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Escherichia coli MutY protein has a guanine-DNA glycosylase that acts on 7,8-dihydro-8-oxoguanine:guanine mispair to prevent spontaneous G:C→C:G transversions

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ABSTRACT

Low rates of spontaneous G:C→C:G transversions would be achieved not only by the correction of base mismatches during DNA replication but also by the prevention and removal of oxidative base damage in DNA. *Escherichia coli* must have several pathways to repair such mismatches and DNA modifications. In this study, we attempted to identify mutator loci leading to G:C→C:G transversions in *E.coli*. The strain CC103 carrying a specific mutation in *lacZ* was mutagenized by random miniTn*10* insertion mutagenesis. In this strain, only the G:C→C:G change can revert the glutamic acid at codon 461, which is essential for sufficient β-galactosidase activity to allow growth on lactose. Mutator strains were detected as colonies with significantly increased rates of papillae formation on glucose minimal plates containing P-Gal and X-Gal. We screened ~40 000 colonies and selected several mutator strains. The strain GC39 showed the highest mutation rate to Lac$^+$. The gene responsible for the mutator phenotypes, *mut39*, was mapped at around 67 min on the *E.coli* chromosome. The sequencing of the miniTn*10*-flanking DNA region revealed that the *mut39* was identical to the *mutY* gene of *E.coli*. The plasmid carrying the *mutY*$^+$ gene reduced spontaneous G:C→T:A and G:C→C:G mutations in both *mutY* and *mut39* strains. Purified MutY protein bound to the oligonucleotides containing 7,8-dihydro-8-oxo-guanine (8-oxoG):G and 8-oxoG:A. Furthermore, we found that the MutY protein had a DNA glycosylase activity which removes unmodified guanine from the 8-oxoG:G mispair. These results demonstrate that the MutY protein prevents the generation of G:C→C:G transversions by removing guanine from the 8-oxoG:G mispair in *E.coli*.

INTRODUCTION

Errors during DNA replication and spontaneous DNA damage are potential sources of spontaneous mutations (1–3). However, the critical cause of each spontaneous base substitution is not completely understood. In particular, the pathways leading to G:C→C:G transversions and their biological importance remain uncertain (3). Spontaneous G:C→C:G transversions are rare events. However, the frequency of the transversions significantly increases upon exposure to ionizing radiation and oxidizing chemicals (4–7). Active oxygen species can be produced as an inescapable by-product by the incomplete reduction of oxygen during aerobic metabolism, and their production is further enhanced by exposure to various oxidative stress conditions (8–10). Active oxygen species are potential causes for spontaneous DNA damage inducing G:C→C:G transversions (11–14). In fact, G:C→C:G transversions are predominant in spontaneous mutations in an *Escherichia coli sodAsodB* mutant (our unpublished results), which is fully defective in superoxide dismutase activities (15). Base mismatches during DNA replication are also potential sources of spontaneous mutations (3,16–19). The G:G mispair, for instance, could assume a relatively stable conformation in duplex DNA (20,21). If unrepaired, these mismatches and oxidative DNA damage could have mutagenic consequences. Therefore, low rates of spontaneous G:C→C:G transversions (3) would be achieved by the prevention and repair of oxidative base damage and by the correction of base mismatches.

Mutants with higher than normal rates of spontaneous mutations, termed mutators, have facilitated the understanding of mutational pathways. Mismatch repair directed by *dam* methylation and requiring the products of the *mutH*, *mutL* and *mutS* genes predominantly corrects transition mutations in *E.coli* (3,16–18,22,23). Several mutators, *mutT*, *mutM*, *mutY*, *mutA* and *mutC*, show elevated frequencies of specific transversions (24–28). However, these mutator strains do not have significantly enhanced levels of G:C→C:G transversions (3). This implies that additional repair systems correct mispairing that leads to the G:C→C:G transversions. Hence, we attempted to isolate and characterize additional mutators in order to clarify the pathways of G:C→C:G transversions.

We used a sensitive screening method to detect mutators that revert to defined mutations in *lacZ* by a limited number of base substitutions (3,24,25). Strains CC101–CC106 all carry a specific *lacZ* mutation affecting residue 461 in *lacZ* on an F′*lacproAB* episome (29). In the CC103 strain, only a G:C→C:G change can restore the wild-type codon at position 461 in the *lacZ* gene (29). We mutagenized the CC103 strain by random miniTn*10-tet*

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insertion mutagenesis (30). The strain GC39 was selected by higher rates of papillae formation and reversion to Lac^+. The sequence of the miniTn*10*-flanking region indicated that the mutator locus was identical to that of the *mutY* gene. Purified MutY protein bound to 7,8-dihydro-8-oxo-guanine (8-oxoG):G as well as to 8-oxoG:A mispairs. In addition, we found that the MutY protein had a DNA glycosylase activity to remove unmodified guanine from the 8-oxoG:G mispair. These results demonstrate that the MutY protein prevents the generation of G:C→C:G transversions by removing the unmodified guanine from the 8-oxoG:G mispair in *E.coli*.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli strains CC103 and CC104 are derivatives of P90C [*ara*Δ(*lacproB*)$_{XIII}$] with an F′*lacproAB* episome (29). Each strain carries a different *lacZ*$^-$ mutation affecting residue 461 in β-galactosidase (3,29). A series of isogenic mapping strains containing Tn*10kan*, Tn*5* or Tn*9* at specific sites in the genome (31) were used to map mutator loci on the *E.coli* chromosome. The transduction experiments with $P1_{vir}$ phage were carried out according to Miller (32) with a slight modification.

