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# Molecular dissection of in vivo DNA rearrangements induced by radiation and chemical mutagens

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**Abstract.** Cellular DNA is continuously exposed to radiation and chemicals. To analyze the deletion mutations in a whole body system, *gpt* delta mice has been established. In this mouse model, deletions in lambda phage DNA integrated in the chromosome are preferentially selected as Spi$^-$ (*s*ensitive to *P*2 *i*nterference) phages, which can then be subjected for molecular analysis. Here, we report the sequence characteristics of deletions induced by ionizing radiations in liver, ultraviolet light B (UVB) in epidermis, mitomycin C (MMC) in bone marrow and heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine (PhIP) in colon. About half of the large deletions occur between short direct-repeat sequences and the remainder had flush ends, suggesting that they are generated during the repair of double-strand breaks in DNA. Radiations, UVB and MMC efficiently induced the large deletions whereas PhIP mainly generated one base deletions in runs of guanine bases. The most predominant mutations in untreated mice were one base deletions in runs of adenine bases. Possible mechanisms of the intra-chromosomal deletion mutations are discussed. 

*Keywords:* Deletion; Point mutation; Genome instability; Transgenic mouse; Transgenic rat

## 1. Introduction

Humans are exposed to various DNA-damaging agents, which induce oxidation, methylation and deamination of DNA, adduct formation and strand sessions. Of the various DNA lesions, the most detrimental is probably double-strand breaks (DSBs) in DNA. DSBs can be induced by ionizing radiations (IRs), anti-cancer therapeutic agents

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such as mitomycin C (MMC) or when replicative DNA polymerases encounter single-stranded breaks or DNA lesions [1]. DSBs in DNA trigger a set of cellular responses such as delay of cell cycle and apoptosis. However, if not properly repaired or eliminated, DSBs enhance the frequency of illegitimate recombination, i.e., deletions, amplifications and translocations of chromosomes, leading to genome instability and tumorigenesis. Human cells are estimated to suffer more than 2000 imperfect repair sites in the chromosome at the end of 70 years of life because of the spontaneous DSBs [2].

To gain insights into the mechanisms of deletion mutations in vivo, we have established a novel transgenic mouse, named *gpt* delta [3,4]. The mice carry tandem repeats of lambda EG10 DNA in the chromosome 17, which are retrievable as phage particles by in vitro packaging reactions. The rescued phages are then subjected to Spi$^-$ (*s*ensitive to *i*nterference) selection. This selection takes advantage of the restricted growth of wild-type lambda phage in P2 lysogens. Only mutant lambda phages that are deficient in the functions of both the *gam* and *redBA* genes can grow well in P2 lysogens and display the Spi$^-$ phenotype. Simultaneous inactivation of both the *gam* and *redBA* genes is usually induced by deletions in the region. Using the shuttle vector mutation assay, deletions can be analyzed at a molecular level in various organs of mice. In addition, 6-thioguanine (6-TG) selection has been incorporated for the detection of point mutations, i.e., base substitutions and frameshifts. Here, we report the characteristics of deletion mutations induced by radiation and chemicals. The mutation spectra of *gpt* mutations have been reported previously [5].

## 2. Molecular nature of Spi$^-$ mutations

We have analyzed more than 400 independent Spi$^-$ mutants at a sequence level. They were rescued from the liver, spleen, bone marrow, epidermis, colon and kidney of *gpt* delta mice and the mutations were induced by IRs (heavy ion, X-ray and gamma-ray radiations), ultraviolet light B (UVB), MMC and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine (PhIP). Mutants rescued from untreated mice were also analyzed. We categorized the mutants into five classes (classes I to V) based on the deletion sizes and the sequence characteristics of the junctions (Fig. 1).

Class I mutants include large deletions exceeding 1 kb. The maximum length of the deletions is about 10 kb. Almost half of Class I mutants has short homologous sequences at the junctions of mutants. Thus, we subdivided Class I mutants into Class I-A and I-B. Mutants of Class I-A are large deletions that exhibit short homologous sequences of 1 to 12 bp at the junctions. Class I-B mutants are large deletions without short homologous sequences. Intriguingly, about 10% of Class I-A and I-B mutants have insertion sequences in the junctions. These extra nucleotides are often called "filler DNA" and the genetic rearrangements that arise by illegitimate recombination in mammalian cells have such sequences at about 10% [6]. The length of the insertion sequence is usually 1 or 2 bp but the maximum insertion was 14 bp. Class I-A and I-B mutants delete regions of both the *gam* and *redBA* genes or a region in the *gam* gene and the upstream. They are induced by IRs, UVB and MMC.

Class II include middle-size deletions of more than 1 bp but less than 1 kb. Some have short homologous sequences but others not as in the case of Class I mutants. Mutations of

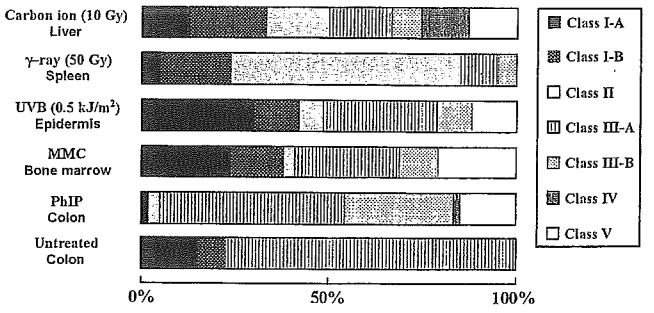


Fig. 1. Schematic representation of various classes of Spi⁻ mutations. Class I-A, large deletions with short homologous sequences at the junctions; Class I-B, large deletions without short homologous sequences; Class II, middle size (more than 1 bp but less than 1 kb) deletions; Class III-A, single bp deletions at run sequences; Class III-B, single bp deletions at non-run sequences; Class IV, complex mutations; Class V, miscellaneous mutations. The references of each treatment are as follows: carbon ion [9]; gamma-ray [7]; UVB [10]; MMC [11]; PhIP[8]; untreated [8].

more than 20 bp but less than 600 bp are frequently identified in the spleen of mice irradiated with gamma-ray at a dose of 50 Gy.

Class III mutants are 1 bp deletions in the *gam* gene. These small deletions are not supposed to induce Spi⁻ mutations. However, translation of the *gam* and *redBA* genes is probably linked, and the *gam* gene is first transcribed so that the 1 bp deletions in the *gam* gene may interfere with the start of translation of the downstream *redBA* genes, thereby functionally inactivating not only *gam* but also *redBA* [7]. We subdivided Class III mutants into Class III-A and III-B: the former mutations occur at run sequences such as AAAAAA and the latter at non-repetitive sequences. Class III-A mutants occurring at A:T repetitive sequences are the most predominant type of mutations in untreated mice. PhIP induces Class III-A deletions. However, most of them occur at G:C repetitive sequences or beside run sequences [8].

