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Specialized mismatch repair function of Glu339 in the Phe-X-Glu motif of yeast Msh6

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Abstract

The major eukaryotic mismatch repair (MMR) pathway requires Msh2-Msh6, which, like *E. coli* MutS, binds to and participates in repair of the two most common replication errors, single base-base and single base insertion-deletion mismatches. For both types of mismatches, the side chain of *E. coli* Glu38 in a conserved Phe-X-Glu motif interacts with a mismatched base. The Oε of Glu38 forms a hydrogen bond with either the N7 of purines or the N3 of pyrimidines. We show here that changing *E. coli* Glu38 to alanine results in nearly complete loss of repair of both single base-base and single base deletion mismatches. In contrast, a yeast strain with alanine replacing homologous Glu339 in Msh6 has nearly normal repair for insertion-deletion and most base-base mismatches, but is defective in repairing base-base mismatches characteristic of oxidative stress, e.g., 8-oxo-G•A mismatches. The results suggest that bacterial MutS and yeast Msh2-Msh6 differ in how they recognize and/or process replication errors involving undamaged bases, and that Glu339 in Msh6 may have a specialized role in repairing mismatches containing oxidized bases.

Introduction

The stability of bacterial and eukaryotic genomes partly depends on the ability of the general DNA mismatch repair (MMR) machinery to correct mismatches that escape exonucleolytic proofreading during DNA replication (reviewed in [1,2]). Inactivation of MMR strongly elevates spontaneous mutation rates for single base substitutions and single base insertion-deletions, indicating that under normal circumstances, MMR efficiently repairs the mismatches responsible for both types of mutations. In bacteria, MMR is initiated by binding of homodimeric MutS protein to mismatched DNA. MutS is required to repair a wide variety of polymerization errors and can bind all possible single base-base mismatches and duplexes containing one to three extra bases in one strand [2,3]. Crystal structures of *E. coli* and *Taq* MutS bound to mismatched DNA [4–7] reveal that most contacts with the DNA are to the backbone. Only a phenylalanine and a glutamate in one monomer of MutS directly contact the mismatched base, and both are part of a Phe-X-Glu motif that is conserved in many bacterial and eukaryotic MutS proteins [4–7]. The phenylalanine stacks with a mismatched or unpaired

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base and is essential for mismatch binding and for MMR function in *E. coli* and in eukaryotes [8–11]. The side chain of the glutamate also interacts with the mismatched base, including forming a hydrogen bond to purines or pyrimidines in base-base mismatches as well as in single-base insertion-deletion mismatches [4–7]. The conservation of this glutamate and the structural similarities in base specific contacts among *E. coli* and *Taq* MutS bound to several different single base mismatches [4–7], implies that this glutamate participates in repairing both single base substitution and single base insertion-deletion mismatches in these two bacteria. Previous studies have shown that replacement of Glu38 in *E. coli* MutS with other amino acids results in defective repair of base substitution mismatches [4,12,13]. One objective of the present study is to test the prediction that non-conservative replacements of Glu38 in *E. coli* MutS result in defective repair of the other common replication error, single base insertion-deletion mismatches. A second objective is to determine if the repair of different types of mismatches depends on the nature of the glutamate interactions with DNA, i.e., charge repulsion with the DNA backbone and/or hydrogen bonding to the base ([4–7,12,13], and see Discussion). This objective can be examined by analyzing the effects of an alanine versus a glutamine replacement for Glu38.

Eukaryotes contain two MMR heterodimers that are homologs of bacterial MutS. The Msh2-Msh3 heterodimer can bind to and is largely responsible for the repair of insertion-deletion mismatches containing multiple extra bases in one strand (reviewed in [1,2,14,15]. Neither Msh2 nor Msh3 has the conserved Phe-X-Glu motif. The other eukaryotic MutS heterodimer is Msh2-Msh6, whose primary role is repairing single base-base mismatches and insertion-deletion mismatches containing one extra base. Like bacterial MutS, Msh2-Msh6 can bind to a variety of single base mismatches. Only Msh6 has the Phe-X-Glu motif containing the conserved phenylalanine and glutamate residues that make base specific contacts with mismatched DNA in bacterial MutS, implying that Msh6 is the subunit responsible for contacting mismatched bases in the Msh2-Msh6 heterodimer. This implication is strongly supported by studies demonstrating that substituting other amino acids for the conserved phenylalanine in Msh6 results in reduced Msh2-Msh6 binding to mismatched DNA and strongly elevated mutation rates in cells [8,9,16]. In other studies, replacing the conserved glutamate in *Saccharomyces cerevisiae* Msh6 (Glu339) with alanine resulted in an increase in the rate of single base deletions in a run of 14 A-T base pairs [16,17]. Interestingly, this E339A-dependent increase was much smaller than that observed when *MSH6* was disrupted altogether, indicating partial retention of MMR function in the E339A mutant [16]. This result is surprising given the strong inactivation of MMR of base-base mismatches conferred by the E38A change in *E. coli* MutS. One explanation for the small mutator effect of the E339A mutation in yeast *Msh6* is that the role of Glu339 in recognition and repair of mismatches may vary depending on the type of mismatch, e.g., whether base-base or insertion-deletion, or perhaps may depend on mismatch base composition or location [16,17]. Here we test these possibilities by examining the specificity of mutagenesis *in vivo* resulting from E38A and E38Q substitutions in *E. coli* MutS and from E339A and E339Q substitutions in yeast *Msh6*. The results with *E. coli* MutS are consistent with structural information on bacterial MutS and indicate that Glu38 is critical for repair of both single base-base and single base deletion mismatches *in vivo*. In contrast, Glu339 in yeast *Msh6* appears to have a minor role in repair of most single base mismatches, but has a substantial role in repairing a subset of base-base mismatches that may result from oxidative damage.

