The Shizosaccharomyces pombe Homolog (SpMYH) of the Escherichia coli MutY Is Required for Removal of Guanine from 8-Oxoguanine/Guanine Mispairs to Prevent G:C to C:G Transversions

Takashi DOI¹, Shin-Ichiro YONEKURA¹, Keizo TANO², Shinji YASUHIRA², Shuji YONEI¹ and Qiu-Mei ZHANG¹*

8-oxoguanine/Base excision repair/MutY/S. pombe/Base substitution.

The frequency of G:C→C:G transversions significantly increases upon exposure of cells to ionizing radiation or reactive oxygen species. Transversions can be prevented by base excision repair, which removes the causative modified bases from DNA. Our previous studies revealed that MutY is responsible for removing guanine from 7,8-dihydro-8-oxoguanine/guanine mispairs (8-oxoG/G) and prevents the generation of G:C→C:G transversions in E. coli. SpMYH, a homolog of E. coli MutY, had been identified and characterized in the fission yeast S. pombe. Purified SpMYH has adenine DNA glycosylase activity on A/8-oxoG and A/G mismatch-containing oligonucleotides. In this study, we examined whether SpMYH has a similar activity allowing it to remove G from 8-oxoG/G in DNA. The purified SpMYH tightly bound to duplex oligonucleotides containing 8-oxoG/G and removed the unmodified G from 8-oxoG/G as efficiently as A from 8-oxoG/A. The activity was absent in the cell extract prepared from an SpMYH-knockout strain of S. pombe. The expression of SpMYH markedly reduced the frequency of spontaneous G:C→C:G transversions in the E. coli mutY mutant. These results demonstrate that SpMYH is involved in the repair of 8-oxoG/G, by which it prevents mutations induced by oxidative stress in S. pombe.

INTRODUCTION

Reactive oxygen species (ROS) are generated in living cells as inescapable by-products of the incomplete reduction of molecular oxygen during normal cellular metabolism.¹-³ The production of ROS is further enhanced by the exposure of cells to exogenous stimuli such as ionizing radiation and various chemical oxidants. One prominent target for oxidation in cells is the DNA. The spectrum of ionizing radiation- and ROS-induced damage to DNA is broad and includes a wide variety of oxidative modifications to purine and pyrimidine bases.⁴-⁷ 7,8-dihydro-8-oxoguanine (8-oxoG) is an abundant and deleterious lesion that causes G:C→T:A base substitution mutations.⁸,⁹ The biological impact and mechanisms of repair of 8-oxoG have been extensively investigated.¹⁰-¹³ Base excision repair is an important mechanism for preventing mutations by removing such causative base lesions from the DNA.¹⁰,¹¹,¹³-¹⁶

In Escherichia coli, several DNA glycosylases are involved in the 8-oxoG repair processes. MutM (formamidopyrimidine-DNA glycosylase) removes 8-oxoG preferentially from 8-oxoG/C pairs whose formation in duplex DNA is induced by in situ oxidation.¹⁰,¹²,¹⁷-¹⁹ Unrepaired 8-oxoG forms mutagenic 8-oxoG/A mispairs during subsequent DNA replication.⁷,¹²,¹⁷,¹⁹,²¹ MutY removes A from 8-oxoG/A and thus provides a second opportunity to prevent mutations.¹⁰,¹²,¹⁷,²¹ Therefore, mutations of the mutM and mutY genes significantly increase G:C→T:A transversions in E. coli.²⁵-²⁷

Spontaneous G:C→C:G transversions are rare events.²⁸ The low frequency of spontaneous G:C→C:G transversions is achieved by the prevention and repair of oxidative base damage.²⁹,³⁰ However, the frequency of the transversions significantly increases upon exposure of the cells to ionizing radiation or ROS.³¹-³³ Oxidatively damaged guanine induced by ROS is primarily responsible for the G:C→C:G transversions.³⁴,³⁵ Recently we found that MutY had DNA glycosylase activity that removes G from 8-oxoG/G and prevents the generation of G:C→C:G transversions in E. coli.³⁶ The frequency of spontaneous G:C→C:G transversions is increased in E. coli mutY mutants compared with the wild-type strain.³⁶

*Corresponding author: Phone: +81-75-753-4097, Fax: +81-75-753-4087, E-mail: qmzhang@kingyo.zool.kyoto-u.ac.jp

¹Laboratory of Radiation Biology, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan;
²Division of Radiation Life Science, Research Reactor Institute, Kyoto University, Kumatori, Osaka 590-0494, Japan.

