by the Ethics Review Committees for Animal Experimentation of the participating institutions.

gpt *assay*

The *gpt* assay (Figure 1) was performed as described previously (Nohmi *et al.*, 1996). Briefly, the phages rescued from genomic DNAs were transfected into *E. coli* YG6020 expressing Cre recombinase. Infected cells were cultured at 37°C on plates containing chloramphenicol (Cm) and 6-thioguanine (6-TG) for 3 days until 6-TG-resistant colonies appeared. To confirm the 6-TG-resistant phenotype, colonies were restreaked on plates containing Cm and 6-TG. Confirmed 6-TG-resistant colonies were cultured, and bacterial pellets were collected by centrifugation. The average background mutant frequency in the *gpt* assay was 1×10^{-6}. The predominant type of the background mutations is insertion of transposable element, IS*1* of *E. coli*, and is distinguishable from the mutations generated in mice (Masumura *et al.*, 1999). As no IS*1* insertion mutations were observed in our collection of *gpt* mutations recovered from mice, they were suggested to be originated in mice but not in *E. coli* host. The data for mutant frequencies in *gpt* assay were therefore presented without subtracting the average background mutant frequencies.

A 739 bp DNA fragment encompassing the *gpt* gene was amplified by PCR as described previously (Masumura *et al.*, 1999). DNA sequencing of the *gpt* gene was performed with a CEQ™ DTCS Quick Start Kit (Beckman Coulter).

Spi⁻ assay

An Spi⁻ assay (sensitive to P2 interference) (Nohmi *et al.*, 1996) was carried out with a slight modification as described previously (Shibata *et al.*, 2003). The frequencies of background mutants were less than 10^{-8} in Spi⁻ assay (Nohmi *et al.*, 1999) and was neglectable. The data for Spi⁻ mutant frequencies were therefore presented without subtracting the background mutant frequencies. Spi⁻ mutant phages were infected to *E. coli* LE392 and phage DNA was purified by Quantum Prep® AquaPure Genomic DNA Kit (Bio-Rad).

Analysis of mutation spectra in the Spi⁻ mutants

To determine the mutated region, lysates of Spi⁻ mutant phage or the phage DNA were used and subjected to PCR analysis with various sets of primers (Nohmi *et al.*, 1999; Okada *et al.*, 1999). To narrow down the mutated region, the structure of each mutation was also analysed by digesting Spi⁻ mutant DNA with restriction enzymes and by comparing the lengths of the produced fragments with those expected for wild-type lambda EG10 DNA. We also adapted a Southern blot hybridization method that uses oligonucleotide DNA probes (Shibata *et al.*, 2004). Briefly, 10 μg of oligomers, 25–36 nucleotides long, were spotted onto Hybond™-N (Amersham Pharmacia Biotech), and after crosslinking by UV (1200 J/m², Stratagene) the membrane was soaked into a hybridization buffer consisting of 10 × Denhardt's solution, 2 × SSC, and 100 μg/ml denatured salmon sperm DNA at 42°C for 2 h. As the probe of each clone, the 15 kb region spanning the *red/gam* genes of each Spi⁻ mutant was amplified by PCR with the primers 18874R and 32890F (Shibata *et al.*, 2004), and was labeled with [α^{32}P]dCTP using the Megaprime DNA Labeling System (Amersham Pharmacia Biotech). Hybridization was performed overnight at 37°C, and the membrane was washed three times each for 1 h at 37°C with a buffer containing 2 × SSC, 0.1% SDS, and 7.5 mM pyrophosphate, and the radioactivity of each oligomer spot was analysed by BAS 2500 (Fuji Film). This method permits the identification of where a deletion occurred and its approximate length. When there is a deletion associated with an insertion, then one or more of the probes does not give a signal, but the PCR product is bigger than expected.

Statistical analysis

The statistical significance of differences in mutant or mutation frequencies between the two groups was analysed using the Mann–Whitney *U*-test. The differences of the frequencies of simple deletion and complex-type deletion between genotypes were analysed by Fisher's exact test.

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Research Articles

Light-Dependent Mutagenesis by Benzo[a]pyrene is Mediated via Oxidative DNA Damage

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Benzo[a]pyrene (B[a]P) is an environmental carcinogenic polycyclic aromatic hydrocarbon (PAH). Mammalian enzymes such as cytochrome P450s and epoxide hydrase convert B[a]P to reactive metabolites that can covalently bind to DNA. However, some carcinogenic compounds that normally require metabolic activation can also be directly photoactivated to mutagens. To examine whether B[a]P is directly mutagenic in the presence of light, we exposed *Salmonella typhimurium* strains with different DNA repair capacities to B[a]P and white fluorescent light at wavelengths of 370–750 nm. B[a]P plus light significantly enhanced the number of His^+ revertants. Mutagenesis was completely light-dependent and required no exogenous metabolic activation. The order of mutability of strains with different DNA repair capacities was strain YG3001 (*uvrB*, *mutM*$_{ST}$) $\gg$ strain TA1535 (*uvrB*) > strain YG3002 (*mutM*$_{ST}$) > strain TA1975. The *uvrB* gene product is involved in the excision repair of bulky DNA adducts, and the *mutM*$_{ST}$ gene encodes 8-oxoguanine (8-oxoG) DNA glycosylase, which removes 8-oxoG from DNA. Introduction of a plasmid carrying the *mOgg1* gene that is the mouse counterpart of *mutM*$_{ST}$ substantially reduced the light-mediated mutagenicity of B[a]P in strain YG3001. B[a]P plus light induced predominantly G:C → T:A and G:C → C:G transversions. We propose that B[a]P can directly induce bulky DNA adducts if light is present, and that the DNA adducts induce oxidative DNA damage, such as 8-oxoG, when exposed to light. These findings have implications for the photocarcinogenicity of PAHs. Environ. Mol. Mutagen. 46:141–149, 2005.

Key words: photocarcinogenesis; benzo[a]pyrene; polycyclic aromatic hydrocarbon; photosensitization; reactive oxygen species

INTRODUCTION

Benzo[*a*]pyrene (B[*a*]P) is a ubiquitous environmental pollutant and a potent mutagen and carcinogen. The carcinogenicity of B[*a*]P is believed to be initiated by its metabolic activation to electrophilic metabolites capable of covalent binding to DNA [Conney et al., 1994]. The best-characterized pathway of metabolic activation involves oxidation by cytochrome P450 to form the 7,8-epoxide, hydrolysis by epoxide hydrase to the *trans*-7,8-diol, and oxidation to the 9,10-epoxide by cytochrome P450 to give diol epoxides, i.e., (+)-*anti*-B[*a*]P-7,8-dihydrodiol-9,10-epoxide (B[*a*]PDE) [Harvey, 1991]. The major DNA reaction product of B[*a*]PDE is the (+)-*trans*-*anti*-B[*a*]P-N^2-dG lesion, which accounts for ~90% of total adducts [Jeffrey et al., 1976; Osborne et al., 1976; Weinstein et al., 1976; Cheng et al., 1989]. B[*a*]PDE produces mostly G:C → T:A transversions [Moriya et al., 1996; Fernandes et al., 1998]. (±)-*anti*-B[*a*]PDE forms adducts preferentially at mutational hot spots in the *p53* gene [Denissenko et al., 1996], which is mutated in many human tumors [Harris, 1993].

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TABLE I. *S. typhimurium* Strains Used in This Study

Name	Description	Source
TA1975	As LT2 but *hisG46 rfa*	Maron and Ames [1983]
TA1535	As TA1975 but Δ(*gal bio chl uvrB*)	Maron and Ames [1983]
YG3001	As TA1975 but Δ(*gal bio chl uvrB*) Δ$mutM_{ST}$::Km^r	Suzuki et al. [1997]
YG3002	As TA1975 but Δ$mutM_{ST}$::Km^r	Suzuki et al. [1997]
TA100	As TA1535 but harboring pKM101	Maron and Ames [1983]

Comparative carcinogenicity studies suggest that multiple mechanisms of activation may be involved in B[*a*]P carcinogenesis. For instance, metabolic activation may also generate reactive oxygen species (ROS), including singlet oxygen, hydrogen peroxide, and superoxide anion radicals [Leadon et al., 1988]. B[*a*]P and other polycyclic aromatic hydrocarbons (PAHs) such as 7,12-dimethylbenz[*a*]anthracene (DMBA) generate a substantial amount of ROS during their metabolism [Von Sonntag, 1987; Cadet and Vigny, 1990]. In addition, some B[*a*]P metabolites are capable of producing ROS. For example, the 6-oxo-B[*a*]P radical can produce B[*a*]P diones [Nagata et al., 1974; Lorentzen and Ts'o, 1977], which can enter a redox cycle between diones and diols, producing ROS as a byproduct [Cerutti et al., 1978]. The B[*a*]P-7,8-dione also generates ROS in the presence of NADPH and $CuCl_2$ [Flowers et al., 1997].

Interestingly, several carcinogens that normally need metabolic activation can be photoactivated to directly-acting mutagens by exposure to light. In fact, several PAHs are mutagenic to *Salmonella typhimurium* strain TA102 without metabolic activation if UVA and visible light are present [Yan et al., 2004]. In addition, B[*a*]P can act as a photosensitizer that generates ROS and binds covalently to DNA on exposure to light in vitro [Kagan et al., 1989]. Exposure of mammalian cells to B[*a*]P and fluorescent light generates oxidative damage in DNA [Mauthe et al., 1995]. Thus, in the cell, both covalent PAH DNA adducts and DNA oxidative damage may be induced after exposure to B[*a*]P and light. This damage occurs even without the metabolic conversion of B[*a*]P.

To examine the mechanisms underlying the light-dependent mutagenesis of B[*a*]P, we exposed a set of *S. typhimurium* strains having different DNA repair capacities to B[*a*]P plus white fluorescent light. The bacterial mutation assay was chosen because the bacteria lack all the enzyme activities required to metabolically activate B[*a*]P. Exposure of the strains to low doses of B[*a*]P plus fluorescent light significantly increased the frequency of His^+ reversion. The mutagenicity was substantially modulated by the ability of the strains to repair bulky DNA lesions and oxidative damage, and a *uvrB* $mutM_{ST}$ double mutant displayed the highest mutability. The *uvrB* gene product is involved in nucleotide excision repair of bulky DNA adducts, and the $mutM_{ST}$ gene encodes 8-oxoguanine (8-oxoG) DNA glycosylase, which removes 8-oxoG in DNA. We propose that B[*a*]P can bind to DNA upon light exposure even without metabolic activation, and that the adducts in DNA, rather than a free form of B[*a*]P, generate mutagenic oxidative DNA damage such as 8-oxoG in the presence of light. We discuss the implications of these findings in terms of the photocarcinogenicity of B[*a*]P.

MATERIALS AND METHODS

Strains and Plasmids

The *S. typhimurium* strains used in this study are listed in Table I. The plasmid carrying *mOgg1* was kindly provided by Drs. T. Kohno and J. Yokota, Department of Biology, National Cancer Center Research Institute, Tokyo, Japan. The *mOgg1* gene was expressed as a GST-fusion protein using a pGEX-1λT system [Tani et al., 1998]. The pGEX-1λT vector was purchased from Amersham Biosciences (Piscataway, NJ) and used as a control.

