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Integration of *In Vivo* Genotoxicity and Short-term Carcinogenicity Assays Using F344 *gpt* Delta Transgenic Rats: *In Vivo* Mutagenicity of 2,4-Diaminotoluene and 2,6-Diaminotoluene Structural Isomers

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An important trend in current toxicology is the replacement, reduction, and refinement of the use of experimental animals (the 3R principle). We propose a model in which *in vivo* genotoxicity and short-term carcinogenicity assays are integrated with F344 *gpt* delta transgenic rats. Using this model, the genotoxicity of chemicals can be identified in target organs using a shuttle vector λ EG10 that carries reporter genes for mutations; short-term carcinogenicity is determined by the formation of glutathione *S*-transferase placenta form (GST-P) foci in the liver. To begin validating this system, we examined the genotoxicity and hepatotoxicity of structural isomers of 2,4-diaminotoluene (2,4-DAT) and 2,6-diaminotoluene (2,6-DAT). Although both compounds are genotoxic in the Ames/*Salmonella* assay, only 2,4-DAT induces tumors in rat livers. Male F344 *gpt* delta rats were fed diet containing 2,4-DAT at doses of 125, 250, or 500 ppm for 13 weeks or 2,6-DAT at a dose of 500 ppm for the same period. The mutation frequencies of base substitutions, mainly at G:C base pairs, were significantly increased in the livers of 2,4-DAT–treated rats at all three doses. In contrast, virtually no induction of genotoxicity was identified in the kidneys of 2,4-DAT–treated rats or in the livers of 2,6-DAT–treated rats. GST-P–positive foci were detected in the livers of rats treated with 2,4-DAT at a dose of 500 ppm but not in those treated with 2,6-DAT. Integrated genotoxicity and short-term carcinogenicity assays may be useful for early identifying genotoxic and nongenotoxic carcinogens in a reduced number of experimental animals.

***Key Words:* *gpt* delta transgenic rat; diaminotoluenes; genotoxicity; carcinogenicity; 3R principle.**

Transgenic rodent models have advanced the field of *in vivo* genotoxicity studies (Nohmi and Masumura, 2005; Nohmi *et al.*, 2000). In these models, λ phage DNA carrying reporter genes for mutations are integrated into the chromosomes of transgenic rodents; the phage DNA is retrieved in phage particles by *in vitro* packaging reactions. The rescued phages are introduced into *Escherichia coli* cells, and mutants that were generated in the rodents are selected. With the shuttle vector system, one can examine the mutagenicity of chemicals in any rodent organ or tissues, including germ cells (Eastmond *et al.*, 2009; Hashimoto *et al.*, 2009). In addition, the mutants recovered from the rodents can be characterized by DNA sequencing (Heddle *et al.*, 2000). Transgenic genotoxicity assays are a reliable method for determining whether genotoxicity is involved in chemical carcinogenicity in the target organs of rodents (Thybaud *et al.*, 2003).

In 1996, we developed the novel transgenic mouse *gpt* delta for *in vivo* genotoxicity assays (Nohmi *et al.*, 1996). These mice have approximately 80 copies of λ EG10 DNA at a single site in chromosome 17 of C57 BL/6J mice (Masumura *et al.*, 1999). A feature of this transgenic mouse is that two mutant selections can be performed instead of just one, to identify a wider spectrum of *in vivo* mutations: *gpt* selection to identify point mutations such as base substitutions and frameshift mutations and Spi$^-$ selection to identify deletion mutations. Because of their sensitivity to deletion-type mutations, *gpt* delta mice have been utilized for radiation biology, cancer research, and regulatory toxicology (Aoki *et al.*, 2007; Masumura *et al.*, 2002; Shibata *et al.*, 2009; Xu *et al.*, 2007). In 2003, we established *gpt* delta rats in a Sprague-Dawley (SD) background by introducing λ EG10 DNA into fertilized SD rat eggs (Hayashi *et al.*, 2003). This *gpt* delta rat carries approximately five copies of λ EG10 DNA at a single site in chromosome 4 and is sensitive to induction of point mutations and deletions by benzo[*a*]pyrene and potassium bromate (Hayashi *et al.*, 2003; Umemura *et al.*, 2009).

