Base excision repair enzyme endonuclease III suppresses mutagenesis caused by 8-hydroxy-dGTP

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Abstract

To examine whether base excision repair suppresses mutations induced by oxidized deoxyribonucleotide 5'-triphosphates in the nucleotide pool, 8-hydroxy-dGTP (8-OH-dGTP) and 2-hydroxy-dATP were introduced into *Escherichia coli* strains deficient in endonucleases III (Nth) and VIII (Nei), and MutY, and mutations in the chromosomal *rpoB* gene were analyzed. The spontaneous *rpoB* mutant frequency was also examined in *mutT*/*nth* and *mutT*/*nei* strains, to assess the influence on the mutations induced by the endogenous 8-OH-dGTP accumulated in the *mutT* mutant. The mutations induced by exogenous 2-hydroxy-dATP were similar in all of the strains tested. Exogenous 8-OH-dGTP increased the *rpoB* mutant frequency more efficiently in the *nth* strain than that in the wild-type strain. The spontaneous mutant frequency in the *mutT*/*nth* strain was 2-fold higher than that in the *mutT* strain. These results suggest that *E. coli* endonuclease III also acts as a defense against the mutations caused by 8-OH-dGTP in the nucleotide pool.

Keywords: base excision repair; nth; 8-OH-dGTP; 2-OH-dATP

1. Introduction

Reactive oxygen species (ROS) are generated not only by exogenous environmental factors, such as ionizing radiation and redox-cycling chemicals, but also through endogenous cellular oxygen metabolism [1,2]. ROS react with DNA, RNA, and their precursors. The damaged nucleic acids and nucleotides cause mutations, resulting in inheritable diseases, cancer, and aging [3,4]. Organisms possess elaborate mechanisms to prevent the mutations caused by the oxidized nucleobases in both the DNA and free nucleotide forms [4-6]. Among the mechanisms, base excision repair (BER), which is initiated by excision of the damaged base by a DNA glycosylase, is the major pathway for the repair of the damaged bases directly formed by the oxidation of nucleobases in DNA [5,6].

8-hydroxy-dGTP (8-OH-dGTP) and 2-hydroxy-dATP (2-OH-dATP) are the major oxidized products of dGTP and dATP, respectively [7]. The physiological importance of these damaged deoxyribonucleotides is supported by the presence of their specific hydrolyzing enzymes [8-10]. *E.coli* MutT and Orf135 (NudG), and mammalian MTH1 eliminate 8-OH-dGTP and 2-OH-dATP in the nucleotide pool, and thereby prevent the misincorporation of these damaged deoxyribonucleotides during DNA replication. When these deoxyribonucleotides are not eliminated from the nucleotide pool, they can be potent mutagenic substrates in DNA synthesis, which is one of the pathways generating oxidatively damaged bases in DNA [8,11-13]. BER enzymes may prevent the mutagenesis by oxidized deoxyribonucleotides after they are incorporated into DNA. However, it is unclear whether the DNA lesions derived from the oxidized deoxyribonucleotides are actually eliminated by BER enzymes.

We have focused on the *E.coli* DNA glycosylases that act on base pairs involving oxidative DNA lesions: endonucleases III (Nth) and VIII (Nei), and MutY. Endonucleases III and VIII excise oxidized pyrimidines, such as thymine glycol and 5-hydroxyuracil [14-16]. In addition, 8-hydroxyguanine (8-OH-Gua) paired with A and G is reportedly removed by these enzymes *in vitro* [17,18]. MutY removes adenine in A:8-OH-Gua pairs as well as in A:G pairs in DNA duplexes [19-21]. In this study, we introduced 8-OH-dGTP and 2-OH-dATP into *E. coli* strains deficient in

these enzymes and examined the mutagenesis caused by 8-OH-dGTP and 2-OH-dATP with an *rpoB* (rifampicin-resistance) assay. Furthermore, we used *mutT*/*nth* and *mutT*/*nei* double mutants to examine the influence of the deficiencies in endonucleases III and VIII on the mutations induced by endogenous 8-OH-dGTP. The exogenous 8-OH-dGTP was more mutagenic in the *nth* strain than in the wt strain. In addition, the spontaneous mutant frequency in the *mutT*/*nth* strain was higher than that in each single mutant. Our results suggest that endonuclease III is involved in protection against the mutations induced by 8-OH-dGTP. This constitutes the first evidence that a BER enzyme suppresses the mutagenesis induced by an oxidized deoxyribonucleotide *in vivo*.

