— Minireview —

Spontaneous Mutagenesis in Rodents: Spontaneous Gene Mutations Identified by Neutral Reporter Genes in *gpt* Delta Transgenic Mice and Rats

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Transgenic rodents are valuable models for investigation of genotoxicity of chemicals *in vivo*. We have developed *gpt* delta transgenic mice (C57BL/6J background) and rats (Sprague-Dawley, SD), which have the ability to identify both point mutations by the *gpt* assay [6-thioguanine (6-TG) selection] and certain types of deletions by the Spi$^-$ (Spi, sensitive to P2 interference) assay. Recently, the *gpt* delta SD rat was backcrossed with the Fisher 344 (F344) rat to establish an *gpt* delta F344 rat. The average spontaneous *gpt* mutation frequencies (MFs) are about 4.5×10^{-6} in both SD and F344 *gpt* delta rats as well as in *gpt* delta mice. The G:C to A:T transitions at 5′-CpG-3′sites and G:C to T:A transversions are the predominant spontaneous *gpt* mutations in rats and mice. However, there is one false mutation (*e.g.* A:T to T:A at position 299) in the rats. The base substitution may have arisen when the lambda EG10 transgene was introduced into the genome of the SD rat during transgenesis. In the Spi$^-$ assay, 1-bp deletions in repetitive sequences are predominantly observed in both mice and rats. Possible mechanisms underlying the spontaneous mutations in *gpt* delta rodents are discussed.

Key words —— *gpt* delta transgenic rodent, spontaneous mutation, mutation spectrum, *gpt* assay, Spi$^-$ assay

OVERVIEW OF *gpt* DELTA TRANSGENIC RODENTS

Gene mutations play an important role in the etiology of many human diseases including cancer. Since humans are exposed to a variety of endogenous and exogenous mutagens, there has been considerable interest in the relationship between exposure, genotoxic effects, and cancer incidence. To assess the risk of mutagens to the human genome, genotoxicity tests have been developed, including *in vivo* mutation assays using experimental animals, which play a crucial role in risk assessment. To investigate *in vivo* genotoxicity, a number of transgenic rodent mutation assays have been developed by introducing reporter transgenes into the chromosome of every cell of the animal.[1,2] Using these systems, mutagenic events induced in a rodent can be detected by recovering the transgene and analyzing the phenotype of the reporter gene in a bacterial host. These models permit quantitation of mutations and identification at the sequence level in any tissue or organ in the body. *lacZ*, *lacI* or *cII* have been employed as reporter genes in transgenic rodents, such as the Muta^{TM} mouse, and the Big Blue^{R} mouse and rat.[3–7] Despite differences in size and sequence context, spontaneous mutation frequencies of these reporter genes are in the mid-10^{-5} range and those are predominantly base substitutions in most tissues. This high background of base substitutions may make it difficult to detect rare mutations such as deletions induced by ionizing radiation.[8,9] To overcome this limitation, a transgenic "*gpt* delta" assay system has been developed for the efficient detection of both point mutations and deletions.[1,10] A unique feature of the lambda EG10 phage vector constructed for this system is the incorporation of two different positive selection methods: the *gpt* assay [6-thioguanine (6-TG) selection] using the *gpt* gene of *Escherichia coli* (*E. coli*) that detects mainly point mutations such as base substitutions and frameshifts, and the Spi$^-$ (Spi, sensitive