Here, we report the establishment of *gpt* delta rat in a Fischer 344 background by backcross of SD *gpt* delta rats with F344 rats for 15 generations. We generated F344 *gpt* delta rats because this background is frequently used for 2-year cancer bioassays. In addition, glutathione *S*-transferase placenta form (GST-P)–positive preneoplastic hepatic foci can be analyzed in the rats (Ito *et al*., 2000). The results of bioassay using GST-P–positive foci show good correlation with those of 2-year cancer bioassay (Ito *et al*., 2000; Ogiso *et al*., 1985). Therefore, GST-P–positive foci formation assay was used as short-term carcinogenicity assay in this study. We hypothesized that we could integrate a genotoxicity assay with a short-term carcinogenicity assay utilizing GST-P foci in F344 *gpt* delta rats. This would reduce the number of animals required for both assays and would allow for examination of the relationship between genotoxicity and preneoplastic lesion formation within the same organs and tissues of chemically treated F344 *gpt* delta rats.

To begin validating this system, we examined the *in vivo* genotoxicity and hepatotoxicity of 2,4-diaminotoluene (2,4-DAT) and 2,6-diaminotoluene (2,6-DAT). The first chemical, 2,4-DAT, is used as an intermediate of the production of toluene diisocyanate, which is a monomer for the production of polyurethane, while 2,6-DAT is an intermediate of dyes, rubber chemicals, and various polymers (NTP 1979, 1980). Although both are genotoxic *in vitro* (Cunningham *et al*., 1989), only 2,4-DAT is carcinogenic in the livers of female mice and male and female rats (NTP, 1979). 2,4-DAT also induces lymphoma in female mice and mammary and subcutaneous tumors in rats. 2,6-DAT is not carcinogenic in mice and rats, regardless of their sex (NTP, 1980). Previous studies with MutaMouse (Kirkland and Beevers, 2006) and Big Blue mouse (Cunningham *et al*., 1996) indicate that 2,4-DAT is mutagenic in the liver, while 2,6-DAT is not. However, the transgenic mice employed for these studies were males, in which the hepatocarcinogenicity of 2,4-DAT is not observed. In addition, there are no reports on *in vivo* gene mutations in rats. Thus, we decided to examine the *in vivo* genotoxicity of both compounds in the liver, as carcinogenic target organ of 2,4-DAT, and kidney, as non-carcinogenic target, along with immunohistochemical analyses. We chose 500 ppm as the highest dose for both DATs according to the dose used in the National Toxicology Program 2-year cancer bioassay (NTP, 1979, 1980). We treated the rats with chemicals for 13 weeks because this period is customarily used to determine the appropriate doses for 2-year cancer bioassays; furthermore, shorter term treatments (e.g., treatments with potassium bromate for 5 weeks [Umemura *et al*., 2006]), sometimes do not induce detectable mutations *in vivo*.

MATERIALS AND METHODS

Establishment of F344 gpt delta rats. All the animals were maintained at Japan SLC (Shizuoka, Japan). The F344 *gpt* delta transgenic rat strain was developed by backcrosses of the original SD *gpt* delta transgenic rat with wild-type F344 rats. In brief, male SD *gpt* delta transgenic rat was mated with F344 female rat to produce an F1 generation. Offspring from the F1 generation were mated with F344 rats to yield an F2 generation. All offspring from successive backcrosses were examined for the possession of the *gpt* gene by PCR (Hayashi *et al*., 2003). After 15 successive backcrosses, identity of the resulting rats to F344 recipient is more than 99.9%. Thus, they were referred to as F344 *gpt* delta rats.

Chemicals. 2,4-DAT (purity 95%) and 2,6-DAT (purity 98%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Diethylnitrosamine (DEN) was obtained from Sigma-Aldrich Japan (Tokyo, Japan).

Bacterial reverse mutation test (Ames test). The mutagenic activities of 2,4-DAT and 2,6-DAT were assayed in a bacterial reverse mutation assay using *Salmonella typhimurium* tester strains TA98 and YG1024, an *O*-acetyltransferase (OAT)–overexpressing derivative. The test was conducted by the preincubation method (Maron and Ames, 1983) in the presence or the absence of S9 mix. At least two plates were used for each dose, and the mean values of the number of revertants per plate were calculated. Chemicals were dissolved in dimethyl sulfoxide, which was used as the negative control.

Animals, diet, and housing conditions. Male 6-week-old F344 *gpt* delta transgenic rats were obtained from Japan SLC and housed five animals per polycarbonate cage under specific pathogen-free standard laboratory conditions: room temperature, 23°C ± 2°C; relative humidity, 60 ± 5%; with a 12:12-h light-dark cycle; and free access to CRF-1 basal diet (Oriental Yeast Company, Tokyo, Japan) and tap water. After a 1-week acclimation period, the animals were used for the experiments.

