACTTAATCCCCCTTCAAATGAG-3′) as primers (long PCR). The length of the long PCR product from wild-type DNA was about 7.5 kb, as expected. The PCR fragment was cloned in pBluescript II SK(−) (Stratagene, La Jolla, CA) for restriction enzyme analysis and DNA sequencing. The regions covering the breakpoint junction in intron 4 and 5 were also amplified using primers shown in Fig. 1.

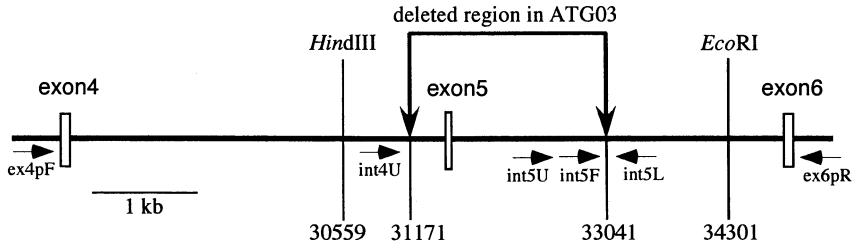
Two loci linked to the *HPRT* locus, *DXS79* and *DXS86*, were analyzed using PCR, as described previously.[32, 33]

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′-CACCTAAATTTCTCTGATAGACTAAGG-3′), ex3pF (5′-GTGGAAGTTTAATGACTAAGAG-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′-GATACTAAGTAATTAGTAAGGCC-3′), ex3pR (5′-GTATATATCCTCCAAGGTGACTAG-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).



Primers

Name	Sequence (5' to 3')	Position & Purpose
int4U	GTTTTAGTGTAGTCCAAGAAT	31114-31134 sense upstream primer PCR and sequencing of the deletion junction
int5L	ATGCAAAAGGTCCAAGTGAAT	33135-33155 antisense downstream primer PCR of the deletion junction
int5U	CCCATCTGAAGTTGAAAATA	32841-32860 sense upstream primer PCR of the 3' breakpoint in the wild-type gene
int5F	CAGACGTTAGGTTGTTTCCA	32986-33005 sense primer sequencing of the 3' breakpoint in the wild-type gene

Fig. 1. Primers used for amplification and sequencing of deletion breakpoints in mutant ATG03. The top portion represents approximate positions of *HPRT* exons 4, 5 and 6, and primers used, together with the deleted region in ATG03. Arrows indicate the direction of DNA synthesis from primers. The sites of *Hin*dIII and *Eco*RI used for subcloning are also shown. The lower portion shows sequences and purposes of primers used. Numbers represent the nucleotide positions according to the published sequence.[36]

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-

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.[37] 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

