A Mutation in the MSH6 Subunit of the *Saccharomyces cerevisiae* MSH2-MSH6 Complex Disrupts Mismatch Recognition*

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In yeast, MSH2 interacts with MSH6 to repair base pair mismatches and single nucleotide insertion/deletion mismatches and with MSH3 to recognize small loop insertion/deletion mismatches. We identified a *msh6* mutation (*msh6-F337A*) that when overexpressed in wild type strains conferred a defect in both MSH2-MSH6- and MSH2-MSH3-dependent mismatch repair pathways. Genetic analysis suggested that this phenotype was due to *msh6-F337A* sequestering MSH2 and preventing it from interacting with MSH3 and MSH6. In UV cross-linking, filter binding, and gel retardation assays, the MSH2- *msh6-F337A* complex displayed a mismatch recognition defect. These observations, in conjunction with ATPase and dissociation rate analysis, suggested that MSH2-*msh6-F337A* formed an unproductive complex that was unable to stably bind to mismatch DNA.

DNA mismatches can arise through DNA replication errors, physical damage, and heteroduplex formation during genetic recombination. If left unrepaird, these mismatches become fixed in the genome as mutations. The best understood mismatch repair system is the *Escherichia coli* mutHLS long-patch repair pathway (1). A model for the initiation of mismatch repair by MutH, MutL, and MutS immediately after passage of a DNA replication fork has been developed based upon *in vivo* studies and an *in vitro* mismatch repair system reconstituted from purified components (1–6). In this model, a mismatch is first recognized and bound by a dimer of MutS that displays an intrinsic ATPase activity. In a reaction that requires ATP, a dimer of MutL binds to MutS and then activates the methylation sensitive endonuclease MutH. Activation of MutH results in cleavage of the unmethylated DNA strand at hemimethylated d(GATC) sites that are transiently present after replication fork passage, providing an entry point for excision and replication proteins to remove the mismatch and repair the resulting DNA gap using the parental DNA strand as a template.

The ability of MutS to both recognize base pair mismatches and trigger downstream events that can be located several kilobases away from a mismatch site suggests that it can bind to DNA in at least two different modes. The first mode allows mismatch recognition, and the second mode allows MutS protein to use the energy of ATP hydrolysis to translocate along DNA with MutL so that it can activate MutH at GATC sites (1, 7, 8). Support for the presence of multiple MutS DNA binding modes was initially obtained from DNA binding assays showing that MutS protein can specifically recognize base pair mismatches in the absence of ATP and that the addition of ATP resulted in the loss of mismatch binding specificity (8). An elegant electron microscopy analysis performed by the Griffith laboratory (7) and the Modrich laboratory (9) provides further evidence for this idea. They showed that MutS can form ATP-dependent loop structures on DNA substrates that contain a mismatch site. The size of the loop was dependent on incubation time and the presence of a mismatch site. They hypothesized that MutS can bind to a mismatch substrate in an ATP independent step. After recognition, a second binding mode is activated through an ATP-dependent conformational change in MutS resulting in the loss of its mismatch recognition functions and a shift into a mode that allows it to bidirectionally translocate along DNA away from a mismatch site. This activity results in the formation of loop structures that are thought to serve as topological intermediates for strand excision and allow MutS to scan along DNA until it identifies and activates MutH bound at GATC sites.

At present, little is known about which domains in MutS are important for mismatch recognition. Recently, a DNA cross-linking analysis performed by Malkov et al. (10) revealed that the phenylalanine 39 residue in *Thermus aquaticus* MutS was critical for photocross-linking of MutS to a mismatch substrate. They also showed that a mutant derivative of MutS, mutS-F39A, displayed a reduced affinity for mismatch substrate. Although this study identified a domain that is important for mismatch recognition, it did not address whether this domain functions in general DNA binding and/or in mismatch binding and whether it functions during one or more DNA binding modes.

Whereas studies in bacteria have greatly increased our understanding of mismatch repair, analogous repair systems in eukaryotes appear to be more complex (1, 11, 12). Several homologs of *mutS* and *mutL* have been identified in eukaryotic organisms. A feature of the MutS homologs is they all contain a highly conserved ATP binding domain. In the yeast *Saccharomyces cerevisiae*, six *mutS* homologs and four *mutL* homologs have been identified, and the gene products of the *MSH2, MSH3, MSH6, MLH1*, and *PMS1* genes have been identified as components of nuclear mismatch repair. Several studies have suggested that during mismatch recognition, MSH2 acts a scaffold for mismatch binding, whereas MSH6 and MSH3 act as specificity factors (13–17). This hypothesis was based on genetic studies showing that *msh2* mutants are defective in the repair of both base pair and loop insertion/deletion mismatches, whereas *msh3* mutants are principally defective in...
were obtained by determining the median frequency of frameshift forward mutations to canavanine resistance and DNA slippage rates formed with pEAE9 and pEAE88, and msh2-Y42A-MSH6 was from pRS424 (13, 15). Strains were transformed with pRS424 and purified from the Yeast Genetic Stock Center and was used for the overexpression experiments described in this paper. Dominance, complementation, and suppression studies were performed using the S. cerevisiae strain FY23 (MATa, ura3–52, leu2Δ1, trp1Δ63) (23) and msh2Δ-MSH6 (EAY28-MATa, ura3–52, leu2Δ1, trp1Δ63, msh2Δ::hisG) derivatives. Strain were transformed using the lithium acetate method (24) with the following episcopal vectors individually or in combination: pEAE51 (GAL10-MSH6, TRP1, 2 μ) (13), pEAE84 (GAL10-MSH6:G987D, TRP1, 2 μ) (15), pEAE20 (GAL10-MSH6, URA3, 2 μ) (25), pEAE86 (GAL10-MSH6, TRP2, 2 μ) (15), pEAE95 (GAL10-MSH6, URA3, 2 μ; this paper), pEAE82 (GAL10-MSH6, LEU2, 2 μ; this paper), pEAE89 (GAL10-MSH6:F337A/G987D, TRP1, 2 μ; this paper), pP2 (GAL10, URA3, 2 μ; kindly provided by Arlen Johnson), pRS424 (TRP1, 2 μ) (26), and pEAE91 (GAL10-msh6-Y42A, URA3, 2 μ; this paper). The S. cerevisiae strain BJ5464 (MATa, ura3–52, trp1, leu2Δ1, his32300, pep4::HIS3, pro31Δ16R, can1, GAL) was obtained from the Yeast Genetic Stock Center and was used for the overexpression and purification of MSH2-MSH6 and the mutant derivative complexes. MSH2-MSH6 complex was purified from BJ5464 transfected with pEAE9 and pEAE51, MSH2-msh6-F337A was from BJ5464 transformed with pEAE9 and pEAE84, and msh2-Y42A-MSH6 was from BJ5464 transformed with pEAE90 and pEAE51. Mutation rates were obtained after determining the median frequency of frameshift events (of 11 colonies) that resulted in resistance to 5-fluoroorotic acid (5-FOA)3 in FY23-derived strains containing pAAE69 (TG)9-T-URA3, ARSH4, CEN6, LEU2 (15, 27, 28).