Media and Chemicals

Nutrient broth (Difco, Detroit, MI) was used for overnight cultures. Vogel-Bonner minimal agar plates and top agar used for the reversion assay (described below) were prepared as described by Maron and Ames [1983]. Soft agar contained 0.6% Bacto™ Agar (Difco) and 0.6% sodium chloride. After sterilization, 0.05 mM L-histidine and 0.05 mM D(+)-biotin were added.

The sources of the chemicals used in this study were as follows: B[*a*]P (CAS 50-32-8), DMBA (CAS 57-97-6), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx; CAS 77500-04-0), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ; CAS 76180-96-6), 2-acetylaminofluorene (2-AAF; CAS 53-96-3), and dimethylnitrosamine (DMN; CAS 62-75-9) were from Wako Pure Chemicals (Osaka, Japan); 2-aminofluorene (2-AF; CAS 153-78-6) was from Nacalai tesque (Kyoto, Japan).

Bacterial Reversion Assay

The mutagenicity assay included a preincubation procedure [Maron and Ames, 1983]. Briefly, the chemicals were dissolved in dimethylsulfoxide (0.1 ml), and incubated with cells (0.1 ml) for 20 min at 37°C in pH 7.4 phosphate buffer (0.5 ml) with shaking. The treated cells were mixed with soft agar (2 ml) containing histidine and biotin, and poured onto Vogel-Bonner agar plates. Kanamycin (25 μg/ml) was added to the medium for the overnight culture of the $mutM_{ST}$-deletion strains YG3001 and YG3002. Ampicillin (50 μg/ml) was added to the medium for the overnight culture of the strains harboring pGEX-1λT or GST-*mOgg1*. Each chemical was assayed at least twice, mostly with duplicate plates. The numbers of His^+ revertants per plate were counted after a 2-day incubation at 37°C with and without visible light. Means and standard deviations were calculated from the revertants on the four or five plates used in two experiments to examine the light-mediated mutagenesis by B[*a*]P. Means and standard deviations were also calculated from the revertants on six plates used for three experiments to examine the suppressive effects of *mOgg1* on the light-dependent mutagenesis by B[*a*]P. No statistical tests were employed to evaluate the results.

White Fluorescent Light Irradiation

Plates were irradiated with white fluorescent light delivered by a fluorescent lamp (15W, 370–750 nm wavelength) during the two-day incubation at 37°C. The plates were placed upside down at a distance of 50 cm from the light source. The light intensity was 1,000 lux as measured with an IM-1 Illumination meter (Tokyo kogaku kikai K.K., Tokyo, Japan). Plates not exposed to light were covered with sheets of aluminum foil during incubation.

DNA Sequencing Analysis

To analyze the mutations, a part of *hisG* gene was amplified by PCR of colonies with primers 5′-GAT TGA TAT CCT GCG CGT GCG TG-3′ (forward, 23mer) and 5′-TCG TCA ACC GGT GTT GCC AGC G (reverse, 22mer), using an MJ Research DNA Engine PTC-200 (GMI, Ramsey, MN). The primers amplify 201 base pairs (bp) of a DNA fragment flanking the 69th codon of the *hisG* gene, the site of *hisG46* mutation. DNA sequencing of the PCR products was carried out with the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems Japan, Tokyo, Japan), using the forward primer (mentioned earlier), and an ABI PRISM 310 DNA sequencer (Applied Biosystems Japan).

RESULTS

Light-Dependent Mutagenicity of B[a]P Depends on the DNA Repair Abilities of *S. typhimurium* Strains

To examine the light-dependent mutagenicity of B[*a*]P, *S. typhimurium* strains YG3001 (*uvrB*, *mutM*$_{ST}$), TA1535 (*uvrB*), YG3002 (*mutM*$_{ST}$), and TA1975 were exposed to B[*a*]P and incubated for 2 days with and without fluorescent light radiation. In these experiments, no exogenous metabolic activation enzymes or co-factors were incorporated in the reaction mixtures. Consistent with the known requirement for metabolic activation for B[*a*]P mutagenesis, virtually no revertants were recovered in the absence of light exposure (data not shown). In contrast, the number of his$^+$ revertants per plate increased when the plates were exposed to light during the 2-day incubation (Fig. 1, Table II). Mutagenesis was dependent on the dose of B[*a*]P and was significantly affected by the ability of the bacteria to repair DNA damage. The most sensitive strain was YG3001 (*uvrB, mutM*$_{ST}$), and the ratios of the sensitivities, i.e., the number of induced His$^+$ revertants per plate per dose, were 112 (YG3001), 31 (TA1535), 2 (YG3002), and 1 (TA1975), compared at a dose of 5 μg B[*a*]P in the presence of fluorescent light.

To examine whether other chemical carcinogens can be similarly photoactivated, we examined the effects of DMBA, 2-AF, MeIQ$_X$, 2-AAF, IQ, and DMN in the presence of fluorescent light. Among these compounds, only DMBA exhibited light-dependent mutagenicity (Table II). Although the light-mediated mutagenicity of DMBA was less than that of B[*a*]P and not dose-responsive at the concentrations that we assayed, it was reproducible. As with B[*a*]P, YG3001(*uvrB, mutM*$_{ST}$) was the bacterial strain most sensitive to the mutagenicity of DMBA plus light.

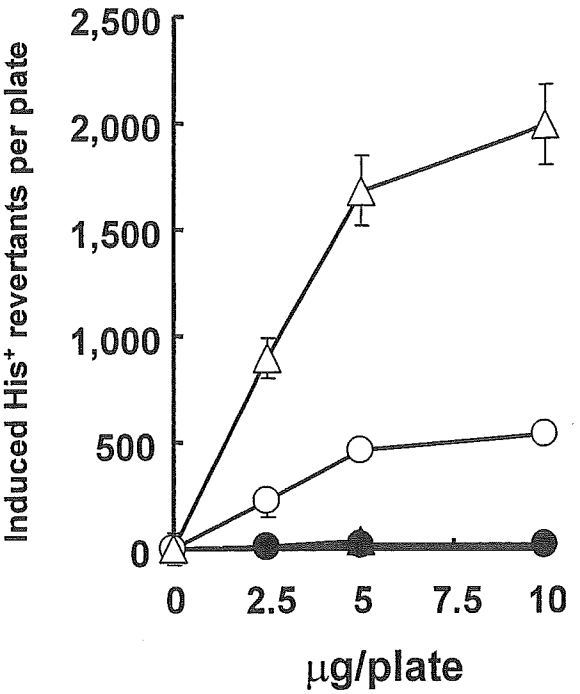

Fig. 1. Mutagenicity of B[*a*]P in the presence of fluorescent light in *S. typhimurium* strains with various DNA repair capacities. The numbers of induced His$^+$ revertants per plate were calculated from the revertants on four or five plates of Exp. I and II by subtracting the mean control revertants (15 for TA1975, 33 for YG3002, 191 for TA1535, and 567 for YG3001 in Table II). The mean and standard deviation of the numbers of induced His$^+$ revertants per plate were plotted vs. the dose of B[*a*]P. Open triangle, YG3001; open circle, TA1535; closed triangle, YG3002; closed circle, TA1975.

In addition to B[*a*]P- and DMBA-induced mutagenesis, the mutations in control plates were also enhanced by the exposure to fluorescent light (Table II). The fold-increases in the number of revertants in the control plates produced by irradiation were 18 (545 in light vs. 31 in dark) for YG3001; 12 (187 in light vs. 15 in dark) for TA1535; 5 (31 in light vs. 6 in dark) for YG3002; and 8 (15 in light vs. 2 in dark) for TA1975.

Suppression of Reversion with Introduction of the *Ogg1* Gene into *S. typhimurium*

The *mOgg1* gene is a mouse counterpart of the *mutM*$_{ST}$ gene and encodes 8-oxodG DNA glycosylase that removes 8-oxoG from DNA. To clarify the contribution of 8-oxodG to the light-dependent mutagenicity of B[*a*]P, a plasmid carrying *mOgg1* and the control vector pGEX-1λT were introduced into YG3001, and the resulting strains were exposed to B[*a*]P and fluorescent light. Expression of *mOgg1* substantially reduced the number of His$^+$ revertants and the resulting mutagenicity was similar to that observed in strain TA1535 harboring the empty vector pGEX-1λT (Fig. 2, Table III). Comparing the numbers of revertants at a dose of 5 μg B[*a*]P per plate in

TABLE II. Light-Dependent Mutagenesis of B[*a*]P and DMBA in *S. typhimurium* Strains With Different DNA Repair Capacities

	TA1975 ($mutM_{ST}^+$, $uvrB^+$)		YG3002 ($\Delta mutM_{ST}$, $uvrB^+$)		TA1535 ($mutM_{ST}^+$, $\Delta uvrB$)		YG3001 ($\Delta mutM_{ST}$, $\Delta uvrB$)	
Dose	Rev./plate[b]	Mean	Rev./plate[b]	Mean	Rev./plate[b]	Mean	Rev./plate[b]	Mean
B[*a*]P[a]								
Exp. I								
0	13, 19	16	34, 38	36	178, 149	164	480, 519	500
2.5	28, 27	28	44, 48	46	327, 490	409	1,412, 1356	1,384
5	33, 27	30	82, 61	72	612, 632	622	2,440, 2328	2,384
10	35, 41, 39	38	78, 82, 82	81	700, 776, 676	717	2,840, 2496, 2384	2,573
Exp. II								
0	11,17	14	26, 32	29	220, 218	219	637, 633	635
2.5	22, 21	22	39, 41	40	407, 431	419	1544, 1542	1543
5	30, 29	30	75, 62	69	680, 687	684	2120, 2104	2112
10	33, 39	36	76, 89	83	726, 699	713	2492, 2408	2450
DMBA[a]								
Exp. I								
0	10, 16	13	21, 17	19	131, 170	151	357, 338	348
25	21, 20	21	34, 34	34	224, 225	225	647, 652	650
50	12, 15	14	29, 28	29	255, 254	255	625, 677	651
100	9, 8, 10	9	21, 24, 39	28	212, 196, 216	208	535, 504, 640	560
Exp. II								
0	20, 14	17	35, 41	38	198, 229	214	694, 701	698
25	20, 27	24	49, 39	44	295, 279	287	1000, 980	990
50	17, 11	14	38, 49	44	284, 323	304	1120, 904	1012
100	20, 18	19	39, 43	41	269, 295	282	844, 920	882

[a]The assays were carried out twice (Exp. I and II) to confirm the initial results.
[b]Spontaneous numbers of His^+ revertants per plate in the dark were 2 for TA1975, 6 for YG3002, 15 for TA1535, and 31 for YG3001.

strain YG3001 harboring the control vector and the strain harboring plasmid carrying *mOgg1* (Table III), the expression of *mOgg1*appeared to reduce the mutagenesis by 50%. A similar reduction in mutagenicity was observed with human *OGG1* [Kim et al., 2004]. Interestingly, the expression of *mOgg1* suppressed reversion in control plates in the presence of light by 45% (255 His^+ revertants per plate in strain YG3001 harboring the control vector vs. 139 His^+ revertants per plate in strain YG3001 harboring the plasmid carrying *mOgg1,* Table III). These results suggest that 8-oxoG in DNA accounts for about half of the light-dependent mutagenesis by B[*a*]P as well as reversion in control plates of YG3001. Since the pGEX-1λT vector carries the gene encoding glutathione S-transferase, this enzyme may be involved in the reduction of mutagenicity observed in strains YG3001 and TA1535 (Fig. 2 and Table III).

