The β Sliding Clamp Binds to Multiple Sites within MutL and MutS*

Francisco J. López de Saro†1, Martin G. Marinus‡, Paul Modrich§, and Mike O’Donnell¶

From †The Rockefeller University and ‡Howard Hughes Medical Institute, New York, New York 10021, the §Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, and the ¶Department of Biochemistry, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710

The MutL and MutS proteins are the central components of the DNA repair machinery that corrects mismatches generated by DNA polymerases during synthesis. We find that MutL interacts directly with the β sliding clamp, a ring-shaped dimeric protein that confers processivity to DNA polymerases by tethering them to their substrates. Interestingly, the interaction of MutL with β only occurs in the presence of single-stranded DNA. We find that the interaction occurs via a loop in MutL near the ATP-binding site. The binding site of MutL on β locates to the hydrophobic pocket between domains two and three of the clamp. Site-specific replacement of two residues in MutL diminished interaction with β without disrupting MutL function with helicase II. In vivo studies reveal that this mutant MutL is no longer functional in mismatch repair. In addition, the human MLH1 has a close match to the proliferating cell nuclear antigen clamp binding motif in the region that corresponds to the β interaction site in *Escherichia coli* MutL, and a peptide corresponding to this site binds proliferating cell nuclear antigen. The current report also examines in detail the interaction of β with MutS. We find that two distinct regions of MutS interact with β. One is located near the C terminus and the other is close to the N terminus, within the mismatch binding domain. Complementation studies using genes encoding different MutS mutants reveal that the N-terminal β interaction motif on MutS is essential for activity in vivo, but the C-terminal interaction site for β is not. In light of these results, we propose roles for the β clamp in orchestrating the sequence of events that lead to mismatch repair in the cell.

The process of chromosomal replication can generate small deletions, insertions, or mismatches in newly synthesized DNA. If left unrepaired, post-replicative DNA damage can lead to genomic instability and increased rates of mutation (1, 2). These types of replicative errors are corrected by a set of enzymes that are specialized in performing DNA mismatch repair (MMR) and are conserved across all domains of life. In *Escherichia coli* it is estimated that the fidelity of replication is increased >100-fold by the action of MMR (3). In humans a deficiency in MMR proteins typically bind to the clamp via N- or C-terminal flexible extensions containing a short motif (20, 21), and the interaction takes place at a hydrophobic pocket near the C terminus of the clamp. Detailed studies of the contact between clamps and their partners show that these interactions are often complex, with involvement of more than one binding surface, and in the case of PCNA even with mechanisms that regulate the interaction by post-translational modifications (22–25). In addition, because PCNA is trimeric and β dimeric, it has been speculated that the ring could accommodate more than one ligand at the same time and therefore serve as a mobile platform that coordinates multiple enzymes performing sequential actions on DNA (22, 26).

What is the precise role of processivity clamps in mismatch repair? Because clamps are typically associated with DNA polymerases, and...
clamp loaders are integral components of the replication fork, clamps could be used for targeting the MMR machinery to "replication factories" that could therefore contain synthetic errors. A second possibility is that they participate directly in the mechanism of action of mismatch repair. For example, MSH2–MSH6 binds to PCNA in the absence of a mismatch but not in its presence, suggesting that PCNA could help the MSH complex locate the mismatch on DNA (12, 27). In addition, because clamps used by DNA polymerase have a distinct orientation on DNA, they could provide the MMR machinery with the means to discriminate between the parental DNA and the newly synthesized strands (2, 4).

In an effort to gain a deeper understanding of the interplay between MMR and replication, we investigated in greater detail the physical interactions between E. coli MutL, MutS, and the &beta; clamp of DNA polymerase. We find that &beta; binds directly to MutL. Interestingly, MutL only interacts with &beta; in the presence of single-stranded (ss) DNA. Studies herein demonstrate that the site of contact with &beta; is located on a loop in the N-terminal ATP-binding domain of MutL. Mutation of two residues within this loop reduces interaction with &beta; but does not influence MutL ATPase and function with helicase II. Mutation of these two residues abolishes mismatch repair in vivo. In addition, we have identified two points of contact on MutS that bind to &beta;, one at the N terminus and another at the C terminus. The C-terminal site has a strong affinity for &beta;, whereas the interaction with the N-terminal site is weak. Interestingly, only the N-terminal site is essential for mismatch repair in vivo. This work provides new insights into the role of &beta; in mismatch repair in the cell, and suggests that the clamp is an essential player. We propose that &beta; helps to order the sequence of events in this multistep repair pathway.

