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Physical and Functional Interactions between *Escherichia coli* MutY Glycosylase and Mismatch Repair Protein MutS▽†

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Escherichia coli **MutY and MutS increase replication fidelity by removing adenines that were misincorporated opposite 7,8-dihydro-8-oxo-deoxyguanines (8-oxoG), G, or C. MutY DNA glycosylase removes adenines from these mismatches through a short-patch base excision repair pathway and thus prevents G:C-to-T:A and A:T-to-G:C mutations. MutS binds to the mismatches and initiates the long-patch mismatch repair on daughter DNA strands. We have previously reported that the human MutY homolog (hMYH) physically and functionally interacts with the human MutS homolog, hMutSα (Y. Gu et al., J. Biol. Chem. 277:11135–11142, 2002). Here, we show that a similar relationship between MutY and MutS exists in *E. coli*. The interaction of MutY and MutS involves the Fe-S domain of MutY and the ATPase domain of MutS. MutS, in eightfold molar excess over MutY, can enhance the binding activity of MutY with an A/8-oxoG mismatch by eightfold. The MutY expression level and activity in *mutS* mutant strains are sixfold and twofold greater, respectively, than those for the wild-type cells. The frequency of A:T-to-G:C mutations is reduced by two- to threefold in a *mutS mutY* mutant compared to a *mutS* mutant. Our results suggest that MutY base excision repair and mismatch repair defend against the mutagenic effect of 8-oxoG lesions in a cooperative manner.**

7,8-Dihydro-8-oxo-guanine (8-oxoG or GO) is a common, but highly mutagenic, base lesion that arises from oxidative attack on DNA (48, 67). *Escherichia coli* has a defense system against GO lesions which involves MutT, MutM (Fpg), MutY, MutS, and Nei (endonuclease VIII) (reviewed in references 21 and 41). MutT is a nucleoside triphosphatase that hydrolyzes 8-oxo-dGTP in the nucleotide pool to 8-oxo-dGMP (Fig. 1, reaction 1) (43). MutM glycosylase removes the GO from GO/C base pairs (Fig. 1, reaction 2) (68). Adenine is frequently misincorporated opposite GO during DNA replication (48, 62, 67) if MutT and MutM fail. If an A/GO mismatch is left unrepaired, a G:C-to-T:A transversion will occur following DNA replication. In this case, MutS and MutY play a key role by removing the misincorporated adenine opposite GO (48, 67) (Fig. 1, reaction 3), therefore reducing G:C-to-T:A transversions. Another *E. coli* enzyme, Nei, can excise GO when GO is opposite a cytosine or adenine (Fig. 1, reactions 2 and 6) and can serve as a backup pathway to repair GO in the absence of MutM and MutY (6, 21).

MutY glycosylase efficiently hydrolyzes adenines from A/GO mispairs (47, 48). MutY can also remove adenines from A/G, A/C, and A/5-hydroxyuracil (2, 3, 39, 47, 69) and can excise guanines from G/GO mismatches (33, 75) at lower rates. To be successful in repairing these replication errors, however, MutY adenine glycosylase activity must be directed to the newly synthesized DNA strand. If MutY activity targets the adenines on the template DNA strand, A:T-to-C:G transversions will arise (Fig. 1, reaction 5), and repair is mutagenic. The mechanisms that modulate the MutY glycosylase activity and allow it to target only newly synthesized DNA remain elusive. It has been suggested that base excision repair by the human MutY homolog (hMYH or hMUTYH) is coupled to DNA replication through docking with hPCNA and hRPA (7, 8, 57). However, it has not been reported that this mechanism exists in *E. coli*.

Methylation-dependent mismatch repair (MMR) is a process used to remove mismatched bases on daughter DNA strands (reviewed in references 51 and 61). In *E. coli*, mismatch repair has been suggested in the removal of either A or GO from A/GO mismatches depending on the transient unmethylated state of the daughter strand (73). MMR requires MutS, MutL, MutH, DNA helicase II, single-stranded DNA binding protein, four exonucleases, and DNA polymerase III holoenzyme. MutS recognizes short insertion-deletion loops (IDLs) up to five unpaired nucleotides and all base-base mismatches except for C/C. The ATPase activity of MutS is essential for mismatch repair function. There are several MutS homologs in eukaryotes. The human mismatch recognition complex hMutSα (hMSH2/hMSH6) recognizes base-base mismatches and small IDLs while hMutSβ (hMSH2/hMSH3) mainly targets larger IDLs (23, 29, 51).

MMR and MutY repair pathways share many common features. First, both pathways take place immediately after DNA replication to increase the fidelity of DNA replication (29, 36, 51, 56, 64–66, 74). As a result of their timing immediately postreplication, both pathways need to be able to distinguish newly synthesized DNA strands from their parental counterparts. Both hMYH and hMSH6 interact with replication proteins PCNA and RPA (10, 28, 57). In addition, hMYH and hMSH2/hMSH6 colocalize with PCNA at replication foci (7, 10, 15, 28, 57). In *E. coli*, the β clamp has been shown to interact with MutS (35) and MutY (C.-Y. Lee and A.-L. Lu, unpublished data). Second, both pathways recognize base/base mismatches such as A/G and A/C. The MutY pathway is one of

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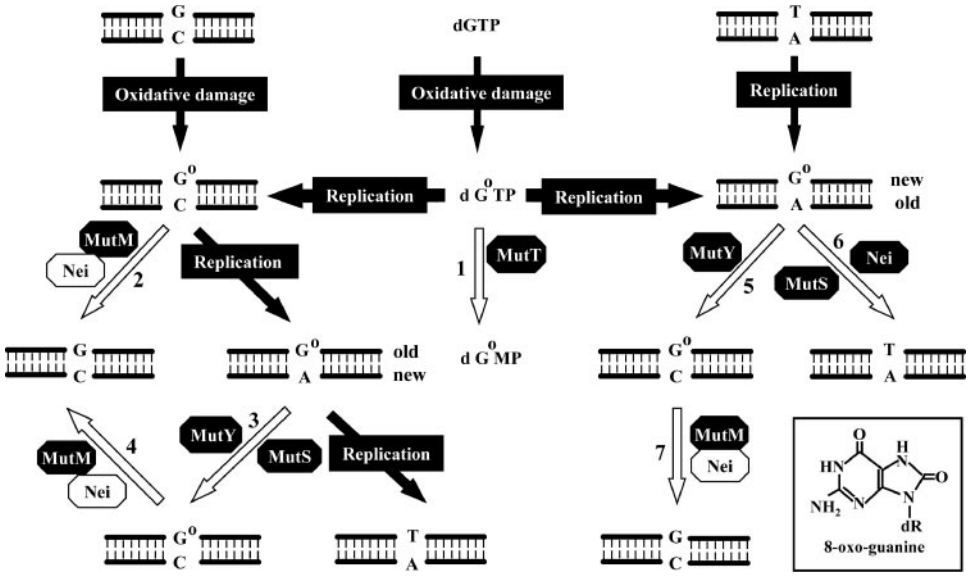

