Identification and Characterization of a Thermostable MutS Homolog from Thermus aquaticus*

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Recognition of mispaired or unpaired bases during DNA mismatch repair is carried out by the MutS protein family. Here, we describe the isolation and characterization of a thermostable MutS homolog from Thermus aquaticus YT-1. Sequencing of the mutS gene predicts an 89.3-kDa polypeptide sharing extensive amino acid sequence homology with MutS homologs from both prokaryotes and eukaryotes. Expression of the T. aquaticus mutS gene in Escherichia coli results in a dominant mutator phenotype. Initial biochemical characterization of the thermostable MutS protein, which was purified to apparent homogeneity, reveals two thermostable activities, an ATP hydrolysis activity in which ATP is hydrolyzed to ADP and Pi and a specific DNA mismatch binding activity with affinities for heteroduplex DNAs containing either an insertion/deletion of one base or a GT mismatch. The ATPase activity exhibits a temperature optimum of approximately 80 °C. Heteroduplex DNA binding by the T. aquaticus MutS protein requires Mg²⁺ and occurs over a broad temperature range from 0 °C to at least 70 °C. The thermostable MutS protein may be useful for further biochemical and structural studies of mismatch binding and for applications involving mutation detection.

Mismatch repair plays a critical role in maintaining genetic stability in both prokaryotes and eukaryotes (reviewed in Ref. 1). The mismatch repair pathway removes mismatches arising from errors of replication that escape proofreading functions, from chemical modification of bases, notably the deamination of 5-methylcytosine, and from homologous recombination between divergent sequences giving rise to heteroduplex DNA containing mispaired or unpaired bases. Mismatch repair also serves as a barrier to interspecies recombination by inhibiting recombination between divergent sequences (2–4). The most extensively studied long-patch mismatch repair systems are the methyl-directed mismatch repair pathway of Escherichia coli and Salmonella typhimurium (5) and the Hex-dependent mismatch repair pathway of Streptococcus pneumoniae (6). The E. coli methyl-directed mismatch repair pathway has been reconstituted in vitro using purified components and has been shown to require 10 proteins, MutS, MutL, MutH, DNA helicase II, SSB, exonuclease I, exonuclease VII, RecJ, DNA polymerase III holoenzyme, and DNA ligase (7, 8).

Among the proteins required for methyl-directed mismatch repair, MutS alone recognizes and binds to heteroduplex DNA containing mispaired or unpaired bases. The in vitro affinity of MutS protein for mismatches and small insertion/deletions of one to four bases roughly parallels the in vivo efficiency of repair (9, 10). In the presence of ATP, a complex consisting of MutS bound to a mismatch site together with MutL activates the MutH endonuclease which incises the unmethylated strand at hemimethylated d(GATC) sites. In this way, repair in E. coli is directed to the newly synthesized strand (reviewed in Ref. 11). In S. pneumoniae, mismatch repair is initiated by MutS and MutL homologs, HexA and HexB, respectively; however, no MutH equivalent has been identified, and repair is thought to be directed by single-strand breaks (6).

Genetic and biochemical studies of mismatch repair establish that many of the essential features of mismatch repair in prokaryotes have been conserved in eukaryotes. A number of eukaryotic MutS and MutL homologs have been identified including at least five MutS homologs (12–15) and three MutL homologs (16–18) in yeast. Defects in genes encoding some of these homologs in humans have been implicated in hereditary nonpolyposis colon cancer in which tumor cells are characterized by instability of microsatellite repeats (19–23).

Elucidating the molecular mechanism of mismatch repair is important given the essential nature of the process in virtually all organisms. Studies of prokaryotic mismatch repair enzymes may be advantageous in certain respects due to their relative simplicity. For example, whereas repair in prokaryotes involves a single MutS species and a single MutL species, repair in eukaryotes involves complex interactions among different MutS and MutL homologs. Studies of two human MutS homologs, hMSH2 and GTBP/p160, indicate that a heterodimer of both proteins is required for optimal binding and correction of mismatches and insertions/deletions of 1 or 2 bases in vitro (24, 25), a finding that has been substantiated in vivo (26).

