AN ABSTRACT OF THE THESIS OF

Gautam Naresh Mankaney for the degree of Honors Baccalaureate of Science in Biochemistry and Biophysics presented on June 5, 2007. Title: Investigating the Role of the MutLα Endonuclease.

Abstract Approved:________________________________________________________

Andrew Buermeyer

Lynch individuals have a predisposition to developing colorectal and other cancers due to inherited defects in their mismatch repair (MMR) system. Although mutations in MMR have been directly implicated in Lynch Syndrome, the precise mechanism(s) of MMR functions have yet to be elucidated. One essential complex, MutLα (a dimer of PMS2 and MLH1), contributes to the correction of base insertion and deletion loops, single-base mismatches, and is necessary to trigger apoptosis in response to several types of DNA damage. A recent study by Kaydrov and colleagues showed that MutLα has an endonucleolytic activity only necessary for providing a loading site for the 5’-3’ directed ExoI in a reconstituted in vitro MMR and that a recombinant MutLα deficient for endonucleolytic activity (PMS2-E705K) was unable to repair a 3’ nicked substrate. In related studies, Deschenes et al. showed that hMutLα-E705K was unable to restore MMR activity to cytoplasmic extracts from hMutLα-deficient cells using a 5’-nicked substrate. Based on the contradictory findings by Kadyrov et al. and Deschenes et al., we investigated the ability of MutLα-E705K, in comparison to MutLα-WT, to restore repair activity in a reconstituted MMR system using a 3’, 5’, or a 3'/5’ nicked substrate. We present preliminary data showing that hMutLα-WT does and hMutLα-E705K does not support a reconstituted in vitro repair system when provided with a 3’, 5’ or 3’/5’-nicked substrate, suggesting additional roles for MutLα endonucleolytic activity in in vitro MMR.

Key Words: DNA Mismatch Repair, MutLα-E705K, MutLα endonucleolytic activity

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Investigating the Role of the MutLα Endonuclease

by

Gautam Naresh Mankaney

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Gautam Naresh Mankaney, author
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To all of you
INTRODUCTION

Lynch Syndrome, also referred to as hereditary nonpolyposis colorectal cancer (HNPCC), is characterized by an autosomal dominant inheritance of early-onset colorectal cancer and an increased risk of other cancers, including cancers of the urinary tract, pancreas, endometrium, stomach, small bowl and ovaries (1-3). The majority of individuals with Lynch Syndrome inherit a mutation in one of several DNA mismatch repair (MMR) genes (4-5). These genes encode for proteins involved in the MMR system, an evolutionarily conserved process essential for maintaining the high fidelity of DNA replication through a surveillance mechanism that corrects mistakes made by replicating DNA polymerases. Eighty percent of Lynch individuals do develop some form of cancer at an average age of forty-four, while the average age of onset for non-Lynch individuals is sixty-four (6-7). Of the 160,000 new colorectal cancer cases that arise in the United States each year, about five percent can be attributed to Lynch Syndrome with an additional ten to twelve percent of sporadic colorectal cancer cases also displaying defects in DNA mismatch repair (MMR) (8-9). The association of MMR with sporadic and hereditary cancers has greatly intensified research into the biochemical and cellular mechanisms of MMR.

DNA mismatch repair is one of several DNA repair mechanisms used by cells for DNA metabolism and genomic stabilization. MMR is involved in repairing incorrectly paired bases arising during DNA replication or recombination or from spontaneously deaminated or chemically damaged bases (4, 10). The integrity of DNA is crucial to a cell’s viability and ability to correctly function. With the high rate of spontaneous
chemical damage and degradation present in cells, it is inevitable that the accurate
information content of the genome degrades over time; the modification of just one of the
three billion bases in the human genome can result in severe consequences (11). Thus, it
is imperative that a cell has DNA repair mechanisms that maintain the integrity of its
DNA by restoring the vital chemical information stored in its genome.

In addition to correction of replication errors, MMR components contribute to the
induction of apoptosis in response to DNA damage and to the controlling of cell cycle
checkpoints. Inactivation of this system leads to a several hundred-fold increase in a
cell’s mutation rate, reduced apoptotic responses, and an increased risk of multistage
carcinogenesis (12-13). Understanding the structural and functional consequences of
mutations in MMR genes will aid us in determining whether a certain cancer is MMR
deficient, in prescribing the best treatment for that cancer, and performing genetic
analyses to predict whether an individual or an individual’s offspring will be likely to
develop a MMR deficient cancer.

The mechanism of MMR has been extensively studied in *E. coli*, but has yet to be
completely elucidated in eukaryotes. However, the discovery of the components and
mechanisms of *E. coli* MMR (Figure 1) have helped in the investigation of eukaryotic
MMR.
Figure 1. Prokaryotic mismatch repair. Mismatches are identified by MutS, which then forms a complex with MutL. The complex recruits and activates the MutH endonuclease. An exonuclease and helicase II excise the portion of DNA containing the mismatch and finally, DNA polymerase restores the original region. Proper strand discrimination occurs by recognition of adenine methylation of GATC sequences on the parental strand by the MutH endonuclease. (Figure source - 14)

The three homodimeric proteins MutS, MutL, and MutH, are key components to the prokaryotic MMR system (15). In the first step, the ATPase MutS identifies the mismatch and binds to the DNA (16). Due to adenine methylation of the parent DNA strand at GATC sites and the transient lack of methylation on the nascent strand, MMR can distinguish and direct repair towards the newly replicated strand (17-18). MutS recruits MutL in an ATP-dependent manner and the MutS-MutL complex subsequently activates the specific GATC-endonucleolytic activity of MutH. MutH can cleave and form a DNA strand discontinuity oriented either 5’ or 3’ to the mismatch and up to one kilobase away, reflective of MMR’s bidirectional capabilities (19-21). One of several exonucleases (dependent upon the orientation of the nick) excises a portion of the DNA, subsequently to be resynthesized by DNA polymerase and sealed by DNA ligase, completing the repair
process. Ultimately, the newly replicated and corrected strand becomes fully methylated and will serve as parental DNA for future post-replicative MMR processes (22-23).

There are several proteins in eukaryotic MMR homologous to the MutS and MutL proteins found in bacteria (*Table 1*). Together, they form higher order complexes whose functions include recognizing and correcting both single-base mismatches and small loops formed by DNA insertion and deletion (typical of errors generated during DNA replication), identification of the DNA strand to be excised, and activation of the excision process (24-25). Although these roles are similar to their prokaryotic counterparts, the homodimeric MutS and MutL complexes have evolved into several heterodimers.

Eukaryotic MutS exists as a heterodimer of MSH2 and MSH6 (abbreviated as MutSα), of MSH2 and MSH3 (MutSβ), or in plants, as MSH2 and MSH7 (MutSγ). MutSα can bind all eight base-base mismatches and small (less than 10 nucleotides in length) DNA insertion deletion loops (IDLs). MutSβ preferably binds IDLs of all sizes but does not appear to bind base mismatches, and MutSγ, unique to plants, binds most basepair mismatches (26-28).

