Affinity of mismatch-binding protein MutS for heteroduplexes containing different mismatches

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We have used bandshift analysis to measure the interaction between the *Escherichia coli* mismatch-binding protein MutS and synthetic DNA fragments containing all possible DNA mismatches as well as an unpaired T (ΔT). The order of affinity is found to be ΔT > GT > GG > AA ≈ TT ≈ TC > CA > GA > CC > GC. We find that the affinity for GT mismatches is affected by the flanking sequence and decreases in the order $G_nC_n > (GC)_n > A_nT_n > (AT)_n$. Studies with base analogues show good binding to φT (where φ represents 1′,2′-dideoxyribose), but much weaker binding to Gφ.

Key words: DNA mismatches, DNA recognition, mismatch repair.

INTRODUCTION

Successful replication requires that DNA be copied without the introduction of errors, which would generate mutations. Although DNA polymerases are very accurate, and possess their own proof-reading activities, mistakes do occur that need to be corrected after replication. In addition, genetic recombination produces regions of heteroduplex DNA that contain mismatched nucleotides. Further damage to the DNA is caused by environmental factors. To prevent the formation of these mutations, all organisms express enzymes that repair mispaired and damaged DNA [1–4].

One of the best characterized mismatch repair systems is the MutHLS enzyme complex from *Escherichia coli* [4–6]. In this complex, MutS is the 97 kDa protein that recognizes and binds to DNA mismatches [7]. The MutS–mismatch complex then recruits MutH and MutL, leading to excision of the DNA at hemimethylated GATC sites [8,9]. The section of DNA between the MutH incision site and the mismatch (up to 1000 bases long) is subsequently removed and resynthesized, replacing the mismatch with the correct base.

Several studies have shown that MutS has variable affinity for different mismatches [10–14]. *In vivo*, transition mismatches are usually repaired more efficiently than transversions. MutS forms the strongest complexes with GT mismatches and single unpaired bases [14,15], whereas poor binding is observed at CC mismatches. In general the efficiency of correction by MutHLS systems is GT ≈ GG ≈ CA ≈ AA > TT ≈ TC ≈ AG > CC. This repair efficiency is correlated with the mismatch binding affinity of MutS *in vitro*, although the enzyme binds to GT much better than it does to AC [16]. Mismatch binding is also thought to be affected by the base sequence in the vicinity of the mismatch, such that repair efficiency increases with increasing GC content in the neighbouring sequence [17]. Studies by electron microscope and surface plasmon resonance have revealed that MutS binds to heteroduplex DNA as a homodimer [18,19], although MutS-heteroduplex complexes demonstrate more complicated behaviour when analysed by sedimentation [7,20]. Recent gel-filtration studies have suggested that the MutS dimer might assemble into a hexameric species in a concentration-dependent fashion [21]. In addition to mismatches and insertion/deletion loops, MutS also binds to alkylated bases [22,23] and single-stranded DNA [24].

Here we have used a bandshift analysis with synthetic DNA fragments to measure the binding of MutS to all eight mismatches

A)
Duplexes containing single mismatches

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5'-GATCGATCGGACTCCGAGTACCGCAATGAACTTAGCACCGAGTGAATTGCTAGCCAGTCGAATT
3'-CTAGCTAGCCTGAGGCTCATGGCGTTA[     ]TCGTGGCTCACTTAACGATCGGTCAGCTTAA
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Mismatch	lower strand (3'-5')	Mismatch	lower strand (3'-5')
WC	CTTGAA	AC	C<u>C</u>TGAA
GT	<u>T</u>TTCTA	CT	CTT<u>T</u>AA
GA	<u>A</u>TTCTA	CC	CTTCAA
GG	<u>G</u>TTCTA	TT	CTTGTA
AA	C<u>A</u>TGAA	ΔT	CTTGAAT

B)
Duplexes containing GT mismatches in different sequence contexts

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5'-GATCGATCGGACTCCGAGTACCGCAAT[X]ACCGAGTGAATTGCTAGCCAGTCGAATT
3'-CTAGCTAGCCTGAGGCTCATGGCGTTA[X]TGGCTCACTTAACGATCGGTCAGCTTAA
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$(GC)_n$	GCGCG<u>G</u>CGCG/CGCGTGCGC
G_nC_n	GGGGG<u>G</u>GGGG/CCCCTCCCC
$(AT)_n$	ATATG<u>T</u>ATA/TATATATAT
A_nT_n	AAAAG<u>A</u>AAA/TTTTTTTTT