Class IV is a complex type of mutations where the exact junctions could not be identified because of the genome rearrangements. This class of mutants is frequently observed in p53 knockout mice irradiated with heavy ion [9]. Class V includes miscellaneous mutations.

## 3. Possible mechanisms of deletion mutations in vivo

Deletions with short (Class I-A) or no (Class I-B) homologous sequences at their junctions have been observed in a number of mutant genes implicated in human diseases including cancer. Indeed, about 40% of large deletions in human disorders are characterized by the presence of very short sequence homologies at the breakpoints [12]. On the basis of the sequence characteristics observed in the junctions of Spi⁻ mutants, we suggest that non-homologous end joining (NHEJ) repair plays an important role in the generation of intra-chromosomal deletions such as Spi⁻ Class I mutants. This pathway involves the DNA end-binding heterodimer Ku70/Ku80, DNA-$PK_{CS}$, XRCC4 and DNA ligase IV. Although some of these proteins play an essential role in the maintenance of genome stability and suppression of tumorigenesis, NHEJ repair pathway

has the potential to induce deletion mutations. If two incompatible ends are generated by IRs, they first have to be converted to ligatable ends by enzymatic processing which often causes deletions.

Genome rearrangements associated with oxidative stress are important in carcinogenesis. In this regard, *gpt* delta rat [13] could be important because most of carcinogenesis studies are undertaken in rats rather than in mice. The genome rearrangements can directly be examined in the target organs of carcinogenesis in *gpt* delta rats kept under various nutritional conditions or exposed to radiation and chemicals.

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Environmental and Molecular Mutagenesis 45:150–161 (2005)

*Review Article*

# Molecular Nature of Intrachromosomal Deletions and Base Substitutions Induced by Environmental Mutagens

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Cellular DNA is exposed to a variety of exogenous and endogenous mutagens. A complete understanding of the importance of different types of DNA damage requires knowledge of the specific molecular alterations induced by different types of agents in specific target tissues in vivo. The *gpt* delta transgenic mouse model provides the opportunity to characterize tissue-specific DNA alterations because small and large deletions as well as base substitutions can be analyzed. Here, we summarize the characteristics of intrachromosomal deletions and base substitutions induced by ionizing radiation in liver and spleen, ultraviolet B (UVB) radiation in epidermis, mitomycin C (MMC) in bone marrow, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in colon, and aminophenylnorharman (APNH) in liver of *gpt* delta mice. Carbon-ion radiation, UVB, and MMC induced large deletions of more than 1 kb. About half of the large deletions occurred between short direct-repeat sequences and the remainder had flush ends, suggesting the involvement of nonhomologous end joining of double-stranded breaks (DSBs) in DNA. UV photoproducts and interstrand crosslinks by MMC may block DNA replication, thereby inducing DSBs. In contrast, PhIP and APNH mainly generated 1 bp deletions in runs of guanine bases. As for base substitutions, UVB and MMC induced G:C→A:T transitions at dipyrimidine sites and tandem base substitutions at GG sites, respectively. PhIP and APNH induced G:C→T:A transversions. Translesion DNA synthesis across the lesions, i.e., UV photoproducts, intrastrand crosslinks by MMC, and guanine adducts by the heterocyclic amines, may be involved in the induction of base substitutions. These results indicate the importance of sequence information to elucidate the mechanisms underlying deletions and base substitutions induced in vivo by environmental mutagens. Environ. Mol. Mutagen. 45:150–161, 2005.


**Key words: deletion; base substitutions; transgenic mouse; environmental mutagens; tissue specificity**

## INTRODUCTION

The human genome is continuously exposed to various DNA-damaging agents, which induce oxidation, methylation and deamination of DNA, adduct formation, and DNA strand breaks. Of the various DNA lesions, the double-strand breaks (DSBs) in DNA may be the most genotoxic. DSBs can induce delays in cell cycle progression and apoptosis, which contribute to the repair of DSBs and the elimination of damaged cells, respectively [Zhou and Elledge, 2000; Khanna and Jackson, 2001]. However, if not properly repaired or eliminated, DSBs enhance the frequency of illegitimate recombination, i.e., deletion, amplification, and translocation of chromosomes, leading to genome instability and tumorigenesis [Vamvakas et al., 1997; Richardson and Jasin, 2000; Zhou and Elledge, 2000; Khanna and Jackson, 2001].

Homologous recombination (HR) and nonhomologous recombination repair pathways have evolved to counteract the genotoxicity of DSBs in DNA [Takata et al., 1998; Haber, 2000; van Gent et al., 2001; Jackson, 2002]. The latter pathway is also referred to as nonhomologous end joining (NHEJ) repair. HR repair copies information from the



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homologous sequence in sister chromatids or homologous chromosomes to restore the original sequence at the DSB. In yeast, this pathway is the major mechanism of DNA DSB repair, and it is also important in vertebrate cells [Tsuzuki et al., 1996; Sonoda et al., 1998]. NHEJ repair appears to be the prevailing mechanism of DSB repair in mammalian cells. This repair system can rejoin DSB ends directly without sequence homology or by using a short (1 to about 10 bp) region of homologous sequence [Morris and Thacker, 1993; Critchlow and Jackson, 1998; Tsukamoto and Ikeda, 1998; Thacker, 1999; Pfeiffer et al., 2000]. The NHEJ pathway is critical where no homologous sequences are available in $G_0$ and $G_1$ cells. Since the NHEJ pathway involves modification of the two broken ends in DNA to make them compatible, nucleotides at each end are often lost. This processing of the terminal bases is particularly important when DSBs are induced by IR because the broken termini often have modified bases [Henner et al., 1983]. Thus, NHEJ is an imperfect process in terms of preserving genetic information [Pfeiffer et al., 2000; Ferguson and Alt, 2001; Lieber et al., 2003]. It is estimated that human cells have more than 2,000 imperfect repair sites in their chromosomes at the end of 70 years of life because of spontaneous DSBs [Lieber et al., 2003].

In addition to the HR and NHEJ pathways, cells possess specialized DNA polymerases to prevent DSBs in DNA by bypassing DNA lesions that block DNA replication [Friedberg and Gerlach, 1999]. DSBs are induced when DNA replication forks stall at pyrimidine dimers, interstrand crosslinks, apurinic sites, or modified bases. The specialized DNA polymerases would replace stalled replicative DNA polymerases at DNA-blocking lesions, interacting with the primer strand to continue DNA synthesis [Pages and Fuchs, 2002]. After short tracts of bypass DNA synthesis, replicative DNA polymerases again take over and finish the remaining chromosome replication. In some bypass reactions that the specialized DNA polymerases catalyze, correct bases are incorporated opposite template lesions, while others incorporate incorrect bases. Thus, translesion DNA synthesis appears to contribute to both DNA damage tolerance and the induction of point mutations. Human cells possess four Y-family DNA polymerases (eta, iota, kappa, and hREV1) and one B-family DNA polymerase (zeta) as specialized DNA polymerases [Rattray and Strathern, 2003]. Since there are at least 15 DNA polymerases in human cells, other DNA polymerases may act as bypass polymerases as well. This bypass mechanism could be more important than NHEJ in mammals since it prevents the induction of DSBs while NHEJ rejoins the broken ends.

