**Saccharomyces cerevisiae** MutLα IS A MISMATCH REPAIR ENDONUCLEASE*

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Abstract

MutL homologs are crucial for mismatch repair and genetic stability, but their function is not well understood. Human MutLα (MLH1-PMS2 heterodimer) harbors a latent endonuclease that is dependent on integrity of a PMS2 DQHA(X)₂E(X)₄E motif (Kadyrov et al. (2006) Cell 126, 297-308). This sequence element is conserved in many MutL homologs, including the PMS1 subunit of **Saccharomyces cerevisiae** MutLα, but is absent in MutL proteins from bacteria like *Escherichia coli* that rely on d(GATC) methylation for strand directionality. We show that yeast MutLα is a strand-directed endonuclease that incises DNA in a reaction that depends on a mismatch, yMutSα, yRFC, yPCNA, ATP, and a pre-existing strand break, whereas *E. coli* MutL is not. Amino acid substitution within the PMS1 DQHA(X)₂E(X)₄E motif abolishes yMutLα endonuclease activity in vitro and confers strong genetic instability in vivo, but does not affect yMutLα ATPase activity or the ability of the protein to support assembly of the yMutLα•yMutSα•heteroduplex ternary complex. The loaded form of yPCNA may play an important effector role in directing yMutLα incision to the discontinuous strand of a nicked heteroduplex.

Mismatch repair is a conserved process that guards genome stability (reviewed in (1-4)). A major function of mismatch repair is the correction of DNA replication errors, a reaction that has been most thoroughly studied in *E. coli*, where replication error correction is directed to the daughter strand by virtue of the transient absence of d(GATC) methylation on newly synthesized DNA (reviewed in (1,2,5)). Eleven activities have been implicated in methyl-directed mismatch repair, which has been reconstituted in a purified system (6-9). The reaction is initiated via mismatch recognition by MutS, which recruits MutL to the heteroduplex. Assembly of MutS-MutL-heteroduplex complex activates MutH endonuclease, which cleaves the unmethylated strand at a hemimethylated d(GATC) site (10). This strand break, which may reside 3’ to 5’ or 5’ to 3’

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excision system, which removes that portion of the incised strand spanning the two DNA sites (7-9). DNA polymerase III holoenzyme is sufficient to support repair of the ensuing gap, and ligase restores the covalent integrity to the product (6).

While the *E. coli* methyl-directed system has served as the paradigm for studies of mismatch repair, several lines of evidence suggest that this reaction may differ significantly from that in other organisms in which mismatch repair has been studied. For example, *Streptococcus pneumoniae* does not encode a MutH homolog, and MutH and d(GATC) methylase homologs have not been identified in humans, *Drosophila melanogaster*, or *Saccharomyces cerevisiae* genomes (NCBI BLAST search of protein databases, not shown). Furthermore, we have recently found that human MutLo (MLH1-PMS2 heterodimer) is a latent endonuclease that incises the discontinuous strand of a nicked heteroduplex in a mismatch- MutSa-, RFC-, PCNA-, and ATP-dependent manner (11). Activity of this endonuclease is dependent on the integrity of a DQHA(X)$_2$E(X)$_4$E metal-binding motif located within the C-terminal portion of PMS2 (11). This motif is highly conserved in eukaryotic and eubacterial MutL proteins, but is lacking in MutL proteins from bacteria like *E. coli* that rely on d(GATC) methylation for strand discrimination during mismatch repair (11). Although *E. coli* MutH and human MutLo are both latent endonucleases, these activities play functionally distinct roles in the two repair systems. Whereas MutH incision provides a nick which serves as the actual strand signal that directs repair (6,12), incision by MutLo depends on preexistence of a signaling strand break (11).

Yeast *S. cerevisiae* contains four MutL homologs: MLH1, MLH2, MLH3, and PMS1 (the homolog of human PMS2), which form three heterodimeric complexes with MLH1 being a common subunit (1-3). These complexes are referred to as MutLa (MLH1-PMS1 heterodimer), MutLβ (MLH1-MLH2 heterodimer), and MutLγ (MLH1-MLH3 heterodimer). Of these three complexes, MutLa and MutLγ contain the DQHA(X)$_2$E(X)$_4$E motif within their PMS1 and MLH3 subunits, respectively. The MLH2 subunit of MutLβ has an ENFV(X)$_2$E(X)$_4$D sequence that is partially homologous to the DQHA(X)$_2$E(X)$_4$E motif (11). Genetic studies have shown that yeast MutLa plays a major role in mismatch repair, whereas yeast MutLγ and MutLβ have more specialized roles (1).

We show here that yMutLa, like its human homolog, is a latent endonuclease, but *E. coli* MutL is not. As in the case of the human protein, yMutLa incises the discontinuous strand of 5’- or 3’- nicked heteroduplex DNA in a mismatch, yMutSa- yPCNA-, yRFC-, and ATP-dependent fashion. Amino acid substitution within the DQHA(X)$_2$E(X)$_4$E motif of yPMS1 inactivates endonuclease activity and confers high mutation rates *in vivo*. We also present evidence suggesting that yPCNA may play an important role in directing yMutLa endonuclease incision to the nicked heteroduplex strand.