C)
Duplexes containing various base analogues

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5'-CTGACGATGTGAACTATGACGCTCCTAGGCTGATTACTGACTGCTGGACT
3'-GACTGCTACACTTGATACTGCGAGGGTCCGACTAATGACTGACGACCTGA
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Figure 1 Sequences of the oligonucleotides used in this work

(**A**) Duplexes for examining single base mismatches. (**B**) Duplexes for examining the effect of sequence context on the binding to a GT mismatch. (**C**) Duplexes containing various base analogues in which the GT mispair is replaced by GU, Gφ, IT, IU, 2APT, NebT, φT or φφ. In each case the upper strand of the duplex was labelled at the 5′ end. Abbreviation: WC, Watson–Crick homoduplex.

Abbreviations used: ΔT, unpaired T; φ 1′,2′-dideoxyribose; 2AP, 2-aminopurine; Neb, nebularine (deoxypurine).

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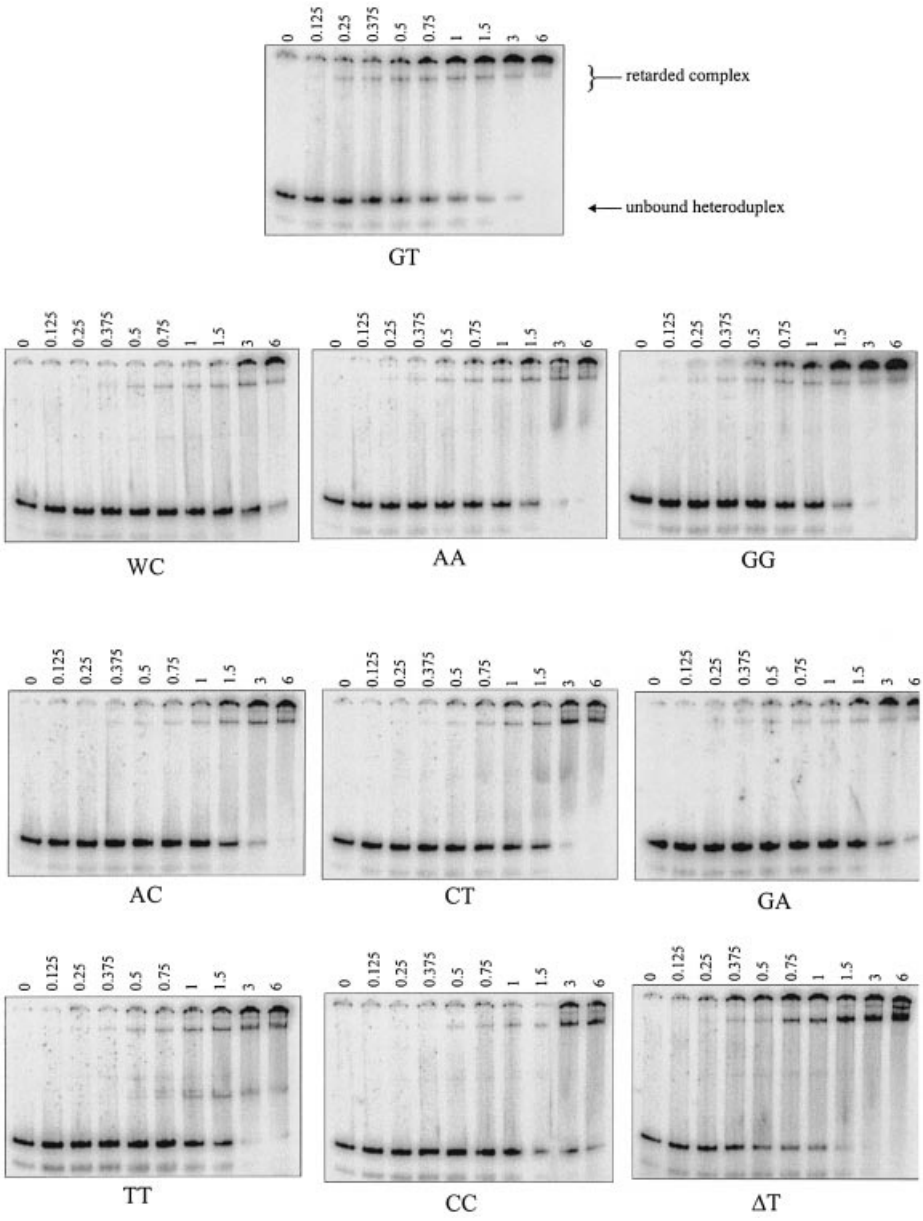

