MutS homolog sliding clamps shield the DNA from binding proteins

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MutS homolog (MSH) proteins initiate mismatch repair (MMR) by recognizing mispaired nucleotides and in the presence of ATP form stable sliding clamps that randomly diffuse along the DNA. The MSH sliding clamps subsequently load MutL homolog (MLH/PMS) proteins that form a second extremely stable sliding clamp, which together coordinate downstream MMR components with the excision-initiation site that may be hundreds to thousands of nucleotides distant from the mismatch. Specific or nonspecific binding of other proteins to the DNA between the mismatch and the distant excision-initiation site could conceivably obstruct the free diffusion of these MMR sliding clamps, inhibiting their ability to initiate repair. Here, we employed bulk biochemical analysis, single-molecule fluorescence imaging, and mathematical modeling to determine how sliding clamps might overcome such hindrances along the DNA. Using both bacterial and human MSH proteins, we found that increasing the number of MSH sliding clamps on a DNA decreased the association of the Escherichia coli transcriptional repressor LacI to its cognate promoter LacO. Our results suggest a simple mechanism whereby thermal diffusion of MSH sliding clamps along the DNA alters the association kinetics of other DNA-binding proteins over extended distances. These observations appear generally applicable to any stable sliding clamp that forms on DNA.

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This article contains supporting information, Figs. S1–S8 and Tables S1–S3.

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Sliding clamps on DNA have been conserved throughout evolution and play essential roles in coordinating DNA replication, repair, and the cellular damage response (1–3). Among notable examples are the MutS homolog (MSH)4 proteins, which operate as a homo- or hetero-dimer and specifically recognize mismatched nucleotides, lesions, or structures within the DNA (4). MSH proteins bind and hydrolyze ATP (5–7). Importantly, DNA mismatch or structure recognition provokes ATP binding by MSH proteins that ultimately results in the formation of a stable sliding clamp (8–13). These MSH sliding clamps move by one-dimensional (1D) thermal (Brownian) diffusion with a lifetime of up to 10 min while in intermittent contact with the DNA backbone (14–17). The MSH sliding clamps that function in mismatch repair (MMR) act as a platform to load MutL homolog (MLH/PMS) proteins, which then form a second extremely stable freely diffusing ATP-bound sliding clamp that communicates mismatch recognition along the DNA to an excision-initiation site, which may be hundreds to thousands of nucleotides distant from the mismatch (4, 18).

Unrelated DNA-binding proteins may become roadblocks if they bind between the mismatch and the downstream excision-initiation site. Single-molecule imaging of molecular motors that use the energy of ATP hydrolysis to move along the DNA have demonstrated that these proteins can actively remove roadblocks (19). Alternatively, some proteins that rely on thermal fluctuation–driven motion may hop over roadblocks on DNA (20). Remarkably, MSH sliding clamps that rely entirely on 1D thermal diffusion to move along the DNA have been shown to disassemble nucleosomes (21). The mechanism of MSH-dependent nucleosome disassembly was not immediately obvious since the numerous histone octamer–DNA interactions that comprise a nucleosome would appear to require significant energy to release (22).

One hypothesis is that multiple randomly diffusing MSH sliding clamps might increasingly occupy nucleosomal DNA that is transiently unwrapped as a result of thermal fluctuation.

4 The abbreviations used are: MSH, MutS homolog; SPR, surface plasmon resonance; dig-antidig, digoxigenin-antidigoxigenin; smTIRF, single-molecule total internal reflection fluorescent; smPIFE, single-molecule protein-induced fluorescence enhancement.
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(23). Such 1D thermal motion of multiple MSH sliding clamps was projected to provide an explicit mechanism for influencing the binding of other proteins over distances that are larger than the total DNA footprint of the MSH particles (21, 23). Here we have used bulk analysis and single-molecule fluorescence imaging to examine the properties of MSH sliding clamps on DNA. We found that increasing the numbers of MSH sliding clamps on DNA decreased LacI repressor association to its cognate LacO promoter. We also show that the number of MSH sliding clamps already bound to the DNA affected the loading of additional MSH sliding clamps at a mismatch. Together these observations appear consistent with the ability of MSH sliding clamps to affect the association kinetics of additional DNA-binding proteins.

Results

ATP-bound MutS homologs reduce LacI binding to its cognate LacO site

The random diffusion characteristics of MSH sliding clamps on DNA suggested that they might influence the association of heterologous DNA-binding proteins over distances greater than their footprint. To address this possibility we examined the effect of three different MSH proteins, *Thermus aquaticus* TaMutS, *E. coli* EcMutS, and human (*Homo sapiens*) HsMSH2-HsMSH6, on the binding of the *E. coli* transcription factor LacI to its cognate LacO site using surface plasmon resonance (SPR) (Fig. 1, *a–c*, left panels; supporting Fig. S1). In this system the proximal end of a 98-bp mismatched DNA was attached to the SPR surface utilizing a biotin-streptavidin linkage. The mismatch was located 15 bp from the surface and the 21-bp LacI LacO site (24–26) was located 12 bp from the distal end that was blocked by digoxigenin-antidigoxigenin (dig-antidig). This DNA substrate could retain up to three MSH sliding clamps with a footprint of ~26 bp in the presence of ATP (9, 14, 27–30). However, there is only enough space for LacI to bind if there are two or less MSH sliding clamps present on the DNA. Previous studies have demonstrated that single or multiple sliding clamps are not formed on duplex DNA in the absence of a mismatch (9, 11). Following subtraction of the intrinsic MSH dissociation kinetics, we find that the association rate of LacI ($k_{off,LacI}$) decreased with increasing MSH concentration in the presence of ATP (Fig. 1, *a–c*, middle and right panels; supporting Fig. S1). As a control we show that increasing MSH concentration in the absence of ATP does not affect LacI binding (supporting Fig. S2). These observations are consistent with the conclusion that $k_{off,LacI}$ is inhibited with increasing ATP-bound MSH sliding clamps on the DNA.

