

tase- or *O*-acetyltransferase-deficient strains suggest that nitro PAHs seem to be strongly associated with the genotoxicity observed in the urban and industrial regions (9).

To sensitively detect the genotoxicity of B[a]P and other PAHs in complex mixtures extracted from various polluted environmental sources, Matsui *et al.* (10) have recently engineered *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) strain YG5161, which harbored plasmid pYG768 carrying *dinB* encoding *Escherichia coli* DNA polymerase IV in standard Ames tester strain TA1538. The DNA polymerase can bypass a variety of DNA lesions such as those induced by B[a]P (11,12). Thus, the expression of the polymerase enhances B[a]P-induced -2 frameshift events in CGC-GCGCG repetitive sequences in the *hisD* gene in strain TA1538, which reverse the phenotype from His<sup>-</sup> to His<sup>+</sup> (13). In fact, the strain exhibits several times higher sensitivity to the genotoxicity of B[a]P than does strain TA1538 or another standard strain TA98 (10). Strain TA98 is the same as strain TA1538 but harbors plasmid pKM101 carrying *mucAB* encoding another Y-family DNA polymerase, i.e., DNA polymerase RI (14). Strain YG5161 also exhibits higher sensitivity to 10-azabenz[a]pyrene (10-AzaB[a]P), 3-methylcholanthrene (3-MC) and 3-nitrobenzo[a]pyrene (3-NB[a]P) than does strain TA1538 or TA98.

Despite the high sensitivity, strain YG5161 possesses a potential problem that is cross sensitivity to genotoxic nitroaromatics and aromatic amines. *S. typhimurium* has strong metabolic activation enzymes for nitroaromatics and aromatic amines, i.e., nitroreductase and *O*-acetyltransferase (15,16). The former is required for the reductive activation of nitroaromatics, and the latter is involved in the activation of *N*-hydroxy compounds derived from nitro- and amino-aromatics. Because of the potent enzyme activities, the genotoxicity of nitroaromatics and aromatic amines is very sensitively detected with *S. typhimurium* tester strains (17). As a consequence, the genotoxic PAHs can be veiled in complex mixtures extracted from various polluted environmental sources if genotoxic nitroaromatics or aromatic amines are contaminated.

In this study, we disrupted the *nfsB* and *oat* genes encoding nitroreductase and *O*-acetyltransferase, respectively, to decrease the cross sensitivity to nitro- and amino-aromatics. Introducing plasmid pYG768 into the  $\Delta nfsB\Delta oat$  strain resulted in strain YG5185, which retained the high sensitivity to PAHs but exhibited much reduced sensitivity to nitro- and amino-aromatics. We propose that the novel strain YG5185 is useful to detect genotoxic PAHs in the complex mixtures specifically and sensitively.

## Materials and Methods

**Strains and plasmids:** The strains and the plasmids used in this study are listed in Table 1.

**Chemicals:** The names, CAS registry numbers, abbreviations and sources of the chemicals assayed in this study are as follows: B[a]P (50-32-8), 7,12-dimethylbenz[a]anthracene (57-97-6, DMBA), 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (67730-11-4, Glu-P-1), and 2-aminoanthracene (613-13-8, 2-AA) from Wako Pure Chemicals (Osaka, Japan); 3-MC (56-49-5) and 1-aminoanthracene (610-49-1, 1-AA) from Sigma-Aldrich Japan K. K. (Tokyo, Japan); 1-nitropyrene (5522-43-0, 1-NP) and 1,8-dinitropyrene (42397-65-9, 1,8-DNP) from Tokyo Kasei Kogyo (Tokyo, Japan). 10-AzaB[a]P (189-92-4) and 1-nitrobenzo[a]pyrene (70021-99-7, 1-NB[a]P) were provided by Drs. Ken-ichi Saeki, Nagoya City University, Nagoya, Japan, and Kiyoshi Fukuhara, National Institute of Health Sciences, Tokyo, Japan, respectively. *N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine (4245-77-6, ENNG) is a laboratory stock.

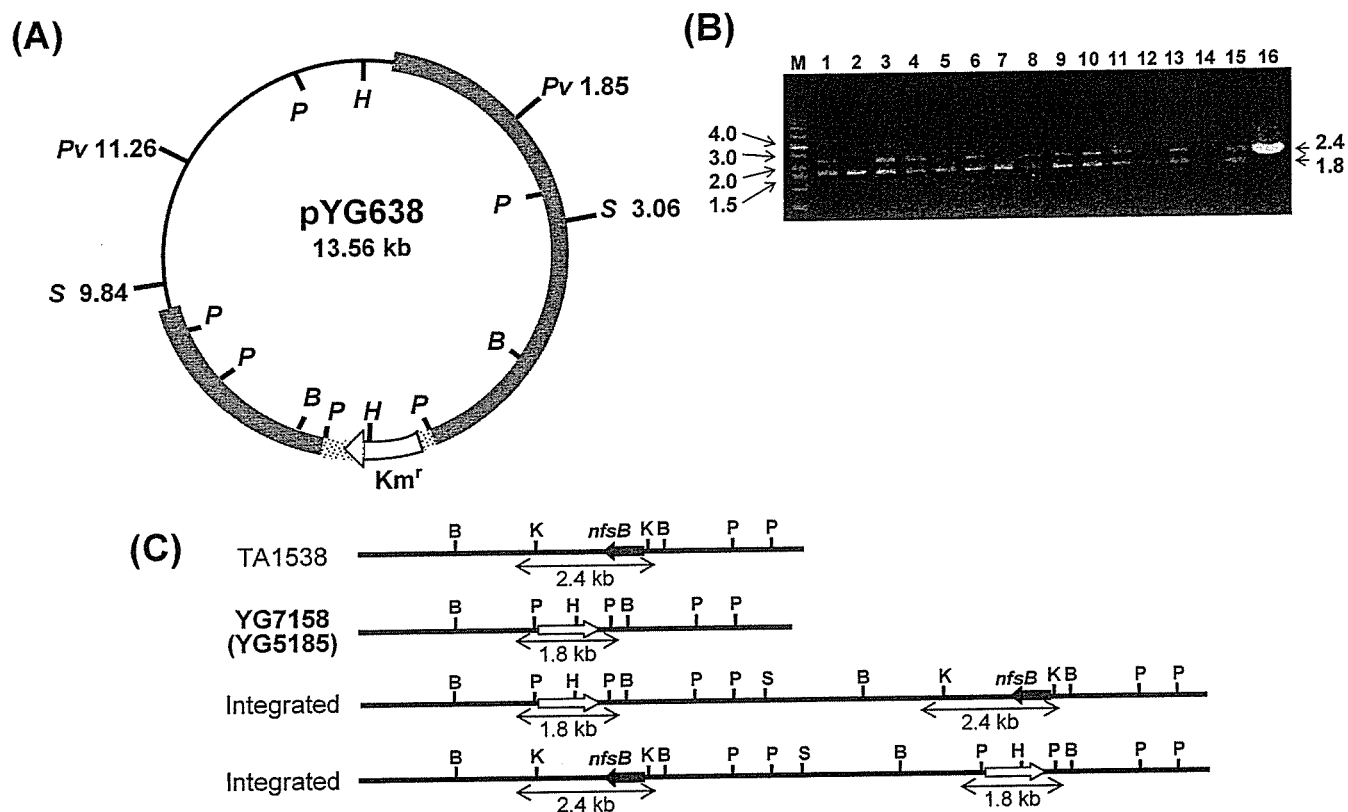
**Media:** Luria-Bertani broth and agar were used for bacterial culture. Vogel-Bonner minimal agar plates and top agar were prepared as previously described, and used for the His<sup>+</sup> reversion assay with *S. typhimurium* (13). Nutrient broth (Difco, MI, U.S.A.) with ampicillin (AP, 50  $\mu$ g/ml) was used for pre-cultures of strain YG5161 and YG5185 for the reversion assay.

**Construction of nitroreductase deficient strain:** Plasmid pYG638 (Fig. 1A) was digested with *SalI* and *PvuII* (New England Biolabs, MA, U.S.A.) to remove the replication origin (18), and the 6.8-kb linear *SalI*-*SalI* DNA fragment containing the kanamycin-resistance (*Km*<sup>r</sup>) gene between two flanking regions of the *nfsB* gene was purified with JET Sorb extraction kit (Genomed GmbH, Bad. Oeynhausen, Germany) after agarose gel electrophoresis. The purified DNA fragment was treated with T4 DNA ligase (Nippon Gene, Tokyo, Japan), and introduced into  $\Delta oat$  derivative of strain TA1538, i.e., strain YG7129, by electroporation (19,20). Colonies resistant to kanamycin were selected, and replacement of the *nfsB* gene with the DNA fragment carrying the *Km*<sup>r</sup> gene was examined by PCR (primers; 5'-TGGAAGCTGCTTTTACCGAACACT-3' and 5'-CCCGGACATAATAGAAAACCGGT-3') followed by 0.8% agarose gel electrophoresis.