FIG. 1. 8-OxoG repair in *E. coli*. MutT, MutM, MutS, MutY, and Nei (endonuclease VIII) are involved in defending against the mutagenic effects of 8-oxoG lesions (structure is shown in the inset). The MutT protein hydrolyzes 8-oxo-dGTP (dG^{o}TP) to 8-oxo-dGMP (dG^{o}MP) and pyrophosphate (reaction 1). GO (G^o) in DNA can be derived from oxidation of guanine or misincorporation of dG^{o}TP during replication. The MutM glycosylase removes GO adducts while it is paired with cytosine (reactions 2, 4, and 7). Nei can function as a backup for MutM to remove GO from GO/C. When C/GO is not repaired by MutM, adenines are frequently incorporated opposite GO bases by DNA polymerase III during DNA replication. A/GO mismatches are repaired to C/GO by the MutY-dependent or MutS-dependent pathway (reaction 3). When dG^{o}TP is incorporated opposite adenine during DNA replication, MutY repair on GO/A can cause more mutation (reaction 5) while GO/A repair by MutS and Nei can reduce mutation (reaction 6). This figure is adapted from the work of Lu et al. with permission of the publisher (41).

the two short-patch mismatch repair pathways in *E. coli* (38). Finally, both pathways are involved in mutation avoidance of DNA oxidation. The most frequent mutations observed in *mutY* mutants are G:C-to-T:A transversions consistent with MutY's adenine specificity to A/G and A/GO (47, 52, 59). Germ line mutations in the *hMYH* gene can cause autosomal recessive colorectal adenomatous polyposis (1, 20, 24, 60, 63). Tumors from these affected patients contain somatic G:C-to-T:A mutations in the adenomatous polyposis coli gene, k-*ras*, and other genes (1, 25, 34). Inherited mutations in MMR genes are associated with hereditary nonpolyposis colorectal cancer (58). Wyrzykowski and Volkert (73) have suggested that the *E. coli* MMR may prevent oxidative mutagenesis by removing either adenine or GO from A/GO mispairs. When cells deficient in MMR are grown anaerobically, spontaneous mutation frequencies are reduced compared with those of the same cells grown aerobically (73). In addition, a *dam* mutant has an increased sensitivity to hydrogen peroxide treatment. This sensitivity can be suppressed by mutations that inactivate MMR (73). Overexpression of MutM can suppress the MutH-dependent increase in transversion mutations (73). Although the most frequent mutations observed in *mutS* mutants are A:T-to-G:C and G:C-to-A:T transitions, overexpression of MutS protein significantly decreases the rate of G:C-to-T:A transversions in the wild type and *mutY* and *mutM* mutants (76). Yeast (14, 22, 53) and mammalian (11, 13, 46) mismatch repair pathways are also involved in reducing the mutagenesis caused by GO. The extensively overlapping functions of the MMR and MutY base excision repair pathways offer an intriguing molecular relationship to study.

Our previous studies have shown that hMYH interacts with hMutSα via the hMSH6 subunit and also that it does not interact with hMutSβ (18). Moreover, we showed that the binding and glycosylase activities of hMYH with an A/GO mismatch are enhanced by hMutSα. In this report, we observe that *E. coli* MutS interacts with MutY by stimulating the DNA binding activity of MutY with A/GO mismatches. Further, we found that the expression level of MutY is upregulated in *mutS* cells compared to wild-type cells. Unexpectedly, inactivation of MutY in a *mutS* background reduces the mutation frequency of *mutS* single mutants by half. Overall, our findings suggest that the MutY base excision repair pathway may cooperate with the mismatch repair pathway to achieve antimutagenic functions.

MATERIALS AND METHODS

***E. coli* strains.** AB1157 [*ara-14 argE3* Δ(*gpt-proA*)*62 galK2 hisG4 kdgK51 leuB6 lacY1 mtl-1 rac* mutant *rfbD1 rpsL31 thr-1 tsx-33 supE44 xyl-5*), KM75 (*mutS*::Tet derived from AB1157), GM7724 (*mutY*::Cam derived from AB1157), and GM7726 (*mutS*::Tet and *mutY*::Cam derived from AB1157) were generous gifts from Martin Marinus. Strains with DE3 lysogen were constructed according to the procedures recommended by Invitrogen (Carlsbad, CA). CC106 (12) and P1 phage were kindly provided by Michael Volkert. The *mutS*::Tet, *mutY*::Cam, and *mutS*::Tet *mutY*::Cam derivatives of CC106 were made by P1 transduction (49) from KM75 and GM7724.

MutS and MutY deletion constructs. The MutS wild type and deletion construct Δ1–120 in pET15b were kindly provided by Peggy Hsieh. MutS deletions, Δ1–311, Δ261–556, and Δ680–853, were obtained from Martin Marinus (72). MutS(Δ557–853) was made by PCR using the wild-type *mutS* gene as a template and Chang380 and Chang420 primers (all primers are listed in Table S1 in the supplemental material). The PCR products were digested with BamHI and XhoI and ligated into BamHI-XhoI-digested pET21a (Novagen, Darmstadt, Germany). The PCR primers used for the intact *mutS* gene were Chang462 and Chang465. The PCR product was then digested with EcoRI and XhoI and ligated into EcoRI-XhoI-digested pASK-IBA33plus (IBA BioTAGnology, Gottingen,

Germany) to obtain pASK-IBA33plus-MutS. All the MutS constructs were fused with a six-His tag.

