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# New functional sites in MutS affect DNA mismatch repair

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The MutS protein plays an important role in the DNA mismatch repair system. Mutations in the *mutS* gene can lead to genome instability and ultimately cell malfunction. Here we have established a method for identifying functional defective mutants of MutS by random mutation and rifampicin screening. Some novel functional sites in MutS were identified. The MutS mutant strains were analyzed using surface plasmon resonance, gel filtration and far-western methods to determine the molecular mechanisms behind the DNA mismatch repair function of MutS.

**DNA mismatch repair, MutS, random mutation, functional defective mutants**

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The DNA mismatch repair system maintains the genomic stability of cells by correcting errors in DNA replication. MutS, a crucial protein in the DNA mismatch repair system, specifically binds to mismatched DNA to trigger downstream events in mismatch repair [1]. Although MutS is vital for mismatch repair, the mechanism by which it detects mismatch sites remains unclear. Furthermore, the role of MutS oligomerization in mismatch repair is still under debate [2–5]. Here, we address these questions by establishing a random mutation method to detect MutS mutants that are defective in mismatch repair. By examining functional changes in these mutants, we provide new insight into the function of MutS in mismatch repair. Mutations in the human homologue of MutS (MSH) are associated with cancer [6], and deletion or mutation of hMSH2 leads directly to hereditary nonpolyposis colorectal cancer (HNPCC) [7]. Therefore, the identification of novel functional sites in MutS will aid the discovery of cancer-related mutations in MSH and help elucidate the molecular mechanisms involved in MutS function.

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Random mutation is a technique widely used in protein engineering to improve the function of enzymes [8]. Although this technique has rarely been used to study the functional mechanisms of proteins, it offers a more effective method than site-directed mutation, especially when structural information is unavailable. In this study, a library of *mutS* gene mutants was constructed by error-prone PCR [9]. The MutS defective mutants, obtained via rifampicin screening [10], failed to complement the *mutS* deficiency in KM75 cells [11]. The main functional properties of MutS in the mismatch repair system, namely its mismatch binding activity, oligomerization state and MutL binding activity, were analyzed using various methods to determine the mechanism by which mutation of MutS results in mismatch repair dysfunction.

## 1 Construction and screening of defective mutants of MutS

Mutations were introduced into the *mutS* gene randomly using error-prone PCR. The resulting DNA library was then

ligated to vector pET15b [12] and transformed into KM75 cells. Cells were shaken overnight in liquid media and then plated on solid media containing rifampicin. Strains harboring a functionally defective MutS protein grew on the selective media because the high genomic mutation frequency led to rifampicin resistance. Mutants Y145H, A272T, I475V and T838I were identified by DNA sequencing and analyzed further.

## 2 Spontaneous mutation frequency

Using AB1157 (standard strain) [11], KM75 (*mutS*::Tet derived from AB1157), KM75/pET15b and KM75/pET15b-*mutS* as controls, the spontaneous mutation frequencies of mutants Y145H, A272T, I475V and T838I were analyzed by ANOVA [11,13]. The spontaneous mutation frequency was expressed as the ratio of rifampicin-resistant colonies to viable colonies, and the relative mutation frequency was expressed as the ratio of the average mutation frequency of a given strain to AB1157 (Table 1). The results indicated that KM75/pET15b-*mutS* resumed mismatch repair function due to its basal expression of wild type MutS. Compared with strains harboring wild type MutS, all strains containing mutants Y145H, A272T, I475V and T838I showed a high spontaneous mutation frequency due to defects in the mismatch repair system.

**Table 1** Mutation frequencies of strains[a)]

| Strain | Spontaneous mutation frequency ($10^{-6}$) | Relative mutation frequency[b)] |
|---|---|---|
| AB1157 | 0.020±0.011 | 1 |
| KM75 | 2.5±1.3* | 125 |
| KM75/pET15b | 2.0±0.63* | 101 |
| KM75/pET15b-*mutS* | 0.018±0.017 | 1 |
| KM75/pET15b-*mutS*-Y145H | 0.28±0.087* | 14 |
| KM75/pET15b-*mutS*-A272T | 1.8±0.95* | 88 |
| KM75/pET15b-*mutS*-I475V | 0.81±0.16* | 40 |
| KM75/pET15b-*mutS*-T838I | 1.0±0.34* | 50 |

a) Experiments were repeated at least four times. b) The ratio of the average mutation frequency of each strain compared with that of strain AB1157. *, $P<0.05$ indicated a significant difference in the spontaneous mutation frequency compared with that of strain KM75/pET15b- *mutS*.