**EXPERIMENTAL PROCEDURES**

**Baculoviruses**

Baculovirus constructs expressing yMLH1 and yPMS1 or yMLH1 and yPMS1-E707K were prepared using the pFastBac Dual vector (Invitrogen). The yMLH1 gene was PCR-amplified from an pCYB2-yMLH1 template (13) using primers d (GTCCTCGAGGCCCCACATTGTCTCTCAGAATAAAGACCTTTGGATC) and d (GTCGCATGCTATATAAACACTCTCAAAAAACCTTTGTATAGATC). The PCR product was cleaved with XhoI and SphI and then cloned under p10 promoter control by insertion into XhoI- and SphI-cleaved pFastBac Dual to yield pyMLH1. yPMS1 and yPMS1E707K genes were PCR-amplified from pMH8 (13) or an E707K mutant derivative of pMH8 prepared using the mutagenic oligonucleotides d(CGAAATTATCTTTTATCATG) and its complement. PCR amplification primers were d
(GTCGGATCCGCCACCATGACACAAATTCATCAGATAAACG) and d (GTCAGGCCTTATCATATTTCGTAATCCTTCGAAAATGAGC). The yPMS1 or yPMS1E707K PCR products were cleaved with BamHI and StuI and then inserted into BamHI- and StuI-cleaved pyMLH1 to place yPMS1 expression under control of polyhedrin promoter (plasmids pyMutLa and pyMutLaE707K). Sequences of yMLH1 and yPMS1 genes in these transfer vectors were confirmed by DNA sequencing. The Bac-to-Bac baculovirus expression system (Invitrogen) was used to produce recombinant viruses.

**Protein Preparations**

*E. coli* MutS (14), MutL (15), and DNA helicase II (16) were prepared by published methods. Exonuclease VII and SSB were obtained from USB. *E. coli* β-clamp, the γ clamp-loader complex, and DNA polymerase III core were isolated as described (17-19). yRFC (95% pure) and yMutSα (95% pure) were isolated by minor modifications of the published procedures (20). Purification of yMutLa, yMutLaE707K, and yPCNA were carried out at 0-4 °C. During isolation of the latter three activities as detailed below, all buffers contained 1 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride, and fractions were collected into tubes containing a set of protease inhibitors to yield final concentrations of aprotinin, leupeptin, E64 and pepstatin of 1 μg/ml, 2 μg/ml, 0.5 μg/ml and 0.7 μg/ml, respectively. Proteins were identified in column fractions by SDS-PAGE followed by Coomassie R-250 staining.

yMutLa and yMutLaE707K were purified from baculovirus-infected SF9 cells (MOI of 3-6, 52 h infection), which were collected by centrifugation (1160×g, 10 min) and frozen in liquid N2. Cell pellets from 1-liter cultures were thawed and suspended in 20 ml of buffer A (20 mM HEPES, pH 7.4, 0.5 mM EDTA, 0.01% NP40, 5% glycerol (w/v)) containing 200 mM NaCl. The suspension was clarified by centrifugation at 40,000×g for 25 min. Ionic strength of the supernatant was adjusted to that of 150 mM NaCl, and the supernatant loaded onto a 5-ml heparin HiTrap column (GE HealthCare), equilibrated with the buffer A containing 150 mM NaCl at flow rate of 1.5 ml/min. After a 15 ml wash with equilibration buffer, the column was eluted with a 60-ml linear gradient of NaCl (150 - 1000 mM) in buffer A. yMutLa and yMutLaE707K fractions, which eluted at 470 mM NaCl, were diluted to 180 mM NaCl with buffer A and loaded at 1 ml/min onto a 1-ml Mono Q column (GE HealthCare) equilibrated with buffer A containing 180 mM NaCl. After a 5 ml wash with starting buffer, the column was eluted with a 20-ml gradient of NaCl (180 - 500 mM) in buffer A. yMutLa and yMutLaE707K fractions, which eluted at 240 mM NaCl, were diluted to 120 mM NaCl with buffer A and loaded at 1 ml/min onto a 1-ml Mono S column (GE HealthCare), equilibrated with buffer A containing 120 mM NaCl. After a 5 ml wash with starting buffer, the column was eluted with a 20-ml NaCl gradient (120 - 500 mM) in buffer A. yMutLa and yMutLaE707K peak fractions, which eluted at ~260 mM NaCl and were 99% pure, were pooled, quick-frozen in liquid N2 in small aliquots, and stored at -80°C.

Yeast PCNA was expressed in *E. coli* according to Ayyagari et al (21), and cells collected and frozen in liquid N2. Cells obtained from a 1.2-liter culture were thawed and suspended in 40-ml buffer B (25 mM HEPES, pH 7.4, 5% glycerol (w/v), 0.02% NP40, 0.5 mM EDTA) containing 50 mM KCl. After disruption by sonication, the lysate was clarified by centrifugation at 40,000×g for 20 min. The supernatant was adjusted to 150 mM KCl and loaded at 5 ml/min onto a 10-ml HiTrap Q column (GE HealthCare) equilibrated with buffer B containing 150 mM KCl. After a 20-ml wash with starting buffer, the column was eluted with a 100-ml linear gradient of KCl (150-1000 mM) in buffer B. Fractions containing yPCNA, which eluted at 400 mM KCl, were pooled, supplemented with (NH₄)₂SO₄ to 2 M, and loaded at 0.5 ml/min onto a 1-ml Resource Phe column (GE HealthCare) equilibrated with buffer B containing 2 M (NH₄)₂SO₄. After a 10-ml wash with equilibration buffer, the column was eluted with buffer B. yPCNA fractions were pooled and dialyzed against buffer B containing...
30 mM KCl for 2.5 h. The dialysate was adjusted with KCl to a conductivity equivalent to that of 40 mM KCl and loaded at 1 ml/min onto a 5-ml heparin HiTrap column (GE HealthCare), equilibrated with buffer B containing 40 mM KCl, followed by 15-ml wash with the equilibration buffer. yPCNA does not bind to the heparin column under these conditions. After adjustment of the KCl concentration of the heparin column pass through to 250 mM, the fraction was loaded at 1.5 ml/min onto a 1-ml MonoQ column (GE HealthCare) equilibrated with buffer B containing 250 mM KCl. The column was washed with 5-ml of equilibration buffer and eluted with a 10-ml linear gradient of KCl (250 - 600 mM) in buffer B at a flow rate of 0.5 ml/min. yPCNA eluted at 0.42 M KCl. Peak fractions (99% pure) were pooled and aliquots were quick-frozen in liquid N2 and stored at -80°C.