Materials and Methods

Strains

E. coli strain NR13138 is a *trpE9777* derivative of KA796 (*ara*, *thi*, Δ *prolac*), which has been described [18]. Mutant *mutS* strains carrying E38Q or E38A substitutions were created by site-

specific mutation and chromosomal replacement as described below. A *mutS215::Tn10* derivative of NR13138 was prepared by P1 transduction [19], using strain ES1481 as donor [20]. NR13138 (*mutS*⁺) and its *mutS::Tn10*, *mutS*(E38Q), and *mutS*(E38A) derivatives were then used as recipients in an F' cross (conjugation) introducing F'*prolac* (F'128–27) [21] into each strain. This F' carries a *lac* operon used routinely for selection of *E. coli lacI* mutants and determination of the specificity of mutation using the *lacI* gene as a target [21]. For studies in *Saccharomyces cerevisiae*, the strains, plasmids, and media have been described previously [16,22].

Plasmids

pMQ315, which bears the *mutS*⁺ gene, has been described previously [23]. pMAKSACA, the suicide replacement vector used to modify the *mutS*⁺ gene in the genome, has been described previously [24]. It expresses a thermosensitive replication initiation protein (repA_{ts}) such that replication from its origin occurs at 30°C but not at 43°C [24]. It also contains a chloramphenicol-resistance gene and the *sacB* gene, which confers sucrose sensitivity to the host cells [24].

Media and Reagents

LB broth was as described by [25]. Phenyl-β-D-galactoside (P-gal) plates contained 1× VB salts [26], 750 μg/mL P-gal, and 1 μg/mL thiamine. The restriction enzymes AvrII, BamHI, and NheI, and the T4 DNA ligase were obtained from New England Biolabs. The XL-10-gold ultracompetent cells and the Quickchange Site-Directed Mutagenesis Kit were obtained from Stratagene. The TOPO TA Cloning kit and all oligonucleotides used were obtained from Invitrogen.

Making E38A/Q Mutations and Cloning into pMAKSACA

The Quickchange Site-directed Mutagenesis kit from Stratagene was used to introduce mutations coding for the E38A (codon change of gaa to gca) and E38Q (codon change of gaa to caa) changes into the *mutS*⁺ gene in pMQ315. These mutant *mutS* genes were then PCR amplified using the following primers to engineer either NheI or AvrII restriction enzyme sites at the 5' end and a BamHI restriction enzyme site at the 3' end of the gene:

NheI primer: 5' – ATA GCT AGC AAC TGC AAA TTG CCG GAC – 3'

AvrII primer: 5' – ATA CCT AGG AAC TGC AAA TTG CCG GAC – 3'

BamHI primer: 5' – TAT GGA TCC AAG CTT TCC TAT TAT ACA GGG TG – 3'

The PCR products were then cloned into the TOPO vector (Invitrogen) according to the manufacturer's instructions. The PCR products were ligated into the TOPO vector because the ensuing restriction digests were more efficient than with the PCR products alone. The TOPO vectors with the MutS inserts were digested with AvrII and BamHI or NheI and BamHI, depending on the primers used. The gene replacement vector pMAKSACA was also digested with the same pairs of enzymes. The pMAKSACA linearized vector and the MutS insert were gel purified and ligated together using T4 DNA ligase. The ligation products were transformed into XL-10-Gold Ultracompetent cells (Stratagene) and plated on LB containing 17 μg/mL chloramphenicol (LB-Cm). The complete plasmid was then purified and electroporated into NR13138 cells.

Integrating the MutS E38A/Q Mutations into the Chromosome of NR13138 Cells

The *mutS* genes were integrated into the chromosome of strain NR13138 using the suicide vector pMAKSACA according to the method of [24]. Briefly, after electroporation, the cells

were plated on LB-Cm and grown overnight at 30°C. Single Cm^r colonies were selected and subjected to two rounds of growth at 43°C (non-permissive conditions for replication from the repA^{ts} origin) in liquid LB-Cm to select for cells in which the pMAKSACA/*mutS*(E38A/Q) plasmids had integrated into the bacterial chromosome. The resulting partial *mutS* diploids were then plated at 30°C on LB plates without NaCl containing 5% sucrose (LBS-N). The LBS-N medium selects for cells that have eliminated the pMAKSACA vector along with one of the *mutS* copies from the co-integrate molecule, since pMAKSACA contains the *sacB* gene, which confers sucrose sensitivity to the host cells [24]. After overnight growth, the LBS-N plates were replica-plated onto LB-Cm and LB plates, which were grown at 30°C overnight. Several colonies that were chloramphenicol-sensitive were chosen and their genomic DNA was isolated. The *mutS* gene was PCR amplified off the genomic DNA and sequenced to identify isolates containing the E38A or E38Q mutation and to verify that no other mutations were introduced. Additionally, the cells were also checked to make sure that no contaminating plasmid DNA was present.