2. Materials and methods

2.1. Nucleotide and bacteria strains

8-OH-dGTP was purchased from TriLink BioTechnologies (San Diego, CA, USA). 2-OH-dATP was prepared by an oxidation reaction of dATP with Fe(II)-EDTA-O_2, and was purified by HPLC as described previously [11]. Oligodeoxyribonucleotides were purchased from Sigma Genosys Japan (Ishikari, Japan) in purified forms.

The *E. coli* strains used in this study are listed in Table 1. The AB1157 strain [22] (*F*$^-$: *thr*-1 *leuB*6 *thi*-1 *lacY*1 *galK*2 *ara*-14 *xyl*-5 *mtl*-1 *proA*2 *his*-4 *argE*3 *rpsL*31 *tsx*-33 *supE*44 *flaND*) was obtained from the National Institute of Genetics, Stocks Research Center (Mishima, Japan), and was used as a wild-type (wt) strain in the mutagenesis experiments. Other strains used in this study were derived from AB1157. NKJ1002 (*nth*::Cm^r), NKJ1003 (*Δnei*::Km^r) and MK609 (*mutY zgd*::Tet^r) were used as strains deficient in endonuclease III, endonuclease VIII and MutY, respectively [23,24]. The *leu*$^+$ and *leu*$^+$ *mutT* loci were transduced from MK602 into NKJ1002 and NKJ1003 by P1 transduction to yield ST001-ST004 (Table 1).

2.2. Measurement of nucleotide amounts introduced into E.coli

dATP was introduced by the calcium chloride method as described previously, with a slight modification [25-27]. Single colonies of each strain were inoculated into 5 mL of LB medium and then cultured at 37°C until the turbidity at 570 nm reached 0.6. Competent cells were prepared by a treatment with 0.1 M calcium chloride. dATP (5 mM, 2.5 μL) including 2.5 μCi [α-^{33}P]dATP was added to 50 μL of the *E. coli* suspension. The mixtures were placed on ice for 3 hr. The cells were washed by ice-cold LB and then lysed by alkali-SDS solution. The lysate was neutralized by HCl and centrifuged. The supernatant was spot on DEAE-filter paper. The paper was dried and radioactivities were quantitated with a Fujifilm BAS-2500 Image Analyzer (Fuji Photo Film, Tokyo, Japan).

2.3. Introduction of deoxyribonucleotides into E. coli and analysis of mutant frequency

8-OH-dGTP and 2-OH-dATP were introduced by the calcium chloride method as described above. The deoxyribonucleoside 5'-triphosphates (2.5 μL) were added to 50 μL of the *E. coli* suspension. AB1157 and the other strains were treated with 25 nmol and 12.5 nmol of the deoxyribonucleotides, respectively (see Results). The mixtures were placed on ice for 3 hr. Pre-warmed SOC medium (450 μL) was then added, and the cells were cultured at 37°C for 1 hr with agitation.

A portion of each culture was appropriately diluted with ice-cold LB, spread onto an LB plate, and incubated at 37°C for 12 hr (the titer plate). Another portion of the culture was spread onto an LB plate supplemented with 100 μg/mL rifampicin, which was incubated at 37°C for 20 hr to select *rpoB* mutants (the selection plate). The mutant frequency was calculated by dividing the numbers of colonies on the selection plates by those on the titer plates.

2.4. Analysis of mutant frequency in wt and mutT strains deficient in Nth or Nei

A single colony of *E. coli* was inoculated in 5 mL of LB and then grown at 37°C to a cell

density of ~10^9 cells/mL (OD_{570} = 0.8). A portion of each culture was diluted with ice-cold LB, spread onto an LB plate, and incubated at 37°C for 12 hr (the titer plate). Another portion of the culture was spread onto an LB plate supplemented with 100 μg/mL rifampicin, which was grown at 37°C for 24 hr to select *rpoB* mutants (the selection plate). The mutant frequency was calculated as described above.

2.5. Sequence analysis of rpoB gene

The fragment corresponding to positions 1519-1725 of the *rpoB* gene was amplified by PCR, using the primers 5'-dACAGGATATGATCAACGCAA-3' and 5'-dCGATACGGAGTCTCTCAAGGCC-3', as described previously [28]. The amplified DNA fragment was analyzed by sequencing with the former primer, a Big Dye Terminator Cycle Sequencing Kit, and an ABI model 377 DNA sequencer (Applera, Norwalk, CT, USA).

