Discrete *in vivo* roles for the MutL homologs Mlh2p and Mlh3p in the removal of frameshift intermediates in budding yeast

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The DNA mismatch repair machinery is involved in the correction of a wide variety of mutational intermediates. In bacterial cells, homodimers of the MutS protein bind mismatches and MutL homodimers couple mismatch recognition to downstream processing steps [1]. Eukaryotes possess multiple MutS and MutL homologs that form discrete, heterodimeric complexes with specific mismatch recognition and repair properties. In yeast, there are six MutS (Msh1–6p) and four MutL (Mlh1–3p and Pms1p) family members [2,3]. Heterodimers comprising Msh2p and Msh3p or Msh2p and Msh6p recognize mismatches in nuclear DNA [4,5] and the subsequent processing steps most often involve a Mlh1p–Pms1p heterodimer [6,7]. Mlh1p also forms heterodimeric complexes with Mlh2p and Mlh3p [8], and a minor role for Mlh3p in nuclear mismatch repair has been reported [9]. No mismatch repair function has yet been assigned to the fourth yeast MutL homolog, Mlh2p, although *mlh2* mutants exhibit weak resistance to some DNA damaging agents [10]. We have used two frameshift reversion assays to examine the roles of the yeast Mlh2 and Mlh3 proteins *in vivo*. This analysis demonstrates, results implicating Mlh3p in mismatch repair.

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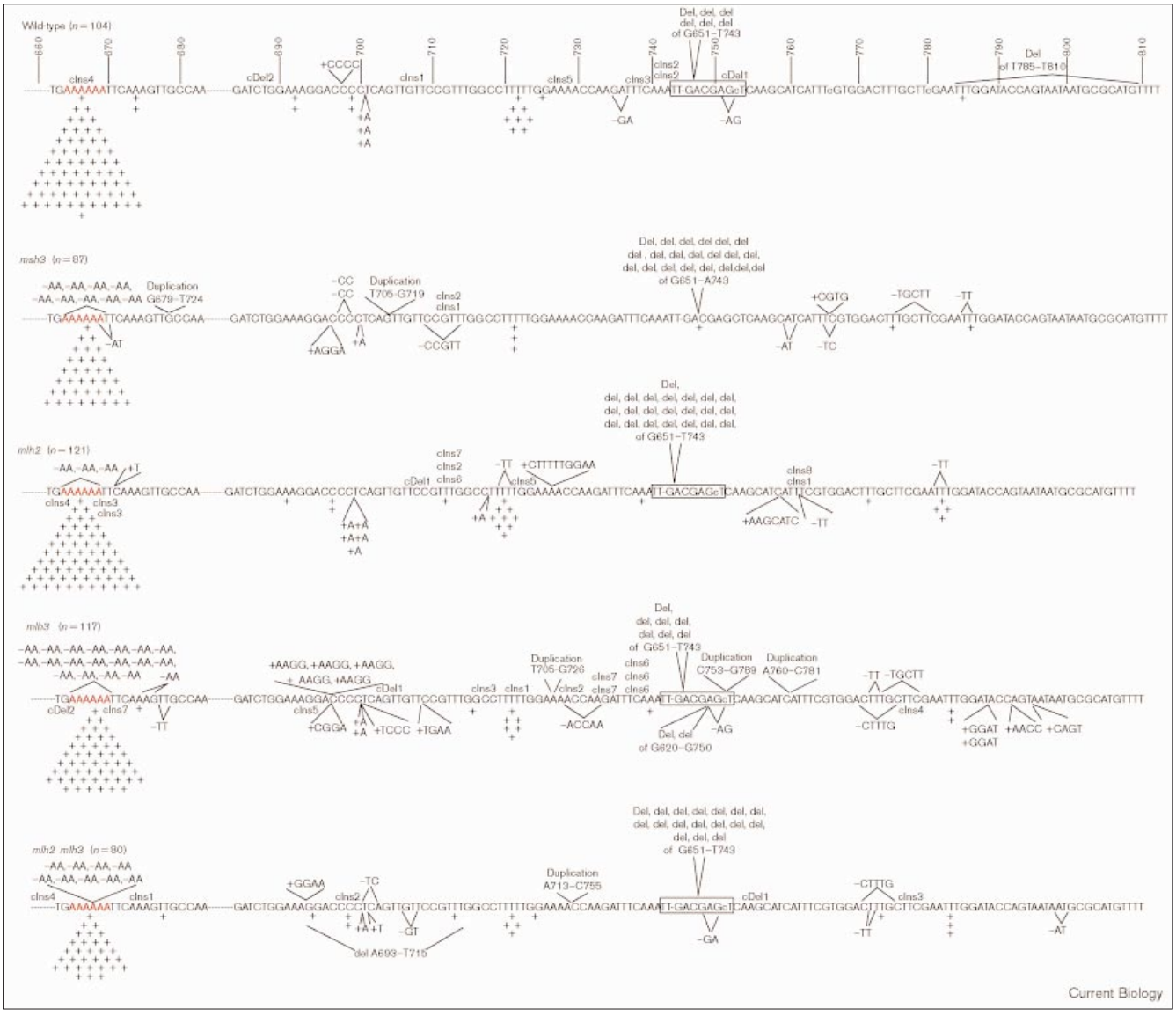
Results and discussion

