**Table 4** Difference in the $C_T$ of real-time amplification in the microdissected samples from those of the lower limit of amplification in the standard samples[a]

| | Tissue area (μm in radius) | Corresponding cell numbers[b] | No. of samples | Difference in $C_T$[c] | CV |
|---|---|---|---|---|---|
| Gene | | | | | |
| CYP2B1 | 100 × 4 pieces | 208 | 6 | 3.9 ± 1.3[d] | 33.3 |
| | 100 × 2 pieces | 104 | 9 | 2.8 ± 2.0 | 71.4 |
| | 100 × 1 piece | 52 | 8 | 1.6 ± 1.5 | 93.8 |
| | 50 × 1 piece | 13 | 16 | −1.7 ± 1.7 | Not available |
| | 30 × 1 piece | 5 | 6 | −2.1 ± 2.5 | Not available |
| GAPDH | 100 × 4 pieces | 208 | 5 | 7.9 ± 0.4 | 5.1 |
| | 100 × 2 pieces | 104 | 10 | 5.3 ± 1.5 | 28.3 |
| | 100 × 1 piece | 52 | 9 | 1.9 ± 2.1 | 110.5 |
| | 50 × 1 piece | 13 | 14 | 1.5 ± 1.9 | 126.7 |
| | 30 × 1 piece | 5 | 5 | −2.4 ± 1.3 | Not available |

[a]Liver tissue of a rat treated with PB at 80 mg/kg body weight/day for 3 days was used for both standard and microdissected samples. Unfixed frozen tissue was used for standard and hematoxylin-stained 10-μm-thick sections were subjected to analysis. With 5 μl of total RNA extracted by RNAqueous Micro, one-step RT-PCR was performed on GAPDH with the TaqMan probe detection system and CYP2B1 with the SYBR Green detection system.
[b]Cell numbers in each sample were calculated from the mean liver cell numbers in the circle area of 100 μm radius (52 ± 3; $n$=10).
[c]Values were calculated by subtracting the $C_T$ of each sample from the $C_T$ of the standard sample showing the lower limit of amplification. Expressed as mean ± SD.
[d]Amount of total RNA and its $C_T$ (mean ± SD) of the standard sample at the lower limit of amplification within the dynamic range was respectively 0.4 pg and 32.4 ± 1.3 cycles for CYP2B1 ($n$=3), and 0.4 pg and 38.1 ± 0.9 cycles for GAPDH ($n$=4).

tion of the integrity and yield of extracted DNA (Uneyama et al. 2002). Hematoxylin has been reported to influence divalent cations ($Mg^{2+}$) that are important for maintaining Taq DNA polymerase activity (Chen et al. 1996), and this was reported to be apparent when manually dissected large tissue samples are subjected to PCR analysis (Burton et al. 1998; Murase et al. 2000). However, such an inhibitory effect might be negligible when microdissected small tissue specimens are analyzed (Ehrig et al. 2001). In line with the present study results, hematoxylin staining did not affect RT-PCR when microdissected small tissue specimens were analyzed in a previous study (Imamichi et al. 2001).

The cell number required for mRNA expression analysis in microdissected tissue specimens is primarily dependent on the expression level of the target genes of interest. In the case of cyclin D1 in primary tumor tissues, Specht et al. (2002) reported that transcripts could be measured in a minimum of 20 microdissected tumor cells from formalin-fixed PET specimens with an improved extraction protocol and the TaqMan PCR method in combination, but they also found 2000 cells to be suitable to obtain reproducible real-time PCR results. Although the expression values did not greatly vary (CV <20) from 100 pg of total RNA in the present experiment examining relative abundance of amplifiable mRNAs (Table 2), ~200 cells (corresponding to 2 ng based on the RNA yield data in Table 5) can be considered as a minimum for the practical expression analysis of mRNA species, judging from the very small variation of difference in $C_T$ values of GAPDH gene with the corresponding tissue size (100 μm in radius × 4 pieces) reflecting homogeneity in the expression between samples as well as very small technical variation (Table 4). Expression levels of CYP2B1 varied between samples, even with a tissue area of 208 cells. With PB treatment, graded expression of CYP2B1 in the liver lobule occurs in the rat, with pronounced induction in the periportal region (Bühler et al. 1992). In the present study, midzonal areas of hepatic lobules were subjected to analysis, and therefore the variability of CYP2B1 expression in each sample might rather reflect a local event due to the graded expression profile of the transcript within this area.

In the present study, the concentration of total RNA was measured by RiboGreen fluorescent dye, with 1 ng as the lower detection limit in a 1-ml assay volume, and we could obtain ~1 ng of total RNA from 100 microdissected liver cells. If normalization of mRNA expression level to the input amount of total RNA is intended, a total of 3 ng or more (corresponding to >300 cells in a 10-μm-thick section of the rat liver)

**Table 5** Total RNA yields in microdissected unit areas of methacarn-fixed rat liver PET sections[a]

| Microdissected area[b] | No. of samples | RNA yield (ng/tissue) |
|---|---|---|
| 1000 × 1000 μm (1669 cells)[c] | 5 | 34.1 ± 5.81 |
| 500 × 500 μm (417 cells) | 4 | 5.8 ± 2.89 |
| 250 × 250 μm (104 cells) | 5 | 1.3 ± 0.16 |

[a]Liver of a rat treated with PB at 80 mg/kg body weight/day for 3 days. Total RNA (20 μl) extracted with RNAqueous-Micro was subjected to determination of RNA yield by RiboGreen RNA Quantitation kit.
[b]Tissue sections 10 μm thick were stained with hematoxylin before microdissection.
[c]Numbers of cells contained in each area were calculated from the mean cell numbers in the circle area of 100 μm radius as estimated in Table 4.

would be necessary for measurement of RNA concentration (1 ng) and after real-time RT-PCR of several genes (2 ng) under the present experimental conditions. If a fluorescence microplate reader is available, the total volume for assay of total RNA concentration could be minimized. As another normalization method, the expression level of a reference gene can be used. Housekeeping genes, such as GAPDH in the present study, are usually selected for this purpose. However, disadvantages with a single housekeeping gene were recently reported (Lee et al. 2002; Tricarico et al. 2002), and sexual dimorphism exists in GAPDH expression in several brain regions of developing rats (Perrot-Sinal et al. 2001). Sexual dimorphism of ERα expression in MPOA was apparent here, irrespective of the normalization method.

In conclusion, we have now demonstrated that methacarn-fixed PET allows practical mRNA expression analysis in microdissected areas with real-time RT-PCR after hematoxylin staining. Although recent studies have also demonstrated good performance of mRNA expression measurement with microdissected formalin-fixed PETs (Specht et al. 2001,2002), formaldehyde causes modification of nucleotides and therefore a high frequency of non-reproducible sequence alteration and amplification of only short fragments (reviewed by Srinivasan et al. 2002), suggesting limited utility for molecular analysis. Considering the availability for both DNAs and proteins in PET sections (Shibutani et al. 2000; Shibutani and Uneyama 2002; Uneyama et al. 2002), methacarn should prove to be a versatile tool for multipurpose analysis of target genes in specific cell populations. We are now applying methacarn for global gene expression analysis using a microarray technique with microdissected PET specimens. The question of how long molecules are retained intact in methacarn-fixed PET should now be addressed. Although we do not have data for archival tissues stored for several years/decades, mRNA levels could be measured with methacarn-fixed PET that had been prepared 6 months previously in the present study.

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# Dose-related changes of oxidative stress and cell proliferation in kidneys of male and female F344 rats exposed to potassium bromate

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**It is still of importance to investigate renal carcinogenesis by potassium bromate ($KBrO_3$), a by-product of water disinfection by ozonation, for assessment of the risk to man. Five female F344 rats in each group were given $KBrO_3$ at a dose of 300 mg/kg by single i.g. intubation or at a dose of 80 mg/kg by single i.p. injection, and were killed 48 h after the administration for measurements of thiobarbituric acid-reactive substances (TBARS) and 8-oxodeoxyguanosine (8-oxodG) levels in the kidney. Both levels in the treated animals were significantly elevated as compared with the control values. In a second experiment, 5 male and female F344 rats in each group were administered $KBrO_3$ at concentrations of 0, 15, 30, 60, 125, 250 and 500 ppm in the drinking water for 4 weeks. $KBrO_3$ in the drinking water did not elevate TBARS in either sex at any of the doses examined, but 8-oxodG formation in both sexes at 250 ppm and above was significantly higher than in the controls. Additionally, the bromodeoxyuridine-labeling index for proximal convoluted tubules was significantly increased at 30 ppm and above in the males, and at 250 ppm and above in the females. α2u-Globulin accumulation in the kidneys of male rats was increased with statistical significance at 125 ppm and above. These findings suggest that DNA oxidation induced by $KBrO_3$ may occur independently of lipid peroxidation and more than 250 ppm $KBrO_3$ in the drinking water can exert a carcinogenic effect by way of oxidative stress. (Cancer Sci 2004; 95: 393–398)**

Potassium bromate ($KBrO_3$) was at one time widely used as a maturing agent for flour and as a dough conditioner.[1] It was, however, demonstrated to induce renal cell tumors in male and female F344 rats after oral administration for 2 years in the drinking water[2] and the use of $KBrO_3$ as a food additive is now limited or prohibited, so that exposure of humans via food is very low.[3] Nevertheless, there is still concern regarding this chemical in the environment. In order to avoid the formation of trihalomethanes, major by-products in the process of drinking water chlorination[4] that are carcinogenic in rodents,[5] ozone disinfection has been proposed as an alternative method.[6] However, it has been shown that ozonation of surface water can generate bromate as one of various by-products in treated drinking water,[7] implying a potential hazard.