***lacI* mutant frequencies and *lacI*^d mutational spectra**

lacI mutant frequencies were determined from 20 independent cultures grown overnight in LB medium. Aliquots were plated on P-gal medium to determine the total number of *lacI* mutants per culture and aliquots of a 10⁶ dilution were plated on MM glucose plates to determine the total number of cells. Independent *lacI* mutants for sequencing were obtained as described [21,27]. In brief, 10 independent overnight cultures for each of the four strains (NR13138 (*mutS*⁺), NR13138::*mutS*:Tn10, NR13138:*mutS*E38A, and NR13138:*mutS*E38Q) were diluted to about 10³ cells/mL in LB medium. This solution was used to start 96 cultures (0.2 ml each) in wells of 96-well micro-titer dishes (960 cultures total for each strain). The cultures were grown to saturation at 37°C on a shaking platform. A one-μL aliquot of each culture was spread on a quarter section of P-gal plates, which were incubated at 37°C for 60 hours. One mutant was picked randomly from each quarter section. These independent mutants (960 for each strain) were placed by toothpicking in a gridded pattern (50 per plate) on Pgal plates. The *F*^{prolac} of these mutants was then transferred by replica-mating into strain CSH52 for determination of the subclass of *lacI*^d mutants [21]. The fraction of *lacI*^d mutants, as determined in CSH52, was 0.16 for the NR13138 (*mutS*⁺) strain, 0.57 for the NR13138::*mutS*:Tn10 strain, 0.50 for the NR13138:*mutS*E38A strain, and 0.45 for the NR13138:*mutS*E38Q strain.

Measuring mutation rates in yeast

For phenotypic testing of the mutator phenotypes, yeast strains were grown overnight in liquid media. After growth for two to three days at 30°C, cells were plated onto selective media and analyzed after additional growth at 30°C, as described before [28,29]. For fluctuation analysis, at least twelve yeast cultures per strain were started from single colonies and grown to stationary phase in YPDA. Cells were plated after appropriate dilutions onto selective medium lacking lysine or histidine for revertant count, complete medium containing canavanine and lacking arginine for Can^r mutant count, and complete medium for viable count. The mutation rates were then determined as previously described [28,30].

DNA sequencing

For sequencing, DNA of the *lacI*^d mutants was PCR amplified directly from colonies on LB plates using two primers, 5'-GTGATTGGCTGTCTGAATCTGG-3' and 5'-AGGCAGCTTCCACAGCAATGG-3', yielding a 661-bp fragment that included the *lacI* region of interest (nucleotides 1–250). This region was sequenced using the primer 5'-GGCACCCAGTTGATCGGC-3' to obtain the spectra shown in Figure 1. Table 1 shows the total number of sequences identified for each strain. For canavanine-resistant yeast mutants, genomic DNA was prepared using the MasterPureTM yeast purification kit (Epicentre

Technologies). The *CAN1* gene was amplified using primers CanF 5'-TCTGTCGTCGAATCGAAAG-3' and CanR 5'-TTCGGTGTATGACTTATGAGGGTG-3' and AmpliTaq (Applied Biosystems). The PCR product was purified with the Qiaquick PCR Purification kit (Qiagen) and the entire gene sequenced using the following six primers: SF-1: 5'-ATTCTGTACGCAGTCCT-3', SF-2: 5'-GAACTAGTTGGTATCACT-3', SF-3: 5'-CTCAATCTCGCACATCAG-3', SR-1: 5'-TGTCTCCATGTAAGCCAA-3', SR-2: 5'-ATATTATACCTGGACCCC-3', SR-3: 5'-ATGAAAAGACCTGTACCA-3'. All sequencing was performed using an ABI Prism dRhodamine Terminator Cycle Sequencing Kit (Applied Biosystems) and manufacturer-recommended protocols. Sequences were aligned and compared to wild type sequence using AutoAssembler, Sequence Navigator software (Applied Biosystems), and SeqLab (Wisconsin Package).

Results

Contribution of *E. coli* MutS Glu38 to MMR in vivo

Replacing Glu38 in *E. coli* MutS with alanine or glutamine has previously been shown to elevate the frequency of mutations that lead to resistance to rifampicin [4,12,13]. Rifampicin resistance results from single base substitutions; therefore, these data indicate that Glu38 is important for MMR of single base-base mismatches. However, in those studies, the type and location of the base substitutions as well as the contribution of Glu38 to MMR of insertion-deletion mismatches was not examined. In order to define more precisely the contribution of Glu38 to MMR of a variety of mismatches, we examined the mutational specificity in the various *mutS* mutants using the *E. coli lacI* reporter system [21]. This reporter system monitors numerous different base substitution and frameshift mutations (primarily single-base deletions) in many different sequence contexts within a 200-nucleotide sequence encoding the N-terminal portion of the *E. coli lacI* gene [21]. Using this system, we compared the mutational spectra and mutant frequencies of a wild type *MutS* strain, a *mutS::Tn10* strain, a *mutSE38A* strain, and a *mutSE38Q* strain (see Materials and Methods).

***mutS::Tn10* Strain**—As expected based on previous studies [31], complete disruption of *MutS* results in a strong mutator phenotype (Table 1). Sequence analysis of 216 independent *lacI^d* mutants revealed that, in comparison to wild type *E. coli*, complete loss of MutS function resulted in a large increase of transition base substitutions (100-fold) and single-base insertion-deletions (45-fold), and a smaller increase (12-fold) of transversion base substitutions (Table 1). Single base mutations occurred throughout the N-terminal coding sequence of the *lacI* gene (Figure 1A), and they were distributed in a non-random manner similar to that observed previously [31].