To investigate the mechanisms underlying repair and mutagenesis associated with DNA damage in vivo, we have developed a transgenic mouse named *gpt* delta [Nohmi et al., 1996, 2000]. A feature of the mouse model is the capacity to detect small and large deletions as well as base substitutions in various organs of mice. The sequence alterations can be characterized at the molecular level. Here, we summarize the characteristics of deletions and base substitutions induced by ionizing radiation (IR) in liver and spleen [Nohmi et al., 1999; Masumura et al., 2002; Yatagai et al., 2002], ultraviolet B (UVB) light in epidermis [Horiguchi et al., 2001], mitomycin C (MMC) in bone marrow [Takeiri et al., 2003], 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in colon, and aminophenylnorharman (APNH) in liver [Masumura et al., 1999a, 2000, 2003b] and discuss the possible mechanisms of mutation induction.

## *gpt* DELTA TRANSGENIC MICE

*gpt* delta transgenic mice were generated by microinjection of λEG10 phage DNA (48 kb) into the fertilized eggs of C57BL/6J mice [Nohmi et al., 1996]. The mice carry about 80 copies of the transgene in a head-to-tail fashion at a single site in chromosome 17, and the transgene is maintained as a homozygote (the mice carry about 160 copies of λEG10 DNA per diploid genome) [Masumura et al., 1999b]. The model uses $Spi^-$ selection to detect and analyze in vivo deletion mutations ($Spi^-$ stands for "sensitive to P2 interference"; Fig. 1) [Nohmi et al., 1996, 1999]. This selection takes advantage of the restricted growth of wild-type λ-phage in P2 lysogens [Sambrook et al., 1989; Ikeda et al., 1995]. Only mutant λ-phages that are deficient in the functions of both the *gam* and *redBA* genes can grow well in P2 lysogens and display the $Spi^-$ phenotype as long as the phages carry a *chi* site and the host strain is $recA^+$. Simultaneous inactivation of both the *gam* and *redBA* genes is usually induced by deletions in the region.

The product of the *gam* gene inactivates the exonuclease V encoded by the *recBCD* genes of *E. coli* [Smith, 1983]. In the prophage P2, the *old* gene product kills the host *E. coli* cell if the host's exonuclease V is inactivated. Thus, when P2 lysogens are infected with wild-type λ-phage, the *old* gene product kills the host cell, thereby preventing the propagation of incoming λ-phage. In the absence of the *gam* and *red* gene products, the defective phage can replicate its DNA in the host cell because exonuclease V is not inactivated. However, the phages must carry a *chi* sequence (GCTGGTGG) to prevent their DNA from digestion by the potent exonuclease V. The *chi* sequence inactivates the *recD* gene product, thereby inhibiting the exonuclease digestion.

Because of the size limitation for in vitro packaging reactions (there must be two *cos* sites separated by 38–51 kb of DNA), the size of deletions detectable by $Spi^-$ selection is less than 10 kb (actually the maximum size of deletion we have identified is 9,198 bp). Thus, the mutants are mostly intrachromosomal deletions. However, the tandem array of 80 copies of λ EG10 DNA in the *gpt*

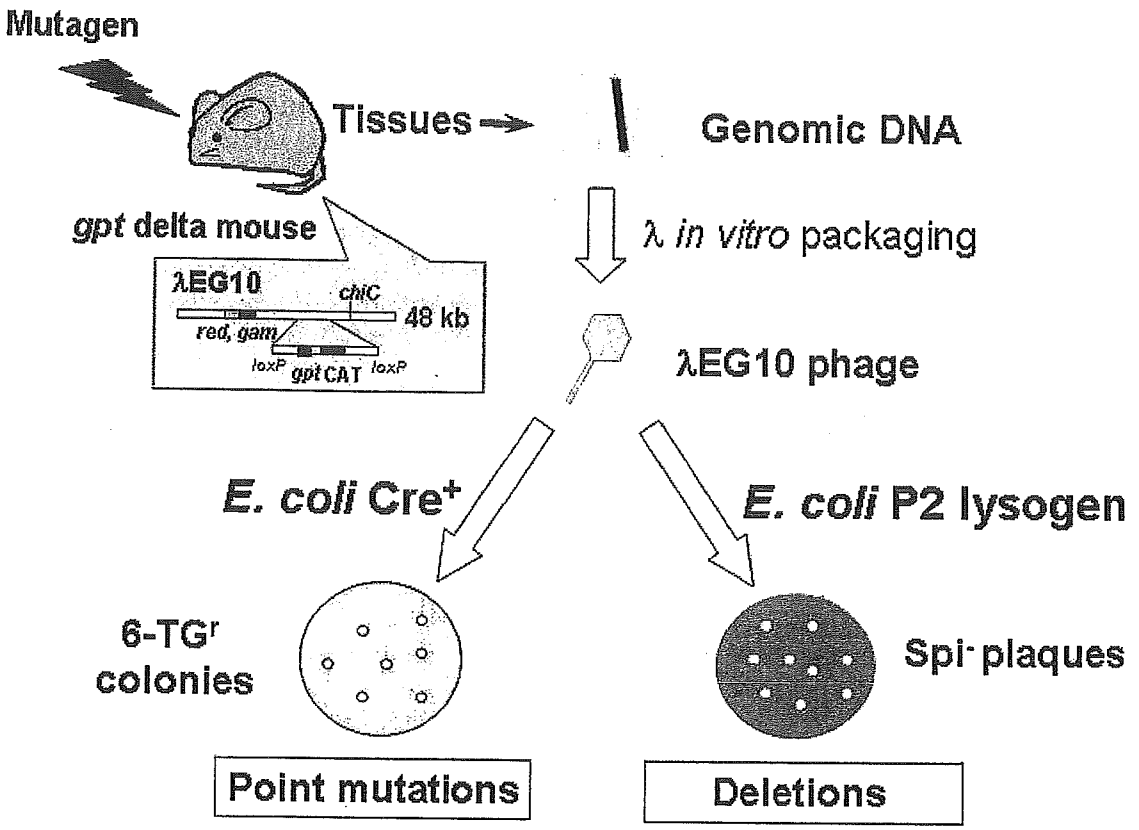


**Fig. 1.** Protocols for the *gpt* delta transgenic mouse mutagenicity assay [Nohmi et al., 2000]. Two distinct *E. coli* host cells are infected with the rescued λ EG10 phages; one is *E. coli* strain YG6020 expressing Cre recombinase for 6-TG selection, and the other is a P2 lysogen for $Spi^-$ selection. In the cells expressing Cre recombinase, λ EG10 DNA is converted to a plasmid carrying the *gpt* and chloramphenicol acetyltransferase (*cat*) genes. The *E. coli* cells harboring the plasmid carrying mutant *gpt* can be positively selected as bacterial colonies on plates containing chloramphenicol and 6-TG. Mutant λ EG10 phages lacking *red/gam* gene functions can undergo positive selection as $Spi^-$ plaques in P2 lysogens. Inclusion of $MgSO_4$ (10 mM) in the plates during the plaque formation process increases the size of $Spi^-$ plaques [Shibata et al., 2003]. Using *gpt* and $Spi^-$ selection, the frequencies of point mutations and deletions in vivo can be compared in the same DNA sample.

delta mouse amounts to a potential target of approximately 3.8 megabases.

