The Effect of O\textsuperscript{6}-methylguanine DNA adducts on the Adenosine Nucleotide Switch Functions of hMSH2-hMSH6 and hMSH2-hMSH3

Mark Berardini, Anthony Mazurek and Richard Fishel\textsuperscript{*}

Genetics and Molecular Biology Program, Kimmel Cancer Center, Thomas Jefferson University,

233 S. 10th Street, Philadelphia, PA 19107

Running Title: Recognition of O\textsuperscript{6}-methylguanine DNA by Mismatch Repair Proteins

\textsuperscript{*} Corresponding Author: rfishel@hendrix.jci.tju.edu
ABSTRACT

The human homologs of prokaryotic mismatch repair (MMR)\(^1\) have been shown to mediate the toxicity of certain DNA damaging agents; cells deficient in the MMR pathway exhibit resistance to the killing effects of several of these agents. While previous studies have suggested that the human MutS homologs, hMSH2-hMSH6, bind to DNA containing a variety of DNA adducts as well as mispaired nucleotides, a number of studies have suggested that DNA binding does not correlate with repair activity. In contrast, the ability to process adenosine nucleotides by MutS homologs appears to be fundamentally linked to repair activity. In this study, oligonucleotides containing a single well-defined O\(^6\)-methylguanine adduct were used to examine the extent of lesion provoked DNA binding, single-step ADP→ATP exchange, and steady-state ATPase activity by hMSH2-hMSH3 and hMSH2-hMSH6 heterodimers. Interestingly, O\(^6\)-methylguanine lesions when paired with either a C or T were found to stimulate ADP→ATP exchange as well as the ATPase activity of purified hMSH2-hMSH6, while there was no significant stimulation of hMSH2-hMSH3. These results suggest that O\(^6\)-methylguanine uniquely activates the Molecular Switch functions of hMSH2-hMSH6.

\(^1\) Abbreviations used are: MMR, mismatch repair; ATPase, ATP hydrolysis activity; bp, basepairs; \(k_{\text{cat}}\), catalytic rate constant; \(K_m\), Michaelis-Menten constant; \(K_{1/2}\), concentration of substrate at 1/2 maximal velocity; MNU, N-methyl-N-nitrosourea; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.
INTRODUCTION

DNA mismatch repair is the cellular process by which mispaired nucleotides in DNA are corrected (1-5). Mispaired bases in DNA can occur as a result of polymerase errors during DNA replication, recombination between non-homologous parental DNA sequences, or replication past bases containing covalent DNA adducts (6). In human cells, mispaired bases in DNA are recognized by heterodimeric homologs of the bacterial MutS protein, hMSH2-hMSH3 or hMSH2-hMSH6. Mutations in hMSH2 and one of the human homologs of the bacterial MutL protein, hMLH1 have been associated with a majority of hereditary non-polyposis colon cancer (HNPCC) cases (2).

Previous studies have shown that the hMSH2-hMSH6 complex recognizes primarily single base mispairs while hMSH2-hMSH3 recognizes larger insertion deletion loops (IDL) in DNA (7-10). In addition to recognizing mispaired bases in DNA, hMSH2-hMSH6 has also been shown to bind DNA containing a variety of covalent adducts including O6-methylguanine (11) and the clinically useful antitumor drug cisplatin (12,13). Furthermore, loss of MMR activity has been associated with cellular resistance to these agents (14-17). For example, a human lymphoblastoid cell line deficient for hMSH2-hMSH6 activities has been shown to be dramatically resistant to the methylating agent MNNG and failed to undergo apoptosis (18,19). Moreover, the repair of O6-methylguanine adducts by methylguanine methyltransferase (MGMT) precluded MMR proficient cells from undergoing apoptosis (20). The reintroduction
and expression of a wild type MMR gene has been shown to restore the sensitivity to DNA
damaging agents (21-23). These and other findings suggest a direct role for the MMR system in
potentiating the toxicity of DNA damaging drugs such as MNU and MNNG.

The mechanism of MMR potentiated toxicity to O6-methylguanine lesions remains
enigmatic although a number of models have been proposed. One model is based on the
observation that replication past O6-methylguanine lesions results in the misincorporation of a
nucleotide opposite the lesion (24). The mismatched nucleotide, often a thymidine, then serves
as a substrate for repair directed to the newly replicated strand by hMSH2-hMSH6. This newly
synthesized region of DNA continues to misincorporate a nucleotide opposite the O6-
methylguanine lesion, which is subsequently excised and re-synthesized. Repeated cycles of
binding, excision and re-synthesis is suggested to result in the accumulation of strand breaks, cell
cycle arrest and apoptosis: termed the "futile-cycle model" (17,25,26). Cells lacking MMR are
thought to escape such futile cycles of repair and display a damage tolerance (resistant)
phenotype. A second model proposes that the binding of mismatch repair proteins to damaged
bases may shield the adduct from cellular repair processes allowing lesions to persist in the
genome: termed "binding-occlusion model" (27). Yet a third model has suggested that MMR
proficient cells are capable of inhibiting replication past certain DNA adducts leading to cell
cycle arrest and a cytotoxic response (28). Finally, an alternative to these models suggests that
specific types of DNA damage are recognized by the MutS homologs, which provokes the
exchange of ADP→ATP resulting in the formation of a sliding clamp associated with the DNA
adjacent to the lesion. In the absence of a targeted repair event, a threshold number of DNA-bound sliding clamps are proposed to ultimately signal apoptosis (29).