***mutS(E38A/Q)* Strains**—*E. coli* strains in which MutS Glu38 has been replaced with either alanine or glutamine had *lacI^d* mutant frequencies of 140×10^{-8} and 68×10^{-8} , respectively (Table 1). Both the *mutS(E38A)* and *mutS(E38Q)* mutant frequencies are nearly as high as the *mutS::Tn10* strain that completely lacks functional MutS (Table 1). Sequence analysis of *lacI^d* mutants indicates that the alanine replacement strongly inactivates repair of single base-base and single base insertion-deletion mismatches to extents similar to those seen in the *mutS* null strain (Figure 1). The glutamine replacement partially inactivates repair of single base insertion-deletion mismatches and single base-base mismatches that result in transitions, but to a lesser extent than observed with the alanine replacement (Figure 1 and Table 1). Only a minor effect on repair of mismatches that result in transversions was observed in the *mutS(E38Q)* strain (Table 1). Additionally, fewer single base insertions and deletions were observed compared to the other mutant strains (Table 1). Single base mutations in both the *mutS(E38A)* and *mutS(E38Q)* strains were distributed in a non-random manner similar to that observed in

the *mutS* null strain (Fig. 1). These data demonstrate that the Glu38 has a major role in MMR of a wide variety of single base mismatches.

Contribution of Glu339 in yeast Msh6 to MMR in vivo

Next, we examined the role of the conserved glutamate (Glu339) in the Phe-X-Glu motif of yeast Msh6 (Glu339) in MMR of a wide variety of single base-base and single base insertion-deletion mismatches. We used three mutational reporter genes that monitor repair of different types of mismatches. Spontaneous mutation rates were analyzed using (i) *lys2:A₁₄* and *lys2:A₇* reporters that revert to Lys⁺ via deletion of a single A-T base pair in a homopolymeric run of 14 or seven A-T base pairs, respectively [30], (ii) a *his7-2* reporter that reverts to His⁺ primarily via addition of a single A-T base pair in a homopolymeric run of 7 A-T base pairs [29], and (iii) the *CAN1* reporter that permits scoring of a wide variety of mutations in the *CAN1* gene resulting in canavanine resistance. In the latter instance, the identity of the mutations is established by sequence analysis of independent *can^r* mutants.

Complementation of mutator phenotype by *msh6 E339A*—Initially, we analyzed the effects of substituting Glu339 with alanine in an experiment in which *MSH6* was present in a single-copy ARS-CEN plasmid in a strain defective in both *msh3* and *msh6*. The reversion rate at *lys2:A₁₄* is very high in the *msh3 msh6* strain (1900×10^{-6} , Table 2) due to inactivation of both the Msh2-Msh3- and Msh2-Msh6-dependent MMR pathways. Expression of wild type *MSH6* from the ARS-CEN plasmid strongly complemented the MMR defect, reducing the mutation rate by 590-fold (Table 2). Expression of *msh6 E339A* also strongly reduced the mutation rate, to within 4-fold of that observed with wild type *MSH6* (Table 2). Strong complementation by *msh6 E339A* was also observed at the *his7-2* locus, where the mutation rate was significantly reduced to within 3-fold of wild type (Table 2). These data indicate that the E339 side chain is not essential for MMR of most single-base deletion and single base insertion mismatches, respectively, at the *lys2:A₁₄* and *his7-2* loci.

In order to monitor a wider variety of mutations including base substitutions, mutation rates were also analyzed at the *CAN1* locus. Expression of wild type *MSH6* reduced the *can^r* mutation rate of the *msh3 msh6* strain by 21-fold (Table 2). Expression of the *E399A* allele also reduced the mutation rate, but only by 2-fold (from 10×10^{-6} to 4.8×10^{-6} , Table 2) with overlapping confidence limits. The *E339Q* allele reduced the mutation rate by 3-fold to 3×10^{-6} (not shown), indicating that, as in *E. coli*, a glutamine replacement in Msh6 retains slightly more MMR function than does an alanine replacement (Tables 1 and 2). Importantly, both missense alleles complement the mutator effect at *CAN1* to a lesser extent than at the *lys2:A₁₄* and *his7-2* loci (Table 2).

To determine if this reduced efficiency reflected a reduction in overall mismatch repair capacity or perhaps a selective effect on a subset of mismatches, we compared the sequences of *can^r* mutants recovered from strains complemented by wild type *MSH6* and *msh6E339A* (Table 2). Among 22 sequenced mutants from the *msh3 msh6* strain (listed in [32]), 15 contained single base indels, three contained single base transitions, and four contained single base transversions, corresponding to respective predicted mutation rates of 6.8×10^{-6} , 1.4×10^{-6} and 1.8×10^{-6} (Table 2). Sequence analysis of 14 *can^r* mutants from the *msh3 msh6* strain expressing wild type *MSH6* (Table 2) revealed that six mutants contained single base insertion-deletion mutations, three contained single base transitions and one contained a single base transversion. The corresponding rates indicate that wild type *MSH6* suppressed the insertion-deletion rate by 32-fold, the transition rate by 14-fold, and the transversion rate by 53 fold (Table 2). Sequence analysis of 25 *can^r* mutants from the strain expressing *msh6E339A* (Table 2) revealed no single base insertion-deletion mutations, indicating that *msh6E339A* suppresses the rate of single base insertion-deletion mutations by at least 32-fold (Table 2). These data