The *gpt* delta mouse assay also employs 6-thioguanine (6-TG) selection for the detection of point mutations, i.e., base substitutions and frameshifts, in the transgene (Fig. 1). 6-TG selection utilizes the *gpt* gene of *E. coli* as a reporter. Since the product of wild-type *gpt* converts 6-TG to a toxic substance, only cells deficient in the *gpt* gene can survive on a plate containing 6-TG. Thus, *E. coli gpt*-mutant cells can be positively selected using 6-TG. The coding size of *gpt* is 456 bp. Hence, two distinct types of mutation, i.e., deletion and point mutation, are identified by $Spi^-$ and 6-TG selection, respectively.

## MOLECULAR NATURE OF $Spi^-$ MUTATIONS

Sequencing of the break points of chromosomes is most helpful in identifying the nature of processing DSBs. We have analyzed more than 400 independent $Spi^-$ mutants at the sequence level. Mutants were rescued from the liver, spleen, bone marrow, epidermis, colon, and kidney of *gpt* delta mice; the mutations were induced by IR (carbon particle, X-ray and γ-ray radiation), UVB, MMC, PhIP, and APNH. Mutants rescued from untreated mice were also analyzed. We categorized the mutants into five classes based on the deletion sizes and the sequence characteristics of the junction regions. We speculated that the size distribution of mutations might reflect differences in the mechanisms of their induction.

Class I mutants included large deletions exceeding 1 kb. The cutoff size of 1 kb was chosen because the size of most large deletions was more than 1 kb and deletions between 600 bp and 1 kb were very rare (3 out of 476 total $Spi^-$ mutants). About half of class I mutants have short homologous sequences at the junctions of mutants. Thus, we subdivided class I mutants into class IA and IB. Mutants of class IA are large deletions that exhibit short homologous sequences of 1–12 bp at the junctions (Fig. 2). The length of the homologous sequence is not related to the size of deletions. Class IB mutants are large deletions without short homologous sequences. Intriguingly, about 10% of class IA and IB mutants have inser-

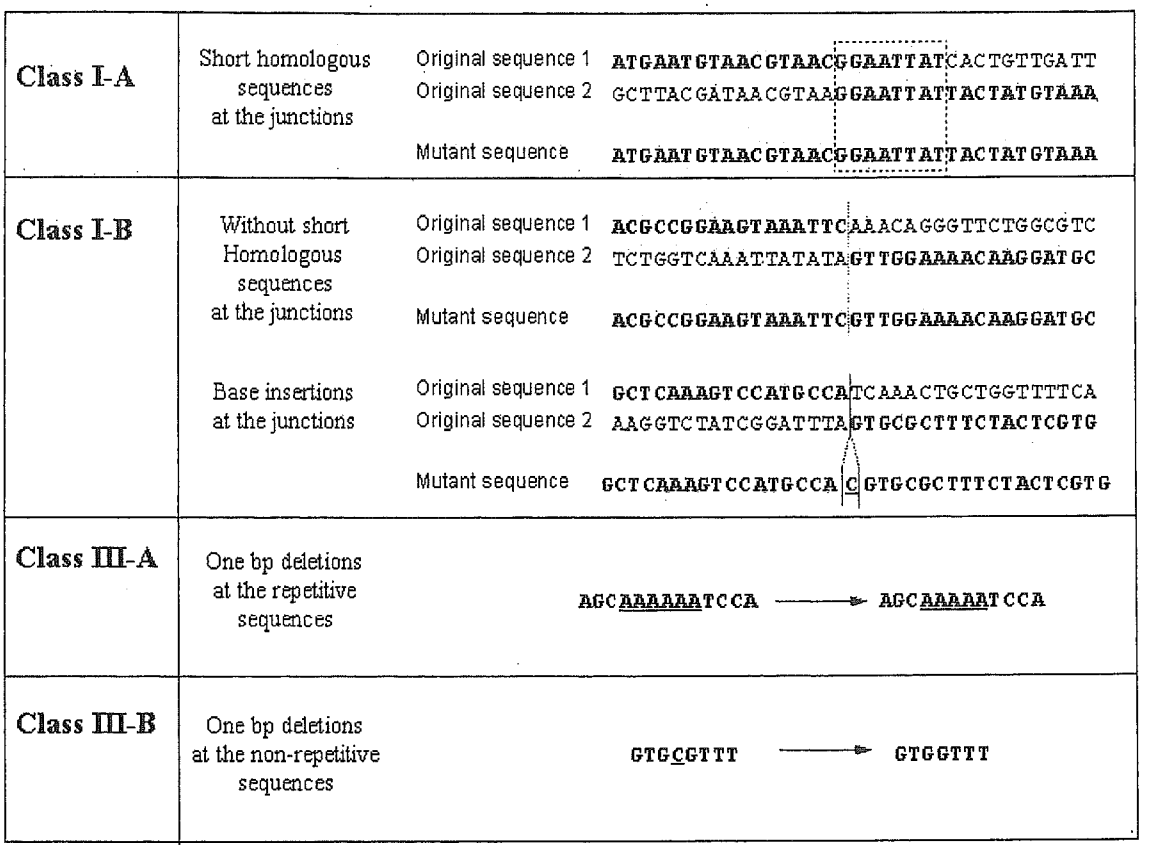


**Fig. 2.** Sequence characteristics of Spi$^-$ mutations. Class IA mutants are large deletions exceeding 1 kb in size and have short homologous sequences at the junctions. The homologous sequences (8 bp) are boxed. DNA sequences generated by the deletion between original sequences 1 and 2 are referred to as mutant sequence. Class IB large deletions do not have short homologous sequences. The junction is highlighted with a dotted line. About 10% of class I mutants possess base insertions at the junctions. An example of insertion of C is presented. Class IIIA mutants are single bp deletions at run sequences, such as AAAAAA. Class IIIB single bp deletions occur at nonrun sequences.

tion sequences in the junctions. These extra nucleotides are often called "filler DNA" and the genetic rearrangements that arise by illegitimate recombination in mammalian cells have such sequences at a frequency of about 10% [Roth et al., 1991]. The length of the insertion sequence is usually 1 or 2 bp, but the maximum insertion was 14 bp. These inserted bases or sequences are mostly the same as or complementary to the neighboring sequences. Class IA and IB mutants delete regions of both the *gam* and *redBA* genes or a region in the *gam* gene and upstream sequence.

