hMSH2 and hMSH6 play distinct roles in mismatch binding and contribute differently to the ATPase activity of hMutS α

Ingram Iaccarino, Giancarlo Marra, Fabio Palombo¹ and Josef Jiricny²

Institute of Medical Radiobiology, P.O. Box 424, August Forel-Strasse 7, CH-8029, Zürich, Switzerland and ¹Istituto di Ricerche di Biologia Molecolare 'P. Angeletti', Via Pontina Km 30 600, I-00040 Pomezia, Italy

²Corresponding author
e-mail: jiricny@imr.unizh.ch

In extracts of human cells, base–base mismatches and small insertion/deletion loops are bound primarily by hMutS α , a heterodimer of hMSH2 and hMSH6 (also known as GTBP or p160). Recombinant hMutS α bound a G/T mismatch-containing oligonucleotide with an apparent dissociation constant $K_d = 2.6$ nM, while its affinity for a homoduplex substrate was >20-fold lower. In the presence of ATP, hMutS α dissociated from mismatched oligonucleotide substrates, and this reaction was attenuated by mutating the conserved lysine in the ATP-binding domains of hMSH6, hMSH2 or both to arginine. Surprisingly, this reaction required only ATP binding, not hydrolysis. The ATPase activity of hMutS α variants carrying the Lys→Arg mutation in hMSH2 or in hMSH6 was severely affected, but these mutants were still proficient in mismatch binding and were able to complement, albeit to different extents, mismatch repair-deficient cell extracts. The mismatch binding-proficient, ATPase-deficient double mutant was inactive in the complementation assay and its presence in repair-proficient extracts was inhibitory. We conclude that although the ATPase activity of hMutS α is dispensable for mismatch binding, it is required for mismatch correction.

Keywords: ATPase activity/hMutS α /mismatch-binding proteins/mismatch repair

Introduction

To date, ~40% of kindreds afflicted with hereditary non-polyposis colon cancer (HNPCC) (for reviews on HNPCC see, e.g. Marra and Boland, 1995; Jiricny, 1996) have inherited mutations in the *hMSH2* gene (Liu *et al.*, 1996; Marra and Boland, 1996). As this gene encodes a homologue of the bacterial mismatch-binding protein MutS (Leach *et al.*, 1993), it was widely assumed that hMSH2 is the mismatch-binding factor in human cells; indeed, recombinant hMSH2 has been shown to bind to mispairs and insertion/deletion loops (IDLs) *in vitro* (Fishel *et al.*, 1994a,b). However, recent biochemical evidence showed that hMSH2 exists predominantly in the form of a heterodimer with a second MutS homologue, hMSH6 (Drummond *et al.*, 1995; Palombo *et al.*, 1995; Acharya *et al.*, 1996). This factor, referred to as hMutS α , binds

base–base mismatches and small IDLs in band-shift assays (Hughes and Jiricny, 1992; Drummond *et al.*, 1995; Palombo *et al.*, 1996) and restores mismatch repair to extracts of cells mutated in *hMSH2* or *hMSH6* (Drummond *et al.*, 1995).

Functionally, MutS and hMutS α appear to be rather similar. MutS binds mismatch-containing DNA as a homodimer (Allen *et al.*, 1997) and has a weak ATPase activity (Haber and Walker, 1991). Addition of ATP to the binding reaction results in a dissociation of the protein–DNA complex, as seen both in band-shift experiments (our unpublished observations) and in DNase I footprinting assays (Grilley *et al.*, 1989). This may be only an artefact of oligonucleotide-based *in vitro* assays, however. On circular, mismatch-containing heteroduplex molecules, MutS remains associated with the DNA and appears to form an α -loop (so-called because it resembles the Greek letter α), where it remains bound at the crossover point (Allen *et al.*, 1997). This reaction, as well as the recruitment of the downstream mismatch repair factors MutL and MutH, was proposed to require ATP hydrolysis. The ATPase activity of MutS was shown to reside in the C-terminal domain of the protein, which houses the so-called 'type A' consensus motif GxxxxGKS/T (Walker *et al.*, 1982; Gorbalenya and Koonin, 1990). The pioneering study of Haber and Walker (1991) demonstrated that mutating the invariant lysine residue to alanine (K622A) in the MutS homologue of *Salmonella typhimurium* resulted in a 30-fold increase in K_m and a 5-fold reduction in K_{cat} . The DNA-binding activity of the mutant was also affected, albeit to a lesser extent. When overexpressed, this mutant had a dominant-negative phenotype. Interestingly, a number of dominant-negative MutS mutants, identified subsequently in *Escherichia coli* (Wu and Marinus, 1994), had mutations in the vicinity of the ATP-binding motif.

The binding of hMutS α or of *Saccharomyces cerevisiae* MutS α to mismatch-containing oligonucleotide heteroduplexes was also shown to be sensitive to ATP (Hughes and Jiricny, 1992; Drummond *et al.*, 1995; Palombo *et al.*, 1995; Iaccarino *et al.*, 1996). This was not unexpected, as the C-terminal portions of all the MutS homologues identified to date are highly conserved (Fishel and Kolodner, 1995) and the ATP-binding sites are almost invariant (Figure 1). It therefore seemed reasonable to assume that the ATPase activities of hMSH2 and hMSH6 reside within their respective type A motifs. However, in the context of the hMutS α heterodimer, the contribution of the two individual ATP-binding sites to the total ATPase activity of hMutS α could be different. Moreover, following mismatch binding, the hMutS α –DNA complex presumably interacts with hMutL α , a heterodimer of hMLH1 and hPMS2, the human functional homologue of the MutL homodimer. Thus, unlike in bacteria, where mismatch-bound MutS homodimer interacts with the MutL homo-

hmsH3	I	T	G	P	N	M	G	G	K	S	S	Y	I	K	Q	V	A	L	L	T	913
ymsh3	I	T	G	P	N	M	G	G	K	S	S	Y	I	R	Q	V	A	L	L	T	837
hmsH2	I	T	G	P	N	M	G	G	K	S	T	Y	I	R	Q	T	G	V	I	V	686
ymsh2	I	T	G	P	N	M	G	G	K	S	T	Y	I	R	Q	V	G	V	I	S	705
mutS	I	T	G	P	N	M	G	G	K	S	T	Y	M	R	Q	T	A	L	I	A	631
hmsH6	V	T	G	P	N	M	G	G	K	S	T	L	M	R	Q	A	G	L	L	A	1151
hexa	V	T	G	P	N	M	G	G	K	S	T	Y	M	R	Q	L	A	M	T	A	619
ymsh4	I	T	G	C	N	M	G	G	K	S	V	Y	L	K	Q	V	A	L	I	C	651
ymsh5	V	T	G	A	N	A	S	G	K	S	V	Y	L	T	Q	N	G	L	I	V	660
ymsh6	L	T	G	A	N	A	G	K	S	S	T	I	L	R	M	A	C	I	A	V	999