indicate that Glu339 is largely dispensable for repair of mismatches containing a single unpaired base. Significantly, 22 of 25 *can^r* mutants from the strain expressing *msh6E339A* contained a single base substitution, suggesting that Glu339 does play a significant role in MMR of single base-base mismatches (Table 2). In marked contrast to the strong transition base substitution bias in the *E. coli mutSE38A* strain (Table 1), 18 of the 22 substitutions observed in the yeast *msh6E339A* strain were transversions (Table 2), representing a 100-fold increase over wild type (Table 2). As discussed below, 17 of these transversions are characteristic of replication errors involving 8-oxo-G•A mismatches (Table 2).

Mutation rates of strains with *msh6E339A* at its endogenous locus—Next, we examined strains with *MSH6* present in its natural chromosomal location. Spontaneous mutation rates were compared for wild type (*MSH6*), *msh6*, and *msh6E339A* strains that were either wild type for *MSH3* or that contained an *msh3* null mutation, the latter to eliminate any contribution of the Msh2-Msh3 repair pathway to the rates. In the *MSH3* background (Table 3, Experiment 1), disruption of *msh6* resulted in a 15-fold mutator phenotype at *his7-2*. However, no mutator effect was observed at either *his7-2* or *lys2:A₇* in the *msh6E339* strain (Table 3, experiment 1). Similar results were obtained in the *msh3* background (Table 3, Experiments 2 and 3), i.e., the *msh6* strain had clear mutator phenotypes at *his7-2* and *lys2:A₇* that were not seen in the *msh6E339A* strain. These data further support the conclusion from the above experiments indicating that Glu339 is largely dispensable for repair of mismatches containing a single unpaired base.

The results were different at *CAN1*, where mutation rates were elevated in both the *msh6* and *msh6E339A* strains in all three experiments (Table 3). The mutator effect was consistently greater for complete loss of Msh6 than for replacing Glu339 with alanine, indicating that the *msh6E339A* strain retains some MMR function. Sequence analysis of 45 and 46 *can^r* mutants, respectively, from the *MSH6* and *msh6E339A* strains (Table 3) permitted calculation of rates for mutations resulting from different types of mismatches (Table 3). The results indicate that substitution of alanine for Glu339 in yeast Msh6 has little effect on MMR of single base insertion-deletions or transition base substitutions, but strongly increases the rate of G-C to T-A transversions (Table 3, Experiment 3).

To further explore the role of Glu339 in repairing mismatches resulting from replication of oxidized bases, we determined mutation rates in an *ogg1* deficient strain background. This deficiency eliminates the main pathway for removing 8-oxo-G from DNA, resulting in 8-oxo-G-A mismatches due to replication of unrepaired 8-oxo-G that arises spontaneously. In an *ogg1* strain, mutation rates at *his7-2* and *lys2:A₇* were not further increased by the *msh6E339A* mutation, confirming that Glu339 is not involved in repair of single base insertion and deletion mismatches (Table 4). However, the mutation rate at the *CAN1* locus was increased in an *ogg1msh6E339A* strain as compared to an *ogg1* strain (Table 4), and sequence analysis of *can^r* mutants revealed that the rate of G-C to T-A transversions in the *ogg1msh6E339A* strain was nearly as high as in an *ogg1msh6* strain. Moreover, the rates of single base insertions and deletions and transition substitutions in the *ogg1msh6E339A* strain were similar to the wild type strain (*ogg1MSH6*) (Table 4). These data strongly support the idea that the Glu339 residue is specifically involved in the recognition and repair of oxidative damage-induced 8-oxo-G-A mispairs.

Discussion

MMR is an evolutionarily conserved repair process that has numerous features in common in organisms from bacterial to humans (reviewed in [1,2]. These include conservation of MutS proteins, which share conserved sequence motifs that are logically thought to have common functions in MMR. The present study reveals that a residue in one such conserved motif in

MutS has an important general role in MMR in *E. coli*, while the homologous residue in yeast Msh6 has a more specialized role in MMR. This greater specialization of function in eukaryotic MMR at the level of an individual residue is consistent with generally greater specialization of MMR protein functions in eukaryotes, which have multiple MSH proteins as compared to a single MutS protein in *E. coli*.

The function of Glu38 in *E. coli* MutS

The results in Tables 1 and 3 can be used to calculate the percent of replication errors that are not repaired in each *mutS* strain (Figure 2). The *E. coli mutSE38A* strain is strongly defective in repair of single base-base mismatches, confirming earlier studies that used resistance to rifampicin to monitor base substitutions [4,12,13], but whose identity was not determined. Our data extend the MMR defect to numerous base-base mismatches in the variety of sequence contexts in the *lacI* reporter gene (Figure 1). The results also reveal that substitution of alanine for Glu38 largely inactivates MMR of single base deletion mismatches.