Media, Reagents, and Chemicals—E. coli strains were grown in LB broth or on LB agar that was supplemented with 100 μg/ml ampicillin when required (29). Yeast strains were grown in either YPD or minimal selective media (30). 2% glucose, 2% sucrose, 3% glycerol, 2% lactate, and 2% galactose were included as carbon sources as indicated. When required, canavanine (Sigma) was included in minimal selective media lacking arginine at 60 mg/liter. 5-FOA (U. S. Biological, San Antonio, TX) plates were prepared as described (30). Polyclonal antibodies raised against MSH2 and MSH6 were obtained as described previously (15). Recombinant human MSH2 and MSH6 to create the msh2-Y42A, msh6-F337A, msh6-G335D, and msh6D343A alleles was performed using overlapping polymerase chain reaction mutagenesis (31). Oligonucleotide synthesis and double-stranded DNA sequencing of the entire subcloned fragments used to make the msh2 and msh6 mutant alleles were performed at the Cornell Biotechnology Analytical-Synthesis Facility (Ithaca, NY). All restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase, T4 DNA polymerase, and Vent polymerase were from New England Biolabs (Beverly, MA) and used according to the manufacturers’ specifications. Plasmid DNA was isolated by alkaline lysis, and all DNA manipulations were performed as described previously (32).

Oligonucleotides Used In DNA Binding Studies—Duplex oligonucleotide DNA substrates used in filter binding, ATPase and UV cross-linking studies were created from the following oligonucleotides: 1) 5’-ATGTTGATCTGATGTTGATCTGCTGAAAGATAT-3’, 2) 5’-ATTCCCCAGCGGATGAAACCTAATCATTACCATAT-3’, 3) 5’-ATGTTGATCTGATGTTGATCTGCTGAAAGATAT-3’, 4) 5’-ATTCCCCAGCGGATGAAACCTAATCATTACCATAT-3’, and 5) 5’-ATTCCCCAGCGGATGAAACCTAATCATTACCATAT-3’. All oligonucleotides were synthesized by Operon (Alameda, CA). In oligonucleotide 4, X refers to the position of an iododeoxyuridine residue. Iododeoxyuridine (5′-IUrD) substituted derivatives were synthesized by Operon using 5′-I-Deoxyribose phosphatephoramide (Glen Research Corp.). 5′-IUrD oligonucleotides were shielded from light and handled according to Operon specifications. Homoduplex and +1 mismatch DNA substrates (13) used in the filter binding and ATPase studies were created by annealing oligonucleotides 1 and 2 and oligonucleotides 3 and 2, respectively (32). In the UV cross-linking studies, the AT-I homoduplex substrate was formed by annealing oligonucleotides 4 and 5, and the Δ1-I mismatch substrate was formed by annealing oligonucleotides 4 and 6. Duplex DNA substrates were 5′-P-labeled by 5′-end labeling oligonucleotides with γ[32P]ATTP and T4 polynucleotide kinase prior to the annealing reaction.

Biochemical Techniques—Overexpression and purification of MSH2-MSH6 and msh2-Y42A-MSH6 was performed as described previously (13, 15). MSH2-msh6-F337A and MSH2-msh6-G335D were purified using the same procedure with the exception that these purifications were performed on an 100 ml NaCl Buffer A (25 mM Tris, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) instead of the 200 mM salt conditions used to load and wash the MSH2-MSH6 complex. In cells extracts obtained from overexpression strains, the levels of soluble wild type and mutant MSH2-MSH6 proteins were indistinguishable and were found to be equivalent to 3% of total protein. This was determined in SDS-PAGE by directly comparing the intensity of MSH2 and MSH6 bands in crude extracts to known amounts of purified complex. Through Western blot analysis, we determined that the number of MSH2 monomers per wild type mid-log cell was approximately 400; a 700-fold increase in this level was observed in strains that overexpressed MSH2 using the GAL10 promoter.4 The purity of protein preparations was monitored by SDS-PAGE (8% gels) (33) (Fig. 2A). The approximate molecular weight of the MSH2-MSH6 and MSH2-msh6-F337A was determined by measuring the time required to elute polypeptides from a Superose 6HR gel filtration column as described previously (13). This analysis was kindly performed by William Enslow at the Cornell Biotechnology Analytical-Synthesis Facility.

UV Cross-linking—In the UV cross-linking studies, 120 ml (1.5 μg) MSH2-MSH6 or msh2-msh6-F337A was incubated in 50-μl reactions for 15 min at 30 °C with 20 μM 5′-P-labeled duplex DNA substrate in buffer containing 25 mM Tris, pH 7.5, 0.01 mM EDTA, 0.1 mM dithiothreitol, and 2.0 mM MgCl2. The immunoprecipitation reactions presented in Fig. 1B and the cross-linking reactions presented in Fig. 4A were performed in the absence of MgCl2. Samples were then transferred to 100 μl of a Rayonet Photochemical reactor, model RPR100 (Branford, CT) equipped with 350 nm bulbs for 0–60 min of reaction time. After UV irradiation, samples were immediately boiled for 3 min in SDS-PAGE sample buffer and loaded onto an 8% SDS-PAGE gel for analysis by gel electrophoresis. Following

3 The abbreviations used are: 5-FOA, 5-fluoroorotic acid; hMSH, human MSH; PAGE, polyacrylamide gel electrophoresis; 5′-IUrD, iododeoxyuridine; ssDNA, single-stranded DNA.

4 T. Sokolowski and E. Alani, unpublished data.
electronophoresis, the SDS-PAGE gel was stained with Coomassie Blue, dried, and visualized using a phosphorimager screen. Immunoprecipitations of denatured cross-linked samples were carried out using methods described by Iaccarino et al. (17). 10 μl of cross-linked protein-DNA samples were boiled for 5 min in SDS sample buffer (New England Biolabs) and then added at a 10% final concentration to reactions containing anti-MSH2, anti-MSH6, or no antibody suspended in 0.1 M NaCl Buffer A. After a 45-min incubation at 4 °C, 30 μl of protein A-Sepharose were added to each reaction followed by another 45-min incubation. Protein A beads were spun down at 2500 rpm for 45 s. Supernatant was removed from the beads, which were then washed with 200 μl of 0.1 M NaCl Buffer A. Beads were spun again, supernatant removed, and the beads were resuspended in SDS sample buffer, boiled for 5 min, and loaded onto SDS-PAGE gels. All gels were visualized using a phosphorimager screen (Molecular Dynamics), and bands were quantified using Mac IQ Imagequant software.