The binding of LacI is altered by the number of MutS sliding clamps on DNA

SPR studies are unable to determine how the MSH sliding clamps affect the ability of LacI to bind the mismatched DNA. We consider at least two possibilities: 1) MSH sliding clamps saturate the DNA effectively obscuring the LacO site, or 2) sub-saturating MSH sliding clamps affect LacI binding at a distance. We used a single-molecule total internal reflection fluorescent (smTIRF) microscope system to determine the number of MSH sliding clamps on the mismatched DNA and their effect on LacI binding. Cy3-labeled TaMutS was utilized in these studies because we have previously demonstrated specific mismatch binding and the formation of stable ATP-bound sliding clamps on defined mismatched DNA substrates (14, 17). The 98-bp DNA was attached to a passivated surface via a biotin-NeutrAvidin linkage and modified to contain a +dT mismatch 15 bp from the proximal end with a Cy5 fluorophore 9 bp distal of the mismatch. The LacO site was located similarly to the SPR mismatched DNA substrate (Fig. 2a; supporting Table S1). The Cy5 location was previously shown to have no influence on mismatch binding activity or the formation of TaMutS sliding clamps (14, 17). The position of the LacO ensured that when bound by LacI the TaMutS sliding clamps may access only 65 bp of the 98-bp mismatched DNA.

LacI binding was determined by observing the time-averaged FRET produced by Cy3–TaMutS on the Cy5-labeled LacO-mismatched DNA (14, 17). FRET is inversely correlated with the length of accessible DNA and thus depends on the presence or absence of bound LacI. We calculate time-averaged FRET efficiencies of Cy3–TaMutS on the full-length 98 bp LacO-mismatched DNA of $E_1 \sim 0.32, E_2 \sim 0.34, and E_3 \sim 0.30$ for one, two, and three single fluorophore-labeled sliding clamps, respectively (Fig. 2; supporting Fig. S3). The binding of LacI to the LacO-mismatched DNA reduces the effective length that Cy3–TaMutS sliding clamps may occupy, resulting in a calculated increase in the time-averaged FRET efficiency of $E_{1,1-LacI} \sim 0.60$ and $E_{2,2-LacI} \sim 0.46$ for one and two single-labeled Cy3–TaMutS sliding clamps, respectively. The site size of three TaMutS sliding clamps on the LacO-mismatched DNA is expected to fully exclude LacI binding.

The number of TaMutS sliding clamps on single LacO-mismatched DNA molecules was determined by counting the number of Cy3 photobleaching steps (Fig. 2b; supporting Fig. S3). These were separated into single ($E_1$), double ($E_2$), and triple ($E_3$) fluorophores with associated FRET efficiency ($E$). Based on the labeling efficiency we expected 30% of the TaMuS dimers might contain two Cy3 fluorophores, potentially influencing the sliding clamp count. However, in the absence of LacI the binned FRET efficiencies fit to normal curves with means of $E_1 = 0.31 \pm 0.08, E_2 = 0.30 \pm 0.12, and E_3 = 0.21 \pm 0.08$, which closely correlated with the calculated time-averaged FRET efficiencies (Fig. 2b; supporting Fig. S3). Prebinding LacI to the LacO-mismatched DNA followed by loading Cy3–TaMutS sliding clamps resulted in DNA molecules that contained one or two fluorophores with FRET efficiencies that correlated extremely well with the calculated time-averaged FRET efficiencies (see Fig. 2a compared with Fig. 2b for one fluorophore, $E_{1,1-LacI} = 0.60 \pm 0.10$, or compared with supporting Fig. S3 for two fluorophores, $E_{2,2-LacI} = 0.47 \pm 0.07$). We conclude that simple Cy3 fluorophore counting provides a reasonable approximation of the number of TaMutS sliding clamps, and that time-averaged FRET efficiency is a practical indicator of the presence or absence of LacI on the LacO-mismatched DNA.

We noted that the distributions of binned FRET efficiency with and without LacI overlapped for both the one- or two-fluorophore cases. A stringent criterion for distinguishing LacI-bound DNA is to identify molecules with a FRET efficiency that is greater than 2 S.D. from the mean ($E_{1,1-LacI} > 0.4, E_{2,2-LacI} > 0.6$).
Figure 1. SPR analysis of LacI binding in the presence of MSH sliding clamps. A 5'-biotin 98-bp duplex DNA (supporting Table S1) containing a +dT (TaMutS) or G/T (EcMutS and HsMSH2-HsMSH6) mismatch was anchored to the surface of the streptavidin-coated SPR (Biacore) chip and the remaining end blocked by dig-antidig as described previously (38). Each curve is color-coded and comes in pairs ± LacI injection. Association curves were processed as described in supporting Fig. S1 and supporting information to extract $k_{on}$ LacI. a, TaMutS (0, 20, 50, 100, 200, 500, and 800 nM) ± LacI (0.5 nM). Subtracted LacI association curve in the presence of TaMutS (middle) and calculated $k_{on}$ LacI plotted against TaMutS concentration (right). b, EcMutS (0, 10, 30, 60, 100, and 200 nM) ± LacI (1.5 nM). LacI association curve in the presence of EcMutS (middle) and calculated $k_{on}$ LacI plotted against EcMutS concentration (right). c, HsMSH2-HsMSH6 (0, 10, 30, 60, 100, and 200 nM) ± LacI (1.5 nM). Left and right, LacI association curve in the presence of HsMSH2-HsMSH6 (left) and calculated $k_{on}$ LacI plotted against HsMSH2-HsMSH6 concentration (right).
We then examined 40 μm × 80 μm fields containing ~60 well-resolved molecules and found that when one Cy3 fluorophore was associated with the Cy5-labeled LacO-mismatched DNA, 55% of the molecules displayed a time-averaged FRET efficiency consistent with LacI bound to the DNA (E_{1,LacI} = 0.60) (Fig. 2a). When two Cy3 fluorophores were associated with the Cy5 LacO-mismatched DNA, 37% of the molecules displayed a time-averaged FRET efficiency consistent with LacI bound to the DNA (E_{2,LacI} = 0.47) (Fig. 2c). As expected, we did not observe any molecules that displayed altered time-averaged FRET when three or four Cy3 fluorophores were associated with the Cy5 mismatched DNA, although these events were