$KBrO_3$ has been classified as a genotoxic carcinogen based on positive mutagenicity in the Ames,[8] chromosome aberration[9] and micronucleus tests.[10] It has the potential to induce 8-oxodeoxyguanosine (8-oxodG) formation both *in vitro* and *in vivo*,[11–14] and since ribo- and deoxyribonucleosides of 8-oxodG induce sister chromatid exchange in human lymphocytes[15] and 8-oxodG pairs with adenine as well as cytosine, generating GC-to-TA transversion upon replication by DNA polymerases,[16] it has been postulated that this oxidized base is responsible for the mutagenicity and carcinogenicity.[17,18] The formation of oxidized base also indicates that the intra-nuclear redox status is altered in an oxidative direction, and this may lead to the induction of aberrant transcriptional events. However, except for our previous paper,[19] we know of no data showing a direct correlation between actual carcinogenic doses and 8-oxodG formation in kidney DNA. Likewise, although it has been proposed that reactive free radicals resulting from the oxidizing property of $KBrO_3$ also attack membrane lipids to induce cellular lipid peroxidation (LPO) in male rats,[20,21] it remains uncertain whether LPO indeed occurs concomitantly with DNA oxidation during carcinogenesis. In view of the possible role of various reactive aldehydes as end products of LPO in tumorigenesis,[22,23] it is necessary to assess their participation in $KBrO_3$ carcinogenesis.

A two-stage model using *N*-ethyl-*N*-hydroxyethyl-nitrosamine (EHEN) as an initiator has supplied clear evidence that $KBrO_3$ has promoting activity for renal carcinogenesis in male and female rats.[24,25] We have also shown that numbers of bromodeoxyuridine (BrdU)-incorporating cells in kidney tubules are elevated in male and female rats exposed to $KBrO_3$ at a dose of 500 ppm in the drinking water.[19,26] While we have hypothesized the involvement of oxidative stress induced by $KBrO_3$ in the promoting activity, our previous data also suggest that the promoting action observed in male rats may be dependent on cell proliferation due to accumulation of α2u-globulin, a male rat specific urinary protein.[26] Elimination of this possibility as a factor contributing to $KBrO_3$ promoting activity is a prerequisite for accurate assessment of the carcinogenic risk in humans.

In the present study, in order to confirm a positive correlation between oxidized DNA base formation and occurrence of LPO, we measured the levels of 8-oxodG and thiobarbituric acid-reactive substances (TBARS) in kidneys of F344 female rats given $KBrO_3$ by single administration at high doses. Secondly, we examined the dose-response effects with reference to 8-oxodG levels, TBARS, BrdU-labeling and α2u-globulin accumulation in kidney, as well as serum creatinine (CRN) level, of male and female rats, employing the same doses and route as used in the previous carcinogenicity tests and promoting activity assays. The aim was to clarify the possibility that LPO and oxidative DNA damage participate in $KBrO_3$ initiation and to cast light on the effects of oxidative stress in the promotion phase.

## Materials and Methods

**Chemicals.** $KBrO_3$ was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Alkaline phosphatase and BrdU were obtained from Sigma Chemical Co. (St. Louis, MO) and nuclease P1 was from Yamasa Shoyu Co. (Chiba).

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**Animals, diet and housing conditions.** The protocols for this study were approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Five-week-old male and female F344 rats (specific pathogen-free) were purchased from Charles River Japan (Kanagawa) and housed in polycarbonate cages (5 rats per cage) with hardwood chips for bedding in a conventional animal facility maintained under conditions of controlled temperature (23±2°C), humidity (55±5%), air change (12 times per h) and lighting (12 h light/dark cycle). The animals were given free access to CRF-1 basal diet (Charles River Japan) and tap water, and were used after a 1-week acclimation period.

**Animal treatments.**

Experiment I: Five female rats in each group were given $KBrO_3$ at a single dose of 300 mg/kg by i.g. administration or 80 mg/kg by i.p. injection. Control animals received saline at the same volume as the i.p. administration group. All animals were killed 48 h after the administration under ethyl ether anesthesia, and the right and half of the left kidneys were immediately removed and frozen with liquid nitrogen and stored at −80°C until measurement of 8-oxodG in nuclear DNA and TBARS levels. The remaining kidney tissue was fixed in buffered formalin and then routinely processed for embedding in paraffin, sectioning and H&E staining. The doses and experimental period followed reported conditions under which the 8-oxodG and TBARS levels in kidney were significantly increased.[27]

Experiment II: Five male and female rats in each group were administered $KBrO_3$ solution at concentrations of 0, 15, 30, 60, 125, 250 and 500 ppm in the drinking water for 4 weeks. All animals were injected with BrdU (100 mg/kg) i.p. twice a day for the final 2 days of the exposure and once on the day of termination, 2 h before killing. For analysis of CRN, the animals were anesthetized with ethyl ether and blood was collected from the aorta. Determination of CRN was carried out at SRL, Inc. (Tokyo). At necropsy, the right kidneys were fixed in ice-cold acetone for 3 days and processed for embedding in paraffin, sectioning (4 μm), and immunostaining for BrdU after histochemical demonstration of γ-glutamyltranspeptidase (γ-GT) activity. The left kidneys were frozen and stored as in Experiment I until measurement of 8-oxodG in nuclear DNA, and TBARS levels and α2u-globulin contents in the homogenates.

**Measurement of nuclear 8-oxodG.** The 8-oxodG levels in kidney DNA were determined according to the method of Nakae *et al.*[28] Nuclear DNA was extracted with a DNA Extracter WB Kit (Wako Pure Chemical Industries, Ltd., Osaka). The DNA was digested to deoxynucleotides with nuclease P1 and alkaline phosphatase and levels of 8-oxodG (8-oxodG/$10^5$ deoxyguanosine) were assessed by high-performance liquid chromatography (HPLC) with an electrochemical detection system (Coulochem II, ESA, Bedford, MA).

**Measurement of TBARS.** Malondialdehyde (MDA, nmol/g) was assessed as an index of LPO by the method of Uchiyama and Mihara.[29] A 0.1 g portion of kidney was homogenized with 0.9 ml of 1.15% KCl solution and the TBARS content was measured.

**α2u-Globulin content.** α2u-Globulin accumulation in kidneys was measured using a commercially available ELISA kit (Quatikinine M, R&D Systems, Inc., MN). Absorbance at 450 nm was determined using a microplate reader (Thermo Labsystems, Vantaa, Finland), with the reference wavelength set at 590 nm.

**Immunohistochemical procedures.** For immunohistochemical staining of BrdU, sections were treated sequentially with normal horse serum, monoclonal mouse anti-BrdU (Becton Dickinson) (1:100), biotin-labeled horse anti-mouse IgG (1:400) and avidin-biotin-peroxidase complex (ABC) after denaturation of DNA with 4 *N* HCl. Before the denaturation step, sections were processed histochemically for demonstration of γ-GT activity by the method of Rutenburg *et al.*[30] using L-glutamyl-4-methoxy-β-naphthylamide (Polysciences, Ltd., Warrington, PA) as a substrate in order to assist in distinguishing the three kinds of tubules, as previously described.[26] The sites of peroxidase binding were demonstrated by incubation with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). The immunostained sections were lightly counterstained with hematoxylin for microscopic examination.

**Cell proliferation quantification.** Cells of the three kinds of tubules in the kidney were identified on the basis of γ-GT activity and morphology as previously described.[31] At least 3000 tubular cells in each kidney were counted. The labeling index (LI) was calculated as the percentage of cells positive for BrdU incorporation.

