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Abstract Approved: _____

Andrew Buermeyer

Genomic integrity is crucial for the viability and function of a cell. One key pathway that acts to maintain genomic integrity is DNA mismatch repair (MMR). MMR acts to correct base pair mismatches hat have escaped proofreading during DNA replication. The process of MMR is dependent on the protein heterodimer MutLα, composed of the proteins MLH1 and PMS2. Mutations in MLH1 are linked to a condition known as Lynch syndrome, which is characterized by a predisposition to early-onset colorectal cancer. Because MLH1 is a frequent target of mutations that disable MMR and therefore cause Lynch syndrome, research on mutations in MLH1 is of significant interest. Other laboratories have used an *in vitro* approach to study MMR, however these studies generally were not quantitative and too labor intensive for the analysis of multiple variants of MLH1. We present preliminary data detailing a novel approach to the biochemical analysis of DNA mismatch repair. In our assay, MutLα is prepared from MLH1 and PMS2-deficient cells transfected to express wild type PMS2 and wild type or mutant MLH1. This recombinant MutLα is then used to complement a cellular extract from cells deficient in MLH1 and PMS2, reconstituting a complete repair system. Our findings demonstrate that such an approach is capable of supporting mismatch repair *in vitro* with sufficient precision and reproducibility to support the comparative analysis of multiple mutants of MLH1 and PMS2.

Key Words: DNA Mismatch Repair, MMR, MutLα, MLH1

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A Novel Approach to the Biochemical Analysis of Variants in the Essential DNA Mismatch Repair Protein MLH1

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

Andrew Nguyen, Author

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LIST OF ABBREVIATIONS

MMR	Mismatch Repair
HNPCC	Hereditary Nonpolyposis Colon Cancer
MLH	MutL Homolog
MSH	MutS Homolog
PMS	Post-Meiotic Segregant
MEF	Mouse Embryonic Fibroblast
TOE	Transiently Overexpressed
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
DTT	Dithiothreitol
PMSF	Phenylmethanesulphonylfluoride
UvrD	DNA Helicase II
PCNA	Proliferating Cell Nuclear Antigen
EXO1	Exonuclease 1

INTRODUCTION

Cancer is a disease of uncontrolled cellular growth caused in part by an accumulation of mutations in DNA affecting specific growth-regulatory tumor-suppressors and oncogenes. One key pathway that limits the accumulation of mutations is DNA mismatch repair (MMR), in which the base pair mismatches generated during DNA replication are recognized, excised, and then corrected. The MutLα heterodimer, consisting of the proteins MLH1 and PMS2 is essential for MMR. The failure of MMR is linked to a significantly increased risk of several cancers, as in an autosomal dominant condition known as hereditary nonpolyposis colon cancer (HNPCC) or Lynch Syndrome (*1*). Lynch Syndrome is responsible for at least 5% of colorectal cancer cases and patients with Lynch syndrome develop cancer at a relatively early age (*2*).

DNA mismatch repair is an evolutionarily conserved process that acts to maintain genomic integrity through improving the fidelity of DNA replication. Through the proofreading and nucleotide selection actions of DNA polymerases, the probability of an erroneous base being incorporated during DNA replication is already very low, on the order of 10^{-7} per base pair per replication (5). MMR further improves replication fidelity by correcting replication errors such as base substitution mismatches that are not detected and corrected by the DNA polymerases. Thus, the accuracy of replication is improved by 50-1000 times because of mismatch repair (*6*).

Although DNA mismatch repair has been comprehensively studied in prokaryotes, the process is still not fully understood in eukaryotes. However, studying the mechanism of MMR in *E. coli* (Figure 1) has been a great help in understanding the process in eukaryotes. Prokaryotic MMR depends on three homodimeric proteins – MutS, MutH, and MutL. MutS is an ATPase that is responsible for initial mismatch recognition and binding. After MutS binds to the

mismatch, it forms a complex with MutL, and this complex activates an endonuclease activity of MutH. The nick generated by MutH can be located either 3' or 5' to the mismatch, at which point one of four single-strand exonucleases will excise the DNA past the mismatched nucleotide with the help of single-stand binding protein and UvrD helicase. After DNA resynthesis and ligation, the repair process is complete (*7*).

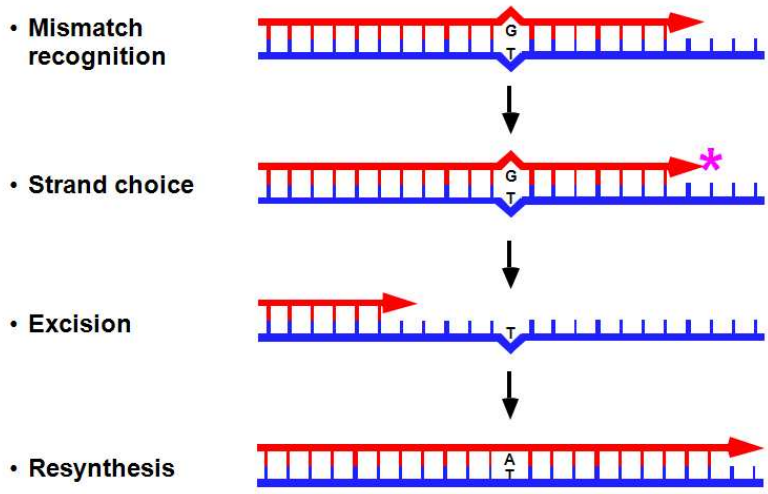


Figure 1. General Mechanism of DNA Mismatch Repair. Mismatch recognition takes place via MutS. MutL is involved in strand discrimination, and excision is accomplished by various exonucleases. Resynthesis of DNA copying the template stand and subsequent ligation of the remaining nick completes the repair process. Figure by AB Buermeyer, reprinted with permission.

"Mut" homologs		
E. coli	***S. cerevisiae***	***H. sapiens***
MutS	Msh2, Msh6, Msh3	Msh2[a], Msh6[a], Msh3
	Msh1	-[b]
	Msh4, Msh5	Msh4, Msh5
MutL	Mlh1	Mlh1[a]
	Pms1	Pms2[a]
	Mlh2, Mlh3	Pms1[a]
MutH	-[b]	-[b]
MutU (UvrD)	-[b]	-[b]

[a]Mutations found in cancer families.

[b]Not identified.

Table 1. "Mut" Homologs. *E.coli* genes that function in MMR and their eukaryotic counterparts in yeast and humans. The fact that multiple homologs of both MutS and MutL exist in eukaryotes suggests that the process of mismatch repair has been conserved from prokaryotes to eukaryotes (*7*).