Class I mutants are induced by IR, UVB, and MMC (Fig. 3). IR induces DSBs through direct deposition of energy on DNA and through oxidation of DNA [Masumura et al., 2002; Yatagai et al., 2002]. We suggest that UVB and MMC induce lesions that block the progression of the replication fork, thereby inducing DSBs in DNA [Horiguchi et al., 2001; Takeiri et al., 2003]. UVB and MMC induce pyrimidine dimers and interstrand crosslinks in complementary DNA strands, respectively, which strongly block DNA replication [De Silva et al., 2000; McHugh et al., 2000; Limoli et al., 2002]. In the case of MMC, DSB formation could be caused either by two closely separated incisions on opposite strands or by replication of nicked DNA [Dronkert and Kanaar, 2001]. Alternatively, DSBs may occur as a result of cleavage of Holliday junctions formed by the unwinding of stalled replication forks [McGlynn and Lloyd, 2002]. DSBs may also be induced in the single-stranded DNA region created by DNA damage [Livneh, 2001], possibly through the attack of an endonuclease specific for single-stranded DNA [Ukita and Ikeda, 1996]. UVB may also induce the single-stranded DNA region by UV photoproducts. The resulting DSB ends can be digested by exonucleases, generating 3′ or 5′ protruding ends. The deletion sizes of class I mutants induced by UVB and MMC are more than 1 kb [Horiguchi et al., 2001; Takeiri et al., 2003]. Thus, the deletion sizes, i.e., more than 1–2 kb, may represent the minimal unit of exonuclease digestion once DSBs are induced in the chromosomes. The last step is rejoining of the ends. In the case of class IA mutants having short homologous sequences at the junctions, the joining of the ends would take place by annealing of the complementary short homologous sequences, which would be followed by

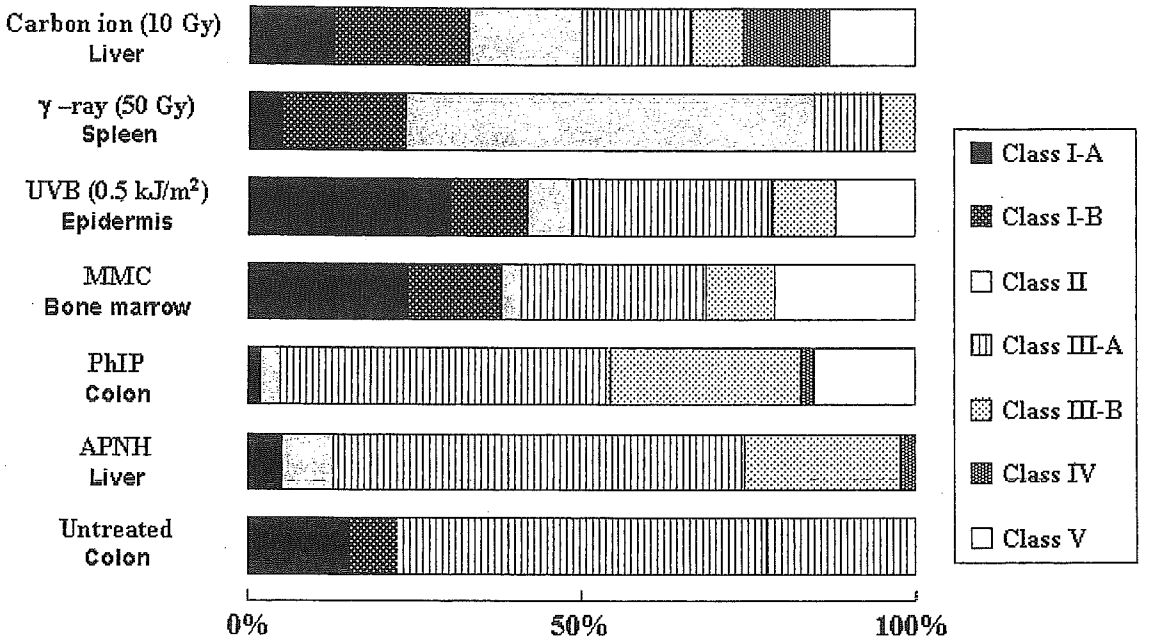


Fig. 3. Schematic representation of various classes of Spi⁻ mutations. Class IA, large deletions with short homologous sequences at the junctions; class IB, large deletions without short homologous sequences; class II, middle-size (more than 1 bp but less than 1 kb) deletions; class IIIA, single bp deletions at run sequences; class IIIB, single bp deletions at nonrun sequences; class IV, complex mutations; class V, miscellaneous mutations. The references for each treatment are as follows: carbon ion [Yatagai et al., 2002]; γ-ray [Nohmi et al., 1999]; UVB [Horiguchi et al., 2001]; MMC [Takeiri et al., 2003]; PhIP [Masumura et al., 2000]; APNH [Masumura et al., 2003b]; untreated [Masumura et al., 2000]. The sequence characteristics except for that of APNH are based on the results without the confirmation assay using WL95 (P2) strain. Thus, class V base substitution mutants are not included in the APNH spectrum. Class V mutants are not identified in untreated mice even without confirmation with WL95(P2) strain.

gap filling and ligation. In the case of class IB mutants having flush ends, the double-stranded ends would be joined without the short direct-repeat sequences.

Class II mutants included middle-size deletions of more than 1 bp but less than 1 kb. Some had short homologous sequences but others did not, as in the case of class I mutants. Mutations of more than 20 bp but less than 600 bp were frequently identified in the spleen of mice irradiated with γ-ray at a dose of 50 Gy (Fig. 3). These middle-size deletions may be induced by clustered DNA damage, which is often induced by high-dose radiation [Gulston et al., 2002].

Class III mutants were 1 bp deletions in the *gam* gene. These small deletions should not induce Spi⁻ mutations. Translation of the *gam* and *redBA* genes is probably linked, but the *gam* gene is transcribed first. Therefore, 1 bp deletions in the *gam* gene may interfere with the start of translation of the downstream *redBA* genes, functionally inactivating not only *gam* but also *redBA* [Nohmi et al., 1999]. This accounts for how class II mutants having deletions with a size of $3n + 1$ ($n = 0, 1, 2, 3 \ldots$) in the *gam* gene display Spi⁻ phenotypes. We subdivided class III mutants into class IIIA and IIIB; the former mutations occur at runs of the same base, such as AAAAAA, and the latter at nonrepetitive sequences (Fig. 2). Class IIIA mutants occurring at A:T repetitive sequences are the predominant type of mutation in untreated mice (Fig. 3). PhIP and APNH induce class IIIA deletions, but most of the induced mutations occur at repetitive G:C sequences or immediately flanking run sequences [Masumura et al., 2000, 2003b]. We hypothesize that −1 bp deletions induced by PhIP and APNH are induced by translesion DNA synthesis. Since large deletions were not frequently observed in the Spi⁻ mutants recovered from PhIP- or APNH-treated mice, we suggest that PhIP and APNH mainly induces single bp deletions rather than large deletions in vivo. This is consistent with the report that PhIP exhibits only weak or negative genotoxicity in chromosome aberration and micronucleous assays in the bone marrow and peripheral blood cells using C57BL/6 mice [Tucker et al., 1989].