Molecular Analysis of Mutations Induced by B[a]P Plus Visible Light

In the *hisG46* allele, 5′-CCC-3′ (proline) replaces the wild-type sequence of 5′-CTC-3′ (leucine) at the 69th codon. Besides true reversion (CCC to CTC), several base substitutions at the CCC sequence can revert the phenotype to His^+. To investigate the mutations induced by B[*a*]P plus fluorescent light at the molecular level, the spectrum of mutations in the *hisG46* allele was determined for the His^+ revertants of YG3001 (Table IV). As controls, we analyzed revertants of YG3001 exposed to light but not to B[*a*]P and of TA100 exposed to B[*a*]P (10 μg/plate) with S9 mix in the dark. TA100 was chosen because *S. typhimurium* strains lacking pKM101, such as TA1535 or YG3001, are not mutable by B[*a*]P even in the presence of S9 mix (data not shown). We could not use strain YG3001 harboring pKM101 because the strain displayed an extremely high frequency of spontaneous mutation. Since guanine bases are major targets of both B[*a*]PDE and ROS, we designated the target sequence as 5′-$G_1G_2G_3$-3′ in the opposite strand. Interestingly, the positions of the mutations were dependent on how B[*a*]P had been activated (Table IV). More mutations were induced at G_3 than at G_2 when strains YG3001 were exposed to B[*a*]P and fluorescent light. In contrast, the opposite tendency, i.e., more mutations at G_2 than at G_3, was observed when strain TA100 was treated with B[*a*]P plus S9 mix. The types of mutations were also different. B[*a*]P plus light induced both G:C → C:G and G:C → T:A transversions, whereas no G:C → C:G transversions were induced by metabolically activated B[*a*]P. The mutational spectrum of YG3001 revertants exposed to light alone was similar to that of the same strain treated with photoactivated B[*a*]P. G:C → C:G mutations were, however, less frequent in the absence of B[*a*]P treatment, although the difference was not statistically significant ($P = 0.07$ by Fischer's exact test). We conclude that G_3 in 5′-$G_1G_2G_3$-3′ is particularly susceptible to reactions depending on both light and B[*a*]P, whereas G_2 in 5′-

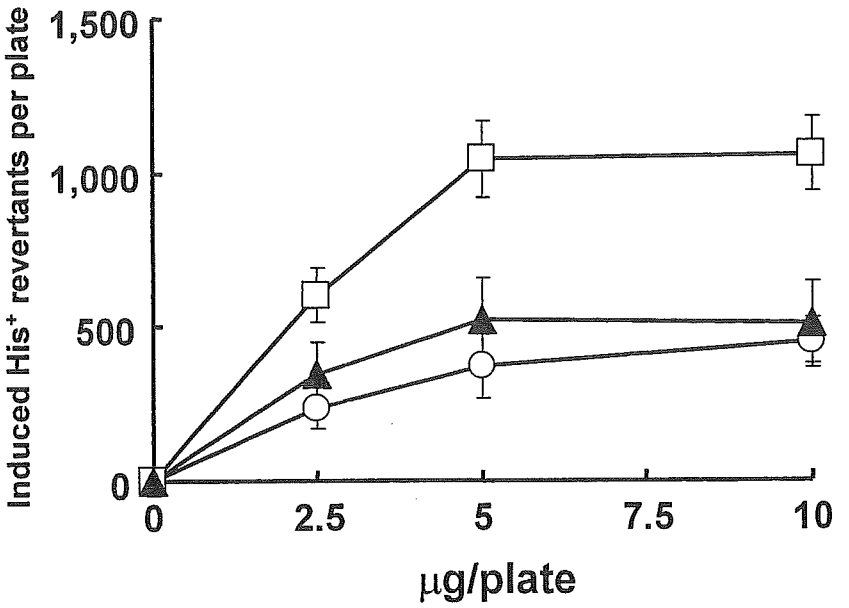

Fig. 2. Suppression of the light-dependent mutagenicity of B[*a*]P with *mOgg1*. The numbers of induced His^+ revertants per plate were calculated from the revertants on six plates from three experiments by subtracting the mean control revertants (146 for TA1535/pGEX-1λT, 255 for YG3001/pGEX-1λT, and 139 for YG3001/GST-*mOgg1* in Table III). The mean and standard deviation of the numbers of induced His^+ revertants per plate were plotted vs. the dose of B[*a*]P. Open circle, TA1535 harboring empty vector pGEX-1λT; open square, YG3001 harboring pGEX-1λT; closed triangle, YG3001 harboring GST-*mOgg1*.

TABLE III. Suppressive Effects of *mOgg1* on Light-Dependent Mutagenesis by B[*a*]P[a]

Dose (μg/plate)	TA1535/ pGEX-1λT	YG3001/ pGEX-1λT	YG3001/ GST-*mOgg1*
0	146 ± 47	255 ± 39	139 ± 34
2.5	383 ± 70	858 ± 87	485 ± 101
5	519 ± 104	1300 ± 125	661 ± 135
10	594 ± 81	1314 ± 123	649 ± 135

[a]Experiments were conducted three times with duplicate plates. The means and standard deviations were calculated from the numbers of revertants on six plates from three experiments.

$G_1G_2G_3$-3′ is more susceptible to mutation by metabolically activated B[*a*]P in the presence of pKM101 in the host strain.

DISCUSSION

We present evidence that B[*a*]P is mutagenic even without metabolic activation if fluorescent light is present and that the mutagenicity is substantially affected by the DNA repair capacities of the exposed cells (Fig. 1). The predominant mutations induced by photoactivated B[*a*]P are G:C → T:A and G:C → C:G transversions, preferentially at the G_3 position in the 5′-$G_1G_2G_3$-3′ sequence (Table IV). Our findings indicate that oxidative DNA damage—in particular 8-oxoG in DNA—plays an important role in the light-dependent mutagenesis by B[*a*]P (Fig. 2, Table III).

The mutagenicity of photoactivated B[*a*]P was synergistically enhanced by disruption of the $mutM_{ST}$ and *uvrB* genes (Fig. 1, Table II). Deletion of $mutM_{ST}$ alone increased the mutagenicity only slightly, but when combined with Δ*uvrB*, the effect was more pronounced. We suggest that a photoactivated form of B[*a*]P can bind to DNA. The resulting bulky DNA adducts would then behave as powerful photosensitizers to generate mutagenic oxidative damage in nearby regions of DNA (summarized in Fig. 3). This model is supported by the following observations. First, free B[*a*]P behaves as a photosensitizer that generates ROS [Kagan et al., 1989]. Second, ROS is involved in the nonenzymatic binding of B[*a*]P to DNA in vitro [Bryla and Weyand, 1991]. In addition, 1-hydroxypyrene, a PAH metabolite, forms covalent DNA adducts upon UVA irradiation [Dong et al., 2000]. We assume that a free form of B[*a*]P could generate ROS and self-oxidize to bind to DNA in the presence of fluorescent light. Because 8-methoxypsoralen intercalates into DNA and becomes activated to form DNA adducts upon light irradiation [Averbeck, 1989], we speculate that B[*a*]P might form covalent adducts in DNA more easily if it first intercalates into DNA. Alternatively, exposure of B[*a*]P to light may result in reactive radical intermediates, such as the 6-oxo-B[*a*]P radical, which bind to DNA [Inomata and Nagata, 1972]. Whatever the mechanisms are, B[*a*]P appears to form bulky DNA adducts without metabolic conversion if fluorescent light is present. It follows,

TABLE IV. Mutation Spectra and Position of the *hisG46* Target Site

hisG46[a] (5′-$G_1G_2G_3$-3′, 3′-$C_1C_2C_3$-5′)	YG3001 (0 μg B[*a*]P/plate) with light without S9 mix	YG3001 (10 μg B[*a*]P/plate) with light without S9 mix	TA100 (10 μg B[*a*]P/plate) with S9 mix without light
	No. (%)	No. (%)	No. (%)
G_2	9 (19)	4 (8)	35 (75)
G:C → T:A	4 (8)	3 (6)	28 (60)
G:C → C:G[b]	0 (0)	0 (0)	0 (0)
G:C → A:T	5 (11)	1 (2)	7 (15)
G_3	37 (77)	43 (92)	12 (25)
G:C → T:A	26 (54)	23 (49)	10 (21)
G:C → C:G	10 (21)	20 (43)	0 (0)
G:C → A:T	1 (2)	0 (0)	2 (4)
Others	2 (4)	0 (0)	0 (0)
	48 (100)	47 (100)	47 (100)

[a]Alteration of G_1 does not produce revertants because of wobbling

[b]The revertants have no G:C → C:G alterations at G_2 because the CGC codon (arginine) does not restore activity to HisG protein [Eisenstadt et al., 1989]

therefore, that inactivation of the pathway for the excision of bulky DNA adducts would increase the steady-state level of B[*a*]P adducts. This, in turn, would generate more oxidative damage in adjacent regions of DNA. The B[*a*]P adducts in DNA may, at least in part, account for the mutagenicity observed in strain TA1535 (Δ*uvrB*). Deletion of $mutM_{ST}$ in the Δ*uvrB* background would prevent the removal of the oxidative DNA damage, thereby enhancing the light-dependent mutagenesis.

Although a free form of B[*a*]P generates ROS in a light-dependent manner [Kagan et al., 1989], it does not seem to induce substantial amounts of oxidative DNA damage because the inactivation of $mutM_{ST}$ alone enhanced the mutagenicity only slightly (Table II). Because of their extreme reactivity, ROS generated from B[*a*]P by light may react only with B[*a*]P itself or closely neighboring molecules. Since DMBA also displayed photoactivated mutagenicity in strain YG3001 (Table II), we speculate that DMBA-DNA adducts and perhaps other PAH-DNA adducts might induce oxidative DNA damage by generating ROS in the presence of light. Light-dependent mutagenicity of DMBA also has been demonstrated in *S. typhimurium* TA98 and TA1537 without metabolic activation, which suggests the formation of DNA adducts inducing frameshift mutations [Utesch and Splittgerber, 1996]. In this respect, it is important to identify the chemical structures of B[*a*]P- and DMBA-DNA adducts that are generated by photoactivation.