**Figure 2.** The frequency of LacI-binding events is reduced with the number of Cy3–TaMutS fluorophores on a mismatch DNA. **a,** an illustration of the mismatched DNA substrate. The 95-bp substrate contains a mismatch at 15T+ and a Cy5 fluorophore at the 24th nucleotide from the 5'-biotin bound to the smTIRF flow cell surface with the remaining end blocked by dig-antidig (supporting Table S1). Binding of Cy3–TaMutS to the mismatch results in a high FRET signal (E = 0.8) that in the presence of ATP resolves into a sliding clamp with time-averaged FRET (E = 0.3) as described previously (17). The binding of LacI and/or multiple sliding clamps alter the time-averaged FRET efficiency as shown below the middle and right molecules and as described in the text. **b,** representative trace showing the anti-correlation between Cy3–TaMutS (green) and Cy5–DNA (red) intensities caused by time-averaged FRET (blue) in the absence of a bound LacI (left) and in the presence of a bound LacI (right). Either natural or laser-intensity driven fluorophore photobleaching was recorded to determine the number of fluorophores bound to the mismatched DNA. Representative single step photobleaching of Cy3–TaMutS emission is shown. Binned histogram insets fit to normal distributions indicate mean FRET efficiency for one-fluorophore + LacI and −LacI with indicated number (N) of events. **c,** the frequency of LacI-bound DNA molecules. Total observations are shown above the data points from two separate experiments of a 40 μm × 80 μm field of view containing ~60 well-resolved molecules. LacI-binding events were distinguished by their characteristic time-averaged FRET value and then binned with the number of photobleached fluorophores (see text).
relatively rare (Fig. 2c). We note that with the four Cy3 fluorophore case at least one of the TaMutS sliding clamps must contain two fluorophores. These results support the conclusion that increased numbers of MSH sliding clamps exclude LacI transcription factor binding to DNA.

**MutS sliding clamps on the DNA affect the $k_{on}$ of LacI**

To determine whether MutS sliding clamps affect the on or off rate of LacI-binding kinetics, we developed a single-molecule protein-induced fluorescence enhancement (smPIFE) system (31). A Cy3 fluorophore was placed 2 bp distal to the LacO site on the 98-bp mismatched DNA (Fig. 3a; supporting Table S1). LacI binding induced a PIFE signal (supporting Fig. S4a) in which the $\tau_{off,LacI}$ depended upon protein concentration and could be used to determine the on rate ($k_{on,LacI}$; supporting information and supporting Fig. S4b) and off rate ($\tau_{on,LacI} = 1/k_{off,LacI}$) to calculate the equilibrium dissociation constant ($K_D$) = $k_{off,LacI}/k_{on,LacI} = 119 \pm 22$ pm, which was similar to historical reports (24). To count the number of ATP-bound sliding clamps we labeled TaMutS or EcMutS with Alexa Fluor 647 (Thermo Fisher Scientific) as described previously (14, 17).

For these studies the $\tau_{off,LacI}$ was determined with 1 nM LacI, which was then followed by counting the number of Alexa Fluor 647 photobleaching events to determine the number of fluorophores on single LacO-mismatched DNA molecules (Fig. 3b; supporting Fig. S5 and supporting information). The resulting $\tau_{off,LacI}$ histograms were individually binned for one- and two-fluorophore photobleaching events (17). To account for the possibility of unlabeled and double-labeled MutS dimers the $\tau_{off,LacI}$ histograms were fit to a double exponential (Fig. 3c). We then normalized the total probability density to 1 and based on the experimentally determined labeling efficiency, fixed the ratio of double fluorophore bleaching events to single fluorophore bleaching events to reduce the number of variables to two (supporting Fig. S5 and supporting information). These fits could either be used directly or subjected to maximum likelihood estimation to garner the $\tau_{off,LacI}$ when one ($\tau_{off,LacI,1}$) or two ($\tau_{off,LacI,2}$) MutS sliding clamps were present on the LacO-mismatched DNA (supporting Table S3). These $\tau_{off}$ values were then used to calculate the $k_{on,LacI}$ in the presence of one MutS sliding clamp or two MutS sliding clamps and displayed individually with standard deviation error for experimental comparison (Fig. 3c). These results demonstrate that increased numbers of MutS sliding clamps decrease the $k_{on}$ of LacI association to its cognate LacO-binding site.

**Reduction in LacI binding is consistent with a predictive model for freely diffusing MutS sliding clamps on DNA**

We developed a model that quantitatively describes the reduction of $k_{on,LacI}$ as a result of any number of sliding clamps and DNA lengths. This model treats MSH sliding clamps as particles that engage in free 1D diffusion along the DNA, with important, the properties of a 1D Tonks Gas/Sliding Clamp can be systematically calculated with this model (supporting information, model for freely diffusing MutS sliding clamps, and supporting Table S2).

