Validation study of the combined repeated-dose toxicity and genotoxicity assay using \textit{gpt} delta rats

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Transgenic rodents carrying reporter genes to detect organ-specific \textit{in vivo} genetic alterations are useful for risk assessment of genotoxicity that causes cancer. Thus, the Organization for Economic Co-operation and Development has established the guideline for genotoxicity tests using transgenic animals, which may be combined with repeated-dose toxicity studies. Here, we provide evidence to support equivalence of \textit{gpt} delta and wild type (WT) rats in terms of toxicological responses to a genotoxic hepatocarcinogen, \textit{N}-nitrosodimethylamine (DEN), and a non-genotoxic hepatocarcinogen, di(2-ethylhexyl)phthalate (DEHP). \textit{gpt} delta rats treated with DEHP showed similar increases in liver and kidney weights, serum albumin, albumin/globulin ratios, and incidence of diffuse hepatocyte hypertrophy compared to WT F344 and Sprague–Dawley (SD) rats. DEN-treated \textit{gpt} delta rats showed equivalent increases in the number and area of precancerous GST-P-positive foci in the liver compared to WT rats. The livers of DEN-treated \textit{gpt} delta rats also showed increased frequencies of \textit{gpt} and \textit{Sp}i mutations; such changes were not observed in DEHP-treated \textit{gpt} delta rats. These results indicated that \textit{gpt} delta rats (both F344 and SD backgrounds) showed comparable DEHP-induced toxicity and DEN-induced genotoxicity to those observed in WT rats. With regard to the administration period, the general toxicity of 1.2% DEHP was evident throughout the experimental period, and the genotoxicity of 10 p.p.m. DEN could be detected after 2 weeks of administration and further increased at 4 weeks. These results suggested that combined assays using \textit{gpt} delta rats could detect both general toxicity and genotoxicity by the canonical 4-week administration protocol. Therefore, this assay using \textit{gpt} delta rats would be applicable for risk assessment including early detection of genotoxic carcinogens and ultimately serve to reduce cancer risks in humans from environmental chemicals.

Carcinogenicity is one of the most serious hazards of chronic exposure to chemicals. Carcinogens are classified into two major groups: genotoxic carcinogens and non-genotoxic carcinogens. As genotoxicity is not thought to have a threshold, it is important to determine the genotoxicity of chemicals for risk assessment. Therefore, a number of \textit{in vitro} and \textit{in vivo} genotoxicity tests have been developed. Among them, transgenic \textit{in vivo} genotoxicity assays, which use transgenic rodents carrying reporter genes to detect genetic alterations, enable us to evaluate organ-specific \textit{in vivo} genotoxicity. Thus, the Organisation for Economic Co-operation and Development (OECD) has established the guideline for genotoxicity testing using transgenic animals. The \textit{gpt} delta rats and mice are considered one of the established transgenic models, which have provided sufficient data to support their use in the OECD test guideline. These rodents carry the \textit{gpt} transgene, which detects point mutations, and the \textit{red}/\textit{gam} transgenes, which detect deletion mutations.\textsuperscript{(2,3)}

At present, \textit{in vivo} genotoxicity studies are carried out independently of repeated-dose toxicity studies. Therefore, both transgenic animals (for evaluation of genotoxicity) and wild type (WT) animals (for evaluation of general toxicity) are required. Therefore, combining transgenic rodent gene mutation assays\textsuperscript{(1)} with repeated-dose toxicity studies would conform to the 3Rs principle (Replacement, Refinement, and Reduction) of animal use in laboratory experiments. To accomplish this objective, data are required to ensure that transgenic gene mutation assays are efficiently sensitive in the protocol used for repeated-dose toxicity studies and to verify that the performance of the repeated-dose assay is not adversely affected by using a transgenic rodent strain rather than the parental WT strain.\textsuperscript{(1)} Therefore, in this study, we compared the general toxicity of a genotoxic hepatocarcinogen, \textit{N}-nitrosodimethylamine (DEN), and a non-genotoxic hepatocarcinogen, di(2-ethylhexyl)phthalate (DEHP), in F344/\textit{gpt} delta and Sprague–Dawley (SD)/\textit{gpt} delta rats with their parental WT F344 and SD rats to determine whether \textit{gpt} delta rats were equivalent to WT rats in terms of toxicological responses. In addition to general toxicity, we also compared the carcinogenic effects of DEN between \textit{gpt} delta and WT rats by examining the appearance of procarcinogenic GST-P-positive lesions, which are detected in the liver after genotoxic treatment.\textsuperscript{(1)}
In addition to the ability of gene mutation assays in transgenic animals to detect organ-specific genotoxicity, these assays are also able to detect mutations after only a short duration of repeated exposure because clonal expansion of mutant cells is not necessary. Therefore, we also examined the appropriate administration duration for simultaneous detection of both general toxicity and genotoxicity by killing animals after 2, 4, or 8 weeks of treatment.