Inactivation of repair of both base substitution and insertion-deletion mismatches when alanine replaces Glu38 is anticipated by structure-function studies of the conserved glutamate in *E. coli* and *Taq* MutS. *E. coli* and *Taq* MutS bind to mismatched DNA as homodimers in which only the phenylalanine and glutamate in the highly conserved Phe-X-Glu motif of only one monomer make specific contacts with the mismatched base [4–7]. The phenylalanine stacks with one of the two bases of a base-base mismatch or with the unpaired base of a single base insertion-deletion mismatch. Those same mismatched bases also form a hydrogen bond with the glutamate, Glu38 in *E. coli* MutS. MutS binding to mismatched DNA results in a 60° kinking of the DNA at the mismatched site. It has been proposed that kinking is facilitated by electrostatic repulsion between the negatively charged side chain of Glu38 and the phosphate backbone of the DNA [5–7,13]. Replacing Glu38 with alanine removes the negative charge and eliminates this repulsion, explaining the loss of discrimination during initial binding of MutSE38A to matched versus mismatched DNA [4,12,13].

Replacing Glu38 with alanine also eliminates the hydrogen bond to the mismatched thymine in a G-T mismatch [4]. Recent studies of *E. coli* MutS with Glu38 replaced with alanine, glutamine, or threonine have led to the suggestion that this hydrogen bond makes a small contribution to discrimination during initial binding [4]. These studies indicate that the hydrogen bond is particularly important for post-binding transmission of conformational changes within MutS that induces formation of a stable MutS-ATP complex capable of forming a sliding clamp that participates in downstream events in MMR [4]. Thus, the strong inactivation of repair of all types of single base mismatches in the *mutSE38A* strain (Table 1) may reflect loss of charge repulsion and loss of hydrogen bonding by Glu38, diminishing repair capacity at two or more steps in the MMR reaction pathway.

Some indication of the relative importance of charge repulsion and hydrogen bonding to the efficiency and the mismatch specificity of repair is provided by the results in the *mutS*(E38Q) strain, which retains more repair capacity than does the *mutS*(E38A) strain (Figure 2). While replacing glutamate with glutamine eliminates the negative charge, a recent structural study shows that the glutamine side chain in an *E. coli* E38Q mutant MutS protein forms two hydrogen bonds with the thymidine in a G-T mismatch [4]. These hydrogen bonds involve a side chain conformation similar to that used when glutamate hydrogen bonds to an A-A mismatch [6], a transversion mismatch. This type of hydrogen bonding may be relevant to the observation that 94% of mismatches leading to transversions are repaired in the *mutS*(E38Q) strain (Figure 2). Transition mismatches are somewhat less efficiently repaired in the *mutS*(E38Q) strain (Figure 2), suggesting that the identity of the base-base mismatch may determine the contribution of hydrogen bonding to MMR efficiency. Interestingly, this glutamate residue was found to interact strongly with a cisplatin DNA substrate, indicating that its mode of

interaction may contribute significantly to the initiation of MMR and/or to a cell death response [17].

The function of Glu339 in yeast Msh6

In the absence of a structure of Msh2-Msh6 bound to a mismatch, many studies have logically used the structures of *E. coli* and *Taq* MutS as surrogates to interpret genetic and biochemical data on eukaryotic MMR. Particularly, amino acid residues that are conserved across species are inferred to have equivalent functions. Surprisingly, despite conservation of *E. coli* Glu38 as Glu339 in yeast Msh6, the consequences of an alanine replacement for the glutamate in the highly conserved Phe-X-Glu motif of Msh6 are different. Specifically, the *msh6E339A* mutant yeast strain retains robust capacity to repair insertion-deletion mismatches and transition mismatches, while it is strongly defective in repairing mismatches resulting in G-C to T-A transversions (Fig. 2). Thus, the defects conferred by replacing the conserved glutamate with alanine are mismatch specific to an even greater degree in Msh6 than in MutS.

In addition to correcting single base replication errors involving undamaged bases, several studies have shown that Msh6 (but not Msh3) also participates in correcting mismatches resulting from replication of 8-oxo-G, a common lesion generated by oxidative stress [33–38]. A signature of oxidative stress-induced substitutions is a G-C to T-A transversion, which results when dAMP is incorporated opposite 8-oxo-G in its *syn* conformation in the template strand [39]. This G-C to T-A transversion accounts for the majority of base substitutions in the *msh6E339A* mutant yeast strain (Tables 3 and 4), despite the fact that it is only one of among eight possible single base substitutions. The rate of this event in the *msh6E339A* mutant yeast strain is ~50-fold higher than in wild type yeast, reflecting nearly complete loss of MMR (Table 3, Figure 2). These data suggest that Glu339 may be particularly important for repairing mismatches resulting from replication of oxidized bases. Additionally, when *msh6E339A* was expressed from an *ARS-CEN* vector in an *msh3 msh6* strain, three of 25 *can^r* mutants contained C-G to G-C transversions that could result from bypass of lesions resulting from secondary oxidation of 8-oxo-G [40], and four others contained A-T to C-G transversions that are signatures of misincorporation of 8-oxo-dGTP opposite template adenine [41–43] (Table 3).

One possible explanation for why Glu339 may be important for repairing oxidized mismatches is that Glu339 may participate in a base flipping mechanism. The idea that MutS proteins may flip mismatched bases has been proposed earlier based on several lines of evidence [2,44]. It is well known that several other DNA repair enzymes excise damaged DNA bases only after flipping them out of the helix [45–48]. Replacing Glu339 with an alanine could impair flipping of bases in mismatches that have nearly normal geometry and/or high stability, e.g., 8-oxo-G_{syn}•A mismatches. This explanation, among others, for the highly specific MMR defect conferred by the E339A mutation would undoubtedly benefit from structural information on Msh2-Msh6 bound to different mismatches and from functional studies involving initial binding and post-binding events. Studies of the phenotypes of mice containing the equivalent of the yeast Msh6 E339A mutation could also be informative regarding the contribution of substitution mutations caused by oxidative stress to disease (e.g., see [38]).