Enzymes and chemicals

Phenyl-β-D-galactoside (P-Gal) was obtained from Sigma Chemicals. Tetracycline hydrochloride, kanamycin and chloramphenicol were purchased from Wako Pure Chemicals. Ampicillin and rifampicin were the products of Meiji Seika and Boehringer-Mannheim, respectively. 5-Bromo-4-chloro-3-indolyl-D-galactoside (X-Gal), restriction enzymes and the BcaBest sequencing kit were obtained from Takara Shuzo. *Taq* DNA polymerase and isopropyl-1-thiol-β-D-galactopyranoside (IPTG) were purchased from Toyobo. Oligonucleotide containing a single 8-oxoG residue was obtained from Trevigen, Inc. Plasmid pGEX-4T-3, glutathione–Sepharose 4B and thrombin protease were obtained from Pharmacia Biotech. [α-^{32}P]dCTP (>111 TBq/mmol) and [γ-^{32}P]ATP (>259 TBq/mmol) were the products of ICN Biomedicals Inc.

Selection of mutator strains

Random Tn*10* insertion mutagenesis was performed in *E.coli* CC103 by infection with a derivative of λ phage, λ1098, which carries a Tn*10*-derived transposon (miniTn*10-tet*) that can transpose the Tn*10* from the phage into the *E.coli* chromosome (3,30). The cells of *E.coli* CC103 were grown in λym broth (30) and infected with λ1098 at the multiplicity of infection of ~5. The infected cells were plated on λym agar plates containing 15 μg/ml tetracycline and incubated at 39.5°C for ~20 h. To select G:C→C:G mutator strains, ~40 000 colonies were replica-plated to glucose minimal plates (3) containing 15 μg/ml tetracycline, 40 μg/ml X-Gal and 500 μg/ml P-Gal. Plates were incubated at 37°C for ~10 days. We selected several colonies that had a rate of papillae formation significantly higher than normal. P1 lysates were prepared on these mutants and the transposon was again transduced into *E.coli* CC103 to ensure that only one transposon was present in each mutant derivative.

Spontaneous mutation assays

A single colony was inoculated into glucose minimal medium and cultured at 37°C to the stationary phase. The reversion to Lac^+ was assayed as follows: 0.1 ml of cell suspensions were plated on minimal agar plates containing 1% lactose (lactose minimal plates) and then incubated at 37°C for ~10 days. The normal plating density was ~2×10^8 cells per plate. The mutation to rifampicin resistance was assayed as follows: 0.1 ml of cell suspensions (~2×10^8 cells) were plated on LB plates (3) containing 100 μg/ml rifampicin and then incubated for ~24 h. The number of mutant colonies on the plates was counted to estimate the mutation frequency.

Genetic mapping

The gene responsible for the mutator phenotypes was mapped on the *E.coli* chromosome using the P1 mapping kit, a series of isogenic mapping strains containing Tn*10kan*, Tn*5* or Tn*9* at specific sites in the genome (31). P1 transduction was performed according to Miller (32) with a slight modification. The concentrations of tetracycline, kanamycin and chloramphenicol, when added, were 15, 50 and 30 μg/ml, respectively. Kanamycin- or chloramphenicol-resistant colonies were tested for growth on tetracycline plates at 37°C. Individual Kan^rTet^s or Cm^rTet^s colonies were then tested for growth on Lac indicator plates (glucose minimal plates containing P-Gal and X-Gal) that monitor papillation.

Sub-cloning and sequencing of the *mut39*::miniTn*10-tet* gene

The chromosomal DNA of *E.coli* GC39 carrying the *mut39*::miniTn*10-tet* was digested by *Bam*HI and ligated into the *Bam*HI site of pUC118. The resulting plasmids were introduced to *E.coli* JM109 and then plated on LB plates containing 50 μg/ml ampicillin and 15 μg/ml tetracycline. The nucleotide sequence of the miniTn*10*–*mut39* junction was determined by the dideoxynucleotide chain termination method (33). Sequencing reactions were carried out with the BcaBest^{TM} sequencing kit and the primer 5′-CCAAAATCATTAGGGGATTCATCAG-3′ (the miniTn*10-tet* end) (34). The sequencing products were resolved on denaturing 6% polyacrylamide gels. After electrophoresis, the gels were dried and then autoradiographed using Fuji RX films at –80°C.