In this paper, we characterize a MutS homolog from the thermophilic eubacterium Thermus aquaticus YT-1. The highly conserved sequence of the thermostable MutS protein and its heteroduplex binding properties, namely the involvement of a single polypeptide species and mismatch binding over a wide temperature range up to 70 °C, indicate that it will be a useful reagent in biochemical and structural studies directed toward a mechanistic understanding of mismatch repair. In addition, the thermostable MutS protein may be useful in applications involving mutation detection.

MATERIALS AND METHODS

DNA Substrates—Oligodeoxynucleotides were synthesized using an Applied Biosystems 380B synthesizer followed by purification on denaturing polyacrylamide gels. Purified oligonucleotides were annealed in 10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, and 100 mM NaCl followed by purification on native polyacrylamide gels (27). Oligonucleotide duplexes were stored in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. DNA substrates were 5′-end-labeled using T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (DuPont NEN, 3000 Ci/mmol). DNA
Cloning of T. aquaticus mutS Gene—A 366-bp fragment, 348 bp excluding the EcoRI cloning sites, was amplified from T. aquaticus YT-1 genomic DNA (28) by the polymerase chain reaction, PCR (Perkin-Elmer, Norwalk, CT). The fragments were digested against EcoRI and SacI, separated on an agarose gel, and cloned into the SacI site of the pBluescript SKII (Stratagene) vector. Sequencing revealed that this clone contained the 5′-end of a 1.0 kb fragment which was cloned subsequently from a SacI-digested genomic library of T. aquaticus. Southern hybridization of T. aquaticus genomic DNA with the 32P-labeled 348-bp probe identified a 215-bp fragment corresponding to the 5′-end of the mutS gene. An appropriate dilution was plated on LB or LB-ampliCin plates to determine the number of viable cells and on either LB or LB-ampliCin containing 100 μg rifampicin (Sigma) to score for rifampicin-resistant cells. All growth was at 37 °C. ATPase Assays—Assays were performed in 20 mM Tris-HCl, pH 7.5 at 25 °C, 0.5 mM CaCl2, 5 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 100 μg bovine serum albumin per ml, and 20 μg/ml ampicillin. Appropriate dilutions were plated on LB or LB-ampliCin plates to determine the number of viable cells and on either LB or LB-ampliCin containing 100 μg rifampicin (Sigma) to score for rifampicin-resistant cells. All growth was at 37 °C. ATPase Assays—Assays were performed in 20 mM Tris-HCl, pH 7.5 at 25 °C, 0.5 mM CaCl2, 5 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 100 μg bovine serum albumin per ml, and 20 μg/ml ampicillin. Appropriate dilutions were plated on LB or LB-ampliCin plates to determine the number of viable cells and on either LB or LB-ampliCin containing 100 μg rifampicin (Sigma) to score for rifampicin-resistant cells. All growth was at 37 °C.

**RESULTS**

Cloning of T. aquaticus mutS Homolog—Degenerate oligo(deoxynucleotide) primers corresponding to two highly conserved amino acid sequences found in bacterial, yeast, and human MutS homologs were used in a polymerase chain reaction (PCR) to amplify genomic DNA from T. aquaticus YT-1 (see Fig. 1 and "Materials and Methods"). PCR primers derived from the synthetic HindIII end contain-
from these highly conserved peptide sequences were previously used to isolate mutS-related sequences in S. cerevisiae (12). Based on the E. coli mutS and S. cerevisiae MSH2 sequences, we predicted a 351-bp amplification product corresponding to 117 amino acid residues. The PCR product from T. aquaticus genomic DNA yielded a single product corresponding to 348 bp amplified from T. aquaticus genomic sequence (data not shown). DNA sequencing of the amplified sequence revealed a single continuous open reading frame corresponding to 116 amino acid residues with extensive homology to E. coli MutS protein (80% identity).