**EXPERIMENTAL PROCEDURES**

*Materials*—High pressure liquid chromatography purified N-terminal biotinylated peptides were purchased from Bio-synthesis Inc. All other peptides were purchased from Chiron Mimotopes, which utilizes a synthetic method that leaves a diketopiperazine linker at the C terminus of the peptide. Streptavidin was purchased from Sigma. Labeled nucleotides were from PerkinElmer Life Sciences. Unlabeled nucleotides and AMPPNP were from Amersham Biosciences. Proteins were purified as follows: &beta; as described (28); &beta; containing a C-terminal 6-residue kinase recognition sequence and human PCNA with a 6-residue N-terminal tag were purified and labeled using [γ-32P]ATP as described (29); pol III core was reconstituted from isolated subunits and purified from unbound proteins as described (30).

*MutS and MutL Overexpression and Purification*—The MutS gene was cloned into pET11c (Novagen) to yield pET-mutS. MutS mutants, MutSSN (pET-mutSSN) and MutSC (pET-mutSC), were generated using site-directed mutagenesis by the QuikChange method (Stratagene, La Jolla, CA). Constructs were sequenced before use. Purification of MutS and MutL derivatives was based on the procedure described earlier (31). For MutS and its derivatives, plasmids were transformed in E. coli BL21(DE3) and 12L of cells grown at 37 °C in LB media supplemented with 0.2% glucose and 50 µg/ml ampicillin. When the cell culture reached an A600 of 0.6, 1 mM isopropyl 1-thio-β-D-galactopyranoside was added and after a further 2-h incubation the cells were harvested by centrifugation. Cells were resuspended in 50 ml of lyso buffer (20 mM KPO4, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, 100 mM NaCl) and lysed using a French press at 4 °C. The lysate was clarified by centrifugation and the extract was mixed with a solution of 25% streptomycin sulfate (0.4 ml/1 ml of extract). After stirring, the precipitate was pelleted by centrifugation and the supernatant was treated with solid ammonium sulfate (0.180 g/ml) at 4 °C. After centrifugation, the precipitate was resuspended in buffer A (20 mM KPO4, pH 7.5, 1 mM EDTA, 10 mM dithiothreitol, 25 mM NaCl, 5% glycerol) and dialyzed in the same buffer. This dialysate was applied to a heparin-Sepharose chromatography column (150-ml bed volume) and eluted with a 500-ml linear gradient of NaCl (25–500 mM). Fractions were analyzed on a SDS-polyacrylamide gel and those containing MutS were pooled, dialyzed against Buffer A, and then applied to a 200-ml column of fast-flow S-Sepharose (Amershams Biosciences) equilibrated in Buffer A. The S-Sepharose column was eluted with a 600-ml NaCl gradient (25–500 mM) in buffer A. MutS-containing fractions were pooled, precipitated with ammonium sulfate, dialyzed against buffer A, and then stored at −80 °C.

The MutL gene was cloned in pET11c to yield pET-mutL. MutLLF in which residues Leu-150 and Phe-151 were replaced by alanine was constructed by site-directed mutagenesis (QuikChange) to yield pET-mutLLF. E. coli BL21(DE3) was transformed with pET-mutL or pET-mutLLF and grown, induced, and harvested as described above for MutS. MutL and MutLLF were purified as described (32) except that a fast-flow Q (Amersham Biosciences) column replaced the hydroxyapatite column.

*Protein Gel Mobility Shift Assays*—Native polyacrylamide gel electrophoresis assays using [32P]β (150 dpm/mmol) were performed as described by López de Saro and O’Donnell (17). Reactions (15 µl) contained 20 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, 4% glycerol, 50 µg/ml bovine serum albumin, 100 mM NaCl, 5 mM DTT, and 50 mM [32P]β. Reactions also included peptide, streptavidin, MutS, DNA, MutL, or AMPPNP as indicated in the figure legends. Duplex DNA was a 300-mer duplicated by PCR and gel purified, and ssDNA was a 90-mer synthetic oligonucleotide (5'-CCCCCTATTAGGTTGTGACATTTCTCATATATCAAAATC-ACCCGAACAGGCACCCAGGAAACCGCTCCTCAGAG-CGCACACCT-3'). After incubation at 37 °C for 5 min, 5 µl of the reaction was applied to a 4% native polyacrylamide gel (4% acrylamide-bisacrylamide 29:1, 1X TBE buffer, 5% glycerol). Electrophoresis was performed in 1X TBE buffer (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA) at 19 mA for 90 min (4 °C). Gels were dried and detection of [32P]β was performed using a PhosphorImager. Free and bound forms of [32P]β were quantitated using ImageQuant software (Amersham Biosciences) and the percentage of bound [32P]β obtained using the formula [32P]β Bound = [32P]β Bound × 100/[32P]β Total. Concentrations of the addition of [32P]β ligands are indicated in each figure.