**DNA Binding, Dissociation, and ATPase Assays**—DNA filter binding assays were performed as described previously (25). The standard buffer for the DNA binding assay contained 25 mM Tris, pH 7.5, 0.1 mM dithiothreitol, 0.01 mM EDTA, and 40 μg/ml bovine serum albumin. When indicated, ATP was added to a final concentration of 1.5–2.0 mM. Binding was performed at 30 °C for 15 min in a 60-μl reaction. This time point was chosen because maximal binding for MSH2-MSH6 to oligonucleotide substrate was observed at 15 min, and no change in binding was observed in incubations that ranged from 15 to 90 min. Each reaction contained 16.7 nM 32P-labeled +1 substrate, 25 mM Tris, pH 7.5, and 0.1 mM dithiothreitol. Unlabeled +1 and homoduplex substrates were added as competitor as indicated in Fig. 3. After incubation, samples were analyzed by filter binding to KOH-treated oligonucleotide substrate was observed at 15 min, and no change in time point was chosen because maximal binding for MSH2-MSH6 to incubation. Protein A-Sepharose were added to each reaction followed by another 45-min incubation. Protein A beads were spun down at 2500 rpm for 45 s. Supernatant was removed from the beads, which were then washed with 200 μl of 0.1 M NaCl Buffer A. Beads were spun again, supernatant removed, and the beads were resuspended in SDS sample buffer, boiled for 5 min, and loaded onto SDS-PAGE gels. All gels were visualized using a phosphorimager screen (Molecular Dynamics), and bands were quantified using Mac IQ Imagequant software.

**RESULTS**

**UV Cross-linking Indicates That Both MSH2 and MSH6 Are Cross-linked to a Duplex Oligonucleotide at the Site of a Mismatch**—Previous studies have led to the proposal that the MSH6 subunit of the *S. cerevisiae* MSH2-MSH6 complex, which is principally involved in repairing base-base and single insertion/deletion mismatches, acts as a mismatch recognition specificity factor for MSH2 (13–15). A similar proposal for the human mismatch repair proteins, based on UV cross-linking analysis of hMSH2-hMSH6 to mismatch substrates, was made by Iaccarino et al. (17), in which it was found that only the hMSH6 subunit could be cross-linked to a mismatch substrate. Recently, Malkov et al. (10) demonstrated that mismatch DNA substrates containing an iododeoxyuridine residue (5-IdUrd) at the site of the mismatch could be specifically cross-linked to *T. aquaticus* MutS at phenylalanine 39, which is located in a region that is highly conserved among MutS homologs. Malkov et al. (10) also showed that a substitution of alanine at this position (mutS-F39A) abolished binding of MutS to mismatch substrates.

We performed a similar analysis to identify residues involved in yeast MSH2-MSH6 mismatch recognition. Of the 38-mer homoduplex (AT-1) and single nucleotide loop substrates (Δ1-I), were synthesized that contained single 5-IdUrd substitutions at the indicated thymine residues (see under “Experimental Procedures”). In the Δ1-I substrate, the 5-IdUrd substitution constitutes an extrahelical loop of one nucleotide (see under “Experimental Procedures”). In filter binding competition assays (see below), MSH2-MSH6 displayed a 10-fold binding specificity for the Δ1-I substrate compared with the AT-1 substrate: this specificity was similar to that observed with +1 and homoduplex substrates tested previously (see Fig. 3A and data not shown).

UV-dependent cross-linking of mismatch and homoduplex substrates to the MSH2-MSH6 complex was observed by incubating 32P-labeled Δ1-I or AT-1 substrate with MSH2-MSH6 under standard binding conditions (Fig. 1; see under “Experimental Procedures”). After incubation, samples were irradiated with UV light for 0–60 min, resolved by SDS-PAGE, and then exposed to film to detect radioactively labeled protein-DNA complexes. As shown in Fig. 1A, in binding reactions involving either the Δ1-I or the AT-1 substrates, a major band migrating at ~190 kDa (Band B) was observed that was dependent on MSH2-MSH6 and UV exposure and migrated in SDS-PAGE slightly above the position of MSH6 (142 kDa). The size of the labeled complex was consistent with a Δ1-I or AT-1 substrate (~30 kDa) cross-linked to MSH6 (142 kDa). At UV exposures 15 min and longer, a band migrating at greater than 205 kDa (Band A) was observed, as well as a faint band migrating at ~116 kDa (Band C). At 30 min of UV exposure, a cross-linking efficiency of 17% (as measured by densitometry) was observed in the Δ1-I reactions; this cross-linking efficiency was 7-fold higher than that found in reactions involving the AT-1 substrate.

Immunoprecipitation analysis was performed on denatured cross-linked species to determine whether Bands A–C contained MSH2 or MSH6. As shown in Fig. 1B, Band B was recognized and immunoprecipitated only by MSH6 antibody (lane 2), and Band C was immunoprecipitated only by MSH2 antibody (lane 3). Band A was recognized by both MSH2- and MSH6-specific antibodies, suggesting that this species contained cross-links that physically joined all three components of the MSH2-MSH6-Δ1-I complex. It is important to note that in addition to Bands A–C, lower intensity bands were observed in the cross-linking reactions shown in Fig. 1A; based on immunoprecipitation analysis and the mobility of these bands relative to Bands A–C in SDS-PAGE, we hypothesize that they represent complexes containing photocleavage products of the 5-IdUrd DNA substrates.

The msh6-F337A Mutation Conferred a Mismatch Repair Defect—We constructed alanine substitutions at residue 42 in MSH2 (msh2-Y42A) and residue 337 in MSH6 (msh6-F337A) based on the fact that these residues can be aligned with the *T. aquaticus* MutS Phe-39 residue (Fig. 2A) (10). The msh2-Y42A and msh6-F337A alleles were cloned into expression plasmids; galactose induction studies indicated that each mutant was overexpressed to levels similar to that observed for the corresponding wild type proteins (Fig. 2B and data not shown). The effect of these mutations was then assessed for defects in mismatch repair.

