

The *Escherichia coli* MutL Protein Physically Interacts with MutH and Stimulates the MutH-associated Endonuclease Activity*

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All possible pairwise combinations of UvrD, MutL, MutS, and MutH were tested using the yeast two-hybrid system to identify potential interactions involving mismatch repair proteins. A two-hybrid screen previously identified a physical interaction between MutL and UvrD. Although several other known interactions were not observed, a novel interaction between MutL and MutH was detected. A series of truncations from the NH₂ and COOH termini of MutL demonstrated that the COOH-terminal 218 amino acids were sufficient for the two-hybrid interaction with MutH. Removal of a small number of residues from either the NH₂ or COOH termini of MutH eliminated the two-hybrid interaction with MutL. Protein affinity chromatography experiments confirmed that MutL, but not MutS, physically associates with MutH. Furthermore, MutL greatly stimulated the d(GATC)-specific endonuclease activity of MutH in the absence of MutS and a mispaired base. Stimulation of the MutH-associated endonuclease activity by MutL was dependent on ATP binding but not ATP hydrolysis. Further stimulation of this reaction by MutS required the presence of a DNA mismatch and a hydrolyzable form of ATP. These results suggest that MutL activates the MutH-associated endonuclease activity through a physical interaction during methyl-directed mismatch repair in *Escherichia coli*.

The methyl-directed mismatch repair pathway in *Escherichia coli* functions to correct DNA biosynthetic errors that arise during chromosomal replication and to discourage recombination between substantially diverged DNA sequences (1). Inactivation of the mismatch repair system results in elevated spontaneous mutation rates (2). The pathway has been reconstituted *in vitro* and involves the action of eight proteins (3).

Initiation of mismatch repair requires MutS, MutL, and MutH in addition to a DNA mismatch, ATP, and Mg²⁺, and results in the generation of a nick in the unmethylated (nascent) strand of a nearby hemimethylated d(GATC) sequence (4). The transient hemimethylated state of d(GATC) sequences after replication serves as a signal to direct repair to the nascent DNA strand (5, 6). MutS recognizes and binds the mismatched base (7, 8). MutL binds the MutS-mismatch complex (9), and MutH is stimulated to catalyze the endonucleolytic cleavage at the d(GATC) site in the presence of MutL and MutS

(4). After the initiation stage of mismatch repair, DNA unwinding is initiated at the nick by DNA helicase II (UvrD) and proceeds to a point beyond the error (10, 11). Excision of the error-containing DNA strand is facilitated by the action of one of several exonucleases (depending on the polarity of the reaction) which serve to degrade the single-stranded DNA (ssDNA)¹ as it is unwound by UvrD (11, 12). In the presence of ssDNA-binding protein, DNA polymerase III holoenzyme catalyzes repair synthesis on the resulting gapped DNA molecule to restore the correct sequence, and DNA ligase seals the final nick (3).

The *E. coli* MutH protein possesses a weak endonuclease activity that is specific for unmethylated d(GATC) sequences (13). In the presence of ATP, MutS, MutL, and a hemimethylated DNA substrate containing a mismatched base pair, the MutH-associated endonuclease activity is greatly stimulated (4). However, the mechanism by which the MutH endonuclease activity is activated by the MutS-MutL complex is not known.

Recently, we identified a physical interaction between the MutL and UvrD proteins using a yeast two-hybrid screen with UvrD as bait (14). Simultaneously, a biochemical interaction was reported between MutL and UvrD (15). To identify other potential interactions involving *E. coli* mismatch repair proteins, all possible pairwise combinations of MutS, MutL, MutH, and UvrD were tested for interactions using the yeast two-hybrid system. An interaction was identified between MutL and MutH which was subsequently confirmed by affinity chromatography. The weak endonuclease activity of MutH on unmethylated d(GATC) sequences was greatly stimulated by MutL. Surprisingly, this stimulation of the activity of MutH occurred in the absence of MutS and a mismatched base pair, suggesting that MutL is the component of the MutS-MutL complex responsible for activating MutH during mismatch repair *in vivo* and that the activation occurs via a direct physical interaction. In addition, the stimulation of MutH by MutL was dependent on ATP but not ATP hydrolysis. These results suggest an additional role for the MutL protein in coordinating activities during mismatch repair in *E. coli*.

EXPERIMENTAL PROCEDURES

Materials

pGAD424 and pGBT9, and yeast HF7c and SFY526 were from the Matchmaker two-hybrid system (CLONTECH). pCYB1, pCYB2, and all components of the Impact I protein purification system were from New England Biolabs. *E. coli* GE1752ΔuvrD (16) and GE1752mutS::Tn5 (17) were constructed previously in this laboratory. HMS174 (*recA1 hsdR* (r_{K12}-m_{K12}⁺) Rif^r) was from Novagen.

BL21(DE3)mutS::Tn5 was constructed by P1 transduction (18) using GE1752mutS::Tn5 as the donor strain and BL21(DE3) as the recipient.

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¹ The abbreviations used are: ssDNA, single-stranded DNA; PCR, polymerase chain reaction; BSA, bovine serum albumin; ATPγS, adenosine 5'-O-(3-thiotriphosphate); AMP-PNP, adenosine 5'-(β,γ-imino)triphosphate; MES, 4-morpholineethanesulfonic acid.

Several Kan^r transductants were selected, colony purified, and screened for a mutator phenotype. To ensure that the mutator phenotype was caused by the mutant *mutS* allele, complementation experiments were performed using a high copy number plasmid that expressed MutS.