Figure 2 Effect of His_6-MutS on the gel mobility of heteroduplexes containing single base mismatches

The sequences of the duplexes are shown in Figure 1(A); the individual mismatches are shown below each panel. The MutS concentration (in μM) is shown at the top of each gel lane. Abbreviation: WC, Watson–Crick homoduplex.

and also to a fragment containing an unpaired T. We have also measured the affinity for GT mismatches in different sequence environments and have examined binding to base analogues of G and T.

MATERIALS AND METHODS

His_6-MutS

The TX3149 cell line containing plasmid pTX412, encoding the mutS gene as a His-tag protein, was a gift from Dr G. Feng and Dr M. E. Winkler (University of Texas, Houston Medical School, Houston, TX, U.S.A.). MutS, obtained as a His-tag protein, was prepared by the method of Feng and Winkler [25]. Culture (100 ml) was grown until mid-exponential phase (D_{600} approx. 0.6) and MutS production was induced by adding isopropyl β-D-thiogalactoside to a concentration of 1 mM. After 3 h of growth at 37 °C, the cultures were cooled on ice for 10 min and the cells were harvested by centrifugation at 5000 ***g*** for 5 min. The cell pellet was washed with deionized water, resuspended in 20 mM Tris/HCl, pH 7.9, containing 0.5 M NaCl, 5 mM imidazole and 10 % (v/v) glycerol, after which cells were lysed by sonication. After centrifugation to remove the insoluble material, the protein was purified on Novagen His·bind Quick 900 cartridges and dialysed against 40 mM Tris/HCl, pH 7.6, containing 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 13.3 % (v/v) glycerol. We typically obtained approx. 1 mg of purified protein (approx. 95 % pure) from 100 ml of culture.

DNA fragments

Oligonucleotides were obtained from Oswel DNA Service. These were synthesized on the 0.2 μmol scale, purified by HPLC, dissolved in water and stored at -20 °C. One strand of each duplex (3 pmol) was labelled at the 5′ end with [γ-^{32}P]ATP (3 pmol) by using polynucleotide kinase. The labelled oligonucleotide was purified on a 15% (w/v) polyacrylamide gel containing 8 M urea. After the DNA had been eluted from the gel, it was precipitated with ethanol and resuspended in 20 μl of 10 mM Tris/HCl buffer containing 0.1 mM EDTA. To this was added a slight molar excess of the unlabelled complementary strand, and the duplexes were annealed by slowly cooling them from 100 to 25 °C. The annealed duplexes were purified on 10% (w/v) polyacrylamide gels.

Three different types of heteroduplex were used in these studies; the sequences are shown in Figure 1. For studies with single mismatches we used the sequences shown in Figure 1(A), so that, as far as possible, all mismatches were in the same sequence context. Studies investigating the effects of sequence context on the interaction with GT mismatches used the sequences shown in Figure 1(B). These sequence are similar to those in Figure 1(A), except that the central nine bases have been altered, generating GT mismatches in alternating or homopolymeric tracts of AT or GC base pairs. For the studies with different base analogues we used sequences that were originally designed for work with Vsr endonuclease [26], shown in Figure 1(C). In these oligonucleotides, G was replaced in turn by 2-aminopurine (2AP), nebularine (deoxypurine, Neb), inosine or a pseudo-abasic site (1′,2′ dideoxyribose, φ), whereas T was replaced by either U or φ.

Band-shift analysis

Protein–DNA samples were prepared by mixing 1 μl of labelled DNA (less than 15 fmol) with various amounts of His_6-MutS in 10 μl of 20 mM Tris/HCl, pH 7.6, containing 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.01 mM EDTA and 10% (v/v) glycerol. Unlabelled poly(dI)·poly(dC) (15 pmol) was included to decrease non-specific binding, and the samples were equilibrated on ice for 20 min. The samples were applied to 6% (w/v) polyacrylamide gels prepared in 1 × TAM buffer (50 mM Tris/acetate, pH 7.6, containing 5 mM $MgCl_2$) and were run at 4 °C for approx. 4 h. Gels were fixed in 10% (v/v) acetic acid/15% (v/v) propan-2-ol, transferred to Whatmann 3MM paper and dried under vacuum at 80 °C for 1.5 h. Dried gels were exposed to a Kodak Phosphor Storage Screen, which was scanned with a Molecular Dynamics Storm 860 PhosphorImager. The results were analysed with Molecular Dynamics ImageQuant software.

