MSH2-MSH3 directs the repair of insertion/deletion loops of up to 13 nucleotides in vivo and in vitro. To examine the biochemical basis of this repair specificity, we characterized the mispair binding and ATPase activity of hMSH2-hMSH3. The ATPase was found to be regulated by a mismatch-stimulated ADP → ATP exchange, which induces a conformational transition by the protein complex. We demonstrated strong binding of hMSH2-hMSH3 to an insertion/deletion loop containing 24 nucleotides that is incapable of provoking ADP → ATP exchange, suggesting that mismatch recognition appears to be necessary but not sufficient to induce the intrinsic ATPase. These studies support the idea that hMSH2-hMSH3 functions as an adenosine nucleotide-regulated molecular switch that must be activated by mismatched nucleotides for classical mismatch repair to occur.

Mismatched nucleotides arise in DNA as a result of chemical and physical damage, recombination between parental DNAs containing sequence heterologies, and misincorporation errors during replication (1). Elevated rates of spontaneous mutations may result if such mismatched nucleotides are not correctly repaired. The idea that an excision repair system exists that recognizes and repairs mismatched nucleotides was developed independently by Pauling and Hanawalt (2) and Witkin (3). The best understood mismatch repair (MMR)1 pathway is the MutHLS system of Gram-negative bacteria (for review see Refs. 4–6). The MutHLS system of Gram-negative bacteria (for review see Refs. 4–6). The nascent DNA strand may “slip,” forming insertion/deletion loops (IDL) that would then result in the lengthening or shortening of these sequences if left unrepaired (17). The MMR pathway appears largely responsible for the repair of IDLs as well as simple mismatched nucleotides that presumably arise as a result of misincorporation errors during DNA replication (for review see Ref. 15). Interestingly, the E.coli MutHLS pathway will recognize and repair all combinations of single nucleotide mismatches as well as IDLs containing one through four nucleotides but not IDLs containing five nucleotides (18).

The MMR system has been highly conserved through evolution, with multiple MutS and MutL homologs having been identified in yeast and humans (for review see Ref. 16). In Saccharomyces cerevisiae, the MutS homologs scMSH2, scMSH3, and scMSH6 have been shown to be responsible for the recognition and repair of mismatches and IDLs. The scMSH2-scMSH6 heterodimer recognizes mismatched nucleotides and a subset of single- and dinucleotide IDLs, whereas the scMSH2-scMSH3 heterodimer is responsible for the repair of a overlapping set of single-nucleotide IDLs as well as IDLs of up to 13 nucleotides (19–22). The repair of a presumed 26-base pair IDL by an MSH2-dependent process has been reported (23). However, these experiments were performed by examining post meiotic segregants following mating and could be interpreted to result from altered recombination conversion tracts as suggested by Alani et al. (24), which is underlined by the experiments of Tran et al. (44), who demonstrate a dependence of IDL repair on the Rad52 recombination pathway.

Under protein limiting conditions, the human MutS homologs (hMSH2, hMSH3, and hMSH6) appear very similar to their yeast counterparts with respect to mismatch binding specificity and repair (25–30). However, under conditions where the hMSH2-hMSH6 heterodimer exceeds the mismatch by 10-fold, there is evidence that binding and repair of IDLs outside the traditionally accepted range may occur (31).

The bacterial MutS proteins and their eukaryotic MutS homologs (MSH) contain Walker A and B consensus adenosine nucleotide and magnesium binding motifs (32). Furthermore, the homology between the 48 known MSH proteins is confined to this region, suggesting that ATP binding and hydrolysis play a pivotal role in mismatch repair functions. Recently, the intrinsic ATP hydrolysis activity (ATPase) associated with the adenosine nucleotide binding domains of the hMSH2-hMSH6 protein complex was found to regulate mismatch binding similar to a molecular switch (33). The hMSH2-hMSH6 molecular switch is ON (bound to a mismatch) in the ADP-bound form and OFF in the ATP-bound form. Hydrolysis of the ATP molecule resulted in the recovery of mismatch binding capability, whereas ADP → ATP exchange resulted in dissociation from the mismatch. The hMSH2-hMSH6 switch appears to be regulated by the exchange of ADP → ATP, which is uniquely provoked by mismatch recognition/binding. Such a mechanism
is strikingly similar to that displayed by the G protein family of purine nucleotide-binding proteins (34).