To prepare M13mp18 ssDNA, phage infection of *E. coli* XL-1 Blue (Stratagene) and collection of phage particles were performed as described (19). Phage particles were purified on a CsCl gradient (0.438 g of CsCl/ml; 83,000 × *g* for 24 h at 25 °C). After isolation from the gradient and dialysis against 10 mM Tris-HCl (pH 8.0) to remove CsCl, the phage particles were treated with 200 µg/ml proteinase K and 0.1% SDS for 1 h at 50 °C. M13mp18 ssDNA was purified from phage particles by sequential extractions with buffered phenol, 25:24:1 phenol:chloroform:isoamyl alcohol, and 24:1 chloroform:isoamyl alcohol followed by ethanol precipitation. M13mp18 RF DNA was prepared from phage-infected XL-1 Blue cells as described (20).

T7 DNA polymerase was purified previously according to a published procedure (21). All enzymes used for cloning and PCR were from New England Biolabs with the exception of T4 DNA ligase, which was from Boehringer Mannheim. Nucleotides were from Amersham Pharmacia Biotech.

Methods

Cloning Mismatch Repair Genes—Construction of pGAD424-UvrD, pGAD424-MutL, pGBT9-UvrD, and pGBT9-MutL was described previously (14). The coding regions of *mutS* and *mutH* were amplified by PCR from *E. coli* K-12 genomic DNA using Vent DNA polymerase. Oligonucleotide primers for amplifying the *mutS* gene contained restriction enzyme sites that allowed cloning of the *mutS* coding sequence into the *EcoRI* and *BamHI* sites of pGAD424 and pGBT9, creating a translational fusion with the Gal4 transcriptional activation domain and DNA binding domain, respectively, for use in the yeast two-hybrid system. In addition, these primers contained restriction enzyme sites that allowed cloning of *mutS* into the *NdeI* and *SmaI* sites of pCYB2 for overexpression and purification of MutS using the Impact I protein purification system. Likewise, primers for amplifying the *mutH* coding sequence contained restriction enzyme sites that allowed cloning of *mutH* into the *EcoRI* and *BamHI* sites of pGAD424 and pGBT9 and into the *NdeI* and *SapI* sites of pCYB1. pET3c-MutL was constructed by subcloning the *NdeI*-*BamHI* fragment containing the *mutL* coding sequence from pGAD424 into the *NdeI* and *BamHI* sites of pET3c (Novagen).

Deletion Constructions—Deletions from each end of the *mutL* gene in pGAD424 were constructed previously (14). To construct *mutHΔ38N* and *mutHΔ10C*, the appropriate portion of the *mutH* gene was amplified by PCR using Vent DNA polymerase. The oligonucleotide primers used in these reactions contained restriction enzyme sites that allowed cloning of each PCR product into the *EcoRI* and *BamHI* sites of pGBT9, creating a translational fusion with the Gal4 DNA binding domain.

Yeast Two-hybrid Assays—Potential interactions between mismatch repair genes were tested in yeast HF7c by cotransformation of all possible combinations of pGAD424 and pGBT9 harboring the *uvrD*, *mutL*, *mutS*, and *mutH* genes. After selection of cotransformants on complete synthetic media lacking tryptophan and leucine, cells were transferred to complete synthetic media lacking tryptophan, leucine, and histidine and supplemented with 1 mM 3-amino-1,2,4-triazole. HF7c contains a *HIS3* reporter gene that requires a two-hybrid interaction for expression. Yeast SFY526 containing a *lacZ* reporter gene was used to confirm any interactions by monitoring β-galactosidase activity in the presence of the color-producing substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. β-Galactosidase activity was quantified (where indicated) as described by the supplier using the substrate *o*-nitrophenyl β-D-galactopyranoside, and results were expressed as Miller units (18).

Protein Purifications—Purification of MutL from GE1752Δ*uvrD* was described previously (14). To overexpress MutL in an *E. coli* strain lacking a functional *mutS* gene product, a 10.5-liter culture of BL21(DE3)*mutS*::Tn5 containing pET3c-MutL was grown at 30 °C in 2 × YT medium. Protein expression was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside at an A_{600 nm} of 2.0, and incubation at 30 °C was continued for 5 h. Cells (118 g) were harvested by centrifugation and washed with M9 minimal medium salts. Cell lysis and protein purification were performed essentially as described previously (9) with a few exceptions. First, 10% glycerol was included in all column buffers. Second, a Bio-Rex 70 column was used as the initial chromatographic step. Subsequently, the first hydroxylapatite chromatographic step was used, and the second hydroxylapatite column was eliminated. Third, a

Superose 12 HR 10/30 high performance liquid chromatography sizing column (Amersham Pharmacia Biotech) was used for the final purification step instead of a Sephadex G-150 column. Storage buffer for MutL was 25 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM 2-mercaptoethanol, 0.1 mM EDTA, and 50% glycerol.

To overexpress MutH, three 1-liter cultures of HMS174 containing pCYB1-MutH were grown at 37 °C to an A_{600 nm} of 1.2 in 2 × YT medium. Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to 0.5 mM, and growth was continued at 30 °C for 5 h. Cells (12 g) were collected by centrifugation and resuspended in column buffer (20 mM Tris-HCl (pH 8.0), 750 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride). Cells were lysed by sonication, and protein purification was performed using a 20-ml chitin column (4.1 cm × 4.9 cm²) equilibrated in column buffer essentially as recommended for the Impact I system.

To overexpress MutH in an *E. coli* strain lacking a functional *mutS* gene product, a 10.5-liter culture of GE1752*mutS*::Tn5 containing pCYB1-MutH was grown at 30 °C to an optical density of 3.0 in 2 × YT medium. Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to 0.5 mM, and growth was continued for 5 h at 30 °C. Cells were harvested by centrifugation, washed once with M9 minimal medium salts, and resuspended in column buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.2% Triton X-100, 0.5 mM EDTA, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride). Cells (70 g) were lysed by sonication and purified on a 20-ml chitin column (4.1 cm × 4.9 cm²) according to the Impact I purification protocol. The chitin column was equilibrated and washed with column buffer, and intein-induced self-cleavage was initiated with cleavage buffer (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.1 mM EDTA, 10% glycerol, and 100 mM 2-mercaptoethanol). The cleavage reaction was allowed to proceed for 72 h before elution of MutH from the chitin column. Pooled MutH (18 mg in 27.5 ml) was dialyzed extensively against 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.1 mM EDTA, and 10% glycerol to remove the 2-mercaptoethanol. MutH was precipitated with 60% ammonium sulfate and resuspended in 4 ml of 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.1 mM EDTA, and 10% glycerol and dialyzed against two 500-ml volumes of this buffer to remove ammonium sulfate. MutH was loaded in 1-ml aliquots onto a Superose 12 HR 10/30 sizing column at a flow rate of 0.2 ml/min to separate MutH from a prominent contaminating protein of approximately 75 kDa. Both preparations of MutH were stored in 25 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.1 mM EDTA, and 50% glycerol.

