Specific Binding of Human MSH2-MSH6 Mismatch-Repair Protein Heterodimers to DNA Incorporating Thymine- or Uracil-containing UV Light Photoproducts Opposite Mismatched Bases*

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Huxian Wang‡, Christopher W. Lawrences‡, Guo-Min Lif‡, and John B. Hays¶

From the ¶Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, Oregon 97331-7301, the ¶Department of Biophysics and Biochemistry, University of Rochester Medical Center, Rochester, New York 14642-8408, and the §Department of Pathology, University of Kentucky Medical Center, Lexington, Kentucky 40536

Previous studies have demonstrated recognition of DNA-containing UV light photoproducts by bacterial (Feng, W.-X., Lee, E., and Hays, J. B. (1991) Genetics 128, 1007–1020) and human (Mu, D., Tursun, M., Duckett, D. R., Drummond, J. T., Modrich, F., and Sancar, A. (1997) Mol. Cell. Biol. 17, 780–789) long-patch mismatch-repair systems. Mismatch repair directed specifically against incorrect bases inserted during semi-conservative DNA replication might efficiently antagonize UV mutagenesis. To test this hypothesis, DNA 5'-mers containing site-specific T-T cis-syn-cyclobutane pyrimidine-dimers or T-T pyrimidine-(6-4)pyrimidinoine photoproducts, with all four possible bases opposite the respective 3'-thymines in the photoproducts, were analyzed for the ability to compete with radiolabeled (T/G)-mismatched DNA for binding by highly purified human MSH2-MSH6 heterodimer protein (hMutSα). Both (cyclobutane-dimer)/AG and ((6-4)photoproduct)/AG mismatches competed about as well as non-photoproduct T/T mismatches. The two respective pairs of photoproduct/AA "matches"; the apparent affinity of hMutSα for the (6-4)photoproduct)/AA, "matched" substrate was actually less than that for TT/AA homoduplexes. Surprisingly, although hMutSα affinities for both non-photoproduct UU/AG double mismatches and for (uracil-cyclobutane-dimer)/AG single mismatches were high, affinity for the (uracil-cyclobutane-dimer)/GG mismatch was quite low. Equilibrium binding of hMutSα to DNA containing (photoproduct/base) mismatches and to (T/G)-mismatched DNA was reduced similarly by ATP (in the absence of magnesium).

In most prokaryotes, and in all eukaryotes examined, highly conserved protein systems that recognize DNA mismatches and certain DNA lesions play critical roles in maintenance of genetic stability. These long-patch mismatch-repair systems decrease DNA replication error rates 100–1000-fold, by recognizing and correcting mismatches that escape proofreading by DNA polymerases (1). In Escherichia coli (2), homodimers of MutS protein bind preferentially to mismatches; MutS and MutL homodimers then activate MutH protein to nick specifically the unmethylated DNA strand at the nearest adenine-hemimethylated d(GATC) sequence, during the interval before adenes in newly replicated d(GATC) sequences are methylated. This most likely involves a translocation/search process that requires ATP hydrolysis (3). MutH thus specifically directs incision and subsequent excision to the nascent DNA strand, so replicative errors are always corrected rather than fixed. After excision via exonucleolytic cleavage from the initial incision past the mismatch, the replicative polymerase (here E. coli DNA polymerase III) fills the gap. The MutH/d(GATC)-hemimethylation strand-specificity mechanism is not found in eukaryotes or in some bacteria, and repair directionality remains poorly understood in these organisms.

The MutS/MutL recognition/coupling paradigm is retained in eukaryotes but is now more complex (4, 5). The single eubacterial MutS is replaced by at least six MutS homologs (MSHs)† in yeast, human beings, and probably other eukaryotes. Recognition of nuclear-DNA mismatches is accomplished by two distinct heterodimers, comprised of MSH2 plus MSH6 or MSH2 plus MSH3 polypeptides; these show different but overlapping specificities for base/base mismatches and various insertion/deletion loop-outs (6–9). Other MutS homologs, MSH1, and MSH4 and MSH5, play less well characterized roles in mitochondrial stability (10) and meiotic recombination (11, 12) respectively. Two of the several eukaryotic MutL homologs, in the form of an MLH1-PMS2 heterodimer, couple mismatch recognition to subsequent processing steps (13); this again appears to involve ATP-triggered translocation of MSH2-MSH6 (or MSH2-MSH3) (14, 15).

Accumulating evidence for interaction of mismatch-repair systems with a surprising variety of DNA lesions now points to additional roles for mismatch repair. These lesions include UV light photoproducts (16, 17), O6-MeG residues (18, 19), cisplatin G-G intrastrand cross-links (18), adriamycin (20) and acetyl-aminofluorene and aminofluorene (AAF/AF) adducts.