**Statistics.** The significance of differences in the results from Experiment I was evaluated with Student's *t* test. For Experiment II ANOVA was used, followed by Dunnett's multiple comparison test.

## Results

**Experiment I.** The data for 8-oxodG and TBARS levels in kidneys of female rats given $KBrO_3$ by single administration at doses of 300 mg/kg (i.g.) or 80 mg/kg (i.p.) are summarized in Fig. 1. Values for both parameters were significantly ($P<0.01$) elevated as compared with the controls, in line with previous data. Histopathological examination revealed severe nephrotoxicity characterized by hemorrhage and protein diapedesis in Bowman's capsule, accumulation of hyaline droplet-like material and basophilic alteration in proximal tubules, and extensive necrosis of collecting ducts (Fig. 6, A and B).

**Experiment II.** As shown in Fig. 2, $KBrO_3$ in the drinking water did not cause elevation of TBARS in kidneys of either sex at any of the doses examined. However, 8-oxodG levels in male

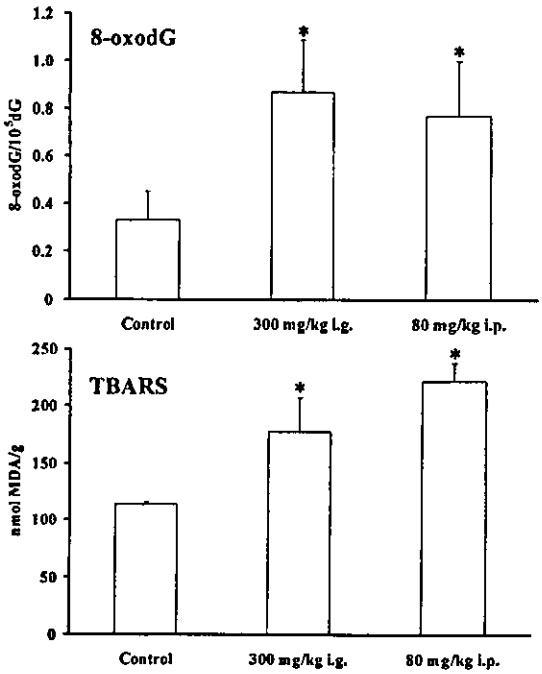


Fig. 1. 8-OxodG and TBARS levels in kidneys of female rats 48 h after single i.g. or i.p. administration of $KBrO_3$ at the dose of 300 or 80 mg/kg, respectively. Values are means±SD of data for 5 rats. Significantly different (* $P<0.01$) from the control group treated with saline alone.

rats exposed to $KBrO_3$ in the drinking water were elevated at concentrations of 250 ppm and above in a clearly dose-dependent manner (250 ppm, 0.57±0.19/$10^5$ dG, $P<0.01$; 500 ppm, 0.71±0.21/$10^5$ dG, $P<0.01$) as compared to the control value (0.31±0.06/$10^5$ dG). Likewise, 8-oxodG levels in female rats were 0.51±0.10/$10^5$ dG at 250 ppm and 0.70±0.16/$10^5$ dG at 500 ppm, which were statistically significantly higher ($P<0.01$) than the control value (0.25±0.05/$10^5$ dG). Histopathologically, although degeneration of proximal tubules was dose-dependently observed in the males at 60 ppm and above, there were no overt nephrotoxicity in the females at any of the doses examined. Fig. 3 illustrates changes in BrdU-LI for each tubule type in male and female rats treated with $KBrO_3$ in the drinking water at concentrations of 0, 15, 30, 60, 125, 250 and 500 ppm for 4 weeks. BrdU-LIs of proximal convoluted tubular cells (PCT) in the males were elevated in a dose-dependent manner, with significant increases at 30 ppm (1.69±0.32%, $P<0.01$), 60 ppm (2.67±0.45%, $P<0.01$), 125 ppm (4.23±0.80%, $P<0.01$), 250 ppm (6.11±2.23%, $P<0.01$) and 500 ppm (9.10±1.40%, $P<0.01$), as compared to the control value (0.87±0.32%). In the females, although there was no change up to 125 ppm, dose-dependent increase was subsequently observed to 1.29±0.39% at 250 ppm and 2.22±0.37% at 500 ppm, both of which were statistically siginificant ($P<0.01$) as compared to the control value (0.59±0.14%) (Fig. 6C). On the other hand, no change in BrdU-LIs for other tubules was found at any dose in either sex. Fig. 4 summarizes data for α2u-globulin accumulation in kidneys of male and female rats given $KBrO_3$ in the drinking water. In the males, increase was evident at 30 ppm and above in a dose-dependent fashion, the elevation being statistically significant at 125 ppm (1.37±0.18 mg/ml, $P<0.01$), 250 ppm (1.96±0.24 mg/ml, $P<0.01$) and 500 ppm (3.50±0.26 mg/ml, $P<0.01$) as compared to the control value (0.80±0.16 mg/ml). In contrast, α2u-globulin contents in the females were much lower than those in the males and were not changed by $KBrO_3$ exposure. Fig. 5 shows the changes of serum CRN levels in rats of both sexes given $KBrO_3$ in the drinking water. In contrast to the male data, revealing a slight, but statistically significant elevation at 250 ppm and above, there was no change among the female groups.

## Discussion

It is generally accepted that oxygen radicals can attack DNA to produce damaged bases, including 8-oxodG, and/or initiate the oxidative decomposition of cellular membranes by LPO,[23]

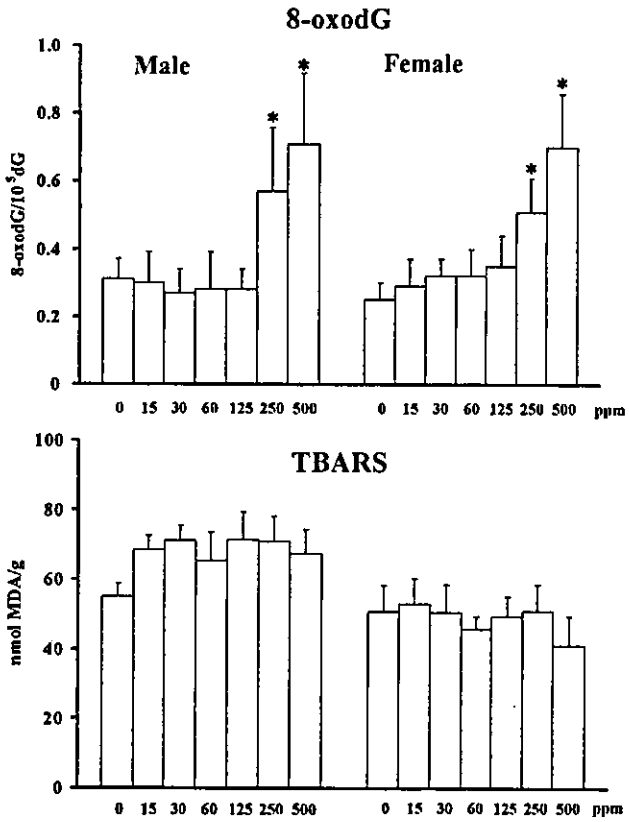


Fig. 2. 8-OxodG and TBARS levels in kidneys of male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Significantly different (* $P<0.01$) from the control group (0 ppm).

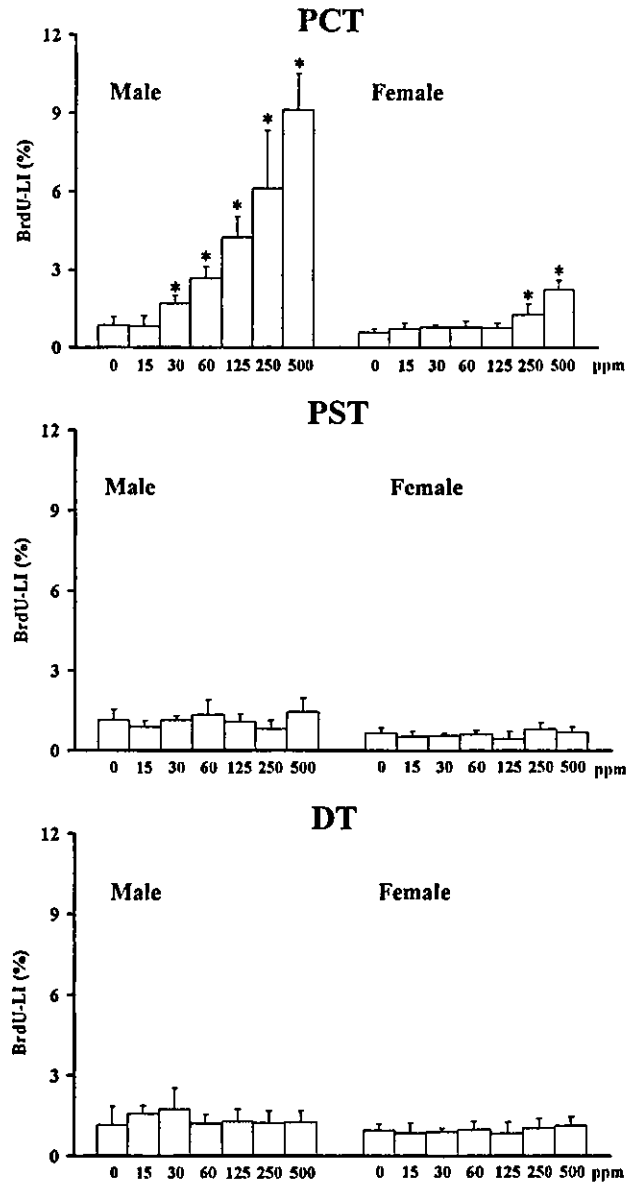


Fig. 3. BrdU-LIs for the proximal convoluted, straight and distal tubules (PCT, PST, DT) of male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Significantly different (* $P<0.01$) from the control group (0 ppm).

