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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

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The *Escherichia coli* MutT protein hydrolyzes 8-hydroxy-dGTP (8-OH-dGTP) *in vitro*, and *mutT* gene deficiencies cause increased spontaneous A:T→C:G mutations. However, no direct evidence exists for enhanced mutagenicity of 8-OH-dGTP in *mutT* cells. In this study, 8-OH-dGTP was introduced into wild type and *mutT E. coli* cells, and mutations of a chromosomal gene were monitored. 8-OH-dGTP induced mutations of the *rpoB* gene, the degree of the mutation induction in the *mutT* strain being ~6-fold higher than that in the wild type strain. On the other hand, 2-hydroxy-dATP, which is not a substrate of the MutT protein, increased the mutation to similar degrees in the two strains. These results constitute the first evidence that the MutT protein suppresses mutation by 8-OH-dGTP *in vivo*.

Key words: 8-hydroxy-dGTP, MutT, mutation, nucleotide pool sanitization, oxidized nucleotide.

Abbreviations: 2-OH-dATP, 2-hydroxy-2′-deoxyadenosine 5′-triphosphate; 8-OH-dGTP, 8-hydroxy-2′-deoxyguanosine 5′-triphosphate; LB, Luria-Bertani; wt, wild type.

Nucleotide pool sanitization is an important mechanism for organisms to prevent mutagenesis caused by damaged DNA precursors (*1*, *2*). The *Escherichia coli* MutT protein was the first enzyme found to degrade a mutagenic oxidized DNA precursor, and its substrate is 8-OH-dGTP (*3*). The mammalian MTH1 and MTH2 proteins hydrolyze the same compound, suggesting the importance of this type of enzyme (*4*, *5*). Additionally, the MTH1 protein hydrolyzes other oxidized DNA precursors, such as 2-OH-dATP and 8-hydroxy-2′-deoxyadenosine 5′-triphosphate (*6*). 2-OH-dATP is degraded by the *E. coli* Orf135 protein *in vitro* (*7*).

MutT hydrolyzes 8-OH-dGTP to the corresponding monophosphate, and its Michaelis constant (K_m) is 0.48 μM *in vitro* (*3*). The amount of 8-hydroxyguanine in the chromosomal DNA is higher in the *mutT* strain than in the wt strain (*8*). 8-OH-dGTP is mutagenic and elicits A:T→C:G transversions when introduced directly into bacterial cells (*9*). In agreement with these findings, A:T→C:G transversions are induced with high frequency in the *mutT* strain (*10*). These results strongly suggest that the MutT protein suppresses mutations by eliminating 8-OH-dGTP from the nucleotide pool *in vivo*. However, no direct evidence exists for enhanced mutagenicity of 8-OH-dGTP in *mutT* cells.

In this study, we introduced 8-OH-dGTP into the wt and *mutT E. coli* strains, mutations of the chromosomal *rpoB* gene being monitored. 8-OH-dGTP significantly induced mutations, and the degree of the mutation induction was ~6-fold higher in the *mutT* strain than in the wt strain. On the other hand, 2-OH-dATP, which is not a substrate of the MutT protein (*6*), increased the mutation to similar degrees in the two strains. These results constitute the first evidence that the MutT protein suppresses the mutagenesis induced by 8-OH-dGTP *in vivo*.

MATERIALS AND METHODS

Materials—*E. coli* strain AB1157 (*F*: *thr*-1, *leuB*6, *thi*-1, *lacY*1, *galK*2, *ara*-14, *xyl*-5, *mtl*-1, *pro*A2, *his*-4, *argE*3, *rpsL*31, *tsx*-33, *supE*44, *flaND*) was used for the mutagenesis experiments. This strain was obtained from the National Institute of Genetics, Stocks Research Center (Mishima, Japan). AB1157-derived strain MK602 (*mutT*) was a gift from Dr. Yusaku Nakabeppu of Kyushu University. Oligodeoxyribonucleotides were purchased from sigma Genosys Japan (Ishikari, Japan) in purified forms. 8-OH-dGTP was prepared as previously described (*11*). 2-OH-dATP was prepared by the treatment of dATP with Fe(II)-EDTA-O_2, and was purified by high-performance liquid chromatography as described (*12*). These purified nucleotides were each eluted as a single peak on both reverse-phase and anion-exchange HPLC (data not shown).