The msh2-Y42A and msh6-F337A alleles were tested to de-
MSH2-MSH6 Mismatch Recognition

FIG. 1. MSH6 specifically cross-links to a Δ1-I mismatch substrate containing an iodouracil residue at the site of the mismatch. A, SDS-PAGE analysis of MSH2-MSH6 complexes cross-linked to mismatch substrates. 20 nm [32P]-labeled Δ1-I and AT-I homoduplex substrates were incubated in the presence or absence of 100 nm MSH2-MSH6 complex for 15 min at 30 °C as described under "Experimental Procedures." Samples were irradiated with 350 nm UV light as indicated, subjected to SDS-PAGE (8% gels), and visualized using a phosphorimager. Lanes 1–5 display the indicated control reactions. Lanes 6 and 7 indicate the position of Coomassie Blue-stained MSH2-MSH6 complex and molecular weight standards (205, 116, 97, 66, 43 kDa), respectively. MSH2 and MSH6 are 109 and 142 kDa, respectively. Lanes 8–15 display a UV irradiation time course for MSH2-MSH6 binding to the Δ1-I (lanes 8–11) and AT-I (lanes 12–15) substrates. B, immunoprecipitation analysis. 32P-Labeled Δ1-I heteroduplex was incubated with MSH2-MSH6 as above and irradiated with 350 nm UV light for 15 min. UV-irradiated samples were denatured and immunoprecipitated with MSH6-specific (lane 2) or MSH2-specific (lane 3) antibodies, and the immunoprecipitate was subjected to SDS-PAGE (8% gels) (see under "Experimental Procedures"). Lane 1 displays the autoradiogram of a cross-linked sample that was directly loaded onto SDS-PAGE gels, and lane 4 displays the results of an immunoprecipitation reaction performed with cross-linked sample in the absence of antibody. Bands A–C are described in the text.

term whether they can complement the mutator phenotype exhibited by msh2Δ and msh6Δ strains, respectively (14). In an assay that assesses mutation rate (principally base-base and single nucleotide insertion/deletion mutations) through the acquisition of resistance to the arginine analog canavanine, msh2Δ strains exhibit a 13–20-fold higher mutation rate than either wild type strains or msh2Δ strains that overexpress MSH2 (14, 38, 39). The msh2-Y42A allele fully complemented the msh2Δ mutator phenotype when its protein product was expressed in both GAL10 promoter inducing (galactose) and repressing (glucose) conditions (Table I) (10) (data not shown). These data indicated that the msh2-Y42A mutation does not disrupt mismatch repair. In the same assay, the msh6-F337A allele failed to complement the modest mutator phenotype exhibited in msh6Δ strains and instead conferred a mutation rate equivalent to that observed in msh2Δ strains (a 20-fold increase for a msh6Δ strain containing pGAL10-msh6-F337A versus a 3.8-fold increase for a msh6Δ strain containing a dummy plasmid).

The msh6-F337A Mutation Confers a Dominant Negative Mutator Phenotype That Is Similar to the Mutator Phenotype Observed in msh2Δ Strains—Recently, Studamire et al. (15) analyzed a mutation in the MSH6 ATP binding domain (msh6-G987D) that severely inhibited the ATPase activity of the MSH2-MSH6 complex. When overexpressed in wild type strains, the msh6-G987D allele conferred a weak dominant negative mutator phenotype that was similar to the phenotype found in msh6Δ strains, as it was limited to defects in the MSH2-MSH6 repair pathway (Table I) (15). In contrast, when the msh6-F337A allele was similarly overexpressed in wild type strains, a mutator phenotype was observed that was similar to that observed in msh2Δ strains. In the canavanine assay, wild type strains overexpressing msh6-F337A displayed a 21-fold higher mutation rate than strains lacking this plasmid and a 5-fold higher rate than msh6Δ strains or wild type strains that overexpressed msh6-G987D (Table I). In DNA slippage assays (26) that detect 2–4 insertion/deletion mismatches formed in an in-frame (TG)16T cassette placed within the URA3 gene, msh2Δ strains exhibited an approximately 17-fold higher rate of slippage events, as measured by resistance to 5-fluoroorotic acid, than wild type. msh6Δ strains or a wild type strain overexpressing MSH6 displayed DNA slippage frequencies that were indistinguishable from wild type. However, strains overexpressing msh6-F337A displayed a DNA slippage rate that was similar to that observed in msh2Δ strains (Table I). It is important to note that the overexpression of all msh6 single and double mutant protein combinations in this study was similar to that observed for overexpression of wild type MSH6 (data not shown).

MSH2 Overexpression Suppresses the Dominant Negative Phenotype Conferred by Overexpressing msh6-F337A—As described above, the msh6-F337A mutation conferred a dominant negative phenotype that was similar to the mutator phenotype observed in msh2Δ strains. We hypothesized that this resulted from msh6-F337A sequestering MSH2 in an inactive complex that was prevented from interacting with either MSH6 or MSH3 subunits. We tested this idea by performing canavanine mutator assays on wild type strains overexpressing the msh6-F337A, MSH2, and MSH6 subunits individually or in combination. As shown in Table I, co-overexpression of MSH2 or MSH6 with msh6-F337A fully suppressed the dominant negative phenotype observed in strains that only overexpressed msh6-F337A, consistent with the idea that MSH2 was sequestered from interacting with MSH3 or MSH6. This experiment, however, did not rule out the possibility that excess MSH2 suppressed the dominant negative phenotype conferred by the
msh6-F337A allele by suppressing its mismatch repair defect. To test this, mutator assays were performed in a msh6Δ strain that overexpressed both MSH2 and msh6-F337A. As shown in Table I, this strain displayed a mutation rate that was similar to that observed in msh6Δ strains, indicating that overexpression of MSH2 cannot suppress the msh6-F337A mismatch repair defect to achieve wild type mismatch repair function.

Analysis of msh2-Y42A-MSH6 and MSH2-msh6-F337A Complexes—MSH2-msh6-F337A and msh2-Y42A-MSH6 complexes were purified as described in Fig. 2B and under “Experimental Procedures.” The complexes each contained a subunit stoichiometry that was similar to the wild type complex, suggesting that the site-specific mutations did not compromise interactions between subunits or disrupt the overall structure of the mutant proteins. Approximately the same yield of purified protein per unit of weight of induced cells was observed for each of the wild type and mutant heterodimers, and coimmunoprecipitation and gel filtration analysis indicated that the mutant complexes behaved similarly to wild type in these assays and formed stable heterodimers (data not shown) (13, 15).

In ATPase assays performed in the absence of DNA, the ATPase activities of MSH2-msh6-F337A and MSH2-MSH6 were identical (see Fig. 5) (15) (Km and Vmax were identical; data not shown). The msh2-Y42A-MSH6 complex was indistinguishable from wild type in all genetic and biochemical assays described in this report (data not shown).