Biochemical analysis has demonstrated that mismatched nucleotides stimulate the intrinsic ATPase activity of both hMSH2-hMSH3 and hMSH2-hMSH6 (10,30-32). This intrinsic ATPase is controlled by mismatch-provoked ADP→ATP exchange which results in a conformational transition and the formation of a hydrolysis-independent sliding clamp on the DNA. However, it is not clear whether the same biochemical process(es) operate on DNA containing adducts such as O6-methylguanine. Here we have explored the biochemical interaction of purified hMSH2-hMSH3 and hMSH2-hMSH6 heterodimeric complexes with oligonucleotides containing a single O6-methylguanine adduct at a defined position. It was our goal to understand the distinctions between mispair and lesion recognition by the human MutS homologs toward clarifying the models which have been proposed to explain the influence of MMR on the toxicity of such lesions. It would appear that the defining biochemical feature that distinguishes these models is the ability of the MutS homologs to recognize O6-methylguanine lesions in the context of a homoduplex substrate and to be displaced by ADP→ATP exchange. Our results appear most consistent, but not uniquely confined to, the direct apoptosis signaling model.
MATERIALS AND METHODS

Protein Purification: Recombinant hMSH2-hMSH3 and hMSH2-hMSH6 protein complexes were overexpressed in SF9 cells using the pFastBac dual expression vector (GIBCO-BRL) and purified as previously described (10,30). Protein concentration was determined by the Bradford assay using globular protein standards (Biorad).

Preparation of DNA Substrates: Oligonucleotides were obtained from Midland Certified Reagent Company (Midland, TX). Unlabelled duplex oligonucleotides were prepared by annealing equal amounts of gel purified single stranded DNA’s (Figure1). The purity and consistency of the annealed duplex DNA was monitored by radiolabel phosphorylation with T4 polynucleotide kinase (New England Biolabs) and [γ\(^{32}\)P]-ATP (NEN Life Science Products) as recommended by the manufacturer. Unincorporated [γ\(^{32}\)P]-ATP was removed using Centri-Sep spin columns (Princeton Separations). In all cases, >99% of the substrate was found to be duplex as monitored by native gel electrophoresis.

Gel Shift Analysis: Reactions were carried out with 0.5 nM (~0.27 ng) of \(^{32}\)P end-labelled duplex DNA substrate in 20 µl of buffer containing 25 mM Hepes, pH8.1, 100 mM NaCl, 15% glycerol, 25 µM ADP, 2 mM MgCl\(_2\) and 25 ng (~923 fmol) of unlabelled G/C duplex competitor. Samples
were incubated for 15 minutes at 37°C, placed on ice and loaded onto a 5% polyacrylamide TBE gel containing 5% glycerol. Gels were dried onto Whatmann 3MM paper and exposed to a Molecular Dynamics phosphorimaging screen overnight.

**DNA Competition Analysis.** Reactions were carried out using 20 nM (~10 ng) of \(^{32}\)P end-labelled duplex DNA substrate in 20 µl of buffer containing 25 mM Hepes, pH 8.1, 100 mM NaCl, 15% glycerol, 25 µM ADP, 2 mM MgCl\(_2\), and the indicated amounts of unlabelled duplex DNA competitors. Samples were incubated for 15 minutes at 37°C, placed on ice and diluted into 4 ml of ice cold wash buffer containing 25 mM Hepes, pH 8.1, 100 mM NaCl, 2 mM MgCl\(_2\), and 15% glycerol. Samples were applied to pre-wet nitrocellulose filters (Millipore HAWP02500, 25mm, 0.45um) and washed with 4 ml of wash buffer. Filters were dried and Cerenkov counted. Protein concentrations were set to 10-20% total binding in the absence of any unlabelled DNA competitor which was 12.5 nM hMSH2-hMSH6 for radiolabelled G/T and 25 nM hMSH2-hMSH3 for radiolabelled +(CA) DNA substrates.

**ATPase Activity:** The ATPase assay was performed using the method described by Gradia, et al., (30). hMSH2-hMSH3 and hMSH2-hMSH6 were incubated with 240 nM (~125ng) duplex DNA substrate at 37°C for 30 minutes in 20 µl of buffer containing 25 mM Hepes, pH8.1, 100 mM NaCl, 15% glycerol, 2 mM MgCl2, 0.33 pmol of gamma \(^{32}\)P-ATP and various concentrations of...
unlabelled ATP. Reactions were stopped by the addition of 400 μl of 10% (w/v) activated charcoal containing 1 mM EDTA. Samples were centrifuged in 0.5 ml tubes for 10 minutes at 14000 rpm in a tabletop microfuge. Aliquots of 100 μl were removed and counted in a RackBeta 1209 liquid scintillation counter (Pharmacia). Protein concentration was varied (15nM-30nM for hMSH2-hMSH6 and 10nM-50nM for hMSH2-hMSH3) in order to maintain the total amount of ATP hydrolyzed under 20%. The ATPase velocity is expressed in min⁻¹ values [(mol ATP hydrolyzed) (mol protein)⁻¹ (minute)⁻¹] as a function of ATP concentration (Figure 4) and DNA concentration (Figure 5). For DNA concentration dependent ATPase assays, reactions were carried out as described above except 75nM hMSH2-hMSH6 was incubated with the DNA substrates in a final volume of 20 μl containing 100 μM ATP.