## 3 Mismatch binding activity

DNA binding activity was detected by surface plasmon resonance. Table 2 shows that MutS bound to GT mismatches more specifically than to AT matched DNA. Mutant A272T completely lost the ability to bind both matched and mismatched DNA, leading to a defect in mismatch repair function. Mutants Y145H and I475V showed significantly reduced binding to matched and mismatched DNA. Mutant T838I showed no obvious changes in its binding to mismatched DNA compared with that of wild type MutS.

**Table 2** Surface plasmon resonance analysis of DNA binding activity[a)]

| Strain | GT mismatched | AT matched |
|---|---|---|
| MutS | 253±10 | 16±2.0 |
| Y145H | 86±12 | 10±0.6 |
| A272T | 4±1 | 0.41±0.1 |
| I475V | 43±8 | 1.4±1.0 |
| T838I | 240±9 | 28±1.5 |

a) Experiments were carried out in triplicate. Binding response values (RU) were recorded.

## 4 Protein oligomerization

Gel filtration (Figures 1A and B) was used to analyze the oligomerization of proteins. SDS-PAGE (Figures 1C and D) showed that before gel filtration the purified protein consisted of a single, non-dispersed band. The purity of the target proteins was shown to be over 90% when analyzed by AlphaImage2000. Figure 1A shows that wild type MutS mainly formed tetramers and dimers. Mutants Y145H, A272T and I475V exhibited a similar oligomerization state to that of wild type MutS (data not shown). However, the oligomerization state was altered in mutant T838I, with the tetramer protein being virtually undetectable (Figure 1B). Because oligomerization is important for the mismatch repair function of MutS, this change might result in the loss of mismatch repair activity in the T838I mutant.

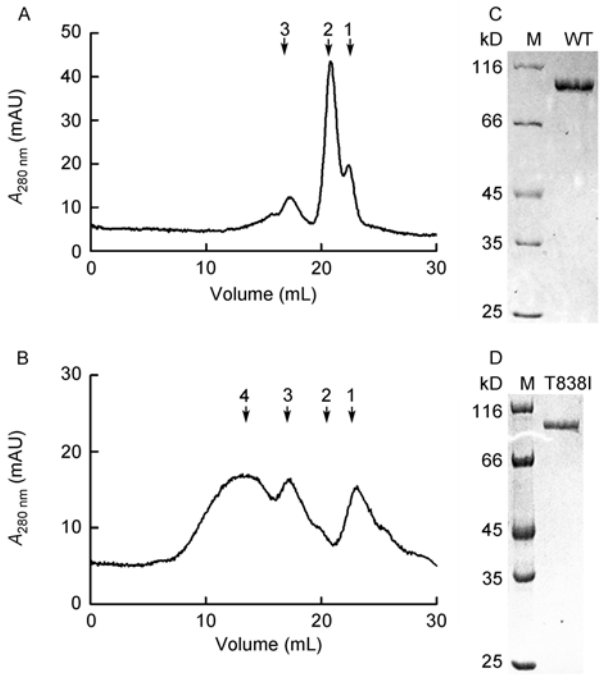


**Figure 1** Oligomerization of proteins as detected by gel filtration. Gel filtration of wild type MutS (A) and T838I (B). Peak 1, dimer; peak 2, tetramer; peak 3, octamer; peak 4, aggregate. SDS-PAGE analysis of wild type MutS (C) and T838I (D) before gel filtration.

## 5 Interactions between MutS and MutL

Far-Western experiments were carried out to detect the interactions between the MutS and MutL proteins. Target proteins (wild type or mutant MutS) were spotted onto a nitrocellulose membrane, followed by addition of the SBP-tagged MutL protein (MutL-SBP) [13] at a final concentration of 800 nmol $L^{-1}$. Binding of MutL-SBP to the target proteins was measured by the colorimetric analysis of the protein spots. Figure 2 shows that although mutant T838I exhibited slightly weaker binding activity than wild type MutS, the binding of other mutants to MutL-SBP was similar to that of wild type MutS.