† The abbreviations used are: MSH, MutS homologs; bp, base pairs; BSA, bovine serum albumin; CPD, cis-syn-cyclobutane-pyrimidine-dimers; (6-4)photoproduct, pyrimidine-(6-4)pyrimidinoine photoproduct; hMutSα, human MSH2-MSH6 protein heterodimer; T/<<-T/AA, T/<<-AG, T/<<-AC, T/<<-T/A, C/>>-C/GG, U/>>-U/AA, U/>>-U/AG, U/>>-U/AG, U/>>-U/AC, U/>>-U/AT, thymine-, cytosine-, or uracil-containing cyclobutane-dimers (5′–5′ as shown), appearing in DNA opposite indicated (3′–5′) nucleotides; T/6-4/T/AA, T/6-4/T/AG, T/6-4/T/AC, T/6-4/T/T (thymine-containing) (6-4)photoproducts (shown 5′–3′) appearing in DNA opposite indicated (3′–5′) dinucleotides; Pu, purine nucleotide; Py, pyrimidine nucleotide; BND-cellulose, benzoylated naphthoylated DEAE-cellulose.

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¶ To whom correspondence should be addressed. Tel.: 541-737-1777; Fax: 541-737-0497; E-mail: haysj@bcc.orst.edu.
(21), and S<sup>6</sup>-methylthioguanine/base mismatches (22). In particular, recombination of nonreplicating UV-irradiated phage λ DNA in (unirradiated) <i>E. coli</i> mutants deficient in excision repair (Uvr<sup>α</sup>) was found to depend mostly on <i>E. coli</i> MutS, MutL, and MutH functions, on the presence of unmethylated d(GATC) sequences in the phage DNA (16), and on helicase and exonuclease processes previously implicated in mismatch repair (17). Analysis of intracellular DNA revealed extensive MutS-dependent breakdown of the nonreplicating UV-irradiated DNA (16). Studies of transcription-coupled nucleotide excision repair of cyclobutane-pyrimidine-dimers (CPDs) in <i>E. coli</i> and in human cells have, respectively, implicated MutS and MutL (23) and MSH2 and MLH1 (24) functions in these processes.

A recent study by Mu et al. (25) has provided direct evidence for binding of human MSH2-MSH6 heterodimers (conveniently abbreviated hMutS<sub>a</sub>) to DNA-containing UV photoproducts (or cisplatin lesions). Binding to DNA-containing CPD/base mismatched TTTGAGGAGAAGGAAATGGGACGATTGACTCCCTTGCCACTATGATTGCTAACCATT moieties was roughly 1/3 as high as binding to DNA-containing CPD/base mismatches (or cisplatin lesions). Binding to DNA-containing CPD/base mismatches (T<>T/TAG) was roughly 1/3 as high as binding to non-photoproduct base/base mismatches (T/TAG). The marginal binding to matched CPDs (T<>T/TAG) and (6-4) photoproducts (T<>T/TAG) could not be clearly distinguished from the nonspecific background. No data for (T<>T/TAG) substrates were reported. In parallel experiments, T<>T/TAG moieties proved to be markedly better substrates for nucleotide excision repair than T<>T/TAG.

We show here that hMutS<sub>a</sub> binds with high specificity to both T<>T and T6-4T photoproduct/base mismatches; binding to T6-4T/TAG moieties actually appears less high than to TA homoduplexes. Although hMutS<sub>a</sub> recognizes all photoproduct/base mismaps, the highest affinity is for guanine opposite the 3'-thymine in either photoproduct. Affinity for single mismatches opposite U<>U photoproducts is at least as high as for mismatched T<>T photoproducts.

## EXPERIMENTAL PROCEDURES

**Cell Extracts and Proteins**—HeLaS<sub>3</sub> cells were either purchased from the National Cell Culture Center (Minneapolis, MN) or grown in Joklik modified medium (Life Technologies Inc.) supplemented with 5% calf serum, at the Cell Culture Facility, Oregon State University Environmental Health Science Center. Nuclear extracts were prepared from HeLaS<sub>3</sub> cells as described (26). In brief, proteins were extracted with 0.15 M salt from nuclei released from the cells broken by homogenization and then precipitated with ammonium sulfate. hMutS<sub>a</sub> protein heterodimers were prepared essentially as described (21, 26). Briefly, the 30–65% ammonium sulfate fraction from HeLaS<sub>3</sub> nuclear extracts was passed through a single-stranded DNA-cellulose column, and the fraction subsequently eluted with 1 M ATP, then chromatographed on a 1-ml Amersham Pharmacia Biotech HR 5/5 Mono Q column. Purified hMutS<sub>a</sub> was supplemented with 1.0 mg/ml bovine serum albumin (BSA) and 10% (w/v) sucrose, frozen in liquid nitrogen, and stored at −80 °C. SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining indicated that the purified protein was near homogeneity (>98% pure). This procedure yielded 30–40 μg of protein from 10 to 15 liters of cells, as determined by the Bradford assay using BSA standards (33).