Treatments of animals. The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Thirty male F344 *gpt* delta rats were randomized by weight into six groups. 2,4-DAT and 2,6-DAT were each mixed into Oriental CRF-1 powdered basal diet (Oriental Yeast Company) and stored at 4°C in the dark before use. Starting at 7 weeks of age, the rats were fed diets containing 0, 125, 250, or 500 ppm 2,4-DAT or 500 ppm 2,6-DAT for 13 weeks. There was also a positive control group; these rats were received a once-a-week ip injection of 20 mg/kg body weight DEN for 13 weeks. Parameters monitored included clinical signs, body weight, and food intake. The highest dose of 2,4-DAT was reduced from 500 to 400 ppm at week 9 because the dose at 500 ppm reduced the body weight of rats at week 8. All the surviving animals were killed under ether anesthesia at the end of the experiments. The liver and kidneys were isolated from each animal and were immediately excised, weighed, and cut into 2- to 3-mm-thick slices. The slices were fixed in 10% buffered formalin solution and routinely processed to paraffin blocks for histopathological examination as well as immunohistochemistry. Hematoxylin and eosin-stained tissue preparations cut from the blocks were examined by light microscopy.

Micronucleus assay. At autopsy, 60 μl of peripheral blood was obtained from the tail veins of all animals. The samples were processed according to the instructions supplied with the MicroFlow^{PLUS} kit (Litron, Rochester, NY), fixed in ultra-cold methanol, and stored immediately after fixation at −80°C until flow cytometry analysis was performed. Approximately 20,000 reticulocytes were counted for each sample using Becton-Dickinson FACSCalibur flow cytometer (Franslin Lakes, NJ) to detect the presence of micronuclei (MNs).

Immunohistochemical procedures. Liver sections of 3-μm thickness were treated with rabbit anti-rat GST-P antibody (1:1000; Medical & Biological Laboratories, Nagoya, Japan) and monoclonal mouse anti-Ki67 (MIB-5) antibody (1:50; Dako, Tokyo, Japan) (1:50), respectively. Areas and numbers of GST-P–positive foci larger than 0.1 mm in diameter of the liver sections were quantitatively measured with an image processor for analytical pathology (IPAP-WIN; Sumika Technos Company, Osaka, Japan). To investigate proliferative activity, we counted at least 1000 hepatocyte nuclei in each liver; labeling indices were calculated as the percentage of cells positive for Ki67

staining. The remaining tissues were immediately frozen in liquid nitrogen and stored at −80°C for subsequent mutation assays.

DNA isolation and in vitro packaging of λ phage DNA. High–molecular-weight genomic DNA was extracted from the liver and kidneys using the RecoverEase DNA Isolation kit (Stratagene, La Jolla, CA). λ EG10 phages were rescued using Transpack Packaging Extract (Stratagene).

gpt Mutation assay. The assay was conducted according to previously published methods (Nohmi *et al*., 1996). All the confirmed *gpt* mutants recovered from the livers were sequenced; identical mutations from the same rat were counted as one mutant. The mutant frequencies of the *gpt* gene (*gpt* MFs) in the liver and kidney were calculated by dividing the number of confirmed 6-thioguanine–resistant colonies by the number of rescued plasmids. DNA sequencing of the *gpt* gene was performed with the BigDye Terminater Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Spi⁻ assay. The Spi⁻ assay was conducted according to previously published methods (Masumura *et al*., 2002). To confirm the Spi⁻ phenotype of the candidates, suspensions were spotted on three types of plates on which XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains were spread with soft agar. True Spi⁻ mutants, which made clear plaques on all of the plates, were counted. Spi⁻ mutant lysates were obtained by infecting *E. coli* LE392 with the recovered Spi⁻ mutants. The lysates were used as templates for PCR and sequencing analysis to determine the deleted regions (Masumura *et al*., 2002). The Spi⁻ mutants were categorized into three classes: one base pair (bp) deletions, deletions of more than 1 bp, and complex mutations. The entire sequence of λ EG10 is available at http://dgm2alpha.nihs.go.jp/default.htm.

Statistical analysis. The statistical significance of the difference in the value of MFs between treated groups and negative controls was analyzed by Student's *t*-test. A *p* value less than 0.05 denoted the presence of a statistically significant difference. Variances in values for body weight, organ weight, and immunohistochemical data were examined by one-way ANOVA with Dunnett's multiple test to compare the differences between control and treated groups.