The 348-bp fragment was used to probe restriction endonuclease digests of T. aquaticus genomic DNA in Southern blots. The probe hybridized to a single 2.67-kb BamHI fragment which was subsequently cloned from a genomic library of T. aquaticus (see "Materials and Methods"). A probe derived from the 5' end of the BamHI fragment was used to isolate a 1.0-kb SacI fragment containing the 5' end of the putative mutS gene.

DNA sequencing revealed that the full-length T. aquaticus mutS gene has a single, large open reading frame of 2,436 bp predicting a polypeptide of 811 amino acids corresponding to 89.3 kDa (Fig. 1). The DNA sequence has a 69% GC content reflecting the high overall GC content, 67.4%, of T. aquaticus (32). A presumptive ribosome binding site, 5'GAGG3', is located just upstream of the first AUG start codon.

Sequence comparison of the translated open reading frame of T. aquaticus mutS with E. coli, Saccharomyces cerevisiae, and human MutS homologs predicts extensive sequence homology, 63% identity with E. coli MutS, 56% identity with S. cerevisiae MSH2, and 55% identity with human MSH2 (Fig. 2). Within the putative nucleotide binding region corresponding to the region amplified by PCR, the sequence conservation is even more extensive with T. aquaticus MutS sharing 80% identity with E. coli MutS. The consensus sequences for the Mg2+-ATP binding site, GKS/T and DE invariant sequences (33, 34), are present in the T. aquaticus translated sequence at positions Gly588 and Asp653, respectively, corresponding to positions C2269 and T2490, respectively, in the nucleotide sequences shown in Fig. 1. In addition, a putative helix-turn-helix domain (35) is present near the carboxyl terminus of the T. aquaticus protein at positions Gly738 through Ala757.

Expression of T. aquaticus mutS in Repair-proficient E. coli Confers a Mutator Phenotype—

Sequence comparison of the translated open reading frame of T. aquaticus mutS with E. coli, Saccharomyces cerevisiae, and human MutS homologs predicts extensive sequence homology, 63% identity with E. coli MutS, 56% identity with S. cerevisiae MSH2, and 55% identity with human MSH2 (Fig. 2). Within the putative nucleotide binding region corresponding to the region amplified by PCR, the sequence conservation is even more extensive with T. aquaticus MutS sharing 80% identity with E. coli MutS. The consensus sequences for the Mg2+-ATP binding site, GKS/T and DE invariant sequences (33, 34), are present in the T. aquaticus translated sequence at positions Gly588 and Asp653, respectively, corresponding to positions C2269 and T2490, respectively, in the nucleotide sequences shown in Fig. 1. In addition, a putative helix-turn-helix domain (35) is present near the carboxyl terminus of the T. aquaticus protein at positions Gly738 through Ala757.

Expression of T. aquaticus mutS in Repair-proficient E. coli Confers a Mutator Phenotype—A functional role in mismatch repair for the presumptive T. aquaticus MutS protein was assessed in vivo by measuring the frequency of spontaneous mutation to rifampicin resistance in mismatch repair-proficient E. coli harboring the T. aquaticus mutS gene. The T. aquaticus mutS gene cloned into the T7 expression vector...
Table I

| Strain          | Rif^R mutants per 10^8 cells |
|-----------------|-----------------------------|
| BL21(DE3)       | 0.75 ± 0.45                 |
| BL21(DE3) (pET3a) | 0.29 ± 0.10                 |
| BL21(DE3)       | 10.7 ± 4.9                  |
| (pETMutS)       |                             |

FIG. 2. Comparison of predicted amino acid sequence of T. aquaticus MutS with MutS homologs. Sequences were aligned using the PILEUP program (Genetics Computer Group, Madison). Boxed residues indicate conserved positions. Bold indicates positions conserved among all four MutS proteins. Abbreviations: TaqMutS, T. aquaticus MutS; EcoMutS, E. coli MutS; Msh2, S. cerevisiae MSH2; hMsh2, human hMSH2. Sequences for E. coli and eukaryotic MutS proteins are from GenBank.