*Superdex 200 Chromatography*—A 25-ml FPLC Superdex 200 column (Amersham Biosciences, separation range 10,000–600,000 Da) was used to analyze MutS and its derivatives. The column buffer contained 50 mM Hepes (pH 7.4), 1 mM EDTA, 100 mM NaCl, and 2 mM DTT. Wild-type MutS, MutS800, and MutS6 were each loaded at a concentration of 25 µM in a volume of 200 µl. Fractions (420 µl) were collected and protein content measured by the Bradford assay (Bio-Rad), using bovine serum albumin as a standard.

*Determination of Mutant Frequency*—The wild-type strain used for the mutagenesis assay was AB1157 (F− thr-1 araC14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rfbD1 mgl-51 rpoS396 rpsL31 kdgK51 xylA5 mtl-1 argE3 thi-1) obtained from E. A. Adelberg (Yale University). Strains KM52 and KM75 (from K. C. Murphy, University of Massachusetts Medical School) were derived from AB1157 by replacement of the mutL and mutS coding regions by genes encoding chloramphenicol and tetracycline resistance, respectively (33). Strains were grown at 37 °C to saturation from single colonies in Brain Heart Broth (20 g/liter) supplemented with ampicillin (100 µg/ml) when required. To determine the frequency of rifampicin resistance, aliquots were spread on L plates with or without ampicillin (100 µg/ml) and onto L
β Clamp Interactions with MutL and MutS

plates containing rifampicin (100 μg/ml) with or without ampicillin (100 μg/ml). The plates were incubated at 37 °C overnight before scoring. To determine Gal+ papillation, 10-μl aliquots of the overnight cultures were spotted on MacConkey agar plates containing 0.2% galactose as the sole carbon source. The plates were scored after incubation for 3 days at 37 °C.

Construction of the mutSC Chromosomal Mutant—Strain GM8607, containing the mutS gene chromosomal deletion was constructed as described by Calmann et al. (34) except that the strain used for Red-mediated recombination was GM8496, which contains the cat gene inserted between codons 820 and 821 of the mutS gene (mutS820-cat). Amp⁵-Cam⁵ and Amp⁸ Cam⁸ recombinants were recovered after electroporation, which contained the mutS chromosomal deletion or mutS820-cat, respectively, as determined by DNA sequencing. P1vir transduction was used to move the mutS gene into AB1157 using Amp⁸ as the selective marker and the resultant strain was designated GM8607.

ATPase Assays—Wild-type MutL or MutL⁵F (5 μM, as dimer) where incubated with or without a synthetic gel-purified 100-mer ssDNA oligonucleotide (6.6 μM) in buffer containing 20 mM Tris (pH 7.5), 4% glycerin, 40 μg/ml bovine serum albumin, 5 mM MgCl₂, and 5 mM DTT. Reactions (15 μl) were supplemented with 3 mM ATP and 2.5 μCi of [α-³²P]ATP, and incubated at 37 °C for 15 min. Reactions were stopped by addition of 25 mM EDTA, and 0.4 μl were spotted on PEI thin-layer chromatography (TLC) plates to separate ATP from ADP. TLC plates were developed with 0.6 M potassium phosphate (pH 3.4). TLC plates were dried and radioactivity was visualized and quantified using a PhosphorImager (Amersham Biosciences).

DNA Gel Mobility Shift Assay—DNA containing a mismatch (heterodu- plex DNA) was generated by annealing of two complementary 68-mer synthetic oligonucleotides (A: 5'-GGTCCAGCTCTAGAGGATCCC-CCGGTACCGACTTTGAATTCGTTGAACATCTCGATCCCAT-TCATAATCAAAATCACCGGAACCAGAGCCACCAGCTCGA-

RESULTS

MutL interacts with the β Clamp only in the Presence of ssDNA—MutL protein is thought to act as a bridge between the site of DNA damage, recognized by MutS, and enzymes that subsequently process the DNA, like MutH endonuclease, helicases, and exonucleases (1, 7). Structural analysis of MutL indicates that it is a highly flexible molecule with two distinct regions connected by a proline-rich, 100-residue long disordered linker (7, 35). Because the C-terminal domain contains a strong dimerization surface and the N-terminal domains dimerize upon ATP binding, the MutL dimer contains a very large cavity (∼100 Å) that is sufficiently large to possibly accommodate 2–4 DNA helices at the same time (7). The N-terminal domain contains the ATP-binding site, and MutL is a weak ATPase that is stimulated by ssDNA; a putative ssDNA-binding site is located at the interface of two N-terminal domains as suggested by structural studies and mutational analysis (36).