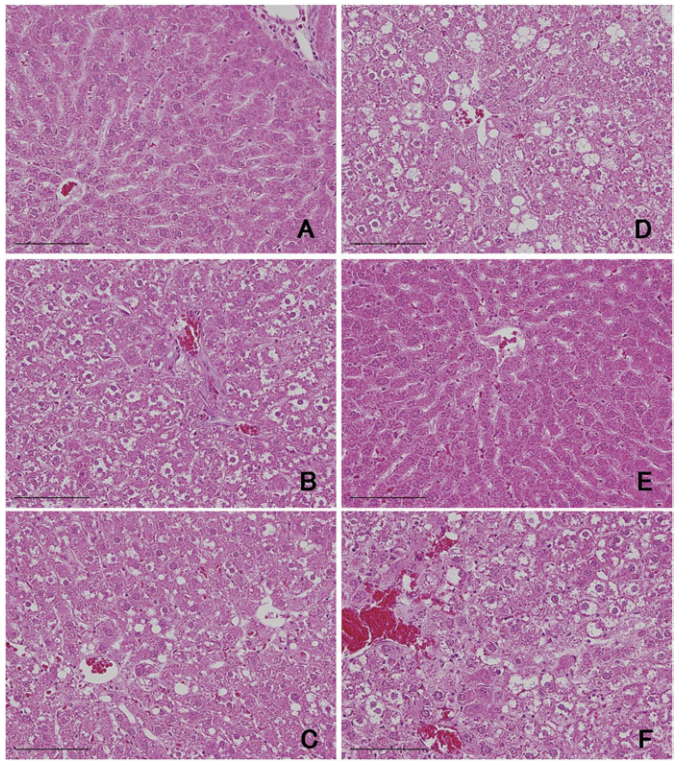

FIG. 1. Histological comparison of rat livers treated with 0 ppm 2,4-DAT (A), 125 ppm 2,4-DAT (B), 250 ppm 2,4-DAT (C), 500 ppm 2,4-DAT (D), 500 ppm 2,6-DAT (E), and DEN (F). Hepatotoxicity was observed in rats administered 2,4-DAT and DEN. Bar = 100 μm.

RESULTS

Dietary Treatment with 2,4-DAT Induced Preneoplastic Lesions in the Livers of F344 gpt Delta Rats

Dietary treatment with 2,4-DAT reduced body weight significantly at all three doses, while dietary treatment with 2,6-DAT did not (Supplementary table 1). Treatments with 2,4-DAT, but not 2,6-DAT, increased the relative weight of the livers and kidneys in a dose-dependent manner. Hypertrophy and vacuolar degeneration of hepatocytes was observed in the livers of rats in the 2,4-DAT treatment groups (Fig. 1). Cell proliferation was significantly enhanced by 2,4-DAT at a dose of 250 ppm but not by treatment with 2,6-DAT or other doses of 2,4-DAT (Table 1). That labeling index was twofold higher compared with that of basal diet group. GST-P–positive foci were induced by treatment with 2,4-DAT at a dose of 250 or 500 ppm and by the positive control treatment with DEN (Table 2). There were significant differences in number of foci and area of foci between rats treated with 2,4-DAT at 500 ppm and those of the basal diet group and between rats treated with DEN and the control group. No histopathological changes were observed in the kidneys of rats that were fed 2,4-DAT or 2,6-DAT. These results suggest that 2,4-DAT, but not 2,6-DAT, induced preneoplastic lesions in the livers of F344 *gpt* delta rats.

Both 2,4-DAT and 2,6-DAT Induced Mutations In Vitro

We confirmed that both 2,4-DAT and 2,6-DAT were mutagenic in *S. typhimurium* strain TA98 in the presence of S9 activation (Fig. 2). Treating cells with either of the DATs in the absence of S9 mix did not produce any increase in the number of revertants per plate. Similar, but less significant, results were obtained with another standard *S. typhimurium* strain TA100 (Supplementary table 2). These observations suggest that DAT metabolites were responsible for the mutagenic effects. To explore the metabolic activation pathways *in vitro*, we employed strain YG1024, which overproduces OAT, a phase II enzyme. Strain YG1024 detects frameshift mutations because it was derived from strain TA98 (Watanabe *et al*., 1994). As shown in Figure 2, YG1024 exhibited enhanced sensitivity to the mutagenicity of both 2,4-DAT and 2,6-DAT in the presence of S9 activation. The mutagenicity of 2,6-DAT was similar to that of 2,4-DAT in the presence of S9 activation (in strain TA98, 1036 vs. 1316 His^+ revertants per plate at 625 μg of 2,4-DAT and 2,6-DAT, respectively; in strain YG1024, 3460 vs. 3896 His^+ revertants per plate at 156 μg of 2,4-DAT and 2,6-DAT, respectively).