Another family of complexes necessary for eukaryotic MMR include the heterodimers MLH1-PMS2 (MutLα; found in eukaryotes), MLH1-PMS1 (MutLα; found in yeasts), MLH1-MLH3 (MutLβ found in eukaryotes), and MLH1-MLH2 (MutLβ found in yeasts) (29-30). MutLα has been more extensively studied than MutLβ, and based on biochemical and genetic studies in yeast and mammalian systems, is the primary complex involved in mismatch repair initiated by MutSα or MutSβ (31). Recent studies illustrate that MutLα can interact with MSH2, MutSα, MutSβ, proliferating cell nuclear antigen (PCNA), and ExoI, reflecting the ability of the MutLα-MutS complex to form higher
order complexes and recruit other proteins to the site of repair and activating repair events (32-35).

Table 1. The table above outlines the mismatch complexes and their substrate specificities present in E. coli, S. cerevisiae, and humans. Question marks indicate unidentified or unspecified complexes and functions of complexes involved in mismatch repair. (Table source - 36)

<table>
<thead>
<tr>
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<th>S. cerevisiae</th>
<th>Human</th>
<th>Functions of Eukaryotic Proteins</th>
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<td>MLH3</td>
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<tr>
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<td>?</td>
<td>?</td>
<td>?</td>
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<tr>
<td>uvrD</td>
<td>?</td>
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<td></td>
<td>Exonuclease I</td>
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<td>RFC, PCNA, Polyγ</td>
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The mechanism of strand discrimination in all organisms excluding E. coli is unknown. In vitro repair systems (Figure 2) successfully direct MMR excision to a certain strand through a single DNA strand discontinuity either 5’ or 3’ to the mismatch (37). This perhaps signifies a mechanism for the identification of the nascent strand in eukaryotic MMR. During replication, breaks between Okazaki fragments or the 3’ end of the leading strand may serve as the strand discontinuities required for initiation of repair (38). One model, based on the extensive involvement of PCNA during MMR, suggests that a PCNA-DNA polymerase–MMR protein complex may help discriminate between the two strands during DNA replication (10). A recent study, discussed in further detail below, suggests that MutLα is a eukaryotic homolog of MutH, generating a nick on the nascent DNA strand to prepare it for excision and re-synthesis (39).

Once repair has been initiated, one or more exonucleases are necessary to excise the mistake-containing DNA strand. Three exonuclease activities have been implicated in
DNA strand excision during MMR, including the 5’-3’ directed activity of ExoI and the proofreading functions of DNA polymerases δ and ε (10). DNA polymerase δ is primarily implicated in strand re-synthesis and DNA ligase completes the MMR reaction by sealing the nick on the 3’ end of the resynthesized strand (40).

**Figure 2.** Reaction schematic of *in vitro* MMR for a 5’ or 3’ nicked mismatch substrate. The complexes necessary for MMR identify the single mismatch present on the plasmid substrate, excise a portion of the DNA containing the mismatch, and resynthesize the strand. Resynthesis of the strand introduces a restriction site that can be used, along with another restriction site already present on the plasmid, to evaluate levels of MMR. *(Figure source - 41)*

Recent studies have identified purified proteins sufficient for eukaryotic MMR in a biochemically reconstituted system. 5’ directed MMR is observed in the presence of
MutSα, ExoI, RPA, DNA polymerase δ, and DNA ligase; supplementation of MutLα, PCNA, and RFC enhances 5’ directed MMR activity and is required for 3’ directed repair, thus supporting a MMR system with bidirectional capabilities in human extracts (42, 43, 39). 5’, but not 3’, MMR is observed in the same mixtures in the absence of MutLα, however, the requirement for MutLα in vivo is without dispute. It has been shown that the 5’ to 3’ directed hydrolytic activity of ExoI is responsible for basepair excision in 5’ directed MMR, but the activity responsible for 3’-directed hydrolysis in this reconstituted system has not been identified. Interestingly, the purified in vitro repair system is capable of 3’ directed repair solely in the presence of ExoI (39, 43). Essential for both 5’ and 3’ directed MMR, ExoI may have a 3’ to 5’ cryptic activity or extensive involvement and interaction with other MMR proteins and processes (44). Remaining key questions concerning the mechanism of MMR include the requirement of MutLα and PCNA for MMR, the mechanism for strand identification, and the relevance of 5’ directed ExoI activity necessary for 3’ directed MMR.

With approximately fifty percent of Lynch syndrome cancers having mutations in MLH1 and around five percent in PMS2 (8, 9), the study of MutLα and its role in MMR has been of special importance. Although MutLα plays a critical role in MMR, the molecular functions of the protein are only partially understood. MLH1 has an N-terminal domain that catalyzes the hydrolysis of ATP, a C-terminal domain (CTD) essential for interaction with and stabilization of PMS2, and can bind DNA (both double- and single-stranded) in a sequence-independent manner (Figure 3) (45, 46). Over 250 MLH1 mutations have been associated with human cancers, and until recently, mutations in PMS2 were thought to play minimal roles in the initiation of tumorigenesis. Nakagawa
and others have shown that PMS2 can behave according to the Knudson model (the Knudson model predicts that children with familial tumors have inherited an initial genetic alteration and require only one additional, rate-limiting genetic alteration to initiate tumorigenesis.) and that mutations in PMS2 are often overlooked (47). Thus, a mutation or loss of the wild-type allele in patients heterozygous for a recessive PMS2 mutation can be associated with autosomal dominant inheritance of Lynch Syndrome. Homozygous recessive and compound heterozygosity in PMS2 mutations also are associated with Turcot syndrome, characterized by the presence of brain tumors in young colorectal cancer patients (47, 48, 49).

Kadyrov and others recently discovered that MutLα has an endonucleolytic activity necessary for in vitro MMR and that the PMS2-E705K mutation (the glutamic acid residing at position 705 is substituted with a lysine) inactivates the MutLα heterodimer’s ability to sufficiently function as an endonuclease. The glutamic acid at residue 705 is part of a highly conserved amino acid motif (DQHA(X)2E(X)4E) in the

**Figure 3.** The proteins involved in the human MutLα heterodimer. The grey regions indicate specified functional domains of MLH1 (756 amino acids in length) and PMS2 (862 amino acids in length). (Figure source - 51)
active site of the latent endonuclease. The endonucleolytic activity of MutLα is activated during a repair reaction, and is dependent upon the presence of and complex formed with MutSα, RFC, and PCNA and can form nicks either 3’ or 5’ of the basepair mismatch (Discussion, Figure 13). Kaydrov and others aver that MutLα is the eukaryotic homolog of MutH, the protein that nicks the nascent DNA strand and prepares it for strand excision and synthesis. According to this hypothesis, the endonucleolytic activity of MutLα explains how the 5’-3’ specific exonuclease, ExoI, can excise the portion of DNA containing the mismatch when only a 3’ nick is provided on a substrate for in vitro MMR (39).

