Repair of Base-Base Mismatches and Four-Base Loops Formed During
Meiotic Recombination in *S. cerevisiae*

Jana E. Stone

A dissertation submitted to faculty of the University of North Carolina at Chapel
Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy
in the Curriculum in Genetics and Molecular Biology

Chapel Hill

2006

Approved by:

Thomas D. Petes

Patricia J. Pukkila

Dale A. Ramsden

Gwendolyn B. Sancar

Jeff J. Sekelsky

ABSTRACT

Jana E. Stone
Repair of Base-Base Mismatches and Four-Base Loops Formed During
Meiotic Recombination in *S. cerevisiae*
(under the direction of Thomas D. Petes)

DNA mismatches are generated when heteroduplexes formed during

recombination involve DNA strands that are not completely complementary. I

used tetrad analysis in *Saccharomyces cerevisiae* to examine the meiotic repair

of a base-base mismatch and a four-base loop in a wild-type strain and in strains

with mutations in genes implicated in DNA mismatch repair. Efficient repair of

the base-base mismatch required Msh2p, Msh6p, Mlh1p, and Pms1p, but not

Msh3p, Msh4p, Msh5p, Mlh2p, Mlh3p, Exo1p, Rad1p, Rad27p, or the

proofreading exonuclease of DNA polymerase δ. Efficient repair of the four-base

loop required Msh2p, Msh3p, Mlh1p, and Pms1p, but not Msh4p, Msh5p, Msh6p,

Mlh2p, Mlh3p, Exo1p, Rad1p, Rad27p, or the proofreading exonuclease of DNA

polymerase δ. I find evidence that a novel Mlh1p-independent complex

competes with an Mlh1p-dependent complex for the repair of a four-base loop. I

also found that the frequency and position of local double-strand DNA breaks

affect the ratio of mismatch repair events that lead to gene conversion versus

restoration of Mendelian segregation.

Mutations in *POL30*, which encodes PCNA (*p*roliferating *c*ell *n*uclear *a*ntigen)

in *S. cerevisiae*, increase the rate of mutations in vegetative cells.

Collaborators found that mitotic recombination between homeologous sequences is slightly elevated in *pol30-52* and *pol30-201* strains. I showed that repair of base-base mismatches generated during meiotic recombination is decreased in both *pol30* mutants; however, the repair defect associated with the hypomorphic *pol30* mutants is not as severe as that observed when *MSH2* is deleted. Aberrant segregation and crossovers are also decreased in the *pol30* mutants.

To address the role of ATP binding/hydrolysis in MMR-related processes, I examined mutations known to compromise the ATPase activity of Pms1p and Mlh1p. The results of these analyses confirm a differential requirement for the Pms1p ATPase activity in replication *vs.* recombination processes, while demonstrating that the Mlh1p ATPase activity is important for all examined MMR-related functions.

ACKNOWLEDGEMENTS

First of all, I'd like to thank my advisor, Tom Petes, for his ongoing guidance, support, insight, and impatience. His enthusiasm for science has been a daily inspiration. Thanks to all of the members of the Petes lab, past and present, for being so generous with their knowledge and encouragement. Special thanks to Francene Lemoine and Anne Casper, for helpful comments on this dissertation and for the requisite four laughs a day. Thanks also to the members of my committee, Pat Pukkila, Dale Ramsden, Gwen Sancar, and Jeff Sekelsky, for their time and support.

I have been very lucky to have a number of friends who have cheered me along the way. Thanks to all of them, they know who they are. Many, many thanks to Rebecca, Heather, and Gwyn for always lending me an ear when I needed it most.

Finally, I thank my family for absolutely everything, most especially my mother, for being both a great role model and one of my best friends.

TABLE OF CONTENTS

Page

ix

CHAPTER I. GENERAL INTRODUCTION

The DNA mismatch repair (MMR) proteins have a number of functions that are important for maintaining genomic stability (reviewed by Harfe & Jinks-Robertson 2000a). First, MMR corrects errors made during DNA replication. This function of MMR, known as the "spellchecker" activity, is responsible for removing misincorporated bases from the newly-synthesized DNA strand. Mutations that eliminate this function result in a global mutator phenotype and, in mammals, are associated with certain types of colorectal cancers. A second function of the MMR machinery is to recognize mismatches created during recombination between DNA strands that are not completely identical. This function, known as the "anti-recombination" activity, forces the dissolution of the recombination event. Third, MMR is responsible for repairing mismatches generated during meiotic recombination, and this repair generates gene conversion events. Fourth, some MMR-related proteins are involved in the processing of meiotic recombination intermediates to generate crossovers. Finally, in mammalian cells, the MMR proteins are involved in the DNA damage checkpoint (reviewed by Stojic *et al.* 2004); no similar role for the MMR proteins has been described in yeast.

A. Repair of replication errors – the "spellchecker" function

   1. Replication fidelity. If replication errors are not repaired, mutations will be created during the next round of cell division. DNA polymerases commonly make two types of replication errors: nucleotide misincorporation and insertion/deletion errors. Nucleotide misincorporation can occur either when an incorrect base is inserted opposite the template strand or when the template base is chemically damaged. Insertion/deletion errors can occur when a DNA polymerase "slips" off of its template and the DNA strands are subsequently reannealed incorrectly (Levinson & Gutman 1987, Trinh & Sinden 1991). Such slippage events most often occur during the replication of microsatellites and minisatellites. The rate of base misincorporation by the replicative DNA polymerase in the absence of correction systems is about one in 10^{-4} to 10^{-5} base pairs. However, these mistakes are usually corrected by the proofreading exonuclease activities of the replicative DNA polymerases δ and ϵ (reviewed by Kunkel 2004). This proofreading activity improves the fidelity of DNA replication to a level of one error in 10^{-7} base pairs. The MMR machinery is responsible for repairing mismatches that escape proofreading. The combined, protective functions of proofreading and MMR make mutations rare events, occurring at a rate of one in 10^{-9} to 10^{-10} base pairs per cell division.

   2. The *E. coli* paradigm for mismatch repair. *In vitro* and *in vivo* studies of MMR in *E. coli* have revealed the steps involved in the repair process and the proteins required to catalyze each of these steps (Modrich 1991, Iyer *et al.* 2006). Repair of replication errors occurs in the following steps: (1) the recognition of the mismatch, (2) the identification of the newly-synthesized strand (strand

discrimination), (3) the nicking of the identified newly-synthesized strand, (4) the

excision of the DNA between the nick and the mismatch, and (5) the repair of the

resulting gap by DNA synthesis, followed by ligation (Figure 1.1). In *E. coli*, MMR is

initiated when a MutS homodimer binds to a mismatch. The signal that distinguishes

the newly-synthesized strand from the template strand is DNA methylation of the

adenine in GATC sequences (Fig. 1.1). Since there is a lag between DNA

replication and DNA methylation, the newly-synthesized strand is transiently

unmethylated. MutH binds to hemi-methylated GATC sites. Next, a MutL

homodimer mediates the association between MutS and MutH in an ATP-dependent

manner. This association stimulates MutH to nick the newly-synthesized, or non-

methylated, strand. The resulting nick then provides the site where a helicase

(UvrD) is loaded onto the DNA by MutL. After the DNA is unwound by this helicase,

the nicked strand is then removed either by a 5' to 3' exonuclease (exonuclease VII

or RecJ) or by a 3' to 5' exonuclease (exonuclease I or X), depending on the

orientation of the nick relative to the mismatch. DNA synthesis across this excised

region by the replicative DNA polymerase and the subsequent ligation completes the

repair process.

   3. **Eukaryotic mismatch repair differs from the *E. coli* paradigm.** Although

the *E. coli* MMR paradigm has been useful for understanding some aspects of MMR

in eukaryotes, there are a number of differences between MMR in *E. coli* and

eukaryotes. First, all eukaryotes analyzed thus far have multiple MutS and MutL

homologues, but no MutH homologues (Table 1.1). Second, physical studies have
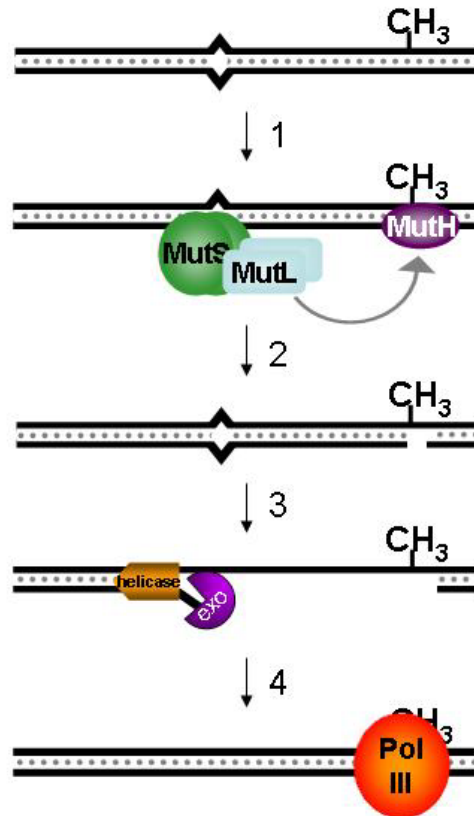
FIGURE 1.1. Repair of replication errors in *E. coli*. A mismatch reflecting a misincorporated base is shown. In *E. coli*, adenine residues are methylated in the GATC motif. After DNA synthesis, the newly-synthesized strand is transiently unmethylated. This lack of methylation is used as a signal for the removal of the misincorporated base. MMR is initiated when MutS binds the mismatch and MutH binds to hemimethylated sites (step 1). MutL brings MutS and MutH together and stimulates MutH to make a single-strand break on the unmethylated, newly-synthesized strand (step 2). The MutH-catalyzed incision site can be either 5' or 3' of the mismatch. The DNA between the incision site and the mismatch is then unwound and excised, using the appropriate combination of a helicase and an exonuclease (step 3). Finally, DNA replication and ligation fill in the gap to complete the repair process (step 4).