Not only chemically-induced mutagenesis, but also reversion in control plates was enhanced by the exposure to fluorescent light. Previous studies report the light-dependent enhancement of reversion in control plates in several *S. typhimurium* and *Escherichia coli* strains, but there was no discussion in relation to oxidative DNA damage [Henderson et al., 1994; Utesch and Splittgerber, 1996]. Our observation that the expression of *mOgg1* substantially suppressed reversion in control plates of YG3001 (*uvrB*, $mutM_{ST}$) strongly suggests that 8-oxoG in DNA plays a significant role in the light-dependent mutagenesis in the control cells. We suspect that some natural compounds in the cell or components in the medium might act as photosensitizers and contribute to the mutagenesis in the strain. These compounds might modulate the mutagenicity of light-irradiated B[*a*]P as well. Besides YG3001, reversion in control plates of TA1535 (*uvrB*) was also enhanced by exposure of light. In a separate experiment, we observed that reversion in control plates of strain TA100, which is the same as TA1535 but harbors plasmid pKM101, was about five times higher than that in control plates of TA1535 when exposed to fluorescent light (unpublished results). Plasmid pKM101 encodes DNA pol RI, which bypasses a variety of DNA adducts [Goldsmith et al., 2000]. Thus, we suggest DNA adducts that are excised by excision repair and bypassed by DNA pol RI could be formed when the cells are exposed to fluorescent light. The slight reduction of reversion in control plates of TA1535 by the vector plasmid pGEX-1λT might be due to the suppression of adduct formation by the glutathione S-transferase carried by the vector. This enzyme is known to behave as a scavenger of electrophiles [Landi, 2000].

The mutation spectra shown in Table IV are consistent with the idea that ROS contribute to the mutagenicity of light-irradiated B[*a*]P in strain YG3001 ($mutM_{ST}$ Δ*uvrB*). The predominant mutation was G:C → T:A transversion, which is a signature mutation of 8-oxoG in DNA [Wood et al., 1990; Moriya et al., 1991; Cheng et al., 1992]. It is reported that 8-oxoG in DNA can be further photooxidized to several compounds, some of which induce G:C → C:G transversion in SOS-induced *E. coli* [Buchko et al., 1995]. In particular, one of the photoproducts, i.e., 2-amino-5-((2-deoxy-β-D-*erythro*-pentofuranosyl)amino)

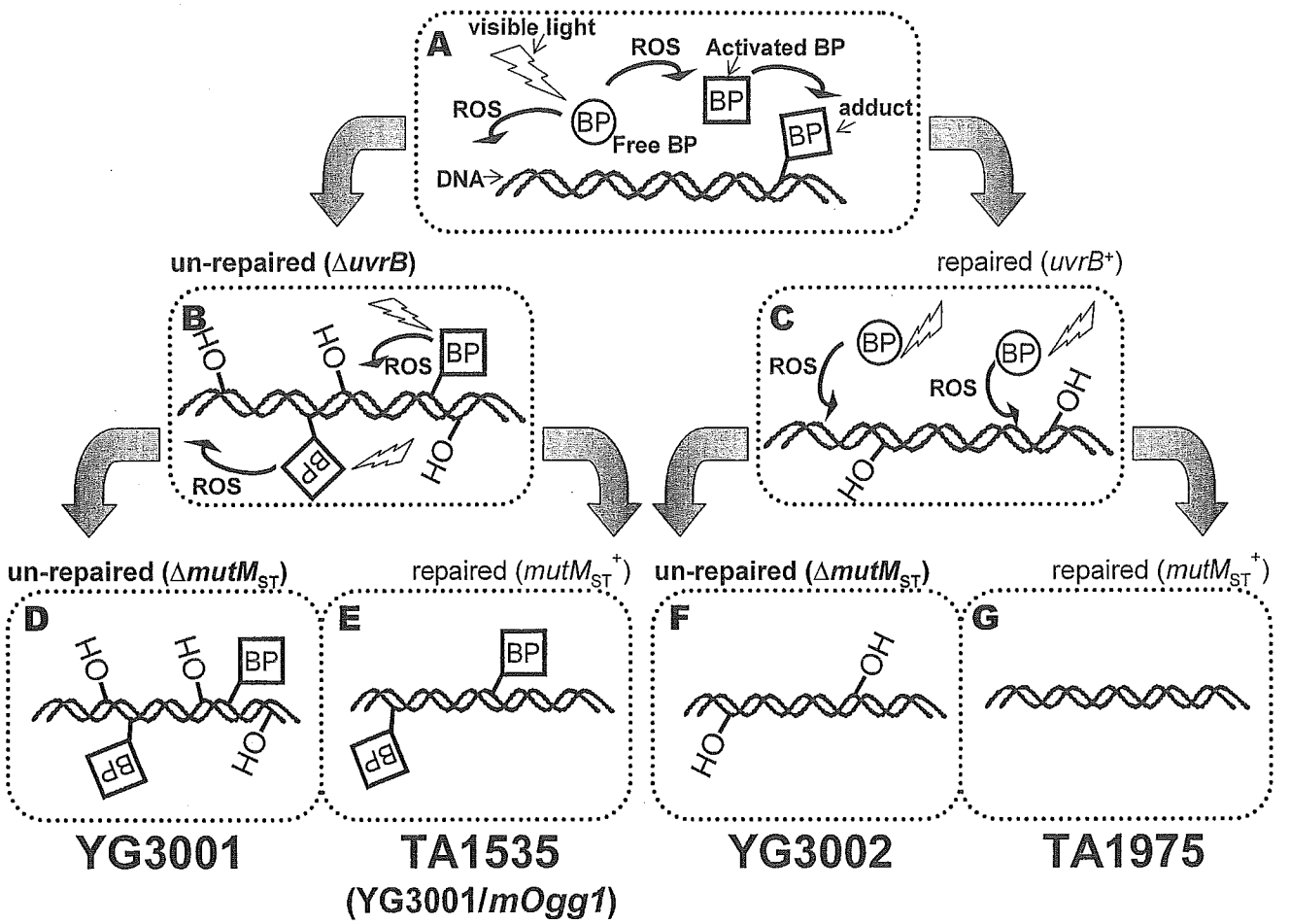

Fig. 3. A proposed mechanism of light-dependent mutagenesis by B[*a*]P in *S. typhimurium* strains having different DNA repair capacities. B[*a*]P in a free form or intercalated in DNA is photoactivated and binds to DNA (**A**). The binding may be mediated through the generation of ROS. The resulting B[*a*]P-DNA adducts behave as photosensitizers that generate ROS in the presence of light (**B**). The ROS generated from the adducts induce oxidative damage such as 8-oxoG in DNA, thereby promoting mutagenesis in strain YG3001 (*uvrB, mutM*$_{ST}$) (**D**). In strains TA1535 (*uvrB*) and YG3001 harboring the plasmid carrying *mOgg1*, most of 8-oxoG in DNA is removed and thus the mutagenicity is reduced (**E, G**). Most of the B[*a*]P-DNA adducts are removed by Uvr-dependent excision repair in strains YG3002 (*mutM*$_{ST}$) and TA1975 (**C, F, G**). ROS generated from the free form of B[*a*]P induces 8-oxoG in DNA in strain YG3002 (**F**). However, the contribution to mutagenesis is small because deletion of *mutM*$_{ST}$ alone enhances the light-dependent mutagenicity of B[*a*]P only slightly.

4*H*-imidazol-4-one (dIz), preferentially forms bp with G [Kino and Sugiyama, 2001]. Primer extension experiments using a template containing dIz demonstrate that dGTP is selectively incorporated opposite dIz. Thus, the second major mutation, i.e., G:C → C:G, may also be explained by ROS-induced DNA damage such as dIz. Although the repair mechanism for dIz in DNA is poorly understood, it seems reasonable to assume that deletion of *mutM*$_{ST}$ increases the amount of dIz in DNA, at least in part, by increasing the level of 8-oxoG in DNA.

The mutational spectra (Table IV) also indicate that the mechanisms of mutagenesis by metabolically activated B[*a*]P and light-activated B[*a*]P may be different. The G_2 position in 5′-$G_1G_2G_3$-3′ is susceptible to metabolically activated B[a]P whereas the G_3 position in 5′-$G_1G_2G_3$-3′ is susceptible to photoactivated B[*a*]P. An analysis of the mutation spectrum induced by B[*a*]PDE in the *supF* gene of an *E. coli* plasmid revealed hot spots in 5′-GG-3′ sequences, with more mutations occurring at the 5′-G than at the 3′-G [Rodriguez and Loechler, 1993; Hanrahan et al., 1997]. This is consistent with our findings that G_2 is more susceptible to metabolically activated B[*a*]P than G_3. If the G_2 is particularly reactive toward photoactivated B[*a*]P, it is most likely that, as the nearest G base, G_3, would be the most susceptible site to the oxidative damage by ROS derived from the bound B[*a*]P-DNA adducts. It should be noted, however, that the presence of pKM101 plasmid in the TA100 strain could affect the mutation spectrum induced by metabolically activated B[*a*]P. More work is needed to clarify the mechanisms underlying the different hot spots for metabolically activated and photoactivated B[*a*]P.

Considering the formation of ROS from B[*a*]P adducts, it is an open question whether ROS generated from the B[*a*]P adducts can oxidize bases at a remote site. Hall et al. reported that oxidative damage to DNA may be promoted from a remote site as a result of hole migration through DNA [Hall et al., 1996]. Kino and Sugiyama [2001] showed that oxidation of 8-oxoG to dIz by ROS generated from the photosensitizer, anthraquinone, can occur six bases distant from the 8-oxoG in the opposite DNA strand. It will be important to examine to what extent distant bases are oxidized by ROS generated by B[*a*]P and other PAH adducts.

The ubiquity of B[*a*]P and visible light in the environment suggests the cooperative genotoxicity shown in this study might have implications for the carcinogenicity of PAHs. Enhancement of skin tumors in mice exposed to B[*a*]P and near ultraviolet light has been reported [Santamaria et al., 1966; Cavalieri and Calvin, 1971]. Some light-absorbing pharmaceuticals, such as psoralens and chlorpromazine derivatives, cause photogenotoxic effects, and the fluoroquinolone antibiotics have recently been recognized as being photomutagens [Gocke, 2001]. Our results suggest that not only free forms of chemicals but also DNA adducts could be powerful photosensitizers that generate oxidative damage in DNA. Although bulky adducts in transcriptionally active regions of the chromosome are preferentially removed by nucleotide excision repair, those in transcriptionally inactive regions, which account for most of the genome, are excised less quickly [Svejstrup, 2002]. Our findings suggest that some PAH-DNA adducts in transcriptionally inactive regions have the potential to generate ROS in the presence of light. Their carcinogenic potential would be enhanced by the ability to cause DNA damage in remote chromosomal sites. Thus, in addition to their documented activity as metabolically activated carcinogens and their ability to alter the methylation status of DNA [Turk et al., 1995], PAHs may contribute to age-related human diseases, including cancer, through photochemical activation [Cerutti et al., 1985].

