

The DNA binding properties of the MutL protein isolated from *Escherichia coli*

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ABSTRACT

The *mutL* gene of *Escherichia coli*, which is involved in the repair of mispaired and unpaired nucleotides in DNA, has been independently cloned and the gene product purified. In addition to restoring methyl-directed DNA repair in extracts prepared from *mutL* strains, the purified MutL protein binds to both double and single stranded DNA. The affinity constant of MutL for unmethylated single stranded DNA was twice that of its affinity constant for methylated single stranded DNA and methylated or unmethylated double stranded DNA. The binding of MutL to double stranded DNA was not affected by the pattern of DNA methylation or the presence of a MutHLS-repairable lesion.

INTRODUCTION

Mispaired and unpaired bases can arise in DNA as a result of DNA polymerase infidelity, genetic recombination, and damaged bases (e.g., spontaneous deamination of 5-methylcytosine to thymine). In *Escherichia coli*, the repair of G·T, A·C, C·T, A·A, T·T, and G·G mispairs (1, 2, 3), as well as potential frameshift lesions (4, 5), are repaired by a methyl-directed DNA repair (MDDR) pathway (for Review see 6). Unlike other DNA repair pathways (e.g. UvrABC) that recognize damaged nucleotides, the MDDR pathway must differentiate between the 'correct' genetic information (present in the parent strand of replicating DNA) from the potential mutation (present in the daughter strand), and remove the latter. Wagner and Meselson (7) proposed that this discrimination is achieved by recognizing the undermethylation of the newly synthesized strand. Several genes are involved in this repair pathway including *mutH*, *mutL*, *mutS*, *uvrD*, and *dam* (6). All have been cloned and the gene products purified (6). Dam methylates the adenine in the sequence 5-GATC_3 (8), and UvrD is DNA helicase II (9). MutH is a 25 kd protein (10) that nicks DNA at unmethylated 5-GATC_3 sites (11) and, consequently, is believed to be involved in targeting the daughter strand for repair. MutS is a 97 kd protein that binds to double stranded DNA at the site of a mismatch (1, 12). Although MutS has also been reported to complex with single

stranded M13 phage DNA (13), it is possible that MutS is binding to hairpin regions within the single stranded DNA. MutL is a 66–70 kd protein that binds to the MutS-mismatch DNA complex (14). In this report the effect of DNA methylation and the influence of a MDDR repairable lesion on the binding properties of MutL to double stranded and single stranded DNA were investigated. To our knowledge this is the first report of the binding of MutL to DNA in the absence of MutS.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and plasmids

Bacteriophage M13mp9 (15) was a gift from Dr. K. Abremski (E.I. duPont de Nemours and Co., Inc., Wilmington, DE). Phage M13mp9FS1 contains a one base pair insertion into the *PstI* site of M13mp9 and phage M13mp9FS3 contains a three base pair insertion into the *SalI* site (5). pRK12 was the gift of Dr. R. Hoess (E.I. duPont de Nemours and Co., Inc., Wilmington, DE). Strain RG905 contains the *mutL* gene under the control of the λ_{PL} promoter in the λ lysogen KA1298 (C_{1857}) which was also obtained from Dr. K. Abremski.

Enzymes and chemicals

Restriction endonucleases were obtained from either Bethesda Research Laboratories (Gaithersburg, MD), New England Biolabs (Beverly, MA), or Boehringer Mannheim Biochemicals (Indianapolis, IN). Proteinase K and calf intestinal phosphatase were obtained from Boehringer Mannheim Biochemicals. T4 polynucleotide kinase was obtained from either Boehringer Mannheim Biochemicals or Bethesda Research Laboratories. NA-CS-52 was obtained from Bethesda Research Laboratories. Streptomycin sulfate was purchased from Sigma Chemical Co. (St. Louis, MO). Bio-Gel HT (hydroxylapatite), Bio-Rex 70, and Bio-Gel A-15m were purchased from Bio-Rad Laboratories (Richmond, CA). Nitrocellulose type RS for column chromatography was the generous gift of Hercules Powder Co. (Wilmington, DE). Dialysis membranes (type VS, 0.025 μ pore size) were obtained from Millipore Corporation (Bedford, MA). Protein determinations were performed using the Protein Assay Kit from Bio-Rad Laboratories.