### Preparation of DNA 51-mer Top Strands Containing UV Photoproducts

All oligonucleotide sequences used here were designed to be free from significant secondary structure, using CPrimer program (free software from Regents of University of California). DNA 11-mers containing no photoproducts (Table I, oligomers 1 and 4) or single site-specific T<>T, T6-4T, or U<>U photoproducts (oligomers 2, 3, and 5) were prepared and purified by high pressure liquid chromatography as described (28–30). The U<>U oligomers were then 5'-phosphorylated using phosphorylase-T4 polynucleotide kinase and unlabeled ATP. The T<>T and T6-4T oligomers were purified in phosphorylated form. Chromatographic analyses indicated that T<>T, T6-4T, and U<>U oligomers were >95%, >99, and >95% pure, respectively. The "top right" flanking 20-mer 7 was purchased from Life Technologies, Inc., phosphorylated, and high pressure liquid chromatography-purified. The "top left" flanking 20-mer 6 and the bottom "scaffold" 41-mer 8 were synthesized at the Oregon State University Central Services Facility, 16895

### Table I

**Oligonucleotides used in this study (5’–3’)**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. TT 11-mer</td>
<td>GCAAGTTGGAG</td>
</tr>
<tr>
<td>2. T&lt;&gt;T 11-mer</td>
<td>GCAAGTTGGAG</td>
</tr>
<tr>
<td>3. T&lt;&gt;T 11-mer</td>
<td>GCAAGTTGGAG</td>
</tr>
<tr>
<td>4. TT 11-mer</td>
<td>GCAAGTTGGAG</td>
</tr>
<tr>
<td>5. U&lt;&gt;U 11-mer</td>
<td>GCAAGTTGGAG</td>
</tr>
<tr>
<td>6. 20-mer 1</td>
<td>AATGGTTAGCAATCATATG</td>
</tr>
<tr>
<td>7. 20-mer 2</td>
<td>TCAATCTGCTTCGTATTTC</td>
</tr>
</tbody>
</table>
| 8. 41-mer       | AGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
purified by electrophoresis in 20 or 10% denaturing gels (polyacrylamide gels containing 7 M urea), and recovered from gel by standard procedures (34). To prepare top strand 51-mers (oligomers 9–13), we used about 2.5 mmol each of oligomers 1–5, respectively. The latter were mixed with oligomers 6–8 (1.5–2-fold excess, respectively) in DNA Ligation Buffer (50 mM Tris-HCl, pH 7.5; 7.5 mM MgCl2; 10 mM dithiothreitol; 1 mM ATP; 25 μg/ml BSA) and heated at 85 °C for 5 min; after slow cooling to 16 °C, mixtures were incubated overnight with 15 units of DNA ligase (New England Biolabs, Beverly, MA). The product 51-mer top strands were separated from shorter oligomers by electrophoresis in 10% denaturing gels and recovered as described above.

Single-strand 51-mers containing T<<T or U<<U CPDs were efficiently cleaved by phage T4 endonuclease V, whereas T (6-4)T 51-mers were completely resistant to endonuclease V but were digested by the 3’-5’exonuclease activity of T4 DNA polymerase up to the product positions, yielding 5’-end-labeled 26- and 25-mers. These were mixed with various photoproduct-containing and non-photoproduct 51-mers in Annealing Buffer (10 mM Tris-HCl, pH 8.0; 1 mM Na2EDTA; 50 mM NaCl), heated at 85 °C for 5 min, and slowly cooled to room temperature. After addition of 0.2 volume of BND-cellulose (equivalent to 0.1 volume of settled resin), 5 mM NaCl was added to a final concentration of 1. Mixtures were incubated 5 min and then layered onto Sephadex G-50 Nick Spin Columns (Amersham Pharmacia Biotech, Uppsala, Sweden) that had previously been equilibrated in DNA Buffer (10 mM Tris-HCl, pH 8.0; 1 mM Na2EDTA). Duplexes were recovered after centrifugation, according to the instructions of the manufacturer. To test for removal of single-stranded DNA, a small aliquot was treated with polynucleotide kinase (which prefers 5’-single-stranded DNA ends (35)) and [γ-32P]ATP (3000 Ci/mmol, NEN Life Science Products) and electrophoresed in non-denaturing 10% polyacrylamide gels. If necessary, the BND-cellulose and spin-column steps were repeated. No single-stranded DNA contaminants were apparent in the final autoradiographs. Conductivity of spin-column pass-through solutions were found to be the same as for DNA buffer, i.e. essentially all NaCl had been removed. Final DNA concentrations (typically 0.25–0.5 μg/ml) were determined by the Hoechst-33258 dye-binding fluorometric technique of Labarca and Paigen (36), using a series of DNA standards and a TKO 100 Fluorometer (Hoefer Scientific Instruments) according to the instructions of the manufacturer. We prepared 32P-end-labeled non-photoproduct T/G heteroduplexes similarly, except that the top 51-mer 9 was 32P-5’-end-labeled before mixing with bottom 51-mer 16.

