DNA-dependent Activation of the hMutSα ATPase*

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ATP hydrolysis by MutS homologs is required for function of these proteins in mismatch repair. However, the function of ATP hydrolysis in the repair reaction is controversial. In this paper we describe a steady-state kinetic analysis of the DNA-activated ATPase of human MutSα. Comparison of salt concentration effects on mismatch repair and mismatch-provoked excision in HeLa nuclear extracts with salt effects on the DNA-activated ATPase suggests that ATP hydrolysis by MutSα is involved in the rate determining step in the repair pathway. While the ATPase is activated by homoduplex and heteroduplex DNA, the half-maximal concentration for activation by heteroduplex DNA is significantly lower under physiological salt concentrations. Furthermore, at optimal salt concentration, heteroduplex DNA increases the \( k_{cat} \) for ATP hydrolysis to a greater extent than does homoduplex DNA. We also demonstrate that the degree of ATPase activation is dependent on DNA chain length, with the \( k_{cat} \) for hydrolysis increasing significantly with chain length of the DNA cofactor. These results are discussed in terms of the translocation (Allen, D. J., Makhov, A., Grilley, M., Taylor, J., Thresher, R., Modrich, P., and Griffith, J. D. (1997) EMBO J. 16, 4467–4476) and the molecular switch (Gradia, S., Acharya, S., and Fishel, R. (1997) Cell 91, 985–1005) models that invoke distinct roles for ATP hydrolysis in MutS homolog function.

As originally demonstrated in bacteria (1–3), mismatch repair also plays an important role in stabilization of the genome of eukaryotes by correcting DNA biosynthetic errors and by participating in the cellular response to some types of DNA damage (reviewed in Refs. 4–8). Hereditary and somatic defects in this system have also been implicated in cancer development (7). Bacterial MutS and MutL play critical roles in the initiation of the prokaryotic mismatch repair reaction. Mismatch recognition is mediated by a MutS homodimer (8–11), and MutL, which exists as a homodimer in solution, binds to the MutS-DNA complex in a reaction that requires ATP but is apparently independent of nucleotide hydrolysis (12). MutL is thought to interface mismatch recognition by MutS to activities that function subsequent to mismatch recognition (3, 13). Thus, both MutS and MutL are required for the mismatch-dependent activation of the MutH (d(GATC) endonuclease (14), which serves to identify the newly synthesized DNA strand, and for activation of DNA helicase II, which initiates excision at the nick introduced by MutH into an unmethylated d(GATC) sequence on the newly synthesized strand (15, 16). MutL appears to play a direct role in the latter reaction since the protein has been shown to physically interact with and to dramatically activate helicase II on conventional helicase substrates (15, 17).

Homologs of bacterial MutS and MutL have been implicated in the initiation of mismatch repair in eukaryotes. Mismatch recognition in yeast and mammalian cells is mediated by MutSα, a heterodimer of MutS homologs MSH2 and MSH6 that recognizes base-base and insertion/deletion mismatches (18–23), and by MutSβ, a MSH2-MSH3 heterodimer that recognizes insertion/deletion mispairs (22–26). MutL function in eukaryotes is also provided by a heterodimeric MutLa complex, an MLH1-PMS1 heterodimer in yeast and an MLH1-PMS2 complex in mammalian cells (27, 28). Although little is known about the activities of MutLa in eukaryotic mismatch repair, yeast MutLa has been shown to form a ternary complex with MutSα and a mismatch, a reaction that like assembly of the bacterial MutL-MutS-heteroduplex ternary complex, requires ATP but apparently does not depend on ATP hydrolysis (29).

In addition to its mispair recognition activity, bacterial MutS has an associated ATPase activity that is required for function of the protein in mismatch repair. Genetic inactivation of the nucleotide binding site results in a dominant negative phenotype (30, 31), and nonhydrolyzable ATP analogues block MutS- and MutL-dependent activation of MutH in vitro (14). The nucleotide binding site consensus sequence is conserved in eukaryotic MutS homologs, and mutant studies have implicated the nucleotide binding centers of yMSH2, hMSH2, and hMSH6 in mismatch repair (32, 33).

There is general agreement in the literature that prokaryotic and eukaryotic MutS homologs are responsible for the initial recognition of mismatches, with subsequent repair complex assembly leading to excision and resynthesis steps of the reaction (4–6). Because MutS homologs bind with high specificity to heteroduplex DNA in the absence of ATP (11, 18, 20, 21, 34, 35), ATP hydrolysis by these proteins presumably functions at steps subsequent to mismatch recognition.