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Preparation of heteroduplex DNA substrates

Nicked circular, hemimethylated, heteroduplex DNA substrates for use in the *in vitro* repair assay or in the preparation of fragments for gel mobility shift assays were prepared as described previously (2) using unmethylated phage strand and methylated complementary strand. Briefly, methylated M13mp9 wildtype RF, prepared by equilibrium density gradient centrifugation in cesium chloride-ethidium bromide (16), was linearized with *Bgl*II, phenol extracted, and precipitated with ethanol. Hybridization reactions contained 2.4 μ g of linearized RF and 24 μ g of the appropriate unmethylated single stranded mutant phage DNA in a final volume of 150 μ l of 1 \times SSC. After boiling the mixture for 10 min to denature the double stranded DNA, the DNA was hybridized for 1 h at 65°C. NaCl was added to 0.3 M and the volume was adjusted to 3 ml by addition of a solution of 20 mM Tris hydrochloride (pH 7.2), 2 mM Na₂EDTA, 0.3 M NaCl. Unhybridized single stranded DNA was removed by passing the mixture over a 3 ml nitrocellulose column as described (5, 17). The double stranded DNA, which was contained in the eluate, was diluted one third with TE and applied to a NACS-52 column (0.2 ml of resin) equilibrated with TE containing 0.2 M NaCl. After washing the column with 9 ml of equilibration buffer, DNA was eluted with 3–200 μ l aliquots of TE containing 1M NaCl. The eluates were pooled, precipitated with ethanol, and resuspended in TE.

In vitro DNA repair assay

A previously described *in vitro* repair assay (5, 18) was used for the determination of MutL activity. Cell extracts for *in vitro* DNA repair assay were prepared as described (18), but with modifications (5). Reactions (10 μ l) contained 180 ng of hemimethylated heteroduplex DNA (37.6 fmol molecules containing the + 1 frameshift) and 15–30 μ g of crude extract. The reactions were stopped after 1 h of incubation at 37°C by the addition of 90 μ l of 10 mM Tris hydrochloride (pH 7.6), 25 mM Na₂EDTA, 1.2% *N*-lauroylsarcosine and 1 μ l of 250 μ g/ml proteinase K. After incubation at 55°C for 1 h, the mixture was extracted twice with an equal volume of phenol, precipitated with ethanol, and dissolved in 20 μ l of 50 mM Tris hydrochloride (pH 8.0), 10 mM MgCl₂, 50 mM NaCl. *Pst*I (10 units) was added and the reactions incubated for 1 h at 37°C. The reaction products were separated by agarose gel electrophoresis at 5 mA/cm to separate nicked circular molecules from linears (19). The DNA was visualized by staining with ethidium bromide.

Purification of MutL

Strain RG905 which is capable of overproducing MutL was constructed by placing the 2.4 kb *Sal*I-*Bam*HI fragment containing the *mutL* gene under the control of the λ_{pL} promoter in the expression vector pRK12 which is a derivative of pKC30 (20). An overnight culture (10 ml) of strain RG905, grown in LB medium containing 200 μ g/ml ampicillin at 30°C was diluted into 1 liter of LB medium containing 200 μ g/ml ampicillin and was grown at 30°C to an OD₅₉₀ of 0.5. The culture was then shifted to 42°C and incubated for an additional 4 h. Bacteria were harvested by centrifugation at 6900 \times g for 20 min at 4°C. Cell pellets were resuspended in 0.05 M K₂HPO₄ (pH 7.4), 0.1 mM Na₂EDTA, 0.5 mM dithiothreitol. After centrifugation at 6900 \times g for 15 min at 4°C, the supernatant was discarded and cell pellets were stored at –80°C until further use.

MutL was purified essentially as described (14) except that the final step (Sephadex G-150 chromatography) was omitted. A summary of the purification is presented in Table 1 and an SDS-polyacrylamide gel of the various steps of the purification shown in Figure 1. Densitometric scan of Figure 1 revealed that MutL comprises >98% of the Coomassie Brilliant Blue stained material in Fraction V (not shown). Approximately 45-fold purification was achieved which resulted in a 20% yield and final specific activity of 9,664 units/mg. One unit of MutL activity is defined as that required to convert 1 pmol (4.78 μ g of nucleotide) of hemimethylated heteroduplex substrate DNA molecules to repaired homoduplex per hour under standard assay conditions. These results are in excellent agreement with those reported previously (14).