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Mutagenesis Induced by Oxidized DNA Precursors: Roles of Y Family DNA Polymerases in *Escherichia coli*

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To reveal the roles of Y family DNA polymerases in the mutagenesis induced by oxidatively damaged DNA precursors, 2-hydroxy-dATP (2-OH-dATP) and 8-hydroxy-dGTP (8-OH-dGTP) were introduced into *Escherichia coli* strains deficient in the Y family polymerases, DNA polymerase IV (pol IV, encoded by the *dinB* gene) and DNA polymerase V (pol V, encoded by the *umuDC* locus). The mutation induced by 2-OH-dATP, but not that induced by 8-OH-dGTP, occurred less frequently in the *dinB*$^-$ strain than in the wild-type (wt) strain, suggesting the involvement of pol IV in the mutagenesis by 2-OH-dATP. Expression of pol IV from plasmid enhanced the mutagenesis by 2-OH-dATP in the *dinB*$^-$ strain. This enhancement depends on the polymerase activity since the expression of a mutant pol IV lacking the polymerase activity did not increase the mutations induced by 2-OH-dATP. In contrast, both 2-OH-dATP and 8-OH-dGTP caused mutations more efficiently in the *umuDC*$^-$ strain than in the wt strain, suggesting that the *umuDC* gene products suppressed the mutagenesis by these oxidized DNA precursors. The DNA polymerase activity was not required for the suppressive effects because expression of the *umuDC* gene products lacking the polymerase activity also suppressed the mutagenesis. These results suggest that the *E. coli* pol IV was involved in mutagenesis by 2-OH-dATP and that the *umuDC* gene products play suppressive role(s) in the mutagenesis by damaged nucleotides.

Introduction

Reactive oxygen species (ROS)[1] are generated endogenously and exogenously and are believed to be an important source of the mutations that cause various diseases, aging, and neurodegeneration (*1*). Many of the lesions formed on DNA by ROS result in the alteration of genetic information (*2*). 2-Hydroxyadenine (2-OH-Ade) and 8-hydroxyguanine (8-OH-Gua), which are mutagenic oxidative DNA lesions (*3*), are likely to be produced in the DNA through the following two pathways. One is the direct modification of DNA residues, and the other is the incorporation by DNA polymerase(s) (pol) of oxidatively damaged DNA precursors, 2-hydroxy-2′-deoxyadenosine 5′-triphosphate (2-OH-dATP) and 8-hydroxy-2′-deoxyguanosine 5′-triphosphate (8-OH-dGTP), which are produced in the nucleotide pool. Both of the pathways contribute similarly to the formation of 8-OH-Gua (*4*). In addition, the involvement of 8-OH-dGTP as well as 8-OH-Gua in DNA in mutagenesis was indicated by analysis of bacterial strains lacking MutT, MutM, and/or MutY (*5*). The incorporation of oxidatively damaged precursor might be more important in the case of 2-OH-Ade, because its formation by the Fenton type reaction is much more efficient in the monomeric form than in DNA in vitro (*6*).

The significance of these damaged nucleotides is supported by the presence of their specific hydrolyzing enzymes (*7*−*9*). The MutT protein removes 8-OH-dGTP from the nucleotide pool by hydrolysis to the corresponding monophosphate derivative (*7*). In fact, a *mutT* deficient *Escherichia coli* strain shows a 1000-fold increase in the mutation rate as compared to the wild-type (wt) strain (*4*). In addition, the human MutT homologue, hMTH1, hydrolyzes 2-OH-dATP as well as 8-OH-dGTP (*8*, *10*), and the knock-out mice of this gene exhibit increased occurrences of carcinogenesis in the lung, liver, and stomach (*11*). Moreover, both the 2-OH-dATPase and the 8-OH-dGTPase activities of hMTH1 suppressed H_2O_2-induced cell death (*12*). Thus, the incorporation of 2-OH-dATP and 8-OH-dGTP seems to strongly affect cellular functions.

These oxidized DNA precursors are misincorporated by DNA pol(s) opposite incorrect bases and form mispairs. 8-OH-dGTP is incorporated opposite A, thus inducing A:T→C:G transversions in *E. coli* (*7*). In contrast, the misincorporation mode of 2-OH-dATP is highly DNA pol specific. *E. coli* DNA pols misincorporate 2-OH-dATP opposite G, whereas mammalian DNA pol α incorporates 2-OH-dATP opposite C on template DNA in vitro (*6*, *13*). These variations generate the different mutation spectra of 2-OH-dATP, G:C→T:A transversions, and G:C→A:T transitions in *E. coli* and in mammalian nuclear extracts, respectively (*14*, *15*), although the actual mutation

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[1] Abbreviations: HE, holoenzyme; 2-OH-Ade, 2-hydroxyadenine; 2-OH-dATP, 2-hydroxy-2′-deoxyadenosine 5′-triphosphate; 8-OH-Gua, 8-hydroxyguanine; 8-OH-dGTP, 8-hydroxy-2′-deoxyguanosine 5′-triphosphate; pol, polymerase; ROS, reactive oxygen species; TLS, translesion synthesis; wt, wild-type.

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spectrum of 2-OH-dATP in living mammalian cells is unknown.

The Y family of DNA pols, a new class that lacks intrinsic exonuclease activity, has been found in various species (*16*). This family of DNA pols seems to be involved in error-free or error-prone translesion synthesis (TLS) (*17*). Recently, the involvement of Y family DNA pols in the incorporation of damaged DNA precursors was also suggested by in vitro experiments using purified DNA pols. The archeal Y family DNA pols P1 and P2 and the human pol η exclusively incorporate 8-OH-dGTP opposite A in the template DNA and incorporate 2-OH-dATP opposite G more efficiently than opposite T (*18*). Thus, it would be quite interesting to examine the in vivo roles of Y family DNA pols in the incorporation of oxidatively damaged deoxyribonucleotides.

Here, we examined the effects of *E. coli* Y family DNA pols, DNA pol IV (pol IV, encoded by the *dinB* gene) (*19*), and DNA pol V (pol V, encoded by the *umuDC* locus) (*20*, *21*) on the mutagenesis induced by 2-OH-dATP and 8-OH-dGTP. 2-OH-dATP induced mutations less efficiently in the *dinB*$^-$ strain than in the wt strain, and both 2-OH-dATP and 8-OH-dGTP induced mutations more efficiently in the *umuDC*$^-$ strain than in the wt strain. Interestingly, these effects of pol IV and the *umuDC* gene products seemed to be DNA pol activity-dependent and -independent, respectively. These results suggest that the *E. coli* Y family DNA pols, pol IV, and the *umuDC* gene products, enhanced and suppressed, respectively, the mutagenesis induced by oxidatively damaged DNA precursors.

Materials and Methods

Materials. The FPLC grade nucleoside triphosphates were from Amersham Bioscience (Piscataway, NJ). 2-OH-dATP and 8-OH-dGTP were prepared from dATP and dGTP, respectively, and were purified by HPLC as described (*6*, *22*). The purified nucleotides showed the same ultraviolet spectra as those of the corresponding deoxyribonucleosides in the literature (*23*, *24*) and were eluted as a single peak in both reverse phase and anion exchange HPLC (data not shown). Their purities were estimated to be more than 99%. Purified oligonucleotides were from Hokkaido System Science (Sapporo, Japan) and Sigma Genosys Japan (Ishikari, Japan).

The *E. coli* strain YG7207 (*ΔdinB::kan*) is a derivative of the AB1157 strain (*25*). The *E. coli* strains YG7209 and YG7210 are derivatives of AB1157 and YG7207, respectively, in which *ΔumuDC::Cat* (*26*) was transferred by P1 transduction. Plasmids pYG768 and pYG779, respectively, carry *dinB* and *dinB*003, of which the latter encodes a mutant (D103N) pol IV lacking the pol activity (*26*, *27*). The amount of *dinB* mRNA in AB1157 containing pYG768 was more than 10-fold as abundant as that in AB1157 without the plasmid (data not shown). The pSE117 plasmid contains the entire *umuDC* operon (*28*), and the amount of *umuC* mRNA in AB1157 containing pSE117 was more than 10-fold as abundant as that in AB1157 without the plasmid (data not shown). The conversion of the 101st codon from GAT (Asp) to AAT (Asn) of the *umuC* gene in pSE117 was performed according to the protocol for the Altered Sites II in vitro Mutagenesis System (Promega, Madison, WI). The mutagenic oligonucleotide used was 5′-dAGATTTACAGTATTAATGAGGCATTCTGC-3′, where the mismatched base is underlined. The mutation was confirmed by DNA sequencing. This plasmid (pUE101) expresses a mutant UmuD′$_2$C protein (pol V), which cannot perform TLS (*20*). Plasmid pUE102 was constructed by deleting the *Hin*d III fragment (about 3 kb) including 2/3 of the *umuC* gene (amino acid residues 149–422) from pSE117. Plasmids pKMTa1 and pKMTb1, derivatives of pYG768, were constructed by *Sac* I-*Eco*R I (including the lexA binding region and the *dinB* gene) and *Sna*B I-*Eco*R I (including the *dinB* gene) digestions, respectively, followed by end filling of the 3′ overhangs using a Blunting High kit (Toyobo, Osaka, Japan) prior to religation of the blunt-ended DNA. Plasmids pGW2101 and pGW2133 are *rop*$^-$ plasmids encoding UmuDC and UmuD′C, respectively (*29*). The plasmid DNAs used in this study are shown in Table 1.

Table 1. Plasmids Used in This Study

plasmids	relevant genotype or characteristics	source
pYG768	pWSK29 derivative, DinB	*27*
pYG779	pWSK29 derivative, DinB (D103N)	*25*
pKMTa1	pYG768 derivative, *lexA*$^-$, *dinB*$^-$	this study
pKMTb1	pYG768 derivative, *dinB*$^-$	this study
pSE117	pBR322 derivative, UmuDC	*28*
pUE101	pSE117 derivative, UmuDC (D101N)	this study
pUE102	pSE117 derivative, UmuD, UmuC (1–148)	this study
pGW2101	pBR322 derivative, UmuDC, *rop*$^-$	*29*
pGW2133	pGW2101 derivative, UmuD′C	*29*

Mutation Assays. The introduction of 2-OH-dATP and 8-OH-dGTP was performed essentially as described (*14*). Single colonies of each strain on optimal selection plates were suspended in 7.5 mL of prewarmed LB medium and incubated at 37°C until the cultures were growing exponentially (ca. 0.6 OD_{610}), and competent cells were prepared by a treatment with 0.1 M calcium chloride. The nucleotide solution (1.25 μL) was added to a final concentration of 125 or 250 μM to 50 μL of the *E. coli* suspension, which was placed on ice for 20–25 min. After heat shock treatment (42 °C for 45 s and then 0 °C for 2 min), SOC medium (450 μL) was added, and the cells were incubated at 37 °C for 2 h with shaking. LB and SOC media containing 150 μg/mL ampicillin were used for *E. coli* strains harboring the vector plasmids in this paper. A portion of the culture was diluted with ice-cold LB, plated on an LB agar plate, and incubated at 37 °C overnight (the titer plate). Another portion of the culture was plated on rifampicin (100 μg/mL) plates, which were incubated at 37 °C for 24 h to select *rpoB* mutants. The mutant frequency was calculated according to the numbers of colonies on the titer and selection plates. The statistical significance of the results was examined by the Student's *t*-test.