The cloning of the MutY expression plasmid pET-MYW1 has been described in a previous study (71). The constructs MutY-M25 (residues 1 to 226) and MutY-M15 (residues 216 to 350) fused with glutathione *S*-transferase (GST) have also been described previously (42). GST-tagged MutY-M25Δ1 containing residues 1 to 25 and residues 135 to 226 of MutY was subcloned into the BamHI and XhoI sites of pGEX-4T-2 (GE Health, Waukesha, MI) by a PCR method using the *mutY*(Δ26–134) gene (31) as a template and the primers ChangYGSTF and Chang363. Similarly, GST-tagged MutY-M25Δ2 containing residues 26 to 148 of MutY was subcloned into the BamHI and XhoI sites of pGEX-4T-2 by the PCR method using primers Chang404 and Chang406. The wild-type MutY gene was subcloned into the NcoI-XhoI site of pACYCDuet-1 (Novagen, Darmstadt, Germany) by PCR using the primers Chang448 and Chang449. The MutY protein expressed from pACYCDuet-1-MutY was S tagged at its C terminus.

Partial purification of MutS and its deletion mutant proteins. KM75(DE3) cells containing wild type and deletion *mutS* constructs were grown in Luria-Bertani (LB) broth containing 100 μg/ml ampicillin at 37°C. The cultures were shifted to 20°C at an A_{590} of 0.6 to induce protein expression by the addition of isopropylthiogalactoside (IPTG) to a final concentration of 0.2 mM. The cells were harvested 16 h later by centrifugation at 10,000 × *g* for 20 min. The cells were then resuspended in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole). After sonication and centrifugation, the supernatants were incubated with nickel agarose (Ni-NTA; QIAGEN, Valencia, CA) at 4°C for 1 h. After washing, His-tagged proteins were eluted from the resin following the manufacturer's protocol. Purified proteins were visualized by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and fractions containing MutS were pooled, dialyzed with buffer A (20 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride), divided into small aliquots, and stored at −80°C. Wild-type MutS and all deletion mutants were at least 90% pure as judged by Coomassie blue staining of a polyacrylamide gel.

GST pull-down assay. Expression and immobilization of GST-MutY constructs on glutathione-Sepharose 4B (GE Health, Waukesha, WI) were performed according to the procedures previously described (18). Intact or mutant MutS proteins (200 ng) were incubated with GST-MutY constructs (400 ng) and immobilized on beads in 180 μl of buffer G (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 2 mM EDTA) containing 20 μg bovine serum albumin with rotation at 4°C for 4 h. After centrifugation at 1,000 × *g*, the supernatants (20 μl) were saved, and the pellets were washed five times with 800 μl of buffer G containing 0.1% Nonidet P-40. The pellets and supernatants were fractionated on an 8% SDS-polyacrylamide gel. The proteins were then transferred to a membrane and allowed to react with anti-His antibody (BD Biosciences, San Diego, CA). Western blotting was performed using the Enhanced Chemiluminescence analysis system (GE Health, Waukesha, WI). The quantification of Western blot signal was performed with LabWorks analysis software (UVP, Inc., Upland, CA).

Coimmunoprecipitation. pASK-IBA33plus-MutS and pACYCDuet-1-MutY were cotransformed into BL21(DE3) (Stratagene, La Jolla, CA) cells. The cells were grown in 100 ml LB broth containing 100 μg/ml ampicillin and 75 μg/ml chloramphenicol. The MutS and MutY protein expression was induced at an A_{590} of 0.6 by adding 0.2 mM isopropylthiogalactoside and 50 ng/ml anhydrotetracycline. The cells were harvested 12 h later, and the cell paste was resuspended in 8 ml of buffer G (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA). After sonication, the mixture was centrifuged at 10,000 × *g* for 20 min. An 0.5-ml amount of the cell extract was precleared by adding 40 μl protein A-Sepharose (GE Health, Waukesha, MI) at 4°C for 1 h. After centrifugation at 1,000 × *g*, the supernatant was incubated with either 1 μg of anti-S-tag antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or 1 μg of rabbit anti-mouse immunoglobulin G (Calbiochem, Darmstadt, Germany) for 4 h at 4°C. After centrifugation at 1,000 × *g*, the pellets were washed six times with 800 μl of buffer G containing 0.1% Nonidet P-40. The pellets were fractionated on a 10% SDS-polyacrylamide gel. The proteins were transferred to a membrane and allowed to react with anti-His antibody (BD Biosciences, San Diego, CA) for detecting MutS and anti-S-tag antibody for detecting MutY. Western blotting was performed by the enhanced chemiluminescence analysis system (GE Health, Waukesha, WI).

Preparation of *E. coli* cell extracts. AB1157, KM75, and GM7724 cells were cultured in 10 ml of LB broth to an A_{590} of 1.0 to 1.2. After centrifugation, the cell pellets were washed with 20 ml 1× M9 salts (42 mM Na_2HPO_4, 24 mM KH_2PO_4, 9 mM NaCl, and 19 mM NH_4Cl) followed by a second wash with 1 ml 1× M9 salts. The cells were then lysed by procedures described by Li et al. (32) to obtain whole-cell extracts. Soluble cell extracts of AB1157, KM75, and GM7724 were prepared as previously described (40). The protein concentration was measured by the Bradford protein assay (Bio-Rad, Philadelphia, PA). The MutS and MutY protein levels in the *E. coli* cell extracts were determined by Western blotting with antibodies to MutS and MutY, respectively. Quantification of Western blotting signals was done with LabWorks analysis software (UVP, Inc., Upland, CA).

RNA isolation and reverse transcription-PCR. AB1157 and KM75 cells were cultured in 10 ml of LB broth to an A_{590} of 1.2. The culture (2.5 ml) was diluted with 12.5 ml of LB broth, mixed with 30 ml of RNAprotect Bacteria Reagent (Qiagen, Valencia, CA) by vortexing for 5 s, and incubated for 5 min at room temperature. The RNA was isolated according to the instruction manual for the MasterPure RNA purification kit (Epicentre, Madison, MI). The RNA concentration was determined by A_{260}. RNA (1 μg) was reverse transcribed using Thermoscript reverse transcriptase (Invitrogen, Carlsbad, CA) with primer Chang388. The product (2 μl) of the reverse transcription-PCR was then amplified by PCR with primers Chang388 and Chang404 for 25 cycles. A control reverse transcription-PCR was run concurrently, omitting reverse transcriptase to confirm that the PCR products were not derived from genomic DNA. The PCR products were resolved on a 1% agarose gel, visualized by ethidium bromide staining, and quantified by LabWorks analysis software (UVP, Inc., Upland, CA).