Preparation of GST fusion protein

A plasmid expressing the glutathione-S-transferase (GST)-MutY fusion protein was constructed as follows: the plasmid pLC20-5 bearing *E.coli mutY* gene (35) was amplified with two PCR primers; one containing an *Eco*RI site followed by the sequence around the putative start codon (5′-AACAACAGTGAATTCG-GTGACCAT-3′) and the other containing a *Sal*I site followed by the sequence around the stop codon (5′-ATATAGTCGACGTTG-CAGGAAAGTA-3′). A standard PCR was performed with 100 μl standard reaction mixture containing 20 mM Tris–HCl (pH 8.4), 1.5 mM $MgC1_2$, 200 μM each dNTP, 0.5 mM of primer and 5 U of *Taq* DNA polymerase. The amplified 1147 bp fragment was digested with *Eco*RI and *Sal*I, and then the *Eco*RI/*Sal*I fragment containing the whole coding region of the *mutY* gene was inserted into *Eco*RI/*Sal*I-digested pGEX-4T-3. The resulting plasmid was named pGEX-MutY. The pGEX-MutY could reduce the frequency of G:C→T:A transversions in the *mutMmutY* mutant.

Escherichia coli BL21 was transformed with the plasmid pGEX-MutY. The cells were grown at 22–24°C in 1 l of LB medium containing 100 μg/ml ampicillin until the absorbance at

600 nm reached ~0.8. Expression of the GST-MutM fusion protein was induced by the addition of 0.1 mM IPTG, and growth was continued at 22–24°C for an additional 8 h. Cells were then harvested by centrifugation and resuspended in phosphate-buffered saline (PBS) containing 1% Triton X-100. A cell extract was prepared by sonication of the cell suspensions, followed by centrifugation at 15 000 *g* for 30 min at 4°C. The supernatant was applied to a glutathione–Sepharose 4B column. The column was washed with 10 column vol of PBS, and the bound protein was eluted with 15 mM glutathione in 50 mM Tris–HCl (pH 8.0). The eluted samples were concentrated by ultrafiltration, and the buffer was replaced by PBS. The GST-MutY fusion protein was cleaved by thrombin protease by incubation for 12–14 h at 4°C. Proteins were assayed by Coomassie blue staining after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Gel mobility shift assay

The oligonucleotide containing a single 8-oxoG residue was the product of Trevigen, Inc. Each of the 24mer oligonucleotides was labeled at the 5′-end with [γ-^{32}P]ATP by T4 polynucleotide kinase and then annealed with its complementary oligonucleotides as previously described (36). The binding reactions were carried out in 25 mM Tris–HCl (pH 8.0), 0.5 mM dithiothreitol, 0.5 mM EDTA, 10% glycerol, 25 mM NaCl, 25 mM KCl, 10 mM $ZnCl_2$, 0.125 mM each dNTP, 4 mM spermidine, 50 µg/ml calf thymus DNA and various amounts of MutY protein in a final volume of 5 µl. After an incubation for 30 min at 4°C, the reaction mixtures were electrophoresed on 12% non-denaturing polyacrylamide gels in TBE buffer (25 mM Tris, pH 8.0, 24 mM boric acid and 0.5 mM EDTA) at 100 V at room temperature. After electrophoresis, the gels were dried and then autoradiographed using Fuji RX films at –80°C.

8-oxoG-containing DNA cleavage assay

Duplex oligonucleotides containing a single 8-oxoG residue at position 10 were constructed as mentioned above (Fig. 2). The reaction mixtures contained 20 fmol substrate oligonucleotides and various amounts of MutY protein in 10 µl of the reaction buffer containing 20 mM Tris–HCl (pH 7.6), 0.5 µg of BSA and 10 mM EDTA. After an incubation at 37°C, the reaction was terminated by the addition of 2 µl of 2.5 M NaOH and heated at 95°C for 5 min. This served to cleave any apyrimidinic/apurinic (AP) sites generated by DNA glycosylase reactions, followed by the addition of a solution (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol and 20 mM EDTA). After heating at 98°C for 5 min, the samples were cooled and loaded onto 20% polyacrylamide gels in the presence of 7 M urea. After electrophoresis at 1800 V, the gels were dried and then autoradiographed using Fuji RX films at –80°C.

RESULTS

Isolation of mutator strains

To isolate mutator strains with a higher than normal rate of G:C→C:G transversions, the *E.coli* strain CC103 was mutagenized by random miniTn*10-tet* insertion mutagenesis. In this strain, only a G:C→C:G change can restore the wild-type codon at position 461 in the *lacZ* gene on F′*lacproAB* episome (29). Mutators were selected by screening the rates of papillae formation on glucose minimal plates containing P-Gal and X-Gal. Colonies grow until they exhaust the glucose. Lac$^+$ revertants, which can utilize the P-Gal as a carbon source, grow out as papillae and are stained blue by X-Gal (3). We screened ~40 000 colonies and found five mutator strains that generate papillae at higher rates than normal (Fig. 1).