To overexpress MutS, four 750-ml cultures of HMS174 containing pCYB2-MutS were grown at 37 °C to an A_{600 nm} of 0.7 in 2 × YT medium. Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to 0.5 mM, and growth was continued for an additional 5 h at 30 °C. Cells were harvested by centrifugation and resuspended in column buffer (20 mM Tris-HCl (pH 8.0), 750 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride). Cells (9 g) were lysed by sonication and purified using a 20-ml chitin column (4.1 cm × 4.9 cm²) essentially as recommended for the Impact I system. Purified MutS was stored in 25 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.1 mM EDTA, and 50% glycerol. All protein concentrations were determined using the Bio-Rad protein assay. Because of the nature of the intein cleavage reaction in the Impact I system, purified MutS contained two extra amino acids on the COOH terminus (proline and glycine). The amino acid sequence of MutH purified using the Impact I system was identical to native MutH.

Preparation of DNA Substrates—The oligonucleotides 5'-GGTACCGAGTTTCGAATTTCG-3' and 5'-GGTACCGAGCTCGAATTTCG-3' were used to generate covalently closed duplex M13mp18 DNA containing a single G-T mismatch (heteroduplex) and no mismatch (homoduplex), respectively. The G-T mismatch in the heteroduplex substrate disrupted a *SacI* site in the polylinker of M13mp18. The presence of the mismatch was confirmed by digestion of the heteroduplex substrate with *SacI*.

Both oligonucleotides anneal to identical positions in the M13mp18 polylinker, and all manipulations used to generate the heteroduplex and homoduplex substrates were identical. Before annealing, oligonucleotides were phosphorylated using T4 polynucleotide kinase. Annealing mixtures (65 µl) contained 100 mM Tris-HCl (pH 8.0), 20 mM MgCl₂, 140 pmol of oligonucleotide, and 4.6 pmol of M13mp18 ssDNA molecules. These mixtures were heated to 94 °C for 3 min and cooled 1 °C/min to 30 °C in a Perkin-Elmer 2400 thermal cycler. Components of the extension reaction were added to the annealing mixtures such that a final volume of 130 µl was achieved containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 8 µg/ml bovine serum albumin (BSA), 500 µM each dNTP, and 8.3 mM dithiothreitol. Extension reactions were incubated at

30 °C for 30 min with enough T7 DNA polymerase to achieve complete conversion of M13mp18 ssDNA to duplex molecules. To achieve covalently closed molecules, 750 μ M ATP and 1 unit of T4 DNA ligase (Boehringer Mannheim) were added to each extension reaction. Extension reactions were pooled and covalently closed, and nicked circular DNA was separated on a CsCl/EtBr gradient as described (19).

Endonuclease Assays—MutH-catalyzed endonuclease reactions (16 μ l) contained 20 mM Tris-HCl (pH 7.6), 4 mM MgCl₂, 20 mM NaCl, 50 μ g/ml BSA, and 50 ng of the appropriate DNA substrate. When present, ATP, ATP γ S, and AMP-PNP were 1.25 mM. When present, MutL and MutS were added immediately before initiation of the reactions with the indicated concentration of MutH. All protein dilutions were made in 10 mM Tris-HCl (pH 8.0). All reactions were incubated at 37 °C for 15 min and quenched with 4 μ l of 5 \times dye solution (25% glycerol, 100 mM EDTA, and 0.025% bromophenol blue). Reaction products were subjected to electrophoresis on 0.8% agarose gels in the presence of 0.5 μ g/ml EtBr to separate covalently closed and nicked circular DNA species. Agarose gels were subsequently irradiated with a hand-held UV (254 nm) lamp for 30 min, restained for 30 min with 0.5 μ g/ml EtBr, and destained with deionized and distilled water. Gels were illuminated with UV light and photographed using an Eagle Eye II still video imaging system (Stratagene).

Affinity Chromatography—4.5 mg of purified MutH was covalently coupled to approximately 750 μ l of Affi-Gel 10 resin as described by the supplier (Bio-Rad) in 25 mM MES (pH 6.4), 200 mM NaCl, and 20% glycerol for 12 h at 4 °C. The coupling reaction was quenched with 25 mM ethanalamine (pH 8.0) for 1 h, and the resin was transferred to a chromatography column (inner diameter = 0.75 cm). The coupling efficiency was greater than 50% based on quantitation of protein in the initial column flow-through using the Bio-Rad protein assay. The column was equilibrated with affinity buffer (25 mM Tris-HCl (pH 7.5), 10% glycerol, 2.5 mM 2-mercaptoethanol, and 3 mM MgCl₂) containing 50 mM NaCl. Approximately 100 μ g of the indicated protein, diluted to a 1-ml volume in affinity buffer plus 50 mM NaCl, was applied to the MutH affinity column at a flow rate of 10 ml/h. The column was washed four times with 500 μ l of affinity buffer plus 50 mM NaCl, collecting each wash as an individual fraction. The column was eluted with four 500- μ l volumes of affinity buffer plus 1 M NaCl, collecting each as an individual fraction. Fractions were analyzed for protein content by electrophoresis on a 10% polyacrylamide gel in the presence of SDS followed by staining with Coomassie Brilliant Blue. A control column containing chicken egg white lysozyme covalently coupled to Affi-Gel 10 resin was constructed previously (14). Experiments using this column were performed exactly as described for the MutH affinity column.