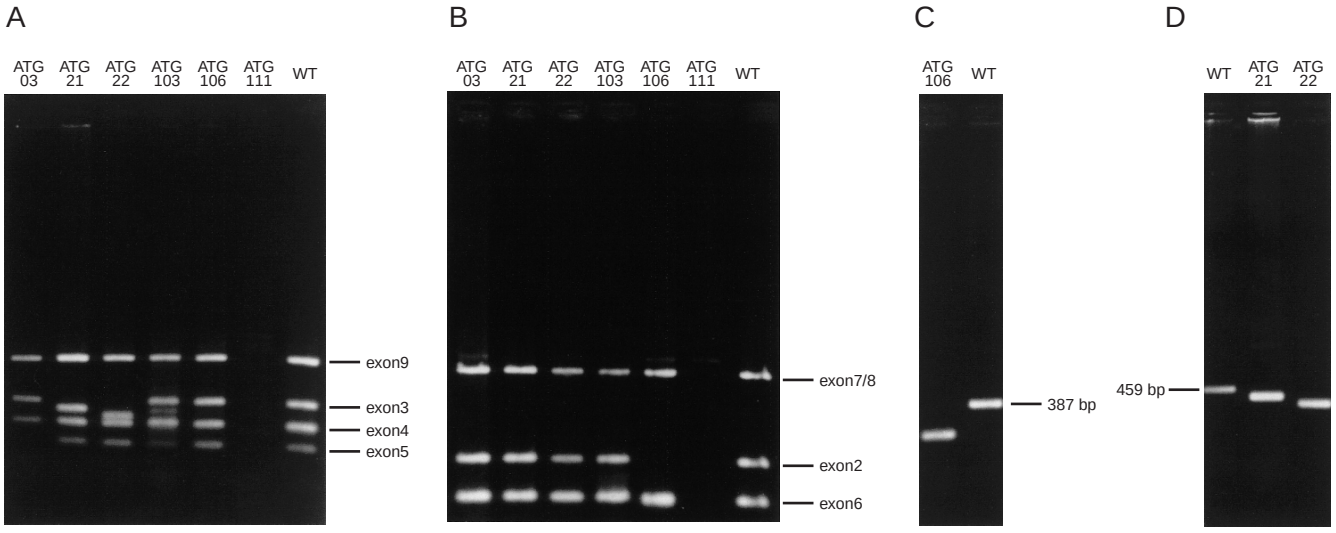


Fig. 2. PCR analysis of the *HPRT* gene in 6TG-resistant mutants of GM2783 cells induced by γ-rays. (A) *HPRT* exons 3, 4, 5 and 9 were amplified in four separate fragments using multiplex PCR. Although the bands of exon 5 in mutant clones ATG21, ATG22 and ATG103 are very faint in this figure, another 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.

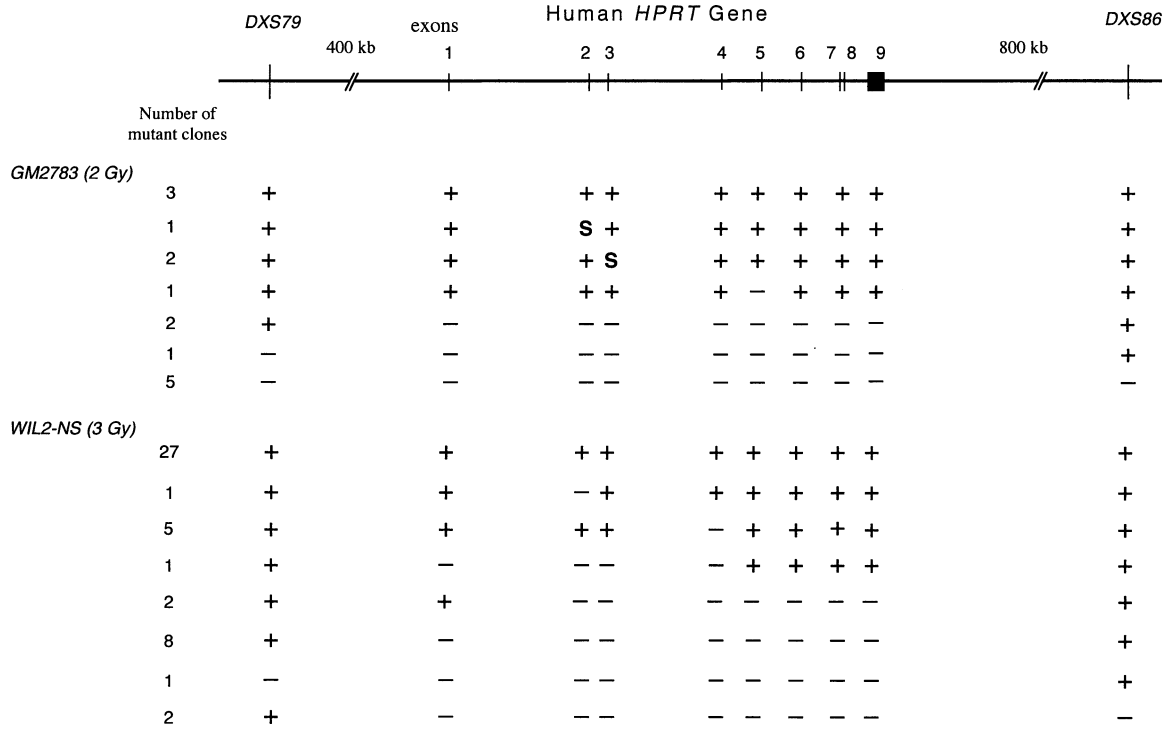


Fig. 3. Extent of deletions in GM2783 and WIL2-NS cells. The exons of the *HPRT* gene and the *DXS79* and *DXS86* markers were examined in mutant clones of GM2783 and WIL2-NS cells induced by γ-rays. "+" denotes the presence of bands of normal size, "S," the presence of bands of smaller size, and "−," the absence of bands.