Materials and Methods

Experimental animals. Five-week-old male specific pathogen-free C57BL/6J (F344/WT (Slc:SD), F344/gpt delta (F344/Slc-Tg [gpt delta]), SD/WT (Slc:SD), and SD/gpt delta (Slc:SD-Tg [gpt delta])) rats were purchased from Japan SLC (Shizuoka, Japan) and used after a 1-week acclimatization period. The animals were housed in polycarbonate cages (five rats per cage) with soft chips for bedding. The animals were maintained in a room with a barrier system under conditions of controlled temperature (22 ± 3°C), humidity (55 ± 15%), air changes (more than 10 times/h), and lighting (12:12 h light : dark cycle) and were given free access to an MF basal diet (Oriental Yeast, Tokyo, Japan) and tap water.

Test chemicals. N-nitrosodiethylnitramine (CAS: 55-18-5, >99% pure) was purchased from Wako Pure Chemical Industry (Tokyo, Japan) (Lot: PEI3F-ES), Di-(2-ethylhexyl) phthalate (CAS: 117-81-7, 97% pure) was purchased from Tokyo Chemical Industry (Tokyo, Japan). GST-P expression was visualized using diaminobenzidine (Dojindo, Kumamoto, Japan). The DNA was purified by ethanol precipitation. In the DEHP treatment experiment, the DNA was dialyzed against TE buffer using a RecoverEase DNA Isolation Kit (Agilent Technologies, Santa Clara, CA, USA). The gpt and Spi− assays were carried out as described previously. Briefly, λEG10 phage was reconstructed from genomic DNA with Transpack Packaging Extract (Agilent Technologies). For gpt gene mutation assays, E. coli YG6020 were transformed with the reconstructed phage and then plated onto M9 minimum agar plates supplemented with chloramphenicol (Cm) with or without 6-thioguanine (6-TG). The transformants carrying mutant gpt genes, which grew on M9+Cm−6-TG plates, were subjected to colony-direct PCR with primers designed to amplify the gpt transgene for sequencing analysis. Frequencies of gpt gene mutations were calculated by the number of 6-TG/Cm− colonies harboring independent mutations divided by the number of Cm− colonies. For Spi− mutant assays, E. coli XL1-Blue MRA and XL1-Blue MRA (P2) were infected with the phage and then plated onto λ-tryptase plates. The Spi− candidates, which formed plaques on the XL1-Blue MRA (P2) plates, were confirmed by resporiting onto E. coli XL1-Blue MRA, XL1-Blue MRA (P2), and WL95 (P2) strains. Plaques that appeared on the three E. coli strains were counted as Spi− mutants. Spi− mutant frequencies were calculated by the number of Spi− mutants divided by the number of plaques on XL1-Blue MRA.

Statistical analysis. Significant differences in the data for body weights, organ weights, serum biochemistry, numbers and areas of GST-P-positive foci in the liver, frequencies of mutations in the gpt gene, and Spi− mutant frequencies in red/gam genes were evaluated by Tukey’s test. Significant differences in the incidences of histopathological findings were evaluated by Fisher’s exact probability test.

Results

Clinical signs of toxicity prior to euthanasia. In both DEN and DEHP experiments, no clinical signs were observed throughout the experimental period. All animals survived until the scheduled necropsy. Average intakes of DEN and DEHP per body weight were considered to be dose-dependent (Tables 1, 2).

As WT and gpt delta rats were not littermates, the F344/gpt rats used in DEN treatment and the SD/gpt delta rats used in both DEN and DEHP treatments were relatively smaller than their corresponding WT rats at the start of treatment (6 weeks old). Administration of DEN at 0.1, 1, or 10 p.p.m. in drinking water had no effect on body weight gain (Figs 1a,b,5) in all.
strains. Administration of DEHP at 1.2% in food caused significant decreases (or decreasing trends) in body weight gain compared to the corresponding controls from weeks 3 to 8 (Fig. 1c,d). The magnitudes of the observed decreases were 4.7% (F344/WT), 7.6% (F344/gpt delta), 7.2% (SD/WT), and 6.9% (SD/gpt delta) of the mean control body weight at 4 weeks and 6.6% (F344/WT), 10.5% (F344/gpt delta), 9.5% (SD/WT), and 12.2% (SD/gpt delta) of the mean control body weight at 8 weeks (Fig. 1).