To test the application of the Tonks Gas/Sliding Clamp model in the absence of additional proteins and to establish its main parameter, the footprint size ($\sigma$) of the MutS sliding clamps, single-molecule fluorescence imaging was used to count the number of MutS sliding clamps on a DNA containing a single mismatch (Fig. 4a; supporting Table S1). In these studies a 95-bp DNA was linked at the proximal end to a passivated...
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(a) Schematic of DNA with fluorophores labeled with Cy5 and Cy3. 15dT+ and dT+ indicate the DNA ends.

(b) Graphs showing FRET efficiency and intensity over time for different concentrations of fluorophores.

(c) Plots of fraction of molecules vs. number of fluorophores for 10nM, 50nM, and 300nM calculations.

(d) Graphs showing DNA length vs. ratio of certain reaction rates for one, two, and three MutS complexes.

(e) Graphs showing reaction rate ratios for different numbers of MutS complexes.
surface via 5′-biotin-NeutrAvidin and the distal end was blocked by 3′-dig-antidig antibody (Fig. 4a). Blocking both ends (surface and dig-antidig) effectively retains freely diffusing MutS sliding clamps on the mismatched DNA as described previously (14, 17).

An extrahelical +dT mismatch was positioned at 15 bp from the proximal end and the DNA was labeled 7 bp from the distal end with Cy5 (Fig. 4a). Cy3-labeled TaMutS was used in this analysis because we have previously demonstrated specific mismatch binding and the formation of stable ATP-bound sliding clamps on similar mismatched DNA substrates (14, 17).

We first determined that the location of the Cy5 does not influence TaMutS mismatch binding or the formation of an ATP-bound sliding clamp by SPR (supporting Fig. S6). These results suggest the ATP-bound TaMutS sliding clamps loaded onto the DNA are the consequence of genuine mismatch recognition events.

Our previous studies showed that ATP-bound Cy3–TaMutS sliding clamps rapidly diffuse along the length of short oligonucleotides producing a time-averaged FRET with the Cy5-labeled DNA (Fig. 4b) (14, 17). These observations indicated that the number of ATP-bound TaMutS sliding clamps could be unequivocally determined by monitoring the FRET signal results from Cy3–TaMutS located on the Cy5-mismatched DNA and then counting the number of Cy3 photobleaching events over a 100-s observation (Fig. 4b; supporting Fig. S7).

We found that the fraction of TaMutS sliding clamp–associated fluorophores depended on protein concentration (Fig. 4c, data points). The Tonks Gas/Sliding Clamp model predicts that the effective on-rate for binding a new TaMutS clamp will be affected by number of MutS sliding clamps already diffusing on the DNA. This prediction allows for calculation of the concentration-dependent number of MutS sliding clamp on the DNA (supporting information). The general agreement with the measured and calculated distribution of MutS sliding clamps provides support for the Tonks Gas/Sliding Clamp model (Fig. 4c, lines). In addition, the interpolated footprint size (σ = 26 bp) corresponds well with previous biochemical determinations (33).

The Tonks Gas/Sliding Clamp model may also be used to calculate the $k_{on,Lac}$ in the presence of MutS sliding clamps that may transiently occlude the LacO-binding site and reduce accessibility to LacL (Fig. 3; supporting information). Moreover, the model permits calculation of on-rates for LacL ($k_{on,Lac}$) at different DNA lengths and numbers of MSH sliding clamps bound to the DNA (Fig. 4d). The ratio of $k_{on,Lac}$ with zero MSH sliding clamps ($k_{on,Lac,0}$) to that with N number of MSH sliding clamps on the DNA ($k_{on,Lac,N}$) provides a convenient measure of any reduction in LacL binding (Fig. 4d).

For example, the Tonks Gas/Sliding Clamp model predicts at least a 2-fold reduction in $k_{on,Lac,N}$ ($k_{on,Lac,0}/k_{on,Lac,N} = 2$) when two MutS sliding clamps are confined to 160 bp or less, and when three MutS sliding clamps are confined to 235 bp or less. A 10-fold reduction in $k_{on,Lac,N}$ ($k_{on,Lac,0}/k_{on,Lac,N} = 10$) is predicted when two MutS sliding clamps are confined to 100 bp or less, and three MutS sliding clamps are confined to 140 bp or less.

For the specific DNA used in Fig. 3, we calculated a distribution of predictions using several hypothetical MSH footprints (σ = 24, 25, or 26 bp) and LacL footprints (σ = 21 or 25 bp) and assuming that either the 5′-biotin-NeutrAvidin or dig-antidig linkages increased the DNA length by an additional 2 bp, which resulted in a mean with upper and lower quartile for $k_{on,Lac,0}/k_{on,Lac,N}$ (Fig. 4e; supporting information). We find excellent agreement with the theoretical mean predicted by the Tonks Gas/Sliding Clamp model for two MutS sliding clamps when compared with the experimentally determined average of the $k_{on,Lac,2}$ shown in Fig. 3c ($k_{on,Lac,0}/k_{on,Lac,2} = 9.6 ± 2.0$ S.D.; Fig. 4e, red asterisk in two MutS). For one MutS sliding clamp, we noted a larger effect on the experimental ratio than predicted by the Tonks Gas/Sliding Clamp model ($k_{on,Lac,0}/k_{on,Lac,1} = 2.6 ± 0.9$ S.D.; Fig. 4e, red asterisk in one MutS). Our calculations using this model assume that the DNA is a stiff rod and the MutS proteins are inelastic spheres with defined site size. However, small changes on the effective DNA length that may be occupied by a MutS sliding clamp, the site size of either the MutS or the LacI proteins (elasticity or hydration) and the mechanical properties of the DNA compared with lattice models (34) may account for the differences in experimental and predicted effects of one MutS sliding clamp on $k_{on,Lac}$.