Protein concentrations were estimated using the Bio-Rad Protein Assay with bovine serum albumin (BSA, Pierce) as standard and are expressed as moles of heterodimer for yMutSα and yMutLα, homotrimer for yPCNA, and heteropentamer for yRFC.

Mismatch-provoked DNA incision assay
3'-G-T heteroduplex, 3'-A•T homoduplex, relaxed covalently closed G-T DNA, and relaxed covalently closed A•T homoduplex were prepared from f1MR phages as described (11,22, 23). 3'-substrates contained a single-strand break 141 base pairs 3' to the location of the mismatch (or A•T base pair in homoduplex controls; shorter path in the circular molecule). In 5'-substrates, the separation distance between the two sites was 128 base pairs. Mismatch-provoked incision reactions (40 μl) contained 20 mM HEPES-KOH (pH 7.6), 140 mM KCl, 5 mM MgCl2, 2 mM ATP, 1 mM DTT, 0.2 mg/ml BSA, 1.2% (w/vol) glycerol, and 1.2 nM nicked 5'- or 3'-DNA. yMutSα (25 nM), yMutLα (2 nM or as indicated), yRFC (12.5 nM), and yPCNA (30 nM) were present as indicated. After incubation at 30°C for 10 min, reactions were terminated by the addition of 30 μl of 0.35% SDS, 0.3 mg/ml Proteinase K, 0.4 M NaCl, 0.3 mg/ml glycogen, and 13 mM EDTA, followed by incubation of the samples at 55°C for 15 min. After extraction with phenol/chloroform and isopropanol precipitation, recovered DNA was analyzed by Southern analysis as described previously (11).

E. coli mismatch repair reactions
The possibility that E. coli MutL might harbor endonuclease activity was tested in reactions (20 μl) containing 50 mM HEPES-KOH (pH 8.0), 50 mM KCl, 6 mM MgCl2, 1.4 mM DTT, 0.1 mM EDTA, 0.2 mg/ml BSA, 2 mM ATP, 1.2 nM nicked 5'-G-T heteroduplex or homoduplex DNA, and as indicated: 18.4 nM MutS (as monomer), 12 nM MutL (as monomer), 11.6 nM β-clamp (as dimer), and 4 nM γ clamp-loader complex. After incubation at 37°C for 20 min, reactions were quenched by addition of 90 μl of 10 mM Tris-HCl (pH 8.0), 20 mM EDTA, extracted with phenol/chloroform, and precipitated with isopropanol. Recovered DNA was subjected to electrophoresis through alkaline agarose and Southern analysis as above. Mismatch repair was scored under similar conditions except that reactions were supplemented with 0.1 mM each of the four dNTPs, 265 nM SSB (single-stranded DNA binding protein), 7 nM DNA helicase II, 16.5 nM exonuclease VII, and 75 nM DNA polymerase III core, consisting of α, ε, and θ subunits. Repair was scored by cleavage of reaction products with ClaI and HindIII as described (24).

ATP-Mn2+-dependent endonuclease assays
Reactions (40 μl) contained 20 mM HEPES-NaOH (pH 7.6), 20 mM NaCl, 1 mM MnSO4, 0.5 mM ATP, 1 mM DTT, 0.2 mg/ml bovine serum albumin, 1 % (w/vol) glycerol, 1.2 nM f1MR59 supercoiled DNA, and yMutLo or yMutLoE707K as indicated. Incubation was at 30°C for 20 min. Reactions with E. coli MutL (80 nM) were performed in a similar manner, except that 23 mM KCl was substituted for NaCl, BSA was present at 0.5 mg/ml, and incubation was at 37°C. Reactions were terminated and DNA products analyzed as previously described (11).
Other biochemical methods
Surface plasmon resonance spectroscopy was performed as described (25) in 20 mM Tris-HCl, pH 7.5, 140 mM KCl, 1 mM DTT, 0.005% Surfactant P-40, 5 mM MgCl$_2$, and 1 mM ATP. The SA sensor chip (GE HealthCare) was derivatized with 149 resonance units of a 201-bp G-T heteroduplex and with 150 resonance units of an otherwise identical A•T homoduplex.

Initial rates of ATP hydrolysis by yMutL$_\alpha$ were determined under buffer conditions used for mismatch-provoked DNA incision assays except that 111 mM KCl and 29 mM NaCl were substituted for 140 mM KCl, wild type or mutant yMutL$_\alpha$ was present at 1 $\mu$M, ATP concentration was varied between 0.005 and 2 mM, and reactions contained 16.7 $\mu$Ci/ml [$\gamma$-32P]ATP (6000 Ci/mmol, GE Healthcare). Reactions were terminated and ATP hydrolysis quantitated as previously described (11). $K_m$ and $k_{cat}$ values were determined by non-linear least squares fit to a hyperbola.

Yeast strains and plasmids
_Saccharomyces cerevisiae_ haploid strain E134 (MATa ade5 lys2-4nsE$_A_{14}$ trp1-289 his7-2 leu2-3,112 ura3-52) has been described previously (26,27). Haploid strains DAG634 and DAG629 are isogenic to E134 but MATa.