Fig. 1. Alignment of the amino acid sequences of the ATP-binding motifs of MutS homologues. mutS, *E. coli* MutS; hexa, *Streptococcus pneumoniae* HexA; ymsh2, ymsh3, ymsh4 and ymsh6, *S. cerevisiae* MSH2, MSH3, MSH4 and MSH6 respectively; hmsH2, hmsH3 and hmsH6, human hMSH2, hMSH3 and hMSH6, respectively. The alignment was carried out using the Pileup program with a Prettybox plotting option in the GCG package (University of Wisconsin). The numbers on the right refer to the position of the last amino acid residue of the shown sequence in the respective full-length protein. Identical amino acids are shown in black boxes, similar ones in different shades of grey.

dimer in the presence of ATP (Grilley *et al.*, 1989; Allen *et al.*, 1997), the eukaryotic mismatch repair complex is asymmetric, and it is at present unclear how the four proteins interact with DNA and with each other. Due to this asymmetry, it is reasonable to assume that each subunit of the two heterodimers will play a specific role in the correction process. In an attempt to elucidate these roles, we expressed the four human proteins in a baculovirus system and are currently studying the protein–protein and protein–DNA interactions in *in vitro* mismatch repair and mismatch-binding assays. As part of this programme, it was of interest to study how hMutS α interacts with mismatched DNA substrates, as well as to determine the relative contribution of the individual ATPase activities of hMSH2 and hMSH6 to mismatch repair.

Results

Recombinant hMutS α heterodimers carrying Lys→Arg mutations in the ATP-binding sites of hMSH2, hMSH6 or both polypeptides can be overproduced in a baculovirus expression system

In order to study the individual contributions of hMSH2 and hMSH6 to the ATPase activity of hMutS α , we decided to inactivate the ATP-binding domains of the two proteins by site-directed mutagenesis, and to reconstitute the heterodimeric factor in all the possible combinations of wild-type and mutant forms. MutS homologues belong to a superfamily of NTP-binding proteins that share at least four conserved motifs (Gorbalenya and Koonin, 1990). Consensus site I contains the sequence GxxxxGKS/T (Figure 1), which has been shown to form a flexible loop implicated in the binding of the γ -phosphoryl moiety of ATP or GTP. Mutation of the lysine residue in this motif to alanine (K622A) was shown to drastically reduce the ATPase activity of the *S. typhimurium* MutS protein (Haber and Walker, 1991). In an earlier study, concerned with another member of this superfamily, the *S. cerevisiae* RAD3 protein, mutation of Lys48 to arginine was reported to abolish its ATPase activity, but not its ability to bind ATP (Sung *et al.*, 1988). We decided to use the latter mutation (K→R) to inactivate the ATPase activity of hMSH2 and hMSH6, because it might allow us to differentiate between ATP hydrolysis and ATP binding. The two

mutants, hMSH2 K675R and hMSH6 K1140R, were obtained by site-directed mutagenesis (see Materials and methods).

The cDNAs encoding the wild-type and mutant forms of hMSH2 and hMSH6 were cloned into baculovirus expression vectors, and the recombinant viruses were used to infect Sf9 cells. Co-infection of the cells with equal plaque-forming units of these vectors (see also Palombo *et al.*, 1996) yielded the recombinant hMutS α complexes in large amounts; they represented ~10% of the total proteins present in the infected Sf9 extracts. The following heterodimers were obtained by this method: hMSH2–hMSH6 (hMutS α), hMSH2 K675R–hMSH6 (hMutS α 2KR), hMSH2–hMSH6 K1140R (hMutS α 6KR) and hMSH2 K675R–hMSH6 K1140R (hMutS α 2,6KR). The previously reported purification strategy for hMutS α (Drummond *et al.*, 1995) was based on elution of the complex from a single-stranded DNA–cellulose matrix with ATP. Since we intended to study mutants lacking ATPase activity, and since these would most likely fail to elute with ATP from this matrix, we based our purification scheme on the use of heparin–Sepharose and anion-exchange matrices. This enabled us to use the same procedure for all the combinations of mutant and wild-type proteins, thus also reducing the likelihood of having different contaminants in the various preparations.

The chromatographic behaviour of the wild-type and mutant hMutS α complexes was similar, indicating that the general folding of the proteins and the heterodimer formation were not affected by the targeted mutations. Analysis of ATPase activities was performed with the Resource Q fractions. A distinct peak of ATPase activity co-eluted with the peak corresponding to the wild-type recombinant heterodimer (data not shown). Other ATPase activities detected in the column fractions were clearly separated from the hMutS α peak.

hMSH2 alone was expressed in large amounts and was largely soluble, such that it could be purified using a chromatographic strategy similar to that used for hMutS α , except that a Mono Q column was used in the last step of the purification (see Materials and methods).

In contrast to hMSH2, hMSH6 displayed poor solubility in the absence of hMSH2, and was highly sensitive to proteolytic degradation during purification (our unpublished observations). For this reason, hMSH6 used in this study was only ~80% pure. The purity of the recombinant proteins was judged by electrophoresis on SDS–polyacrylamide gels (Figure 2).

Wild-type hMutS α , but not hMSH2 or hMSH6, bind to oligonucleotide substrates containing the G/T mismatch

In an earlier study, the binding of hMutS α to heteroduplex DNA was shown to be dependent on both hMSH2 and hMSH6, using *in vitro* translated proteins and specific polyclonal antibodies against the two polypeptides (Palombo *et al.*, 1995). In this study, we used purified recombinant proteins to analyze the DNA-binding activity of the two polypeptides, either alone or as components of the hMutS α heterodimer.

Neither the partially purified preparation of hMSH6, nor the purified hMSH2 alone were able to bind a 34mer oligonucleotide containing a G/T mismatch (Figure 3,

Fig. 2. SDS-PAGE profile of the recombinant, baculovirus-expressed hMSH2, hMSH6 and hMutS α wild-type and mutant variants. hMutS α is designated as hMutS α 2KR when mutated in hMSH2, as hMutS α 6KR when mutated in hMSH6, and as hMutS α 2,6KR when mutated in both hMSH6 and hMSH2. The migration of the molecular size standards is shown on the right.