Deschenes and others have also characterized the E705K mutation in PMS2, and have shown that hPMS2-E705K is a recessive loss-of-function allele rather than a dominant allele. Located in the highly conserved CTD region of PMS2, E705K does not appear to significantly affect the stability of the MutLα heterodimer, however, cells expressing PMS2-E705K show high mutation rates similar to cells lacking PMS2. Furthermore, a MutLα complex containing PMS2-E705K (MutLα-E705K) was unable to restore MMR activity to a MutLα-deficient cellular protein extract in an in vitro repair assay using a 5’-nicked plasmid substrate (50). This later finding appears inconsistent with Kaydrov's conclusion that the MutLα endonuclease activity is necessary to form a single-strand discontinuity in order to load the 5’-3’ specific ExoI. If, as Kaydrov proposes, the only role of the endonucleolytic activity is to generate a 5’ nick for loading ExoI, then the endonucleolytic activity of MutLα should not be required in 5’-directed repair. Deschenes and colleagues show that PMS2-E705K fails to show significant levels of repair on a substrate with a 5’ nick (5’ directed repair). Thus, the provision of a 5’ nick
apparently does not obviate the need for the endonucleolytic activity of MutLα, contrary to the prediction of the Kaydrov model. The Deschenes' finding instead suggests additional roles for MutLα endonucleolytic activity.

The goal of this study was to test Modrich’s proposed mechanism (39) by comparing the repair efficiencies of a recombinant MutLα lacking endonucleolytic activity (MutLα-E705K) to a wild type MutLα in an in vitro repair system. Three different substrates were used: one with the DNA single strand discontinuity oriented 5’ to the mismatch (5’ substrate), one with the discontinuity on the 3’ side of the mismatch (3’ substrate), and finally, one with both the 5’ and 3’ nicks (doubly nicked substrate). We hypothesized that the provision of a 5’ nick would obviate the need for MutLα endonucleolytic activity, and that MutLα-E705K should show levels of repair comparable to WT on the 5’ substrate and doubly nicked substrates.

We present preliminary results indicating that the WT MutLα was capable of sufficient in vitro MMR with all three substrates while the MutLα E705K exhibited little to no repair on all three substrates.
MATERIALS AND METHODS

Cell Culture

The cell line (MP1) used in this study was a doubly mutant (\textit{Mlh1}^{-/-}, \textit{Pms2}^{-/-}) immortalized mouse embryonic fibroblast line (MEF). Cells were maintained in DMEM containing 10% bovine calf serum (HyClone), 4.5g/L L-glutamine, 100 U/mL penicillin and streptomycin (Invitrogen), and 1X non-essential amino acids (all media and reagents were from Cellgro, Mediatech Inc., Herndon, Va.). The cells were maintained in a 5% CO2 atmosphere at 38°C in sterile culture flasks (Greiner Bio-One, Longwood, Fl.).

Mammalian Expression Vectors

The pCMV expression vectors, containing either the human (\textit{h}) MLH1 WT or \textit{h}PMS2 WT cDNA insert, were already available in the lab (Dr. Andrew Buermeyer, Department of Environmental and Molecular Toxicology, Oregon State University). The pCMV expression vector containing the \textit{h}PMS2-E705K insert was a generous gift from Dr. Sue Deschenes (Dept. of Biology, Sacred Heart University).
Transient Transfections

2.0-2.2 x 10^6 MP1 cells were plated into 100 mm plates, such that cultures were 70-80% confluent at the time of transfection (18-24 hours later). A total of 24 μg of pCMV-\textit{h}MLH1 WT and either 24 μg of pCMV-\textit{PMS2} - E705K or pCMV-\textit{PMS2} WT purified plasmid DNA (Maxiprep kit, Qiagen, Valencia, CA) were transfected into cells using 40 μl LipofectAmine (Invitrogen) and 56 μl Plus reagent per plate according to manufacturer’s instructions. Cells were incubated with the transfection mixture for 3 h at 38°C and rinsed with DMEM before the growth media was replaced. Cells were incubated overnight in complete medium to allow expression and accumulation of recombinant MLH1 and PMS2.

Cells were harvested with trypsin, pelleted by centrifugation at 2000 x g, for 4 minutes at 4°C, and resuspended with 1 mL of isotonic buffer (20 mM Hepes pH 7.9, 1 mM MgCl2, 5 mM KCl, 250 mM sucrose), 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Cells were pelleted again as above and resuspended in 1 mL of hypotonic buffer (20 mM Hepes pH 7.9, 1 mM MgCl2, 5 mM KCl), 1 mM DTT and PMSF. Cells were pelleted as before, resuspended in 200 μL hypotonic buffer, lysed in a Potter-Elvehjem dounce homogenizer, and placed on ice for 30 minutes. Finally, cell lysates were centrifuged for 10 min, at 22,000 x g, at 4°C to remove nuclei and other insolubles and frozen in liquid nitrogen in 25 μL aliquots. Samples were stored at 80°C and thawed on ice before use.
Analysis of Protein Expression

Total protein concentration of whole cell lysates were determined by the Bio-Rad Pierce BCA Protein Assay (Pierce Biotechnology, Rockford, IL), following the manufacturer protocol. Data was collected on a SpectraMax UV Plate reader at 595 nm and analyzed with Microsoft Excel software.

Concentrations of recombinant MLH1 and PMS2 in cell lysates were determined by immunohistochemical (western) blot analysis. 5-10 μg of total cellular protein was separated by denaturing polyacrylamide gel electrophoresis (Criterion Gel System, Bio-Rad, San Francisco, CA) for one hour at 200 V. Following SDS-PAGE, the gels were soaked in transfer buffer (25 mM Tris, 250 mM glycine pH 7.4, 10% v/v methanol) for 15 minutes. The proteins were transferred to Immobilon-P PVDF membrane (Millipore, Danvers, PA) in a Criterion transfer blotter at 100 V for 30 minutes. The membrane was blocked with 5% w/v powdered milk in TBST (90 mM NaCl, 1 mM KCl, 17 mM Tris, 0.1% Tween, pH 7.6) for 20 minutes and probed overnight at 4 °C with monoclonal antibodies at concentrations suggested by the manufacturer (0.5 μg / mL anti-MLH1 and 0.5 μg/mL anti-PMS2, both from PharmMingen, San Diego, CA). Blots were washed in TBST three times, 5 minutes each, and placed in blocking solution containing secondary antibody (horseradish peroxidase – conjugated goat anti-mouse IgG, Pierce, Rockford, IL) and Precision Streptactin-HRP (Biorad, Hercules, CA) for 1 h. Blots were washed for 15 minutes and signal was developed using the enhanced chemiluminescence substrate (SuperSignal West Pico Chemiluminescent substrate, Pierce Biotechnology, Rockford, IL). Individual band intensities were quantified by exposing the blot for 20
minutes using a Chemi Genius Bio Imaging System and analysis with Gene Tools software (Synoptics LTD, Cambridge, England). Concentrations of MLH1 and PMS2 were determined by comparison of band intensities in extract samples with those of known masses of purified MutLα (45) diluted into MP1 extracts.