The S. cerevisiae and human MSH2-MSH3 heterodimers have been purified, and their mismatch binding specificities have been partially characterized. The yeast MSH2-MSH3 heterodimer appears to recognize IDLs of up to 14 nucleotides in vitro, and this binding activity has been reported to be insensitive to ATP (19). The mispair binding specificity of the human hMSH2-hMSH3 heterodimer remains unclear as a result of the fact that nearly all of the oligonucleotides utilized in the study appear to contain single-nucleotide mismatches in addition to IDL nucleotide mismatch (21). In this study, we report the DNA binding specificity and ATPase activity of the hMSH2-hMSH3 protein complex. In large part, the mechanism of mispair recognition by hMSH2-hMSH3 heterodimer appears similar to the hMSH2-hMSH6 heterodimer except with respect to which nucleotide mismatches are capable of provoking ADP → ATP exchange. These results indicate that the hMSH2-hMSH3 mismatch repair complex functions as an adenosine nucleotide-regulated molecular switch and that mispair binding appears to be a necessary but not sufficient step in the repair of IDLs by the classical mismatch repair system. We entertain the possibility that hMSH2-hMSH3 may retain some functions that are independent of adenosine nucleotide exchange and hydrolysis.

**Materials and Methods**

**Overexpression and Purification of hMSH2-hMSH3—hMSH2 and hMSH3 clones have been described previously (29). hMSH2 and N-terminal His	extsubscript{6}-tagged hMSH3 recombinant proteins were overexpressed in SF9 insect cells utilizing the pFastBac dual expression vector (Life Technologies, Inc.). Infected SF9 cells were harvested and suspended in buffer A (300 mM NaCl, 20 mM imidazole, 25 mM HEPES-NaOH (pH 8.1), 10% glycerol, and protease inhibitors (0.5 mM phenylmethysulfonyl fluoride, 0.8 μg/ml pepstatin, and 0.8 μg/ml leupeptin)) followed by rapid freezing in liquid nitrogen. The following purification procedures were carried out at 4 °C. The cells were thawed on ice and disrupted by passage through a 25-gauge needle, and the resulting crude extract was cleared by ultracentrifugation at 40,000 × g. The supernatant was loaded onto a nickel nitrilotriacetic acid Superflow column (Quiagen), washed with buffer A, and eluted with a linear gradient of imidazole from 20 mM to 200 mM. The hMSH2-hMSH3 complex eluted at approximately 70 mM imidazole. Peak fractions were loaded directly onto a hMSH2-hMSH3 complex eluted at approximately 600 mM NaCl. Positions of hMSH2 (104.7 kDa) and hMSH3 (126.75 kDa) are indicated.

**Gel Mobility Shift Assay—** This assay was performed with 9 fmol of labeled DNA substrate in a buffer containing 100 mM NaCl, 25 mM HEPES-NaOH (pH 8.1), 1 mM dithiothreitol, 0.1 mM EDTA, 15% glycerol, and 20 ng of 200 base pair homoduplex competitor in a final reaction volume of 20 μl. The reactions were incubated at 25 °C for 10 min and immediately placed on ice. The samples were electrophoresed on a 5% polyacrylamide (29:1 bis), 4% glycerol gel in Tris-bUFFERED EDTA buffer. The gels were dried and then quantitated using a Molecular Dynamics PhosphorImager. Concentrations of adenosine nucleotides and magnesium were as noted in the figure legends. Dissociation constants (K	extsubscript{d}) for hMSH2-hMSH3 binding to the various DNA substrates were determined utilizing a Molecular Dynamics PhosphorImager as the DNA concentration at which half-maximal binding occurs and are presented with standard deviations.