Sequence Analysis of the rpoB Gene. Portions of the *rpoB* gene were amplified by PCR, using *Taq* DNA pol (Toyobo) and sets of primers as follows: 5′-dACAGGATATGATCAACGCCAA-3′ and 5′-dCGATACGGAGTCTCAAGGAA-3′ for positions 1467–1775 and 5′-dTGGTGATCTATGAGCGCGAA-3′ and 5′-dACCAGTTCCATCTGCAGCTT-3′ for positions 305–725, as described (*30*, *31*). The closest 2–5 colonies to the center of a rifampicin plate were selected for sequencing analysis, to avoid any picking bias based on colony size. The amplified PCR products were sequenced using the former primers, an ABI PRISM Big Dye Terminator Cycle Sequencing Kit, and an ABI model 377 DNA sequencer (Applera, Norwalk, CT).

Results

Effects of DNA Pols IV and V on Mutations by Damaged DNA Precursors. 2-OH-dATP and 8-OH-dGTP, the oxidized forms of dATP and dGTP, respectively, show high mutagenicity in *E. coli* (*14*). To investigate the effects of Y family DNA pols on mutagenesis by these oxidatively damaged DNA precursors, we introduced 2-OH-dATP and 8-OH-dGTP by the $CaCl_2$ method into *E. coli* strains deficient in DNA pol IV (pol IV, encoded by the *dinB* gene) and/or DNA pol V (pol V, encoded by the *umuDC* locus). Moreover, *E. coli* strains containing the episomal *dinB* gene and the *umuDC* locus were used. After 2-OH-dATP and 8-OH-dGTP were added to a $CaCl_2$-treated *E. coli* suspension, the cells were cultured at 37 °C for 2 h after heat shock. The chromosomal *rpoB* gene was used as the mutagenesis target,

Table 2. Effects of Pol IV and Pol V Expression on Mutagenesis by Oxidized Nucleotides

	mutant frequency ($\times 10^{-8}$)[a]					
	2-OH-dATP added[b]			8-OH-dGTP added[b]		
	0 μM	125 μM	250 μM	0 μM	125 μM	250 μM
AB1157 (wt)	5.7 (2.9)	9.0 (4.4)	21.5 (8.1)	6.0 (2.2)	13.0 (5.8)	18.1 (7.6)
YG7207 (*dinB*$^-$)	2.8 (1.6)	5.1 (2.8)	11.4 (6.4)	3.0 (1.3)	9.5 (3.6)	15.8 (7.2)
YG7209 (*umuDC*$^-$)	3.8 (1.6)	12.7 (6.7)	32.7 (10.4)	3.9 (1.3)	19.1 (4.5)	31.8 (8.3)
YG7210 (*dinB*$^-$, *umuDC*$^-$)	3.2 (2.1)	11.4 (6.5)	31.6 (13.6)	5.1 (2.9)	18.2 (7.6)	25.6 (8.9)
AB1157/pYG768 (DinB)	5.1 (1.1)	10.9 (3.9)	23.6 (7.6)	7.0 (2.6)	17.1 (7.9)	23.4 (9.4)
AB11157/pSE117 (UmuDC)	11.1 (2.7)	13.1 (3.6)	19.4 (6.6)	14.1 (5.2)	19.3 (4.9)	21.8 (6.1)

[a] All data are represented as means (SD) of 11−26 independent experiments. [b] Amount of 1.25 μL, to a final concentration of 125 or 250 μM of each oxidized nucleoside triphosphate, added to 50 μL of a 0.1 M calcium chloride-treated *E. coli* suspension. The control experiment (0 μM) was acomplished with the same volume of water.

and single base substitutions in various sequence contexts could be monitored by the selection with rifampicin (*30*).

In the control experiment, in which an equal volume of water instead of the damaged nucleotide solution was added to the bacteria, the observed mutant frequency was 2-fold lower in the pol IV deficient strain, YG7207, as compared to that in AB1157, the wt strain (Table 2). The pol V deficient strain YG7209 also showed a slightly decreased mutant frequency. Conversely, the additional expression of pol V by the pSE117 plasmid containing the *umuDC* genes in the wt strain exhibited more than a 2-fold increase in the mutant frequency in the control experiments. Although the overexpression of pol IV reportedly causes large elevations of the mutant frequency (*25*, *32*), only a slight increase was observed in this study, possibly because of the presence of the *lexA* operating region on the pYG768 plasmid. LexA keeps the expression of *dinB* low by transcriptional regulation, even on an episomal plasmid (*27*). The mutagenic effect of pol V expressed from pSE117 might be limited similarly. We used these *lexA* binding site-containing plasmids to avoid too much of an increase in the background mutant frequencies by the error-prone DNA pols, for the evaluation of the mutagenesis by damaged DNA precursors.

Next, we compared the frequencies of the mutations induced by these damaged nucleotides in various strains. The mutant frequencies were increased by the addition of 2-OH-dATP and 8-OH-dGTP in all of the strains used, in a dose-dependent manner (Table 2). In contrast, the treatment with unmodified dATP and dGTP did not increase mutant frequencies in all of the strains tested (data not shown), as described previously (*14*). Thus, mutations by the addition of 2-OH-dATP and 8-OH-dGTP were not due to mere nucleotide imbalance. To evaluate the actual frequency of mutations induced by the exogenous nucleotides, we subtracted the mutant frequency of the control experiments from the value obtained in the presence of 250 μM of an oxidized nucleotide. This subtracted mutant frequency was effective when strains with different background mutant frequencies were compared (*33*). Each subtracted frequency was calculated from the 250 μM nucleotide and control experiments using the same original single colonies.

The subtracted mutant frequencies in the wt strain were 15.8×10^{-8} and 12.1×10^{-8} with the addition of 2-OH-dATP and 8-OH-dGTP, respectively (Figure 1). 2-OH-dATP induced mutations less efficiently (8.6×10^{-8}, $P < 0.001$, examined by the Student's *t*-test) in the pol IV deficient strain than in the wt strain, suggesting that pol IV enhanced mutagenesis by 2-OH-dATP. The statistical significance ($P < 0.01$) was also obtained by the Mann−Whitney test. However, the additional expression of pol IV from plasmid in the wt strain did not

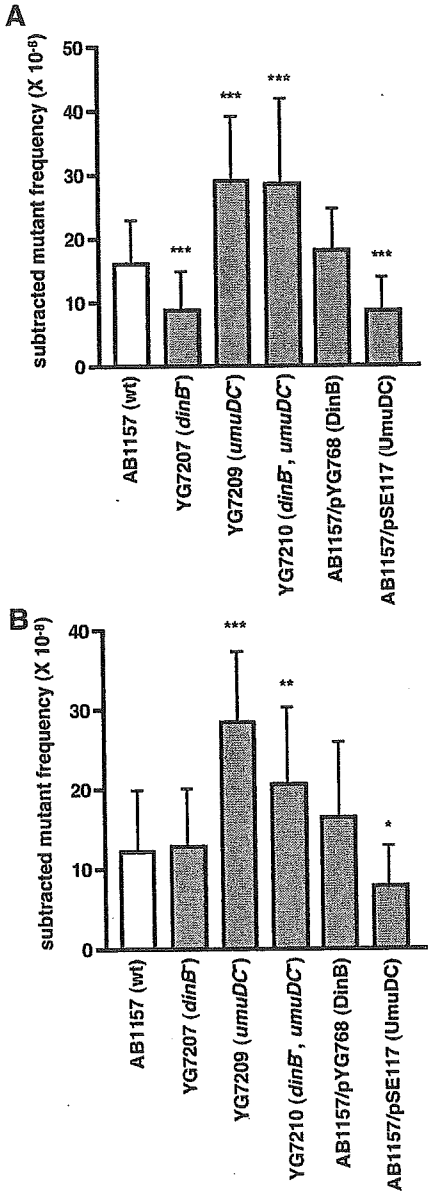

Figure 1. Mutant frequencies induced by (A) 2-OH-dATP and (B) 8-OH-dGTP in various *E. coli* strains. Values were calculated by subtracting the mutant frequencies of control experiments from the values obtained in the presence of 250 μM 2-OH-dATP or 8-OH-dGTP. This subtraction was done for the 250 μM nucleotide and control experiments, using the same original single colonies, and each subtracted frequency was considered as a single datum. The presented data are the means of 11−26 independent experiments. Error bars represent SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (significant difference vs the wt strain).

Table 3. Spectra of Mutations Induced by Damaged Nucleotides in *E. coli*[a]

	AB1157 (wt)			YG7207 (*dinB*⁻)			YG7209 (*umuDC*⁻)		
	control	2-OH-dATP	8-OH-dGTP	control	2-OH-dATP	8-OH-dGTP	control	2-OH-dATP	8-OH-dGTP
single base substitution									
transition									
A:T→G:C	7 (5)	2 (2)	0 (0)	18 (13)	2 (3)	1 (1)	25 (20)	2 (3)	0 (0)
G:C→A:T	98 (64)	26 (31)	22 (28)	57 (38)	15 (19)	7 (8)	34 (28)	5 (6)	4 (5)
transversion									
A:T→T:A	15 (10)	0 (0)	1 (1)	15 (11)	3 (4)	1 (1)	20 (16)	1 (1)	2 (3)
A:T→C:G	9 (6)	1 (1)	55 (71)	31 (19)	6 (8)	71 (83)	20 (16)	3 (4)	66 (89)
G:C→T:A	20 (13)	54 (64)	0 (0)	26 (17)	53 (66)	2 (2)	17 (14)	65 (83)	2 (3)
G:C→C:G	3 (2)	1 (1)	0 (0)	4 (2)	1 (1)	3 (4)	3 (3)	2 (3)	0 (0)
others	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3[b] (3)	0 (0)	0 (0)
total	152 (100)	84 (100)	78 (100)	151 (100)	80 (100)	85 (100)	122 (100)	78 (100)	74 (100)

	YG7210 (*dinB*⁻,*umuDC*⁻)			AB1157 /pYG768 (DinB)			AB1157 /pSE117 (UmuDC)		
	control	2-OH-dATP	8-OH-dGTP	control	2-OH-dATP	8-OH-dGTP	control	2-OH-dATP	8-OH-dGTP
single base substitution									
transition									
A:T→G:C	10 (11)	0 (0)	2 (3)	9 (10)	0 (0)	1 (2)	9 (7)	1 (1)	3 (6)
G:C→A:T	23 (24)	1 (2)	4 (6)	44 (51)	10 (17)	11 (17)	68 (52)	29 (40)	25 (48)
transversion									
A:T→T:A	16 (17)	1 (2)	5 (7)	11 (13)	1 (2)	2 (3)	8 (6)	2 (3)	1 (2)
A:T→C:G	30 (32)	7 (12)	55 (81)	16 (18)	1 (2)	47 (73)	0 (0)	0 (0)	16 (31)
G:C→T:A	10 (11)	50 (83)	2 (3)	5 (6)	48 (80)	1 (2)	33 (25)	39 (54)	6 (12)
G:C→C:G	4 (4)	1 (2)	0 (0)	1 (1)	0 (0)	2 (3)	14 (11)	1 (1)	1 (2)
others	0 (0)	0 (0)	0 (0)	1[b](1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
total	93 (100)	60 (100)	68 (100)	87 (100)	60 (100)	64 (100)	132 (100)	72 (100)	52 (100)

[a] All data are represented as cases found (%). [b] See the Supporting Information Table 1 legend.

significantly increase the mutagenesis by 2-OH-dATP (Figure 1A). In contrast, neither the deficiency nor the additional expression of pol IV affected the mutation frequency of 8-OH-dGTP (Figure 1B).