**Cytoplasmic Extracts**

Cytoplasmic extracts for repair reactions were obtained from MP1 cells grown in 40 p150 plates until 60-80% confluent. The cells were washed with PBS (Mediatech Inc., Herndon, VA) and removed with 2 mL of trypsin (0.5 g/L porcine trypsin in 137 mM NaCl, 5 mM KCl, 6 mM dextrose, 7 mM sodium bicarbonate, 0.7 mM EDTA, and 5 μM phenol red). The cells were then transferred to a 50 mL conical tube, pelleted at 2000 x g for 4 minutes at 4°C, resuspended in 50 mL of cold isotonic buffer (20 mM Hepes pH 7.9, 1 mM MgCl₂, 5 mM KCl, 250 mM sucrose), 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Cells were pelleted again as above and resuspended in 50 mL of hypotonic buffer (20 mM Hepes pH 7.9, 1 mM MgCl₂, 5 mM KCl), 1 mM DTT and 0.5 mM PMSF. Cells were pelleted as before, resuspended at density of 1.5 x 10⁸ cells / mL hypotonic buffer, lysed in a Potter-Elvehjem dounce homogenizer, and placed on ice for 30 m. Finally, cell lysates were centrifuged for 10 minutes, at 22,000 X g, at 4°C to remove nuclei and other insolubles and frozen in liquid nitrogen in 200 μL aliquots. Samples were stored at 80°C and thawed on ice before use and concentrations (measured by the Pierce Protein Assay as outlined previously) ranged from 9-10 μg / μl.
Substrate Preparation

Plasmid substrates containing an A/C mismatch were constructed using methods outlined by Wang (52) with some variations (Figure 5). The pRO1 plasmid was generously provided by Dr. John B. Hays (Dept. of Mol. Tox., Oregon State University). pRO1 with a G/T mismatch and 3’ nick (using same methodology) was already available in the lab.

The pRO1 was transformed into a competent ER2925 E. coli culture (New England BioLabs, Ipswich, MA) deficient for both the Dam and Dcm methyltransferases, a condition required for formation of the single strand discontinuities using the N.AlwI enzyme. Colonies were grown on LB-agar plates containing 100 μg / mL chloramphenicol and 100 μg / ml ampicillin at 38°C overnight. 18 colonies were selected and grown in the same conditions, but in 2 mL of LB growth medium. Plasmids were purified from each culture using the Qiagen spin column mini kit and analyzed on a 1.5% agarose TAE gel with 0.5 μg / mL ethidium bromide. The E. coli strain that generated the highest ratio of purified plasmids (supercoiled pRO1 migrates at approximately 1500 basepairs) to anomalous DNA, likely representing plasmid dimers, was inoculated into 1 L of LB with 100 μg / mL chloramphenicol and 100 μg / ml ampicillin. The 1 L culture was grown overnight at 37°C, purified using a QIAGEN Plasmid Mega Kit, with the pRO1 (Figure 5, structure A) eluted into 1 mL deionized, nanopure H2O.

Next, to generate a gapped intermediate (Figure 5, structure C), 500 μg of pRO1 was nicked with N.BstNB1 (New England BioLabs, Ipswich, MA) using 2 U / μg of plasmid for 2 hours at 55°C in a 1mL reaction with restriction buffer (0.1 mM Tris, pH
7.5, 0.1 mM MgCl₂, 1.5 mM KCl, and 0.01 mM DTT). Once the pRO1 was totally relaxed (migrates at approximately 4000 basepairs on 1.5% agarose gel), the 32 basepair sequence located between the two nicks was removed adding a 50-fold molar excess of competitor oligonucleotide (complementary to the sequence to be removed; 5’ GAGCGACTCGTTAAGCTCGAGGTGAAT-3’) and placing the 1.5 mL eppendorf tube containing the sample into 80-85°C 1 L water bath to cool overnight. Excess oligonucleotide and annealed oligonucleotide, resulting in gapped pRO1, was removed by ultrafiltration on a Centricon YM-100 filter at 300 x g. TE was added to the sample until the volume reached 2 mL, and the sample was centrifuged 5 times (with the initial volume adjusted to 2 mL with TE) in a fixed angle rotor for 30 minutes each.

To linearize any non-gapped DNA, the gapped preparation of pRO1 was digested with 100 U of Hpa1 for 3 h at 37°C using New England BioLabs buffer 4. A 1.5% agarose gel revealed the relative amounts of relaxed (gapped) and linear DNA which migrate at 4 kb and 2.2 kb, respectively; no supercoiled DNA was present. Plasmid-Safe ATP-Dependent DNase (Epicentre, Madison, WI) was used to degrade the contaminating linearized DNA (represents homoduplex from incomplete production of gapped molecule). Adenosine triphosphate was added to reach a final concentration of 1 mM and Plasmid-Safe was added at 3 units / μg linearized DNA (estimated by analyzing band intensities of agarose gel), followed by incubation for 2 h at 37°C. The gapped plamids were converted to mismatch substrates by ligating an oligonucleotide with a sequence containing a single, non-complementary base at the position of the mismatch (Figure 5, structure D). The A/C mismatch was created by using the oligonucleotide, 5’-ATTACCTCGA(A)GCTTAGTTAACGAGTCGCTC-3’, with the non-complementary
base in parenthesis. A ten-fold excess of oligonucleotide was added to the reaction, which was then placed in a bath of 1 L 55-60°C H₂O and allowed to cool for 4 h. ATP to a final concentration of 1 mM, T4 DNA ligase (Fermentas) at a concentration of 0.5 U / μg gapped plasmid (based on gel quantification), and T4 DNA ligase buffer at 1x were added. The ligation reaction was incubated at room temperature overnight. Conversion of the 4 kb-migrating band to a 1.5 kb-migrating band on a 1.5 % agarose gel indicated successful ligation to generate a closed-circle, mismatched plasmid.

Cesium chloride banding was used to separate the supercoiled plasmid with a single mismatch from other present DNA. 2.5 mg ethidium bromide and 1.05g CsCl / mL TE were added to the solution containing the plasmids until the final volume equaled that of the tube to be centrifuged. The tube was centrifuged under a vacuum at 60,000 x g for 5 h at 4°C in a Beckman VTi80 rotor. Since the mismatch substrate (supercoiled) bound less ethidium bromide than any open circle substrate present (not ligated and nicked or gapped), it had a higher relative density and formed the lower of the two bands in the CsCl solution after centrifugation. The lower of the two bands (the mismatch substrate), visible under UV light, was removed with a needle. The DNA was extracted using water-saturated N-butanol and dialyzed against 4 L TE pH 8.0 at 4°C overnight. The DNA was precipitated by centrifugation for 10 minutes at 14,000 x g, rinsed with 70% ethanol, air-dried, and resuspended in 50 μL TE.