4

TABLE 1.1. **MutS and MutL homologues from eukaryotic model organisms**

E. coli	S. cerevisiae	Human	C. elegans	D. melanogaster	A. thaliana
MutS	Msh1	hMsh1	N.I.	N.I.	MSH1
	Msh2	hMsh2	MSH2	SPEL1	MSH2
	Msh3	hMsh3	N.I.	N.I.	MSH3
	Msh4	hMsh4	MSH4	N.I.	MSH4
	Msh5	hMsh5	MSH5	N.I.	N.I.
	Msh6	hMsh6	MSH6	MSH6	MSH6
					MSH7
MutL	Pms1	hPms2	PMS1	PMS2	PMS2
	Mlh1	hMlh1	MLH1	MLH1	MLH1
	Mlh2	hPms1	N.I.	N.I.	N.I.
	Mlh3	hMlh3	Ced H12	N.I.	MLH3

N.I. indicates none identified.

demonstrated that MutS homologues function as heterodimers, as do MutL

homologues. Each set of heterodimers is thought to recognize a specific substrate

(Fig 1.2). Third, the signal by which eukaryotes discriminate between the template

and the newly-synthesized strands is not understood, as eukaryotes do not have a

MutH homologue and eukaryotes do not use methylated DNA as the strand

discrimination signal. It has been proposed that eukaryotic strand discrimination

involves the recognition of DNA nicks created during DNA replication. Such

recognition may be facilitated by the known interactions between the DNA replication

and MMR machines. As observed for *E. coli* MMR, eukaryotic MMR can be directed

in vitro by a nick located either 5' or 3' to the mismatch. Finally, the exonucleases

required for the excision step of MMR in eukaryotes have not been unambiguously

identified, although it is likely that Exo1p is involved (Umar *et al.* 1996).

    4. Genetic assays for the spellchecker function in yeast. Three types of

genetic assays are used to examine yeast MMR *in vivo*: assays of forward mutation

rates, reversions, and microsatellite instability (Sia *et al.* 1997a, Harfe & Jinks-

Robertson 2000b). Forward mutation rate assays detect any mutation that

eliminates the function of a specific gene, while reversion and microsatellite

instability assays detect only specific types of mutations. The most commonly used

forward mutation rate assay involves selecting for resistance to the arginine

analogue canavanine. This resistance is acquired through mutations in the *CAN1*

gene, which encodes an arginine permease (for examples see Marsischky *et al.*

1996, Kokoska *et al.* 2000).

Reversion rate assays are used to detect a specific type of mutations. For

example, the *cyc1* system can be used to detect single base substitutions. The wild-

type Cyc1p has a cysteine at position 22, encoded by TGC. A cysteine at this

position is essential for function of the protein. Thus, a mutant allele containing the

codon CGC at this position can revert only by a change from C to T or from G to A

(Earley & Crouse 1998). Other reversion assays detect frameshift mutations (for

examples see New *et al.* 1993, Tran *et al.* 2001). For example, the *hom3-10* allele

has a single base addition in a short mononucleotide run. Thus, revertants

(detected as Thr[+]) colonies usually reflect a one bp deletion. A second assay

involves strains that contain frameshift mutations in the *lys2* gene. Lys[+] revertants

have compensating frameshifts (Harfe & Jinks-Robertson 1999). Finally, in-frame

insertions of microsatellite sequences have been inserted into the *URA3* gene.

Alterations that result in loss of the correct reading frame can be selected by plating

cells on medium containing 5-fluoro-orotate (Sia *et al.* 1997b).

5. Yeast mismatch repair proteins.

MutS homologues: In *S. cerevisiae*, six MutS homologues (*MSH1-6*) and four

MutL homologues (*MLH1-3* and *PMS1)* have been identified through various genetic

and genomic assays (Harfe & Jinks-Robertson 2000b). The MutS homologues

function as heterodimers, and each heterodimer acts on a specific set of substrates

during MMR processes (Fig 1.2). Of the six MutS homologues, only Msh2p, Msh3p,

and Msh6p are involved in the MMR spellchecker function. Msh4p and Msh5p lack

the domain necessary to recognize mismatches, and are instead involved in
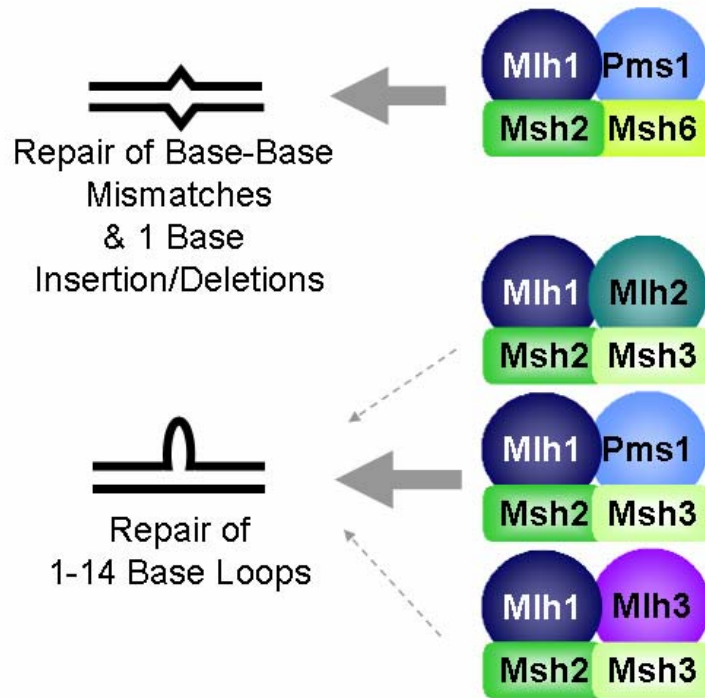
FIGURE 1.2. Substrates specificities of yeast MutS and MutL homologues in the spellchecker function of MMR. The relative contributions of each complex to the repair of base-base mismatches and small (1-14 bp) loops are indicated by the size of the arrow. Msh2p/Msh6p/Mlh1p/Pms1p is involved in MMR of base-base mismatches. Msh2p/Msh3p/Mlh1p/Pms1p repairs the majority of small loops. Msh2p/Msh3p/Mlh1p/Mlh2p and Msh2p/Msh3p/Mlh1p/Mlh2p are involved in the repair of a small fraction of small loops resulting from DNA polymerase slippage in homopolymeric nucleotide runs.

processing of meiotic recombination intermediates that are resolved to generate crossover events (discussed below in section I.C.). Msh1p is only involved in maintaining the stability of the mitochondrial DNA (Reenan & Kolodner 1992, Sia & Kirkpatrick 2005). Because Msh1p has no effect on the repair of nuclear DNA, it will not be discussed further in this dissertation.

In all forward mutation assays, the deletion of *MSH2* results in a strong mutator phenotype, whereas the effect of deletion of either *MSH3* or *MSH6* are more assay specific (Harfe & Jinks-Robertson 2000b). This finding suggests that Msh2p is required for the detection of all DNA mismatches, while Msh3p and Msh6p are required for the detection of only specific types of mismatches. Using the *cyc1* reversion assay which selects for single base mutations, the deletion of either *MSH2* or *MSH6* was shown to substantially increase the rate of base substitutions, while the deletion of *MSH3* had no effect (Earley & Crouse 1998). This finding suggests that MMR for base-base mutations requires both Msh2p and Msh6p, but not Msh3p. Subsequent analysis demonstrated that most base-base MMR required a heterodimer of Msh2p and Msh6p. Although Msh3p is not involved in the repair of base-base mismatches, it is likely that it can detect such mismatches because (as described below in section I.B.) Msh3p is involved in reducing recombination between genes that differ by base substitutions (Nicholson *et al.* 2000, see section I.B. below).

In reversion rate assays that measure single base insertions or deletions, both *msh3* and *msh6* mutations have weak mutator effects relative to the *msh2* mutation. However, *msh3 msh6* double mutants have a mutator effect similar to that of *msh2*

single mutants (Marsischky *et al.* 1996, Greene & Jinks-Robertson 1997, Harfe & Jinks-Robertson 1999). This finding suggests that Msh2p/Msh3p and Msh2p/Msh6p heterodimers compete for the repair of single base loops formed as a consequence of DNA polymerase slippage.

Sia *et al.* (1997b) systematically examined the effects of mutations in *MSH2, MSH3,* and *MSH6* on microsatellites in which the repeat units varied between one and twenty bp. For microsatellites with repeat units of one or two bp, mutants in all three genes led to increased instability. For repeat units of 4 bp or more, *msh6* had no effect, and *msh2* and *msh3* had equivalent effects. The simplest interpretation of this result is that DNA loops of one or two bases are repaired by two complexes, one containing Msh2p and Msh6p and one containing Msh2p and Msh3p. DNA loops greater than two bases are repaired exclusively by the Msh2p-Msh3p complex.

In vitro binding studies have confirmed that Msh2p/Msh6p heterodimers bind to all DNA base-base mismatches, although they have a weak affinity for C/C mismatches (Marsischky *et al.* 1996, Alani 1996); C/C mismatches are inefficiently repaired in both prokaryotes and eukaryotes (Fogel *et al.* 1981, White *et al.* 1985, Dohet *et al.* 1985, Detloff *et al.* 1991). Msh2p/Msh6p heterodimers can also recognize small DNA loops *in vitro*, with a much higher affinity for one-base loops than for larger (2-8 base) loops (Marsischky *et al.* 1996, Alani 1996, Marsischky & Kolodner 1999). Affinity of the Msh2p/MSh6p heterodimer for mismatches was found to be increased if other mismatches were within 18 bp (Marsischky & Kolodner 1999). Msh2p/Msh3p heterodimers can recognize small loops of up to about 10 bases *in vitro* (Habraken *et al.* 1996). The binding of the Msh2p/Msh3p heterodimer

to one-base loops confirms that the Msh2p/Msh3p heterodimer and the Msh2p/Msh6p heterodimer are functionally redundant with respect to recognition of one-base insertion/deletion errors (Marsischky *et al.* 1996).