We previously described a frameshift reversion assay that specifically detects +1 frameshift mutations at the yeast *LYS2* locus [11]. The +1 assay is based on reversion of the –1 frameshift allele *lys2ΔA746*, which is constrained to occur in an approximately 150 base pair (bp) window surrounding the original mutation. In a wild-type strain, 85% of reversion events were simple 1 bp insertions, with the majority occurring in a mononucleotide run of six adenines (Figure 1). Although deletion of *MSH3* did not alter the *lys2ΔA746* reversion rate, two distinct classes of frameshift events were elevated in an *msh3Δ* strain, but not in the other mismatch-repair-defective strains analyzed (*msh2Δ*, *msh6Δ*, *pms1Δ* and *mlh1Δ* strains; [11]). First, 2 bp deletions increased from 2% of the total frameshift events in a wild-type strain to 17% of the total events in an *msh3Δ* mutant (Figure 1 and see Supplementary material). The vast majority of these 2 bp deletions were in the mononucleotide run of six adenines. The occurrence of a large deletion with endpoints in 10 bp direct repeats was also elevated, increasing from 7% of the total frameshift events in a wild-type strain to 24% of the total events in an *msh3Δ* mutant (Figure 1 and see Supplementary material). Because the yeast MutS homologs function in a multiprotein complex with MutL homologs [7,12,13], the increased occurrence of specific classes of frameshifts in the *msh3Δ* strain suggested that specific MutL homologs might also be involved in removing the novel mutational intermediates. We therefore examined *lys2ΔA746* reversion rates and spectra in *mlh2Δ* and *mlh3Δ* mutants.

in the reversion rate of the *lys2ΔA746* allele was detected (see Supplementary material). Because *msh3Δ* and *msh6Δ* single mutants typically exhibit a weak mutator phenotype in frameshift assays [4,5,11,14,15], we also examined the mutator phenotypes of *mlh2Δ msh3Δ*, *mlh3Δ msh3Δ*, *mlh2Δ msh6Δ* and *mlh3Δ msh6Δ* double mutants for possible synergistic effects. Relative to the *msh3Δ* and *msh6Δ* single mutants, no increase in the reversion rate of the *lys2ΔA746* allele was evident in any of the double mutants (data not shown). Although the *mlh2Δ* and *mlh3Δ* mutants did not exhibit an increase in reversion rate, we did observe a striking difference in the reversion spectrum of the *lys2ΔA746* allele (Figure 1 and see Supplementary material). In *mlh2Δ* cells, relative to wild-type cells, there was a significant, 2.7-fold increase (from 7% to 18% of the total events) in the large deletion class of frameshift events. The proportion of reversion events in the large deletion class was statistically the same in the *mlh2Δ* mutant as in the *msh3Δ* mutant. In *mlh3Δ* cells the opposite pattern was observed, with the –2 bp frameshift class exhibiting a significant, 10-fold increase (from 2% of the total in wild-type cells to 19% of the total in *mlh3Δ* cells) and the large deletion class remaining unchanged. The proportion of –2 events in the *msh3Δ* and *mlh3Δ* mutants was the same. In the *mlh2Δ mlh3Δ* double mutant, the reversion spectrum was similar to what would be expected if