3. Results

3.1. Effects of base excision repair enzymes on mutagenesis by oxidized deoxynucleotides

To investigate the effects of BER on the mutagenicity of 8-OH-dGTP and 2-OH-dATP, we first introduced these deoxyribonucleotides into *E.coli* strains deficient in endonucleases III (NKJ1002) and VIII (NKJ1003), and MutY (MK609) (Table 1). We did not examine the corresponding *mutM* strain, due to its significant growth delay as compared to the wt strain (data not shown). To examine the amounts of deoxyribonucleotides incorporated into the strains, competent cells were preliminarily treated with [α-^{33}P]dATP, and the radioactivities in the treated cells were measured after thorough washing and lysis. The incorporation of [α-^{33}P]dATP into the AB1157 (wt) cells was unexpectedly less than in the other strains. Similar amounts of incorporation were observed when the AB1157 and the other strains were treated with 25 and 12.5 nmol of [α-^{33}P]dATP,

respectively (data not shown). Thus, we treated the *E. coli* cells with 8-OH-dGTP and 2-OH-dATP at these concentrations.

We compared the frequencies of the *rpoB* mutants induced by these damaged deoxyribonucleotides in various strains. The mutant frequencies were increased by the addition of 8-OH-dGTP and 2-OH-dATP in all of the strains used (Table 2). On the other hand, the mutant frequencies were not enhanced by the addition of unmodified dGTP and dATP (data not shown). To assess the actual frequency of the mutations induced by 8-OH-dGTP and 2-OH-dATP in the BER-defective strains, we subtracted the mutant frequencies of the control experiments from the values obtained in the presence of an oxidized deoxyribonucleotide (Table 3). Each subtracted mutant frequency was calculated from the deoxyribonucleotide and control experiments using the same original colonies. This subtracted frequency was used when strains with different background mutant frequencies were compared [26,27]. The subtracted mutant frequency in the *nth* strain treated with 8-OH-dGTP (2.2×10^{-7}) was increased 2.4-fold in comparison with that in the wt strain (0.91×10^{-7}, $P<0.01$) (Table 3). This result suggests that endonuclease III could prevent the mutagenesis by 8-OH-dGTP *in vivo*. On the other hand, the 8-OH-dGTP-induced mutant frequencies in the *nei* and *mutY* strains were insignificantly different from that in the wt strain, although they seemed to be higher. When 2-OH-dATP was introduced into each strain, the subtracted mutant frequencies were not significantly different from that in the wt strain (Table 3). These results suggest that endonucleases III and VIII, and MutY did not prevent the mutagenesis by 2-OH-dATP.

3.2. Mutation spectra of BER-defective strains treated with oxidized deoxyribonucleotides

We then analyzed the mutation spectra in the *rpoB* gene in each strain (Table 4). 8-OH-dGTP induced A:T→C:G transversions (39%) in the wt cells, as expected. Nearly 60% of the mutations were G:C→T:A transversions when 2-OH-dATP was introduced. Various substitution mutations were observed in the controls of the *nth* and *nei* strains. The ratios of A:T→C:G and G:C→T:A transversions were increased when 8-OH-dGTP and 2-OH-dATP, respectively, were

introduced in both strains. Almost all of the mutations were G:C→T:A transversions in the *mutY* cells treated with water, 2-OH-dATP, and 8-OH-dGTP (Table 4). A:T→C:G transversions were observed only in the 8-OH-dGTP experiments. The distributions of the mutations detected in the *rpoB* gene are summarized in Supplemental Table 1.

3.3. Effects of endonuclease III- and VIII-deficiencies in the mutT mutant

The results described above prompted us to investigate the influence of endonuclease III- and VIII-deficiencies on the mutations caused by endogenous 8-OH-dGTP. This form of oxidized dGTP is believed to accumulate in *mutT* strains, since MutT is the major hydrolyzing enzyme specific for 8-OH-dGTP, and more 8-OH-Gua in DNA is detected in the *mutT* cells than in the wt cells [8,24,26]. We examined the spontaneous *rpoB* mutant frequency in the *mutT*, *mutT*/*nth*, and *mutT*/*nei* strains (Table 5).