Reconstructing the MMR system *in vitro* with mammalian cell extracts has proven helpful in understanding eukaryotic MMR. Previous studies have demonstrated that mismatch repair is well conserved from prokaryotes to eukaryotes, however there are some distinct differences. The homodimers MutS and MutL present in prokaryotes are represented by several

heterodimers in eukaryotes. MutS has evolved into two heterdimeric complexes, MutSα and MutSβ. These two heterodimers share a common subunit, MSH2 (MutS Homolog). MutSα pairs MSH2 with MSH6, while MutSβ pairs MSH2 with MSH3 (*17-19*). These higher order complexes function to recognize and bind both base-pair mismatches and insertion-deletion loops of DNA (*20, 21*). The activities of MutSα and MutSβ are similar but still distinct. MutSα, which contains approximately 80-90% of cellular MSH2, is responsible for recognizing base-pair mismatches and small (1-2 nucleotide) insertion-deletion loops. MutSβ is responsible for locating larger (2-10 nucleotide) insertion-deletion loops, and is relatively inactive at recognizing base-pair mismatches (*16*).

As in prokaryotes, the MutL family is also represented in eukaryotic MMR. There are three heterodimeric complexes that belong to the MutL family in eukaryotes: MutLα, MutLβ, and MutLγ. Each one of these complexes consists of Mlh1 (MutL Homolog) and either PMS2 (MutLα), PMS1 (MutLβ), or MLH3 (MutLγ). Of the three MutL complexes, the consensus is that MutLα is the primary heterodimer involved in mismatch repair (*11, 12*). Like its prokaryotic counterpart, MutLα serves a variety of roles in mismatch repair. It has been shown that MutLα associates with other complexes during the repair process – these include MutSα, MutSβ proliferating cell nuclear antigen (PCNA) and EXO1. The ability of MutLα to complex with several other proteins suggests that its inclusion in higher level structures is critical for repair (*14, 15*). In addition to these functions, MutLα has several other roles. Research has shown that MutLα has a latent endonuclease activity that, when activated, introduces single-strand breaks in proximity to a mismatched nucleotide. In doing so, new sites for the aforementioned exonuclease EXO1 are created to excise the strand containing the mismatch (*13*).

There are other similarities between prokaryotic and eukaryotic mismatch repair. Firstly, base-pair mismatches in eukaryotes are repaired at an efficiency similar to that of prokaryotes, and in a strand-specific fashion. The mechanism by which strand choice takes place in eukaryotes is still under debate, but it has been demonstrated *in vitro* that a strand can be targeted for repair by a nick in the mismatched DNA substrate (*8*), and likely involves activation of the latent endonuclease activity of MutLα. Furthermore, repair is bidirectional for both prokaryotes and eukaryotes (*9*).

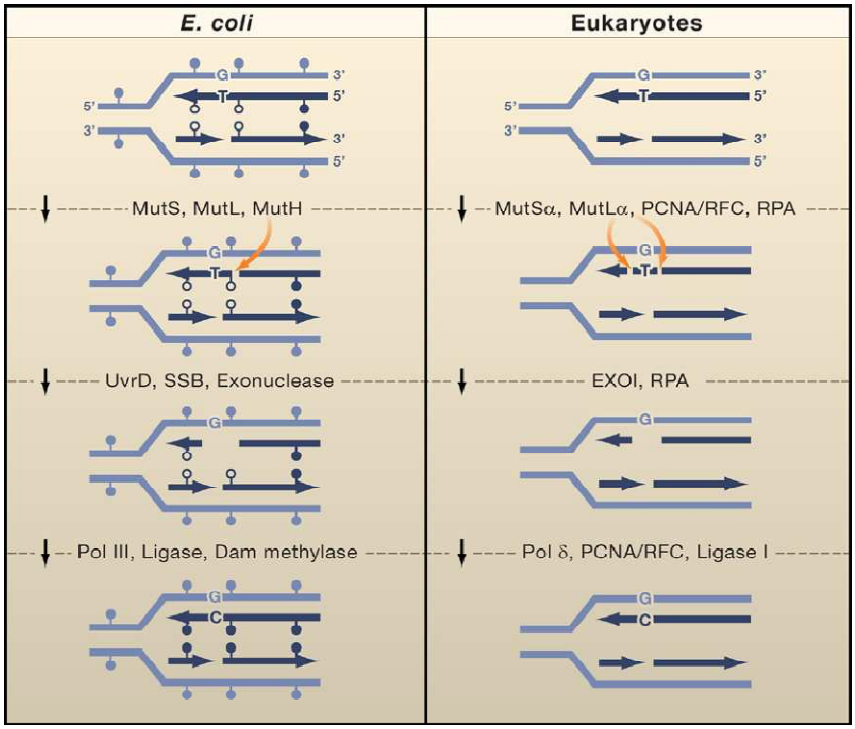


Figure 2. DNA Mismatch Repair in *E. coli* (left) and Eukaryotes (right). In *E. coli*, recognition of the G/T mismatch brings the MutS, MutL and MutH homodimers to the site of the mismatch where MutH nicks the target strand at GATC motifs either 5' or 3' to the mismatch. Next UvrD and exonuclease will bind, excising past the mismatch. Resynthesis and ligation, followed by methylation completes the repair process. The repair process is similar in eukaryotes. First, MutSα, MutLα, and PCNA will assemble at the G/T mismatch. MutLα will nick the target strand at random sites both 5' and 3' to the mismatch, enabling the loading of EXO1. After excision and resynthesis, repair is complete.

Besides its role in correcting base-pair mismatches, MMR also functions to induce apoptosis in response to some types of irreparable DNA damage. In doing so, MMR is able to trigger cell death. One DNA lesion that has been extensively studied with relation to MMR is

O^6-methylguanine. When MMR recognizes an O^6-methylguanine-thymine mismatch and attempts repair only to have DNA polymerase δ repeatedly try to place thymine opposite O^6-methylguanine, the resulting futile cycling can lead to double strand breaks in the DNA and thus activation of apoptosis signaling cascades. An alternative possibility is that MMR proteins might activate signaling pathways for apoptosis via direct protein-protein interactions with other signaling factors (*10*).