**ADP Exchange:** ADP exchange assays were carried out using a modification to the method of Gradia et al. (30). Reactions were carried out with 60 nM (32.5ng) DNA substrate in a buffer containing 25 mM Hepes, pH 8.1, 100 mM NaCl, 2mM MgCl₂, 1mM DTT, 15% glycerol and 2.3 μM [³H]-ADP. Protein (60 nM) was incubated with [³H]-ADP for 15 minutes at 25°C. The DNA substrate was then added and the mixture placed on ice for 5 minutes. The exchange reaction was initiated by the addition of ATP to a final concentration of 25 μM. The reaction was carried out at 25°C and stopped at the times indicated in Figure 6 by dilution of the sample into 4 ml of ice cold stop buffer containing 25 mM Hepes, pH8.1, 100 mM NaCl, 2mM MgCl₂
and 15% glycerol. The sample was then immediately filtered through a nitrocellulose filter (Millipore HAWP02500, 25mm, 0.45μm) and washed with 4 ml of ice-cold stop buffer. Five ml of scintillation fluid was added to the dried filters and the samples were quantitated by liquid scintillation counting. The amount of [³H]-ADP bound by hMSH2-hMSH6 and hMSH2-hMSH3 before the addition of DNA and ATP was used as the zero time point and normalized to 100% binding.

RESULTS

Recognition of DNA Substrates - Purified hMSH2-hMSH6 and hMSH2-hMSH3 were assessed for their ability to bind to duplex DNA oligonucleotides containing a single O⁶-methylguanine adduct at a defined position paired with either cytosine (mG/C) or thymine (mG/T) nucleotides (Figure 1). A number of reports have demonstrated that hMSH2-hMSH6 binds primarily to single base mispairs in DNA with the highest apparent binding affinity for G/T mismatches (33-36). In addition, hMSH2-hMSH6 has been shown to bind DNA containing a variety of adducts including O⁶-methylguanine (11). In contrast, hMSH2-hMSH3 recognizes primarily larger insertion deletion loop-type (IDL) mismatched DNA (34). While biochemical studies of hMSH2-hMSH3 on IDL’s have been reported (10), its affinity for DNA adducts is not known. We examined the relative gel-shift binding of hMSH2-hMSH6 and hMSH2-hMSH3 to the five
model DNA substrates (Figure 1 and 2). A comparison of hMSH2-hMSH6 binding affinity suggests G/T >> +CA > mG/T > mG/C ≈ G/C. In contrast, the hMSH2-hMSH3 binding affinity suggests +(CA) >> mG/T ≈ mG/C ≈ G/T ≈ G/C. However, these results should be tempered by the lack of equivalent binding saturation by the DNA substrates which suggests complex binding functions (see discussion).

We have used competition studies to overcome such complex binding activities and to gauge the relative binding specificity of the human MutS homologs for DNA (33). Our previous studies demonstrated that hMSH2-hMSH3 exhibited a substantial affinity for DNA containing a +(CA) mismatch (10) while hMSH2-hMSH6 exhibited a substantial affinity for DNA containing a G/T mismatch substrates (33). Thus, we assessed the relative affinity of O6-methylguanine containing DNA substrates for hMSH2-hMSH3 and hMSH2-hMSH6 relative to their binding to 20 nM of the 32P-labelled model high affinity substrates, respectively (Figure 3). We determined that the amount of unlabelled test DNA competitor required to reduce binding of hMSH2-hMSH6 to 32P-labelled oligonucleotide containing a G/T mismatch (G/T) by 50% (IC50) was: G/T (45 nM), mG/T (360 nM), mG/C (560 nM), +(CA) (870 nM), and G/C (1550 nM).

In contrast, the IC50 for unlabelled competitors with hMSH2-hMSH3 binding to the 32P-labelled +(CA) substrate were: +(CA) (40 nM), mG/T (275 nM), G/T (285 nM), G/C (365 nM) and mG/C (375 nM). These observations suggest that hMSH2-hMSH6 displays a significant
affinity for both mG/C and mG/T containing DNA substrates while hMSH2-hMSH3 does not appear to exhibit any significant affinity for the O\textsuperscript{6}-methylguanine DNA substrates.