TABLE 1
Quantification of Hepatocyte Proliferation

	No. of Rats	No. of Total Nuclei	No. of Ki-67–Positive Nuclei	Index
Basal diet	5	2170.8 ± 890.9	27.4 ± 8.1	0.013 ± 0.004
Basal diet (DEN)	5	1749.6 ± 729.2[a]	73.8 ± 19.7	0.042 ± 0.009*
125 ppm 2,4-DAT	5	1700.2 ± 700.1[a]	14.0 ± 6.6	0.008 ± 0.005
250 ppm 2,4-DAT	5	1436.4 ± 596.7[a]	44.4 ± 10.5	0.031 ± 0.007*
500 ppm 2,4-DAT	5	1308.6 ± 537.6[a]	20.4 ± 9.0	0.015 ± 0.006
500 ppm 2,6-DAT	5	2048.8 ± 860.8	17.8 ± 7.4	0.014 ± 0.004

[a]Total number of nuclei was significantly decreased compared to the basal diet treatment group.
*Significantly different from the basal diet group ($p < 0.01$).

These results suggest that both DATs are mutagenic in the presence of S9 activation *in vitro* and also that *O*-acetylation is important for the metabolic activation.

In Vivo Mutagenicity of 2,4-DAT

For the initial *in vivo* genotoxicity assay, we examined MN formation in the peripheral blood of F344 *gpt* delta rats treated with 2,4-DAT or 2,6-DAT. However, no significant increase in MN frequency was observed in any of the treated groups (Supplementary table 4).

Next, we examined the mutagenicity of the DATs in the livers and kidneys of the rats. *gpt* MFs were significantly increased in the livers of 2,4-DAT–treated rats at all three doses and in the DEN-positive control, compared to the control group (Fig. 3, Supplementary table 3). No increases in MFs were observed in the livers of 2,6-DAT–treated rats or in the kidneys of either 2,4-DAT– or 2,6-DAT–treated rats. To characterize the *gpt* mutations in the liver, we performed DNA sequencing (Table 3). The predominant base substitutions were G:C-to-A:T transitions and G:C-to-T:A and G:C-to-C:G transversions in the 2,4-DAT–treated groups. In addition, base substitutions at A:T bps were also induced. In the DEN-treated positive control group, A:T-to-T:A transversions were the most predominant type of mutation. Spi$^-$ MFs in the liver were also significantly increased in 2,4-DAT treatment groups at doses of 250 and 500 ppm and in the DEN-treated group (Table 4). They were not increased by treatment with 2,6-DAT. DNA sequence analysis revealed that the specific mutant frequency (SMF) of a −1 frameshift at run sequences such as GGGG in the *gam* gene was increased more than fourfold after treatment with 500 ppm 2,4-DAT, while the SMF of deletions of more than two bps was not enhanced at this dose (Supplementary table 5). Thus, most of the Spi$^-$ mutations were −1 frameshift mutations at run sequences, and large deletion mutations were not significantly induced by treatment with 2,4-DAT.

TABLE 2
Quantification of GST-P–Positive Foci

	No. of Rats	No. of Foci (No./cm^2)	Area of Foci (mm^2/cm^2)
Basal diet	5	0.00 ± 0.00	0.000 ± 0.000
Basal diet (DEN)	5	78.92 ± 17.70**	1.924 ± 0.655**
125 ppm 2,4-DAT	5	0.00 ± 0.00	0.000 ± 0.000
250 ppm 2,4-DAT	5	1.19 ± 1.21	0.022 ± 0.023
500 ppm 2,4-DAT	5	6.05 ± 3.93*	0.502 ± 0.476*
500 ppm 2,6-DAT	5	0.00 ± 0.00	0.000 ± 0.000

*Significantly different from the basal diet group ($p < 0.05$).
**Significantly different from the basal diet group ($p < 0.01$).

DISCUSSION

In the regulatory sciences, a default assumption is that genotoxic carcinogens have no thresholds for their activities, and thus, no acceptable daily intake can be set for these chemicals when they are used as food additives, pesticides, or veterinary medicines (Kirsch-Volders *et al*., 2000; Nohmi, 2008). It is thought that single molecules of genotoxic compounds can induce mutations, and thus, genotoxic carcinogens impose carcinogenic risks to humans even at very low doses. However, how the genotoxicity of chemicals should be defined is not entirely clear. Currently, more than 200 genotoxicity assays have been proposed (Preston and Hoffmann, 2007). Unsurprisingly, the results among the various genotoxicity assays are inconsistent. The aromatic amine structural isomers 2,4-DAT and 2,6-DAT are an interesting reference pair that illustrates the inconsistency between *in vitro* and *in vivo* results (Cunningham *et al*., 1989). Both 2,4-DAT and 2,6-DAT are mutagenic *in vitro* in *S. typhimurium* strains, but only 2,4-DAT is carcinogenic in mice and rats (NTP, 1979).