MutL homologues: Four MutL homologues have been identified in *S. cerevisiae: MLH1*, *MLH2*, *MLH3*, and *PMS1.* Yeast two-hybrid and co-immunoprecipitation assays have demonstrated that, in vegetative cells, Mlh1p/Mlh2p, Mlh1p/Mlh3p, and Mlh1p/Pms1p heterodimers are formed (Wang *et al.* 1999). Deletion of either *MLH1* or *PMS1* results in the same mutator phenotype as deletion of *MSH2* (Kramer *et al.* 1989a, Greene & Jinks-Robertson 1997, Harfe & Jinks-Robertson 1999), indicating that the initiation of MMR by either Msh2p/Msh6 or by Msh2p/Msh3p also requires an Mlh1p/Pms1p heterodimer. Deletion of either *MLH2* or *MLH3* generally has no effect on the mutation rate assays, although weak phenotypes have been detected in assays that detect frameshifts in 7-10 bp homopolymeric runs (Flores-Rozas & Kolodner 1998, Harfe *et al.* 2000). Thus, Mlh1p/Mlh2p and Mlh1p/Mlh3p heterodimers are thought to act in concert with Msh2p/Msh3p heterodimers for the repair of some insertion/deletion errors, although the contributions of the Mlh1p/Mlh2p and Mlh1p/Mlh3p heterodimers are minor compared to that of Mlh1p/Pms1p heterodimers.

Protein alignments have placed MutL and its homologs in the GHL (gyrase b, Hsp90, MutL) family of ATPases (Dutta & Inouye 2000), a group of proteins that contain highly conserved amino acid motifs that mediate ATP-binding and hydrolysis. The crystal structure of MutL revealed that ATP-binding induces N-terminal conformational changes that allow for homodimerization (Ban *et al.* 1999).

Mlh1p and Pms1p have a similar ATP-dependent conformational cycle that mediates their heterodimerization (Tran & Liskay 2000). Additionally, the binding of ATP to Mlh1p and Pms1p has been shown to be essential for MMR (Hall *et al.* 2002). Interestingly, mutations in the ATPase domain of Mlh1p have a much stronger phenotype than comparable mutations in Pms1p (Tran & Liskay 2000). For example, two different *mlh1* ATPase mutants have approximately one-third of the effect of an *mlh1* null mutant in both *CAN1* forward mutation and *hom3-10* reversion rate assays. In contrast, two analogous *pms1* ATPase mutants have no effect on *CAN1* forward mutation rates and about one-hundredth of the effect of a *pms1* null mutant on the *hom3-10* reversion rate. Additional analysis revealed that when any of these *mlh1* or *pms1* ATPase mutations are combined with a deletion of *EXO1*, which encodes for one of the nucleases implicated in MMR, there is a synergistic effect on mutation rates in both assays (Tran *et al.* 2001). However, the ATPase-deficient Mlh1p retains the ability to interact with Exo1p (Tran *et al.* 2001). These data suggest that the ATP-dependent conformational changes of Mlh1p and Pms1p may be necessary for the coordination of a nuclease activity that is functionally redundant with Exo1p.

Nucleases: *In vitro* experiments using nicked substrates containing a mismatch have demonstrated that the excision step of eukaryotic MMR can be directed by a nick that is either 5' or 3' of the mismatch (Fang & Modrich 1993, Dzantiev *et al.* 2004, Constantin *et al.* 2005). These results suggest that both 5' to 3' and 3' to 5' exonucleases are involved in eukaryotic MMR. Of the numerous nucleases identified in yeast, only a few have been implicated as involved in MMR on the basis

of causing a mutator phenotype when inactivated. Only these MMR-associated

nucleases will be considered in this dissertation: Exo1p, Rad27p, Rad1p/Rad10p,

and the proofreading activity of DNA polymerases δ and ϵ (reviewed by Fleck *et al.*

1999). Mutations that inactivate these individual nucleases have a less extreme

mutator phenotype than *msh2* strains. It is likely, therefore, that the nuclease that is

involved in MMR is functionally redundant with other nucleases. Unfortunately,

strains with double mutants of the nucleases are often inviable (for example, *rad27*

pol3-01; Kokoska *et al.* 1998), making it difficult to systematically investigate this

redundancy.

   Exo1p is likely to be one of the nucleases involved in eukaryotic MMR. It has 5'

to 3' exonuclease and endonuclease activities and is a member of the *FEN1* family

of flap endonucleases (Tishkoff *et al.* 1997a). Exo1p was originally isolated in *S.*

cerevisiae based on its two-hybrid interaction with Msh2p, and it has subsequently

been shown to interact with Msh3p, and Mlh1p (Tishkoff *et al.* 1997a, Tran *et al.*

2001). Despite the known physical interactions between Exo1p and MMR proteins,

genetic evidence for a role of Exo1p in MMR is somewhat limited. The spectrum of

mutations observed in *exo1* mutants is different from that observed in either *msh2* or

mlh1 mutants; there is a bias for base substitution errors over frameshift errors in

exo1 strains, an effect similar to that observed in MMR-proficient strains (Tran *et al.*

2001). Also, the mutator phenotype of an *exo1* strain is much weaker than those of

either *msh2* or *mlh1* strains (Tishkoff *et al.* 1997a, Tran *et al.* 2001). This weak

mutator phenotype makes it difficult to determine whether *exo1* and *msh2* function in

the same repair pathway because additive effects are not easily distinguished from

13

epistasis. However, the *exo1* mutation has been found to be epistatic to mutations that affect the ATPase activities of Mlh1p and Pms1p (Tran *et al.* 2001). While Exo1p mutant strains do not display many of the phenotypes associated with MMR-deficiencies in yeast, human Exo1p has been shown to support nick-directed MMR *in vitro* (Dzantiev *et al.* 2004, Constantin *et al.* 2005). Yeast Exo1p also stimulates meiotic crossovers (discussed below in section I.C.), and has a role in telomere maintenance (Tran *et al.* 2004).

Rad27p is the yeast homologue of the human FEN1 5' to 3' flap endonuclease, that is responsible for the removal of the RNA tails from Okazaki fragments (Liu *et al.* 2004). Deletion of *RAD27* elevates forward mutation rates, although the spectrum of the mutations differs from the base substitutions and frameshift mutations that are typically observed in canonical MMR-deficient strains. The most frequent mutation in *rad27* strains is the duplication of sequences flanked by short, direct repeats (Tishkoff *et al.* 1997b). This type of mutation is not observed in MMR-deficient strains. Microsatellite instability has also been detected in *rad27* strains. The mutant strains have an elevated rate of insertions within the microsatellites, (Kokoska *et al.* 1998).

It has also been suggest that the 3' to 5' proofreading exonucleases associated with DNA polymerase δ and ϵ may have a role in MMR in yeast. Strains lacking the exonuclease activity of either of these polymerases have low mutation rates. However, there is a synergistic effect on the mutator phenotype when strains lack both Exo1p and one of the proofreading activities (Tran *et al.* 1999). One interpretation of this result is that Exo1p and the proofreading activities of DNA

polymerases δ and ϵ compete in the excision step of MMR. The proofreading exonuclease activity of DNA polymerase δ may also compete with Rad27p in mutation avoidance, as mutations that eliminate both of these nuclease activities are synthetically lethal (Kokoska *et al.* 1998).

Rad1p and Rad10p (yeast equivalents of human XPF and ERCC1) function together as the nuclease required for nucleotide excision repair (NER). The NER pathway is responsible for removing a wide variety of bulky lesions in DNA, such as UV-induced pyrimidine dimers. By two-hybrid analysis, Msh2p has been shown to interact with Rad10p as well as a number of other NER proteins (Bertrand *et al.* 1998). However, deletion of *RAD10* or any other NER gene has only a very weak effect on mutation rate as determined by either the forward or reversion rate assays (Bertrand *et al.* 1998). Therefore, it is difficult to determine if the *rad10* mutation is epistatic or additive to the *msh2* mutation. In *S. pombe*, an Msh2-independent NER pathway has been demonstrated to be involved in the removal of C/C mismatches created during meiotic recombination, but it is unknown if this activity is also plays a role in the repair of replication errors (Fleck *et al.* 1999).

It should be noted that recent evidence suggests that Pms1p (hPms2p) has a latent endonuclease activity in humans (Kadyrov *et al.* 2006) and yeast (P. Modrich and T. Kunkel labs, unpublished results). Previous studies have shown that the human Mlh1p/Pms2p heterodimer is required for the excision step in an *in vitro* nick-directed MMR assay when the nick is located 3' to a mismatch, but not when the nick is located 5' to a mismatch (Dzantiev *et al.* 2004, Constantin *et al.* 2005). Kadvrov *et al.* (2006) showed Pms2p is activated to make an additional incision on

the nicked strand, which usually located distal to the mismatch. This incision

occurred when the initial nick was located either 3' or 5' to the mismatch. The

subsequent excision of DNA between the nicks was carried out by the 5' to 3'

exonuclease activity of Exo1p. Since the endonuclease activity of human Pms2p

was found to require ATP-Mn^{2+} (Kadyrov *et al.* 2006), a mutation was made in yeast

PMS1 that was predicted to disrupt the interaction between Pms1p and the metal

ion. This mutation has the same effect on mutation rate as does the deletion of

PMS1, suggesting that the endonuclease activity of Pms1p is important for MMR *in*

vivo (P. Modrich and T. Kunkel labs, unpublished results). Interestingly, Mlh3p

contains a domain that is similar to the one mutated in the *pms1* endonuclease-

deficient allele described above. The effects of mutations of this domain have not

been examined.

   PCNA: As described above, it is not yet understood how eukaryotic cells

distinguish between the newly-synthesized and the template DNA strands. It has

been suggested that PCNA (<u>p</u>roliferating <u>c</u>ell <u>n</u>uclear <u>a</u>ntigen) may play a role in this

process. PCNA is the homotrimeric sliding clamp that promotes processive DNA

replication via its associations with DNA polymerases δ and ϵ. It is also a

multifunctional matchmaker protein involved in coordinating a number of DNA repair

processes, such as MMR, NER, base excision repair, and translesion DNA

synthesis (Maga & Hubscher 2003). PCNA was identified as an MMR-associated

factor because of its interactions with Msh2p and Mlh1p in yeast, as determined by

two-hybrid and co-IP assays (Umar *et al.* 1996, Johnson *et al.* 1996a, Gu *et al.*

1998). PCNA interacts with Msh2p/Msh3p and Msh2p/Msh6p heterodimers via

motifs located at the N-termini of Msh3p and Msh6p (Clark *et al.* 2000). These

motifs are characteristic of other known PCNA-interacting proteins. Mutations that

eliminate these motifs were shown to disrupt the physical interaction between Msh3p

or Msh6p and PCNA and to elevate mutation rates (Clark *et al.* 2000).