Introduction of Deoxyribonucleotides—A single colony was taken from an LB agar plate and then inoculated into 7 ml of LB medium. The *E. coli* culture was incubated at 37°C until the turbidity at 570 nm reached 0.6. The culture was placed on ice for 10 min and then divided into 0.5 ml aliquots in microtubes. Each sample was centrifuged at 13,000 × *g* for 2 min at 4°C. Competent cells were prepared by treatment with 0.1 M calcium chloride. To 50 μl of the *E. coli* suspension, 2.5 μl of either a 5 mM (total 12.5 nmol) deoxyribonucleotide solution or H_2O

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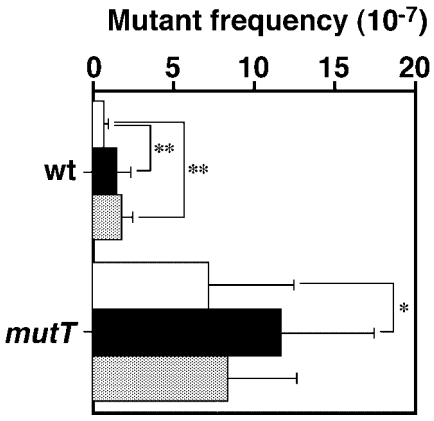

Fig. 1. **Mutant frequency in wt and *mutT E. coli* cells treated with deoxyribonucleotides.** Competent cells were prepared, and after 8-OH-dGTP and 2-OH-dATP had been added to the *E. coli* suspensions, the mixtures were treated as described under "MATERIALS AND METHODS." Open bars, control experiment (H_2O treatment); closed bars, 8-OH-dGTP treatment; hatched bars, 2-OH-dATP treatment. Experiments were repeated at least nine times, and data are expressed as means ± standard deviation. Asterisks indicate a significant difference *versus* with H_2O treatment, $^{*}p < 0.05$ or $^{**}p < 0.01$.

was added, and then the mixture was placed on ice for 180 min. Prewarmed SOC medium (450 μl) was then added, and the cells were incubated at 37°C for 60 min.

A portion of the suspension was diluted with ice-cold LB medium, transferred to an LB agar plate (a titer plate), and then incubated at 37°C for 12 h. Another portion of the suspension was transferred to an LB agar plate containing rifampicin (100 μg/ml) (a selection plate) and then incubated at 37°C for 20 h. 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 means of Student's *t*-test.

Sequence Analysis—The fragment corresponding to positions 1519–1725 of the *rpoB* gene was amplified by PCR, using the primers 5′-dACAGGATATGATCAACGCCAA-3′ and 5′-dCGATACGGAGTCTCAAGGAA-3′, as described (*13*). The amplified DNA fragment was analyzed by sequencing using the former primer, a Big Dye Terminator Cycle Sequencing Kit, and an ABI model 377 DNA sequencer (Applera, Norwalk, CT, USA).

RESULTS AND DISCUSSION

Increased Mutations on Direct Incorporation of Oxidized Deoxyribonucleotides—It was previously demonstrated that the direct incorporation of an oxidized deoxyribonucleotide added to an *E. coli* suspension induced chromosomal gene mutations (*9*, *14*). Thus, we treated the wt and *mutT* strains with the oxidized deoxyribonucleotide 8-OH-dGTP, which is degraded by the MutT protein *in vitro* (*3*). Additionally, 2-OH-dATP, another mutagenic oxidized deoxyribonucleotide, was also examined.

When the wt (AB1157) bacteria were treated with 8-OH-dGTP and 2-OH-dATP, mutations were elicited, as reported previously (*9*). For this strain, the *rpoB* mutant frequencies were 14.5×10^{-8} and 18.3×10^{-8} upon treatment with 12.5 nmol of 8-OH-dGTP and 2-OH-dATP, respectively (Fig. 1). On the other hand, the mutant frequency was 6.6×10^{-8} in the control experiments. Thus, the induction was 7.9×10^{-8} and 11.7×10^{-8} by 8-OH-dGTP and 2-OH-dATP, respectively, in the wt strain.

The mutant frequency in the control experiments was 7.2×10^{-7} for the *mutT* strain (Fig. 1), indicating its mutator character. For this strain, the mutant frequencies were 11.7×10^{-7} and 8.5×10^{-7} upon treatment with 12.5 nmol of 8-OH-dGTP and 2-OH-dATP, respectively (Fig. 1). Thus, the induction was 4.5×10^{-7} and 1.3×10^{-7} by 8-OH-dGTP and 2-OH-dATP, respectively. The subtracted mutant frequency of 8-OH-dGTP, 4.5×10^{-7} for the *mutT* strain, was ~6-fold higher than that for the wt strain (7.9×10^{-8}), and the two values were statistically significant ($p < 0.05$). Thus, these results constitute the first evidence that MutT prevents the mutations induced by 8-OH-dGTP *in vivo*. In contrast, the subtracted mutant frequencies of 2-OH-dATP were similar for the two strains, in agreement with the fact that MutT does not act on 2-OH-dATP *in vitro* (*6*).