Fig. 3. Mismatch-binding activities of native and recombinant hMutS α and its constituents hMSH2 and hMSH6, as assayed in a band-shift experiment. The ^{32} P-labelled 34mer oligonucleotide probes, either containing a single mismatch (G/T) or perfectly matched (G/C), were incubated either with nuclear extracts of the mismatch repair-deficient colon cancer line HCT15 or the mismatch repair-proficient cervical cancer line HeLa, or with the purified polypeptides as indicated above the lanes (see Materials and methods for experimental details). The abundant, fast migrating non-specific band is due to the Ku70–Ku80 heterodimer present in the extracts. The weak, slowest migrating band present in the HeLa G/C and the HCT15 G/T lanes is of unknown origin, but is frequently observed when no specific complex is formed in the extracts.

lanes hMSH6 and hMSH2, respectively). The inability of the recombinant hMSH2 to bind mismatched DNA under our assay conditions was in agreement with the finding that nuclear extracts of the colorectal tumour line HCT15 (as well as those of the isogenic DLD1 cells) lack specific mismatch-binding activity (Figure 3, lane HCT15 NE). This line has mutations in both *hMSH6* alleles and is thus devoid of hMSH6, but the extracts contain nearly wild-type levels of hMSH2 (Palombo *et al.*, 1995). However, as this finding was contrary to previous reports (Fishel *et al.*, 1994a,b), we wanted to ascertain that our hMSH2 preparation was biologically active. To this end, we premixed the purified recombinant hMSH2 with the crude extract of hMSH6-infected SF9 cells. As shown in Figure 3 (lane hMSH2 + hMSH6), the protein–DNA complex in this lane had the same electrophoretic mobility as that in lanes hMutS α and HeLa NE, where the G/T-containing

Fig. 4. Affinity of the recombinant hMutS α for a DNA substrate containing a single G/T mismatch. The ^{32} P-labelled 34mer oligonucleotide probe was incubated with increasing amounts of the recombinant mismatch-binding factor in the absence of non-specific competitor DNA. The protein–DNA complex was separated from the free probe by non-denaturing polyacrylamide gel electrophoresis (A) and the relative amounts of free and bound probe were quantitated using a STORM phosphorimager with ImageQuant software (Molecular Dynamics, Inc.). The dissociation constant, K_d , was estimated from the plot shown in (B), using the equations described in Materials and methods.

34mer substrate was incubated with the purified recombinant hMutS α or with HeLa extracts, respectively. This result shows that the recombinant hMSH2 and hMSH6 protein preparations were able to interact to form a hMutS α complex capable of binding mismatch-containing duplexes.

Increasing amounts of hMutS α were used to estimate its affinity for an oligonucleotide duplex containing a G/T mismatch or for a perfectly matched control substrate. The titration shown in Figure 4A (substrate G/T) is represented graphically in Figure 4B. The results of a similar titration experiment, using the G/C probe, are also shown (Figure 4B). The quantification of both data sets revealed that hMutS α has an affinity 20-fold higher for the G/T heteroduplex than for the G/C homoduplex, the apparent dissociation constants (K_d) for the two oligonucleotide substrates being 2.6 and 54 nM, respectively.

hMutSα bound to a mismatch-containing substrate contacts DNA via the hMSH6 subunit

To study the mode of binding of hMutS α to the mismatched oligonucleotide, we employed UV cross-linking experiments, using a substrate substituted with 5-bromouracil. As shown in Figure 5 (lane 'no Ab'), cross-linking of the oligonucleotide probe with the purified recombinant hMutS α gave rise to a species of approximate molecular

Fig. 5. UV cross-linking of hMutS α to mismatch-containing DNA and immunoprecipitation of the protein–DNA complex with specific antibodies. The ^{32} P-labelled 34mer oligonucleotide duplex, containing a G/T mispair and a single 5-bromouracil residue (see Materials and methods), was incubated with the recombinant hMutS α either in the presence (lanes ‘+ trypsin’) or absence (lanes ‘– trypsin’) of 50 ng of trypsin. Following UV cross-linking, the protein–DNA complexes were immunoprecipitated with anti-hMSH2 or anti-hMSH6 polyclonal antisera under native or denaturing conditions. The figure is an autoradiograph of an SDS–polyacrylamide gel.

size 180 kDa, which agrees well with the size of hMSH6 covalently linked to the radiolabelled oligonucleotide. In order to show conclusively that the cross-linked protein is indeed hMSH6, we carried out immunoprecipitation experiments with polyclonal antibodies raised against hMSH6 and hMSH2. As shown in Figure 5 (lanes ‘– trypsin’), immunoprecipitation with anti-hMSH6 antibodies under denaturing conditions resulted in the precipitation of the 180 kDa radioactive species (lane ‘ α -hMSH6-den’), while no radioactivity was brought down with the anti-hMSH2 antibody under the same conditions (lane ‘ α -hMSH2-den’). When native immunoprecipitation conditions were employed, the anti-hMSH2 antibody could precipitate the protein–DNA complex, indicating that hMSH2 and hMSH6 were both present in the protein–DNA complex, but that only hMSH6 was able to make a covalent link with the DNA.

In earlier experiments (Jiricny *et al.*, 1988), we observed that cross-linking of the G/T-binding factor present in HeLa extracts gave rise to two bands, of ~180 and 120 kDa, especially in extracts that were fractionated. At that time, we assumed that the G/T-binding factor was a single protein; we therefore proposed that the smaller species was the product of the proteolytic degradation of the larger polypeptide (Hughes and Jiricny, 1992). This could be substantiated by further studies, which showed that partial proteolytic digests of the purified G/T-binding factor gave rise to protein–DNA complexes of ~120 kDa (Hughes and Jiricny, 1992). However, since the discovery that the factor was a heterodimer, there existed the possibility that the 180 and 120 kDa species were due to cross-linking of the DNA substrate with hMSH6 and hMSH2, respectively. We therefore decided to resolve this situation by carrying out the above-described immunoprecipitation experiments with recombinant, DNA-bound hMutS α , subjected to partial proteolysis with trypsin, followed by DNA cross-linking. As shown in Figure 5 (lanes ‘+ trypsin’), the protein complex could be degraded to a smaller species, which behaved identically in terms of substrate specificity and ATP sensitivity (data not

shown) to the undegraded complex (lanes ‘– trypsin’). These data demonstrate that even in the degraded complex, the DNA substrate is contacted by hMSH6. Although we have no direct evidence which part of hMSH6 is susceptible to proteolysis, we were able to show earlier that the N-terminal 114 amino acids of hMSH6 were dispensable for DNA binding (Palombo *et al.*, 1995). Moreover, the highly conserved C-terminal portions of the two polypeptides contain the ATP-binding sites and are most likely involved in protein–protein interactions; it is improbable that they are dispensable for DNA binding. Taken together, our data suggest that the two constituent proteins of hMutS α interact to form a globular complex, in which the N-terminus of hMSH6 is exposed and thus susceptible to proteolysis.