Plasmids pAG32, pCORE, and the URA3-based yeast integrative plasmid YIpPMS1 have been described previously (26,28-30). E707K and D706N-E707Q substitution mutations for genetic studies were introduced into the yPMS1 gene using the yeast integrative plasmid YIpPMS1 and the Quikchange Site-directed Mutagenesis kit (Stratagene). Oligonucleotide d(CGAAATTATACCTTTATCATATGTCATGC) with its complement and oligonucleotide d(GTTTATTGTCGATCAGCATGCAAGTAATCAAAAGTATAATTTCGAAACACGCTGA) with its complement were used to introduce E707K and D706N-E707Q mutations, respectively.

Construction of haploid strains with mutations in _S. cerevisiae_ PMS1
The chromosomal _PMS1_ gene in strain E134 was replaced with the mutant _pms1-E707K_ and _pms1-D706N-E707Q_ alleles using the mutant derivatives of plasmid YIpPMS1 cut with HpaI as described previously (26). The _pms1_ derivatives of E134, DAG634, and DAG629 were created by disrupting the _PMS1_ gene with an open reading frame conferring resistance to hygromycin B (hphMX4). The hphMX4 cassette was PCR-amplified from the pAG32 plasmid (28) using primers d(ATGACACAAATTCATCAGATAAAGCAGATATAGTGTTCATCGAATTACATCTGGATTGAATTCGAGCTCG) and d(TCATATTTCGTAATCCTTCGAAATGACGCTCAAAATCAGTCGTCGAC) that are complementary to _PMS1_ gene flanking region sequences. The resulting PCR product was then transformed into E134, DAG634, and DAG629 using the lithium acetate method, and _pms1_ derivatives were selected for by resistance to hygromycin B and verified by PCR using primers d(CGATAAAATGTTTCCACCA) and d(TATCCATCAAGCATCCTTCA).

Creating diploid strains with mutations in _S. cerevisiae_ PMS1
To create diploid strains, the _MET2_ gene was deleted in the haploid strains DAG629 and DAG634, and the _MET6_ gene was deleted in the haploid strain E134, using the _delitto perfetto_ method. Briefly, the KanMX4-URA3 cassette was PCR-amplified from the pCORE plasmid (29,30) using the following sets of primers specific to _MET2_ gene (d(ATGTCGATACCTTTAAAAATATGAAACGCCTCAAGAGCTGACATTGAGGAGAT TAAAGGAAGGCTCGTCTTCCGACACTGG)) and d(...
or MET6 gene (d
(ATGGTTCAATCTGCTGTCTTAGGGTTCCCAAGAATCGGTCCAAACAGAGAATTA
AAGAAGGCGAGCTCGTTTTCGACACTGG) and d
(TTAATTCTTGTATTTGTCACGGAA
TACTTGGCGGCTACCATATGAGTCAAAGACAATCTCCTTACCATTAAGTTG
ATC)). The PCR products were then transformed as described (29,30), and disruption of MET2 or MET6 was verified. The haploid strains were then mated and diploids containing at least one genetic copy of either MET2 or MET6 were selected on media lacking methionine. Strains E134, DAG629, and DAG634 containing wild type PMS1, pms1E707K, pms1D706N/E707Q, or pms1Δ were mated with each other to obtain the desired homozygous and heterozygous strains.

**S. cerevisiae mutation rates**

Mutation rates were measured by fluctuation analysis (27,31). 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 either lysine or histidine for revertant count, complete medium containing canavanine and lacking arginine for Can' mutant count, and complete medium for viable count. Mutation rates and 95% confidence intervals were then calculated as previously described (27,32).

**RESULTS**

**S. cerevisiae MutLα is an endonuclease, but E. coli MutL is not**

Integrity of a hPMS2 DQHA(X)2E(X)4E metal-binding motif is required for hMutLα endonuclease activity (11). This motif is also present in the *S. cerevisiae* PMS1 subunit (the homolog of hPMS2) of yMutLα, but is absent in *E. coli* MutL (Fig. 1A). Activation of hMutLα endonuclease at physiological ionic strength is dependent on a mismatch, a strand break, hMutSα, hRFC, hPCNA, and ATP•Mg2+; however, an ATP-stimulated mismatch-independent activity that does not require other protein cofactors is readily detectable at low ionic strength in the presence of Mn2+ (11). Near homogeneous yMutLα displays a similar ATP-stimulated, Mn2+-dependent endonuclease that nicks mismatch-free supercoiled DNA at low ionic strength (Fig.1B). This Mn2+-dependent activity is further stimulated by yeast RFC and PCNA, an effect that requires the presence of both proteins (Supplemental Fig. 1). By contrast, we have been unable to detect Mn2+-dependent endonuclease activity associated with *E. coli* MutL (Fig. 1C).

As observed with the corresponding human proteins at physiological ionic strength, yMutSα, yMutLα, yRFC, and yPCNA support a mismatch- and ATP•Mg2+-dependent nucleolytic reaction that degrades the incised strand of 5’- or 3’-nicked heteroduplex DNA, an effect that requires all 4 proteins and ATP (Fig. 2A-D and Table 1). This reaction depends on a pre-existing DNA break because no detectable hydrolysis was observed under these conditions when the nicked substrate was replaced by a relaxed, covalently closed circular heteroduplex (Supplemental Fig.2A). Furthermore, because the reaction products remain fully sensitive to several restriction endonucleases that cleave near the mismatch or the original heteroduplex strand break (Supplemental Fig. 2B), we conclude that hydrolysis in this system does not produce single-stranded gaps and hence that it occurs by an endonucleolytic mechanism. As shown previously with the corresponding human activities (11), incision by yMutLα in the presence of yMutSα, yRFC, and yPCNA can occur throughout the nicked heteroduplex strand (Supplemental Fig. 3), although there is a clear preference for incision on the distal side of
mismatch relative to the location of the original heteroduplex strand break when these two sites are separated by 128 (5'-heteroduplex) or 141 bp (3'-heteroduplex) (Fig. 2A and C).