DNA binding studies

Gel mobility shift assays. The binding of MutL to small DNA fragments was determined using the gel electrophoretic mobility shift assay (21, 22). Binding reactions (10 μ l) contained 3 ng of ³²P end-labeled DNA in 50 mM Tris hydrochloride (pH 8.0), 50 mM NaCl. MutL protein (Fraction V), at the concentrations indicated in the Figure legends, was added to the reaction in a

Table 1. Purification of MutL protein from strain RG905. MutL was purified from strain RG905 thermally induced for expression of the product of the cloned *mutL* gene. Protein content and Specific Activity (using the *in vitro* repair assay) were determined as described in *Materials and Methods*

Fraction	Protein	Specific Activity	Recovery
	mg	units/mg	%
I. Cleared lysate	210	219	100
II. Streptomycin sulfate	195	171	73
III. Ammonium sulfate	191	164	68
IV. Hydroxylapatite	14	1,577	48
V. Bio-Rex 70	1	9,664	21

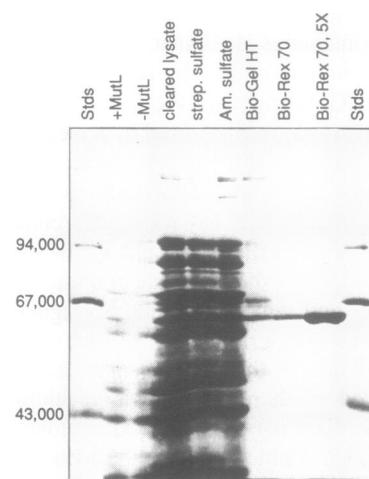


Figure 1. SDS-PAGE analysis of fractions from MutL purification. An aliquot of each fraction from the MutL purification was analyzed by SDS-polyacrylamide (8%) gel electrophoresis and proteins visualized by staining with Coomassie Brilliant Blue. Lanes: 'Stds' contain the molecular weight standards: phosphorylase B (97.4 kd), BSA (67 kd), and ovalbumin (43 kd); '+MutL' contains crude extract from strain RG905 induced to overproduce MutL; '-MutL' contains crude extract of strain containing the vector alone. The other lanes contain samples from the pooled fractions at each step in the purification as indicated in Table 1 and lane 'Bio-Rex 70, 5X' contains five times as much sample as the 'Bio-Rex 70' lane.

volume of 3 μ l, adjusted when necessary with MutL dilution buffer [0.02 M K_2HPO_4 (pH 7.4), 0.05 M KCl, 0.1 mM Na_2EDTA , 1 mM DTT and 0.1 mg/ml bovine serum albumin]. After incubation on ice for 30 min, 2 μ l of a 50% glycerol solution containing xylene cyanol FF was added and the mixture was separated by electrophoresis at 250 V at 4°C on a 5% polyacrylamide gel (19% acrylamide: 1% bisacrylamide) in TBE until the bromophenol blue present in the lanes containing the molecular weight markers reached approximately 1 cm from the bottom of the gel. After drying, the gel was analyzed by autoradiography.

Several different DNA's were used as substrates in the gel mobility shift assay. A 302 bp *PvuII* fragment from M13mp9 RF devoid of *dam* methylation or methylated on both strands was used to determine the effect of *dam* methylation on the interaction of MutL with DNA. To discern the effect of the presence of a MutHLS-repairable lesion on the binding of MutL, the hemimethylated 302 bp *PvuII* fragment from either the homoduplex or the 3 base addition heteroduplex (5) was used as substrate. The single *Dam* methylation site in this fragment was located at nucleotide 203; the three base addition was at position 196. The 72 and 118 bp fragments were obtained from *HaeIII* digests of ϕ X174; the 187 bp substrate is the *EcoRI-EcoRV* fragment from pBR322. All substrates were 5' end-labeled with [γ - ^{32}P]ATP and T4 polynucleotide kinase using standard procedures (16).

Gel chromatography of MutL-DNA complexes. During chromatography through Bio-Gel A-15m, either M13 RFI or phage DNA will elute much more quickly than MutL because of the large difference in their sizes. However, if MutL binds to DNA, then the formation of this complex will result in MutL eluting more rapidly than MutL alone. The affinity constants for the binding of MutL to high molecular weight single and double stranded DNA were determined by this gel exclusion chromatography technique (23). Fraction V (250 μ l) was dialyzed against 100 ml ice-cold binding assay buffer [50 mM Tris hydrochloride (pH 8.0), 50 mM NaCl] for 30 min using a Millipore Type VS membrane. MutL (240 μ l, 28 μ g protein) was incubated with M13mp9 DNA (10 μ g/ml) in a binding mixture (400 μ l final volume) which also contained 5% glycerol for 30 min on ice. The mixture was chromatographed at 4°C over a 12 cm \times 1 cm Bio-Gel A-15m column that had been equilibrated with binding assay buffer containing the substrate DNA at 10 μ g/ml. Fractions (0.46 ml) were collected and 75 μ l of each fraction was analyzed by SDS-PAGE; protein was visualized by staining the gel with Coomassie Brilliant Blue. The elution volume of MutL in the absence of substrate was determined by omitting DNA from the binding reaction and column buffer. Fractions were analyzed for protein by SDS-PAGE and proteins visualized

Table 2. Association Constants of MutL-DNA Interactions. ^aAssociation constant was calculated from the equation as described in *Materials and Methods*. Each value represents the average of three independent experiments. ^bRelative binding affinity was calculated by dividing the K_a obtained with the other substrates by the K_a obtained with methylated single stranded DNA.