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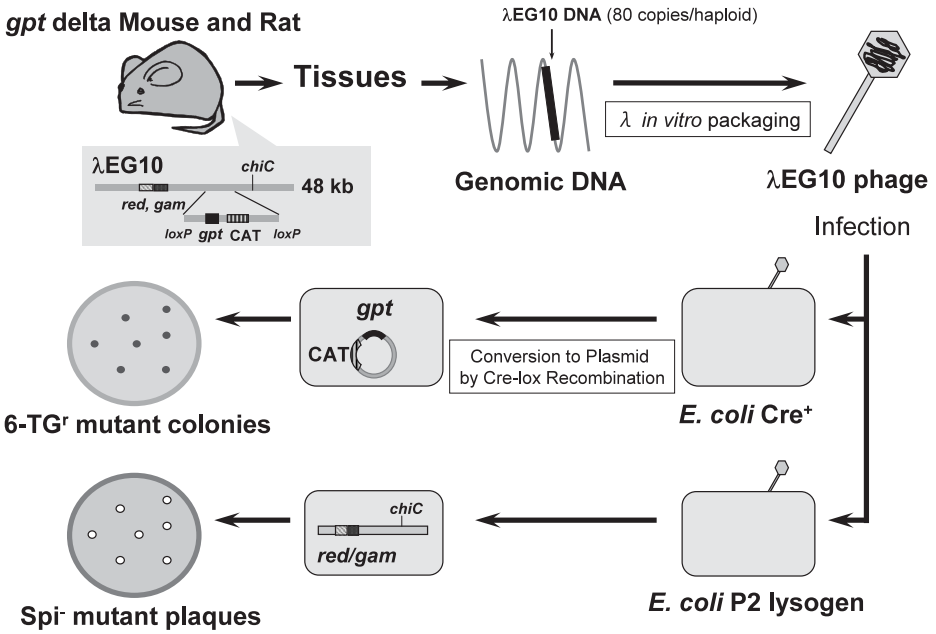

Fig. 1. Principle of *gpt* Delta Transgenic Rodent Mutation Assays[1, 10, 13, 14]

Genomic DNA is extracted from tissue of a transgenic rodent. Lambda EG10 DNA is recovered by *in vitro* packaging and rescued as phage particles. Two distinct *E. coli* host strains are infected with the rescued lambda EG10 phages: one is *E. coli* YG6020 expressing Cre recombinase for the *gpt* assay and the other is a P2 lysogen for the Spi$^-$ assay. In the *gpt* assay, lambda EG10 DNA is converted to plasmid carrying *gpt* and CAT by cre-lox recombination. Bacteria harboring the plasmids carrying mutant *gpt* are positively selected as colonies on plates containing chloramphenicol and 6-thioguanine. In the Spi$^-$ assay, mutant lambda EG10 phages lacking *red/gam* gene functions are positively selected as Spi$^-$ plaques on lawns of P2 lysogens. The same DNA prepared from identical tissue sample is applied to both *gpt* and Spi$^-$ assays.

to P2 interference) assay (Spi$^-$ selection) using the *red/gam* genes of lambda phage that detects deletions including frameshifts (Fig. 1).

To accomplish the *gpt* and Spi$^-$ assay, the *gpt* delta transgenic mouse has been developed.[1, 10] This mouse was established by microinjection of lambda EG10 phage DNA into the fertilized eggs of C57BL/6J mice. It carries about 80 copies of the transgene in a head to tail fashion at a single site in chromosome 17 and is maintained as a homozygote (carrying 160 copies of transgene per diploid).[11] The same lambda EG10 transgene used in the *gpt* delta mouse was integrated into the genome of Sprague-Dawley (SD) rat to establish the *gpt* delta rat.[12] The *gpt* delta rat harbors about 5–10 copies of the transgene in chromosome 4 and is maintained as a heterozygote. The *gpt* and Spi$^-$ assay systems have been validated primarily in mice with many chemical mutagens/carcinogens, UV and ionizing radiation, for which mutagenicity, organ specificity and mutation spectrum have been thoroughly characterized.[1, 10–28] Recently, the outbred *gpt* delta SD rat was backcrossed with Fisher 344 (F344) rat, to establish an inbred *gpt* delta rat (F344). In this review, we focus on the spontaneous mutations detected by the *gpt* and Spi$^-$ assays in *gpt* delta mice and rats and discuss possible mechanisms underlying these *in vivo* mutations.