The mutY homolog gene (SpMYH) from a cDNA library of S. pombe encodes a polypeptide of 461 amino acids that shows 28 and 31\% identity with E. coli MutY and the human MutY homolog, respectively.\(^{37}\) Purified SpMYH has adenine DNA glycosylase activity toward A/G and A/8-oxoG mismatch-containing oligonucleotides like E. coli MutY.\(^{37}\) However, E. coli MutY and SpMYH have slightly different substrate specificities. SpMYH has higher activity toward 2-aminopurine/G and A/2-aminopurine, and lower activity forward A/C, than MutY.\(^{37,38}\) Therefore, it is of interest to examine whether or not SpMYH has similar DNA glycosylase activity toward 8-oxoG/G as E. coli MutY. In this study we showed that SpMYH efficiently removes G from 8-oxoG/G and demonstrated that the expression of SpMYH markedly reduced the high frequency of spontaneous G:C→C:G transversions in an E. coli mutY mutant.

MATERIALS AND METHODS

Enzymes and chemicals

Ampicillin and rifampicin were obtained from Wako Pure Chemicals (Osaka, Japan). Restriction enzymes were obtained from Takara Shuzo (Kyoto, Japan). Taq DNA polymerase and isopropyl-1-thiol-ß-D-galactopyranoside (IPTG) were purchased from Toyobo (Osaka, Japan). Plasmid merase and isopropyl-1-thiol-ß-D-galactopyranoside (IPTG) were obtained from Takara Shuzo (Kyoto, Japan). Restriction enzymes were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). [\(^{32}\)P]-ATP (>259 TBq/mmol) was obtained from ICN (Costa Mesa, CA).

Strains and media

S. pombe Y31 cells (h\(^{+}\) ade6-M120 leu1-32 ura-D18 hisD1) were cultured at 30°C in YES medium supplemented with 0.5% yeast extract, 3% glucose, 40 g/ml of adenine, 20 g/ml of L-histidine, 60 g/ml of L-leucine, 30 g/ml of L-lysine and 20 g/ml of uracil. E. coli CC103 and CC104 are derivatives of P90C (ara (lac proB)xaI) with an F'lac proAB episome.\(^{39}\) Each strain carries a different lacZ mutation affecting the activity of ß-galactosidase. Only a G:C→C:G and G:C→T:A change can restore the wild-type codon at position 461 in the lacZ gene on the episome in the CC103 and CC104 strains, respectively.\(^{39}\) E. coli cells were grown in LB broth at 37°C with aeration, unless otherwise stated. When necessary, ampicillin (50 g/ml) was added to the medium.

Cloning of the S. pombe MYH gene

An S. pombe cDNA library was obtained from Stratagene (La Jolla, CA) and used as a template for PCR to amplify the S. pombe SpMYH gene. We synthesized two PCR primers, SpMYH-For (5'-CCCCCGATCCCTCGGATTCAAATCATTCTTTAG-3') and SpMYH-Rev (5'-GGGGGCTC-GAGGCGTATTAGCACTTCG-3'). The PCR product was digested by BamHI and XhoI, and the digested fragment was inserted into pBluescript II SK\(^{+}\). The sequence was checked to verify that no mutations had been generated by the PCR. The resulting plasmid was named pBlu-SpMYH.