**Mutagenicity assay:** The mutagenicity assay was carried out with a pre-incubation procedure (13). Briefly, 0.1 mL overnight culture was incubated with the chemicals dissolved in 0.1 mL solvent and 0.5 mL of S9 mix for 20 min at 37°C. When S9 mix is not required, 0.5 mL of 1/15M phosphate buffer pH7.4 was added. The mixture was then poured onto agar plates with soft agar and incubated for 2 days at 37°C. Each chemical was assayed with 4-7 doses on triplicate plates with four

**Table 1.** Strains and plasmids used in this study

Strain	Genetic characteristic	Source
TA1538	<i>hisD3052</i> , <i>gal</i> , $\Delta(chl, uvrB bio) rfa$	(13)
YG5161	the same as TA1538, but harbors pYG768; Ap <sup>r</sup>	(10)
YG7129	the same as TA1538, but deficient in <i>oat</i> ; Cm <sup>r</sup>	(19)
YG7158	the same as YG7129, but deficient in <i>nfsB</i> ; Cm <sup>r</sup> Km <sup>r</sup>	this study
YG5185	the same as YG7158, but harbors pYG768; Ap <sup>r</sup> Cm <sup>r</sup> Km <sup>r</sup>	this study
<i>plasmid</i>		
pYG638	Derivative of pBR322 for the disruption of the <i>nfsB</i> gene; Km <sup>r</sup> Ap <sup>r</sup> (see Fig. 1)	(18)
pYG768	Derivative of pWSK29 with <i>dinB</i> gene; Ap <sup>r</sup>	(34)



**Fig. 1.** Disruption of the *nfsB* gene. (A) Physical map of pYG638 (18). The thin and thick gray lines indicate DNA of plasmid pBR322 and the chromosome DNA derived from *S. typhimurium* TA1538, respectively. The dotted region shows the *Pst*I fragment derived from plasmid pUC-4k, which contains the Km<sup>r</sup>-gene cassette, whose transcriptional direction is indicated by the arrow head. Symbols for restriction enzyme sites: B, *Bam*HI; H, *Hind*III; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I. A *Sal*I-*Sal*I fragment containing the Km<sup>r</sup> cassette with the size of 6.78 kb was used for targeted disruption of the *nfsB* gene by pre-ligation method. (B) Result of PCR. PCR products were subjected to 0.8% agarose gel electrophoresis. M indicates size marker and lane numbers are indicated at the top of the gel image. Lanes 2, 5, 7 and 12 indicate the clone whose *nfsB* gene has been replaced with the Km<sup>r</sup> gene and lane 16 shows the proper size of the band including the *nfsB*<sup>+</sup> gene. Other lanes exhibit the clones that have the *nfsB*<sup>+</sup> gene as well as the Km<sup>r</sup>-gene fragment integrated into the chromosome. (C) Partial restriction maps of the *nfsB* gene and the surrounding chromosomal region in strain TA1538 and its Km<sup>r</sup> recombinants. Closed and open arrows indicate the position and the transcriptional direction of the *nfsB* gene and the Km<sup>r</sup> cassette, respectively. Thin arrows indicate the size of the bands amplified by PCR in (B).

strains, i.e., TA1538, YG7158, YG5161, YG5185, in parallel.

## Results

### Establishment of *S. typhimurium* strain YG5185:

To reduce the cross sensitivity to aromatic amines and

nitroaromatics, we disrupted the *nfsB* gene of *S. typhimurium* strain YG7129, which is the same as strain TA1538 but the *oat* gene is already disrupted (Table 1). After electroporation of the DNA fragments containing the Km<sup>r</sup> gene instead of the *nfsB* gene into strain YG7129, Km<sup>r</sup> colonies were selected. Chromosome

**Table 2.** Mutagenicity of 11 chemicals in *S. typhimurium* strains with and without plasmid carrying the *dinB* gene encoding *E. coli* DNA pol IV in the presence and the absence of the *nfsB* and *oat* genes

Chemicals	Group	S9	TA1538	YG5161	YG7158	YG5185	Dose
				+DNA pol IV	$\Delta nfsB\Delta oat$	$\Delta nfsB\Delta oat$ +DNA pol IV	
10-AzaB[a]P	A	+	2 (1.0)	21 (10.5)	2 (1.0)	28 (14.0)	25 $\mu$ g/plate
B[a]P	A	+	8 (1.0)	103 (12.9)	9 (1.1)	126 (15.8)	10 $\mu$ g/plate
3-MC	A	+	8 (1.0)	51 (6.4)	5 (0.6)	48 (6.0)	10 $\mu$ g/plate
2-AA	A	+	531 (1.0)	1,111 (2.1)	432 (0.8)	1,680 (3.2)	2 $\mu$ g/plate
DMBA	A	+	5 (1.0)	15 (3.0)	4 (0.8)	9 (1.8)	5 $\mu$ g/plate
ENNG	A	-	2 (1.0)	11 (5.5)	2 (1.0)	12 (6.0)	10 $\mu$ g/plate
1-AA	B	+	18 (1.0)	53 (2.9)	5 (0.3)	14 (0.8)	10 $\mu$ g/plate
1-NB[a]P	B	-	192 (1.0)	2,042 (10.6)	72 (0.4)	326 (1.7)	0.5 $\mu$ g/plate
1-NP	C	-	145 (1.0)	230 (1.6)	23 (0.2)	29 (0.2)	1 $\mu$ g/plate
1,8-DNP	C	-	130,560 (1.0)	218,040 (1.7)	2,040 (0.02)	3,120 (0.02)	25 ng/plate
Glu-P-1	C	+	41,240 (1.0)	45,550 (1.1)	1,710 (0.04)	1,670 (0.04)	100 ng/plate

Each chemical was assayed with 4–7 doses on triplicate plates with four strains in parallel. The numbers of His<sup>+</sup> revertants per plate per  $\mu$ g are calculated at the doses indicated with arrows in Fig. 2. The numbers in parenthesis represent the values relative to the numbers of His<sup>+</sup> revertants per  $\mu$ g in TA1538. Differences of relative mutability more than two fold or less than half were regarded as significant effects of the introduction of plasmid pYG768 carrying *dinB* encoding DNA pol IV or the deletion of the *oat* and *nfsB* genes on the mutability. Group A: chemicals whose mutagenicity was enhanced by DNA pol IV but was not reduced by  $\Delta nfsB\Delta oat$ ; Group B: chemicals whose mutagenicity was enhanced by DNA pol IV and was reduced by  $\Delta nfsB\Delta oat$ ; Group C: chemicals whose mutagenicity was not enhanced by DNA pol IV but was reduced by  $\Delta nfsB\Delta oat$ .

DNA surrounding the *nfsB* gene was amplified from the colonies by PCR using primers flanking the *nfsB* gene, and the products were analyzed by agarose gel electrophoresis (Fig. 1B). If the chromosomal *nfsB* gene is correctly replaced with the introduced DNA fragments carrying the Km<sup>r</sup> gene by recombination using the flanking homologous sequences, the Km<sup>r</sup> colonies will exhibit single DNA bands of 1.8 kb. If no such true replacement occurs and the introduced DNA fragments are only integrated into the vicinity of the chromosomal *nfsB* gene, they will exhibit double bands of 2.4 kb (the intact *nfsB* gene) and 1.8 kb (the Km<sup>r</sup> gene). As expected, some Km<sup>r</sup> colonies exhibited single 1.8-kb bands while others exhibited double bands of 2.4 kb and 1.8 kb. We suggested that the Km<sup>r</sup> colonies exhibited single 1.8-kb bands were deficient in the *nfsB* gene as well as the *oat* genes, and named the resulting strain YG7158. Plasmid pYG768 carrying the *dinB* gene encoding *E. coli* DNA polymerase IV was introduced and the resulting strain was referred to as YG5185,

which was used for the subsequent mutation assays.

**Specificity and sensitivity of strain YG5185 to genotoxic PAHs:** We compared the sensitivity of strain YG5185 to 11 mutagens with those of three reference strains: the parent strain, i.e., TA1538, the derivative of strain TA1538 harboring plasmid pYG768, i.e., YG5161, and the  $\Delta nfsB\Delta oat$  derivative of strain TA1538, i.e., YG7158 (Table 2). Introduction of plasmid pYG768 did not affect the spontaneous mutation frequencies in strains TA1538 and YG7158. Based on the order of sensitivity of the strains, we classified the chemicals into Group A to C as follows. For Group A compounds, i.e., 10-AzaB[a]P, B[a]P, 3-MC, 2-AA, DMBA and ENNG, deletion of the *nfsB* and *oat* genes did not reduce the sensitivity, and introduction of plasmid pYG768 substantially enhanced it (Fig. 2A). Thus, YG5185 exhibited similar or comparable sensitivity to Group A compounds as did strain YG5161, and the order of the sensitivity was YG5185 = YG5161 > TA1538 = YG7158. For Group B