**Discussion**

Our results provide consistent evidence that stable freely diffusing ATP-bound MSH sliding clamps can influence the association of other binding proteins with the DNA. The major effect of the multiple MSH sliding clamps is to reduce the $k_{on}$ of a DNA-binding protein. This would effectively increase the $K_D$ of a DNA-binding protein ultimately reducing its equilibrium binding as well as its localization to the region occupied by stable sliding clamps. There appeared to be little effect on the $k_{off}$ of LacL when one MSH sliding clamp was present, although the number of observations was low as a result of the $k_{off}$ of LacL. A unique effect on $k_{on}$ implies that the exclusion effect introduced by multiple MSH sliding clamps would only affect unbound DNA-binding proteins or previously bound DNA-binding proteins immediately following equilibrium dissociation.

Figure 4. Concentration dependence of loaded TaMutS sliding clamps compared with theoretical Tonks Gas/Sliding Clamp model predictions. a, an illustration of the substrates utilized to count TaMutS sliding clamps on the DNA. A 95-bp duplex DNA was anchored to the smTIRF flow cell surface by a biotin-NeutrAvidin at one end while the other end was blocked by a dig-antidig complex. Cy5 was linked to the 5th base from the dig-antidig and the mismatch was located at the 15th bp position from the 5′-biotin end. b, representative traces showing the anti-correlation between Cy3–TaMutS and Cy5–DNA. This prediction allows for calculation of the concentration-dependent number of MutS sliding clamp on the DNA (supporting information). The general agreement with the measured and calculated distribution of MutS sliding clamps provides support for the Tonks Gas/Sliding Clamp model (Fig. 4c, lines). In addition, the interpolated footprint size (σ = 26 bp) corresponds well with previous biochemical determinations (33).

The Tonks Gas/Sliding Clamp model may also be used to calculate the $k_{on,Lac}$ in the presence of MutS sliding clamps that may transiently occlude the LacO-binding site and reduce accessibility to LacL (Fig. 3; supporting information). Moreover, the model permits calculation of on-rates for LacL ($k_{on,Lac}$) at different DNA lengths and numbers of MSH sliding clamps bound to the DNA (Fig. 4d). The ratio of $k_{on,Lac}$ with zero MSH sliding clamps ($k_{on,Lac,0}$) to that with N number of MSH sliding clamps on the DNA ($k_{on,Lac,N}$) provides a convenient measure of any reduction in LacL binding (Fig. 4d). For example, the Tonks Gas/Sliding Clamp model predicts at least a 2-fold reduction in $k_{on,Lac,N}$ ($k_{on,Lac,0}/k_{on,Lac,N} = 2$) when two MutS sliding clamps are confined to 160 bp or less, and when three MutS sliding clamps are confined to 235 bp or less. A 10-fold reduction in $k_{on,Lac,N}$ ($k_{on,Lac,0}/k_{on,Lac,N} = 10$) is predicted when two MutS sliding clamps are confined to 100 bp or less, and three MutS sliding clamps are confined to 140 bp or less.

For the specific DNA used in Fig. 3, we calculated a distribution of predictions using several hypothetical MSH footprints (σ = 24, 25, or 26 bp) and LacL footprints (σ = 21 or 25 bp) and assuming that either the 5′-biotin-NeutrAvidin or dig-antidig linkages increased the DNA length by an additional 2 bp, which resulted in a mean with upper and lower quartile for $k_{on,Lac,0}/k_{on,Lac,N}$ (Fig. 4e; supporting information). We find excellent agreement with the theoretical mean predicted by the Tonks Gas/Sliding Clamp model for two MutS sliding clamps when compared with the experimentally determined average of the $k_{on,Lac,2}$ shown in Fig. 3c ($k_{on,Lac,0}/k_{on,Lac,2} = 9.6 ± 2.0$ S.D.; Fig. 4e, red asterisk in two MutS). For one MutS sliding clamp, we noted a larger effect on the experimental ratio than predicted by the Tonks Gas/Sliding Clamp model ($k_{on,Lac,0}/k_{on,Lac,1} = 2.6 ± 0.9$ S.D.; Fig. 4e, red asterisk in one MutS). Our calculations using this model assume that the DNA is a stiff rod and the MutS proteins are inelastic spheres with defined site size. However, small changes on the effective DNA length that may be occupied by a MutS sliding clamp, the site size of either the MutS or the LacI proteins (elasticity or hydration) and the mechanical properties of the DNA compared with lattice models (34) may account for the differences in experimental and predicted effects of one MutS sliding clamp on $k_{on,Lac}$.
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These observations support a previous hypothesis that the disassembly of nucleosomes by HsMSH2-HsMSH6 was the result of MSH sliding clamps inhibiting nucleosome DNA rewrapping kinetics around the histone octamer (21, 23). This concept also explains the synergistic effect of HsMSH2-HsMSH6–catalyzed nucleosome disassembly when histone post-translational modifications that reduced nucleosome DNA wrapping stability were present (21, 23). Recent studies from our group demonstrated that MSH sliding clamps load MLH/PMS proteins onto the DNA in a cascade of extremely stable ATP-bound sliding clamps (18). It seems possible that these two sliding clamps together may synergistically reduce the localized association of proteins that could inhibit communication and/or excision processes during mismatch repair.

There are a number of other stable sliding clamps that associate with DNA in all organisms. In eukaryotes these include the replicative processivity factor proliferating cell nuclear antigen (PCNA) and the DNA damage response complex RAD9-HUS1-RAD1 (9-1-1 complex) that are ubiquitous in dividing cells and are essential for genome maintenance and stability (1, 2). It is likely that biological evolution has selected for these stable sliding clamp complexes based at least in part on the useful physical property that they may freely diffuse over long distances.