Preparation of GST-SpMYH fusion protein

A plasmid expressing the glutathione-S-transferase (GST)-SpMYH fusion protein was constructed as follows: the plasmid pBlu-SpMYH bearing the SpMYH gene was amplified with two PCR primers, one containing a BamHI site followed by the sequence around the putative start codon (5'-CGGGATCCATGTCGGATTCAAATCATTCTTTAG-3') and the other containing an XhoI site followed by the sequence around the stop codon (5'-CGGCTCGAGGCGT-GATTAGCACTTCG-3'). The amplified PCR fragment containing the whole coding region of the SpMYH gene was digested with BamHI and XhoI and inserted into BamHI/XhoI-digested pGEX-4T-1. The resulting plasmid was named pGEX-SpMYH. The E. coli SY5 mutJ::Tet mutant was transformed with the plasmid pGEX-SpMYH. The cells were grown at 37°C in 500 ml of LB medium containing 50 g/ml of ampicillin until the absorbance at 600 nm reached 0.6. Expression of the GST-SpMYH fusion protein was induced by the addition of 0.1 mM IPTG and the cells were further grown at 26°C for 3 hr. The cells were harvested by centrifugation and resuspended in 20 ml of buffer A (10 mM Na\(_2\)HPO\(_4\), 1.8 mM KH\(_2\)PO\(_4\), 140 mM NaCl, 2.7 mM KCl and 0.1% Triton X-100). The cell extract was prepared by sonication of the cell suspension, followed by centrifugation at 25,000 g for 20 min at 4°C. The supernatant was applied to a glutathione-Sepharose 4B column. The column was washed with 10 volumes of PBS and then the fusion protein was cleaved by the addition of 160 units of thrombin to the column followed by incubation at 22°C for 12 h. The SpMYH was eluted with buffer A and stored in buffer A containing 0.2 mM PMSF and 20% glycerol at –80°C.

Preparation of substrate DNA

Oligonucleotide containing a single 8-oxoG residue was obtained from Trevigen, Inc. (Gaithersburg, MD). Other oligonucleotides were synthesized by Takara Shuzo. The nucleotide sequences of oligonucleotides used in this study are shown in Fig. 1.

Cloning of the S. pombe MYH gene

An S. pombe cDNA library was obtained from Stratagene (La Jolla, CA) and used as a template for PCR to amplify the S. pombe SpMYH gene. We synthesized two PCR primers, SpMYH-For (5'-CCCCCGATCCCTCGGATTCAAATCATTCTTTAG-3') and SpMYH-Rev (5'-GGGGGCTC-GAGGCGTATTAGCACTTCG-3'). The PCR product was digested by BamHI and XhoI, and the digested fragment was inserted into pBluescript II SK\(^{+}\). The sequence was checked to verify that no mutations had been generated by the PCR. The resulting plasmid was named pBlu-SpMYH.

Construction of SpMYH-knockout strain of S. pombe

The SpMYH-knockout strain was constructed as follows, a linear SpMYH::ura4\(^{+}\) fragment was used to disrupt the
SpMYH gene in S. pombe Y31 cells. PCR analyses of several colonies with a ura4Δ phenotype revealed that they had integrated the ura4Δ marker in the SpMYH gene. Among the knockout strains obtained, K1-3 cells were haploid and the ura4Δ insertion site was in the SpMYH gene. A single gene replacement was sufficient to eliminate the function of SpMYH in the yeast cells.

Preparation of S. pombe crude extract

Single colonies of S. pombe Y31 and K1-3 were inoculated into flasks containing YES medium and cultured at 30°C to stationary phase with shaking. The cultures were diluted 100 fold into 250 ml of fresh YES medium and then grown to mid-log phase (optical density of 1.5-1.8 at 600 nm) at 30°C. The cells were collected by centrifugation at 6,000 rpm for 5 min. The pellets were washed twice with distilled water and resuspended in 2 volumes of buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM MgCl₂, 50 mM KCl, 5% glycerol, 3 mM dithiothreitol (DTT), 1 mM PMSF and 1 mg/ml pepstatin A). The cells were then disrupted by vortex-mixing (15 rounds of 30-sec each) with an equal volume of glass beads (diameter 0.35–0.5 mm) with cooling on ice for 60 sec between rounds. Then a saturated solution of ammonium sulfate was added to a final concentration of 0.3 M, and the mixture was allowed to stand on ice until use. The cells were then disrupted by vortex-mixing (15 rounds of 30-sec each) with an equal volume of glass beads (diameter 0.35–0.5 mm) with cooling on ice for 60 sec between rounds. Then a saturated solution of ammonium sulfate was added to a final concentration of 0.3 M, and the mixture was allowed to stand on ice until use.