RESULTS

MutL and MutH Interact in the Yeast Two-hybrid System—Previously, we identified a physical interaction between the methyl-directed mismatch repair proteins MutL and UvrD using a yeast two-hybrid screen of an *E. coli* genomic library with UvrD as bait (14). To identify other potential interactions between components of the mismatch repair system, the *mutS* and *mutH* genes were amplified by PCR from *E. coli* K-12 genomic DNA and cloned into the two-hybrid system vectors pGAD424 and pGBT9 as described under “Experimental Procedures.” All possible pairings of *uvrD*, *mutL*, *mutS*, and *mutH* were tested for interactions in yeast HF7c cells containing a *HIS3* two-hybrid reporter gene. Although the previously described dimerization of MutL (9), oligomerization of MutS (7), and interaction between MutL and MutS (9) were not detected using the yeast two-hybrid system (data not shown), a potential interaction between MutL and MutH was observed (Fig. 1).

Interestingly, the interaction between MutL and MutH was only observed when MutL was fused to the Gal4 transcriptional activation domain and MutH was fused to the Gal4 DNA binding domain. To confirm that the pGAD424-MutH construct expressing MutH as a fusion with the Gal4 transcriptional activation domain did not contain a mutation resulting in the loss of an interaction with the MutL-Gal4 DNA binding domain fusion, *mutH* was subcloned from pGBT9-MutH into pGAD424. Again, an interaction was not observed with the MutL-Gal4 DNA binding domain fusion. These results were confirmed using yeast SFY526, containing a *lacZ* reporter gene under the

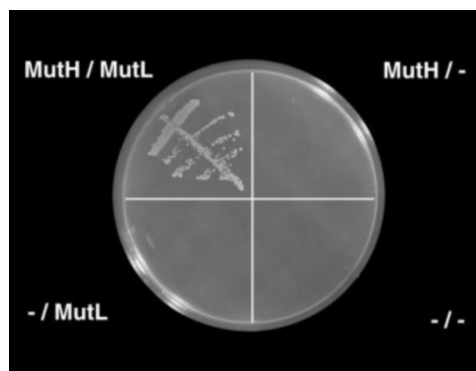


FIG. 1. **MutL and MutH interact in the yeast two-hybrid system.** Yeast HF7c cells containing pGBT9 and pGAD424 with or without the *mutH* and *mutL* genes were grown at 30 °C on complete synthetic media lacking tryptophan, leucine, and histidine and supplemented with 1 mM 3-amino-1,2,4-triazole. Cells in each quadrant were streaked from a single transformant that was colony-purified. Labels represent the fusion proteins present in the HF7c cells in the order: DNA binding domain fusion/transcriptional activation domain fusion. A minus sign indicates the absence of MutH or MutL from the fusion protein.

control of a promoter other than the *HIS3* reporter gene in HF7c. The reason for the observed “polarity” in the two-hybrid interaction between MutL and MutH is not known.

Purified MutL Is Specifically Retained on a MutH Affinity Column—Purified MutH protein (Fig. 2) was covalently coupled to an activated agarose resin (Affi-Gel 10) as described under “Experimental Procedures.” To confirm a physical interaction between MutL and MutH *in vitro*, 100 μ g of purified MutL was applied to the MutH affinity column. The column was washed with buffer containing 50 mM NaCl, and bound protein was eluted with buffer containing 1 M NaCl. A large fraction of the applied MutL was retained on the MutH column after the 50 mM NaCl wash steps and was eluted with 1 M NaCl (Fig. 3A). In contrast, when an identical experiment was performed using an Affi-Gel 10 column covalently coupled to chicken egg white lysozyme, the applied MutL was found exclusively in the flow-through and 50 mM NaCl wash fractions (Fig. 3B). Therefore, MutL was specifically retained on the MutH affinity column because of a physical interaction with MutH.

To ensure further that the interaction between MutL and MutH observed using affinity chromatography was specific, 100 μ g each of BSA and MutS were applied to the MutH affinity column. Using the same experimental protocol used for MutL, neither BSA nor MutS was retained to a significant extent on the column (Fig. 3, C and D). These results support the yeast two-hybrid results and suggest that a physical interaction exists between MutL and MutH.

The COOH Terminus of MutL Contains the MutH Interaction Domain—To identify the regions of MutL and MutH responsible for the two-hybrid interaction, a series of truncations was made from the NH₂ and COOH termini of both proteins. Truncated *mutL* alleles were generated in pGAD424 and tested for an interaction in the presence of pGBT9-MutH in yeast SFY526 (Fig. 4). Likewise, truncated *mutH* alleles were generated in pGBT9 and tested for an interaction in the presence of pGAD424-MutL. SFY526 contains a *lacZ* reporter gene encoding β -galactosidase. The relative strengths of interactions were measured using a spectrophotometric assay that monitors the cleavage of *o*-nitrophenyl β -D-galactopyranoside by β -galactosidase. Results are reported as Miller units (18).

Removal of 293, 344, or 397 amino acids from the NH₂ terminus of MutL (MutL Δ 293N, MutL Δ 344N, and MutL Δ -397N) did not eliminate the two-hybrid interaction with MutH. In contrast, removal of 438 amino acids from the NH₂ terminus

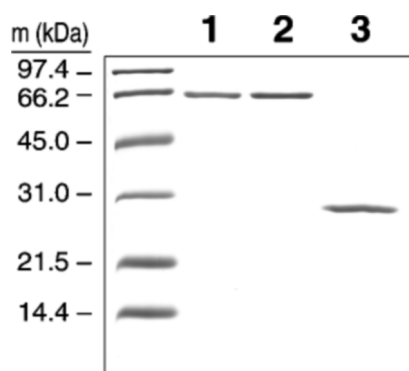


FIG. 2. **Purified MutL and MutH proteins.** Proteins were subjected to electrophoresis through a 12% polyacrylamide gel in the presence of SDS and visualized with Coomassie Brilliant Blue. *Lane 1*, 1 μ g of MutL purified using the Impact I system. *Lane 2*, 1 μ g of MutL purified from GE1752mutS::Tn5 as described under "Experimental Procedures." *Lane 3*, 1 μ g of MutH purified using the Impact I system. Molecular mass markers were: rabbit muscle phosphorylase b, 97.4 kDa; BSA, 66.2 kDa; hen egg white ovalbumin, 45.0 kDa; bovine carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa.