Analysis of results

Apparent dissociation constants for the protein–DNA complex can be obtained by quantitative analysis of the concentration dependence of the bandshifts. This analysis assumes that the complex does not dissociate during gel electrophoresis and requires that the DNA concentration be very low, much lower than the dissociation constant. It is usual to calculate the amount of radiolabel in the retarded species as a function of protein concentration. However, because the MutS–DNA complex has a high molecular mass and a very low gel mobility, we encountered some problems with DNA remaining in the wells. We therefore chose to measure the disappearance of free DNA with increasing concentrations of His_6-MutS. The amount of free DNA was plotted against the MutS concentration, and the data points were initially fitted to a simple binding equation by using FigP for Windows (Biosoft). However, this consistently produced a poor fit to the data points, which seemed to follow a sigmoidal rather than a hyperbolic curve. The results were therefore described by a co-operative binding curve:

$$[DNA]_f/[DNA]_T = 1-[MutS]^h/(K_d+[MutS]^h)$$

where $[DNA]_f$ is the concentration of free DNA, $[DNA]_T$ is the total DNA concentration, [MutS] is the MutS concentration and K_d is the apparent dissociation constant.

RESULTS

Binding of MutS to heteroduplexes containing single mismatches

Figure 2 shows the bandshifts for the interaction of His_6-MutS with heteroduplexes containing different mismatches, in addition to a Watson–Crick homoduplex. In all cases the addition of MutS caused a large decrease in the mobility of the labelled DNA. These changes were dependent on the concentration of MutS, so that in several instances almost no free DNA was evident at the highest protein concentration. In all cases most of the retarded radiolabel had a very low mobility and hardly entered the gel matrix. We assume that this very low mobility arose from the high molecular mass of the MutS homodimer–DNA complex (230 kDa). In addition, in some instances several retarded species can be seen. Because of these difficulties we measured the disappearance of free DNA with increasing concentrations of His_6-MutS, plotting the amount of free DNA remaining against the protein concentration, rather than estimating the amount of labelled DNA in the retarded species. This quantitative analysis yielded the binding plots shown in Figure 3. It is clear that these plots cannot be described by simple hyperbolic binding curves, and so were fitted by a co-operative binding equation as described in the Materials and methods section. The binding parameters obtained are presented in Table 1. From these values it can be seen that MutS bound best to fragments that contained an unpaired T (ΔT) or a GT mismatch. MutS also bound well to the GG, AA, CT and TT mismatches. As expected, the weakest binding was to the Watson–Crick homoduplex (approx. 1/100 of that to a GT-mismatch). Weak binding was also observed to fragments containing CC, GA and AC mismatches. The order of affinity of MutS for these mismatches was $\Delta T > GT > GG > AA \approx TT \approx TC > CA > GA > CC > GC$.

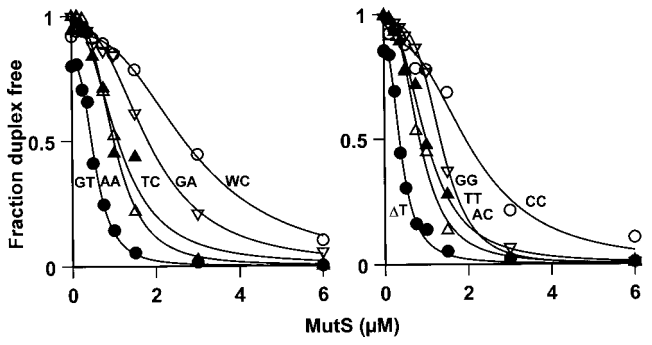

Figure 3 Binding plots showing the interaction of MutS with heteroduplexes containing different base mismatches

The data were obtained from a quantitative analysis of the gels shown in Figure 2. Left panel: ●, GT; △, AA; ▲, TC; ▽, GA; ○, Watson–Crick homoduplex (WC). Right panel: ●, △T; △, GG; ▲, TT; ▽, AC; ○, CC. The full sequences of the duplexes are shown in Figure 1(A).