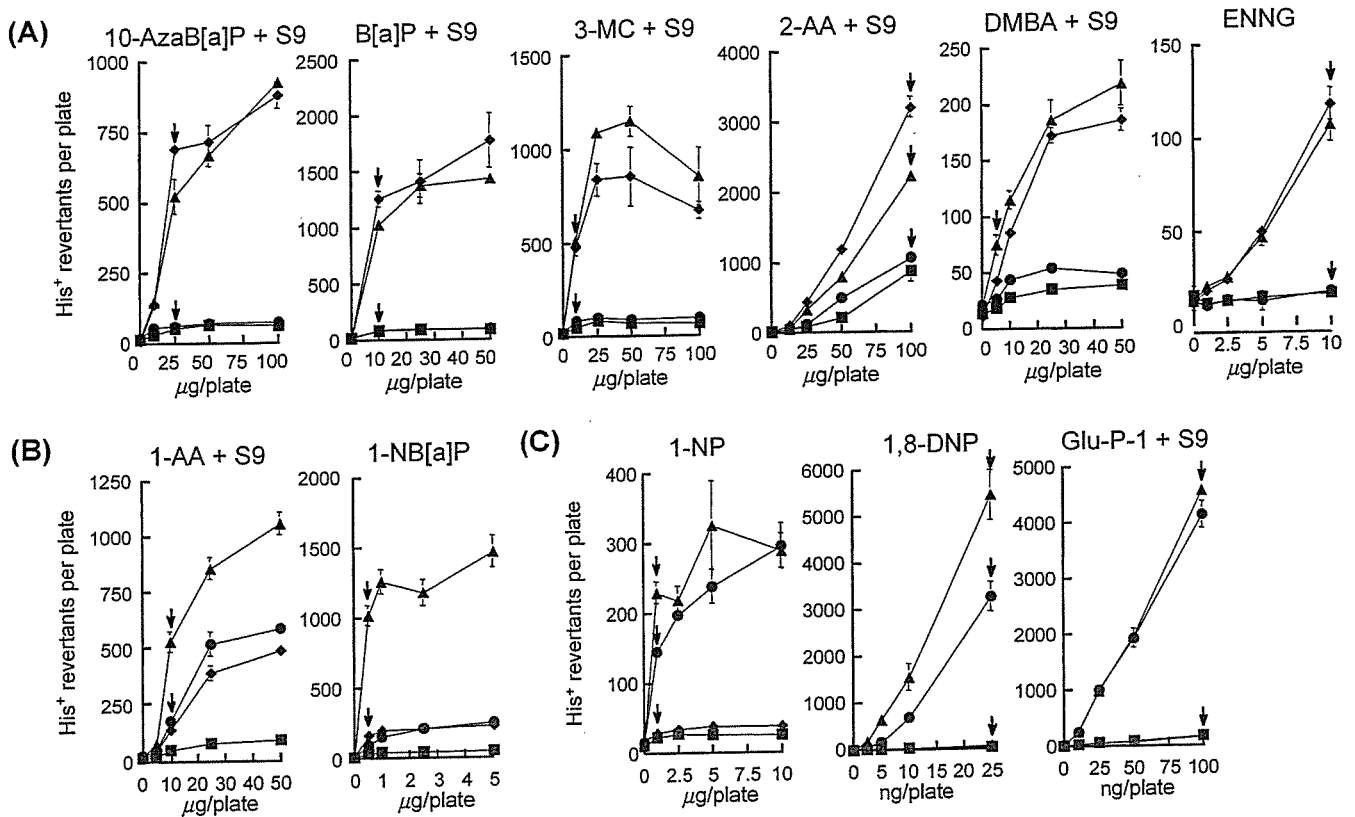


Fig. 2. Mutagenic responses of *S. typhimurium* newly constructed strains in the Ames test. The test chemicals are indicated on each panel. The chemicals are categorized into three Groups A, B and C, which are mentioned in the text and Table 2. Symbols are: ●, TA1538; ▲, YG5161; ■, YG7158; ◆, YG5185. The arrow indicates the dose that was used for the calculation of His<sup>+</sup> revertants per µg per plate in Table 2.

compounds, i.e., 1-AA and 1-NB[a]P, deletion of the *nfsB* and *oat* genes substantially reduced the sensitivity, and introduction of plasmid pYG768 enhanced it (Fig. 2B). Thus, the order of the sensitivity was YG5161 > TA1538 > YG5185 > YG7158. For Group C compounds, i.e., 1-NP, 1,8-DNP and Glu-P-1, deletion of the *nfsB* and *oat* genes severely reduced the sensitivity, and introduction of plasmid pYG768 did not substantially enhance it (Fig. 2C). The order of the sensitivity was YG5161 = TA1538 > YG5185 = YG7158.

## Discussion

Genetically engineered Ames tester strains have been proven to be useful in environmental genotoxicology due to their extreme sensitivity and the mechanistic information they can provide (17). For example, *S. typhimurium* strain YG1021, 1024, 1026 and 1029 have been widely used for environmental research for their hypersensitivity to the genotoxic action of nitroarenes and aromatic amines (21). The strains harbor plasmids carrying *nfsB* or *oat*, conferring high enzymatic activities of nitroreductase or *O*-acetyltransferase in strain TA98 or TA100 (15,16,22,23). Strains YG7104 and YG7108, whose repair systems for the damage by alkylating agents are disrupted, exhibit hyper sensitivity

to alkylating agents, such as methyl methanesulfonate and dimethylnitrosamine (24,25), and they are used for mechanistic analyses for chemical mutagenesis and carcinogenesis (26–28). Strain YG3001, YG3002 and YG3003 are highly sensitive to oxidative mutagens due to the lack of the *mutM* gene encoding 8-hydroxyguanine DNA glycosylase (29), and are shown to be useful for the studies on oxidative DNA damage (30,31).

To expand this line of research, we have previously established *S. typhimurium* strain YG5161 by introduction of plasmid carrying *dinB* encoding *E. coli* DNA polymerase IV into strain TA1538 to increase the sensitivity to genotoxic PAHs (10). Because of the error-prone nature of DNA polymerase IV, strain YG5161 exhibited higher sensitivity to PAHs such as B[a]P, 10-AzaB[a]P and 3-NB[a]P than did standard Ames strain TA1538 or TA98. Nevertheless, strain YG5161 has a potential defect as a bio-detector of genotoxic PAHs in complex mixtures, which is the cross sensitivity to other classes of genotoxic compounds, i.e., nitroaromatics and aromatic amines. Since these compounds are ubiquitously present in the environment and their genotoxicity in *S. typhimurium* is extremely amplified by the presence of intracellular metabolic activation enzymes, i.e., nitroreductase and *O*-acetyltran-

sferase, they can veil the potential genotoxicity of PAHs in the complex mixtures (17). For example, 1,8-DNP could be dominantly detected as a principle genotoxic compound in complex mixtures if 1,8-DNP and B[a]P were present at a weight ratio of 1:1,000. This is because the genotoxicity of 1,8-DNP, i.e., the numbers of His<sup>+</sup> revertants per plate per  $\mu\text{g}$ , is more than 15,000 times higher than B[a]P in strain TA1538 (Table 2). Despite the potent genotoxicity in *S. typhimurium*, 1,8-DNP is categorized into Group 2B (possible human carcinogens) by International Agency for Research on Cancer (IARC) while B[a]P is classified into Group 2A (probable human carcinogens) (32). Hence, we found it important to increase the specificity of tester strains to genotoxic PAHs.

Here, we disrupted the *nfsB* and *oat* genes of strain TA1538, introduced plasmid pYG768 carrying *dinB* into the  $\Delta nfsB\Delta oat$  strain and established novel *S. typhimurium* strain YG5185 (Table 1). When compared the sensitivity of strain YG5185 and YG5161, they exhibited comparative sensitivity to Group A compounds where four out of six compounds, i.e., 10-azaB[a]P, B[a]P, 3-MC and DMBA, were PAHs (Table 2, Fig. 2A). Thus, strain YG5185 appears to be able to detect the genotoxic PAHs with similar high sensitivity as does strain YG5161. The remaining two compounds in Group A are 2-AA and ENNG. It cautions that the compounds that are more sensitively detected by strain YG5161 or YG5185 compared with strain TA1538 are not necessarily PAHs. They can be aromatic amines or alkylating agents.

For Group B compounds, i.e., 1-AA and 1-NB[a]P, the genotoxicity was significantly reduced by the deletion of *nfsB* and *oat*, as in the case of Group C compounds (Fig. 2B and C). Unlike Group C compounds, however, the genotoxicity of 1-AA and 1-NB[a]P was three to 10 times enhanced by the introduction of plasmid carrying *dinB* (Fig. 2B). Actually, the genotoxicity of two compounds was more sensitively detected with strain YG5161 compared with standard strain TA98 (10). Thus, it seems that DNA lesions induced by 1-AA and 1-NB[a]P are more efficiently bypassed by DNA polymerase IV in an error-prone manner than by DNA polymerase RI encoded by *mucAB* carried by plasmid pKM101 in strain TA98. Interestingly, the genotoxicity of 1-AA was reduced by more than 70% by the deletion of *nfsB* and *oat* while the genotoxicity of 2-AA was not (Table 2, Fig. 2A and B). These results suggest that the intracellular metabolic activation mechanisms are markedly different between two aromatic amino compounds despite the structural similarity.

In Group C compounds, the deletion of *nfsB* and *oat* reduced the genotoxicity of 1-NP by more than 85% and those of 1,8-DNP and Glu-P-1 by more than 95%

(Table 2). Strain YG5185 exhibited much lower sensitivity to the genotoxicity of nitroaromatics and aromatic amine than did strain YG5161 (Fig. 2C). Thus, we concluded that strain YG5185 more specifically detects the genotoxicity of PAHs than does strain YG5161. Strain YG5185 could help and facilitate the successful isolation of genotoxic PAHs in complex mixtures. We have to point out, however, that the genotoxicity of 1,8-DNP and Glu-P-1 in strain YG5185 is still more than 25 times and 10 times higher than that of B[a]P. Hence, there is a possibility that genotoxic nitroaromatics or aromatic amines can be detected as principle mutagens if the complex mixtures are heavily contaminated with nitroaromatics or aromatic amines.

During the strain construction, we noticed that *S. typhimurium* TA1538/1,8-DNP could have mutations in the genes other than the *oat* gene. This is because introduction of plasmid pYG768 carrying *dinB* did not enhance the sensitivity of the strain to B[a]P while introduction of the same plasmid into YG7158 ( $\Delta nfsB\Delta oat$  strain) or YG7129 ( $\Delta oat$  strain) enhanced it more than 10 times. It may not be surprising that strain TA1538/1,8-DNP has unexpected mutations because it was generated by random mutagenesis with 1,8-DNP (33). To avoid such confusion by extra mutations, we specifically disrupted the *oat* and *nfsB* genes by a targeting method, i.e., the pre-ligation method, which has been developed in this laboratory (20).