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 *Hin*dIII site at n.t. 30 559 in intron 4 and the *Eco*RI site at n.t. 34 301 in intron 5, but the digestion with both *Hin*dIII and *Eco*RI generated a 1.8 kb fragment (Fig. 1). Sequencing of the *Hin*dIII-*Eco*RI 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. 33 046, 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.

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.

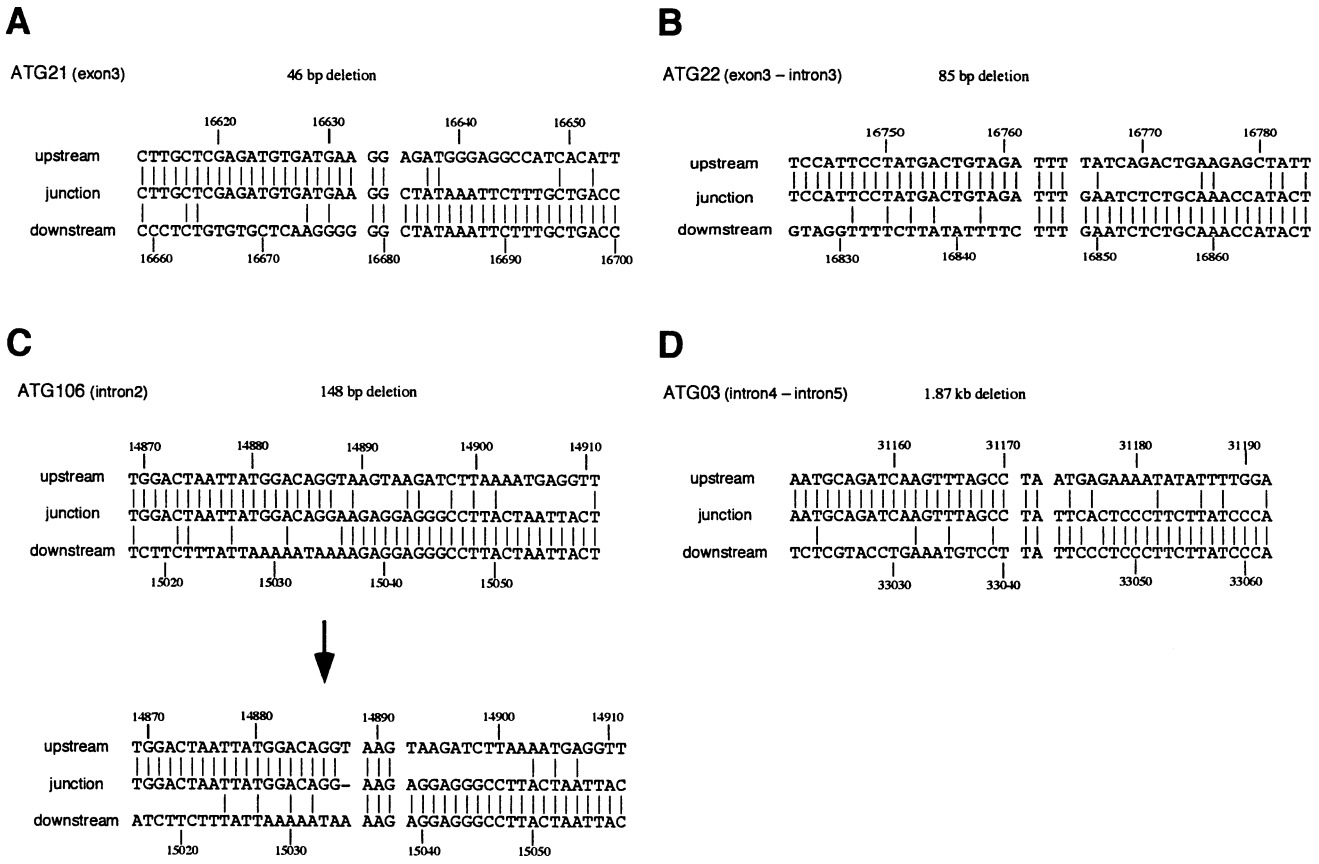


Fig. 4. Alignments of breakpoint and junction sequences in deletion mutants of GM2783 cells. Junction sequences are aligned with 5′ (upstream) and 3′ (downstream) donor nucleotide sequences for each junction. Only the upper (5′ to 3′) strand is indicated. (A) Junction sequences of mutants ATG21 (A), ATG22 (B), ATG106 (C) and ATG03 (D). In (C), the upper portion represents the direct alignment of the junction sequence with 5′ (upstream) and 3′ (downstream) donor sequences, showing that there is no repeat sequence at the junction. The lower portion represents the alignment of the same sequence assuming a loss of T at n.t. 14 888, showing a direct repeat of AAG. The numbers indicate positions of the sequences within the *HPRT* gene.[36]

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 have examined the molecular nature of γ-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 γ-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 γ-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.