Class IV contains complex mutations where the exact junctions could not be identified because of genome rearrangements. This class of mutants was observed in *p53*-knockout mice irradiated with heavy ions [Yatagai et al., 2002]. Class V includes miscellaneous mutants, mainly base substitution mutants in the *gam* gene. These mutants have no deletions in the *gam* gene, and they are not expected to be Spi⁻ mutants. Class V base substitution mutants could be the result of the leakiness of the P2 lysogen, i.e., the XL-1 Blue MRA (P2) strain. In fact, the class V base substitution mutants do not display Spi⁻

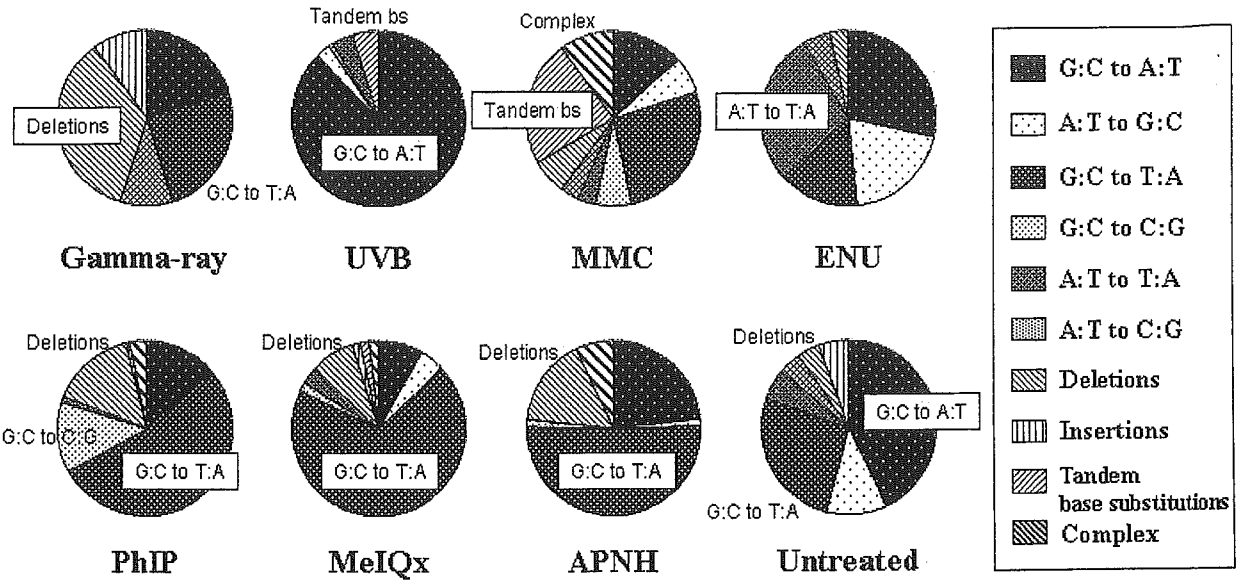


Fig. 4. Schematic representation of various types of *gpt* mutations. The organ where mutations were analyzed and the references are as follows: γ-ray in the liver [Masumura et al., 2002]; UVB in the epidermis [Horiguchi et al., 1999]; MMC in the bone marrow [Takeiri et al., 2003]; ENU in the bone marrow [Masumura et al., 1999b]; PhIP in the colon [Masumura et al., 2000]; MeIQx in the liver [Masumura et al., 2003a]; APNH in the liver [Masumura et al., 2003b]; untreated in the colon [Masumura et al., 2000].

phenotypes in another P2 lysogen, i.e., *E. coli* WL95 (P2). Most of the class V mutants make plaques on a *recA*$^-$ *E. coli*. The phenotype of λ-phage that cannot produce a plaque on a *recA*$^-$ strain is called Fec$^-$. λ *red* and *gam* double-mutants are Fec$^-$. To eliminate the base substitution mutants, we recently introduced the WL95 (P2) strain to confirm the phenotype of the Spi$^-$ candidates recovered from mice [Masumura et al., 2003b].

## Spi$^-$ MUTATIONS IN UNTREATED MICE

We have analyzed more than 50 Spi$^-$ mutants rescued from the liver, epidermis, bone marrow, and colon of untreated *gpt* delta mice [Masumura et al., 2000, 2002, 2003b; Horiguchi et al., 2001; Takeiri et al., 2003]. The predominant mutation is 1 bp deletion in repetitive sequences, i.e., in the *gam* gene, namely, class IIIA (Figs. 2 and 3). There are several hot spots of spontaneously occurring Spi$^-$ mutations. The most prominent one leads to the formation of class IIIA mutants, A6 to A5 at position 295–300 in the *gam* gene (the numbering starts from the first ATG of the *gam* gene). The mutation has been repeatedly identified in all organs examined. Other examples include 1 bp deletions of A5 to A4 at position 227–231, G4 to G3 at position 286–289, and C4 to C3 at position 238–241 in the *gam* gene. We suggest these events are most likely induced by slippage errors of DNA polymerases during DNA replication [Streisinger et al., 1966]. We have not identified a hot spot for large deletions (class IA or IB) in either untreated or treated mice. This may indicate that DSBs are randomly induced in the transgene region. However, further work is required before reaching any conclusion.

## MOLECULAR CHARACTERISTICS OF *gpt* MUTATIONS

In addition to Spi$^-$ selection, we have analyzed in vivo point mutations by 6-TG selection. Here, we briefly summarize the sequence characteristics of these *gpt* mutations (Fig. 4).

### γ-Ray Radiation

The most characteristic mutation of γ-ray mutagenesis was a short deletion of mostly less than 10 bp [Masumura et al., 2002]. These deletions occur at nonrepetitive sequences. Short deletions in nonrepetitive sequences are also observed in *lacZ* and *lacI* transgenic mice exposed to X-rays and γ-rays, respectively [Winegar et al., 1994; Ono et al., 1999]. Another characteristic mutation was G:C→T:A transversion. Since it is a typical mutation induced by 8-oxo-guanine in DNA [Kasai et al., 1993], we suggest that γ-irradiation induces mutations in the *gpt* gene primarily through the generation of reactive oxygen species (ROS).

