MutL Involvement in two DNA Repair Pathways: Methyl-Directed Mismatch Repair and Very Short Patch Repair

Adam Brian Robertson

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology.

Chapel Hill
2007

Approved by:
Steven W Matson
Patricia Pukkila
Jeff Sekelsky
Dorothy Erie
Matthew Redinbo
Roel Schaaper
MutL Involvement in two DNA Repair Pathways: Methyl-Directed Mismatch Repair and Very Short Patch Repair

(Under the direction of Steven Matson)

MutL is an essential component of the *Escherichia coli* mismatch repair (MMR) pathway and has been shown to interact with nearly every protein involved in the MMR pathway including MutS, MutH and UvrD.

The DNA binding properties of MutL in MMR have been controversial for nearly two decades. The DNA binding requirement by MutL was addressed using a point mutant of MutL—MutL-R266E—which binds DNA poorly. Our results demonstrate, using data obtained from biochemical and genetic analyses with MutL-R266E, that MutL must bind DNA to function in MMR. Therefore, we have attempted to resolve the controversial issue regarding the DNA binding properties of MutL.

MutL is thought to be the master coordinator of MMR, accomplishing this regulatory role through its ATP binding, hydrolysis, and release cycle. Using a point mutant of MutL that cannot bind ATP—MutL-D58A, we show that this mutant is unable to interact with MutS, and MutH and UvrD. Additionally, we have identified the step in MMR that MutL must hydrolyze its bound ATP in order to continue its normal function in MMR. We accomplished this using another point mutant of MutL that cannot hydrolyze ATP—MutL-E29A. These results lend support for the hypothesis of temporal regulation of MMR by the
MutL ATPase. Using MutL-E29A and MutL-D58A in biochemical and genetic assays, we have attempted to identify a role for the MutL ATPase in MMR. These data suggest that MutL must be bound to ATP to function in MMR prior to the loading of the UvrD helicase. Additionally, these data implicate ATP hydrolysis by MutL at a step in the MMR pathway after the UvrD helicase is loaded onto the DNA by MutL.

Previous studies have shown that MutL serves to augment the repair efficiency of the very short patch (VSP) repair pathway. We have reconstituted the minimal VSP repair pathway \textit{in vitro}. We suggest a biochemical role for MutL in VSP repair. The reconstitution of this system will allow further studies addressing the function of MutL in VSP repair.
For mom
ACKNOWLEDGEMENTS

The work presented in this dissertation represents slightly more than four years of research, and an even larger portion of my life has been devoted to the creation of this book. Even though my name appears on the title page of this work I would be remiss if I believed that many other people did not have a large part in the production of this dissertation. A great number of individuals have indeed made this work possible and while there are simply too many people to mention individually I would like to mention some of the individuals who had an extremely significant role in the production of this dissertation.

Of course I must begin by acknowledging my family beginning with my mother and father, not just because without them I would not be on the planet, but because they have shaped me into the person that I am today. My father instilled in me the value of hard work and I believe that his influence can be seen in this dissertation—one thing that I will always remember from my father is that he said “if something is worth doing, it is worth doing right.” Of course I can never forget the influence of my brother—Mark—on everything that I do. I have the greatest respect for my brother and my brother’s opinion; he believes that my endeavor to obtain my PhD in biology is significant. I have always tried to make my brother proud and he is a tough man to please and is always pushing me to be the best that I can be. Mark is enthused about my work and I believe that I have lived up to his expectations with the completion of this dissertation.

I would like to extend a warm thank you to each of the members of my committee—Steve Matson, Jeff Sekelsky, Patricia Pukkila, Dorothy Erie, Roel Schaaper, and Matthew
Redinbo. I should note that everyone on my committee served voluntarily, which alone is extraordinary. My committee is a truly wonderful group; they seem to always have time to speak with me about my research despite their incredibly busy schedules. Their helpful scientific discussions have made the achievements in my graduate career possible. I simply cannot thank these outstanding individuals enough for all their help in making this dissertation possible.

During my graduate career I have worked with many individuals each of whom have had an incredible influence on me aiding to my success in graduate school and personal growth. Danny Monroe and Kambiz Tahmaseb have both proved to be an invaluable resource for scientific discussions and they have had a profound effect on my ability to think like a scientist. Steven Pattishall and Carly Shanahan have helped me during every step of my graduate career by providing assistance with my work and by engaging in meaningful scientific discussions with me. Mike Villareal, Neeta Goli, Dana Clifton, and Annamarie Carter have the incredible ability of making work fun and I must thank them for their help in making this dissertation possible. Debi Haisch has perhaps given me the single most important trait making the actual writing of this dissertation possible—she helped me become a better writer, teaching me that the best way to improve my writing skills is to read non-scientific books.

There are of course so many more people that have made this work possible; however, this list is so extensive that I could easily write an entire book about these individuals. Because of the vast number of people to thank, I would like to simply give a collective thank you to all my friends who have helped to make this work possible
LIST OF TABLES

Table

1. Mismatch repair proteins ............................................................... 24
2. Mutation frequencies and rates ...................................................... 55
3. MutL-E29A and MutL-D58A exhibit a mutator phenotype ............... 94
LIST OF FIGURES

Figure

1. Model for *Escherichia coli* methyl-directed mismatch repair ........................................... 25
2. Model for Human DNA Mismatch Repair ........................................................................ 26
3. Model for *Escherichia coli* very short patch repair ........................................................... 27
4. SDS-PAGE analysis of purified proteins ........................................................................... 56
5. ATP hydrolysis by MutL-R266E is not stimulated by DNA ............................................. 57
6. DNA binding by MutL and MutL-R266E ......................................................................... 59
7. Helicase II stimulation by MutL and MutL-R266E ........................................................... 61
8. ELISA measuring the affinity of MutL and MutL-R266E for helicase II ............................. 62
9. MutL-R266E partially complements a MutL deletion ....................................................... 63
10. MutL-R266E interacts with MutH ................................................................................... 64
11. SDS-PAGE analysis of MutL and MutL mutants ............................................................ 95
12. ATP hydrolysis by MutL, MutL-N33A and MutL-E29A .................................................... 96
13. MutL-E29A stimulates the helicase reaction catalyzed by UvrD ........................................ 97
14. MutL and MutL-E29A bind DNA in the presence of AMP-PNP .................................... 98
15. MutL-E29A binds ATP .................................................................................................... 99
16. MutL-E29A stimulates MutH-catalyzed nicking of heteroduplex DNA ......................... 100
17. MutL-D58A does not interact with MutS ........................................................................ 101
18. A model for MMR .......................................................................................................... 103
19. SDS-PAGE analysis of DNA polymerase I, DNA ligase I, and the Vsr endonuclease 135
20. pUC19-VSR mismatch substrate ................................................................................... 136
21. Vsr-catalyzed nicking of covalently closed circles ....................................................... 137
22. The Vsr endonuclease catalyzes the formation of a nick 5′ to the mismatched thymine within the Vsr recognition sequence ................................................................. 138

23. DNA Polymerase I and the Vsr endonuclease are sufficient to repair a G:T mismatch in the Vsr endonuclease recognition sequence .................................................................................................................. 139

24. DNA ligase I is able to seal the nick created by DNA polymerase I nick translation... 140

25. DNA ligase I competes with DNA polymerase I for the nick created by the nick translation catalyzed by DNA polymerase I ................................................................................. 142

26. MutL and/or MutS do not stimulate or inhibit the Vsr endonuclease-catalyzed nicking reaction................................................................................................................................. 143

27. MutS and MutL do not shorten VSP repair tract lengths in vitro ......................................................... 145

28. A model for VSP repair .......................................................................................................................... 146
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-[³²P]ATP</td>
<td>Adenosine-5′-Triphosphate $^{32}$P labeled on the $\alpha$ phosphate</td>
</tr>
<tr>
<td>$\alpha$-[³²P]dCMP</td>
<td>2′-Deoxycytidine-5′-Monophosphate $^{32}$P labeled on the $\alpha$ phosphate</td>
</tr>
<tr>
<td>$\alpha$-[³²P]dCTP</td>
<td>2′-Deoxycytidine-5′-Triphosphate $^{32}$P labeled on the $\alpha$ phosphate</td>
</tr>
<tr>
<td>$\gamma$-[³²P]ATP</td>
<td>Adenosine-5′-Triphosphate $^{32}$P labeled on the $\gamma$ phosphate</td>
</tr>
<tr>
<td>⁵meC</td>
<td>5-Methyl cytosine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine-5′-Diphosphate</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>Adenosine-5′-(β,γ-imido)triphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5′-Triphosphate</td>
</tr>
<tr>
<td>b</td>
<td>base</td>
</tr>
<tr>
<td>βME</td>
<td>2-Mercaptoethanol</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>CBD</td>
<td>Chitin Binding Domain</td>
</tr>
<tr>
<td>CC</td>
<td>Covalently Closed</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-Coupled Device</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>Chloroform</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>CsCl</td>
<td>Cesium Chloride</td>
</tr>
<tr>
<td>dATP</td>
<td>2′-Deoxyadenosine-5′-Triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>2′-Deoxycytidine-5′-Triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>destilled deionized water</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>dGTP</td>
<td>2′-Deoxyguanidine-5′-Triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-Pi</td>
<td>DNA Phosphates</td>
</tr>
<tr>
<td>dNTPs</td>
<td>2′-Deoxyribonucleic acid-5′-Triphosphates</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>2′-Deoxythymine-5′-Triphosphate</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetraacetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>fmol</td>
<td>femtomole</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GHKL</td>
<td>Gyrase B, Heat Shock Protein, Histidine Kinase, MutL ATPase</td>
</tr>
<tr>
<td></td>
<td><strong>Superfamily</strong></td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>H₃PO₄</td>
<td>Phosphoric Acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HCOOH</td>
<td>Formic Acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-Hydroxyethyl]piperazine-N′-[2-Ethanesulfonic Acid]</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary Nonpolyposis Colon Cancer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>IDL</td>
<td>Insertion/deletion loop</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-Thiogalactopyranoside</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>K_{1/2}</td>
<td>½ maximal stimulation</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>kcal</td>
<td>kilocalorie</td>
</tr>
<tr>
<td>k_{cat}</td>
<td>Turnover number</td>
</tr>
<tr>
<td>K_D</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium Hydroxide</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani Broth</td>
</tr>
<tr>
<td>LC20</td>
<td>MutL Carboxyl terminal 183 amino acids</td>
</tr>
<tr>
<td>LiCl</td>
<td>Lithium Chloride</td>
</tr>
<tr>
<td>LN40</td>
<td>MutL Amino terminal 331 amino acids</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MgCl_2</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>MgOAc</td>
<td>Magnesium Acetate</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
</tbody>
</table>
Mlh  MutL Homologue
mM  millimolar
MMR  Mismatch Repair
MutL-D58A  Point Mutant of MutL glutamic acid 58 substituted with alanine
MutL-D58N  Point Mutant of MutL glutamic acid 58 substituted with asparagine
MutL-E29A  Point Mutant of MutL aspartic acid 29 substituted with alanine
MutL-E32K  Point Mutant of MutL aspartic acid 32 substituted with lysine
MutL-N33A  Point Mutant of MutL asparagine 33 substituted with alanine
MutL-R266E  Point Mutant of MutL arginine 266 substituted with aspartic acid
MutL-R95F  Point Mutant of MutL arginine 95 substituted with phenylalanine
MWCO  Molecular Weight Cut Off
NaCl  Sodium Chloride
NAD⁺  β-Nicotinamide adenine dinucleotide
NaF  Sodium Flouride
NaOH  Sodium Hydroxide
NaPi  Sodium Phosphate
NaPO₄  Sodium Phosphate
ng  nanogram
nm  nanometer
nM  nanomolar
nmol  nanomole
°C  degrees Celsius
PCR  Polymerase Chain Reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Power of the hydrogen ion concentration</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>pmol</td>
<td>picomole</td>
</tr>
<tr>
<td>Pms</td>
<td>Postmeiotic segregation</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PNPP</td>
<td>p-Nitrophenyl Phosphate</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>Rif&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Rifampicin resistant</td>
</tr>
<tr>
<td>Rif&lt;sup&gt;s&lt;/sup&gt;</td>
<td>Rifampicin sensitive</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sec-Butanol</td>
<td>secondary butanol</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single stranded DNA</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris[hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Polyoxyethylene (20) sorbitan monolaurate</td>
</tr>
<tr>
<td>μcal</td>
<td>microcalorie</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microliter</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>USP</td>
<td>United States Parmaconeia</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UvrD-K35M</td>
<td>Point mutant of UvrD lysine 35 substituted with a methionine</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>VSP</td>
<td>Very short patch</td>
</tr>
<tr>
<td>W</td>
<td>Watt</td>
</tr>
<tr>
<td>W (DNA sequences)</td>
<td>Weak (A or T)</td>
</tr>
</tbody>
</table>
Chapter 1
MutL and Genomic Stability

Deoxyribonucleic acid (DNA) is an information carrying polymer that contains the genetic instructions for cellular developmental and physiological processes. Because DNA is such an essential component of most organisms the information that this molecule carries must remain intact. Nearly every organism studied to date has developed at least one mechanism to maintain the integrity of DNA and many organisms have evolved several biochemical pathways designated to maintaining genomic integrity. The Escherichia coli bacterium makes a useful model organism for the study of many of these mechanisms that maintain the integrity of the information contained in the DNA. I have studied two pathways that maintain genetic integrity in the E. coli genome—methyl-directed mismatch repair (MMR) and very short patch (VSP) repair. The focus of my work has been to understand the role of the MutL protein in these two repair pathways.

Discovery of MutL

MutL was initially discovered in a genetic screen to identify mutator genes/proteins in E. coli (1). Since then the MutL protein has been extensively studied in both prokaryotic and eukaryotic systems (reviewed by (2-4)). MutL has been most extensively studied for its role
in methyl-directed mismatch repair. However, MutL has also been shown to be necessary or has the ability to enhance many other processes in a bacterial cell. These processes include: an essential role in MMR (for recent reviews see (2-4)), suppression of homologous recombination (5,6) and homeologous recombination (7), suppression of the excision of certain transposons from the bacterial chromosome (8), and enhancement of the VSP repair pathway (9).

The MutL Protein

The MutL protein in E. coli forms a homodimer (10). As shown by mutational analysis, a monomer of MutL cannot function in the methyl-directed mismatch repair pathway (11,12) and it remains unknown if a MutL monomer can function in very short patch repair. The MutL protein is one of the founding members of the GHKL (Gyrase B, Heat Shock Protein, Histidine Kinase, and MutL) family of ATPases (13). MutL has a DNA-stimulated ATPase activity which is essential for the protein to function in MMR (14,15).

MutL interacts with single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) with a low affinity (16-18). This affinity for DNA is greatly increased by the addition of an adenine nucleotide (16,18). MutL has been shown to contain interaction domains that allow it to interact with the MMR proteins MutS (10,19), MutH (20-22), and UvrD (23) and the VSP repair protein—the Vsr Endonuclease (24-26). This dissertation will discuss the involvement of the MutL protein in two excision repair pathways—methyl-directed mismatch repair and very short patch repair.
*E. coli* MutL has been crystallized in two fragments—the first fragment crystallized is the amino terminal 331 residues, termed the LN40 based upon the molecular mass crystallized (27). The second fragment crystallized is the carboxyl terminal 183 amino acids, termed the LC20 (28). The LN40 fragment contains the ATP binding and hydrolysis domain of MutL (discussed in chapter 3). This fragment also has the ability to bind to ssDNA and can activate the MutH endonuclease in the presence of a mismatch, MutS, and ATP. The LN40 fragment of MutL is a monomer in solution however upon the addition of a nonhydrolyzable analogue of ATP—AMP-PNP the LN40 fragment forms a dimer (27). The LC20 forms a dimer in solution and contains no intrinsic ATPase activity. Additionally, the LC20 fragment is unable to interact with ssDNA. The two fragments of MutL, when superimposed upon each other, form a central channel that is large enough to accommodate between 2 and 4 duplexed DNA strands. Facing into this central channel are two arginine residues (R266), one from each subunit of the dimer (28), which are known to interact with DNA (16) (discussed in chapter 2) and it is also possible that other residues inside this channel interact with DNA.

Eukaryotes have many homologues of the MutL protein—Mlh1, Mlh2, and Mlh3 (*MutL Homologue*) which were identified by sequence homology to *E. coli* MutL and Pms1 and Pms2 (*Post meiotic segregation*) which were initially identified because mutants of these proteins gave rise to an increased number of post meiotic segregation events and showed sequence homology to *E. coli* MutL (29). In eukaryotes, these MutL homologues heterodimerize to form three discrete heterodimers—MutLα (hMlh1-hPms2 dimer in humans, yMlh1-yPms1 in yeast) (30-35), MutLβ (hPms1-hMlh1 in humans, yMlh1-yMlh3) (31,36,37), and MutLγ (hMlh1-hMlh3 in humans, yMlh1-yMlh2) (38). MutLα repairs
approximately 90% of the mismatches and insertion/deletion loops (IDLs) created during replication (36,38). MutLγ has also been shown to assist in the repair of mismatched bases and IDLs (38). It remains unclear what role, if any, MutLβ plays in mismatch repair.

**Methyl-Directed Mismatch Repair**

The methyl-directed mismatch repair (MMR) system in *E. coli* functions to correct base mismatches and IDLs that arise due to the incorporation of an incorrect base or slippage by a DNA polymerase during DNA replication (reviewed in (39)). Thus, the MMR pathway increases the fidelity of *E. coli* replication by 2-3 orders of magnitude (40-42) thereby increasing genomic stability.

The MMR pathway in *E. coli* utilizes the activities of more than 10 proteins (listed in Table 1) (for a recent review see (3,43)). A current model for MMR (14,44) (Figure 1) suggests that when an *E. coli* DNA polymerase introduces an error during replication—either a mispaired base or IDL—the asymmetric MutS homodimer recognizes and binds (45,46) to the mispaired base or IDL (47-50). MutS recruits the MutL homodimer to the mismatch-MutS complex (51). After MutL is recruited to the MutS-mismatch complex MutL interacts with the MutH monomer bound at the nearest hemi-methylated d(GATC) sequence causing MutH to nick the nascent unmethylated DNA strand, resulting in strand discrimination (20,52-55) as the newly synthesized daughter strand is transiently unmethylated after replication. After MutH catalyzes the formation of a nick MutL binds to the nick and catalyzes the loading of the UvrD helicase onto the appropriate DNA strand. UvrD unwinds the duplex in the 3’ to 5’ direction (56) towards the mismatched base until the mismatched
base has been excised (57,58). The UvrD helicase has a reported processivity of 40-50 nucleotides acting as a helicase on dsDNA (59) and approximately 3 kb as a DNA translocase on ssDNA (60). Repair tracts in *E. coli* have been measured to be 2 kb (54) but can be up to 3 kb in length (61). To account for the low relative processivity of UvrD on dsDNA versus the length of a repair tract, it has been hypothesized that many molecules of UvrD are loaded onto the duplex to unwind the duplex beyond the mismatch (58). An alternative hypothesis is that the MutL protein interacts with the UvrD helicase acting as a processivity factor for UvrD so that UvrD can unwind 3 kb of duplex DNA (62).

As UvrD unwinds the duplex towards the mismatch the appropriate exonuclease degrades the displaced ssDNA (55,63) while the single stranded binding protein binds to and protects the continuous ssDNA strand. DNA polymerase III fills in the gap created by the repair event (64) and the resulting nick is sealed by a DNA ligase (64)—restoring the integrity of the duplex. Finally the Dam methyltransferase methylates the N6 position of the adenine residue in the sequence d(GATC) (65-67), fully Dam methylating the duplex making it refractory to MMR (20,68).

**Eukaryotic Mismatch Repair**

The MMR system is conserved from bacteria to plants to humans. Many of the bacterial MMR proteins have homologues in eukaryotes (see Table 1) (reviewed by (2,43,69)). MMR in eukaryotes not only repairs nuclear replication errors but mitochondrial genomes contain a MMR system to increase the fidelity of replication of the mitochondrial genome (70), although the details of mitochondrial MMR remain poorly understood.
A model for MMR (Figure 2) in yeast and humans is similar to that of *E. coli* MMR. The primary differences between *E. coli* MMR and MMR in human and yeast cells are i) the lack of methylation as a signal for strand discrimination, ii) the substrate specificities of the MutS homologues, iii) the lack of a DNA helicase and, iv) the protein that catalyzes the formation of a nick in the duplex—in humans this role is filled by MutLα (71).

The strand discrimination signal in yeast and humans is thought to be due to strand breaks. In the lagging strand of DNA synthesis single stranded DNA breaks result from discontinuity of DNA replication. Strand discrimination on the leading strand may be the result of a recently discovered weak endonucleolytic activity intrinsic to MutLα (71) producing single stranded breaks on the leading strand of DNA synthesis. Since MutLα physically associates with the replication machinery (72), this interaction could lead to the identification of the newly synthesized strand by MutLα causing it to create a strand break on the nascent strand. The hypothesis that strand breaks direct MMR in eukaryotic cells is drawn from the observation that a covalently closed circular heteroduplex incubated in *Xenopus*, yeast and human cell extracts exhibits little or no strand repair bias (73-77). However, when a circular heteroduplex substrate containing a nick is incubated in extracts from either *Drosophila* and human cells, repair is strongly biased to the strand containing the nick (76-79). An alternative possibility that was experimentally evaluated was that 5-methylcytosine hemi-methylation in CpG sequences served as a signal for strand discrimination (80), similar to the strand discrimination signal provided by 6-methyl adenine hemi-methylation in *E. coli*. The hypothesis that 5-methyl cytosine hemi-methylation directs strand discrimination may be plausible for human cells, but it cannot account for organisms that do not contain cytosine
bases modified by methylation at the N5 position—such as *Drosophila* and yeast (81,82). To date the strand discrimination signal in eukaryotic cells remains somewhat of a mystery.

As for the differences between substrate specificity of the MutL homologues—MutSα binds to mismatches and IDLs that are 1-2 bases in length (83-85), MutSβ binds to IDLs that are greater than 2 bases in length (84,86). The absence of a DNA helicase is reconciled by the ability of the ExoI—in a mismatch, MutLα, MutSα dependent fashion—to degrade nicked DNA in the 5′ to 3′ direction (87-89) while DNA polymerase δ (72,90,91) fills the gap created by ExoI excision.

Human cells, as well as most eukaryotic cells, contain short repeated DNA sequences or microsatellites (92,93). These sequences are prone to polymerase slippage—the template strand is displaced and not copied or is copied more than once resulting in a contraction or expansion, respectively, of the DNA. The structure resulting from polymerase slippage is a loop (mentioned previously as IDLs). If this IDL is not repaired a contraction or expansion of the microsatellite DNA is produced, this phenomenon is one of the phenotypes of genomic instability (reviewed by (94)). These loop structures are effectively repaired by the mismatch repair system (reviewed by (2)) and have been studied extensively because these expansions and contractions can give rise to certain types of cancers and other heritable diseases.

**Mismatch Repair and Cancer**

The initial link between human cancer and DNA mismatch repair was demonstrated by examining a large number of families having Hereditary Nonpolyposis Colon Cancer (HNPCC or Lynch Syndrome, an autosomal dominant genetic disorder (95)). It was seen
that many of these HNPCC patients contained many mutations in repeated DNA sequences or microsatellite DNA (96-98). It was shown that individuals diagnosed with HNPCC had genomic instability in the form of expansions or contractions (to a lesser extent) of dinucleotide and trinucleotide repeats (97,98). This observation led to the hypothesis that HNPCC was caused by replication errors (98). At that time no individual gene or set of genes had been identified that give rise to genomic instability. Later, mutations in the MutS homologue—hMSH2 (99,100) and the MutL homologues—hMLH1, hPMS1, and hPMS2 (101-103) were identified that give rise to a high frequency of HNPCC linking replication errors and mismatch repair to HNPCC.

A mechanism has been proposed to explain why a deletion or a mutation in mismatch repair genes gives rise to a tumor. This mechanism proposes that IDLs and mismatches that are not repaired by a nonfunctional mismatch repair system give rise to loss of function mutations in tumor suppressor genes and/or gain of function mutations in proto-oncogenes (97,98,104). One study (104), looking at families that have a nonfunctional hMSH2 gene, found mutations in the tumor suppressor genes p53 and APC. In these families several patients had more than one mutation in the p53 or APC genes, and one had six mutations in a single tumor suppressor gene. Additionally, in benign tumors analyzed from these patients it was shown that these tumors contained only one mutation in the p53 or APC genes, leading the authors to suggest that the accumulation of mutations in these tumor suppressor genes, in the absence of mismatch repair, leads to the formation of malignant tumors.
Overview of Very Short Patch Repair

Very Short Patch (VSP) repair is a DNA repair pathway that is apparently unique to prokaryotic systems. In *E. coli* the VSP repair pathway functions to correct G:T mismatches that result from the deamination of a cytosine residue that has been modified at the N5 position by the Dcm methyltransferase (105-107). Repair tracts are estimated to be about 10-15 nucleotides in length which led to the naming of the system as very short patch repair (108,109).

Necessity for Very Short Patch Repair

Adenine, cytosine, and the Dcm methyltransferase-modified base 5-methyl cytosine are labile to spontaneous hydrolytic deamination because they contain an exocyclic amine group (110-114). Adenine upon deamination results in an hypoxanthine base (114) producing an hypoxanthine:thymine mismatch. Strand discrimination of this mismatch is possible because hypoxanthine is not a normal base in DNA. This lesion is removed by the hypoxanthine DNA glycosylase (115) and subsequently repaired by base excision repair (reviewed in (116)). When a cytosine residue undergoes spontaneous hydrolytic deamination a uracil base is produced (113) resulting in a uracil:guanine mismatch. Again strand discrimination is made possible due to the fact that uracil is not a normal base in DNA. The uracil DNA glycosylase acts on the uracil base and produces an abasic site, which is then processed by base excision repair (reviewed in (116)).
The situation is different and more complex for 5-methyl cytosine. When 5-methyl cytosine undergoes a spontaneous hydrolytic deamination event a thymine base is produced (111) yielding a thymine:guanine mismatch. Because both thymine and guanine are normal bases in DNA a separate repair pathway is required such that the thymine is always recognized as the incorrect base and repaired to the correct cytosine. The pathway responsible for this event is the very short patch repair pathway ((106,107) reviewed in (117,118)).