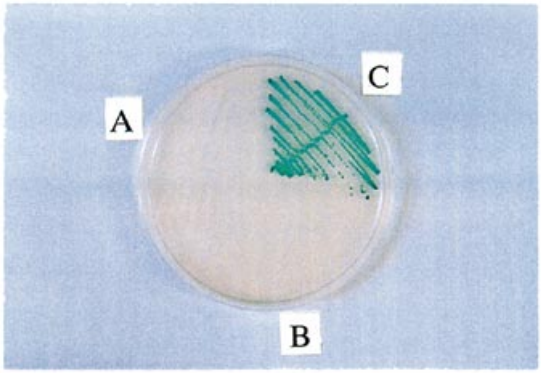

Figure 1. The GC39 mutator strain displayed more papillae than did the CC103 and CC103*mutM* strains. (**A**) *Escherichia coli* CC103, (**B**) CC103*mutM* and (**C**) CC103*mut39* were inoculated to glucose minimal plates containing 40 µg/ml X-Gal and 500 µg/ml P-Gal. Plates were incubated at 37°C for 6 days.

Of the five G:C→C:G mutator strains isolated, strain GC39 showed the highest rate of papillae formation, all colonies of which had >20 papillae on glucose minimal plates containing P-Gal and X-Gal (Table 1). Two tests were run to further assay the properties of the mutator strains. The five mutator strains were placed into three groups based on three tests for papillae formation, generation of Lac$^+$ and rifampicin resistance (Table 1). Strain GC39 was put into Group I. The spontaneous reversion frequency to Lac$^+$ was increased ~50–100-fold in strain GC39, compared with that of the wild-type CC103. The spontaneous mutation to rifampicin resistance was also increased ~10–20-fold in the GC39 strain over the control level. The group II mutators also stimulated the Lac$^+$ reversion and Rif^r mutation in the two strains at relatively high rates (Table 1). Therefore, we selected the GC39 strain for further studies.

Table 1. Characteristics of different mutator strains

Group	Strain	Papillation	Mutants per 10^8 cells	
			Lac$^+$	Rif^r
I	GC39	++	148	188
II	GC9	++	125	1550
	GC24	++	101	1460
III	GC40	+	15	1280
	GC41	+	28	2560
–	CC103	–	3	11

The cell suspensions of mutator strains and the parent strain were plated on lactose minimal agar plates and incubated at 37°C for 6 days. Of the five G:C→C:G mutator strains isolated, strain GC39 showed the highest rate of papillae formation, all colonies of which had >20 papillae on glucose minimal plates containing P-Gal and X-Gal. Papillation is indicated qualitatively. Strong, ++; moderate, +.

Genetic mapping of the mutator locus in GC39 strain

We used a kit of Singer's P1 mapping for *E.coli* consisting of >90 donor strains to determine the location of the mutator locus on the chromosome of *E.coli* (31). Sections of the *E.coli* chromosome

were transferred to *E.coli* GC39 with P1 phage. Antibiotic-resistant transductants (Kan^r or Cm^r) were replica-plated on LB plates containing tetracycline to determine the rate of cotransduction. The cotransduction frequencies of Kan^r Tet^s were 90, 23 and 19% for *nupG3157::*Tn*10kan* at 66.9 min, *metC3158::*Tn*10kan* at 67.9 min and *zgd3156::*Tn*10kan* at 66.0 min, respectively. The location of transposon insertion sites was recalibrated by using cotransduction frequencies for nearby genes, as done previously (37,38). Kan^{r}Tet^s colonies were further replica-plated on glucose minimal plates containing P-Gal and X-Gal. They showed a significant reduction of papillae formation. No transductants containing DNA from other donor strains reduced the rate of papillation. These results indicated that the gene responsible for the mutator phenotypes, named *mut39*, was localized around 67 min on the *E.coli* chromosome.

Sub-cloning and sequencing of the *mut39*::miniTn*10-tet* gene

The nucleotide sequence of the junction between the *mut39* gene and the miniTn*10* insertion was determined using the sequence 5′-CC-AAAATCATTAGGGGATTCATCAG-3′ (nucleotides 1–25 from the miniTn*10-tet* end) (34) as a primer. The sequence determined was identical to the complementary strand of *mutY* gene (26,39). The sequences were compared with the databases of the National Center of Biotechnology Information by using the BLAST server. No other sequences could be found with significant sequence matches.

In addition, the expression of *mutY*$^+$ gene markedly reduced the spontaneous frequency in the *mutY* and *mut39* mutants of *E.coli* (Table 2). These results indicated that *mut39* was the same gene as the *mutY* gene.

Table 2. The effect of *mutM*, *mutY* and *mut39* mutations on the frequency of Lac$^+$ reversion in different mutator strains with or without pGEX-MutY plasmid

Strain	Number of Lac$^+$ revertants per 10^8 cells
CC103	1
CC103*mutY*	120
CC104*mutY*/pGEX-MutY	18
CC103*mutM*	2
CC103*mut39*	146
CC103*mut39*/pGEX-MutY	13
CC103*mutMmut39*	149
CC104	6
CC104*mut39*	232
CC104*mutY*	204
CC104*mutY*/pGEX-MutY	18

The cell suspensions were plated on lactose minimal agar plates and then incubated at 37°C for 2 days (CC104 and its derivatives) or 6–10 days (CC103 and its derivatives). The introduction of the vector pGEX-4T-3 did not affect the frequency of Lac$^+$ revertants.