Gel mobility shift assay

The oligonucleotide containing a single 8-oxoG was labeled at the 5'-end with [³²P]ATP by T4 polynucleotide kinase and then annealed with the complementary oligonucleotides (Fig. 1). The reaction was carried out in 25 mM Tris-HCl (pH 8.0), 0.5 mM DTT, 0.5 mM EDTA, 10% glycerol, 25 mM NaCl, 25 mM KCl, 10 mM ZnCl₂, 0.125 mM each dNTP, 4 mM spermidine, 50 g/ml of calf thymus DNA and various amounts of SpMYH in a final volume of 10 l. After incubation for 30 min at 4°C, the reaction mixtures were electrophoresed on 10% non-denaturing polyacrylamide gels in TBE buffer (25 mM Tris-HCl, 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 film at –80°C.

DNA cleavage assay

DNA cleavage assay was carried out at 30°C for 1 h in a reaction mixture (10 l) containing 10 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM DTT, 50 g/ml of BSA and 1.45% glycerol. The reaction was terminated by the addition of 1 M NaOH and heating at 95°C for 5 min. This served to cleave any apyrimidinic/apurinic (AP) sites generated by DNA glycosylase reactions, and was followed by the addition of gel-loading solution (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol and 20 mM EDTA). After heating at 95°C for 5 min, the samples were cooled and loaded onto 20% polyacrylamide gels in the presence of 7 M urea. After electrophoresis at 1,350 V, the gels were dried and then autoradiographed using Fuji RX film at –80°C.

Spontaneous mutation assay

A single colony of E. coli was inoculated into glucose minimal medium and cultured at 37°C to stationary phase. The reversion to Lac⁺ was determined as previously described.³⁶ One-tenth of a milliliter aliquots of the cell suspensions were plated on minimal agar plates containing 1% lactose (lactose minimal plates) and then incubated at 37°C for 48 hr (E. coli CC104) or 7 days (E. coli CC103).

RESULTS

Binding activity of SpMYH to 8-oxoG/A- and 8-oxoG/G-containing oligonucleotides

In this study we first examined whether purified SpMYH binds to double-stranded oligonucleotides containing 8-oxoG/A and 8-oxoG/G mispairs. Gel mobility shift assays were performed to assess the binding activity of SpMYH. The entire open reading frame of the SpMYH gene was amplified by PCR and subcloned into pGEX-4T-1 to obtain the GST-SpMYH fusion protein. The introduction of pGE-SpMYH reduced the high frequencies of spontaneous G:C →T:A transversions in CC104 mutY (Table 1). The fusion protein was expressed in E. coli SY5 cells treated with IPTG and purified by means of glutathione-Sepharose 4B column chromatography. As shown in Fig. 2, purified SpMYH gave a single Coomassie brilliant blue-stained band (50 KDa) on sodium dodecyl sulfate/polyacrylamide gels.

SpMYH strongly bound to the duplex oligonucleotides containing an 8-oxoG/G as well as to the oligonucleotide...
containing 8-oxoG/A, as shown in Fig. 3a. The binding activity was determined by quantifying the band intensities using NIH Image 1.63. The purified SpMYH bound to the 8-oxoG/G-containing oligonucleotide with similar efficiency as to the 8-oxoG/A-containing oligonucleotide (Fig. 3b). These results indicate that SpMYH has a high efficiency of recognizing 8-oxoG/G mispairs in DNA.

We next performed a competition assay for G:C, 8-oxoG/A and 8-oxoG/G-containing oligonucleotides against the 8-oxoG/G-substrate to determine the substrate specificity of SpMYH. An excess of unlabeled oligonucleotide was added to the binding reaction mixture as competitor. The shifted band was specifically abolished by the addition of unlabeled 8-oxoG/G- or 8-oxoG/A-containing oligonucleotide (Fig. 4). Similar competition was obtained when an excess of unlabeled 8-oxoG/G was added to the reaction mixture of SpMYH and 32P-labeled duplex oligonucleotide containing 8-oxoG/A. Such competition was not observed with the G:C-containing oligonucleotide (Fig. 4).
Repair of 8-oxoG/G by SpMYH


Binding activity to 8-oxoG/A- and 8-oxoG/G-containing oligonucleotides in crude extract from SpMYH-knockout S. pombe