**O\textsuperscript{6}-methylguanine-DNA Stimulation of the ATPase Activity** - DNA containing mispaired nucleotides has been shown to stimulate the intrinsic ATPase activity associated with both hMSH2-hMSH3 and hMSH2-hMSH6 (10,30). However, it is unclear whether DNA containing nucleotide damage stimulates the intrinsic ATPase activity of the heterodimeric complexes in a similar manner. We determined the ability of the mG/C and mG/T substrates to stimulate the ATPase activity of hMSH2-hMSH3 and hMSH2-hMSH6 (Figure 4 and Table 1). The steady-state ATPase activity of hMSH2-hMSH6, relative to that of homoduplex (G/C) DNA, was increased for both mG/C and mG/T substrates. It is interesting to note that when O\textsuperscript{6}-methylguanine was paired with T (mG/T), the steady-state ATPase was dramatically reduced compared to the G/T substrate, while the mG/C substrate increases steady-state ATPase compared to the G/C substrate. Surprisingly, we find the k_{cat} value for hMSH2-hMSH6 in the presence of the +CA substrate was approximately half of that reported by Gradia (33) while the values for the G/T mismatch substrate and homoduplex DNA were similar. Comparison of the oligonucleotides used in these two studies suggested that the sequence context surrounding the +CA mismatched base pairs was different and likely to account for the differences between experiments (data not shown). Studies are in progress to understand the sequence context effects associated with ATPase activation of the hMSH2-hMSH3 and hMSH2-hMSH6 molecular
switch. Importantly, we found that mG/C and mG/T only slightly elevated the ATPase activity of hMSH2-hMSH3 over that of the G/C DNA substrate.

We find a continuous discrimination of O\textsuperscript{6}-methylguanine-containing substrates versus homoduplex DNA by hMSH2-hMSH6 over a wide variety of DNA concentrations (Figure 5 and Table 2). Taken together with the single DNA concentration kinetic data, these results are consistent with the idea that hMSH2-hMSH6 (and not hMSH2-hMSH3) is specifically activated by O\textsuperscript{6}-methylguanine containing DNA.

\textit{O\textsuperscript{6}-methylguanine-DNA Stimulation of ADP→ATP Exchange} - The rate-limiting step for the intrinsic ATPase of MutS and its homologs is ADP→ATP exchange (30). Moreover, ADP→ATP exchange by hMSH2-hMSH3 and MSH2-hMSH6 has been shown to be specific and hierarchical for individual mismatched nucleotides (10,30). We examined the ability of O\textsuperscript{6}-methylguanine containing oligonucleotides to provoke ADP→ATP exchange (Figure 6).

Relative to the homoduplex DNA, the presence of the O\textsuperscript{6}-methylguanine group resulted in a significantly faster rate-of-exchange with both mG/C and mG/T with hMSH2-hMSH6. However, the rate of ADP→ATP exchange provoked by mG/C and mG/T was nearly identical to that of homoduplex DNA with hMSH2-hMSH3. These results are consistent with the steady-state ATPase activities induced by O\textsuperscript{6}-methylguanine containing oligonucleotides and suggest
that hMSH2-hMSH6 is activated by O\textsuperscript{6}-methylguanine in homoduplex or heteroduplex DNA while hMSH2-hMSH3 is largely inactive in the presence of O\textsuperscript{6}-methylguanine lesions.

In order to compare the efficiency that these substrates induce the steady-state ATPase of the human MutS homologs we calculated the $k_{cat}/K_m$ (Table I). Although the $k_{cat}$ for the methylated substrates relative to the homoduplex DNA appeared to increase with hMSH2-hMSH3, little change was observed in the $k_{cat}/K_m$ values (even for the +CA which is a known substrate for hMSH2-hMSH3). These results contrast the ADP→ATP exchange data which clearly demonstrate that the +CA substrate provokes ADP→ATP exchange, while the G/T, mG/T, mG/C, and G/C substrates appeared nearly identical. Moreover, while we find the $k_{cat}$ and ADP→ATP exchange data are concordant, the $K_m$ for the +CA substrate increases significantly relative to the G/T, mG/T, mG/C, and G/C substrates. These results suggest that there is likely to be little or no discrimination of O\textsuperscript{6}-methylguanine containing DNA over homoduplex DNA by the hMSH2-hMSH3 heterodimer, and that the ATPase efficiency (as measured by the $k_{cat}/K_m$) of hMSH2-hMSH3 for a recognized substrate may indicate an alternative feature distinct from hMSH2-hMSH6. In contrast, the $k_{cat}/K_m$ and ADP→ATP exchange for both mG/C and mG/T increased relative to that of the homoduplex G/C for hMSH2-hMSH6.
DISCUSSION