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Figure 1. Spectra of *lacI*^d Single Base Mutations in *mutS*::Tn10, *mutS*(E38A) and *mutS*(E38Q) *E. coli* Strains

Spectra were obtained as described in Materials and Methods. Only the N-terminal portion (nucleotides 1–250) of the *lacI* gene is shown. Single base deletions are shown with an open triangle below the sequence and single base insertions are shown with a closed inverted triangle above the sequence. Single base replacements are indicated above the sequence. Note that the G-C → A-T hotspot at position 120, while previously detected [21,27], was not observed in previous mismatch repair deficient strains, including *mutS* [31].

Figure 2. Percent of Replication Errors Unrepaired by MutS or Msh6 Mutant Enzymes

The percentage of replication errors that are unrepaired by the mutant MutS or Msh6 enzymes was calculated as follows: $((\text{MR mutant} - \text{MR wild type}) / (\text{MR null} - \text{MR wild type})) \times 100$, where MR stands for MR of the specific mutations, i.e. transitions, transversions, or insertions/deletions and mutant stands for either the *E. coli* MutS E38A or E38Q mutant or the *S. cerevisiae* Msh6 E339A mutant. Null stands for either the *E. coli* *mutS::Tn10* or the *S. cerevisiae* *msh3msh6* strains. Instead of transversions, the percentage of G-C to T-A mutations was used for the calculation for *S. cerevisiae* Msh6 E339A. Data used in the calculations were taken from Tables 1 and 3.

Table 1**Mutagenesis in the *lacI* Gene in *E. coli mutS* Mutant Strains**

The mutation frequency (MF) (see Materials and Methods) and the number of *lacI^d* mutants sequenced are shown as well as the nature of the mutation. The 95% confidence limits are shown in parentheses below the frequencies and the relative frequencies of each type of mutation are shown in reference to the wild type in brackets.

Total	Wild Type		<i>mutS::Tn10</i>		<i>mutSE38A</i>		<i>mutSE38Q</i>	
	Sequenced	MF (10 ⁻⁸)	Sequenced	MF (10 ⁻⁸)	Sequenced	MF (10 ⁻⁸)	Sequenced	MF (10 ⁻⁸)
	88	5.8 (1.2–10)	216	250 (140–340)	227	140 (95–190)	224	68 (59–81)
Substitutions	41 (47%)	2.7 [1]	165 (76%)	190 [70]	171 (75%)	110 [41]	192 (86%)	58[21]
Transitions	27 (31%)	1.8 [1]	156 (72%)	180 [100]	160 (70%)	99 [55]	187 (83%)	57 [32]
Transversions	14 (16%)	0.9 [1]	9 (4%)	11 [12]	11 (5%)	6.8 [8]	5 (2%)	1.5 [1.7]
Indels	20 (23%)	1.3 [1]	50 (23%)	58 [45]	53 (23%)	33 [25]	32 (14%)	10 [8]
Other	27 (31%)	1.8 [1]	1 (0.5%)	1.2 [1]	3 (1%)	1.9 [1]	0	≤0.3 [≤1]

Table 2

Complementation of Mutator Phenotype of an *msh3msh6* Strain by *MSH6* or *msh6E339A* Genes Expressed from an *ARS-CEN* Vector
MR: mutation rate, CL: 95% confidence limits, RR: relative rate in bold in reference to the *MSH6* wild type strain. The specific nature of the *can^r* mutants resulting in single base mismatches or insertions/deletions from wild type *MSH6* and *msh6E339A* strains is shown at the bottom of the table.

Reporter Gene	MSH6			msh6E339A			none		
	MR (10 ⁻⁶)	CL (10 ⁻⁶)	RR	MR (10 ⁻⁶)	CL (10 ⁻⁶)	RR	MR (10 ⁻⁶)	CL (10 ⁻⁶)	RR
<i>lysA₁₄</i>	3.3	0.7–8.7	1	14	6.2–29	4	1900 ^a	1400–3800	590
<i>his7-2</i>	0.5	0.2–0.7	1	1.3	0.4–2.7	3	15	14–35	30
<i>Can^r</i>	0.48	0.35–0.78	1	4.8	3.6–7.6	10	10	5.4–17	21
±1	6/14	MR (10 ⁻⁶)	RR	Occurrence/Total	MR (10 ⁻⁶)	RR	Occurrence/Total	MR (10 ⁻⁶)	RR
Transitions	3/14	0.21	1	0/25	≤0.19	≤1	15/22 ^b	6.8 ^b	32 ^b
Transversions	1/14	0.10	1	4/25	0.77	7.7	3/22 ^b	1.4 ^b	14 ^b
Base Substitutions	Occurrence/Total	0.034	1	18/25	3.5	100	4/22 ^b	1.8 ^b	53 ^b
	Occurrence/Total	Mutation	Occurrence/Total	Mutation					
	2/14	G-C → A-T	1/25	G-C → A-T					
	1/14	T-A → C-G	3/25	T-A → C-G					
	1/14	G-C → T-A	10/25	G-C → T-A					
			4/25	A-T → C-G					
			3/25	C-G → G-C					
			1/25	T-A → A-T					
Insertion/ Deletion	3/14	- A							
	2/14	- G							
	1/14	+ T							