**Electrophoretic Mobility Shift Competition Assays—**Reaction mixtures (25 μl) contained 1.32 nM 32P-5’-end-labeled TT/AG (oligomers 9/16) duplex 51-mers in Binding Buffer (10 mM Hepes-KOH, pH 7.5; 110 mM KCl; 1 mM dithiothreitol; 0.4% glycerol), plus particular competing 51-mer duplexes at various concentrations, and hMutSα protein at 3 nM. After incubation for 20 min on ice, mixtures were supplemented with 5 μl of 50% sucrose (w/v) in Binding Buffer, immediately loaded onto 6% polyacrylamide gels (36:1 acrylamide:bisacrylamide (w/w); 6.7 mM Tris acetate, pH 7.5; 1 mM Na2EDTA), and electrophoresed at 8 V/cm for 240 min, with recirculation of room temperature buffer. Gels were dried onto Whatman 3MM paper and visualized by autoradiography at room temperature, using Kodak Biomax x-ray film with intensifying screens. To determine relative amounts of bound (mobility-shifted) and free [32P]DNA, we measured band intensities using a Molecular Dynamics PhosphorImager. We define fractional binding $FB(c)$ as intensity of shifted [32P]DNA band)/(total intensity of all [32P]DNA bands), in the presence of competitor DNA at concentration c, and we define $c_{50}$, by $FB(c_{50})/FB(0) = 0.5$. We determined $c_{50}$ values for the various competitors from straight lines fitted to $FB(c)$ versus c plots; correlation factors for the fits were >0.97.

**Direct Binding Experiments—**Titration with hMutSα of 32P-labeled TT/AG 51-mer (prepared as described above), on ice and at 37 °C, were performed as described under “Electrophoretic Mobility Shift Competition Assay,” except that protein concentrations were varied from 0.2 to 5 nM and no additional competitor DNA was present. For testing effects of ATG on direct equilibrium binding of hMutSα to TT/AG, T<<T/AG, and T<<T/AG 51-mers, respectively unlabeled competitor duplexes were preincubated using overnight incubation with polynucleotide kinase plus [γ-32P]ATP, and higher ATP:DNA ratios than employed with single-stranded DNA, then purified using Sephadex G-50 spin columns. Mixtures containing 1.32 nM 51-mers, 3 nM hMutSα, and various concentrations of ATP were incubated 20 min on ice, and then analyzed as described under “Electrophoretic Mobility Shift Competition Assays,” except that no competitor DNA was present.

**RESULTS**

**Binding Properties of hMutSα Protein—**Because we expected that hMutSα might bind relatively weakly to some photoproduct base pairs, we sought to maximize the sensitivity of binding assays. Use of purified hMutSα of high specific activity minimized both spurious bands due to binding by protein fragments and nonspecific binding by partially inactive protein, making unnecessary the excess carrier DNA employed in some previous studies. Free single-stranded DNA, for which hMutSα shows significant affinity, was completely removed by BND-cellulose chromatography, as described under “Experimental Procedures.” These precautions made it possible to accurately determine absolute concentrations of both bound and unbound DNA. We initially tested the hMutSα preparation by using it to titrate radiolabeled DNA 51-mers incorporating a T/G mismatch, typically the base mispair recognized best by MutS-like proteins (26, 37, 38), employing electrophoretic mobility shift assays (Fig. 1A). Previous workers have performed such assays at various temperatures. Here titrations at 4 and 37 °C yielded nearly identical binding curves (Fig. 1B). Saturation was at 88 and 93% DNA binding, respectively, and apparent half-satura-