Two mutually exclusive models have been proposed for the role of nucleotide binding and hydrolysis by MutS activities. In the translocation model, MutS uses energy derived from ATP hydrolysis to translocate along the DNA contour, a mechanism that would permit coupling of mismatch recognition to recognition of the secondary site that determines strand specificity of the reaction (11, 14–16). In the molecular switch model, the process of ATP binding and hydrolysis serve to switch the protein between two states, one of which is active in mismatch binding and one of which is not (34). This model invokes mismatch recognition by the MutSα-ADP complex, which serves to recruit downstream repair activities to the mismatch. Subsequent binding of ATP results in release of MutSα from the

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¶ Yeast and human mismatch repair proteins are distinguished by y or h prefix, respectively, e.g. yMSH2 and hMSH2.
that ADP is not required for mismatch recognition by hMutS maxime DNA repair in vitro
demonstrate using human MutS the mismatch upon ATP challenge, directly into solution or
these two models is the path by which the MutS homolog leaves plex DNA results in departure of the protein from the mis-
challenge of specific complexes between MutS and heterodu-
plex oligonucleotide substrates.
A
were prepared as described previously (36). hMutS
identied by UV absorbance, combined, and DNA concentrated by
eluted duplex was combined with 0.5 ml (packed volume) of strepta-
for 30 min at 37 °C. Samples from the reaction (2
m for 30 min at 37 °C. Samples from the reaction (2 µl) were combined
with 3 µl of 50% formamide, 0.01% xylene cyanol, and 0.01% bromphe-
Free phosphate was separated from ATP by gel electrophoresis through a 20% acrylamide gel containing 7 µ urea in 89 mM Tris, 89 mM
ate, and resuspended in 10 ml of 10 mM Tris-HCl, pH 8.0, and 1
complexes between MutS and heterodu-
plexes is blocked by physical barriers placed at
the ends of the DNA, consistent with a translational mechanism.
EXPERIMENTAL PROCEDURES
Proteins, Extracts, and DNA Substrates—HeLa cell nuclear extracts were prepared as described previously (36). hMutS was isolated in
>95% pure form according to the procedure of Drummond et al. (18). Poly(dT) and oligo(dT)18 were purchased from Sigma, and synthetic oligonucleotides were purchased from Oligos Etc. (Wilsonville, OR). Phage T7RF DNA and heteroduplex f1MR DNA were prepared as de-
scribed (37). A self-complementary hybrid duplex of the viral and complemen-
tary strands of phages M13 and fd, respectively (M13/10), containing 225 mispaired bases, was a gift from Dr. Leroy Worth (NIEHS, National
Institutes of Health, Research Triangle Park, NC). Duplex oligonucleotide
substrates were prepared as described in the accompanying paper (35).
Two hundred and sixty-three base pair heteroduplexes containing 23 mismatches were constructed by annealing appropriate single strands isolated from DNA fragments generated by PCR amplification of re-
ions of bacteriophages M13 (4591–4854) and fd (4592–4855) RF. The forward primer (5'-GTTTCCCGAATGATAAGT) and the reverse primer (5'-GCATACCTTGGCTGAACCTC) were constructed with or without a 5'-biotin tag. PCR reactions (1 µl) were performed as de-
scribed in the accompanying paper (35), and products designed such that one of the strands would be biotinylated. After amplification, reactions were diluted to 5 µl with 100 mM KPO4 (pH 6.8) and loaded onto a 0.2-mL hydroxyapatite column equilibrated in the same buffer. The column was washed with 10 column volumes of 100 mM KPO4 (pH 6.8), and DNA eluted with 200 mM KPO4 (pH 6.8) in a 0.5-mL volume. The eluted duplex was combined with 0.5 µl (packed volume) of strepta-
vidin-agarose (Sigma) pre-equilibrated in 200 mM KPO4 (pH 6.8), and mixed at room temperature for 3 h. The streptavidin-agarose resin was then placed in an empty column (0.8 x 4 cm, Bio-Rad), allowed to settle, and washed with 5 µl of a buffer containing 25 mM Tris-HCl, pH 8.0, 1
mM EDTA, and 0.5 M NaCl. The nonbiotinylated strand was eluted with 250 mM Tris base (adjusted to pH 12.4 with NaOH), 1 mM EDTA, and 0.5 M NaCl, collecting 0.1-mL fractions. DNA-containing fractions were identied by UV absorbance, combined, and DNA concentrated by
ethanol precipitation. From approximately 20 µg of PCR product, 7–10
µg of nonbiotinylated single strand was recovered. The viral strand of the PCR product derived from M13 was annealed to the complementary
strand of either M13 or fd to generate homoduplex (263-bp M13/M13) and heteroduplex (263-bp M13/10) substrates, respectively.
Mismatch Repair and Gap Formation Assays—Mismatch repair re-
actions (20 µl) containing 100 ng of f1MR G-T heteroduplex DNA contain-
ing a strand break at the Sau96I site 125 bp 5' to the mismatch in the complementary strand (38) and 100 µg of HeLa nuclear extract were performed as described (38), except that the KCl concentration was adjusted from 10 to 30 mM as indicated. Reactions to score mis-
match-provoked gap formation were performed in a similar manner, except that 90 µM aphidicolin was included to inhibit repair DNA synthesis (36, 38). Presence of a single-stranded gap in the DNA was determined by virtue of resistance of gapped DNA to NheI, whose site is 6
nucleotides 5’ to the mismatch (Fig. 1). After incubation with the
HeLa extract, DNA was precipitated, and resuspended in 10
µl of 10 mM Tris-HCl, pH 7.0, and 1 mM EDTA. Dissolved DNA was mixed with an equal volume of a solution containing 10 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 50 mM NaCl, 1 mM dithiothreitol, 2 units each of Bsp1016 and NheI endonuclease, 0.1
mg/ml RNase A, and 1.5 µg/ml bovine serum albumin. Digestion was for 1 h at 37 °C.
ATPase Assay—Hydrolysis of ATP by hMutS was determined by libera-
tion of phosphate from [γ-32P]ATP. Unless specified otherwise, reaction mixtures (20 µl) contained 20 mM Tris-HCl, pH 7.6, 1 mM
dithiothreitol, 50 µg/ml bovine serum albumin, 1 mM MgCl2, 50 mM
KCl, 50 µM [γ-32P]ATP (20 Ci/mmol), and DNA substrates as indicated.
Reactions were initiated by addition of hMutS (38 nm) and incubated for 30 min at 37 °C. Samples from the reaction (2 µl) were combined with 3 µl of 100% formamide, 0.01% xylene cyanol, and 0.01% bromphe-
nol blue. Free phosphate was separated from ATP by gel electrophoresis through a 20% acrylamide gel containing 7 µ urea in 89 mM Tris, 89 mM
boric acid, 2 mM EDTA at 14 V/cm (39). Gels were quantitated using a Molecular Dynamics PhosphorImager. All measurements were in the linear range of the assay.
Nitrocellulose Filter Binding Assay—Reactions (20 µl) contained 20
mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 50 µg/ml bovine serum albumin, 1 mM MgCl2, 0.2 µM [γ-32P]ATP-labeled 50-bp heteroduplex contain-
ing a centrally located G-T mismatch (Fig. 1), 380 ng of hMutS and KCl as indicated. After incubation for 10 min at 37 °C, reactions were passed through 0.45-µm nitrocellulose membranes (Millipore) preincubated in reaction buffer. Membranes were washed with 1 µl of reaction buffer, dried, and the radioactivity determined by scintillation counting.