Relative organ weights. Administration of DEN at 0.1, 1, or 10 p.p.m. in drinking water had no significant effect on organ weights, regardless of the genotype or strain (Table S1). Administration of DEHP at 1.2% in the diet caused a significant increase in liver weight compared to the corresponding controls. The magnitudes of the increases ranged from 1.9- to 2.0-fold (F344/WT), 1.6- to 2.0-fold (F344/gpt delta), 1.7- to 1.9-fold (SD/WT), and 1.7- to 1.9-fold (SD/gpt delta) throughout the experimental period. We also observed significant increases (or increasing trends) in liver weights of rats consuming 0.12% DEHP. Administration of DEHP at 1.2% in both genotypes and strains also caused significant increases (1.1-1.3-fold) in kidney weight compared to the corresponding controls throughout the experimental period (Fig. 2, Table S2).

Serum biochemistry. Administration of DEN at 0.1, 1, or 10 p.p.m. in drinking water had no significant effect on serum biochemistry in all strains (Table S3). Regardless of the genotype and strain, administration of DEHP at 1.2% in the diet caused significant increases in serum albumin (1.1-1.3-fold) and albumin/globulin ratios (1.3-2.0-fold) compared to the corresponding controls. At 1.2%, DEHP also caused a 1.3-1.5-fold increase in serum alkaline phosphatase (ALP) at 8 weeks (Fig. 3, Table S4).

Histopathological findings. Administration of DEN at 0.1, 1, or 10 p.p.m. in drinking water had no significant effect on the incidences of lesions in the liver, spleen, kidneys, lung, heart, or stomach, as indicated by H&E staining of tissue sections (Table 3). Administration of DEHP at 1.2% in the diet caused diffuse hepatocyte hypertrophy in all rats throughout the experimental period. Significant increases in alveolar foamy cell infiltration were found in SD/WT rats treated with 1.2% DEHP at week 8. However, this may have been incidental as this lesion was also frequently found in control groups. Although significant increases in kidney weights were observed in rats treated with 1.2% DEHP, no treatment-related changes were observed under microscopic examinations (Table 4).

Immunohistochemical analysis of GST-P-positive liver foci. Due to the short duration of administration, the majority of GST-P-positive foci contained few cells, particularly at week 2. Therefore, we counted all GST-P-positive hepatocytes as GST-P-positive foci, without cell number/size thresholds. Administration of DEN at 10 p.p.m. caused a significant increase in the number of foci and a significant increase (or increasing trend) in the area of foci beginning at week 2 in all strains (Fig. 4). With regard to both the number and area of GST-P-positive foci, no significant differences were observed between gpt delta and WT rats for both F344 and SD strains at all doses and for all administration durations. In F344/gpt delta rats, although evaluation was carried out with a limited number of initial samples, statistical significance was evident. The additional F344/gpt delta rats treated with 0 or 10 p.p.m. DEN for 8 weeks showed comparable data for the number (2.9 or 164.4 foci/cm², respectively) and area (8.1 × 10⁻⁴ or 7.7 × 10⁻² mm²/cm², respectively) of foci (Fig. S2). It was established that GST-P-positive foci were not induced by peroxisome proliferators, such as DEHP. Indeed, our previous study showed that a carcinogenic dose (12 000 p.p.m., the same as this study) of DEHP did not induce GST-P foci in gpt delta rats. Thus, we considered that GST-P-positive foci was not a suitable marker for comparison between gpt delta and WT rats on the effect of DEHP exposure. Therefore, examination of GST-P-positive foci was carried out in the DEN treatment experiment only.

In vivo mutation assays. Administration of DEN induced significant increases in gpt mutation frequencies (Fig. 5a, Tables S5,S6) and Spi− mutant frequencies (Fig. 5b, Tables S7,S8). In the 10 p.p.m. DEN groups, mutation frequencies of the gpt transgene were 1.62 × 10⁻⁵, 4.05 × 10⁻⁵, and 3.79 × 10⁻⁵.

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Table 1. Water consumption and chemical intake during the entire treatment period with N-nitrosodiethylamine (DEN)

| Strain/genotype | DEN, p.p.m. | Water consumption, g/animal/day | Chemical intake, μg/kg BW/day |
|-----------------|------------|--------------------------------|-------------------------------|
| F344/WT         | 0          | 20.5                           | 0.00                          |
|                 | 0.1        | 19.9                           | 9.00                          |
|                 | 1          | 20.6                           | 90.0                          |
|                 | 10         | 20.7                           | 906                           |
| F344/gpt delta  | 0          | 20.3                           | 0.00                          |
|                 | 0.1        | 19.2                           | 9.50                          |
|                 | 1          | 19.5                           | 96.3                          |
|                 | 10         | 19.1                           | 932                           |
| SD/WT           | 0          | 30.6                           | 0.00                          |
|                 | 0.1        | 30.7                           | 8.38                          |
|                 | 1          | 28.1                           | 78.8                          |
|                 | 10         | 29.9                           | 841                           |
| SD/gpt delta    | 0          | 30.6                           | 0.00                          |
|                 | 0.1        | 32.9                           | 9.38                          |
|                 | 1          | 29.6                           | 85.0                          |
|                 | 10         | 28.3                           | 836                           |

†Additional experiment. BW, body weight; SD, Sprague-Dawley.