Although incision in this four-protein yeast system is strongly activated by a single G-T mismatch, significant incision of control A•T homoduplex was also observed and displayed a similar dependence on yMutSα, yMutLα, γRFC, and γPCNA (Fig. 2A and C; Table 1). At the 140 mM KCl concentration used in our standard reactions, a nicked G-T heteroduplex was preferred over the corresponding nicked A•T homoduplex control by a factor of 5 - 6 (Table 1). This preference was increased to 8 - 9-fold upon increase in KCl concentration to 170 mM, but the reaction was dramatically attenuated at higher salt concentrations (Fig. 2E and F). Some of this background cleavage of homoduplex DNA is likely due to the presence of a mismatch in a small fraction of the molecules; the denaturation-annealing protocol used to prepare control homoduplex DNA is known to introduce mismatches into 5 - 10% of the molecules, an effect that has been attributed to natural variation in the DNA populations employed (10). The residual background of the four-protein system on homoduplex DNA could involve activation by a lesion-independent mechanism (33) and may be due to the absence of one or more additional factors that act to enhance mismatch dependence of the reaction.

As mentioned above and in contrast to human and yeast MutLα, E. coli MutL does not display endonuclease activity in the presence of Mn⁡²⁺ (Fig. 1C). We have also tested the corresponding four-component E. coli system for its ability to support mismatch-dependent incision of nicked heteroduplex DNA. As shown in Fig. 3A, no detectable incision of a nicked 5'-G-T heteroduplex was observed in the presence of MutS, MutL, the β-clamp, and the γ-complex clamp loader. These four activities were nevertheless fully functional in mismatch rectification as judged by their ability to support repair of this heteroduplex upon supplementation with DNA helicase II, exonuclease VII, DNA polymerase III core enzyme, and SSB (Fig. 3B).

**Integrity of the PMS1 DQHA(X)₂E(X)₄E motif is essential for endonucleolytic and genetic stabilization functions of yeast MutLα**

Integrity of a metal-binding DQHA(X)₂E(X)₄E motif within the PMS2 subunit of human MutLα is required for the latent endonuclease function of this activity (11). To assess involvement of the yPMS1 DQHA(X)₂E(X)₄E motif in yeast MutLα endonuclease activity, we prepared a mutant form of the heterodimer containing a Lys substitution for the internal Glu of the DQHA (X)₂E(X)₄E motif (E707K substitution, Fig. 1A). This mutation corresponds to a PMS2 missense mutation that has been identified in a Turcot syndrome family (34). The mutant yeast protein fractionated like wild type yMutLα, displayed normal levels of yMutLα-associated ATP hydrolytic activity (Kₘ = 35 μM and kₗ = 1.0 min⁻¹ as compared to 40 μM and 1.2 min⁻¹ for wild type protein), and was fully functional in its ability to support mismatch-dependent assembly of the yMutα•yMutSα•heteroduplex ternary complex (Fig. 4A). However, the mutant protein was highly defective in its ability to support yMutSα-, γRFC-, γPCNA-, and ATP-dependent incision of nicked heteroduplex DNA (Fig. 4B) and was also deficient in ATP-stimulated endonuclease activity determined in the presence of Mn⁡²⁺ (Fig. 4C). The simplest interpretation of these findings is that the endonuclease observed in these experiments resides within yMutLα and that the yPMS1 DQHA(X)₂E(X)₄E motif may comprise part of the active site.

We have also evaluated the effects of the pms1 E707K change and a D706N-E707Q double substitution mutation on the genetic stabilization activities of yeast MutLα in vivo. Spontaneous mutation rates were analyzed using three different reporter genes: (i) a lys2:A₁₄ reporter that scores reversion to Lys⁺ via deletion of a single A-T base pair in an (A)₁₄ homopolymeric run (32); (ii) a his7-2 reporter that scores reversion to His⁺ primarily via addition of a single A-T base pair in an (A)₇ homopolymeric run (27); and (iii) a CAN1 reporter that scores a wide variety of mutations that result in canavanine resistance. These experiments used yeast strains
with wild type or mutant alleles of PMS1 in its natural chromosomal location. With each of the three reporter systems, disruption of pms1 (pms1Δ) in haploid yeast results in strongly elevated spontaneous mutation rates (Table 2). High mutation rates were also observed in strains harboring the pms1 E707K or D706N/E707Q mutations (Table 2), indicating that these residues, which are in the DQHA(X)2E(X)4E motif, are essential for correction of spontaneous replication errors. While the rates of the pms1 E707K and D706N/E707Q mutant strains are consistently slightly higher than those of the pms1Δ strain, the basis of these small differences is unclear at this point.

To determine if the pms1 E707K or D706N/E707Q mutations are dominant, we constructed diploid strains that were heterozygous or homozygous for these mutations. The D706N/E707Q and E707K homozygotes (+/-) exhibited very high reversion rates at lys2:A14 and his7-2, similar to the pms1Δ homozygote (Table 2). By contrast, the reversion rates of the pms1Δ, pms1 E707K and pms1 D706N/E707Q heterozygous strains are not significantly higher than those of the wild type diploid strain (Table 2), indicating that the missense mutations are not dominant and that one copy of wild type Pms1 is sufficient to complement the mutation. During preparation of this study for publication, Deschenes et al. (35) reported similar effects of the E707K change in vivo. The E707K mutation was found to be a recessive, resulting in mutation rates slightly higher than those conferred by a pms1Δ mutation. Thus, two independent studies have described similar in vivo effects of the E707K mutation.