**ATPase and ADP Exchange Assays—** ATPase and ADP → ATP exchange assays were performed as described previously utilizing the
results

purification of the hMSH2-hMSH3 protein complex—hMSH2 and hMSH3 proteins were overexpressed in Sf9 insect cells as a heterodimer utilizing a dual expression baculovirus vector (Life Technologies, Inc.). This complex was purified to greater than 95% homogeneity by affinity chromatography (Fig. 1). The hMSH2-hMSH3 complex was determined to be at an approximate 1:1 molar ratio as judged by quantitative denaturation.

TABLE I

kinetic parameters of the hMSH2-hMSH3 heterodimer

The kinetic parameters were determined for hMSH2-hMSH3 on DNA substrates by varying the substrate concentration and performing Michaelis-Menton analysis. All values are presented with standard deviations.

| DNA Substrate | $V_{max}$ (×10⁻¹⁵ mol/min) | $K_M$ (×10⁻⁶ M) | $K_{cat}$ (×min⁻¹) | $K_d$ (×10⁻⁹ M) |
|---------------|-----------------------------|------------------|-------------------|------------------|
| +CA           | 2.53 ± 0.24                 | 7.56 ± 0.64      | 1.41 ± 0.13       | 1.6 ± 0.14       |
| (+CA)₄       | 2.49 ± 0.26                 | 7.49 ± 0.45      | 1.38 ± 0.14       | 1.8 ± 0.09       |
| (+CA)₈       | 1.25 ± 0.08                 | 4.71 ± 0.51      | 0.69 ± 0.046      | 2.2 ± 0.19       |
| (+CA)₁₂      | 1.04 ± 0.066                | 3.80 ± 0.32      | 0.57 ± 0.03       | 0.8 ± 0.06       |
| G/C           | 0.74 ± 0.05                 | 3.51 ± 0.07      | 0.41 ± 0.029      | 1.29 ± 0.05      |
| No DNA        | 0.48 ± 0.05                 | 1.39 ± 0.05      | 0.27 ± 0.028      |                  |

insertion/deletion DNA substrates described above (33). Standard deviations are noted.

Partial Proteolysis Assay—One μg of protein per reaction was digested with 0, 40, 80, or 160 ng of trypsin (Promega) in buffer A (25 mM HEPES, pH 8.1, 100 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 20% glycerol) at 37 °C for 45 min. The reactions were stopped by the addition of 2× sample loading buffer and boiling for 10 min. The proteolyzed products (0.5 μg each sample) were separated on 6% SDS-polyacrylamide gels (35) and analyzed by silver staining and by Western analysis utilizing anti-hMSH2 polyclonal antibody (Ab-3; Oncogene Research Products). When indicated, proteolysis was performed with 1 mM ATP·γS or ADP.

Insertion/Deletion Loop Binding by the hMSH2-hMSH3 Complex—The yeast MSH2-MSH3 complex has previously been demonstrated to bind to IDLs containing up to 14 nucleotides by gel shift analysis (19). The human hMSH2-hMSH3 complex has been shown to repair an IDL containing 12 nucleotides but not an IDL containing 27 nucleotides (31), although these experiments are complicated by a general increase in hMSH2-independent repair of the larger IDLs. To determine the binding capacity of the purified hMSH2-hMSH3 protein complex to IDLs, gel shift analysis was performed utilizing DNA substrates containing IDLs that contained CA repeats of 2, 8, 16, and 24 nucleotides. The apparent dissociation constants ($K_d$) were determined to be 1.6 ± 0.14 nM for the +CA, 1.8 ± 0.09 nM for the (+CA)₄, 2.2 ± 0.19 nM for the (+CA)₈, and 0.8 ± 0.06 nM for the (+CA)₁₂ IDL (Fig. 2; Table I). Binding of hMSH2-hMSH3 to homoduplex DNA was not saturable up to 160 nM. Both hMSH2 and hMSH3 appear to be required for the specific high affinity binding to IDLs because purified hMSH2 (alone) displays at least a 50-fold reduced affinity for mismatched nucleotides ($K_d$ ~ 50–100 nM) (36). Taken together with the recent findings that the yeast and human MSH2-MSH3 complexes appear incapable of efficiently directing the repair of IDLs containing 16 or 27 nucleotides, respectively, these data appear to suggest that simple binding of hMSH2-hMSH3 to IDLs is not sufficient to initiate mismatch repair (22, 31).