Mutations in *POL30* (the gene that encodes yeast PCNA) elevate forward

mutation rates, reversion rates, and microsatellite instability (Ayyagari *et al.* 1995,

Kokoska *et al.* 1999, Chen *et al.* 1999, Lau *et al.* 2002). Epistasis analyses of

mutator phenotypes suggest that PCNA functions in the same pathway as Msh2p

and Mlh1p for the repair of replication errors (Umar *et al.* 1996, Johnson *et al.*

1996a, Chen *et al.* 1999). Mutations in *pol30* have also been shown to result in a

variety of other phenotypes such as cold sensitivity and increased sensitivity to the

DNA damaging agents methyl methanesulfonate, ultraviolet light, and hydroxyurea

(Ayyagari *et al.* 1995, Lau *et al.* 2002). These data suggest that the *pol30* mutants

can cause defects in replication and checkpoint control, as well as defects in MMR.

These effects are somewhat separable, as *pol30* mutants have been isolated that

have defects in MMR but not replication (Lau *et al.* 2002).

Umar *et al*. (1996) demonstrated that PCNA affects two different steps in MMR,

one preceding the excision of the mismatch and another during resynthesis after the

mismatch has been excised. PCNA is also involved in the recognition of

mismatches, since PCNA increases the capacity of Msh2p/Msh6p heterodimers to

bind to mismatches and can transfer Msh2p/Msh6p heterodimers to mispaired DNA

(Lau & Kolodner 2003). Recent studies of human nick-directed MMR *in vitro* have

demonstrated another role for PCNA. It was found that PCNA was required for the

3' to 5' but not the 5' to 3' excision of the DNA between a nick and a mismatch (Dzantiev *et al.* 2004). Collectively, the research described above suggests that PCNA may be involved in the coordination of the entire MMR process.

B. Prevention of recombination between diverged sequences –

   the "anti-recombination" function of mismatch repair

Divergent sequences have reduced levels of recombination. For example, recombination rates between *E. coli* and *S. typhimurium*, which have about 20% DNA sequence divergence, recombine at rates 10^5-fold less than observed in intraspecies crosses (Rayssiguier *et al.* 1989). Recombination rates, however, are increased 1000-fold if either MutS or MutL are disrupted in the recipient *E. coli* strain (Rayssiguier *et al.* 1989). It is believed that this "anti-recombination" function of MMR does not destroy heteroduplex DNA, because plasmids containing 18% mismatches can be efficiently transformed into *E. coli* (Westmoreland *et al.* 1997). Instead, it is believed that these recombination events are either blocked or reversed in an MMR-dependent manner.

In bacteria, anti-recombination is clearly involved in preventing different species from exchanging genetic information. In yeast, this MMR function prevents recombination between diverged sequences during both mitosis and meiosis (reviewed by Harfe & Jinks-Robertson 2000b). The presence of even a single mismatch lowers the rate of mitotic recombination in a MMR-dependent manner, and the recombination rates decrease further as the number of mismatches increase

18

(Datta *et al.* 1997, Chen & Jinks-Robertson 1998). For example, sequences that are 25% non-identical have a 5000-fold lower mitotic recombination rate than do sequences that are 100% identical (Datta *et al.* 1997). In organisms, such as yeast, that have dispersed repeated genes with diverged DNA sequences, this anti-recombination is important in inhibiting the ectopic recombination events that would generate chromosome deletions, duplications, and translocations.

Msh2p/Msh6p and Msh2p/Msh3 heterodimers generally have the same substrate specificities for the spellchecker and the anti-recombination functions (Nicholson *et al.* 2000). Mlh1p and Pms1p are also involved in anti-recombination; however, the rate of recombination between diverged sequences is much higher in *msh2* mutants than in *mlh1* or *pms1* mutants (Chen & Jinks-Robertson 1999, Nicholson *et al.* 2000). This unequal effect suggests that mismatch binding alone is capable of activating the anti-recombination function to a certain extent. In addition to the MutS and MutL homologues, the Rad1p/Rad10p and Exo1p nucleases are also thought to be involved in anti-recombination (Nicholson *et al.* 2000).

C. Roles of mismatch repair proteins during meiosis

1. Introduction to meiosis. Meiosis is the complex cellular process by which sexually reproducing organisms generate haploid gametes from a diploid cell. As much of the machinery required for meiosis is conserved in all eukaryotes, insights gained from the study of meiosis in *S. cerevisiae* are likely to be applicable to higher eukaryotes. During meiosis, a single round of DNA replication is followed by two rounds of cell division, which results in the formation of four haploid cells. Prior to

the first meiotic division, chromosomes align and undergo meiotic crossing over.

Such crossovers create physical connections between the homologs that, in

combination with cohesins, are required for accurate chromosome segregation.

During the first cell division, homologs are segregated without separating the sister

chromatids. During the second cell division, these sister chromatids are separated.

    2. Meiotic recombination models. In most organisms, meiotic recombination

events are initiated by double-strand breaks that are catalyzed by Spo11p (Keeney

et al. 1997). Figure 1.3A illustrates a modified version of the canonical double-

strand break repair (DSBR) model of meiotic recombination (Szostak *et al.* 1983,

Sun *et al.* 1991). The DNA ends generated by Spo11p are resected 5' to 3', leaving

3'-ended, single-stranded tails. One of these 3' ends then invades the homologous

chromosome to form a region of heteroduplex DNA (a duplex derived by strands

from two different chromosomes). Evidence suggests that the initial strand invasion

step generates only a very short region of heteroduplex DNA, and that the length of

this region is not dependent on the extent of the resection (Merker *et al.* 2003). The

invading strand then primes DNA synthesis that further displaces the opposing

strand. This displaced strand then captures the second 3' end, thereby forming a

second region of heteroduplex DNA on the other homolog. Therefore, the resulting

molecule consists of two regions of heteroduplex DNA that flank the site of the

original DSB with Holliday junctions (HJs) on either side. Genetic evidence indicates

that the length of the heteroduplex DNA is much greater on one side of the original

DSB (Merker *et al.* 2003). Any sequence difference between the homologous

FIGURE 1.3. Meiotic recombination models.

A. A modified form of the double-strand break (DSB) repair model (Szostak *et al.* 1983, Sun *et al.* 1991) is illustrated. Recombination occurs between the two chromosomes (depicted in red and blue with 3' ends flagged) that differ by a heterozygous marker (alternative alleles represented by open and closed circles). In step 1, recombination is initiated by a DSB (indicated by large arrowhead) on the blue chromosome and the broken ends are then resected 5' to 3', leaving two single-stranded 3' ends. In step 2, one of the 3' ends invades the red chromosome, creating a short region of heteroduplex DNA. In step 3, the invading strand primes repair DNA synthesis (represented by a stippled line) that further displaces the red strand. This displaced "D loop" pairs with the right 3' end, creating a second region of heteroduplex DNA that contains a mismatch (represented by pairing of open and closed circles). Repair synthesis primed by the blue strand also occurs. In step 4, the HJs are resolved; by cutting the strands indicated by the small arrowheads, the recombination event can be resolved with (left) or without (right) an associated crossover. In step 5 and 5', the nicks created during HJ resolution are ligated.

B. Synthesis-dependant strand-annealing (SDSA) is illustrated. Steps 1 - 3 are the same as above, except that the D loop is not captured by the right 3' end. In step 4, the strand invasion is reversed. In step 5, repair synthesis fills in the remaining gap. SDSA always results in recombination events that are not associated with crossovers (step 6).
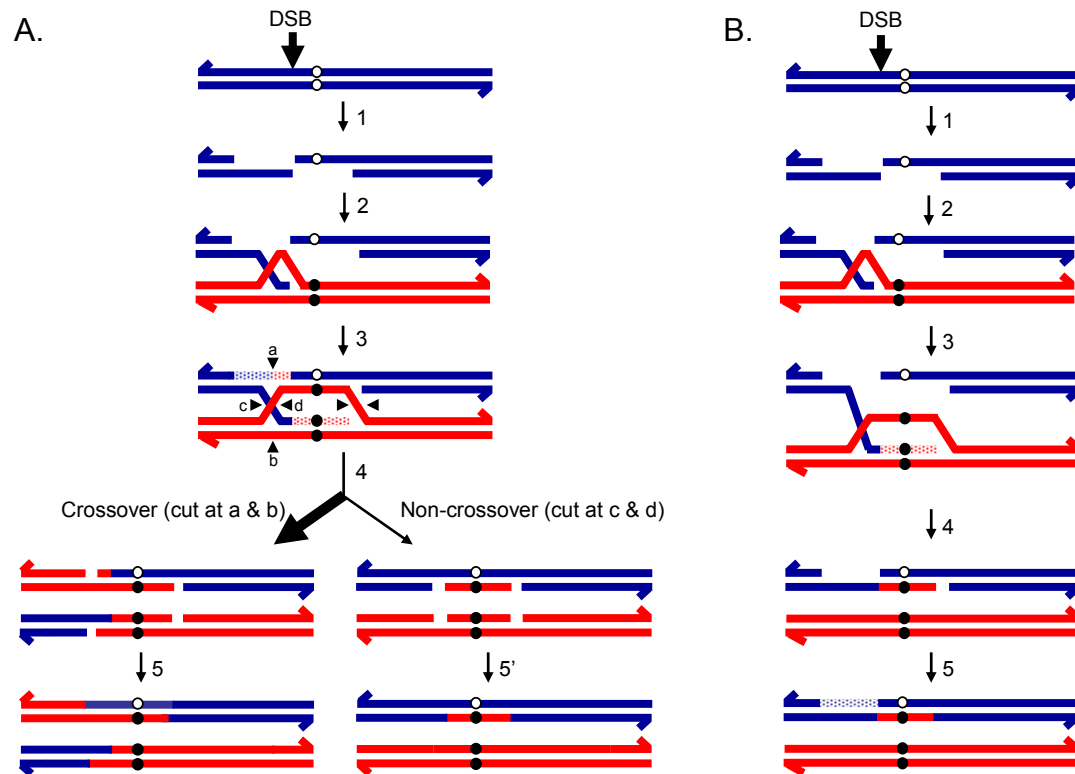
chromosomes in heteroduplex regions will result in DNA mismatches. Resolution of the two HJs that flank the heteroduplexes completes the recombination process and can result in an associated crossover, or not, depending on which strands are cut (Fig. 1.3A).