The loaded form of PCNA may function as an effector that directs MutLα endonuclease to the nicked heteroduplex strand

As mentioned above, low level incision of nicked homoduplex DNA by the four-component yMutSα, yMutLα, yRFC, and yPCNA system is detectable under standard assay conditions (Fig. 2A and C, Table 1). This effect is particularly evident at a MutLα concentration 10 times that used in the standard assay (“Experimental Procedures”). Incision of nicked homoduplex DNA under these conditions is strongly biased to the incised strand and requires yMutLα, RFC and yPCNA (Supplemental Fig. 4), but is independent of the presence of yMutSα. To determine whether yMutLα alone is capable of discriminating nicked from continuous DNA strands, we exploited the observation that the endonuclease activity of the protein is demonstrable in the presence of ATP•Mn2+ in the absence of other proteins, provided that the ionic strength is very low (Fig 1B). Under these conditions, MutLα cleaves the continuous and discontinuous strands of a nicked circular homoduplex with similar efficiencies (compare lanes 1-5 of Fig. 5A and B). Supplementation of MutLα and ATP•Mn2+ with PCNA and RFC had little effect on endonucleolytic incision of the continuous homoduplex strand (Fig. 5B, compare lanes 1-5 with 6-10). However, the clamp and clamp loader markedly potentiated incision of the nicked strand (Fig. 5A, compare lanes 1-5 with 6-10), an effect that was dependent on presence of both activities (Fig. 5A, compare lanes 11 and 12 with lane 7). These results, which are quantitated in Fig. 5C, demonstrate substantial strand bias of incision by the yMutLα endonuclease in the presence of yPCNA and yRFC, but in the absence of yMutSα. The possible significance of this finding will be considered below.

DISCUSSION

MutL homologs have been implicated in early steps of both E. coli and human mismatch repair (1-4). E. coli MutL couples mismatch recognition by MutS to activation of downstream activities. These downstream activities include MutH endonuclease, which incises an unmethylated d(GATC) sequence located either 3’ or 5’ to the mismatch, as well as 3’ to 5’ or 5’ to 3’ excision systems that load at 3’ or 5’ MutH strand breaks, respectively. Despite several similarities between the E. coli and human mismatch repair systems, there is no evidence indicating that eukaryotic MutLα provides a similar function. Other than its ATPase function,
the only molecular activities that have been attributed to MutLα, which have been documented only in the case of the human activity, are an ability to suppress hExo1 hydrolysis on homoduplex DNA (36,37) and an associated nick-directed endonuclease that is activated in a mismatch-, hMutSα-, hPCNA-, and hRFC-dependent manner (11). The latter activity requires integrity of a DQHA(X)E(X)4E metal binding motif located near the C-terminus of the hPMS2 subunit, which has been suggested to comprise part of the endonuclease active site.

In this report, we have demonstrated that S. cerevisiae MutLα also harbors a mismatch-dependent endonuclease that requires yMutSα, yRFC, and yPCNA for its activation. Amino acid substitution within the DQHA(X)E(X)4E motif of the yPMS1 subunit abolishes endonuclease activity but is without discernible effect on yMutLa ATPase activity or the ability of the protein to support mismatch-dependent assembly of the yMutLa•yMutSα•heteroduplex complex. As in the case of human MutLa (11), these findings suggest that the DQHA(X)2E(X)4E motif may comprise part of the endonuclease active site. By contrast, we have been unable to detect endonuclease activity associated with E. coli MutL despite the use of several approaches. We have noted previously that the DQHA(X)2E(X)4E element is conserved in eukaryotic homologs of hPMS2 and found in many prokaryotic MutL proteins, but is conspicuously absent in MutL proteins of eubacteria like E. coli that rely on d(GATC) methylation to direct mismatch repair (11).

These observations indicate existence of two distinct classes of mismatch repair systems: one exemplified by the E. coli methyl-directed pathway and a second that is characterized by its dependence on MutL homolog endonuclease activity, as typified by the human and yeast systems. As noted above, E. coli methyl-directed mismatch repair relies on distinct 3’ to 5’ or 5’ to 3’ excision systems, depending on whether the MutH-produced strand break is located 3’ or 5’ to the mismatch. However, several recent reconstitution studies using purified human repair proteins have shown that mismatch correction need not require distinct 3’ to 5’ and 5’ to 3’ excision activities if the MutL homolog supports endonuclease function. Thus, MutSα, Exo1 and RPA are sufficient to support mismatch-dependent excision directed by a 5’-strand break (23,36,38,39); however, the 5’ to 3’ hydrolytic activity of Exo1 is also sufficient for mismatch removal from a 3’-heteroduplex provided that MutSa, Exo1, and RPA are supplemented with MutLa, RFC, and PCNA (11,23). This is because MutLa endonuclease can introduce a strand break 5’ to the mismatch, which then serves as an entry site for MutSα-activated Exo1. While work with these purified human systems has demonstrated that Exo1 can suffice as the hydrolytic activity for mismatch removal from a 5'- or 3'-heteroduplex, it is important to note that they do not rule out the possible involvement of other excision activities in eukaryotic mismatch repair.

