FOOD SAFETY


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Review (Invited)

# Past, Present and Future Directions of *gpt* delta Rodent Gene Mutation Assays

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Genotoxicity is a critical endpoint of toxicity to regulate environmental chemicals. Genotoxic chemicals are believed to have no thresholds for the action and impose genotoxic risk to humans even at very low doses. Therefore, genotoxic carcinogens, which induce tumors via genotoxic mechanisms, are regulated more strictly than non-genotoxic carcinogens, which induce tumors through non-genotoxic mechanisms such as hormonal effects, cell proliferation and cell toxicity. Although Ames bacterial mutagenicity assay is the gold standard to identify genotoxicity of chemicals, the genotoxicity should be further examined in rodents because Ames positive chemicals are not necessarily genotoxic *in vivo*. To better evaluate the genotoxicity of chemicals in a whole body system, gene mutation assays with *gpt* delta transgenic mice and rats have been developed. A feature of the assays is to detect point mutations and deletions by two distinct selection methods, ie, *gpt* and Spi$^-$ assays, respectively. The Spi$^-$ assay is unique in that it allows analyses of deletions and complex DNA rearrangements induced by double-strand breaks in DNA. Here, I describe the concept of *gpt* delta gene mutation assays and the application in food safety research, and discuss future perspectives of genotoxicity assays *in vivo*.

**Key words:** *gpt* delta, transgenic, gene mutation, deletion, food safety

## 1. Introduction

Humans are continuously exposed to a variety of chemicals, some of which interact with DNA, thereby inducing mutations and cancer. The most notable example of environmental mutagens and carcinogens is cigarette smoke, which plays a major role in the etiology of lung cancer and a variety of chronic diseases[1]. Cigarette smoke is a mixture of 4,000 chemicals and contains more than 60 known carcinogens[2,3]. The International Agency for Research on Cancer (IARC) has evaluated nearly 1,000 chemicals for their potential to induce cancer in humans and concluded that about 100 chemicals are carcinogenic to humans (Group 1)[4]. The conclusion is supported by epidemiological evidence as well as rodent carcinogenicity assays and mechanistic studies. Group 1 chemicals including aflatoxin $B_1$ and 4-aminobiphenyl and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (nicotine-derived nitrosaminoketone (NNK)) are all potent mutagens and carcinogens.

To identify mutagenic and carcinogenic chemicals in the environment and effectively evaluate the health effects, international organizations have set up guidelines of genotoxicity assays. The examples are guidelines by International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) for

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The contents of this article reflect solely the view of the author(s).
**Abbreviations:** 6-TG, 6-thioguanine; CA, chromosome aberration assay; CAT, chloramphenicol acetyltransferase; DMH, 1,2-dimethylhydrazine; DSS, dextran sodium sulfate; F344, Fischer 344; Hprt, hypoxanthine phosphoribosyltransferase; ICH, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; MLA, mouse lymphoma gene mutation assay; MMC, mitomycin C; MN, micronucleus assay; NNK, nicotine-derived nitrosaminoketone; OECD, Organization for Economic Co-operation and Development; P-gal, phenyl-β-D-galactoside; SD, Sprague Dawley

**Table 1.** Remodeling of the standard battery of genotoxicity assays

| Classical battery | Alternative battery |
|---|---|
| 1. Ames test | 1. Ames test |
| 2. CA, MN or MLA *in vitro* | |
| 3. MN or CA *in vivo* | 2. MN *in vivo* |
| | 3. The second *in vivo* test |

These batteries are recommended by ICH as guideline S2(R1)[8], where the classical battery and the alternative battery are called option 1 and 2, respectively. In the alternative battery, it is recommended that two different tissues are examined with two *in vivo* assays, such as bone marrow by micronucleus test (MN) and liver by the second *in vivo* test. Chromosome aberration test (CA) *in vivo* is not recommended in the alternative battery because CA does not detect aneuploidies. In the classical battery, MN *in vitro* can detect aneuploidies and thus CA *in vivo* is included in the battery.

pharmaceuticals[5] and those by the Organization for Economic Co-operation and Development (OECD) for industrial chemicals[6]. In general, (1) a bacterial reverse mutation assay (Ames test), (2) a chromosome aberration assay (CA), a micronucleus assay (MN) or a mouse lymphoma gene mutation assay *in vitro* (MLA) and (3) an MN assay *in vivo* constitute a standard battery for genotoxicity assays (**Table 1**). However, it has been reported that CA, MN and MLA *in vitro* have many false positives, ie, positive in the assays but negative in rodent two-year cancer bioassays[7]. In other words, these assays have low specificity to predict rodent carcinogens. Therefore, an alternative battery that skips CA, MN and MLA *in vitro* but includes a second *in vivo* assay has been proposed by ICH[8]. Transgenic rodent gene mutation assays, which I will describe below, are one of the candidates for the second *in vivo* assay. The assays detect heritable genetic changes, ie, gene mutations, and thus are suitable to examine the results of Ames test, which also detects gene mutations.