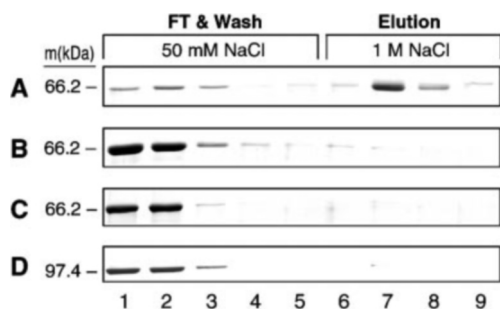


FIG. 3. **MutL is specifically retained on a MutH affinity column.** Approximately 100 μ g of MutL (*panel A*), BSA (*panel C*), or MutS (*panel D*) was applied to a 750- μ l Affi-Gel 10 column to which purified MutH had been covalently coupled as described under "Experimental Procedures." Likewise, 100 μ g of MutL was applied to an Affi-Gel 10 column containing covalently coupled chicken egg white lysozyme (*panel B*). In all panels: *lane 1*, flow-through (FT); *lanes 2-5*, 50 mM NaCl wash fractions; *lanes 6-9*, 1 M NaCl elution fractions. Each lane contains 36 μ l of the corresponding fraction. All fractions were 500 μ l with the exception of the flow-through, which was 1 ml. Molecular mass markers were: rabbit muscle phosphorylase b, 97.4 kDa; BSA, 66.2 kDa.

(MutL Δ 438N) or 59 amino acids from the COOH terminus (MutL Δ 59C) of MutL completely eliminated the two-hybrid interaction with MutH. These results indicate that the COOH-terminal 218 amino acids of MutL are necessary and sufficient to maintain this interaction and therefore contain the MutH interaction interface.

Removal of 38 amino acids from the NH₂ terminus (MutH Δ 38N) or 10 amino acids from the COOH terminus (MutH Δ 10C) of MutH eliminated the two-hybrid interaction with MutL. Thus, we were unable to define the interaction interface of MutH in more detail. It is possible that both ends of MutH contribute to the interaction domain. Alternatively, one or both of these truncation mutants may not be expressed or maintained as stable proteins in the yeast cells. These results are strikingly similar to those observed for the MutL-UvrD interaction (14). The COOH-terminal 218 amino acids of MutL were also sufficient to maintain the two-hybrid interaction with UvrD, whereas both the NH₂ and COOH termini of UvrD were required for the interaction with MutL.

Purified MutL Stimulates the Endonuclease Activity of Purified MutH in the Absence of MutS or a Mismatched Base—The d(GATC)-specific endonuclease activity of MutH is relatively weak in the absence of other components of the mismatch

repair system (13). However, this activity is markedly stimulated in the presence of MutS, MutL, ATP, Mg²⁺, and a DNA substrate containing a mismatched base (4). MutL is known to stimulate the helicase activity of UvrD (15), with which it physically interacts (14). In an effort to identify the functional role of the interaction between MutL and MutH, we examined the effect of MutL on the endonuclease activity of MutH in the absence of MutS, ATP, and/or a mismatch-containing DNA substrate.

The MutH endonuclease activity is specific for unmethylated d(GATC) sequences (13) and is not dependent on superhelicity in the DNA (4). One strand of the homoduplex substrate (see "Experimental Procedures"), which was synthesized *in vitro* using T7 DNA polymerase, was completely unmethylated. The template strand was at least partially methylated since it was prepared directly from a *dam*⁺ *E. coli* strain (XL-1 Blue). MutH endonuclease activity was evaluated by monitoring the conversion of covalently closed M13mp18 molecules to nicked circular molecules based on their different migration rates during agarose gel electrophoresis in the presence of EtBr. At high enzyme concentrations purified MutH catalyzed the complete conversion of the homoduplex substrate to nicked circular and a small fraction of linear molecules, as expected (data not shown). The appearance of a linear species in the reaction products was likely caused by incomplete methylation of the M13mp18 ssDNA molecules, leaving a fraction of available d(GATC) sites subject to cleavage on both strands of the substrate. MutH exhibited no activity on a fully methylated M13mp18 circular duplex (data not shown). A concentration of MutH (1.7 nM) which catalyzed barely detectable conversion of covalently closed homoduplex molecules to nicked circular molecules in a 15-min reaction at 37 °C was chosen to examine the potential stimulation of this reaction by MutL (Fig. 5, *lane 2*).

The addition of MutL greatly stimulated the level of MutH endonuclease activity but only in the presence of ATP (Fig. 5, *lanes 5 and 6*). Purified MutL alone exhibited no detectable endonuclease activity (Fig. 5, *lane 3*), and MutS had no effect on the MutH endonuclease activity in the presence of ATP (Fig. 5, *lane 7*). The level of endonuclease activity in reactions containing MutH alone was not altered by the presence of ATP (data not shown).

A titration with MutL demonstrated that stimulation of the MutH-associated endonuclease activity was dependent on MutL concentration (Fig. 6). The maximal level of conversion of covalently closed molecules to nicked circular molecules occurred at a MutL concentration of 38 nM. The specificity of the MutH-catalyzed endonuclease reaction for unmethylated d(GATC) sequences has been well defined (4). Consistent with this, stimulation of MutH endonuclease activity by MutL was dependent on the presence of unmethylated d(GATC) sites in the DNA because use of M13mp18 RF DNA prepared from a *dam*⁺ *E. coli* strain did not result in significant endonuclease activity (Fig. 7B). Results were identical using a heteroduplex DNA substrate that differed only in the existence of a single G-T mismatch (data not shown).