Treatment with 8-OH-dGTP or 2-OH-dATP did not severely affect the total number of colonies of either strain.

Table 1. **The distribution of mutations elicited by 8-OH-dGTP and 2-OH-dATP detected in the *rpoB* gene.**

wt[a]			*mutT*[a]		
None[b]	8-OH-dGTP[b]	2-OH-dATP[b]	None[b]	8-OH-dGTP[b]	2-OH-dATP[b]
1532 T→G (1)	1535 C→T (1)	1534 T→C (1)	1538 A→C (11)	1538 A→C (11)	1538 A→C (8)
1546 G→A (6)	1535 C→A (1)	1535 C→T (1)	1714 A→C (2)	1714 A→C (3)	1546 G→T (1)
1547 A→G (1)	1538 A→C (1)	1546 G→A (3)			1714 A→C (1)
1576 C→T (1)	1546 G→A (1)	1546 G→T (6)			1715 T→G (2)
1586 G→A (3)	1547 A→G (2)	1548 C→A (1)			
1592 C→T (4)	1565 C→T (1)	1576 C→T (2)			
1714 A→C (1)	1586 G→A (2)	1586 G→T (1)			
	1587 T→G (1)	1592 C→T (1)			
	1592 C→T (3)	1691 C→T (1)			
	1691 C→T (1)				
	1714 A→C (2)				
	1715 T→G (1)				

A portion of the gene (nucleotides 1519–1725) was analyzed. The sequence of the sense strand is shown. The numbers of colonies are shown on the right side in parentheses. [a]Strain. [b]Nucleotide added.

Table 2. **Spectrum of mutations induced by 8-OH-dGTP and 2-OH-dATP in the wt and *mutT* strains.**

	wt[a]						*mutT*[a]					
	None[b]		8-OH-dGTP[b]		2-OH-dATP[b]		None[b]		8-OH-dGTP[b]		2-OH-dATP[b]	
	Cases found (*n* (%))	MF ($\times 10^{-8}$)[c]	Cases found (*n* (%))	MF ($\times 10^{-8}$)[c]	Cases found (%)	MF ($\times 10^{-8}$)[c]	Cases found *n* (%)	MF ($\times 10^{-7}$)[c]	Cases found (*n* (%))	MF ($\times 10^{-7}$)[c]	Cases found (*n* (%))	MF ($\times 10^{-7}$)[c]
Transition												
G:C→A:T	14 (82)	5.4	9 (53)	7.7	8 (47)	8.6	0 (0)	0.0	0 (0)	0.0	0 (0)	0.0
A:T→G:C	1 (6)	0.4	2 (12)	1.7	1 (6)	1.1	0 (0)	0.0	0 (0)	0.0	0 (0)	0.0
Transversion												
G:C→T:A	0 (0)	0.0	1 (6)	0.9	8 (47)	8.6	0 (0)	0.0	0 (0)	0.0	1 (8)	0.7
A:T→C:G	2 (12)	0.8	5 (29)	4.3	0 (0)	0.0	13 (100)	7.2	14 (100)	11.7	11 (92)	7.8
Total	17 (100)	6.6	17 (100)	14.5	17 (100)	18.3	13 (100)	7.2	14 (100)	11.7	12 (100)	8.5

[a]Strain. [b]Nucleotide added. [c]MF means mutation frequency.

8-OH-dGTP- and 2-OH-dATP-introduction decreased colonies by ~15% for the wt strain, and only 8-OH-dGTP-treatment decreased colonies, by ~20%, for the *mutT* strain.

Mutation Spectra—Rifampicin-resistant colonies isolated from independent cultures derived from the wt and *mutT* bacteria were selected, and the nucleotide sequence of the *rpoB* gene, which is responsible for rifampicin-sensitivity, was analyzed. A portion (nucleotides 1519–1725) of the gene was sequenced, because more than 90% of mutations reportedly occur in this amplified region (*13*, *15*). As shown in Tables 1 and 2, A:T→C:G and G:C→T:A transversion mutations were induced by 8-OH-dGTP and 2-OH-dATP, respectively, in the wt strain, as described previously (*9*). A:T→C:G mutations were detected at four sites upon treatment with 8-OH-dGTP (Table 1). A hotspot was observed at position 1546 in the case of G:C→T:A mutations elicited by 2-OH-dATP. G:C→A:T transitions were detected most frequently in the control experiment.