Finally, the resuspended mismatch DNA (approximately 14 μg) was nicked (Figure 5, structure E) using the appropriate restriction endonucleases (3’ nick - N.AlwI, New England Biolabs; 5’ nick – N.Bbvc1A, New England Biolabs). 9.4 μg of mismatch substrate were nicked with 150 U of N.AlwI for 2h at 37°C and the conversion of the
supercoiled plasmid to nicked substrate was evaluated on a 1.5% gel as before. 4.6 μg of the 3’ nicked substrate and 4.6 μg of the mismatch substrate were then incubated with 75 units of N.Bbvc1A at 37°C for 2 h. The complete conversion of the mismatch substrate to the 5’ nicked substrate was assumed to reflect the same conversion of the 3’ nicked substrate to the 3’ + 5’ doubly nicked substrate. The final concentrations of the three substrates were 100 ng / μL.

**In Vitro MMR Assays**

Each repair reaction contained 100 ng of the mismatch substrate, 4 μL of the 10x reaction buffer (1.1M KCl, 50 mM MgCl2, 10 mM reduced glutathione, 15 mM ATP, 1 mM dNTP, 200 mM Tris pH 7.6, and 500 μg/ml bovine serum albumin), 25 μl of cytoplasmic extracts, 100-300 fmol MutLα, and hypotonic buffer to a final volume of 40 μl.

Upon addition of the MutLα, the reactions were incubated for 15 minutes at 37°C and stopped with 60 μl stop buffer (1.2% sodium dodecylsulfate, 25 mM EDTA, 0.3 μg/μl Proteinase K – Fermentas). The reactions were then incubated for 30 minutes at 37°C and the DNA was extracted using an equivalent volume of phenol/chloroform (100 μl) and Phase Lock Gel (Eppendorf) to help separate the aqueous and organic phases. The aqueous phase was removed and the DNA was ethanol precipitated (10 μl sodium acetate 3 M pH 5.2 and 220 μL 100% EtOH) for at least 45 minutes at -20°C. The precipitated DNA was washed with 70% EtOH, air-dried, and resuspended in 10 μl of 10 μg / mL RNase/ H2O solution. The samples were incubated at 37°C for 15 minutes to assist with
RNase activity and DNA resuspension. Finally, the samples were incubated with 2.5 U BanI and 2.5 U HindIII in a final volume of 20 μl (in New England BioLabs Buffer 2) for 2h at 37°C. The control samples (G/T mismatch substrates), previously demonstrated as successful substrates for *in vitro* MMR, were similarly incubated with BanI and XhoI in New England BioLabs Buffer 4. (All restriction enzymes were purchased from InVitrogen.)

The reactions were analyzed on a 1.5% agarose gel run at 185 V for 30 minutes, stained in dilute ethidium bromide for 30 minutes, and washed twice in water for 15 minutes each time. Band intensities were quantified using a Chemi Genius Bio Imaging System, and Gene Tools software. Successful repair was marked by two linear fragments at approximately 1.1 kb, while unsuccessful repair was marked by a single, linear band at 2.2 kb. Repair efficiency (calculated as percent of substrate sensitive to digestion with HindIII or XhoI) was determined by dividing the sum of the intensities of the two 1.1 kb fragments by the sum of the intensities of all three fragments (the two 1.1 kb fragments and the 2.2 kb fragment).
RESULTS

Generation of Substrates for *in vitro* Mismatch Repair

MutLα endonuclease activity is believed necessary during 3’-directed MMR to generate a nick 5’ to the mismatch as an entry point for the 5’-3’-specific exonuclease activity of ExoI (39). However, other results suggest additional roles for the endonuclease activity during MMR (50). To determine if the presence of a preexisting 5’ nick would obviate the requirement for MutLα endonuclease, we compared the *in vitro* repair activities of MutLα-WT and the endonucleolytic deficient MutLα-E705K.

We generated three pRO1 substrates containing an A/C mismatch. Successful repair was ascertained by the substitution of guanine for adenine and the restoration of a diagnostic HindIII restriction site in the plasmid substrate. The three substrates differed in the location of the single-strand discontinuities (nicks) necessary for strand identification *in vitro* repair. The 3’-substrate had a nick oriented 3’ to the mismatch, the 5’-substrate had a nick oriented 5’ to the mismatch, and the doubly-nicked substrate contained both the 3’ and 5’ nicks. In order to best reflect physiological conditions, and as previously shown by other studies, the single strand discontinuities were located within 150 bases of the mismatch. Specifically, we used a doubly-nicked substrate with the nicks located approximately 50 base pairs from the mismatch in either direction.

The pRO1 plasmid used in generation of the substrate was propagated in the ER2925 *E. coli* strain deficient for both Dam and Dcm methyltransferase, a condition necessary for the proper functioning of the nicking enzyme N.AlwI. Following
transformation, small scale DNA isolations were performed using cultures initiated with 24 individual colonies from the ER2925 \textit{E. coli} strain (Figure 4, lanes 1-12 & 13-24). The presence of a band migrating at approximately 1500 bp reflected the successful propagation of pRO1 and its purification as a closed circle (supercoiled) molecule. Also present was a band migrating around 4,000 bp, typically observed in other \textit{E. coli} strains transformed with pRO1 (data not shown) and most likely indicative of plasmid dimers. In most of the ER2925 strains, this band was light in comparison to the 1500 bp band. Colony #4 was selected to inoculate a 1L LB culture for large scale DNA preparation due to its high pRO1 copy number and relatively low dimer contamination in comparison to the other colonies.

\textbf{Figure 4.} Small scale DNA preparations from 24 ER2925 colonies (lanes 1-24) transformed with pRO1. The band migrating at 1500 bp represents closed circle (supercoiled) pRO1 while the bands above 10,000 bp and at 4000 bp reflect genomic DNA and possible plasmid dimers, respectively.
In order to generate mismatch substrate for the \textit{in vitro} repair reactions, the preparation scheme outlined in \textit{Figure 5 (Materials and Methods)} was followed.

\textbf{Figure 5}. A schematic outlining the generation of mismatch substrate containing a single strand discontinuity. First, the pRO1 substrate (A) is nicked twice (B - the bases between the nicks are shown in red). Then, the bases are removed using a complementary oligonucleotide (shown in purple), creating the gapped substrate (C). The non-complementary oligonucleotide (green) is then inserted to generate the mismatch substrate (D - mismatch is indicated by the triangular kinks). Finally, the single strand discontinuity is created by adding the appropriate nicking restriction endonuclease (E).