gpt ASSAY (6-TG SELECTION) FOR POINT MUTATIONS

The principles and method of the *gpt* assay have been described previously (Fig. 1).[1, 10, 15] Briefly, the *gpt* gene encodes guanine phosphoribosyltransferase that is involved in the purine salvage pathway of *E. coli.* This enzyme phosphoribosylates not only guanine, but also 6-TG, which is toxic to cells when it is incorporated into DNA. Thus, *E. coli* cells expressing wild-type *gpt* cannot survive on the plates containing 6-TG. Only *gpt* mutants can form colonies on plates containing 6-TG. The coding region of *gpt* is 456 bp, which is convenient for sequence analysis of the mutants. When *E. coli* strain YG6020 expressing Cre recombinase is infected with lambda EG10 rescued from *gpt* delta rodents, the plasmid region is efficiently excised, circularized and propagated as multi-copy number plasmid carrying *gpt* and chloramphenicol acetyltransferase

(CAT). Bacteria harboring mutated *gpt* genes can be positively selected as colonies on plates containing 6-TG and chloramphenicol (Cm). The number of rescued phages can be determined by plating the cells on the plates containing Cm alone. The *gpt* mutant frequency (MF) is calculated by dividing the number of the *gpt* mutant colonies by the number of rescued Cm-resistant colonies.

The spontaneous *gpt* MFs of *gpt* delta mice (C57BL/6J background) have been previously reported. In most experiments, the values of spontaneous *gpt* MFs are in the range of 5×10^{-6}. This is less than that of other transgenic rodent mutation assay systems, such as Muta mice and Big Blue mice, in which spontaneous MFs are usually greater than 1×10^{-5}. Different selection method and sequence context of the *gpt* gene might account for the lower MF of the *gpt* assay. Even if some types of mutation occurred in the *gpt* gene, residual enzymatic activity may still catalyze enough amount 6-TG to kill the cells. Because the *gpt* mutant cells are grown on M9 minimal medium agar plate containing 6-TG for 3–4 days, phenotypic selection of 6-TG resistance could require complete loss of enzymatic activity of the mutated *gpt* gene product as a selectable phenotype. There are no clearly observed tissue differences in the spontaneous MFs in *gpt* delta mice. Although the number of studies in which multiple tissues types have been analyzed is limited, similar spontaneous MFs were observed in six tissues (liver, spleen, colon, testis, brain and bone marrow) collected from the same animals.[11] No significant differences between male and female were observed in the spontaneous and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced MFs in liver and colon.[11] In *gpt* delta mice, spontaneous MF in the liver at 85 weeks of age increased by a factor of 2 over that at 19 weeks of age.[22] It is also reported that spontaneous MFs increase with age in most somatic cells in Muta mice and Big Blue mice.[29–32] However, the trend could be different in brain and germ tissue, where MFs don't increase with age in adult mice.[33–35] Interestingly, it has been reported that some gene knockout mice show different spontaneous MFs. Spontaneous *gpt* MF in the liver is significantly higher in *ogg1* gene knockout mice, which lack 8-oxo-guanine DNA glycosylase activity.[36] Interleukin-10 (IL-10)-deficient mice, which spontaneously develop intestinal inflammation, have a *gpt* mutation frequency in the colon about five times higher than that in wild type mice.[37] In transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2)-null mice, the spontaneous *gpt* mutation frequency in the lung was approximately three times higher in *nrf2*-null (*nrf2*−/−) mice than in *nrf2* heterozygous (*nrf2*+/−) or wild-type (*nrf2*+/+) mice; whereas in the liver, the mutation frequency was higher in *nrf2*−/− and *nrf2*+/− mice than in *nrf2*+/+ mice. In contrast, no difference in mutation frequency was observed in testis between the three genotypes.[38] These results suggest that the intracellular environment contributes to spontaneous mutations, perhaps through oxidative stress and/or detoxification systems.