## 2. Gene Mutation Assays *in Vivo*

Before establishment of transgenic rodent gene mutation assays in 1980s and 1990s, *in vivo* mutation assays were limited to particular tissues such as melanoblasts or spleen lymphocytes. An example of the classical *in vivo* gene mutation assays is “mouse spot test” where developing embryo are exposed to test chemicals[9]. If mutations are induced in the genes that control the pigmentation of coat color of mice, the offspring will have spots of changed color in the coat. The frequency of such spots in the treated group is compared with that of spots in the control group. Although this assay surely detects various gene mutations *in vivo*, the target cells are restricted to melanoblasts in embryo. The assay was deleted from OECD test guidelines in 2014, because few people used it for evaluation of genotoxicity of chemicals. Another example is “mouse spleen lymphocytes assay”, where mice are treated with chemicals and then the spleen lymphocytes are cultured *in vitro* to select 6-thioguanine (6-TG) mutants[10]. If mutations occur in the hypoxanthine phosphoribosyltransferase (*Hprt*) locus in the spleen, the mutated lymphocytes should be resistant to 6-TG *in vitro*. Although this assay allows detection of mutations in adult mice, the target organ is only spleen and the mutant selection *in vitro* takes about 10 days. No molecular analyses of the 6-TG mutations are allowed. Thus, the assay is not popular in the field of genotoxicology. The transgenic rodent gene mutation assays are superior to the classical *in vivo* assays in that they allow detection of mutations in any organ of rodents including germ cells and molecular analyses of the mutants by DNA sequencing[11].

Key technological innovation for the transgenic rodent gene mutation assays is rescue of lambda phage vectors from mammalian chromosomes by *in vitro* packaging reactions. Mutation is a very rare event, ie, $1 \times 10^{-5}$ to $10^{-6}$ or 0.001 to 0.0001%. Thus, reporter genes for mutations should be rescued from mammalian chromosomes with high yield and high fidelity. The innovative idea to rescue lambda phage DNA carrying reporter genes for mutations by *in vitro* packaging reactions was first reported by Glazer et al[12]. They introduced multiple copies of a lambda phage vector containing *supF* in *Escherichia coli*, a reporter gene for mutations, into the chromosomes of mouse L-cells and irradiated the cells with ultraviolet light (UV). Then they rescued the phage with *in vitro* packaging extracts and introduced the rescued phages to *E. coli* host cells to identify mutant phages. This strategy worked well, and they identified a characteristic mutation of C to T in the reporter gene after recovery from mammalian cells irradiated with UV. The *in vitro* packaging extracts are extracts of *E. coli* having defective lambda phage, which enable excision of lambda DNA from the mammalian chromosome and promote packaging of the excised DNA into phage particles[13].

The idea to rescue the lambda vector DNA carrying reporter genes for mutations from mammalian cells by the *in vitro* packaging reactions was applied to rescue the reporter genes from transgenic mice. Two prototypes of transgenic mice for mutation assays were established in late 1980s. Muta™ Mouse has a lambda vector carrying *lacZ*[14] and Big Blue® Mouse has a lambda vector with *lacI*[15]. The *lacZ* and *lacI* are genes of *lac* operon of *E. coli*, where *lacI* encodes the repressor protein LacI, and *lacZ* encodes β-galactosidase, whose expression is repressed by LacI. Initially, the Muta™ Mouse assay was very time consuming because one had to find a very small number of colorless mutant plaques among millions of blue plaques. The *lacZ* mutants are colorless because they cannot hydrolyze X-gal, which is a substrate of β-galactosidase and produces blue color after the hydrolysis, while the wild-type phage produces blue plaques because of the intact enzyme activity. The mutant selection in Big Blue® Mouse was better than that of Muta™ Mouse because the mutant plaques were blue and the wild-type plaques were colorless. The intact *lacI* gene produces a repressor protein LacI, which shuts down the expression of β-galactosidase, resulting in colorless plaques. On the contrary, the *lacI* mutants allow the expression of β-galactosidase, which results in blue plaques. Nevertheless, both selections were time-consuming and expensive because X-gal is an expensive agent. To circumvent the problem, a positive selection for *lacZ* mutations was introduced. *E. coli* mutants deficient in *galE* are sensitive to galactose because they accumulate a toxic intermediate, ie, galactose-6-phosphate, generated from galactose[11]. The *galE* gene encodes galactose epimerase, which converts the toxic galactose-6-phosphate to non-toxic glucose-6-phosphate. This fact was employed for the development of the positive selection. *E. coli galE* cells infected with lambda phage having wild-type *lacZ* metabolize phenyl-β-D-galactoside (P-gal) into the toxic intermediate and result in cell death with no phage propagation. Only *lacZ* mutant phage can form plaques in the lawn of *E. coli galE* cells in the presence of P-gal, which is converted to galactose by the action of β-galactosidase. This positive selection substantially enhanced the performance of *lacZ* mutation assay and reduced the cost. Nevertheless, the coding size of *lacZ* is about 3 kilo base pairs (kb), which is not short enough for routine DNA sequencing for identification of mutations. In 1996, the *cII* gene of lambda phage was introduced as a novel reporter gene for mutations[16]. The *cII* gene encodes a repressor protein involved in lambda lysogenic/lytic cycle. In the *hfl*$^-$ *E. coli*, phages with active *cII* gene cannot enter a lytic cycle and form no plaques because of the deficit of *Hfl* protease. This protease normally degrades CII protein and lets the phage enter a lytic cycle. The only phages with inactive *cII* mutants can make plaques with the *E. coli hfl*$^-$ cells, which lack Hfl protease that degrades CII protein. Thus, this is a positive selection. The coding size of *cII* is about 300 base pairs (bp), which is about 1/10 of *lacZ* and 1/3 of *lacI*. In addition, the *cII* selection is applicable to both Big Blue® Mouse and Muta™ Mouse. Therefore, the *cII* selection is much more frequently used for Big Blue® and Muta™ Mouse assays than the original *lacI* or *lacZ* selections.