Upon binding to ATP, several disordered loops (L₁, L₂, L₃, L₄5, and the N-terminal domain dimerize upon ATP binding). Although ATP has no apparent effect on ssDNA binding, ssDNA shifts were readily apparent in the presence of β labeled with a Kᵣ value of ∼250 nM (each as dimer) (Fig. 1B). These results suggest that the interaction of MutL with β may require a conformational change in MutL brought about by ssDNA binding.

Next we tested the effect of the non-hydrolyzable nucleotide analog, AMPPNP, on the binding of MutL to β in the presence of ssDNA (Fig. 1B). AMPPNP decreases the affinity of MutL for β in the presence of ssDNA by ∼3-fold (Fig. 1B). AMPPNP binding to MutL causes partial dissociation of β from the N-terminal domains of the MutL dimer (36), and therefore this result suggests that the interaction of MutL with β may be partially regulated by conformational changes in the N-terminal domains of MutL.

To determine whether MutL binds to the same C-terminal face of β as MutS and other proteins, we examined the interaction by the kinase protection assay (Fig. 1C) (17, 37). The rate of phosphorylation of the 6-residue kinase tag attached to the C-terminus of β was followed in the presence or absence of MutL and the ssDNA oligonucleotide. As a con-

Microtiter Plate Assays—N-terminal biotinylated peptides (100 pmol) were added to streptavidin-coated 96-well plates in 30 μl of PBST buffer (0.01 M phosphate-buffered saline, pH 7.2, supplemented with 0.1 (v/v) Tween 20) and incubated 1 h at 23 °C. Wells were then washed three times by addition and removal of 100 μl of PBST buffer. [³²P]β or human [³²P]PCNA were added (90 nM) directly to wells in a volume of 30 μl and incubated for 1 h, after which each well was washed three times with 50 μl of PBST buffer. Plates were analyzed using a Phospho- rimager (Amersham Biosciences).
Clamp Interactions with MutL and MutS

mutL interacts with β in the presence of ssDNA. A native PAGE was used to analyze the interaction of \( \text{[32P]} \)β with MutL (2 μM) in the presence or absence of ssDNA (10 μM as 300-mer), ssDNA (10 μM as 100-mer), and AMPPNP (1 mM). MutS was included in lanes 8 and 9 to demonstrate that AMPPNP does not affect the ability of MutS to bind β under these conditions. B, a titration of MutL and MutL \( ^{\text{LF}} \) (0.150–5 μM) into reactions containing 50 nM \( \text{[32P]} \)β was performed to estimate the affinity of the complexes in the presence of ssDNA and AMPPNP. Complexes were separated by native PAGE and bound and free \( \text{[32P]} \)β was quantified for each reaction using a PhosphorImager.

C, kinase protection assay. Protein kinase labels the C-terminal kinase tags of β that protrude from the face to which DNA polymerase binds these (see diagram). In the absence of additional factors, both clamps are phosphorylated equally (lanes 1–3). Addition of MutL-ssDNA prevents labeling of MutL (lanes 4 and 6) but not of the PCNA control (lanes 2 and 5). This result demonstrates that the 20-mer peptide readily dissociates complexes of pol III α subunit as well as all five DNA polymerases of E. coli (38). The result demonstrates that the 20-mer peptide readily dissociates complexes of pol III α subunit as well as all five DNA polymerases of E. coli (38). The sequence used to design peptides for experiments described in panel B is underlined. β, biotinylated peptides were attached to an enzyme-linked immunosorbent assay plate coated with streptavidin, and tested for retention of \( \text{[32P]} \)PCNA (right) or \( \text{[32P]} \)PCNA (left) as described (38). In the left panel the peptide used was a 20-mer derived from the E. coli sequence in panel A. In the right panel the peptide used was a 20-mer derived from the human MLH1 sequence in panel A. In both mutant peptides the LF sequence (boxed in panel A) was mutated to alanines in the peptide experiments shown in panel B are boxed. The sequence used to design peptides for experiments described in panel B is underlined.
β Clamp Interactions with MutL and MutS