**Experimental procedures**

**Proteins and DNA**

*T. aquaticus* TaMutS(C42A,T469C) was expressed, purified, and labeled with Cy3 or Alexa Fluor 647 as described previously with a labeling efficiency per monomer of 54.5 or 45%, respectively (14, 17). *E. coli* EcMutS(DB35R,R840E) containing a C-terminal formylglycine-generating enzyme (FGE) hexa-amino acid recognition sequence and a hexa-histidine tag was expressed, purified, and labeled with Alexa Fluor 647 by Hydrazinyl-iso-Pictet-Spengler (HIPS) ligation as described previously (35, 36) with a labeling efficiency per monomer of 26%. The human HsMSH2-HsMSH6 heterodimer was purified as described previously (37). Mismatch binding and ATP-dependent sliding clamp formation was determined by surface plasmon resonance (SPR), as described previously (8, 38). PAGE gel purified DNA oligonucleotides (supporting Table S1) were purchased from Midland Reagents (Midland, TX) or Integrated DNA Technologies (Coralville, IA). Fluorophore labeling of DNA and purification by reverse phase HPLC on a C18 column (Agilent) was performed as described previously (14, 17). Complementary oligonucleotides were annealed and purified by HPLC on a Gen-Pack ion exchange column (Waters) as described previously (39).

**SPR-binding kinetic analysis**

A 98-bp mismatched DNA containing a 5′-biotin at one end and a 5′-dig at the other end was attached to a streptavidin-coated SPR chip (Biacore) as described previously (supporting Table S1) (38). MSH protein binding at indicated concentrations was performed in SM Buffer (20 mM Tris–HCl, pH 7.8, 100 mM NaCl, 5 mM MgCl2, 0.1 mM DTT, 0.1 mM EDTA, 0.5 mM ATP) and minus glucose, glucose oxidase/catalase (GOD/CAT), and trolox, but with 0.005% Surfactant P20 (GE Healthcare, BR100054), 0.2 mg/ml acetylated BSA (Promega, R3961), and 25 nM antidig (Roche) at 10 μl/min and 23 °C (EcMutS and HsMSH2-HsMSH6) or 35 °C (TaMutS). For studies that examined the effect of MSH sliding clamps on LacI binding, following MSH binding in the presence of ATP (see MutS/MSH Injection, Fig. 1; supporting Fig. S1), LacI (0.5 nM for TaMutS; 1.5 nM for EcMutS and human HsMSH2-HsMSH6) was injected (see LacI Injection, Fig. 1; supporting Fig. S1) and binding compared with the absence of LacI. The MSH dissociation curve in the absence of LacI was subtracted from the rate curve in the presence of LacI to obtain the LacI association curve (supporting Fig. S1). The LacI association curve was fit to a single component binding exponential to obtain the $k_{on}$ LacI at each concentration of initial MSH binding/loading (supporting Fig. S5).

**Single-molecule FRET and photobleaching analysis**

Cy3–TaMutS (100 nM) was incubated with indicated Cy5–DNA (supporting Table S1) for 5 min in SM Buffer (plus 0.0025% P20) to load TaMutS sliding clamps. After washing away free MutS with SM Buffer plus 0.0025% P20 with oxygen scavenging system (OSS) (0.8% w/v glucose, 146 units/ml glucose oxidase, 2170 units/ml catalase, 2 mM trolox) and 1 nM LacI, time-averaged FRET was detected after 1 min as described previously using a prism-type laser excitation smTIRF microscopy system (14, 17) containing an Olympus IX-71 with water-type 60× objective (N.A. 1.2), a Photometrics DV2 two-channel dual color separation system and a Princeton Instruments ProEM 512 Exelon charge-coupled device recorder. The number of Cy3 fluorophore photobleaching steps were counted as described previously (14, 17) and compared with the number and distribution of fluorophores predicted based on the TaMutS monomer labeling efficiency. Briefly, laser intensity may be increased and the resulting step loss of fluorophore signal(s) until zero fluorescence is an indicator of the number of fluorophores on a single molecule. Fluorescent images and FRET following 532 nm DPSS LASER excitation were analyzed using IDL software (ITT VIS) and MATLAB (The MathWorks) scripts. Data analysis methods may be found in the supporting information.

**Single-molecule PIFE**

We assembled a prism-type laser excitation total internal reflection fluorescence (TIRF) microscopy system (14, 17) containing an Olympus IX-71 with water-type 60× objective (N.A. 1.2), a Photometrics DV2 two-channel dual color separation system and a Princeton Instruments ProEM 512 Exelon charge-coupled device recorder. The DNA substrate was identical to the SPR substrate except it was labeled by Cy3 near the LacO-binding site that when bound by LacI induced protein-induced fluorescence enhancement (PIFE) (supporting Table S1) (31). Fluorescent images were analyzed using IDL (ITT VIS) and MATLAB (The MathWorks) scripts following 532 nm (PIFE) and 635 nm (MH fluorophore photobleaching and counting) DPSS laser excitation (Crystal Laser, 2 milliwatt) at 250–400 ms time resolution. The $\tau_{off}$ (period of protein-free DNA) and $\tau_{on}$ (period of protein-bound DNA) were determined by measuring fluorescence intensity changes upon LacI binding (supporting Fig. S4a), where the cumulative distributions at several
concentrations of LacI were fit to a single exponential decay (supporting Fig. S5a). The $k_{on\text{-LacI}}\left(\tau_{off\text{-LacI}}^{-1}\right)$ and $k_{off\text{-LacI}}\left(\tau_{on\text{-LacI}}^{-1}\right)$ were found to be linear and used to obtain the $K_a$ values (supporting Fig. S4, b and c). We note that two measures for $\tau_{off}$, the time between the initial injection to the first LacI-binding event or the time between LacI-binding dissociation and reassociation, gave nearly identical $k_{on}$ between 0 and 1 nm LacI (compare supporting Fig. S4b and supporting Fig. S4c). Above 1 nm LacI the $\tau_{off}$ was increasingly short relative to our time resolution and fluorophore PIFE intensity fluctuations, thus escalating the measurement error.