In the *gpt* delta rat, the spontaneous *gpt* MFs we have obtained from more than 40 samples from various organs were between 0.9×10^{-6} and 8.5×10^{-6} (4.5×10^{-6} in average). No difference was observed between outbred SD rats (4.5×10^{-6}, calculated from 31 samples) and inbred F344 rats (4.4×10^{-6}, calculated from 10 samples). Although the data represent males and females, different organs (liver, kidney and mammary gland), and different ages (between 10 and 52 weeks old), these values are comparable to those in mice. On the other hand, Hayashi *et al*. reported that the spontaneous *gpt* MF in the liver of the rats was lower than that of mice.[12] The similar observation was also reported in Big Blue mice and rats.[7] Additional studies are needed to investigate the effect of genetic strain background, tissue type, and age in *gpt* delta rat in order to validate these findings.

Spontaneous *gpt* mutation spectra of *gpt* delta rodents are shown in Table 1. Regardless of species or strain background, the proportions of mutations are remarkably similar, although the sample size for F344 rats is small. The most frequent mutations are G:C to A:T transitions. More than half of these occur at 5′-CpG-3′ sequences in mice and SD rats. This suggests that deamination of methylated cytosines at CpG sites contributes to spontaneous mutations in the *gpt* gene *in vivo*.[39] Beside transitions, G:C to T:A transversions are frequently observed base substitutions. This type of mutations may reflect oxidative damage in DNA, such as 8-oxoguanine lesions[40] or abasic sites.[41] Other mutations were frameshifts, short deletions and insertions. These findings were largely consistent between different tissues. Although lower spontaneous *gpt* MFs than Muta mouse and Big Blue mouse are observed, the characteristic mutation spectra are similar, and the similarity of spontaneous mutation spectra in different tissues and different rodent strains are also observed with the

Table 1. Spontaneous *gpt* Mutation Spectra of *gpt* Delta Mice and Rats

	C57BL/6J mouse			SD rat			F344 rat		
	No.	CpG	%	No.	CpG	%	No.	CpG	%
Base substitutions									
Transitions									
G:C → A:T	59	(32)	33.3	39	(22)	43.8	12	(3)	40.0
A:T → G:C	19		10.7	6		6.7	1		3.3
Transversions									
G:C → T:A	31		17.5	18		20.2	5		16.7
G:C → C:G	4		2.3	1		1.1	1		3.3
A:T → T:A	13		7.3	1		1.1	1		3.3
A:T → C:G	11		6.2	2		2.2	1		3.3
Deletions	24		13.6	15		16.9	9		30.0
1 bp	18			12			5		
> 2 bps	6			3			4		
Insertions	10		5.6	7		7.9	0		0.0
Others[a)]	6		3.4	0		0.0	0		0.0
Total No. of Mutations	177		100.0	89		100.0	30		100.0
No. of animal	23			31			10		
Sex	male			male and female			male		
Tissues	liver			liver, kidney, mammary gland			liver		
Age	10–20 weeks old			10–52 weeks old			20–24 weeks old		

a) Multiple base substitutions, bese substitutions coupled with deletion or insertion.

lacI transgene.[42,43)] De Boer *et al.* reported the spontaneous *lacI* mutations for liver, spleen, bladder, stomach, kidney, bone marrow, lung and skin of Big Blue mice. They showed the similarity of the *lacI* mutational spectra in all tissues.[42)] Basically, the predominant class of spontaneous mutations was G:C to A:T transitions, most of which occurred at CpG sites. The second most common class was G:C to T:A transversions. All other base substitution classes contributed less than 10% each. Of the non-base substitution events, the loss of a single base pair was the most frequently occurring event. Zhang *et al.* compared *lacI* spontaneous mutation spectra in the liver of C57BL/6, B6C3F1 and BC-1 mice and F344 rats and concluded that spontaneous mutations appear to be similar, regardless of genetic location, rodent strain, or species.[43)]