which not only act as intermediates for free radical chain reactions, but also generate various reactive aldehydes, such as malondialdehyde and *trans*-4-hydroxy-2-nonenal, which directly form exocyclic DNA adducts.[22,32] A single exposure of male rats to $KBrO_3$ at high doses causes an increase of TBARS along with 8-oxodG formation,[27] which was also confirmed in the present study using female rats. However, exposure to carcinogenic doses in the drinking water failed to increase TBARS, in spite of the elevation of 8-oxodG levels. Another group has also reported that a single dose of $KBrO_3$ at a low dose did not elevate etheno-DNA adducts formation or TBARS levels in the kidneys of male rats.[21] In the light of the finding of no initiating activity of $KBrO_3$ with a single i.g. administration at 300 mg/kg,[33] our present data indicate that LPO might not be involved in the renal carcinogenesis due to this compound. Instead, histological findings in the present study suggest an involvement of LPO in the nephrotoxicity induced by $KBrO_3$. It has recently been reported that reduction of $KBrO_3$ by sulfhydryl compounds such as glutathione and cysteine yields bromine oxides and bromine radicals, which can effectively oxidize guanine.[34] A large amount of cysteine is supplied as a result of metabolism of glutathione by γ-glutamyltransferase on the proximal tubule brush borders,[35] where $KBrO_3$ reduction might give rise to bromine oxides. Since they are stable in comparison with radicals, they might move into the nuclei, where further reduction could generate bromine radicals in close proximity to nuclear DNA, leading to formation of 8-oxodG without any necessity for intervention of cellular LPO.

Kurokawa *et al.* earlier reported significantly elevated incidences of renal cell tumors in male and female rats given $KBrO_3$ at 250 and 500 ppm in the drinking water for 110 weeks.[2] A further dose-response study using only male rats showed 125 ppm to also be a carcinogenic dose.[36] However, a recent study by another group demonstrated that while $KBrO_3$

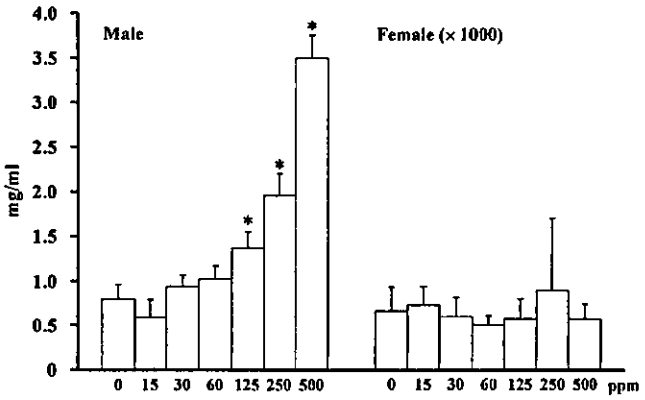


Fig. 4. α2u-Globulin levels in kidneys of male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Note the values for females are μg/ml. Significantly different (* $P<0.01$) from the control group (0 ppm).

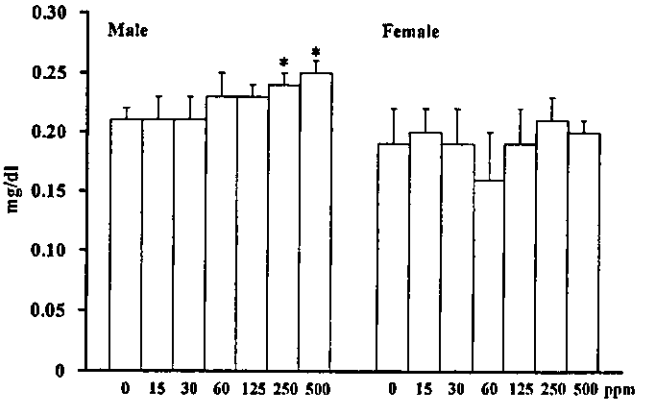


Fig. 5. Serum CRN levels in male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Significantly different (*, ** $P<0.05$, 0.01) from the control group (0 ppm).

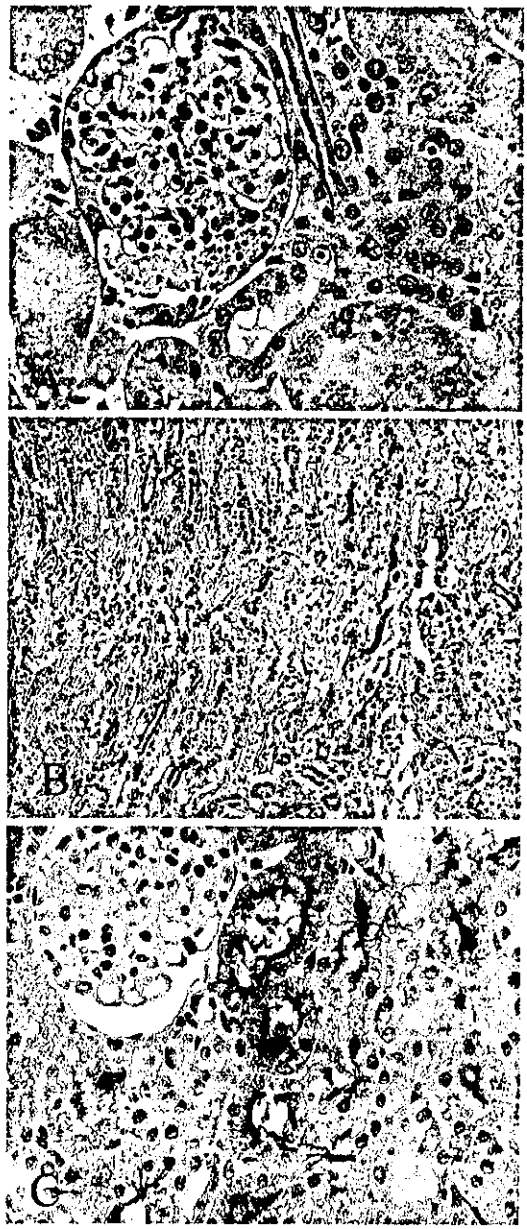


Fig. 6. (A) Renal cortex of a female rat treated with $KBrO_3$ at 80 mg/kg by single i.p. injection. Note hemorrhage and protein diapedesis in Bowman's capsule, accumulation of hyaline droplet-like material and basophilic alteration in proximal tubules. H&E staining at ×720 original magnification. (B) Renal medulla of a female rat treated with $KBrO_3$ at 80 mg/kg by single i.p. injection. Note extensive necrosis of collecting ducts. H&E staining at ×180 original magnification. (C) Renal cortex of a female rat treated with $KBrO_3$ at 500 ppm in drinking water for 4 weeks. BrdU-positive cells were seen in PCT (positive enzymatic reaction for γ-GT), but not in DT (negative enzymatic reaction for γ-GT). γ-GT-BrdU immunohistochemical staining at ×720 original magnification.

at 400 ppm in the drinking water was able to induce tumors in male rats with significant incidences, this was not the case with 200 ppm.[37] For the present, it seems equivocal whether 125 ppm has a carcinogenic potential. Accordingly, the present demonstration of increased 8-oxodG formation in kidney DNA of male and female rats given $KBrO_3$ at 250 and 500 ppm, but not at 125 ppm and below, seem to be in accordance with the carcinogenic data. In addition, the fact that $KBrO_3$-induced renal cell tumors originate from the proximal tubules[37] allows us to hypothesize that oxidative stress participates in the carcinogenesis. In a previous carcinogenicity study, the mean induction time for tumors in males was much shorter than in females,[2] but there was no sex difference with regard to doses inducing 8-oxodG formation in the present study. Therefore, variation in the tumor latency period might be explained by differential susceptibility to $KBrO_3$-induced cell proliferation, rather than oxidative stress.