The eukaryotic MMR system has been shown to be involved in the cellular processing of DNA damage induced by methylating agents such as MNU and MNNG. These alkylating agents are capable of forming covalent adducts at the O\textsuperscript{6} position of guanine which are known to be mutagenic and cytotoxic if not properly repaired. In this study, oligonucleotides containing a single O\textsuperscript{6}-methylguanine lesion at a defined position were used as substrates for the purified human DNA mismatch recognition proteins hMSH2-hMSH3 and hMSH2-hMSH6 in an effort to understand the underlying biochemical mechanism by which they mediate the cytotoxicity of O\textsuperscript{6}-methylguanine lesions. The O\textsuperscript{6}-methylguanine was placed opposite both a C (mG/C) and a T (mG/T) on the complementary strand. It has been reported that DNA replication past an O\textsuperscript{6}-methylguanine frequently inserts a T opposite the G containing the O\textsuperscript{6}-methyl group. We found that the O\textsuperscript{6}-methylguanine adduct was a better substrate when paired with a T than when paired with a C for hMSH2-hMSH6 in both the gel shift and competition binding analysis. These results are similar to other reports which have shown an increased binding affinity of hMSH2-hMSH6 for O\textsuperscript{6}-methylguanine and cisplatin intrastrand cross-links when opposite a T (11,12). These data support the idea that translesion DNA replication increases the efficiency of damage recognition leading either to repair and/or other downstream signaling. Interestingly, little or no binding to O\textsuperscript{6}-methylguanine lesions was observed with hMSH2-hMSH3, which is consistent
with the observation that cells carrying homozygous mutations in hMSH6 are more resistant to the killing effects of methylating agents while cells carrying homozygous mutations in hMSH3 appear similar to wild type (37).

Previous studies have shown that mismatched nucleotides in DNA induce the intrinsic ATPase activity of both hMSH2-hMSH3 and hMSH2-hMSH6. We have demonstrated here that DNA containing a single O6-methylguanine lesion is also capable of stimulating the ATPase activity hMSH2-hMSH6. This steady-state ATPase activity was consistent with the rate at which a particular lesion/mismatch provokes ADP→ATP exchange by either hMSH2-hMSH3 or hMSH2-hMSH6. However, the role for the ATPase activity of these complexes in mismatch repair has been somewhat controversial. It has been proposed that ATP hydrolysis is necessary for the translocation of the hMSH2-hMSH6 heterodimer along the DNA backbone following recognition of mispaired nucleotides (38). An alternative model suggests that mismatched or lesion-containing DNA provokes the exchange of ADP→ATP resulting in a conformational transition and in the formation a hydrolysis independent sliding clamp on DNA (31). In this model, it is the increased local concentration of sliding clamps associated DNA adjacent to the lesion/mismatch that serve as a first step for downstream signaling events (29). While O6-methylguanine lesions appear to stimulate hMSH2-hMSH6 adenosine nucleotide processing activity, the modest stimulation of cognate hMSH2-hMSH3 activities suggests only a weak or modest overlapping and redundant function; similar to mispair recognition (39). However, it is worth noting that the ratio of hMSH2-hMSH6 to hMSH2-hMSH3 in mismatch proficient HeLa
cells has been estimated to be 10:1 (34). Together with the observation that hMSH2-hMSH3
displays a near order-of-magnitude weaker ATP processing activity than hMSH2-hMSH6, these
results suggest that processing of $O^6$-methylguanine lesions by hMSH2-hMSH3 is unlikely to
contribute to the cytotoxicity of these lesions.

The mechanism by which loss of DNA mismatch repair results in resistance $O^6$-
methylguanine and cisplatin lesions is not completely understood. However, it is becoming
increasingly clear that methylation damage (likely $O^6$-methylguanine lesions) induce apoptosis
through a MMR-dependent signaling pathway (19,20). A recent study has shown that cells
treated with MNU induce the phosphorylation and stabilization of the p53 tumor suppressor,
which appears to require a functional MMR system (40). However, other studies have clearly
shown that apoptosis induced by methylating agents is dependent on MMR but independent of
p53 (18,19). Interestingly, a p53 related gene, p73, appears to be over-expressed and directly
linked to the MMR-dependent apoptotic response induced by cisplatin (41). These studies are
complicated by the observation that, initiation of apoptosis appears to be lesion specific, since
cells treated with ionizing radiation undergo apoptosis regardless of MMR status (18).

The observation that hMSH2-hMSH6 adenosine nucleotide processing is stimulated by
mG/C reduces the likelihood of a model for methylation sensitivity in mammalian cells that
uniquely depends on replication misincorporation followed by a "futile-cycle" of repair.
Moreover, the significant steady-state ATPase implies cycles of lesion recognition, ADP→ATP
exchange, and the formation of an hMSH2-hMSH6 sliding clamp that diffuses away from the lesion containing oligonucleotide; thus reducing the likelihood of a "binding-occlusion" model. These observations are consistent with a model in which a portion of the sensitivity to O\(^6\)-methylguanine occurs via recognition of mG/C by the MMR machinery and direct transduction of this lesion recognition to the apoptotic machinery (although we can not completely eliminate an intertwined lesion-dependent replication-block model). The importance of MMR complexes in the direct initiation of apoptosis has been underlined by the observation that over-expression of hMSH2 or hMLH1, but not hMSH3, hMSH6 or hPMS2, in primary mouse embryo fibroblasts provokes apoptosis in both repair proficient and deficient lines (19). The precise mechanism of MMR signaling to downstream effectors, such as the repair or apoptotic processes, remains to be established. It is tempting to speculate that two separate signal transduction pathways exist for the MMR machinery, one to signal DNA mismatch/lesion repair and one, in the case of high lesion loads, to signal apoptosis.