Out of the mutations linked with Lynch syndrome, most (>95%) are in only three mismatch repair genes – *MLH1*, *MSH2*, and *MSH6*. Of these three, the majority of known mutations occur in *MLH1* (*3*), and a significant percentage (~30%) are point mutations that change a single amino acid in the protein. The effects of such point mutations can be difficult to predict. There are several rare mutations that are known to cause Lynch Syndrome, but it is not known if common polymorphisms are correlated with a higher cancer risk. Knowing which individuals are at increased risk for cancer via MMR deficiency influences treatment options for cancer patients (22) or cancer surveillance strategies for individuals at higher risk. Previous studies have screened potentially pathogenic mutations and identified numerous mutations that destabilize MLH1 and some that interfere with heterodimer formation with PMS2 (*4*). Point mutations that destabilize MLH1 or block interaction with PMS2 (therefore preventing formation of MutLα) are very likely to cause MMR defects. However, there are several mutants identified with no known biochemical defects previously characterized. Either these mutations cause MMR defects and therefore increased risk of cancer, or they may be rare, non-pathogenic polymorphisms.

Previous studies have used an *in vitro* approach to study mismatch repair. Tomer and others reconstituted a MMR system by using purified MutLα expressed in insect (*spodoptera*

frugiperda) cells (24). Raevaara and others used cellular extracts from both human and insect cells in a complementation approach (25). These and other studies have shown that it is possible to study MMR *in vitro*.

The goal of my research was to characterize a novel approach for biochemical analysis of DNA mismatch repair and establish the limits of its quantitative precision. Whereas other studies have used purified MutLα or cellular extract derived from insect cells, our new approach would use extracts from mouse embryonic fibroblasts (MEFs) transfected to transiently express wild-type and variant forms of MutLα. The goal was to develop a relatively fast and qualitative screening assay that would be used to determine the consequences of specific point mutations in *MLH1* for MMR capacity. The secondary goal was to apply the assay and measure the relative capacity of wild-type versus mutant MLH1 to support error-correction.

In this study I present preliminary results demonstrating that an *in vitro* DNA mismatch repair assay conducted via a complementation approach of MutLα deficient cytoplasmic extract with MutLα transfected cell extract is feasible and warrants further study.

MATERIALS and METHODS

Tissue culture

Mouse embryonic fibroblasts deficient in MLH1 and PMS2 (*Mlh1*$^{-/-}$, *Pms2*$^{-/-}$, known as the MP1 cell line (*24*)) were grown in a 5% CO_2 atmosphere at 38°C as previously described (*27*). Cells were grown in "10% complete medium", consisting of DMEM (Dulbecco/Vogt modified Eagle's minimal essential medium, Mediatech), 10% (v/v) bovine calf serum (HyClone), 1X non-essential amino acids (Mediatech), and 100 U/ mL penicillin and streptomycin (Invitrogen).

Cell extracts

MP1 (null) extracts were prepared from growing cells at 60-80% confluency in 150 mm plates as described (*24*). Approximately 7.5 x 10^8 cells were harvested with trypsin and then pelleted at 800 x g at 4°C. Cells were resuspended in isotonic buffer (1 mM $MgCl_2$, 5 mM KCl, 20 mM Hepes pH 7.9, 250 mM sucrose, 1 mM DTT) then pelleted again at 800 x g and 4°C. Cell pellets were rinsed in hypotonic buffer (1 mM $MgCl_2$, 5 mM KCl, 20 mM Hepes pH 7.9, 1 mM DTT), then pelleted once more at 800 x g. The cell pellets were then resuspended to achieve a concentration of 1.5 x 10^8 cells/mL in hypotonic buffer including 100µg/mL PMSF, and allowed to swell for 15 minutes. After swelling, the cells were lysed in a dounce homogenizer and

allowed to sit on ice for 30 minutes. The extract was then centrifuged at 22,000 x g for 10 minutes at 4°C, and then aliquoted and stored at -80°C.

Transiently overexpressed (TOE) cell extracts were prepared from growing MP1 cells transfected to transiently express hMLH1 and hMPS2. Cells were seeded at a density of 1.8 x 10^6 cells per 100 mm dish and allowed to grow (typically overnight, ~16-18 h) until each dish was 70-80% confluent. Each dish received a transfection mix containing 48 µg of DNA, which was divided as follows: 24 µg of pCMV hMLH1 WT or mutant, and 24 µg of pCMV hPMS2 WT. The other contents of the transfection mix were 40 µl of Lipofectamine (Invitrogen) and 56 µl of Plus reagent (Invitrogen) in DMEM (Mediatech). Each dish was incubated with transfection mix for 3 hours at 37°C, whereupon each dish was re-fed with 10% complete medium after a DMEM wash.

After 24 hours of growth, cells were harvested with trypsin and then pelleted at 800 x g at 4°C. Cells were resuspended in isotonic buffer (1 mM $MgCl_2$, 5 mM KCl, 20 mM Hepes pH 7.9, 250 mM sucrose, 1 mM DTT) then pelleted again at 800 x g and 4°C. Cell pellets were then resuspended in hypotonic buffer (1 mM $MgCl_2$, 5 mM KCl, 20 mM Hepes pH 7.9, 1 mM DTT), then pelleted once more at 800 x g. The cell pellets were then resuspended to achieve a concentration of 1.5 x 10^8 cells / mL in hypotonic buffer including 100µg / mL PMSF, and allowed to swell for 15 minutes. After swelling, the cells were lysed in a dounce homogenizer and allowed to sit on ice for 30 minutes. The extract was then centrifuged at 22,000 x g for 10 minutes at 4°C, and then aliquoted and stored at -80°C.

Quantification of MutLα

The protein concentrations of whole cell lysates were determined using the Bicinchoninic Acid Protein Assay Kit (Pierce). Sample absorbances at 595nm were collected on 96 well plates on a SpectaMax UV plate reader and compared to absorbances from a standard curve generated with bovine serum albumin.

Concentration of MutLα in each TOE prep was determined by quantitative immunoblot (western blot) analysis. Each sample was prepared such that total protein equaled approximately 5 µg in 1X sample buffer (200 mM Tris-Cl pH 6.8, 400 mM DTT, 8% SDS, 0.4% Bromophenol Blue, 50% Glycerol). Samples included aliquots of TOE extract diluted 1:10 or 1:5 with MP1 extract or standards consisting of purified MutLα (*24*) similarly prepared (0-18 ng total was diluted into ~5 µg of MP1 extract). The samples were denatured at 95°C for 5 minutes, and then loaded onto 18 or 24-well Criterion 4-12% XT Bis-Tris gels (Bio-Rad) and electrophoresed for one hour at 200 V. After electrophoresis was complete, the gel was soaked in 1x transfer buffer (25 mM Tris, 250 mM glycine pH 7.4, 10% v/v methanol) for 15 minutes. The proteins were blotted onto an Immobilon-P PVDF membrane (Millipore) in a Criterion blotter for 30 minutes at 100 V. Blocking of the membrane was accomplished by a 20 minute wash with 5% w/v powdered milk in TBST (90 mM NaCl, 1 mM KCl, 17 mM Tris, 0.1% Tween pH 7.6). The blot was probed overnight at 4°C with anti-MLH1 (Pharmingen) and anti-PMS2 (Pharmingen), both at 0.5 µg/ml. After the primary probing, the blot was washed 3X with TBST (5 minutes per wash) and probed with secondary antibody for an hour. The secondary antibody used was a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Pierce) at a 1:7500 dilution.