2 H. Wang and J. Hays, unpublished observations.
tion was at 1.3 nM hMutSa. Only negligible levels of complexes moving faster than the major band were apparent (Fig. 1A). To eliminate the possibility that the major band corresponded to highly cooperative binding of two hMutSa heterodimers, which would result in a sigmoidal binding curve, we measured binding at numerous low protein concentrations. The data (Fig. 1B) correspond to a strictly hyperbolic (non-cooperative) curve. Calculation of dissociation constants (Kd values) from concentrations of bound and unbound DNA at hMutSa concentrations below 2 nM yielded 1.2 nM. A similar titration experiment (data not shown) yielded a half-saturation concentration of 1.3 nM. The apparent Kd of 1.2 nM for this hMutSa preparation is at the extreme lower range of values reported for previous binding studies. Previous studies showed apparent saturation at various hMutSa:DNA ratios as follows: 2–4 for hMutSa purified from HeLa cells in the presence (26) or absence (21) of (10-fold) competitor, and 40–50 (18, 25), or more than 600 (15) for recombinant proteins. The high concentrations of nonspecific carrier DNA employed in some of these cases might have affected apparent hMutSa binding properties. At hMutSa concentrations above 2 nM, increasing levels of a slower moving complex appeared, corresponding to about 8% of bound DNA at the highest protein concentrations. This might reflect nonspecific binding of a second hMutSa molecule to regions of the 51-bp substrate outside of the 25–35-bp region expected to be covered by a single heterodimer bound to the central mismatch (15). The titration curve does not extend far enough to determine unequivocally whether binding of this additional protein is cooperative, i.e. facilitated by the presence of the mismatch-bound hMutSa, or essentially independent. The apparent absence of such multi-protein complexes in previous studies may have been due to the smaller size of the heteroduplex substrates employed and/or the presence of excess carrier DNA.

Relative Affinities of hMutSa for Base-Base and Photoprod-
uct-Base Mismatches—To maximize sensitivity to small differences in hMutSa affinity among different substrates, we compared the abilities of various unlabeled DNA 51-mers to compete with 1.32 nM radiolabeled (T/G)-51-mer in mobility shift assays (in the absence of other nonspecific carrier DNA), rather than directly measuring binding affinities. Fig. 2A shows typical competition analyses, for unlabeled 51-mers containing a TT/AG base/base mispair (identical to the radiolabeled probe), a T<>T/AG (cyclobutane-pyrimidine-dimer)/base mispair, and a T(6-4)/T/AG (T(6-4)/T) (6-4)photoproduct)/base mispair. Binding to radiolabeled T/G 51-mer was reduced 50% by an equal concentration of unlabeled T/G 51-mer, as expected. Fig. 2B shows a typical plot of the reciprocal of the fractional binding of radiolabeled (T/G)-51-mer by hMutSa at various competitor concentrations, relative to binding in the absence of competitor. The concentrations corresponding to 50% reduction in binding (ordinate value of 2.0 in Fig. 2B), for a variety of substrates with TT, T<>T, or T(6-4)/T upper strands, are summarized in Fig. 3. Affinities relative to TT/AA homoduplexes appear in parentheses. Several results are of particular interest. First, all base-base mismatches analyzed here were specifically bound by hMutSa, consistent with previous binding studies (26, 37, 38) and with observations that all base or base/base mismatched 51-mer, plus 3 nM hMutSa, purified as described under “Experimental Procedures,” were analyzed as described under “Electrophoretic Mobility Shift Competition Assays.” A, representative autoradiograph for binding of radiolabeled TT/AG 51-mer in the presence of indicated concentrations of unlabeled base/base and photoproduct/base 51-mers. B, plots (data from A) of the fraction of DNA bound (amount of [32P]DNA showing altered mobility divided by total [32P]DNA in the absence of unlabeled competitor, divided by the fraction bound at indicated concentrations of unlabeled TT/AG 51-mer) vs. competitor DNA concentrations (nM). Indicated straight lines were fitted to the respective data sets.

Fig. 2. Competition analysis of hMutSa binding to mis-
matched DNA substrates. Mixtures of 1.32 nM [32P]-5’-end-labeled (T/G) 51-mer, a particular concentration of a particular photoproduct/base or base/base mismatched 51-mer, plus 3 nM hMutSa, purified as described under “Experimental Procedures,” were analyzed as described under “Electrophoretic Mobility Shift Competition Assays.” A, representative autoradiograph for binding of radiolabeled TT/AG 51-mer in the presence of indicated concentrations of unlabeled base/base and photoproduct/base 51-mers. B, plots (data from A) of the fraction of DNA bound (amount of [32P]DNA showing altered mobility divided by total [32P]DNA in the absence of unlabeled competitor, divided by the fraction bound at indicated concentrations of unlabeled TT/AG 51-mer) vs. competitor DNA concentrations (nM). Indicated straight lines were fitted to the respective data sets.