Substrate	K_a , M^{-1a}	Relative Binding Affinity ^b
unmethylated M13mp9 RFI	7.3×10^6	1.0
methylated M13mp9 RFI	7.3×10^6	1.0
unmethylated M13mp9 phage	1.5×10^7	2.1
methylated M13mp9 phage	7.1×10^6	1.0

by staining with Coomassie Brilliant Blue. The elution volume of the DNA alone was determined by omitting MutL from the binding mixture and DNA from the column buffer. Fractions were analyzed for the presence of DNA by running an aliquot of each fraction on an agarose gel (0.8%) in TAE and staining with ethidium bromide. Substrate DNA was either methylated or unmethylated double stranded M13mp9 RF DNA or single stranded M13mp9 phage DNA. From the elution volumes of the protein and the DNA alone, and of the protein in the presence of DNA, the association constant (K_a) can be determined using the following equation (23):

$$K_a [D] = (V_p - V_e) / (V_e - V_d)$$

where [D] is the DNA site concentration, V_p is the elution volume of the protein in the absence of DNA, V_e is the elution volume of the protein in the presence of DNA, and V_d is the elution volume of the DNA in the absence of protein.

RESULTS AND DISCUSSION

In vitro DNA repair activity of purified MutL protein

To demonstrate that strain RG905 was capable of overproducing a functional MutL protein an *in vitro* repair assay (18) was used to follow the purification of MutL. This assay, developed by Modrich and colleagues, depends on the ability of a mutated restriction enzyme site in a hemimethylated heteroduplex DNA to be restored to a cleavable site after DNA repair. This assay has been modified slightly in that the hemimethylated DNA substrate used in the purification of MutL was a nicked circular substrate containing a 1 base addition in the *PstI* site of M13mp9 (5). These insertions have created a new restriction site in the

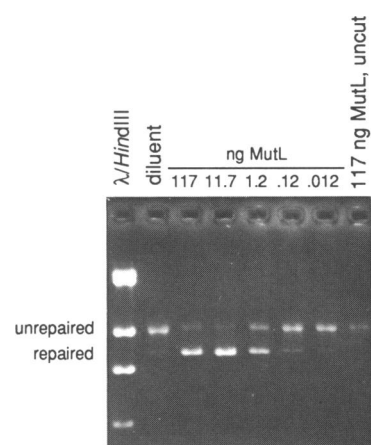


Figure 2. *In vitro* DNA repair of a hemimethylated heteroduplex containing a single base addition by Fraction V. A single base addition heteroduplex (5) was incubated with *mutL* extracts supplemented with the indicated amounts of MutL protein from Fraction V. Repair products were analyzed by digestion with *PstI* which only cleaves repaired molecules. Lanes: ' λ /*HindIII*' contains a *HindIII* digest of λ DNA as size standards; 'diluent' contains MutL dilution buffer; and '117, uncut' contains 117 ng of MutL protein but was not treated with *PstI*. The +1 frameshift, hemimethylated heteroduplex substrate (5) is methylated on the complementary strand (c) and is not methylated on the phage strand (p). The sequence at the site of the +1 frameshift is:



where the respective restriction endonuclease sequences are underlined. The extra 'A' residue in the mutant phage DNA is indicated in bold.

DNA such that repair of either strand is readily detected (5). As expected, extracts prepared from *mutL* strains (Fig. 2, lane 2) are defective in methyl directed DNA repair (5, 18). An activity that can complement this defect (Fig. 2, lanes 3 to 7) was overproduced from the λ_{PL} expression vector and copurified with the M_r 66,000 protein during Bio-Gel HT and Bio-Rex 70 chromatography (Fig. 1). The level of repair was proportional to the amount of the MutL protein added and was dependent on the addition of the restriction enzyme *Pst*I (compare lanes 3 and 8). The apparent discrepancy in the level of DNA in each lane