ATP Hydrolysis Is Not Required for Stimulation of the MutH Endonuclease Activity by MutL—The clear requirement for ATP to achieve MutL stimulation of the MutH endonuclease activity was surprising because neither protein has been previously demonstrated to bind or hydrolyze ATP. We were unable to detect ATP hydrolysis in either the purified MutH or MutL preparations (data not shown). Furthermore, no detectable ATP hydrolysis was observed during the MutL-stimulated endonuclease assays described above (data not shown). To determine if ATP hydrolysis was required for the MutL-dependent stimulation of the MutH endonuclease activity, AMP-PNP

A

Protein (amino acids)		Interaction with MutH	β -Galactosidase Activity (Miller units)
MutL (1-615)	N ██████████ C	+	5.4
MutL Δ 293N (294-615)	██████████ N ██████████ C	+	12.7
MutL Δ 344N (345-615)	██████████ N ██████████ C	+	9.6
MutL Δ 397N (398-615)	██████████ N ██████████ C	+	8.1
MutL Δ 438N (439-615)	██████████ N ██████████ C	-	ND
MutL Δ 59C (1-556)	N ██████████ C	-	ND

B

Protein (amino acids)		Interaction with MutL	β -Galactosidase Activity (Miller units)
MutH (1-229)	N ██████████ C	+	5.4
MutH Δ 38N (39-229)	██████████ N ██████████ C	-	ND
MutH Δ 10C (1-219)	N ██████████ C	-	0.076

FIG. 4. **The COOH terminus of MutL is sufficient for the two-hybrid interaction with MutH.** Yeast SFY526 cells containing pGBT9 and pGAD424 with various alleles of the *mutH* and *mutL* genes were grown at 30 °C on complete synthetic media lacking tryptophan and leucine. β -Galactosidase activity was measured as described under "Experimental Procedures." *Panel A*, truncations of the *mutL* gene were constructed in pGAD424 and were tested for an interaction in the presence of pGBT9-MutH. *Panel B*, truncations of the *mutH* gene were constructed in pGBT9 and tested for an interaction in the presence of pGAD424-MutL. A *plus sign* indicates the presence of an interaction, and a *minus sign* indicates the absence of one. Results represent the average of at least three trials using independent transformants when an interaction was detected and at least two trials using independent transformants when an interaction was not detected. *ND*, not detectable.

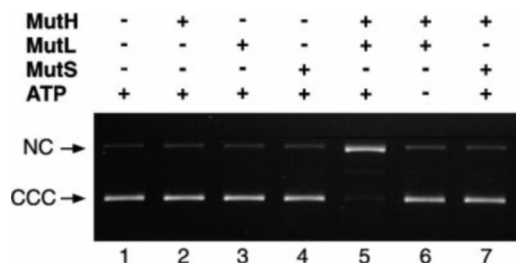


FIG. 5. **MutL, but not MutS, stimulates the endonuclease activity of MutH.** Endonuclease assays containing the components indicated above each *lane* were performed as described under "Experimental Procedures." When present, MutH, MutL, and MutS were at concentrations of 1.7, 19.0, and 33.8 nM, respectively. When present, ATP was 1.25 mM. Each reaction contained 50 ng of the M13mp18 homoduplex DNA substrate. *NC*, nicked circular DNA. *CCC*, covalently closed circular DNA.

and ATP γ S were substituted for ATP. AMP-PNP and ATP γ S are either not hydrolyzed or are poorly hydrolyzed by most ATPases. Fig. 7A demonstrates that both ATP analogs supported the MutL-stimulated endonuclease activity of MutH, suggesting that ATP hydrolysis was not required. The apparent K_m for ATP in the MutL-stimulated nicking reaction was approximately 265 μ M (data not shown). A titration of the endonuclease reaction with AMP-PNP was qualitatively identical to that with ATP (data not shown), ruling out the possibility that contaminating ATP present in the AMP-PNP affected the results.

Purified MutS Has No Effect on the MutL-stimulated MutH Endonuclease Activity in the Absence of a Mismatched Base or ATP—Because it was believed that MutS, MutL, and a DNA mismatch were required for stimulation of the MutH-associated endonuclease activity, it was necessary to rule out the possibility that a MutS contaminant existed in the MutL and/or MutH preparations. To address this concern MutL and MutH

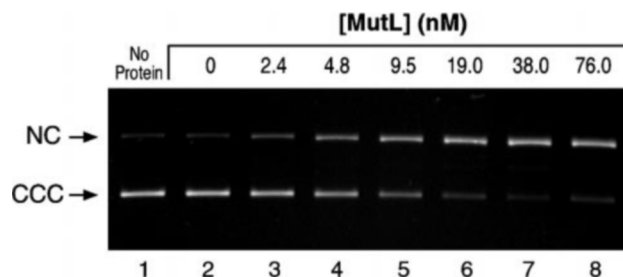


FIG. 6. **Stimulation of MutH is dependent on the MutL concentration.** The endonuclease activity of MutH in the presence of increasing concentrations of MutL was measured as described under "Experimental Procedures." *Lane 1*, unreacted DNA. All reactions contained 1.7 nM MutH, 50 ng of the M13mp18 homoduplex DNA substrate, and 1.25 mM ATP. The concentration of MutL in each reaction is indicated. *NC*, nicked circular DNA. *CCC*, covalently closed circular DNA.

were purified from an *E. coli* strain containing an insertion in the *mutS* gene (see "Experimental Procedures"). Results using these protein preparations were indistinguishable from those using the original preparations, eliminating the possibility that MutS was a contributing factor.