Dissociation of hMutS α from mismatch-containing oligonucleotides requires ATP binding, not ATP hydrolysis

The mismatch-binding activity of hMutS α could be abolished when ATP or its poorly hydrolysable analogue, ATP γ S, were present in the DNA-binding reaction (Hughes and Jiricny, 1992). These early results suggested that ATP binding rather than ATP hydrolysis is required for dissociation of the protein–DNA complex. However, recent studies showed that the non-hydrolysable ATP analogue, AMP-PNP, had no effect on the mismatch-binding activity of hMutS α purified from HeLa cells (Drummond *et al.*, 1995) or expressed in the baculovirus system (our unpublished observations), suggesting the need for ATP hydrolysis in this process. In an attempt to investigate this phenomenon more closely, we first decided to eliminate the possibility that AMP-PNP failed to affect dissociation of the complex because it was not bound by hMutS α . To this end, ATP, ATP γ S and AMP-PNP were used to displace radioactively labelled ATP from a complex with the purified heterodimer. As the ability of hMutS α to hydrolyse [γ - ^{32} P]ATP could be inhibited by all three nucleotides (Figure 6A), we concluded that all were capable of interacting with the ATP-binding pockets of hMSH2 and hMSH6. We next decided to address the problem of ATP hydrolysis. As purified hMutS α could be shown to hydrolyse ATP γ S, albeit ~10-fold more slowly than ATP (data not shown), we argued that if ATP hydrolysis was necessary to displace the heterodimer from a complex with mismatched DNA, this dissociation process should be affected differently in the presence of increasing amounts of the two nucleotides. Because ATP and ATP γ S had a similar effect on the dissociation of the DNA–protein complex in a band-shift assay (Figure 6B), we concluded that the displacement of hMutS α from mismatch-containing DNA requires ATP binding but not ATP hydrolysis. This finding could be substantiated further in a duplicate experiment, carried out in the presence of 5 mM EDTA (Figure 6B), i.e. under conditions where the ATPase activity of hMutS α was completely abolished (data not shown). Again, both triphosphates had virtually indistinguishable effects on the dissociation reaction. It is not clear in this regard why the non-hydrolysable AMP-PNP failed to behave similarly to ATP and ATP γ S. We can only assume that it interacts with the ATP-binding site in a manner that does not permit the protein complex to undergo a conformational change required for dissociation.

Fig. 6. Effect of ATP and its analogues ATPγS and AMP-PNP on hMutSα-mediated ATP hydrolysis and DNA binding. (A) Excess unlabelled ATP or its analogues ATPγS and AMP-PNP were used as competitors for [γ - ^{32} P]ATP. (B) Dissociation of hMutSα from DNA in the presence of ATP or ATPγS, in the presence or absence of divalent cations. See Materials and methods for experimental details.

Table I. Kinetic parameters for ATP hydrolysis and DNA binding of the wild-type and mutant hMutSα heterodimers

	K_m (μ M ATP)	V_{max} (pmol P_i /min)	K_{cat} (per min)	K_{cat}/K_m	K_d^a (nM)
hMutSα	0.4	0.5	0.5	1.25	2.6
hMutSα 6KR	4.7	0.18	0.18	0.04	11.1
hMutSα 2KR	1.39	0.16	0.16	0.11	6.7
hMutSα 2,6KR	3.7	0.08	0.08	0.02	17.7

^aThe dissociation constants were calculated using a G/T heteroduplex.

Mutations of the conserved lysine residues in the ATP-binding domains of hMSH6 and hMSH2 attenuate ATP binding and ATPase activity of the mutant hMutSα heterodimers

The single mutants, hMutSα 6KR and hMutSα 2KR, as well as the double mutant hMutSα 2,6KR, were produced and purified to near homogeneity as described in Materials and methods. As shown in Table I, the rates of ATP hydrolysis of all the mutant heterodimers were severely affected, the Michaelis–Menten constant being 4- to 13-fold higher. Interestingly, the Lys→Arg mutation in hMSH6 incapacitated the heterodimer to almost the same extent as the double mutation, while the single amino acid change in hMSH2 had a somewhat smaller effect on ATP hydrolysis (Table I). These experiments show that, in the

Fig. 7. The Lys→Arg mutation in the conserved nucleotide-binding motif of hMSH2 and hMSH6 reduces the affinity of the mutated protein for ATP. In this experiment, [γ - ^{32} P]ATP was cross-linked to the recombinant wild-type or mutant heterodimers. The figure is an autoradiograph of an SDS–polyacrylamide gel.

absence of mismatched substrate, the ATP-binding sites of hMSH2 and hMSH6 in the context of hMutSα are actively involved in ATP hydrolysis.

In order to establish whether the Lys→Arg mutations also affected ATP binding, UV light was used to covalently cross-link [γ - ^{32} P]ATP to the ATP-binding pocket(s) of hMutSα. As shown in Figure 7, wild-type hMSH2 was labelled with an efficiency significantly higher than wild-type hMSH6. This effect could be explained in two ways. First, it could indicate that hMSH2 binds the nucleotide with an affinity higher than hMSH6. Second, assuming that the efficiency of the cross-linking reaction increases with the length of the residence time of ATP within the active site of a protein, the result would imply that hMSH6 has a faster turnover than hMSH2. We favour the latter possibility, because the kinetic data (Table I) show that the ATPase activity of hMutSα mutated in hMSH6 (hMutSα 6KR) was more severely affected than that of hMutSα mutated in hMSH2 (hMutSα 2KR). This suggests that hMSH6 contributes more to the total ATPase activity of the heterodimer and should thus have higher turnover rates. Control experiments, in which the cross-linking was carried out with hMSH2 and hMSH6 separately, gave results similar to those observed with the hMSH2–hMSH6 heterodimer, indicating that the effect was not due to a difference in accessibility of the respective ATP-binding pockets in the heterodimer (data not shown). The efficiency of ATP cross-linking of the mutated complexes is also shown (Figure 7). hMutSα 6KR displayed a pattern similar to the wild-type heterodimer. Due to the low efficiency of cross-linking of ATP to hMSH6, no significant effect of the mutation was apparent. In contrast, the efficiency of ATP cross-linking to hMSH2, as a component of heterodimers mutated either in hMSH2 alone or in both hMSH2 and hMSH6, was strongly reduced by the Lys→Arg mutation in position 675 of hMSH2. As ATP hydrolysis was very inefficient in the case of the double mutant, the decreased efficiency of binding could not be due to an increased turnover of the nucleotide. These results therefore demonstrate that, contrary to our expectations, changing the conserved lysine to arginine in the nucleotide-binding loop of the constituent proteins of hMutSα affected not only their ATPase activity, as had been shown for RAD3 (see above), but also their ability to bind the nucleotide.

The Lys→Arg mutations in the ATP-binding sites of hMSH2 and hMSH6 have little effect on mismatch-binding activity

Mutation of Lys622 of *S. typhimurium* MutS protein to alanine also affected its mismatch-binding activity (Haber and Walker, 1991). We wished to determine whether our mutant hMutS α factors were similarly affected. This study was made more interesting by the fact that the two proteins, hMSH6 and hMSH2, appeared to play somewhat distinct roles in ATP hydrolysis and in DNA binding. As in the case of the bacterial MutS protein, the dissociation constant of the hMutS α complex mutated in both ATP-binding loops was increased >6-fold with respect to the wild-type factor (Table I). Interestingly, the complex mutated solely in hMSH6, the hMutS α subunit that was implicated by cross-linking studies to be directly involved in DNA binding, had a $K_d = 11.1$ nM, which was almost 2-fold higher than that exhibited by the complex mutated only in hMSH2. The mutation of the ATP-binding sites of hMutS α therefore does appear to affect the affinity for mismatch-containing DNA, albeit only to a small extent. However, unlike the case of the bacterial K622A MutS mutant (Haber and Walker, 1991), we failed to observe differences in the migration of the wild-type and mutant protein–DNA complexes in polyacrylamide gels.