RESULTS
Salt Dependence of the in Vitro Mismatch-dependent Repair Reaction—A classical approach to correlating a measured en-
zyme activity to an enzymatic pathway is to alter the reaction conditions of both the enzyme and the entire system and phe-
nomenologically establish whether conditions that activate or in-
hibit the particular enzyme correspondingly activate or inhibit the enzymatic pathway is to alter the reaction

2 The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s).
Identical to that of the mismatch repair reaction, with maximal shown in Fig. 2.

Gap formation were performed in an identical manner except that 90 circular f1MR G-T heteroduplex containing a strand break at the recognition site for which is 6 bp 5' to the mismatch in the complementary strand (38) and 100 µg of HeLa nuclear extract. KCl concentration was varied as indicated (0–300 mM). Lower panel, reactions for mismatch-provoked gap formation were performed in an identical manner except that 90 µM aphidicolin was included. Gap formation was scored by conversion of the DNA to a form resistant to NheI endonuclease, the recognition site for which is 6 bp 5' to the mismatch (Fig. 1).

proven excision reaction (36, 38) was measured under identical conditions as a function of the [KCl]. The excision step of the reaction can be resolved from repair DNA synthesis in the presence of aphidicolin, which inhibits the latter step of repair (38). In the presence of the drug, mismatch-provoked excision results in a single-stranded gap spanning a region from the nick to approximately 90–170 nucleotides beyond the mispair (36, 38). This gap can be scored by measuring the fraction of DNA molecules that become resistant to NheI endonuclease, the recognition site for which is 6 bp 5' to the mismatch (38). As shown in Fig. 2B, the salt profile for gap production is nearly identical to that of the mismatch repair reaction, with maximal efficiency occurring at 130 mM KCl. Significantly, the absolute molar efficiencies of the two reactions were very similar, suggesting that steps subsequent to gap formation proceed with high efficiency, independent of the salt concentration.