The hMSH2-hMSH3 complex was found to bind to the (+CA)₁₂ in multiple slower-migrating forms at concentrations above 10 nM. Similar slower migrating forms of hMSH2-hMSH6 bound to a G/T mismatch were detected at concentrations above 400 nM (33). These slower migrating forms may indicate mispaired oligonucleotides-containing multiple protein complexes or alternate binding mechanisms.

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Fig. 3. hMSH2-hMSH3 insertion/deletion loop binding sensitivity to ATP and ATP·γS. The hMSH2-hMSH3 heterodimer was incubated with 9 fmol of either +CA, +(CA)₁₂, IDL, or G/C DNA substrate in the presence of 500 μM ATP or ATP·γS and increasing concentrations of magnesium: A, +CA DNA substrate with ATP·γS; B, +(CA)₁₂ DNA substrate with ATP·γS; C, G/C DNA substrate with ATP; D, +CA DNA substrate with ATP·γS; E, +(CA)₁₂ DNA substrate with ATP·γS; and F, G/C DNA substrate with ATP·γS.
Effect of ATP on hMSH2-hMSH3 Mispair Binding—The addition of ATP to MutS homologs has been shown to result in its dissociation from mismatched nucleotides. Interestingly, we found that ATP does not alter the binding of hMSH2-hMSH3 to IDL heteroduplex DNA in the absence of magnesium. However, in the presence of 1 to 10 mM magnesium, the binding of the hMSH2-hMSH3 complex to a $^{1}$CA IDL was found to be sensitive to ATP (Fig. 3A). The ATP-dependent dissociation of hMSH2-hMSH3 from the $^{+}$CA IDL reached the background level of homoduplex (G/C) binding at 10 mM magnesium (Fig. 3C). In contrast, the binding of the protein complex to a $^{+}$CA $^{12}$ IDL was relatively stable in the presence of ATP (Fig. 3B). The differential sensitivity of the hMSH2-hMSH3-DNA complex to the presence of magnesium is in contrast to results obtained for the hMSH2-hMSH6 heterodimer (33). Additionally, the finding that ATP is capable of dissociating hMSH2-hMSH3 from small IDLs differs from studies in yeast in which the MSH2-MSH3 complex was found to remain stably bound to these substrates in the presence of ATP (19). This disparity may reflect differences between the yeast and human hMSH2-hMSH3 homologs and/or purification schemes. The addition of ADP had no effect on binding regardless of magnesium concentration and is similar to that reported for the hMSH2-hMSH6 complex (data not shown) (33).

Dissociation of hMSH2-hMSH3 from the $^{+}$CA but not the $^{+}$CA $^{12}$ IDL substrates also occurred in the presence of the poorly hydrolyzable ATP analog ATPγS (Figs. 3, D and E). We have found that hMSH2-hMSH3 does not significantly hydrolyze ATPγS (data not shown), supporting the conclusion that the release of hMSH2-hMSH3 from the +CA DNA duplex occurs independent of hydrolysis. Similar observations have been made for the bacterial MutS and the hMSH2-hMSH6 heterodimer (data not shown (33).