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REFERENCES

1. Kolodner, R. (1996) *Genes & Development* **10**(12), 1433-42
2. Fishel, R., and Wilson, T. (1997) *Current Opinion in Genetics & Development* **7**(1), 105-13
3. Modrich, P., and Lahue, R. (1996) *Annual Review of Biochemistry* **65**, 101-33
4. Jiricny, J. (1996) *Cancer Surveys* **28**, 47-68
5. Fishel, R. (1998) *Genes & Development* **12**(14), 2096-101
6. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) *DNA Repair and Mutagenesis*, American Society for Microbiology, Washington, D.C.
7. Acharya, S., Wilson, T., Gradia, S., Kane, M. F., Guerrette, S., Marsischky, G. T., Kolodner, R., and Fishel, R. (1996) *Proceedings of the National Academy of Sciences of the United States of America* **93**(24), 13629-34
8. Risinger, J. I., Umar, A., Boyd, J., Berchuck, A., Kunkel, T. A., and Barrett, J. C. (1996) *Nature Genetics* **14**(1), 102-5
9. Palombo, F., Iaccarino, I., Nakajima, E., Ikejima, M., Shimada, T., and Jiricny, J. (1996) *Current Biology* **6**(9), 1181-4
10. Wilson, T., Guerrette, S., and Fishel, R. (1999) *Journal of Biological Chemistry* **274**(31), 21659-64
11. Duckett, D. R., Drummond, J. T., Murchie, A. I., Reardon, J. T., Sancar, A., Lilley, D. M.,
12. Yamada, M., O'Regan, E., Brown, R., and Karran, P. (1997) *Nucleic Acids Research* **25**(3), 491-6

13. Mu, D., Tursun, M., Duckett, D. R., Drummond, J. T., Modrich, P., and Sancar, A. (1997) *Molecular & Cellular Biology* **17**(2), 760-9

14. Branch, P., Aquilina, G., Bignami, M., and Karran, P. (1993) *Nature* **362**(6421), 652-4

15. Humbert, O., Fiumicino, S., Aquilina, G., Branch, P., Oda, S., Zijno, A., Karran, P., and Bignami, M. (1999) *Carcinogenesis* **20**(2), 205-14

16. Lage, H., and Dietel, M. (1999) *Journal of Cancer Research & Clinical Oncology* **125**(3-4), 156-65

17. Vaisman, A., Varchenko, M., Umar, A., Kunkel, T. A., Risinger, J. I., Barrett, J. C., Hamilton, T. C., and Chaney, S. G. (1998) *Cancer Research* **58**(16), 3579-85

18. Hickman, M. J., and Samson, L. D. (1999) *Proceedings of the National Academy of Sciences of the United States of America* **96**(19), 10764-9

19. Zhang, H., Richards, B., Wilson, T., Lloyd, M., Cranston, A., Thorburn, A., Fishel, R., and Meuth, M. (1999) *Cancer Research* **59**(13), 3021-7

20. Meikrantz, W., Bergom, M. A., Memisoglu, A., and Samson, L. (1998) *Carcinogenesis* **19**(2), 369-72

21. Hawn, M. T., Umar, A., Carethers, J. M., Marra, G., Kunkel, T. A., Boland, C. R., and
Koi, M. (1995) *Cancer Research* **55**(17), 3721-5

22. Umar, A., Koi, M., Risinger, J. I., Glaab, W. E., Tindall, K. R., Kolodner, R. D., Boland, C. R., Barrett, J. C., and Kunkel, T. A. (1997) *Cancer Research* **57**(18), 3949-55

23. Fink, D., Nebel, S., Aebi, S., Zheng, H., Cenni, B., Nehme, A., Christen, R. D., and Howell, S. B. (1996) *Cancer Research* **56**(21), 4881-6

24. Singer, B., Chavez, F., Goodman, M. F., Essigmann, J. M., and Dosanjh, M. K. (1989)

   *Proceedings of the National Academy of Sciences of the United States of America* **86**(21), 8271-4

25. Goldmacher, V. S., Cuzick, R. A., Jr., and Thilly, W. G. (1986) *Journal of Biological Chemistry* **261**(27), 12462-71

26. Karran, P., and Bignami, M. (1992) *Nucleic Acids Research* **20**(12), 2933-40

27. Mello, J. A., Acharya, S., Fishel, R., and Essigmann, J. M. (1996) *Chemistry & Biology* **3**(7), 579-89

28. Moreland, N. J., Illand, M., Kim, Y. T., Paul, J., and Brown, R. (1999) *Cancer Research* **59**(9), 2102-6

29. Fishel, R. (1999) *Nature Medicine* **5**(11), 1239-1241

30. Gradia, S., Acharya, S., and Fishel, R. (1997) *Cell* **91**(7), 995-1005

31. Gradia, S., Subramanian, D., Wilson, T., Acharya, S., Makhov, A., Griffith, J., and Fishel, R. (1999) *Molecular Cell* **3**(2), 255-61

32. Blackwell, L. J., Bjornson, K. P., and Modrich, P. (1998) *Journal of Biological Chemistry*
273(48), 32049-54

33. Gradia, S., Acharya, S., and Fishel, R. (2000) J. Biol. Chem. 275(6), 3922-3930

34. Genschel, J., Littman, S. J., Drummond, J. T., and Modrich, P. (1998) Journal of Biological Chemistry 273(31), 19895-901