The loading of MSH sliding clamps followed by LacI protein binding was carried out in SM Buffer plus 0.0025% P20 with the Alexa Fluor 647–TaMutS (250 nm, or 315 nm) or Alexa Fluor 647–EcMutS (30 nm, or 40 nm) added to the flow cell for 10 min, followed by a 10× volume wash with SM Buffer to eliminate free MSH protein, followed by LacI (1 nM) injection. The $\tau_{off\text{-SH}}$ in the presence of MSH sliding clamps was obtained as the time between the initial LacI injection and the first LacI-binding event PIFE induced by LacI binding. The number of Alexa Fluor 647 fluorophores was obtained from the number of photo-bleaching steps as described previously (14, 17). The methods utilized for data analysis may be found in the supporting information.

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References
1. Doré, A. S., Kilkenny, M. L., Rzechorzek, N. J., and Pearl, L. H. (2009) Crystal structure of the rad9–rad1–hus1 DNA damage checkpoint complex—implications for clamp loading and regulation. Mol. Cell 34, 735–745 CrossRef Medline
2. Hedglin, M., Kumar, R., and Benkovic, S. J. (2013) Replication clamps and clamp loaders. Cold Spring Harb. Perspect. Biol. 5, a010165 CrossRef Medline
3. Lee, J. B., Cho, W. K., Park, J., Jeon, Y., Kim, D., Lee, S. H., and Fishel, R. (2014) Single-molecule views of MutS on mismatched DNA. DNA Repair (Amst.) 20, 82–93 CrossRef Medline
4. Fishel, R. (2015) Mismatch repair. J. Biol. Chem. 290, 26395–26403 CrossRef Medline
5. Gradia, S., Acharya, S., and Fishel, R. (1997) The human mismatch recognition complex hMSH2-hMSH6 functions as a novel molecular switch. Cell 91, 995–1005 CrossRef Medline
6. Haber, L. T., and Walker, G. C. (1991) Altering the conserved nucleotide binding motif in the Salmonella typhimurium MutS mismatch repair protein affects both its ATPase and mismatch binding activities. EMBO J. 10, 2707–2715 Medline
7. Hughes, M. J., and Jiricny, J. (1992) The purification of a human mismatch–binding protein and identification of its associated ATPase and helicase activities. J. Biol. Chem. 267, 23876–23882 Medline
8. Acharya, S., Foster, P. L., Brooks, P., and Fishel, R. (2003) The coordinated functions of the E. coli MutS and MutL proteins in mismatch repair. Mol. Cell 12, 233–246 CrossRef Medline
9. Gradia, S., Subramanian, D., Wilson, T., Acharya, S., Makhot, A., Griffith, J., and Fishel, R. (1999) hMSH2-hMSH6 forms a hydrolys-is-independent sliding clamp on mismatched DNA. Mol. Cell 3, 255–261 CrossRef Medline
10. Mazur, D. J., Mendillo, M. L., and Kolodner, R. D. (2006) Inhibition of Msh6 ATPase activity by mispaired DNA induces a Msh2(ATP)–Msh6(ADP) state capable of hydrolys-is-independent movement along DNA. Mol. Cell 22, 39–49 CrossRef Medline
11. Mendillo, M. L., Mazur, D. J., and Kolodner, R. D. (2005) Analysis of the interaction between the Saccharomyces cerevisiae MSH2–MSH6 and MLH1–PMIS1 complexes with DNA using a reversible DNA end-blocking system. J. Biol. Chem. 280, 22245–22257 CrossRef Medline
12. Snowden, T., Acharya, S., Butz, C., Berardini, M., and Fishel, R. (2004) hMSH4–hMSH5 recognizes Holliday Junctions and forms a meiosis-specific sliding clamp that embraces homologous chromosomes. Mol. Cell 15, 437–451 CrossRef Medline
13. Wilson, T., Guerrette, S., and Fishel, R. (1999) Dissociation of mismatch recognition and ATPase activity by hMSH2–hMSH3. J. Biol. Chem. 274, 21659–21664 CrossRef Medline
14. Cho, W. K., Jeong, C., Kim, D., Song, K. M., Hanne, J., Ban, C., Fishel, R., and Lee, J. B. (2012) ATP alters the diffusion mechanics of MutS on mismatched DNA. Structure 20, 1264–1274 CrossRef Medline
15. Gorman, J., Wang, F., Redding, S., Prys, A. J., Fazio, T., Wind, S., Alani, E. E., and Greene, E. C. (2012) Single-molecule imaging reveals target-search mechanisms during DNA mismatch repair. Proc. Natl. Acad. Sci. U.S.A. 109, E3074–E3083 CrossRef Medline
16. Honda, M., Okuno, Y., Hengel, S. R., Martin-Lopez, J. V., Cook, C. P., Amunugama, R., Soukop, R. J., Subramanyam, S., Fishel, R., and Spies, M. (2014) Mismatch repair protein hMSH2–hMSH6 recognizes mismatches and forms sliding clamps within a D-loop recombination intermediate. Proc. Natl. Acad. Sci. U.S.A. 111, E316–E325 CrossRef Medline
17. Jeong, C., Cho, W. K., Song, K. M., Cook, C., Yoon, T. Y., Ban, C., Fishel, R., and Lee, J. B. (2011) MutS switches between two fundamentally distinct clamps during mismatch repair. Nat. Struct. Mol. Biol. 18, 379–385 CrossRef Medline
18. Liu, J., Hanne, J., Britton, B. M., Bennett, J., Kim, D., Lee, J. B., and Fishel, R. (2016) Cascading MutS and MutL sliding clamps control DNA diffusion to activate mismatch repair. Nature 539, 583–587 CrossRef Medline
19. Yardimci, H., Loveland, A. B., van Oijen, A. M., and Walter, J. C. (2012) Single-molecule analysis of DNA replication in Xenopus egg extracts. Methods 57, 179–186 CrossRef Medline
20. Gorman, J., Prys, A. J., Visnapuu, M. M., Alani, E., and Greene, E. C. (2010) Visualizing one-dimensionally diffusion of eukaryotic DNA repair factors along a chromatin lattice. Nat. Struct. Mol. Biol. 17, 932–938 CrossRef Medline
21. Javadi, S., Manohar, M., Punja, N., Mooney, A., Ottozen, J. I., Poirier, M. G., and Fishel, R. (2009) Nucleosome remodeling by hMSH2–hMSH6. Mol. Cell 36, 1086–1094 CrossRef Medline
22. Locke, G., Tolkunov, D., Moqtaderi, Z., Struhl, K., and Morozov, A. V. (2010) High-throughput sequencing reveals a simple model of nucleosome energetics. Proc. Natl. Acad. Sci. U.S.A. 107, 20998–21003 CrossRef Medline
23. Forties, R. A., North, J. A., Javadi, S., Tabbaa, O. P., Fishel, R., Poirier, M. G., and Bundschuh, R. (2011) A quantitative model of nucleosome dynamics. Nucleic Acids Res. 39, 8306–8313 CrossRef Medline
24. Gilbert, W., and Maxam, A. (1973) The nucleotide sequence of the lac operator. Proc. Natl. Acad. Sci. U.S.A. 70, 3581–3584 CrossRef Medline
25. Gilbert, W., and Müller-Hill, B. (1966) Isolation of the lac repressor. Proc. Natl. Acad. Sci. U.S.A. 56, 1891–1898 CrossRef Medline
26. Jacob, F., and Monod, J. (1961) Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3, 318–356 CrossRef Medline