Genetic and physical evidence has indicated that not all meiotic recombination events proceed through the canonical DSBR pathway. Allers and Lichten (2001) provided physical evidence that heteroduplex products that give rise to noncrossovers are formed temporally before heteroduplexes that give rise to crossovers. This finding conflicts with the DSBR model that predicts that crossover and noncrossover products arise from the same heteroduplex intermediate. The authors suggest that early-forming heteroduplexes are the result of the synthesis-dependant strand-annealing (SDSA) pathway; such a pathway was originally proposed to explain mitotic recombination events that are not associated with crossovers (reviewed by Paques & Haber 1999). In the SDSA pathway, after strand invasion and repair synthesis have occurred, the invading strand disassociates and reanneals with the other 3' end (Fig. 1.3B). As a result, heteroduplex DNA is formed on only one side of the DSB, and the recombination event is resolved as a noncrossover. Merker *et al.* (2003) provided genetic evidence of SDSA events by demonstrating that recombination events that have heteroduplex DNA on only one side of the DSB are less often associated with crossovers than recombination events that have heteroduplex DNA on both sides of the DSB. Based on the rate with which such events were observed, they predicted that about two-thirds of recombination events are processed via the DSBR pathway and the remaining one-

third are processed via alternate pathways, including SDSA and break-induced replication.

   3. **Measuring repair of mismatches generated during meiotic recombination.** In *S. cerevisiae*, the four haploid products (spores) of a single meiosis are packaged together into a single tetrad, and each of these spores is a viable haploid organism. These features allow one to easily distinguish reciprocal and non-reciprocal recombination events. It is convenient to use heterozygous auxotrophic markers to study meiotic recombination in yeast, as the phenotypes of the spore colonies can be easily analyzed by replica plating onto appropriate omission media. For example, in our experiments, we frequently use strains that are heterozygous for mutations in the *HIS4* and *ARG4* genes, since these genes are required for the biosynthesis of histidine and arginine, respectively. These genes are particularly informative in studies of meiotic recombination because they are located near hotspots for meiosis-specific DSBs. The DSB site upstream of *HIS4* is the second hottest of the 6000 *S. cerevisiae* ORFs, while the DSB site upstream of *ARG4* is 134th hottest (Gerton *et al.* 2000).

   Figure 1.4 illustrates the possible segregation events resulting from sporulation of a diploid heterozygous for a mutation in the initiating codon of *HIS4*, resulting in a T to A change. If this base-substitution is included within a heteroduplex, an A/A or a T/T mismatch will result, depending upon which strand is transferred. Repair of the A/A mismatch can lead to two possible results. If the mismatch is repaired such that the wild-type information is duplicated, a gene conversion event will result. This type of event will produce three His[+] spore colonies and one His[-] spore colony (a 6:2

event, using nomenclature for eight-spored fungi). Alternatively, if the mismatch is repaired such that the mutant (*his4-AAG*) allele is duplicated, then Mendelian segregation is restored. It should be noted that Mendelian segregation events that result from restoration-type repair cannot be directly distinguished from canonical Mendelian segregation (i.e. no recombination event).

If the A/A mismatch is not repaired, then one spore will receive both mutant and wild-type information. The first mitotic division, following meiosis, will produce one cell with the wild-type phenotype and one with the mutant phenotype. Such events are called "post-meiotic segregation events" (PMS) (Petes *et al.* 1991). In this example, one would observe a tetrad with two His$^+$ spore colonies, one His$^-$ spore colony, and one spore colony that is sectored His$^+$/His$^-$ (a 5:3 event). Similarly, repair or failure to repair the T/T mismatch will result in a 2:6 gene conversion or a 3:5 PMS event, respectively. As PMS events result from mismatches that are not repaired and gene conversion events reflect mismatches that are repaired, the ratio of PMS events to total aberrant segregation events (conversion + PMS) can be used as a measure of the efficiency of meiotic MMR. Using this type of measurement, it has been shown that all types of base-base mismatches (with exception of C/C mismatches) are efficiently repaired in wild-type strains (Detloff *et al.* 1991).

In addition to the base-base mismatches described above, loops of various sizes can be incorporated into meiotic heteroduplexes. Such loops are formed by constructing strains that are heterozygous for deletions or insertions. If the mutant contains an insertion, when the mutant and wild-type sequences pair to form a

FIGURE 1.4. Segregation patterns resulting from repair or lack of repair of a meiotic mismatch. This figure shows a modified form of the double-strand break (DSB) repair model (Szostak *et al.* 1983, Sun *et al.* 1991) in which recombination is initiated by a DSB (indicated by the black triangle) on the wild-type chromosome (left side of figure) or the mutant chromosome (right side of figure). Open and closed circles represent wild-type and mutant alleles, respectively. The specific mutation is a T-to-A substitution. After DSB formation (step 1), the broken ends are resected, leaving 3' ended, single-stranded tails (step 2). A limited amount of strand invasion occurs, followed by new DNA synthesis (indicated with stippled lines) that displaces a "D loop" (step 3). The heteroduplex DNA formed when the D loop is displaced contains a mismatch (A/A on the left, T/T on the right), as indicated by the pairing of open and closed circles. Failure to repair the mismatch results in a 3:5 PMS event (if the initiating DSB occurred on the wild-type chromosome) or a 5:3 PMS event (if the initiating DSB occurred on the mutant chromosome). Repair of this mismatch can result in a 2:6 gene conversion (if the initiating DSB occurred on the wild-type chromosome), a 6:2 gene conversion (if the initiating DSB occurred on the mutant chromosome), or restoration of Mendelian segregation (4:4).

1

2

3

No repair Repair Repair No repair

Post-meiotic segregation (3:5)

Gene conversion (2:6)

Restoration of Mendelian segregation (4:4)

Gene conversion (6:2)

Post-meiotic segregation (5:3)

27
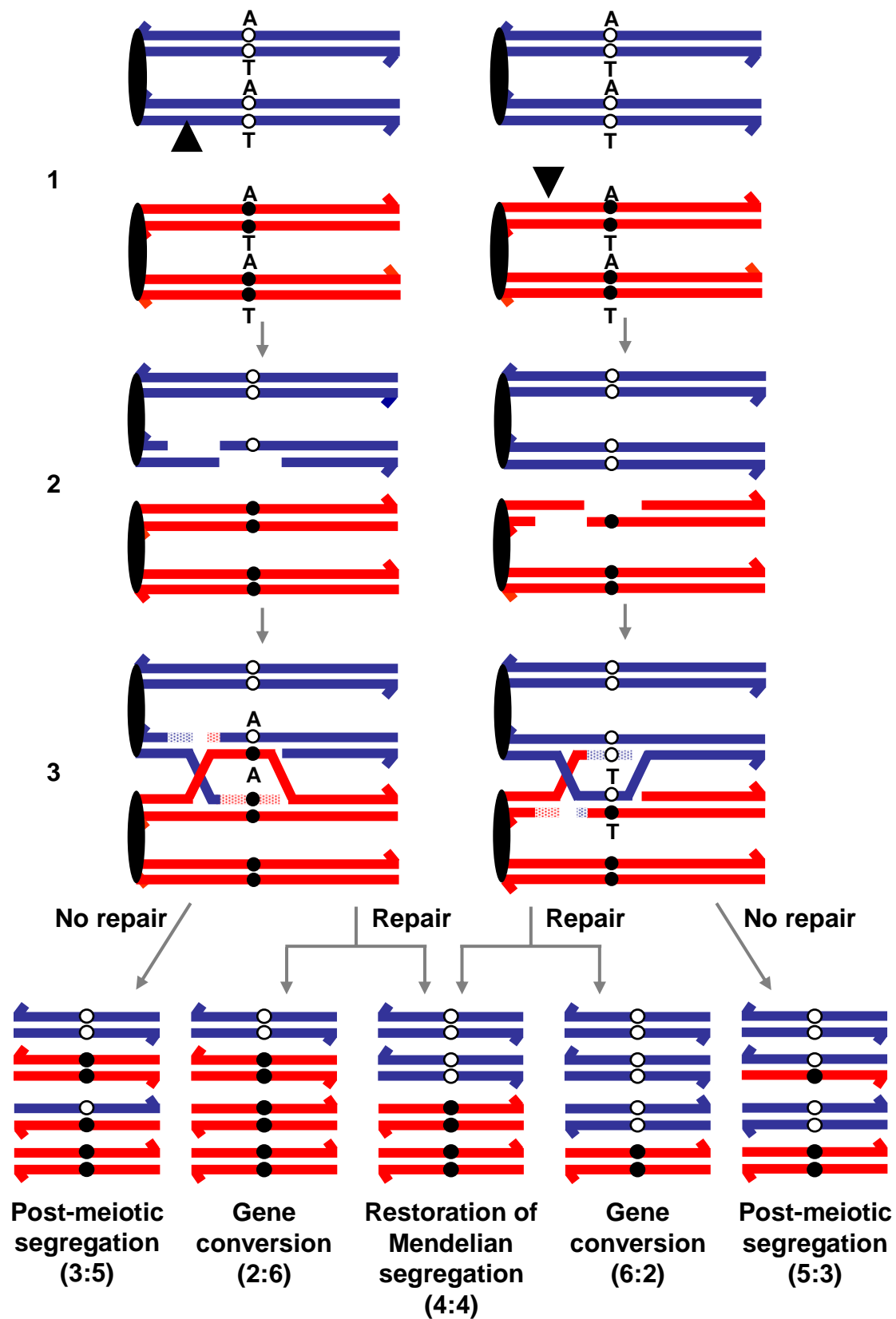
heteroduplex, the inserted sequence will form a loop. Small (4-27 bp) loops with no

secondary structures are generally repaired very efficiently in wild-type strains;

however, palindromic sequences that can form hairpin structures are not efficiently

repaired (Nag & Petes 1991, Detloff *et al.* 1992).