E. coli lacking the MutT protein exhibited a quite strong mutator phenotype, as reported previously (10.9×10^{-7}, Table 5) [24,29]. In the *mutT* background, the endonuclease III-deficiency increased the spontaneous mutant frequency by 1.8-fold (18.9×10^{-7}, $P<0.001$). However, the *nth* mutation alone affected the *rpoB* mutant frequency only slightly. In contrast, the endonuclease VIII-deficiency did not affect the spontaneous mutant frequency in the *mutT* background. These results support our finding that the presence of endonuclease III suppressed the mutagenesis caused by 8-OH-dGTP. Almost all of the mutations observed in the *mutT*, *mutT*/*nth*, and *mutT*/*nei* strains were A:T→C:G transversions (Table 6 and Supplemental Table 2). Hotspots were observed at positions 1538 and 1714 as reported previously [30].

4. DISCUSSION

We investigated the roles of BER on the mutagenesis by 8-OH-dGTP and 2-OH-dATP generated by ROS in the nucleotide pool. We introduced these oxidized deoxyribonucleotides into *E.*

coli strains deficient in DNA glycosylases, endonucleases III and VIII, and MutY, and examined the *rpoB* mutant frequencies. In addition, we examined the influence of the deficiency in these BER enzymes in the *mutT* background.

The subtracted mutant frequency in the *nth* strain was significantly higher than that in the wt strain, when 8-OH-dGTP was introduced into these strains (Table 3). In addition, the deficiency in endonuclease III in the *mutT* cells enhanced the mutant frequency (Table 5). These results suggest that endonuclease III could suppress the mutations caused by 8-OH-dGTP. The major mutations induced by 8-OH-dGTP are A:T→C:G transversions [25-27, also observed in this study]. This type of mutation seems to occur by the incorporation of 8-OH-dGTP opposite A residues in DNA, and the subsequent insertion of C opposite the 8-OH-Gua bases during the next round of replication. Thus, the induction of A:T→C:G transversions is suppressed when the 8-OH-Gua base in the 8-OH-Gua:A pairs is removed by a DNA glycosylase. The results obtained in this study are consistent with the previous report that endonuclease III excises 8-OH-Gua in DNA containing an 8-OH-Gua:A pair *in vitro* [17]. On the other hand, endonuclease VIII, as well as endonuclease III, also reportedly excises 8-OH-Gua in DNA containing an 8-OH-Gua:A pair *in vitro* [18]. This implies that the deficiency in the *nei* gene enhances the mutagenicity of exogenous and endogenous 8-OH-dGTP. However, the introduction of 8-OH-dGTP into the *nei* strain caused only a slight increase in the *rpoB* mutant frequency (Table 3). The deficiency in endonuclease VIII in the *mutT* strain had no effect on the mutant frequency (Table 5). In contrast to the report by Hazra *et al*. [18], Blaisdell *et al*. found no cleavage of DNA containing an 8-OH-Gua:A pair by endonuclease VIII [31]. Thus, the excision by endonuclease VIII may be highly sequence-dependent, and this may be the reason for the modest effect of its deficiency.

MutY excises A residues from A:8-OH-Gua pairs and prevents the G:C→T:A transversions generated by the direct oxidation of G residues in DNA [19,24,32,33]. The action of MutY on the A:8-OH-Gua pairs formed by the incorporation of 8-OH-dGTP would enhance the A:T→C:G mutations. Thus, fewer mutations by 8-OH-dGTP were expected in the *mutY* strain. However, we did not observe the expected reduction under our experimental conditions (Table 3). Previously, it was

found that the MutY-deficiency reduced the number of A:T→C:G mutations in the *mutT* background [24,33,34]. We cannot explain these discrepancies at this time. Further studies are necessary to reveal the reason(s) for the MutY effect on the mutagenesis by 8-OH-dGTP.

Endonuclease III and MutY recognize an identical base pair, 8-OH-Gua:A. The biological significances of these BER enzymes seem to be that endonuclease III removes 8-OH-Gua formed by the incorporation of 8-OH-dGTP opposite A and that MutY removes A formed by copying 8-OH-Gua. Thus, it is required that either or both of the enzymes recognize(s) the nascent chain. Recently, Bai and Lu reported interactions between MutY and mismatch repair protein, MutS [35]. MutY might distinguish the newly synthesized strand by interaction with mismatch repair complex that distinguishes the nascent chain in a methylation dependent-manner [36]. Endonuclease III also excises oxidized pyrimidines [14,15] and thus, this type of interaction may not be present.