Briefly, the pRO1 obtained from the large scale DNA preparation was purified and then nicked by incubation with Nt.BstNB1 (\textit{Figure 6}), forming two nicks enclosing a 32 bp sequence. The sequence was removed in the presence of excess oligonucleotide complementary (referred to as competitor oligonucleotide in \textit{Materials and Methods}) and replaced by an identical nucleotide sequence containing a single non-complementary base (resulting in mismatch formation). Finally, the mismatch substrate was nicked with N.Alw1, N.Bbvca1A, or a combination thereof to form the 3’, 5’, and doubly nicked substrates.
Additional steps were interjected in the scheme described above in order to ensure a pure A/C substrate. Upon creation of the gapped substrate, any contaminating non-gapped plasmid was linearized with HpaI (restriction site located in the sequence of the gapped closed circular plasmid), marked by the appearance of an additional band migrating at 2.2 kb (equal to size of plasmid; data not shown). The linearized bands were degraded by ATP-dependent DNase (Epicentre, Madison, WI).

The non-complementary oligonucleotide was then ligated into the gapped pRO1, forming the mismatched plasmid (Figure 7). The successful insertion of the oligonucleotide was marked by the appearance of a band migrating at 1500 bp (closed circle plasmid), and the disappearance of a band migrating approximately band at 3000 bp (compare lanes 1 and 2, Figure 7). In lane 1, there was a 1500 bp migrating band, a contaminant of unknown origin. The impurity was probably also present in lane 2, indicating that the amount of mismatch pRO1 produced was slightly less than the amount reflected by the intensity of the 1500 bp band in the gel. The pRO1 substrate and other
impurities were then cesium banded to purify the supercoiled, mismatch plasmid away from the other impurities.

In the final step, the mismatch substrate was either nicked with N.AlwI (3’), N.Bbvc1A (5’), or both enzymes (Figure 8). Complete conversion of the 1500 bp band (supercoiled plasmid) to a 4000bp band (open circle) marked the successful nicking of the substrate. An unidentified band migrating at 2200 bp also was visible in all three nicked substrates. The substrates were still used for repair reactions because the amount of impurity represented a relatively small percentage (7 – 15 %) of the total mismatch substrate.
Figure 8. Nicking of the mismatch pRO1 substrate. The mismatch pRO1 (lane 1) was either digested with N.AlwI to form the 3’ nick (lane 2), N.Bbvc1A to form the 5’ nick (lane 3), or both enzymes to form the doubly nicked substrate (lane 4). An unidentified band, not present in the originally supercoiled mismatch substrate, was observed in all three nicked substrates.

In vitro Mismatch Repair Reactions

Testing the substrate for repair

After generation of the A/C substrate with the necessary nicks, we used in vitro MMR reactions to investigate the ability of the MutLα-E705K mutant to repair a mismatch substrate containing nicks both 3’ and 5’ to the mismatch. We hypothesized that the provision of a 5’ nick should obviate the need for the endonucleolytic activity of MutLα, and thus similar levels of repair should be observed between MutLα-WT and MutLα-E705K mutant complexes. The inability, or reduced ability, of E705K to repair the doubly nicked substrate would be consistent with other requirements for MutLα endonucleolytic activity during MMR. Described below are the preliminary results for the in vitro reactions with the wildtype and mutant forms. To compare wildtype and mutant forms of MutLα, repair reactions were reconstituted by combining protein
extracts of MutLα – deficient cells with MutLα-WT or MutLα-E705K. The MutLα-deficient extract (present in excess) contained all factors needed for repair except MutLα-WT or MutLα-E705K. Successful repair (substitution of a guanine for the mismatched adenine) was visualized by the restoration of a diagnostic HindIII restriction site in the plasmid substrate. All substrates also were linearized by BanI digestion to facilitate quantification of the repaired fraction. In the absence of repair, a single band migrating at 2.2 kb was expected, whereas in the case of successful repair, two bands migrating around 1100 bp (the result of the double BanI and HindIII digest) were visible. Repair levels equaled the sum of the intensities of the 1100 bp bands divided by the sum of the intensities of the 1100 bp bands and the 2200 bp band (pRO1 linearized only by BanI, since repair did not restore a HindIII site).

As predicted, MutLα-WT was capable of supporting repair on the 3’ A/C, 5’ A/C, and doubly nicked A/C substrates (Figure 9). It was also capable of supporting repair on an additional substrate containing a G/T mismatch and a 3’ nick (generated by S. Nelson in the Buermeyer lab), which was included as a positive control (Figure 9). Levels of repair on the different substrates ranged from 25% to 37% and also reflect the bidirectional repair capabilities of MMR in cytoplasmic extracts. Although previous experiments (Buermeyer lab) have shown repair levels of up to 70% using the 3’ nicked G/T substrate and MutLα-WT, the repair levels demonstrated here should be sufficient to allow comparison of repair efficiencies between MutLα-WT and MutLα-E705K on the three substrates.
Comparing repair levels of MutLα-WT and MutLα-E705 (deficient for endonucleolytic activity) on four different substrates.

We next performed repair reactions reconstituted with MutLα-WT or MutLα-E705K (Figure 10). In this experiment, MutLα-WT supported repair levels of 14%, 5%, and 10% on the A/C 3’, A/C 5’, and A/C doubly nicked substrates, respectively. In
comparison, MutLα-E705K demonstrated little to no repair on the same three substrates. Although this suggests an inability of MutLα-E705K (similar levels of repair in the presence of no MutLα) to support repair of the three substrates, the repair levels of MutLα-WT were too low to show significant differences between the wildtype and mutant complexes. The reason for the difference in apparent repair capacity of MutLα-WT is not known. Since it was demonstrated earlier that MutLα-WT was capable of supporting levels of repair significant enough to make a comparison between the two complexes (between 25 – 38%) on the three substrates (Figure 9), the experiments were repeated several times. In the following experiments, reactions reconstituted with MutLα-WT, MutLα-E705K, or no MutLα were compared with the A/C 3’, A/C 5’, A/C doubly nicked, and G/T 3’ substrates.

In the subsequent experiments, two observations were made. First, there was incomplete digestion of the A/C pRO1 substrate possibly due to incomplete resuspension of the plasmid following phenol extractions when resuspended in RNase A and incubated with both BanI and HindIII. Incomplete resuspension and digestion of the substrates used...
in the repair reactions resulted in an inability to determine if there was repair (Figure 11). Additional experiments verified that the A/C substrate was able to completely linearize in the presence of BanI and HindIII, indicating that either the presence of reagents unique to the repair reactions inhibited the enzymatic activity of the restriction enzymes or that incomplete resuspension was the primary problem (data not shown). Interestingly, no digestion or resuspension problems were experienced with the G/T 3’ substrate (Figure 11). The difference between the A/C and G/T substrates was that the A/C substrate was purified from a Dcm<sup>−/−</sup>, Dam<sup>−/−</sup> E. coli (deficient for both the Dam and Dcm methyltransferases) and that the G/T substrate was incubated with BanI and XhoI instead of BanI and HindIII (correction of the G/T mismatch resulted in the restoration of a diagnostic XhoI restriction site).