Table 1 Apparent dissociation constants for His_6-MutS bound to heteroduplexes containing different mismatches

The values were obtained from the bandshift experiments as described in the text according to the equation $[DNA]_f/[DNA]_T = 1 - [MutS]^h/(K_d + [MutS]^h)$. Results are presented as means ± S.E.M.

Mismatch	Apparent K_d (μM)	h
WC	15.0 ± 2.3	2.6 ± 0.1
GT	0.19 ± 0.03	2.7 ± 0.3
GA	4.8 ± 0.9	2.5 ± 0.2
GG	0.62 ± 0.07	2.7 ± 0.3
AA	1.0 ± 0.1	2.9 ± 0.2
AC	3.4 ± 0.4	3.9 ± 0.3
CT	1.3 ± 0.3	2.3 ± 0.4
TT	1.0 ± 0.1	2.5 ± 0.2
CC	6.9 ± 2.4	2.6 ± 0.4
ΔT	0.12 ± 0.02	2.3 ± 0.2

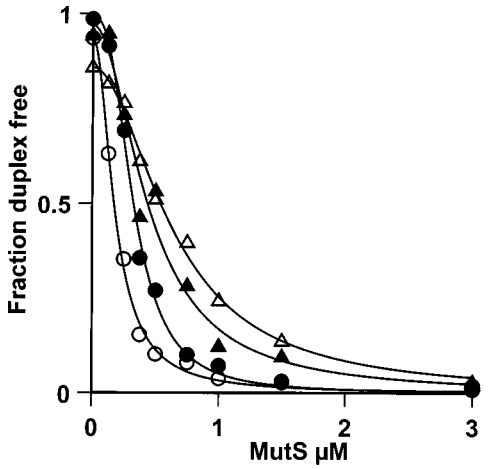

Figure 5 Binding plots showing the interaction of MutS with heteroduplexes containing GT mismatches in different sequence contexts

The data were obtained from a quantitative analysis of the gels shown in Figure 4. Symbols: ○, G_nC_n; ●, $(GC)_n$; ▲, A_nT_n; △, A_nT_n. The full sequences of the duplexes are shown in Figure 1(B).

Effect of sequence context on binding to GT mismatches

The effect of sequences flanking a GT mismatch was investigated by using a series of heteroduplexes containing GT mismatches in different environments: surrounded by poly(dG)·poly(dC), alternating G and C, poly(dA)·poly(dT) and alternating A and T. The sequences of these heteroduplexes are shown in Figure 1(B). Figure 4 shows the gel-shift assays for the interaction of His_6-MutS with these heteroduplexes; the binding plots are presented in Figure 5. The binding parameters derived from these results are presented in Table 2. As expected, MutS bound strongly to all these heteroduplexes, because they all contained a GT mismatch. Binding was better when the surrounding sequence contained GC residues rather than AT. In addition, the protein bound better to GT sites flanked by homopurine·homopyrimidine tracts than to regions of alternating purines and pyrimidines. The affinity for the single GT mismatch in a mixed-