We obtained evidence that the MSH2-msh6-F337A complex was defective in DNA binding during the purification of the complex. Under conditions where the wild type complex was retained on a ssDNA cellulose column (200 mM NaCl), the MSH2-msh6-F337A complex was not retained. The purification procedure for the MSH2-msh6-F337A complex was then modified by loading MSH2-msh6-F337A fractions onto the ssDNA column at 100 mM NaCl (see under “Experimental Procedures”). To better quantify the binding of MSH2-MSH6 and MSH2-msh6-F337A complexes to DNA containing an insertion mismatch or homoduplex DNA, filter binding assays were performed with 111 and homoduplex DNA substrates (Fig. 2C; Refs. 13 and 33; see under “Experimental Procedures”). The 37-mer homoduplex substrate is identical to the 111 substrate with the exception that the 111 substrate contains an adenine insertion after base pair 15. In this assay, increasing amounts of protein were added to a constant amount (16.7 nM) of 32P-labeled 111 or homoduplex DNA. As seen in Fig. 2C, MSH2-MSH6 binding to 111 substrate was maximal at 3.6 pmol (60 nM) of protein with nearly 50% of total counts bound. In contrast, less than 10% of total counts were bound in a binding reaction containing 60 nM MSH2-msh6-F337A complex and...
either homoduplex or +1 substrate. In addition to a general DNA binding defect, the MSH2-msh6-F337A complex appeared to display a defect in discriminating between +1 and homoduplex substrates. At 0.6 pmol (10 nM) of complex, 33 and 17% of +1 and homoduplex substrate, respectively, were bound to MSH2-MSH6 complex. In contrast, 8.5 and 7.5% of +1 substrate was an approximately 20-fold more effective competitor than homoduplex substrate. In reactions involving MSH2-msh6-F337A, a discrete gel shift was not observed (Fig. 2C and data not shown).

**MSH2-msh6-F337A Is Defective in Both Mismatch Binding and in General DNA Binding**—To further explore the mismatch recognition properties of wild type and mutant complexes, the DNA binding activity of MSH2-MSH6 and MSH2-msh6-F337A were analyzed in filter binding and gel retardation competition assays. In the filter binding assays (Fig. 3, A and B) 20 nM (0.3 μg) MSH2-MSH6 or MSH2-msh6-F337A complex was incubated with 16.7 nM 32P-labeled +1 substrate in the presence and absence of various concentrations of unlabeled +1 or homoduplex competitor. In these reactions the molar ratio of protein to DNA substrate was approximately 1:1 and was chosen based on the binding titrations shown in Fig. 2C. Mismatch binding between the two competitors was measured by determining the maximal horizontal separation between the binding curves (13, 33). As shown for the MSH2-MSH6 complex in Fig. 3A, the +1 substrate is an approximately 8-fold more effective competitor than homoduplex substrate; this result is consistent with that found previously (13, 15). In competitions involving the MSH2-msh6-F337A complex (Fig. 3B), the +1 and homoduplex competitions were similar and suggested a dramatic decrease in mismatch binding specificity compared with the MSH2-MSH6 complex. A loss in mismatch binding specificity was also observed in MSH2-MSH6 binding experiments performed in the presence of 2.0 mM ATP; ATP addition was previously shown to eliminate mismatch recognition (13) (data not shown).

In gel retardation assays, 360 nM (0.9 μg) MSH2-MSH6 or MSH2-msh6-F337A complex was incubated with 10 nM 32P-labeled +1 substrate in the presence and absence of various concentrations of unlabeled +1 or homoduplex competitor substrates. As shown in Fig. 3, D and E, in the absence of unlabeled competitor, MSH2-MSH6 completely retarded the mobility of the 32P-labeled +1 substrate; in competition reactions, the +1 unlabeled substrate was an approximately 20-fold more effective competitor than homoduplex substrate. In reactions involving MSH2-msh6-F337A, a discrete gel shift was not observed; instead, a diffuse gel shift pattern was observed that spanned from the position of unbound 32P-labeled +1 substrate to the position of the MSH2-MSH6+1 complex (Fig. 3D).

**ATP Enhances UV Cross-linking and Binding of MSH2-msh6-F337A to DNA**—UV DNA cross-linking studies were performed to examine in greater detail the mismatch binding properties of the wild type and MSH2-msh6-F337A complexes. These experiments were carried out as described under “Experimental Procedures.” 24 nM (0.3 μg) MSH2-MSH6 or MSH2-msh6-F337A was incubated with 20 nM 32P-labeled Δ1-1 DNA at 30 °C for 15 min. UV-irradiated for 15 min, electrophoresed on 8% SDS-PAGE gels, and then quantified by using a phosphorimager (see under “Experimental Procedures”). As shown in Fig. 4A, the MSH2-msh6-F337A complex displayed a 6-fold lower level of cross-linking to the Δ1-1 substrate compared with MSH2-MSH6 (Fig. 4A, lanes 1 and 2). When 1.5 mM ATP was included in the binding reaction prior to UV cross-linking, a 2.7-fold reduction in the level of MSH2-MSH6 cross-linking to the Δ1-1 substrate was observed (lane 4). However, in the MSH2-msh6-F337A reactions, ATP enhanced cross-linking of the complex to the Δ1-1 substrate by 2.5-fold (Fig. 4A, lanes 1 and 3).

In filter binding assays, we compared the effect of 1.5 mM ATP on the binding of +1 and homoduplex DNA by the MSH2-

### Table I

Average rates of spontaneous mutations and DNA slippage events in msh2Δ, msh6Δ, and wild type strains bearing the msh6-Y42A, msh6-F337A, and msh6-G987D alleles on GAL10, 2μ plasmids

| Strain relevant genotype | GAL10, 2μ vector relevant gene(s) | Canavamine resistance assay | (TG)16T tract alteration assay |
|--------------------------|---------------------------------|-----------------------------|-----------------------------|
|                          | (x 10^-5)                       | Relative to wild type       | Relative to wild type       |
| msh2Δ                    | MSH2                            | 6.2 ± 3.0                   | NT                          |
| msh2Δ                    | None                            | 19 ± 5.3                    | 13                          |
| msh2Δ                    | msh2-Y42A                       | 4.1 ± 1.7                   | 0.7                         |
| msh6Δ                    | MSH6                            | 14 ± 10                     | 2.3                         |
| msh6Δ                    | None                            | 24 ± 2.6                    | 3.8                         |
| msh6Δ                    | msh6-F337A                      | 122 ± 60                    | 20                          |
| msh6Δ                    | MSH2                            | 29 ± 5.0                    | 4.7                         |
| msh6Δ                    | msh6, msh6-F337A                | 31 ± 5.9                    | 5.0                         |
| Wild type                | MSH2                            | 5.5 ± 3.2                   | 0.9                         |
| Wild type                | MSH6                            | 14 ± 12                     | 2.2                         |
| Wild type                | msh6-F337A                      | 132 ± 29                    | 21                          |
| Wild type                | msh6-G987D                      | 25 ± 8.3                    | 4.1                         |
| Wild type                | MSH6, msh6-F337A                | 8.0 ± 6.6                   | 1.3                         |
| Wild type                | MSH2, msh6-F337A                | 10 ± 4.1                    | 1.6                         |

* NT, not tested.