## 6 Discussion

In this study, new defective mutants of MutS were obtained by random mutation and rifampicin screening. These mutants were then analyzed by various methods to determine whether the main properties of MutS that affect its mismatch repair function had been affected by these mutations. In addition, circular dichroism was used to detect the secondary structure of the proteins, and the results indicated that the secondary structure of all of the mutants was the same as that of wild type MutS (data not shown). Taking all of our experimental data into account, reduced mismatched DNA binding was caused by deficiencies in mismatch repair induced by the Y145H, A272T and I475V mutations, and changes in the oligomerization of the MutS protein in the T838I mutant likely led to malfunction of the mismatch repair system. The relationship between the structure and the function of the MutS protein is discussed below.

Residue Y145 is located in a conserved β-sheet within the protein. The substitution of tyrosine with an alkaline histidine might change the polarity of the β-sheet, thus reducing mismatched DNA binding function. The D167H mutation of hMSH2 in HNPCC families, corresponding to residue D149 in *E. coli*, results in reduced mismatch binding activity [7]. Residues Y145 and D149 in *E. coli* are located in the same β-sheet. Our data suggest that this β-sheet is important for the binding of MutS to mismatched DNA and that this function is conserved between different species. These findings indicate that using random mutation to discover new functionally defective mutants of MutS is a feasible method for uncovering potential mutations in hMSH2 that are related to disease.

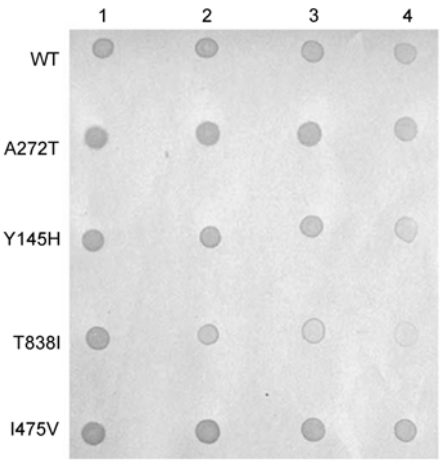


**Figure 2** Far-Western analysis of the interactions between wild type or mutant MutS and the MutL-SBP protein. Colored spots indicate positive reactions. Columns 1–4 represent the concentration of wild type or mutant MutS protein: 5, 2.5, 1.25 μmol $L^{-1}$and 650 nmol $L^{-1}$, respectively.

According to the crystal structure of MutS, residue A272 is situated distantly from the mismatch binding sites and it is therefore unlikely that this residue is directly involved in binding DNA [14,15]. However, mutant A272T completely lost its ability to bind both matched and mismatched DNA, and exhibited impaired mismatch repair. The importance of the A272 site supports the idea that random mutation can be employed to discover novel functional sites that might not be detected by site-directed mutagenesis studies.

Residue I475 is located within the four antiparallel β-strands of the MutS protein which contact the backbone of the mismatched DNA in a sequence-independent manner [14]. Though the effect of substitution of isoleucine to valine on structure was subtle, the I475V mutation resulted in a marked reduction in DNA binding and led to defects in mismatch repair.

It has been reported that the dimer-forming MutS protein (CF/D835R) is impaired in the mismatch-dependent activation of MutH [3]. However, other evidence suggests that a chromosomal MutS truncation mutation eliminating the dimerization/tetramerization domain eliminates mismatch repair, whereas the tetramer-disrupting MutS D835R and R840E mutations only modestly affect MutS function [2]. Mutant T838I obtained in this study not only disrupted tetramer formation but also showed impaired mismatch repair, demonstrating the importance of MutS tetramerization. Moreover, the T838I mutant showed slightly weaker MutL binding activity, possibly affecting the efficiency of mismatch repair. Further studies are required to fully address the effect of this mutation.

In conclusion, we have established a random mutation method for studying the mismatch repair function of MutS. The novel functional sites of MutS identified in this study provide new insight into the function of mismatch binding and oligomerization of MutS in mismatch repair.

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