To obtain further evidence that SpMYH plays a critical role in base excision repair in S. pombe, an SpMYH-knockout strain was constructed. A linear SpMYH::ura4+ fragment was used to disrupt the SpMYH gene in S. pombe Y31 cells. The resultant SpMYH-knockout strain, K1-3, had no adenine DNA glycosylase activity (data not shown). Disruption of SpMYH caused increased sensitivity to H2O2 (data not shown), as previously reported by Chang et al.40

Cell extracts from the wild-type Y-31 and mutant K1-3 cells were used to examine the binding activities to 8-oxoG/A- and 8-oxoG/G-oligonucleotides. As shown in Fig. 5, the cell extract from the SpMYH-knockout strain lost the ability to bind to both 8-oxoG/A- and 8-oxoG/G-containing duplex oligonucleotides. These results demonstrate that SpMYH is critical for the repair of 8-oxoG/G as well as 8-oxoG/A in DNA in vivo.

Cleavage activity of purified SpMYH for 8-oxoG/G-containing duplex oligonucleotides

SpMYH has been shown to possess adenine DNA glycosylase activity that catalyzes the cleavage of the glycosyl bond to release A from A/8-oxoG and A/G mispairs in DNA.37,38,40 In this study, we examined whether SpMYH has DNA glycosylase activity for 8-oxoG/G as well. 32P-labeled oligonucleotides containing G or 8-oxoG at position 10 were annealed to the complementary strand to construct G/N (N=G, A, T and C) or 8-oxoG/N base pair-containing duplex oligonucleotides. Each duplex oligonucleotide was incubated with 1 pmol of purified SpMYH at 30°C for 30 min in a reaction mixture (10 μl) containing 10 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM DTT, 50 g/ml of BSA and 1.45% glycerol. After incubation, the mixtures were electrophoresed on 20% polyacrylamide/7 M urea gels at 1,350 V, the gels were dried and then autoradiographed using Fuji RX films at –80°C. The positions of intact oligonucleotides and cleaved products are indicated. The substrates contained G/C (lane 1), G/T (lane 2), G/A (lane 3), G/G (lane 4), 8-oxoG/C (lane 5), 8-oxoG/T (lane 6), 8-oxoG/A (lane 7) or 8-oxoG/G (lane 8).
incubated with purified SpMYH at 30°C for 30 min. The reaction mixtures were electrophoresed on denaturing polyacrylamide gels. As shown in Fig. 6, the SpMYH DNA glycosylase was active on the duplex oligonucleotides containing 8-oxoG/A mispairs, in accord with the findings of Lu and Fawcell.37) Furthermore, Sp-MYH was found to efficiently remove unmodified G from 8-oxoG/G as efficiently as A from G/A mispairs. The cleavage site of the 8-oxoG/G-containing oligonucleotide was the same as that of 8-oxoG/A-containing oligonucleotide.

**Relative cleavage activity of SpMYH glycosylase on 8-oxoG/A and 8-oxoG/G substrates**

Double-stranded oligonucleotides (24-mer) containing 8-oxoG/G and 8-oxoG/A at position 10 were used as the substrates for the SpMYH DNA glycosylase assay. The progress of the DNA glycosylase reaction was monitored by the change in the migration of the substrate oligonucleotides. Increasing amounts of SpMYH were incubated with the 8-oxoG/G- and 8-oxoG/A-containing oligonucleotides at 30°C for 60 min. The results are shown in Fig. 7. SpMYH efficiently cleaved the oligonucleotide containing 8-oxoG/G. However, the cleavage activity for 8-oxoG/G was slightly lower than that for 8-oxoG/A. The $k_{cat}/K_m$ values of SpMYH were calculated to be 0.04 and 0.13 M$^{-1}$ sec$^{-1}$ for 8-oxoG/G and 8-oxoG/A substrates, respectively (Table 2). Therefore, the specific activity of SpMYH for removing G from 8-oxoG/G was about 1/3 of that for removing A from 8-oxoG/A mispairs.

**Table 2.** The kinetic parameters of DNA glycosylase activity of SpMYH for 8-oxoG/A and 8-oxoG/G mispairs.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (M)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-oxoG/A</td>
<td>4.1</td>
<td>0.52</td>
<td>0.13</td>
</tr>
<tr>
<td>8-oxoG/G</td>
<td>3.1</td>
<td>0.12</td>
<td>0.04</td>
</tr>
</tbody>
</table>

The Michaels constant ($K_m$) and the catalytic constant ($k_{cat}$) were estimated from Lineweaver-Burk plots.