Gel mobility shift assay. The DNA substrate is a 44-mer duplex DNA containing an A/8-oxoG mismatch, 5′-AATTGGGCTCCTCGAGGAATT**A**GCCT TCTGCAGGCATGCCCCGG-3′ and 3′-TTAACCCGAGGAGCTCCTTAA**O** CGGAAGACGTCCGTACGGGGCC-5′, where O represents 8-oxoG.

The DNA substrate was 5′ labeled with [γ-^{32}P]ATP as described previously (37). The reaction mixture (20 μl) contained 0.05 nM of purified MutY, varied amounts of purified MutS, 0.09 nM of A/8-oxoG-containing DNA substrate, 75 μg/ml bovine serum albumin, 20 mM Tris-HCl (pH 7.6), 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.5 μg/ml poly(dI-dC), and 2.9% glycerol. When cell extracts were used in the assay, the EDTA concentration was increased to 10 mM to inhibit nonspecific nuclease activity. The reactions were performed at 37°C for 30 min, and the reaction mixtures were supplemented by adding 2 μl of 50% glycerol. The protein-DNA complexes were separated on a 4% or 6% polyacrylamide gel in 50 mM Tris-borate buffer, pH 8.3, containing 2.5% glycerol. Electrophoresis was carried out at 4°C with a 20-mA current with 50 mM Tris-borate buffer. The gel was dried and exposed to a PhosphorImager screen. The percentages of bound DNA were analyzed by ImageQuant (GE Health, Waukesha, WI).

MutY glycosylase assay. The glycosylase assay was carried out in a 10-μl reaction mixture containing 1.8 fmol of DNA substrate, 20 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol, 1 mM EDTA, 2.9% glycerol, and 50 μg/ml of bovine serum albumin. After incubation at 37°C for 30 min, the reaction mixtures were supplemented with 1 μl of 1 M NaOH and heated at 90°C for 30 min. Five microliters of formamide dye (90% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue) was added to the sample, and 5 μl of this mixture was loaded onto a 14% polyacrylamide sequencing gel containing 7 M urea.

Mutation frequency measurement. Strains were grown overnight at 37°C in LB broth containing appropriate antibiotic(s). Four independent overnight cultures (0.1 ml) were plated onto rifampin-containing plates, and resistant colonies were scored the next day. For the Lac$^+$ reversion assay, four independent overnight cultures (0.1 ml) were plated onto minimal agar plates (49) containing 0.2% lactose. The colonies grown on the minimal agar plates were scored 2 days after plating. The cell titer of each culture was determined by plating a 10^{-6} dilution of the culture onto LB agar plates. Mutation frequencies were expressed as ratios of Rif^r or Lac$^+$ cells to total cells. For each measurement, the experiments were repeated at least three times.

Sequencing of the *rpoB* gene. *E. coli* chromosomal DNA was isolated using a genomic DNA purification kit (Gentra System, Minneapolis, MN). The main group of mutations (cluster II) of the *rpoB* gene was PCR amplified using Chang440 and Chang441 primers (see Table S1 in the supplemental material) as described previously (27). The PCR product was purified with the QIAquick PCR purification kit (QIAGEN, Valencia, CA) and sequenced directly with Chang442 primer.

RESULTS AND DISCUSSION

Physical interaction of MutY and MutS. The MutY repair pathway and the mismatch repair pathway both remove replication errors immediately after DNA replication. Gu et al. have shown that hMYH is physically associated with hMutSα via the hMSH6 (18). We investigated whether there is a similar interaction in *E. coli*. The recombinant MutY and MutS proteins fused with S tag and His tag, respectively, were coex-

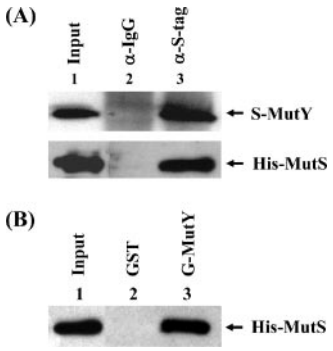

FIG. 2. Physical interaction of MutY and MutS. (A) Coimmunoprecipitation of MutY with MutS. Immunoprecipitation was performed with rabbit anti-mouse (lane 2) or S-tag (lane 3) antibody and extracts from BL21(DE3) cells expressing S-tagged MutY and His-tagged MutS proteins. Western blotting was performed with S-tag antibody (upper panel) or with His-tag antibody (lower panel). Lane 1 of the upper panel represents 15% of input extract, and lane 1 of the lower panel represents 0.5% of input extract. (B) Binding of MutS to GST-tagged MutY. GST (lane 2) or GST-MutY (lane 3) immobilized to glutathione-Sepharose beads was used to pull down His-tagged MutS protein. The pellets were fractionated on an 8% SDS-polyacrylamide gel followed by Western blot analysis to detect MutS with the antibody to His tag. Lane 1 contains 10% of input MutS protein.

pressed in the BL21(DE3) cells. With quantitative Western blotting analysis and comparison to a known amount of purified MutY and MutS, there was an 18-fold excess of His-MutS over S-MutY (data not shown). Because MutS is a dimer (30, 55), the molar ratio of MutS dimer over MutY monomer is 9. The cell extract was subjected to immunoprecipitation with anti-S-tag antibody for MutY. Western blotting was performed against anti-S- and anti-His-tag antibodies. As shown in Fig. 2A, about 30% of MutY was immunoprecipitated by anti-S antibody (lane 3, upper panel). Small amounts of MutS protein (~0.5% of input) were found to associate with MutY protein in the immunoprecipitants (lane 3, lower panel) but not with the control immunoglobulin G (lane 2, lower panel). Because His-MutS dimer is in a ninefold excess over S-MutY, about 15% of MutY molecules were associated with MutS. To further demonstrate the physical interaction between MutY and MutS, purified His-tagged MutS protein was incubated with GST-MutY fusion protein that was immobilized on glutathione-Sepharose. As shown in Fig. 2B, approximately 9% of the input His-MutS was found to associate with the immobilized GST-MutY fusion protein (lane 3) but not with GST alone (lane 2). With the same method, we showed that there was no interaction between MutY and MutL (data not shown).