In the two-stage rat renal carcinogenesis model using EHEN as an initiator, promoting activity of $KBrO_3$ was apparent in both sexes of rats.[24,25] In particular, in males, a dose of 30 ppm in the drinking water was sufficient for development of dysplastic foci from initiated cells. We also showed, in the present study, that $KBrO_3$ at the same dose was able to cause degeneration and increase of BrdU-LI in the PCT in the males. α2u-Globulin accumulation in the kidney of male rats exposed to $KBrO_3$ was also observed in a dose-dependent manner at 30 ppm and above, even though the increases at 30 and 60 ppm were not statistically significant. It has been established that this is associated with eventual cell death and subsequent cell proliferation.[38] Despite negative mutagenicity,[39,40] exposure to this kind of chemical can lead to renal cell tumors in male rats, which implies that α2u-globulin-mediated cell proliferation might be sufficient for tumor development.[41] Thus, it is highly probable that $KBrO_3$-induced cell proliferation in PCT and subsequent tumor-promoting activity observed in males might involve α2u-globulin accumulation. However, the finding that $KBrO_3$ exposure of female rats also increases BrdU-LI in PCT at doses of 250 and 500 ppm in spite of the absence of α2u-globulin indicates an involvement of some other mechanism. Considering that $KBrO_3$ might be reduced to form more reactive species at PCT,[34] the good correlation between the doses inducing 8-oxodG formation and elevation of BrdU-LI enables us to hypothesize that the cell proliferation observed in female rats might result from oxidative stress.[19,25] Since the histopathological findings and serum biochemical parameters indicate no obvious nephrotoxicity in female rats treated with $KBrO_3$ in the drinking water at any dose tested, oxidative stress might act via mitogenic stimulation.[42–44]

Judging from the female data, it appears that the cell proliferation observed in male rats at 125 ppm and below might be attributed to α2u-globulin accumulation and not to oxidative stress. In other words, the increase at 250 ppm and above in the males might reflect the combined effects of the two. For risk assessment of $KBrO_3$ in the human situation, it is essential to focus on oxidative stress and to ignore α2u-globulin-mediated effects.[45] The overall data allow us to hypothesize that more than 250 ppm of $KBrO_3$ in the drinking water is able to exert both initiating and promoting activities in the kidney of rats of both sexes by means of the generated oxidative stress. Long-term studies now appear warranted for confirmation.

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MOLECULAR CARCINOGENESIS 42:9–17 (2005)

# In Vivo Mutational Analysis of Liver DNA in *gpt* Delta Transgenic Rats Treated With the Hepatocarcinogens *N*-Nitrosopyrrolidine, 2-Amino-3-Methylimidazo[4,5-*f*]Quinoline, and Di(2-Ethylhexyl)Phthalate

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In order to cast light on carcinogen-specific molecular mechanisms underlying experimental hepatocarcinogenesis in rats, in vivo mutagenicity and mutation spectra of known genotoxic rat hepatocarcinogens *N*-nitrosopyrrolidine (NPYR), and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), as well as the nongenotoxic hepatocarcinogen di(2-ethylhexyl)phthalate (DEHP) and the noncarcinogen acetaminophen (AAP), were investigated in guanine phosphoribosyltransferase (*gpt*) delta transgenic rats, a recently developed animal model for genotoxicity analysis. After 13-wk treatment, glutathione *S*-transferase placental form (GST-P)-positive liver cell foci were significantly increased in NPYR-treated and IQ-treated rats. In the DEHP-treated rats, marked hepatomegaly with centrilobular hypertrophy of hepatocytes occurred, although GST-P staining was consistently negative. Positive mutagenicity was detected in IQ- and NPYR-treated rats. Mutant frequencies (MFs) in the liver DNA were $188.0 \times 10^{-6}$ and $56.5 \times 10^{-6}$, approximately 35-fold and 10-fold higher, respectively, than that of nontreatment control rats ($5.5 \times 10^{-6}$). There were no increases in MFs in the DEHP- or AAP-treated rats as compared to the nontreatment control value. IQ induced mainly base substitutions leading to G:C to T:A transversions (56.9%) and deletions of G:C base pairs. In contrast, NPYR primarily caused specific A:T to G:C transitions (49.3%), which are very rare in the other groups. These data provided support for the conclusion that IQ and NPYR hepatocarcinogenesis depends on genotoxic processes and specific DNA adduct formation while DEHP exerts its influence via a nongenotoxic promotional pathway. Our data also indicate that analysis of specific in vivo mutational responses with transgenic animal models can provide crucial information for understanding the molecular mechanisms underlying chemical carcinogenesis. 

Key words: hepatocarcinogens; in vivo mutation assay; *gpt* delta rats

## INTRODUCTION

Environmental carcinogens are classified into genotoxic and nongenotoxic types based on in vitro bacterial mutagenicity. However, it is well-documented that in vitro mutagenicity does not always reflect in vivo mutagenicity and carcinogenicity in rodents [1–4]. The discrepancy between in vivo and in vitro models may result from organ-specific pathways of xenobiotic metabolism and DNA repair in vivo. To accomplish in vivo detection of gene mutations in multiple organs, transgenic rodents carrying reporter genes such as *lacI*, *lacZ*, and guanine phosphoribosyltransferase (*gpt*) have been developed [5]. These model animals can provide crucial information for understanding the in vivo mechanism of organ-specific mutagenesis induced by various carcinogens present in the human environment.

In spite of the toxicological importance of rat species, in vivo mutagenicity has been extensively studied in transgenic mice because of their availability. Recently, a novel transgenic rodent for genotoxicity analysis, named the *gpt* delta rat, has been developed [6]. Advantageous features allow positive detection of different types of mutations, including point mutations and deletions, as also shown with *gpt* delta mice [7]. Point mutations are

Abbreviations: *gpt*, guanine phosphoribosyltransferase; 6-TG, 6-thioguanine; NPYR, *N*-nitrosopyrrolidine; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; DEHP, di(2-ethylhexyl)phthalate; AAP, acetaminophen; MEHP, mono(2-ethylhexyl)phthalate; 8-OHdG, 8-hydroxydeoxyguanosine; MF, mutant frequency; GST-P, glutathione *S*-transferase placental form.

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detected by 6-thioguanine (6-TG) selection with the *gpt* gene of *E. coli* and deletion mutations are identified by Spi$^-$ selection with the *red/gam* genes of lambda phage [5]. In the present study, aimed at elucidating carcinogen-specific mutagenic mechanisms underlying experimental hepatocarcinogenesis, in vivo mutation spectrum of *N*-nitrosopyrrolidine (NPYR), a carcinogenic cyclic nitrosamine present in processed food and tobacco smoke in the human environment [8,9], was investigated in *gpt* delta rats. NPYR is metabolically activated by microsomal P450, and its metabolites have been shown to form guanine adducts mainly, due to simple alkylation in the in vitro system [10]. The in vitro mutagenicity is detected only in the presence of an activating system such as rat liver homogenate [11]. Although the bacterial systems with *lacI* gene and M13mp2 phage DNA have shown the predominant base substitutions at G:C base pair [12,13], the in vivo mutation spectrum of NPYR has not yet been determined. Therefore, with *gpt* delta rats, the present study aimed to elucidate the in vivo mechanism of mutagenesis induced by NPYR in comparison with the mutation spectra of other hepatocarcinogens. For this purpose, well-studied rodent hepatocarcinogens 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and di(2-ethylhexyl)phthalate (DEHP) were also subjected to the in vivo mutation assay. In addition, a nonmutagenic hepatotoxic compound, acetaminophen (AAP), was used as a negative control chemical for the mutagenicity assay.

Heterocyclic aromatic amines are the major class of genotoxic hepatocarcinogens in rodents, as well as *N*-nitroso compounds, which may be active in humans [14,15]. IQ is one of the most carcinogenic and mutagenic heterocyclic aromatic amine present in cooked-foods and cigarette smoke [14,16]. In rodents, it exerts multipotential carcinogenicity in various organs [17], including the liver of nonhuman primates [18]. Potent mutagenicity with DNA adduct formation has been shown both in vitro and in vivo [19] and mutational analysis conducted in BigBlue rats has revealed characteristic G:C transversions and 1 bp G:C deletions in the liver, colon, and kidney [20]. Thus, because of abundant background data of carcinogenicity and mutagenicity, IQ can be used as an appropriate standard mutagen for the mutational analysis.