4. **Mismatch repair proteins involved in the repair of mismatches in meiotic**

heteroduplexes. Holliday originally proposed that gene conversion events result

from the repair of mismatches whereas PMS events result from unrepaired

mismatches (Holliday 1964). This prediction was first supported by Fogel and

coworkers who demonstrated that a mutaton in *PMS1* resulted in an increase in

PMS events and a decrease in gene conversion events (Williamson *et al.* 1985,

Kramer *et al.* 1989b). Similar types of studies have demonstrated that Msh2p and

Mlh1p, and to a lesser extent Msh3p and Msh6p, play roles in meiotic MMR (New *et*

al. 1993, Alani *et al.* 1994, Prolla *et al.* 1994, Tornier *et al.* 2001). It has been

generally accepted that the same DNA repair complexes function for both the

spellchecker function and for meiotic MMR. Specifically, it is believed that repair of

base-base mismatches in heteroduplexes required Msh2p/Msh6p and Mlh1p/Pms1p

heterodimers, while repair of small loops (generally generated using strains

heterozygous for four-base restriction site fill-ins) in heteroduplexes requires

Msh2p/Msh3p and Mlh1p/Pms1p. There are, however, two issues related to this

conclusion. First, this conclusion is based on studies done with a variety of different

strain backgrounds with a variety of different mismatches located in a variety of

different contexts. Second, it is clear that the repair of large DNA loops (greater than

27 bases) requires a different type of repair complex that involves both MMR

proteins (Msh2p and Msh3p) and proteins involved in nucleotide excision repair (Rad1p and Rad10p) (Kirkpatrick & Petes 1997, Sugawara *et al.* 1997, Kearney *et al.* 2001). In Chapter II, I describe my study of meiotic MMR that involves examining the effects of all MMR proteins on a defined type of mismatch in the same context in an isogenic strain background.

    5. Mismatch repair and polarity gradients. Mutations in MMR genes influence the overall levels of aberrant segregation in a manner that is dependent on the location of the marker within the gene. Studies of a number of DSB hotspots have demonstrated that the levels of aberrant segregation of efficiently-repaired markers close to the DSB are higher than those for markers located further away. Such gradients of aberrant segregation have been termed "polarity gradients" (reviewed by Fogel *et al.* 1981, Petes *et al.* 1991). Both *ARG4* and *HIS4* have well-defined polarity gradients. The *HIS4* polarity gradient is much steeper when looking at efficiently-repaired markers compared to inefficiently-repaired markers (Detloff *et al.* 1992). Similarly, these gradients are much steeper in MMR-proficient strains than in MMR-deficient strains (Alani *et al.* 1994, Hunter & Borts 1997, Vedel & Nicolas 1999, Argueso *et al.* 2002).

    It has been suggested that polarity gradients reflect a gradient in the ratio of conversion-type to restoration-type repair rather than a gradient in the formation of heteroduplex DNA (Detloff *et al.* 1992). If this prediction is correct, there will be a bias for conversion-type repair of markers that are closer to the DSB site, while markers further away from the DSB site can undergo either conversion-type repair or

FIGURE 1.5. Nick-directed repair of mismatches within recombination

intermediates. Recombination is initiated by a DSB (indicated by large arrowhead)

on the blue chromosome. Following resection of the DSB ends, (step 1), strand

invasion (step 2) primes repair DNA synthesis. Repair DNA synthesis displaces a

D-loop of DNA that anneals to the right 3' end (step 3). As the homologues differ by

a single mutation, a mismatch is formed in the resulting heteroduplex DNA

(represented by pairing of open and closed circles).

   A. MMR is directed by the nick remaining at the site of the DSB (the Initiation or

"I-nick") (Detloff *et al.* 1992, Porter *et al.* 1993).   During MMR, DNA between the I-

nick and the mismatch is excised (step 4A). Repair synthesis is then completed

(step 5A) and HJs are resolved (step 6A). The end result of I-nick-directed MMR is

a gene conversion event.

   B. MMR is directed by a nick created during resolution of the right HJ (the

Resolution or "R-nick"). During HJ resolution, the strands indicated by the small

arrowheads are nicked (step 4B). The DNA between the R-nick and the mismatch is

then excised (step 5B). DNA synthesis then duplicates the sequences from the blue

chromosome, resulting in a restoration event.

A.　　　　　　　　　　　　B.

↓ 1

↓ 2

↓ 3

I

↓ 4A

↓ 5A

↓ 6A

Gene Conversion

→

↓ 4B

R

↓ 5B
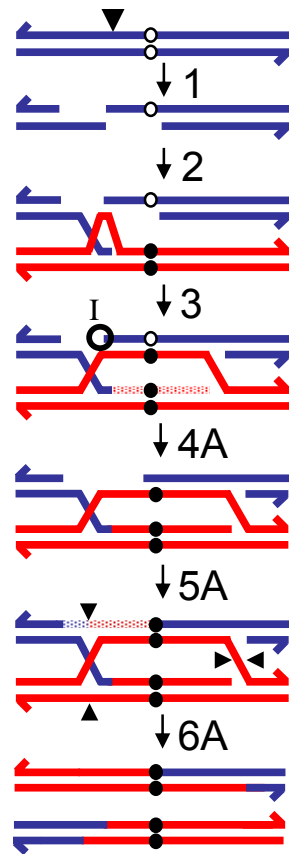
↓ 6B
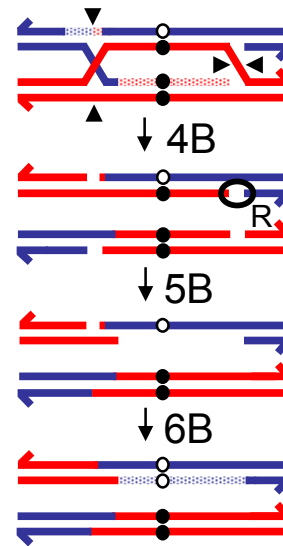
Restoration

restoration-type repair. The mechanistic explanation of this model relies on the use of two types of nicks in recombination intermediates to direct the excision step of meiotic MMR (Fig 1.5). Mismatches located close to the DSB site would be most likely to be repaired using the nick resulting from the initial formation of the recombination intermediate to direct MMR (Porter *et al.* 1993). This pattern of repair has been termed "early repair" (Foss *et al.* 1999) and would result in gene conversion events (Fig 1.5A). Alternatively, the nick resulting from the resolution of the HJ that is displaced from the position of the initiating DSB could also be used to direct MMR (termed "late repair"; Fig. 1.5B). A mismatch located near this nick (i.e. far away from the original DSB site) would be repaired with a bias favoring restoration-type repair over conversion-type repair.

6. Involvement of MutS and MutL homologues in crossover regulation.

MSH4 and *MSH5* were identified as genes with homology to the previously identified MutS homologs. The Msh4 and Msh5 proteins lack the mismatch-binding domain that is characteristic of other MutS homologs and have no role in the MMR spellchecker function. Deletion of either *MSH4* or *MSH5*, however, result in a significant reduction in the levels of meiotic crossovers (Ross-Macdonald & Roeder 1994, Hollingsworth *et al.* 1995, Wang *et al.* 1999). Similarly, deletions of the MMR genes *MLH1, MLH3,* and *EXO1* genes reduce crossovers (Hunter & Borts 1997, Wang *et al.* 1999, Kirkpatrick *et al.* 2000, Khazanehdari & Borts 2000). One interpretation of these results is that crossovers are promoted by a complex of Msh4p/Msh5p/Mlh1p/Mlh3p. Exo1p is in the same epistasis group as Msh4p/Msh5p for crossover regulation, but in a different epistasis group for spore

viability (Kirkpatrick *et al.* 2000, Khazanehdari & Borts 2000, Abdullah *et al.* 2004). It has been speculated that Exo1p might have a general role for processing of recombination intermediates prior to HJ resolution; however, the exact nature of this role remains unclear.

Recently, the structure-specific endonuclease Mus81p/Mms4p has also been implicated in crossover formation (de los Santos *et al.* 2003). Thus, crossovers in *S. cerevisie* are generated by two pathways: the Msh4p/Msh5p pathway, and the Mus81p/Mms4p pathway (de los Santos *et al.* 2003, Abdullah *et al.* 2004, Argueso *et al.* 2004). In some other organisms, only one pathway of crossover regulation exists (reviewed by Hoffmann & Borts 2004). For instance, all crossovers in *C. elegans* are regulated by the Msh4p/Msh5p pathway. In contrast, *S. pombe* and *D. melanagastor* lack *MSH4* and *MSH5* and crossover regulation in these organisms requires their Mus81p orthologues.

7. Summary of research in this dissertation: In the following chapters of this dissertation, I describe my analysis of DNA mismatch repair proteins, various cellular exonucleases, and the PCNA processivity factor in the meiotic repair of DNA mismatches and in regulating meiotic crossovers. In chapter 2, I determine which MutS homologs, MutL homologs, and nucleases are involved in the repair of base-base mismatches and four-base loops. The specific types of base-base mismatches (A/A and T/T) were chosen because it was possible to examine the same types of base-base mismatches at two different loci in our genetic background. This allowed me to compare meiotic MMR at two different sites. The four-base loop substrate was chose for convenience; a strain carrying a four-base restriction site fill-in already

existed in the lab. In chapter 3, I find evidence that PCNA is involved in the anti-recombination function, the repair of meiotic mismatches, and the processing of heteroduplex DNA generated during meiotic recombination, in addition to spellchecker function of MMR. In chapter 4, I present a collaboration which examined how mutations in the ATPase domains of Mlh1p and Pms1p affect the various functions of MMR.