To evaluate the effect of purified MutS on the MutL-stimulated endonuclease activity directly, reactions containing either homoduplex or heteroduplex DNA were titrated with MutS in the presence of either ATP or AMP-PNP. The concentration of MutH was 0.7 nM, and the concentration of MutL was 9.5 nM in these reactions and resulted in a low level of endonuclease activity. MutS had no effect on the stimulation of MutH endonuclease activity by MutL when the homoduplex substrate was used in the presence of ATP (Fig. 8A). In stark contrast, MutS further stimulated the endonuclease activity of MutH when the heteroduplex DNA substrate containing a single G-T mismatch was used (Fig. 8B). However, stimulation of the endonuclease activity by MutS using the heteroduplex re-

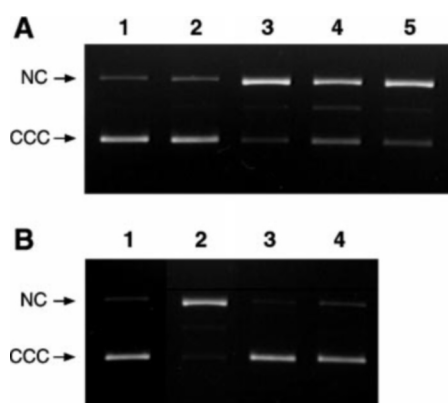


FIG. 7. Stimulation of the MutH endonuclease activity by MutL does not require ATP hydrolysis and is dependent on the methylation state of d(GATC) sites. Endonuclease reactions were performed as described under "Experimental Procedures." *Panel A*, all reactions contained 50 ng of homoduplex DNA substrate and 19.0 nM MutL. *Lanes 2–5*, 1.7 nM MutH was also present in the reactions. *Lane 2*, nucleotide was omitted from the reaction. *Lanes 3, 4, and 5*, ATP, AMP-PNP, and ATP-PNP were 1.25 mM each, respectively, in the reactions. *Panel B*: *lane 1*, unreacted homoduplex DNA substrate; *lane 3*, unreacted M13mp18 RF DNA. *Lanes 2 and 4* contained 50 ng of the homoduplex substrate or M13mp18 RF DNA, respectively, 1.7 nM MutH, 19.0 nM MutL, and 1.25 mM ATP. *NC*, nicked circular DNA. *CCC*, covalently closed circular DNA.

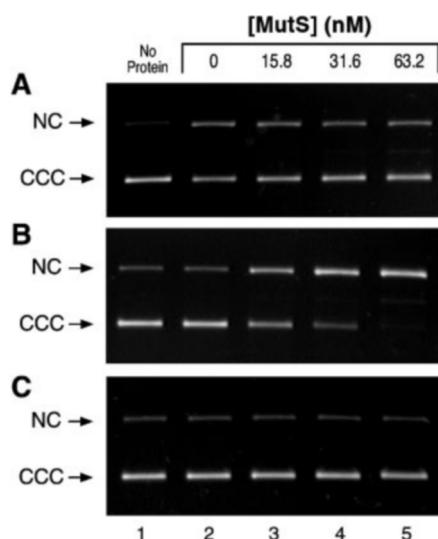


FIG. 8. MutS has no effect on the stimulation of MutH endonuclease activity by MutL in the absence of ATP or a DNA mismatch. Endonuclease assays were performed as described under "Experimental Procedures." The concentration of MutH in all reactions was 0.7 nM. The concentration of MutL in all reactions was 9.5 nM. When present, MutS was included at the concentration indicated. *Lane 1* in all three panels represents 50 ng of unreacted DNA. *Panel A*, all reactions contained 50 ng of the homoduplex DNA substrate and 1.25 mM ATP. *Panel B*, all reactions contained 50 ng of the heteroduplex DNA substrate and 1.25 mM ATP. *Panel C*, all reactions contained 50 ng of the heteroduplex DNA substrate and 1.25 mM AMP-PNP. *NC*, nicked circular DNA. *CCC*, covalently closed circular DNA.

quired ATP hydrolysis because substitution of AMP-PNP for ATP eliminated the stimulatory effect (Fig. 8C). The fact that MutS required a hydrolyzable form of ATP, coupled with the observation that MutS had no effect on reactions containing the homoduplex substrate, provided further support that MutL alone is capable of stimulating the MutH-associated endonuclease activity in the absence of MutS and a DNA mismatch.

DISCUSSION

In this report, a physical interaction between the *E. coli* methyl-directed mismatch repair proteins MutL and MutH

was demonstrated using the yeast two-hybrid system and protein affinity chromatography. MutL also interacts with MutS (9) and with UvrD (14, 15). Taken together, these results support the previously proposed hypothesis that MutL acts to bring together other protein components of the mismatch repair pathway (15, 22, 23). Interestingly, MutL has been demonstrated to stimulate the helicase activity of UvrD (14, 15), the rate of MutS-mediated DNA loop formation at the site of a mismatched base (24), and in this report, the endonuclease activity of MutH. Thus, one can envision MutL as a master coordinator of the mismatch repair pathway. Its ability to interact with the other mismatch repair proteins and stimulate their respective activities, presumably in a coordinated fashion, might serve to restrict these activities to the mismatch repair pathway itself. For example, inappropriate endonucleolytic cleavage by the MutH protein when action of the mismatch repair pathway is not required would be prevented. The observation that the MutL-stimulated MutH endonuclease activity is further enhanced in the presence of MutS, a mismatch, and ATP supports this notion.