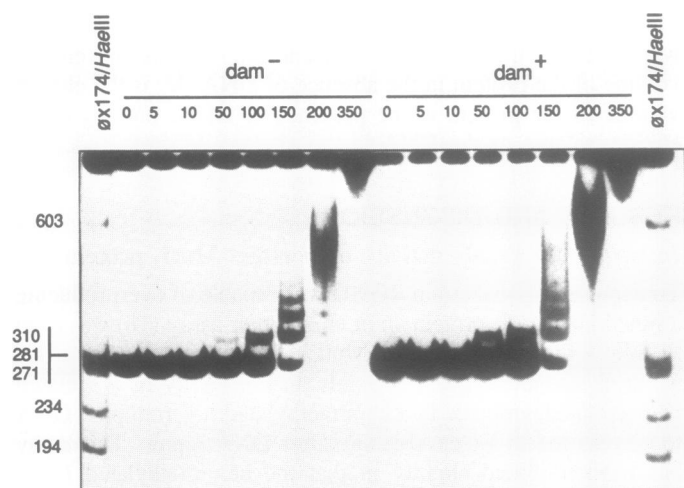


Figure 3. Gel mobility shift assay using methylated and unmethylated DNA as substrate. 3 ng (15.1 fmol molecules) of ^{32}P end-labeled 302 bp *Pvu*II fragment (*dam*⁺ or *dam*⁻ as indicated) from M13mp9 RFI, containing a single *Dam* methylation site, was incubated with the indicated amounts of MutL protein and the binding reaction analyzed by polyacrylamide gel electrophoresis and DNA visualized by autoradiography as described in the *Materials and Methods*. Lanes: ' ϕ X174/*Hae*III' contain *Hae*III digested, double stranded ϕ X174 DNA molecular weight standards that were end-labeled with [^{32}P]ATP (numbers along the left indicate the size in bp of the fragments); numbers at the top of each lane indicate the amount in ng of MutL (Fraction V) included in the binding reaction.

is due to the fact that the nicked circular DNA substrate is being converted into covalently closed circular DNA (by the *E. coli* DNA ligase present in these extracts and NAD added as a cofactor) and is migrating as a disperse band (5).

DNA binding studies

Previous reports suggest that MutL only binds specifically to DNA containing a mismatch in the presence of MutS and ATP (14). However, since these experiments measured the protection of DNA from digestion with pancreatic DNase I, alternative possibilities were also worthy of consideration. For instance, the interaction of MutL with DNA in a sequence independent manner would not have been detected using DNA footprinting experiments. Consequently, the ability of MutL (Fraction V) to bind to DNA was tested using gel electrophoretic mobility shift assays. The interaction of a protein with a specific DNA fragment changes the electrophoretic mobility of the DNA when analyzed by non-denaturing PAGE (21, 22). Figure 3 (lanes 2 to 9) shows that incubation of Fraction V with an unmethylated double stranded DNA fragment results in decreased electrophoretic mobility of the DNA. The extent of the shift increases with increasing amounts of added MutL protein. The binding is independent of ATP, is stable at room temperature, and is only slightly affected by the $MgCl_2$ between 0.2 mM and 10 mM (data not shown).

The binding of MutL to the DNA fragment appears to occur in very discrete steps. Typically, over five discrete bands can be detected but the major ones are always the first three suggesting that three MutL physical units can bind to the 302 bp fragment. Consequently, the effect of fragment size on the binding of MutL was examined using three different double stranded DNA fragments: 72 bp, 118 bp and 187 bp (Figure 4). MutL did not bind to the 72 bp fragment and bound only weakly to the 118 bp fragment. However, full binding (as compared with the 302 bp fragment, Figure 3) was seen with the 187 bp fragment. Interestingly, the 118 bp fragment showed only one predominate band while the 187 bp fragment shows two predominate bands. Taken together, these results suggest that the