II. ANALYSIS OF THE PROTEINS INVOLVED IN THE *IN VIVO* REPAIR OF BASE-BASE MISMATCHES AND FOUR-BASE LOOPS FORMED DURING MEIOTIC RECOMBINATION IN THE YEAST *SACCHAROMYCES CEREVISIAE*[1]

A. Introduction

DNA mismatch repair (MMR) is the process by which base-base mismatches and certain other types of DNA lesions are corrected (reviewed by Harfe & Jinks-Robertson 2000). DNA mismatches can be formed either by errors made during DNA replication (polymerase slippage or misincorporation of bases by DNA polymerase) or by formation of recombination-associated heteroduplexes in which the DNA strands are not perfectly complementary. Correction of replication errors is sometimes termed the "spellchecker" function of MMR. The related process of correction of DNA mismatches within heteroduplexes results in the non-reciprocal recombination process of gene conversion. The mismatch repair proteins also have several roles that are not directly related to correction of mismatches (reviewed by Harfe & Jinks-Robertson 2000). First, a number of the mismatch repair proteins are involved in reducing the rate of recombination between diverged repeated sequences. Second, some MMR proteins act as DNA damage sensors in DNA damage checkpoint pathways (Stojic *et al.* 2004). Third, some MMR proteins promote crossing-over (Hoffmann & Borts 2004, Börner *et al.* 2004). Finally, some

---

[1] This chapter has been published previously (Stone & Petes 2006).

MMR proteins participate in the removal of single-stranded "tails", allowing recombination between repeated genes by the single-strand annealing pathway (Sugawara *et al.* 1997). Our study is focused on the role of the MMR proteins in DNA mismatch repair.

In vivo and *in vitro* studies of mismatch repair in *E. coli* (reviewed by Modrich & Lahue 1996) have defined the steps in mismatch repair and the proteins involved in catalyzing these steps. These steps include (1) the recognition of the DNA mismatch, (2) the identification of the newly-synthesized DNA strand, (3) the nicking of the newly-synthesized strand near the mismatch, (4) the excision of the mispaired DNA on the nicked strand, and (5) DNA synthesis and ligation to fill in the gap on the nicked strand. To initiate the process of repair, MutS binds to the DNA mismatch, and MutL facilitates a MutS/MutH interaction. This MutS/MutH complex stimulates MutH-mediated nicking of the non-methylated, newly-synthesized DNA strand. The nicked strand then is removed via helicase and exonuclease activities. DNA synthesis across the excised region and subsequent DNA strand ligation complete the repair process.

The process of mismatch repair is much more complicated in eukaryotes. Our understanding of the steps in the process and the proteins involved for each step is based on genetic studies (types of mutations observed in strains lacking MMR and the efficiency of correction of various types of mismatches formed during recombination) and biochemical studies. All steps in the process have not yet been defined. In *Saccharomyces cerevisiae*, six MutS homologues (Msh1-6p), four MutL homologues (Mlh1-3p, Pms1p), and no MutH homologues have been identified

36

(reviewed by Harfe & Jinks-Robertson 2000). Most eukaryotic MMR and related processes are carried out by different combinations of a heterodimer of MutS homologues and a heterodimer of MutL homologues. For instance, base-base mismatches as well as one base loops are repaired by an Msh2p/Msh6p/Mlh1p/Pms1p complex, whereas small (one to fourteen bases) loops are repaired by an Msh2p/Msh3p/Mlh1p/Pms1p complex. Heterodimers of Mlh1p and Mlh2p or Mlh1p and Mlh3p play a minor role in the repair of frameshift mutations generated as a result of polymerase slippage at mononucleotide runs (Harfe *et al.* 2000). In contrast, Msh4p and Msh5p do not have any known spellchecker activity, but these proteins (as well as Mlh1p and Mlh3p) are involved in promoting crossovers (Hunter & Borts 1997, Wang *et al.* 1999; Börner *et al.* 2004). Finally, Msh1p functions exclusively in the mitochondria (Reenan & Kolodner 1992, Sia and Kirkpatrick 2005).

While mismatch recognition has been well characterized, less is understood regarding the strand discrimination signal and subsequent processing steps of MMR in eukaryotes. It has been proposed that PCNA (Proliferating Cell Nuclear Antigen), the sliding clamp associated with DNA polymerase, may differentiate which strand is the template for repair (Umar *et al.* 1996). In an *in vitro* system of MMR, the 3' to 5' excision reaction requires PCNA and the clamp loader RFC (Replication Factor C) (Dzantiev *et al.*, 2004). It is also possible that single strand nicks left during Okazaki fragment formation might direct repair (Genschel *et al.* 2002).

A number of nucleases have been implicated as involved in MMR: Rad1p/Rad10p (the yeast equivalent of XPF/ERCC1), Exo1p, Rad27p (the yeast

homologue of the Fen1 flap endonuclease), and the proofreading activity of DNA polymerase δ (reviewed by Marti & Fleck 2004). Of these nucleases, Exo1p is the one most likely to have a direct role in MMR, since mutations in *EXO1* result in a mutator phenotype (Tishkoff *et al.* 1997). Since this mutator phenotype is much weaker than that observed in *msh2*, *mlh1*, or *pms1* strains, and since the types of mutations observed in *exo1* strains are substantially different from those observed in strains with other MMR mutations, the Exo1p activity is likely to be partly functionally redundant with that of one or more other nucleases (Tishkoff *et al.* 1997; Tran *et al.* 2001). It is also likely that at least part of the mutator phenotype of *exo1* mutants is independent of an effect on MMR (Tran *et al.* 2001). In addition, Exo1p physically interacts with Msh2p, Msh3p, and Mlh1p (Tishkoff *et al.* 1997, Tran *et al.* 2001). Finally, *in vitro* studies indicate that Exo1p is required for bidirectional repair of a mismatch (Constantin *et al.* 2005).

Mismatches resulting from misincorporation errors of DNA polymerase are highly variable in type and position. In contrast, the mismatches in heteroduplexes formed during meiotic recombination are completely defined in type and position. In this study, we construct yeast strains that form either base-base (A/A or T/T) mismatches or four-base loops in heteroduplexes generated during meiotic recombination. By tetrad analysis (details explained in the Results section), we examine the efficiency of repair of these two types of mismatches in wild-type strains and in strains with MMR defects. We investigate the effects of all of the MutS and MutL homologues, except the mitochondria-specific *MSH1* gene product. In addition, we analyzed the effects of mutations in the nuclease-encoding *RAD1*,

38

EXO1, and *RAD27* genes, and a mutation in the proofreading nuclease of DNA polymerase δ.

This study is the first in which the effects of all of the genes previously implicated in DNA mismatch repair are analyzed for specific types of DNA mismatches in one isogenic genetic background. Our results confirm the importance of the Msh2/Msh6/Mlh1/Pms1 proteins in the repair of base-base mismatches and the Msh2/Msh3/Mlh1/Pms1 proteins in the repair of a four-base loop. We show that Mlh3p also has a significant role in the repair of four-base loops, and demonstrate the existence of a complex that repairs four-base loops that is dependent on Msh2p and Msh3p, but independent of Mlh1p. Lastly, we demonstrate that the ratio of the types of repair (conversion versus restoration) can be altered by changing the level of local double-strand breaks.

B. Materials and Methods

1. Strains: All haploid yeast strains were derivatives of AS4 (α *arg4-17 trp1 tyr7 ade6 ura3*) or AS13 (a *leu2 ade6 ura3*) (Stapleton & Petes 1991). The genotypes and constructions of haploids and diploids are described in Tables 2.1 and 2.2, respectively. All mutant derivatives were constructed by one-step or two-step transplacement or by crosses with isogenic strains. All transformants were verified using PCR analysis. As diploid AS4 x AS4 strains have a sporulation deficiency caused by a mutation within *STP22*, an *STP22*-containing plasmid (pDJ173; provided by D. Jenness, University of Massachusetts Medical School) was maintained during AS4 x AS4 crosses. Haploids were cured of pDJ173 prior to use

39

in experiments. pDJ173 was created by inserting the 2kb *Hind*III/*Sal*I fragment of

pDJ166 (Li *et al.* 1999) into *Hind*III/*Sal*I-cut YCp50.

2. Genetic Techniques: Standard media and genetic methods were used

(Sherman *et al.* 1982) except where indicated. Some of the mutant strains

examined have mutator phenotypes, resulting in increased levels of spore inviability.

To maximize spore viability, we generated diploids by mating haploid strains

overnight, and sporulating the diploids on the following day without purification. As

in previous studies, diploids were sporulated at 18° C to maximize meiotic

recombination at *HIS4* (Fan *et al.*, 1995). Following tetrad dissection on plates

containing rich growth media (YPD), we replica-plated the resulting spore colonies to

various omission media to score segregating markers. Spore colonies from strains

carrying the *his4-Sal* mutation were examined microscopically for small sectors on

both media lacking histidine and media lacking arginine. Spore colonies from strains

carrying the *his4-AAG* allele, with a few exceptions, were examined microscopically

only on medium lacking histidine.

In order to detect chromosome III nondisjunction events, spore colonies were

replica-plated to tester strains of the *a* and α mating types. After overnight

incubation, the mated cells were replica-plated to omission medium lacking adenine.

Tetrads were considered to have meiosis I nondisjunction events involving

chromosome III when only two spores were viable and both lacked the ability to

mate. This procedure will underestimate the frequency of nondisjunction, if the

nondisjunction events are unrelated to a lack of meiotic crossovers.

3. Statistical Analysis: Comparisons were made using the Fisher exact test with two-tail p values or by Chi-square analysis (for comparisons involving more than two experimental parameters or comparisons with numbers too large for the Fisher exact test) using the statistical analysis program on the VassarStats website (http://faculty.vassar.edu/lowry/VassarStats.html). Whether one- or two-tailed tests were done is indicated in the Tables. Because of the large numbers of comparisons performed, we used the sequential p value procedure (Benjamini & Hochberg 1995) to limit the false discovery rate. For each set of comparisons, we first determined the uncorrected p values and then used the Benjamini-Hochberg procedure with an initial value of $\alpha = 0.05$ to determine which p values were statistically significant.