Data from Studamire et al. (15).
MSH2-MSH6 Mismatch Recognition

MSH6 and MSH2-msh6-F337A complexes (Fig. 4B). In reactions containing 16.7 nM +1 substrate and 20 nM (0.3 μg) MSH2-MSH6, 34% of total 32P-labeled +1 substrate was bound; in identical reactions containing MSH2-msh6-F337A, 7% total binding was observed. The addition of ATP to the MSH2-MSH6 +1 binding reaction resulted in a significant reduction in binding from 34 to 25% (Fig. 4B). In reactions involving MSH2-msh6-F337A, the addition of ATP resulted in an increase in +1 substrate binding from 7 to 14%. In binding reactions involving homoduplex DNA that were performed under the same conditions, the addition of ATP to either the MSH2-MSH6 or MSH2-msh6-F337A reactions resulted in both cases in an increase in homoduplex binding (Fig. 4B). These experiments were performed at several different ATP concentrations with qualitatively similar results (data not shown). Taken together, the filter binding results are consistent with those obtained in the UV-cross-linking experiments.

Mismatch Substrates Specifically Stimulate the ATPase Activity of MSH2-MSH6-FA337 While Inhibiting the ATPase Activity of MSH2-msh6-F337A—Previous analysis of the yeast MSH2-MSH6 complex indicated that under low salt conditions (<50 mM NaCl), the ATPase activity of MSH2-MSH6 was reduced in the presence of homoduplex DNA and reduced even further in the presence of +1 substrate (15, 39) (Fig. 5). Genetic analysis of the msh6-F337A mutation and UV cross-linking analysis of the MSH2-msh6-F337A complex encouraged us to examine the effect of DNA substrate on the ATPase activity of the MSH2-msh6-F337A complex. As described earlier, in the absence of DNA, the ATPase activity of MSH2-msh6-F337A was identical to that observed for the MSH2-MSH6 complex (Fig. 5B). However, unlike the MSH2-MSH6 complex, +1 substrate addition (167 nM) to the MSH2-msh6-F337A ATPase reaction resulted in a nearly 4-fold increase in its ATPase activity (Fig. 5). The addition at 167 nM single-stranded oligonucleotide or a mismatch substrate (+2) that is not recognized by the MSH2-MSH6 complex (13) did not affect the ATPase activity of the MSH2-msh6-F337A complex, suggesting that the enhanced ATPase activity was not the result of a contaminating activity such as that conferred by a helicase (data not shown).

The stimulation of the MSH2-msh6-F337A ATPase activity by the +1 substrate was further investigated in experiments performed at a constant concentration of ATP (33 μM) and varying amounts of +1 or homoduplex substrate. As shown in Fig. 5C, at all concentrations tested, the +1 substrate displayed a stronger inhibition of MSH2-MSH6 ATPase activity than the homoduplex substrate, and increasing concentrations of either DNA substrate resulted in a gradual reduction in ATPase activity (Fig. 5). In contrast, increasing concentrations of homoduplex DNA had no effect on the ATPase activity of the MSH2-msh6-F337A complex; however, the +1 substrate displayed a concentration-dependent effect on ATPase activity. At low +1 substrate concentrations, the +1 substrate stimulated the ATPase activity of the MSH2-msh6-F337A complex with maximal stimulation observed at 167 nM +1 substrate. At +1 substrate concentrations greater than 167 nM, the ATPase activity progressively decreased; at 660 nM +1 substrate, the ATPase activity of the MSH2-msh6-F337A complex was inhibited to a level below that observed with homoduplex substrate.
tions performed in the absence (lane 1), and presence (lane 3) of 1.5 mM ATP. Lanes 2, 4, and 5, MSH2-MSH6 cross-linking reactions performed in the absence (lane 2) and presence (lane 4) of 1.5 mM ATP and in the absence of UV irradiation (lane 5). Bands A–C are described in the text. B, filter binding analysis of MSH2-MSH6 (2-6) and MSH2-msh6-F337A (2-6FA) complexes incubated in the presence (hatched columns) or absence (filled columns) of 1.5 mM ATP. DNA binding assays were performed by filter binding as described under “Experimental Procedures” with 16.7 nM 32P-labeled +1 substrate and 20 nM MSH2-MSH6 or MSH2-msh6-F337A. Duplicate experiments were performed, and the difference between the two values is shown with error bars.

Studamire et al. (15) showed that the ATPase activity of complexes defective in the MSH2 ATPase was still modulated by mismatch and homoduplex substrates; this modulation, however, was not observed in complexes deficient in the MSH6 ATPase activity. To test whether the enhancement of the MSH2-msh6-F337A ATPase activity by +1 substrate was due to the ATPase activity of the msh6-F337A subunit, we purified a complex containing both the msh6-F337A mutation and a msh6 mutation (msh6-G987D) that was shown previously to severely inhibit the MSH6 ATPase activity (15). MSH2-msh6-F337A/G987D complex was purified as described for MSH2-msh6-F337A. A similar yield, purity, and subunit ratio were obtained for purified MSH2-msh6-F337A/G987D compared with MSH2-MSH6 and MSH2-msh6-F337A (data not shown). We also found that the ratio of MSH2 to MSH6 in immunoprecipitations involving MSH2-specific antibody and crude extracts was indistinguishable for wild type and MSH2-MSH6 and MSH2-msh6-F337A (data not shown). In DNA binding assays, the MSH2-msh6-F337A/G987D complex displayed a defect in mismatch binding that was indistinguishable from that observed for the MSH2-msh6-G987D complex characterized previously (15). The ATPase activity of the MSH2-msh6-F337A/G987D complex was unaffected by the presence of either homoduplex or +1 substrate; this result is consistent with the MSH6 ATPase playing an important role in the enhancement of the MSH2-msh6-F337A ATPase by +1 substrate and also supports the idea that the MSH6 ATPase plays a critical role in signaling mismatch recognition (15).

Dissociation of the MSH2-MSH6 and MSH2-msh6-F337A-Oligonucleotide Complexes—The finding that MSH2-msh6-F337A was defective in mismatch binding but was capable of detecting mismatch substrates as demonstrated by mismatch-dependent changes in its ATPase activity suggested that it was incapable of forming stable complexes with mismatch substrates. We tested this idea by performing the dissociation experiments presented in Fig. 7. In these studies, 5.7 nM (0.3 μg) MSH2-MSH6 and MSH2-msh6-F337A were preincubated with either 1.4 nM 32P-labeled homoduplex or +1 substrate for 15 min, after which a 300-fold excess of unlabeled competitor was added. The stability of the original protein-DNA complexes was then measured as a function of time in filter binding assays.