Fig. 3. High level expression of the cloned T. aquaticus mutS gene in E. coli. A 2.9-kb genomic fragment containing the coding region of T. aquaticus mutS was cloned into the T7 expression vector pET3a and transformed into E. coli BL21(DE3) as described under "Materials and Methods." Upon induction of T7 polymerase with IPTG, a 90-kDa polypeptide was synthesized at high levels as judged by electrophoresis on a 12% SDS-polyacrylamide gel (lanes 1 and 2). The 90-kDa polypeptide was partially purified from crude cell extracts by centrifugation at 100,000 g followed by a heat denaturation step, precipitation in ammonium sulfate, and chromatography on a Mono Q anion exchange column (lanes 3–6). A molecular mass standard, lysozyme, 14 kDa, co-migrated with the tracking dye to within 1.6 cm of the bottom of the gel.

pET3a was transformed into E. coli strain BL21(DE3) (mutS^+). In mutator assays of BL21 cells harboring either the pET3a vector alone or pETMutS containing the T. aquaticus mutS gene, high level induction of T7 polymerase in the presence of IPTG proved lethal. Therefore, the plate assay for a mutator gene, high level induction of T7 polymerase in the presence of IPTG and relied on basal levels of T7 polymerase expression in these strains.

The presence of the T. aquaticus mutS gene in repair-proficient E. coli conferred a dominant mutator phenotype as judged by elevated frequencies of spontaneous mutation to rifampicin resistance (Table I). Whereas the pET3a vector alone yielded a mutation frequency similar to that of the host E. coli BL21, the presence of T. aquaticus mutS resulted in a 14.3-fold increase in the frequency of spontaneous mutation. This result is analogous to that previously observed for other heterologous mutS genes from humans and S. pneumoniae (19, 36) although the magnitude of the increase in the spontaneous mutation frequency is lower in the case of T. aquaticus mutS.

Not surprisingly, we were unable to demonstrate direct complementation of an E. coli mutS^- strain, GW3732 (mutS201::Tn5) (37) by expression of the heterologous T. aquaticus mutS gene cloned in an IPTG-inducible expression vector, pTrc 99 A (data not shown). Such complementation is unlikely given the temperature optimum of the thermophilic protein, see below, as well as the requirement in E. coli for MutS-mediated activation of MutH cleavage at hemimethylated d(GATC) sites which are absent in T. aquaticus.

Overproduction of the T. aquaticus MutS Protein—High level expression of the T. aquaticus mutS gene in E. coli was achieved using a T7 expression system (see "Materials and Methods"). Upon induction by IPTG, a 90-kDa polypeptide was synthesized at high levels (Fig. 3, lanes 1 and 2). Following a 100,000× g centrifugation of the crude cell extract, the vast majority of the 90-kDa polypeptide remained in the soluble fraction. Subsequent purification steps involved heating of the resultant supernatant at 70 °C for 45 min to denature host proteins, precipitation of the 90-kDa polypeptide in 30% saturated ammonium sulfate, and chromatography on a Mono Q anion exchange column (Fig. 3, lanes 3–6). The 90-kDa protein eluted from the Mono Q column at approximately 260 mM NaCl as judged by SDS-polyacrylamide gel electrophoresis analysis of column fractions (data not shown). The partially purified polypeptide is estimated by Coomassie staining of gels to be in excess of 90% pure after the Mono Q chromatography step.

Identification of the 90-kDa protein as that encoded by the cloned T. aquaticus mutS gene was determined by N-terminal amino acid sequencing of the polypeptide excised from an SDS
Sequencing of the first 15 amino acid residues yielded an exact match with the predicted sequence shown in Fig. 2.