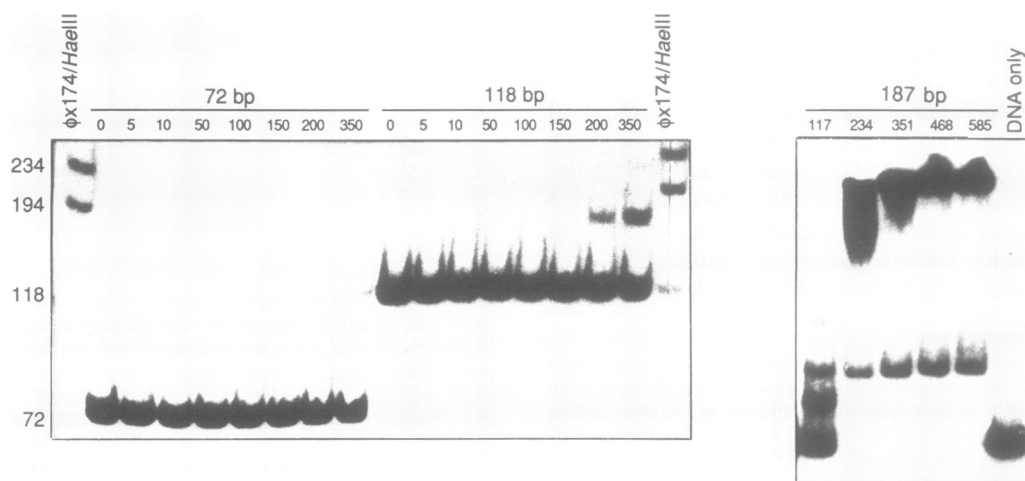


Figure 4. The effect of fragment size on the binding of MutL to DNA. Binding reactions contained 3 ng of the indicated ^{32}P end labeled DNA fragment. The amount (ng) of MutL (Fraction V) added to each reaction mix is indicated above each lane. Binding reactions were analyzed by polyacrylamide gel electrophoresis and DNA visualized by autoradiography as described in *Materials and Methods*. Both the 72 bp and 118 bp fragments were from a *Hae*III digest of ϕ X174; the 187 bp fragment is the *Eco*RI-*Eco*RV fragment of pBR322. The lanes ' ϕ X174/*Hae*III' contains double stranded DNA molecular weight markers which are ϕ X174 DNA digested with *Hae*III and end-labeled with [^{32}P]ATP.

minimal binding site of MutL is approximately 100 bp in length the binding is independent of γ GATC₃ sequences since none of these three fragments contain a γ GATC₃ sequence.

Although γ GATC₃ sequences are not required for the binding of MutL to DNA, the effect of methylation of the adenine in the single γ GATC₃ site in the substrate on MutL binding was tested since Dam methylation plays a key role in MDDR. Comparison of unmethylated DNA (lanes 2 to 9) in Figure 3 with the methylated DNA (lanes 10 to 17) shows that there is no significant difference in the shift of each substrate at similar amounts of added MutL. The above results (Figures 3 and 4) suggest that MutL can bind to double stranded DNA, that this

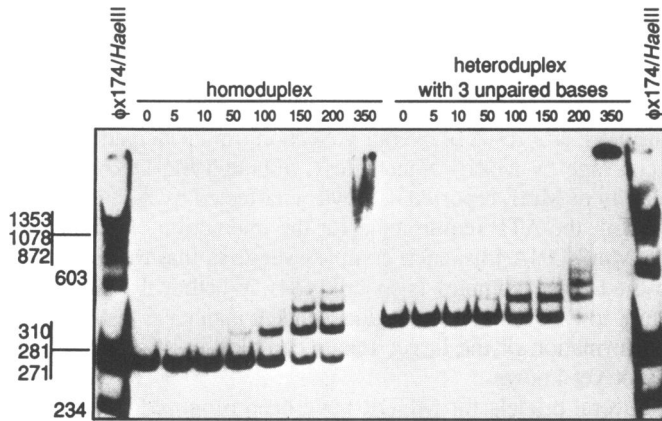


Figure 5. The effect of a MutHLS-repairable lesion on the binding of MutL to hemimethylated DNA. Either a hemimethylated homoduplex *PvuII* DNA fragment or the same fragment containing a 3-base addition on the unmethylated phage strand (3 ng, 15.1 fmol molecules) was incubated with increasing amounts (ng) of MutL (Fraction V) as indicated above each lane. Binding reactions were analyzed by polyacrylamide gel electrophoresis and DNA visualized by autoradiography as described in *Materials and Methods*. Lanes: ' ϕ X174/*HaeIII*' contains double stranded DNA molecular weight markers which are ϕ X174 DNA digested with *HaeIII* and end-labeled with [³²P]ATP. The sequence at the site of the +3 frameshift heteroduplex (5) is:

c 3' ACCA--GCTGC *SalI*
p 5' TGGTTAACGACG *HpaI*

where the other symbols and abbreviations are as described in the legend to Figure 2.

binding is independent of the state of Dam methylation of the DNA or even the presence of γ GATC₃ sequences, and that MutS is not required for the sequence independent binding of MutL. Finally, comparison of the binding seen between 50 ng and 200 ng of MutL (Figure 3) suggests that the binding is cooperative: more total DNA is bound in going from 100 ng to 150 ng than from 50 ng to 100 ng and the formation of the larger complexes occurs only after the smaller ones.