After washing in TBST for 15 minutes, the blot was developed in 10 ml of SuperSignal West Pico chemiluminescent agent (Pierce) for 3 minutes and then exposed for 45 minutes in the ChemiGenius (Synoptics) imaging station. Band intensities were quantified with the GeneTools application (Synoptics) and analysis was performed with Microsoft Excel and/or Prism 4.0 (Graphpad). Band intensities for MLH1 and PMS2 in TOE extract samples were compared to a standard curve of purified MutLα. This comparison allowed an extrapolation of the unknown amount of MutLα in each TOE extract.

Mismatch substrate

At the time of this research the G/T mismatch and CT loop substrates were available in the Buermeyer laboratory. The pRO1 plasmid used for construction of the mismatch substrate was provided by Dr. John Hays (Department of Environmental and Molecular Toxicology, Oregon State University), using the methodology described by Wang and Hays in (*31*).

***In vitro* repair assay**

All reaction mixtures were assembled on ice. Each reaction mixture had a total final volume of 40 µl, and was assembled in two parts. The first part of the mixture consisted of 250-275 ng of null extract, and 0-25 ng of TOE prep diluted into variable volumes of hypotonic buffer. The second part of the reaction contained 100 ng of mismatch substrate and 4 µl of 10X reaction buffer (1.1 M KCl, 50 mM $MgCl_2$, 10 mM glutathione, 15 mM ATP, 1 mM dNTPs, 200 mM Tris pH 7.6, 500 µg/ml BSA). The mixture of mismatch substrate and reaction buffer was

added to the cytoplasmic extracts and hypotonic buffer to initiate the repair reaction, and reaction mixtures were incubated in a 37°C water bath.

Reactions were halted after 0-25 minutes by addition of 60 µl of stop buffer (1.2% sodium dodecylsulfate, 25 mM EDTA, 0.3 µg/µl Proteinase K). The mixtures were incubated in a 37°C water bath for 30 minutes, at which point the reaction substrate/product DNA was purified by phenol/chloroform extraction and then ethanol precipitated as follows: 50 µl of both phenol and chloroform were added to each reaction along with Phase Lock gel (Eppendorf) and vortexed to mix. After mixing, the mixtures were centrifuged at 22,000 x g for 10 minutes. The aqueous layer was removed and the DNA precipitated by addition of 10 µl of 3 M sodium acetate pH 5.2 and 220 µl of 100% ethanol. The precipitation mixes were incubated at -20°C for at least 10 minutes, followed by centrifugation at 22,000 x g for 10 minutes at room temperature. After precipitation the DNA pellet was washed with 70% ethanol and then resuspended in 10 µl of 10 µg/ml RNase and dissolved in water. The newly resuspended samples were incubated at 37°C for 30 minutes, then doubly digested with 2.5 U of both BanI and XhoI. Each digest reaction also included 2 µl of 10X NEBuffer4 (New England Biolabs) and water to make a final reaction volume of 20 µl. The digest reactions were incubated at 37°C for two hours.

After digestion the samples were run on a 1.5% agarose gel for 45 minutes at 150V. Once the electrophoresis finished the gels were stained with ethidium bromide and then destained with water. The gel was then imaged using the ChemiGenius (Synoptics) imaging station. Band intensities were quantified using the GeneTools software by Synoptics. The extent of repair was calculated as the summed intensity of the 1050 and 1150 bp bands divided by the sum of these bands plus the intensity of the 2200 bp band (unrepaired).

RESULTS

Rationale and General Description of Experimental Approach

The ultimate goal of this study was to determine the effect of mutations in *MLH1* on DNA mismatch repair. Our approach was to use an *in vitro*, biochemical assay to compare the ability of wild-type versus mutant MLH1 to repair defined base-pair mismatches. Similar approaches used previously demonstrate that mismatch correction can be measured using extracts of human or mouse cells (*23, 25*), indicating that such extracts contain all factors necessary for mismatch repair. Similar approaches have also shown that mismatch correction in mammalian cell extracts is MutLα-dependent, and that the activity of wild-type versus mutant MutLα can be compared using recombinant MLH1 and PMS2 (*23, 24*). To compare wild-type and mutant MLH1, we chose to use a complementation approach in which recombinant MutLα (constituted with wild-type or mutant MLH1) was added to MutLα-deficient base extract containing all other factors required for MMR. Our source of recombinant MutLα was extracts prepared from MLH1- and PMS2- deficient mouse embryonic fibroblasts (MP1 cells) (*24*) transfected to express human MLH1 and PMS2 (*4*). The base, MutLα-deficient extract was prepared from non-transfected MP1 cells. Although previous studies have used purified MutLα (*24*), we chose to use extracts of transfected cells for two reasons. The first reason was to develop a rapid, screening assay that did not require cloning of baculovirus vectors for each *MLH1* mutant and multiple, extensive protein purifications. The second reason was because preliminary experiments indicated the MutLα expressed in transfected cells retained a higher specific activity than the purified MutLα.

Mismatch repair *in vitro* generally requires a DNA plasmid substrate containing a single, defined mismatch and a single, defined strand discontinuity (nick), and a cell-free protein extract

containing all necessary protein factors for MMR. The mismatch is localized in the plasmid substrate such that it disrupts a diagnostic restriction site, and the nick is used to direct repair to one specific strand of the DNA. Repair progress is monitored by digestion of the reaction substrate and subsequent analysis by agarose gel electrophoresis. Successful repair of the G/T mismatch substrate we used results in the restoration of an Xho1 restriction site by way of replacement of the offending guanine nucleotide for an adenine. Similarly, the repair of the CT loop substrate results in the restoration of a diagnostic Xho1 restriction site. In our assay, mismatch substrates also contain a Ban1 site approximately 1kb away from the mismatch site that is unaffected by (un)successful repair, and is used to linearize the plasmid to facilitate analysis of repaired versus unrepaired products. Following electrophoresis, unsuccessful repair is detected as a single band migrating at 2.2kb, resulting from the Ban1 digest. Successful repair results in two additional bands migrating at approximately 1050 and 1150 bp, and their intensities are related to the extent of mismatch repair that took place. Repair was quantified by summing the intensities of the two ~1.1kb bands and dividing by the sum of the intensities of the 2.2kb band and the two ~1.1kb bands (Figure 3).