## 3. Establishment of *Gpt* Delta Mice

Despite the advancement of the positive selections, both assays with Muta™ Mouse and Big Blue® Mouse were insensitive to deletions, which are induced by radiation or chemical treatments with cross-linking agents such as mitomycin C (MMC)[17,18]. This insensitivity may be due to the high spontaneous background levels of *lacZ, lacI* and *cII*, ie, in the mid-$10^{-5}$. The spontaneous mutations are mainly due to deamination of 5-methylcytosine to thymine in the CpG sites, ie, C to T transitions, since bacterial transgenes are highly methylated in mammalian cells[19]. Because of the high levels of spontaneous base substitutions, rare mutations such as deletions are overlooked by the selections. For example, when the level of base substitutions is $5 \times 10^{-5}$ and deletion is $5 \times 10^{-7}$, small number of deletion mutations will be hard to be identified. To effectively detect deletion mutations, I decided to use Spi$^-$ selection when I started to develop *gpt* delta transgenic mouse assay[20–22] (**Fig. 1**). Spi$^-$ stands for *S*ensitive to *P*2 *I*nterference. Wild-type lambda phages can lyse *E. coli* and make phage plaques. However, if P2 phage is already in the chromosome of *E. coli* (P2 lysogen), the wild-type lambda phage cannot lyse P2 lysogen. This phenomenon is called P2 interference. Interestingly, defective lambda phages, which lack the functions of the *red* and *gam* genes, can lyse P2 lysogen, thereby making phage plaques. These plaques are called Spi$^-$ plaques. The *gam* gene product inactivates exonuclease V, which is encoded by *recBCD* genes in *E. coli*. In P2 lysogen, there is a gene *old*, which kills the host *E. coli* when the exonuclease V is inactivated. When P2 lysogen is infected with wild-type lambda phage, the gene product of *old* kills the host cells, thereby preventing the propagation of incoming lambda phage. In the absence of the *red* and *gam* genes, the defective phage has a chance to propagate in P2 lysogen because the exonuclease V is not inactivated. However, the incoming lambda phage must have a Chi sequence (GCTGGTGG) to prevent the DNA from digestion by the exonuclease V. The *chi* sequence inactivates the *recD* gene product, thereby inhibiting the exonuclease. The *red* gene is composed of *redA* and *redB*. They are involved in recombination that resolve the replication form of phage DNA. The Spi$^-$ phage that are *red*$^-$ and *gam*$^-$ must be

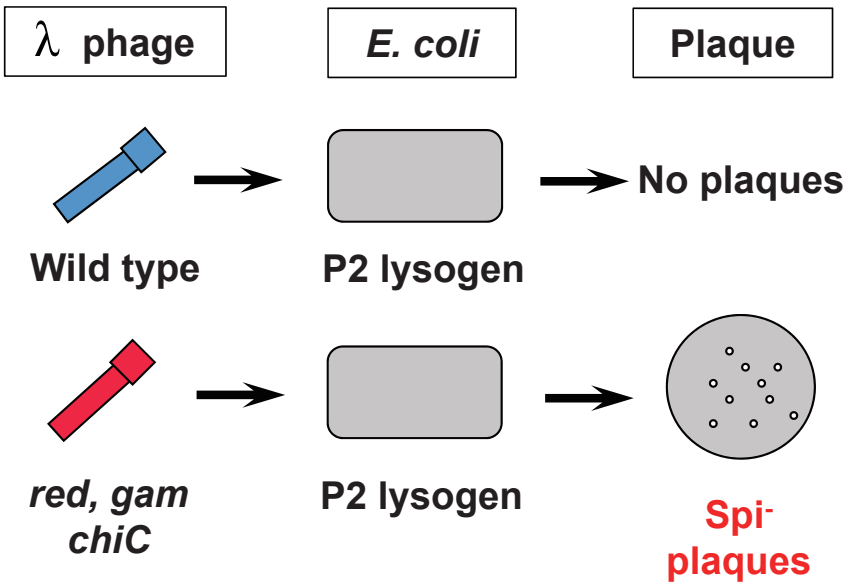


**Fig. 1.** Principle of Spi⁻ selection.
This selection takes advantage of the fact that propagation of wild-type lambda phage is restricted in P2 lysogen, which is *E. coli* having P2 prophage. Defective lambda phage deficient in the functions of both the *gam* and *red* genes can propagate well in P2 lysogen and display Spi⁻ plaques as long as they carry the *chiC* mutation and the host strain is *recA*⁺.

propagated in *recA*⁺ *E. coli* to allow efficient packaging DNA. No satisfactory explanation of Red in Spi⁻ selection has been offered.

Since the *red* and *gam* genes are located side-by-side in the lambda DNA and inactivation of two genes by two independent point mutations is very rare, the inactivation of both *red* and *gam* genes is most likely induced by deletions in the region. The *chiC* sequence makes the Spi⁻ plaques bigger and visible. Spi⁻ selection is unique in that it preferentially and positively selects deletion mutations. As expected, deletion mutations and complex gene rearrangements were successfully identified by Spi⁻ selection when *gpt* delta mice were irradiated with heavy ion or γ-ray, or treated with MMC[23,24]. Molecular analyses of the Spi⁻ mutants revealed the characteristics of junctions of the deletion mutants, ie, some deletion mutants have short overlapping sequences, ie, 1–8 bp, which is sometimes called microhomology, while others have no such sequences. Because of the size limitation of *in vitro* packaging, the size of deletions detectable by Spi⁻ selection is less than 10 kb. Lambda phage should have two *cos* sites for the packaging at the both ends and the sites should be separated by 38–51 kb in DNA. In other words, too small or too large lambda DNA cannot be packaged to the phage particles even when they have two *cos* sites at the end. Therefore, deletions detectable by Spi⁻ selection are mostly intragenic deletions. Nevertheless, *gpt* delta mice have tandem array of more than 50 copies of about 48 kb lambda EG10 in the chromosome 17[25]. Thus, they have potentially detect large deletions of approximately 2.5 mega bps (Mb).