The subtracted mutant frequencies induced by 2-OH-dATP in the *nth*, *nei*, and *mutY* strains were similar to that in the wt strain (Table 3). These results suggest that endonucleases III and VIII, and MutY did not prevent the mutations by 2-OH-dATP, although they might perform backup functions for each other. However, double-stranded oligonucleotides containing 2-hydroxyadenine were very poor substrates for the purified endonucleases III and VIII (Kamiya, Kasai, Terato and Ide, unpublished results) and MutY [37]. The Orf135 (NudG) protein catalyzes the hydrolysis of 2-OH-dATP *in vitro*, and its induced mutagenesis is more frequent in the *orf135*$^-$ strain than in the wt strain [9,38]. Thus, the nucleotide pool sanitization is the only mechanism that has been revealed so far as a defense against 2-OH-dATP-generated mutations.

In this study, we found that endonuclease III was involved in the prevention of the mutations by 8-OH-dGTP generated in the nucleotide pool by ROS, when 8-OH-dGTP evades hydrolysis by MutT. This finding constitutes the first evidence that a BER enzyme suppresses the mutagenesis induced by an oxidized deoxyribonucleotide *in vivo*. We have recently established an assay system for the mutations generated by an oxidized deoxyribonucleotide in living mammalian cells [39]. Further studies will reveal whether the mammalian BER enzyme(s) can function as a defense against the damaged deoxyribonucleotides formed by ROS. Studies along these lines are in

progress.

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REFERENCES

[1] S. B. Farr, T. Kogoma. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*, Microbiol. Rev. 55 (1991) 561-585

[2] B. Halliwell, O. I. Aruoma. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems, FEBS Lett. 281 (1991) 9-19

[3] B. N. Ames, M. K. Shigenaga, T. M. Hagen. Oxidants, antioxidants, and the degenerative diseases of aging, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 7915-7922

[4] H. Kamiya. Mutagenic potentials of damaged nucleic acids produced by reactive oxygen/nitrogen species: approaches using synthetic oligonucleotides and nucleotides: survey and summary, Nucleic Acids Res. 31 (2003) 517-531

[5] B. Demple, L. Harrison. Repair of oxidative damage to DNA: enzymology and biology, Annu. Rev. Biochem. 63 (1994) 915-948

[6] J. Cadet, A. G. Bourdat, C. D'Ham, V. Duarte, D. Gasparutto, A. Romieu, J. L. Ravanat. Oxidative base damage to DNA: specificity of base excision repair enzymes, Mutat. Res. 462 (2000) 121-128

[7] N. Murata-Kamiya, H. Kamiya, M. Muraoka, H. Kaji, H. Kasai. Comparison of oxidation products from DNA components by gamma-irradiation and Fenton-type reactions, J. Radiat. Res. (Tokyo) 38 (1997) 121-131

[8] H. Maki, M. Sekiguchi. MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis, Nature 355 (1992)273-275

[9] H. Kamiya, N. Murata-Kamiya, E. Iida, H. Harashima. Hydrolysis of oxidized nucleotides by the *Escherichia coli* Orf135 protein, Biochem. Biophys. Res. Commun. 288 (2001) 499-502

[10] J. Y. Mo, H. Maki, M. Sekiguchi. Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: sanitization of nucleotide pool, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 11021-11025

[11] H. Kamiya, H. Kasai. Formation of 2-hydroxydeoxyadenosine triphosphate, an oxidatively

damaged nucleotide, and its incorporation by DNA polymerases. Steady-state kinetics of the incorporation, J. Biol. Chem. 270 (1995) 19446-19450

[12] Y. I. Pavlov, D. T. Minnick, S. Izuta, T. A. Kunkel. DNA replication fidelity with 8-oxodeoxyguanosine triphosphate, Biochemistry 33 (1994) 4695-4701

[13] K. Satou, H. Harashima, H. Kamiya. Mutagenic effects of 2-hydroxy-dATP on replication in a HeLa extract: induction of substitution and deletion mutations, Nucleic Acids Res. 31 (2003) 2570-2575

[14] A. Katafuchi, T. Nakano, A. Masaoka, H. Terato, S. Iwai, F. Hanaoka, H. Ide. Differential specificity of human and *Escherichia coli* endonuclease III and VIII homologues for oxidative base lesions, J. Biol. Chem. 279 (2004) 14464-14471

[15] L. H. Breimer, T. Lindahl. DNA glycosylase activities for thymine residues damaged by ring saturation, fragmentation, or ring contraction are functions of endonuclease III in *Escherichia coli*, J. Biol. Chem. 259 (1984) 5543-5548