**Reduction of the spontaneous mutation frequency by pGEX-SpMYH in E. coli mutY mutant**

To demonstrate that SpMYH is a functional homolog of MutY in vivo, we measured the mutation frequency of SpMYH-expressing E. coli strains. We previously found that the mutY-deficient mutant also showed a considerably higher frequency of G:C→C:G transversions in E. coli.36) Hence,

**Table 3.** Spontaneous mutation to Lac$^+$ in E. coli CC103 mutY with plasmid carrying SpMYH.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lac$^+$ mutants/10$^8$ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC103</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>CC103 mutY</td>
<td>64 ± 6</td>
</tr>
<tr>
<td>CC103 mutY/pGEX-4T-1</td>
<td>67 ± 7</td>
</tr>
<tr>
<td>CC103 mutY/pGEX-SpMYH</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

0.1 ml of aliquot of overnight culture was plated on lactose minimal medium and incubated at 37°C for 7 days. Mutant colonies on the plates were counted to estimate mutation frequency. Five independent cultures were assayed in each experiment.
we next examined whether or not SpMYH could reduce the high frequency of Lac<sup>+</sup> reversion in the E. coli CC103 mutY. E. coli CC103 mutY was transformed with pGEX-SpMYH. E. coli CC103, CC103 mutY, CC103 mutY vector and CC103 mutY/pGEX-SpMYH were plated on lactose minimal plates then incubated at 37°C for up to 14 days. The introduction of the SpMYH gene into E. coli CC103 mutY markedly reduced the frequency of spontaneous G:C→C:G transversions (Table 3). Thus, SpMYH is indeed a functional homolog of E. coli MutY.

**DISCUSSION**

Purines undergo oxidation of the ring atoms leading to various chemical modifications. 8-oxoG is formed in large quantities in response to ionizing radiation and ROS and has strong mispairing properties. Therefore, the biological impact of the oxidation products of guanine has been extensively investigated. DNA polymerase extension experiments with a template containing 8-oxoG have revealed that dATP is preferentially incorporated opposite 8-oxoG. 8-oxoG can form a base-pair with A in the Hoogsteen mode, and 8-oxoG/A base-pairs are responsible for G:C→T:A transversions.

Studies of 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 formamidopyrimidines and 8-oxoG, from DNA. MutY is also a DNA glycosylase that efficiently removes A incorporated opposite an 8-oxoG. Such mispairs are formed if 8-oxoG remains in the template during DNA replication. The MutT protein hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP, thus preventing 8-oxo-dGTP from being incorporated during replication.

The frequency of G:C→C:G transversions is considerably higher under oxidative stress than under non-oxidative conditions. Oxidative guanine damage in DNA is predominantly responsible for not only G:C→T:A but G:C→C:G transversions. Our previous study showed that spontaneous G:C→C:G transversions were also accumulated in strains defective in MutY function. MutY is able to bind to 8-oxoG/G as tightly as to 8-oxoG/A mispairs, and has guanine-DNA glycosylase activity toward 8-oxoG/G mispairs. In contrast to G:C→T:A transversions, the oxidative lesion that causes G:C→C:G transversions has not yet been identified. Oxidatively damaged bases such as 8-oxoG in general arise via the following two processes: 8-oxoG is generated in DNA *in situ* by the attack of a hydroxyl radical on G. It is or is incorporated into nascent DNA opposite A and G in the template from the 8-oxo-dGTP pool, when MutT fails to destroy 8-oxo-dGTP. In this study, we calculated the energy of 8-oxoG/G base-pair formation according to Sugiyama *et al.* The results suggested that G (enol)/8-oxoG (-8.2 kcal) base-pairs are remarkably stable. Hence, DNA polymerases may incorporate G opposite 8-oxoG in the template during DNA replication. The misincorporation of 8-oxo-dGTP would increase the frequency of A:T→C:G and G:C→C:G transversions.