### UVB

The most frequent *gpt* mutations in the epidermis of UVB-irradiated transgenic mice were G:C→A:T transitions at dipyrimidine sites such as 5′-TC-3′ and 5′-CC-3′ (underlined C is changed to T) [Horiguchi et al., 1999]. CC→TT double transitions were also identified. The C→T and CC→TT transitions at dipyrimidine sites have been identified as common mutations in the *p53* tumor suppressor gene in human skin cancers [Kress et al., 1992]. We suggest that these mutations are generated by

translesion bypass across UV-induced lesions by specialized DNA polymerases. Although UVB induces deletions, the MF of *gpt* base substitution mutations is more than 75 times higher than that of class I large deletions. We speculate that the high frequency of base substitution might be due to efficient translesion bypass across UV-induced lesions by specialized DNA polymerases.

### MMC

MMC specifically induced tandem-base substitutions, mainly in 5′-GG-3′ sequences in the bone marrow of mice. No single-base substitutions or single-guanine-base deletions were induced by the treatment [Takeiri et al., 2003]. Like UVB-induced base substitutions, translesion bypass across the lesions may be involved in the induction of base substitutions. However, the ratio of the specific MFs for tandem base substitutions and large deletions is almost 1:1. We suggest that MMC induces deletions more frequently than does UVB because inter- and intra-strand crosslinks are difficult lesions to bypass, even for specialized DNA polymerases.

### N-Ethyl-nitrosourea (ENU)

ENU induced A:T→T:A transversion mutations, suggesting the importance of modified thymine base such as $O^2$-ethylthymine for the induction of mutations [Masumura et al., 1999b].

### PhIP and APNH

Treatment of mice with PhIP- or APNH-containing diet induced G:C→T:A transversion mutations in the colon and liver, respectively [Masumura et al., 2000, 2003b]. Single bp frameshifts at G:C were also identified. More than half of these single bp frameshifts occurred at G:C sites in 5′-GGG-3′ or 5′-GG-3′ sequences. The base substitutions and frameshifts could be induced by translesion bypass replication of template dG-C8-adducts.

### 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx)

Treatment of mice with a diet containing MeIQx induced G:C→T:A mutations in the liver [Masumura et al., 2003a]. MeIQx as well as APNH induced more mutations in the liver than the colon, with the opposite being true for PhIP [Masumura et al., 1999a]. This organ specificity for genotoxicity is consistent with the organ specificity of carcinogenesis by the heterocyclic amines: the target organs for the carcinogenicity of MeIQx and APNH are liver while the target organ for PhIP in male rats is colon. The liver *gpt* MF induced by 300 ppm MeIQx in the diet is similar to the liver MF induced by 20 ppm APNH.

### Oxidative DNA Damage

OGG1 is a glycosylase that excises 8-oxo-guanine in DNA. *gpt* delta mice were crossed with $Ogg1^{-/-}$ mice [Minowa et al., 2000]. The spontaneous *gpt* MF in the liver was significantly higher in $Ogg1^{-/-}$ mice than in the wild-type mice. In addition, administration of potassium bromate, which induces oxidative damage in DNA, enhanced the amount of 8-oxo-guanine in the kidney and liver DNA of $Ogg1^{-/-}$ mice and increased the *gpt* MF [Arai et al., 2002, 2003]. Further studies are necessary to investigate whether oxidative damage in DNA induces deletion mutations and determine the molecular characteristics of such deletions.

### Spontaneous Mutations

The mutations most commonly observed in untreated mice were G:C→A:T transition mutations. More than half of these occurred at 5′-CpG-3′ sites, namely, at positions 64, 110, and 115 of the *gpt* gene (the numbering starts from the first ATG of the *gpt* gene). This suggests that deamination of 5-methylcytosine at CpG sites contributes to spontaneous *gpt* mutations. Besides G:C→A:T mutation, G:C→T:A transversions were also frequently observed among spontaneous *gpt* mutants. The remaining mutants were frameshifts or short deletions. These characteristics are largely consistent with the spontaneous mutation spectra of the *lacI* gene of Big Blue mice [de Boer et al., 1998].

## POSSIBLE MECHANISMS OF INTRACHROMOSOMAL DELETION MUTATION IN VIVO

Deletions with short (class IA) or no (class IB) homologous sequences at their junctions have been observed in a number of mutant genes implicated in human diseases, including cancer. Examples include the retinoblastoma [Canning and Dryja, 1989], α-galactosidase A [Kornreich et al., 1990], β-globin [Henthorn et al., 1990], factor VIII [Woods et al., 1991], and aspartylglucosaminidase genes [Jalanko et al., 1995]. Indeed, about 40% of large deletions in human disorders are characterized by the presence of very short sequence homologies at the breakpoints [Morris and Thacker, 1993]. On the basis of the sequence characteristics observed in the junctions of $Spi^-$ mutants, we suggest that NHEJ repair plays an important role in the generation of intrachromosomal deletions such as $Spi^-$ class I mutants. This pathway involves the DNA end-binding heterodimer KU70/KU80, DNA-$PK_{CS}$, XRCC4, and DNA ligase IV [Kanaar et al., 1998; van Gent et al., 2001]. Although some of these proteins play an essential role in the maintenance of genome stability and suppression of tumorigenesis, the NHEJ repair pathway has the potential to induce deletion muta-

tions. If two incompatible ends are generated by IR, they first have to be converted to ligatable ends by enzymatic processing, which often causes small deletions [Pfeiffer et al., 2000]. Since the protein-coding regions are only a few percent of mammalian DNA and expressed essential genes are rare, many of the small deletions associated with NHEJ repair may be tolerated in the mammalian genome. KU70/80 heterodimer proteins may function as the alignment factor by protecting DNA ends from excess degradation and enhancing the ligation [Feldmann et al., 2000].

## EFFECTS OF p53 AND ATM ON DELETION MUTATIONS

To gain insight into the NHEJ process, *gpt* delta mice were crossed with $p53^{-/-}$ mice [Tsukada et al., 1993] or $Atm^{-/-}$ mice [Barlow et al., 1996], and $Spi^-$ mutations were analyzed following IR. The tumor suppressor gene *p53* is involved in various aspects of genome stability, such as signal transduction, cell cycle checkpoint control, apoptosis, and DNA repair, and is referred to as "the guardian of the genome" [Lakin and Jackson, 1999]. To examine the roles of p53 in in vivo mutations, *lacI* transgenic mice were crossed with $p53^{-/-}$ mice. Contrary to expectation, no statistical difference in *lacI* MF and spectrum was observed between $p53^{+/+}$ and $p53^{-/-}$ mice [Buettner et al., 1997]. It is known, however, that *p53* expression is regulated in an organ-specific manner [Komarova et al., 2000]. Indeed, the *p53* defect enhanced the $Spi^-$ MF in the kidney of mice [Yatagai et al., 2002]. Sequence analysis of the $Spi^-$ mutants indicated that the enhancement was primarily due to an increase in complex (class IV) deletions. In contrast to the kidney, the *p53* defect had no effect on $Spi^-$ MF in the liver. These results suggest that p53 suppresses deletion mutations in an organ-specific manner.