For the *mutT* strain, a mutational hotspot appeared at position 1538 without the oxidized deoxyribonucleotide treatment (Table 1). As reported, only A:T→C:G transversions were observed. Importantly, very similar distribution patterns of A:T→C:G mutations in the *mutT* strain were obtained in the presence and absence of 8-OH-dGTP, while the total mutant frequency was increased 1.6-fold. Thus, these mutation spectra data also support the idea that the spontaneous mutations in the *mutT* strain were induced by the accumulation of intracellular 8-OH-dGTP.

We multiplied the total mutant frequency by the ratio of A:T→C:G transversions (Table 2). The frequencies of A:T→C:G transversions upon 8-OH-dGTP treatment increased by 3.5×10^{-8} for the wt strain, and by 45×10^{-8} for the *mutT* strain. Again, this indicates that 8-OH-dGTP elicits this type of mutation more frequently in the absence of the MutT protein.

Interestingly, an A:T→C:G transversion at position 1538 was detected for only one case among the total five A:T→C:G mutations when the wt strain was treated with 8-OH-dGTP (Table 1). This distribution pattern is statistically different from those of the *mutT* strain with and without the 8-OH-dGTP treatment ($p < 0.05$ or $p < 0.01$, respectively, χ^2 test for independence). This result is somewhat unexpected, since the 8-OH-dGTP inside the cell was regarded as the common cause of the A:T→C:G transversions. The A:T→C:G mutations could be generated through the incorporation of 8-OH-dGTP opposite A in the template DNA (*3*, *16–19*). It is possible that DNA polymerase(s) misinsert 8-OH-dGTP at various A residues with different affinities. The A:T→C:G mutation (misincorporation of 8-OH-dGTP) at position 1538 might occur only when the intracellular 8-OH-dGTP concentration is high. Mutational hotspots could be generated due to the sequence-preference of DNA repair protein(s) (*20*). In the case of the A:T→C:G mutation, the A:8-hydroxyguanine base pair is a very poor substrate for the MutM protein (*21*). In contrast, the MutY protein fixes the A:T→C:G mutation by removing the A base (*18*, *19*, *22–24*). It is also possible that the MutY protein has different affinities for A:8-hydroxyguanine base pairs.

In the wt strain, G:C→A:T transition mutations appeared to be induced by 8-OH-dGTP, in addition to A:T→C:G mutations (Table 2). The reason for this result is currently unknown, but it may result from disturbance of the nucleotide pool balance on introduction of exogenous 8-OH-dGTP.

Role of MutT—Various findings suggesting the involvement of the MutT protein in 8-OH-dGTP degradation *in vivo* have been reported. The MutT protein hydrolyzes 8-OH-dGTP to the monophosphate *in vitro* (*3*). The 8-hydroxyguanine content of the chromosomal DNA is higher in the *mutT* strain than in the wt strain (*8*). A:T→C:G transversions, which are the mutations elicited by 8-OH-dGTP in bacterial cells, are induced with high frequency in the *mutT* strain (*9*, *10*). However, a failure to detect 8-OH-dGTP in extracts of *mutT* strains was recently reported, which cast some doubt on the expected MutT function (*25*).

In this study, we introduced 8-OH-dGTP into the wt and *mutT* strains, and demonstrated for the first time that the presence of the MutT protein actually suppressed the A:T→C:G transversions induced by this oxidized deoxyribonucleotide (Tables 1 and 2). Therefore, the MutT protein suppresses mutations by eliminating 8-OH-dGTP from the nucleotide pool *in vivo*.

In addition to MutT, two other proteins in *E. coli* seem to remove 8-OH-dGTP. Kobayashi *et al.* found that the *E. coli ribA* gene product, GTP cyclohydrolase II, hydrolyzed 8-OH-dGTP (*26*). We previously reported that the Orf135 protein, which contains a region homologous to MutT, hydrolyzed 8-OH-dGTP *in vitro* (*7*). However, MutT appears to act as the major hydrolyzing enzyme specific

for 8-OH-dGTP. The overproduction of the *ribA* gene product reduces the increased MF level in the *mutT* strain but the extent of suppression by the *ribA* gene is lower than the effect of the authentic *mutT* gene. In the *mutT*$^+$ background, a deficiency of the *ribA* gene has no significant effect on the mutation frequency. In contrast to the case of MutT, effects of a deficiency of the Orf135 protein are not evident upon treatment with 8-OH-dGTP (*27*). Thus, the GTP cyclohydrolase II and Orf135 proteins may serve as back-up enzymes for the MutT protein.

On the other hand, the mutant frequency was slightly increased upon the treatment with 25 nmol of 8-OH-dGTP. The effect of the Orf135 protein is not evident in this case.

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