In summary, we established novel *S. typhimurium* strain YG5185 by introduction of plasmid carrying *dinB* encoding *E. coli* DNA polymerase IV into  $\Delta nfsB\Delta oat$  derivative of standard Ames tester strain TA1538. The newly constructed strain exhibited higher sensitivity to the genotoxic compounds including PAHs but reduced sensitivity to nitroaromatics and aromatic amines. We propose that strain YG5185 is useful to detect the genotoxic PAHs in complex mixtures extracted from various polluted environmental sources.

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## Involvement of Y-Family DNA Polymerases in Mutagenesis Caused by Oxidized Nucleotides in *Escherichia coli*

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***Escherichia coli* DNA polymerase IV incorporated 2-hydroxy-dATP opposite template guanine or thymine and 8-hydroxy-dGTP exclusively opposite adenine in vitro. Mutator phenotypes in *sod/fur* strains were substantially diminished by deletion of *dinB* and/or *umuDC*. DNA polymerases IV and V may be involved in mutagenesis caused by incorporation of the oxidized deoxynucleoside triphosphates.**

Excess oxidation is a major threat to the genomic integrity of most living organisms. Reactive oxygen species oxidize deoxynucleoside triphosphates (dNTPs), as well as DNA, and some of the oxidized dNTPs have been shown to be mutagenic when they are incorporated in DNA. 8-Oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP) leads to A · T-to-C · G transversions when it is incorporated opposite adenine (A) in the template (5, 14). To counteract the mutagenic 8-OH-dGTP, *Escherichia coli* has a sanitizing enzyme, MutT, that hydrolyzes 8-OH-dGTP (20). When the *mutT* gene is inactivated, the frequency of mutation of A · T to C · G increases more than a thousandfold compared with the wild-type frequency (35). In the case of 2-oxo-1,2-dihydro-2'-deoxyadenosine 5'-triphosphate (2-OH-dATP), G · C-to-T · A transversions occur when it is incorporated opposite guanine (G) in the template (14, 16). Another sanitizing enzyme, Orf135, degrades 2-OH-dATP in *E. coli*, and G · C-to-T · A mutations occur in an *orf135*-deficient strain more frequently than in the wild-type strain (15, 17).

The members of the Y family of DNA polymerases (DNA Pols) are involved in error-free and error-prone translesion synthesis (TLS) of damaged template DNA in various species, including humans (13, 26). Recently, involvement of Y-family DNA polymerases in the incorporation of damaged dNTPs was suggested by in vitro experiments performed with purified DNA Pols (28). The archaeal Y-family DNA Pols from *Sulfolobus* sp. and the human DNA Pols exclusively incorporate 8-OH-dGTP opposite A in the template DNA and incorporate 2-OH-dATP opposite G and thymine (T). Thus, it would be interesting to examine the in vivo roles of Y-family DNA Pols in the incorporation of mutagenic dNTPs into DNA. *Escherichia coli* strain QC1736 seems to be an appropriate background to investigate the roles of Y-family DNA Pols (DNA Pol IV and Pol V encoded by *dinB* and *umuDC*, respectively) in the mutagenesis

caused by oxidized nucleotides. Iron metabolism is deregulated in this strain due to the lack of the Fur protein, a negative regulator of iron uptake (29). This strain also lacks both superoxide dismutases (SodA and SodB), which cata-

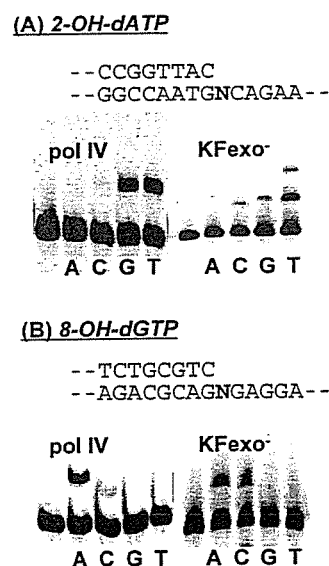


FIG. 1. Incorporation of oxidized nucleotides by DNA polymerases. The incorporation of 2-OH-dATP (A) and 8-OH-dGTP (B) into DNA by DNA Pol IV and KF *exo*<sup>-</sup> of *E. coli* was assayed as described previously (28). Cy3-conjugated primer, annealed to the template at a 1:1 ratio (0.1 μM), was incubated with DNA Pol IV (0.1 μM) or KF *exo*<sup>-</sup> (0.02 U), and then 50 μM 2-OH-dATP (A) or 50 μM 8-OH-dGTP (B) was added. No other dNTPs were added to the reaction mixtures. All the reactions were carried out at room temperature for 30 min. The reaction products were analyzed on 15% denatured polyacrylamide gels, and the bands were visualized using a Molecular Imager FX Pro system (Bio-Rad, Richmond, CA). The oligonucleotide sequences of the primer and template were 5'-Cy3-CGCGCGAAGAC CCGTTAC-3' and 5'-GAAGGGATCCTTAAGACNGTAACCGGTCTT CGCGCG-3', respectively, for 2-OH-dATP and 5'-Cy3-CGGAGCTCGGT CGGCGTCTGCGTC and 5'-AGCCGCAGGAGNGACGCAGACGCC GACCGAGCTCCG-3', respectively, for 8-OH-dGTP (N = A, C, G, or T). Parts of the sequences of the primer and template are shown. The unlabeled lanes on the left indicate the positions of Cy3-labeled primers without extension.

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TABLE 1. Strains used in this study<sup>a</sup>

Strain	Characteristics	P1 transduction or conjugation	Reference or source
CC101	Derivative of strain P90C [ <i>araA(lac proB)</i> <sub>XIII</sub> ] carrying F' <i>lacIZ-proB</i> <sup>+</sup> ; <i>lacZ</i> has a mutation (GAG to TAG) at codon 461		7
CC104	Derivative of strain P90C [ <i>araA(lac proB)</i> <sub>XIII</sub> ] carrying F' <i>lacIZ-proB</i> <sup>+</sup> ; <i>lacZ</i> has a mutation (GAG to GCG) at codon 461		7
AR30	<i>ΔdinB61::ble sulA211</i>		4
DE2302	<i>thr-1 ara-14 leuB6 Δ(gpt proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 arg3 thi-1 uvrA6 Δ(umuDC)595::cat fadR615::Tn10 purB58</i>		34
EC8	<i>thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 argE3 thi-1 uvrA6 Δ(umuDC)596::ermGT fadR<sup>+</sup> purB<sup>+</sup></i>		11
KY1056sFtet101	AB1157 derivative; harboring F' derived from CC101, which has Tn10 in it for selection of F'		K. Yamamoto
KY1056sFtet104	AB1157 derivative; harboring F' derived from CC104, which has Tn10 in it for selection of F'		K. Yamamoto
YG6125A	AB1157 derivative; harboring F' derived from CC101, which has Tn10 in it for selection of F' and <i>ΔdinB::kan</i>		This study
YG6125B	AB1157 derivative; harboring F' derived from CC104, which has Tn10 in it for the selection of F' and <i>ΔdinB::kan</i>		This study
QC1736	<i>Δ(argF-lac)U169 rpsL ΔsodA3 sodB::MudPR fur::kan; Cm<sup>r</sup> Km<sup>r</sup></i>		29
YG6177	Like QC1736 but <i>ΔdinB61::ble; Cm<sup>r</sup> Km<sup>r</sup> Zc<sup>r</sup></i>	AR30 (P1) → QC1736	This study
YG6180	Like QC1736 but <i>ΔumuDC(596)::ermGT; Cm<sup>r</sup> Km<sup>r</sup></i>	DE2302/EC8 (P1) → QC1736	This study
YG6124	Like QC1736 <i>ΔdinB61::ble; ΔumuDC(596)::ermGT; Cm<sup>r</sup> Km<sup>r</sup> Zc<sup>r</sup></i>	DE2302/EC8 (P1) → YG6177	This study
YG6175 <sup>b</sup>	Like QC1736 but harboring F' from CC101; Cm <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup>	KY1056sFtet101 → QC1736	This study
YG6176 <sup>b</sup>	Like QC1736 but harboring F' from CC104; Cm <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup>	KY1056sFtet104 → QC1736	This study
YG6178 <sup>b</sup>	Like QC1736 but <i>ΔdinB61::ble</i> and harboring F' from CC101; Cm <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup> Zc <sup>r</sup>	YG6125A → YG6177	This study
YG6179 <sup>b</sup>	Like QC1736 but <i>ΔdinB61::ble</i> and harboring F' from CC104; Cm <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup> Zc <sup>r</sup>	YG6125B → YG6177	This study
YG6181 <sup>b</sup>	Like QC1736 but <i>ΔumuDC(596)::ermGT</i> and harboring F' derived from CC101; Cm <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup>	KY1056sFtet101 → YG6180	This study
YG6182 <sup>b</sup>	Like QC1736 but <i>ΔumuDC(596)::ermGT</i> and harboring F' from CC104; Cm <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup>	KY1056sFtet104 → YG6180	This study
YG6126 <sup>b</sup>	Like QC1736 but <i>ΔdinB61::ble</i> and <i>ΔumuDC(596)::ermGT</i> and harboring F' from CC101; Cm <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup> Zc <sup>r</sup>	YG6125A → YG6124	This study
YG6127 <sup>b</sup>	Like QC1736 but <i>ΔdinB61::ble</i> and <i>ΔumuDC(596)::ermGT</i> and harboring F' from CC104; Cm <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup> Zc <sup>r</sup>	YG6125B → YG6124	This study

<sup>a</sup> The deletion strains for *dinB* encoding DNA Pol IV were constructed by P1 transduction as indicated. The *umuDC* deletion encoding DNA Pol V was introduced into QC1736 and YG6177 by two-step P1 transduction (11). (P1) indicates that P1vir phage lysate was prepared in the strain. F' with a mutation for specific detection of changes from G · C to T · A or from A · T to C · G was separately introduced by conjugation as indicated. The arrows indicate the directions of transfer for P1 transduction and conjugation. Chloramphenicol, kanamycin, tetracycline, and zeocin were used at concentrations of 10 μg/ml, 25 μg/ml, 10 μg/ml, and 50 μg/ml, respectively. Cm<sup>r</sup>, chloramphenicol resistance; Km<sup>r</sup>, kanamycin resistance; Tc<sup>r</sup>, tetracycline resistance; Zc<sup>r</sup>, zeocin resistance.