**T. aquaticus MutS Has a Thermostable ATPase Activity—**

Thin layer chromatography analysis of ATPase assays revealed that the T. aquaticus MutS protein hydrolyzes ATP to ADP and Pi at elevated temperatures. ATPase assays containing T. aquaticus MutS protein and [α-32P]ATP were incubated at 70°C for 15 min. Reaction products were analyzed on polyethyleneimine-cellulose TLC plates. Incubation of [32P]ATP with increasing amounts of T. aquaticus MutS protein resulted in extensive hydrolysis at 70°C (Fig. 4A and B). Spontaneous hydrolysis of ATP in the absence of any protein at 70°C was less than 5%. A time course of ATP hydrolysis at 70°C in the presence of 770 ng of MutS protein is shown in Fig. 4C. To estimate a specific activity for ATP hydrolysis by this MutS protein preparation, we have measured the extent of ATP hydrolysis at early time points under conditions described above (data not shown). The extents of ATP hydrolysis after 60 s, 90 s, and 120 s are 9.4 ± 0.4%, 14.2 ± 0.7%, and 21.9 ± 2.2%, respectively. Assuming the MutS protein preparation is 95% pure, we estimate a specific activity of 774 ± 82 pmol of ATP hydrolyzed to ADP and Pi per pmol of MutS protein after 30 min at 70°C.

The thermostability of the ATPase activity of T. aquaticus MutS was assessed over a wide temperature range (Fig. 5). Consistent with the 70–75°C temperature optimum for T. aquaticus growth in vivo (32), the temperature optimum for the ATPase activity as judged by the extent of ATP hydrolysis after 15 min was 80°C although ATP hydrolysis mediated by MutS occurred at lower temperatures. Thus, 72% of the ATP was hydrolyzed at 80°C, whereas 22% of the ATP was hydrolyzed at 37°C. At 90°C, the extent of ATP hydrolysis was reduced to only 15%. In a control experiment, nonenzymatic hydrolysis of ATP at 80°C in the absence of MutS protein was less than 4% (data not shown). These assays were conducted in the presence of 20 mM Tris-HCl, pH 7.5 at 25°C. At 70°C, the pH would be expected to drop about 1.3 pH units to pH 6.2. Subsequent ATPase experiments have been repeated at 70°C in the presence of 20 mM Hepes buffer, pH 7.5 at 25°C, instead of Tris-HCl with no discernible effect on the extent of ATP hydrolysis (data not shown). Reactions carried out in the presence of Hepes buffer would be expected to show a considerably smaller effect of temperature on pH, pH 6.8 at 70°C, as opposed to pH 7.5 at 25°C (38).

**Thermostable Binding to a GT Mismatch—**

The ability of T. aquaticus MutS protein to bind specifically to DNA mismatches was determined in filter binding assays using 36-bp heteroduplex substrates containing a single GT mismatch (see Fig. 6). Filter binding assays utilized a two-filter system in which protein-DNA complexes were detected on nitrocellulose filters while free DNA was trapped on DEAE-cellulose (31). Heteroduplex binding assays were carried out in varying concentra-
1 heteroduplex greatly reduced binding, 16% heteroduplex bound. In contrast, binding to a heteroduplex containing a single-base deletion with respect to a homoduplex control (Fig. 6, B and C). Only 5% of a single-strand DNA substrate was bound by MutS demonstrating that, under these conditions, T. aquaticus MutS protein has very low affinity for single-strand DNA. This result makes it unlikely that MutS protein is recognizing single-strand regions of heteroduplex substrates containing a GT mismatch or a deletion of 1 base (see below) even at elevated temperatures.