In addition to deletion mutations, point mutations such as base substitutions and frameshift should have been detected by a positive selection with a reporter gene having a convenient size for DNA sequencing. To this end, I decided to use the *gpt* gene of *E. coli* as the reporter[11,20,23]. The gene encodes guanine phosphoribosyltransferase, which is a bacterial counterpart of mouse *Hprt*. The coding size is 456 bp, which is appropriate for identification of mutations by DNA sequencing. The *gpt* mutants of *E. coli* can be positively selected with medium containing 6-TG because the wild-type enzyme metabolizes 6-TG to a toxic substance and kills the host bacterial cells. However, the *gpt* selection is effective only when the gene is on a multi-copy-number plasmid. Thus, the lambda phage vector carrying the *gpt* gene should have been converted into plasmid DNA when the phage was introduced into host bacterial cells. To solve this problem, I took an advantage of the *cre-lox* system. The *cre* gene encodes a site-specific recombinase that excises the *loxP* sites. If the target DNA sequence is flanked by two *loxP* sites, the target sequence will be excised and circulated carrying the target sequence by Cre recombinase. We made a novel lambda vector EG10 carrying a linearized plasmid DNA where the *gpt* gene, the chloramphenicol acetyltransferase (CAT) gene and a ColE1 replication origin are flanked by two direct repeat sequences of *loxP* (**Fig. 2**). This lambda vector also carries the *red/gam* genes and the *chiC* sequence necessary for Spi⁻ selection. We also engineered the host *E. coli* strain YG6020, which originally lacks the *gpt* gene in the chromosome but has the *cre* gene[20]. The strain also lacks *recA* to suppress *ex-vivo* mutations after introduction of the reporter gene in the host *E. coli* cells. The "*ex-vivo* mutations" are mutations that occur in *E. coli* but not in mice or rats. They occur

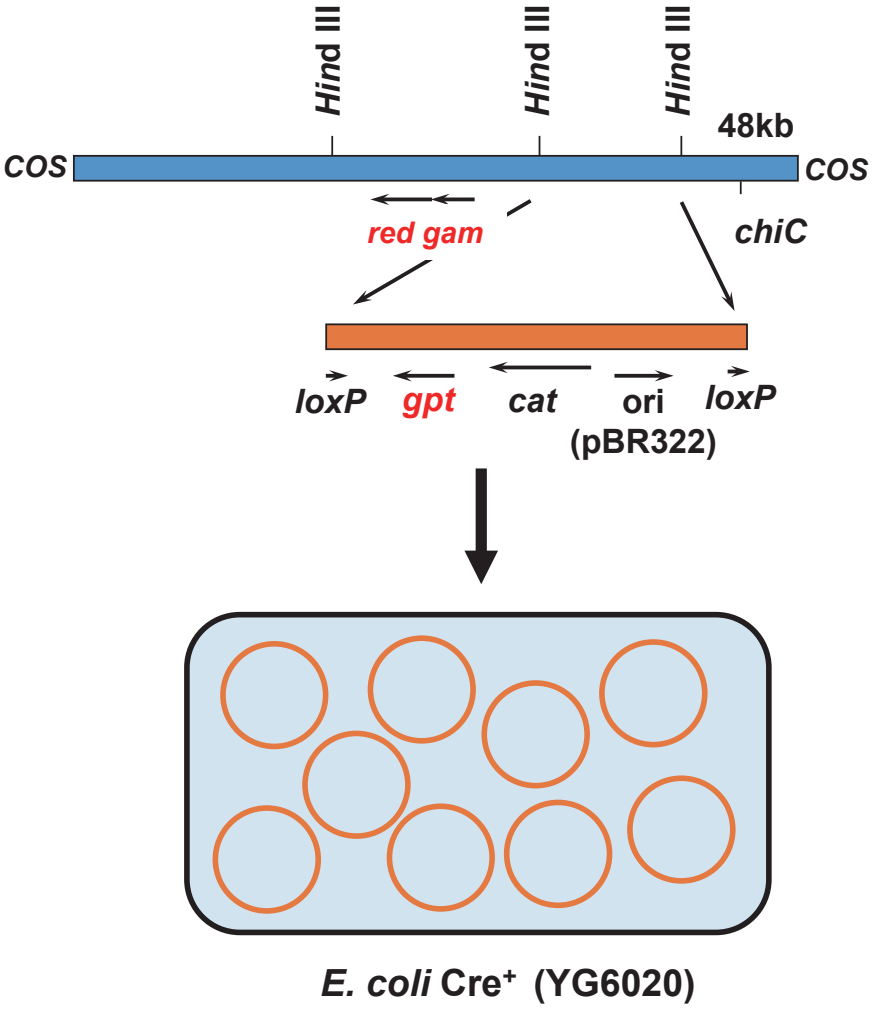


**Fig. 2.** Structure of lambda EG10 and its conversion to plasmid pYG142 in *E. coli* YG6020 Cre$^{+}$.
The lambda EG10 is about 48 kb, which is composed of lambda 2001 and the linearized plasmid pYG144. The linearized plasmid region of the vector has been expanded for detail. The vector carries the *red* and *gam* regions and a *chiC* mutation involved in Spi$^{-}$ selection. The linearized plasmid region contains a ColE1 replication origin (ori), a chloramphenicol resistance gene (*cat*), the *gpt* gene of *E. coli* (*gpt*), and two direct-repeat sequences of *loxP*, which are recognition sequences of Cre recombinase. The linearized plasmid region can be excised and converted to the multi-copy-number plasmid pYG142 carrying a single *loxP* sequence when the lambda EG10 is introduced to *E. coli* YG6020 expressing Cre recombinase.