The deficiency in pol V significantly facilitated the mutagenicity of 2-OH-dATP ($P < 0.001$) and 8-OH-dGTP ($P < 0.001$) (Figure 1). A similar enhancement was obtained in the *dinB umuDC* mutant strain YG7210. This result suggests that pol V suppressed the mutagenesis induced by these oxidized nucleotides. When pol V was additionally expressed from plasmid in the wt strain, the subtracted mutant frequencies were decreased to 8.3×10^{-8} and 7.7×10^{-8} ($P < 0.001$ and $P < 0.05$), in the cases of 2-OH-dATP and 8-OH-dGTP, respectively.

To rule out the possibility that the permeation efficiency of the damaged nucleotides into cells causes the difference in the mutant frequencies, we introduced ^{33}P-labeled dATP into the wt, pol IV and pol V deficient strains to check the membrane permeability. As expected, there were no differences in the incorporation amount among the strains tested (data not shown). In addition, cell division occurred about twice in all of the strains, from the time of the introduction of damaged nucleotides to the plating, indicating that the growth rate did not vary considerably between the wt and its derivative strains. Thus, the difference in the induced mutant frequency was not due to the frequency of DNA replication.

These results suggest that the mutagenicity of 2-OH-dATP was enhanced and suppressed in the presence of pol IV and pol V, respectively. The mutagenesis by 8-OH-dGTP was also suppressed by pol V, although a deficiency in pol IV did not affect it in this case.

Mutation Spectra of 2-OH-dATP and 8-OH-dGTP. We then analyzed the sequences of the *rpoB* genes in a total of 1628 colonies, obtained with the control, 2-OH-dATP, and 8-OH-dGTP experiments in various strains. Between two and five colonies per plate were picked and sequenced, based on the number of mutants on the rifampicin plates. When two colonies were picked from one plate, each colony had different mutations. Five colonies were picked from one plate when more than 50 mutant colonies appeared on the plate. Because the *E. coli* suspension seems to have been plated before the third cell division as described above and because the mutations are fixed during the second replication, most of the nucleotide-induced mutants would be derived from different *E. coli* cells, which incorporated the nucleotide.

Supporting Information Tables 1–3 and Table 3 show the results of the sequencing analysis of the *rpoB* mutants. In the control experiment, 64% of the mutants contained G:C→A:T transitions in the case of the wt strain (Supporting Information Table 1 and Table 3). The ratio of these mutations was greatly decreased in the pol IV and pol V deficient strains. Only the additional expression of pol V enhanced this type of mutation, when we consider the increase in the total mutant frequency. The absence of an obvious effect of pol IV expression from plasmid in the wt strain may be due to the existence of a relatively large number of pol IV molecules in cells (250 molecules/cell), even without induction by the SOS response (*27*). The number of A:T→C:G and A:T→G:C mutations, which were shown to be induced on the *cII* gene by the overexpression of pol IV (*32*), seemed to increase in the AB1157/pYG768 combination. The frequencies of G:C→C:G and G:C→T:A transversions were increased in the pol V-overexpressing strain, as reported previously (*34*). A deficiency in pol V increased the occurrence of A:T→C:G transversions, while this type of mutation was not observed when pol V was additionally expressed in the wt strain. Unexpectedly, one and three examples of 3 or 9 bp deletions and insertions were detected in the pol IV-overexpressing strain (TAT insertion at positions between 1541 and 1542) and the pol V deficient strain (TTT insertion at positions between 1540 and 1543, 9 bp deletion from 1546 to 1554, and GCA deletion from 1594 to 1596), respectively.

Table 4. Effects of the *dinB* and *umuDC* Gene Products

		mutant frequency ($\times 10^{-8}$)[a]			
		2-OH-dATP added		8-OH-dGTP added	
strain	plasmid	0 μM	250 μM	0 μM	250 μM
YG7207	none	2.8 (1.6)	11.4 (6.4)	3.0 (1.3)	15.8 (7.2)
	pYG768 (DinB)	3.1 (1.6)	20.2 (9.6)	4.4 (2.1)	23.3 (4.0)
	pYG779 (DinB D103N)	4.1 (2.1)	5.7 (2.1)	4.4 (2.2)	8.0 (3.6)
YG7209	none	3.8 (1.6)	32.7 (10.4)	3.9 (1.3)	31.8 (8.3)
	pSE117 (UmuDC)	6.3 (3.8)	18.9 (7.8)	5.8 (4.3)	24.3 (5.2)
	pUE101 (UmuDC D101N)	2.8 (1.6)	12.4 (6.9)	3.0 (1.6)	17.8 (9.3)
	pUE102 (UmuD)[b]	2.4 (0.9)	34.2 (5.6)	2.4 (1.1)	28.3 (7.7)
	pGW2101 (UmuDC)	2.4 (0.8)	12.0 (1.4)	5.1 (4.5)	16.7 (5.4)
	pGW2133 (UmuD'C)	14.0 (6.9)	32.8 (5.9)	10.5 (4.6)	24.6 (3.6)

[a] All data are represented as means (SD) of 3–26 independent experiments. [b] UmuD, UmuC (1–148).

The addition of 250 μM 2-OH-dATP specifically induced G:C→T:A transversions in all strains examined (Supporting Information Table 2 and Table 3). Similarly, 8-OH-dGTP induced A:T→C:G transversions (Supporting Information Table 3 and Table 3). These results suggest that 2-OH-dATP and 8-OH-dGTP were misincorporated opposite G and A, respectively, independent of the expression of pol IV or pol V. The G:C→T:A transversions induced by 2-OH-dATP formed some minor hotspots (Supporting InformationTable 2). It has been reported that 2-OH-dATP and 8-OH-dGTP preferentially induce transversions in 5′-GG-3′ and 5′- TAA-3′ sequences, respectively (*14*). The sites of the G:C→T:A mutations induced by 2-OH-dATP were mostly within 5′-GG-3′ sequences (25/54 in the wt strain), whereas 8-OH-dGTP induced A:T→C:G transversions mostly in 5′-C/GA-3′ sequences. The reason for this contradictory result with 8-OH-dGTP might be due to the paucity of detectable A sites with T in the 5′-flanking sequence in the *rpoB* gene. No remarkable difference in the incidence of G:C→T:A or A:T→C:G mutations in these hotspots was observed with the expression of pol IV and pol V.

Pol Activity-Dependent and -Independent Effects on Mutagenesis by Damaged DNA Precursors. The plasmids pYG779 (*19*) and pUE101, respectively, encode mutant pol IV and pol V, with an amino acid substitution in the highly conserved region of the UmuC analogues (Asp-103→Asn and Asp-101→Asn, respectively). To examine the mechanism by which pol IV and pol V affect the mutagenesis by damaged DNA precursors, we introduced these plasmids into the pol IV and polV deficient strains YG7207 and YG7209, respectively, and the mutations induced by 2-OH-dATP and 8-OH-dGTP were measured (Table 4). Because these mutant proteins lack their pol activities, the expression of their derivatives cannot induce SOS mutagenesis (*19–21, 35*). The mutant UmuC (D101N) product forms the $UmuD_2C$ (D101N) complex with two molecules of UmuD and confers the cold sensitivity in a *lexA* deficient strain (*35*), which means that the folding of the mutant protein is probably normal. We measured the mutant frequency in strains harboring various plasmids, including pYG779 and pUE101, with and without the treatment with damaged nucleotides (Figure 2).

The expression of pol IV in YG7207 restored the mutant frequency to a level comparable to that in the wt strain, when 2-OH-dATP was added ($8.6–17.1 \times 10^{-8}$) (Figure 2A). Surprisingly, the mutant pol IV (DinB D103N) reduced the mutant frequency, as compared to the pol IV deficient strain without the plasmid. This reduction also occurred in the case of 8-OH-dGTP.

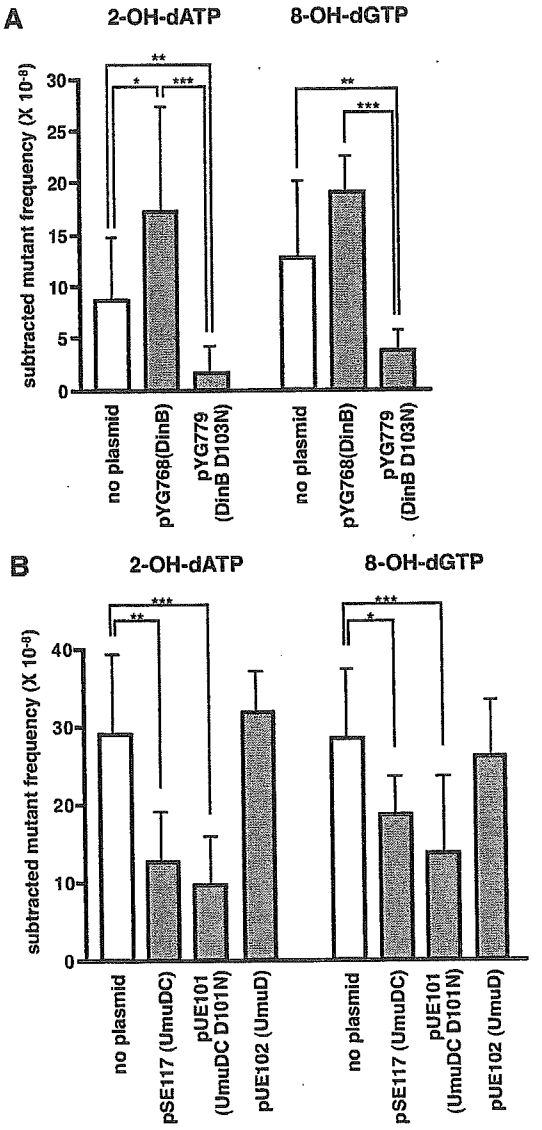

Figure 2. Effect of the pol IV and pol V pol activities. Subtracted mutant frequencies, calculated as in the Figure 1 legend, were examined (A) in a pol IV deficient strain (YG7207) expressing DinB (pol IV) and DinB D103N (mutant DinB lacking the pol activity) and (B) in a pol V deficient strain (YG7209) expressing UmuDC (pol V), UmuDC D101N (mutant UmuC with UmuD lacking the pol activity), and UmuD with UmuC (1–148). Data are the means of at least five independent experiments. Error bars represent SD. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Control mock vectors, constructed by the deletion of only the *dinB* coding region, and *dinB* and *lexA* from pYG768 (pKMTb1 and pKMTa1, respectively), did not influence