In *E. coli* the second cytosine in the sequence 5′-CCWGG-3′/5′-CCWGG-3′ is methylated at the N5 position by the Dcm methyltransferase (105). This cytosine is the only place in the *E. coli* genome that normally contains a 5-methyl cytosine moiety (119). Because this sequence is the only place in the *E. coli* genome that a 5-methyl cytosine is located, it follows that this sequence is the only place in the *E. coli* genome that would produce a thymine:guanine mismatch due to hydrolytic deamination of a 5-methyl cytosine residue (112). The sequence specificity of the methylation of this particular cytosine residue allows for the specific recognition of the thymine as the incorrect base within this sequence context and therefore makes strand discrimination possible.
Model for Very Short Patch Repair

VSP repair has been shown to absolutely require four proteins: the Vsr endonuclease, DNA polymerase I, a DNA ligase, and the Dcm methyltransferase (120-122). The presence of these four proteins repairs between 30 % and 67 % of the G:T mismatches in a genetic system (9,109,123)—the VSP Repair pathway is greatly enhanced by the addition of MutS and MutL increasing repair of the target mismatch to nearly 100 % (107,123).

A current model for VSP repair (figure 3) starts with the spontaneous hydrolytic deamination of a 5-methyl cytosine within the sequence 5′-C5meCWGG-3'/5′-C5meCWGG-3′ producing a thymine:guanine mismatch 5′-CTWGG-3'/5′-C5meCWGG-3′. MutS recognizes and binds this G:T mismatch in a similar manner as it would bind a G:T mismatch in mismatch repair (47-49). MutL is then recruited to the MutS-mismatch complex (50). MutL in the mismatch-MutS-MutL complex then recruits the Vsr endonuclease, DNA polymerase I, and DNA ligase I to the mismatch. It is worthy to note here that MutL has been shown to increase the Vsr endonuclease’s affinity for heteroduplex DNA in this specific sequence context (26). Additionally, one study has shown that MutL stimulates the Vsr catalyzed nicking reaction (24); however, the experimenters in this study used MutL concentrations that are well beyond physiological concentrations. It is also important to note that the Vsr endonuclease both recognizes and cleaves at a G:T mismatch in this sequence context in the absence of MutS. Thus, MutS recognition of the G:T mismatch is not essential—consistent with the fact that the mutS gene does not appear to be essential for VSP repair (See Chapter 4).
The Vsr endonuclease catalyzes the formation of a nick 5′ to the mismatched thymine residue in the sequence 5′-CTWGG-3'/5'-C\textsuperscript{5me}CWGG-3′ (24,121,124-126). After the formation of a nick by the Vsr endonuclease, DNA polymerase I is loaded onto the duplex at the nick. DNA polymerase I nick translates in the 5′ to 3′ direction using its 5′ to 3′ exonuclease activity and its 5′ to 3′ DNA polymerase activity approximately 10-15 base pairs beyond the nick (107,122,123) repairing the G:T mispair to a canonical Watson-Crick G:C base pair and leaving a ligatable nick (108,109). DNA ligase I, which has already been recruited to the repair site by MutS and MutL, then seals the nick created by the nick translation of DNA polymerase I. Finally, the repaired sequence 5′-CCWGG-3'/5'-C\textsuperscript{5me}CWGG-3′ is again methylated by the Dcm methyltransferase producing the initial sequence 5′-C\textsuperscript{5me}CWGG-3'/5'-C\textsuperscript{5me}CWGG-3′ (105).

An aspect of this model that remains unclear is how the MutS dimer, if bound to the mismatch, is physically moved from the mismatch so that the Vsr endonuclease is able to move into position such that it has access to the phosphodiester backbone allowing the Vsr endonuclease to cleave 5′ to the mismatched thymine. It is possible that the Vsr endonuclease is physically able to remove the MutS dimer from the mismatch by some yet unknown mechanism. Additionally, we believe that the VSP repair reaction is dependent upon the β-clamp protein; however, the role of the β-clamp in VSP has not been studied.

**Reason for Dcm methylation**

The necessity of Dcm methylation in *E. coli* remains unclear. Dcm methylation may actually prove to be mutagenic in *E. coli* producing higher than expected levels of G:C to
T:A transition mutations (127,128)—however, there are conflicting reports on this issue (129). It is important to note here that without Dcm methylation the VSP repair pathway is apparently useless in the *E. coli* cell since the VSP repair substrate cannot produced—there would be no 5-methyl cytosines available to undergo spontaneous hydrolytic deamination and therefore no G:T mismatches resulting from deamination.

One possible role for Dcm methylation may be that this methylation can impart resistance to cleavage by the restriction enzyme *EcoR*II and therefore this restriction modification system would protect the bacterial cell from invading parasitic DNA, such as viruses (130,131). *EcoR*II cleaves both sides of unmethylated duplex DNA containing the sequence 5′-CCWGG-3′/5′-CCWGG-3′ (132), effectively destroying the invading DNA. The *E. coli* genome is unable to be cut by the *EcoR*II endonuclease because digestion of DNA by this enzyme is blocked by Dcm methylation (133-135). The necessity of this restriction modification system for cellular defense may explain the necessity for the VSP repair pathway.

**Human T/G Repair**

Human DNA contains cytosines that are methylated at the N5 position. Approximately 5% of cytosines in higher eukaryotes are methylated (136), methylation occurs on the cytosine in the sequence CpG (where p represents a phosphate group) (137,138). Not every cytosine in a CpG sequence context is methylated (139-141). In humans this methylation is thought to be involved in gene regulation (reviewed by (142)). Cytosines in eukaryotes that are methylated at the N5 position are also labile to spontaneous hydrolytic deamination.
events—yielding a T:G mispair. Humans have no homologous VSP repair pathway; however, the repair of this deamination damage is facilitated by the thymine DNA glycosylase (143-147). The thymine DNA glycosylase removes the incorrect mispaired thymine base to produce an abasic site which is then processed by the human base excision repair pathway (for a review of human base excision repair see (148)). Effectively, the thymine DNA glycosylase and base excision repair in human cells takes the place of the VSP repair pathway.

The subject of this dissertation will revolve primarily around the involvement of the MutL protein in the methyl directed mismatch repair pathway and the very short repair pathway of *E. coli*. The second\(^1\) and third\(^2\) chapters will address intermolecular interactions of the MutL protein with DNA and ATP, respectively (Chapters 2 and 3 have been published in peer reviewed journals (14,16)). These chapters will include discussions of how these interactions affect the protein’s ability to function *in vivo*. The fourth chapter of this dissertation will demonstrate a complete reconstitution of the very short repair pathway *in vitro* including a possible role for MutL in VSP repair.

---

\(^1\) Chapter 2 has been published (Robertson, A et al. (2006) *J Biol Chem* **281**(29), 19949-19959), A significant contribution to this publication was made by Steven Pattishall.

\(^2\) Chapter 3 has been published (Robertson, A. B. et al. (2006) *J Biol Chem* **281**(13), 8399-8408), A significant contribution to this publication was made by Steven Pattishall and Erin Gibbons.
References


Table 1. Mismatch repair proteins

<table>
<thead>
<tr>
<th></th>
<th>Yeast</th>
<th>Human</th>
<th>Plants</th>
<th>Dimerization partner</th>
</tr>
</thead>
<tbody>
<tr>
<td>MutL</td>
<td>PMS1</td>
<td>PMS2</td>
<td>PMS2</td>
<td>Heterodimerizes with MLH1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Heterodimerizes with PMS1, MLH2, and MLH3</td>
</tr>
<tr>
<td></td>
<td>MLH1</td>
<td>MLH1</td>
<td>MLH1</td>
<td>MLH3</td>
</tr>
<tr>
<td></td>
<td>MLH2</td>
<td>PMS1</td>
<td>N/A</td>
<td>Heterodimerizes with MLH1</td>
</tr>
<tr>
<td></td>
<td>MLH3</td>
<td>MLH3</td>
<td>MLH3</td>
<td>Heterodimerizes with MLH1</td>
</tr>
<tr>
<td>MutS</td>
<td>MSH1</td>
<td>N/A</td>
<td>MSH1</td>
<td>Mitochondrial MMR</td>
</tr>
<tr>
<td></td>
<td>MSH2</td>
<td>MSH2</td>
<td>MSH2</td>
<td>Heterodimerizes with MSH3 and MSH6</td>
</tr>
<tr>
<td></td>
<td>MSH3</td>
<td>MSH3</td>
<td>MSH3</td>
<td>Heterodimerizes with MSH2</td>
</tr>
<tr>
<td></td>
<td>MSH4</td>
<td>MSH4</td>
<td>MSH4</td>
<td>Heterodimerizes with MSH5</td>
</tr>
<tr>
<td></td>
<td>MSH5</td>
<td>MSH5</td>
<td>MSH5</td>
<td>Heterodimerizes with MSH4</td>
</tr>
<tr>
<td></td>
<td>MSH6</td>
<td>MSH6</td>
<td>MSH6</td>
<td>Heterodimerizes with MSH2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MSH7</td>
<td>Unknown</td>
</tr>
<tr>
<td>MutH</td>
<td>N/A</td>
<td>MLH1-PMS2</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>UvrD</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>ExoI</td>
<td>N/A</td>
<td>ExoI</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>ExoX</td>
<td>N/A</td>
<td>ExoI</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>RecJ</td>
<td></td>
<td></td>
<td>5' to 3' Exonuclease of Pol δ</td>
<td></td>
</tr>
<tr>
<td>ExoVII</td>
<td></td>
<td></td>
<td>5' to 3' Exonuclease of Pol δ</td>
<td></td>
</tr>
<tr>
<td>DNA Pol III</td>
<td></td>
<td></td>
<td>Pol δ</td>
<td></td>
</tr>
<tr>
<td>SSB</td>
<td></td>
<td></td>
<td>DNA Ligase</td>
<td>DNA Ligase</td>
</tr>
</tbody>
</table>

Figure 1. Model for Escherichia coli methyl-directed mismatch repair
Figure 2. Model for Human DNA Mismatch Repair (Taken from Modrich, P. J. Biol. Chem. 2006;281:30305-30309)
Figure 3. Model for *Escherichia coli* very short patch repair
Chapter 2

The DNA Binding Activity of MutL is Essential for Methyl-directed Mismatch Repair in *Escherichia coli*

The DNA binding properties of the DNA mismatch repair protein MutL and their importance in the repair process have been controversial for nearly two decades. We have shown, through the use of a single point mutant of MutL (MutL-R266E), that DNA binding by MutL is required for dam-directed mismatch repair *in vivo*. We demonstrate that purified MutL-R266E retains wild-type biochemical properties that do not depend on DNA binding, such as basal ATP hydrolysis in the absence of DNA and the ability to interact with other mismatch repair proteins. However, purified MutL-R266E binds DNA poorly in vitro as compared to MutL and, consistent with this observation, its DNA-dependent biochemical activities, like helicase II stimulation and DNA-stimulated ATP hydrolysis, are severely affected. Finally, genetic assays show that MutL-R266E has a strong mutator phenotype demonstrating that the mutant is unable to function in dam-directed mismatch repair *in vivo*. Based upon the evidence collected we have determined that DNA binding is essential for mismatch repair function.
Introduction

Methyl-directed mismatch repair (MMR) is the primary pathway for correcting replication errors and unwanted recombination events in the *E. coli* bacterial cell (1-3). Therefore, a functional MMR pathway is essential for ensuring the integrity of the chromosome, as well as maintaining an acceptable cellular mutation rate. The primary components of the bacterial MMR system are the mutator proteins (MutL, MutS, MutH, and UvrD), several exonucleases, including ExoI, ExoVII, and RecJ (4-6), DNA polymerase III, single-stranded DNA binding protein and DNA ligase. These proteins act in concert to correct base-base mismatches after passage of the replication fork (7).

A current model of MMR (for reviews see (2,8-10) posits that the MutS protein recognizes and binds the mismatched base pair, followed by the binding of MutL to form a ternary complex (11). In *E. coli* both of these proteins function as homodimers (12,13). This complex forms a loop in the DNA (14) in search of the nearest hemi-methlyated d(GATC) site where MutH binds to provide the strand discrimination essential in methyl-directed mismatch repair. Once the MutS-MutL complex locates a MutH-bound hemi-methylated d(GATC) site, MutH is stimulated, presumably by MutL (15,16), to nick the unmethylated DNA strand resulting in discrimination of the nascent and parental DNA strands (4,17). The use of the nearest hemimethylated d(GATC) as the site of MutH-directed incision provides MMR with a bidirectional capability since this site could be located on either side of the mismatch. Helicase II (UvrD gene product) is then loaded on the appropriate strand at the nick and translocates in a 3’ to 5’ direction (18) unwinding the DNA duplex until the mismatch has been removed (19). The signal indicating sufficient DNA has been unwound to complete the repair process is not
known. One of the several nucleases, with an appropriate polarity, degrades the nascent DNA strand and single-stranded DNA binding protein binds and stabilizes the single-stranded DNA (ssDNA) template until DNA polymerase III is recruited to fill the gap. The resulting nick is sealed by DNA ligase, completing the repair process and restoring the integrity of the DNA (13).

The role of MutL protein in MMR remains to be completely defined on a mechanistic level. The protein was originally purified, using a biochemical complementation assay, as an essential component for partially reconstituted mismatch repair in cell extracts lacking MutL (12). Subsequent experiments demonstrated its interaction with MutS at a mismatch (13,20) and the solved crystal structure of the amino-terminal domain of MutL demonstrated an ATP binding/hydrolysis fold common to the GHKL group of ATP hydrolyzing enzymes (21). Purified MutL catalyzes a slow ATP hydrolysis reaction that is stimulated by the presence of ssDNA and is essential for MMR (20-22). In addition, MutL has been shown to interact with and stimulate the hemi-methylated d(GATC)-directed nicking reaction catalyzed by MutH (15,16) as well stimulating the unwinding catalyzed by UvrD (19). Thus, MutL has been characterized as the master regulator of MMR in view of its interaction with many of the proteins required for MMR.

The stimulation of MutL-catalyzed ATP hydrolysis by the addition of DNA has prompted an investigation of the DNA binding properties of MutL. Several groups have demonstrated that MutL binds to both ssDNA and double-stranded DNA (dsDNA) (20,23), while others report that MutL does not bind DNA (24). Recently the DNA binding activity of MutL has been suggested to be an artifact of in vitro assays designed to measure DNA binding (25). Indeed,
a current model for MMR postulates that DNA binding by MutL is not required for the MMR processes (26).

Using a combination of biochemical and genetic assays we present evidence to suggest that DNA binding by MutL is an absolute requirement for MMR. These studies take advantage of a mutL point mutant (MutL-R266E) in which the arginine at position 266 has been altered to a glutamic acid. Expression of this protein in a cell strain lacking functional MutL results in a mutator phenotype similar to that observed with a complete deletion of the mutL gene. The purified mutant protein, MutL-R266E, exhibits a weak ssDNA binding affinity, a weak ssDNA-stimulated ATPase activity and poor stimulation of UvrD-catalyzed DNA unwinding. Taken together, these data are consistent with a primary DNA binding defect since the purified protein is able to interact with the other MMR proteins as effectively as wild-type MutL protein. We conclude that the DNA binding activity of MutL is essential for MMR.

**Materials and Methods**

_Bacterial strains and plasmids_ – _E. coli_ BL21(DE3) (F’ _ompT_ [lon] _hsdSB_Br−_mB−_gal λDE3) was from Novagen. A derivative of the strain, BL21(DE3)_uvrD::Tn5 mutL::Tn10 has been described previously (27). The MutL gene was cloned into the pET15b expression vector (Novagen) by removing the mutL gene from pET3C-MutL (28) with BamHI and inserting the gene in pET15b cut with the same restriction enzyme. This plasmid is referred to as pET15b-MutL. The pET15b-MutL-R266E construct was a gift from Peggy Hsieh (National Institutes of Health). Constructs were sequenced to verify the coding sequence of the gene and to ensure the absence of any unintended mutations.
Protein Purification – MutL and MutL-R266E were expressed in E. coli BL21(DE3) uvrD::Tn5 mutL::Tn10 cells containing the appropriate MutL expression vector. Two liters of cells were grown at 37°C to an A<sub>600</sub> of 0.8 and protein expression was induced by the adding IPTG to 0.5 mM. After an additional four hour incubation at 37°C the cells were harvested by centrifugation, washed with cold H<sub>2</sub>O, suspended in binding buffer (50 mM NaPO<sub>4</sub> (pH 7.0), 500 mM NaCl, 5 mM imidazole, 10 % (v/v) glycerol) and frozen at –75°C until future use. Cells were thawed on ice and lysed by the addition of lysozyme to 200 μg/ml followed by incubation at 0°C for 60 min. The lysate was sonicated briefly to reduce the viscosity, applied to a TALON metal affinity resin (BD Biosciences - 1 ml of resin/L cells) and extensively washed with binding buffer. To decrease the NaCl concentration before elution, the column was washed with several column volumes of binding buffer containing 225 mM NaCl instead of 500 mM NaCl. The column was eluted using 200 mM imidazole in the low NaCl binding buffer. This step results in a substantial purification of MutL but contaminants were still present. The protein was further purified using a MonoQ ion exchange column. The protein that eluted from the TALON column was diluted three-fold with MonoQ buffer (25 mM Tris-HCl (pH 7.0), 0.1 mM EDTA (pH 8.0), 1 mM DTT, and 10 % (v/v) glycerol) containing no added NaCl to achieve a final NaCl concentration of approximately 75 mM and applied to a MonoQ column equilibrated in MonoQ buffer containing 75 mM NaCl. The protein was eluted using a gradient from 75 mM NaCl to 500 mM NaCl in MonoQ buffer. MutL was detected by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The protein eluted at approximately 180 mM NaCl and fractions containing MutL were pooled. To achieve greater than 95% purity the proteins were further purified by gel filtration using a Superdex 200 column. The
pooled fractions from the MonoQ column were concentrated using solid polyethylene glycol 20,000 and loaded onto a Superdex 200 column equilibrated with Superdex Buffer (50 mM NaPO₄ (pH 7.0), 250 mM NaCl, 0.5 mM EDTA (pH 8.0), and 10 % (v/v) glycerol). Fractions containing MutL were identified by SDS-PAGE and MutL eluted at the position expected for a MutL dimer. Final purity was assessed by SDS-PAGE (see Fig. 4). 

UvrD was purified a previously described (27) from a strain that contained an insertion in mutL to ensure that purified UvrD was not contaminated with MutL. The protein was greater than 95% pure as determined by SDS-PAGE.

**DNA substrates** – The various DNA substrates (ligands) used in DNA binding assays and DNA unwinding assays were prepared using synthetic DNA oligomers. The sequences and sources of the oligomers used in these studies were as follows: 50-mer – 5’- TTT TGG GGC GAA GTT TTA TGG TCT CCT ACC TAG GCC GGC TAT TCA GGG TT-3’ (MWG Biotech); 91-mer – 5’-AGT AGC ACC ATT ACC ATT AGC AAG GCC GGA AAC GTC ACC AAT GAA ACC ATC GAT AGC AGC ACC GTA ATC AGT AGC GAC AGA ATC AAG TTT G-3’ (Integrated DNA Technologies); Complement to 50-mer – 5’-AAC CCT GAA TAG CCG GCC TAG GTA GGA GAC CAT AAA ACT TCG CCC CAA AA-3’ (MWG Biotech); 3’ overhang 70-mer – 5’-AAC CCT GAA TAG CCG GCC TAG GTA GGA GAC CAT AAA ACT TCG CCC CAA AAT CCC TAA TAA TCC AAT CAA A-3’(MWG Biotech). To construct DNA binding ligands, 2.3 pmol (molecules) of 50-mer was labeled on the 5’-end using 3.33 μM [γ-³²P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (New England Biolabs) using reaction conditions suggested by the supplier. Free nucleotides were removed using a Qiagen Nucleotide Removal Kit. The 50 bp homoduplex and 50/70 3’overhang duplex DNAs were made by annealing 2.8 pmol of the
50-mer complement or the 3’ overhang 70-mer to the labeled 50-mer in annealing buffer (40 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 50 mM NaCl). The DNAs were mixed and heated to 100°C for 5 min, cooled to 65°C and incubated for 30 min, and then cooled to 25°C and incubated for 10 min. The substrate DNA was purified from the unannealed 50-mer on a 10% native polyacrylamide gel run in TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, (pH 8.3)) at 3.1 V/cm for 12 hours. DNA was extracted from the gel slice by electroelution in 1 X TBE. An electric potential of 3.1 V/cm was passed across the gel slice for 8 hrs at 4°C. The electroeluted DNA was then dialyzed against TEN buffer (40 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl) buffer. The eluted DNA was concentrated as previously described (29) using sec-butanol.

The 93 bp partial duplex DNA substrate was prepared as previously described (30). Briefly, 2 μg M13mp7 ssDNA, 40 ng 91-mer, annealing buffer, and water were added to a final volume of 50 μl. The mixture was heated to 100°C for 5 min, cooled to 65°C and incubated for 30 min and finally incubated at room temperature for 10 min. [α-³²P]-dCTP (3.33 μM) and 5 units of DNA polymerase I (Klenow fragment, New England Biolabs) were added. The extension reaction was allowed to proceed for 30 min at 25°C, chased by the addition of 20 nmoles of dCTP and allowed to incubate at 25°C for an additional 10 min. The volume of the solution was increased to 100 μl with STE buffer (100 mM NaCl, 10 Tris-HCl (pH 7.5), 1 mM EDTA), phenol/CHCl₃ extracted and free nucleotides were removed using an A5M column (Biorad). The partial duplex substrate eluted in the void volume and was used directly in helicase assays. The concentration of the DNA substrate is estimated at 20 μM DNA-Pi.
The 710 bp blunt duplex was prepared by digesting pLitmus28 with ScaI and XbaI (New England Biolabs). The resulting 710 base fragment was labeled using DNA polymerase I (Klenow fragment, New England Biolabs). The reaction contained 87.7 nM 710 bp pLitmus28 fragment (DNA molecules), 50 mM NaCl, 10 mM Tris HCl (pH 7.9), 10 mM MgCl$_2$, 1mM dithiothreitol, 200 μM dATP, 200 μM dGTP, 200 μM dTTP, [α-$^{32}$P]-dCTP, and 5 units of DNA polymerase I (Klenow Fragment, New England Biolabs) (30 μl). The reaction was incubated at 25°C for 20 min, chased by the addition of 20 nmols of dCTP at 25°C for 15 min. Free nucleotides were removed using a nucleotide removal kit (Qiagen).

**Mutator Assays** – Nine independent cultures of GE1752, GE1752 mutL::Tn10, GE1752 mutL::Tn10/pET15b-MutL and GE1752 mutL::Tn10/pET15b-MutL-R266E were grown to saturation at 37°C in the presence of appropriate antibiotics. Appropriate dilutions of each cell strain were made and cells were plated on LB plates containing appropriate antibiotics to determine a cell titer and on LB plates containing rifampicin (100 μg/ml) to measure the number of rifampicin resistant colonies. Plates were incubated at 37°C overnight and colonies were counted. Mutation frequencies and rates were calculated using the method of the mean as previously described (31-34).

**Small Scale Mutator Assays** - Two cultures each of BL21(DE3), BL21(DE3)mutL::Tn10, BL21(DE3) mutL::Tn10/pETcoco-2, BL21(DE3) mutL::Tn10/pETcoco-2-MutL, BL21(DE3) mutL::Tn10/pETcoco-2-MutL-R266E and BL21(DE3) mutL::Tn10/pETcoco-2-MutL-E29A were grown for 15 hrs and 30 min at 37°C in the presence of the appropriate antibiotics (pETcoco-2 from Novagen). One of the two cultures was grown in the absence of IPTG while the other culture was grown in the presence of 250 μM IPTG. Five μl of serial dilutions (0, 1:10, and 1:100) were plated on an LB plate containing rifampicin (100 μg/ml).
The plate was incubated at 37°C overnight and the plate was then analyzed qualitatively to determine the mutator phenotype of the particular mutant.

To confirm that the appropriate MutL protein was being expressed a Western blot of each of the plated samples was run. One ml of cells were centrifuged to pellet the cells. The cells were resuspended in TE buffer according to the following formula to ensure equal loading: resuspension volume of TE buffer = \( \frac{A_{600} \times 100}{0.5} \). 12.5 μl of the cells were then lysed in an equal volume of SDS gel loading buffer (32 mM Tris HCl (pH 6.8), 3.2 % (v/v) SDS, 1.2 M BME, 20 % (v/v) glycerol) and boiled for 5 min. The samples were run on SDS-PAGE at 14.5 V/cm to separate the proteins in the bacterial lysate. The proteins were transferred to nitrocellulose by applying an electric potential of 14.5 V/cm across the gel for 60 min at 4°C. The nitrocellulose filter paper was blocked with 2 % (w/v) powdered milk (Carnation) in 25 ml TTBS (10 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.1 % (v/v) Tween-20) for 60 min while shaking. The nitrocellulose filter paper was then washed 4 times with TTBS for 15 min while shaking. Twenty five ml of rabbit anti-MutL in TTBS (1:25,000) was incubated with the nitrocellulose filter paper for 2 hours. The nitrocellulose filter paper was washed 4 times with 25 ml TTBS. 25 ml of goat anti-rabbit conjugated to alkaline phosphotase in TTBS (1:10,000) was added to the nitrocellulose filter paper for 30 min. The nitrocellulose filter paper was washed 4 times with 25 ml TTBS. The blot was then reacted with 5 ml Western Blue (Promega) alkaline phosphotase substrate until a color reaction was observed (~5 min).