when lambda DNA having DNA lesions such as DNA adducts are propagated in *E. coli*. If the *recA* gene is activated, such unwanted "*ex-vivo*" mutations may occur. Therefore, the host *E. coli* must be *recA*$^{-}$ in the *gpt* selection. In the assay, rescued lambda EG10 phages are introduced into distinct *E. coli* host strains for Spi$^{-}$ and *gpt* selections (**Fig. 3**). In Spi$^{-}$ selection, the rescued phages are infected into *E. coli* strain XL1-Blue MRA (P2), which is a P2 lysogen, to select Spi$^{-}$ mutants. In *gpt* selection, the rescued phages are infected to strain YG6020, where the lambda phage is converted to a multi-copy-number plasmid carrying the *gpt* gene plus the drug resistance gene, and the colonies harboring plasmid carrying the mutated *gpt* genes are selected with plates containing 6-TG and chloramphenicol. Transgenic mice were established by microinjection of lambda EG10 DNA into fertilized eggs of BDF1 or C57BL/6J mice[20]. We made more than 30 transgenic lines and selected one that was originated from C57BL/6J mice, ie, #30. The mice exhibited very high rescue efficiency and low spontaneous mutation frequencies of *gpt* and Spi$^{-}$. Because the reporter gene for point mutations is *gpt* and deletions can be detected by Spi$^{-}$ selection, we named the transgenic mice "*gpt* delta".

So far, *gpt* delta mice have been utilized mainly in two research areas. One is radiation biology and another is chemical safety evaluation[23]. In the field of radiation biology, *gpt* delta mice were irradiated and Spi$^{-}$ and *gpt* mutations were analyzed. Interestingly, the spectra of mutations induced by irradiation are varied depending on the radiation species[26]. For example, Spi$^{-}$ mutation frequency was higher for carbon particle irradiation than γ-ray irradiation while

*gpt* mutation frequency was higher for γ-ray than carbon particle. DNA sequence analyses revealed that carbon particle irradiation induces deletions with the size more than 1 kb, whereas gamma-ray irradiation induces smaller sized deletions and base substitutions. The results suggest that heavy-ion beam irradiation is effective at inducing deletions via double-strand breaks in DNA but ineffective at producing oxidative DNA damage by free radicals. In addition, *gpt* delta mice were used to study combined effects of radiation and chemical exposure, eg, low dose γ-ray irradiation plus NNK exposure[27], and X-ray irradiation plus *N*-ethyl-*N*-nitrosourea (ENU) exposure[28].

In the field of chemical safety evaluation, the mice are exposed to chemical carcinogens and the *in vivo* mutations are analyzed to examine whether genotoxicity is involved in the mechanisms of carcinogenesis. One interesting example is dicyclanil, a pyrimidine-derived insect growth regulator[29]. This chemical is all negative in various genotoxicity assays including Ames test while it is a hepatocarcinogen in female mice. The mutagenicity of dicyclanil was examined in the liver of male and female *gpt* delta mice by Umemura et al and it was found that the chemical induces *gpt* mutations, ie, mainly G:C to T:A, in female mice but not in male. 8-Oxo-dexoxyguanosine, an oxidative lesion in DNA, was induced in both genders, and cell proliferation and increases in liver weights were enhanced only in female. It was concluded that the female specific cell proliferation plus oxidative damage in DNA induces gender-specific *gpt* mutations in the liver. The results caution that decision whether the carcinogen is genotoxic or not should be made based on the results of *in vivo* mutagenicity assays in the target organ of carcinogenesis in rodents.

## 4. Establishment of *Gpt* Delta Rats

Although mice are predominantly used as a tester rodent in genotoxicology assays, rats are routinely used for toxicology including two-year cancer bioassays. In addition, there are a number of species-specific carcinogens, which are carcinogenic in rats but not in mice and *vice versa*[30]. An example is aflatoxin $B_1$, a mycotoxin, whose carcinogenic potential varies among species[31]. Aflatoxin $B_1$ is carcinogenic in the liver of humans and rats but mice are known to be relatively resistant. The species difference may be caused by the different metabolic activation and detoxication capacities in the species. Therefore, it was expected that transgenic rats for gene mutation assays *in vivo* should have been established. In fact, Big Blue® Rat had been established[32,33]. In 2003, lambda EG10 DNA was introduced into fertilized eggs of Sprague Dawley (SD) rats, thereby establishing *gpt* delta rats[34]. The DNA was introduced in the chromosome 4 with 5 to 10 copies per haploid. An attempt to make homozygous rats having lambda EG10 in both chromosomes was failed because the teeth development of the homozygous rats was retarded. The rats could not take the diet and survive. The established hemizygous *gpt* delta rats exhibited a reasonable sensitivity to mutagenicity of benzo[*a*]pyrene in the liver. The *gpt* and $Spi^-$ mutant frequencies of liver of rats treated with benzo[*a*]pyrene were increased in a dose dependent manner. Later, SD *gpt* delta rats were used to examine the relationship between mutagenicity and carcinogenicity of phenacetin, an analgesic drug, in long-term repeated dose studies[35]. The rats were fed diet containing 0.5% phenacetin for 26 or 52 weeks and the mutations were examined in the liver, a non-target organ of carcinogenesis, and the kidney, the target organ. The *gpt* and $Spi^-$ mutant frequencies were enhanced by the administration and the frequencies were higher in 52 weeks than in 26 weeks. Interestingly, both *gpt* and $Spi^-$ mutant frequencies were much higher in the liver than in the kidney. The results suggest that the intensity of genotoxicity does not correlate with the induction of tumor formation, and also that an organ where the highest mutation frequency was observed is not necessarily a target organ for carcinogenesis.