MutY contains two structural domains. The N-terminal domain of MutY has catalytic activity (17, 33, 44, 45, 54) and contains two subdomains: an iron-sulfur module and a six-helix barrel module containing the conserved helix-hairpin-helix motif (16, 19). The C-terminal domain of MutY has been shown by nuclear magnetic resonance to have a structure similar to that of MutT (70) and plays an important role in the recognition of GO lesions (9, 17, 33, 54). A MutY construct without the six-helix barrel module has been characterized (31). Thus, four GST constructs containing different domains of MutY were made to determine the MutY region involved in interacting with MutS. All the truncated MutY proteins were well expressed and soluble in *E. coli* cell extracts. The results are shown in Fig. 3A and summarized in Fig. 3B. The strength of binding was calculated as the ratio of the amount of MutS in the pellet to that of input MutS. The N-terminal domain of MutY (M25, residues 1 to 226) retained the ability to associate with MutS (Fig. 3A, lane 4). A weak interaction was also detected with the C-terminal domain of MutY (Fig. 3A, lane

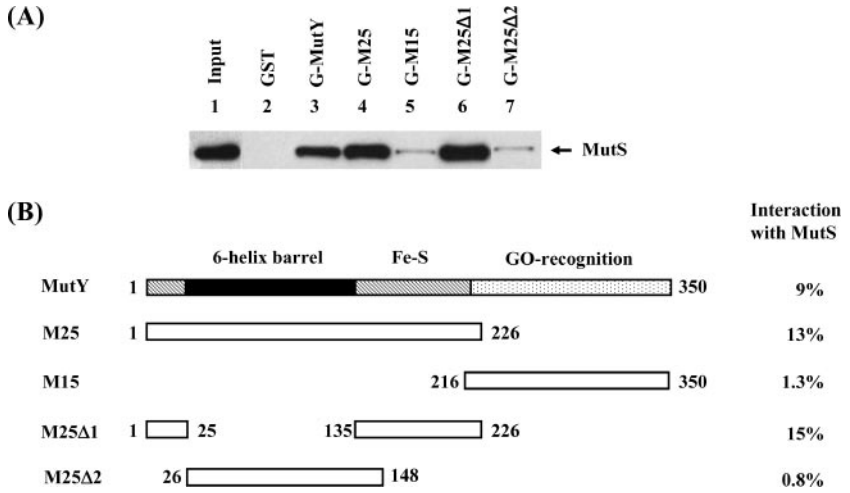

FIG. 3. Determination of regions of MutY involved in MutS binding. (A) Various GST-MutY constructs were immobilized to glutathione-Sepharose beads and used to pull down His-tagged MutS protein. A control was run concurrently with immobilized GST alone (lane 2). Lane 1 contains 10% of input His-tagged MutS protein. The same amounts of GST fusion proteins were used in the experiments by normalizing with the corresponding protein bands on a Coomassie blue-stained 12% SDS-polyacrylamide gel (data not shown). The pellets were fractionated on an 8% SDS-polyacrylamide gel followed by Western blot analysis with the antibody to His tag. (B) Graphic depiction of GST-MutY constructs and the binding with MutS proteins. The region shaded in black is the six-helix barrel (residues 26 to 134). The Fe-S domain consists of residues 1 to 25 and 135 to 226. The C-terminal domain of MutY is involved in GO recognition. The portions of protein present in the MutY deletion constructs are indicted by boxes and numbers of amino acid residues. The strength of binding, presented at the right, was calculated as the ratio of the amount of MutS in the pellet to that of input MutS (10% in lane 1).

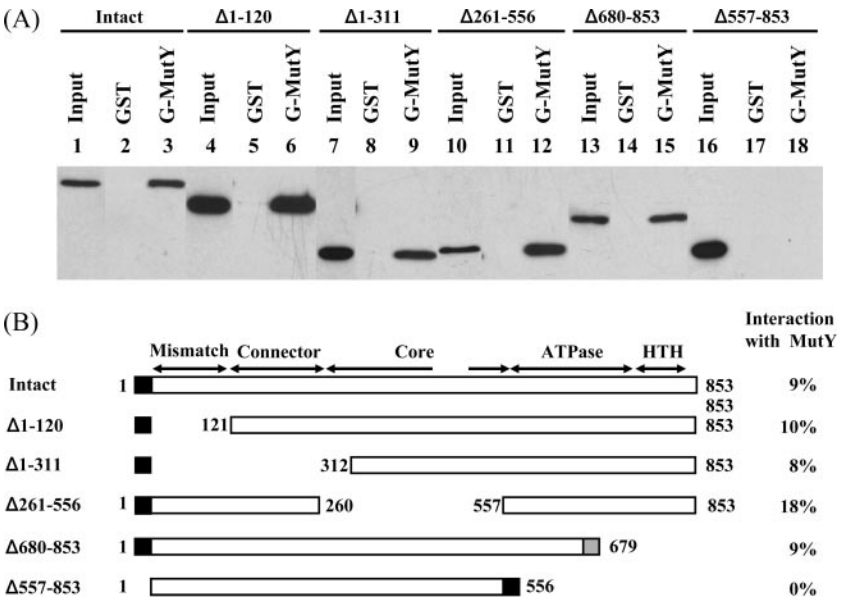

FIG. 4. Determination of regions within MutS involved in MutY binding. (A) The GST pull-down assays were performed with different MutS constructs as indicated and GST-MutY immobilized on glutathione-Sepharose (lanes 3, 6, 9, 12, 15, and 18). Controls were run concurrently with immobilized GST alone for each MutS construct (lanes 2, 5, 8, 11, 14, and 17). Ten percent of each input MutS construct was loaded to lanes 1, 4, 7, 10, 13, and 16. Western blot analyses of the pellets were performed with antibody to His tag. (B) Graphic depiction of MutS constructs and the binding to GST-MutY fusion protein. The amino-terminal mismatch recognition domain (residues 2 to 115), the connector domain (residues 116 to 266), the core domain (residues 267 to 443 and 504 to 567), the ATPase domain (residues 568 to 765), and the HTH domain (residues 766 to 800) are indicated. Residues 444 to 503 are the clamp domain. The names of MutS domains with their corresponding residues were adapted from the work of Lamers et al. (30). In addition, the extreme C-terminal domain (residues 801 to 853) is responsible for the tetramer formation (5). The portions of protein present in the MutS deletion constructs are indicted by boxes and numbers of amino acid residues. The black and gray boxes at the N or C terminus of MutS represent His tag and an additional 13 amino acids, respectively. The strength of binding, presented at the right, was calculated as the ratio of the amount of MutS in the pellet to that of input MutS constructs.