Conserved Loop L2 of the N-terminal Domain of MutL Is Involved in β Binding—Inspection of an alignment of diverse bacterial MutL proteins for candidate β-interaction sequences did not reveal an obvious 4- or 5-residue consensus β-binding motif. However, loop 2 in the N-terminal ATP-binding domain of MutL contains a highly conserved LF motif reminiscent of the one present in the δ subunit of the γ-complex that, despite its deviation from the consensus, binds the hydrophobic pocket in β. We tested β binding to a series of peptides derived from sequences comprising loops L1 and L3 of MutL, with negative results (data not shown), but a 20-mer peptide corresponding to loop 2 (L2) (Fig. 2A) in the N-terminal domain tested positive for binding to β (Fig. 2B, left panel). To examine the peptide for interaction with β, an N-terminal biotinylated 20-mer peptide corresponding to loop 2 of MutL was attached to wells of a microtiter plate coated with streptavidin, and the wells were probed with [32P]β (Fig. 2B). The presence of the peptide resulted in retaining [32P]β in the well. A double mutation to alanine of residues Leu-150 and Phe-151 within the peptide (residue number corresponding to full-length MutL) abolished the interaction. Interestingly, the homologous region in human MLH1 contains a sequence (QXXVXXLF) that may be predicted to bind PCNA (Fig. 2A). A peptide derived from the human MLH1 protein was capable of forming complexes with human [32P]PCNA (Fig. 2B, right panel). Mutation of residues Leu-155 and Phe-156 to alanine in the peptide derived from human MLH1 abolished the interaction.

These results indicate that loop L2 in MutL is a candidate site for interaction with β and that the homologous residues in human MLH1 could bind PCNA. Mutation of the two residues to alanine renders the MutL mutant inactive in complementing a strain of E. coli in which the normal mutL gene is inactivated by replacement of the coding sequence with the chloramphenicol acetyltransferase (cat) gene (Table 1). The mutant protein was purified (referred to herein as MutL(F)) and tested for binding to β (Fig. 1B). MutL(F)-ssDNA shows decreased binding to β with respect to wild-type MutL-ssDNA. This effect may be due to residual binding at the mutated site on MutL(F), or may be explained by the presence of a second site of interaction elsewhere in MutL. Interestingly, the affinity of MutL(F)-ssDNA for β does not decrease in the presence of AMP-PNP and is similar to that of wild-type MutL in the presence of AMP-PNP. To examine whether the double mutation causes a defect in the ATPase rate of MutL, we performed ATPase assays in the presence and absence of ssDNA. The results in Fig. 2C show that the ATPase activity of MutL(F) is indistinguishable from that of wild-type MutL. Stimulation of helicase activity by UvrD is also unaffected by the double mutation in MutL(F), which shows a similar activity in this assay as wild-type MutL (Fig. 2D). This study suggests that MutL binds β only in the presence of ssDNA, and that a site of contact between MutL and β is located in the conserved loop L2 on the N-terminal domain of the protein.

Peptides Derived from N- and C-terminal Sequences of MutS Interact with β—We have shown previously that E. coli MutS interacts with β in solution (17). Many of the proteins that bind to β do so via extreme N- or C-terminal residues (21, 23, 38). To investigate whether MutS utilizes...
from both the N terminus (MutS1–21) and a region very near the C terminus (MutS802–820) bind to DNA by wild-type MutS and MutSN shows that certain residues between Met-13 and Arg-19 are important to preserve the interaction with β. Amino acid replacements Q15A, L18A, and Q16A have an intermediate effect. In contrast, peptides with replacements Y17A or L20A bind to β similar to wild-type peptide.

Next, we investigated the effect of the MutS β-binding peptides on the mobility of preformed MutSβ complexes. Interestingly, the results, in Fig. 3B, demonstrate that both the N- and C-terminal MutS peptides disrupt the MutSβ complex (Fig. 3B, lanes 1–4), suggesting that both bind to β at the same location. We then tested the effect of the two MutS peptides on preformed DNA polymerase III (core)-β complexes (Fig. 3B, lanes 5–7). The result shows that the two peptides are both capable of disrupting the pol III core-β complex, implying that, despite their lack of sequence similarity, they both bind to the same sites on β that are contacted by pol III core.

Studies of the interaction between pol III core and β indicate the presence of at least two contact points on β, possibly the two identical hydrophobic pockets in the two protomers of the β dimer (21, 38, 39). Taken together, these results suggest that MutS binds to β in a manner similar to that of the pol III core and is consistent with our previous result that MutS binds β on the same face of the ring as the α subunit of DNA polymerase III and the β subunit of the γ-complex (17).

The N-terminal β-Binding Site in MutS Is Essential in Vivo—The studies herein using peptides derived from the MutS sequence suggest that one putative β-binding site is located in the N terminus, which is a part of the mismatch DNA-binding domain of MutS (Domain 1). To obtain a more detailed view of this interaction, nested peptides were synthesized spanning the first 21 amino acids of MutS (Fig. 4A). As shown in Fig. 4B, peptides corresponding to N-terminal sequences of MutS can efficiently compete the MutS-β interaction by binding to β, except peptide MutS1–11. This result defines a particular region of interaction spanning residues Pro-12 to Lys-21. To identify the particular residues in the MutS peptide that are essential for the interaction with β, residues were mutated one at a time. The results (Fig. 4C) show that certain residues between Met-13 and Arg-19 are important to preserve the interaction with β. Amino acid replacements Q15A, L18A, and R19A show a greatly reduced binding to β, whereas M13A, M14A, and Q16A have an intermediate effect. In contrast, peptides with replacements Y17A or L20A bind to β similar to wild-type peptide.