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 γ-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.[38] 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 γ-rays induce large 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 γ-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 γ-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 mutants arising spontaneously or induced by ionizing radiation.[39–41] The mechanism of rejoining DNA ends with little or no sequence homology is called illegitimate recombination or non-homologous end-joining.[39, 42, 43] 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.[44] 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*.[22–24] 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.[45] The complex shows an exonuclease activity that is important for processing the ends of DNA in the non-homologous end-joining pathway.[46, 47] Recently, it has been shown that the ATM protein phosphorylates and activates the Nbs1 protein in the response to DNA damage.[25–28] 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.[18]

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REFERENCES

1) Gatti, R. A., Boder, E., Vinters, H. V., Sparkes, R. S., Norman, A. and Lange, K. Ataxia-telangiectasia: an interdisciplinary approach to pathogenesis. *Medicine*, **70**, 99–117 (1991).
2) Taylor, A. M., Metcalfe, J. A., Thick, J. and Mak, Y. F. Leukemia and lymphoma in ataxia telangiectasia. *Blood*, **87**, 423–438 (1996).
3) Shiloh, Y., Tabor, E. and Becker, Y. Cellular hypersensitivity to neocarzinostatin in ataxia-telangiectasia skin fibroblasts. *Cancer Res.*, **42**, 2247–2249 (1982).
4) Taylor, A. M., Harnden, D. G., Arlett, C. F., Harcourt, S. A., Lehmann, A. R., Stevens, S. and Bridges, B. A. Ataxia telangiectasia: a human mutation with abnormal radiation sensitivity. *Nature*, **258**, 427–429 (1975).
5) Taylor, A. M., Rosney, C. M. and Campbell, J. B. Unusual sensitivity of ataxia telangiectasia cells to bleomycin.