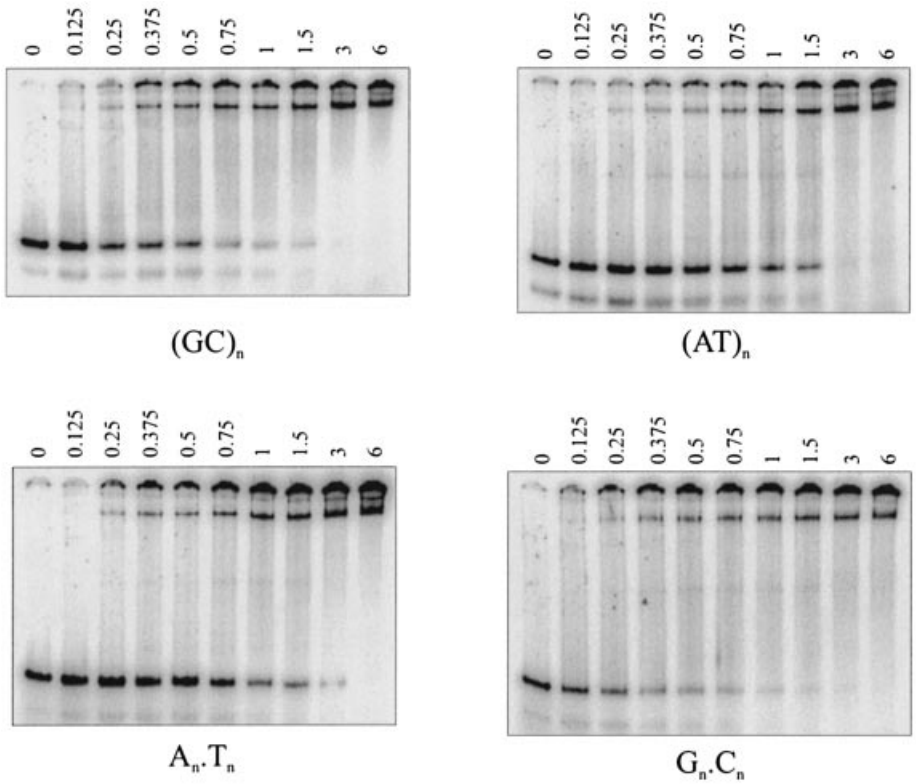

Figure 4 Effect of His_6-MutS on the gel mobility of heteroduplexes containing GT mismatches in different sequence contexts

The sequences of the duplexes are shown in Figure 1(B); the flanking sequences are shown below each panel. The MutS concentration (in μM) is shown at the top of each gel lane.

Table 2 Apparent dissociation constants for His_6-MutS bound to heteroduplexes containing GT mismatches in different sequence contexts

Results are presented as means ± S.E.M.

Mismatch	Apparent K_d (μM)	h
$(GC)_n$	0.05 ± 0.01	2.6 ± 0.2
$(AT)_n$	0.42 ± 0.03	1.9 ± 0.1
$A_n \cdot T_n$	0.21 ± 0.05	1.9 ± 0.3
$G_n \cdot C_n$	0.035 ± 0.006	2.0 ± 0.1

sequence environment presented above (0.19 ± 0.03 μM; mean ± S.E.M.) fell between those for a flanking GC or AT content. The apparent K_d values suggest that the order of affinity of MutS for the GT mismatch in varying sequence context was G_nC_n > $(GC)_n$ > mixed sequence > A_nT_n > $(AT)_n$.

Role of functional groups in GT mismatch recognition

We examined the role of various substituents on the GT mismatch in determining MutS binding by measuring the interaction of His_6-MutS with fragments containing base analogues at this position (Figure 1C). These base analogues are not themselves of biological significance but they allowed us to probe the possible interactions between mismatches and the enzyme. These fragments were originally prepared for related studies with the Vsr endonuclease [26]; the GT mismatch is located in the sequence context CTAGG. Previous studies at single protein concentrations suggested that IT was bound less well than GT and that the NebT mispair was bound less well [16]. Figure 6 shows the gel-shift assays for the interaction of His_6-MutS with these different heteroduplexes. The binding parameters obtained from a quantitative analysis of these results are presented in Table 3. As expected, MutS bound well to the GT mismatch, although the heteroduplex with a pseudo-abasic site opposite T (φT) was

Table 3 Apparent dissociation constants for His_6-MutS bound to heteroduplexes containing various base analogues

Results are presented as means ± S.E.M.

Mismatch	Apparent K_d (μM)	h
GT	1.3 ± 0.2	1.8 ± 0.2
IT	12.1 ± 1.7	2.5 ± 0.1
2APT	9.0 ± 2.9	2.1 ± 0.3
NebT	10.2 ± 3.3	2.7 ± 0.3
φT	0.70 ± 0.1	2.3 ± 0.3
φφ	44 ± 23	2.9 ± 0.4
Gφ	5.2 ± 0.6	2.4 ± 0.1
GU	5.5 ± 1.3	2.5 ± 0.3
IU	2.1 ± 0.3	1.5 ± 0.2