For the MSH2-MSH6-homoduplex complex, a rapid decay was observed immediately after unlabeled homoduplex substrate was added. As shown in Fig. 7A, approximately 70% of the MSH2-MSH6-homoduplex complexes dissociated within 1 min after unlabeled homoduplex was added. For the MSH2-MSH6-+1 complex, the addition of unlabeled +1 substrate resulted in a biphasic decay, indicating the presence of two types of complexes. One complex was unstable, with a t1/2 of ~5 min, and the other was stable, with a t1/2 of ~80 min (Fig. 7A). These results indicated that ~40% of the MSH2-MSH6-+1 complexes formed stable complexes. In experiments involving MSH2-msh6-F337A, similar dissociation profiles were observed for the MSH2-msh6-F337A-homoduplex and MSH2-msh6-F337A-+1 complexes, and the dissociation of these complexes was more rapid than that observed for the MSH2-MSH6-homoduplex complex (Fig. 7B). The inclusion of 2.0 mM ATP during the MSH2-MSH6-+1 preincubation phase resulted in a dissociation profile that was similar to that found for the MSH2-MSH6-homoduplex preincubated in the presence or absence of ATP (Fig. 7C). The addition of ATP during the formation of MSH2-msh6-F337A-homoduplex and MSH2-msh6-F337A-+1 complexes had no effect on the dissociation of these complexes (data not shown).

The finding that MSH2-msh6-F337A complexed with +1 or homoduplex substrate displayed a dissociation profile that was even more rapid than was observed for MSH2-MSH6 provides an explanation for why we were able to detect MSH2-msh6-F337A-+1 complexes in filter binding but were unable to observe discrete complexes in the gel shift assay. We hypothesize that the MSH2-msh6-F337A-+1 complexes were detected in filter binding because these unstable complexes have a greater chance to be recovered on nitrocellulose filters, as the filter binding step was rapid and was performed in standard DNA binding buffer (see under “Experimental Procedures”). We believe that these unstable complexes were not observed in the gel shift assay because the gel electrophoresis step provides an extended time period to allow for dissociation of the complex. This argument can also explain why a greater mismatch binding specificity for MSH2-MSH6 was observed in the gel shift assay compared with the filter binding assay. An important caveat in our hypothesis is that unlike gel retardation analysis, in which specific protein-DNA complexes can be identified, it is difficult to determine in filter binding whether the complexes that were formed resulted from specific protein-DNA interactions or resulted from nonspecific protein-DNA aggregates.
nM (0.3 µM) activity of MSH2-msh6-F337A was specifically stimulated by ATP hydrolysis observed that in contrast to wild type complex, the ATPase activity of complexes in the presence of DNA substrate, we examined the effect of ATP on the mismatch binding (Figs. 2 and 3). In one set of experiments that involved measuring the ATPase activity of wild type and MSH2-msh6-F337A complexes. In another set of experiments that examined the effect of ATP on the mismatch binding activity, we incubated the presence of the 33.3 µM [γ-32P]ATP, and the indicated concentrations of +1 or homoduplex substrate and the amount of ATP hydrolyzed were determined after a 15-min incubation. A–D: open triangles, no DNA substrate; filled squares, homoduplex substrate; open squares, +1 substrate.

![Image](89x347 to 257x480)

**FIG. 5.** The effect of +1 and homoduplex substrate on the ATPase activity of MSH2-MSH6 and MSH2-msh6-F337A. In A and B, 20 nM (0.3 µg) MSH2-MSH6 (A) or MSH2-msh6-F337A (B) was assayed for ATPase activity as described under “Experimental Procedures” in the presence of 167 nM +1 or homoduplex substrate. The amount of ATP hydrolyzed was determined after 15 min at 30 °C for duplicate reactions, and the differences between these two values are shown with error bars. In C and D, 20 nM (0.3 µg) MSH2-MSH6 (C), or MSH2-msh6-F337A (D) was incubated in the presence of the 33.3 µM [γ-32P]ATP, and the indicated concentrations of +1 or homoduplex substrate and the amount of ATP hydrolyzed were determined after a 15-min incubation. A–D: open triangles, no DNA substrate; filled squares, homoduplex substrate; open squares, +1 substrate.

![Image](254x493 to 554x729)

**FIG. 6.** The effect of +1 and homoduplex substrate on the ATPase activity of MSH2-msh6-F337A/G987D and MSH2-msh6-F337A. 20 nM (0.3 µg) MSH2-msh6-F337A/G987D or MSH2-msh6-F337A was assayed for ATPase activity as described under “Experimental Procedures” in the presence of 100 µM [γ-32P]ATP and 167 nM +1 or homoduplex substrate (homo). The amount of ATP hydrolyzed was determined after 15 min at 30 °C for duplicate reactions, and the differences between the duplicates are shown with error bars.

DISCUSSION

The msh6-F337A Mutation Confers a Mismatch Recognition Defect—We analyzed mutations in MSH2 (msh2-Y42A) and MSH6 (msh6-F337A) that are analogous to a mutation in T. aquaticus MutS that disrupts binding to mismatch DNA (10). Genetic analysis indicated that the msh6-F337A but not the msh2-Y42A mutation conferred a mismatch repair defect. In UV cross-linking and DNA binding assays that involved titration analysis and substrate competitions, MSH2-msh6-F337A displayed defects in both general and mismatch-specific DNA binding (Figs. 2 and 3).

Additional support for the idea that the msh6-F337A mutation conferred a mismatch binding defect came from experiments that examined the effect of ATP on the mismatch binding activity of wild type and MSH2-msh6-F337A complexes. In one set of experiments that involved measuring the ATPase activity of complexes in the presence of DNA substrate, we observed that in contrast to wild type complex, the ATPase activity of MSH2-msh6-F337A was specifically stimulated by +1 substrate (Fig. 5). However, as the concentration of +1 substrate was raised above 167 nM, the ATPase activity of the MSH2-msh6-F337A complex decreased and eventually reached a level that was below that observed in the absence of +1 substrate. Homoduplex DNA did not affect ATPase activity, suggesting that msh6-F337A confers specific defects in mismatch recognition that can be overcome in the presence of a sufficiently high concentration of +1 substrate, leading to a wild type response (decrease) in ATPase activity.