Next, we constructed MutS mutants by site-directed mutagenesis of the mutS gene and tested the mutant proteins for binding to β in vitro. A double mutant, Q15A/L18A, or a quadruple mutant Q15A/Q16A/L18A/R19A (referred to below as MutSβ) did not show a significant decrease in binding to β, suggesting that the interaction at the N terminus is not necessary for MutS to bind β in solution (data not shown). In solution the MutS-β interaction may be dominated by the C-terminal region of MutS, which is a strong interaction (see below). It also remains possible that the N-terminal residues identified here could become important for the interaction when MutS and β are both bound to DNA. MutSβ binds to heteroduplex DNA containing a G-T mismatch with an affinity similar to wild-type MutS (Fig. 4D), and its ATPase activity is also indistinguishable from wild-type MutS (data not shown).

To determine whether the MutS mutants were functional in vivo, the mutant genes were tested for their ability to complement E. coli KM75, a mismatch repair-deficient strain in which a tetracycline-resistance cassette replaces the chromosomal mutS gene. Plasmids that contain a copy of wild-type MutS restore mismatch repair (Table 1). Consistent
with the in vitro studies above, the plasmid containing the quadruple mutant MutS\(^N\) gene is unable to complement the repair pathway, suggesting that \(\beta\) interaction at the N-terminal site on MutS is important to function (Table 1).

The C-terminal Domain of MutS Contains a Strong \(\beta\)-Binding Site—Alignment of bacterial MutS sequences from different bacteria allowed Dalrymple and co-workers (21, 40) to identify a putative \(\beta\)-binding motif about 30 residues from the C terminus of \(E.\ coli\) MutS, and they showed that a peptide derived from this region could bind to \(\beta\). This peptide contains a short sequence, QMSLL, that is related to the \(\beta\)-binding motifs of other \(E.\ coli\) proteins. In light of the experiments described above with peptide MutS\(_{802-820}\) (Fig. 3), we tested binding of \(\beta\) to two mutant forms of the MutS protein defective in this region near the C terminus. One form of MutS, MutS\(_{800}\) (or MutS800), contains a 53-amino acid C-terminal truncation and has been described and characterized previously (41, 42). The other contains a 5-residue deletion in the putative \(\beta\)-binding motif (QMSLL) within the C terminus (MutS\(_{812-818}\)), which will be referred to here as MutS\(^C\). As shown in Fig. 5A, neither MutS800 nor MutS\(^C\) interact with \(\beta\) under the conditions of the native polyacrylamide gel-shift assay, suggesting that the motif located at the C terminus is an important site for binding to \(\beta\) in solution.

MutS binds to \(\beta\), with a \(K_d\) value of \(~250\) nM (as dimer) (Fig. 5B), as determined by the native gel electrophoresis assay, and the presence of homo- or heteroduplex DNA, or ATP, did not alter this equilibrium (data not shown). For comparison, a similar experiment was performed using pol III core, which is known to bind \(\beta\) with a \(K_d\) of \(~250\) nM using other methods (43). In solution MutS is a tetramer, but MutS800 is dimeric, indicating that the missing 53 residues of MutS800 not only contain a \(\beta\)-binding site, but are also required for tetramerization. We tested the oligomeric state of MutS\(^C\) by gel filtration chromatography (Fig. 5C) but find that MutS\(^C\) co-migrates with wild-type MutS and that therefore MutS\(^C\) appears to be a tetramer. We conclude that the deleted \(\beta\)-binding motif is not a determinant of the oligomeric status of MutS.

Although previous reports have shown that MutS800 is functional in vivo and can complement a null MutS mutant when expressed from plasmids (41, 44), more recent work has shown that the C-terminal domain of MutS is critical for mismatch repair in vivo when the protein is expressed from a single copy gene in the chromosome (34). However, strain GM8607, containing a deletion of five residues within the C-terminal domain of MutS (MutS\(^C\)), can indeed complement the MutS-null strain when in a plasmid or in single-copy in the chromosome (Table 1). We conclude that, whereas the N-terminal motif seems to be essential for mismatch repair in vivo, the C-terminal one is not.