Salt Dependence of the DNA-stimulated ATPase Activity of hMutSα—One of the defining characteristics among MutS homologs is a nucleotide binding motif that is required for ATPase activity and function of the proteins in mismatch repair (14, 21, 30, 33, 34, 40). Examination of several eukaryotic MutS homologs has yielded dissimilar findings with respect to the degree of DNA activation of the ATPase and the effect of a mismatch on the reaction. In the case of the yeast mitochondrial MutS homolog MSH1, modest DNA activation (~2-fold) has been observed with both homo- and heteroduplex DNAs, with the former being the more effective activator at low salt and the latter at high salt (40). Limited activation by homoduplex DNA has also been reported for γMutSα, but this activity has been found to be slightly inhibited by heteroduplex DNA (21). By contrast, hMutSα has been reported to be activated by heteroduplex DNA (34). It is evident that determination of reaction parameters effecting the ATPase activity of MutS homologs are appropriate in order to resolve apparently conflicting reports of the activating effect of DNA. The following experiments attempt to define the DNA-stimulated activation of hMutSα in terms of salt dependence, effect of a mispair, and nature of the DNA cofactor.

We observe 10–20-fold DNA activation in the rate of hMutSα ATPase activity from a basal $k_{cat, ATP}$ of 0.2 ADP/min to 2.4–3.9 ADP/min at saturating [ATP] and [DNA]. The $K_{cat, ATP}$ for the DNA-stimulated reaction is ~30 µM, similar to that reported by Gradia et al. (34) for the recombinant protein. Fig. 3 shows the salt dependence of hMutSα ATPase activity at saturating [ATP] and a concentration of 50-bp homo- or heteroduplex DNA (Fig. 1, 10 µM) that saturates stimulation of ATPase activity at low salt. At relatively low [KCl], both homo- and heteroduplex DNA activate hMutSα to the same degree. Increasing the [KCl], however, increases the rate of ATP hydrolysis, which reaches a maxima between 100 and 150 mM, with the heteroduplex DNA consistently yielding a higher degree of ATPase activation at KCl concentrations above 100 mM. Further increases in the [KCl] mitigate activation, and activity falls to the basal rate of ATP hydrolysis measured in the absence of added DNA co-factor. This intrinsic level of hMutSα ATPase activity was found to be independent of the KCl concentration over the range tested. Significantly, the ATPase activity determined with saturating heteroduplex exhibits a maximum at 150 mM KCl, similar to the [KCl] at which maximal repair and mismatch-provoked excision was observed (compare with Fig. 2, A and B). Therefore, conditions that favor optimal hydrolysis of ATP correlate with those of mismatch repair. Inhibition of the bacterial MutS ATPase by ATPγS corresponded to a similar degree of inhibition of the mismatch-provoked MutS, MutL, and MutH strand nicking reaction (14). Of the three proteins involved, MutS is the only protein that has been demonstrated to have ATPase activity (14, 30).

Salt Concentration Dependence of the $K_{1/2, DNA}$ and $k_{cat}$ for the DNA-stimulated hMutSα ATPase Activity—In order to clarify the nature of the salt optima observed in the DNA-stimulated ATPase reaction, the rate of ATP hydrolysis was measured as a function of heteroduplex concentration at different salt concentrations (Fig. 4A). Data were fit to a square hyperbola and the corresponding $k_{cat, ATP}$ and $K_{1/2, DNA}$ (DNA concentration that gave at half-maximal activation) values are plotted as a function of the [KCl] in Fig. 4 (B and C, respectively). The $k_{cat, ATP}$ determined at saturating concentrations of heteroduplex DNA is ~2-fold higher than that at low salt. In contrast, the DNA concentration at which half-maximal activation is observed increases with increasing [KCl]. The double-reciprocal plots (Fig. 4B) also display a significant increase in $K_{1/2, DNA}$ with increasing [KCl].