In this study, we confirmed *in vitro* genotoxicity with *S. typhimurium* TA98 and YG1024 and explored *in vivo* genotoxicity with F344 *gpt* delta rats. Both DATs were mutagenic in the *S. typhimurium* strains *in vitro* when the S9 activation system was present (Fig. 2). In contrast, only 2,4-DAT was mutagenic in the livers of rats (Fig. 3, Table 4, Supplementary table 4). Both *gpt* and Spi$^-$ MFs in the liver were significantly increased in 2,4-DAT–treated rats compared to those in the control group. We did not observe any increase in *gpt* MFs in the livers of 2,6-DAT–treated rats or in the kidneys of 2,4- or 2,6-DAT–treated rats (Fig. 3, Supplementary table 4). Kidney may not have capacity to activate 2,4-DAT as in the case of bone marrow (see below). We identified preneoplastic lesions (i.e., GST-P–positive foci) in the livers of rats treated with 250 and 500 ppm 2,4-DAT (Table 2) but not in the livers of rats treated with 2,6-DAT. Generally, proliferation is activated in cancer cells. Ki-67 is a nuclear marker of cell proliferation and detectable in cells at all phases of the cell cycles except G_0 (Gerdes *et al*., 1983). The Ki-67

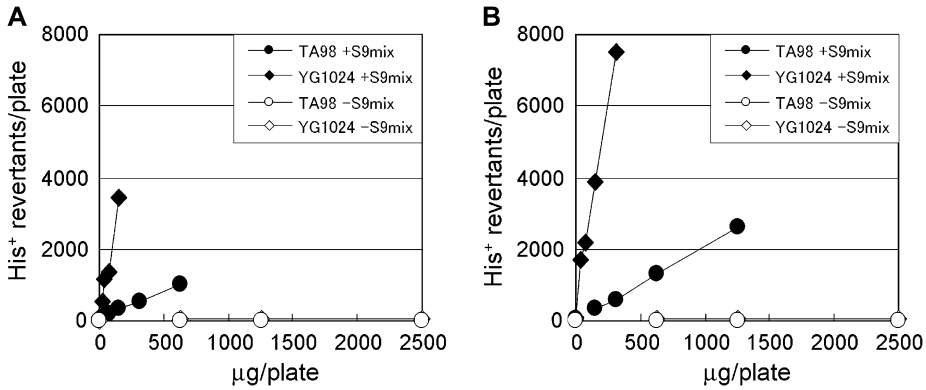

FIG. 2. Mutagenic activity of 2,4-DAT (A) and 2,6-DAT (B) in *Salmonella typhimurium* strains TA98 (circle) and YG1024 (rhombus). Filled circle and rhombus assayed with S9 mix; open circle and rhombus assayed without S9 mix.

labeling index is a measure of tumor proliferation and reported the association with liver and breast cancer outcome (de Azambuja *et al*., 2007; Nolte *et al*., 1998). The increase in Ki-67 index suggested the precancerous status of liver of rats treated by 2,4-DAT (Table 1, Fig. 1). We conclude from these results that genotoxicity assays (i.e., *gpt* and Spi$^-$ assays) and short-term carcinogenicity assays (i.e., GST-P–positive foci formation) can be conducted with F344 *gpt* delta rats. Because we observed genotoxicity in the target organ of carcinogenicity, these results strongly suggest that the carcinogenicity of 2,4-DAT is due to genotoxic activities. Integration of the genotoxicity assay with the pathological assay including GST-P–positive foci formation in *gpt* delta rats could reduce the number of animals necessary for these assays; this would contribute to the adoption of the 3R (reduction, replacement, and refinement) principle for animal use in the life sciences (Balls, 1997). It should be mentioned, however, that GST-P–positive foci formation often needs long treatment periods, for example, 16 and 24 weeks, respectively, for 2-amino-3,8-dimethylimidazo[4,5-*f*]-quinoxaline and 2-acetylaminofluorene (Bagnyukova *et al*., 2008; Tsuda *et al*., 2003) and such long treatments may increase the risk of false-positive results of mutations due to nongenotoxic mechanisms caused by chronic toxicity, for example, tumor induction and inflammatory responses (Thybaud et al., 2003).