<sup>b</sup> Strain used for the LacZ reversion assay.

lyze the breakdown of the superoxide anion. Thus, both iron overload and superoxide stress occur in strain QC1736, which leads to high rates of spontaneous mutation from A · T to C · G and from G · C to T · A (24). The hot spots and sequence contexts of A · T-to-C · G mutations are almost identical to those in a *mutT* strain (25). In contrast, the hotspots of G · C-to-T · A mutations are very different from those in *mutM mutY* strains, in which 8-OH-G in DNA acts as a major mutagenic lesion. Expression of the cDNA of the human counterpart of *E. coli* MutT, MTH1, which hydrolyzes both 8-OH-dGTP and 2-OH-dATP (12), suppresses the mutator phenotype of the strain. Thus, it has been concluded that the targets contributing to the oxidative mutagenesis in the *sodI/fur* mutant are oxidized dNTPs, such as 8-OH-dGTP and 2-OH-dATP, rather than DNA (25).

We first examined the specificity with which the purified native form of DNA Pol IV (31) incorporates 2-OH-dATP and 8-OH-dGTP in vitro. DNA Pol IV predominantly incorporated 2-OH-dATP opposite template G and T, and the fre-

quency of incorporation opposite G was almost equal the frequency of incorporation opposite T (Fig. 1 A). In contrast, Klenow fragment *exo<sup>-</sup>* (KF *exo<sup>-</sup>*) (New England Biolabs, Massachusetts) predominantly incorporated 2-OH-dATP opposite T. DNA Pol IV almost exclusively incorporated 8-OH-dGTP opposite A, and KF *exo<sup>-</sup>* incorporated 8-OH-dGTP opposite A and cytosine (C) (Fig. 1 B). These in vitro results suggest possible involvement of Y-family DNA polymerases in oxidative mutagenesis through misincorporation of the oxidized dNTPs during DNA synthesis in *E. coli*.

To examine the in vivo roles of Y-family DNA Pols, mutation frequencies were compared for *sodI/fur* strains with and without Pol IV and Pol V (Table 1). Both A · T-to-C · G and G · C-to-T · A transversion frequencies were reduced by 80 to 90% by deletion of either *dinB* or *umuDC* or both in the *sodI/fur* strains (Table 2). Interestingly, the double mutants (*ΔdinB ΔumuDC*) exhibited levels of mutation frequency similar to those of single mutants (*ΔdinB* or *ΔumuDC*). These results suggest that the base substitutions by erroneous incorporation

TABLE 2. Mutation frequencies for the *sodAB fur* strains with and without DNA Pol IV and DNA Pol V<sup>a</sup>

Expt	Mutation frequency (10 <sup>-6</sup> )							
	Pol IV <sup>+</sup> /Pol V <sup>+</sup> (YG6176)	Pol IV <sup>-</sup> /Pol V <sup>+</sup> (YG6179)	Pol IV <sup>+</sup> /Pol V <sup>-</sup> (YG6182)	Pol IV <sup>-</sup> /Pol V <sup>-</sup> (YG6127)	Pol IV <sup>+</sup> /Pol V <sup>+</sup> (YG6175)	Pol IV <sup>-</sup> /Pol V <sup>+</sup> (YG6178)	Pol IV <sup>+</sup> /Pol V <sup>-</sup> (YG6181)	Pol IV <sup>-</sup> /Pol V <sup>-</sup> (YG6126)
G · C → T · A								
Expt I	28.2 ± 3.5	3.66 ± 0.32	2.72 ± 0.20	3.57 ± 0.42				
Expt II	35.6 ± 2.5	2.61 ± 0.12	2.82 ± 0.15	3.23 ± 0.30				
A · T → C · G								
Expt I					12.8 ± 0.61	2.49 ± 0.18	3.24 ± 0.25	2.43 ± 0.15
Expt II					10.0 ± 0.60	2.08 ± 0.16	3.7 ± 0.21	1.86 ± 0.19
Expt III					9.1 ± 0.80	2.38 ± 0.47		

<sup>a</sup> The mutagenicity assay was carried out as described previously (32). Briefly, a single colony was inoculated into 2 ml of M9-glucose minimal medium, and then the overnight culture was diluted 1,000-fold. Eight to twelve diluted cultures were prepared, and they were cultivated overnight. One milliliter of each culture was harvested and washed twice with phosphate buffer (pH 7.4), and then the cell pellet was suspended in phosphate buffer. All of the suspension was spread on one plate for mutation, and a portion of the diluted suspension was used for determining survival. Twenty amino acids were added in the assays (both liquid medium and plates) for growth of *sodAB fur* strains because the production of amino acids is inhibited by oxygen radicals (3). The values are means ± standard errors.

of 2-OH-dATP or 8-OH-dGTP require both DNA Pol IV and DNA Pol V functions.

The dNTP pool and DNA are continuously exposed to a variety of exogenous and endogenous damaging agents, including reactive oxygen species, and the incorporation of oxidized dNTPs into DNA is a major source of spontaneous mutagenesis and carcinogenesis (1). Here we obtained biochemical and genetic evidence that DNA Pol IV and Pol V may be involved in oxidative mutagenesis through misincorporation of altered nucleotides (i.e., 2-OH-dATP and 8-OH-dGTP) during DNA synthesis. This is consistent with the report by Satou et al. (27) that DNA Pol IV promotes mutation of G · C to T · A in *E. coli* when 2-OH-dATP is directly introduced into cells by CaCl<sub>2</sub> treatment. It has also been suggested that SOS-inducible polymerases, including Pol IV and Pol V, are involved in mutagenesis caused by increases in the normal levels of dNTPs (33). It has been reported that more than one DNA polymerase is involved in mutagenesis when the Y-family DNA polymerases are involved in TLS. For benzo[a]pyrene-induced mutagenesis, both Pol IV and Pol V are required for a -1 frameshift TLS (23). DNA lesions induced by other chemicals, including 3-methylcholanthrene or dimethylbenzo[a]anthracene, also require both DNA Pol IV and Pol V for a -2 frameshift in a CG repetitive sequence in *Salmonella enterica* serovar Typhimurium (18, 21). Thus, we speculate that DNA Pol IV and Pol V are involved in sequential biochemical steps, such as incorporation and extension of oxidized dNTPs during chromosome replication. One of these polymerases might incorporate oxidized dNTPs into DNA in an erroneous manner, and the other might extend the mutagenic primer termini containing the oxidized deoxynucleoside monophosphate, thereby inducing base substitutions. It is obvious, however, that more experiments are needed to elucidate the precise mechanisms.

DNA Pol IV is controlled by  $\sigma^S$ , and the level of expression of Pol IV in the stationary phase decreases significantly when the *rpoS* gene encoding  $\sigma^S$  is defective (10, 19). Thus, Pol IV appears to be regulated not only by the SOS response but also by the  $\sigma^S$ -dependent stress response. In stationary-phase cells, the amount of cellular mismatch repair proteins decreases at least 10-fold (8). Hence, the error-prone nature of Pol IV is expected to be more significant. In fact, DNA Pol IV is responsible for some of the adaptive mutations in stationary-

phase cells (9, 22). Interestingly, adaptive mutagenesis is approximately fourfold more frequent in a *sodA sodB* strain than in the parental strain, and this mutagenesis is suppressed under anaerobic conditions (2). Therefore, DNA Pol IV might be involved in stationary-phase mutagenesis by either incorporation of oxidized dNTPs or extension of primers having oxidized deoxynucleoside monophosphates or both, although it is possible that DNA Pol IV induces mutations by error-prone bypass across oxidized bases in template DNA.

The oxidized nucleotide pools also cause a problem in mammalian cells. Spontaneous tumorigenesis in lungs, livers, and stomachs is enhanced in mice that are deficient in *Mth1* (30). In addition, a recent study suggested that the majority of mutations in human cells that are deficient in mismatch repair do not arise from spontaneous replication errors but from the incorporation of oxidized dNTPs (6). Thus, it might be interesting to examine the roles of mammalian Y-family DNA Pols in genome instability caused by oxidation of the nucleotide pool.

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## Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells

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### Abstract

The recent finding that acrylamide (AA), a potent carcinogen, is formed in foods during cooking raises human health concerns. In the present study, we investigated the genotoxicity of AA and its metabolite glycidamide (GA) in human lymphoblastoid TK6 cells examining three endpoints: DNA damage (comet assay), clastogenesis (micronucleus test) and gene mutation (thymidine kinase (TK) assay). In a 4 h treatment without metabolic activation, AA was mildly genotoxic in the micronucleus and TK assays at high concentrations (>10 mM), whereas GA was significantly and concentration-dependently genotoxic at all endpoints at  $\geq 0.5$  mM. Molecular analysis of the TK mutants revealed that AA predominantly induced loss of heterozygosity (LOH) mutation like spontaneous one while GA-induced primarily point mutations. These results indicate that the genotoxic characteristics of AA and GA were distinctly different: AA was clastogenic and GA was mutagenic. The cytotoxicity and genotoxicity of AA were not enhanced by metabolic activation (rat liver S9), implying that the rat liver S9 did not activate AA. We discuss the *in vitro* and *in vivo* genotoxicity of AA and GA.