$^3$L. Blackwell and P. Modrich, unpublished results.
The presence of a G-T mismatch in the 50-bp substrate resulted in a decrease in DNA binding. The ATPase activity of hMutSα is activated by a variety of DNA cofactors (summarized in Table I). Oligonucleotide and natural duplex DNAs are both effective activators of the ATPase activity. In general, the $K_{1/2,\text{DNA}}$ value is lower for the smaller duplex substrates compared with those for the large natural DNAs. An exception is the full-length M13/fd hybrid molecule, which contains numerous mismatches (average of 1 mismatch/30 bp) and which displays a $K_{1/2,\text{DNA}}$ value similar to those obtained with oligonucleotide duplexes. These compiled kinetic results were obtained under identical conditions (50 mM KCl) to permit comparisons between the different DNA cofactors. At this relatively low salt concentration, no difference is observed for the $K_{1/2,\text{DNA}}$ value between the hetero- and homoduplex oligodeoxyribonucleotides ≈70 bp in length. We note however, that the apparent $K_{1/2,\text{DNA}}$ expressed on a per-molecule basis (~2 nm) is actually below the enzyme concentration used in the assay (38 nm), which is indicative of very tight (stoichiometric) binding that would mask modest differences in affinity caused by the presence of a mismatch. Certainly, at higher salt concentrations that weaken protein-DNA interactions (cf. Fig. 4, A and 4C), a clear specificity for the mismatch
**TABLE I**

Substrate Specificity of hMutSa ATPase

| Substrates          | \( K_{1/2} \) | \( k_{cat} \) |
|---------------------|--------------|--------------|
| Natural DNAs        | \( \mu M \)  | min \(^{-1} \) |
| f1RF                | 1.9 ± 0.42   | 3.9 ± 0.12   |
| f1MR G-T heteroduplex | 1.8 ± 0.52   | 2.9 ± 0.11   |
| M13/fd              | 0.17 ± 0.13  | 3.8 ± 0.04   |
| Oligonucleotide substrates |          |              |
| 31-bp A-T          | 0.22 ± 0.02  | 2.4 ± 0.16   |
| 50-bp A-T          | 0.12 ± 0.04  | 2.4 ± 0.04   |
| 50-bp G-T          | 0.13 ± 0.02  | 2.7 ± 0.03   |
| 70-bp A-T          | 0.11 ± 0.01  | 3.1 ± 0.05   |
| 263-bp homoduplex | 1.1 ± 0.34   | 3.0 ± 0.16   |
| 263-bp heteroduplex| 0.79 ± 0.13  | 3.9 ± 0.11   |
| sDNAs              |              |              |
| M13                | 0.21 ± 0.08  | 3.7 ± 0.16   |
| Poly(dT)           | 0.07 ± 0.02  | 3.3 ± 0.08   |
| Oligo(dT)\(_{18}\) | 1.3 ± 0.24   | 3.6 ± 0.05   |
| No DNA             |              | 0.2 ± 0.01   |

is observed as demonstrated in Fig. 6 (at 150 mM KCl).

In contrast to the findings of Gradia et al. (34), we have also observed a significant and reproducible DNA chain length effect on \( k_{cat,ATP} \) measured at saturating DNA concentrations (Table I). The \( k_{cat,ATP} \) for a 31-bp oligoduplex is 2.4 ± 0.16 ADP/min compared with that measured at saturating concentrations of the 262-bp fragment of 3.9 ADP/min. Further increases in the DNA length (e.g. f1RF, 6.2 kilobase pairs) has no significant effect on the \( k_{cat,ATP} \). Furthermore, high molecular weight single-stranded DNA is also an effective activator of hMutSa ATPase both in terms of its apparent affinity (\( K_{1/2,\text{DNA}} \)) and stimulatory ability (\( k_{cat,ATP} \)). Single-stranded oligo(dT)\(_{18}\) yields a comparable \( k_{cat,ATP} \) value but displays a weaker apparent affinity as judged by the much larger \( K_{1/2,\text{DNA}} \) concentration. Clearly, further experiments will be necessary to determine if the stimulatory effect of single-stranded DNA on hMutSa ATPase activity is mechanistically relevant or simply represents an uncoupled ATPase activity.

**DISCUSSION**

The requirement for ATP hydrolysis by MutS homologs during mismatch repair is clearly documented (30–33), and the modulating effects of ATP and ATP analogs on complex formation between MutS homologs and heteroduplex DNA are well established. However, the interpretation of these effects is a matter of some debate. The translocation model invokes ATP binding and hydrolysis in movement of the protein along the helix. The molecular switch model posits that ATP hydrolysis by hMutSa fulfills a signaling function, somewhat analogous to the role GTP hydrolysis plays in signaling by the G-protein family.