ATP-dependent dissociation of hMutS α complexes with mismatch-containing oligonucleotides is severely affected by the Lys→Arg mutations in hMSH2 and hMSH6

Electron microscopic studies carried out with the *E. coli* MutS protein bound to a mismatch-containing circular heteroduplex (Allen *et al.*, 1997) showed that the polypeptide initially binds at the mismatch site, but that it translocates in the presence of ATP to form a so-called α -loop structure, where it interacts with the MutL and (presumably) MutH proteins. In band-shift assays, the ATP-dependent movement from the bound mismatch results in a dissociation of the protein–DNA complex, due to the small size of the oligonucleotide substrate. In a recent study, Alani demonstrated that mutation of the second invariant glycine to aspartate (G693D) in the ATP-binding motif GxxxxGKS/T of *S. cerevisiae* MSH2 (Alani *et al.*, 1997) resulted in an apparent stabilization of the scMutS α –DNA complex in the presence of ATP. This residue is adjacent to the lysine mutated in our studies and contributes to the formation of the flexible loop responsible for ATP binding. The yeast factor is a heterodimer of MSH2 and MSH6 and is thus highly homologous to the mammalian hMutS α ; we therefore expected to see a similar effect with our mutants. The effect of increasing ATP concentration on the stability of the protein–DNA complexes formed between the mismatch-containing oligonucleotide and the three mutant heterodimers, hMutS α 2KR, hMutS α 6KR and hMutS α 2,6KR, was compared with the stability of a similar complex involving the wild-type hMutS α heterodimer. The results (Figure 8) show that the mutant heterodimers are significantly less sensitive to the presence of ATP with respect to the wild-type protein. As anticipated, mutation of both hMSH2 and hMSH6 resulted in a complex that appeared largely insensitive to the nucleotide, the hMutS α 2,6KR protein remaining stably bound to DNA even in the presence of

Fig. 8. Dissociation of the wild-type hMutS α or its mutant variants hMutS α 2KR, hMutS α 6KR and hMutS α 2,6KR from a mismatch-containing probe is affected differently by increasing concentrations of ATP. The nucleotide (0–5 mM) was added to binding mixtures, containing the recombinant proteins and the ^{32} P-labelled 34mer G/T oligonucleotide, at the beginning of the incubation period. Following separation of the free and bound forms by native polyacrylamide gel electrophoresis, the radioactivity in the respective bands was quantified using a STORM phosphorimager with ImageQuant software (Molecular Dynamics, Inc.).

10 mM ATP (data not shown). Under similar experimental conditions, the ATP sensitivity of the hMutS α complexes mutated in only one component of the heterodimer appeared to be intermediate between that of the double mutant and the wild-type complex (Table I). Because we could show that the above process does not require ATP hydrolysis (Figure 6B), the effect of the mutations on ATP-dependent dissociation from mismatched DNA must be due to reduced affinity of the mutant proteins for ATP, a conclusion substantiated by the results of the UV cross-linking experiments (Figure 7).

The double mutant hMutS α 2,6KR is inactive in an *in vitro* mismatch repair assay

The biological activities of the recombinant heterodimers were measured in terms of their ability to complement extracts of hMutS α -deficient cell lines in an *in vitro* mismatch repair assay (Thomas *et al.*, 1991), using M13 heteroduplexes containing a single G/T mispair and a strand discrimination signal (a nick), situated 3' or 5' from the mismatch (see Materials and methods). Using extracts of HCT15 cells, a colon tumour line lacking hMSH6, we could show (Figure 9) that the purified recombinant hMutS α could restore full repair activity in this extract, and similar results were obtained with the hMutS α 2KR mutant. The single mutant hMutS α 6KR was slightly affected in repair complementation under these conditions. Although small, this effect was reproducible (e.g. using other hMutS α -deficient extracts and/or different mismatched substrates) and most likely reflects the lower ATPase activity of the hMutS α 6KR heterodimer as compared with hMutS α 2KR (Table I). We attempted to substantiate this hypothesis further by carrying out the *in vitro* mismatch correction assays in the presence of limiting amounts of ATP. Indeed, reducing the concentration of added ATP in the assay from 4 to 0.2 mM resulted in a decrease in the complementation efficiency of the wild-type hMutS α factor by only 12%, while the complementation efficiency of the mutant hMutS α 2KR and

Fig. 9. ATPase activity of hMutS α is required for mismatch correction. The wild-type recombinant hMutS α or the single and double mutants hMutS α 2KR, hMutS α 6KR or hMutS α 2,6KR were used to complement extracts of mismatch repair-deficient HCT15 cells. The substrates were M13 heteroduplexes carrying either the G/T or the T/G mismatch and a strand discrimination signal (a nick) 3' or 5' from the mismatch.

hMutS α 6KR heterodimers was lowered by 21 and 33%, respectively. It could be argued that the less severe effect of the hMSH2 mutation could be due to a limited exchange of the mutated form of this protein with the wild-type hMSH2 present in HCT15 extracts. This is not the case, as identical results could be obtained using extracts of HEC59 cells, an endometrial cancer line mutated in hMSH2 but lacking both hMSH2 and hMSH6 polypeptides (data not shown).

In contrast to the single mutants, the double mutant hMutS α 2,6KR was unable to complement the mismatch repair defect of HCT15 extracts (Figure 9). As this heterodimer is proficient in mismatch recognition (Figure 8), the defect in complementation of the correction process is most likely due to the insensitivity of its complex with mismatched DNA to ATP (see also Discussion).

The hMutS α 2,6KR double mutant inhibits the repair capacity of a mismatch correction-proficient cell extract

The severely impaired ability of the doubly mutated heterodimer to hydrolyse ATP (Table I) and to be displaced from the mismatched oligonucleotide substrate in a band-shift assay (Figure 8) implied that this mutant should fail to translocate from the mismatch, and should thus interfere with normal mismatch repair. We tested this hypothesis by adding the hMutS α 2,6KR mutant heterodimer to an *in vitro* mismatch repair assay carried out with extracts of mismatch repair-proficient HeLa cells. As shown in Figure 10, the efficiency of correction of the G/G substrate decreased upon the addition of increasing amounts of the recombinant, doubly mutant mismatch-binding factor. Based on these results, we would expect hMutS α 2,6KR to display a dominant-negative phenotype *in vivo*.