5). In the N-terminal domain of MutY, MutS interacts mainly with the Fe-S module (M25Δ1, residues 1 to 25 and 135 to 226) (Fig. 3B, lane 6) and very weakly with the six-helix barrel domain (M25Δ2, residues 26 to 148) (Fig. 3A, lane 7). The reason for the slightly stronger affinities of M25 and M25Δ1 than of full-length MutY to MutS is unknown. It may be caused by more exposure of the MutS binding domain in the truncated MutY proteins without the C-terminal domain. Gu et al. (18) have found that the hMSH6-interacting region on hMYH is present in residues 232 to 254, which correspond to residues 148 to 170 of *E. coli* MutY. This region is within the Fe-S module.

By using immobilized GST-MutY fusion protein to pull down constructs containing different domains of MutS, we mapped the region of MutS that interacts with MutY. The X-ray crystal structure of MutS (30, 55) reveals that it contains six domains: the amino-terminal mismatch-recognition domain (residues 2 to 115), the connector domain (residues 116 to 266), the core domain (residues 267 to 443 and 504 to 567), the clamp domain (residues 444 to 503), the ATPase domain (residues 568 to 765), and the helix-turn-helix (HTH) domain (residues 766 to 800). In addition, the extreme C-terminal domain (residues 801 to 853), which is not present in the MutS crystal structure, is responsible for tetramer formation (5). The MutS deletion constructs, therefore, were made according to the domain structure. The results are shown in Fig. 4A and summarized in Fig. 4B. Intact MutS, MutS(Δ1–120), MutS(Δ1–311), and MutS(Δ680–853) showed binding ability similar to that of MutY. MutS(Δ261–556), in comparison to the wild-type MutS, showed a twofold-greater affinity to MutY. The reason for this stronger affinity to MutY of Δ261–556 than of full-length MutS is unknown. Biswas et al. (4) have shown that the HTH motif is important for dimerization, which is in turn critical for both the ATPase and DNA mismatch binding activities of MutS. Wu and Marinus (72) have shown that MutS(Δ261–556) and MutS(Δ680–853) affect the dimerization process. The oligomeric status of MutS, therefore, does not affect its association with MutY. MutS(Δ557–853) is expected to be a monomer. Because MutS(Δ680–853) retained interaction (Fig. 4A, lane 15) and MutS(Δ557–853) showed no interaction with MutY (Fig. 4A, lane 18), residues 557 to 679 of MutS are important for the MutY interaction. This region is located within the ATPase domain of MutS and is highly conserved among MutS family members. It is interesting that the hMSH6 missense mutations D1213V and V1260I, found in MT1 cells, reduce interaction with hMYH (18). These two mutations of hMSH6 have been suggested to affect the ATPase activity of hMutSα (26). The corresponding residues in *E. coli* MutS, D693 and E740, are located proximally to residues 557 to 678 (30).

Functional interactions of MutY with MutS. Gu et al. (18) have shown that hMutSα can stimulate both hMYH binding and glycosylase activities towards DNA containing A/GO mispairs. However, the stimulation effect of hMutSα on hMYH glycosylase activity is much weaker than that on hMYH substrate binding (twofold increase in glycosylase versus eightfold

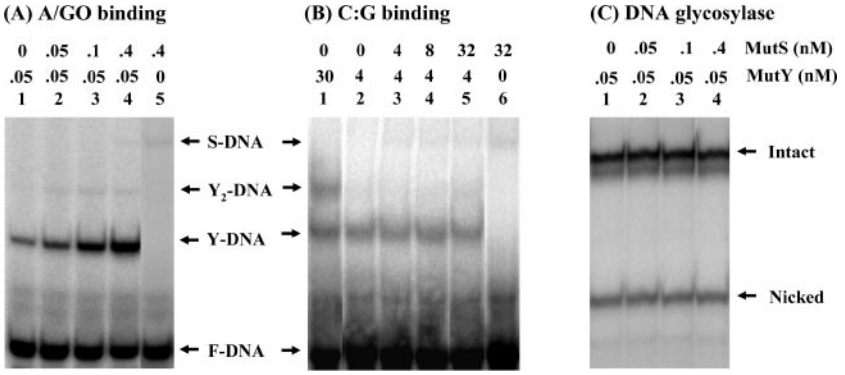

FIG. 5. MutS stimulates MutY binding activity towards A/8-oxoG-containing DNA. (A) DNA substrates (0.09 nM) containing A/GO mismatches were incubated with MutY (0.05 nM) (lane 1) and increasing amounts of MutS (lanes 2 to 4). The reaction mixture in lane 5 contains 0.4 nM MutS and no MutY. The samples were fractionated on a nondenaturing 4% polyacrylamide gel. The arrows indicate the positions of MutY-DNA complex (Y-DNA), MutS-DNA complex (S-DNA), and free DNA substrate (F-DNA). The faint band (marked by Y_2-DNA) between the Y-DNA and S-DNA bands may be a complex of MutY dimer and DNA (31). (B) Homoduplex DNA substrates (0.09 nM) containing C:G were incubated with MutY as indicated (lanes 1 and 2) and increasing amounts of MutS (lanes 3 to 5). The reaction mixture in lane 6 contains 32 nM MutS and no MutY. Reaction mixtures are similar to those of panel A. (C) A/8-oxoG-containing DNA substrates (0.09 nM) were incubated with MutY (0.05 nM) (lane 1) and increasing amounts of MutS (lanes 2 to 4). After MutY incubation, the reaction mixtures were heated at 90°C for 30 min in the presence of 0.1 M NaOH. The samples were fractionated on a denaturing 14% polyacrylamide gel. The arrows indicate the positions of intact and nicked DNA.

increase in binding). Therefore, we tested the influence of MutS on MutY activity. As shown in Fig. 5A, the affinity of MutY for A/GO was much higher than that of MutS (compare the Y-DNA complex in lane 1 to the S-DNA complex in lane 5). MutS, at an eightfold molar excess over MutY, could enhance the binding affinity of MutY towards A/GO-containing oligonucleotides by approximately eightfold (Fig. 5A, compare lanes 1 and 4). The binding of MutS to A/GO was not affected by the addition of MutY (compare the S-DNA complex in lanes 4 and 5 in Fig. 5A). The faint band (marked by Y_2-DNA) between the Y-DNA and S-DNA bands was also increased when MutS concentrations increased. This band may be a complex of MutY dimer and DNA as shown by Lee et al. (31). The Y_2-DNA complex was more apparent when MutY concentrations were high as shown in lane 1 of Fig. 5B. However, neither the nonspecific binding for homoduplex DNA (Fig. 5B, lanes 2 to 5) nor the glycosylase activity of MutY on an A/GO mismatch (Fig. 5C) was enhanced by MutS. Thus, MutS facilitates the lesion-specific recognition of MutY. By comparison to a known amount of purified MutY and MutS, the copy numbers of MutY and MutS in AB1157 cells were measured. The cells in log phase contained ~300 molecules of MutS protein per cell and ~20 molecules of MutY protein per cell. Because the ratio of MutS dimer to MutY monomer is 7.5 in *E. coli* cells, MutS may enhance the binding affinity of MutY towards A/GO mismatches approximately eightfold in vivo based on the result of the in vitro assay (Fig. 5A).