DEHP is a widely used plasticizer for vinylchloride products, which has been found to cause liver tumors in rats and mice in long-term feeding assays [21]. Because no mutagenicity has been detected in in vitro mutagenicity tests [22,23], DEHP has been categorized as a nongenotoxic carcinogen. However, several studies have suggested possible mutagenicity of mono(2-ethylhexyl)phthalate (MEHP), a principal hydrolysis metabolite of DEHP, in the reverse mutation assay (*E. coli*) and the Rec$^-$ assay (*B. subtilis*) [24,25]. Moreover, significant increases of 8-hydroxydeoxyguanosine (8-OHdG), a premutagenic DNA adduct formed by oxidative stress, have been observed in hepatic DNA of rats treated with DEHP, suggesting the involvement of oxidative DNA damage in its hepatocarcinogenesis [26,27]. Thus, in the present study DEHP was included in order to clarify its in vivo mutagenicity in the *gpt* delta system, which can widely and efficiently detect both point mutations and deletions with two different types of selection.

Accumulated studies have suggested that the transgenic animals are useful models for evaluating carcinogenic risk and chemopreventive potential of environmental materials to which humans are exposed [28–30]. Moreover, analyses of mutation spectra provide important information for understanding the molecular mechanisms underlying chemical carcinogenesis [31]. In the present study, in vivo mutagenicity analyses of major classes of rodent hepatocarcinogens were, therefore, performed in *gpt* delta rats and their possible mechanisms of hepatocarcinogenesis are discussed.

## MATERIALS AND METHODS

### Animals and Treatments

Twenty-five female Sprague-Dawley *gpt* delta rats carrying about ten tandem copies of the transgene lambda EG10 per haploid genome obtained from Japan SLC (Shizuoka, Japan) were randomized by weight into five groups. They were housed in a room with a barrier system, and maintained under the following constant conditions: temperature of 24 ± 1°C, relative humidity of 55 ± 5%, ventilation frequency of 18 times/h, and a 12 h light-dark cycle with free access to Oriental MF basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water.

Starting at 11-wk of age the rats were treated with test chemicals or maintained as controls for 13 wk. Groups of five animals were fed IQ (Toronto Research Chemical, Inc., Ont., Canada), DEHP (Wako Pure Chemical, Osaka, Japan), and AAP (Sigma Chemical Co., St. Louis, MO) at doses of 300, 12000, and 10000 ppm in MF basal diet, respectively. Another five rats were given NPYR (Aldrich Chemical Co., Milwaukee, WI) dissolved in a small quantity of ethanol and diluted in their drinking water at a dose of 200 ppm. The five nontreatment control rats received MF basal diet alone and tap water. At the end of the experiment, all animals were killed, and a part of the left lateral lobe of the liver was preserved at −80°C for subsequent in vivo mutation assays. The rest of the lobes were fixed in 10% buffered formalin for histopathological examination. Immunostaining of glutathione *S*-transferase placental form (GST-P) was performed by using polyclonal anti-GST-P (MBL, Nagoya, Japan) as the primary antibody, and goat IgG raised against rabbit

IgG as the secondary antibody. The signals were amplified with ABC KIT (DAKO, Kyoto, Japan), and detected with 3,3'-diaminobenzidine (DAB). The numbers and areas of GST-P positive liver cell foci comprising ten or more cells were measured by using an Image Processor for Analytical Pathology (IPAP, Sumika Technos, Osaka, Japan).

### In Vivo Mutation Assays

The 6-TG and Spi$^-$ selections were performed as previously described [5]. Briefly, genomic DNA was extracted from the liver, and lambda EG10 DNA (48 kb) was rescued as the lambda phage by in vitro packaging. For 6-TG selection, the packaged phage was incubated with *E. coli* YG6020, which expresses Cre recombinase, and converted to a plasmid carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. In order to determine the total number of rescued plasmids, 3000-fold diluted phages were used to infect YG6020, and were poured on the plates containing chloramphenicol without 6-TG. The plates were incubated at 37°C for the selection of 6-TG-resistant colonies. Positively selected colonies were counted on d 3 and collected on d 4. The MF was calculated by dividing the number of *gpt* mutants after clonal correction by the number of rescued phages.

For the Spi$^-$ selection, the packaged phage was incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase soft agar and poured onto lambda-trypticase agar plates. Next day, plaques (Spi$^-$ candidates) were punched out with sterilized glass pipettes and the agar plugs were suspended in SM buffer. In order to confirm the Spi$^-$ phenotype of candidates, the suspensions were spotted on three types of plates where XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains were spread with soft agar. The real Spi$^-$ mutants, which made clear plaques on every plate, were collected and stored as phage lysates at 4°C. Approximate deletion sizes of the Spi$^-$ mutants were determined by agarose gel electrophoresis of the PCR-amplified target sequence.

For characterizing the mutation spectra of *gpt* mutants, a 739 bp DNA fragment containing the 456 bp coding region of the *gpt* gene was amplified by PCR as described previously [5]. DNA sequencing was performed with Big Dye™ Terminater Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA) on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems).

### Statistical Evaluation

For statistical analysis, the Student's *t*-test was used to compare liver and body weights, and quantitative data for GST-P positive liver cell foci and MFs between groups.

## RESULTS

### Growth of Animals, Liver Weights, and Chemical Intake

Data for final body and organ weights, and intake of test chemicals are shown in Table 1. The final body weight was not affected by any treatment during the experiment. Daily intakes of IQ, NPYR, and DEHP calculated from the consumption of diet or water were comparable to those in previous studies which showed significant carcinogenicity in rats [8,17,21]. Liver/body weight ratios were significantly ($P < 0.01$) increased in all rats that received chemicals, suggesting sufficient dosing. Especially, marked hepatomegaly was observed in the DEHP-treated rats ($P < 0.01$) with an increase to 183% of the nontreatment control value.

### Histopathology and Immunohistochemical Analysis of GST-P

Histopathologically altered, mostly clear, hepatocellular foci were frequently observed in the IQ-treated and NPYR-treated rats (Figure 1A and C).

Table 1. Body and Liver Weights, and Food, Water, and Chemical Intake Data

| Treatment | Number of rats | Body weight (g)[a] | Liver/body weight ratio (%)[a] | Food intake (g/rat/d) | Water intake (mL/rat/d) | Chemical intake | |
|---|---|---|---|---|---|---|---|
| | | | | | | Total (mg/rat) | Daily (mg/rat/d) |
| IQ | 5 | 264.6 ± 22.7 | 3.32 ± 0.11* | 14.3 | — | 362 | 4.0 |
| NPYR | 5 | 257.8 ± 10.9 | 3.25 ± 0.16* | 13.6 | 17.1 | 310 | 3.4 |
| DEHP | 5 | 279.3 ± 36.6 | 4.98 ± 0.28** | 14.0 | — | 17 010 | 187.0 |
| AAP | 5 | 266.5 ± 11.4 | 3.40 ± 0.29* | 15.6 | — | 12 740 | 140.0 |
| Control | 5 | 269.6 ± 27.0 | 2.72 ± 0.21 | 13.3 | 23.2 | — | — |

IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; NPYR, *N*-nitrosopyrrolidine; DEHP, di(2-ethylhexyl)phthalate; AAP, acetaminophen.
[a]Data are mean ± SD values.
*$P < 0.01$ (vs. control).
**$P < 0.01$ (vs. IQ, NPYR, AAP, and control).