ATP Hydrolysis Assays – Reaction mixtures (120 μl) contained 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 20 mM NaCl, 50 μg/ml BSA, 5 mM 2-mercaptoethanol, 3 mM ATP ([γ-³²P] ATP). For reaction mixtures containing DNA the 50-mer was added at concentrations
varying from 0 μM to 4 μM as indicated in the figure legends. Reactions were initiated by the addition of purified MutL or MutL-R266E to a final concentration of 1 μM followed by incubation at 37°C. Aliquots (20 μl) were removed at 0, 15, 30, 45, and 60 min and added to 280 μl quench solution (20 mM H₃PO₄, 1 mM EDTA (pH 8.0), 5% (w/v) Norit activated charcoal USP). The quenched reactions were incubated on ice for 15 min and the activated charcoal was sedimented in a microfuge. A 200 μl aliquot of the supernatant was removed, added to 3 ml scintillation cocktail (Ecoscint A, National Diagnostics) and counted in a liquid scintillation counter.

**DNA Binding Assays** – Double filter DNA binding assays were as described (35) Briefly, nitrocellulose (Millipore) and DEAE (DE81, Whatman) filters (2.5 cm) were prepared as previously described (35) with the exception that the filters were incubated at 4°C with MutL reaction buffer (25 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 20 mM NaCl, 5 mM 2-mercaptoethanol) prior to use. Reaction mixtures (20 μl) contained 25 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 20 mM NaCl, 5 μg/ml BSA, 5 mM 2-mercaptoethanol, 3 mM AMP-PNP, 1 nM oligonucleotide 50-mer, 1 nM 50 homoduplex, or 1 nM 70/50 3’-overhang DNA, or 4 uM DNA Pi 93 bp partial duplex DNA, and protein as indicated. The reactions were incubated at 37°C for 10 min, diluted with 1 ml MutL reaction buffer and then filtered across treated nitrocellulose and DEAE filters in a vacuum manifold. The filters were washed twice with reaction buffer, dried, and counted in a liquid scintillation counter. Data was analyzed as previously described (35).

**Helicase Unwinding Assays** – Reaction mixtures (20 μl) contained 25 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 20 mM NaCl, 5 mM 2-mercaptoethanol, 50 μg/ml BSA, 3 mM ATP, 93
bp partial duplex DNA (final concentration of 2 μM DNA-Pi) or 710 bp blunt duplex DNA (final concentration of 0.5 nM DNA molecules), 1.25 or 16 nM UvrD, and a titration of MutL or MutL-R266E. Reactions were initiated by the addition of ATP after prewarming the reaction solution to 37°C for 5 min. The reactions were terminated after 10 min for the 93 bp partial duplex or 20 min for the 710 bp blunt duplex by the addition of 10 μl of stop solution (50% (w/v) glycerol, 68 mM EDTA (pH 8.0), 0.022 % (w/v) xylene cyanole, 0.022 % (w/v) bromophenol blue, 0.3 % (w/v) SDS, 44.5 mM Tris base, and 44.5 mM boric acid). The reaction products were resolved on an 8% SDS polyacrylamide gel run in 0.5X TBE and 0.1 % (w/v) SDS. The gel was imaged using a phosphor screen (Molecular Dynamics, GE Healthcare), imaged by Storm (Molecular Dynamics, GE Healthcare), and quantified using ImageQuant (Molecular Dynamics, GE Healthcare).

*MutH Affinity Column Chromatography – 560 μg MutH in MutH affinity chromatography buffer (100 mM Hepes-KOH (pH 7.5), 25 mM NaCl, 100 μM EDTA, 10 % (v/v) glycerol) was bound to 1.5 ml Affi-Gel 10 Activated Immunoaffinity Support overnight at 4°C. The resin was blocked with 100 μl of 1 M ethanolamine (pH 8.0) for 1 hr at 4°C, added to a column and washed in MutH affinity chromatography buffer until the flow through contained no detectable protein (determined by measuring the A$_{280}$ of the flow-through). 100 μg of MutL, 80 μg MutL-R266E, or 100 μg BSA was loaded onto the column in binding buffer. The column was washed with 4 column volumes of binding buffer and eluted with increasing concentrations of NaCl. Five 300 μl fractions were collected at each NaCl concentration (50 mM, 200 mM, 1 M, 2 M NaCl) in MutH affinity chromatography buffer and analyzed by a Bradford protein assay and SDS-PAGE. The gels were analyzed by densitometry, measuring the relative density of protein on the gel.*
Enzyme Linked Immunosorbent Assay (ELISA) – 625 fmol UvrD or 625 fmol BSA in solution with 25 mM Hepes-KOH (pH 7.5), 50 mM NaCl and 1.6 mM Sulfo N-hydroxysuccinimide were added to each of 16 wells (100 μl/well) on a CovaLink plate (Nunc). To initiate the binding reactions of UvrD or BSA, 6.5 mM N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride was added to each of the wells (50 μl/well). The binding reaction was carried out for 2 hrs at 25°C. The wells were washed four times with 350 μl wash buffer (25 mM Hepes-KOH (pH 7.5), 50 mM NaCl, 0.1 % (v/v) Tween 20, 2 % (w/v) BSA (Fraction V RIA grade)). The wells were blocked with 350 μl 25 mM Hepes-KOH ((pH 7.5), 50 mM NaCl, 3 % (w/v) BSA and 0.1 % (v/v) Tween 20) overnight at 4°C. The wells were washed four times with 350 μl wash buffer and a titration of MutL or MutL-R266E in wash buffer was added to the wells (100 μl/well) as indicated and incubated for 2 hours at 25°C. The wells were rinsed four times with 350 μl wash buffer. 100 μl Rabbit anti-MutL IgG (1:10,000) in wash buffer was added to the wells and incubated at 25°C for 2 hrs. The wells were washed with 350 μl wash buffer. 100 μl goat anti-rabbit IgG conjugated to alkaline phosphatase (1:10,000) in wash buffer, was added to the wells for 30 min at 25°C. The wells were washed with 350 μl wash buffer. 100 μl PNPP alkaline phosphotase substrate (1 M diethanolamine, one mg/ml p-nitrophenyl phosphate disodium salt (Pierce)) was added to the wells for 30 min and quenched with 50 μl of 2 M NaOH. The color reaction was read at A$_{405}$ using a MicroQuant plate reader. The data was analyzed by subtracting the background MutL or MutL-R266E bound to BSA from the MutL or MutL-R266E bound to UvrD.
Results

Since its discovery several years ago, the DNA binding activity of MutL has been investigated in several laboratories with conflicting results (11,23-25,28,36,37). Taken together, most investigations show that MutL binds ssDNA, and probably duplex DNA, but the biological significance of this result remains to be demonstrated. Most recently, the ssDNA binding activity of MutL has been suggested to be an artifact of in vitro assays and not essential for the role of MutL in MMR (25). We (28), and others (19,28), have shown that MutL stimulates the unwinding of duplex DNA by helicase II, presumably by facilitating the loading of helicase II at an appropriate site to initiate DNA unwinding. It has been presumed that an interaction between MutL and DNA is important in this reaction. However, the DNA binding activity of MutL has never been shown to have a direct role within the context of the bacterial MMR system in vivo. We have used a MutL mutant that has a single point mutation at position 266 in which the basic arginine residue has been changed to an acidic glutamic acid (MutL-R266E) to investigate the role of DNA binding by MutL in MMR. Previous studies (20,36) have shown this mutant to exhibit a DNA binding defect. We have extended those studies here and suggest that DNA binding by MutL is essential for MMR.

*E. coli* mutL-R266E exhibits a mutator phenotype – The ability of the mutL-R266E allele to substitute for the wild-type *mutL* gene in MMR was evaluated in a genetic complementation assay (Table 2). This assay measures the ability of plasmid-borne *mutL* alleles to substitute for the wild-type gene in a strain containing an insertion in the chromosomal *mutL* gene. As expected, the mutation frequency was elevated approximately
100-fold in the absence of MutL as demonstrated by the increased frequency of Rif\textsuperscript{r} cells. Addition of the vector alone (pET15b) to the strain lacking MutL did not significantly alter either the mutation frequency or the mutation rate. The addition of wild-type \textit{mutL} on the pET15b expression plasmid restored both the mutation rate and frequency to that observed in the wild-type strain indicating that the plasmid-borne copy of \textit{mutL} could effectively replace the chromosomal copy of the gene. It is important to note that expression of MutL was not induced in these experiments and, therefore, a basal level of MutL expression from this plasmid is sufficient to restore MMR to wild-type levels. The \textit{mutL-R266E} mutant allele failed to complement the loss of the chromosomal copy of \textit{mutL} indicating that this protein was not functional in MMR. Since the expression of the \textit{mutL-R266E} allele in these cells was confirmed by Western blot, we conclude that \textit{mutL-R266E} encodes a mutant MutL protein that is not functional in MMR. Similar results have been obtained by us (E.A. Gibbons and S.W. Matson, unpublished results) and others (22,38) with regard to the role of the ATP hydrolysis reaction catalyzed by MutL.

\textit{Purification and biochemical characterization of MutL-R266E} – Since MutL-R266E was defective in mismatch repair, the mutant protein was purified and characterized using a variety of biochemical assays. The purification schemes for MutL and MutL-R266E were identical. Both proteins were overexpressed using the pET15b vector in the BL21(DE3) \textit{uvrD::Tn5 mutL::Tn10 E. coli} cell strain. This strain was chosen to ensure there was no contamination by either wild-type MutL or helicase II. The latter has been shown to interact with and, in some cases, copurify with MutL and its presence in final preparations of MutL-R266E could confound the interpretation of biochemical results.
Purification of each protein was facilitated by the presence of an \( \text{NH}_2 \)-terminal six histidine tag that was provided on the vector chosen for expression. It is important to note that the histidine tagged protein was also used in the complementation assays described above indicating that the purification tag was not interfering with the ability of the protein to function in MMR. The histidine-tagged protein was bound to TALON resin and eluted using imidazole. The protein was further purified using a MonoQ ion exchange column followed by a Superdex 200 gel filtration column to remove essentially all impurities (for details see “Experimental Procedures”). The purified proteins are shown in Figure 4. Importantly, the purified proteins are shown to be MutL using polyclonal antibodies directed against MutL and the protein preparations lack any detectable helicase II as demonstrated by Western blot (data not shown). It should also be noted that both MutL and MutL-R266E elute from the Superdex 200 column in the position expected for a dimer of MutL. Thus, the dimerization properties of MutL-R266E were not affected by the mutation.

\textit{ATP hydrolysis by MutL-R266E is not stimulated by DNA} – DNA has been shown to increase the low basal rate of ATP hydrolysis catalyzed by MutL (20,21). This finding implies there is an interaction between DNA and the MutL dimer since DNA-bound MutL hydrolyzes ATP at a faster rate. However, to date the available direct evidence for DNA binding is considered controversial. Purified MutL and MutL-R266E exhibited equivalent levels of basal ATP hydrolysis (MutL ATPase rate\(_{\text{No DNA}}\) = 3.0 ± 0.6 pmol/min, MutL-R266E ATPase rate\(_{\text{No DNA}}\) = 2.9 ± 1.4 pmol/min) that compare favorably with values for the rate of basal MutL hydrolysis that have been reported previously (21,22) (Fig. 5). This finding indicates that the mutant protein is able to bind and subsequently hydrolyze ATP. Together with the purification properties of the protein, this suggests that MutL-R266E is properly
folded and observed in vivo defects in MMR cannot be attributed to improper folding of the protein.

When ssDNA was added to the reaction containing wild-type MutL the rate of ATP hydrolysis increased as the ssDNA concentration was increased (Fig. 5). The rate dependence on the presence of ssDNA was well described by a rectangular hyperbola where the ssDNA-stimulated ATP hydrolysis reaction was maximal at a DNA concentration of 4 μM and a rate of MutL-catalyzed hydrolysis of 14.8 ± 0.26 pmol/min. In a similar experiment the rate of MutL-R266E catalyzed ATP hydrolysis did not increase as a function of increasing ssDNA concentration. In fact, the MutL-R266E hydrolysis rate remained at the basal level. While indirect, this data suggests that the mutant protein was unable to productively interact with ssDNA since the rate of ATP hydrolysis was not stimulated by the addition of DNA.

MutL-R266E is defective for ssDNA binding – To directly test the possibility that MutL-R266E fails to bind ssDNA, or binds ssDNA with a significantly lower affinity than wild-type MutL, we performed double filter DNA binding assays (35). Previously we have shown that MutL is able to bind a 92 bp partial duplex DNA with an apparent K_D of 180 nM in the presence of the nonhydolyzable ATP analogue AMP-PNP (28). We were able to recapitulate this finding which is shown in Figure 6. MutL is able to bind to a 93 bp partial duplex with an apparent K_D of 2.8 nM. MutL-R266E is also able to bind to this substrate in the presence of AMP-PNP with a severely decreased affinity. To more accurately describe the binding of MutL-R266E we observed that at low concentrations of MutL-R266E almost no DNA was bound; however, at significantly elevated concentrations of MutL-R266E some of this
substrate was bound yielding a $K_D$ of 1.4 $\mu$M. This suggests that MutL-R266E has a very low affinity for DNA although it is not completely DNA binding deficient.

A 93 bp partial duplex DNA ligand has the characteristics of ssDNA, dsDNA, and 3’overhang ssDNA. To further characterize the DNA substrate preference of MutL and MutL-R266E for these varied DNA secondary structures, three DNA ligands were generated using oligonucleotides; a 50-mer ssDNA, a 50-mer dsDNA, and a substrate with a 50 bp duplex region containing a 20 base 3’ overhang ssDNA region. The binding of MutL and MutL-R266E was tested using the double filter DNA binding assay and each of these ligands (ssDNA, dsDNA, 3’overhang DNA) in the presence and absence of AMP-PNP (Fig. 3). Wild-type MutL exhibited a preference for binding the DNA ligands in the following order: ssDNA ($K_D = 246.7$ nM) > 3’overhang DNA ($K_D = 4.05$ $\mu$M ) > dsDNA ($K_D > 5000$ $\mu$M) suggesting that ssDNA is the preferred ligand for DNA binding. Under the same conditions, MutL-R266E was unable to bind significantly to any of these DNA ligands. The absence of DNA binding by the mutant protein is a direct demonstration that MutL-R266E cannot bind DNA or binds DNA very poorly (see Figure 6).

*MutL-R266E is able to stimulate helicase II-catalyzed unwinding* –To address the issue of MutL-R266E and its interaction with helicase II we compared the ability MutL and MutL-R266E to stimulate UvrD in helicase unwinding assays using both a 93 bp partial duplex and a 710 bp blunt duplex. As shown here (Fig. 7) and in previous reports (19) MutL stimulates the helicase II-catalyzed unwinding of these DNA substrates with an apparent $K_{1/2, 93 \text{ bp PD}}$ of 28.4 nM MutL and a $K_{1/2, 710 \text{ bp blunt}}$ of 208 nM ($K_{1/2}$ defined as the MutL concentration required for ½ maximal stimulation). Under the same conditions, MutL-R266E was also capable of stimulating the helicase II unwinding reaction on a 93 bp partial duplex substrate,
albeit very poorly ($K_{1/2} = 305$ nM), and was unable to stimulate the helicase II unwinding reaction on a 710 bp blunt duplex. Again, this is indirect evidence of the poor DNA binding activity associated with the mutant protein. It is likely that MutL-R266E is able to stimulate the helicase II unwinding reaction due to the fact that a single point mutation in MutL does not abolish DNA binding, and therefore MutL-stimulated unwinding, but does diminish the DNA binding activity significantly.

*MutL-R266E partially complements the mutL deletion when overexpressed in vivo* – The data presented above are consistent with the possibility that MutL-R266E is able to interact with ssDNA, but with a much lower affinity than the wild-type protein. We reasoned that if this were the case then overexpression of the mutant protein *in vivo* might rescue the defect observed in a strain that does not express MutL in genetic complementation assays. The notable ability of MutL-R266E to stimulate helicase II to nearly the same extent as wild-type MutL, but at very high concentrations of protein, is consistent with this possibility. To test this idea a single copy vector (pETcoco-2) was utilized to reduce basal expression from the T7 promoter in the absence of IPTG and to allow better control of the expression of protein in the cell by addition of low concentrations of IPTG. The final IPTG concentration chosen to induce protein expression was 250 μM and the cell cultures were grown for exactly 15 hours and 30 minutes before plating. Figure 8 shows the results of these experiments with *E. coli* BL21(DE3) *mutL::Tn10* and the indicated plasmid. All the cell strains used in this experiment are mutators in the absence of IPTG, as expected, since MutL is not expressed. However, in the presence of IPTG, protein expressed from the pETcoco-2-MutL plasmid is able to fully complement the MutL deletion and, importantly, the plasmid-borne MutL-R266E was able to partially complement the deletion such that the mutator phenotype was
not as severe. This result is consistent with the interpretation that MutL-R266E has a decreased DNA binding affinity that can be partially overcome by increased expression of the mutant protein and that MutL DNA binding is essential for MMR in vivo.

The R266E mutation does not abrogate any interaction in the MMR pathway – MutL is known to interact with several proteins involved in MMR (12,15,24,36,39,40). Thus, the mutator phenotype associated with MutL-R266E might be due to an inability to interact with one or more of the MMR proteins. We tested directly the ability of MutL-R266E to interact with both helicase II and MutH as indicated below. Previous studies (36) have shown that MutL-R266E interacts with MutS.

The interaction between MutL-R266E and MutH was assessed by affinity chromatography. As shown in Figure 9, MutL-R266E binds an affinity column constructed using purified MutH and elutes at a NaCl concentration of 200 mM. This is identical with the elution pattern observed for wild-type MutL. We conclude that MutL-R266E interacts with MutH and it is unlikely that the mutator phenotype associated with the mutL-R266E allele is due to an inability to interact with MutH.

The interaction between MutL-R266E and helicase II has been shown earlier in the helicase II stimulation assays (see Fig. 7). To more directly assess the interaction between MutL and helicase II we performed a double sandwich ELISA (Fig. 10). In this experiment 625 fmol of helicase II was bound to an ELISA plate and a titration of MutL or MutL-R266E was performed. Bound protein was detected using an antibody directed against MutL. The data indicate that the interaction between MutL-R266E and helicase II is similar to that of MutL and helicase II (K_D, MutL = 527 nM, K_D, MutL-R266E = 685 nM). While these values are unlikely to represent true dissociation constants it is clear that both proteins interact with
UvrD with similar affinity. Thus, the mutator phenotype observed in cells expressing MutL-R266E is not due to a defect in the interaction between MutL-R266E and helicase II.

**Discussion**

The role of DNA binding by MutL in the process of mismatch repair has been debated in the literature for the last 14 years. While there is considerable evidence suggesting that MutL binds DNA (20,23,28), there is also evidence to the contrary (24) and recent experiments have suggested that DNA binding may be an artifact of in vitro experiments (25). Here we have used biochemical and genetic assays to characterize a MutL point mutant in an effort to evaluate the biological importance of DNA binding by MutL. Taken together, the *in vivo* and in vitro results presented in this study strongly suggest that MutL must bind DNA as part of the MMR process.

The *in vitro* DNA binding results clearly show that, under the conditions used here, MutL-R266E is unable to bind DNA to any significant extent. Wild-type MutL, on the other hand, does bind DNA under these conditions. In fact, the mutant protein failed to exhibit measurable levels of DNA binding, using a nitrocellulose filter binding assay, to any of the substrates tested. Importantly, the mutant protein failed to bind the ssDNA and 3’overhang DNA for which wild-type MutL has a demonstrated affinity. This result is consistent with the lack of DNA simulation in the ATP hydrolysis reactions catalyzed by MutL-R266E. However, these conditions are clearly not physiological and, therefore, it remained possible that the binding of wild-type MutL to DNA measured in these experiments was an artifact of
the in vitro conditions. Thus, a careful analysis of the other biochemical properties of MutL-R266E and its function in vivo were undertaken.

Purified MutL-R266E retains the biochemical properties of wild-type MutL that do not involve DNA binding. These properties include: (i) basal ATP hydrolysis, (ii) an ability to interact with MutH, MutS, and helicase II and (iii) the ability to dimerize. These results demonstrate that the protein retains both its tertiary structure and its dimeric form. In addition, it is able to catalyze the hydrolysis of ATP. However, the properties dependent upon DNA binding were severely affected. The basal ATPase activity exhibited by the mutant protein was not stimulated by the addition of ssDNA whereas the basal ATPase of wild-type MutL was significantly stimulated by the addition of ssDNA (see Fig. 5). The mutant protein was also compromised in its ability to stimulate the unwinding reaction catalyzed by helicase II although, at high concentrations, it was able to stimulate helicase II to a significant extent on a partial duplex DNA substrate. The latter result was, perhaps, unexpected since our previous study suggested that MutL loaded helicase II, perhaps at the junction of ssDNA-duplex DNA, in a reaction directed by the binding of MutL to DNA (28).

To explain these results we suggest that a complex of MutL and helicase II actually binds the DNA and that the DNA binding affinity of both proteins is critical for this interaction with DNA. In fact, it is possible that the affinity of the complex for DNA is higher than the affinity of either protein in isolation. If this is the case, then the MutL-R266E-helicase II complex might retain significant DNA binding affinity based on the interaction of helicase II with ssDNA. This would be consistent with the fact that MutL-R266E can stimulate helicase II-catalyzed unwinding but only at higher concentrations of the mutant MutL. With these characteristics in mind, it is not unexpected that the MutL-R266E has a strong mutator
phenotype and we conclude, based on both genetic and biochemical data, that DNA binding by MutL is critical for MMR.

There is significant support for this conclusion. In yeast, it has been demonstrated that MutL homologues must bind DNA in order for MMR to occur (41). Point mutations in the yeast homologues (PMS1-K328E and MLH1-R273E, R274E), similar to the point mutation analyzed here, increase the mutation frequencies and rates in vivo. However, these point mutants have yet to be characterized biochemically and their DNA binding properties have not been evaluated. Based on the data shown here with MutL-R266E it is likely that these mutants will exhibit a DNA binding defect.

At least one report has suggested that the binding of MutL to DNA is independent of the presence of ATP (23). However, we have shown that MutL does not bind DNA in the absence of a non-hydrolyzable analogue of ATP (AMP-PNP) under our conditions. This strongly suggests that DNA binding is dependent on ATP binding. We hypothesize that ATP-bound MutL interacts with DNA and remains bound to the DNA until one or both of the bound ATP molecules are hydrolyzed to ADP and Pi. After an ATP hydrolysis fueled conformational change MutL is released from the DNA. Due to the slow ATPase intrinsic to MutL we also postulate that the MutL ATPase acts as a molecular timer to load helicase II molecules onto the DNA. The processivity of helicase II-catalyzed unwinding of duplex DNA has been measured to at 40-50 base pairs in one study (42) and approximately 250 base pairs in another study (43). However, repair tracts in vivo can be up to 2 kb in length (44,45) and we have demonstrated significant unwinding of a 710 bp duplex DNA substrate by helicase II in the presence of MutL. In a recent study by Lohman et al. the processivity of helicase II was determined to be 2400 ± 600 bases as the protein translocates along ssDNA
We suggest that MutL binds to the DNA and continuously loads helicase II molecules for 6-7 seconds at which point MutL hydrolyzes its ATP and is subsequently released from the DNA. MutL, no longer associated with the DNA, is not able to load helicase II onto the DNA. The multiple helicase II molecules that are loaded onto the duplex are able to unwind up to 2-3 kb of duplex DNA even though the processivity of helicase II for unwinding duplex DNA is much less than 2 kb. As one molecule of helicase II dissociates from the DNA, a second molecule of helicase II takes its place and unwinding continues uninterrupted. This helps to insure that, no matter what the distance between the initiating nick and the mismatch, adequate DNA is unwound by helicase II to insure repair of the mismatch. This model does not, however, address the termination of the repair track which has been shown to extend approximately 100 base pairs past the mismatch (17). This remains unresolved.

The crystal structure of dimeric MutL (21) positions arginine 266 facing inward and previous reports (33) have suggested it could interact with the negatively-charged backbone of DNA. Thus, arginine 266, may be a primary amino acid involved in recognizing the DNA through electrostatic interactions. After the DNA is bound, a conformational change in the dimer may occur allowing MutL to ‘clamp’ onto the DNA, forming a stable complex. We suggest that the most stable form of MutL bound to DNA is with ATP bound to one or both of the dimeric MutL ATP binding sites. Altering the basic arginine residue to an acidic residue may severely disturb this interaction so that the DNA rarely induces this conformational change and forms this more stable complex.