The MutY activity and expression level are dependent on MutS in vivo. We hypothesized that MutY base excision repair might be inefficient in the *mutS* cells due to the lack of MutS stimulation, assuming that MutY was expressed at the same levels in the wild-type and the *mutS* cells. Therefore, cell extracts of the wild-type and the *mutS* strains were prepared to assay the MutY activity. Surprisingly, the MutY activity in the *mutS* extracts was approximately twofold higher than that in the wild-type extracts (Fig. 6A). Next, we checked the MutY protein levels in these cell extracts by Western blotting. As shown in Fig. 6B, the MutY protein level in the *mutS* extracts was sixfold higher than that in the wild-type extracts. Thus, the specific substrate binding ability of MutY (the activity per microgram of total protein) in the *mutS* cells was actually

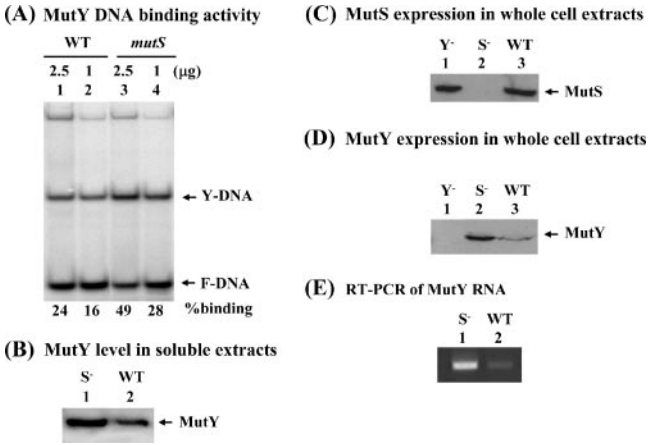

FIG. 6. The DNA binding activity and expression level of MutY are upregulated in the *mutS* cells. (A) MutY binding activity towards A/8-oxoG-containing DNA with extracts from AB1157 (wild type [WT], lanes 1 and 2) and KM75 (*mutS*, lanes 3 and 4). The assay was performed with 1 and 2.5 μg of cell extracts. The samples were fractionated on nondenaturing 6% polyacrylamide gels. The arrows indicate the positions of MutY-DNA complex (Y-DNA) and free DNA substrate (F-DNA). The numbers at the bottom of the figure indicate the percentages of DNA substrate bound by MutY. (B) Western blot analysis for MutY with 80 μg soluble extracts from AB1157 (WT) and KM75 (*mutS*, S^-) cells. (C) Western blot analysis for MutS was performed with 60 μg total cell proteins from AB1157 (WT), KM75 (*mutS*, S^-), and GM7724 (*mutY*, Y^-) cells. (D) Western blot analysis for MutY was performed with 60 μg total cell proteins from AB1157 (WT), KM75 (*mutS*, S^-), and GM7724 (*mutY*, Y^-) cells. (E) MutY mRNA was quantitated by reverse transcriptase PCR (RT-PCR) with isolated RNA from AB1157 (WT) and KM75 (*mutS*, S^-) cells. The DNA products were resolved on a 1% agarose gel and visualized by staining with ethidium bromide.

TABLE 1. Mutation frequencies of *mutS*, *mutY*, and *mutS mutY* mutants

Strain	No. of Rif^r cells/ 10^8 cells	Increase (fold)
AB1157 (wild type)	0.6 ± 0.7	1
KM75 (*mutS*)	299 ± 64	498
GM7724 (*mutY*)	14 ± 13	23
GM7726 (*mutS mutY*)	128 ± 24	213
AB1157 plus pET-MYW1	2.5 ± 0.8	4.2
KM75 plus pET-MYW1	359 ± 53	598
GM7724 plus pET-MYW1	2.1 ± 2.3	3.5
GM7726 plus pET-MYW1	357 ± 41	595

TABLE 2. Mutation distribution of *rpoB* in *mutS* and *mutS mutY* mutants

Mutation	No. of clones with *rpoB* mutation (%[a]) in mutant type			
	mutS		*mutS mutY*	
	This study	Kim et al. (27)	This study	Kim et al. (27)
A:T→G:C	26 (87)	72 (84)	21 (70)	35 (94)
G:C→A:T	3 (10)	14 (16)	8 (27)	1 (3)
G:C→T:A	1 (3)	0 (0)	0 (0)	1 (3)
A:T→T:A	0 (0)	0 (0)	1 (3)	0 (0)
Total	30	86	30	37

[a] The numbers inside parentheses represent the percentage which was calculated by dividing the number of each mutation type by the number of total sequenced clones.

threefold lower than that of the wild-type cells. Because the cell extracts represent soluble proteins, MutY and MutS expression levels in these cells were detected using Western blotting in whole-cell extracts. There was no MutS protein in the *mutS* cells (Fig. 6C, lane 2). The protein levels of MutS were similar in both the wild type and the *mutY* cells (Fig. 6C, compare lanes 1 and 3). However, both the protein (Fig. 6D, lanes 2 and 3) and mRNA (Fig. 6E) levels of MutY in the *mutS* cells were sixfold higher than those in the wild-type cells. Taken together, in a *mutS* mutant, the mRNA and protein levels of MutY are increased to compensate for the lack of MutS function in mismatch repair and result in the enhancement of MutY activity. The mechanism by which the transcription or stability of MutY mRNA is regulated in the *mutS* cells requires further investigation. This is in contrast to the human system, in which the protein level of hMYH is not upregulated in the MMR-defective cells (18).