Cancer Res., **39**, 1046–1050 (1979).
6) Aurias, A., Dutrillaux, B., Buriot, D. and Lejeune, J. High frequencies of inversions and translocations of chromosomes 7 and 14 in ataxia telangiectasia. *Mutat. Res.*, **69**, 369–374 (1980).
7) Oxford, J. M., Harnden, D. G., Parrington, J. M. and Delhanty, J. D. Specific chromosome aberrations in ataxia telangiectasia. *J. Med. Genet.*, **12**, 251–262 (1975).
8) Taylor, A. M., Metcalfe, J. A., Oxford, J. M. and Harnden, D. G. Is chromatid-type damage in ataxia telangiectasia after irradiation at G0 a consequence of defective repair? *Nature*, **260**, 441–443 (1976).
9) Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T., Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S. R., Simmons, A., Clines, G. A., Sartiel, A., Gatti, R. A., Chessa, L., Sanal, O., Lavin, M. F., Jaspers, N. G. J., Taylor, A. M. R., Arlett, C. F., Miki, T., Weissman, S. M., Lovett, M., Collins, F. S. and Shiloh, Y. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science*, **268**, 1749–1753 (1995).
10) Lavin, M. F. and Shiloh, Y. The genetic defect in ataxia-telangiectasia. *Annu. Rev. Immunol.*, **15**, 177–202 (1997).
11) Shiloh, Y. Ataxia-telangiectasia and the Nijmegen breakage syndrome: related disorders but genes apart. *Annu. Rev. Genet.*, **31**, 635–662 (1997).
12) Lavin, M. F. and Khanna, K. K. ATM: the protein encoded by the gene mutated in the radiosensitive syndrome ataxia-telangiectasia. *Int. J. Radiat. Biol.*, **75**, 1201–1214 (1999).
13) Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y. and Ziv, Y. Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science*, **281**, 1674–1677 (1998).
14) Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B. and Siliciano, J. D. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science*, **281**, 1677–1679 (1998).
15) Matsuoka, S., Huang, M. and Elledge, S. J. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science*, **282**, 1893–1897 (1998).
16) Carr, A. M. Piecing together the p53 puzzle. *Science*, **287**, 1765–1766 (2000).
17) Kaufmann, W. K. and Paules, R. S. DNA damage and cell cycle checkpoints. *FASEB J.*, **10**, 238–247 (1996).
18) Jeggo, P. A., Carr, A. M. and Lehmann, A. R. Splitting the ATM: distinct repair and checkpoint defects in ataxia-telangiectasia. *Trends Genet.*, **14**, 312–316 (1998).
19) Cornforth, M. N. and Bedford, J. S. On the nature of a defect in cells from individuals with ataxia-telangiectasia. *Science*, **227**, 1589–1591 (1985).
20) Pandita, T. K. and Hittelman, W. N. Initial chromosome damage but not DNA damage is greater in ataxia telangiectasia cells. *Radiat. Res.*, **130**, 94–103 (1992).
21) Sasaki, M. S. and Taylor, A. M. Dissociation between radioresistant DNA replication and chromosomal radiosensitivity in ataxia telangiectasia cells. *Mutat. Res.*, **307**, 107–113 (1994).
22) Carney, J. P., Maser, R. S., Olivares, H., Davis, E. M., Le Beau, M., Yates, J. R., 3rd, Hays, L., Morgan, W. F. and Petrini, J. H. The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. *Cell*, **93**, 477–486 (1998).
23) Matsuura, S., Tauchi, H., Nakamura, A., Kondo, N., Sakamoto, S., Endo, S., Smeets, D., Solder, B., Belohradsky, B. H., Der Kaloustian, V. M., Oshimura, M., Isomura, M., Nakamura, Y. and Komatsu, K. Positional cloning of the gene for Nijmegen breakage syndrome. *Nat. Genet.*, **19**, 179–181 (1998).
24) Varon, R., Vissinga, C., Platzer, M., Cerosaletti, K. M., Chrzanowska, K. H., Saar, K., Beckmann, G., Seemanova, E., Cooper, P. R., Nowak, N. J., Stumm, M., Weemaes, C. M., Gatti, R. A., Wilson, R. K., Digweed, M., Rosenthal, A., Sperling, K., Concannon, P. and Reis, A. Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell*, **93**, 467–476 (1998).
25) Gatei, M., Young, D., Cerosaletti, K. M., Desai-Mehta, A., Spring, K., Kozlov, S., Lavin, M. F., Gatti, R. A., Concannon, P. and Khanna, K. ATM-dependent phosphorylation of nibrin in response to radiation exposure. *Nat. Genet.*, **25**, 115–119 (2000).
26) Lim, D. S., Kim, S. T., Xu, B., Maser, R. S., Lin, J., Petrini, J. H. and Kastan, M. B. ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature*, **404**, 613–617 (2000).
27) Wu, X., Ranganathan, V., Weisman, D. S., Heine, W. F., Ciccone, D. N., O'Neill, T. B., Crick, K. E., Pierce, K. A., Lane, W. S., Rathbun, G., Livingston, D. M. and Weaver, D. T. ATM phosphorylation of Nijmegen breakage syndrome protein is required in a DNA damage response. *Nature*, **405**, 477–482 (2000).
28) Zhao, S., Weng, Y. C., Yuan, S. S., Lin, Y. T., Hsu, H. C., Lin, S. C., Gerbino, E., Song, M. H., Zdzienicka, M. Z., Gatti, R. A., Shay, J. W., Ziv, Y., Shiloh, Y. and Lee, E. Y. Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. *Nature*, **405**, 473–477 (2000).
29) Arlett, C. F. and Harcourt, S. A. Cell killing and mutagenesis in repair-defective human cells. *In* "DNA Repair Mechanisms," ed. P. C. Hanawalt, E. C. Friedberg and C. F. Fox, pp. 633–636 (1978). Academic Press, Inc., New York.
30) Tatsumi, K. and Takebe, H. γ-Irradiation induces mutation in ataxia-telangiectasia lymphoblastoid cells. *Gann*, **75**, 1040–1043 (1984).
31) Gibbs, R. A., Nguyen, P. N., Edwards, A., Civitello, A. B. and Caskey, C. T. Multiplex DNA deletion detection and exon sequencing of the hypoxanthine phosphoribosyltransferase gene in Lesch-Nyhan families. *Genomics*, **7**, 235–244 (1990).