The effect of a MutHLS-repairable lesion on the interaction of MutL with double stranded DNA

Although the evidence indicates that MutS is involved in mismatch recognition (12, 24), and that MutL alone is not sufficient to recognize a mismatch (14), the effect of a frameshift type lesion on the interaction of MutL with DNA was examined using the gel mobility shift assay since MutL binds to single stranded DNA (see below). The substrate for these experiments was isolated from hemimethylated heteroduplexes constructed from unmethylated M13mp9 mutant phage containing 3 additional nucleotides hybridized to methylated wildtype M13mp9 RFI complementary strand (5). This is the same *PvuII* fragment as used in the gel mobility shift experiments described in Figure 3. The control DNA for these experiments was the hemimethylated *PvuII* fragment in homoduplex form. The data in Figure 5 show that, as anticipated, the 3-base addition heteroduplex migrates anomalously even in the absence of MutL (compare lanes 2 and 10). DNA fragments containing unpaired bases migrate more slowly through polyacrylamide gels as a result of DNA bending imparted by the frameshift lesion (25). Nonetheless, the effect of MutL binding to the hemimethylated heteroduplex containing the 3-base addition (lanes 10 to 17) is similar to that of the hemimethylated homoduplex (lanes 2 to 9). No reproducible difference in either the quantity or the pattern of the shift was detected for either of these substrates. These results indicate that neither a MutHLS-repairable lesion nor hemimethylation significantly alters the interaction of MutL with double stranded DNA.

Gel chromatography

Since the above data show that Fraction V contained a DNA binding activity, a gel exclusion chromatography technique (23)

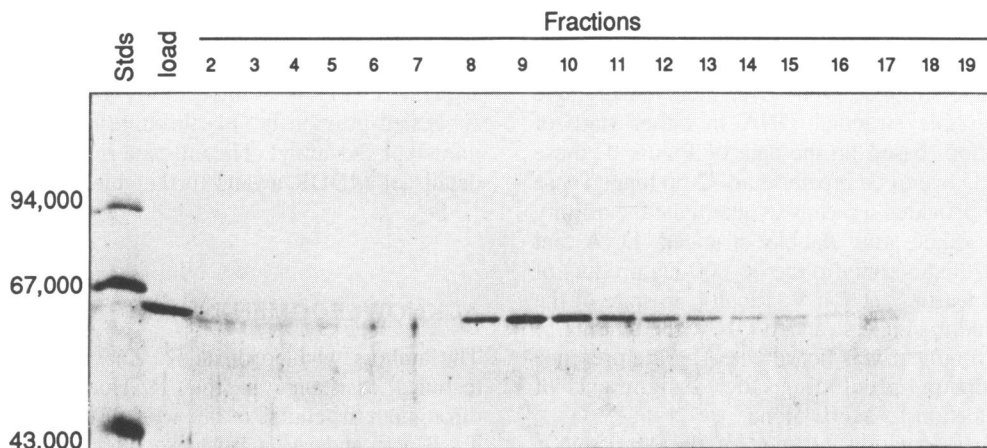


Figure 6. Gel exclusion chromatography analysis of MutL in the presence of unmethylated single stranded M13mp9 phage DNA. Binding reactions, performed as described in the *Materials and Methods*, were analyzed by SDS-PAGE and the protein visualized by staining with Coomassie Brilliant Blue. Lanes: '*Stds*' contains molecular weight markers as in Figure 1; '*load*' contains 6 μ l of MutL (Fraction V).

was employed to measure the association constant of MutL for DNA. Binding reactions containing MutL and DNA were chromatographed over a Bio-Gel A-15m column as described in the Materials and Methods. MutL alone elutes from this column in fractions 17 and 18 indicating an elution volume of 8.1 ml. Double stranded M13mp9 RFI DNA (4.8×10^6 Da) has an elution volume of 4.1 ml while single stranded M13mp9 phage DNA has an elution volume of 3.7 ml. When a binding reaction of MutL and either methylated or unmethylated double stranded DNA was chromatographed over the a column equilibrated with buffer containing the same DNA, the peak of MutL protein was contained in fraction 13 which corresponds to an elution volume of 6.0 ml. Thus, these data confirm the results of the gel mobility shift experiment (Figure 3) that MutL can bind to both methylated and unmethylated double stranded DNA, and indicate that methylation at Dam sites (M13mp9 contains seven 5GATC_3 sequences) does not affect the interaction of MutL with double stranded DNA.