The specificity of binding of MutS to heteroduplex DNAs was assessed in competition experiments (Fig. 6C). Binding to a 32P-labeled GT mismatch substrate, 62% substrate bound, was poorly competed by a hundredfold molar excess of AT homoduplex competitor, 52% substrate bound. In contrast, binding to the 32P-labeled GT heteroduplex was competed efficiently by a hundredfold molar excess of unlabeled GT heteroduplex, 7% heteroduplex bound. The binding by MutS to a single-base deletion heteroduplex likewise exhibited great specificity since a hundredfold molar excess of AT duplex failed to reduce binding to the 32P-labeled A1 substrate, 89% and 85% heteroduplex bound in the absence and presence of homoduplex competitor, respectively. In contrast, a hundredfold molar excess of unlabeled A1 heteroduplex greatly reduced binding, 16% heteroduplex bound.

The specificity of binding of MutS to the 36-bp A1 heteroduplex containing the single-base deletion was also monitored in a band mobility shift assay (Fig. 8D). DNA binding assays were carried out essentially as described for filter binding assays except that, after incubation of MutS protein and DNA substrates for 15 min at 60 °C, the reactions were electrophoresed on a nondenaturing polyacrylamide gel in the presence of MgCl2 at room temperature. As expected, no significant binding to either single-strand DNA or homoduplex DNA was observed. In contrast, MutS protein efficiently bound to the 32P-labeled A1 substrate resulting in the formation of a single, slower migrating species. The formation of a MutS-A1 DNA complex was not affected by the presence of a 133-fold molar excess of unlabeled AT homoduplex competitor, but was essentially abolished by the presence of a 133-fold molar excess of unlabeled A1 heteroduplex. Under these same conditions, we are unable to demonstrate a gel shift with the GT mismatch substrate although MutS-mismatch complexes are formed efficiently as judged by filter binding (data not shown). This result suggests that MutS-A1 complexes are more stable than MutS-GT complexes under these electrophoretic conditions.

Heteroduplex DNA Binding by MutS Exhibits a Wide Temperature Optimum—Filter binding assays were carried out over a wide range of temperatures to assess the thermostability of MutS binding to a 61-bp A1 heteroduplex DNA containing a single-base insertion with respect to a homoduplex control (Fig. 6). T. aquaticus MutS bound the 61 bp A1 heteroduplex DNA over a wide range of temperature up to 70 °C, between 70–79% heteroduplex bound versus 8–12% homoduplex bound (Fig. 9). Binding to either DNA substrate in the absence of any MutS protein at 60 °C, lanes C, was 2%. Due to the relatively large effect of temperature on pH of Tris-HCl buffers, see above, we have repeated heteroduplex DNA binding assays at 60 °C in the presence of Hepes buffer, pH 7.8 at 25 °C, instead of Tris-HCl, pH 7.5 at 25 °C, with no discernible difference in the extent of binding to either the GT or A1 heteroduplex substrates (data not shown). Whether 70 °C represents the upper temperature limit for DNA binding by MutS protein is unclear. Melting temperatures of the 61-bp A1 heteroduplex and homoduplex substrates were determined to be 80.8 °C and 82.2 °C, respectively (data not shown). The instability of the DNA substrates at temperatures above 70 °C obviously precludes more precise determination of the upper temperature limit for heteroduplex binding. We observed that, even at 70 °C, the discrimination between homoduplex and A1 heteroduplex DNAs by T. aquaticus MutS remained high, 70% binding to the A1 substrate versus 12% binding to the corresponding homoduplex. Surprisingly, both the extent of binding and the discrimination between heteroduplex and homoduplex DNA remained essentially unchanged from 0 °C to 70 °C.