32) Tachibana, A., Tatsumi, K., Masui, T. and Kato, T. Large deletions at the *HPRT* locus associated with the mutator phenotype in a Bloom's syndrome lymphoblastoid cell line. *Mol. Carcinog.*, **17**, 41–47 (1996).
33) Morris, T., Masson, W., Singleton, B. and Thacker, J. Analysis of large deletions in the *HPRT* gene of primary human fibroblasts using the polymerase chain reaction. *Somat. Cell Mol. Genet.*, **19**, 9–19 (1993).
34) Higuchi, R. G. and Ochman, H. Production of single-stranded DNA templates by exonuclease digestion following the polymerase chain reaction. *Nucleic Acids Res.*, **17**, 5865 (1989).
35) Takagi, S., Kimura, M. and Katsuki, M. Direct sequencing of PCR products using unlabeled primers. *Biotechniques*, **14**, 218–221 (1993).
36) Edwards, A., Voss, H., Rice, P., Civitello, A., Stegemann, J., Schwager, C., Zimmermann, J., Erfle, H., Caskey, C. T. and Ansorge, W. Automated DNA sequencing of the human *HPRT* locus. *Genomics*, **6**, 593–608 (1990).
37) Furuno-Fukushi, I., Tatsumi, K., Takahagi, M. and Tachibana, A. Quantitative and qualitative effect of gamma-ray dose-rate on mutagenesis in human lymphoblastoid cells. *Int. J. Radiat. Biol.*, **70**, 209–217 (1996).
38) Yamada, Y., Park, M. S., Okinaka, R. T. and Chen, D. J. Molecular analysis and comparison of radiation-induced large deletions of the *HPRT* locus in primary human skin fibroblasts. *Radiat. Res.*, **145**, 481–490 (1996).
39) Meuth, M. The structure of mutation in mammalian cells. *Biochim. Biophys. Acta*, **1032**, 1–17 (1990).
40) Miles, C., Sargent, G., Phear, G. and Meuth, M. DNA sequence analysis of gamma radiation-induced deletions and insertions at the *APRT* locus of hamster cells. *Mol. Carcinog.*, **3**, 233–242 (1990).
41) Morris, T. and Thacker, J. Formation of large deletions by illegitimate recombination in the *HPRT* gene of primary human fibroblasts. *Proc. Natl. Acad. Sci. USA*, **90**, 1392–1396 (1993).
42) Lieber, M. R., Grawunder, U., Wu, X. and Yaneva, M. Tying loose ends: roles of Ku and DNA-dependent protein kinase in the repair of double-strand breaks. *Curr. Opin. Genet. Dev.*, **7**, 99–104 (1997).
43) Roth, D. and Wilson, J. Illegitimate recombination in mammalian cells. *In* "Genetic Recombination," ed. R. Kucherlapati and G. R. Smith, pp. 621–653 (1988). American Society for Microbiology, Washington, DC.
44) Dar, M. E., Winters, T. A. and Jorgensen, T. J. Identification of defective illegitimate recombinational repair of oxidatively-induced DNA double-strand breaks in ataxia-telangiectasia cells. *Mutat. Res.*, **384**, 169–179 (1997).
45) Petrini, J. H. The mammalian Mre11-Rad50-nbs1 protein complex: integration of functions in the cellular DNA-damage response. *Am. J. Hum. Genet.*, **64**, 1264–1269 (1999).
46) Paull, T. T. and Gellert, M. The 3′ to 5′ exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks. *Mol. Cell*, **1**, 969–979 (1998).
47) Trujillo, K. M., Yuan, S. S., Lee, E. Y. and Sung, P. Nuclease activities in a complex of human recombination and DNA repair factors Rad50, Mre11, and p95. *J. Biol. Chem.*, **273**, 21447–21450 (1998).