The accompanying paper describes the effects of nucleotides on the ATPase both in terms of its apparent affinity (\( K_{1/2,\text{DNA}} \)) and stimulatory ability (\( k_{cat,ATP} \)). Further experiments will be necessary to determine some of the puzzling effects alluded to above with respect to DNA effects on this MutS homolog activity. We have shown that the DNA activation of the ATPase is largely due to an increase in \( k_{cat,ATP} \), with decrease in activity at elevated salt concentrations due to turnover rate as well as a reduced apparent affinity for DNA. The resulting salt profile for the ATPase is strikingly similar to that of mismatch repair and mismatch-activated excision reactions, strongly suggesting that hMutSa ATP hydrolysis is involved in the slow step that determines the overall rate of repair.

The effects of salt concentration on the DNA-activated hMutSa ATPase reaction described may serve to clarify some of the puzzling effects alluded to above with respect to DNA effects on this MutS homolog activity. We have shown that the DNA activation of the ATPase is largely due to an increase in \( k_{cat,ATP} \) with decrease in activity at elevated salt concentrations due to turnover rate as well as a reduced apparent affinity for DNA. The resulting salt profile for the ATPase is strikingly similar to that of mismatch repair and mismatch-activated excision reactions, strongly suggesting that hMutSa ATP hydrolysis is involved in the slow step that determines the overall rate of repair.

These kinetic studies are also relevant to the question of the role ATP hydrolysis by hMutSa plays in mismatch repair. We have shown that conditions which maximize the DNA-stimulated ATPase activity of hMutSa also maximize mismatch repair. This is in agreement with the concept that ATP hydrolysis is performing an active translocation role, which is necessary and limiting for subsequent steps in the repair pathway. However, these findings are difficult to reconcile with an information-based role for ATP hydrolysis. The role of ATP hydrolysis in the molecular switch model is to regenerate the “on” form of hMutSa (e.g. on a per-molecule basis, hMutSa must bind and hydrolyze ATP to regenerate a hMutSa-ADP complex in order to participate in further rounds of mismatch recognition and signaling). However, in the extract systems used to study in vitro mismatch repair, hMutSa is in 20-fold excess over heteroduplex (23) and is not limiting for repair (26). Consequently, there is no need to regenerate an “on” signal for efficient repair to occur in these cell-free systems. The limiting nature of hMutSa, therefore, is not in its solution concentration (e.g. rate or level of binding) but in its function subsequent to mismatch binding.

Inasmuch as ATP binding is sufficient to promote the dissociation of specific hMutSa-mismatch complexes (18, 33, 34), the requirement for ATP hydrolysis cannot be attributed to its function in release of hMutSa from the mismatch. At the saturating concentrations of ATP used in these studies, release of hMutSa from the mismatch will be independent of the rate of ATP hydrolysis. Moreover, single-turnover ATP hydrolysis experiments have shown that the rate of ATP hydrolysis is fast (e.g. chemistry is not rate-limiting) relative to the steady-state rate of ATP turnover (34). Upon binding ATP, hMutSa rapidly hydrolyzes the nucleotide, and the ADP complex is relatively long lived. At saturating ATP, the steady-state concentration of hMutSa-ADP will be unaffected by subtle changes in the steady-state turnover rate of ATP. Thus, in a model where the steady-state concentration of hMutSa-ADP is the only relevant species that interacts with heteroduplex, there is no expected correlation between the rate of ATP turnover and the rate of repair.

We have also observed chain length effects with respect to DNA cofactor activation of the ATPase, with longer DNAs
consistently found to be better activators than oligonucleotide duplexes due to a \( k_{at} \) effect. While these data cannot be definitively interpreted in terms of mechanism, it is interesting to note that an increase in \( k_{at,ATP} \) with increased DNA chain length is the expected result for a translocation mechanism (42, 43). However, it is difficult to rationalize these findings with the molecular switch model, which does not invoke a functional role for sequences external to the mispair.

We thus regard the results presented here as inconsistent with a nucleotide signaling role in hMutS function. Indeed, in the accompanying paper (35), we show that free DNA termini are required for ATP-promoted hMutS dissociation from a small heteroduplex, a finding which implies that hMutS does in fact translocate along the helix in the presence of the nucleotide.

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