Spontaneous mutations in *mutY* and *mut39* strains of *E.coli*

Strain GC39 was initially isolated by increased rates of G:C→C:G transversions in *E.coli* CC103 (Table 1). We examined whether the *mut39* mutation can affect the rate of Lac$^+$ reversion in the *E.coli* strain CC104. In this strain, the G:C→T:A change can restore the wild-type codon at position 461 in the *lacZ* gene (29). The CC104 cells were infected with P1 phage multiplied in *E.coli* GC39 and plated on lactose minimal plates, followed by an incubation at 37°C for ~2 days. The *mut39* mutation resulted in an enhanced frequency of spontaneous G:C→T:A transversions (Table 2). The properties of *E.coli* CC104*mut39*::miniTn*10* were comparable with those of the CC104*mutY*::Tn*10* strain (25). In addition, the introduction of the *mutY*::Tn*10* mutation into *E.coli* CC103 also resulted in increased rates of papillae formation and reversion to Lac$^+$ (Table 2).

The MutM and MutY DNA glycosylases are both parts of the 8-oxoG repair system in *E.coli*, which prevents the generation of G:C→T:A mutations caused by spontaneous 8-oxoG formation in DNA (13,14,28). The *mutM* and *mutY* mutants show an increased rate of spontaneous G:C→T:A mutations compared with the wild-type strain (25,28,40). When *mutM* and *mutY* were combined, the mutator activity is more striking (25,28,40). In contrast, the *mutM* mutation did not increase the spontaneous G:C→C:G transversions in CC103. Furthermore, the addition of the *mutM* mutation to the CC103*mut39* led to no significant increase in mutation rates (Table 2).

Construction of GST-MutY fusion plasmid and purification of MutY protein

The entire open reading frame of the *mutY* gene was amplified by PCR and sub-cloned into pGEX-4T-3 to obtain a GST-MutY fusion construct. The sequence was checked to verify that no mutations had been generated by the PCR. The construct pGEX-MutY obtained was able to reduce the high rates of spontaneous G:C→T:A transversions in CC104*mutMmutY*. The fusion protein was over-expressed in *E.coli* BL21/pGEX-MutY treated with IPTG and then purified by means of a glutathione–Sepharose 4B column chromatography. The synthesis of MutY was confirmed by SDS–PAGE. The 39 KDa MutY protein was present on the gels after cleavage of the GST-MutY fusion protein with thrombin protease.

Gel mobility shift assay

The gel mobility shift assay was done to assess the binding activity of *E.coli* MutY protein to a series of double-stranded oligonucleotides containing various base mispairs. The 24mer oligonucleotides, including the one containing 8-oxoG, were synthesized and ^{32}P-labeled at the 5′-terminus with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. The sequences of the oligonucleotides are shown in Figure 2. The double-stranded oligonucleotides containing G:C, 8-oxoG:A, G:G, 8-oxoG:G and G:A were constructed by annealing ^{32}P-labeled oligonucleotide 2 with complementary oligonucleotide 6 (labeled 2/6), 3/5, 2/4, 3/4 and 2/5, respectively. MutY protein strongly bound to duplex oligonucleotides containing an 8-oxoG:G in addition to an 8-oxoG:A mispair, whereas it did not bind to other mispairs, as shown in Figure 3A. The binding activity was calculated by quantifying the band intensities by densitometry of the autoradiograms. The purified MutY protein bound to the 8-oxoG:G mispairs with efficiency similar to the 8-oxoG:A mispairs (Fig. 3B). These results suggested that MutY protein is capable of repairing an 8-oxoG:G mispair in DNA.

Cleavage activity of MutY protein on 8-oxo-G:G mispair-containing duplex DNA

Double-stranded oligonucleotides (24mer) containing a set of four DNA mispairs (G:G, 8-oxoG:G, 8-oxoG:A and G:A) at

1 5'-GAACTAGTGCATCCCCCGGGCTGC-3'
2 5'-GAACTAGTGGATCCCCCGGGCTGC-3'
3 5'-GAACTAGTGOATCCCCCGGGCTGC-3'

4 3'-CTTGATCACGTAGGGGGCCCGACG-5'
5 3'-CTTGATCACATAGGGGGCCCGACG-5'
6 3'-CTTGATCACCTAGGGGGCCCGACG-5'

Figure 2. Sequences of oligonucleotides used to compare substrate specificity. O, 8-oxoG.