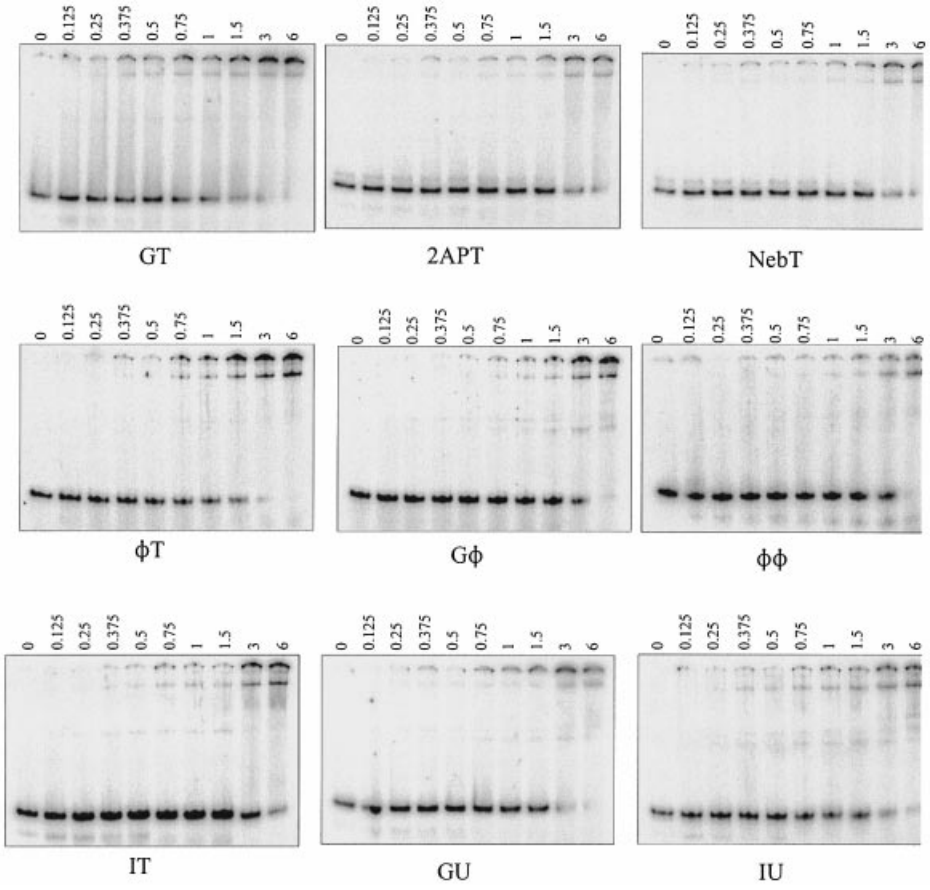

Figure 6 Effect of His_6-MutS on the gel mobility of heteroduplexes containing GT mismatches with various base analogues

The sequences of the duplexes are shown in Figure 1(C); the base mismatches are shown below each panel. The MutS concentration (in μM) is shown at the top of each gel lane.

bound even better, with a halving of the K_d. IU was bound almost as well as GT, whereas IT, 2APT and NebT showed much weaker binding. The most poorly recognized lesion was that containing two pseudo-abasic sites. The order of affinity of MutS for the various analogues, as revealed by these apparent K_d values, was φT > GT > IU > Gφ ≈ GU > 2APT ≈ NebT ≈ IT > φφ. Surprisingly, we found that IT was a poorer substrate than IU, even though GT was better than GU.

DISCUSSION

Mismatch recognition

The results presented here show that MutS binds to all the heteroduplex DNA fragments, with the best binding to ΔT and GT. The order of binding affinities to different mismatches is ΔT > GT > GG > AA ≈ TT ≈ TC > CA > GA > CC > GC, in good agreement with previous studies [10]. MutS has been shown to repair transition mismatches better than transversions, with GT and AC mismatches being well repaired [17,27]. It is therefore surprising that the AC mismatch binds MutS much less well than GT, as noted in other studies [16]. The AC mismatch can adopt a pH-dependent wobble conformation (as for GT), generating an A+C base pair [28–30]. If the adenine is not protonated then there is only one hydrogen bond between the A and C; this can be easily deformed, so that its structure will be especially sensitive to the sequence of the surrounding base pairs. Because GT is also the most stable mismatch, it seems unlikely that the protein functions by sensing the thermodynamic stability of each mismatch. The protein binds poorly to pyrimidine·pyrimidine mispairs, correlating with their poor repair *in vivo*. The structure of the GA mismatch is also dependent on sequence context [29], and it is generally not well repaired by the *E. coli* mismatch repair system [11]. The observation that ΔT is well recognized is interesting, although we do not know whether it forms an extrahelical loop or is accommodated into the helix to form a bulge. However, it has been suggested [12] that the *E. coli* mismatch repair system recognizes and repairs intrahelical but not extrahelical (looped-out) bases. These studies confirm earlier suggestions that the binding affinity of MutS is not the sole determinant in the efficiency of mismatch repair.