^afrom reference [16]

^bfrom reference [32]

Table 3**Mutation Rates in Strains Expressing *MSH6* or *msh6E339A* From its Endogenous Chromosomal Locus**

MR: mutation rate, CL: 95% confidence limits, RR: relative rate in bold in reference to the *MSH6* wild type strain. Experiment 1 represents experiments performed in a strain with a wild type *MSH3* background. Experiments 2 and 3 represent two independent experiments both performed in a strain with an *msh3Δ* background. The specific nature of the *can^r* mutants resulting in single base mismatches or insertions/deletions from Experiment 3 for wild type *MSH6* and *msh6E339A* strains is shown at the bottom of the table.

Reporter Gene	MR (10 ⁻⁷)	<i>MSH6</i> CL (10 ⁻⁷)	RR	MR (10 ⁻⁷)	<i>msh6E339A</i> CL (10 ⁻⁷)	RR	MR (10 ⁻⁷)	<i>msh6A</i> CL (10 ⁻⁷)	RR
Experiment 1. <i>MSH3</i> strain									
<i>lys:A₇</i>	0.17	0.09–0.13	1	0.21	0.10–0.26	1	0.27	0.23–0.33	2
<i>his7-2</i>	0.11	0.09–0.13	1	0.13	0.11–0.16	1	0.33	0.25–0.38	3
<i>Can^r</i>	2.1	1.4–2.6	1	11.0	8.4–25	5	20	14–31	10
Experiment 2. <i>msh3</i> strain									
<i>lys:A₇</i>	0.45	0.32–0.53	1	0.85	0.34–1.8	2	33	23–52	190
<i>his7-2</i>	0.96	0.75–1.3	1	2.0	1.1–3.2	2	14	9.5–19	15
<i>Can^r</i>	1.7	1.3–3.5	1	7.7	4.6–13	5	76	48–110	42
Experiment 3. <i>msh3</i> strain									
<i>lys:A₇</i>	0.26	0.20–0.33	1	0.56	0.51–0.67	2	20	10–24	77
<i>his7-2</i>	0.90	0.79–1.0	1	0.60	0.48–0.72	1	5.9	3.6–7.6	7
<i>Can^r</i>	2.6	1.8–3.4	1	8.3	7.1–12	3	48	39–68	18
Occurrence/ Total									
±1	1.4	24/45	1	0.9	5/46	1	25	13/25	18
Transitions	0.46	8/45	1	1.3	7/46	3	7.7	4/25	17
G-C to T-A	0.12	2/45	1	5.8	32/46	48	3.8	2/25	32
Base Substitution									
Mutation		Occurrence/ Total	Mutation	Occurrence/ Total					
G-		6/45	G-	6/46					
C → A-T		2/45	C → A-T	1/46					
T-			T-						
A → C-G		2/45	A → C-G	32/46					
G-			G-						
C → T-A		4/45	C → T-A	1/46					
C-			A-						
G → G-C		1/45	T → C-G						
T-									
A → A-T									
Insertion/ Deletion									
-C		15/45	-C	2/46					
-G		4/45	-T	1/46					
-T		2/45	-A	1/46					
+A		1/45	+T	1/46					
+C		1/45							
+G		1/45							

Table 4

Mutation Rates in *ogg1* Strains

MR: mutation rate, CL: 95% confidence limits, RR: relative rate in bold in reference to the *ogg1/MSH6* wild type strain. *MSH6* or *msh6E339A* was expressed from its endogenous chromosomal locus. The specific nature of the *can'* mutants resulting in single base mismatches or insertions/deletions for all of these strains is shown at the bottom of the table.

Reporter Gene	MR (10^{-7})	MSH6 CL (10^{-7})	RR	MR (10^{-7})	msh6E339A CL (10^{-7})	RR	MR (10^{-7})	msh6A CL (10^{-7})	RR
<i>lys:A₇</i>	0.14	0.13-0.19	1	0.18	0.10-0.21	1	0.21	0.16-0.24	2
<i>his7-2</i>	0.10	0.06-0.14	1	0.11	0.06-0.13	1	0.21	0.17-0.36	2
<i>Can'</i>	3.1	2.8-4.1	1	9.6	7.7-16	3	35	28-41	11
		Occurrence/Total			Occurrence/Total			Occurrence/Total	
±1	0.38	4/33	1	0.37	1/26	1	2.8	2/25	7
Transitions	0.85	9/33	1	1.1	3/26	1	1.5	11/25	18
G-C to T-A	0.94	10/33	1	7.0	19/26	7	13	9/25	14
Base Substitution		Occurrence/ Total		Occurrence/ Total					
	Mutation		Mutation		Mutation				
	G-C → T-A		G-C → T-A		G-C → T-A				
	G-C → A-T		G-C → A-T		A-T → G-C				
	G-C → C-G		A-T → C-G		G-C → A-T				
	A-T → T-A		A-T → G-C		A-T → C-G				
	A-T → C-G				A-T → T-A				
Insertion/ Deletion	-G	2/33	-C	1/26	-T				
	-A	1/33							
	-T	1/33							