position 10 were constructed by annealing oligonucleotide 2 with complementary oligonucleotide 4 (labeled 2/4), 3/4, 3/5 and 2/5, respectively, and used as a substrate for MutY DNA glycosylase activity assay. Each duplex oligonucleotide was incubated at 37°C with purified MutY protein. Reactions were terminated by the addition of NaOH and subsequently heated at 95°C for 5 min. This served to cleave any AP sites generated by the DNA glycosylase. The progress of the glycosylase reaction can therefore be monitored by a change in the migration of the product. The results are shown in Figure 4. The MutY glycosylase was active on the duplex oligonucleotides containing the 8-oxoG:A and G:A mispairs (lanes 9–12). The cleavage of the 24mer substrates to 14mer products was due to the ability of the MutY protein to remove adenine from the 8-oxoG:A and G:A mispairs (26,41–43). In addition, the MutY protein was found to efficiently remove unmodified guanine from 8-oxoG:G (lanes 7 and 8). The cleavage site of the 8-oxoG:G-containing oligonucleotide was the same as those of the 8-oxoG:A and G:A-containing oligonucleotides. In contrast, the MutY protein did not remove the 8-oxoG from 8-oxoG:G mispair-containing DNA (lanes 5 and 6) and did not repair the G:G mispair (lanes 1–4). Figure 5 shows an incubation time-dependent cleavage of 8-oxoG:G-containing oligonucleotides by purified MutY protein. The cleavage reaction proceeded at a slow rate, compared with that for 8-oxoG:A mispair. Complete cleavage was achieved after 8 h of incubation for the 8-oxoG:G, and after ~1 h for the 8-oxoG:A (data not shown).

The relative effects of MutY glycosylase on the 8-oxoG:G and 8-oxoG:A mispairs were determined. Increasing amounts of MutY protein were incubated with the 8-oxoG:G- and 8-oxoG:A-containing oligonucleotides for 8 and 1 h, respectively. The results are shown in Figure 6. The specific activities of the MutY protein on 8-oxoG:G and 8-oxoG:A substrates were 0.1 and 0.7 nmol/mg protein/h, respectively. Therefore, the activity of the MutY protein to remove unmodified guanine from the 8-oxoG:G was ~1/7 of that for removing adenine from the 8-oxoG:A mispair.

DISCUSSION

The *mut39* locus responsible for the mutator phenotypes in GC39 was located around 67 min on the *E.coli* chromosome. The *mutY* gene is also mapped near 67 min (25). The sequence of the miniTn*10*-flanking region was identical to that of the *mutY* gene. The results indicated that *mut39* is an allele of the *mutY* gene. This was supported by the finding that G:C→T:A transversions were also accumulated in *E.coli* CC104*mut39*, as in the CC104*mutY* strains. In addition, the introduction of *mutY*::Tn*10* mutation into *E.coli* CC103 resulted in increased rates of reversion to Lac$^+$.

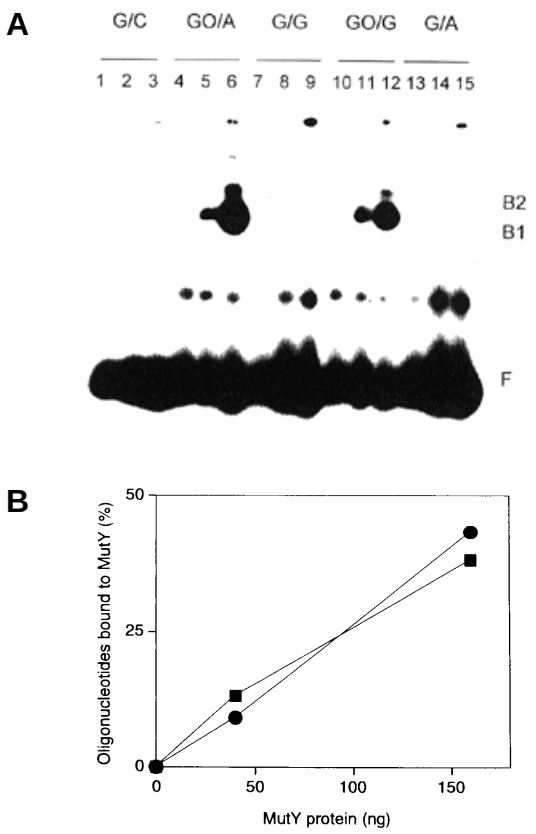

Figure 3. Binding activity of purified MutY protein to double-stranded oligonucleotides with various mispairs. (**A**) ^{32}P-labeled double-stranded oligonucleotides (10 fmol) containing G:C (lanes 1–3), 8-oxoG:A (lanes 4–6), G:G (lanes 7–9), 8-oxoG:G (lanes 10–12) and G:A (lanes 13–15) were incubated with MutY protein purified from the GST-MutY in the presence of sonicated calf thymus DNA. After incubation for 30 min at 4°C, the reaction mixtures were electrophoresed on 12% non-denaturing polyacrylamide gels in TBE buffer at 100 V. After electrophoresis, the gels were dried and then autoradiographed using Fuji RX films at –80°C. F, free oligonucleotide; B1, MutY protein–oligonucleotide complex; B2, additional MutY protein–oligonucleotide complex. Lanes 1, 4, 7, 10 and 13, without MutY; lanes 2, 5, 8, 11 and 14, with 40 ng MutY; lanes 3, 6, 9, 12 and 15, with 160 ng MutY. (**B**) Increasing amounts of the MutY protein were incubated with double-stranded oligonucleotides. The extent of the MutY binding to the 8-oxoG:A (●) and 8-oxoG:G (■) was quantified by densitometry.