Table 2. Food consumption and chemical intake during the entire treatment period with di(2-ethylhexyl)phthalate (DEHP)

| Strain/genotype | DEHP, % | Food consumption, g/animal/day | Chemical intake, mg/kg BW/day |
|-----------------|--------|-------------------------------|------------------------------|
| F344/WT         | 0      | 15.7                          | 0.00                         |
|                 | 0.012  | 16.6                          | 8.75                         |
|                 | 0.12   | 16.6                          | 89.1                         |
|                 | 1.2    | 16.8                          | 914                          |
| F344/gpt delta  | 0      | 18.0                          | 0.00                         |
|                 | 0.012  | 16.9                          | 9.05                         |
|                 | 0.12   | 16.8                          | 90.8                         |
|                 | 1.2    | 16.4                          | 920                          |
| SD/WT           | 0      | 21.0                          | 0.00                         |
|                 | 0.012  | 20.6                          | 7.24                         |
|                 | 0.12   | 21.2                          | 73.9                         |
|                 | 1.2    | 25.3                          | 838                          |
| SD/gpt delta    | 0      | 19.3                          | 0.00                         |
|                 | 0.012  | 19.9                          | 7.24                         |
|                 | 0.12   | 19.5                          | 70.6                         |
|                 | 1.2    | 20.6                          | 857                          |
at 2, 4, and 8 weeks, respectively, in F344/\(\text{gpt}^+\) delta rats and 1.36 \(\times\) \(10^{-3}\), 3.18 \(\times\) \(10^{-3}\), and 4.06 \(\times\) \(10^{-3}\) at 2, 4, and 8 weeks, respectively, in SD/\(\text{gpt}^+\) delta rats. These values were 16.0- to 48.8-fold higher than those of the corresponding control groups. In the 1 p.p.m. DEN groups, although statistical significance was not achieved, the mutation frequencies were 3.9- to 8.8-fold higher than those of the corresponding control groups. Sequencing analysis of \(\text{gpt}^+\) mutants revealed that administration of DEN caused significant increases in GC-TA, AT-TA, and AT-CG transversions and GC-AT and AT-CG transitions (Fig. 6, Tables S9, S10). In addition to \(\text{gpt}^+\) mutation frequencies, Spi/\(C_0\) mutant frequencies, which are mainly induced by large structural mutations or frameshift mutations, were also dramatically increased by DEN administration. In the 10 p.p.m. DEN groups, Spi\(^\text{−}\) mutant frequencies were 0.92 \(\times\) \(10^{-5}\), 1.59 \(\times\) \(10^{-5}\), and 2.73 \(\times\) \(10^{-5}\) at 2, 4, and 8 weeks, respectively, in F344/\(\text{gpt}^+\) delta rats and 0.99 \(\times\) \(10^{-5}\), 1.75 \(\times\) \(10^{-5}\), and 2.68 \(\times\) \(10^{-5}\) at 2, 4, and 8 weeks, respectively, in SD/\(\text{gpt}^+\) delta rats. These values were 2.5- to 9.4-fold higher than those of control groups. Comparable results were obtained from the additional experiment. Individual data are shown in Tables S5–S10. Administration of DEHP had no effect on the \(\text{gpt}^+\) mutation frequencies, \(\text{gpt}^+\) mutation spectra, or Spi\(^\text{−}\) mutant frequencies (Fig. 5c,d, Tables S11–S16).