Fig. 10. The double mutant hMutS α 2,6KR inhibits mismatch correction *in vitro*. Amounts of 0.14, 0.28, 0.56, 0.84 and 1.12 μ g of the wild-type (white columns) or the mutant (black columns) heterodimer were added to the mismatch repair-proficient extracts of HeLa cells. The M13 substrate contained a G/G mispair and a strand discrimination signal 3' from the mismatch.

Discussion

Despite the fact that the constituent proteins of the *E. coli* post-replicative mismatch repair pathway have been identified (Modrich, 1991; Modrich and Lahue, 1996), and that the system has been reconstituted from its individual purified components *in vitro* (Lahue *et al.*, 1989), our knowledge of the mechanism of the repair reaction is limited. Data that help elucidate at least the initial events of this complex process come from a recent electron microscopic study, which suggested that following binding to the mismatch, the MutS protein, presumably in the form of a homodimer, translocates along the DNA in an ATP-dependent reaction to form the α -loop, where it rests at the crossover point (Allen *et al.*, 1997). Although direct evidence that would implicate the ATPase activity of MutS in protein translocation is not available at present, variants of this polypeptide containing mutations in or near the consensus ATP-binding site have been shown to have a dominant-negative phenotype when overexpressed in mismatch-proficient bacteria (Wu and Marinus, 1994).

In eukaryotes, the predominant mismatch-binding activity in cell extracts is a heterodimer of MSH2 and MSH6. As mentioned in the Introduction, the C-terminus of the MutS homologues of all species is highly conserved and the ATP-binding site is almost invariant (Figure 1; see also Fishel and Kolodner, 1995). Based on this high degree of conservation, it can be anticipated that changes in the ATPase activity of all these proteins will display a similar phenotype. However, due to the heterodimeric nature of the eukaryotic mismatch-binding factors, the question arises of whether the inactivation of ATPase activity of only a single component of the heterodimer will lead to mismatch repair deficiency. In yeast, this would appear to be the case. When the ATP-binding site was mutated in the MSH2 protein, the MSH2-MSH6 heterodimer was insensitive to ATP in a filter-binding assay, and overexpression of the mutant protein in a wild-type *S. cerevisiae* strain brought about a mutator phenotype (Alani *et al.*,

1997). An analogous situation could exist also in human cells. Mutations in the vicinity of the ATP-binding motif were identified in both *hMSH2* (Orth *et al.*, 1994) and *hMSH6* (Papadopoulos *et al.*, 1995) genes, and it is therefore possible that these mutations affect the ATPase activity of hMutS α in these cells. Indeed, in the mismatch repair-deficient cell line MT1, which contains missense mutations in both alleles of *hMSH6* (Papadopoulos *et al.*, 1995), the hMSH2–hMSH6 heterodimer has been shown to be defective, as the extracts of this cell line can be complemented with purified wild-type hMutS α (Drummond *et al.*, 1995). However, these results do not directly implicate the ATPase activity of hMutS α in the observed repair defect. We therefore decided to address this issue in a series of biochemical experiments. While detailed analysis of the causes underlying the phenotype of MT1 cells is currently in progress in our laboratory, we wanted to study the effect of ATPase-inactivating mutations on the human mismatch repair reaction *in vitro*. The primary questions that we wished to address were the following: (i) what are the relative contributions of the two subunits of hMutS α to ATP hydrolysis? and (ii) is ATP hydrolysis required for mismatch correction?

Our present study demonstrates that, in the context of the human mismatch-binding factor hMutS α and in the absence of DNA, both subunits contribute to the ATPase activity of the complex, albeit to different extents. Thus, while the double mutant was 60-fold less efficient in hydrolysing ATP than the wild-type factor, mutations of the ATP-binding site of hMSH2 and hMSH6 decreased the $K_{cat}/K_m > 10$ - and 33-fold, respectively (Table I). The latter data imply that the two subunits of hMutS α play somewhat different roles in the context of the heterodimer. This hypothesis is further supported by the cross-linking experiments shown in Figure 5, which indicate that only hMSH6 contacts the mismatch-containing DNA duplex, as well as by the higher efficiency of ATP cross-linking to hMSH2 (Figure 7), which suggests that the nucleotide has a longer residence time in the binding site of this polypeptide.

In the presence of mismatch-containing DNA, hMutS α 2KR and hMutS α 6KR were equally resistant to ATP (Figure 8). These findings support the notion that ATP binding, rather than ATP hydrolysis, is required to displace the bound protein from a complex with the mismatched DNA substrate. However, in mismatch correction assays, the hMutS α 6KR was less able to complement the repair-deficient extracts than hMutS α 2KR (Figure 9), which reflected their respective abilities to hydrolyse ATP. Surprisingly, in spite of the fact that the effect of the single mutations on the ATPase activities of hMutS α 2KR and hMutS α 6KR was severe (Table I), their effect on mismatch correction was low. The explanation probably lies in the fact that the repair reaction is carried out in the presence of 4 mM ATP and an ATP-regenerating system. At these concentrations, the singly mutated factors retain some capacity to translocate along, or dissociate from, the DNA (Figure 8), and are thus proficient in mismatch repair. This is not the case for the doubly mutant hMutS α , which remains bound at the mismatch site even in the presence of 10 mM ATP (data not shown). Taken together, we conclude that ATPase activity of hMutS α is necessary for mismatch repair, but not for dissociation of the factor

from DNA, at least in the absence of downstream repair proteins such as the MutL homologues.

Mismatch repair deficiency is closely linked to human malignancy. HNPCC segregates predominantly with mutations in *hMSH2* and *hMLH1* genes, whereby ~70% of HNPCC alleles appear to carry loss-of-function mutations in the form of frameshifts that lead to truncations (Liu *et al.*, 1996; Marra and Boland, 1996). However, missense mutations in mismatch repair genes have also been observed in HNPCC families (Liu *et al.*, 1996; Marra and Boland, 1996). Interpretation of such mutations is sometimes difficult, as it is hard to distinguish between causative mutations and polymorphisms. In cases where such mutations are found in the vicinity of highly conserved motifs, it would appear more likely that such sequence changes will turn out to be phenotypically relevant. Taking the present study as an example, we showed that mutations affecting the ATPase activity of the hMutS α factor are indeed deleterious to mismatch repair *in vitro*, even though the Lys→Arg mutations in the ATP-binding sites of hMSH2 or hMSH6 failed to affect heterodimer formation or mismatch binding significantly. It remains to be seen whether a similar difference in phenotype would also result *in vivo*. Such studies require that the mutant alleles be expressed in cell lines producing no wild-type hMSH6, hMSH2 or both proteins. Although lines mutated in the *hMSH2* and *hMSH6* genes are available, no double mutant has been found to date. The second hurdle lies in the expression of mismatch repair genes in human cell lines; numerous experiments attempted in several laboratories have met with little success to date. The *in vivo* substantiation of our *in vitro* results with the Lys→Arg ATPase mutants will therefore require further long-term studies.