It was noted that the binding of hMSH2-hMSH3 to the +CA DNA duplex was not completely abolished in the presence of magnesium and 500 μM either of ATP or ATPγS. This residual binding appeared similar to the background of hMSH2-hMSH3 binding to homoduplex DNA, which was also refractory to dissociation by ATP and ATPγS (Fig. 3, C and F). These results suggest that the residual binding of hMSH2-hMSH3 to the
+CA DNA duplex in the presence of ATP and ATPγS is the likely result of nonspecific association(s).

ATP Hydrolysis by the hMSH2-hMSH3 Complex Is Stimulated by IDL Mismatched DNA—The bacterial MutS protein is known to possess a low level ATPase activity that has been conserved in the yeast and human homologs (33, 37–39). Although significant literature has begun to emerge regarding the ATPase activity of the MSH2-MSH6 complex, little is currently known about the ATPase activity of the MSH2-MSH3 heterodimer. We found that IDLs stimulate the intrinsic ATPase of the hMSH2-hMSH3 heterodimer (Fig. 4A). Michaelis-Menten and Lineweaver-Burk analysis suggest that the hMSH2-hMSH3 ATPase was most active in the presence of the +CA and +(CA)₄ IDLs, less active in the presence of the +(CA)₆ and +(CA)₁₂ IDLs and homoduplex DNA, and relatively inactive in the absence of DNA (Table I, Fig. 4A). These results demonstrate that IDLs containing 2 and 8 nucleotides stimulate the hydrolysis of ATP by hMSH2-hMSH3 significantly more than loops containing 16 and 24 nucleotides. The stimulation of the hMSH2-hMSH3 ATPase by IDLs largely correlates with their relative repair by the MMR system in vitro and in vivo (22, 30, 31). However, this conclusion must be tempered by the fact that mismatch repair of the sequences context contained in the present oligonucleotides has not been performed.

Adenine Nucleotide Exchange by hMSH2-hMSH3 Is Controlled by Mismatch Recognition—Adenine nucleotide exchange by hMSH2-hMSH3 in the presence of IDL DNA substrates was determined by measuring the exchange of protein bound [³H]ADP for unlabeled ATP (Fig. 4B). Nucleotide exchange was found to be very rapid in the presence of the +CA, +(CA)₄ (t½ ≤ 4 s), and +(CA)₁₂ (t½ = 6 s) IDL mismatched DNAs whereas in the presence of +(CA)₆ (t½ = 20 s) and homoduplex (t½ ≥ 40 s), the exchange was significantly slower. In the absence of DNA, relatively little exchange occurred during the 160-s reaction (t½ > 160 s). These results indicate that the binding of hMSH2-hMSH3 to IDL DNA substrates stimulates the exchange of ADP → ATP resulting in the release from the mismatch and recycling of the protein complex.

The hMSH2-hMSH3 Complex Undergoes Adenosine Nucleotide-regulated Conformational Changes—Protein footprinting by partial proteolysis has been widely performed to determine the structural domains of proteins, protein-protein interaction sites, and conformational changes induced by ligand binding. To detect conformational changes induced by binding of the hMSH2-hMSH3 protein complex to ADP and ATPγS, partial proteolysis was carried out utilizing modified trypsin. We observed different protease accessibility, as exhibited by altered banding patterns on a silver-stained gel, when the hMSH2-hMSH3 complex was bound to magnesium, magnesium/ADP, and magnesium/ATPγS (see arrowheads, Fig. 5A). Western analysis of an identical partial proteolysis experiment (probed with an hMSH2-specific antibody) was also performed (Fig. 5B). By comparing lanes where protein was free of adenosine nucleotide, bound by ADP, or bound by ATP, it was possible to identify several bands that were unique to hMSH2 which were differentially sensitive to proteolytic cleavage (Fig. 5B). This result suggests that the hMSH2-hMSH3 complex undergoes a conformational transition associated with adenosine nucleotide binding that is qualitatively similar to that observed with G proteins. Thus, binding and hydrolysis of ATP by hMSH2-hMSH3 appears to control conformational transitions associated with mispair recognition and perhaps MMR signaling.