**DISCUSSION**

To understand the role of processivity clamps in MMR, we have analyzed the \(E.\ coli\) \(\beta\) clamp for interaction and function with the MutL and MutS proteins. We observe an interaction between MutL and \(\beta\) that is only apparent in the presence of ssDNA. Furthermore, binding of ATP modulates the mutL-\(\beta\) interaction. These results indicate that \(\beta\) binds to MutL while MutL is engaged in the process of mismatch repair. The \(\beta\) interaction site in MutL appears similar to that in the \(\delta\) subunit of the clamp loader, and replacement of 2 amino acids in MutL (MutL\(^L\)) diminishes binding to \(\beta\) similar to analogous mutations in the \(\delta\) subunit. The MutL mutant retains ATPase activity and ability to stimulate helicase II. Expression of the MutL\(^L\) mutant fails to complement a MutL defective strain of \(E.\ coli\), indicating that the interaction of MutL with \(\beta\) is essential to function.

Study of MutS identifies two distinct sites of interaction with \(\beta\). Analysis of MutS deletion mutants indicate that MutS binds to \(\beta\) most strongly via residues in the C-terminal region of MutS. However, as revealed by peptide analysis, an additional contact site with \(\beta\) is located at the extreme N terminus of MutS. In vivo functional studies reveal that
MutS, which is mutated in amino acids required for β binding at the N-terminal site, is no longer capable of complementing a MutS-deficient strain of *E. coli*, indicating that this site is important to mismatch repair in the cell. Overall, the results of the current report highlight the use of processivity clamps in mismatch repair and indicate that they act at more than one step, and possibly coordinating MutS and MutL activities with replication.

Recent evidence has accrued for at least two points of contact between pol IV and the clamp and between pol III and the clamp (23, 39, 45, 46). One may speculate that one contact is strong and allows a change in MutS. This may transfer DNA from the inner channel to the outer channel of MutS, kinking DNA at the damaged site and releasing the clamp (a). C alignment of the β-binding motifs of *E. coli* core proteins: DNA polymerase II C-terminal motif (α); DNA polymerase III C-terminal motif (β); MutS C-terminal motif (γ); MutL DNA polymerase II C-terminal motif, and DNA polymerase IV C-terminal motif.

MutS/β—Two Coupled Rings to Scan the Chromosome—The finding that the C-terminal region of MutS can bind β may seem puzzling, as this domain (53 residues) of MutS, missing in the crystal structure, would appear to be located far from the DNA duplex as illustrated in Fig. 6A (41, 44). Thus, it would seem improbable that a clamp encircling DNA could interact with the C terminus of MutS when MutS is bound to a mismatch. However, the *Thermus aquaticus* crystal structure of free MutS (without DNA) has shown that the N-terminal domains are highly mobile (44) and suggest that the MutS dimer could possibly form a ring for DNA interaction. This hypothesis is consistent with the behavior that MutS exhibits in a number of studies (49, 50). It has been proposed that the MutS ring may be semi-closed and therefore less stable on DNA compared with β and PCNA (51), but may allow for one-dimensional diffusion on DNA before encountering the mismatch.

A clue to how MutS may slide on DNA is provided by a second channel (inner channel) apparent in the crystal structure (Fig. 6A). This channel is distinct from the one in which mismatched DNA is bound and its size and surface electrostatic potential is compatible with DNA contact (51). The paradox of a C-terminal interaction of MutS with β can readily be solved if MutS interacts with β when DNA is threaded through the inner channel. An alignment of the channels in both molecules positions the C-terminal and the N-terminal binding sites of MutS at approximately the same distance as exists between the two hydrophobic binding sites of β, ~60 Å apart (Fig. 6A).

Structural studies of MutS with homoduplex DNA, or of the missing C-terminal extension that interacts with β, will be needed to further evaluate this model. An interesting implication, however, is that upon binding to damaged DNA, the binding surface of MutS to β may disconnect upon the relocation of the DNA duplex to the lower channel (Fig. 6A, left panel). β release from the N-terminal site would be predicted to occur upon binding of the MutS to damaged DNA because the residues implicated in β binding form an α-helix when MutS binds mismatched DNA (41). In addition, when MutS binds the mismatch β it would be expected to detach from the C-terminal site as well, because the new distance would now be too great for the C terminus of MutS to interact with β.