$\begin{align*}
5' \text{GCATACGGAGTAAAGTCGCGACATCTATTGCCGCA} & \text{3'} \\
3' \text{GTATCTGGCTTATCTGCCGCTGTAGTAGACAGCTGC} & \text{5'} \\
5' \text{ATATCGATCGTGAAGATGGGCGCCGAGATGGCGATCGAAGT} & \text{3'} \\
3' \text{TATAGCTATACCGGCTTCCTACGCGTCGCGAGCGCTCTTAC} & \text{5'} \\
5' \text{ATATCGATCGTGAAGATGGGCGCCGAGATGGCGATCGAAGT} & \text{3'} \\
3' \text{TATAGCTATACCGGCTTCCTACGCGTCGCGAGCGCTCTTAC} & \text{5'}
\end{align*}$

**Fig. 6.** DNA substrates used in ATPase and heteroduplex binding assays. Duplex substrates were made by annealing gel-purified oligodeoxynucleotides and were subsequently purified from polyacrylamide gels as described under “Materials and Methods.” Sequences for the 36-bp substrates were described previously (30).
We describe the cloning and overexpression of a MutS homolog from T. aquaticus YT1 and the initial biochemical characterization of the thermostable MutS protein. The gene encodes a 90-kDa polypeptide with DNA mismatch binding and ATPase activities that are active at temperatures as high as 70–80 °C. The deduced amino acid sequence of the T. aquaticus MutS protein exhibits extensive sequence homology with MutS proteins from both prokaryotes and eukaryotes and shares 63% overall identity with E. coli MutS protein. Highly conserved regions include the Walker consensus sequence for nucleotide binding (33, 34) as well as a helix-turn-helix motif near the carboxyl terminus of the protein (12, 39). Expression of T. aquaticus mutS in mismatch-proficient E. coli resulted in a dominant mutator phenotype as judged by the frequency of spontaneous mutation to rifampicin resistance. By analogy to other MutS homologs, notably S. pneumoniae hexA (36) and human hMSH2 (19), the dominant mutator phenotype conferred by T. aquaticus mutS suggests a role for T. aquaticus mutS in mismatch repair in vivo although it has not been shown directly. The basis for the dominant mutator phenotype conferred by the T. aquaticus gene is unknown. The thermostable MutS protein may bind to heteroduplex DNA in the E. coli genome and thereby prevent the endogenous repair machinery from gaining access to mismatches. Alternatively, the thermostable MutS protein may interact nonproductively with other components of the E. coli mismatch repair machinery, for example, the MutL protein.

As others have observed for the S. pneumoniae hexA gene (36, 39), we were unable to demonstrate complementation of the mutator phenotype of an E. coli mutS” mutant by the introduction of the T. aquaticus mutS gene. This is not unexpected given that methyl-directed mismatch repair in E. coli responds to the state of methylation at dam methylation sites. Such a methylation system is not known to exist in thermo-stable bacteria, and, as in the case of S. pneumoniae and eukaryotic systems, it is unlikely that the T. aquaticus MutS protein can activate E. coli MutH, the endonuclease that incises at hemimethylated d(GATC) sites. In addition, a heterologous MutS protein may not be able to facilitate the large number of protein-protein interactions required for mismatch repair in E. coli best documented by interactions between MutS, MutL, and MutH proteins (reviewed in Ref. 5).

The presence in the T. aquaticus mutS gene of highly conserved sequence motifs found in a large number of proteins that bind and hydrolyze nucleotides including the MutS family (33, 34) predicted that the thermostable MutS protein would have an ATP hydrolytic activity. Such an ATPase activity has been demonstrated by others in MutS proteins from both prokaryotes (30, 40) and eukaryotes (41, 42). Here, we demonstrate that T. aquaticus MutS protein exhibits a thermostable ATPase activity in the absence of exogenous DNA resulting in the cleavage of ATP to ADP and P<sub>i</sub> (Fig. 4). The ATPase activity exhibits a temperature optimum of 80 °C although substantial activity is also detected at 60 °C (Fig. 5).