DISCUSSION

The most widely accepted model for mismatch repair suggests that MutS and its homologs bind to mismatched nucleotides, which is followed by the association of MutL or its homologs (5). This multiprotein complex is then proposed to perform bi-directional ATP-dependent translocation on the DNA to the site of incision prior to the excision/reassembly repair reaction (40). The results presented here for the hMSH2-hMSH3 heterodimer and in a previous publication for the hMSH2-hMSH6 heterodimer suggest a somewhat simpler model: MutS homologs function as simple adenosine nucleotide-regulated molecular switches (6, 33). In the molecular switch model, mispair recognition provokes adenosine nucleotide exchange much like ligand binding provokes guanosine nucleotide exchange in G protein-coupled receptor systems. As with G proteins, we have demonstrated that adenosine nucleotide
binding and exchange by the MutS homologs triggers a conformational transition that is the likely signal for downstream events. Furthermore, ATP hydrolysis is proposed to recycle the system following signal transduction such that it may recognize another mismatch.

Here we demonstrate that ADP → ATP exchange by hMSH2-hMSH3 is stimulated by IDLs, illustrating the versatility of the eukaryotic mismatch signaling process. Although bacterial MutS is limited to IDLs of no more than 4 nucleotides, the combination of hMSH2-hMSH3 and hMSH2-hMSH6 heterodimers extends the range of mismatch induced signaling from simple mismatched nucleotides to IDLs of up to 16 nucleotides. More importantly, we have demonstrated strong binding of hMSH2-hMSH3 to a 24-nucleotide loop; yet, this IDL tolerates not only simple mismatched nucleotides but also IDLs of up to 16 nucleotides. More importantly, we have demonstrated strong binding of hMSH2-hMSH3 to a 24-nucleotide loop; yet, this IDL induces little or no ATPase activity and does not effectively stimulate ADP → ATP exchange. This finding appears to explain the relative lack of repair of large IDLs (≥16 nucleotides) by the MMR system in vitro or in vivo (22, 31). Together, these results appear to suggest that mismatch binding by the MutS homologs is necessary but not sufficient for mismatch repair.

The mechanism by which different mismatched or IDL nucleotides induces ADP → ATP exchange is unknown. However, it is becoming increasingly clear that MutS homolog function is tied to the ability of a mismatch, lesion, or DNA structure to provoke ADP → ATP exchange. By comparison, ligand binding by G protein-coupled receptors has been proposed to result in receptor conformational transitions that stimulate GDP → GTP exchange by G proteins. Similarly, our results suggest that the well known subtle and overt conformational alterations that distinguish mismatched DNA from homoduplex DNA is the signal(s) that provokes ADP → ATP exchange within the MutS homologs. Moreover, under physiologically relevant conditions, mispair binding is likely to be transient and merely the exchange factor that stimulates exchange of ADP → ATP, which is followed by rapid dissociation from the mismatch. Our work with the hMSH2-hMSH6 mispair recognition complex suggests that the conformational transition induced by ADP → ATP exchange results in the formation of an ATP hydrolysis-independent sliding clamp that remains stably bound to the DNA following dissociation from the mismatch (41). By analogy, it is likely that hMSH2-hMSH3 retains the same function(s) in transducing the mismatch signal to the repair machinery (see Ref. 42 for an alternate model).

A role for MSH2-MSH3 in homologous recombination has also been proposed based on genetic data in S. cerevisiae (43). It is possible that the ATP-independent binding of hMSH2-hMSH3 to large IDLs provides a target for the recombination-repair machinery and/or repair process that is mechanistically different from those provoked by the hMSH2-hMSH6 adenosine nucleotide molecular switch (33).

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