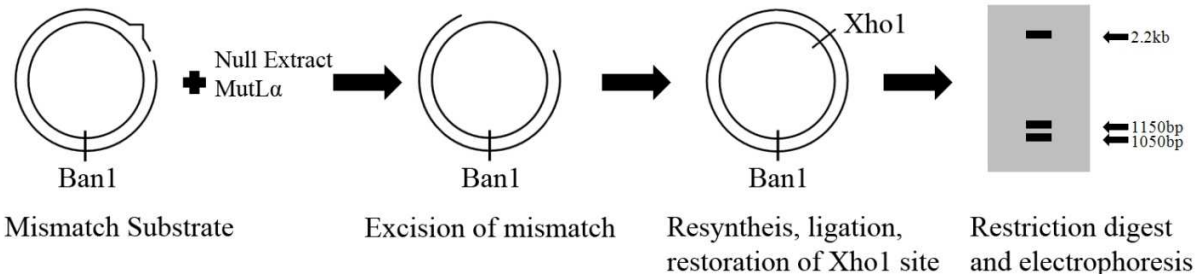


Figure 3. Schematic of the *in vitro* Mismatch Repair Assay. Successful repair of the mismatch restores a Xho1 restriction site, resulting in two bands of 1150 and 1050 bp visible after agarose gel electrophoresis. The band at 2.2 kb represents unrepaired substrate linearized by BanI digestion.

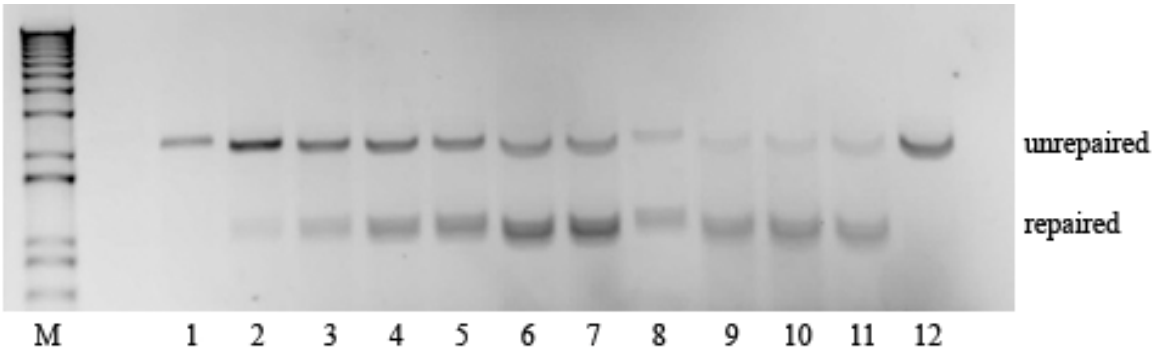
Development and Characterization of the *in vitro* Mismatch Repair Assay

Our goals were first to demonstrate that *in vitro* repair is dependent on both MutLα and null extract (extract deficient in *MLH1*- and *PMS2*-) and then to determine the specific activity of wild type MutLα expressed in terms of fmol of substrate repaired per fmol MutLα per unit time. The specific activity of MutLα would be calculated based on the concentration and time-dependence of repair measured using non-saturating conditions. Although saturating conditions (defined as excess MutLα and/or excess reaction time) can yield maximal levels of repair, such conditions may not detect differences in efficiency between wild type MutLα and mutant. Mutants that are totally deficient for repair can be screened at saturating conditions by detecting a lack of repair at excessive time or concentrations of MutLα.

The first goal was to establish conditions where all protein factors required for MMR except MutLα were in excess. Under these conditions, additional MP1 extract added that contains

recombinant MutLα should not affect levels of repair measured. To establish such conditions, we compared the extent of mismatch repair using a range of different MP1 extract concentrations. In these experiments we used purified, recombinant MutLα (*24*) in significant excess at a constant concentration.

A)



B)

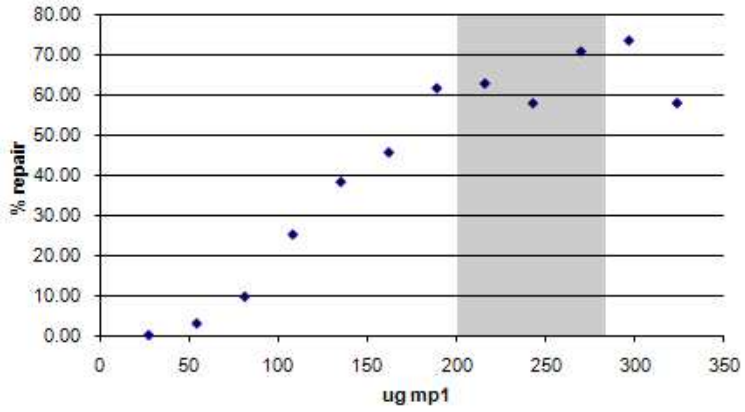


Figure 4. Establishing Conditions for the Biochemical Complementation Assay. A) *In vitro* mismatch repair assays titrating MP1 extract were performed as described in *Materials and Methods*. Reactions included 500 ng of purified, recombinant MutLα. The mixture loaded in lane 1 contained no MP1 extract, and concentration of MP1 in reactions increases in lanes 2-11. The reaction in lane 12 contains no MutLα. Repair was measured after 25 minutes of reaction time. **B)** Extent of repair, expressed as percent of total input substrate detected as XhoI sensitive (i.e. repaired) plotted as a function of mass (µg) of MP1 extract in the reaction. Maximal levels of repair were apparent with $\geq$ 225 µg of MP1 extract.

Results demonstrated that percent of repaired substrate increased with increasing mass of MP1 extract, reaching a maximum of ~60-70% repair with ≥ 225 µg MP1 extract. Further increases in MP1 extract did not appear to yield higher levels of repair. No repair was observed in the absence of either MP1 extract or recombinant MutLα. Thus, this experiment confirms that repair is dependent both on proteins present in null extract and MutLα. Furthermore, we conclude that the extent of repair in complementation reactions containing at keast 225 µg MP1 extract would not be increased by inclusion of an additional 25-50 µg of MP1 extract.