35. Alani, E. (1996) Molecular & Cellular Biology 16(10), 5604-15

36. Jiricny, J., Su, S. S., Wood, S. G., and Modrich, P. (1988) Nucleic Acids Research 16(16), 7843-53

37. de Wind, N., Dekker, M., Claij, N., Jansen, L., van Klink, Y., Radman, M., Riggins, G., van der Valk, M., van't Wout, K., and te Reile, H. (1999) Nature Genetics 23, 359-362

38. Blackwell, L. J., Martik, D., Bjornson, K. P., Bjornson, E. S., and Modrich, P. (1998) Journal of Biological Chemistry 273(48), 32055-62

39. Kolodner, R. D., and Marsischky, G. T. (1999) Current Opinion in Genetics & Development 9(1), 89-96

40. Duckett, D. R., Bronstein, S. M., Taya, Y., and Modrich, P. (1999) PNAS 96(22), 12384-12388

41. Gong, J. G., Costanzo, A., Yang, H. Q., Melino, G., Kaelin, W. G., Jr., Levrero, M., and Wang, J. Y. (1999) Nature 399(6738), 806-9
FIGURE LEGENDS

FIGURE 1. Oligonucleotide DNA substrates (mG = O6-methylguanine).

FIGURE 2. Relative binding of hMSH2-hMSH6 and hMSH2-hMSH3 to oligonucleotide DNA substrates containing O6-methylguanine. Binding of 0, 6.25, and 12.5 nM of hMSH2-hMSH6 (Panel A) and 0, 12.5, and 25 nM of hMSH2-hMSH3 (Panel B) to 0.27 ng of 32P-end labelled 41 base pair duplex oligonucleotide substrates shown in Figure 1. Binding reactions were carried out in the presence of 25 ng of unlabelled G/C homoduplex DNA and separated on a 5% native gel.

Figure 3. Competition binding specificity of hMSH2-hMSH6 and hMSH2-hMSH3 to oligonucleotide DNA substrates containing O6-methylguanine. Panel A) 12.5 nM hMSH2-hMSH6 was incubated with 10 ng of 32P-end labelled G/T substrate in the presence of increasing concentrations of the indicated unlabelled duplex DNA competitor. Panel B) 25 nM hMSH2-hMSH3 was incubated with 10 ng of 32P-end labelled +CA substrate in the presence of increasing concentrations of the indicated unlabelled duplex DNA competitor. Data points and error bars represent the average and standard deviation (respectively) from at least two experiments.
Figure 4. **Stimulation of hMSH2-hMSH6 and hMSH2-hMSH3 ATPase activity by O\(^6\)-methylguanine DNA substrates.** ATP hydrolysis by hMSH2-hMSH6 (Panel A) and hMSH2-hMSH3 (Panel B) was measured in the absence of DNA (no DNA), and in the presence of each of the 41bp duplex DNA substrates (240nM) shown in Figure 1 for the indicated concentrations of ATP. Data points and error bars represent the average and standard deviation (respectively) from at least three experiments. Kinetic parameters were calculated by fitting the data to the Michaelis-Menten equation and presented in Table 1 (see materials and methods).

Figure 5. **DNA concentration-dependent stimulation of the hMSH2-hMSH6 ATPase by O\(^6\)-methylguanine DNA substrates.** DNA concentration profile of the hMSH2-hMSH6 (75 nM) ATPase activity. Reactions were carried using 100 µM ATP with increasing molar concentrations (nM molecules) of the G/C, mG/C and mG/T oligonucleotide substrates. Data points and error bars represent the average and standard deviation (respectively) from at least two experiments.

Figure 6. **Stimulation of ADP-ATP exchange by O\(^6\)-methylguanine DNA substrates.** 60 nM hMSH2-hMSH6 (Panel A) and 60 nM hMSH2-hMSH3 (Panel B) were incubated with 2.3 µM \(^3\)H-ADP and 60 nM of the indicated duplex oligonucleotide substrate (see materials and methods). Exchange reactions were initiated by the addition of ATP to a final concentration of
25 μM and stopped at the indicated time points. Data points and error bars represent the average and standard deviation (respectively) from at least three experiments.
Table 1. Stimulation of hMSH2-hMSH6 and hMSH2-hMSH3 ATPase activity by duplex oligonucleotide substrates.