Why do both 2,6-DAT and 2,4-DAT exhibit mutagenicity *in vitro*? The inconsistency between *in vitro* and *in vivo* results could be due to the different metabolic pathways of 2,6-DAT *in vitro* and *in vivo*. It is plausible that a DAT amino group is first oxidized by a specific cytochrome P450 (e.g., CYP1A2), and the resulting *N*-hydroxy group is further activated by OAT, which leads to the generation of nitrenium ions that can bind to DNA *in vitro* (Watanabe *et al*., 1994). *In vitro,* both DATs were mutagenic only in the presence of S9 activation, and strain YG1024, which overexpresses OAT, exhibited greater sensitivity to the DATs than did strain TA98 (Fig. 2). Both *S. typhimurium* strains possess GC repetitive sequences in the *hisD* gene that serve as target sites for mutations. We speculate, therefore, that 2,4-DAT could be activated *in vivo* via the pathway described above and induce mostly guanine adducts in DNA. In fact, it was reported that 2,4-DAT induces DNA

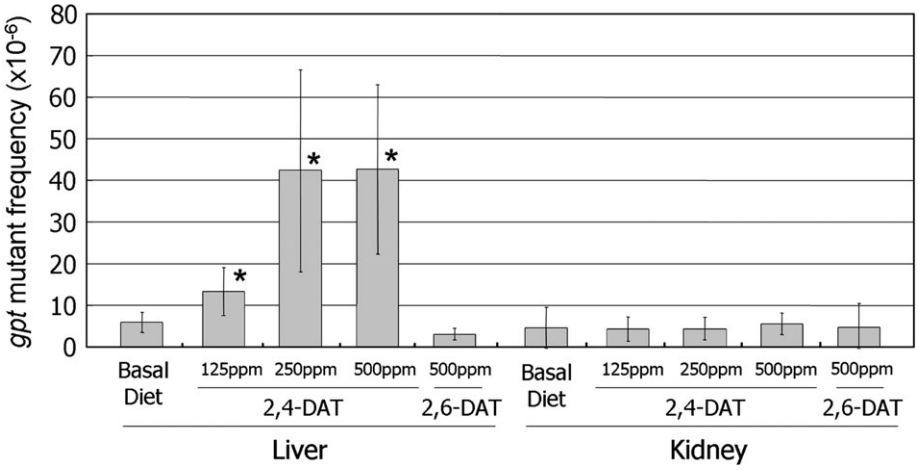

FIG. 3. MFs of *gpt* genes. Values represent mean SD ($n = 5$). Significant differences were observed in 2,4-DAT–treated livers compared to livers from rats fed negative control basal diet. *$p < 0.05$.

TABLE 3
Classification of *gpt* Mutations in *gpt* Delta Rat Livers

Type of gpt Mutation	Basal Diet		125 ppm 2,4-DAT		250 ppm 2,4-DAT		500 ppm 2,4-DAT		500 ppm 2,6-DAT		Basal Diet (DEN)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Base substitution												
Transition												
G:C → A:T	4	22	15	26	18	38	12	35	4	27	13	22
(CpG)	(1)		(6)		(6)		(3)		(1)		(3)	
A:T → G:C	1	6	1	2	5	11	1	3	3	20	12	21
Transversion												
G:C → T:A	4	22	16	28	12	26	6	18	4	27	3	5
G:C → C:G	1	6	7	12	4	9	7	21	0	0	0	0
A:T → T:A	0	0	5	9	4	9	1	3	1	7	23	40
A:T → C:G	1	6	4	7	3	6	1	3	0	0	7	12
Deletion												
−1	4	22	3	5	1	2	2	6	1	7	0	0
>2	2	11	2	3	0	0	1	3	2	13	0	0
Insertion	1	6	3	5	0	0	1	3	0	0	0	0
Others	0	0	2	3	0	0	2	6	0	0	0	0
Total	18	100	58	100	47	100	34	100	15	100	58	100

adducts in the livers of rats over 6000 times more efficiently than does 2,6-DAT (Taningher *et al*., 1995). The sequence analysis that we conducted indicates that most of the mutations induced by 2,4-DAT at 500 ppm were guanine base substitutions; that is, G:C-to-A:T, G:C-to-T:A, and G:C-to-C:G (Table 3). 2,6-DAT might be more efficiently detoxicated than 2,4-DAT *in vivo* because its *para* site at position 4 can be oxidized and subsequently conjugated by phase II enzymes (Cunningham *et al*., 1989). Detoxication of 2,6-DAT by phase II enzymes may be ineffective *in vitro* compared to *in vivo*. Appropriate cofactors, for example, uridine 5′-diphosphoric acid P2-β-D-glucopyranuronosyl ester for glucuronidation, may be needed to effectively detoxify the active metabolites of 2,6-DAT *in vitro*. At present, however, we cannot rule out the possibility that other factors, such as DNA repair, cell proliferation, translesion DNA synthesis, or apoptosis, might be involved in the differences in mutagenicity of 2,6-DAT *in vitro* and *in vivo*.