[16] R. J. Melamede, Z. Hatahet, Y. W. Kow, H. Ide, S. S. Wallace. Isolation and characterization of endonuclease VIII from *Escherichia coli*, Biochemistry 33 (1994) 1255-1264

[17] Y. Matsumoto, Q. M. Zhang, M. Takao, A. Yasui, S. Yonei. *Escherichia coli* Nth and human hNTH1 DNA glycosylases are involved in removal of 8-oxoguanine from 8-oxoguanine/guanine mispairs in DNA, Nucleic Acids Res. 29 (2001) 1975-1981

[18] T. K. Hazra, T. Izumi, R. Venkataraman, Y. W. Kow, M. Dizdaroglu, S. Mitra. Characterization of a novel 8-oxoguanine-DNA glycosylase activity in *Escherichia coli* and identification of the enzyme as endonuclease VIII , J. Biol. Chem. 275 (2000) 27762-27767

[19] M. L. Michaels, C. Cruz, A. P. Grollman, J. H. Miller. Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 7022-7025

[20] A. Gogos, J. Cillo, N. D. Clarke, A-L. Lu. Specific recognition of A/G and A/7,8-dihydro-8-oxoguanine (8-oxoG) mismatches by *Escherichia coli* MutY: removal of the C-terminal domain preferentially affects A/8-oxoG recognition, Biochemistry 35 (1996)

16665-16671

[21] K. G. Au, S. Clark, J. H. Miller, P. Modrich. *Escherichia coli* mutY gene encodes an adenine glycosylase active on G-A mispairs, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 8877-8881

[22] E. A. Adelberg, S. N. Burns. Genetic variation in the sex factor of *Escherichia coli*, J. Bacteriol. 79 (1960) 321-330

[23] Y. Saito, F. Uraki, S. Nakajima, A. Asaeda, K. Ono, K. Kubo, K. Yamamoto. Characterization of endonuclease III (nth) and endonuclease VIII (nei) mutants of *Escherichia coli* K-12, J. Bacteriol. 179 (1997) 3783-3785

[24] T. Tajiri, H. Maki, M. Sekiguchi. Functional cooperation of MutT, MutM and MutY proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in *Escherichia coli*, Mutat. Res. 336 (1995) 257-267

[25] M. Inoue, H. Kamiya, K. Fujikawa, Y. Ootsuyama, N. Murata-Kamiya, T. Osaki, K. Yasumoto, H. Kasai. Induction of chromosomal gene mutations in Escherichia coli by direct incorporation of oxidatively damaged nucleotides. New evaluation method for mutagenesis by damaged DNA precursors *in vivo*, J. Biol. Chem. 273 (1998)11069-11074

[26] H. Kamiya, C. Ishiguro, H. Harashima. Increased A:T-->C:G mutations in the mutT strain upon 8-hydroxy-dGTP treatment: direct evidence for MutT involvement in the prevention of mutations by oxidized dGTP, J. Biochem. (Tokyo) 136 (2004) 359-362

[27] K. Satou, M. Yamada, T. Nohmi, H. Harashima, H. Kamiya. Mutagenesis induced by oxidized DNA precursors: roles of Y family DNA polymerases in *Escherichia coli*, Chem. Res. Toxicol. 18 (2005) 1271-1278

[28] S. K. Petersen-Mahrt, R. S. Harris, M. S. Neuberger. AID mutates *E. coli* suggesting a DNA deamination mechanism for antibody diversification, Nature 418 (2002) 99-103

[29] E. C. Cox, C. Yanofsky. Mutator gene studies in *Escherichia coli*, J. Bacteriol. 100 (1969) 390-397

[30] L. Garibyan, T. Huang, M. Kim, E. Wolff, A. Nguyen, T. Nguyen, A. Diep, K. Hu, A. Iverson, H. Yang, J.H. Miller. Use of the *rpoB* gene to determine the specificity of base substitution

mutations on the *Escherichia coli* chromosome, DNA Repair. 2 (2003) 593-608

[31] J. O. Blaisdell, Z. Hatahet, S. S. Wallace. A novel role for *Escherichia coli* endonuclease VIII in prevention of spontaneous G-->T transversions, J. Bacteriol. 181 (1999) 6396-6402

[32] Y. Nghiem, M. Cabrera, C. G. Cupples, J.H. Miller. The mutY gene: a mutator locus in *Escherichia coli* that generates G.C-->T.A transversions, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 2709-2713