A. hMSH2-hMSH6

| DNA substrate | $k_{cat-DNA}$ (min$^{-1}$) | $K_m$ (M x 10$^{-6}$) | $k_{cat}/K_m$ (min$^{-1}$ • M$^{-1}$ x 10$^{-4}$) |
|---------------|----------------------------|------------------------|---------------------------------|
| G/T           | 17.98 +/- 0.41              | 42.5 +/- 3.0           | 42.4                            |
| mG/T          | 8.72 +/- 0.11               | 28.0 +/- 1.3           | 31.2                            |
| +CA           | 8.44 +/- 0.19               | 24.2 +/- 2.0           | 34.9                            |
| mG/C          | 7.29 +/- 0.12               | 24.4 +/- 1.6           | 29.9                            |
| G/C           | 5.82 +/- 0.08               | 29.0 +/- 1.5           | 20.1                            |
| no DNA        | 1.10 +/- 0.19               | 80.2 +/- 33.8          | 1.4                             |

B. hMSH2-hMSH3

| DNA substrate | $k_{cat-DNA}$ (min$^{-1}$) | $K_m$ (M x 10$^{-6}$) | $k_{cat}/K_m$ (min$^{-1}$ • M$^{-1}$ x 10$^{-4}$) |
|---------------|----------------------------|------------------------|---------------------------------|
| +CA           | 1.92 +/- 0.029             | 4.8 +/- 0.242          | 40.2                            |
| mG/C          | 1.22 +/- 0.022             | 2.4 +/- 0.184          | 50.7                            |
| mG/T          | 1.16 +/- 0.018             | 2.8 +/- 0.174          | 42.1                            |
| G/T           | 1.04 +/- 0.025             | 3.0 +/- 0.283          | 35.1                            |
| G/C           | 0.85 +/- 0.013             | 1.9 +/- 0.133          | 44.7                            |
| no DNA        | 0.28 +/- 0.002             | 0.5 +/- 0.026          | 61.5                            |
Table 2. DNA concentration dependent stimulation of hMSH2-hMSH6 ATPase activity by G/C, mG/C, and mG/T DNA substrates

| DNA substrate | $k_{cat-DNA}$ | $K_{1/2,DNA}$ | $k_{cat} / K_m$ |
|---------------|--------------|---------------|----------------|
| G/C          | 7.89 +/- 0.90 | 77.97 +/- 23.7 | 10.12          |
| mG/C         | 9.77 +/- 0.67 | 63.78 +/- 12.7 | 15.32          |
| mG/T         | 9.89 +/- 0.23 | 33.13 +/- 2.9  | 29.85          |
Figure 1

G/C

GCT TAG GAT CAT CGA GGA TCG AGC TCG GTG CAA TTC AGC GG
CGA ATC CTA GTA GCT CCT AGC TCG AGC CAC GTT AAG TCG CC

mG/C

GCT TAG GAT CAT CGA GGA TCGmG AGC TCG GTG CAA TTC AGC GG
CGA ATC CTA GTA GCT CCT AG C TCG AGC CAC GTT AAG TCG CC

G/T

GCT TAG GAT CAT CGA GGA TCgG AGC TCG GTG CAA TTC AGC GG
CGA ATC CTA GTA GCT CCT AGT TCG AGC CAC GTT AAG TCG CC

mG/T

GCT TAG GAT CAT CGA GGA TCmG AGC TCG GTG CAA TTC AGC GG
CGA ATC CTA GTA GCT CCT AG T TCG AGC CAC GTT AAG TCG CC

+CA

GCT TAG GAT CAT CGA GGA TC g AGC TCG GTG CAA TTC AGC GG
CGA ATC CTA GTA GCT CCT AGAC TCG AGC CAC GTT AAG TCG CC
| DNA substrate | hMSH 2/6 (nM) | G/C | mG/C | G/T | mG/T | +CA |
|---------------|--------------|-----|------|-----|------|-----|
|               |              | 0   | 6.25 | 12.5| 25   |     |
|               |              | 0   | 6.25 | 12.5| 25   |     |
|               |              | 0   | 6.25 | 12.5| 25   |     |
|               |              | 0   | 6.25 | 12.5| 25   |     |
|               |              | 0   | 6.25 | 12.5| 25   |     |
|               |              | 0   | 6.25 | 12.5| 25   |     |

A

| DNA substrate | hMSH 2/3 (nM) | G/C | mG/C | G/T | mG/T | +CA |
|---------------|--------------|-----|------|-----|------|-----|
|               |              | 0   | 12.5 | 25  |      |     |
|               |              | 0   | 12.5 | 25  |      |     |
|               |              | 0   | 12.5 | 25  |      |     |
|               |              | 0   | 12.5 | 25  |      |     |
|               |              | 0   | 12.5 | 25  |      |     |
|               |              | 0   | 12.5 | 25  |      |     |

B
Figure 3

A

hMSH2-hMSH6

% labelled G/T bound

[unlabelled DNA competitor] (nM)

B

hMSH2-hMSH3

% labelled +CA bound

[unlabelled competitor DNA] (nM)
Figure 4

A

hMSH2-hMSH6

ATPase velocity (min⁻¹)

[ATP] μM

B

hMSH2-hMSH-3

ATPase velocity (min⁻¹)

[ATP] μM
Figure 5

[Graph showing the ATPase velocity (min⁻¹) against [DNA] nM for hMSH2-hMSH6 with data points for G-C, mG-C, and mG-T.]
Figure 6

A

hMSH2-hMSH6

B

hMSH2-hMSH3
The Effect of O6-methylguanine DNA adducts on the Adenosine Nucleotide Switch
Functions of hMSH2-hMSH6 and hMSH2-hMSH3
Mark Berardini, Anthony Mazurek and Richard Fishel

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