The model in Fig. 6B implicates β in the earliest stages of mismatch repair in which β targets MutS to sites of DNA replication and helps MutS in one-dimensional scanning along DNA for lesions (Fig. 6B, diagrams a and b). In this hypothesis, we propose that DNA threads through the upper chamber in MutS, allowing both MutS sites to bind the β clamp (as described above). MutS binding to the mismatch would reposition the DNA and break both sites of contact with β, ejecting the clamp from the MutS-DNA complex. Previous studies also suggest that
there is a close relationship between mismatch recognition and processivity clamp binding and release (12, 27). The affinity of β for MutS (Fig. 5B) is similar to that of pol III core in solution (~250 nM (43), Fig. 5B). Targeting of MutS to β could possibly explain the observed reduced mutagenesis rates in the lagging strand versus the leading strand (52, 53), because the mechanism of DNA replication results in the accumulation of processivity clamps on the lagging strand (37). Also, because all processivity clamps loaded at the replication fork have the same orientation with respect to the double helix, β could direct repair to the newly synthesized strands. We show here that the C-terminal site in MutS accounts for the strength of interaction with β in solution. Hence, MutS may first bind to β via the C-terminal motif and then, as MutS encircles DNA, also via the N-terminal motif. Is there any similarity between the β motifs in MutS and pol III? An alignment of the two binding motifs in E. coli pol III α subunit and the two binding motifs in MutS (Fig. 6C) show similarity at positions one and four (Q^1XXL^4), whereas a hydrophobic residue, either Leu or Phe, has been shown to be accommodated at positions 5 or 6 in some β binding motifs (21). This minimal consensus sequence in different ligands of a common binding site reflects the ability of the hydrophobic pocket on the surface of β to interact dynamically with several different partners in the cell (38).

**The MutL-β Interaction Requires ssDNA**—The determination of MutL structures by Yang and collaborators (7, 35, 36, 54) has led to exciting new insights into their role in mismatch repair. However, major questions remain regarding the mechanism by which MutL coordinates mismatch repair. For example, the amino acids in MutL that interact with MutS, MutH, and UvrD have not yet been determined, and there is uncertainty as to the mode in which MutL binds to various DNA species. The study presented here adds yet another protein partner to MutL, the β clamp processivity factor. Studies of eukaryotic MLH1 also indicate that it binds the PCNA clamp (8, 55–57). Our results show that MutL binding to β requires ssDNA. Presumably MutL binding to ssDNA induces a conformational change in the N-terminal domains of the MutL dimer that allow for interaction with β. It is interesting to note the atomic position of MutL residues Leu-150 and Phe-151 implicated in this study in binding to β. The structures of MutL with and without nucleotide are shown in Fig. 7A. The β interactive residues are exposed in the absence of nucleotide, but are sequestered in its presence. It seems likely that this nucleotide-induced change underlies the observation herein that nucleotide diminishes the ability of MutL, to bind β. We also demonstrate that human PCNA binds to a peptide derived from the corresponding region in human MLH1.

The finding that ssDNA is required for β binding by MutL, combined with the fact that β slides only on double-stranded DNA, suggests that the interaction between MutL and β occurs at a junction between ss- and dsDNA. A ss/dsDNA junction is the natural substrate of DNA polymerases (Fig. 7B), and this structure is also present during the excision step in mismatch repair that involves the combined actions of a helicase and exonuclease to degrade the newly replicated DNA strand (Fig. 7C). We demonstrate here that MutL competes with the pol III core for β, and thus one may speculate that MutL may displace pol III core from a primed site by direct competition for the processivity clamp. Alternatively, MutL may bind to one β protomer, whereas pol III binds the other, as demonstrated recently for pol III and pol IV binding to one β dimer simultaneously (22). This may even induce the polymerase to back-track to the point of the mismatch by stimulating the proofreading activity present in all replicative DNA polymerase complexes (i.e. the ε subunit of E. coli DNA polymerase III). For example, in eukaryotic organisms, genetic evidence has implicated the intrinsic 3’ exonucleases of pol δ and pol ε in mismatch repair (58). On the other hand, independent exonucleases may be recruited to the clamp, as has been demonstrated recently for human EXOI and PCNA (56, 59).

DNA loop structures have been implicated in mismatch repair to account for the distance between the site of DNA damage and the place where degradation of the damaged DNA strand starts, which can be on the order of kilobases (2, 7, 60). MutL would be well suited for DNA looping as a consequence of the long connector sequences between the N- and C-terminal domains, and the ability of the N-terminal domains to dimerize in an ATP-dependent manner (36).

The models of Figs. 6 and 7 involve a combination of DNA looping and movements of MutS and MutL along the DNA contour that could eventually reconcile some of the current working models of mismatch repair. Further studies will be needed to reveal the exact roles of β, but the interactions of β to both MutS and MutL described in this study indicate that the clamp has multiple functions in mismatch repair.

**Acknowledgment**—We thank Jeff Finkelstein for the overexpression vectors for wild-type MutS, MutL, and UvrD proteins.
DNA: Replication, Repair, and Recombination: 
The β Sliding Clamp Binds to Multiple Sites within MutL and MutS

Francisco J. López de Saro, Martin G. Marinus, Paul Modrich and Mike O'Donnell

Access the most updated version of this article at doi: 10.1074/jbc.M601264200

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 27 of which can be accessed free at http://www.jbc.org/content/281/20/14340.full.html#ref-list-1