Mutation frequencies of *mutS* single mutants and *mutS mutY* double mutants. To study the biological significance of the MutS-MutY interaction, we measured the mutation frequencies of *mutS*, *mutY*, and *mutS mutY* mutants, checking for rifampin resistance. In the absence of DNA repair, mutations in the rifampin binding site of RNA polymerase render the cell resistant to rifampin. The *mutS* strain had a 498-fold-higher frequency than the wild type (compare rows 1 and 2 in Table 1) while the *mutY* strain had a 23-fold-higher frequency than the wild type (compare rows 1 and 3 in Table 1). However, to our surprise, the mutation frequency of the *mutY mutS* double mutant was approximately twofold lower than that of the *mutS* single mutant (compare rows 2 and 4 in Table 1). The results are reproducible and are statistically significant ($P = 0.008$). These results differ from the findings of Kim et al. (27), who mentioned that the mutation frequencies of *mutS* and *mutS mutY* were similar. No data, however, were shown to support those findings of Kim et al. (27). To confirm that this antimutagenic effect is associated with a *mutY* deficiency, MutY protein was overproduced in *mutY*, *mutS*, and *mutS mutY* strains. The mutation frequency of the *mutY* mutant was complemented by MutY (compare rows 3 and 7 in Table 1); however, overproduction of MutY in the wild-type cells caused an approximately fourfold increase in mutation frequency (compare rows 1 and 5 in Table 1, $P = 0.04$). Overproduction of MutY in the *mutS mutY* double mutant strain increased the mutation frequency to the same level as that of the *mutS* mutant (compare rows 2, 4, and 8 in Table 1, $P = 0.001$). Overproduction of MutY had no detectable effect on the mutation frequency in the *mutS* strain (compare rows 2 and 6 in Table 1, $P = 0.2$). In the MutY-overproducing *mutS* and *mutS mutY* strains, MutY protein is expressed at the same level (checked by Western blotting; data not shown). These data indicate that the high mutation frequency observed in the *mutS* strain (row 2 in Table 1) may be caused, in part, by elevated expression of MutY (Fig. 6).

We further investigated the mutation types that are reduced in *mutS mutY* cells compared to *mutS* cells. We selected 30 rifampin-resistant colonies each from *mutS* and *mutS mutY* cells and sequenced the *rpoB* genes. Our results (Table 2) are similar to previously published data (27, 50) indicating that the majority of Rif^r mutants from the *mutS* and *mutS mutY* strains are A:T-to-G:C transitions. Importantly, the G:C-to-T:A and T:A-to-G:C transversions were very low. However, there is a slight difference between our results and those reported by Kim et al. (27) regarding the relative distribution between A:T-to-G:C and G:C-to-A:T transitions (Table 2). Our results indicate that the percentage of A:T-to-G:C mutations is reduced while that of G:C-to-A:T mutations is increased in *mutS mutY* strains compared to *mutS* strains. The data from Kim et al. (27) showed the opposite trend (Table 2). One of the intermediates of A:T-to-G:C transition mutation is an A/C mismatch which serves as a weak substrate of MutY (69). Kim

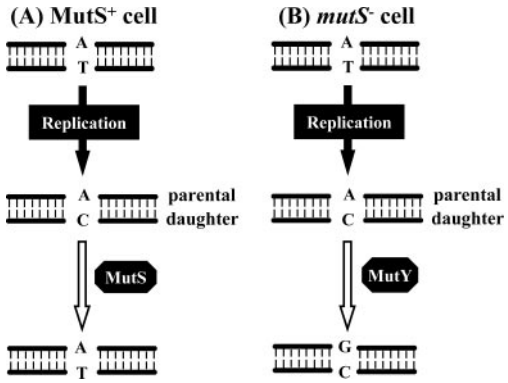

FIG. 7. Overproduction of MutY in *E. coli mutS* cells may have a mutagenic effect on A:T-to-G:C transitions. (A) In MutS$^+$ cells, A/C mismatches generated during DNA replication are mainly repaired by MutS-dependent mismatch repair. (B) In *mutS* cells, overproduced MutY can remove A from an A/C mismatch in which A is on the parental strand and thus cause an A:T-to-G:C transition.

TABLE 3. A:T→G:C transition frequencies of *mutS*, *mutY*, and *mutS mutY* mutants

Strain	No. of Lac$^+$ cells/ 10^8 cells	Increase (fold)
CC106 (wild type)	0.05 ± 0.10	1
CC106 (*mutS*)	38.2 ± 16.8	751
CC106 (*mutY*)	0.06 ± 0.12	1
CC106 (*mutS mutY*)	13.0 ± 1.1	256

et al. (27) suggested that the MutY protein competes with the MMR system for the processing of A/C mispairs. If MutY removes A from an A/C mismatch in which A is on the parental strand, an A:T-to-G:C transition will arise (Fig. 7B). We suspect that the mutagenic effect of MutY on A/C mismatches likely contributes to the twofold-higher mutation frequency in the *mutS* mutant than in the *mutS mutY* double mutant. Thus, we compared the A:T-to-G:C mutation frequencies of *mutS* and *mutS mutY* strains in a CC106 host (12) by Lac$^+$ reversion assay. As shown in Table 3, the *mutS* strain had a 751-fold-higher frequency than the wild-type strain (compare rows 1 and 2 in Table 3), while the *mutS mutY* strain had a 256-fold-higher frequency than the wild-type strain (compare rows 1 and 4 in Table 3). Therefore, the *mutS mutY* strain has a threefold-lower mutation frequency in A:T-to-G:C transitions than the *mutS* strain does. This finding is consistent with the rifampin forward assay which showed that the mutation frequency of the *mutY mutS* double mutant was approximately twofold lower than that of the *mutS* single mutant. The *mutY* strain had a mutation frequency similar to that of the wild-type strain (compare rows 1 and 3 in Table 3), indicating that MutY plays a minimal role in A:T-to-G:C transitions in MutS$^+$ cells.

In summary, our data suggested a cooperation and competition model for the MutS-MutY interaction. In MutS$^+$ cells, A/C mismatches generated during DNA replication are mainly repaired by MutS-dependent mismatch repair. MutS enhances MutY binding to A/GO mismatches (Fig. 7A). In *mutS* cells, MutY is overproduced to repair A/GO mismatches. However, when MutY activity is increased in the *mutS* cells to excise adenines on the template DNA strands, it may further cause A:T-to-G:C transitions (Fig. 7B) and A:T-to-C:G transversions (Fig. 1, reaction 5). An alterative explanation for the decreased mutagenesis in the *mutS mutY* strain compared to the *mutS* mutant is that the strand specificity of MutY is lost in the *mutS* mutant, thus causing increased misrepair at A/C or A/GO mismatches.

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