While this manuscript was under review, Gradia *et al.* (1997) published a comprehensive study of the ATPase activity and DNA-binding properties of recombinant hMutS α . Although their kinetic parameters differ somewhat from our data, this effect could be due to the fact that their factor is not identical to ours (the hMSH6 has a His⁶ tag) and that their purification scheme was different. However, the two studies are in complete agreement regarding the lack of requirement for ATP hydrolysis in the dissociation of the protein factor from mismatch-containing DNA. Their observation that the ATPase activity of the heterodimer is stimulated by the presence of a G/T-containing substrate supports and extends our conclusions that ATP hydrolysis is required for mismatch repair. The proposal that hMutS α acts as a molecular switch, whereby its affinity for mismatched DNA is governed by the bound co-factor (on in the ADP-bound form, off in the ATP-bound form), has provided us with an alternative model of the initial steps of the mismatch repair process in eukaryotes, which may facilitate the identification of further protein–protein interactions involved in this complex pathway.

Materials and methods

Site-directed mutagenesis and production of recombinant baculoviruses

A PCR-based site-directed mutagenesis approach was used to change the conserved lysine present in the nucleotide-binding motif of both

hMSH6 and hMSH2 to arginine. Lys1140 of hMSH6 was mutated to arginine in a single PCR step, using the oligonucleotide primer 5'-TCATAAGCGTAGACCTGCCCAT-3', containing the mutation and the unique restriction site *AccI*, and a primer located upstream from the unique *PstI* site. The PCR product was purified and cloned between the *PstI* and *AccI* sites of hMSH6/BluescriptSK. [This latter plasmid contains the entire hMSH6 open reading frame (ORF) cloned in BluescriptSK, flanked with unique *BamHI* and *XhoI* sites on its 5' and 3' ends, respectively. It was constructed by joining the N-terminal sequence of hMSH6, amplified by RT-PCR from total HeLa mRNA using primers designed according to a published sequence (Nicolaidis *et al.*, 1996), to the N-terminus of clone C1 (Palombo *et al.*, 1995).] After sequencing, the hMSH6 cDNA, either wild-type or carrying the K1140R mutation, was cloned between the *BamHI* and *XhoI* sites of pFastBacI (Gibco-BRL) to give rise to clones pFastBac/hMSH6 or pFastBac/hMSH6/KR.

hMSH2 K675R was obtained by PCR overlap extension mutagenesis (Higuchi *et al.*, 1988) using the following primers: HKR1, 5'-CAGCTA-GATGCTGTTGTC-3'; HKR2, 5'-ATGGGAGGTAGATCAACATA-3'; HKR3, 5'-TATGTTGATCTACCTCCCAT-3' and the T7 universal primer. The PCR fragments obtained with HKR1/HKR3 and HKR2/T7 were used as templates in a second PCR step, using HKR1/T7 primers. The final PCR product was cloned between the *NdeI* and *Bsu36I* sites of hMSH2/BluescriptSK (a kind gift of B. Vogelstein). After sequencing, the *NdeI*-*XhoI* fragment carrying the K675R mutation was inserted between the corresponding *NdeI* and *XhoI* sites of pFastBac/hMSH2 to give rise to clone pFastBac/hMSH2/KR. The vector pFastBac/hMSH2 contains the entire ORF of hMSH2, cloned between the *XbaI* and *XhoI* sites of pFastBacI. The recombinant baculoviruses were obtained using the Bac-To-Bac system according to the manufacturer's (Gibco-BRL) instructions.

Production and purification of recombinant proteins

The same procedure was used for the production and purification of wild-type and mutated hMutS α heterodimers. *Spodoptera frugiperda* 9 (Sf9) cells (typically 1.2×10^8) were co-infected with hMSH6 (or hMSH6-K1140R) and hMSH2 (or hMSH2-K675R) recombinant baculoviruses at a multiplicity of infection of 10. After 72 h, cells were collected and total extracts were prepared as described (Jiricny *et al.*, 1988). The extracts (brought to 1 M salt with 0.1 vol. of 5 M NaCl) were passed through a 1 ml DEAE-Sepharose Fast Flow column (Pharmacia), diluted to 0.25 M salt with 3 vols of buffer A (25 mM HEPES-NaOH pH 7.6, 1 mM EDTA, 2 mM β -mercaptoethanol), and loaded onto a 5 ml Hi-Trap heparin-Sepharose FPLC column (Pharmacia). The protein complex was eluted with a 45 ml linear gradient from 25 to 100% buffer B (25 mM HEPES-NaOH pH 7.6, 1 M NaCl, 1 mM EDTA, 2 mM β -mercaptoethanol). The fractions containing hMutS α heterodimer (eluting at ~40% buffer B) were pooled, diluted with buffer A to a conductivity corresponding to 15% salt, and loaded onto a 1 ml Resource Q FPLC column (Pharmacia). The fractions containing the pure hMutS α complex were pooled, dialysed extensively against buffer A containing 0.11 M NaCl, 10% sucrose, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (storage buffer), and stored in aliquots at -80°C. This procedure yielded 350 μ g of protein.

For the purification of hMSH2, the same infection protocol was used. After chromatography on DEAE-Sepharose FF (see above), the total extract was diluted to 0.2 M salt, loaded onto a 5 ml Hi-Trap heparin-Sepharose FPLC column (Pharmacia) and eluted with a 45 ml linear gradient from 20 to 100% buffer B. The pooled protein was diluted with buffer A to a conductivity corresponding to 15% salt, and loaded on a 1 ml Mono Q column (Pharmacia), equilibrated with 15% buffer B. The pure protein eluted at the beginning of a 30 ml linear gradient from 15 to 70% of buffer B, and was dialysed against storage buffer and stored in aliquots at -80°C.

Band-shift assays

The band-shift assays were performed as described (Jiricny *et al.*, 1988); the protein was incubated with the labelled oligonucleotide substrates for 20 min in a 20 μ l total volume containing 25 mM HEPES pH 8.0, 0.5 mM EDTA, 10% (v/v) glycerol, 0.5 mM dithiothreitol (DTT) and 0.5 mg/ml bovine serum albumin (BSA). After 20 min incubation at room temperature, 5 μ l of each sample were loaded on a 6% non-denaturing polyacrylamide gel run in TAE buffer (pH 7.5). The G/C and G/T substrates were constructed by annealing the synthetic 34mer oligo 5'-AGCTTGGCTGACGGTTCGACGGATCCCCGGGAATT-3' with the complementary strand 5'-AATTCCTGGGGATCCGTCGACCTGC-AGCCAAAGCT-3', labelled at its 5' end with ^{32} P with polynucleotide kinase. Typically, 1 μ g of non-specific competitor poly[d(I-C)-d(I-C)]

was used per 10 μ g of crude extracts, and 5 ng per 200 ng of purified protein; no non-specific competitor was used for the calculation of dissociation constants.