Purines undergo oxidation of the ring atoms leading to various chemical modifications. The highly mutagenic 8-oxoG is formed in large quantities (14,44). The 8-oxoG has strong mispairing properties, and both bacterial and eukaryotic DNA polymerases insert A opposite 8-oxoG at a high frequency (45–47). Consequently, the presence of 8-oxoG residues in the template induces G:C→T:A transversions (28,40,44,48). Studies with *E.coli* have shown that three different repair activities, MutM, MutY and MutT, cooperate to prevent mutations from being formed at 8-oxoG lesions. MutM DNA glycosylase removes purines with either ruptured or intact (but oxidized) imidazole rings, such as fapy and 8-oxoG, from DNA (13,43,49,50). MutY is also a DNA glycosylase which efficiently removes an adenine incorporated opposite an 8-oxoguanine (13,26,27,51). Such mispairs are formed if 8-oxoG remains in the template during DNA replication. The enzyme removes adenine from A:G mispairs as well (20). The MutT protein hydrolyses 8-oxo-dGTP to

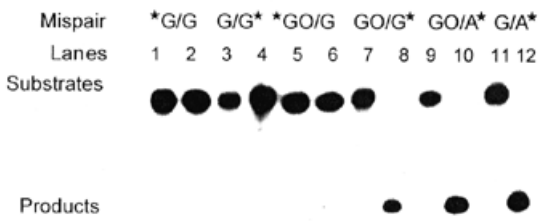

Figure 4. Cleavage of double-stranded oligonucleotides containing a single 8-oxoG mispaired with C, G and A by the MutY protein. Substrate oligonucleotides (20 fmol) containing various mispairs were incubated with 420 ng of MutY protein in 10 µl of the reaction buffer containing 20 mM Tris–HCl (pH 7.6), 0.5 µg of BSA and 10 mM EDTA at 37°C for 14 h. After electrophoresis at 1800 V, the gels were dried and then autoradiographed using Fuji RX films at –80°C. Lanes 1, 3, 5, 7, 9 and 11, without MutY; lanes 2, 4, 6, 8, 10 and 12, with MutY. *, ^{32}P-labeled strand at the 5′-terminus.

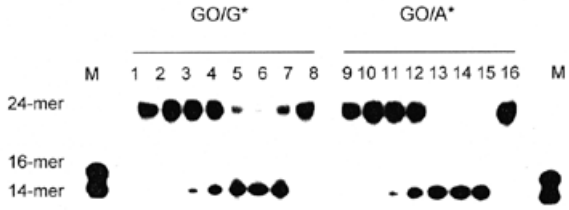

Figure 6. Relative activity of MutY glycosylase on 8-oxoG:A and 8-oxoG:A substrates. Twenty fmol substrate oligonucleotides containing 8-oxoG:G (lanes 1–8) and 8-oxoG:A (lanes 9–16) mispairs were incubated with various amounts of MutY protein in 10 µl of the reaction buffer containing 20 mM Tris–HCl (pH 7.6), 0.5 µg of BSA and 10 mM EDTA for 1 and 8 h, respectively. After electrophoresis at 1800 V, the gels were dried and then autoradiographed using Fuji RX films at –80°C. Lanes 1, 8, 9 and 16, no enzyme; lanes 2 and 10, 1.2 ng; lanes 3 and 11, 2.4 ng; lanes 4 and 12, 6 ng; lanes 5 and 13, 30 ng; lanes 6 and 14, 45 ng; lanes 7 and 15, 60 ng. *, ^{32}P-labeled strand at the 5′-terminus.

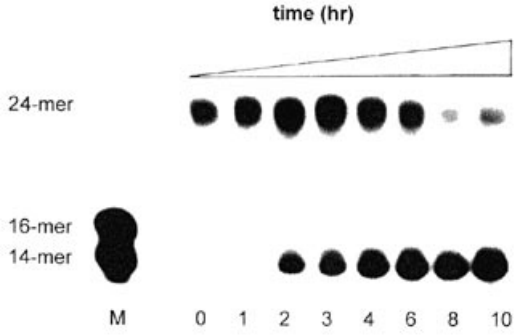

Figure 5. Time course of the MutY glycosylase reaction on 8-oxoG:G substrate. ^{32}P-labeled oligonucleotide 3 was annealed with the complementary oligonucleotide 4. Substrate double-stranded oligonucleotides (20 fmol) containing 8-oxoG:G were incubated with 60 ng of MutY protein in 10 µl of the reaction buffer at 37°C for 0–10 h and analyzed as described in Figure 4.

8-oxo-dGMP, thus preventing 8-oxo-dGTP from being incorporated during replication (52). Strains defective in *mutY* and *mutM* functions accumulate spontaneous G:C→T:A transversion mutations (25,28,40).