Ataxia telangiectasia (AT) is an autosomal recessive hereditary disease characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, immunodeficiency, chromosome instability, sensitivity to IR, and an increased incidence of cancer [Jorgensen and Shiloh, 1996]. The gene responsible for AT was cloned and designated *ATM* (ataxia-telangiectasia-mutated) [Savitsky et al., 1995]. The ATM protein is a member of PI-3 kinase family, which includes DNA-PK. ATM appears to play a crucial role in cell cycle control and DNA repair by phosphorylation of a number of proteins, including p53, CHK2, BRCA1, and NBS1 [Durocher and Jackson, 2001; Shiloh and Kastan, 2001]. ATM is inactive in unirradiated cells as a dimer but it phosphorylates its partner at serine-1981 in a rapid response to IR damage, which causes dimer dissociation [Bakkenist and Kastan, 2003]. The ATM monomers phosphorylate p53 at serine-15 [Banin et al., 1998; Canman et al., 1998]. Thus, if p53 is the guardian of the genome, ATM could be considered as "the guardian of the guardian of the genome" [Gatti et al., 2001].

Knockout *Atm* mice ($Atm^{-/-}$) are cancer-prone, immunodeficient, and sensitive to IR [Barlow et al., 1996]. To examine the roles of ATM in suppressing deletion mutations in vivo, $Atm^{-/-}$ *gpt* delta mice as well as control, i.e., $Atm^{+/+}$ *gpt* delta mice, were exposed to whole-body X-ray radiation at doses of 5, 10, and 50 Gy and $Spi^-$ MFs were determined in the liver 3 days after the irradiation [Furuno-Fukushi et al., 2003]. Dose-response MF curves were virtually identical for $Atm^{-/-}$ and $Atm^{+/+}$ mice, and structural analysis revealed no significant difference in the proportion of large deletions and 1 bp frameshifts between $Atm^{-/-}$ and $Atm^{+/+}$ mice. These results suggest that ATM does not affect IR-induced deletions in the liver. However, this does not necessarily mean that ATM is not involved in the suppression of IR-induced intrachromosomal deletions. As in the case of p53, *Atm* disruption might affect induction of mutations in other organs. It will be interesting to examine the effects of *Atm* deficiency on $Spi^-$ mutation in proliferating organs such as spleen or thymus.

## OTHER TRANSGENIC MICE FOR DETECTION OF MUTATIONS IN VIVO

There are a number of transgenic mouse models to analyze mutation in vivo. The Big Blue *lacI* mouse and *lacZ* Muta mouse are frequently used to detect point mutations in various organs of mice [Gossen et al., 1989; Kohler et al., 1991]. The *cII* gene has also been used to identify relatively small mutations in the assays rapidly [Jakubczak et al., 1996]. The mutation spectra for UV, PhIP, and MeIQx for the *lacI* gene are similar to those of the *gpt* gene [Okonogi et al., 1997; You et al., 1999; Bol et al., 2000]. However, class I-type large deletions detected by $Spi^-$ selection are not identified with the reporter genes when the *lacI* or *lacZ* mice are treated with UV or MMC. The high frequency of base substitution induced by UVB and MMC might mask a low incidence of deletions in the *lacI* or *lacZ* genes. Alternatively, the sequence containing the *red/gam* genes for $Spi^-$ selection might be prone to deletion in the mouse chromosome. Plasmid *lacZ* mice have been engineered to analyze deletion as well as base substitution mutations in the chromosome [Boerrigter et al., 1995], and genome rearrangements associated with aging have been characterized [Dolle and Vijg, 2002]. It would be interesting to compare the spectra of deletions detected with *lacZ* plasmid mice and *gpt* delta mice that are induced by environmental mutagens.

Several transgenic mice have also been developed to analyze recombination in vivo. $Aprt^{+/-}$ and $Tk^{+/-}$ mice were created to measure loss of heterozygosity (LOH) [Engle et al., 1996; Dobrovolsky et al., 1999]. Recombi-

nation at direct-repeat sequences can be analyzed with $p^{un}$ (pink-eyed unstable) mice and FYDR (fluorescent yellow direct repeat) mice [Hendricks et al., 2003; Reliene and Schiestl, 2003]. When recombination occurs at direct repeat sequences comprising two truncated coding regions, the full length of *lacZ* or enhanced yellow fluorescent protein (*eyfp*) can be restored. Since recombination at direct repeat sequences is rare in somatic cells, the former model analyzes recombination during embryonic development to give rise to a visible phenotype in mature animals, while the latter takes advantage of the fluorescent protein, which increases the detection limits. Direct-repeat recombination during spermatogenesis can be measured with other *lacZ* mice [Murti et al., 1994; Moynahan et al., 1996]. The high frequency of recombination in germ cells allows the detection of recombinant cells by flow cytometry with fluorescent substrates. A mouse model that specifically identifies somatic intrachromosomal recombination inversion events has been established and has been used to analyze the effects of radiation and chemicals on genome rearrangements [Sykes et al., 1999].

## CONCLUSIONS

The *gpt* delta mouse provides the opportunity for molecular analysis of both intrachromosomal deletions and base substitutions in tissues and organs of mice treated with mutagens. Previous studies indicate that NHEJ repair of DSBs and bypass DNA synthesis across DNA lesions are involved in the induction of deletions and base substitutions, respectively. However, the maximum size of class I deletions is less than 10 kb. Thus, $Spi^-$ selection only detects a part of the potential genome rearrangements and neglects most interchromosomal translocations and intrachromosomal megabase deletions. Other approaches including FISH analysis might be required to characterize the entire spectrum of chromosome rearrangements. Nevertheless, sequence information of the break points of chromosomes detected by $Spi^-$ selection is helpful for identifying the nature of DSB processing in vivo.

Point mutations in mouse transgenes are regarded as neutral mutations because the transgenes are not expressed in the mouse genome. Hence, point mutations can accumulate in the transgenes as a result of repeated treatments [Heddle, 1999]. Unlike point mutations, which are mostly induced by DNA adducts, $Spi^-$ large deletions are induced by DSBs. Thus, it will be interesting to examine whether deletions can also accumulate over time.

In addition to the *gpt* delta mouse, a transgenic rat carrying the same reporter gene has been developed [Hayashi et al., 2003]. Transgenic rats could be important because most carcinogenesis studies are performed in rats rather than in mice. The molecular nature of deletions and base substitutions can be analyzed in the target organs for carcinogenesis with *gpt* delta rats. This would provide critical information as to whether particular chemicals induce tumors via genotoxic mechanisms or not. Knowledge about the specific molecular alterations induced by chemicals in the various organs of rats will be helpful for understanding the molecular mechanisms underlying environmental mutagenesis and carcinogenesis.

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