At physiological ionic strength and in the presence of ATP•Mg2+, activation of yeast and human MutLa endonucleases is dependent on a mismatch, a pre-existing strand break, MutSa, RFC, and PCNA. A striking feature of this activity is its strong bias for incision of the discontinuous heteroduplex strand. However, at very low salt concentration and in the presence of ATP•Mn2+, the endonucleolytic activity of yMutLa is demonstrable on homoduplex DNA in the absence of other proteins (Figs. 1B and 5). While incision of a nicked homoduplex under these conditions is without strand bias, supplementation with PCNA and RFC substantially activates cleavage of the discontinuous DNA strand (Fig. 5). The finding that both proteins are necessary for this strand bias effect suggests that the loaded form of PCNA may function as an effector that directs MutLa cleavage to a discontinuous DNA strand. It is noteworthy in this regard that the involvement of PCNA during early steps of mismatch repair has led to the proposal that it may assist in communication between the mismatch and the strand signal for the purpose of strand discrimination (40). The fact that MutSa is not necessary for imposition of strand bias in the presence of ATP•Mn2+ implies that interaction of DNA-bound MutLa with PCNA and/or RFC is involved in this effect. While interaction of MutLa with RFC has
not been described, weak but specific interaction between MutLα and PCNA in the absence of DNA has been documented in both the human and yeast systems (23,40,41). Experiments to further evaluate the significance of this interaction are underway.

The strand-directed nature of the MutLα endonuclease can be understood in terms of the role of mismatch repair in the correction of DNA biosynthetic errors, and the fact that amino acid substitution mutations within the PMS1 DQHA(X)2E(X)4E motif result in highly elevated spontaneous mutagenesis rates (Table 2, and ref. (35)) is consistent with a requirement for the endonuclease activity of MutLα in replication error correction. However, MutLα is also involved in several other genetic stabilization pathways, including the suppression of recombination between quasi-homologous sequences (homeologous recombination) and the cellular response to certain types of DNA damage (1-3). Recent experiments from the Liskay laboratory have shown that amino acid substitution within the DQHA(X)2E(X)4E motif also compromises the activity of MutLα as a suppressor of homeologous recombination and severely attenuates its activity in the cellular response to S-glutathione methyltransferase and 6-thioguanine lesions (42). The simplest interpretation of these findings is that the endonuclease activity of MutLα plays an important role in the function of the protein in these pathways as well, although the nature of its involvement remains to be ascertained.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.
Yeast MutLα is an ATP-Mn\(^{2+}\)-dependent DNA endonuclease but \textit{E. coli} MutL is not. (A) Alignment about the DQHA(X)\(_2\)E(X)\(_4\)E metal binding motif for human PMS2, yeast PMS1, and \textit{E. coli} MutL. (B and C) Endonuclease activities of yeast MutLα (B) or \textit{E. coli} MutL (C) on supercoiled homoduplex f\textit{1}MR59 DNA were determined as described under “Experimental Procedures” except that ATP, MnSO\(_4\), and MgCl\(_2\) were varied as shown.
FIG. 2.
Yeast MutLα incises the nicked strand of 3'- and 5'- heteroduplex DNA in a mismatch-, yMutSα-, yPCNA- and yRFC-dependent manner. Incision reactions (“Experimental Procedures”) contained proteins as indicated, nicked 3'-G-T heteroduplex (panels A and B, lanes 1-6), 3'-A•T homoduplex (lanes 7-12), nicked 5'-G-T heteroduplex DNA (panels C and D, lanes 1-6), or 5'-A•T homoduplex DNA (lanes 7-12). DNA products were cleaved with Clal, resolved by alkaline agarose electrophoresis and transferred to nylon membranes, which were probed with 32P-labeled oligonucleotides corresponding to viral strand coordinates 5491-5514 (A), complementary strand coordinates 2505-2526 (B and D) or viral strand coordinates 5732-5755 (C) (11,22,23). Bars with an asterisk indicate the approximate location of probe hybridization. Size markers are shown on the left. (E) Reactions
containing yMutSa, yMutLa, yPCNA, yRFC, and nicked 3’-G-T heteroduplex DNA (●) or 3’-A•T homoduplex DNA (○) were performed as described above, but the KCl concentration was varied as shown. Incision of the nicked strand was quantitated by phosphorimager analysis as described previously (11) after alkaline gel electrophoresis and hybridization with a $^{32}$P-labeled probe corresponding to viral strand coordinates 5491-5514. (F) The mismatch dependence of incision was calculated from the data in panel E.
FIG. 3.  
*E. coli* MutL, MutS, the β clamp and the γ complex do not support incision of a nicked heteroduplex DNA. (A) Reactions contained nicked 5'-G·T or 5'-A·T DNAs and proteins as indicated ("Experimental Procedures"). DNA products were resolved by electrophoresis through alkaline agarose, transferred to a nylon membrane, and probed with $^{32}$P-labeled oligonucleotide corresponding to viral strand coordinates 5629-5652 (indicated by bar with an asterisk). Supplementation of reactions with SSB, DNA helicase II, and DNA polymerase III core did not alter the experimental outcome, and similar results were obtained with nicked 3' heteroduplex or homoduplex DNA (not shown). (B) Left lane - reactions as in panel A were supplemented with dNTPs, SSB, DNA helicase II, exonuclease VII, and DNA polymerase III.
core (“Experimental Procedures”). Repair, which renders the 5’-heteroduplex sensitive to HindIII, was scored by cleavage with HindIII and ClaI. **Right lane** - G-T heteroduplex incubated in the absence of repair proteins was digested with HindIII and ClaI.
Although defective in endonuclease activity, yeast MutLaE707K supports mismatch-dependent yMutLa•yMutSa•heteroduplex ternary complex formation. (A) Surface plasmon resonance spectroscopy was performed as described under “Experimental Procedures.” (Upper) Solutions tested contained 100 nM yMutSa (red lines), 100 nM yMutLa (blue lines), or a mixture of 100 nM of yMutSa and 100 nM yMutLa (green lines). Solid lines are results obtained with a 201 bp G-T heteroduplex, while dashed lines are those obtained with an otherwise identical A•T homoduplex. (Lower) Experimental procedures were as in the upper panel except that wild type yMutLa was replaced with yMutLaE707K. (B) Incision of nicked 3’-G-T heteroduplex DNA in the presence of yMutSa, yRFC, yPCNA, and ATP•Mg$^{2+}$ was performed as described in “Experimental Procedures” except that yMutLa (●) and yMutLaE707K (■) concentrations were varied as indicated. DNA products were visualized and quantitated as in Fig. 2E. (C) ATP•Mn$^{2+}$-dependent endonuclease activity on supercoiled DNA at the low ionic strength and in the absence of other proteins was determined as a function of MutLa (●) and yMutLaE707K (■) concentrations (“Experimental Procedures”).
FIG. 5.
yPCNA in the presence of RFC directs yMutLα incision to a nicked DNA strand. Incision reactions containing nicked 3’-A•T homoduplex DNA were as described in “Experimental Procedures” and Fig. 2, except that yMutSα was omitted, RFC and PCNA were present as indicated, yMutLα was varied as shown, ATP concentration was 0.5 mM, 1 mM MnCl$_2$ was substituted for MgCl$_2$, and total salt concentration was 22 mM (11 mM NaCl and 11 mM KCl). Incision of the nicked (A) and continuous (B) homoduplex strands was visualized as in Fig. 2A-D. (C) Incision of the nicked (●, ■) and continuous (○, □) homoduplex strands of panels A and B was quantitated as in Fig. 2E for reactions that included yMutLα, yRFC and yPCNA (●, ○) or yMutLα only (■, □).
**TABLE 1**