The net goal was to prepare and test recombinant MutLα produced by transfection and transient expression of *hMLH1* and *hPMS2* in MLH1- and PMS2-deficient MP1 cells. Such transient transfections generally result in an overexpression of MutLα (*4*) and were refered to as TOE preparations (TOE = Transiently Overexpressed). We quantified the concentration of MutLα in TOE extracts by immunoblot (western blot) analysis in which the signal intensities of MLH1 and PMS2 present in TOE extracts were compared to a standard curve generated from signal intensities of purified MutLα diluted into extracts of non-transfected MP1 cells.

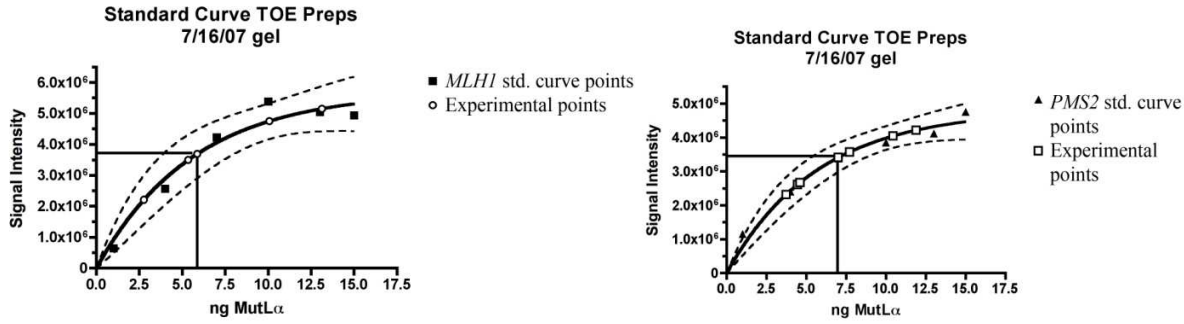


Figure 5. Quantification of MutLα. MutLα concentrations in TOE extracts were determined by quantitative immunoblot analysis. Band intensities for MLH1 and PMS2 present in TOE extracts were compared to a standard curve generated from signal intensities of purified MutLα. Plotted are the MLH1 and PMS2 standard curves with data points representing extracts of unknown [MutLα] lying on the curves.

The results from the western blot quantification showed that MutLα was expressed in the transfected cells and was present in significant concentrations in the TOE extracts. Furthermore, we conclude that the amount of MutLα in each preparation can be determined quantitatively.

To determine whether recombinant MutLα produced via transfection of *MLH1*- and *PMS2*-deficient MEFs (MP1 cells) would support significant MMR, mismatch correction assays were performed. Extract of MP1 cells transfected to transiently expresss wild type *hMLH1* and *hPMS2* were added to null MP1 cell extract and assayed for G/T mismatch correction as described above using purified MutLα (Figure 4). These assays were done using saturating amounts of null extract, with the amount of MutLα-containing extract varied to determine how the extent of repair depended on MutLα concentration. Results are summarized below:

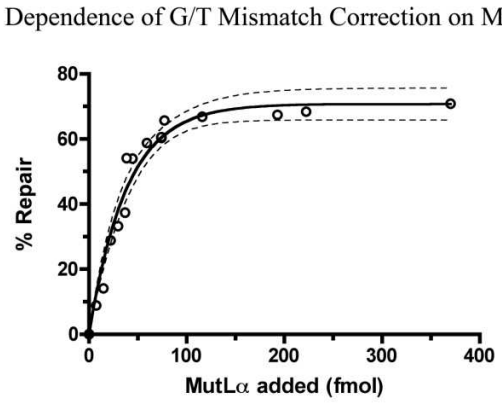


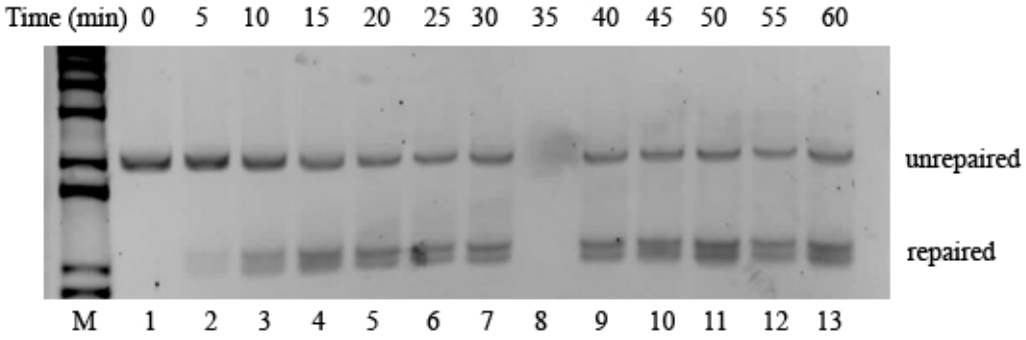
Figure 6. Dependence of G/T Mismatch Correction on MutL*α*. Mismatch repair reactions containing 250 µg MP1 extract and varying amounts of WT MutLα-containing TOE extracts were assayed. Plotted is the data from two different preparations of wild type MutLα-containing extract produced from transfection of *MLH1*- and *PMS2*- deficient MEFs, each analyzed in 2-3 independent experiments. Repair reached a maximum of approximately 70% (representing approximately 50 fmol of plasmid substrate) at levels of MutLα nearing 100 fmol.

The extent of repair increased linearly in reactions containing up to ~50 fmol of recombinant MutLα, and reached maximal levels with $\geq$ 100 fmol. Plateau levels of repair were similar to that achieved with an excess of purified MutLα. Analysis of the slope from linear range of the concentration dependence ($\leq$ 50 fmol MutLα) yielded a specific activity of 0.85 fmol plasmid substrate repaired / fmol MutLα. Significantly, the specific activity of MutLα present in TOE extracts appeared several times higher than that of purified MutLα (Nelson, Buermeyer personal communication).

We conclude that recombinant MutLα present in extracts of transfected MEFs has sufficient specific activity to allow measurement of activity in the complementation assay. Furthermore, analysis of multiple independent preparations (Figure 5) indicates that our transfection and extract preparation procedures are sufficiently reproducible that the complementation assay should be a robust approach for comparing the activity of wild-type and mutant MutLα.

To determine the kinetics of the appearance of repaired, XhoI-sensitive substrate, a time course analysis was done (Figure 7). Following a barely perceptible lag, the extent of repair increased linearly for 10-15 minutes, reaching a plateau after approximately 25 minutes.