Since Fischer 344 (F344) rats are more frequently used for two-year cancer bioassays than SD rats, SD *gpt* delta rats were backcrossed with F344 rats for 15 generations, thereby establishing F344 *gpt* delta rats[36]. 2,4-Diaminotoluene, a liver carcinogen, and the non-carcinogenic structural isomer, 2,6-diaminotoluene, were administered in F344 *gpt* delta rats for 13 weeks. These chemicals were both mutagenic in Ames *Salmonella* strains in the presence of S9 activation. 2,4-Diaminotoluene enhanced *gpt* and $Spi^-$ mutant frequencies in the liver while 2,6-diaminotoluene did not. The results strongly support the notion that Ames positive chemicals are not necessarily positive *in vivo*. The non-carcinogenic 2,6-diaminotoluene was mutagenic *in vitro* because detoxication metabolism may be ineffective in the S9 activation system. F344 *gpt* delta rats were used for chemoprevention studies, too[37]. Male *gpt* delta rats were given a single injection of 1,2-dimethylhydrazine (DMH) and followed by dextran sodium sulfate (DSS) in drinking water for a week. They were fed diets containing silymarin, a natural flavonoid from the seeds of milk thistle, for 4 weeks before the DMH injection and samples were collected at 32 weeks after the DMH treatment. Silymarin suppressed the tumor formation in the colon in a dose dependent manner. In the mutation assays, DMH plus DSS enhanced the *gpt* mutant frequency in the colon, and the silymarin treatments reduced the mutant frequencies significantly. The results suggest that F344 *gpt* delta

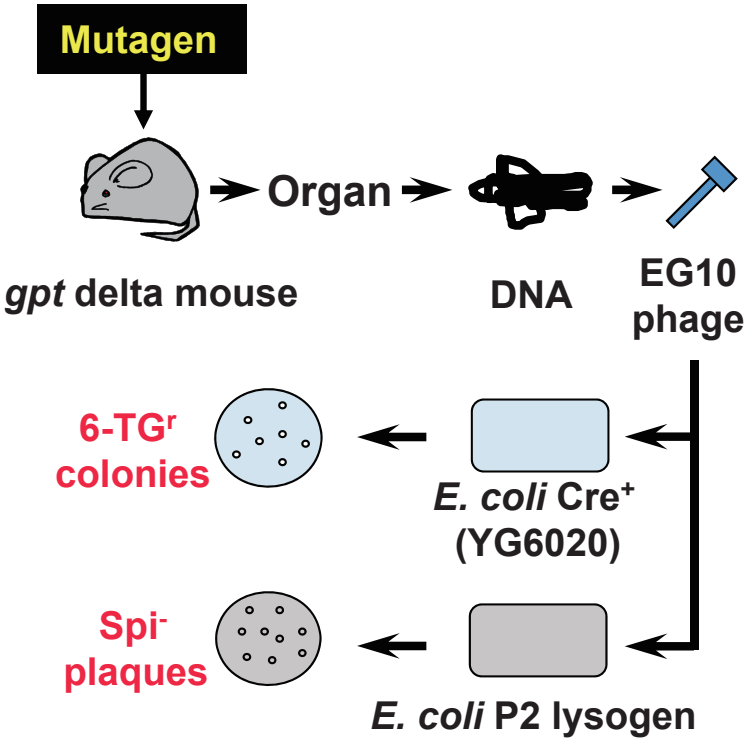


**Fig. 3.** Protocols of *gpt* delta transgenic mouse gene mutation assay.
Two *E. coli* host cells are infected with the rescued lambda EG10 phages. One is *E. coli* YG6020 expressing Cre recombinase for 6-TG selection and the other is P2 lysogen for Spi⁻ selection. In *E. coli* YG6020, lambda EG10 is converted to the multi-copy-number plasmid pYG142 carrying *gpt* and CAT. *E. coli* cells harboring plasmid carrying mutant *gpt* can form colonies on the plates containing chloramphenicol and 6-TG (*gpt* selection). Mutant lambda phage lacking the functions of *red* and *gam* can form Spi⁻ plaques on plates containing P2 lysogen (Spi⁻ selection).

rats allow direct comparison of tumor formation and mutation induction in identical organs in identical rats. This characteristics are useful for making decisions whether the carcinogen is genotoxic or non-genotoxic. The decision is important for food safety because no acceptable daily intake (ADI) can be set for the chemical that induces mutations *in vivo*.

## 5. *Gpt* Delta Rodent Gene Mutations Assays in Food Safety Research

So far, about 20 chemicals have been examined with *gpt* delta mice or rats for food safety (**Table 2**). The chemicals include mycotoxins, food additives, heterocyclic amines and other compounds generated in cooking processes and others. Almost all the chemicals examined are rodent carcinogens. Therefore, the purpose of the assays appears to examine whether the genotoxicity is involved in the carcinogenesis. The experimental conditions were designed to mimic longer-term cancer bioassays. The administration period was 8 weeks, 13 weeks and in one case 78 weeks, which are longer than the standard administration period of 4 weeks[38]. In some cases, C57BL/6J *gpt* delta mice were crossed with C3H/He mice, thereby generating B6C3F1 *gpt* delta mice, which are the same genetic background for two-year cancer bioassays. As a result, citrinin[39], flumequine[40], ginkgo biloba extract[41] and 3-monochloropropane-1,2-diol (3-MCPD) esters[42] were turned out to be negative in the target organ(s) of carcinogenesis in *gpt* delta transgenic assays, and hence they could be non-genotoxic carcinogens. In contrast, estragole[43], madder color[44] and methyleugenol[45] were positive in the transgenic assays, and therefore genotoxic mechanisms may participate in the carcinogenesis. It seems that *gpt* delta transgenic mice/rats gene mutation assays are effective to distinguish genotoxic and non-genotoxic carcinogens *in vivo*.