In a second set of experiments, the binding of MSH2-msh6-F337A to single nucleotide insertion/deletion (+1, Δ1-1) and homoduplex substrates was measured in UV cross-linking and in filter binding assays performed in the presence and absence of ATP (Fig. 4). Previous studies indicated that the specific binding of MutS homolog complexes to mismatch substrates in vitro was abolished by ATP (8, 13, 19, 41, 42). This loss of mismatch binding specificity in the presence of ATP most likely reflects a switch from a recognition mode to a translocation mode (7, 9). In contrast, the binding of MSH2-msh6-F337A complex to both homoduplex and single nucleotide insertion/deletion substrates was improved in the presence of ATP, and approached one-half of the level observed for wild type MSH2-MSH6 (Fig. 4). Based on the two binding mode model for MutS described in the Introduction, we propose that the mutant complex is defective in mismatch recognition but can still function in a second mode that allows it to bind and/or translocate along DNA in an ATP-dependent fashion. It is important to note that in the presence of ATP, the MSH2-msh6-F337A complex did not reach the level of binding to homoduplex DNA that was observed for MSH2-MSH6. This finding can be reconciled in the context of a two binding mode model if the initial loading of the MSH2-MSH6 complex onto DNA is accomplished primarily through the first binding mode, the disruption of which would be expected to reduce both general DNA binding and mismatch recognition. At present it is unclear whether the ATP-dependent binding of the MSH2-msh6-F337A complex is mediated by residues in the conserved domain presented in Fig. 2A or is located elsewhere.

A Model for MSH2-MSH6 Binding to Base Pair Mismatches—The observation that MSH2-MSH6 forms unstable and stable complexes with DNA provides an explanation for how the modest selectivity of the complex for mismatches observed in filter binding assays could result in efficient repair. Based on the off rate studies presented in Fig. 7, we propose that MSH2-MSH6 initially binds to DNA and forms an unstable and non-specific complex that rapidly dissociates, allowing a new cycle
of binding to occur. A proportion of the complexes that form are stable, and the formation of these stable complexes requires the presence of mispaired DNA in the substrate. The stability of the MSH2-MSH6-+1 complex in dissociation experiments suggests that MSH2-MSH6 undergoes a change in binding conformation when it is bound to a mismatch substrate; this results in the stable binding of MSH2-MSH6 to a mismatch and provides a recognition signal for interactions with repair proteins that are involved in subsequent steps. Such a mechanism could amplify the selectivity of the complex for mispaired bases (24, 33). A similar binding mode has been demonstrated for the binding of MSH1, MSH2, and hMSH2-hMSH6 to mismatch DNA (9, 24, 33). We propose that because the MSH2-msh6-F337A complex is unable to form a stable complex on mismatch DNA, it fails to provide a mismatch recognition signal for interactions with mismatch repair proteins and instead undergoes futile cycles of mismatch binding and release that involves ATP hydrolysis by the MSH6 subunit.

The above model for mismatch binding does not address the genetic data presented in Table I that showed that strains overexpressing msh6-F337A displayed a strong dominant negative phenotype in both the mutator and DNA slippage assays. Overexpression of MSH6, on the other hand, did not dramatically affect mismatch repair in either assay. As shown in the biochemical assays presented in this paper, the MSH2-msh6-F337A complex was unable to stably bind to mismatch DNA substrates. A hypothesis that can account for these genetic and biochemical observations is that in wild type cells, MSH2 is able to switch between MSH3 and MSH6 subunits when present in a mismatch binding competent complex. In such a scenario, the presence of a high level of one MSH partner would not prevent interactions with a partner present at lower levels. Based on this idea, we suggest that the inability of MSH2-msh6-F337A to stably bind to DNA prevents MSH2 within the mutant complex from switching between MSH3 and MSH6 subunits and thus withdraws MSH2 from pools available for repair. Experiments to test this idea are in progress.

Does MSH2 Contribute to Mismatch Binding?—The observation that the MSH2-msh6-F337A complex is defective in mismatch binding whereas its ATPase activity responds to the presence of a mismatch suggests that additional residues in the MSH2-MSH6 complex are involved in mismatch recognition; this idea is also supported by the finding that the mutant complex showed residual cross-linking to the +1 substrate (Fig. 4). Does the entire mismatch recognition activity of the MSH2-MSH6 complex reside in the MSH6 subunit, or is MSH2 also involved? Previous analysis of MSH2 and MSH6 purified individually indicated that neither polypeptide demonstrated the mismatch binding specificity displayed by the complex (13, 25). In cross-linking analysis, we observed a Band C at lower intensity that was shown by immunoprecipitation analysis to contain MSH2 (Figs. 1, 4). The intensity of this band could be increased in binding reactions that omitted magnesium chloride or in reactions that involved incubating MSH2-MSH6 complex with DNA substrates containing 5-IdUrd substitutions at different distances away from the mismatch site.3 At present, these data cannot distinguish whether MSH2 is playing a role in general DNA binding or in recognition. In an initial attempt to investigate the putative mismatch binding domain shown in Fig. 2A, we made two substitutions in MSH6 (msh6-G335D and msh6-D343A) that map to residues that are in the vicinity of Phe-337 and are highly conserved in MutS homologs. Unfortunately, these substitutions resulted in the destabilization of the mutant msh6 proteins and prevented their purification (data not shown). We are currently analyzing the contribution of the MSH2 and MSH6 subunits in mismatch recognition by examining a set of msh2 and msh6 mutants that display genetic phenotypes consistent with a defect in mismatch binding.

The MSH6 ATPase Acts as a Signaling Factor in Mismatch Recognition—Previously, Studamire et al. (15) individually mutagenized the ATP binding domains of MSH2 and MSH6 and tested the mutant complexes for ATPase activity in the presence and absence of mismatch substrate. Their results supported a role for MSH6 as a mismatch recognition signaling factor, as complexes containing only a functional MSH6 ATPase could be modulated by mismatch binding in a manner qualitatively similar to that observed for the MSH2-MSH6 complex (Fig. 5). The ATPase activity of complexes that contained only a functional MSH2 ATPase was unchanged by the

3 T. Quach, J. Bowers, and E. Alani, unpublished data.
presence of DNA substrate (15). The data presented in this paper provide further support for this idea, as biochemical analysis showed that the enhanced ATPase activity of the MSH2-mlh1-F337A complex in the presence of a mismatch was eliminated if a second mutation was introduced into the MSH6 subunit that inactivated the MSH6 ATPase (MSH2-msh6-F337A/G987D). We hypothesize that the MSH6-dependent mismatch signaling activity plays an important role in recruiting downstream mismatch repair factors, such as the MLH1-PM1 complex (15). Recent data from the Prakash laboratory (22) are consistent with this idea, as they observed that ATP was required for the assembly of a ternary complex consisting of a DNA mismatch and the yeast MSH2-MSH6 and MLH1-PM1 complexes.

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