affinity of the TT/AG base/base mismatch, both affinities are as high as that of the TT/AT base/base mismatch, which is repaired efficiently in vitro (39) and in vivo (40). However, relative affinities and repair efficiencies may be affected by sequence context. Third, mismatched (6-4)photoproduct substrates showed considerably more affinity than the matched T(6-4)/T/AA substrate, although both photoproduct base combinations are expected to be highly distorted and devoid of hydrogen bonding at the 3’-photoproduct base (48). Again, T(6-4)/T/AG showed the highest affinity. The apparent affinity of a 51-mer containing a single T(6-4)/T/AA moiety was actually significantly less than that of the TT/AA homoduplex (p = 0.0056, based on analysis of variance and pairwise t tests of data for the three matched substrates). The mismatch affinity ratios, i.e. ratios of affinities for TT/AG, T<>T/AG, and T(6-4)/T/AG mismatches relative to affinities for the corresponding matched substrates, were, respectively, 20, 3.6, and 4.3.
U base mismatches appeared higher than the affinity for T/T, and R E different ATP concentrations on direct binding of hMutS ATP sensitivities should be similar. We tested the effect of matches is analogous to its binding to base/base mismatches, 

that the reduction of hMutS matches seen here in the absence of ATP confirms our assumption that the reduction of hMutS binding to T/G in the presence of these photoproduct/base mismatches (Fig. 3) reflects the direct binding affinities of the latter. The amount of T<>T/AG DNA bound at this concentration of hMutS (3 nM) is about 1/2 the amount of TT/AG 51-mer bound, in good agreement with the measurements of Mu et al. (25). The ATP sensitivities for these substrates were quite similar; estimated ATP concentrations at half-maximal binding were, respectively, 58, 71, and 79 μM (Fig. 5). Since these experiments were performed in the absence of magnesium, the results are consistent with previous reports that ATP binding but not ATP hydrolysis is required for the dissociation of hMutSα from mismatched duplexes (14, 15).

**FIG. 3.** Competition for hMutSα binding by DNA containing uracil/base or (photoproduct-uracil)/base mismatches. Binding/competition experiments, similar to those shown and analyzed in Fig. 2, were performed using 1.32 nM 32P-5’-end-labeled T/G 51-mers, 3 nM hMutSα, and 0.33 to 84 nM concentrations of indicated unlabeled competitor 51-mers. Competitor concentrations corresponding to 50% reduction in binding (ordinate value of 2.0 in fitted plots similar to those in Fig. 2B) are shown. Mismatch affinity factors (reciprocals of ratios of concentrations showing 50% reduction relative to concentration for TT/AG (27.8 nM)) are indicated in parentheses. Individual symbols (□, ○, and ●) correspond to independently determined competition curves.

**FIG. 4.** Competition for hMutSα binding by DNA containing uracil/base or (photoproduct-uracil)/base mismatches. Binding/competition experiments, similar to those shown and analyzed in Fig. 2, were performed using 1.32 nM 32P-5’-end-labeled T/G 51-mers, 3 nM hMutSα, and 0.33 to 84 nM concentrations of indicated unlabeled competitor 51-mers. Competitor concentrations corresponding to 50% reduction in binding (ordinate value of 2.0 in fitted plots similar to those in Fig. 2B) are shown. Mismatch affinity factors relative to U/AA (see legend to Fig. 3) are indicated in parentheses. Individual symbols (□ and ○) correspond to independently determined competition curves.

**FIG. 5.** Effect of ATP on binding of hMutSα to base/base and photoproduct/base DNA mismatches. Binding of 1.32 nM 32P-5’-end-labeled TT/AG, T<>T/AG, and T(6-4)/T/AG 51-mer by 3 nM hMutSα at 4 °C, in the presence of indicated concentrations of Na-ATP (in the absence of Mg2+) were performed as described under “Direct Binding Experiments.” The fraction of DNA bound corresponds to the amount of [32P]DNA showing reduced mobility divided by total [32P]DNA in each lane. Means of three independent experiments for TT/AG (●), T<>T/AG (×), and T(6-4)/T/AG (○) and standard deviations are shown.

**DISCUSSION**

We have used equilibrium binding studies to address the hypothesis that eukaryotic MSH2-MSH6 heterodimers, such as the hMutSα protein studied here, bind more strongly to photoproduct/base mismatches, such as T<>T/AG, (6-4)/T/AG, and U<>U/AG, than to the respective matched photoproducts, T<>T/AA, T(6-4)/T/AA, and U<>U/AA. Although our studies have sensitively delineated relative affinities among an extensive set of substrates, equilibrium binding cannot measure the full quantitative range of biological specificity. The T/G 51-mer

Since cytosines in cyclobutane-pyrimidine-dimers deaminate much more rapidly than non-photoproduct cytosines (32), T<>U, U<>T, and U<>U photoproducts might be expected to accumulate in UV-irradiated cells. Where deamination occurred in the vicinity of appropriate signals for initiation of excision, mismatch repair might thus actually fix mutations. We measured the affinity of hMutSα for uracil-containing base/base and photoproduct/base single and double mismatches (Fig. 4). Among base/base mismatches, UU/AG and UU/GG showed, respectively, somewhat less and somewhat more affinity than TT/AG mismatches, relative to the corresponding homoduplexes; the trend UU/AG > UU/AC > UU/AT paralleled that for the TT series. Affinities of hMutSα for all three single U<>Ubase mismatches appeared higher than the affinity for U<>U/AA; the mismatch affinity ratio for U<>U/AG was 6.7, considerably higher than the (T<>T/AG):(T<>T/AA) ratio of 3.6. Surprisingly, even though the UU/GG and U<>U/AG affinity ratios were, respectively, 24 and 6.7, the ratio for the double (U<>U) base mismatch was only 1.4.