Second, the A/C substrates nicked with N.Bbvc1A (5’ nick) consistently developed two unique bands migrating slightly above 1000 bp and below 700 bp after <i>in vitro</i> MMR processing (Figure 11). The 3’ A/C substrate (nicked with N.AlwI) occasionally had the unique band migrating slightly above 1000 bp after repair processing. The intensities of these bands were insignificant in comparison to the A/C substrate; thus, it was unlikely that the source of the bands interfered with the repair reactions.
Figure 11. Lanes 1-3: 3’ A/C; lanes 4-6: 5’ A/C; lanes 7-9: 3’/5’ A/C; lane 10 – linearized 3’ G/T pRO1 unprocessed through MMR reaction; lanes 11-13 – 3’ G/T controls. The bands around 4000 bp and 5-6000 bp reflect incomplete digestion and resuspension of the pRO1 substrate. Also, note the presence of a unique band around above 1000 bp in lanes 4-9 (substrates containing 5’ nicks). In this image, only lanes 8 and 11-13 can be evaluated for repair due to complete resuspension and linearization. E705K did not show repair on the 3’/5’ substrate and the 3’ G/T substrate while WT did repair the 3’ G/T substrate with a 66% repair efficiency.

Although the original experiment comparing the repair levels of MutLα-WT and MutLα-E705K on the 3 A/C substrates was not reproduced in one experiment, the combined data (Figure 12) of the three experiments (each of which had problems with digestion and resuspension) suggested that MutLα-WT showed significantly higher levels of repair on the 3 A/C and G/T substrates, while no repair was detected in reactions reconstituted with MutLα-E705K. The G/T positive and negative controls did work in the experiments (Figure 11, lanes 11-13) – MutLα-WT repaired 3’ G/T, while no MutLα or MutLα-E705K did not, suggesting possibly a problem with the substrates generated in the Dam−/−, Dcm−/− E. coli strain for this work. In addition, all experiments were performed with a single preparation of MutLα-WT and MutLα-E705K. Thus, we cannot rule out the
possible influence of a functionally defective form of the proteins used in the experiments in this report.

Figure 12. Repair reactions on the 3', 5', and doubly nicked substrates in the presence of MutLα-WT, MutLα-E705K, or no MutLα. Results are based on compiled data from three experiments. Resuspension problems with the A/C 3' and A/C 5' substrates in the presence of MutLα-WT resulted in an inability to determine repair levels.

Accepting the limitations and caveats (as described above) of our work, our preliminary data are not in agreement with our initial hypothesis because the presence of a 5' nick did not apparently obviate the need for MutLα endonucleolytic activity. MutLα-E705K, a recombinant MutLα deficient for endonucleolytic activity, did not show repair on the 3' A/C, 5' A/C, doubly nicked A/C, or 3' G/T substrates. This suggests additional needs for MutLα endonucleolytic activity during in vitro MMR.
DISCUSSION

Lynch individuals have a predisposition to developing colorectal and other cancers due to inherited defects in their mismatch repair system (1-7). Although mutations in MMR have been directly implicated in Lynch Syndrome, the precise mechanism(s) of MMR functions have yet to be elucidated. One essential complex, MutLα, has been associated with over fifty percent of all Lynch mutations (8, 9). Despite the fact that the majority of these mutations are found on MLH1 (8, 9), PMS2, and its yeast homolog (pms1), are crucial for the proper functioning of the MutL complexes in eukaryotic MMR (45-49). Most research to date has been focused on the role of MLH1 in MMR because its functions are non-redundant. Nevertheless, mutations in PMS2 can still significantly affect MMR and an investigation of such mutations is crucial to gain a complete understanding of the MMR system.

In mammalian cells, PMS2 dimerizes with MLH1 to form the MutLα complex (29-30), contributing to the correction of base insertion and deletion loops (IDLs) and single-base mismatches (36). In addition, MutLα is necessary to trigger apoptosis in response to several types of DNA damage (12-13). Both functions are likely important in MMR-dependent suppression of cancer risk.

A PMS2 mutation of interest is when residue 705, a glutamic acid, is replaced by a lysine (PMS2-E705K). PMS2 residue 705 is part of a highly conserved motif, the significance of which was unknown until recently (39). Using a reconstituted in vitro MMR system, Kaydrov et al. have determined that MutLα has an endonucleolytic activity necessary for in vitro MMR and that the PMS2-E705K inactivates the hMutLα...
heterodimer’s ability to sufficiently function as an endonuclease. Among other proteins essential for MMR, the reconstituted system included hMutSα, hMutLα, RFC, RPA, the 5’-directed ExoI, and PCNA. Repair activities were measured using a plasmid substrate with a single-strand discontinuity (a condition necessary for directed in vitro MMR) oriented 3’ to the mismatch. According to Kaydrov et al, the endonucleolytic activity, activated in a MutSα-RFC-PCNA dependent manner, was only essential for the formation of a nick 5’ to the mismatch, creating a loading site for the 5’ directed ExoI (39).

In related studies, Deschenes et al further characterized hMutLα-E705K as a stable heterodimer and with the PMS2-E705K monomer encoded for by a recessive allele. They did show, however, that PMS2-deficient mouse embryonic fibroblasts transfected to express PMS2-E705K exhibited increased levels of microsatellite instability in comparison to cells transfected with wildtype PMS2 (PMS2). Furthermore, using an in vitro MMR assay with a single-stranded discontinuity oriented 5’ to the mismatch, they showed that hMutLα-E705K was unable to restore MMR activity to cytoplasmic extracts from hMutLα-deficient cells to display repair activity (50). This finding is somewhat at odds with that of Kaydrov et al. Based on the purified in vitro MMR assay used by Kaydrov, the provision of the 5’ nick in a plasmid substrate should obviate the need for MutLα endonucleolytic activity and similar levels of MMR should be observed in the presence of either hMutLα or hMutLα-E705K.

In this study, we tested Kadyrov and Modrich’s proposed mechanism regarding the need for MutLα endonucleolytic activity by comparing the repair efficiencies of hMutLα-E705K to a wild type MutLα in an in vitro repair system using one of three substrates. A single-strand discontinuity was oriented either 3’ or 5’ to the mismatch on
the 3' and 5' substrates, respectively, whereas a doubly-nicked substrate contained both of the 3' and 5' discontinuities. We hypothesized that the recombinant MutLα deficient for endonucleolytic activity, in comparison to wild type MutLα, should show little to no repair activity on the 3’ substrate, while the provision of a 5’ nick should obviate the need for endonucleolytic activity and thus, repair activity should be observed on the 5’ and doubly-nicked substrates. The hypothesis is based on a parallel model that elucidated the role of the MutH endonuclease in *E. coli*. Hypothesized to be an endonuclease necessary for the loading of one of several exonucleases, similar levels of MMR were measured in both a reconstituted *E. coli* with all necessary MMR complexes and a system excluding MutH, but in the presence of a nicked DNA substrate (20, 22, 53, 54).