In sequence analysis of the *gpt* gene recovered from *gpt* delta rats, we should note that an unexpected A:T to T:A transversions at position 299 (*e.g.* nucleotide 299 from the first codon of ATG, in the *gpt* sequence) was frequently observed. We conclude that this base substitution must have arisen in the lambda EG10 DNA during SD rats transgenesis, and is not induced by spontaneous or induced somatic mutagenesis. Evidence for this includes: (1) base substation is observed in untreated rats as well as mutagen-treated rats at a similar frequency; (2) it typically occurs along with another mutation in any given *gpt* mutant; (3) it was detected in gpt^+ (6-TG sensitive) colonies rescued from both *gpt* delta SD and F344 rats; (4) we observed that 96/473 (20%) *gpt* mutants recovered from *gpt* delta rats contain this base substitution [the frequency should be about 20% (2×10^{-1}) if one of five copies of integrated lambda EG10 has A:T to T:A change at position 299]; and (5) it was never found among 1680 *gpt* mutants we have analyzed in *gpt* delta mice. Thus, an A:T to T:A transversion at position 299 observed only in *gpt* delta transgenic rats is a "false" mutation. In the development of transgenic rodents, multiple copy transgenes are usually integrated at a single site of chromosome in a head-to-tail fashion. We suppose that an unintended point mutation might have been arisen in one copy of the transgenes during the first round of DNA replication when they integrated into the chromosome of *gpt* delta SD rat. Although this *gpt* mutation results in an amino acid substitution from isoleucine (Ile) to asparagine (Asn), it doesn't cause a mutated *gpt* phenotype (*e.g.* is a silent mutation) and therefore doesn't affect the *gpt* MF. Because of this, A:T to T:A mutations at 299 were excluded from the mutation spectra of *gpt* delta rats in Table 1.

Table 2. Spontaneous Spi$^-$ Mutation Spectra of *gpt* Delta Mice and Rats

	C57BL/6J mouse		SD rat		F344 rat	
	No.	(%)	No.	(%)	No.	(%)
One bp deletions	95	70.4	5	50.0	30	76.9
GGGG	21	15.6	2	20.0	12	30.8
GGG	4	3.0	0	0.0	2	5.1
GG	6	4.4	0	0.0	2	5.1
G	10	7.4	1	10.0	3	7.7
AAAAAA	18	13.3	0	0.0	2	5.1
AAAAA	29	21.5	2	20.0	8	20.5
AAA	1	0.7	0	0.0	1	2.6
AA	0	0.0	0	0.0	0	0.0
A	6	4.4	0	0.0	0	0.0
> 2 bp deletions	28	20.7	2	20.0	4	10.3
2 bps–1 kb	8	5.9	1	10.0	1	2.6
> 1 kb	20	14.8	1	10.0	3	7.7
Complex	6	4.4	1	10.0	5	12.8
Others[a)]	6	4.4	2	20.0	0	0.0
Total No. of Mutations	135	100.0	10	100.0	39	100.0
No. of animal	43		3		10	
Sex	male and female		male		male	
Tissues	brain, colon, liver, bone marrow, epidermis, lung		liver		liver, kidney	
Age	10–20 weeks old		24 weeks old		17–30 weeks old	

a) Deletions coupled with insertions and/or base substitutions.

SPI$^-$ ASSAY (SPI$^-$ SELECTION) FOR DELETIONS

The Spi$^-$ assay is a unique selection that can detect deletions, rather than base substitution mutations. The methodology and the characteristics of the chemical- and radiation-induced Spi$^-$ mutations have been described in detail.[1,14)] Spi$^-$ selection takes advantage of the restricted growth of wild type lambda phage in P2 lysogens.[44)] This phenotype is called Spi (sensitive to P2 interference). Only mutant lambda phages that are deficient in the functions of both the *gam* and *red* genes can grow in P2 lysogens and display the Spi$^-$ phenotype. Simultaneous inactivation of both the *gam* and *red* genes is usually induced by deletions. 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 up to 10 kb. Thus, the mutants are mostly intrachromosomal deletions. However, the tandem array of 80 copies of lambda EG10 DNA in the *gpt* delta mouse provides a potential target of approximately 3.8 megabases.