TABLE 4
Spi⁻ Mutant Frequency in Rat Livers

Treatment	No. of Rats	Mutant Frequency ($\times 10^{-6}$) (Mean ± SD)	*p* Value (*t*-test)
Basal diet	5	4.43 ± 1.99	
Basal diet (DEN)	5	341.22 ± 180.91	0.002
125 ppm 2,4-DAT	5	8.20 ± 4.75	0.07
250 ppm 2,4-DAT	5	13.42 ± 4.83	0.003
500 ppm 2,4-DAT	5	15.98 ± 4.45	0.0004
500 ppm 2,6-DAT	5	5.49 ± 2.53	0.241

In addition to the discrepancy between *in vitro* and *in vivo* mutagenicity, neither 2,4-DAT nor 2,6-DAT was genotoxic in the bone marrows of rats according to the MN assay (Supplementary table 3). 2,4-DAT is also not mutagenic when applied to MutaMouse skin (Kirkland and Beevers, 2006). Thus, we suggest that the negative results of the MN assay may be due to inefficient metabolic activation of 2,4-DAT at extrahepatic sites. Alternatively, the active metabolites generated in the liver may not reach the bone marrow. The poor metabolic activation in extrahepatic sites and/or short half-lives of the active metabolites may also account for the negative results of the MN assay with DEN in the bone marrow (Supplementary table 3). The MN assay is usually the first choice for *in vivo* genotoxicity assays in the development of pharmaceuticals; our results indicate that rather than relying on the MN assay in the bone marrow, genotoxicity should be evaluated in multiple organs, including the target organs of carcinogenicity.

The Spi⁻ assay is unique to *gpt* delta mice and rats and identifies deletion-type mutations (Nohmi *et al*., 2000). Previous studies with *gpt* delta mice suggested that genotoxic compounds and physical factors (e.g., radiation) induce different types of deletion mutations *in vivo* (Nohmi and Masumura, 2005). For example, heavy-ion radiation, ultraviolet B radiation, and mitomycin C induce large deletions in the liver, epidermis, and bone marrow, respectively, at molecular sizes of >1 kbp (Horiguchi *et al*., 2001; Masumura *et al*., 2002; Takeiri *et al*., 2003). In contrast, aromatic amines such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and aminophenylnorharman (APNH) induce −1 frameshift mutations in runs of guanine bases in the colon and liver, respectively (Masumura *et al*., 2000, 2003). We characterized Spi⁻ mutants obtained from the livers of rats treated with 500-ppm 2,4-DAT and concluded that, like PhIP and APNH, 2,4-DAT induces mostly −1 frameshift mutations (Supplementary table 5). These results suggest that Spi⁻ assay, as well as the *gpt* assay, is useful for characterizing mutations, which may constitute the molecular basis of chemically induced carcinogenesis.

At the 2006 International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use meeting held in Yokohama, Japan, revisions of the guidelines for a basic test battery of *in vitro* and *in vivo* genotoxicity tests were discussed (Hayashi, 2008). The current guidelines recommend two *in vitro* assays (Ames test and either a mammalian chromosome aberration test or a mammalian gene mutation test) plus one *in vivo* assay (usually MN test). Because of the high rate of false positives with *in vitro* mammalian cell assays (Kirkland *et al*., 2005), however, an alternative test battery was proposed at the meeting (Hayashi, 2008). The new battery is composed of one *in vitro* assay (Ames test) plus two *in vivo* assays (MN test plus a second *in vivo* test such as a transgenic assay or *in vivo* comet assay). It is possible to choose the classical battery of two *in vitro* assays plus one

in vivo assay instead of the alternative new battery. With the 3R principle in mind, integration of *in vivo* genotoxicity assays and a 28-day repeated dose toxicity assay was also discussed. *In vivo* mutagenicity assays and an *in vivo* MN assay can be integrated into a 28-day repeated dose toxicity study when transgenic rodents are used (Thybaud *et al*., 2003). In this study, we found that an *in vivo* genotoxicity assay and a short-term bioassay for liver carcinogenesis using GST-P–positive foci as an end point of preneoplastic lesions can be conducted with F344 *gpt* delta rats. Integration of the two assays using transgenic rats may further facilitate adoption of the 3R principle in regulatory toxicology.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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