We propose a model for MMR that takes into account the DNA binding properties of MutL. This model suggests that MutL may exist in a complex with helicase II in the cell prior to coming in contact with the DNA and MutS. We have preliminary evidence
suggesting that the MutL-helicase II complex exists in the absence of DNA and that this complex has a higher affinity for DNA than either protein alone (data not shown). MutL may release helicase II to unwind the DNA duplex due to a combination of signals, including the binding of MutL to DNA, MutS, MutH, or any combination of the three. Because MutL is unable to bind DNA in the absence of ATP or an ATP analogue, we suggest that the ATP binding/ATP hydrolysis activity of MutL may be linked to its DNA bind and release cycle. When ATP is hydrolyzed the DNA is released by MutL. This release stops the loading of helicase II onto the DNA, in turn preventing a repair tract from becoming excessively long. MutL continues to load helicase II molecules onto the DNA until one or both of its bound ATPs are hydrolyzed. At this point, MutL dissociates from the DNA and the downstream MMR proteins finish the repair of the excised DNA.
References

33. Luria, S. E., and Delbruck, M. (1943) *Genetics* 28, 491-511


Table 2. Mutation frequencies and rates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation Frequency (Mutations/Culture)</th>
<th>Mutation Rate (Mutations/Gen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GE1752</td>
<td>8.00x10^{-9} ± 1.66x10^{-8} ††</td>
<td>9.23x10^{-10}</td>
</tr>
<tr>
<td>GE1752 MutL::Tn10</td>
<td>9.49x10^{-7} ± 3.92x10^{-7}</td>
<td>9.18x10^{-8}</td>
</tr>
<tr>
<td>GE1752 MutL::Tn10/pET15b</td>
<td>2.24x10^{-6} ± 8.97x10^{-7}</td>
<td>2.36x10^{-7}</td>
</tr>
<tr>
<td>GE1752 MutL::Tn10/pET15b MutL</td>
<td>3.78x10^{-8} ± 2.44x10^{-8}</td>
<td>9.20x10^{-9}</td>
</tr>
<tr>
<td>GE1752 MutL::Tn10/pET15b MutL-R266E</td>
<td>1.55x10^{-6} ± 6.45x10^{-7}</td>
<td>1.29x10^{-7}</td>
</tr>
</tbody>
</table>

†† Mark C. Hall – Unpublished data

Mutation Frequencies and rates were determined by in vivo mutator assays as described in the “Materials and Methods” section.
Figure 4. SDS-PAGE analysis of purified proteins. (A) Purified MutL and MutL-R266E were resolved on a 9.6% polyacrylamide gel run in the presence of SDS and stained with Coomassie blue. Lane 1, 1 μg of MutL; lane 2, 1 μg of MutL-R266E. (B) An identical gel was run, transferred to a nylon membrane and probed with antibody directed against MutL. Lane 3, 1 μg of MutL; lane 4, 1 μg of MutL-R266E. The position of molecular weight standards is shown on the left.
Figure 5. ATP hydrolysis by MutL-R266E is not stimulated by DNA. ATP hydrolysis reactions containing 1 μM MutL (closed circles) or 1 μM MutL-R266E (closed squares) were as described under “Materials and Methods”. A rate of ATP hydrolysis was determined at each DNA concentration indicated by taking time points at 0’, 15’, 30’, 45’ and 60’ and determining the amount of ATP hydrolyzed at each time point. A line was fit to this data to determine the rate at the specific DNA concentration. The ssDNA concentrations used were 0 μM, 0.1 μM, 0.2 μM, 0.5 μM, 1.0 μM, 2.0 μM, and 4.0 μM. The ssDNA was the 50-mer oligonucleotide. The data represent the average of at least three rate determinations at each DNA concentration.
Figure 6 Panel A
Panel B
Figure 6. DNA binding by MutL and MutL-R266E. Nitrocellulose filter binding assays were conducted as described under “Materials and Methods” using the indicated concentrations of MutL (closed circles) or MutL-R266E (closed squares). Binding to (A) 4 μM (DNA Pi) partial duplex DNA (B) 1 nM ssDNA 50-mer are shown. All reactions contained 3 mM AMP-PNP and each point represents the average of at least three experiments.
Figure 7 Panel A
Panel B
Figure 7. Helicase II stimulation by MutL and MutL-R266E. Helicase activity assays containing helicase II and MutL or MutL-R266E were as described under “Materials and Methods” using a 93 bp partial duplex substrate (panel A) or a 710 bp blunt duplex DNA substrate (panel B). (A) The fold stimulation of the helicase II unwinding reaction by MutL and MutL-R266E on a 93 bp partial duplex is shown above. All reactions contained 2.5 nM helicase II, 0.5 nM 93 bp partial duplex DNAs and the indicated concentration of MutL (closed circles) or MutL-R266E (closed squares). The fraction of the substrate unwound in the presence of MutL was determined and compared to the fraction of the substrate unwound by helicase II alone to determine the level of stimulation. (B) The fraction of the 710 bp duplex DNA substrate unwound by helicase II stimulated by MutL (closed circles) and MutL-R266E (closed squares). Reactions contained 16 nM helicase II, 0.5 nM 710 bp blunt duplex DNA. The data represents an average of at least three experiments with error bars representing the standard deviation about the mean.
Figure 8. ELISA measuring the affinity of MutL and MutL-R266E for helicase II. Helicase II (6.25 fmol) was bound to a Covalink Plate (Nunc) as described under “Materials and Methods”. Increasing concentrations of MutL (closed circles) and MutL-R266E (closed squares) were then added. The amount of MutL or MutL-R266E that interacted with helicase II bound on the plate was determined as described under “Materials and Methods” using an antibody directed against MutL and a secondary antibody conjugated with alkaline phosphatase.
Figure 9. MutL-R266E partially complements a MutL deletion. Panel A. Cultures of BL21(DE3), BL21(DE3)mutL::Tn10, (3) BL21(DE3)mutL::Tn10/pETcoco-2, BL21(DE3)mutL::Tn10/pETcoco-2-MutL, and BL21(DE3) mutL::Tn10/pETcoco-2-MutL-R266E were grown at 37°C for 15 hrs 30 min under the appropriate antibiotic selection. Cultures on the right were grown in the presence of 250 μM IPTG. Five μl of the indicated serial dilutions were plated on LB plates containing 100 μg/ml rifampacin and incubated overnight at 37°C.
Figure 10. MutL-R266E interacts with MutH. A MutH affinity column was constructed as described under “Materials and Methods”. 100 μg of MutL (solid line) or 80 μg MutL-R266E were loaded on the column in affinity binding buffer containing 25 mM NaCl. The column was washed with affinity buffer containing increasing concentrations of NaCl and 300 μl fractions were collected and evaluated for the presence of MutL by SDS-PAGE. Fractions 1 and 2 represent buffer + 25 mM NaCl, fractions 3 and 4 represent buffer + 50 mM NaCl, fractions 5 and 6 represent buffer + 200 mM NaCl, fractions 7 and 8 represent buffer + 1 M NaCl and fraction 9 represents buffer + 2 M NaCl. Forty μl of each fraction was analyzed by SDS-PAGE. The results were quantified using densitometry measuring the relative density of MutL or MutL-R266E on the SDS gel after staining with Coomassie blue and destaining with 10 % acetic acid.
MutL-catalyzed ATP hydrolysis is required at a post-UvrD loading step in methyl-directed mismatch repair

Methyl-directed mismatch repair is a coordinated process that ensures replication fidelity and genome integrity by resolving base pair mismatches and insertion/deletion loops. This post-replicative event involves the activities of several proteins many of which appear to be regulated by MutL. MutL interacts with and modulates the activities of MutS, MutH, UvrD and perhaps other proteins. The purified protein catalyzes a slow ATP hydrolysis reaction that is essential for its role in mismatch repair. However, the role of the ATP hydrolysis reaction is not understood. We have begun to address this issue using two point mutants, MutL-E29A which binds nucleotide but does not catalyze ATP hydrolysis and MutL-D58A which does not bind nucleotide. As expected, both mutants failed to complement the loss of MutL in genetic assays. Purified MutL-E29A protein interacted with MutS and stimulated the MutH-catalyzed nicking reaction in a mismatch-dependent manner. Importantly, MutL-E29A stimulated the loading of UvrD on model substrates. In fact, stimulation of UvrD-catalyzed unwinding was more robust with MutL-E29A than the wild-type protein. MutL-D58A, on the other hand, did not interact with MutS, stimulate MutH-catalyzed nicking or stimulate the loading of UvrD. We conclude that ATP-bound MutL is required for the incision steps associated with mismatch repair and that ATP hydrolysis by
MutL is required for a step in the mismatch repair pathway subsequent to the loading of UvrD and may serve to regulate helicase loading.

**Introduction**

DNA mismatch repair is the primary mechanism for correcting DNA replication errors (base substitution mismatches and insertion-deletion loops) (1-5) and is also involved in preventing recombination between divergent DNA sequences (6,7). Thus, an active mismatch repair system helps to ensure the fidelity of chromosomal replication and functions to maintain genomic stability in all organisms (5). Consistent with this idea, defects in mismatch repair genes in human cells have been linked to genomic instability and hereditary colon cancer underscoring the importance of this repair pathway (8-14).

The methyl-directed mismatch repair (MMR) pathway in *E. coli* has been reconstituted *in vitro* (15) and the sequence of events in the mismatch repair process has been well described using this purified system (for reviews see 1,16,17). In the first step, MutS recognizes the base pair mismatch (18,19) and then MutL binds to the MutS-DNA complex (20). Both proteins are functional as homodimers and the MutS$_2$-MutL$_2$ complex has been shown to loop out the DNA in what is presumed to be an active search for the nearest d(GATC) methylation site either 5' or 3' to the mismatch (20,21). Then the MutS$_2$-MutL$_2$ complex stimulates MutH, bound to a hemi-methylated d(GATC) site, to nick the nascent, unmethylated DNA strand (22-24). Once the nascent DNA strand has been nicked, DNA helicase II (UvrD) and the appropriate exonuclease excise the error-containing DNA strand (25,26). UvrD-catalyzed unwinding commences at the nick and continues past the mismatch.
providing ssDNA for cleavage by an exonuclease (5). The resulting gap is filled by DNA polymerase III and DNA ligase seals the nick (15).

Importantly, the MMR pathway has bi-directional capability (23,26,27). A nick is generated at the hemi-methylated d(GATC) located closest to the mismatch and, therefore, could exist on either side of the mismatch (25). However, UvrD unwinds DNA with a specific 3' to 5' polarity (28). As a result, UvrD must be loaded on the appropriate DNA strand in order to unwind toward the mismatch. We and others (29,30,31) have demonstrated a physical interaction between UvrD and MutL that results in a significant stimulation of the unwinding reaction catalyzed by UvrD. Notably, MutL fails to stimulate DNA unwinding catalyzed by other superfamily I DNA helicases (Hall, M., Jordan, R. and Matson, S.W. unpublished results) and the unwinding activity of Rep protein (37% identical to UvrD), while slightly enhanced by MutL in helicase assays, is not detectably stimulated on a nicked heteroduplex circular DNA substrate in the presence of MutL and MutS (31). It seems likely that MutL serves to load UvrD onto the nicked DNA substrate with the appropriate polarity to ensure correction of the mismatch although this has not been shown directly.

The mechanism by which the UvrD-catalyzed unwinding reaction is stimulated by MutL is not fully understood although several important details of the reaction have been described. For example, on a nicked, circular molecule containing a mismatch, MutS, MutL, and UvrD initiate unwinding at the nick site and begin helix opening in the direction toward the mismatch. This reaction requires all three protein components and the presence of a mismatch (30,31). Moreover, while MutL dramatically stimulates UvrD-catalyzed DNA unwinding, it does not increase the rate of ATP hydrolysis (29). Recent studies using model DNA substrates are consistent with the notion that MutL acts to load UvrD onto a DNA
substrate. MutL appears to be capable of loading multiple molecules of UvrD onto the DNA to allow the unwinding of long duplex regions. At present, there is no direct evidence for an increase in the processivity of the unwinding reaction catalyzed by UvrD in the presence of MutL (32) and we have suggested that the presence of multiple molecules of UvrD on the DNA allows for the unwinding of long duplex regions.

The biochemical activity associated with MutL protein has been a matter of debate for several years. Several laboratories have demonstrated that MutL binds to both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) (32-34), while others report that MutL does not bind DNA (35). It is now clear that MutL catalyzes a weak ATPase reaction and this activity is required for mismatch repair (32,34,36,37). In this report, we investigate the role of MutL-catalyzed ATP hydrolysis in the stimulation of the unwinding reaction catalyzed by UvrD. Using two MutL point mutants, MutL-E29A and MutL-D58A, we demonstrate that ATP-bound MutL is required for interaction with MutS, stimulation of MutH-catalyzed nicking of a hemi-methylated DNA mismatch-containing substrate and loading of UvrD. MutL-catalyzed ATP hydrolysis is not required for either the stimulation of MutH-catalyzed incision at the hemi-methylated d(GATC) site or the stimulation of UvrD. In addition, we show that MutL-E29A stimulates UvrD-catalyzed unwinding with greater efficiency than wild-type MutL and the ATP bound form of MutL is responsible for stimulation of UvrD. Together, these results demonstrate that while the ATP bound form of MutL is required for all steps prior to and including incision, the MutL-catalyzed ATPase activity is required in a step of mismatch repair subsequent to the loading of UvrD.
Materials and Methods

Bacterial strains and plasmids -- *E. coli* BL21(DE3) (F*ompT* [lon] *hsdSB*β*~gα~gal λDE3*) was from Novagen, Inc.  BL21(DE3)*ΔuvrD* (38), BL21(DE3) *uvrD::Tn5 mutL::Tn10* and GE1752*ΔmutL::Tn10* (32) were constructed previously in this laboratory.

pET11d, pLysS, and pET3c were from Novagen, Inc.  pLitmus28 was from New England Biolabs.  M13mp18 ssDNA was purified as previously described (39).  The UvrD expression plasmid has been described (38).

Oligonucleotides and enzymes – Restriction endonucleases, DNA polymerase I (large fragment), and polynucleotide T4 kinase were from New England Biolabs and used as recommended by the supplier.  Oligonucleotides were from IDT and were purified on a 20% polyacrylamide/8 M urea denaturing gel prior to use.

Protein purification – UvrD was purified as described previously (40).  MutL and its various mutant forms were overexpressed prior to purification in BL21(DE3)*uvrD::Tn5mutL::Tn10* containing pET15b-*mutL* in LB media at 37°C and purified as described (41).  MutH and MutS were expressed and purified as previously described (41).

The concentration of UvrD was determined using the published extinction coefficient of 1.29 ml mg⁻¹ cm⁻¹ (42).  The concentration of MutL, MutL point mutants, MutS and MutH was determined using a Bradford protein assay (Biorad) with bovine serum albumin as a standard.
**DNA Substrates** – The 750 bp blunt duplex DNA fragment was prepared by digestion of pLitmus28 with *Dra*I followed by treatment with Calf intestinal phosphatase (Roche) to produce a 5'-OH group on each end. The fragment was isolated from an agarose gel and was subsequently labeled at the 5' ends using $[\gamma-^{32}P]ATP$ and T4 polynucleotide kinase. The $[^{32}P]DNA$ fragment was separated from unincorporated $[\gamma-^{32}P]ATP$ using an A-5M sizing column (Biorad) equilibrated in 10 mM Tris-HCl (pH 7.5)/100 mM NaCl/1 mM EDTA. The substrate concentration was estimated assuming an 85% recovery of the DNA and the DNA was used directly in helicase assays.

The 93 bp partial duplex substrate used in standard helicase and DNA binding assays was prepared as described previously (43). The heteroduplex DNA substrate was prepared as described (41).

**Mutator Assays** – Seven independent cultures of GE1752, GE1752mutL::Tn10, GE1752mutL::Tn10/pET15b-MutL, GE1752mutL::Tn10/pET15b-MutL-E29A and GE1752mutL::Tn10/pET15b-MutL-D58A were grown overnight to saturation at 37°C in the presence of appropriate antibiotics. Serial dilutions of each cell strain were made, and appropriate dilutions were plated on LB plates containing antibiotics to determine a cell titer and on LB plates containing rifampicin (100 μg/ml) to measure the number of rifampicin resistant colonies. Plates were incubated at 37°C overnight, and colonies were counted.

**DNA Binding Assays** – DNA binding reaction mixtures (20 μl) contained 25 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 20 mM NaCl, 5 mM β-mercaptoethanol, 0.5 nM $[^{32}P]DNA$ (93 base pair partial duplex helicase substrate), 2.9 mM AMP-PNP and the indicated amount of either
MutL or MutL-E29A. The reactions were incubated for 10 min at 37°C, diluted to a final volume of 1 ml with reaction buffer containing 50 μg/ml BSA and filtered onto nitrocellulose and DEAE filters as described (44). Background values representing DNA retention on the nitrocellulose filter in the absence of protein were typically less than 3% and were subtracted from the binding values reported.

Gel mobility shift reaction mixtures (20 μl) contained 25 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 20 mM NaCl, 5 mM β-mercaptoethanol and 1.0 nM [³²P]DNA (5'-end labeled 50 base oligonucleotide, 32) and 1 mM AMP-PNP. Reaction tubes were incubated for 20 min on ice followed by the addition of five μl of 75% [v/v] glycerol; glycerol and loading dyes were added to the control tube that contained only the oligonucleotide. Proteins were diluted in UvrD storage buffer (42).

Samples were immediately loaded onto an 8% polyacrylamide gel (67:1 cross-linking ratio) containing a Tris-borate buffer (50 mM Tris, 50 mM Borate, and 2.5 mM EDTA). Samples were electrophoresed at a constant voltage of 8 V/cm at 4°C until the bromophenol blue marker had migrated to approximately 1 inch from the bottom of the gel. Results were visualized using Storm 840 PhosphorImager (Molecular Dynamics).

**ATPase Assays** – Standard ATPase reaction mixtures (30 μl) contained 25 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 20 mM NaCl, 5 mM β-mercaptoethanol, 50 μg/ml bovine serum albumin, 30 μM (DNA-Pi) M13 ssDNA, 2 mM [α-³²P]ATP and the indicated amount of MutL or one of its mutant derivatives. Reaction mixtures lacking protein were assembled on ice and incubated for 2 min at 37°C prior to the addition of protein to initiate the reaction. Aliquots (5 μl) were removed at the indicated times, quenched with an equal volume of 33
mM EDTA/6 mM ATP/6 mM ADP and spotted on polyethyleneimine thin layer plates. The plates were developed in 1 M HCOOH/0.8 M LiCl, air dried and analyzed using a Phosphorimager.

*Helicase Assays* – Reaction mixtures (20 µl) contained 25 mM Tris-HCl (pH 7.5), 3 mM MgCl$_2$, 20 mM NaCl, 5 mM β-mercaptoethanol, 50 µg/ml BSA, 3 mM ATP, 93 bp partial duplex DNA (final concentration of 2 µM DNA-Pi) or 750 bp blunt duplex DNA (final concentration of 1 nM DNA molecules), 1.25 or 16 nM UvrD, and a titration of MutL, MutL-E29A or MutL-D58A. Reactions were initiated by the addition of ATP after pre-warming the reaction solution to 37°C for 5 min. The reactions were terminated after 10 min for the 93 bp partial duplex or 20 min for the 750 bp blunt duplex by the addition of 10 µl of stop solution (50% (v/v) glycerol, 68 mM EDTA (pH 8.0), 0.022 % (w/v) xylene cyanol, 0.022 % (w/v) bromophenol blue, 0.3 % (w/v) SDS, 44.5 mM Tris base, and 44.5 mM boric acid). The reaction products were resolved on an 8% native polyacrylamide gel run in 0.5X TBE and 0.1% (w/v) SDS. Polyacrylamide gels were imaged using a phosphor screen (Molecular Dynamics, GE Healthcare) and quantified using ImageQuant on a Storm phosphorimager (Molecular Dynamics, GE Healthcare).

*MutL-stimulated MutH Nicking Assays* – Reaction mixtures (16 µl) contained 20 mM Tris-HCl (pH 7.6), 4 mM MgCl$_2$, 20 mM NaCl, 50 µg/ml BSA, 3 mM ATP, 50 ng covalently closed heteroduplex DNA, 33.8 nM MutS, 1.9 nM MutH and the indicated concentrations of MutL, MutL-E29A or MutL-D58A. Reactions were initiated by the addition of MutH, incubated at 37°C for 15 min and terminated by the addition of EDTA to a final
concentration of 12.5 mM. Samples were resolved on a 0.8% agarose gel run in the presence of ethidium bromide (0.5 μg/ml). To obtain quantitative results the gel was irradiated with UV light for 30 min, stained with ethidium bromide and destained. Density in the nicked DNA species and the supercoiled DNA species was determined using an Alpha imager.

**DNase I Footprinting Assays** – Two complementary 91 base pair oligonucleotides (with the exception of a G:T mismatch at position 59 relative to the 5'-end label) were annealed to form a duplex with a specific mismatch. One oligonucleotide (the same oligonucleotide used in the preparation of the 93 bp partial duplex DNA substrate) was phosphorylated using T4 polynucleotide kinase and [γ-32P]ATP, and the two strands were annealed. The annealed DNA substrate was isolated on an 8% native polyacrylamide gel. The appropriate labeled fragment was cut out of the gel and electroeluted at 100 V for 5 hours at 4°C in a TBE buffer. The substrate was then dialyzed overnight at 4°C against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl.

DNase I footprinting reaction mixtures (10 μl) contained 1 nM [32P]DNA, 25 mM Tris-HCl (pH 7.5), 20 mM NaCl, 100 μg/mL BSA, 1 mM DTT, 5 mM MgCl₂, 1 mM ATP, 1 μM MutS and either 1.3 μM, 650 nM, or 325 nM MutL or MutL-D58A. The reactions were incubated at 30°C for 15 minutes to allow protein binding and then 1 μL of CaCl₂ and 1 μL of DNase I were added to final concentrations of 1 mM and 0.5 μg/mL respectively. The reaction was incubated at 30°C for an additional 4 minutes. Reactions were stopped using 12 μl of a stop solution containing 80% (v/v) formamide, 1 mM EDTA (pH 8.0), 10 mM NaOH, and 0.05% (w/v) xylene cyanol and bromophenol blue. The samples were boiled for 3 min, loaded onto an 8% denaturing polyacrylamide gel and electrophoresed at 30 watts for 1.5
hours. The gels were soaked in a drying solution of 40% methanol, 10% acetic acid, and 3% glycerol and then dried for 2 hours under heat and vacuum. Gels were imaged using a Storm PhosphorImager (Amersham Biosciences) and quantified using ImageQuant software.

**Isothermal titration calorimetry** – Experiments were performed in a Microcal VP-ITC microcalorimeter (Northampton, MA, USA). The reaction mixture (2.1 ml) contained 50 mM Na-Pi (pH 7.0), 37 μM MutL-E29A or 26.3 μM MutL-D58A, 200 mM NaCl and 3 mM MgCl₂. ATP (1.43 mM for MutL-E29A or 526 μM for MutL-D58A) in reaction buffer was injected using a 600 μl syringe rotating at 300 rpm. The injection volumes were 2.5 μl added over five seconds for MutL-E29A or 5.0 μl added over 10 seconds for MutL-D58A. The time between injections was 180 seconds. The experiments were performed at 25°C.

**Circular Dichroism** – Circular dichroism measurements were made in an Applied Photosystems Pistar-180 CD spectrometer. MutL and MutL-D58A protein concentrations were 3.8 μM and 3.9 μM, respectively, in CD buffer (150 mM NaF and 50 mM NaPi (pH = 7.0)). Circular dichroism measurements were taken from 185 nm to 260 nm in 0.2 nm increments. Data was analyzed using CDpro (http://lamar.colostate.edu/~sreeram/CDPro/main.html) software using three algorithms (selcon3, CONTINLL, and ProtSS).
Results

Previous studies have shown that MutL catalyzes ATP hydrolysis (36) and that MutL-catalyzed ATP hydrolysis is required for MMR (37,45). However, the rate of MutL-catalyzed ATP hydrolysis is slow in the presence of ssDNA (~ 9 min\(^{-1}\) (37)) and it seems unlikely this fuels active translocation along the DNA lattice suggesting that ATP hydrolysis may play another role (46,47). We and others (29-31) have shown that MutL interacts with and dramatically stimulates the duplex DNA unwinding activity of UvrD. An analysis of this reaction suggested that MutL loads UvrD onto the DNA substrate without significantly increasing the processivity of UvrD-catalyzed unwinding (32). We were interested in determining if stimulation of the UvrD-catalyzed unwinding reaction required the hydrolysis of ATP by MutL. If this were the case, then the MutL-catalyzed ATP hydrolysis requirement in MMR might be explained by the requirement for ATP hydrolysis in loading UvrD to begin resection of the damaged DNA strand. Such a requirement would be consistent with the characterization of MutL as a molecular matchmaker (48).

*MutL-E29A lacks detectable ATPase activity* – To test this hypothesis two MutL point mutants, an ATP hydrolysis defective mutant and an ATP binding defective mutant, were constructed using site-directed mutagenesis. Based on the available three-dimensional structure of the amino-terminal domain of MutL (36) and previous studies (45,50) several mutants were constructed and the mutant proteins purified including MutL-E29A, MutL-E32K, MutL-N33A, MutL-R95F, MutL-D58N and MutL-D58A. Each of the mutant proteins was purified and judged to be greater than 95% pure as determined by SDS-PAGE.
In each case, the purification properties were the same as those of the wild-type protein. The final step in the purification procedure involved gel filtration and all MutL proteins used in this study eluted from this column at the position expected for a MutL dimer. We note that MutL-D58A migrates slightly slower than MutL and the other point mutants shown in SDS-PAGE. The reason for this is not clear. Sequencing of the mutant gene indicates the absence of any unintended mutations and CD spectra indicate the native protein is properly folded.

A ssDNA-stimulated ATPase assay was used to evaluate the ATP hydrolysis reaction catalyzed by each mutant as compared to the wild-type protein (Fig. 12, data not shown). Wild-type MutL catalyzes a weak, but detectable, DNA-stimulated ATP hydrolysis reaction with a $k_{cat}$ of approximately 9 min$^{-1}$. This value is in good agreement with a previously reported value for this protein (37) but higher than a recently reported value (45). The reason for this discrepancy is not clear but may be related to the ssDNA effector used in the studies reported here. We have used circular M13 ssDNA to stimulate the MutL ATPase reaction since several of the substrates used in DNA helicase activity assays are based on circular DNA molecules.

The MutL-N33A mutant protein exhibited a reduced rate of ATP hydrolysis but ATPase activity was still clearly detectable (Fig. 12). MutL-E32K and MutL-R95F also exhibited slow rates of ATP hydrolysis (data not shown). A reduced, relative to wild-type, ATPase activity associated with the MutL-R95F mutant has been reported previously (45). The ATPase activity of MutL-E29A, on the other hand, was essentially undetectable (Fig. 12). Even at high protein concentration a reproducible rate of ATP hydrolysis could not be measured (data not shown). Therefore, MutL-E29A was chosen to analyze the requirement
for MutL-catalyzed ATP hydrolysis on stimulation of the unwinding reaction catalyzed by UvrD.