(A)



(B)

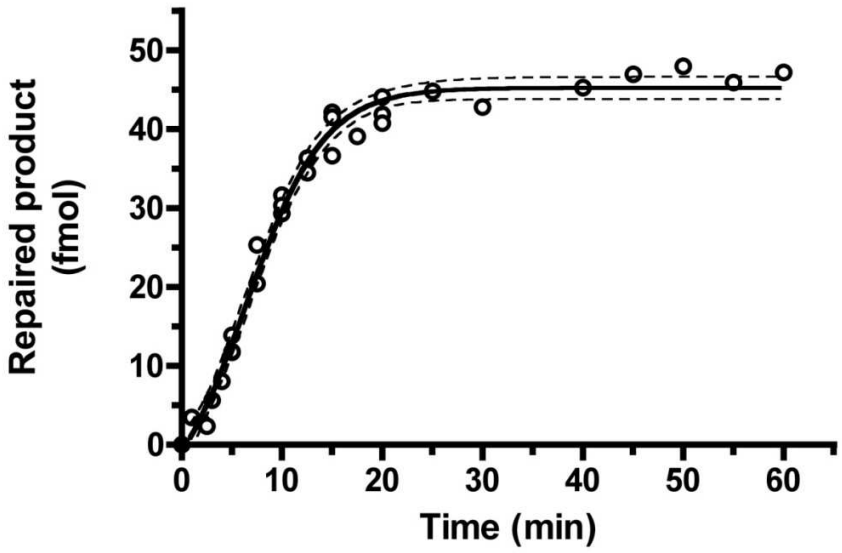


Figure 7. **Time Course Analysis of Wild Type MutLα. A.)** Representative gel image demonstrating time course analysis for appearance of repaired product in an i*n vitro* repair assay using wild type MutLα. One large repair reaction was prepared and aliquots were removed and stopped at various time points. Repair data from 35 minutes is absent in this specific experiment. **B.)** *In vitro* repair reactions using the complementation approach were performed using excess

MP1 extract (250 µg) and recombinant wild type MutLα. Plotted is extent of repair (fmol of repaired product) as a function of time (min). Shown are the results of 4 experiments, using 2 different preparations of MutLα and analyzed together. Data points represent individual reactions; the plotted line was generated by non-linear regression using a sigmoidal fit.

Preliminary Analysis of *MLH1* Mutants

Our ultimate goal was to determine the effect of specific point mutations in *MLH1* on the ability to support mismatch correction. To facilitate the analysis, and to demonstrate that the overall approach is feasible, we performed initial screening of several *MLH1* mutants using saturating amounts of MutLα and extended incubation times. We anticipated that such screening would identify specific mutants that were completely or largely deficient for repair; such mutants would not require additional analysis. Other mutants that retained significant activity would require more careful and extensive analysis to measure the effect of the individual mutations on MutLα activity.

Mlh1-I219V

Previous studies have shown that the I219V mutation ([original amino acid][site of mutation][new amino acid]) is unlikely to affect mismatch repair ability, and that the mutation by itself is not significantly associated with higher cancer risks (*29*, *30*). We hypothesized that I219V should be able to repair mismatch substrate as well wild type MLH1.

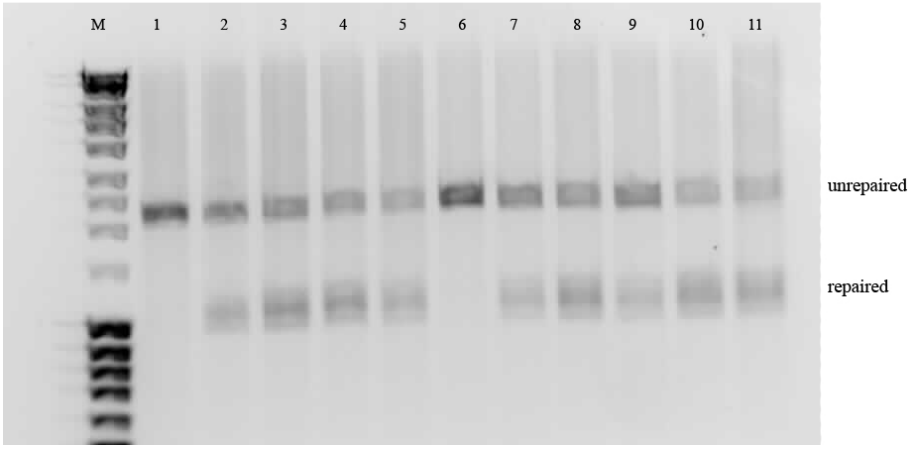


Figure 8. The I219V Variant of MLH1 Shows Levels of Repair Comparable to Wild Type. In this *in vitro* mismatch repair assay, various amounts of I219V MutLα were used with two different preparations of null extract. The reactions in lanes 1 and 6 are negative controls, and the reaction in lane 11 is a control using wild type MLH1.

As expected, I219V did not show a deficiency for repair under conditions saturating for null extract or MutLα at extensive incubation times (Figure 8).

Mlh1-L607H, L582V, K751R, R755W

The four mutants of MLH1 L607H, L582V, K751R and R755W have been identified in human cancers and have been previously characterized as not affecting stability or heterodimer interactions (Hippchen and Buermeyer, unpublished data). We wished to use the *in vitro* MMR assay to determine if they were MMR deficient. We hypothesized that the four mutants would exhibit reduced MMR activity relative to wild type MLH1.

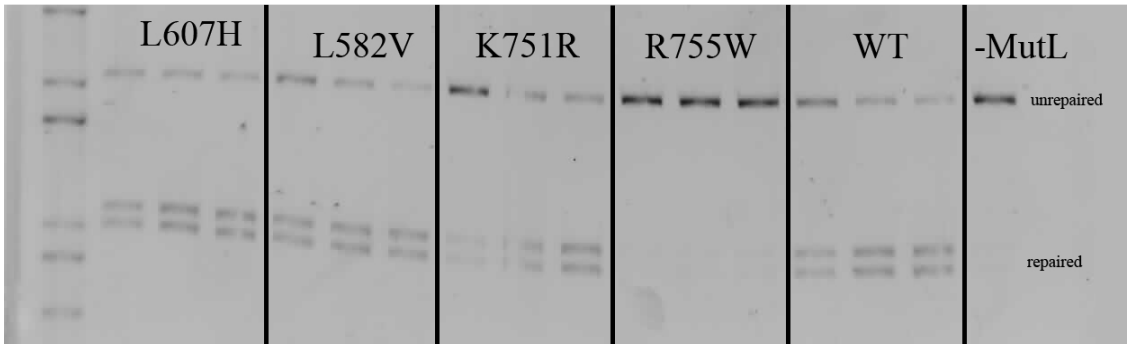


Figure 9. *In vitro* Repair Assay with MLH1-L607H, L582V, K751R, R755W and MLH1-WT. Three reactions were done with each mutant, using varying concentrations of MutLα. Because MutLα is generally well expressed, these reactions were likely done at saturating levels of MutLα. A quantitative analysis of MutLα concentrations in these mutant-containing extracts was not performed.