Using the gel electrophoretic mobility shift assay, no binding of MutL to a chemically synthesized, single stranded 30-mer was detected (data not shown). However, when methylated single stranded M13mp9 phage DNA was used as substrate in the gel exclusion chromatography experiments, MutL bound to single stranded DNA eluting with a peak at fractions 10 and 11. Moreover, when unmethylated M13mp9 phage DNA was used as substrate, MutL eluted with a peak at fractions 9 and 10 (Figure 6). Averaging the values obtained by three separate DNA binding experiments performed with the individual DNA's produced values of 5.1 ml and 4.5 ml for the elution volumes of methylated and unmethylated single stranded DNA, respectively. These results are statistically significant ($P < 0.05$; Z Test) and suggest that MutL has a higher affinity for unmethylated single stranded DNA than methylated single stranded DNA.

The association constant for each of the DNA's tested was calculated as described in the *Materials and Methods* and the results are shown in Table 4. Based on the data from the gel electrophoretic mobility shift assay (see Figures 3 and 4), the minimum site size for the binding of MutL to DNA was estimated to be 100 bp. MutL binds with nearly identical affinity to methylated and unmethylated double stranded DNA and methylated single stranded DNA. However, MutL has an approximately 2-fold higher preference for unmethylated single stranded DNA. Of course, it is possible that MutL is binding to the single stranded phage DNA at hairpin regions. However, such an interpretation cannot account for the 2-fold greater affinity for unmethylated single stranded DNA over methylated single stranded DNA or double stranded DNA in either state of methylation. In addition, based on the data of Figure 4, these hairpin regions would have to be greater than 72 bp long. These experiments not only provided a means to quantitate the affinity of MutL for both single and double stranded DNA but unequivocally assigns that activity to the 66,000 Da product of the *mutL* gene contained in Fraction V. Finally, in spite of the ability of MutL to bind to single stranded DNA, the binding of MutL to the 302 bp fragment was not affected by the presence of a 3 base insertion precluding the assignment of insertion/deletion detection to MutL alone.

Comparison of the association constant of the MutL-DNA interaction with other protein-DNA interactions suggests that MutL is binding in a sequence-independent manner yet more tightly than other non-specific protein-DNA interactions. For

example, the restriction enzyme *HinfI* has a much higher affinity for DNA with its recognition sequence ($K_a \approx 2$ to $4 \times 10^{10} \text{ M}^{-1}$) than DNA lacking it ($K_a \approx 10^4$ to 10^5 M^{-1}) (23). MutS binds most tightly to DNA containing the G·T mispair with a K_a of 2.5 to $5.0 \times 10^7 \text{ M}^{-1}$ and most weakly to the C·C mispair ($K_a \approx 2.08 \times 10^6 \text{ M}^{-1}$) (1, 12). It is interesting to note that the affinity of MutS protein for mismatches is in the same order of magnitude as the interaction of MutL with the various DNA's studied here ($1.5 \times 10^7 \text{ M}^{-1}$).

Previous experiments using DNase I footprinting failed to detect the sequence-independent DNA binding activity of MutL (14) because this technique only detects sequence-specific DNA-protein interactions. The increased protection seen after the addition of MutL to the MutS-DNA mismatch complex could have resulted from this sequence-independent binding. That is, the addition of MutL to the MutS-mismatch complex could have enlarged the specific protected area around the site of the mismatch as a result of overall protection from pancreatic DNase I cleavage by MutL. Nonetheless, although the DNA binding activity of MutL reported here was unaffected by ATP (data not shown), the ATP requirement for the interaction of MutL with the MutS-DNA mismatch complex suggests that the formation of the ternary complex is specific (14). Whether the binding of MutL to DNA in a sequence-independent manner is distinct from the formation of the larger repair complex or stimulated by it is not yet known.

Several models for MDDR have been proposed (6, 26, 27). In one (27) the MutS protein binds to the lesion and serves as a nucleation site for the asymmetric, bidirectional binding of another protein (e.g. MutL). As this second protein binds laterally from the lesion in both directions, it would reach a 5GATC_3 site and provoke incision of the 5GATC_3 by MutH on the unmethylated strand only. Although both *in vivo* and *in vitro* experiments using either mismatch or frameshift substrates (2, 11, 26, 28) have established a role for MutH in strand discrimination, *in vivo* experiments have also implicated MutL in strand discrimination. In these latter experiments (28) unmethylated or hemimethylated heteroduplexes containing 10 bp insertions were not repaired after transformation into an *E. coli mutL* strain whereas fully methylated heteroduplexes were repaired. The small but reproducible preference of MutL for binding unmethylated single stranded DNA over the other forms of DNA reported here is the first biochemical evidence that also supports a role for MutL in strand discrimination. Perhaps the weak endonuclease activity of MutH at unmethylated 5GATC_3 sequences (11) is stimulated by MutL protein as has been suggested previously (14) through the binding of MutL to the unmethylated strand. The ultimate resolution of these molecular details of MDDR awaits further biochemical analysis.

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