Figure 1



Sequence spectra of *lys2ΔA746* events in wild-type and mismatch-repair-defective strains. The sequence of the entire +1 assay reversion window is shown; nucleotides are numbered from the *Xba*I site upstream of the *LYS2* gene and the mononucleotide run of six adenines is shown in red. Nucleotide 746 was deleted and three nucleotides were changed (A767C, T781C, and T753C, lower case letters; see [11]) to create the +1 assay system. Each single base pair insertion within a mononucleotide run is indicated by a plus sign below the run; insertions into nonrepeated sequence are indicated by a plus sign followed by the inserted nucleotide(s). The location of deletion events that occurred between two perfect direct repeats are indicated by 'del'; duplications and the end points of the deletions and duplications are also shown. One copy of the 10 bp direct repeat at the endpoints of the 94 bp deletion is boxed; the second 10 bp direct repeat lies 5′ of the reversion window and is not shown. The deletion in the wild-type spectrum extending from T785 to T810 has 4 bp direct repeats (TTTG) at its ends; the G of the second repeat is outside of the reversion window. The locations of complex events, which are defined as insertion or deletion events accompanied by a base substitution are denoted by cIns and cDel, respectively, with numbers referring to distinct complex events. The sequence of complex events obtained in *mlh2Δ*, *mlh3Δ* and *mlh2Δ mlh3Δ* strains are available upon request. The reversion spectra for the wild-type and *msh3Δ* cells are reproduced from [11].

the mutational spectra of the respective single mutants were combined, and closely resembled the *msh3Δ* spectrum.

Because the large deletions characteristic of the *mlh2Δ* strain and the –2 bp events characteristic of the *mlh3Δ* strain were also increased in an *msh3Δ* mutant, we suggest that both Mlh2p and Mlh3p function within an Msh3p-containing complex to repair the corresponding frameshift intermediates. We further suggest that Mlh2p and Mlh3p each function as a heterodimer with Mlh1p to effect

Msh3p-dependent mismatch repair. This latter point is made on the basis of the observed physical interactions between Mlh1p and either Mlh2p or Mlh3p [8,9]. Flores-Rozas and Kolodner [9] previously suggested that an Mlh1p–Mlh3p heterodimer functions with Msh3p to correct certain types of frameshift intermediates. Using the *hom3–10* and *lys2ΔBgl* frameshift reversion assays, these investigators found that –1 events in short mononucleotide runs were specifically elevated in *mlh3* mutants. Our results support the observations of Flores-Rozas and Kolodner by confirming a role for Mlh3p in mutation avoidance, and expand upon their results by demonstrating, for the first time, a role for Mlh2p in mitotic mismatch repair. These results underscore the importance of using different mutation assays to assess gene function, as roles detected with some assays are not evident with others. Furthermore, although the results reported to date have provided no evidence of interaction between Msh6p and either Mlh2p or Mlh3p, other assays might detect such an interaction.

Table 1

Reversion rates of *lys2* alleles containing 10-nucleotide runs.

Run type	Strain/genotype	Reversion rate
10G	Wild type	5.3×10^{-8} (1.0)
	mlh2Δ	2.1×10^{-7} (4.0)
	mlh3Δ	1.1×10^{-6} (21)
10C	Wild type	8.5×10^{-8} (1.0)
	mlh2Δ	7.9×10^{-7} (9.3)
	mlh3Δ	1.3×10^{-6} (15)
10T	Wild type	2.1×10^{-8} (1.0)
	mlh2Δ	5.4×10^{-8} (2.6)
	mlh3Δ	2.7×10^{-7} (13)

For each type of run, the fold increase in mutation rate relative to the wild-type strain is shown in parentheses.

Mononucleotide runs are known hotspots for polymerase slippage events that generate frameshift mutations, and the mismatch repair machinery appears to be the predominant pathway used to correct slippage events at mononucleotide runs in yeast [14,16]. As originally noted by Tran *et al*. [16], the extreme instability of long mononucleotide runs provides a very sensitive system that can be used to detect weak mutator phenotypes. To test whether Mlh2p or Mlh3p is involved in correcting frameshift intermediates in long mononucleotide runs, we created artificial runs of either ten guanines (10G), ten cytosines (10C) or ten thymines (10T) in the same region of *LYS2* as used above for analyzing +1 frameshift events. The addition of ten nucleotides to the *LYS2* coding sequence is the equivalent of a +1 frameshift event, which is most easily 'reverted' by a compensatory loss of a single nucleotide. Given the length of the runs and the small size of the possible reversion window, one would predict that the majority of reversion events should occur within the run [16]. DNA sequence analysis of at least nine revertants from each of the strains listed in Table 1 confirmed this prediction, with > 98% (148/150) of reversion events being loss of a single nucleotide from the relevant mononucleotide run. The use of 10G, 10C, and 10T runs allowed us to assay the impact, if any, of run composition on DNA polymerase slippage and subsequent mismatch repair.