As expected, MutL-D58A, which does not bind ATP (see Fig. 15), failed to catalyze the hydrolysis of ATP (data not shown). It is important to note that MutL-E29A binds ATP and MutL-D58A fails to bind ATP as will be shown below (see Fig. 15). Therefore, MutL-E29A is ATP hydrolysis defective but ATP binding proficient while MutL-D58A fails to bind nucleotide.

*MutL-E29A and MutL-D58A fail to complement the loss of MutL* – Previous studies (37,45) have shown that MutL-catalyzed ATP hydrolysis is required for MMR. Therefore, neither *mutL-E29A* nor *mutL-D58A* was expected to complement a strain containing a deletion of the *mutL* gene in a genetic complementation assay measuring mutation frequency. In this genetic test the wild-type and mutant proteins were expressed from the expression plasmid that was used for protein purification. It is important to note that neither wild-type nor mutant proteins were overexpressed in these experiments. Rather, expression was dependent on the adventitious use of an RNA polymerase promoter on the plasmid. The *mutL* deletion strain used in these experiments (GE1752mutL::Tn10) does not contain the gene encoding T7 RNA polymerase and, therefore, basal level expression of T7 RNA polymerase cannot explain the expression of MutL observed in these cells. Western blots using polyclonal antisera directed against MutL have shown the *mutL* deletion strain to be deficient in detectable MutL protein and have indicated that plasmid-based expression of both wild-type and mutant MutL is less than 5-fold higher than normal chromosomal levels.
Using a standard fluctuation test (51,52), the frequency of mutation from Rif\textsuperscript{S} to Rif\textsuperscript{R} was measured using a wild-type strain containing a fully intact MMR system, a strain containing a deletion of the mut\textit{L} gene, a strain in which the mut\textit{L} gene was deleted and complemented using an expression plasmid harboring the wild-type mut\textit{L} gene (pET15b-Mut\textit{L}), and strains in which the mut\textit{L} gene was deleted and complementation was tested using an expression plasmid containing either mut\textit{L}-E29A or mut\textit{L}-D58A (Table 3). The mutation frequency increased by approximately 100-fold when the mut\textit{L} gene was deleted, consistent with previous reports (45,53). The mut\textit{L} deletion was effectively complemented by the wild-type protein expressed from pET15b-Mut\textit{L}. Thus, the histidine tag on the amino-terminal end of the protein does not interfere with biological activity as previously shown (53). The Mut\textit{L}-E29A protein and the Mut\textit{L}-D58A proteins did not complement the mut\textit{L} deletion consistent with the previously described requirement for Mut\textit{L}-catalyzed ATP hydrolysis for MMR (37,45). As indicated above, western blots have shown that the mutant proteins are expressed (data not shown) and, therefore, a lack of protein cannot explain the failure to complement MMR as measured by the increase in mutation rate.

\textit{MutL-E29A stimulated UvrD-catalyzed DNA unwinding} – Purified Mut\textit{L}-E29A and Mut\textit{L}-D58A were compared with wild-type Mut\textit{L} in helicase activity assays that measure the ability of Mut\textit{L} to stimulate the unwinding reaction catalyzed by UvrD (Fig. 13). In these experiments two different DNA substrates were utilized. The first was a partial duplex DNA substrate containing 93 bp of duplex DNA on circular M13 ssDNA. The second substrate
was a fully duplex 750 bp DNA fragment. As shown previously (32), MutL dramatically stimulated unwinding of the 93 bp partial duplex substrate by UvrD (Fig. 13A). At a concentration of 1.3 nM UvrD, less than 5% of the substrate was unwound in a 10 minute incubation. The addition of increasing concentrations of MutL stimulated the unwinding reaction and at a concentration of 57 nM MutL nearly 80% of the DNA substrate was unwound. This represents a 20-fold stimulation of the unwinding reaction.

Our previous studies suggest that MutL binds the DNA and loads UvrD onto the substrate (32). Perhaps each loading event is associated with the ATP hydrolysis cycle of MutL. To test this possibility MutL-E29A was used in place of wild-type MutL in these reactions. Remarkably, the hydrolysis defective mutant protein stimulated UvrD-catalyzed unwinding to essentially the same extent as wild-type MutL (Fig. 13A). However, the maximal unwinding reaction was achieved at a lower concentration of the mutant protein. This indicates that MutL-catalyzed ATP hydrolysis is not required for stimulation of the UvrD helicase reaction. Moreover, the ATP hydrolysis defective mutant stimulated unwinding better than the wild-type protein. The ATP binding defective MutL mutant, MutL-D58A, failed to stimulate the unwinding reaction catalyzed by UvrD (Fig. 13C).

UvrD also unwinds both nicked and fully duplex DNA molecules but at higher protein concentrations (42,62). Presumably, thermal denaturation of the DNA at a nick or blunt end allows UvrD to bind and initiate an unwinding reaction. The requirement for an increased UvrD concentration in reactions using these two substrates is similar and the physiologically relevant substrate in MMR is a nicked DNA molecule. In the following experiments we used a blunt duplex DNA substrate with a duplex region of significant length to approximate the conditions that might be encountered in vivo making the assumption that loading at a blunt
duplex end will be similar to loading at a nick. As shown previously (32) and in Figure 13B, wild-type MutL stimulated the unwinding of duplex DNA catalyzed by UvrD. At a concentration of 16 nM UvrD approximately 4-5% of the 750 bp blunt duplex DNA was unwound in the absence of MutL. The addition of increasing concentrations of MutL increased the fraction of substrate unwound to greater than 50% representing a 10-fold stimulation. When wild-type MutL was substituted with the ATP hydrolysis defective MutL-E29A a similar extent of unwinding was achieved but at significantly lower MutL concentrations (Fig. 13B). This is essentially the same result observed using the partial duplex DNA substrate although the effect is more pronounced at low MutL concentrations on this blunt ended DNA substrate. Consistent with the results presented above using a partial duplex substrate, MutL-D58A failed to stimulate the UvrD-catalyzed unwinding reaction (Fig. 13D).

We conclude that ATP hydrolysis catalyzed by MutL is not essential to load UvrD on either a partial duplex substrate or a substrate with a blunt duplex end. Furthermore, an ATPase-deficient MutL mutant is significantly more efficient than the wild-type protein in promoting this reaction. In addition, these data show that the ATP-bound form of MutL participates in the loading of UvrD as demonstrated using the MutL-D58A mutant which failed to stimulate the unwinding reaction using either DNA substrate.

MutL-E29A binds ATP – The data presented above suggest that while ATP hydrolysis by MutL is not required to load UvrD onto a DNA substrate it plays some role in regulating this loading event. That is, loading of UvrD is more efficient in the absence of ATP hydrolysis than it is in the presence of ATP hydrolysis. To gain a better understanding of this reaction it
was important to show that an ATP-bound form of MutL was participating in this reaction. The structure of the amino-terminal ATPase domain of MutL suggests that a mutation in glutamic acid 29 is likely to abrogate ATP hydrolysis but not ATP binding and a previous study has indicated that MutL-E29A binds ATP (45). Importantly, an understanding of the role of ATP binding and hydrolysis by MutL in MMR requires an understanding of the role of the nucleotide in each partial reaction. Therefore, the ability of MutL-E29A to bind ATP was tested.

Previous studies (34,35,41,45) have indicated that MutL binds ssDNA and this binding is dependent on the presence of a nucleotide. Thus, we determined the ability of MutL-E29A to bind DNA in the presence of a non-hydrolyzable ATP analog (AMP-PNP), which has been shown to bind MutL at the ATP binding site (36), and compared this with the ssDNA binding of wild-type MutL (Fig. 14). The data in Figure 14 (upper panel) show the results of gel mobility shift assays using MutL, MutL-E29A and a ssDNA oligonucleotide ligand. Both proteins bind the oligonucleotide in the presence of AMP-PNP (Fig. 14 upper panel, lanes 6-9 and 15-18). Importantly, neither protein bound the DNA to any significant extent in the absence of AMP-PNP (Fig. 14 upper panel, lanes 1-4 and 10-14). This is consistent with our previous studies (32) demonstrating the ability of MutL to bind ssDNA in the presence of AMP-PNP and provides indirect evidence that MutL-E29A binds AMP-PNP. If this were not the case then MutL-E29A would not bind the ssDNA oligonucleotide, even in the presence of AMP-PNP.

The binding of MutL and MutL-E29A to the partial duplex DNA substrate used in unwinding assays was also tested using a nitrocellulose filter binding assay (Fig. 14 lower panel). This ligand contains a variety of secondary structures including both ssDNA and
duplex DNA. Again, both MutL and MutL-E29A bound this DNA molecule in the presence of AMP-PNP. At low protein concentrations MutL-E29A bound this ligand slightly better than wild-type MutL. We conclude, on the basis of DNA binding assays, that MutL-E29A binds ATP as represented by its ability to bind DNA in the presence of AMP-PNP. However, these tests of ATP binding are indirect and we sought a more direct method to test the binding of ATP by MutL-E29A.

The binding of ATP to MutL-E29A was directly measured using isothermal microcalorimetry which measures heat change due to the binding of a ligand to a protein. These data are shown in Figure 15A and clearly indicate the binding of ATP. In this experiment the MutL-E29A concentration was held constant at 37 μM and the ATP concentration was increased. It is important to note that ATP is not hydrolyzed during the course of this experiment since MutL-E29A does not hydrolyze ATP. We conclude that MutL-E29A binds ATP and, under the conditions used in the experiments shown in Fig. 13, the ATP-bound form of MutL is responsible for stimulating the unwinding reaction catalyzed by UvrD.

Similar experiments were performed with MutL-D58A which was predicted to have no ATP binding activity (Fig. 15B). The data indicate no binding of nucleotide to the protein. This is consistent with the lack of ATP hydrolysis activity and provides evidence supporting our conclusion that the ATP-bound form of MutL stimulates the UvrD-catalyzed unwinding reaction while the ATP-free form of MutL does not.

*MutL-E29A stimulates MutH-catalyzed nicking while MutL-D58A fails to simulate nicking*

– In addition to interacting with UvrD, MutL also interacts with MutS (20) and stimulates the
latent endonuclease reaction catalyzed by MutH (20-22). Efficient stimulation of MutH-catalyzed nicking at a hemi-methylated d(GATC) is dependent on the presence of a mismatch base pair and the presence of MutS. However, we have shown that MutL interacts with MutH and is capable of stimulating the MutH-catalyzed nicking reaction on model substrates in the absence of MutS (23). Importantly, this reaction is dependent on the presence of ATP but does not require ATP hydrolysis.

To ensure that MutL-E29A could interact with MutS and MutH, and to evaluate the role of ATP hydrolysis in the MutL-stimulated nicking reaction catalyzed by MutH, we examined the ability of these proteins to interact in a functional assay based on a partial reconstitution of the mismatch repair pathway (Fig. 16). In this assay the ability of MutL to stimulate the latent endonuclease reaction catalyzed by MutH at a hemi-methylated d(GATC) site was evaluated in the presence of a G-T mismatch and MutS. It is clear that MutL-E29A was able to stimulate the MutH endonuclease reaction with essentially the same efficiency as wild-type MutL. Control experiments (data not shown) using DNA lacking a mismatch, with MutL alone, MutS alone and MutH alone indicated this reaction was dependent on all three proteins as well as the mismatch. We conclude that MutL-E29A interacts with both MutS and MutH. In addition, these data indicate that ATP hydrolysis catalyzed by MutL is not required at either the recognition or incision steps in the MMR pathway.

We also evaluated the ability of MutL-D58A to function in this partially reconstituted repair reaction. Since previous results suggested that ATP was required for MutL-stimulated nicking catalyzed by MutH, we expected that MutL-D58A would not substitute for MutL in this reaction. This was indeed the case (Fig. 16). In the presence of MutS, MutH, hemi-methylated DNA and a G-T mismatch there was no observable nicking of the heteroduplex.
DNA molecule when MutL-D58A was added to the reaction. Thus, ATP binding by MutL is essential for the incision step in MMR.

To further investigate the failure of MutL-D58A to function in this partial reconstitution of MMR we investigated the interaction of MutL-D58A with MutS using footprinting experiments (Fig. 17). It has been demonstrated that MutS specifically binds a base pair mismatch and the footprint formed upon MutS binding is extended in the presence of MutL (20). This can be clearly seen in Figure 17 (lanes 5-7). When MutL-D58A is substituted for MutL in this reaction the footprint is essentially identical to that of MutS alone indicating that MutL-D58A does not interact with MutS (Fig. 17, lanes 8-10). We also investigated the interaction of MutL-D58A with MutH using affinity chromatography (data not shown). MutL-D58A does not interact with MutH consistent with the fact it does not stimulate MutH-catalyzed nicking of a heteroduplex DNA substrate. Thus, the ATP bound form of MutL participates in the initial steps of MMR and nucleotide binding by MutL is essential for these steps in the pathway.

**Discussion**

Methyl-directed mismatch repair in *E. coli* is a carefully orchestrated process with remarkable properties that is essential to the fidelity of DNA replication and the maintenance of the genome (1-5). While the activities of more than 10 proteins are essential for MMR, this report focuses on two of those proteins – MutL and UvrD. Both are essential components of the *E. coli* MMR machinery (15). MutL binds DNA (32-35,41), has been shown to interact with MutS, MutH and UvrD (20,23,24,29), and plays a key role in
managing the repair process. In addition, the protein has a DNA-stimulated ATP hydrolysis activity (36) that is essential for MMR (37) but whose molecular role in the process is unknown. UvrD is a 3' to 5' DNA helicase that is essential for displacing the strand to be removed, and ultimately replaced, by the repair process. No other helicase in *E. coli* is able to substitute for UvrD in this role in the cell. Earlier work demonstrated a physical interaction between MutL and UvrD (29) and it has been shown that MutL dramatically stimulates the unwinding activity of UvrD (30-32,45). We have proposed that MutL loads UvrD productively onto the DNA but does not clamp UvrD on the DNA during the unwinding reaction (32). Therefore, the processivity of the unwinding reaction catalyzed by UvrD is not increased. We have also suggested that loading of UvrD by MutL is likely to be a continuous process with multiple molecules of UvrD loaded onto a single DNA substrate. This accounts for the unwinding of long duplex molecules which contain duplex regions far in excess of the processivity of UvrD. We suggest that the additional molecules of UvrD translocate from the initial loading site to the duplex region and thus contribute to the unwinding of longer duplex DNA molecules (32). The processivity of UvrD as a translocase is much higher than as a helicase (57) consistent with this proposed mechanism. In this report we have examined the role of the MutL-catalyzed ATP hydrolysis reaction in the loading of UvrD.

The data presented here clearly show that MutL-catalyzed ATP hydrolysis is not essential for stimulation of UvrD-catalyzed unwinding of duplex DNA (see Fig. 13). Using a MutL point mutant (MutL-E29A) that does not catalyze ATP hydrolysis the stimulation of UvrD-catalyzed duplex DNA unwinding has been directly measured using two DNA substrates. One DNA substrate contained a relatively short region of duplex DNA (93 bp) on a circular
DNA molecule. The other substrate contained a much longer region of duplex DNA (750 bp) that may be more characteristic of the length of many of the repair tracks in MMR. In addition, this DNA substrate contained fully duplex ends and thus lacked a 3'-ssDNA tail to facilitate loading of UvrD. Remarkably, stimulation of the unwinding reaction catalyzed by UvrD was more robust with MutL-E29A than with the wild-type protein. In other words, the stimulation of UvrD-catalyzed unwinding was better in the absence of MutL-catalyzed ATP hydrolysis, particularly on longer duplex regions, than in the presence of MutL-catalyzed hydrolysis. Therefore, the ATPase reaction associated with MutL is not required to load UvrD onto the mismatch repair intermediate to be unwound by UvrD.

In addition, we have shown that the ATP-bound form of MutL is responsible for stimulating UvrD and we suggest that the ATP-bound form of the protein loads UvrD onto the DNA substrate. The ability of MutL-E29A to bind ATP was directly demonstrated by isothermal titration calorimetry (see Fig. 15) and, since the protein fails to hydrolyze ATP, we assume the ATP-bound form of the protein is predominant under the conditions used in the experiments reported here.

To provide additional support for this conclusion MutL-D58A, a mutant that does not bind ATP, was constructed. The purified protein was properly folded, as evidenced by circular dichroism spectroscopy, and was able to bind a partial duplex DNA substrate which does not require binding of ATP by MutL (32). We directly demonstrated that the protein did not bind ATP and it did not stimulate the unwinding reaction catalyzed by UvrD using either a partial duplex substrate or a blunt duplex substrate. Thus, the ATP free form of MutL does not stimulate UvrD-catalyzed unwinding.
The model for the action of MutL presented by Ban and Yang (36) posits that binding of ATP promotes the dimerization of the amino-terminal ATPase domain of MutL. The protein is a dimer in the absence of ATP binding as the protein dimerization interface is in the C-terminal region on the protein (58,59). We suggest that the ATP-induced dimerization of MutL N-terminal domain allows the protein to clamp onto the DNA substrate (41) at the nick generated by MutH. This provides a loading platform for UvrD and helicase molecules are continuously loaded onto the DNA substrate as long as MutL remains clamped on the DNA. In the absence of ATP hydrolysis (i.e. with MutL-E29A) MutL remains bound and loads multiple molecules of UvrD. This results in a very dramatic stimulation of UvrD-catalyzed unwinding at low concentrations of MutL. In this case the most dramatic effect of MutL-E29A on the unwinding reaction would be expected when long duplex substrates are used as is seen here. Since the processivity of UvrD as a helicase has been reported to be approximately 50 bp (56) then perhaps as few as two UvrD molecules must be loaded to unwind the 93 bp partial duplex DNA while many more molecules of UvrD must be loaded onto the 750 bp blunt duplex DNA to effect complete unwinding. This nicely explains the increased stimulation of UvrD-catalyzed unwinding in the presence of MutL-E29A. Importantly, in the absence of ATP-induced dimerization (i.e. with MutL-D58A) the protein does not clamp onto the DNA and does not form a loading platform for UvrD explaining the lack of MutL-D58A-stimulated unwinding.

Furthermore, we suggest that the interaction between MutL and UvrD that results in stimulation of unwinding is abrogated upon ATP hydrolysis by MutL, perhaps due to the release of MutL from the DNA. The result is cessation of loading of UvrD onto the DNA substrate and, ultimately, cessation of unwinding. In the case of MutL-E29A the bound ATP
is not hydrolyzed and MutL continues to load UvrD onto the DNA substrate with a resulting increase in the amount of DNA unwound as compared to wild-type MutL. A model that incorporates these findings is shown in Figure 18.

These results have important implications for our understanding of the process of MMR. First, they serve to further refine our understanding of the role of ATP binding and hydrolysis catalyzed by MutL in MMR. It has been established previously that ATP binding but not hydrolysis by MutL is required for interaction with MutS (60). The requirement for ATP binding by MutL is confirmed in the footprinting assays using MutL-D58A and MutS (see Fig. 17). MutL-D58A does not interact with MutS because it does not bind ATP. In addition, we have shown here (see Fig. 16) and previously (23) that the ATP-bound form of MutL interacts with and stimulates the latent endonuclease reaction associated with MutH. In this report we demonstrate that MutL loads UvrD onto a DNA substrate in the absence of ATP hydrolysis but in a reaction that requires the ATP-bound form of MutL. Thus, the ATP-bound MutL is essential for the initial steps of MMR and MutL-catalyzed ATP hydrolysis is required after strand incision and the beginning stage of strand resection. Second, the data presented here offer the possibility that ATP hydrolysis catalyzed by MutL regulates the amount of UvrD loaded onto the DNA substrate. In the absence of ATP hydrolysis the unwinding step may be uncoupled from the rescission step such that the exonuclease responsible for removing the unwound damage containing nascent strand is unable to keep up with the advancing helicase. If this were the case then repair events might not be properly completed due to the uncoupling and there would be an increase in mutation rate as was observed when mutL-E29A was substituted for mutL. This might result in the genome becoming fragmented resulting in genomic instability. This has not been measured.
Alternatively, MutL-catalyzed ATP hydrolysis may be required for a subsequent loading event in the MMR pathway. For example, MutL might recruit and load the appropriate exonuclease to digest the damaged DNA strand or it might recruit and load the beta clamp as a prelude to loading DNA polymerase onto the available 3’-OH to complete strand resynthesis. In either case, the data presented here are consistent with the view that MutL and its associated ATPase serve as some kind of switch (47) regulating the overall process of MMR. While we have suggested the MutL ATPase reaction might serve to regulate the loading of UvrD this is not the only possibility. Additional data will be required to fully understand the role of the MutL ATPase activity.
References


Table 3. MutL-E29A and MutL-D58A exhibit a mutator phenotype

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Mutation Rate (Mutations/Generation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GE1752</td>
<td>mutL$^+$</td>
<td>9.23 x 10$^{-10}$</td>
</tr>
<tr>
<td>GE1752mutL::Tn10</td>
<td>mutL$^-$</td>
<td>2.36 x 10$^{-7}$</td>
</tr>
<tr>
<td>GEΔmutL/pET15b-MutL</td>
<td>mutL$^+$</td>
<td>9.20 x 10$^{-9}$</td>
</tr>
<tr>
<td>GEΔmutL/pET15b-MutL-D58A</td>
<td>mutL-D58A</td>
<td>5.61 x 10$^{-8}$</td>
</tr>
<tr>
<td>GEΔmutL/pET15b-MutL-E29A</td>
<td>mutL-E29A</td>
<td>1.54 x 10$^{-7}$</td>
</tr>
</tbody>
</table>
Figure 11. SDS-PAGE analysis of MutL and MutL mutants. Proteins (2 μg) were resolved on a 9.6% polyacrylamide gel run in the presence of SDS and stained with Coomassie blue. Lane 1, molecular weight standards (size in kDa indicated on the left); lane 2, MutL; lane 3, MutL-E29A; lane 4, MutL-E32K; lane 5, MutL-N33A; lane 6, MutL-R95F and lane 7, MutL-D58A.
Figure 12. ATP hydrolysis by MutL, MutL-N33A and MutL-E29A. DNA-dependent ATPase reactions were as described under “Materials and methods” using 0.38 μM MutL (●), 0.37 μM MutL-N33A (▲) and 0.5 μM MutL-E29A (■). Reaction mixtures were pre-incubated at 37°C prior to the addition of MutL. Aliquots were removed at the indicated times. The data represents the average of at least 3 experiments; error bars are the standard deviation about the mean.
Figure 13. MutL-E29A stimulates the helicase reaction catalyzed by UvrD. DNA helicase activity assays were as described under “Materials and methods”. Panels A and C – A 93 bp partial duplex DNA was incubated with UvrD (1.3 nM) and the indicated concentrations of MutL (●), MutL-E29A (■, panel A) or MutL-D58A (■, panel C) for 10 min at 37°C. The products of the reaction were resolved on an 8% native polyacrylamide gel and quantified as indicated under “Materials and methods”. The data represent the average of at least 3 experiments with error bars indicating the standard deviation about the mean. Panels B and D – a 750 bp blunt duplex DNA was incubated with UvrD (16 nM) and the indicated concentrations of MutL (●), MutL-E29A (■, panel B) or MutL-D58A (■, panel D) for 20 min at 37°C. The fraction of the substrate unwound in the presence of MutL was determined and compared to the fraction of the substrate unwound by UvrD alone to determine the level of stimulation. All data presented represent the average of at least three experiments. Please note the different titrations of MutL used in the various experiments.
Figure 14. MutL and MutL-E29A bind DNA in the presence of AMP-PNP. **Upper panel** Electrophoretic gel mobility shift assays were as described under “Materials and methods” using a 5'-end labeled ssDNA 50-mer and either MutL or MutL-E29A. The MutL and MutL-E29A concentrations used were 22 nM, 44 nM, 88 nM and 175 nM. AMP-PNP at a final concentration of 3 mM was included in lanes 6-9 and lanes 15-18. **Lower panel** Nitrocellulose filter binding assays using a 93 bp [32P]DNA partial duplex ligand were as described under “Materials and methods” using the indicated concentrations of MutL (●) or MutL-E29A (■). All reaction mixtures contained AMP-PNP at a final concentration of 3 mM. The data represent the average of 3 or more experiments with error bars that represent the standard deviation about the mean.
Figure 15. MutL-E29A binds ATP. Isothermal titration calorimetry was as described under “Materials and methods” using MutL-E29A (panel A) and MutL-D58A (panel B).
Figure 16. MutL-E29A stimulates MutH-catalyzed nicking of heteroduplex DNA. Reaction mixtures containing either MutL (●), MutL-E29A (♦) or MutL-D58A (■) at the indicated concentrations were as described under “Materials and methods”. Reactions were incubated for 15 min at 37°C and terminated by the addition of EDTA. Reaction products were resolved on a 0.8% agarose gel run in the presence of 0.5 µg/ml EtBr. The gels were quantified as described under “Materials and methods”. The data represent the average of at least two determinations.
Figure 17. MutL-D58A does not interact with MutS. DNase I protection assays were as described under “Materials and methods” using 1 μM MutS protein, 1 mM ATP and 1.3 μM, 650 nM, or 325 nM of MutL or MutL-D58A as indicated. The reaction products were resolved on an 8% denaturing polyacrylamide gel. Lane 1, heteroduplex DNA cleaved with Clal (50 bases) and HaeIII (25 bases); lane 2, marker ladder prepared using the [*32P]*91-mer as previously described (63). The position of the G-T mismatch (position 59 relative to the 5'-end) is indicated by an arrow on the left.
Figure 18. A model for MMR. The base pair mismatch shown in (a) is recognized and bound by the homodimer MutS protein (b). (c) The dimeric MutL protein, with ATP bound, mediates communication between the MutS-bound mismatch and MutH bound at the nearest hemi-methylated d(GATC) site. It should be noted that mismatch correction is bidirectional (26) and only a single direction is shown in this model. (d) MutL loads multiple molecules of UvrD onto the nicked intermediate to unwind the damaged DNA strand which is degraded by one of four exonucleases with an appropriate polarity (61). MutL-catalyzed ATP hydrolysis is required for some step in the reaction pathway subsequent to the loading of UvrD. (e) Resection of the damaged DNA strand extends past the mismatch and, presumably, MutL and MutS are displaced by an unknown mechanism. (f) The 3'-OH is then extended by DNA polymerase III, the resulting nick is sealed by DNA ligase (g) to restore the integrity of the DNA strand and (h) Dam methylase methylates the d(GATC) on the repaired DNA strand.
The *Escherichia coli* very short patch (VSP) repair system acts to correct guanine:thymine mismatches that result from spontaneous hydrolytic deamination of 5-methyl cytosine. The VSP repair pathway requires the Vsr endonuclease, DNA polymerase I, a DNA ligase, MutS and MutL to function at its peak efficiency. The biochemical roles of most of these proteins in the VSP repair pathway have been studied extensively; however, these proteins have not been studied together in the context of VSP repair in an *in vitro* system. Through the use of purified components of the VSP repair system we have begun to develop an understanding of the role that each of these proteins has in the VSP repair system. In this report we demonstrate an *in vitro* reconstitution of the minimal VSP repair pathway and propose roles for each of the proteins that are known to function in the VSP repair pathway.