**Discussion**

In this study, we evaluated whether \textit{in vivo} genotoxicity studies and general toxicity studies could be carried out simultaneously in \(\text{gpt}^+\) delta rats, with responses equivalent to those observed in WT rats. In the general toxicological study, there were no significant changes in DEN-treated groups with respect to body weights, relative organ weights, serum biochemistry values, and histopathological examinations. Similarly, serum albumin levels and albumin/globulin ratios were also increased significantly. These changes were consistent with the presence of diffuse

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**Additional experiment.** Because a sufficient number of F344/\(\text{gpt}^+\) delta rats could not be obtained in the DEN treatment study, an additional experiment was carried out to confirm the results of 8-week treatment of the 10 p.p.m. DEN group. There were no significant differences between the initial and additional experiments, and even if data from both experiments were combined, there were no statistical changes in general toxicity or immunohistochemical analysis of GST-P-positive liver foci. The sole statistically significant change was that GC-TA transversion of the 10 p.p.m. DEN group at 8 weeks was changed from \(P < 0.05\) (*) to \(P < 0.01\) (**) when data from these two experiments were combined.
hepatocyte hypertrophy found in all rats treated with 1.2% DEHP. Administration of 1.2% DEHP also caused significant decreases (or decreasing trends) in body weight gain at weeks 3–8. The extents of these hepatomegaly-related changes and body weight reductions found in gpt delta rats were similar to those in WT rats and consistent with those reported in previous studies. Although the testes are thought to be the most sensitive organ to DEHP, for example, exposure to DEHP causes Leydig cell hyperplasia in Long–Evans rats and seminiferous tubule atrophy and Sertoli cell vacuolation in SD

![Image](https://example.com/image.png)

**Fig. 2.** Effects of di(2-ethylhexyl)phthalate (DEHP) on relative organ weights in male F344/wild type (WT), F344/gpt delta, Sprague–Dawley (SD)/WT, and SD/gpt delta rats administered 0, 0.012, 0.12, or 1.2% DEHP in the diet for 2, 4, or 8 weeks. (a) Liver; (b) kidneys. *P < 0.05, **P < 0.01 versus 0% DEHP group (control). *P < 0.05, **P < 0.01, WT versus gpt delta rats under the same conditions. BW, body weight.

![Image](https://example.com/image.png)

**Fig. 3.** Effects of di(2-ethylhexyl)phthalate (DEHP) on serum biochemistry in male F344/wild type (WT), F344/gpt delta, Sprague–Dawley (SD)/WT, and SD/gpt delta rats administered 0, 0.012, 0.12, or 1.2% DEHP in the diet for 2, 4, or 8 weeks. (a) Serum albumin, (b) albumin/globulin (A/G) ratio, and (c) alkaline phosphatase (ALP). Values are mean ± standard deviations. *P < 0.05, **P < 0.01 versus 0% DEHP group (control). ##P < 0.01, WT versus gpt delta rats under the same conditions.
Table 3. Histopathological findings for F344/WT, F344/gpt delta, Sprague-Dawley (SD)/WT, and SD/gpt delta rats treated with N-diethylnitrosamine (DEN)

| Strain/genotype | F344/WT | 0 | 0.1 | 1 | 10 | F344/gpt delta | 0† | 0.1† | 1† | 10† | 0† | 10† |
|-----------------|---------|---|-----|---|----|----------------|----|-----|----|-----|----|-----|
| DEN (p.p.m.)    |         |   |     |   |    |                |    |     |   |     |    |     |
| Weeks           |         | 2 | 4   | 8  | 2  | 4   | 8  | 2   | 4 | 8   | 2  | 4  |
| No. of animals  |         | 5 | 5   | 5  | 5  | 5   | 5  | 5   | 5 | 5   | 5  | 5  |
| Organs and findings |     |     |     |     |     |     |     |     |     |     |     |     |
| Liver           |         | 0 | 0   | 0  | 0  | 0   | 0  | 0   | 1 | (20)| 0  | 0  |
| Focus of cellular alteration |     | 0 | 0   | 0  | 0  | 0   | 0  | 0   | 1 | (20)| 0  | 0  |
| Microgranuloma  |         | 1 | (20)| 3 | (60)| 2 | (40) | 2 | (40)| 1 | (20)| 1 | (20) |
| Lipidosis, focal |       | 0 | 0   | 0  | 0  | 1 | (20) | 0  | 0  | 0  | 0  | 0  | 0  |
| Epidermoid cyst |         | 0 | 0   | 0  | 0  | 0  | 0  | 0   | 0 | 0   | 0  | 0  |
| Pigmentation    |         | 0 | 0   | 0  | 0  | 0  | 0  | 0   | 0 | 0   | 0  | 0  |
| Spleen          |         | 0 | 0   | 0  | 0  | 0  | 0  | 0   | 1 | (20)| 0  | 0  |
| Focal atrophy, acinar |     | 0 | 0   | 0  | 0  | 0  | 0  | 0   | 0 | 0   | 0  | 0  |
| Infarction      |         | 0 | 0   | 0  | 0  | 0  | 0  | 0   | 0 | 0   | 0  | 0  |
| Kidneys         |         | 1 | (20)| 1 | (20)| 3 | (60)| 0  | 0  | 0  | 0  | 1 | (20) |
| Regenerative tubules |     | 0 | 0   | 0  | 0  | 0  | 0  | 0   | 1 | (20)| 0  | 0  |
| Cell infiltration, interstitial, lymphocytic |     | 0 | 0   | 0  | 0  | 0  | 0  | 0   | 0 | 0   | 0  | 0  |
| Lungs           |         | 0 | 0   | 0  | 0  | 0  | 0  | 0   | 0 | 0   | 0  | 0  |
| Cell infiltration, interstitial, lymphocytic |     | 0 | 0   | 0  | 0  | 0  | 0  | 0   | 0 | 0   | 0  | 0  |
| Heart           |         | 0 | 0   | 1 | (20)| 0  | 0  | 0   | 0  | 0  | 0  | 0  |
| Cell infiltration, lymphocytic |     | 0 | 0   | 1 | (20)| 0  | 2 | (40)| 0  | 1 | (25)| 1 | (25) |
| Stomach         |         | 1 | (20)| 0  | 0  | 0  | 0  | 0   | 0  | 0  | 0  | 0  |
| Cysts, glandular glandular stomach |     | 0 | 0   | 0  | 0  | 0  | 0  | 0   | 0 | 0   | 0  | 0  |
| Hyperplasia, squamous cell, forestomach |     | 0 | 0   | 0  | 0  | 0  | 0  | 0   | 0 | 0   | 0  | 0  |
### Table 3. (continued)