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**Keywords:** Acrylamide; Glycidamide; Genotoxicity; TK mutation; Metabolic activation

### 1. Introduction

Acrylamide (AA) is a synthetic chemical that has been produced since the early 1950s. Because AA polymerizes easily to an adhesive gel, it has been widely used in industry for water flocculation, soil coagulation

and grouts. Because it had been believed that humans are rarely exposed to AA under ordinary circumstances, concern was centered only on occupational exposure [1]. In 2000, however, Tareke et al. [2] reported that AA was unexpectedly discovered in cooking foods. It forms during frying and baking principally by a Maillard reaction between asparagine residues and glucose [3,4]. This finding raises concerns about the health risks of AA for the general population [5].

According to toxicological studies, AA is neurotoxic for animals and human [6,7], and the International

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Agency for Research on Cancer classifies it as 2A, a probable human carcinogen [1]. AA is also genotoxic in somatic and germinal cells in *in vitro* and *in vivo* [8]. *In vivo* examination [8] AA is metabolized to the epoxide derivative glycidamide (GA), presumably by cytochrome P4502E1 (CYP2E1) [9]. GA may be more toxic than AA because it reacts quickly with DNA and other biological macromolecules, and it is positive in most genotoxicity tests [8]. AA, on the other hand, is inactive in bacterial and some *in vitro* mammalian gene mutation assays, but it induces sister chromatid exchanges and chromosome aberrations *in vitro* and *in vivo* [8]. AA may have indirect genotoxic mechanisms, such as protein binding, spindle disturbance or hormonal imbalance, which could lead to tumors [10,11]. Thus, the genotoxic mechanism of AA is unclear.

In the present study, we used human lymphoblastoid TK6 cells to investigate the genotoxicity of AA and GA and its mechanisms. TK6 cells are widely used for the thymidine kinase (*TK*) gene mutation assay and can also be used in the *in vitro* micronucleus (MN) and comet (COM) assays. The *TK* gene mutation assay detects a wide range of genetic damage, including gene mutations, large-scale chromosomal deletions, recombination and aneuploidy [12], while other mammalian gene mutation assays, such as the *HPRT* and transgenic *LacZ* and *LacI* gene assays, detect only point mutations and small deletions [13]. Most of the genetic changes observed in *TK* mutants occur in human tumors and are presumably relevant to carcinogenesis. Molecular analysis of the *TK* mutants induced by AA or GA can help elucidate their genotoxic mechanisms. In addition, because it uses a human cell line, the *TK* assay is appropriate for human hazard evaluation.

## 2. Materials and methods

### 2.1. Cell culture, chemicals and treatment

The TK6 human lymphoblastoid cell line has been described previously [14]. The cells were grown in RPMI1640 medium (Gibco-BRL, Life technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JRH Biosciences, Lenexa, KS), 200 µg/ml sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin and maintained at  $10^5$  to  $10^6$  cells/ml at 37 °C in a 5% CO<sub>2</sub> atmosphere with 100% humidity.

AA (CAS # 79-06-1) and GA (CAS # 5694-00-8) were purchased from Wako Pure Chemical Co. (Tokyo). We dissolved them in phosphate-buffered saline just before use. *N*-di-*N*-butylnitrosamine (DBN) (CAS # 924-16-3) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo) and dissolved in DMSO for use. Post-mitochondrial supernatant fractions of

liver homogenate (S9) were purchased from Kikkoman Co. Ltd. (Noda, Chiba, Japan), which were prepared from the liver of phenobarbital- and 5,6-benzoflavone-treated SD rats. We prepared a 10 ml S9 mix with 4 ml S9 fraction and 2 ml each of 180 mg/ml glucose-6-phosphate, 25 mg/ml NADP and 150 mM KCl.

We treated 20 ml aliquots of cell suspensions ( $5.0 \times 10^5$  cells/ml) at 37 °C for 4 h with serially diluted AA or GA, washed them once, re-suspended them in fresh medium, and cultured them in new flasks for the MN and *TK* assays or diluted and plated them for survival measurement (PEO). We treated the cultures with AA both in the absence and presence of 5% S9 mix.

### 2.2. Comet assay

After treating the cells for 4 h with AA or GA, we prepared slides for alkaline COM assay as previously reported [15]. Briefly, the cells were suspended in 0.5% agarose-LGT (Nakalai Tesque Inc., Kyoto, Japan), quickly layered on a slide (Matsunami Glass Ind. Ltd., Osaka, Japan) coated with 1% agarose GP-42 (Nakalai Tesque Inc.), and covered with 0.5% agarose-LGT. We immersed the slide in alkaline lysing solution (pH 13) for 1 h, electrophoresed it for 15 min after the unwinding treatment, fixed the cells with 70% ethanol, and stained them with SYBER green (Molecular Probes, Eugene, OR) according to the manufacturer's recommendation. We observed the cells by an Olympus model BX50 fluorescence microscope. At least 50 cells were captured by CCD camera, and the tail length of the comet image was measured. We statistically analyzed the difference between the non-treated and treated plates with the Dunnett's test after one-way ANOVA [16].

### 2.3. Micronuclei test

Forty-eight hours after treatment, we prepared the MN test samples as previously reported [17]. Briefly, approximately  $10^6$  cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold glacial acetic acid in methanol (1:3), and resuspended in methanol containing 1% acetic acid. We placed a drop of the suspension on a clean glass slide and allowed it to air-dry. We stained the cells with 40 µg/ml acridine orange solution and immediately observed them by Olympus model BX50 fluorescence microscope. At least, 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between non-treated and treated cells were statistically analyzed by Fisher's exact test. The concentration–response relationship was evaluated by the Cochran–Armitage trend test [18].

### 2.4. *TK* gene mutation assay

The TK6 cell cultures were maintained for 3 days after treatment to permit expression of the *TK* deficient phenotype. To isolate the *TK* deficient mutants, we seeded cells from each

culture into 96-microwell plates at 40,000 cells/well in the presence of 3.0  $\mu\text{g/ml}$  trifluorothymidine (TFT). We also plated them at 1.6 cells/well in the absence of TFT for the determination of plating efficiency (PE3). All plates were incubated at 37°C in 5%  $\text{CO}_2$  in a humidified incubator. The TK assay produces two distinct phenotypic classes of TK mutants: normally growing (NG) mutants had the same doubling time (13–17 h) as the wild type cells, and slowly growing (SG) mutants had a doubling time of >21 h. The difference is thought to be due to a putative gene near the TK gene. NG mutants result mainly from intragenic mutations, such as point mutations and small deletions, while SG mutants result from gross genetic changes extending beyond the TK gene [19]. We scored for the colonies in the PE plates and for the colonies for normal-growing TK mutants in the TFT plates at 14th day after plating. We then reseeded the plates containing TFT with fresh TFT, incubated them for an additional 14 days, and scored them for slow-growing TK mutants. Mutation frequencies were calculated according to the Poisson distribution [20]. The data were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson–Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [21].

### 2.5. Molecular analysis of TK mutants

Genomic DNA was extracted from TK mutant cells and used as a template for the polymerase chain reaction (PCR). We analyzed for loss of heterozygosity (LOH) at the human TK gene by PCR products as described previously [22]. A set of primers was used to each amplify the parts of exons 4 and 7 of the TK gene that contains frameshift mutations. Another primer

set for amplifying parts of the  $\beta$ -globin were also prepared. We used quantitative-multiple PCR to co-amplify the three regions and to identify and quantify the PCR products. We analyzed them with an ABI310 genetic analyzer (PE Biosystems, Chiba, Japan), and classified the mutants into "none LOH", "hemizygous LOH" or "homozygous LOH". To determine the extent of LOH, we analyzed 10 microsatellite loci on chromosome 17q by PCR-based LOH analysis described previously [22]. The results were processed by GenoTyper™ software (PE Biosystems) according to the manufacturer's guidelines.

## 3. Results

### 3.1. Cytotoxic and genotoxic responses to AA and GA

Fig. 1a shows the effect of AA on relative survival (RS), mutation frequency (TK assay) and number of micronucleated cells per 1000 cells examined. AA was concentration-dependently cytotoxic, permitting about 20% RS at the maximum concentration (14 mM), while its genotoxicity and clastogenicity were weak. We repeated the experiment because of the weak genotoxicity. AA showed negative in the first TK assay, but positive in the second statistically. In MN test, both experiments showed statistically positive. GA, in contrast, was significantly genotoxic even at concentrations that were not severely cytotoxic (Fig. 1b). At the maximum concentration (2.4 mM), GA induced TK mutation frequencies that were about 20 times and MN fre-

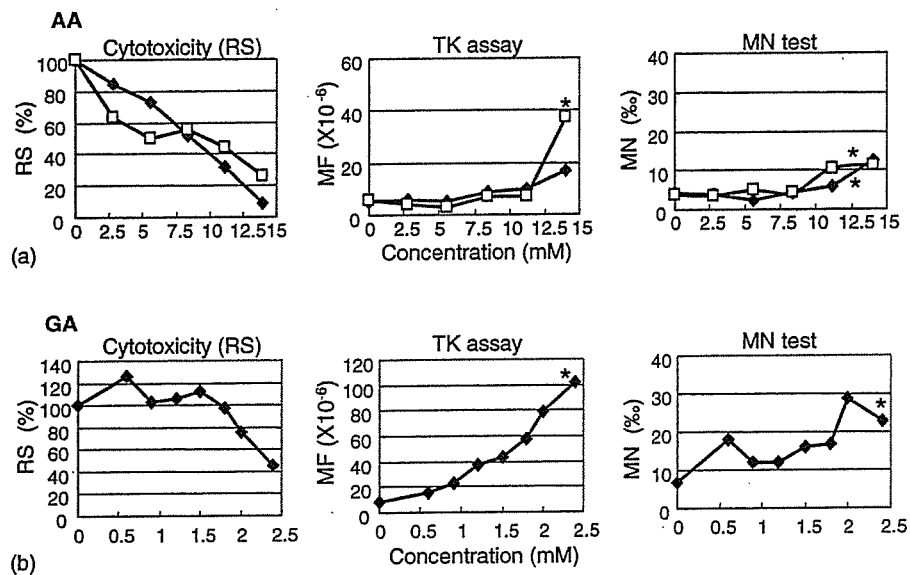


Fig. 1. Cytotoxic (relative survival, RS), genotoxic responses (TK assay and MN test) of TK6 cells treated with AA (a) or GA (b) for 4 h without metabolic activation. The AA experiment was repeated to confirm the result because of the weak genotoxicity. Closed and open symbols are first and second experiment, respectively. Asterisk (\*) statistically significant experiments in both pair-wise comparison and trend test ( $P < 0.05$ ).