**Introduction**

The repair of mismatched bases is of extraordinary importance in maintaining genomic integrity in both eukaryotic and prokaryotic organisms. In *E. coli*, as is also the case in other organisms, there are many endogenous mechanisms that can lead to a base:base mismatch. The primary endogenous mechanisms resulting in base mismatches are errors that result from
DNA replication and spontaneous deamination of DNA bases (1,2). Importantly, in every instance where a mismatch is generated the repair machinery must be able to determine which DNA strand contains the correct base and which DNA strand contains the incorrect base, and subsequently repair the latter—this is known as strand discrimination (reviewed in (3)).

The misincorporation of nucleotides by DNA polymerase III during DNA replication is a well known source of base-base mismatches. These base mismatches are repaired by the mismatch repair pathway (reviewed in (4,5)) which, in E. coli, is directed by the adenine methylation status of the DNA. This repair pathway is able to discriminate between the correct parental strand and the incorrect daughter strand due to the transient unmethylated state at d(GATC) sequences of the daughter strand immediately following replication (6).

Another mechanism leading to base mismatches is the spontaneous hydrolytic deamination of cytosine or adenine residues. The hydrolytic deamination of cytosine produces a uracil:guanine mismatch (7) while the hydrolytic deamination of adenine produces a hypoxanthine-thymine mismatch (8). The correct DNA strand is recognized in each case because uracil and hypoxanthine are not normal bases in DNA. The uracil:guanine mismatch is acted on by the uracil DNA glycosylase, producing an abasic site that is subsequently repaired by base excision repair proteins (reviewed in (9)). The hypoxanthine:thymine mismatch recruits the hypoxanthine DNA glycosylase that removes the hypoxanthine base producing an abasic site (reviewed in (10)). This abasic site is then acted on by base excision repair enzymes to facilitate repair.

In E. coli, the sequence 5′-CCWGG-3′/5′-CCWGG-3′ is methylated, by the Dcm methylase at the second cytosine producing a 5-methyl cytosine (11). When this base
undergoes spontaneous hydrolytic deamination a thymine:guanine mismatch is produced. Both guanine and thymine are naturally occurring bases in *E. coli*, thus this mismatch is intractable to most repair pathways since strand discrimination is not possible. In addition, this thymine:guanine mismatch is refractory to the methyl-directed mismatch repair pathway when the bacterial chromosome is not replicating because the double helix is fully methylated at the d(GATC) sequences that direct the MMR process (12,13). Therefore, this particular mismatch in this sequence context necessitates another DNA repair pathway. This repair pathway in *E. coli* is able to discriminate between the erroneous thymine and the correct guanine, and selectively remove the incorrect thymine. This repair mechanism is known as very short patch (VSP) repair (14).

Hydrolytic deamination of cytosine is a common event in *E. coli*, as it is in all cells containing cytosines, occurring at a rate of one per $10^7$ cytosines per day (15). As noted previously, the deamination of cytosine produces an erroneous uracil base in the double helix, repairable by the uracil DNA glycosylase. Deamination of 5-methyl cytosine occurs at a rate 2-3 times higher than deamination of cytosine (16) and this hydrolytic deamination produces a thymine. The *E. coli* bacterium utilizes the very short patch repair pathway to allow it to specifically repair this G:T mismatch in the 5′-CTWG-3′/5′-CCWG-3′ sequence context (17). It is important to note that this sequence is the only place in the *E. coli* chromosome that would produce a G:T mismatch due to spontaneous hydrolytic deamination of 5-methyl cytosine, because this sequence is the only place where the Dcm methyltransferase acts. The sequence specificity of the deamination of 5-methyl cytosine also provides the sequence specificity for repair and strand discrimination.
As measured in genetic assays, VSP repair requires the Vsr endonuclease, DNA polymerase I, DNA ligase I, MutL, and MutS to function at maximum efficiency. Unlike methyl-directed mismatch repair, deletion of MutS or MutL does not abolish the repair activity of the VSP repair pathway. Rather, these deletions result in a decrease in the efficiency of the pathway. In a wild type genetic background VSP repair corrects a G:T mismatch in the 5′-CTWGG-3′/5′-C^5meCWGG-3′ sequence with nearly 100% efficiency (18,19). However, in a mutL or a mutS genetic background VSP corrects G:T mismatches in the appropriate sequence context with 33 – 67% efficiency (18,20,21). These data suggest that while MutL and MutS are not absolutely required for the function of the VSP repair pathway, they serve to augment the pathway. Therefore, the minimal VSP repair pathway requires three proteins—the Vsr endonuclease, DNA polymerase I, and presumably DNA Ligase I (14,17,22).

The gene product of vsr—the Vsr endonuclease—is implicated as the initiating step in VSP repair. The VSR endonuclease cleaves 5′ to the mismatched thymine leaving a nick with a 3′-OH and a 5′-PO₄ in the DNA (17,23-26). DNA polymerase I is thought to bind at the nick produced by Vsr cleavage and nick translate, using its 5′ to 3′ exonuclease activity and its 5′ to 3′ polymerase activity, 10-15 base pairs beyond the mismatched base (18,19,22). This nick translation reaction leaves a ligatable nick which is presumably sealed by DNA ligase I to restore the integrity of the E. coli genome. The exact functions of MutL and MutS in the pathway remain unknown; however, these proteins are required for VSP repair to function at maximum efficiency (18,19).

In this report we have reconstituted the E. coli VSP repair reaction in vitro using purified proteins and a model substrate. We demonstrate the requirement for the Vsr endonuclease,
DNA polymerase I, and DNA ligase I in a minimal VSP repair reaction. In this reaction the length of the repair patch can be modulated by altering the DNA ligase concentration. Furthermore, we have attempted to elucidate a role for MutS and MutL function in the VSP repair pathway.

**Materials and Methods**

**Cloning**

The gene encoding the Vsr endonuclease (vsr) was amplified from genomic GE1752 DNA using the following primers: 5′-GGG AAT TCC ATA TGG CCG ACG TTC ACG AT-3′ and 5′-TTC CGC TCG AGA GCG AGT AAA TGA ATC CC-3′. These primers were designed to add an NdeI site and an XhoI site to facilitate cloning in an expression vector. The amplified DNA fragment was isolated on a 0.8% (w/v) agarose gel and gel purified using a Qiaquick spin column (Qiagen). The fragment was then digested to completion with NdeI and XhoI and gel purified using a Qiaquick spin column. The overexpression plasmid pTYB1 (New England Biolabs) was also digested to completion with NdeI and XhoI and the plasmid backbone was gel purified using a Qiaquick spin column. The DNA fragment encoding the Vsr endonuclease was cloned into the pTYB1 vector in frame with the intein::CBD fusion creating a carboxyl terminal Vsr intein::CBD fusion gene. This construct was used for purification of the Vsr endonuclease as detailed below.

The polA gene was amplified from genomic GE1752 DNA using the following primers: 5′-TTT TTC ATA TGG TTC AGA TCC CC-3′ and 5′-TTT TTG GAT CCT TAG TGC GCC TGA TCC-3′. These primers were designed to add an NdeI site and a BamHI site to facilitate
cloning in an expression vector. The amplified fragment was isolated on a 0.8% (w/v) agarose gel and purified using a Qiaquick spin column. The DNA fragment was digested with NdeI and BamHI and gel purified as above. The pET15b overexpression plasmid (Novagen) was also digested to completion with NdeI and BamHI and gel purified. The polA DNA fragment was cloned in frame with the amino terminal six histidine tag coded for by the backbone of the pET15b vector. This construct was used for purification of DNA polymerase I as detailed below.

The ligA gene was amplified from genomic GE1752 DNA using the following primers: 5′-GGA ATT CCA TAT GGA ATC AAT CGA ACA ACA-3′ and 5′-CGC GGA TCC TCA GCT ACC CAG CAA ACG C-3′. These primers were designed to add an NdeI site and a BamHI site to facilitate cloning in an expression vector. The PCR product was isolated on a 0.8% (w/v) agarose gel and gel purified using a Qiaquick spin column. The fragment was digested with NdeI and BamHI and gel purified. The pET15b overexpression plasmid was also digested to completion with NdeI and BamHI and gel purified using a Qiaquick spin column. The ligA gene fragment was cloned in frame with the amino terminal six histidine tag coded for by the backbone of the pET15b vector. This construct was used for the purification of DNA ligase as detailed below.

The pUC19-VSR construct was created by digesting pUC19 DNA (NEB) with BamHI and EcoRI. The insert was created by annealing the following synthetic oligonucleotides: 5′-AAT TCC TCA GCA ATC CTC AGC CAG GCC TCA GCT GGC CTC AGC G-3′ and 5′-GAT CCG CTG AGG CCA GCT GCC TGG CTG AGG ATT GCT GAG G-5′. Annealing reactions (50 μL) containing 100 fmol of each oligonucleotide, 50 mM Tris HCl (pH 7.5), 10 mM MgOAc, 5 mM DTT, and 50 mM NaCl were heated to
95°C in a GeneAmp PCR System 2400 Thermocycler (Applied Biosystems), the temperature was then decreased by one degree Celsius per minute to 30°C. The annealing reaction created compatible ends with the BamHI and EcoRI digested pUC19 vector. The insert was ligated into the pUC19 vector and transformed into DH5α cells. Cells were grown in 500 ml LB media containing 100 μg/ml ampicillin at 37°C for 24 hours. The supercoiled plasmid was purified using a CsCl/EtBr gradient as previously described (27).

**Protein Purifications**

BL21(λDE3) (FompT hsdS$_{B}$ r$_{B}^{-}$ m$_{B}^{-}$ gal dcm (λDE3)) cells harboring the pTYB1-Vsr plasmid were grown in 500 ml of LB containing 100 μg/ml ampicillin at 37°C to an A$_{600}$ of 0.6-0.8. The temperature was reduced to 16°C and protein production was induced by the addition of 0.5 mM IPTG. Cells were grown for 16 hours at 16°C and harvested by centrifugation. 500 ml of cells were resuspended in 5 ml VSR lysis buffer (25 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.1 mM PMSF, 0.1 mM benzamidine, 500 mM NaCl, 10% (v/v) glycerol) and frozen for later use.

Cells in VSR lysis buffer were thawed at 4°C. Lysozyme was added to a final concentration of 50 μg/ml and the mixture was incubated at 4°C for 60 min. Triton X-100 was added to a final concentration 0.1% (v/v) and the mixture was heated to 20°C with stirring. The mixture was briefly sonicated to reduce viscosity and then centrifuged at 50,000 g for 60 min to clarify the lysate. The supernatant was applied to a 1 ml chitin resin (NEB) column equilibrated with VSR lysis buffer according to the manufacturer’s instructions. The column was washed with VSR lysis buffer until the flow through contained no detectable protein as measured by a Bradford protein assay. Five ml of VSR lysis buffer with 50 mM
DTT was applied to the column and allowed to flow through the column. The column was then sealed and incubated at 4°C for 48 hours. Ten ml of VSR lysis buffer was applied to the column and 1 ml fractions were collected. Relevant fractions were pooled and dialyzed two times against 100 volumes of VSR Superdex Buffer (250 mM NaCl, 25 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 2 mM DTT, 10% (v/v) glycerol). The dialyzed pool was concentrated using an Amicon Ultra 15 10,000 MWCO (Millipore) centrifugal concentration device to 300 μL. The concentrated pool was applied to an equilibrated Superdex 200 gel filtration column (Amersham) according to the manufacturer’s instructions. Seventy, 0.5 ml fractions were collected and fractions containing the Vsr endonuclease were pooled and dialyzed three times against 100 volumes VSR Storage Buffer (250 mM NaCl, 25 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 2 mM DTT, 50% (v/v) glycerol). Purified Vsr endonuclease was stored at -20°C and was greater than 95% pure as judged by SDS-PAGE. Fresh protein preparations were made every two weeks to prevent possible artifacts resulting from degradation products.

BL21(λDE3) (F'ompT hsdS37(rB mB) gal dcm (λ DE3)) harboring the pET15b-PolA plasmid were grown in 500 ml LB media containing 100 μg/ml ampicillin at 37°C to A600 of 0.6. The production of DNA polymerase I was induced by the addition of IPTG to a final concentration of 0.5 mM. After induction cells were grown for 4 hours at 37°C and harvested by centrifugation. Cells were resuspended in 5 ml Pol I lysis buffer (500 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM imidazole, 10% (v/v) glycerol) and frozen for later use.

Cells expressing DNA polymerase I were thawed at 4°C. After thawing, cells were lysed by the addition of lysozyme to a final concentration of 200 μg/ml and incubated at 4°C for 60
Triton X-100 was added to a final concentration of 0.1% (v/v) and the mixture was heated to 20°C while stirring. The mixture was briefly sonicated to reduce viscosity and centrifuged at 50,000 g for 60 min to clarify the lysate. The supernatant was applied to a 1 ml Talon metal affinity resin (BD) column and washed with lysis buffer until the flow through contained less than 10 μg/ml total protein as assayed by a Bradford protein assay. The column was eluted by the addition of 10 ml Pol I lysis buffer containing 200 mM imidazole. Relevant fractions were pooled and dialyzed twice against 100 volumes of Pol I MonoQ buffer (40 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 8.0), 1 mM DTT, 10% Glycerol). After dialysis the pool was loaded onto a MonoQ HR (10/10) ion exchange column (Amersham) equilibrated with Pol I MonoQ buffer according to the manufacturer’s instructions. The column was washed with Pol I MonoQ buffer until the flow through contained less than 10 μg/ml total protein. The column was eluted with a 160 ml linear NaCl gradient from 40 mM to 500 mM NaCl. DNA Polymerase I eluted from the MonoQ column at approximately 200 mM NaCl. Fractions containing DNA polymerase I were tested for endonuclease activity on supercoiled pUC19 DNA. Fractions that contained no detectable endonuclease activity were pooled and dialyzed three times against 100 volumes Pol I storage buffer (50 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.5 mM EDTA (pH 8.0), 50% (v/v) glycerol) and stored at -20°C. DNA Polymerase I was judged to be greater than 95% pure as judged by SDS-PAGE.

BL21(λDE3) (F' ompT hsdSb(rB-mB') gal dcm (λDE3)) cells harboring pLysS (Novagen) and pET15b-LigA were grown at 37°C in 500 ml LB containing 100 μg/ml ampicillin and 50 mg/ml chloramphenicol to an A600 of 0.6-0.8. Production of DNA ligase I was induced by the addition of IPTG to a final concentration of 0.5 mM. Cells were grown for an additional
four hours at 37°C and harvested by centrifugation. Pellets were resuspended in 5 ml Ligase I lysis buffer (500 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol) and frozen for later use.

Cells expressing DNA ligase I were thawed at 4°C and lysed by the addition of lysozyme to a final concentration of 200 μg/ml and incubated at 4°C for 60 min. Triton X-100 was added to a final concentration of 0.1% (v/v) and the mixture was heated to 20°C. The mixture was briefly sonicated to reduce viscosity and centrifuged at 50,000 g for 60 min to clarify the lysate. The supernatant was applied to a 1 ml nickel-charged His bind resin (Novagen) column equilibrated in Ligase I lysis buffer according to the manufacturer’s instructions. The column was washed with Ligase I lysis buffer until the flow through contained less than 10 μg/ml total protein as measured by a Bradford protein assay. The column was eluted using Ligase I lysis buffer containing 200 mM imidazole. Relevant fractions were pooled and dialyzed twice against Ligase I MonoQ buffer (50 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 8.0), 1 mM DTT, 10% (v/v) glycerol). After dialysis the pool was loaded onto a MonoQ HR (10/10) ion exchange column (Amersham) equilibrated in Ligase I MonoQ buffer according to the manufacturer’s instructions. The column was washed with Ligase I MonoQ buffer until no protein was detectable in the flow through as measured by a Bradford protein assay. The column was eluted with a 160 ml linear NaCl gradient from 50 mM to 500 mM NaCl in Ligase I MonoQ buffer. Ligase I elutes from this gradient at approximately 250 mM NaCl. Relevant fractions were pooled and dialyzed twice against 100 volumes of Ligase I Superdex buffer (250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 2 mM EDTA (pH 8.0), 10% (v/v) glycerol). After dialysis the pool was concentrated using an Amicon Ultra 15 30,000 MWCO (Millipore) centrifugal
concentration device to 300 μL. The concentrated solution was loaded onto a Superdex 200 gel filtration column (Amersham) equilibrated in Ligase I Superdex buffer according to the manufacturer’s specifications. The column was eluted with 35 ml Lig I Superdex buffer. Fractions (0.5 ml) containing DNA ligase I were tested on pUC19 DNA for endonuclease activity. Fractions containing DNA ligase I and lacking detectable endonuclease activity were pooled and dialyzed three times against 100 volumes Ligase I storage buffer (10 mM Hepes-KOH (pH 7.4), 50 mM NaCl, 0.1 mM EDTA (pH 8.0), 1 mM DTT, 50% (v/v) glycerol. After dialysis DNA ligase I was stored at -20°C. DNA ligase I was judged to be greater than 95% pure as measured by SDS-PAGE.

MutS and MutL were expressed and purified as previously described (28,29).

**Endonuclease Activity Assays** Reaction mixtures (20 μL) containing 50 ng pUC19 DNA, one μL of the relevant protein fraction, 6 mM MgCl₂, 25 mM Tris-HCl (pH 7.5), 20 mM NaCl, 6.1 mM βME, and 50 μg/ml BSA were incubated at 37°C for at least three hours. Reactions were stopped by the addition of 5 μL loading dye (50 mM EDTA (pH 8.0), 0.05% (v/v) bromophenol blue, 45% (v/v) glycerol). The entire reaction mixture was loaded onto a 1.4% (w/v) TAE (40 mM Tris base, 20 mM Acetic acid, 2 mM EDTA) agarose gel containing 0.5 μg/ml ethidium bromide. The gel was allowed to electrophorese until the bromophenol blue band migrated ¾ the length of the gel. The gel was photographed using Alpha Imager Software and a CCD camera. Endonuclease activity was scored as the ability of a particular protein fraction to nick or linearize pUC19 DNA. Any protein fraction containing detectable endonuclease activity (greater than 5% pUC19 DNA nicked or linearized) was excluded from any final protein pools.
*pUC19-VSR substrate construction* The pUC19-VSR substrate was created by cloning a DNA fragment into the pUC19 backbone that contains four Nt. *BbvCI* (NEB) nicking enzyme sites, as described above under cloning. Aliquots (50 μL) containing 10 μg pUC19-VSR DNA, 10 units of Nt. *BbvCI* and its reaction buffer were incubated at 37°C for at least ten hours. After the addition of 250 μL buffer PBI (supplied by Qiagen in the PCR clean kit) to each aliquot the reactions were heated to 80°C for 20 minutes. Following heating the reactions were immediately applied to a PCR clean column and centrifuged according to the protocol supplied by Qiagen. The remainder of the Qiagen PCR clean kit protocol was followed according to the manufacturer’s instructions and the gapped pUC19-VSR DNA was eluted from the spin column in 30 μL 10 mM Tris-HCl (pH 7.5). Three synthetic oligonucleotides were designed to be annealed into the gap created by the above procedure: 5′-TCA GCA ATC CTC AGC TAG GCC TCA GCT GGC CTC AGC G-3′, 5′-TCA GCA ATC CTC AGC 5meCAG GCC TCA GCT GGC CTC AGC G-3′, and 5′-TCA GCA ATC TTC AGC CAG GCC TCA GCT GGC CTC AGC G-3′. The first oligonucleotide when annealed to the gapped pUC19-VSR DNA creates a G:T mismatch within the sequence context 5′-CTAGG-3′/5′-CCTGG-3′ where the mismatch is underlined. The second oligonucleotide forms homoduplex DNA when annealed to the gapped pUC19-VSR DNA and serves as a control. The third oligonucleotide when annealed to gapped pUC19-VSR creates a G:T mismatch (underlined) outside the 5′-CCWGG-3′/5′-CCWGG-3′ sequence context also serving as a control. Each of the oligonucleotides was purified on 16% denaturing PAGE prior to annealing. After purification the oligonucleotides were phosphorylated using T4 polynucleotide kinase (NEB) as specified by the manufacturer. ATP, reaction buffer and enzyme were removed from the phosphorylation reaction using a
nucleotide removal kit (Qiagen). The phosphorylated oligonucleotides were then annealed into the gapped pUC19-VSR DNA as follows: annealing reaction mixtures (47 μL) contained 30 μL gapped pUC19-VSR DNA from above, 30.6 pmol phosphorylated oligonucleotide, 50 mM Tris-HCl (pH 7.5), 10 mM MgOAc, 5 mM DTT and 50 mM NaCl and were heated to 80°C in a GeneAmp PCR System 2400 Thermocycler (Applied Biosystems) and the temperature was reduced by one degree Celsius per minute until the reaction temperature reached 16°C. ATP was then added to the reaction to a final concentration of 1 mM and 200 units of T4 DNA ligase (NEB) was added. The final reaction volume was increased to 50 μL with ddH2O. The reaction was allowed to incubate at 37°C for 60 min. Covalently closed DNA was isolated using a CsCl/EtBr gradient or with a gel extraction kit (Qiagen). The concentration of the substrate was measured by A260. To produce a radiolabeled substrate the oligonucleotide that would produce a mismatch in the 5′-CTAGG-3′/5′-CCTGG-3′ sequence was phosphorylated using T4 polynucleotide kinase in the presence of 40 μCi [γ-32P]ATP in the reaction buffer supplied by the manufacturer, otherwise the procedure for production of the substrate is identical procedure to create a mismatched substrate without a radiolabel.

**Vsr Nicking Assays** Reaction mixtures (20 μL) contained 50 ng of the indicated DNA, 6 mM MgCl2, 25 mM Tris-HCl (pH 7.5), 20 mM NaCl, 6.1 mM βME, and 50 μg/ml BSA were preincubated at 37°C for 2 min. If the reactions contained MutS and/or MutL the concentrations are indicated in the figure legend and these reactions were modified to contain 1 mM ATP. Reactions were initiated by the addition of the indicated amount of the Vsr endonuclease. Reactions were stopped after 10 minutes by the addition of 5 μL stop solution (50 mM EDTA (pH 8.0), 0.05% (v/v) bromophenol blue, 45% (v/v) glycerol). Reactions were loaded onto a 1.4% TAE-buffered agarose gel containing 0.5 μg/ml ethidium bromide.
The gel was run until the bromophenol blue band migrated three quarters the length of the gel. The gel was then subjected to UV irradiation at 254 nm for 30 min. The gel was stained in 300 ml TAE buffer containing 0.5 μg/ml ethidium bromide for 30 minutes and destained 3 times in 300 ml ddH₂O for 15 minutes. The intensity of each species on the gel was quantified with Alpha Imager Software using spot the spot densitometry tool and the fraction of the DNA that was nicked was calculated.

**VSR Nicking of pUC19-VSR MM to determine location of nick** Reaction mixtures (20 μL) containing 50 ng pUC19-VSR MM [³²P]DNA, 6 mM MgCl₂, 25 mM Tris-HCl (pH 7.5), 20 mM NaCl, 6.1 mM βME, and 50 μg/ml BSA were preincubated at 37°C for 2 min. Reactions were initiated by the addition of the indicated concentration of the Vsr endonuclease and allowed to proceed at 37°C for 10 minutes. Reactions were terminated by the addition of 180 μL stop solution containing 20 mM EDTA (pH 8.0) and 162 μg/ml glycogen. The Vsr endonuclease was removed by phenol/chloroform extraction followed by an ethanol precipitation as described (27). Pellets were suspended in 17 μL 10 mM Tris-HCl (pH 7.5) followed by a double digestion with EcoRI + BamHI in EcoRI reaction buffer (NEB) at 37°C for 60 min. The double digest was stopped by the addition of an equal volume of 50 mM EDTA (pH 8.0), 85% (v/v) formamide, 0.025% (v/v) xylene cyanole, and 0.025% (v/v) bromophenol blue. Reactions were boiled for 5 minutes and loaded onto a 32 cm 16% denaturing PAGE run at 30 W for 90 minutes. The gel was soaked in 500 ml 40% (v/v) methyl alcohol, 10% (v/v) acetic acid, and 3% (v/v) glycerol for at least 60 minutes. The gel was dried and placed on a phosphor screen (Molecular Dynamics). Data was quantified using ImageQuant software (Molecular Dynamics).
**DNA Polymerase I Repair Assays**

Reaction mixtures (20 μL) containing 50 ng pUC19-VSR MM DNA, 6 mM MgCl₂, 25 mM Tris-HCl (pH 7.5), 20 mM NaCl, 6.1 mM βME, and 50 μg/ml BSA, 50 μM of each dNTP and the indicated concentration of DNA polymerase I were preincubated for 2 min at 37°C. Reactions were initiated by the addition of the Vsr endonuclease to a final concentration of 51.7 nM. Reactions were allowed to proceed at 37°C for 60 min and then terminated by the addition of 180 μL stop solution containing 20 mM EDTA (pH 8.0) and 162 μg/ml glycogen. The Vsr endonuclease and DNA polymerase I were removed by phenol/chloroform extraction followed by an ethanol precipitation. Pellets were suspended in 17.5 μl 10 mM Tris-HCl (pH 7.5) and were subjected to an XcmI + AlwNI double digest in NEB buffer 2 at 37°C for 60 min. Reactions were stopped by the addition of 5 μL stop solution (50 mM EDTA (pH 8.0), 0.05% (v/v) bromophenol blue, 45% (v/v) glycerol). Reactions were loaded onto a 1.4% (w/v) TAE-buffered agarose gel containing 0.5 μg/ml ethidium bromide. The gel was run until the bromophenol blue band migrated three quarters the length of the gel. The intensity of each DNA species on the gel was measured with Alpha Imager Software using the spot densitometry tool. The fraction repaired was calculated as the total DNA cut by both XcmI and AlwNI divided by the total DNA in each lane and multiplied by 100%. Results are reported as % DNA repaired as a function of DNA polymerase I concentration.