In experiments in which nucleotides, ATP or ATP γ S, were added to the binding mixture, an equal volume of NTP solutions of different concentrations was added to the reactions at the beginning of the incubation time.

UV cross-linking experiments

The DNA-protein binding was performed as described above, except for the substrate used. The substrate G/T-U_{Br} was constructed by annealing the 34mer G strand with the 30mer 5'-AGCTTGGCTGCA-GGTT/CGACGGATCCCCGGG-3', where the underlined thymine was substituted with 5-bromouracil. The duplex was labelled by filling-in of the 5'-overhang, using T7 DNA polymerase with dTTP and [α - ^{32} P]dATP. Then 40 μ l of the standard band-shift mixture containing the labelled probe were put on ice and UV irradiated for 5 min in a UV Stratilinker (Stratagene). The sample was divided into four aliquots of 10 μ l each. One was directly denatured in 1 $\times$ SDS sample dye, the others were processed as described below.

Immunoprecipitations

From the material processed as described above, three aliquots were taken for immunoprecipitation. Two aliquots were heated at 95°C for 3 min in the presence of 2% SDS/10 mM DTT and incubated for 40 min at 4°C with 20 μ l of protein A-Sepharose (Pharmacia) pre-coupled with either anti-hMSH2 or anti-hMSH6 antibodies, in 500 μ l of IPP 500 buffer (Harlow and Lane, 1988). The third aliquot was incubated for 40 min at 4°C with 20 μ l of protein A-Sepharose pre-coupled with anti-hMSH2 antibodies in 500 μ l of HEPES-NaOH pH 7.6, 150 mM NaCl, 1% Triton X-100. After incubation, the beads were washed with 3 $\times$ 500 μ l of the respective incubation buffers, resuspended in 20 μ l of 2 $\times$ SDS sample dye, denatured for 5 min at 100°C, and loaded on a 7.5% SDS-PAGE. The fixed and dried gels were autoradiographed for 12 h at -80°C.

Data analysis

The amounts of radioactivity in the free and bound bands in the band-shift assays were quantified using a STORM phosphorimager and ImageQuant software (Molecular Dynamics, Inc.). A Hill plot was used to analyse the data (Brosh and Matson, 1996).

$$K_d = (1 - f)[Pt]/f \log[Pt] = \log[f/(1 - f)] + \log K_d$$

where K_d is the dissociation constant of the DNA-protein complex, [Pt] is the total concentration of wild-type or mutant hMutS α present in the reaction, and f is the ratio of the amount of bound DNA over the total amount of DNA present in the reaction. The logarithm of [Pt] was plotted against the logarithm of $f/(1 - f)$, and the y intercept represented the logarithm of K_d .

ATPase assays

The ATPase assays were carried out at 37°C in a 20 μ l mix containing 25 mM HEPES pH 7.6, 4 mM MgCl₂, 33 nM [γ - ^{32} P]ATP and using 1 pmol of purified protein. At selected time points, 2 μ l aliquots were removed, the reaction was stopped by the addition of an equal volume of formamide dye and loaded onto a 20% sequencing gel, as described previously (Hughes and Jiricny, 1992). For the calculation of the Michaelis-Menten constants, the activities were measured in the presence of 0.5, 1, 2, 3, 4, 6, 10 and 20 μ M ATP.

ATP cross-linking

ATP-protein photocross-linking was performed according to Chen *et al.* (1992). An aliquot (0.1 μ g) of purified protein was incubated in 50 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 10% glycerol, 10% sucrose, 5 mM DTT, 0.5 mg/ml BSA and 0.5 μ M [α - ^{32} P]ATP for 5 min at room temperature, placed on ice and irradiated for 5 min in a UV-Stratilinker (Stratagene). The samples were run on 7.5% SDS-PAGE and analysed by autoradiography.

In vitro mismatch repair assays

The efficiency of cytoplasmic extracts in repairing DNA mismatches was tested as described previously (Thomas *et al.*, 1991). Briefly, cytoplasmic protein extracts were prepared from HCT15, HEC59 or HeLa cell lines using 5×10^8 log phase growing cells. After resuspension in an appropriate volume of ice-cold hypotonic buffer to yield a cell

density of 1×10^8 cells/ml, the cells were allowed to swell for 20 min in a glass Dounce homogenizer (Kontes, Vineland, NJ) on ice, and then lysed mechanically by applying four or more strokes with a tight (type B) pestle. When >80% of cells were lysed, the nuclei were pelleted and the supernatant was centrifuged at 12 000 g for 10 min at 2°C, frozen in liquid nitrogen in aliquots, and stored at -80°C. The protein concentration was analysed by the Bradford method (Bradford, 1976). A M13mp2 DNA heteroduplex, containing a G/T or G/G mispair in the coding sequence of the *lacZ* α complementation gene, was obtained by hybridizing a single-stranded viral (+) DNA with the (-) strand of replicative form DNA as described (Thomas et al., 1991). One fmol of the heteroduplex was used in a repair reaction (25 μ l) with 30 mM HEPES (pH 7.8), 7 mM MgCl₂, 4 mM ATP, 200 μ M each CTP, GTP and UTP, 100 μ M each dATP, dGTP, dTTP and dCTP, 40 mM creatine phosphate, 100 fmol of creatine phosphokinase, 15 mM sodium phosphate (pH 7.5) and 50 μ g of extracted protein. In complementation studies, 0.1 μ g of purified recombinant wild-type or mutant hMutS α factors were added to the extracts. The incubation was carried out at 37°C for 20 min. The repair was directed to the (-) strand of M13mp2 by the presence of a nick. The DNA heteroduplex was then purified and introduced by electroporation into *E. coli* NR9162 (*mutS* strain), plated on minimal medium in a soft agar layer containing 0.5 ml of a log phase culture of CSH50 (the α -complementation strain), 0.5 mg of isopropyl- β -D-thiogalactopyranoside and 2 mg of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside. After incubating for 16–20 h at 37°C, the plaques were assigned to one of the following phenotypes: blue, colourless or mixed. If no repair occurred, mixed plaques were observed containing both blue and colourless progeny. Repair of the substrate in a cell extract reduced the percentage of mixed plaques and increased the percentage of pure-colour plaques.

Acknowledgements

We would like to thank Darinka Bohrer for excellent technical assistance. We are grateful also to our colleagues, Primo Schär and Teresa Lettieri for helpful discussions and for the critical reading of the manuscript, and to Tom Kunkel for the gift of the M13 mp2 phage DNA. This research was supported in part by the Schweizerischer Nationalfonds für die Förderung der wissenschaftlichen Forschung.

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Received January 15, 1998; revised March 3, 1998;
accepted March 4, 1998