| Strain/genotype | SD/WT | SD/gpt delta |
|-----------------|-------|-------------|
| DEN (p.p.m.)    | 0     | 0           |
|                 | 0.1   | 0           |
|                 | 1     | 0           |
|                 | 10    | 0           |
| Weeks           | 0     | 2 4 8 2 4 8 |
|                 | 0.1   | 2 4 8 2 4 8 |
|                 | 1     | 2 4 8 2 4 8 |
|                 | 10    | 2 4 8 2 4 8 |
| No. of animals  | 5     | 5 5 5 5 5 5 |
|                 | 5     | 5 5 5 5 5 5 |
|                 | 5     | 5 5 5 5 5 5 |
|                 | 5     | 5 5 5 5 5 5 |
| Organ and findings
| Liver           |       |             |
| Focal atrophy,  |       |             |
| acinar         |       |             |
| Infarction     |       |             |
| Kidneys        |       |             |
| Regenerative   |       |             |
| tubules        |       |             |
| Cell infiltration, |   |             |
| interstitial,  |       |             |
| lymphocytic    |       |             |
| Lungs          |       |             |
| Cell infiltration, |   |             |
| interstitial,  |       |             |
| lymphocytic    |       |             |
| Heart          |       |             |
| Cell infiltration, |   |             |
| lymphocytic    |       |             |
| Stomach        |       |             |
| Cysts, glandular, |   |             |
| glandular      |       |             |
| stomach        |       |             |
| Hyperplasia,   |       |             |
| squamous cell, |       |             |
| forestomach    |       |             |

†Additional experiment. Data are shown as number of cases (%).
| DEHP (%) | F344/WT | F344/gpt delta | Mice | gpt delta |
|----------|---------|---------------|------|----------|
| Week 2  | 0       | 0             | 5    | 5        |
| Week 4  | 0.012   | 0             | 5    | 5        |
| Week 8  | 0.12    | 0             | 5    | 5        |
| Week 1.2| 1.2      | 0             | 5    | 5        |

| Organs and findings |
|---------------------|
| Liver               |
| Microgranuloma      |
| Hypertrophy, diffuse|
| Single cell necrosis|
| Focal necrosis      |
| Bile duct proliferation|
| Kidneys             |
| Hyaline cast        |
| Regenerative tubules|
| Mineralization, medulla|
| Cell infiltration, interstitial, lymphoctic |
| Tubular dilatation, cortex |
| Tubular vacuolation, distal tubules |
| Cyst                |
| Lungs               |
| Cell infiltration, interstitial, lymphocytes |
| Foamy cell infiltration, alveolar |
| Osseus metaplasia   |
| Granuloma           |
| Testes              |
| Multinucleated cell |
| Tubular cell vacuolation |
| Tubular atrophy     |
| Tubular degeneration|