As shown in Figure 9, R755W showed negligible amounts of repair. MLH1-L607H, L582V, K751R all showed levels of repair comparable to wild type MLH1.

DISCUSSION

Our goal in this study was to develop a novel approach to studying DNA mismatch repair *in vitro*. Specifically, we wanted to develop a relatively fast and quantitative assay that would be used to determine the consequences of specific point mutations in *MLH1* for MMR capacity. Knowing the consequences of specific point mutations in *MLH1* could help predict which individuals are at higher risk for cancer or influence treatment options for current cancer patients. The secondary goal was to apply the assay and study how mutations in *MLH1* affect capacity of mismatch repair compared to wild type. Our approach was to use a biochemical complementation approach using cytoplasmic extracts. Specifically, null extract (extract deficient in MLH1 and PMS2) was prepared from the MP1 cell line, while extract containing recombinant MutLα was generated following transfection of MP1 cells to transiently over-express human MLH1 and PMS2. These two extracts were combined with a DNA plasmid containing a known mismatch, the correction of which would be diagnostic for repair.

We present results demonstrating that a complementation approach combining null extract deficient in MLH1 and PMS2 with recombinant MutLα can support mismatch repair. Furthermore, we are able to conclude that the recombinant MutLα produced by transfection of MEFs has a high enough specific activity to function in the complementation assay. However, there are still issues with the assay that need to be addressed. Due to limitations of time and due to a periodic inconsistency of the assay, we were unable to perform experiments necessary to fully calculate the specific activity of MutLα. An additional time course experiment with at least two different, sub-saturating concentrations wild type MutLα would be required to determine the specific activity in units of (fmol product / fmol MutLα / unit time).

After using the complementation approach with the transiently over expressed (TOE) extracts, several advantages over other published methods became apparent. Firstly, using the TOE cytoplasmic extract instead of purified MutLα cut down on labor required to purify the MutLα. Secondly, using a complementation approach allows us to titrate the amount of MutLα only, which enables the determination of the specific activity of MutLα alone. Using the TOE extract approach also allows for rapid creation of a wide variety of mutant MLH1 containing extracts. Also, it was noted that MutLα prepared via the transfection approach appeared several fold more active than purified MutLα (Buermeyer, personal communication). Perhaps the MutLα present within the cell extracts retains interaction with some cofactor that is lacking in a purified preparation. Another possibility is that the increased handling necessary for a purified preparation decreases activity. An additional benefit of the complementation approach is its potential to be more quantitative than other published approaches because all factors except MutLα are identical. Lastly, a complementation approach enables us to make reagents in bulk.

Because *MLH1* is a frequent target of mutations that disable MMR, research on *MLH1* mutations is of significant interest. Takahashi and others (*23*) used *in vitro* and yeast-based assays to study approximately 100 human *MLH1* variants. They found that the majority of mutations that were functionally inactive affected amino acids near the NH_2-terminal or COOH-terminal of the MLH1 protein. However, their approach lacked a quantification of MutLα; as a result, it is difficult to quantify and compare the effects of different mutations on *MLH1*. Specifically, it is not possible to distinguish between a biochemical defect in MutLα or low levels of expression as the cause of MMR deficiency. Furthermore, in the published experiment, the extent to which MutLα in present in excess in the reactions is not clear. Thus, a hypothetical MLH1 mutant that supports 50% of wild type repair but that is present in several fold excess in

the reaction, would appear to be phenotypically identical to wild type MLH1. In spite of this, the approach used by Takahasi and others was an efficient, qualitative way to screen mutants of MLH1.

Raevaara and others also used an *in vitro* mismatch repair assay to study the *MLH1* mutant K616del (*25*), a deletion of the lysine residue at position 616. They found that MLH1-K616del appeared to support levels of mismatch repair equal to wild type *in vitro.* Defects in repair in cells were associated with low accumulation of the mutant protein in cells. Similar to the Takahashi study described above, a drawback to Raevaara approach was a lack of quantification of MutLα used and a failure to conduct time course experiments or experiments with varied amounts of protein. Without these experiments, it is not possible to quantitatively compare the activity of the mutant MLH1 and wild type.

In another study, Tomer and others (*24*) studied the ATPase activity of the MLH1 protein by using an *in vitro* MMR assay. However, one way in which Tomer study differs from those of Takahashi and Raevaara is that the Tomer group used purified MutLα to study MMR *in vitro,* while Takahashi and Raevaara used cytoplasmic extracts from transfected human or insect cells. While this approach is valid, the infection of insect cells with baculovirus and subsequent extraction and purification of MutLα is time intensive and appears to lead to MutLα with decreased activity.

Using the new approach, we were able to obtain preliminary data on several specific mutants of *MLH1*. Results indicated that *MLH1*-I219V, the most common polymorphism in *MLH1* (*23*), exhibited levels of repair comparable to wild type *MLH1*. Similarly, the L607H, L582V, and K751R variants of *MLH1* supported levels of repair comparable to wild type *MLH1*.

In contrast, the R755W variant of *MLH1* showed an almost complete deficiency for mismatch repair. No repaired product was detected even at saturating concentrations of MutLα and extended incubation times. Further, in-depth study is warranted on the mutants that were able to support repair, but is not a priority for the R755W variant of *MLH1* due to a lack of detected repair at saturating conditions.

In related studies, *MLH1*-I219V was also tested in cellular assays for the ability to complement *MLH1*-deficient MEFs and was found to fully support mismatch repair-dependent suppression of spontaneous mutation and cytotoxic responses to genotoxic DNA damage (Hippchen, Buermeyer, unpublished data). Similar results were observed for the *MLH1* variants L582V and K751R, whereas L607H has not been tested. In contrast, R755W was unable to restore MMR activity to *MLH1*-deficient MEFs. Thus, preliminary data obtained with biochemical complementation are consistent with cellular assays of complementation. The ability of L582V and K751R to complement and support MMR *in vitro* and in cells suggests that they are non-pathogenic polymorphisms of *MLH1* that are not responsible for elevated risk of cancer due to MMR deficiency.

Alternatively, the assays utilized to date both *in vitro* and in cells may not be sensitive enough to detect more moderate decreases in MLH1 activity. Further development of the *in vitro* complementation approach described herein thus is warranted and will contribute to our understanding of the consequences of genetic variation in MLH1 and other MMR genes.

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