[33] R. G. Fowler, S. J. White, C. Koyama, S. C. Moore, R. L. Dunn, R. M. Schaaper. Interactions among the *Escherichia coli* mutT, mutM, and mutY damage prevention pathways, DNA Repair 2 (2003) 159-173

[34] J. J. Vidmar, C. G. Cupples. MutY repair is mutagenic in mutT- strains of *Escherichia coli*, Can. J. Microbiol. 39 (1993) 892-894

[35] H. Bai, A-L. Lu. Physical and functional interactions between *Escherichia coli* MutY glycosylase and mismatch repair protein MutS, J. Bacteriol. 189 (2007) 902-910

[36] T.A. Kunkel, D.A. Erie. DNA mismatch repair, Annu. Rev. Biochem. 74 (2005) 681-710

[37] H. Kamiya, H. Kasai. 2-hydroxyadenine in DNA is a very poor substrate of the *Escherichia coli* MutY protein, J. Radiat. Res. (Tokyo) 41 (2000) 349-354

[38] H. Kamiya, E. Iida, N. Murata-Kamiya, Y. Yamamoto, T. Miki, H. Harashima. Suppression of spontaneous and hydrogen peroxide-induced mutations by a MutT-type nucleotide pool sanitization enzyme, the *Escherichia coli* Orf135 protein, Genes Cells 8 (2003) 941-950

[39] K. Satou, K. Kawai, H. Kasai, H. Harashima, H. Kamiya. Mutagenic effects of 8-hydroxy-dGTP in live mammalian cells, Free Radic. Biol. Med. 42 (2007) 1552-1560

Table 1. *E. coli* strains used in this study.

Strain	Relative genotype	P1 transduction	Reference
AB1157	Wild-type		22
NKJ1002	AB1157 but *nth*::Cm^r		23
NKJ1003	AB1157 but *Δnei*::Km^r		23
MK609	AB1157 but *mutY zgd*::Tet^r		24
MK601	AB1157 but *leu*$^+$		24
ST001	AB1157 but *leu*$^+$ *nth*::Cm^r	MK602→NKJ1002	This study
ST002	AB1157 but *leu*$^+$ *Δnei*::Km^r	MK602→NKJ1003	This study
MK602	AB1157 but *leu*$^+$ *mutT*		24
ST003	AB1157 but *leu*$^+$ *mutT nth*::Cm^r	MK602→NKJ1002	This study
ST004	AB1157 but *leu*$^+$ *mutT Δnei*::Km^r	MK602→NKJ1003	This study

Table 2. Mutant frequencies in the BER-deficient *E. coli* cells treated with oxidized deoxyribonucleotides.[a]

	Mutant frequency ($\times 10^{-7}$)		
	Control	8-OH-dGTP	2-OH-dATP
AB1157 (wt)	0.57 (0.26-1.3)	1.7 [b] (0.75-1.9)	6.9 [c] (2.3-7.9)
MK609 (*mutY*)	5.1 (4.6-7.4)	7.0 (5.0-8.2)	9.4 [b] (7.6-9.9)
NKJ1002 (*nth*)	0.69 (0.16-1.0)	3.1 [b] (1.7-3.3)	9.4 [b] (5.5-10.5)
NKJ1003 (*nei*)	0.20 (0.10-0.54)	1.6 [b] (0.73-2.2)	8.5 [b] (6.1-10.1)

[a] Experiments were repeated ten times. The data are expressed as median and numbers in parentheses represent 95% confidence limits for the median in individual experiments.

[b] $P<0.001$, [c] $P<0.05$, (significant difference versus control , Mann-Whitney U-test)

Table 3. Subtracted mutant frequencies induced by 8-OH-dGTP and 2-OH-dATP in the BER-deficient *E. coli* strains.[a]

	Subtracted mutant frequency ($\times 10^{-7}$)	
	8-OH-dGTP	2-OH-dATP
AB1157 (wt)	0.91 (0.43-1.7)	6.1 (1.3-7.6)
MK609 (*mutY*)	1.2 (0.2-3.0)	3.9 (2.0-4.6)
NKJ1002 (*nth*)	2.2 [b] (0.58-2.5)	8.5 (4.5-10.1)
NKJ1003 (*nei*)	1.4 (0.54-1.5)	8.2 (6.0-10.0)

[a] Experiments were repeated ten times. The data are expressed as median and numbers in parentheses represent 95% confidence limits for the median in individual experiments. Values were calculated by subtracting the mutant frequencies of control experiments from the values obtained upon treatment with 8-OH-dGTP and 2-OH-dATP. This subtraction was done using the same original single colonies, and each subtracted frequency was considered as a single datum.