**VSP Repair Patch Length Assays**

Reaction mixtures (100 μL) containing 200 ng pUC19-VSR MM DNA, 6 mM MgCl₂, 25 mM Tris-HCl (pH 7.5), 20 mM NaCl, 6.1 mM βME, and 50 μg/ml BSA, 10 μM dATP, 0.5 μM dCTP, 10 μM dGTP, 10 μM dTTP, 2 μCi [α-³²P]dCTP, 26 μM NAD⁺, 17.3 nM DNA polymerase I, and the indicated concentrations of DNA ligase I, MutS, and/or MutL were preincubated at 37°C for 2 min. Reactions that
included MutS, MutL, or MutS and MutL also contained 1 mM ATP. Reactions were initiated by the addition of 50 nM Vsr endonuclease and allowed to proceed for 60 minutes. Reactions were stopped by the addition of 200 μL stop solution containing 20 mM EDTA (pH 8.0) and 162 μg glycogen. Proteins were removed by phenol/chloroform extraction followed by an ethanol precipitation. Pellets were suspended in 33 μL 10 mM Tris-HCl (pH 7.5). Each suspended DNA sample was split into three independent reactions—one reaction containing 16.5 μL and two reactions containing 8.25 μL of the initial 33 μL. 11.75 μL 10 mM Tris-HCl (pH 7.5) was added to the first reaction along with 5 μL loading dye (50 mM EDTA (pH 8.0), 0.05% (v/v) bromophenol blue, 0.05% (v/v) xylene cyanol, and 45% (v/v) glycerol). The second reaction was digested with AlwNI and XcmI in NEB buffer 2 (NEB) in a final volume of 20 μL for 60 min at 37°C after which the reaction was terminated by the addition of 5 μL loading dye. The third reaction (19.5 μL) was digested with BfaI and PvuII in NEB buffer 4 at 37°C for 90 min. 0.5 μL TaqI was then added to the reaction and the reaction was allowed to proceed at 65°C for 60 min. The reaction was terminated by the addition of 5 μL loading dye. The first and the second reactions were loaded onto a 1.4% (w/v) TAE-buffered agarose gel containing 0.5 μg/ml ethidium bromide and was allowed to electrophorese until the bromophenol blue dye had migrated ¾ the length of the gel. The gel was subjected to UV irradiation at 254 nm for 30 min, followed by staining in 300 ml ddH₂O containing 0.5 μg/ml ethidium bromide for 30 min. The gel was destained three times in 300 ml ddH₂O for 15 min. The gel was imaged using Alpha Imager Software and a CCD camera and quantified using the spot densitometry tool. The gel was then placed in 300 ml 40% (v/v) methyl alcohol, 10% (v/v) acetic acid, and 3% (v/v) glycerol for 60 min. The gel was dried and placed on a phosphor screen (Molecular Dynamics) and quantified using
ImageQuant (Molecular Dynamics). The third reaction was loaded onto a 16 centimeter 6% (w/v) TBE (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA (pH 8.0)) polyacrylamide gel and allowed to electrophorese at 6.25 V/cm until the xylene cyanole dye migrated the 4 centimeters from the bottom of the gel. The gel was stained in 200 ml ddH$_2$O containing 0.5 μg/ml ethidium bromide for 45 min and was destained 2 times in 250 ml ddH$_2$O for 20 min. The gel was imaged using Alpha Imager Software and a CCD camera. The gel was placed on a phosphor screen (Molecular Dynamics) and quantified using ImageQuant Software (Molecular Dynamics). The specific activity measurement was determined as the number of counts per cytosine per band as a function normalized to the specific activity of Band 2 in the lane containing no ligase or as a function of the total radioactivity per band. The resulting specific activity from the ligase titrations was normalized by using the ethidium bromide stained image to correct for small gel loading inequalities.

**Results**

The components of the VSP repair system have been demonstrated *in vivo* (reviewed by (1)) in genetic studies. These components include the Vsr endonuclease, DNA polymerase I, DNA ligase I, MutL, and MutS. The current model for VSP repair postulates that the Vsr endonuclease incises the DNA backbone 5′ to the mismatched thymine within the sequence context 5′-CTWGG-3'/5′-C$^5$meCWGG-3′ producing a single strand break with a 3′-OH and a 5′-PO$_4$. DNA polymerase I then binds to the nicked site produced by Vsr cleavage and removes the mismatched thymine and between 10 and 15 nucleotides beyond the mismatch via the 5′→3′ nick translation ability of the polymerase. Genetic studies (21,22,30) suggest
the polymerase adds between 10 and 15 nucleotides past the initial nick site and DNA ligase I presumably seals the nicked DNA to restore the integrity of the DNA strand. Genetic studies suggest that both MutL and MutS are also utilized in this pathway although they are not strictly required (19,22,31,32). In the absence of either MutL or MutS the efficiency of the repair reaction is significantly reduced. We have completed a biochemical reconstitution of the minimal VSP repair reaction using purified VSR endonuclease, DNA polymerase I and DNA ligase. Additionally, our results suggest a requirement for MutS and MutL to coordinate the VSP repair pathway.

The minimal VSP repair pathway reconstitution

The Vsr endonuclease, DNA ligase I, and DNA polymerase I were purified as described under “Materials and Methods.” As evaluated by SDS-PAGE, DNA polymerase I, DNA ligase I and the Vsr endonuclease were judged to be greater than 95% homogeneous (Figure 19, lanes 2-4 respectively). The visible protein product migrating below the full length Vsr endonuclease is a well described degradation product of the Vsr endonuclease that occurs during storage (24). The degradation of the amino terminal region of the Vsr endonuclease partially reduces the activity of the protein, while the specificity of the nicking reaction is not altered (24,25,33). None of the purified proteins contained contaminating endonuclease activity greater than 5% when assayed on supercoiled molecules for three hours (data not shown).

The DNA substrate used for reconstitution of the VSP repair pathway is shown in Figure 20. The substrate is a derivative of pUC19 and is fully Dcm and Dam methylated in vivo. A single G:T mismatch exists within the sequence context 5′-CTAGG-3′/5′-C^5meCTGG-3′
making this covalently closed heteroduplex a substrate for the Vsr endonuclease. The G:T mismatch interrupts an XcmI restriction site rendering the substrate resistant to XcmI digestion. If the G:T mismatch is appropriately repaired to a G:C the duplex becomes sensitive to digestion by XcmI (this substrate is referred to henceforth as pUC19-VSR mismatch). Repair in the opposite direction, to an A:T, or no repair leaves the site resistant to cleavage by the XcmI endonuclease. A similar substrate can be created as described under “Materials and Methods” to produce a substrate that has no mismatch (referred to henceforth as pUC19-VSR no mismatch) or a substrate with a G:T mismatch outside the 5'CCAGG-3'/5'-CCTGG-3' sequence context (referred to henceforth as pUC19-VSR wrong Mismatch).

The Vsr endonuclease reaction

Previous reports (17,23) have shown that the Vsr endonuclease can produce a nick 5' to the mismatched thymine within in the sequence context 5'-CTWGG-3'/5'-C5meCWGG-3'. However, the Vsr nicking reaction has only been demonstrated using synthetic oligonucleotides. To demonstrate Vsr-catalyzed nicking on a more biologically relevant substrate the covalently closed circular heteroduplex described above was used (Figure 21). As the Vsr endonuclease concentration increased the fraction of the substrate converted to nicked DNA increased demonstrating a dependence on the Vsr endonuclease for the conversion of covalently closed circles to nicked circles (Figure 21, lanes 2-6). At a concentration of 50 nM Vsr essentially all of the substrate was nicked. We preformed the identical experiment using a substrate that does not contain a mismatch (pUC19-VSR no Mismatch) and using a substrate that contains a G:T mismatch outside the 5'-CTWGG-3'/5'-CCWGG-3’ sequence context. We were unable to detect any measurable nicking catalyzed
by the Vsr endonuclease on fully duplex DNA consistent with previous reports regarding the specificity of the protein (Figure 21, lanes 8-12). We were able to detect a small amount of nicking of the substrate that contained a G:T mismatch outside the 5′-CTWGG-3′/5′-CCWGG-3′ sequence context (Figure 21 lanes 14-18) consistent with previous reports indicating the ability of the Vsr endonuclease to recognize and cleave DNA in sequences that are related to but not identical with the canonical VSR recognition sequence (23,25). However, the fraction of the DNA substrate nicked at this alternate location is significantly reduced. Taken together, these results demonstrate that the preferred substrate for the Vsr endonuclease contains a G:T mismatch within the 5′-CTWGG-3′/5′-CCWGG-3′ sequence.

The experiment described above demonstrates that the Vsr endonuclease produces a nick in the pUC19-VSR mismatch substrate in a Vsr dependent manner. However, this assay does not demonstrate that the nick is produced 5′ to the mismatched thymine within the 5′-CTWGG-3′/5′-CCWGG-3′ sequence. To demonstrate that the nick produced by the Vsr endonuclease occurs 5′ of the mismatched thymine the pUC19-VSR mismatch substrate containing an internal 32P label was prepared (shown with a “*” in Figure 22). The [32P]DNA substrate was incubated with increasing concentrations of the Vsr endonuclease and the plasmid was cleaved with EcoRI and BamHI to release the relevant DNA fragment for analysis by denaturing PAGE. The expected products of Vsr catalyzed cleavage 5′ to the mismatched thymine are two DNA fragments of 43 and 21 bases in length, representing the DNA species that was not nicked by Vsr and the species that was nicked by the Vsr endonuclease, respectively. Figure 22 shows the expected result as well as showing dependence on the concentration of the Vsr endonuclease for nicking of the pUC19-VSR substrate 5′ to the mismatched thymine.
The Vsr endonuclease and DNA polymerase I are able to repair a G:T mismatch in vitro

A previous report (22) speculated that DNA polymerase I removed the mismatched thymine by nick translating past the mismatch and incorporating the correct deoxycytosine opposite the template guanine residue. To test this hypothesis in vitro we incubated the pUC19-VSR mismatched substrate with 50 nM Vsr endonuclease (a concentration that will produce maximal nicking of the substrate) and increasing concentrations of DNA polymerase I. DNA polymerase I and the Vsr endonuclease were removed from the reaction by phenol:chloroform extraction and the resulting product was digested simultaneously with XcmI to confirm repair and AlwNI to linearize the plasmid to increase quantification accuracy. If the substrate is repaired with 100% efficiency then two products are expected on an agarose gel (1888 bp and 820 bp) representing cleavage by both XcmI and AlwNI. A single product at 2708 bp represents an unrepaired species cleaved only by AlwNI. The results of this experiment are shown in Figure 23. It is clear that DNA polymerase I is required for the repair of the mismatched G:T in the pUC19-VSR mismatch substrate since in the absence of DNA polymerase I there is no repair of the heteroduplex DNA substrate (i.e. not cleaved with XcmI; Fig. 23, lane 3). We conclude that DNA polymerase I is able to recognize the 3′-OH produced by the VSR cleavage reaction and extend the end by at least one nucleotide replacing the mismatched G with a T and restoring the XcmI restriction site. In fact, DNA polymerase I catalyzes significant extension of the 3′-OH as shown below.

**DNA Ligase I seals the nick produced by DNA polymerase I-catalyzed nick translation**

A current model of VSP repair proposes that DNA ligase I seals the nick produced after the VSR endonuclease nicks 5′ to the mismatched thymine and DNA polymerase I nick
translates beyond the mismatch (22). We have tested this hypothesis using our *in vitro* system and purified DNA ligase I.

Initial experiments demonstrated that DNA ligase I was able to catalyze the ligation of a Vsr-nicked nicked substrate, albeit poorly, when the mismatched base had not been removed (data not shown). Because of this confounding ligation product we measured ligation after the incorporation of [α-32P]dCMP by DNA polymerase I to ensure that any ligation product visible in the autoradiogram must have undergone the complete VSP repair reaction. We incubated 200 ng of the pUC19-VSR mismatch substrate with 50 nM Vsr, 17.3 nM DNA polymerase I and a titration of DNA ligase I for 60 minutes at 37°C as described under “Materials and Methods.” The result of this experiment, shown in Figure 24, demonstrated that DNA ligase I is able to seal the nick generated as a result of nick translation by DNA polymerase I. Thus, the product of the DNA polymerase I extension reaction must be a ligatable nick. However, as an unexpected result of this DNA ligase I titration we observed much greater incorporation of [α-32P]dCMP at low DNA ligase concentrations than at high DNA ligase concentrations even though all ligase concentrations tested showed some repaired ligation products (Figure 24). This was reflected in the intensity of the covalently closed product (lanes 7, 9, 11, 13, 15 and 17 in the autoradiograph) and in the intensity of the linear products produced after cleavage with *Alw*NI and *Xcm*I (lanes 8, 10, 12, 14, 16 and 18 in the autoradiograph). In fact, the 1888 bp band was barely visible at the highest DNA ligase concentration but increased in intensity and was clearly visible at the lowest DNA ligase concentration.

We suspected that the increased incorporation of [α-32P]dCMP at low DNA ligase concentrations was due to multiple rounds of DNA polymerase I binding and dissociation.
prior to the ligation event. At higher concentrations of DNA ligase I, there were fewer rounds of synthesis and rebinding by DNA polymerase I. This interpretation is consistent with the labeling of the 1888 bp DNA fragment at low DNA ligase concentrations since this DNA fragment is far removed from the site of the G:T mismatch. Effectively, there appeared to be a competition between DNA polymerase I and DNA ligase I for the nick generated by the DNA polymerase I nick translation event. At higher concentrations of DNA ligase I we suspected that DNA ligase I had a higher probability of sealing the nick prior to binding of another molecule of DNA polymerase I. However, at lower concentrations of DNA ligase I we observed greater incorporation of radiolabeled dCMP into the covalently closed circular duplex because there were multiple rounds of DNA polymerase I binding and synthesis prior to the nick being sealed by DNA ligase I.

To test the hypothesis of a competition between DNA polymerase I and DNA ligase I for the nick generated by nick translation we completed the reaction as above with several modifications. The proteins were removed by organic extraction, and then we aliquoted the reaction products into three different reactions. The first reaction was run on an agarose gel containing ethidium bromide to determine if the DNA species was covalently closed. Covalently closed DNA will run in the position of positively supercoiled DNA in the presence of EtBr. The second reaction was digested with AlwNI and XcmI to confirm that the DNA species was indeed repaired. The third reaction was digested with BfaI, PvuII, and TaqI which produces eight restriction fragments resolvable on a 6% polyacrylamide gel each fragment originating at a different position relative to the site at which the repair event initiated (i.e. the location of the mismatched base). By quantifying the relative amount of radioactivity incorporated into each restriction fragment it was possible to determine the
distance over which DNA polymerase I had incorporated nucleotides prior to sealing of the
nick by DNA ligase I. Each of the DNA ligase I concentrations are shown in Figure 25. It is
clear that the length of the synthetic repair track decreases as a function of increasing DNA
ligase I concentrations. This result strongly suggests that DNA polymerase I has the ability
to synthesize a very long repair track, presumably by loading onto the pUC19-VSR mismatch
substrate multiple times, if DNA ligase I is not present at sufficient concentrations to seal the
nick immediately after the dissociation of DNA polymerase I from the substrate. The
reported processivity of DNA polymerase I is approximately 20 nucleotides while nick
translating (34). We presume that DNA polymerase I dissociates after each synthesis event
leaving a substrate suitable for DNA ligase I. If ligase I fails to seal the nick then DNA
polymerase I can bind again to extend the repair track. Thus, in this minimal in vitro
reconstitution it is possible to modulate the length of the repair track by varying the DNA
ligase concentration. Presumably a similar result would be obtained by varying the DNA
polymerase I concentration at a constant DNA ligase concentration. This has not been tested.

*MutS and/or MutL do not stimulate the Vsr catalyzed nicking reaction at physiologically
relevant concentrations*

A previous report (24) has demonstrated that MutL can stimulate the Vsr catalyzed nicking
reactions, at MutL concentrations greater than 3 μM MutL. We have been able to reproduce
this result using MutL concentrations greater than 3 μM MutL (data not shown). To
determine if MutL, MutS, or MutL and MutS in combination could stimulate the Vsr
endonuclease catalyzed nicking reaction we incubated the pUC19-VSR substrate with 0.22
nM Vsr endonuclease 104 nM MutS and a titration of MutL. Figure 26 shows that at lower,
more physiologically relevant, concentrations of MutL and MutS the Vsr catalyzed nicking reaction is not stimulated by the addition of MutL, MutS, or MutS and MutL in combination. We also note that the Vsr catalyzed nicking reaction is not inhibited by MutS, MutL, or the combination of MutS and MutL.

MutS and MutL coordinate the VSP repair reaction in vitro

Based upon previous in vivo work it has been shown that MutS and MutL are required for maximal efficiency of the VSP repair reaction. In the absence of MutS or MutL VSP repair efficiency is decreased to between 30% and 67% (18,20,21). Additionally, genetic evidence suggests that VSP repair tracts are about 10-15 nucleotides in length (21,30). Given these genetic results and the above in vitro observation that repair tracts are of variable length depending upon ligase concentration (see Fig. 25), we reasoned that MutS and MutL might be able to coordinate the VSP repair system. The coordination by MutS and MutL could have the effect of shortening repair tracts by any number of different mechanisms. For example, if MutL and MutS were to increase the local concentration of the Vsr endonuclease, DNA polymerase I, and DNA ligase I at the site of the mismatch this would make DNA ligase I immediately available to seal the nick generated by the DNA polymerase I nick translation reaction and prevent the random loading of another molecule of DNA polymerase I, creating a shorter repair tract nearing that of 10-15 nucleotides. In addition, this would be consistent with previously reported results (24,35,36) indicating that MutL interacts with the Vsr endonuclease and stimulates the binding of this protein to its substrate.

We tested the hypothesis that MutL and MutS may act to decrease the repair patch length by performing the repair tract length assay detailed above using a fixed concentration of 50
nM Vsr, 17.3 nM DNA polymerase I, and 41 nM DNA ligase I with a titration of MutS, MutL, or both MutS and MutL (Figure 27). The results of this experiment did not demonstrate a significantly decreased amount of radioactivity incorporated into the substrate molecule in the presence of MutS and MutL.

**Discussion**

In this paper we have shown that the VSP repair pathway can be reconstituted *in vitro*; the minimal VSP repair reaction requires the Vsr endonuclease, DNA polymerase I, and DNA ligase I. It is important to note that this requirement is for the minimal VSP repair pathway. When we describe the minimal VSP repair pathway we are using only the components of the system believed to be able to initiate and complete the repair of a deaminated 5-methyl cytosine within the sequence context 5′-CTWGG-3′/5′-CCWGG-3′. The data presented in Figures 21 and 23 clearly demonstrate a concentration dependence of the Vsr endonuclease (Figure 21) and DNA polymerase I (Figure 23) for VSP repair. Additionally, we have shown a concentration dependence of DNA ligase I in the VSP repair reaction; this demonstrates a dependence on DNA ligase I for the conversion of the repaired substrate back to a covalently closed circle (Figure 24). Inspection of these data (Figure 25) showed that the length of a repair tract was inversely proportional to DNA ligase I concentration. Importantly, at low concentrations of DNA ligase I the length of the repair tract is quite long on the order of kilobase pairs in length. Because *in vivo* measurements have placed VSP repair tract lengths at 10-15 nucleotides (21,30) we interpreted this result to signify that the minimal VSP repair event was uncoordinated. This result is perhaps not surprising since each of the three
proteins added to the reaction is completely capable of acting independently on the substrate or the intermediate in the reaction pathway. Thus, once the substrate has been nicked by Vsr endonuclease the nick can be sealed by DNA ligase I, leaving the mismatch in place or the 3′-OH can be extended by DNA polymerase I removing the mismatch and, ultimately leaving a ligatable nick.

*In vivo* VSP repair corrects about 30% - 67% of G:T mismatches in the sequence context 5′-CTWGG-3′/5′-C\textsuperscript{5me}CWGG-3′ in the absence of MutS or MutL (18,20,21). In the presence of MutS and MutL the efficiency of VSP repair increases to nearly 100% of G:T mismatches within the 5′CTWGG-3′/5′-C\textsuperscript{5me}CWGG-3′ sequence context (18,19). Therefore, MutS and MutL must have some role in the VSP repair pathway such that these proteins increase the efficiency of VSP repair. Previous results (24) have shown that MutL interacts with the Vsr endonuclease and apparently stimulates binding to the DNA substrate. We have confirmed this result which is only apparent at high concentrations of MutL (data not shown). However, the results shown in Figure 26 demonstrate that neither MutL and/or MutS stimulate the Vsr endonuclease catalyzed nicking reaction. Figure 26 also demonstrates that at physiological concentrations of MutS and MutL the Vsr catalyzed nicking reaction is not inhibited. Because of these results we reasoned that this might not be the primary role of MutL since this left the role of MutS unexplained and the Vsr endonuclease reaction was quite robust in the absence of MutL.

Based on the results of the *in vitro* reconstitution with three proteins we added MutL and MutS to the reaction and measured the length of the repair tract which was clearly much longer than that measured *in vivo* when MutL and MutS were not included. We have shown
that the addition of MutS and MutL in the presence of ATP did not significantly decrease the lengths of *in vitro* repair tracts at relatively low DNA ligase I concentrations (Figure 27).

We speculate on the many ways in which the VSP repair reaction may be coordinated. MutL has been shown to stimulate the nicking reaction catalyzed by the Vsr endonuclease (24). Therefore, it is possible that MutL is able to increase the efficiency of VSP repair by stimulating the nicking reaction catalyzed by the Vsr endonuclease.

The demonstration that MutS and MutL do not shorten VSP repair tract lengths and the finding that MutS and MutL do not stimulate the Vsr catalyzed nicking reaction leaves an unknown role for MutS and MutL in the VSP repair pathway. These two results taken together lead us to propose another model for VSP repair. We speculate that the function of MutS and MutL is to shorten VSP repair tracts by coordinating the VSP repair system. However, we speculate that the reason VSP repair tracts are shorter *in vivo* than in our *in vitro* system is because we are missing a component of the VSP repair system. MutS, MutL, DNA ligase I and DNA polymerase I all interact with the β-clamp dimer (37,38). Because of these facts we propose that the missing protein from our in vitro system is the β-clamp. Upon addition of the β-clamp we can propose a model for how the VSP repair reaction is coordinated by MutS and MutL. We suggest that MutS binds to the G:T mismatch and recruits the MutL dimer. MutL recruits the Vsr endonuclease and the β-clamp. MutL loads the β-clamp onto the helix. The MutS, MutL, β-clamp, Vsr endonuclease, DNA complex then recruits DNA ligase I such that it interacts with the β-clamp. The recruitment of DNA ligase I is followed by the cleavage of the phosphodiester backbone 5’ to the mismatch by the Vsr endonuclease. DNA polymerase I is recruited by this protein complex and is loaded onto the DNA such that it is in contact with the β-clamp. DNA polymerase I then nick translates,
using its 5′ to 3′ exonuclease activity and its 5′ to 3′ polymerase activity, between 10 and 15 bases and subsequently dissociates from the DNA and the β-clamp. Because DNA ligase I travels with the β-clamp it is now poised to ligate the nick created by the nick translation reaction catalyzed DNA polymerase I. After the nick is sealed by DNA ligase I the proteins involved dissociate from the repaired DNA and the Dcm methyltransferase methylates the repaired cytosine.

In vivo and in vitro experiments have shown that the Vsr endonuclease and DNA polymerase I are essential for VSP repair (17,22) and MutS and MutL are required for maximal efficiency of the VSP repair reaction (18,19). However, it has yet to be shown that DNA ligase I is responsible for sealing the nick created by nick translation of DNA polymerase I. A second ligase has recently been identified—DNA ligase II (39)—which could potentially suffice to ligate the nick created by the nick translation of DNA polymerase I. This result remains to be explored further.