T. aquaticus MutS preferentially binds to a heteroduplex DNA containing a single GT mismatch. Binding exhibited a requirement for magnesium ions such that no binding to a GT heteroduplex occurred in the absence of MgCl<sub>2</sub>; however, mismatch binding was substantial upon the addition of 2.5 mM MgCl<sub>2</sub>. Raising the magnesium concentration from 2.5 mM to 10 mM had no significant effect on either the extent of binding or the selectivity of mismatch binding (Fig. 7). The thermostable MutS differs from the S. cerevisiae MSH2 (43) and human hMSH2 (44) proteins with respect to a requirement for Mg<sup>2+</sup>. Neither the MSH2 protein nor hMSH2 requires divalent metal ions for heteroduplex binding, and increasing the concentration of magnesium abolishes the preferential binding of yeast MSH2 to mismatched DNAs as opposed to homoduplex DNA. In contrast, magnesium ions, while not essential, enhance heteroduplex binding by the human GTBP protein (41).

Specific heteroduplex binding mediated by the T. aquaticus MutS protein over a wide temperature range, the extensive sequence homology shared between T. aquaticus MutS and other MutS proteins, and the relative ease with which large amounts of the thermostable MutS protein can be obtained make this protein particularly suitable for mechanistic and structural studies of mismatch repair. In addition, studies of mismatch binding at high temperature may yield novel insights into structural determinants that stabilize such interactions. The properties of the T. aquaticus MutS protein may also

![Image](http://www.jbc.org/)

**Fig. 7.** T. aquaticus MutS binds to GT heteroduplexes. A, the ability of MutS protein to bind specifically to a mismatch was assessed in filter binding assays. Reactions were initiated by the addition of 6.4 nM 32P-labeled homoduplex (AT) or heteroduplex (GT) DNA and 3 μg of MutS protein and were carried out for 15 min at 60 °C in 20 mM Tris-HCl, pH 7.5 (25 °C), 0.1 mM dithiothreitol, 0.1 mM EDTA, 50 μg/ml bovine serum albumin, 5% (v/v) glycerol, 20 μg/ml poly(dI)-poly(dC), and the indicated amounts of MgCl<sub>2</sub> in a 20-μl reaction volume as described under “Materials and Methods.” Reactions were filtered through BAB5 nitrocellulose and NA45 DEAE-cellulose membranes. B, quantitation of the filter binding assays shown in A as described under “Materials and Methods.”
be useful in practical applications. For example, methods have been developed for mutation detection that exploit the ability of E. coli MutS protein to bind specifically to heteroduplex DNA (45, 46). Another application is the use of MutS-mediated mismatch recognition to facilitate genetic linkage mapping (47). Use of the thermostable T. aquaticus protein may be beneficial where enhanced stability of MutS-mismatch complexes is required or where mutation detection at elevated temperatures is desirable.

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FIG. 8. Preferential binding of MutS to a heteroduplex containing a deletion of one base. A and B, MutS binding to a 36-bp heteroduplex containing a deletion of one base, Δ1, was compared to binding of a GT heteroduplex in filter binding assays as described in Fig. 7. C, DNA filter binding assays were carried out as described above with 32P-labeled, 36-bp DNA substrates, AT homoduplex control, GT mismatch, or Δ1 deletion, in the presence or absence of a 100-fold molar excess of unlabeled competitor DNA as indicated. D, specific binding to the Δ1 substrate by MutS protein was demonstrated in band mobility shift experiments. Binding assays containing 2.8 μg of MutS protein and 6.8 nM 36-base single-strand DNA control, 36-bp homoduplex AT control, or 36-bp Δ1 heteroduplex were carried out at 60 °C as described above except that a 133-fold molar excess of cold competitor DNA was added as indicated at the start of the reaction. Binding reactions were electrophoresed on 6% nondenaturing polyacrylamide gels in 5 mM MgCl2.

FIG. 9. Heteroduplex binding as a function of temperature. Filter binding assays were carried out as described in Fig. 7 for 15 min at various temperatures in the presence of 1.4 μg of MutS protein and 6.8 nM 32P-labeled, 61-bp homoduplex or the corresponding 61-bp Δ1 heteroduplex containing an insertion of one base (see Fig. 6). C, incubation without MutS protein at 60 °C. Results are the average of two trials.
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