Yeast MutS\(\alpha\)-, MutL\(\alpha\)-, PCNA-, RFC-, and ATP-dependent incision of the discontinuous strand of 3’- and 5’- heteroduplex DNA

| Reaction   | 5’-G-T  | 5’-A•T  | 3’-G-T  | 3’-A•T  |
|------------|---------|---------|---------|---------|
| Complete   | 24 ± 0.3| 4.4 ± 0.2| 27 ± 3.5| 4.5 ± 1.2|
| - yMutL\(\alpha\) | < 1 | < 1 | 1.5 ± 1.8 | < 1 |
| - yMutS\(\alpha\) | < 1 | < 1 | 1.6 ± 0.4 | < 1 |
| - yPCNA    | < 1 | < 1 | < 1 | < 1 |
| - yRFC     | < 1 | < 1 | < 1 | < 1 |
| - ATP      | < 1 | ND    | < 1 | ND |

Incision data shown were obtained by quantitation of images like those in Fig. 2A-D. Complete reactions (see “Experimental Procedures”) contained yMutS\(\alpha\), yMutL\(\alpha\), yPCNA, yRFC, and ATP, with omissions as noted. Incision values for the continuous strand are in parentheses. Incision is expressed as fmol per 10 min for 40 \(\mu\)l reactions containing 48 fmol substrate DNA. Errors shown reflect the range of values observed in at least two independent experiments. ND – not determined.
## Table 2
Mutation rates in haploid *S. cerevisiae* strains with mutations within the Pms1 DQHA(X)_{2}E(X)_{4}E motif

| Reporter Gene | PMS1 | pms1 D706N/E707Q | pms1 E707K | pms1Δ |
|---------------|------|------------------|------------|-------|
|               | Rate ×10^{-7} | Relative | Rate ×10^{-7} | Relative | Rate ×10^{-7} | Relative | Rate ×10^{-7} | Relative |
| lys:A_{id}    | 1.1  | 1                | 13,000 (9,600-17,000) | 12,000    | 15,000 (5,900-23,000) | 14,000    | 4,900 (3,800-9,200) | 4500     |
| his7-2        | 0.07 | 1                | 3.7 (2.0-5.8)       | 53        | 5.1 (3.4-6.8)         | 73        | 2.2 (2.0-3.5)        | 31       |
| Can'          | 1.7  | 1                | 21 (15-30)          | 12        | 36 (24-68)           | 21        | 15 (11-30)           | 9        |

Mutation rates were measured as described in “Materials and Methods”, with 95% confidence limits shown in parentheses. Rates relative to wild type *PMS1*⁺⁺ are indicated in bold.

## Table 2
Mutation rates in diploid yeast with mutations within the Pms1 DQHA(X)_{2}E(X)_{4}E motif

| Reporter | PMS1⁺⁺ | pms1Δ⁺⁺ | pms1Δ⁻⁻ | pms1 D706N/ E707Q⁺⁺ | pms1 D706N/ E707Q⁻⁻ | pms1 E707K⁺⁺ | pms1 E707K⁻⁻ |
|----------|--------|---------|---------|----------------------|----------------------|--------------|--------------|
|          | rate ×10^{-7} | rate ×10^{-7} | rate ×10^{-7} | rate ×10^{-7} | rate ×10^{-7} | rate ×10^{-7} | rate ×10^{-7} |
| lys:A_{id} | 1.7 | 19,000 (15,000-24,000) | 4.2 (3.0-4.7) | 30,000 (25,000-39,000) | 4.1 (2.7-7.2) | 34,000 (21,000-62,000) | 6.3 (4.4-8.5) |
| his7-2    | 0.2 | 8.0 (0.1-0.2) | 0.05 (0.03-0.08) | 4.6 (3.7-6.6) | 0.07 (0.03-0.2) | 5.7 (0.4-0.4) | 0.06 (0.04-0.09) |

Mutation rates for diploid *S. cerevisiae* strains were measured as described in “Materials and Methods”. The 95% confidence limits are in parentheses and rates relative to wild type *PMS1*⁺⁺ are in brackets.