In previous reports, the spontaneous Spi$^-$ MFs of *gpt* delta mice were around $1–5 \times 10^{-6}$, which is lower than that of other transgenes, such as *lacZ* of Muta mice and *lacI* of Big Blue mice. Because the predominant types of point mutations induced *in vivo* are base substitutions, it could be that the spontaneous Spi$^-$ MF, which only detects deletion mutations but not base substitutions, is generally lower than that of *lacZ*, *lacI* or *gpt* MF. Tissue type and sex differences of spontaneous Spi$^-$ MFs is not clearly observed,[11)] although the number of studies in which multiple tissue types have been compared is very limited. The effect of age has not been well characterized. No significant differences in spontaneous MFs were reported in *p53*, *Atm* or *Parp-1* knockout *gpt* delta mice, although heavy-ion or X-ray irradiation induced more large deletions in knockout mice than in wild type mice.[17,21,26)] In the *gpt* delta rat, the spontaneous Spi$^-$ MFs we have obtained from 13 rat tissues were between 1.3×10^{-6} and 4.4×10^{-6}, similar to those observed in *gpt* delta mice.[12)] No marked strain difference has been observed between SD and F344 rats although additional studies are needed to confirm this finding.

Spontaneous Spi$^-$ mutation spectra of *gpt* delta rodents are shown in Table 2. Although the sample sizes of the rats are small, mutational characteristics of spontaneous Spi$^-$ mutants are similar between

mice and rats. We have analyzed 135, 10 and 39 Spi$^-$ mutants detected in 43 C57BL/6J mice, 3 SD and 10 F344 rats, respectively. These mutants were rescued from brain, liver, epidermis, bone marrow, colon, lung and kidney (and unpublished data in our laboratory).[16,19,20,28,45] The most frequent mutation is a 1 bp deletion in the repetitive sequences in the *gam* gene. These small deletions are not supposed to induce Spi$^-$ mutations. However, translation of the *gam* and *red* 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 *red* gene, thereby functionally inactivating not only *gam* but also *red*.[13] The percentages of the 1 bp deletions are from 50–77%. But these values may be underestimated because we regard identical mutations recovered from the same tissue samples to have resulted from clonal expansion and count them as a single mutation. However, they could also be independent hot spot mutations. If this is the case, they should be counted as multiple independent mutations. Indeed, there are several hot spots of spontaneously occurring Spi$^-$ mutations. Those hot spots are 1-bp deletions of AAAAAA to AAAAA at position 295–300, AAAAA to AAAA at 227–231, GGGG to GGG at 286–289, and CCCC to CCC at 238–241 in the *gam* gene (the number starts from the first ATG of the *gam* gene). We suggest these events are most likely induced by slippage errors of DNA polymerases during DNA replication.

Other than 1 bp deletions, we observed about 20% of the Spi$^-$ mutations are larger deletions more than 2 bps in sizes. In mice, based on the sequence characteristics of the junctions and the neighboring regions, the Spi$^-$ deletions more than 1 kb in size could be classified as either those having junctions exhibiting short homology (1–12 bps) (13/20 = 65%) or those having flush junctions (7/20 = 35%). Deletions with short or no homologous sequences at their junctions have been observed in a number of mutant genes implicated in human diseases, including cancer. About 40% of large deletions in human disorders are characterized by the presence of very short sequence homologies at the breakpoints.[46] We suggest that non-homologous end-joining (NHEJ) repair plays an important role in the generation of intrachromosomal deletions such as Spi$^-$ mutants.[14] This pathway involves the DNA end-binding heterodimer Ku70/Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), X-ray repair cross complementing (XRCC)4, and DNA ligase IV.[47,48] Although some of these proteins play an essential role in the maintenance of genome stability and suppression of tumorigenesis, NHEJ also has the potential to induce deletion mutations. If two incompatible ends are generated by DNA double-strand breaks (DSBs), they first have to be converted to ligatable ends by enzymatic processing, which often causes deletions.[49] The middle size Spi$^-$ mutants (2 bps to 1 kb) might be caused by DNA replication error or by NHEJ. We have also detected complex type deletions containing genome rearrangement. This type of the Spi$^-$ mutation is sometime difficult to analyze by DNA sequencing. Other deletions coupled with short insertions at junctions or base substitutions are also observed in spontaneous Spi$^-$ mutations. We did not identify hot spots in the Spi$^-$ large deletions either in untreated or mutagen-treated mice. This may indicate that DSBs are randomly induced in the neutral transgene region. Further work is required to understand the mechanism by which Spi$^-$ deletions are generated.