In the absence of Mlh2p, we observed four-, nine-, and three-fold increases in the reversion rates of *lys2* alleles containing the 10G, 10C and 10T runs, respectively (Table 1). In *mlh3Δ* strains, reversion rates of *lys2* alleles containing 10G, 10C and 10T runs were elevated 21-, 15-, and 13-fold, respectively (Table 1). Because of the comparable reversion rate increases obtained using different runs, the composition of the run does not appear to greatly affect the ability of either Mlh2p or Mlh3p to correct polymerase slippage errors. We also examined mononucleotide run stabilities in double mutant *mlh2Δ mlh3Δ* strains, and the reversion rates were similar to those in the single mutants (data not shown). Although there were reproducible increases in mononucleotide run instability in *mlh2* or *mlh3* strains, the increases represent only a small fraction of the increases observed when mismatch repair is completely eliminated. Disruption of *MSH2* resulted in an approximately 10,000-fold increase in the reversion rates of *lys2* alleles containing the 10G, 10C or 10T run (data not shown). Use of the extremely sensitive mononucleotide run assays thus has allowed us to identify very subtle contributions of both Mlh2p and Mlh3p to the removal of –1 frameshift intermediates.

A wide variety of frameshift intermediates have the potential to form in yeast and these aberrant DNA structures, if not properly corrected, result in many different types of mutations. The data presented in this report suggest newly identified functions for the MutL homologs Mlh2p and Mlh3p in the correction of frameshift intermediates, presumably in concert with the MutS family member Msh3p. At present, it is not known whether the MutS complex (Msh2p–Msh3p or Msh2p–Msh6p) alone recognizes mismatches and then is joined by a MutL complex (Mlh1p–Pms1p, Mlh1p–Mlh2p or Mlh1p–Mlh3p), or whether the primary recognition complex contains both the MutS and MutL proteins. In the former case, the unique MutS conformation conferred by interaction with specific types of mismatches might determine which MutL complexes are recruited to effect repair. In the latter case, the presence of specific MutL homologs before mismatch binding might alter the mismatch recognition specificity of a particular MutS complex. Although the major role of the mismatch repair machinery is presumably in the correction of mutational intermediates that arise during

DNA replication in S phase, the mismatch repair machinery might also play a critical role in the removal of intermediates that arise as a result of DNA damage or repair. Indeed, yeast gene expression studies indicate that only the *PMS1* mRNA exhibits cell cycle regulation [17,18], thus raising the possibility that different MutL complexes might exist at different points in the cell cycle.

Materials and methods

Mutation rates and spectra

Yeast strains were grown as described previously [11]. Reversion rates to lysine prototrophy were determined by the method of the median [19], using data from 12–24 cultures of each strain. Automated DNA sequence analysis of PCR-amplified genomic fragments was performed as described previously [11].

Introduction of mononucleotide runs into the LYS2 *locus*

A mononucleotide run of either 10 guanines (10G run with guanines on the coding strand), 10 cytosines (10C run) or 10 thymines (10T run) was inserted into a unique *Pfl*MI site ($CCAN_4\downarrow NTGG$; nucleotide 686 relative to the upstream *Xba*I site) located within the region of *LYS2* used in previous frameshift reversion analyses [11,15]. Each run was introduced by annealing two complementary oligonucleotides containing *Pfl*MI-compatible 'sticky' ends, thus allowing directional cloning into the *Pfl*MI site. The oligonucleotides used were: 10C, 5′ACCCCCCCCCCACGAT and 5′GTGGGGGGGGGGTATC; 10G, 5′AGGGGGGGGGGACGAT and 5′GTCCCCCCCCCCTATC; and 10T, 5′ATTTTTTTTTTACGAT and 5′GTAAAAAAAAAATATC. The annealed oligonucleotides were ligated into *Pfl*MI-digested pSR531, a *HIS3* integrating vector containing *LYS2* sequences. The resulting plasmids were used to introduce each run into strain SJR195 (*MATα ade2–101 his3Δ200 ura3ΔNco*) by two-step replacement.

Construction of mismatch-repair-defective strains

MSH2 and *MSH3* were disrupted as described previously [15]. Disruption of *MLH3* was accomplished by replacing base pairs +1 to +2097 of the *MLH3* open reading frame with the bacterial *kan* gene using PCR-mediated gene disruption [20]. *MLH2* was disrupted using a *Hin*dIII fragment from plasmid pmlh2::LEU2 (obtained from M. Liskay), which deletes most of the *MLH2* coding region including the ATG start codon. The *mlh2 mlh3* double mutants were created by disrupting *MLH3* in an *mlh2* strain. All disruptions were confirmed using PCR.

Supplementary material

A table showing the reversion rates of the *lys2ΔA746* allele in wild-type and the mutant yeast strains is available at http://current-biology.com/supmat/supmatin.htm.

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