**Effect of ATP on Binding of hMutSα to Base/Base and Photoproduct/Base Mismatches—**An early step in mismatch-repair pathways is thought to be ATP-triggered translocation/dissociation of MutS-like proteins away from the mismatches to which they had been bound (3, 14, 15). Consequently, the apparent equilibrium binding of these proteins to relatively short linear mismatched oligomers is highly sensitive to ATP (14, 15). If binding of hMutSα to photoproduct/base mismatches is analogous to its binding to base/base mismatches, ATP sensitivities should be similar. We tested the effect of different ATP concentrations on direct binding of hMutSα to radiolabeled TT/AG, T<>T/AG, and T(6-4)/T/AG substrates (Fig. 5). The binding of hMutSα to the photoproduct/base mismatches seen here in the absence of ATP confirms our assumption that the reduction of hMutSα binding to T/G in the presence of these photoproduct/base mismatches (Fig. 3) reflects the direct binding affinities of the latter. The amount of T<>T/AG DNA bound at this concentration of hMutSα (3 nM) is about 1/2 the amount of TT/AG 51-mer bound, in good agreement with the measurements of Mu et al. (25). The ATP sensitivities for these substrates were quite similar; estimated ATP concentrations at half-maximal binding were, respectively, 58, 71, and 79 μM (Fig. 5). Since these experiments were performed in the absence of magnesium, the results are consistent with previous reports that ATP binding but not ATP hydrolysis is required for the dissociation of hMutSα from mismatched duplexes (14, 15).
is only 20 times as effective as a T/A substrate in competing for hMutSa. T/C and T/TT mismatches, known to be corrected efficiently in vitro and in vivo, compete less well. Even if there are occasional altered guanine bases in putative homoduplexes, due to chemical damage during oligonucleotide synthesis, these should be present at the same frequency in the T/G substrate and the photoproduct-containing substrates, so the effects of any altered bases should to a first approximation cancel out in the competition experiments. The single mismatches in these 51-mer substrates are diluted by 50 matched base pairs, but during replication of the human genome, hMutSa (or hMutSβ) protein must initiate correction of perhaps one or two thousand mismatches, scattered among billions of homoduplex pairs. Any significant degree of mismatch-repair incision/excision directed against homoduplex “false positives” would clearly be highly deleterious to the cell. Postulated post-binding steps in the pathway, such as repeated cycles of ATP binding and hydrolysis coupled to hMutS-mediated post-binding steps in the pathway, such as repeated cycles of ATP binding and hydrolysis coupled to hMutS-mediated post-binding steps (41, 42), or ATP-dependent protein recruitment and dissociation from the mismatch (15), or directed helicase/exonuclease action (39, 44), might be more specific, or the initial hMutSa search/recognition process in vivo might be more complex (and specific) than simple equilibrium binding.

Nevertheless, hMutSa shows a clear preference for photoproduct/base mismatches. The mismatch affinity factors of 3.6 and 4.3 for T<>T/AG and T6-4/T/AG (relative to the respective photoproduct/base matches) are less than the factor of 20 for T/T/AG (relative to T/TT/AA) but greater than the factor (2.4) for a non-photoproduct TT/AT mismatch. Since significant hydrogen bonding and Watson-Crick character are retained at the 3' (and 5') position(s) in CPDs (41, 42), it is perhaps not surprising that preferences for recognition of various CPD/base mismatches by hMutSa here roughly parallel the affinity order for base/base mismatches, which is in fact the same order as seen for repair of all eight base/base mismatches in vivo (40). However, recognition of (6-4)photoproduct mismatches was unexpected. These are much more distorted, especially at the 3' position, where there appears to be no hydrogen bonding (43), and an extracyclic oxygen is actually shifted from the 3' - to the 5'-thymine. It is far from clear how a protein binds T(6-4)/T/AG well but T(6-4)/T/AA weakly, when the 3'-photoproduct is so poorly instructive. It may be significant that the affinity trend for intermediate substrates, T(6-4)/T/AT > T(6-4)/T/AC, does not parallel the base/base and CPD/base trends.