GST-P-positive liver cell foci (Figure 1B and D) corresponding to such altered foci were significantly ($P < 0.01$ and $P < 0.05$) increased in the IQ-treated and NPYR-treated groups in terms of number as well as area, but few were observed in the DEHP-treated, AAP-treated, and untreated control rats (Table 2). NPYR at a dose of 200 ppm was much more effective at inducing GST-P-positive foci than IQ at a dose of

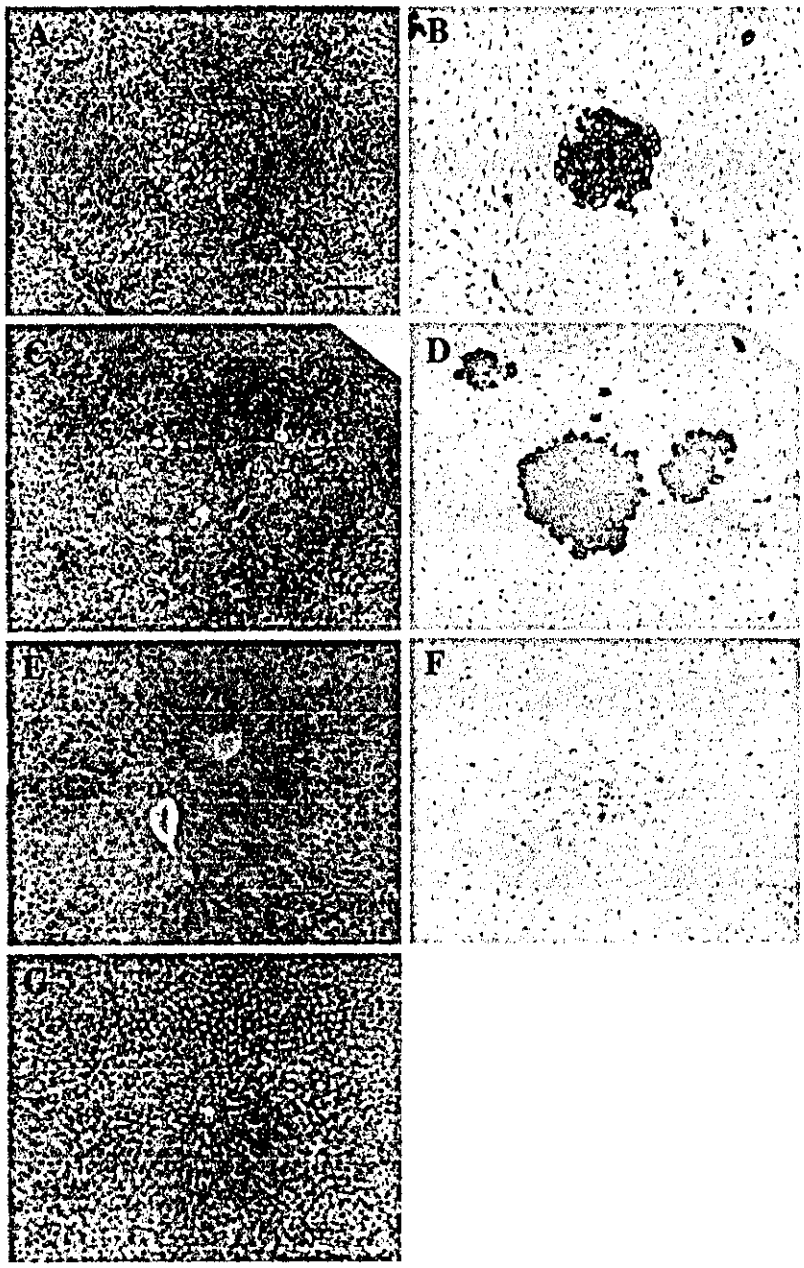


Figure 1. Histopathological findings (A, C, E, G) and immunohistochemistry for glutathione S-transferase placental form (GST-P) (B, D, F). Clear cell foci in the liver of rats given 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (A) and N-nitrosopyrrolidine (NPYR) (C). GST-P stainability corresponding to the foci A and C is evident (B and D). Marked centrilobular hypertrophy of liver cells is observed in a rat treated with di(2-ethylhexyl)phthalate (DEHP) (E). Faint and insignificant stainability of GST-P is found around the central veins of the liver of a rat treated with acetaminophen (AAP) (F). Appearance of the intact liver of a nontreated rat (G). Original magnification: ×180, Bar: 100 μm

Table 2. Numbers and Areas of GST-P Positive Liver Cell Foci

| Treatment | Number of rats | Dose (ppm) | Number of foci/cm$^2$[a] | Area of foci (mm$^2$/cm$^2$)[a] |
|---|---|---|---|---|
| IQ | 5 | 300 | 5.06 ± 1.52** | 0.15 ± 0.06** |
| NPYR | 5 | 200 | 24.05 ± 18.95*,*** | 1.09 ± 1.14* |
| DEHP | 5 | 12 000 | 0.25 ± 0.17 | <0.01 |
| AAP | 5 | 10 000 | 0.62 ± 0.44 | <0.01 |
| Control | 5 | — | 0.48 ± 0.46 | <0.01 |

[a]Data are mean ± SD values.
*$P < 0.05$ (vs. control).
**$P < 0.01$ (vs. control).
***$P < 0.05$ (vs. IQ).

300 ppm. Moreover, it was noteworthy that GST-P-positive single hepatocytes, which were not counted as the foci, were frequently observed in livers of the NPYR-treated rats. In the DEHP-treated rats, marked centrilobular hypertrophy of hepatocytes was observed throughout the whole liver (Figure 1E), although GST-P was consistently negative. In the AAP-treated rats, faint stainability for GST-P was detected in liver cells around the central veins, but these were not regarded as foci because of differences in staining intensity and diffuse location (Figure 1F).

### Mutation Assays

Data for *gpt* MFs analyzed by 6-TG selection are summarized in Table 3. Identical mutations occurring in the same individual were omitted to avoid possible overlapping due to clonal proliferation. In the IQ-treated and NPYR-treated rats, *gpt* MFs in the liver DNA after clonal correction numbered $188.0 \times 10^{-6}$ and $56.5 \times 10^{-6}$, approximately 35-fold and 10-fold higher, respectively, than the nontreatment control value ($5.5 \times 10^{-6}$). There were no increases of *gpt* MFs in the liver DNA of the DEHP-treated ($3.3 \times 10^{-6}$) and AAP-treated rats ($5.5 \times 10^{-6}$) as compared to the nontreatment control value.

The *gpt* mutation spectra were analyzed by sequencing the *gpt* gene amplified from the mutants (Table 4). Because several mutants had two mutations in the *gpt* gene, the total number of mutations was larger than the number of mutants. In the IQ-treated rats, the predominant type of base substitution was the G:C to T:A transversion (62/109 = 56.9%), 45.2% (28/62) of which occurred at 5′-CpG-3′ sites. Most deletions occurred at G:C base pairs (9/10). In the NPYR-treated rats, the predominant type of base substitution was the A:T to G:C transition (33/67 = 49.3%) which was rare in the other groups. The numbers of G:C base substitutions were similar to the control levels. In the nontreatment control, DEHP-treated and AAP-treated rats, the most frequently observed mutation was the G:C to A:T transition. A:T to T:A transversions were observed in common in all the groups at a steady rate (18.2–34.8%). Most of them occurred at the same A:T base pair, the 299th in the *gpt* gene regardless of the experimental treatment.

Spi$^-$ selection for deletion mutations was performed for the DEHP-treated and nontreatment control cases. Spi$^-$ MFs were $4.5 \times 10^{-6}$ and $4.3 \times 10^{-6}$, respectively (Table 5). Percentages of deletions larger than 1 kbp were 24.2 and 23.8 in the DEHP-treated and control rats, respectively. Thus, there were no differences in Spi$^-$ MF and deletion size spectrum between the DEHP-treated and control rats.

## DISCUSSION

In order to understand the molecular mechanisms underlying chemical carcinogenesis in rats, *gpt* delta transgenic rats were here exposed to three established rodent hepatocarcinogens for 13-wk and

Table 3. Guanine Phosphoribosyltransferase (*gpt*) Mutant Frequencies (MFs) in the Liver

| Treatment | Number of rats | Total population | Total *gpt* mutants | *gpt* mutants (clonal correction) | MF ($\times 10^{-6}$)[a] |
|---|---|---|---|---|---|
| IQ | 5 | 525 000 | 133 | 89 | 188.0 ± 44.9* |
| NPYR | 5 | 1 021 500 | 57 | 49 | 56.5 ± 24.5*,** |
| DEHP | 5 | 3 247 500 | 9 | 9 | 3.3 ± 3.8 |
| AAP | 5 | 3 637 500 | 19 | 17 | 5.5 ± 5.6 |
| Control | 5 | 2 976 000 | 19 | 16 | 5.5 ± 2.4 |

[a]Data are mean ± SD values.
*$P < 0.01$ (vs. control).
**$P < 0.01$ (vs. IQ).