We have shown that VSP repair is absolutely dependent on the Vsr endonuclease, DNA polymerase I, and most likely DNA ligase I; however, we did not exclude the possibility that DNA ligase II could substitute for DNA ligase I. Also, we have attempted to explain the role that MutS and MutL play in VSP repair leading us to propose a model involving the β-clamp as to how the VSP repair reaction is coordinated.
References


133


Figure 19. SDS-PAGE analysis of DNA polymerase I, DNA ligase I, and the Vsr endonuclease. The proteins 3 μg were resolved on an 11% polyacrylamide gel in the presence of SDS and stained with Coomassie Blue. Lane 1, molecular mass standards (size in kDa indicated on the left); lane 2, DNA polymerase I; Lane 3, DNA ligase I; Lane 4, the Vsr Endonuclease.
Figure 20. pUC19-VSR mismatch substrate. The G:T mismatch within the Vsr endonuclease recognition sequence is shown as a bulge on the plasmid. Relevant restriction sites are shown, as well as fragments and fragment sizes generated by cleavage by BfaI, PvuII, and TaqI. In parentheses next to each fragment size is the relative distance of each fragment from the mismatch.
Figure 21. Vsr-catalyzed nicking of covalently closed circles. Vsr dependent nicking reactions of covalently closed circles (CC) were performed as described in “materials and methods” A titration of the Vsr endonuclease was used and 50 ng of each plasmid were incubated together for 10 minutes. The reactions were stopped and electrophoresed on a 1.4% agarose gel containing 0.5 μg/ml ethidium bromide. The nicked circles (N) were quantified as the amount of nicked DNA as a function of the total amount of DNA in each lane. Plasmids used – covalently closed DNA containing a G:T mismatch the Vsr recognition sequence (●), covalently closed homoduplexed DNA (■), or covalently closed DNA containing a G:T mismatch outside the Vsr recognition sequence (▲).
Figure 22. The Vsr endonuclease catalyzes the formation of a nick 5′ to the mismatched thymine within the Vsr recognition sequence. Vsr nicking reactions were conducted as described in “materials and methods” the Vsr endonuclease was incubated with radiolabeled covalently closed DNA for 10 minutes at 37°C. The reactions were stopped and the Vsr endonuclease was removed by organic extraction. The circular DNA was incubated with EcoRI and BamHI which flank the Vsr endonuclease recognition sequence. The resulting products were electrophoresed on 16 % denaturing PAGE.
Figure 23. DNA Polymerase I and the Vsr endonuclease are sufficient to repair a G:T mismatch in the Vsr endonuclease recognition sequence. The VSP repair reaction containing 51.7 nM of the Vsr endonuclease and a titration of DNA polymerase I is described under “materials and methods” using 50 ng covalently closed circles containing a G:T mismatch in the Vsr recognition sequence. After incubation at 37°C for 60 minutes the reactions were stopped and DNA polymerase I and the Vsr endonuclease were removed by organic extraction. The circular DNA was digested with XcmI and AlwNI and electrophoresed on a 1.4% agarose gel containing 0.5 mg/ml ethidium bromide. DNA that has been repaired is sensitive to both XcmI digestion and AlwNI digestion producing two fragments 1888 bp and 820 bp. DNA that has not been repaired is resistant to XcmI digestion but sensitive to AlwNI digestion producing a single linear band 2708 bp in length. The data presented represent the results of at least three experiments with error bars representing standard deviations from the means.
Figure 24. DNA ligase I is able to seal the nick created by DNA polymerase I nick translation. Ligation and repair reactions were conducted as described under “materials and methods” using 200 ng covalently closed DNA containing a G:T mismatch within the Vsr endonuclease recognition sequence, 10 μM dATP, dGTP, dTTP, and 0.5 μM α-[32P]-dCTP, 51.7 nM Vsr endonuclease, 17.3 nM DNA polymerase I and a titration of DNA ligase I. After incubation for 60 min at 37°C reactions were stopped and the proteins were removed by organic extraction. One quarter of each reaction was digested with XcmI and AlwNI to confirm that repair had occurred, another quarter of the reaction remained untreated. Both the digested and undigested reactions were run on a 1.4% agarose gel containing 0.5 μg/ml ethidium bromide. The left panel shows the ethidium stained gel confirming that repair had occurred. The right panel shows the autoradiograph demonstrating ligation back to covalently closed circles after repair.
Figure 25. DNA ligase I competes with DNA polymerase I for the nick created by the nick translation catalyzed by DNA polymerase I. Ligation and repair reactions were conducted as described under “materials and methods” using 200 ng covalently closed DNA containing a G:T mismatch within the Vsr endonuclease recognition sequence $10 \mu$M dATP, dGTP, dTTP, and $0.5 \mu$M $\alpha$-[32P]-dCTP, 26 $\mu$M NAD$^+$, 49.5 nM Vsr endonuclease, 17.3 nM DNA polymerase I and a titration of DNA ligase I. After incubation for 60 min at 37°C reactions were stopped and the proteins were removed by organic extraction. One half of the reaction was digested with BfaI, PvuII, and TaqI. The digested fragments were electrophoresed on 6% native PAGE followed by staining with 0.5 μg/ml ethidium bromide. Upper left panel shows the ethidium stained gel and the upper right shows the autoradiograph. Fragments are listed by size with the relative distance from the mismatch shown in parentheses. The bottom panel demonstrates the quantification of the intensity of the radioactivity incorporated into each band normalized to band # 2 in the lane containing no DNA ligase I. Data presented represents the average of at least two experiments with error bars representing standard deviations about the means.
Figure 26. MutL and/or MutS do not stimulate or inhibit the Vsr endonuclease-catalyzed nicking reaction. Nicking reactions, described in “Materials and Methods” were incubated at 37°C with 1 mM ATP, 50 ng covalently closed DNA containing a G:T mismatch within the Vsr endonuclease recognition sequence, 0.22 nM Vsr endonuclease, 104 nM MutS, and a titration of MutL ranging from 500 nM to 2 nM for 10 min. The reactions were stopped and electrophoresed on a 1.4% agarose gel containing 0.5 μg/ml ethidium bromide. The nicked circles (N) were quantified as the amount of nicked DNA as a function of the total amount of DNA in each lane.
Figure 27. MutS and MutL do not shorten VSP repair tract lengths \textit{in vitro}. Ligation and repair reactions were conducted as described under “materials and methods” using 200 ng covalently closed DNA containing a G:T mismatch within the Vsr endonuclease recognition sequence 1 mM ATP, 10 μM dATP, dGTP, dTTP, and 0.5 μM α-[32P]-dCTP, 26 μM NAD⁺, 49.5 nM Vsr endonuclease, 17.3 nM DNA polymerase I, 41 nM DNA ligase I and the indicated concentration of MutS, MutL or both MutS and MutL. After incubation for 60 min at 37°C reactions were stopped and the proteins were removed by organic extraction. One half of the reaction was digested with BfaI, PvuII, and TaqI. The digested fragments were electrophoresed on 6 % native PAGE followed by staining with 0.5 μg/ml ethidium bromide. Upper left panel shows the ethidium stained gel and the upper right shows the autoradiograph. Fragments are listed by size with the relative distance from the mismatch shown in parentheses. The bottom panel demonstrates the quantification of the intensity of the radioactivity incorporated into each band represented as a percentage of the total radioactivity in each lane. Data presented represents the average of at least three experiments with error bars representing standard deviations about the means.
Figure 28. A model for VSP repair. A G:T mismatch is generated by the spontaneous hydrolytic deamination of a 5-methyl cytosine residue. MutS, along with MutL are recruited to the G:T mismatched base. MutS and MutL recruit the Vsr endonuclease, DNA ligase I, and DNA polymerase I, effectively increasing the local concentration of each of the proteins. The Vsr endonuclease catalyzes the hydrolysis of a phosphodiester bond 5’ to the mismatched thymine. DNA polymerase I is then loaded onto the nick and nick translates between 10-15 bases beyond the original mismatch. DNA ligase I seals the nick created by the nick translation reaction catalyzed by DNA polymerase I. Finally, the Dcm methyltransferase methylates the appropriate cytosine on the newly synthesized patch.
Chapter 5
Concluding Remarks

The work presented in this dissertation represents a significant advance in our understanding of the involvement of the MutL protein in the methyl-directed mismatch repair (MMR) pathway. Additionally, this work has provided new insight into the function of MutL in the very short patch repair (VSP) pathway.

Chapter 2 demonstrated, through the use of a MutL point mutant, that the DNA binding activity of MutL is required for the MMR pathway to function. Chapter 3 has shown, through the use of another MutL point mutant, that the MutL protein must be able to bind an adenine nucleotide for MutL to function in MMR. In addition, this chapter demonstrates that the hydrolysis of the adenine nucleotide occurs after MutL has loaded the UvrD helicase onto the DNA helix. This chapter has also provided direct evidence to support evidence from previous reports (1,2) demonstrating that the binding of an adenine nucleotide by MutL is required for MutL to interact with the MutS protein bound at a mismatch. Furthermore, this chapter supports the previously published observation that MutL cannot stimulate the nicking reaction catalyzed by MutH if it cannot bind an adenine nucleotide (3). Additionally, chapter 3 has demonstrated that MutL cannot stimulate the UvrD catalyzed unwinding reaction if it cannot bind an adenine nucleotide. After binding ATP, MutL catalyzes the hydrolysis of ATP. After this MutL-catalyzed ATP hydrolysis step it appears that the MMR pathway is constitutively active due to the \textit{in vitro} observation that UvrD appears to be loaded
indefinitely by MutL onto the duplex—lending support to the hypothesis that hydrolysis of ATP by MutL signals the shut down of the MMR pathway. All this taken together supports the hypothesis that the MutL protein coordinates the MMR reaction and that the MutL bind, hydrolysis, and release cycle with ATP provides the energy necessary for a conformational change in the MutL protein allowing coordination of the MMR pathway by MutL.

The work presented in Chapter 4 has demonstrated a potential function for the MutL protein in the very short patch (VSP) repair pathway. Previously, the function of the MutL protein in VSP repair was unknown other than it served to increase the efficiency of repair events believed to be carried out by the VSP pathway (4-7). The work in this dissertation has shown that MutL, in cooperation with MutS, may function to regulate this repair pathway as evidenced by their effects on the repair tract lengths associated with VSP repair. It appears that MutL functions as a coordinator of the VSP repair pathway. The idea of MutL as a coordination protein in VSP is supported by two important facts—in the absence of MutL VSP repair tracts are greater than 55 nucleotides in length as measured by an in vivo assay and greater than 2.8 kb in length measured by in vitro assay, the other finding is that in the presence of MutL repair tracts in vivo are limited to 10-15 bp (6,8). These two experimental observations strongly support the notion that MutL, by some unknown mechanism, coordinates the VSP repair pathway to effectively regulate the lengths of VSP repair tracts.

The work presented in the preceding chapters has resulted in a greater understanding of the function and role of the MutL protein in both the MMR repair pathway and the VSP repair pathway. However, the work presented in this dissertation has also raised many new questions as to the involvement of the MutL protein in the MMR repair pathway and the VSP
repair pathway. In the following sections I will address some of these unresolved issues and attempt to provide some experimentally testable hypotheses to address these issues.

**Directionality of UvrD loading by MutL in MMR**

The first unresolved issue with regard to the involvement of MutL in the MMR pathway centers on the MutL-stimulated loading of the UvrD helicase onto the DNA. It has been shown that MMR is bidirectional—repair tracts can proceed from either the 3′ or the 5′ direction towards a mismatch, depending upon the location of the closest d(GATC) sequence (9). The UvrD helicase translocates along ssDNA and unwinds dsDNA in the 3′ to 5′ direction (10). Therefore, it follows that there must be some signal that directs the loading of UvrD onto the appropriate DNA strand such that UvrD will always unwind DNA towards the mismatch. I would speculate that this signal is provided by the MutL protein. There are several lines of evidence to support the hypothesis that MutL signals the appropriate strand for UvrD loading—i) MutL physically interacts with the UvrD helicase (11,12), ii) MutL stimulates the UvrD-catalyzed unwinding reaction either by an iterative loading effect (discussed in chapter 3) (13) or by acting as a processivity factor for the UvrD helicase (14), and iii) repair tracts always proceed toward the mismatch signifying that some factor, perhaps MutL, is choosing the appropriate strand on which to load the UvrD helicase (9). Future work in this area could address the issue of the ability of MutL, and not some other MMR protein, to load UvrD onto the appropriate DNA strand such that it always unwinds toward the mismatch.
The ATPase function of MutL in MMR

It is unlikely that the ATPase of MutL has a single function in MMR. The MutL protein that cannot bind ATP has very different biochemical properties from a mutant of MutL that cannot hydrolyze ATP. However both the ATP binding and ATP hydrolysis defective mutants of MutL result in a similar, but not identical, mutator phenotypes (see chapter 3). These mutator phenotypes demonstrate that both ATP binding and hydrolysis by MutL are required for the MMR pathway to function correctly.

The first question this observation raises is how the ATPase of MutL could affect the loading of the UvrD helicase. We have demonstrated that if MutL cannot bind ATP then MutL cannot stimulate UvrD-catalyzed unwinding of longer duplex substrates \textit{in vitro}. We also showed that if MutL could bind ATP, but could not hydrolyze the bound ATP, then MutL protein could stimulate UvrD-catalyzed unwinding of longer duplex substrates better than wild type MutL (see Chapter 3).

MutL has a very slow DNA-stimulated ATPase with a turnover number of about one ATP hydrolyzed per minute (15-17). MutL, when bound to ATP, has a much greater affinity for ssDNA and dsDNA than in the absence of ATP (16). Initially these observations led to the theory that the MutL ATPase may act as a molecular timer regulating the iterative loading of UvrD. This molecular timer—regulated by ATP binding and hydrolysis by MutL—would cause MutL to bind to ssDNA with a higher affinity for about one minute. After MutL hydrolyzes its bound ATP, MutL would be released from the DNA stopping the iterative loading of UvrD. One of our initial models suggested the following series of steps: i) MutL binds to ATP, ii) this MutL-ATP complex then binds to DNA, iii) the stationary MutL-ATP-
DNA complex would then lead to the iterative loading of multiple molecules of UvrD, each UvrD molecule unwinding 40-50 bp of duplex DNA beyond the region of ssDNA created by the previous unwinding event catalyzed by UvrD. This iterative loading of UvrD would continue until MutL hydrolyzed its bound ATP, iv) the ATP bound to MutL is then hydrolyzed to ADP and P$_i$—the hydrolysis of ATP by MutL would cause the release of DNA by MutL, v) after DNA is released by MutL, the loading of the UvrD helicase would cease—effectively stopping the excision phase of the repair event.

Recent work (14) has demonstrated that this hypothesis is at least partially flawed. These researchers have demonstrated that MutL may function as a processivity factor for UvrD. This work shows the first described processivity factor for a helicase. UvrD has been shown to have a processivity of 40-50 bp on duplex DNA (18). In the presence of MutL the UvrD helicase has the ability to unwind much longer duplexes. The current thinking from our laboratory is that MutL serves as a processivity factor for UvrD rather than causing an iterative loading effect although it is formally possible that MutL plays both roles. We still speculate that the ATPase of MutL may function as a timer or switch—whereby hydrolysis of ATP by MutL stops the excision phase of a repair event. However, we now believe that this ATP timer intrinsic to MutL stops the repair event by a different mechanism. In this new model the MutL protein physically associates with the UvrD helicase, where MutL bound to ATP acts as a processivity factor for UvrD, until the ATP bound by MutL is hydrolyzed. We hypothesize that once MutL has hydrolyzed its bound ATP MutL no longer associates with UvrD—UvrD, having lost its processivity factor can no longer unwind long stretches of DNA. UvrD subsequently dissociates from the DNA after unwinding 40-50 base pairs—the intrinsic processivity of UvrD on duplex DNA (18)—stopping the UvrD-catalyzed
unwinding event. The experiments done in our laboratory have used wild type MutL protein. If MutL bound to ATP causes UvrD to be nearly infinitely processive then this produces a testable hypothesis.

Two experimental observations must be true for our hypothesis that MutL bound to ATP increases the processivity of UvrD: (i) UvrD, in the presence of a mutant of MutL that cannot bind ATP, should not have an increased processivity and (ii) UvrD, in the presence of a mutant of MutL that cannot hydrolyze ATP, should be processive on a duplex of infinite length. The mutants of MutL required for these experiments have been constructed and well characterized (19). I believe that conducting these experiments will lend support to the hypothesis that the ATPase intrinsic to MutL is a molecular timer. This timer allows for the effective cessation of the UvrD-catalyzed unwinding during an MMR event.

The ATPase reaction catalyzed by MutL is stimulated by the presence of DNA, more so in the presence of ssDNA than dsDNA (Chapter 2 and (17,20)). This observation leads to another unresolved issue regarding the MutL-catalyzed ATPase reaction. This issue involves two events that seem to contradict one another: (i) the binding to DNA stimulates the MutL-catalyzed ATP hydrolysis reaction and (ii) MutL must be bound to DNA to increase the processivity of UvrD. These two events seem to oppose one another in the context of increasing UvrD-catalyzed DNA unwinding. An increased affinity for DNA by MutL in the presence of ATP would seem to fix the protein to the DNA so that MutL could not translocate with UvrD. The paradox here is that the ATP bound form of MutL is also the form of MutL that is bound to DNA. This interaction with DNA acts to increase the MutL catalyzed ATP hydrolysis reaction. This hydrolysis of ATP by MutL effectively causes the release of UvrD from MutL. To resolve this conflict one may hypothesize that the ATPase
reaction catalyzed by MutL is not increased in the presence of DNA when MutL is bound to both UvrD and DNA. Testing this hypothesis will involve many carefully designed experiments—all designed to measure the ATP hydrolysis reaction catalyzed by MutL in the presence of DNA and a well characterized mutant of UvrD that cannot hydrolyze ATP—UvrD-K35M (21).

The experiments proposed above should help to elucidate the functional role of the MutL catalyzed ATP hydrolysis reaction in the context of MMR. With the proper mutants of MutL and UvrD available, these experiments should and could easily be preformed with the aim of obtaining a better understanding of the molecular timer intrinsic to the MutL ATPase.

**Unresolved issues in the VSP repair pathway**

The reconstitution of the VSP repair pathway opens the door for many questions to be addressed involving the molecular mechanisms involved in the VSP repair pathway. I would first like to address the issue of crosstalk between MMR and VSP repair mechanisms. All these experiments would be predicated with finding that the β-clamp does indeed shorten VSP repair patch lengths in the presence of MutS and MutL.

Hydrolytic deamination of 5-methyl cytosines can and does occur at all stages of the bacterial cell cycle. Repair by MMR would become mutagenic for a deamination event that occurs at 5-methyl cytosine on the fully adenine methylated DNA strand (the template) during or directly after replication. This deamination event should signal the MMR machinery to repair this sequence with an inappropriate strand bias resulting in a transition mutation.
To prevent erroneous MMR events from occurring it seems plausible to hypothesize that there is some communication between the MMR pathway and the VSP repair pathway. One of the thoughts with regard to this mechanism has to do with the ATPase of MutL. The MutL ATPase could provide a signal to determine if the mismatch created is one that should be repaired by MMR or VSP repair. This signal would prevent the transition mutation described above from occurring. It is plausible that when MutS recognizes a mismatch outside of the 5′-CCWGG-3′/5′-CCWGG-3′ mismatch context MutL is then recruited to this mismatch. In the presence of MutL bound to ATP this mismatch-MutS-MutL-ATP complex signals to the cellular MMR machinery to repair the mismatch using the MMR pathway.

Bacterial two hybrid analysis have shown that both MutS and MutL interact with the Vsr endonuclease (22). With this information and the knowledge that MutS binds a mismatch and given that MutS and MutL physically interact with each other when bound to a mismatch it is possible to propose a model for how MutL and MutS recognize and initiate the repair of a G:T mismatch within the 5′-CTWGG-3′/5′-CCWGG-3′ sequence context. First MutS binds the G:T mismatch within the 5′-CTWGG-3′/5′-CCWGG-3′ sequence and MutL is recruited to this mismatch by MutS—leaving two possibilities for how MutL interacts with this mismatch and MutS: (i) MutL interacts with MutS 5′-CTWGG-3′/5′-CCWGG-3′ mismatch complex with a higher affinity when MutL is not bound to ATP or (ii) some other component of the VSP repair pathway incites MutL to hydrolyze its bound ATP. The MutS-MutL-mismatch complex, absent ATP, then recruits the Vsr endonuclease along with the other VSP repair proteins rather than the MMR proteins. This repair complex then repairs this specific G:T mismatch exclusively to a G:C base pair, ensuring the integrity of the genome. Future experiments will utilize the ATP binding and hydrolysis mutants described
in chapter 3, to determine if ATP binding, hydrolysis, and release are necessary for VSP repair to occur.

Another possible way to explain the way in which MutL is able to coordinate the VSP repair pathway would be to speculate that MutS, MutL in the absence of ATP, and the Vsr endonuclease form a ternary complex. This ternary complex would have specificity for repairing a G:T mismatch in the 5’-CTWGG-3’/5’-CCWGG-3’ sequence. It is also reasonable to propose that this ternary complex could not function in MMR due to the presence of the Vsr endonuclease. In effect, this complex could only function to correct G:T mispairs in the context of VSP repair. If MutS bound to a mismatch outside the VSP repair recognition sequence it would cause MutL to bind to ATP and shed the other VSP repair proteins making MutL available to function in MMR. Using the ATP binding and hydrolysis mutants of MutL described in chapter 3 it would be possible to pursue this idea further. These experiments may allow researchers to gain a better understanding of how the MutL protein is able to coordinate two repair pathways. This information would lead to a greater understanding of how most mismatches are repaired correctly regardless of the state of bacterial DNA replication cycle.

The idea of a ternary complex of MutS, MutL and a mismatch formed in the absence of MutL bound to ATP presents several testable hypotheses. To demonstrate that ATP binding, and therefore hydrolysis, by MutL is dispensable for VSP repair it will be essential to demonstrate that an ATP binding defective mutant of MutL can indeed function in VSP repair. It will also be essential to show that a mutant of MutL that cannot hydrolyze ATP—and is therefore almost always bound to ATP—cannot function in VSP repair. It is well known that ATP binding and hydrolysis catalyzed by MutL is essential for MMR (17,19).
Alternatively, it will be imperative to show that a mutant of MutL that can bind, but not hydrolyze ATP, cannot function in VSP repair pathway. Given that an ATP hydrolysis defective mutant of MutL can initiate MMR, the results of these experiments would lend support to the hypothesis that the MutL ATPase acts as a molecular switch between the MMR pathway and the VSP repair pathway. Thus showing that the ATP bound and unbound states by MutL could toggle between the MMR pathway and the VSP repair pathway, respectively.

The interaction domains between MutS and MutL may change depending upon the ATP bound state of MutL—either bound to MutL or not bound to MutL. An experiment could be designed to test this hypothesis. This experiment would involve mapping the interaction domains of MutS and MutL. A dynamic MutS-MutL interface, depending upon ATP binding state of MutL, could signify that MutL is prepared to coordinate a MMR repair event or a VSP repair event. Chemical crosslinking of MutL and MutS with heteroduplex DNA could be employed to examine this potentially dynamic interface. The heteroduplexed DNA would contain a G:T mismatch either in the VSP repair context or in the MMR context. Mapping one or more changes in the two protein’s interaction domains—depending upon the sequence context of the heteroduplexed DNA—would support the hypothesis that ATP binding by MutL acts as a toggle between MMR and VSP repair. Further support for this hypothesis could be demonstrated by using mutants of MutL (characterized in chapter 3) that either cannot bind ATP or cannot hydrolyze ATP. A mutant of MutL that cannot bind ATP should be locked in one conformation such that this mutant can only interact with MutS in one way, regardless of which type of mismatch was encountered—either in the VSP repair sequence or in the MMR sequence. The identical crosslinking experiment with a mutant of
MutL that cannot hydrolyze ATP may yield confounding results. A mutant of MutL that cannot hydrolyze ATP, can still bind ATP and release ATP. Therefore, a change in the interfaces between MutS and MutL in the presence of differing heteroduplex DNAs, VSP repair sequence context or MMR sequence context, does not exclude the possibility that MutL is simply binding ATP and then releasing ATP depending upon which sequence context is encountered. These chemical crosslinking experiments should lead to a greater understanding of how MutL is able to coordinate two repair pathways, both of which could function without temporal separation.

Previously it has been demonstrated that MutL is required for maximal VSP repair efficiency (4,5,23). We have demonstrated in chapter 4 of this dissertation that MutL is required to modulate VSP repair patch lengths. In chapters 2 and 3 we characterized three mutants of MutL—a mutant that cannot bind DNA, a mutant that cannot bind ATP and a mutant that cannot hydrolyze ATP. Based upon our working model of VSP repair summarized in both chapters 1 and 4 we can make certain predictions as to the activity in VSP repair of each of these mutants of MutL.

If DNA binding by MutL is essential for VSP repair to function it would be reasonable to hypothesize that a DNA binding mutant of MutL would not be functional in VSP repair. Because we believe that MutL in complex with MutS allows for short repair tracts in VSP repair it would be necessary to test the activity of the DNA binding mutant of MutL \textit{in vivo} and \textit{in vitro} to determine if DNA binding was required to provide a short patch repair mechanism. If the VSP repair patch lengths with a DNA binding mutant of MutL were similar in length to VSP repair tracts with wild type MutL one could presume that DNA binding was dispensable for patch regulation. Additionally, if the DNA binding mutant of
MutL was nonfunctional in VSP repair then it could reasonably be argued that MutL has an additional, undescribed function in the VSP repair pathway in addition to regulating patch length. The *in vivo* and *in vitro* assays performed above, and described in chapter 4, could also be performed with the ATP binding and ATP hydrolysis defective mutants of MutL, however, in this instance experiments would be aimed at understanding the role of the MutL ATP binding, hydrolysis, and release cycle in VSP repair. These experiments have the propensity to elucidate novel functions of the MutL protein as well as gain a greater understanding of MutL function in VSP repair at the molecular level.

The work presented in this dissertation has allowed for a greater understanding of the fundamentals of DNA repair. More specifically, the text in the preceding chapters has led to many discoveries about the involvement of the MutL protein in two DNA repair pathways—MMR and VSP repair. This dissertation has demonstrated many intermolecular interactions involving the MutL protein and components of the MMR pathway. Of particular significance is the complete reconstitution of the VSP repair pathway *in vitro*. It will be interesting to see what novel discoveries will be made in the future that build upon my work with the MutL protein in MMR and VSP repair.
References