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27. Jeon, Y., Kim, D., Martin-López, J. V., Lee, R., Oh, J., Hanne, J., Fishel, R., and Lee, J. B. (2016) Dynamic control of strand excision during human DNA mismatch repair. Proc. Natl. Acad. Sci. U.S.A. 113, 3281–3286 CrossRef Medline

28. Lamers, M. H., Perrakis, A., Enzlin, J. H., Winterwerp, H. H., de Wind, N., and Sixma, T. K. (2000) The crystal structure of DNA mismatch repair protein MutS binding to a G-T mismatch. [see comments]. Nature 407, 711–717 CrossRef Medline

29. Obmolova, G., Ban, C., Hsieh, P., and Yang, W. (2000) Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA. [see comments]. Nature 407, 703–710 CrossRef Medline

30. Warren, J. J., Pohlhaus, T. J., Changela, A., Iyer, R. R., Modrich, P. L., and Beese, L. S. (2007) Structure of the human MutSα DNA lesion recognition complex. Mol. Cell 26, 579–592 CrossRef Medline

31. Hwang, H., Kim, H., and Myong, S. (2011) Protein induced fluorescence enhancement as a single molecule assay with short distance sensitivity. Proc. Natl. Acad. Sci. U.S.A. 108, 7414–7418 CrossRef Medline

32. Tonks, L. (1936) The complete equation of state of one, two and three-dimensional gases of hard elastic spheres. Phys. Rev. 50, 955–963 CrossRef

33. Biswas, I., and Hsieh, P. (1996) Identification and characterization of a thermostable MutS homolog from Thermus aquaticus. J. Biol. Chem. 271, 5040–5048 CrossRef Medline

34. Chereji, R. V., and Morozov, A. V. (2011) Statistical mechanics of nucleosomes constrained by higher-order chromatin structure. J. Stat. Phys. 144, 379–404 CrossRef Medline

35. Agarwal, P., Kurdirka, R., Albers, A. E., Barfiels, R. M., de Hart, G. W., Drake, P. M., Jones, L. C., and Rabuka, D. (2013) Hydrazino-Pictet-Spengler ligation as a biocompatible method for the generation of stable protein conjugates. Bioconjugate Chem. 24, 846–851 CrossRef Medline

36. Liu, J., Hanne, J., Britton, B. M., Shoffner, M., Albers, A. E., Bennett, J., Zatezalo, R., Barfield, R., Rabuka, D., Lee, J. B., and Fishel, R. (2015) An efficient site-specific method for irreversible covalent labeling of proteins with a fluorophore. Sci. Rep. 5, 16883 CrossRef Medline

37. Heinen, C. D., Wilson, T., Mazurek, A., Berardini, M., Butz, C., and Fishel, R. (2002) HNPCC mutations in hMSH2 result in reduced hMSH2-hMSH6 molecular switch functions. Cancer Cell 1, 469–478 CrossRef Medline

38. Heinen, C. D., Cyr, J. L., Cook, C., Punja, N., Sakato, M., Forties, R. A., Lopez, J. M., Hingorani, M. M., and Fishel, R. (2011) Human MSH2 (hMSH2) protein controls ATP processing by hMSH2-hMSH6. J. Biol. Chem. 286, 40287–40295 CrossRef Medline

39. Mazurek, A., Johnson, C. N., Germann, M. W., and Fishel, R. (2009) Sequence context effect for hMSH2-hMSH6 mismatch-dependent activation. Proc. Natl. Acad. Sci. U.S.A. 106, 4177–4182 CrossRef Medline