A previous study suggested that MutS, MutL, ATP, Mg^{2+} , and a DNA mismatch were all required to stimulate the endonuclease activity of the MutH protein in the context of the mismatch repair system (4). The results presented here are largely in agreement with these results but demonstrate that the MutL subunit of the MutL-MutS complex is responsible for the stimulation of MutH and that this stimulation likely is effected through a protein-protein interaction. Two major lines of evidence indicate that stimulation of the MutH-associated endonuclease activity by MutL is independent of the MutS protein. First, neither ATP hydrolysis nor the presence of a mispaired base was required to observe stimulation of the MutH-associated endonuclease activity by MutL. Thus, the two major activities ascribed to MutS, ATP hydrolysis and mismatch binding, were not required to observe this stimulation. Further stimulation of reactions containing MutL and MutH by MutS did require ATP hydrolysis and a mispaired base. Second, experiments performed using MutL and MutH purified from an *E. coli* strain containing an insertion in the *mutS* gene were indistinguishable from those performed with MutL and MutH purified from *E. coli* containing a wild-type *mutS* gene. Thus, MutS did not contribute to the reaction as a minor contaminant of the purified MutH or MutL preparations. Consistent with these results, we were unable to detect a physical interaction between MutH and MutS using the yeast two-hybrid system and affinity chromatography, and MutS had no effect on the MutH-catalyzed endonuclease reaction in the absence of MutL. We conclude that MutL stimulates the endonuclease activity of MutH and that this stimulation is further enhanced in the complete mismatch repair system. The mechanism by which MutS further stimulates the endonuclease activity of MutH in the presence of MutL is unknown at this time. However, it is clearly related to the mismatch binding activity of MutS because a mismatch must be present to observe this stimulation. It is possible that the MutL-MutH interaction is facilitated when MutL is targeted to a DNA substrate via its interaction with a MutS-DNA mismatch complex. Thus, a series of protein-protein interactions provides a mechanism for specifically targeting MutH-catalyzed DNA incisions to hemimethylated d(GATC) sequences when a mismatch is present.

Surprisingly, the stimulation of MutH by MutL requires the presence of ATP or a nonhydrolyzable ATP analog. The nucleotide dependence of the MutL-stimulated MutH endonuclease activity suggests that either MutH or MutL possesses a nucleotide binding activity. The ability to bind ATP has not been

demonstrated for either of these proteins. However, computer-assisted sequence comparisons have predicted the presence of a nucleotide binding motif in MutL related to those contained by type II topoisomerases and a class of chaperone proteins (25). We were able to demonstrate UV light-induced cross-linking of [α - 32 P]ATP to MutL. However, because the efficiency of cross-linking was extremely low, we were unable to demonstrate convincingly specificity for the cross-linking event. Furthermore, we observed cross-linking of [α - 32 P]ATP to MutH under identical reaction conditions.

In addition to the computer-predicted nucleotide binding motif, two pieces of circumstantial evidence suggest that MutL is the more likely candidate for possession of a nucleotide binding activity. First, the basal endonuclease activity of MutH was not increased in the presence of 1 mM ATP in our reactions. In fact, at high ATP concentrations, an inhibition of activity was observed which may be caused by depletion of free Mg^{2+} , an essential cofactor for the MutH-catalyzed endonuclease reaction (13), or competition between ATP and DNA for the DNA binding site on MutH. Second, ATP binding, but not hydrolysis, was shown to be required for the association of MutL with a MutS-DNA mismatch complex (9). This ATP binding requirement likely is not caused solely by MutS for the following reason. The K_m for ATP hydrolysis catalyzed by our purified MutS preparation was 23 μ M (data not shown), consistent with a recent report (26). The K_m for ATP during the overall mismatch repair reaction in *E. coli* is approximately 300 μ M (4). The large difference between these two values suggests that either the affinity of MutS for ATP is altered in the context of the complete mismatch repair pathway or another protein is contributing to the observed K_m value. The results presented in this study argue in favor of the latter possibility, especially when one considers that the K_m observed here for the MutL-stimulated MutH endonuclease reaction (265 μ M) is very consistent with that for the complete mismatch repair system (300 μ M). Taken together, the available evidence suggests that the MutL protein is more likely to possess a nucleotide binding activity than MutH, although this remains to be demonstrated directly.

The localization of the region of MutL responsible for interacting with MutH to the COOH-terminal 218 amino acids was interesting in light of the fact that this same portion of MutL contains the interface for interacting with UvrD (14). Eukaryotic mismatch repair systems contain homologs of the *E. coli mutL* gene. Extensive amino acid sequence conservation between MutL and its eukaryotic homologs is restricted to a region near the NH_2 terminus of MutL (27, 28). It is likely that the highly conserved regions are involved in activities common to both prokaryotic and eukaryotic MutL proteins such as dimerization and interaction with the mismatch recognition proteins (MutS and MutS homologs). Thus, the presence of a domain at the nonconserved COOH terminus of MutL which mediates interactions with MutH and UvrD suggests that these interactions may be unique to the *E. coli* system. In support of this notion, *mutH* and *uvrD* homologs have not been identified as components of any eukaryotic mismatch repair pathway. Although the region of MutL containing the MutS interaction interface has not been defined, one might expect it to reside near the NH_2 terminus. This line of reasoning also suggests that the prokaryotic and eukaryotic mismatch repair mechanisms diverge extensively after the mismatch recognition steps. Indeed, recent evidence supports a novel model for strand discrimination and mismatch excision in eukaryotes. An

interaction between eukaryotic mismatch repair proteins and proliferating cell nuclear antigen was identified recently in a yeast two-hybrid screen (29). Because this protein acts as a polymerase processivity clamp during DNA replication, this interaction suggests that a physical link may exist between the mismatch repair and replication machinery. Such an interaction provides one possible mechanism by which the newly replicated strand can be identified and may eliminate the need for a helicase dedicated solely to mismatch excision as is the case in *E. coli*. However, other mechanisms are possible given the current data, and additional studies will be required to understand fully the mechanism of mismatch excision in eukaryotic cells.

The mechanism by which MutL stimulates the MutH-associated endonuclease activity is still unknown, although the results presented here suggest that the physical interaction between these two proteins is likely to be involved. In future experiments it will be necessary to examine the correlation between the physical interaction and the biochemical stimulation. In addition, understanding the role of ATP binding in this interaction will be an important goal.

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