Among the chemicals examined, ochratoxin A, a mycotoxin that induces renal tumors in male rats, is interesting because it induces Spi⁻ mutations but not *gpt*[46,47]. At the target site of carcinogenesis, ie, outer medulla of kidney, Spi⁻ mutant frequency was statistically increased when male *gpt* delta rats were treated with ochratoxin A. Large deletion with the size of more than 1 kb was induced by the treatment. However, *gpt* mutation frequency was not increased by the treatment. Further examination with p53 deficient *gpt* delta mice indicates that Spi⁻ mutant frequency, but not *gpt,* was

**Table 2.** Application of *gpt* delta rodent gene mutation assays in food safety research

| Chemical | Species | Organ | Mutagenicity | Carcinogenicity | Administration and Dose | Note | Reference |
|---|---|---|---|---|---|---|---|
| ABAQ*1) | Male mice | Liver, kidney | + (liver) – (kidney) | | Gavage (25, 50 mg/kg/week) for 3 weeks | A product of Maillard reaction between glucose and L-tryptophan | 63 |
| Acrylamide | Mice | Lung | + | + (multiple organs in rats and lung in mice) | Drinking water (100, 200, 400 ppm) for 4 weeks | A product formed in many heat-processed foods | 64 |
| | 3 week- and 11 week-old male rats | Liver, testis | + (testis in young rats) – (liver in young and adult rats) | | Drinking water (20, 40, 80 ppm) for 4 weeks | | 65 |
| Aflatoxin B1 | Male and female B6C3F1 new born mice | Liver | + Infant male and female mice exhibit similar *gpt* mutation frequencies | + (liver) | Intraperitoneal injection on postnatal either day 4 with a single dose of 6 mg/kg or days 4, 7, and 10 with 2 mg/kg (3 × 2 mg/kg) | A mycotoxin that induces liver cancer in humans | 66 |
| APNH*2) | Male mice | Liver, colon | + | + (liver and colon in rats) | Diet (10, 20 ppm) for 12 weeks | A product formed from nonmutagenic norharman and aniline | 67 |
| Arecoline hydrobromide | Male mice | Oral, liver | ± (oral tissues) - (liver) | + (oral tissue) | Drinking water (300, 700 ppm) for 6 weeks | A major alkaloid in areca nut, chewing of which is carcinogenic to humans | 68 |
| Aristolochic acid I and II | Male mice | Kidney | + Aristolochic acid II is more mutagenic than I | + | Gavage (5 mg/kg) for 6 weeks | Products of Aristolochia plant species. They are associated with aristolochic acid nephropathy and Balkan endemic nephropathy. | 69 |
| Citrinin | Male rats | Kidney | - | + (kidney in rats) | Gavage (20, 40 mg/kg/day) for 4 weeks | A food-contaminating mycotoxin | 39 |
| Dicyclanil | Male and female B6C3F1 mice | Liver | + (liver in female mice) | + (liver in female mice) | Diet (0.15%) for 13 weeks | An insect growth regulator | 29 |
| Estragole | Male rats | Liver | + | + (liver in mice) | Gavage (22, 66, 200, 600 mg/kg) for 4 weeks | A natural organic compound used as a food additive | 43 |
| Flumequine | Male and female B6C3F1 mice | Liver | - | + (liver in mice) | Diet (0.4%) for 13 weeks | An anti-bacterial quinolone agent | 40 |
| Ginkgo biloba | Male B6C3F1 mice | Liver | - | + (liver in mice) | Gavage (20, 200, 2000 mg/kg) for 13 weeks | A herbal supplement | 41 |

**Table 2.** Application of *gpt* delta rodent gene mutation assays in food safety research (continued)

| Chemical | Species | Organ | Mutagenicity | Carcinogenicity | Administration and Dose | Note | Reference |
|---|---|---|---|---|---|---|---|
| High fat diet | Male and female mice | liver, kidney, colon | - | | High fat diet for 13 or 26 weeks | | 70 |
| Madder color and lucidin-3-*O*-primeveroside (LuP) | Male rats | Kidney | + | + (for madder color liver and kidney in rats) | Diet (5.0% w/w for madder color or 0.3% w/w for LuP) for 8 weeks | Madder color is a food additive. LuP is a constituent of madder color. | 44 |
| Methyleugenol | Male and female rats | Liver | + | + (liver in rats and mice) | Gavage (10, 30 100 mg/kg) for 13 weeks | A fragrance and flavoring agent | 45 |
| MeIQx[*3)] | Male mice | Liver, colon | + | + (liver and other organs in rats and mice) | Diet (3, 30, 300 ppm) for 12 weeks | A heterocyclic amine present in cooked foods | 71 |
| MX[*4)] | Male and female mice | Liver, lung | - | + (rats) | Drinking water (10, 30, 100 ppm) for 12 weeks, and 100 ppm for 78 weeks | A by-product of water chlorination | 72 |
| 3-MCPD[*5)] | Male rats | Kidney, testis | - | + (kidney and testis of rats) | Gavage (40 mg/kg and the equimolar esters) for 4 weeks | 3-MCPD is a food processing contaminant. The esters are generated in foods. | 42 |
| Ochratoxin A | Male and female rats | Kidney (outer medulla) | + (outer medulla of kidney) Only Spi$^-$ mutant frequency was increased. | + (outer zone of renal medulla in rodents, mainly in male rats) | Diet (5 ppm) for 4 or 13 weeks | A mycotoxin that might be associated with Balkan endemic nephropathy and urinary tract tumors in humans. | 46 |
| | Male rats | | | | Diet (5 ppm) for 4 weeks | | 47 |
| | p53 Proficient and deficient male mice | Kidney | + Spi$^-$ mutant frequency was increased only in p53 deficient mice. | | Gavage (1, 5 mg/kg) for 4 weeks | | 48 |
| | | | | | Gavage (5 mg/kg) for 4 weeks | | 49 |
| PhIP[*6)] | Male and female mice | Colon, spleen, liver, brain, bone marrow, testis | + (colon, spleen, liver) − (testis, brain, bone marrow) No gender difference in mutagenicity | + (colon and prostate of male rats and mammary glands in female rats) | Diet (400 ppm) for 13 weeks | A heterocyclic amine in cooked food. | 73 |
| Potassium bromate | Male and female rats | Kidney | + | + (kidney of male and female rats) | Drinking water (500 ppm) for 9 weeks | A food additive as a flour improvement for bread making | 74 |