Table 1

Cytotoxic and mutational responses to AA and GA, and the results of LOH analysis of normally growing (NG) and slowly growing (SG) TK-mutants

Treatment	Cytotoxic and mutational response			LOH analysis at TK gene			
	RS (%)	MF ( $\times 10^{-6}$ )	% SG	No.	None LOH	Hemi-LOH	Homo-LOH
Vehicle [16]	100	2.19	56	56			
NG mutants				19	14 (74)	3 (16)	2 (11)
SG mutants				37	0 (0)	9 (24)	28 (76)
AA (14 mM, 4 h)	40	18.9	54	48			
NG mutants				22	11 (50)	11 (50)	0 (0)
SG mutants				26	0 (0)	13 (50)	13 (50)
GA (2.2 mM, 4 h)	12	55.5	36	44			
NG mutants				28	26 (93)	2 (7)	0 (0)
SG mutants				16	0 (0)	6 (38)	10 (62)

quencies at about four times the spontaneous level. We detected two distinct phenotypic classes of *TK* mutants in *TK* assay: NG and SG mutants. AA did not affect the proportion of SG mutants, while GA treatment lowered it (Table 1). This implies that GA induced primarily point mutations. In the COM assay, even at the highest concentration, AA did not induce DNA damage, while GA did so strongly starting at 0.6 mM (Fig. 2).

### 3.2. Molecular analysis of *TK* mutants

The *TK* mutants were independently isolated from the cells treated with 14 mM AA or 2.2 mM GA for 4 h. Table 1 shows the cytotoxicity (RS) and *TK* mutation frequency (MF) and proportion of SG mutants (% SG) by the treatment. Genomic DNA extracted from the mutants was subjected by the PCR-based LOH analysis to classify the mutants into three types: non-LOH, hemizygous LOH (hemi-LOH) and homozygous LOH (homo-LOH). In general, hemi-LOH is resulted by deletion and homo-LOH is by inter-allelic homologous recombination [13]. We analyzed 48 AA-induced and 44 GA-induced *TK*

mutants and compared them to those of spontaneously occurring *TK* mutants described previously [16]. The fraction of hemi-LOH in AA-induced mutants, in which 50% each of NG and SG mutants exhibited hemi-LOH, was higher than in spontaneous mutants, indicating that AA-induced primarily deletions. GA, on the other hand, induced primarily NG mutants, and most (93%) of them were the non-LOH type, which is presumably generated by point and other small intragenic mutations. Among 16 GA-induced SG mutants, the percentages that were hemi-LOH (38%) and homo-LOH (62%) were similar to those observed in spontaneous SG mutants. Fig. 3 shows the mutation spectra of *TK* mutants found among treated and untreated TK6 cells. GA and ethyl methane sulfonate, an alkylating agent, produce similar spectra, as do AA and X-radiation.

Fig. 4 shows the distribution of LOH in AA-induced ( $n=37$ ), GA-induced ( $n=17$ ) and spontaneous ( $n=29$ ) LOH mutants. Because the majority of GA-induced mutants were the non-LOH type, we were able to map only 17 GA-induced LOH mutants. As a particular characteristic of AA-induced LOH mutants, we frequently observed small deletions limited to the *TK* locus. The

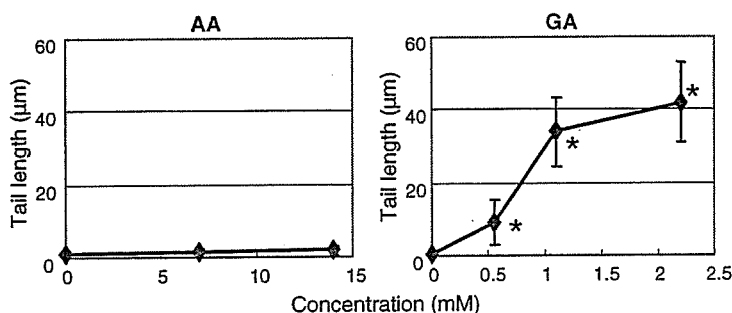


Fig. 2. COM assay results in TK6 cells treated with AA or GA for 4 h without metabolic activation. Asterisk (\*) statistically significant in the Dunnett's tests ( $P < 0.05$ ).

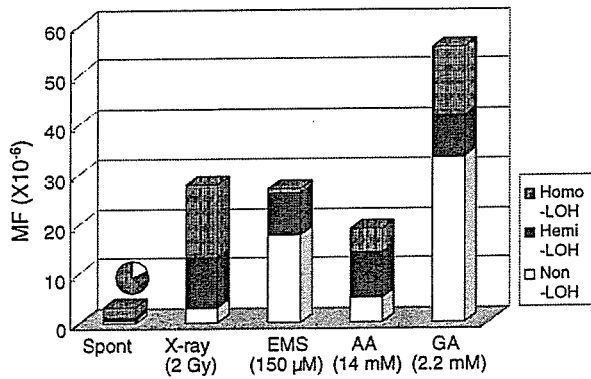


Fig. 3. Frequency and spectra of TK mutations in spontaneous and X-ray-induced (2 Gy), EMS-induced (150 μM, 4 h), AA-induced (14 mM, 4 h) and GA-induced (2.2 mM, 4 h) TK mutants in TK6 cells. The fraction of each mutational event was calculated by considering the ratio of normally growing (NG) and slowly growing (SG) mutants and the results of molecular analysis (Table 1). The data of spontaneous, X-ray-induced and EMS-induced mutation spectra were taken from our previous paper [13].

distribution of LOH in GA-induced and spontaneous LOH mutants was similar.

### 3.3. Cytotoxicity and genotoxicity of AA under metabolic activation

Rat liver S9 mix did not influence the cytotoxicity or genotoxicity of AA but it did enhance the activity of DBN, the positive control chemical (Fig. 5).

## 4. Discussion

A large number of studies about the in vitro genotoxicity of AA have been reported [8]. AA has consistently been negative in bacterial gene mutation assay in both the presence and absence of metabolic activation [23–25] but positive in chromosome aberration and sister chromatid exchange tests in Chinese hamster cell lines [24–26]. In mammalian cell assays, AA induces *Tk* but not *Hprt* gene mutations [24,25,27,28], and is negative in the COM assay even at high concentrations [27]. These results suggest that AA is clastogenic without directly damaging DNA. GA, on the other hand, is positive in most in vitro genotoxicity tests and is recognized as a mutagen [8,27,29]. In the present study, the higher concentrations of AA were positive in the MN and TK assay but negative in the comet assay. According to the in vitro genotoxicity test guideline, however, AA may be negative [30], because the guideline suggests that the maximum concentration should be 10 mM. Because the genotoxic responses at higher concentrations were reproducible, AA may be genotoxic, but its effect is very weak. GA, in contrast, was positive in all the assays, even under conditions of low cytotoxicity. These results are consistent with the reports described above.

The mammalian *TK* gene mutation assay can detect a wide range of genetic changes, including point mutations, small deletions, large-scale chromosomal deletions, inter-allelic recombination and aneuploidy, while