CONCLUSION REMARK

The human genome is continuously exposed to various exogenous and endogenous DNA damaging agents including reactive oxygen species. To survive and protect the genome against DNA damage, cells employ many repair mechanisms such as mismatch repair, base excision repair, nucleotide excision repair, translesion DNA synthesis, and homologous and non-homologous recombination repair mechanisms. However, some repair mechanisms appear to be involved in error-prone DNA replication process or the induction of genome rearrangements such as deletions. To analyze the various types of *in vivo* mutations, *gpt* delta transgenic rodents were established to detect deletions as well as point mutations. Here we reported the characteristics of the spontaneous *gpt* (point mutations) and Spi$^-$ (deletions) mutations obtained from *gpt* delta mice and rats. The results suggested that the assays permit the efficient and quantitative detection of mutations in various tissues of mice and rats, and analysis of mutations at the molecular level. The spontaneous mutations observed in the tissues of mice and rats included both base substitutions and deletions. The predominant types of mutations are G:C to A:T transitions at 5′-CpG-3′ sites, G:C to T:A transversions, 1 bp deletions at repetitive se-

quences, and larger deletions of more than 1 kb. It suggests that deamination of methylated cytosines at CpG sites, oxidative damage to DNA, such as 8-oxoguanine lesions, slippage errors in DNA replication, and error-prone DSB repair may contribute to spontaneous mutations in the rodents. Further studies are necessary to investigate whether oxidative damage in DNA and dNTPs pool induces base substitutions and deletions in mammals and to determine the molecular characteristics of such mutations. Accumulative mutations with age might be related to endogenous oxidative stresses. Genome rearrangements associated with oxidative stress are also important in the field of mutagenesis and carcinogenesis. Because oxidation of DNA is often caused indirectly by malnutrition, the relationship between nutrition and genome rearrangements mediated via oxidative stresses could be an important and interesting topic. To investigate the mechanisms of carcinogenesis in target organs, *gpt* delta rat could be useful because most of carcinogenesis studies are undertaken in rats rather than mice. The mouse model is also useful to investigate specific gene function by crossing with gene knockout mice.

Transgenic mutation assays have the ability to evaluate mutagenesis *in vivo* in a broad range of tissues using neutral reporter genes integrated into genome. Recently, Bielas and Loeb reported a method to directly detect random point mutations in genomic and mitochondrial DNA from mouse and human cells.[50–52] This technique, called random mutation capture, is based on gene capture by hybridization with oligonucleotide probes, followed by cleavage by a restriction enzyme and quantification of the non-cleavable mutants by real-time quantitative PCR. Such a direct detection concept could be extended to quantify mutation in any cell types, at different sites in the genome, in coding and non-coding regions. In another approach, Jiang *et al.* systematically examined the mutational spectrum of the entire human genome and categorized regions using 1.8 million human single nucleotide polymorphisms (SNPs).[53] Although the mutational analysis using SNPs is limited to single base substitutions, extensive and comprehensive sequencing analysis may lead to new perspectives on *in vivo* mutagenesis.

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