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| Stain / genotype | DEHP (%) | Weeks | No. of animals |
|------------------|----------|-------|----------------|
|                  | 0        | 0.012 | 0.12           | 1.2       | 0        | 0.012 | 0.12 | 1.2 |
|                  | 2        | 4     | 8              | 2         | 4       | 8     | 2    | 4   | 8   | 2    | 4   | 8    |

| Organs and findings | SD / WT | SD / gpt delta |
|----------------------|---------|---------------|
| Liver                |         |               |
| Microgranuloma       | 2 (40)  | 0             |
| Hypertrophy, diffuse | 0 (0)   | 0             |
| Single cell necrosis | 0 (0)   | 1 (20)        |
| Focal necrosis       | 0 (0)   | 0             |
| Bile duct proliferation |      |               |
|                    |         |               |
| Kidneys              |         |               |
| Hyaline cast         | 0 (0)   | 1 (20)        |
| Regenerative tubules | 1 (20)  | 1 (20)        |
| Mineralization, medulla | 0   | 1 (20)        |
| Cell infiltration, interstitial, lymphoidic | 0 | 0 |
| Tubular dilatation, cortex | 0 | 0 |
| Tubular vacuolations, distal tubules | 0 | 0 |
| Cyst                 | 0 (0)   | 0             |
| Lungs                |         |               |
| Cell infiltration, interstitial, lymphoidic | 0 | 1 (20) |
| Foamy cell infiltration, alveolar | 0 | 0 |
| Osseous metaplasia   | 0 (0)   | 0             |
| Granuloma            | 0 (0)   | 1 (20)        |
| Testes               |         |               |
| Multinucleated cell  | 1 (20)  | 0             |
| Tubular cell vacuolation | 0 | 0 |
| Tubular atrophy      | 0 (0)   | 1 (20)        |
| Tubular degeneration | 0 (0)   | 1 (20)        |

**p < 0.01 versus control. Data are shown as number of cases (%).
rats (14) even at lower doses than those used in this study, no significant changes were found in the present histopathological examination of both $gpt$ delta and WT rats. This may have been due to the age of the rats at the beginning of administration and/or the administration durations. Although we did observe some statistically significant differences between $gpt$ delta and WT rats in serum biochemistry values in the DEN exposure study and in other organ weights and

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**Fig. 4.** Immunohistochemical analysis of GST-P-positive foci. The multiplicity (no./cm$^2$) (a) and area (mm$^2$/cm$^2$) (b) of GST-P-positive foci in the livers of $N$-nitrosodiethylamine (DEN)-treated rats. $^*$ $P < 0.05$, $^{**} P < 0.01$ versus 0 p.p.m. DEN group (control). SD, Sprague–Dawley; WT, wild type.

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**Fig. 5.** (a) Frequency of mutations in the $gpt$ transgene in the livers of male F344/$gpt$ delta and Sprague–Dawley (SD)/$gpt$ delta rats administered 0, 0.1, 1, or 10 p.p.m. $N$-nitrosodiethylamine (DEN) in drinking water. (b) Spi$^-$ mutant frequencies in the livers of male F344/$gpt$ delta and SD/$gpt$ delta rats administered 0, 0.1, 1, or 10 p.p.m. DEN in the drinking water. (c) Frequency of mutations in the $gpt$ transgene in the livers of male F344/$gpt$ delta and SD/$gpt$ delta rats administered 0, 0.012, 0.12, or 1.2% di(2-ethylhexyl)phthalate (DEHP) in the diet. (d) Spi$^-$ mutant frequencies in the livers of male F344/$gpt$ delta and SD/$gpt$ delta rats administered 0, 0.012, 0.12, or 1.2% DEHP in the diet. $^{**} P < 0.01$ versus 0 p.p.m. DEN group (control).
serum biochemistry values in the DEHP exposure study, no dose-dependent responses were observed, and these differences were sporadic. Thus, we assumed that these differences were incidental. Taken together, our data indicated that gpt delta and WT rats (both F344 and SD strains) showed comparable general toxicity responses to DEN and DEHP.

With regard to the comparison of in vivo genotoxicity, administration of DEN induced the same level of mutations in F344/gpt delta and SD/gpt delta rats in both gpt and Spi assays. The gpt mutation spectra were also similar for both strains. Administration of DEN is known to generate various monoalkylated lesions, that is, N7-ethylguanine, O4-ethylthymine, N3-ethyladenine, and O6-ethylguanine. N7-ethylguanine further undergoes depurination, resulting in the formation of abasic sites.(15) These lesions cause mutations mainly as consequences of error-prone translesion synthesis. Thus, the most frequent mutations were AT-TA transversions, followed by GC-AT and AT-GC transitions and AT-CG and GC-TA transversions. However, the major mutations found in DEN-induced tumors were GC-AT and AT-GC transitions in the Ha-ras gene in C3H/He mice,(16) AT-GC and GC-AT transitions and GC-TA transitions in the K-ras gene in A/J mice(17) and AT-GC transitions in the H-ras gene in B6C3F1 mice.(18) The discrepancy in the mutation spectra between these studies and our results could be due to hotspots of examined genes and results of selective amplification. As endogenous ras genes are functionally expressed in rats, mutations responsible for clonal expansion would be selectively amplified accompanying tumorigenesis. However, the gpt transgene is not expressed in rats, so that it is genetically neutral, which avoids selective pressure in vivo.(10) Thus, gpt assays can detect a wide spectrum of mutations of the gpt transgene that result in loss of its enzymatic activity in E. coli. While gene mutation assays are only available in gpt delta rats, the equivalent formation of procarcinogenic GST-P-positive foci between gpt delta and WT rats implied that similar levels of genotoxicity were induced in both gpt delta and WT rats following administration of DEN. In the DEHP treatment group, mutation frequencies of control groups were much higher than that of DEN treatment. The reason for the difference would be that these experiments were independently carried out by different people. We think it is important that the constant protocol and criteria were kept in each experiment so that we are able to compare to mutation frequencies within respective experiments.