[b] $P<0.01$ (significant difference versus control , Mann-Whitney U-test)

Table 4. Spectra of mutations induced by 8-OH-dGTP and 2-OH-dATP in the BER-deficient *E. coli* cells.[a]

	AB1157 (wt)			NKJ1002 (*nth*)		
	Control	8-OH-dGTP	2-OH-dATP	Control	8-OH-dGTP	2-OH-dATP
transition						
G:C→A:T	44 (88)	20 (49)	15 (37)	27 (73)	24 (59)	4 (11)
A:T→G:C	2 (4)	3 (7)	0 (0)	1 (3)	1 (2)	0 (0)
transversion						
G:C→T:A	1 (2)	1 (2)	26 (63)	5 (14)	1 (2)	34 (87)
G:C→C:G	3 (6)	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)
A:T→C:G	0 (0)	17 (39)	0 (0)	1 (3)	14 (37)	0 (0)
A:T→T:A	0 (0)	1 (2)	0 (0)	1 (3)	1 (2)	1 (3)
total	50 (100)	41 (100)	41 (100)	37 (100)	41 (100)	38 (100)
	NKJ1003 (*nei*)			MK609 (*mutY*)		
	Control	8-OH-dGTP	2-OH-dATP	Control	8-OH-dGTP	2-OH-dATP
transition						
G:C→A:T	17 (44)	7 (17)	3 (8)	3 (8)	2 (5)	0 (0)
A:T→G:C	6 (15)	5 (12)	0 (0)	0 (0)	0 (0)	0 (0)
transversion						
G:C→T:A	4 (10)	2 (5)	35 (90)	36 (90)	36 (88)	36 (97)
G:C→C:G	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
A:T→C:G	5 (13)	26 (63)	1 (3)	0 (0)	3 (7)	0 (0)
A:T→T:A	7 (18)	1 (2)	0 (0)	1 (3)	0 (0)	1 (3)
total	39 (100)	41 (100)	39 (100)	40 (100)	41 (100)	37 (100)

[a]All data are represented as cases found (%).

Table 5. Spontaneous mutant frequencies in *nth*- or *nei*-deficient wt and *mutT* strains.[a]

	Mutant frequency ($\times 10^{-7}$)
MK601 (wt)	0.08 (0.05-0.15)
ST001 (*nth*)	0.50 [b] (0.46-0.79)
ST002 (*nei*)	0.22 [b] (0.19-0.52)
MK602 (*mutT*)	10.9 (8.5-12.2)
ST003 (*mutT/nth*)	18.9 [c] (16.6-23.2)
ST004 (*mutT/nei*)	11.2 (7.8-14.8)

[a] Experiments were repeated fifteen times. The data are expressed as median and numbers in parentheses represent 95% confidence limits for the median in individual experiments.

[b] $P<0.001$ (significant difference versus MK601, Mann-Whitney U-test)

[c] $P<0.001$ (significant difference versus MK602, Mann-Whitney U-test)

Table 6. Spectra of mutations in the *nth*- and *nei*-deficient *mutT* cells.[a]

	MK601	ST001	ST002	MK602	ST003	ST004
	(wt)	(*nth*)	(*nei*)	(*mutT*)	(*mutT*/*nth*)	(*mutT*/*nei*)
transition						
G:C→A:T	8 (21)	26 (72)	10 (29)	0 (0)	5 (7)	1 (1)
A:T→G:C	14 (36)	3 (8)	4 (11)	2 (3)	1 (1)	0 (0)
transversion						
G:C→T:A	7 (18)	2 (6)	1 (3)	0 (0)	1 (1)	0 (0)
G:C→C:G	0 (0)	0 (0)	3 (9)	0 (0)	0 (0)	0 (0)
A:T→C:G	5 (13)	1 (3)	14 (40)	68 (97)	63 (89)	71 (99)
A:T→T:A	2 (5)	4 (11)	3 (9)	0 (0)	0 (0)	0 (0)
others	2 (5)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)
total	39 (100)	36 (100)	35 (100)	70 (100)	71 (100)	72 (100)

[a]All data are represented as cases found (%).