It is also reasonable to assume that once 8-oxoG is formed, the products of further oxidative degradation of 8-oxoG may play a role in the mutagenic process associated with DNA damage. In fact, 8-oxoG has been shown to be readily oxidized. HPLC product analysis demonstrated that 2,5-diamino-4H-imidazol-4-one (Iz) derivative is a key oxidation product of G through 8-oxoG in DNA photosensitized with riboflavin or anthraquinone. Kino and Sugiyama recently found that an oxidized form of 8-oxoG induces insertion of dGMP and dAMP when DNA synthesis is performed with Kfexo-<sup>+</sup>, suggesting that it may be a potent mutagenic lesion leading to G:C→T:A and G:C→C:G transversions. Based on these observations, they proposed that Iz forms base pairs with G to induce G:C→C:G transversions. Ikeda and Saito recently reported that Iz is stabilized in a double-helical DNA structure and that the half-life of Iz under this condition is about 20 hr at 37°C, which is significantly longer than that of dIz monomer. Therefore, DNA replication may cause G:C→C:G transversions.

Takimoto *et al.* previously reported that the majority of base substitutions of the E. coli supF gene induced by riboflavin-mediated photosensitization were G:C→C:G transversions, which are probably caused by 8-oxoG in wild-type and mutM strains. This implies that lesions other than 8-oxoG are produced by riboflavin photosensitization. To characterize the G:C→C:G mutation, riboflavin-photosensitized plasmid DNA carrying the *supF* gene was left at room temperature for 5 hr in the dark before transfection. The delayed transfection gave a mutational spectrum different from that for immediate transfection. G:C→C:G transversions were significantly increased in a mutY mutator strain. Lesions causing G:C→C:G changes increased during 5 hr holding after photosensitization and MutY presumably takes part in this type of base change mutation. Further studies of the biological consequences and repair mechanisms of Iz in duplex DNA by MutY and SpMYH are currently underway in our laboratory.

In E. coli, MutY, MutM and MutT are involved in defending against the mutagenic effects of GO lesions and similar mechanisms to protect cells from the deleterious effects of 8-oxoG and its oxidation products must be present in yeast cells. In *S. pombe*, MutT- and MutM-like proteins have not yet been identified. This led us to study how 8-oxoG and its oxidation products are repaired in this organism. In *S. pombe*, the MutY homolog has been identified and characterized by Lu and Fawcett. SpMYH is responsible for removing misincorporated adenine from A/8-oxoG or A/G mismatches and thus for preventing G:C→T:A trans-
versions.\textsuperscript{37,38} SpMYH-knockout strains show increased mutation rates and increased sensitivity to H$_2$O$_2$.\textsuperscript{49} These results provide evidence supporting the notion that MutY homologs are the major enzymes repairing 8-oxoG and its oxidation products in \textit{S. pombe}. In this study we cloned the SpMYH gene and found that expression of the SpMYH gene markedly reduced the spontaneous mutation frequency in the \textit{mutY} mutant of \textit{E. coli} (Table 3). The purified SpMYH protein recognized and repaired 8-oxoG/G mispairs in DNA (Figs. 3–7), and thus prevented the generation of G:C$\rightarrow$C:G transversions.

G:C$\rightarrow$C:G transversions have been found in important genes in human cells.\textsuperscript{51,52} For example, the GTT to CGT mutation is frequent at codons 12 and 13 of the ras gene.\textsuperscript{52} The human homolog of the \textit{E. coli} \textit{mutY} gene (hMYH) has been cloned and sequenced\textsuperscript{53–55} and is thought to have important functions in the repair of oxidative damage to DNA and in the prevention of mutations resulting from oxidative lesions. We are currently studying whether the human gene is able to reduce the frequency of spontaneous G:C$\rightarrow$C:G transversions and whether the human MutY protein has a guanine-DNA glycosylase activity that removes unmodified guanine from 8-oxoG/G mispairs.

**ACKNOWLEDGEMENTS**

The authors wish to express their gratitude to Drs. J.H. Miller and K. Yamamoto for kindly supplying \textit{E. coli} strains. This work was supported by Grants-in-Aid for Scientific Research and the Grant for the Biodiversity Research of the 21st Century COE (A-14) from the Ministry of Education, Science, Sports and Culture of Japan.

**REFERENCES**

25. Michaels, M. L., Cruz, C., Grollman, A. P. and Miller, J. H.
Repairs of 8-oxoG/G by SpMYH


Received on January 18, 2005
1st Revision received on February 28, 2005
Accepted on February 28, 2005