Table 4. Mutation Spectra of *gpt* Mutant Colonies

| | Treatment | | | | |
|---|---|---|---|---|---|
| Type | IQ | NPYR | DEHP | AAP | Control |
| Base substitution | | | | | |
| Transition | | | | | |
| G:C to A:T | 6 (5.5) | 5 (7.5) | 5 (45.5) | 9 (39.1) | 8 (38.1) |
| A:T to G:C | 1 (0.9) | 33 (49.3) | 0 (0) | 0 (0) | 0 (0) |
| Transversion | | | | | |
| G:C to T:A | 62 (56.9) | 3 (4.5) | 3 (27.3) | 5 (21.7) | 2 (9.5) |
| G:C to C:G | 1 (0.9) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| A:T to T:A | 26 (23.9) | 20 (29.9) | 2 (18.2) | 8 (34.8) | 7 (33.3) |
| A:T to C:G | 1 (0.9) | 3 (4.5) | 0 (0) | 0 (0) | 0 (0) |
| Deletion | 10 (9.2) | 2 (3.0) | 1 (9.1) | 1 (4.3) | 4 (19.0) |
| −1 bp | 9 | 0 | 1 | 1 | 4 |
| >2 bp | 1 | 2 | 0 | 0 | 0 |
| Insertion | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Others | 2 (1.8) | 1 (1.5) | 0 (0) | 0 (0) | 0 (0) |
| Total number of mutation | 109 (100) | 67 (100) | 11 (100) | 23 (100) | 21 (100) |

Numbers in parentheses are percentages.

in vivo mutation assays were performed. GST-P positive liver cell foci were significantly induced by IQ and NPYR, and marked hepatomegaly characterized by centrilobular hypertrophy of hepatocytes was observed in the DEHP-treated rats. Because these alterations are known as early intermediate endpoint lesions for rat hepatocarcinogenesis [32,33], the findings strongly suggest that the doses of these chemicals were sufficient to elicit carcinogenic responses in the livers of *gpt* delta rats in the present study. NPYR at a dose of 200 ppm induced more GST-P lesions than IQ at a dose of 300 ppm, indicating that NPYR is more carcinogenic in the present study, which is compatible with the previous carcinogenicity bioassays reporting 14/15 (93%) and 18/40 (45%) of tumor incidences with NPYR and IQ under the same condition, respectively [8,17]. However, MFs in the *gpt* target gene determined by 6-TG selection were three-times higher in the IQ-treated rats than in the NPYR-treated rats, indicating that the MF itself is not directly correlated with the carcinogenic activity of each compound. In the present mutagenicity assay, the MFs were determined from the mutation occurring in the reporter gene, which has no biological function. Carcinogenesis, however, should require the crucial mutations in the cancer-related genes for the initiation, and also promotional factors for the tumor development. Thus, it is not inconsistent that the carcinogenicity does not necessarily correlate with the in vivo mutagenicity simply in terms of intensity.

In vivo mutagenicity and mutation spectrum of IQ have been investigated in commercially available transgenic rodent models such as MutaMouse and BigBlue [20,34,35]. Because IQ is widely known to be a potent mutagen for the liver and induces mainly G:C to T:A transversion, it was used as an appropriate positive mutagen for validating the mutagenicity assay system. As expected, IQ proved positive, causing G:C to T:A transversions and deletions at G:C base pairs, confirming the reliability of *gpt* delta rats for in vivo mutagenicity assays. In view of the molecular mechanisms, two types of direct DNA adducts, the major *N*-(deoxyguanosin-8-yl)-IQ and the minor 5-(deoxyguanosin-$N^2$-yl)-IQ, are thought to be responsible for IQ-mutagenesis [19,36]. It has also been suggested that oxidative DNA damage with 8-OHdG formation as a result of nonenzymatic reduction of nitro-IQ may play a role in carcinogenesis [37]. Although, we could not determine which

Table 5. Spi$^-$ MFs in the Liver

| Treatment | Number of rats | Total population | Total Spi$^-$ mutants | MF ($\times 10^{-6}$)[a] | Deletion size | |
|---|---|---|---|---|---|---|
| | | | | | <1 kb (%) | >1 kb (%) |
| DEHP | 5 | 7354500 | 33 | 4.5 ± 2.5 | 25 (75.8) | 8 (24.2) |
| Control | 5 | 9340500 | 42 | 4.3 ± 2.5 | 32 (76.2) | 10 (23.8) |

[a]Data are mean ± SD values.

type of DNA lesion was responsible for the IQ-induced hepatocarcinogenesis, recent work with a panel of biomarkers for detecting oxidative stress, DNA damage, and expression of DNA repair enzymes in IQ-treated BigBlue rats pointed to specific DNA adducts rather than oxidative DNA damage as responsible for IQ initiation of hepatocarcinogenesis [38].

DEHP is categorized as a nongenotoxic carcinogen because it induces liver tumors in both sexes of rats and mice in a dose-dependent manner [21], with *Salmonella* in vitro mutagenicity assay being uniformly negative [22,23]. To the best of our knowledge, however, there is only one report testing in vivo mutagenicity of several nongenotoxic carcinogens, including DEHP, in *lacI* transgenic mice, which failed to detect mutagenicity [39]. In the present study, point mutations and deletion mutations were widely screened by 6-TG selection and Spi$^-$ selection, respectively, in order to analyze the possible involvement of genotoxicity caused by reactive metabolites or peroxisomal oxidative stress. However, no mutagenic activity of DEHP was detected in the liver of *gpt* delta rats after 13-wk of treatment. In the DEHP-treated rats, 27.3% of the total mutations were G:C to T:A transversions, known to be caused by 8-OHdG [40], while this was the case for 9.5% in the control rats. Because the *gpt* delta rat has relatively few copies of the transgene lambda EG10, the total number of mutants obtained was too small to allow accurate evaluation of the specific mutation spectrum, but the results do suggest that hepatocarcinogenesis by DEHP in rodents mainly depends on nongenotoxic or promotional mechanisms rather than direct DNA damage.

Despite extensive studies on the metabolism of NPYR or DNA modification by its metabolites, the in vivo mutation spectrum of NPYR in the mammalian species has not yet been determined. In the present study, NPYR predominantly induced A:T to G:C transitions in the liver of the *gpt* delta rats, accounting for 49.3% of all mutations in the *gpt* gene. This was unexpected because there have been a sufficient number of studies for providing specific adduct formations by NPYR with deoxyguanosine. NPYR is metabolically activated via alpha-hydroxylation by cytochrome P450 enzymes to yield reactive intermediates, 4-hydroxybutylaldehyde, and crotonaldehyde [41]. These metabolites can alkylate deoxyguanosine, and mainly result in exocyclic adducts such as $N^7$,C-8 guanine adducts and 1,$N^2$-propanodeoxyguanosine [42]. Because these adducts have also been found in DNA from tissues of NPYR- or crotonaldehyde-treated animals, they might be expected to be the major adducts formed in vivo [43,44]. NPYR-induced mutational spectra have been investigated in bacterial systems with the *lacI* gene and M13mp2 phage DNA as targets [12,13] and base substitutions at G:C base pairs appeared to predominate. In the present study, however, the predominant type of base substitution in the liver of NPYR-treated rats was evidently A:T to G:C, followed by A:T to T:A. The difference between in vitro and in vivo mutagenesis by NPYR may be partly explained by means of specific etheno-adduct formation due to reactions of crotonaldehyde with deoxyadenosine as well as deoxyguanosine, known to yield 1,$N^6$-ethenoadenosine [45]. This latter adduct is formed during lipid peroxidation [46], and has also been detected in tissues from rats treated with vinyl chloride, a hepatocarcinogen in humans and rodents [47]. In the present study, the livers of NPYR-treated rats contained a number of GST-P positive single liver cells which may be indicative of lipid peroxidation and its end products, unsaturated aldehydes such as 4-hydroxynonenal, acrolein, and crotonaldehyde [48]. 1,$N^6$-Ethenoadenosine has shown to be highly mutagenic in mammalian cells such as simian kidney cells, exclusively inducing A:T to G:C base substitutions, while the mutation efficiency in *E. coli* is relatively low [49]. Moreover, Barbin et al. have suggested that concomitant formation of the etheno-adducts may play a role in the hepatocarcinogenesis by vinyl chloride, due to A:T base mutation occurring in the *p53* tumor suppressor gene [50]. In addition to a possible metabolite, crotonaldehyde, from NPYR, these data thus provide a possible mutational mechanism mediated by lipid peroxidation, interpreting our present findings in NPYR-treated rats. For understanding the molecular mechanism of NPYR-induced hepatocarcinogenesis, the levels of mutagenic adenine or thymine adducts and the possible specific mutations in the cancer-related genes need to be further analyzed in the liver of NPYR-treated rats.

In conclusion, based on the characteristic mutation spectra here observed in IQ-treated and NPYR-treated *gpt* delta transgenic rats, the predominant occurrence of A:T to G:C transitions in the NPYR-case suggests a possible contribution of the minor adenine adduct 1,$N^6$-ethenoadenosine to its in vivo mutagenesis in mammals, even though this carcinogen has been reported to form mainly guanine adducts in vitro. No mutagenic activity was detected in DEHP-treated rats, supporting the promotion pathway of DEHP-induced hepatocarcinogenesis rather than direct DNA damage. Our data also indicate that analysis of the specific in vivo mutational spectrum can provide crucial information for understanding the molecular mechanisms underlying chemical carcinogenesis.

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