Appropriately nicked mismatch substrates were generated using a pRO1 plasmid purified from an *E. coli* strain deficient for *Dam*<sup>−/−</sup>, *Dcm*<sup>−/−</sup> methyltransferases as outlined by Hays and Wang (52). We performed the *in vitro* MMR reactions reconstituted with mouse MP1 cell (*Mlh1*<sup>−/−</sup>, *Pms2*<sup>−/−</sup>) cytoplasmic extracts and either *h*MutLα-WT, *h*MutLα-E705K mutant, or no *h*MutLα. Successful MMR was ascertained by the restoration of a diagnostic restriction site in the plasmid substrate, and levels of MMR were analyzed by restriction digest, agarose gel electrophoresis, and digital imaging to measure band intensities.

We present preliminary data showing that *h*MutLα-E705K does not support an *in vitro* repair system when provided with a 3’, 5’ or doubly nicked substrate. In comparison, the *h*MutLα WT protein complex did support significant repair on all three substrates. These findings are consistent with those of Deschenes and colleagues (50) since the provision of a 5’ nicked substrate showed repair in the presence of *h*MutLα WT,
but not in the presence of hMutLα-E705K. Furthermore, the data did not support our hypothesis that the provision of a 5’ nick (on the single and double nicked substrates) would obviate the need for endonucleolytic activity since the recombinant mutant deficient for endonucleolytic activity (hMutLα-E705K) did not show repair on the described substrates.

In order to assess the quality of the MMR assay, the repair levels of the hMutLα WT protein complex were measured on four substrates, including the three substrates used in this experiment and a G/T mismatch substrate with a 3’ nick used in other laboratory experiments to serve as a control (Results, Figure 9B). The repair level of the hMutLα WT protein complex (~36%) on the G/T 3’ substrate was lower than the levels measured previously in the lab (60-70%; data not included). The three substrates generated for this experiment had comparable levels of repair to the G/T substrate (25-38%). Although the repair levels were lower than those usually measured in the lab, they should have been high enough to measure any significant differences between hMutLα-WT and hMutLα-E705K. However, technical difficulties were encountered in performing the repair experiments. In particular, absolute levels of repair with hMutLα-WT varied, and in some experiments, repair could not be evaluated for all reactions due to incomplete digestion of the purified substrates. The quantification of the repair capacities of hMutLα WT and hMutLα-E705K was based primarily on one experiment (Results, Figure 10). However, compiled data from additional experiments were in agreement with the quantification of the repair capacities determined in the initial experiment (Results, Figure 12). hMutLα WT did show repair on the 5’, 3’, and doubly nicked substrates, however, the repair levels were significantly lower than those
measured in other assays (compare Figure 9B and 10 in Results). The addition of
hMutLα-E705K or no hMutLα resulted in a reconstituted MMR system unable to exhibit
repair activity on the three substrates. In the remaining three experiments, complications
arose with the resuspension and digestion of the 3’, 5’, and doubly nicked plasmids for all
three experiments (data included for one – Results, Figure 11). The restriction enzymes
used in the assay were shown to effectively digest the mismatch substrates and the
complications with digestion were unique to the in vitro repair reactions (data not
shown). A preliminary data set, based on the compiled gel images of the three
experiments, showed that hMutLα WT supported repair on all three substrates while
hMutLα-E705K and the lack of hMutLα did not.

In addition, gel images of the 5’ nicked substrates processed for in vitro MMR
exhibited two unique bands (Results, Figure 11). The cause of these bands was not
determined; however, they were present in significantly less quantities than the mismatch
substrate and most likely did not affect the repair reactions.

Finally, the preliminary results in this study were based on one set of transient
overexpression (TOE) preparations of hMutLα-WT and hMutLα-E705K and one
preparation of pRO1-derived substrates. Before validating any conclusions, the
experiments need to be repeated with additional protein and mismatch substrate
preparations.

Despite the technical issues discussed above, the preliminary results obtained
were in agreement with those found by Deschenes et al (50) because the presence of a 5’
nick did not obviate the need for MutLα endonucleolytic activity. The glutamic acid at
residue 705 is part of a highly conserved sequence crucial for the latent, endonucleolytic
activity of MutLα (39); however, alternative explanations for our findings consistent with our initial hypothesis are possible. For example, E705K substitution may interfere with other functions of MutLα necessary for repair.

PMS2-E705K is stabilized by MLH1 and does have weak ATPase activity (45, 46), suggesting that it does still complex with MLH1 in vivo and should be capable of ATP-driven conformational changes in structure. However, the E705K substitution might interfere with other protein–protein interactions. Additional experiments assaying binding to other MMR complexes using recombinant MutLα forms might elucidate such effects. Alternatively, other mutants deficient in endonucleolytic activity could be generated and tested for activity with our doubly nicked substrate.

It also has been shown that MutLα is not needed for in vitro MMR given a 5’ substrate; however, its presence enhances repair activity (42, 43, 39). Genschel and Modrich demonstrate that the base pair hydrolysis of the highly processive ExoI is drastically limited in an in vitro system through supplementation of RPA with MutSα, MutLα, and EXOI. Furthermore, they show that between 190 to 360 nucleotides are removed by the system, consistent with the results obtained with HeLa extracts where excision terminates at several sites on average about 150 beyond the mismatch (in their system, the mismatch and the nick were separated by 128 bases) (42, 55). In the recent study by Kaydrov et al. (39), the latent endonucleolytic activity of MutLα results in the provision of two nicks in the direction of the mismatch (on both a 3’ and 5’ nicked substrate), with the farther nick being termed as the distal nick (Figure 13).
Figure 13. Mechanisms illustrating the role of MutLα endonucleolytic activity in MMR. For both 3’ and 5’ directed repair, the endonuclease creates two single strand discontinuities in the direction of the mismatch, with the distal discontinuity (large red arrow) located on the other side of the mismatch. Using the appropriate single-strand breaks, Deschenes et al. propose that ExoI excises a region of the DNA containing the mismatch, with its processivity limited by the human single-stranded binding protein RPA. After strand excision, the MMR continues as described earlier. (Figure source – 39)

Instead of solely providing loading sites for ExoI, the MutLα endonuclease, in combination with RPA, may play a role in marking the area to be excised. On circular substrates, MMR uses the shortest available route to repair the DNA by excising the path containing the strand break and the mismatch versus the majority of the circular substrate, even though excision in the opposite direction also could be used to excise the mismatch. In a linear mammalian chromosome however, excision of the strand in a direction away from the mismatch would not result in the correction of the mismatch. In essence, it is crucial that the MMR system excises the DNA in the correct direction, perhaps assisted by the DNA discontinuities formed by the endonuclease. Such a role might explain the unexpected requirement for endonucleolytic activity in 5’ nicked substrates. Perhaps
nicking by MutLα is necessary to mark the area to be excised by generating nicks on the opposite side of the mismatch from the preexisting 5’ nick. If true, repair excision in the complete system might not be initiated until additional “marking” nicks are generated. Additional experimentation will be needed to completely elucidate the role(s) of MutLα activity in eukaryotic MMR.
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