It was at first surprising that His_6-MutS bound to the Watson–Crick homoduplex, because several groups have reported that MutS and its counterparts display a low and non-specific DNA binding activity [16,31,32]. The translocation model for MutS ATPase activity proposes the existence of a non-specific DNA-binding site on MutS [18,33,34]. The existence of such a binding site was confirmed by Tachiki et al. [35], who showed that a domain of *Thermus thermophilus* MutS bound non-specifically to double-stranded DNA. In view of these observations, it is likely that the binding seen in these band-shift assays is the non-specific binding of MutS to homoduplex DNA. We might expect that the non-specific binding might increase with DNA length; the DNA fragments used here were longer than those used in other studies [14].

Co-operative binding

All the binding results presented in this paper have been fitted by co-operative binding equations with Hill coefficients (h) between 2 and 3. This might suggest that there is some monomer–dimer or dimer–tetramer equilibrium on binding to DNA. It is known that *E. coli* MutS exists as a homodimer in solution and when binding to DNA. This has been confirmed by studies by electron microscope and surface plasmon resonance [18], although earlier sedimentation studies suggested that MutS showed a more complex behaviour [7], suggesting further oligomerization. Equilibrium sedimentation with *Taq* MutS concluded that this exists in a dimer–tetramer equilibrium, with the dimer favoured at MutS concentrations below 10 μM [20]. The substrate used in these studies was a 37 bp heteroduplex; the authors suggested that the dimer–tetramer equilibrium might be shifted towards tetrameric MutS when binding to larger DNA substrates. Furthermore, a tetrameric MutS species might function to provide an additional DNA-binding site to facilitate translocation along DNA [20]. Gel-filtration studies, with a similar range of MutS concentrations to those employed here, have recently suggested that MutS can form a 580 kDa higher-order structure [21] and that this might be involved in translocation.

Flanking sequences

Experiments *in vivo* [17] have shown that repair efficiency increases with increasing GC content. This effect was more striking for transversion than for transition mismatches [17]. The authors proposed that this effect is due to mismatch repair operating more efficiently in regions of stable heteroduplex, where there is more likelihood of the mispaired bases adopting an intrahelical rather than an extrahelical conformation. The results presented here support the observation that the affinity of MutS for a particular mismatch depends on the surrounding sequence, showing that it binds to GT better when the mismatch is flanked by GC than when it is flanked by AT·GC-rich regions have a wide minor groove and this might be an important factor in recognition if MutS makes contacts with the DNA in this groove, although chemical footprinting studies have indicated that the protein makes contacts with the DNA major groove [36]. Furthermore, the mismatch is recognized better when present in a polypurine·polypyrimidine environment than in a region of alternating purines and pyrimidines. Because local DNA structure depends on sequence [37], this improved recognition might be a consequence of the local DNA structure surrounding the mismatch. It might also be significant that these simple repeating sequences might be especially prone to strand slippage and might therefore generate mismatches during replication.

Base substituents

Previous studies at single protein concentrations suggested that IT is bound less well than GT [16] and that the binding affinities are correlated with the efficiencies of repair *in vivo* [38]. We find that the complete removal of the G from the mismatch (G → φ) results in a doubling of the affinity of His_6-MutS for the heteroduplex. This heteroduplex might be analogous to (ΔT) but is not bound as tightly. However, the removal of the 6-oxo group (G → 2AP), the 2-amino group (G → I) or both (G → Neb) produces a 10-fold decrease in affinity. The similar behaviour of these analogues is surprising, because IT is likely to form a wobble base pair, whereas 2APT and NebT are not wobble pairs. Removal of the pyrimidine base from the GT mismatch (Gφ) resulted in a 4-fold decrease in binding affinity, in contrast with removal of the G (φT), which increased the binding affinity. This suggests that the pyrimidine base is more important than the purine for recognition of the GT mismatch. The role of T is also seen in the fact that removing its 5-methyl group (GU) causes a 4-fold decrease in binding affinity. Surprisingly, the removal of both the 2-amino group of G and the 5-methyl group of T (GT → IU) caused only a halving of affinity.

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