The apparent low affinity of hMutSa for T(6-4)/T/AA, even lower than for TT/AA homoduplexes, hints at complexity in the recognition process. If hMutSa repeatedly searches for mismatches by transiently binding one or a few base pairs in a recognition site, then dissociating when no mismatch is found, a single T(6-4)/T/AA moiety among 50 Watson-Crick pairs would seem unlikely to increase appreciably the average rate of dissociation. However, recognition might involve unidirectional or bi-directional one-dimensional scanning of DNA by hMutSa until a mismatch is recognized and bound. Even in the absence of a mismatch, the loose DNA-protein affinity during the scanning process might cause significant reduction in DNA electrophoretic mobility. However, if hMutSa were to dissociate completely when it encountered a T(6-4)/T/AA moiety, scanning would be disrupted, and there would be no mobility reduction until hMutSa reinitiated the process.

To test the notion that hMutSa recognizes mismatched bases opposite deaminated 3'-cytosines in Py<>C CPDs, leading to fixation rather than correction of these potential mutations, we employed a series of U<>U/AN substrates. (It seems likely that T<>U/AN moieties would show similar results.) Specific recognition of the series of 3'-mismatched U<>U CPDs by hMutSa paralleled recognition of the T<>T series; U<>UAG, a model for mismatch created by deamination, was the best substrate. We also used U<>U substrates to model the product of double deamination, predicted to be significant in vivo (32), which might contribute to the high frequency of CC to TT transitions found in mutation spectra (31). Surprisingly, although non-photoprocess UU/GG double mispairs were bound twice as well as UU/AG single mispairs, no specific affinity for the U<>U/GG double photoprocess/base mismatches was observed. Perhaps the extent of helix distortion/opening here exceeds the hMutSa recognition capability, as appears to be the case for large insertion/deletion loop-outs (6).

The results presented here show that one prerequisite for efficient correction by long-patch mismatch repair of mutations introduced in human cells by error-prone translesion synthesis, namely specific recognition of photoprocess/base mismatches by hMutSa protein, is met. It remains to be determined whether the repair synthesis associated with mismatch repair bypasses the same lesions with reasonable efficiency and fidelity. If so, there would be at least three outcomes after misinsertion opposite photoprocess during semi-conservative replication, the first two resulting in mutation fixation: (i) T<>T/AG to T<>T/AA + TC/AG by replication; (ii) T<>T/AG to TC/AG by excision repair; (iii) T<>T/AG to T<>T/AA by mismatch repair, thus preventing mutation. In case iii, mutation avoidance might therefore proceed by Steps 1–3 as follows.

\[ T<>T/AA \rightarrow T<>T/AG \] (translesion synthesis) \hspace{1cm} (Step 1)
\[ T<>T/AG \rightarrow T<>T/AA \] (mismatch repair) \hspace{1cm} (Step 2)
\[ T<>T/AA \rightarrow T/AA \] (nucleotide excision repair). \hspace{1cm} (Step 3)

Alternatively, blocked repair synthesis might lead to cell death, apparently an important form of mutation avoidance in mammalian cells, and/or recombinational bypass using presumably error-free sister-chromatid DNA. It is now clearly of high interest to determine whether MutS-like proteins in other organisms show the same specificity and whether UV mutagenesis is elevated in prokaryotic and/or eukaryotic mismatch-repair-deficient mutants.

Recent work in our laboratory demonstrates that UV-induced CCC to TCC and TTC to TCC revertant frequencies at lacZ codon 461 are elevated in E. coli mutS, mutL, and mutH mutants, significantly above the higher spontaneous frequencies in these strains. This result is consistent with the hMutSa specificity documented here, but the previously described MutHL-dependent recombination of nonreplating UV-irradiated d(GATC)-undermethylated phage λ DNA (16) now appears paradoxical. Newly irradiated λ virions would be expected to contain far less than one base/base mismatch per (50 kilobase pairs) genome and essentially zero photoprocess/base mismatches. If E. coli MutS, unlike hMutSa, were to initiate mismatch repair in response to matched as well as mismatched photoprocesses, then the apparent antagonism of E. coli UV mutagenesis by mismatch repair cited would imply virtually error-free filling of excision gaps, perhaps by efficient sister-chromatid exchange. Alternatively, deamination of cytosine-containing photoprocesses in the nonreplicating UV-irradiated phage DNA might be accelerated in vivo, by an unknown mechanism, such that recombining Py<>U/PuG or U<>Py/GPu mismatches appeared rapidly enough to stimulate recombination as early as 60 min after infection (16).

\[^3\] P. Modrich, personal communication.

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Specific Binding of Human MSH2-MSH6 Mismatch-Repair Protein Heterodimers to DNA Incorporating Thymine- or Uracil-containing UV Light Photoproducts Opposite Mismatched Bases
Huixian Wang, Christopher W. Lawrence, Guo-Min Li and John B. Hays

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