Collectively, these results suggested that gpt delta rats (both F344 and SD strains) showed comparable general toxicity responses, including the presence of preneoplastic liver lesions and genotoxicity, to WT rats in response to DEN and DEHP treatment. Therefore, these data supported the validity of the combined assay to detect both general toxicity and genotoxicity simultaneously.

In this study, we also examined whether these toxicities could be detected after short durations of administration. Both the general toxicity of 1.2% DEHP and the genotoxicity of 10 p.p.m. DEN could be detected after 2 weeks of administration, suggesting that the combined assay using gpt delta rats was capable of detecting both general toxicity and genotoxicity.
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Supporting Information
Additional supporting information may be found in the online version of this article:

Fig. S1. Body weight gain of F344/gpt delta rats treated with 0 or 10 p.p.m. N-nitrosodiethylamine (DEN) in the additional experiment.

Fig. S2. Immunohistochemical analysis of GST-P-positive foci in the livers of F344/gpt delta rats treated with 0 or 10 p.p.m. of N-nitrosodiethylamine (DEN) for 8 weeks in the additional experiment.

Table S1. Relative organ weights of F344/wild type (WT), F344 gpt delta, Sprague–Dawley (SD)/WT, and SD/gpt delta rats treated with N-nitrosodiethylamine (DEN). For 8 weeks in the additional experiment.

Table S2. Relative organ weights of F344/wild type (WT), F344 gpt delta, Sprague–Dawley (SD)/WT, and SD/gpt delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S3. Serum biochemistry of F344/wild type (WT), F344 gpt delta, Sprague–Dawley (SD)/WT, and SD/gpt delta rats treated with N-nitrosodiethylamine (DEN).

Table S4. Serum biochemistry of F344/wild type (WT), F344 gpt delta, Sprague–Dawley (SD)/WT, and SD/gpt delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S5. Frequency of mutations in the gpt gene in the livers of F344/gpt delta rats treated with N-nitrosodiethylamine (DEN).

Table S6. Frequency of mutations in the gpt gene in the livers of Sprague–Dawley (SD)/gpt delta rats treated with N-nitrosodiethylamine (DEN).
Table S7. Spi− mutant frequencies in the livers of F344/gpt delta rats treated with N-nitrosodiethylamine (DEN).
Table S8. Spi− mutant frequencies in the livers of Sprague–Dawley (SD)/gpt delta rats treated with N-nitrosodiethylamine (DEN).
Table S9. Mutation spectrum of the gpt gene in livers of F344/gpt delta rats treated with N-nitrosodiethylamine (DEN).
Table S10. Mutation spectrum of the gpt gene in livers of Sprague–Dawley (SD)/gpt delta rats treated with N-nitrosodiethylamine (DEN).
Table S11. Frequency of mutations in the gpt gene in livers of F344/gpt delta rats treated with di(2-ethylhexyl)phthalate (DEHP).
Table S12. Frequency of mutations in the gpt gene in livers of Sprague–Dawley (SD)/gpt delta rats treated with di(2-ethylhexyl)phthalate (DEHP).
Table S13. Spi− mutant frequencies in livers of F344/gpt delta rats treated with di(2-ethylhexyl)phthalate (DEHP).
Table S14. Spi− mutant frequencies in livers of Sprague–Dawley (SD)/gpt delta rats treated with di(2-ethylhexyl)phthalate (DEHP).
Table S15. Mutation spectrum of the gpt gene in livers of F344/gpt delta rats treated with di(2-ethylhexyl)phthalate (DEHP).
Table S16. Mutation spectrum of the gpt gene in livers of Sprague–Dawley (SD)/gpt delta rats treated with di(2-ethylhexyl)phthalate (DEHP).