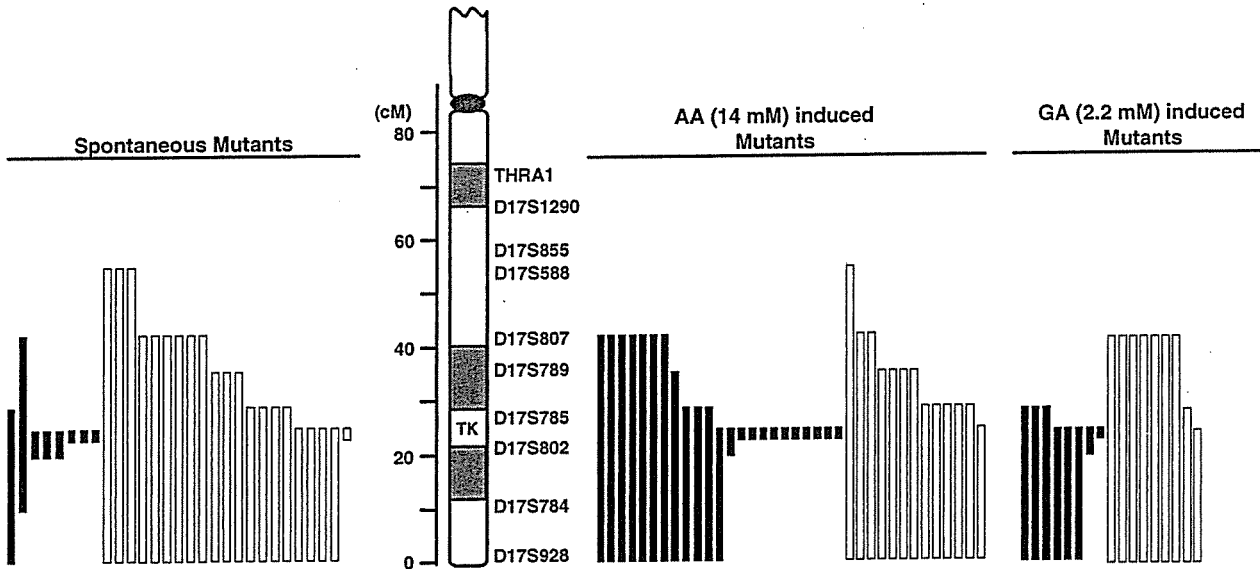


Fig. 4. The extent of LOH in spontaneous, AA-induced and GA-induced LOH mutants from TK6 cells. We examined 10 microsatellite loci on chromosome 17q that are heterozygous in TK6 cells. The human *TK* locus maps to 17q23.2. Open and closed bars represent homo-LOH and hemi-LOH, respectively. The length of the bar indicates the extent of the LOH. We analyzed 29 spontaneous mutants (10 NG and 19 SG mutants), 37 AA-induced mutants (11 NG and 26 SG) and 17 GA-induced mutants (2 NG and 15 SG). The data on spontaneous mutants were taken from our previous paper [13].



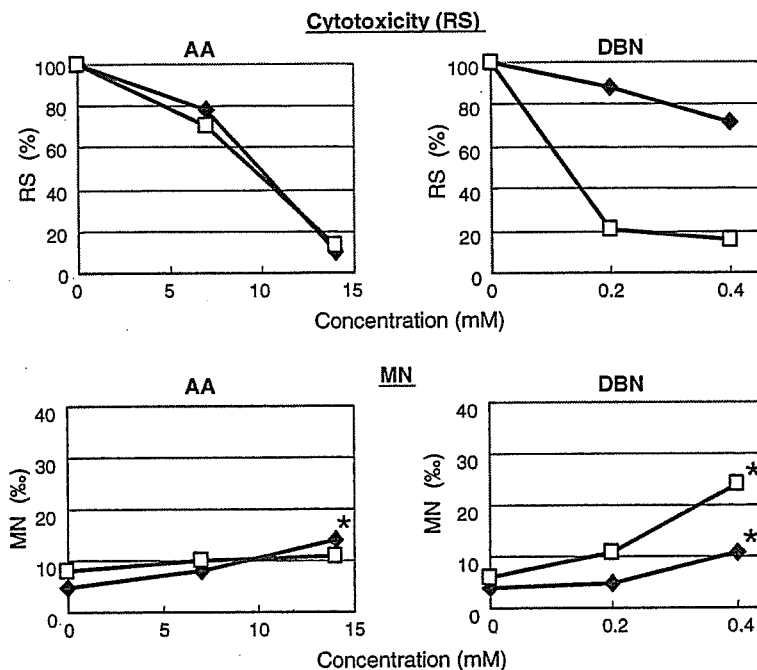


Fig. 5. Cytotoxicity (RS) and MN induction in TK6 cells treated with AA or DBN for 4 h in the presence (open symbol) or absence (closed symbol) of rat liver S9. Asterisk (\*) statistically significant experiments in both pair-wise comparison and trend test ( $P < 0.05$ ).

the bacterial and mammalian *HPRT* gene mutation assays detect only point mutations and small intragenic deletions [13]. AA was positive only in the *TK* mutation assay, suggesting that AA causes predominantly large-scale chromosomal changes. Our molecular analysis of the *TK* mutants supported this hypothesis. The majority of the AA-induced *TK* mutants showed hemi-LOH, which is the result of a deletion, although the other types were also induced (Fig. 3). Deletions are thought to result from the repair of double strand breaks by non-homologous end-joining [13]. Radiation-induced double strand breaks are repaired by non-homologous end-joining, which leads to hemi-LOH. LOH-mapping analysis, however, revealed that AA frequently induces intermediate-sized deletions (100–3000 kb); the deletions encompass exons 4 and 7 of the *TK* locus but do not extend to the microsatellites loci of the vicinity. This type of deletion is rarely observed in radiation-inducing *TK* mutants [13]. Because the COM assay indicated that AA did not induce DNA damage, the deletion may not be caused by DNA damage directly. Mechanisms associated with global genomic instability should also be considered [10] because the LOH patterns, except for the intermediate-sized deletions, are generally similar to those observed in spontaneous mutants. Most GA-induced *TK* mutants, on the other hand, were the non-LOH type, as were most spontaneous ones, strongly

supporting the positive results in bacterial gene mutation assay [29]. In contrast to AA, GA is a mutagen, inducing primarily point mutations.

AA is known to be metabolized to GA by CYP2E1 [9]. GA, an epoxide, forms adducts directly with DNA and protein, causing cytotoxicity and genotoxicity. GA forms mainly *N7*-(2-carbamoyl-2-hydroxyethyl) guanine and *N3*-(2-carbamoyl-2-hydroxyethyl) adenine and reacts with hemoglobin and cytoskeletal proteins [31–33]. Rat S9, however, did not affect AA cytotoxicity or genotoxicity, although it did enhance the cytotoxicity and genotoxicity of DBN, which is also metabolized by CYP2E1. This suggests that rat S9 does not work for activating AA. AA and GA are detoxified through glutathione conjugation, and GA is also detoxified by epoxy hydrolase (EH), which catalyzes the hydrolysis of GA to dihydroxy propionamide [34,35]. Other *in vitro* studies also failed to demonstrate the enhancement of AA genotoxicity by rat S9 [36,37]. Our results do not mean that AA is always detoxified rather than activated because DNA adducts are found in mice and rats given oral AA, and the genotoxicity of AA is consistently observed in *in vivo* studies [8,31,36,37]. Recently, Manjanatha et al. demonstrated in transgenic Big Blue<sup>TM</sup> mice that AA as well as GA induces endogenous *Hpert* and transgenic *cII* mutation at same level, and both chemicals cause predominantly base substitutions and frameshift mutations.

This result may indicate that AA is metabolized to GA in vivo [38]. Tests that use rat liver S9 for metabolic activation may not be appropriate for in vitro investigations of AA genotoxicity and metabolism. Transgenic cells expressing CYP2E1, however, would be useful for demonstrating the in vitro genotoxicity of AA [39].

In conclusion, AA is weakly genotoxic, causing chromosome aberrations and a type of genomic instability. GA, its epoxide metabolite, is highly reactive with DNA. GA is a strong mutagen, inducing predominantly point mutations, and it may contribute to human cancers.

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## Relationship between p53 status and 5-fluorouracil sensitivity in 3 cell lines

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### Abstract

Mouse lymphoma L5178Ytk<sup>+/-</sup> (MOLY) cells and human lymphoblastoid TK6 and WTK-1 cells are widely used to detect mutagens in vitro. MOLY and WTK-1 cells have a p53 mutation, while TK6 cells, which were derived from the same parental line as WTK-1 cells, do not. In this study, we tested the clastogen 5-fluorouracil (5-FU) in the Tk assay and the in vitro micronucleus (MN) assay in MOLY, TK6, and WTK-1 cells to clarify whether differential responses were related to p53 gene status. We also determined the effect of 5-FU on the frequency of apoptotic cells and on cell cycle distribution in each cell line. Furthermore, we measured the activity of the 5-FU metabolizing enzymes (thymidylate synthetase (TS), dihydrouracil dehydrogenase (DPD), orotate phosphoribosyl transferase (OPRT), and thymidine phosphorylase (TP)) in each cell line.

We treated MOLY cells with 1.0–8.0 µg/mL 5-FU for 3 h and TK6 and WTK-1 cells with 1.56–25 and 3.13–50 µg/mL, respectively, for 4 h. In MOLY cells, the mutation frequency (MF) and MN frequency increased. In WTK-1 cells, the MN frequency but not the MF increased. In TK6 cells, neither the MF nor the MN frequency increased. Furthermore, the IC<sub>50</sub> of 5-FU was lower in MOLY cells than in the human cells. The response to 5-FU treatment differed in other ways as well. At the same level of cytotoxicity, the frequency of apoptotic cell was highest in TK6 cells. The cell cycle was delayed just after treatment in MOLY cells while the delay appeared 24 h later in TK6 and WTK-1 cells. Nothing in our analysis, however, revealed marked differences between the cell lines that could account for the severe cytotoxic and mutagenic responses that 5-FU elicited only in MOLY cells.

5-FU is phosphorylated by OPRT and TP and detoxified by DPD. MOLY cells have higher OPRT activity and markedly lower DPD and TP activity than TK6 and WTK-1 cells. The content of TS, however, the target enzyme of 5-FU, was similar in all cell lines, suggesting that 5-FU was more readily phosphorylated and less readily detoxified in MOLY cells than in TK6 and WTK-1 cells.

MOLY cells were more sensitive to 5-FU than WTK-1 cells even though both have a mutated p53 gene, suggesting that the different responses to 5-FU were due to differences in 5-FU metabolism rather than the p53 status.

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### 1. Introduction

Mouse lymphoma L5178Ytk<sup>+/-</sup> (MOLY) cells are widely used in Tk mutation assays [1–5]. MOLY cells are heterozygous at the Tk locus, where loss of heterozygous

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