The present experiments showed that spontaneous G:C→C:G transversion mutations were also accumulated in strains defective in *mutY* function (Table 2). The results enable two main conclusions to be drawn, as represented in the model (Fig. 7). Firstly, damaged bases such as 8-oxoG are generated in glucose-starved cells and give rise to large numbers of G:C→C:G transversions in the absence of MutY protein. The amount of 8-oxoG produced in the DNA of starved cells appears to be about three times greater than in the DNA of growing cells (14). Therefore, 8-oxoG might constitute an important component of the starvation-associated mutations in *E.coli* cells. 8-oxoG could direct the incorporation of dGMP in addition to dAMP opposite the lesion in the template during DNA replication, as suggested by Braun *et al.* (53). The 8-oxoG:G mispair leads to the generation of G:C→C:G transversions if guanine residues incorporated opposite 8-oxoG are left unrepaired. Secondly, MutY protein can recognize and repair 8-oxoG:G mispairs in DNA to prevent the generation of G:C→C:G transversions in *E.coli.* In fact, the purified MutY protein was able to bind to the 8-oxoG:G as tightly as to 8-oxoG:A mispairs and have a guanine-DNA glycosylase active on 8-oxoG:G mispairs.

The *mutM* mutation did not result in enhanced rates of Lac$^+$ reversion in strains CC103 and CC103*mutY* (Table 2). MutM protein is able to remove 8-oxoG from 8-oxoG:G mispair *in vitro* (43,47). The removal of 8-oxoG from 8-oxoG:G mispair by MutM protein might lead to the occurrence of G:C→C:G transversions, because dCMP is incorporated opposite guanine remaining in DNA during repair replication. Hence, it is reasonable that the activity of the MutM protein to remove 8-oxoG from the mispair is repressed before the MutY protein removes guanine from the 8-oxoG:G mispair in *E.coli* cells. The MutY protein might be implicated in this regulation for the MutM activity, as suggested by Bridges *et al.* (14). Braun *et al.* (53) also suggested that due to the action of nucleotide excision repair, oxidatively damaged G* of a G*:G mismatch is removed, and this will lead to G:C→C:G transversions.

Nghiem *et al.* (25), using a detection method relying on a papillation technique, found that the *mutY* mutation resulted in an enhanced rate of spontaneous G:C→T:A transversions, whereas it did not enhance other types of base substitution. In their experiments, cells were incubated on glucose minimal plates for ~3 days to visualize blue papillae. In the present experiments, cells were incubated for ~10 days. Under such conditions, some turnover of cells and a slow increase in cell count are demonstrated (14). The delayed mutation accumulation of G:C→C:G transversions was also described by Mackay *et al.* (54). Lesions such as 8-oxoG may in fact be produced continuously and direct the misincorporation of dAMP and dGMP opposite the lesion in the template DNA. However, the repair of 8-oxoG:G mispair by MutY protein might be less effective than that of 8-oxoG:A mispair.

The human homolog of the *E.coli mutY* gene (*hMYH*) has been cloned and sequenced (55). The human gene encodes a protein of 535 amino acids (55), which is in good agreement with the size of a polypeptide detected in HeLa cells that cross-reacted with the antibody against *E.coli* MutY protein (56). The human *hMYH* gene has 41% identity to the *E.coli* MutY protein (55), suggesting its important functions in the repair of oxidative damage to DNA and in the prevention of mutations from oxidative lesions. We are currently studying whether the human gene is able to reduce the frequency of spontaneous G:C→C:G transversions and whether

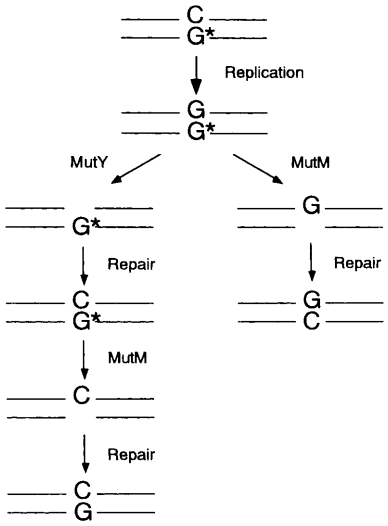

Figure 7. 8-oxoG-related mutagenesis pathways leading to G:C→C:G transversions and the repair mechanism by the MutY protein. Oxidative damage can lead to 8-oxoG lesions in DNA. The asterisk indicates 8-oxoG. If the 8-oxoG lesion is not removed before DNA replication, translesional synthesis by DNA polymerase frequently leads to the misincorporation of G in addition to A opposite the 8-oxoG lesion. MutY protein removes the misincorporated G from the 8-oxoG:G mispairs. 8-oxoG:C mispairs formed during DNA repair replication can be removed by the MutM protein. In contrast, if the MutM protein removes 8-oxoG from the 8-oxoG:G mispair before the action of the MutY protein, the misincorporated G remains, leading to G:C→C:G transversions.

the human MutY protein has a guanine-DNA glycosylase to remove unmodified guanine from 8-oxoG:G mispair.

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