**Table 2.** Application of *gpt* delta rodent gene mutation assays in food safety research (continued)

| Chemical | Species | Organ | Mutagenicity | Carcinogenicity | Administration and Dose | Note | Reference |
|---|---|---|---|---|---|---|---|
| Safrole*7) | Male and female rats | Liver | + | + (liver of rats and mice) | Diet (0.1, 0.5%) for 13 weeks | A natural plant constituent in the essential oils of sassafras, sweet basil, cinnamon and spices | 75 |

Rats are F344 *gpt* delta rats and mice are C57BL/6J *gpt* delta mice unless otherwise indicated.

*1 ABAQ: 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one

*2 APNH: aminophenylnorharman

*3 MeIQx: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline

*4 MX: 3-chloro-4-(dichloromethyl)-5-hydroxy-2(*5H*)-furanone

*5 3-MCPD: 3-monochloropropane-1,2-diol

*6 PhIP: 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine

*7 Safrole: 4-allyl-1,2-methylenedioxybenzene

increased in p53-deficient mice by ochratoxin A[48,49]. No increases in Spi$^-$ and *gpt* mutant frequencies were observed in p53-proficient mice. It seems that ochratoxin A induces double-strand breaks in DNA in the target site of kidney of rats, which resulted in large deletions. The double-strand breaks in DNA may be suppressed by p53. It is puzzling, however, why *gpt* mutant frequency was not increased. It is not uncommon that *gpt* assay and Spi$^-$assay behave differently. However, in most cases, *gpt* mutant frequency was increased more effectively than Spi$^-$ mutant frequency. Only exception was heavy ion irradiation where Spi$^-$ mutant frequency was enhanced without increasing *gpt* mutant frequencies[26]. If ochratoxin A induces mutagenic DNA adducts in the target site, they will induce base substitutions (*gpt*) as well as deletions (Spi$^-$). It is tempting to speculate, therefore, that ochratoxin A may interact with proteins involved in DNA replication, repair or chromosome segregation, thereby inducing double-strand breaks in DNA, which resulted in deletions without inducing point mutations. Alternatively, ochratoxin A may induce DNA adducts, but the DNA adducts are not bypassed by any DNA polymerases in outer medulla of kidney. If so, DNA replication will be completely inhibited and DNA strand breaks will be induced without induction of point mutations. Further molecular analyses may solve the interesting puzzling problem.

## 6. Future Perspectives

A current trend in toxicology is 3R, ie, Replacement, Reduction and Refinement, for research and testing with animals. In ICH guideline, it is recommended to integrate *in vivo* genotoxicity assays into repeated-dose toxicology studies[8]. As described above, many *gpt* delta gene mutation assays, in particular with rats, are already integrated into repeated-dose studies with longer period of administration. The integration enables the direct examination of participation of genotoxicity in carcinogenesis in the target organ(s) of rodents. Recently, a medium-term assay with *gpt* delta rats was proposed for rapid identification of renal carcinogens[50]. In *gpt* delta mice, several knockout or knockin alleles were introduced to clarify the mode of action of test chemicals. Examples include *p53*[48,51], *Atm*[52], *Parp-1*[53], *Ogg1*[54], *Nrf2*[55], *Polk*[56], *IL-10*[57] and P450 reductase-null alleles[58]. Mechanistic understanding of carcinogenesis may be more facilitated when knockout or knockin technologies are introduced into rats. In this regard, CRISPR/Cas9 genome editing system may play an important role in the future[59].

Rapid advancing DNA sequencing technology is a novel approach to analyze mutations. Single cell whole-genome DNA sequencing has a potential to identify mutations in normal somatic cells[60]. In bacteria, furylfuramide-induced mutations in *Salmonella typhimurium* T100 are directly identified by a high-throughput DNA sequencing analysis[61]. Mutations in many types of cancer cells are identified by DNA sequencing and the characteristic mutations are reported[62]. Transition mutations of C to T at CpG site by deamination of 5-methylcytosine, another transition of C to T by UV irradiation and transversion mutations of G to T by smoking are observed in cancer cells. Therefore, mutation spectra associated with specific exposure to chemicals determined by the transgenic assays may help reveal the causes of

human cancer. Although the high throughput DNA sequencing technologies are still under development and need more sophistication to reduce the error rates, we should apply new technologies as much as possible to evaluate the potential genotoxic and carcinogenic risk of chemicals to humans.

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