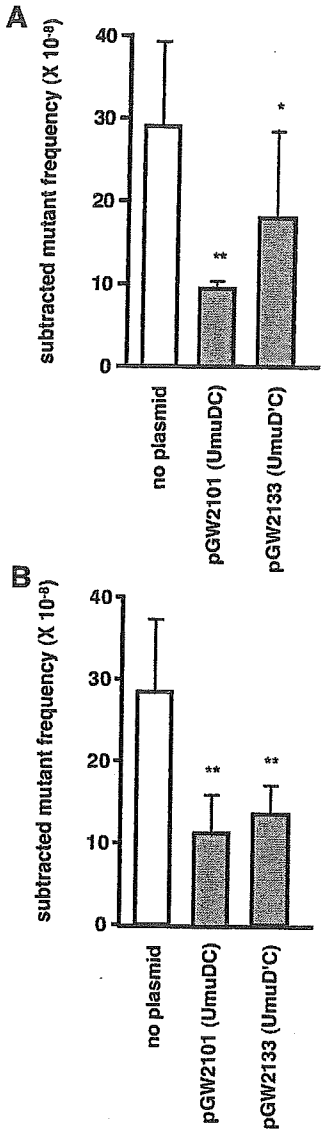

Figure 3. Subtracted mutant frequencies, calculated as in the Figure 1 legend, induced by (A) 2-OH-dATP and (B) 8-OH-dGTP in a pol V deficient strain (YG7209) harboring plasmids encoding UmuDC or UmuD'C. Data are the means of at least three independent experiments. Error bars represent SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (significant difference vs YG7209).

the mutagenicity of the two damaged nucleotides (data not shown). Thus, pol IV exerted the specific facilitation of the mutagenesis by 2-OH-dATP, probably via its pol activity. The reasons why the mutant pol IV (DinB D103N) reduced the mutant frequency will be discussed later.

In contrast, pol V suppressed the mutations induced by 2-OH-dATP and 8-OH-dGTP (Figure 2B). Because the mutant pol V ($UmuD_2C$ D101N) also decreased the subtracted mutant frequency to a similar extent, pol V suppressed the induction of mutations by damaged DNA precursors in a pol activity-independent manner. The expression of UmuD did not affect the mutant frequencies induced by 2-OH-dATP and 8-OH-dGTP. Thus, most of the suppressive effect of pol V was not due to the action(s) of UmuD without UmuC. Moreover, other plasmids encoding UmuDC (pGW2101) and UmuD'C (pGW2133) were introduced into the pol V deficient strain, and the mutant frequencies were measured (Table 4 and Figure 3). The UmuDC expression from pGW2101 considerably decreased the mutant frequency, in good agreement with the expression from another vector, pSE117 (Figure 2). Intriguingly, the expression of UmuD'C, the activated form of UmuDC that actually carries out TLS, also suppressed the mutagenesis by 2-OH-dATP and 8-OH-dGTP, albeit less effectively than that of UmuDC. This unexpected outcome suggests the possibility that both the $UmuD_2C$ and the $UmuD'_2C$ complexes had the suppressing activity or that UmuC suppressed the mutations.

Discussion

The incorporation of oxidatively damaged DNA precursors, 2-OH-dATP and 8-OH-dGTP, by DNA pols appears to be one of the major pathways of 2-OH-Ade and 8-OH-Gua formation in DNA, respectively, which would cause mutations and cancers. These damaged nucleotides are believed to be incorporated mainly by the replicative DNA pol, the DNA pol III holoenzyme (pol III HE) in *E. coli*, since pol III HE and the pol III catalytic subunit incorporate both damaged nucleotides with high error frequencies in vitro (*7*, *13*, *36*). Recently, the highly erroneous incorporation of 2-OH-dATP and 8-OH-dGTP in vitro by Y family DNA pols, such as human DNA pol η or archeal DNA pols P1 and P2, was reported (*18*). Thus, the *E. coli* Y family pols may also incorporate these damaged DNA precursors in vivo. In this experiment, we investigated the mutations induced by 2-OH-dATP and 8-OH-dGTP in *E. coli* strains deficient in the Y family pols, DNA pol IV and DNA pol V.

In this study, 2-OH-dATP, but not 8-OH-dGTP, induced mutations less efficiently in the pol IV deficient strain than in the wt strain, suggesting that pol IV may contribute to the mutagenesis by 2-OH-dATP. Because the expression of the pol IV D101N mutant, which lacks the pol activity, in the pol IV deficient strain did not restore the lower mutagenesis level with 2-OH-dATP, the enhancement by pol IV is dependent on its pol activity. On the basis of these results, pol IV seems to facilitate the mutagenicity of 2-OH-dATP through (i) the misincorporation of 2-OH-dATP and/or (ii) the extension from the 3′-terminal 2-OH-dAMP residue. DNA pol IV incorporates 2-OH-dATP opposite G and T in the template at an almost 1:1 ratio in vitro (Shimizu, M., Gruz, P, and Nohmi, T. Unpublished results). Alternatively, pol IV may be involved in the fixation of mutations by (iii) the incorporation of dTTP opposite 2-OH-Ade during the second round of replication (TLS) and/or (iv) extension from the 3′-terminal dTMP residue incorporated opposite 2-OH-Ade.

The transcriptional regulation of *dinB* by *lexA* is not very strict, so the cellular level of pol IV without SOS induction (250 molecules/cell) is somewhat higher than that of pol III (<20 molecules/cell) or pol V (180 molecules/cell in UmuD, but UmuC and UmuD′ were not detected) (*27*, *37*, *38*). This relative excess of pol IV may enable the efficient misincorporation of 2-OH-dATP, the TLS across 2-OH-Ade, and/or the extension from the mismatched termini involving 2-OH-Ade, and thus the additional expression of pol IV in the wt strain had no obvious effect on 2-OH-dATP mutagenesis (Figure 1). The decreased mutant frequency by the expression of pol IV D103N, the mutant pol IV that lacks the pol activity, may also be explained by the access to the mismatched termini that were left after the incorporation of 2-OH-dATP by pol III and its dissociation. Competition of the pol IV

D103N mutant protein with other pols at the termini may promote the removal of 2-OH-Ade by other repair and/or proofreading proteins that have exonuclease activity, by their physical interaction. Similar explanations are possible for the TLS across 2-OH-Ade.

We expected that pol V, another error-prone DNA pol, would enhance the mutagenesis by the damaged DNA precursors. However, surprisingly, the pol V deficient strain clearly showed augmented mutagenesis by 2-OH-dATP and 8-OH-dGTP, and the mutant frequencies were decreased by the introduction of a plasmid encoding UmuDC (Figures 1−3). This suppression was also observed for UmuDC (D101N), which lacks the pol activity (Figure 2B), thus excluding the possibility of the involvement of pol V-mediated TLS in the observed effects of UmuDC. In addition to their activity in TLS, the *umuDC* gene products are involved in DNA damage checkpoint control (*39*). The suppressive effect of UmuDC observed in this study may be derived from a similar mechanism, although the expression of UmuD′$_2$C, which seemed to be less efficient than UmuDC in DNA damage checkpoint control, decreased the mutant frequency (Figure 3). Alternatively, UmuD$_2$C, UmuD′$_2$C, or UmuC might modulate the replication fidelity or coordination with the repair system by interacting with the replication machinery. The interactions of UmuD and UmuD′ with the pol III α- and ε-subunits, and those of the β-subunit with UmuC, in addition to UmuD and UmuD′, have been reported (*40*, *41*). The reason(s) for the suppressive effect of UmuDC would be revealed with further study.

Previously, Negishi et al. found that the mutagenicity of a bicyclic N^4-oxy-2′-deoxycytidine compound (dP in their report) was lower in a *Salmonella* strain containing the *mucAB* locus, which is homologous to the *umuDC* locus, than in the isogenic strain without *mucAB* (*42*). This result suggests that the suppressive effects of certain Y family DNA pols are common to some extent, at least in bacteria.

Tassotto and Mathews tried to measure the 8-OH-dGTP concentration in *mutT E. coli* and concluded that its intracellular concentration is below 0.34 μM (the limit of detection) (*43*). However, the ratio of 0.34 μM of 8-OH-dGTP to the estimated concentration of dGTP in the bacterium (100 μM) (*44*) is 3.4×10^{-3}, and this ratio (3.4×10^{-3} 8-OH-dGTP/dGTP) seems to be too high, because the yield of 8-OH-dGTP is 3.7×10^{-2} 8-OH-dGTP/dGTP even in an in vitro Fenton type reaction, which occurs under severely oxidative conditions (*6*). In this study, oxidized nucleotides were incorporated into *E. coli* by treating the cells with $CaCl_2$ in the presence of 2-OH-dATP or 8-OH-dGTP. An advantage of this method is that the intracellular concentration of 2-OH-dATP or 8-OH-dGTP is specifically enhanced, and the mutations associated with the specific increase in the concentrations of the oxidized nucleotides can be analyzed. Although the actual intracellular concentrations of the incorporated nucleotides are ambiguous, mutant frequency was enhanced by 1.6-fold upon the 8-OH-dGTP treatment under similar conditions in the *mutT* strain (*33*). This suggests that the amount of 8-OH-dGTP incorporated was ~0.6-fold of that of endogenous 8-OH-dGTP present in the untreated *mutT* cells. Thus, the intracellular concentrations of 2-OH-dATP and 8-OH-dGTP in this study may not be extremely high and may actually be physiologically relevant, considering the situations where cells are exposed to oxidative stress.

In conclusion, we revealed that *E. coli* pol IV was involved in induction of mutations by 2-OH-dATP and that the expression of *umuDC* gene products decreased the mutagenesis induced by 2-OH-dATP and 8-OH-dGTP. This is the first in vivo experiment that demonstrated the roles of Y family DNA pols in mutagenesis by damaged DNA precursors. These pols are regarded as specialized DNA pols that participate in DNA replication only when DNA damage is present. However, if the chromosomal DNA contains many endogenous lesions, and pol IV and pol V are loaded on the replication fork, they will respectively enhance and suppress the incorporation of damaged nucleotides in vivo, as shown in this study. In addition, a recent study suggested that the pol exchange may take place even during normal replication (*45*). In mammalian cells, replication may be rather complicated, because many kinds of pols participate in chromosomal replication. Thus, further studies will be required to elucidate the mechanism of mutagenesis by oxidatively damaged DNA precursors.

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Supporting Information Available: Mutations observed in the control experiments in *E. coli*, mutations induced by 2-OH-dATP in *E. coli*, and mutations induced by 8-OH-dGTP in *E. coli*. This material is available free of charge via the Internet at http://pubs.acs.org.

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