C. Results

1. Experimental System: To examine the roles of the individual MMR proteins on the repair of different types of DNA substrates, we used isogenic diploid *S. cerevisiae* strains heterozygous for either the *his4-AAG* or *his4-Sal* alleles. The *his4-AAG* allele is a T-to-A substitution at the second position of the *HIS4* start codon. A heteroduplex formed between a strand with wild-type information and one with the *his4-AAG* substitution will result in an A/A or T/T mismatch, depending on which strand is transferred in forming the heteroduplex (Detloff *et al.* 1991). We chose to examine this particular mutant substitution because our diploids are also heterozygous for the *arg4-17* allele, which contains a T-to-A mutation at position +127 of the *ARG4* coding sequence (White *et al.* 1985). Thus, by using isogenic strains containing the *his4-AAG* and the *arg4-17* alleles, we are able to determine

whether the same mismatch in different contexts is repaired in the same manner.

The *his4-Sal* allele is a four-base-pair restriction site fill-in located at position +467 of the *HIS4* coding sequence (Nag *et al.* 1989). A heteroduplex formed by one strand containing the wild-type *HIS4* sequences and one strand with the *his4-Sal* sequence results in a four-base loop.

In our genetic background, the *HIS4* locus undergoes a very high rate of meiotic recombination as a consequence of a very strong double-strand break (DSB) located about 250 bp upstream of the *HIS4* initiation codon (Fan *et al.* 1995, Xu & Petes 1996). The high rate of DSB formation results in high rates of heteroduplex formation involving markers within the *HIS4* gene. In a strain heterozygous for the *his4-AAG* mutation, a DSB on the wild-type or mutant chromosomes results in an A/A or a T/T mismatch, respectively. If the heteroduplex reflects a DSB on the wild-type strand, failure to correct the resulting mismatch results in a tetrad with one His$^+$, two His$^-$, and one His$^{+/-}$ sectored spore colonies; such tetrads are termed "3:5", following the nomenclature used for eight-spored fungi. Repair of the mismatch can yield either a gene conversion (2:6) or restore Mendelian segregation (4:4). Similarly, an event initiated on the mutant chromosome will generate a 5:3 or 6:2 tetrad, or restore Mendelian segregation. Since the *HIS4* locus has such a high level of recombination, we also find tetrads with more than one aberrant segregation event. For example, a tetrad with three wild-type spore colonies and one sectored colony is termed a 7:1 segregation event, and assumed to reflect a tetrad with one gene conversion event and one PMS event (Nag *et al.* 1989). Tetrads with more

than one aberrant segregation event are usually less than 10% as frequent as those with one aberrant segregation event.

In many studies, the efficiency of repair of mismatches formed during meiotic recombination is estimated by dividing the number of tetrads with one or more post-meiotic segregation (PMS) events by the total number of aberrant segregation events (tetrads with one or more PMS events, one or more gene conversion events, and tetrads with both gene conversion and PMS events). In the present study, we use a different calculation that gives the appropriate weight to tetrads with two or more aberrant segregation events. More specifically, we calculate the percentage of PMS events per aberrant events by summing the numbers of tetrads with a single PMS spore colony (5:3 + 3:5 + 7:1 + 1:7), two times the number of tetrads with two PMS spore colonies (aberrant 4:4 + aberrant 6:2 + aberrant 2:6), three times the number of tetrads with three PMS spore colonies (deviant 5:3 + deviant 3:5), and four times the number of tetrads with four PMS spore colonies (deviant 4:4). The resulting sum is divided by the sum of aberrant events, calculated by summing the number of tetrads with one aberrant event (6:2 + 2:6 + 5:3 + 3:5), two times the number of tetrads with two aberrant events (7:1 + 1:7 + 8:0 + 0:8 + aberrant 4:4 + aberrant 6:2 + aberrant 2:6), three times the number of tetrads with three aberrant events (deviant 5:3 + deviant 3:5), and four times the number of tetrads with four aberrant events (deviant 4:4). The aberrant segregation classes for tetrads with two or more events are defined in Detloff et al. (1991). The tetrads with three or more aberrant events represent less than 10% of the total aberrant tetrads in all strains examined in this study.

2. Effects of mutations in MutS and MutL homologues on meiotic MMR of the base-base mismatch: As expected from previous studies, the heterozygous *his4-AAG* mutation had a very high rate of aberrant segregation, about 60% of the tetrads of the wild-type strain (PD83; Table 2.3). For most genetic loci in yeast, the frequency of aberrant segregation is 2-10% (Petes *et al.* 1991). In the wild-type strain, repair of the A/A, T/T mismatch at the *HIS4* hotspot is not complete, since 18% of the aberrant segregation events are PMS. Interestingly, for the same mismatch at the *ARG4* locus, repair is complete in the MMR-proficient MW103 strain (Table 2.4), arguing that the efficiency of MMR is somewhat context-dependent. It should be noted that most of the data for the *arg4-17* mismatch came from strains heterozygous for the *his4-Sal* allele.

The effects of the mutations in the MutS and MutL homologues on the repair of the base-base mismatch can be divided into two groups. Deletions of *MSH2, MSH6, MLH1,* and *PMS1* had similar effects, resulting in about 80-90% PMS/aberrant events for *his4-AAG*. Deletions of *MSH3, MSH4, MSH5, MLH2,* and *MLH3* did not significantly affect the ratio of PMS/aberrant events. These results are consistent with the conclusion that most of the meiotic repair events for base-base mismatches are initiated by a complex of the Msh2/Msh6/Mlh1/Pms1 proteins. Similar results were observed for the *arg4-17* marker, although the percentages of PMS/aberrant events are generally lower (45-83%) relative to those seen for the *his4-AAG* marker. Thus, some gene conversion events occur by a mechanism that is independent of the standard MMR pathway, and this mechanism is more efficient at the *arg4-17* site than at the *his4-AAG* site.

Strains with mutations in *msh4, msh5,* and *exo1* had significantly reduced levels of aberrant segregation of the *his4-AAG* marker (Table 2.3). The *exo1* mutants have been previously reported to reduce the frequency of aberrant segregation (Kirkpatrick *et al.* 2000, Khazanehdari & Borts 2000, Abdullah *et al.* 2004). Other researchers found that deletion of *msh4* or *msh5* has little effect on the frequency of aberrant segregation for most markers, although the *arg4-Nsp* allele was reported to have an <u>elevated</u> frequency of aberrant segregation in *msh4* strains (Ross-Macdonald & Roeder 1994, Hollingsworth *et al.* 1995). The reduction in aberrant segregation observed in our study could reflect either a reduced frequency of heteroduplexes that include *his4-AAG* or an increase in the frequency of restoration-type repair relative to conversion-type repair. A reduction in heteroduplex formation by the *msh4* mutation would be expected to reduce the rate of aberrant segregation in a MMR-deficient strain, whereas increased restoration-type repair would have relatively little effect in such a strain. We found a significant ($p = 0.002$ by one-tailed Fisher exact test) reduction in aberrant segregation in the *msh2 msh4* mutant compared to the *msh2* mutant. The level of aberrant segregation was not significantly different in the *msh2 msh4* mutant compared to the *msh4* single mutant. These results argue that the level of heteroduplexes that include the *his4-AAG* mismatch is reduced in the *msh4* strain. If Msh4p/Msh5p-promoted crossovers contribute to heteroduplex formation at the *his4-AAG* mismatch, the observed reduction in aberrant segregation is predicted.

3. Effects of mutations in MutS and MutL homologues on meiotic MMR of a four-base loop: The meiotic repair of the four-base loop was examined in diploids

with the *his4-Sal* allele (Table 2.5). First, as expected from previous studies (reviewed by Surtees *et al.* 2004), the *msh6* mutation had no effect on loop repair, and the *msh3* mutation had approximately the same effect as *msh2*; this result is consistent with the previous conclusion that most of the repair of the four-base loop involves a heterodimer of Msh2p and Msh3p. Second, the effects of the *mlh1* and *pms1* on loop repair were significantly weaker than the effect of *msh2*. Third, the *mlh3* mutation significantly affected the repair of the four-base loop, although the effect was significantly less than those observed for *mlh1* or *pms1*.

To examine further the roles of the various MutL homologues in the repair of the four-base loop, we also examined the efficiency of repair in various double mutant strains. Of the *mlh1 pms1*, *mlh1 mlh2*, and *mlh1 mlh3* double mutants, only the *mlh1 mlh3* double mutant had a significantly greater repair defect that *mlh1* (Table 2.5). The simplest interpretation of these data (to be discussed further below) is that most of the repair of the four-base loops is dependent on a heterodimer of Mlh1p and Pms1p, but some fraction of the repair events require Mlh3p and do not require Mlh1p.

4. Effects of mutations in genes encoding nucleases on meiotic MMR: As discussed in the Introduction, Exo1p has a role in the excision step of MMR, but other nucleases that are partially redundant with Exo1p are also involved. Candidates for these nucleases include Rad1p/Rad10p, Rad27p, and the proofreading activity of DNA polymerase δ. We previously showed that Rad1p and Rad10p were involved in the meiotic repair of 26-base and 1 kb loops, but not in the repair of a base-base mismatch or a four-base loop (Kirkpatrick & Petes 1997,

46

Kearney *et al.* 2001). Since these experiments were done under slightly different conditions from those employed in the present study, we repeated this analysis for the *his4-Sal* and *arg4-17* markers.

As shown in Tables 2.3-2.5, there was no significant effect of *exo1*, *rad1*, *rad27*, or the *pol3-01* (encoding a proofreading exonuclease-deficient DNA polymerase δ) on the efficiency of DNA mismatch repair of the base-base mismatch or the four-base loop. These results confirm previous studies of *exo1* (Kirkpatrick *et al.* 2000, Khazanehdari & Borts 2000, Tsubouchi & Ogawa 2000) and *rad1* (Kirkpatrick & Petes 1997). Although we did not examine the effect of the *rad27* mutation in diploids heterozygous for *his4-AAG*, the mutation had no effect on the repair of the same mismatch (A/A, T/T) at the *ARG4* locus (Table 2.4). The lack of effect of these nucleases on the efficiency of meiotic MMR argues that they have no function in meiotic MMR or their functions are redundant. Unfortunately, the lethality of some double mutant combinations of these nuclease-encoding genes (for example, *rad27 pol3-01*; Kokoska *et al.* 1998) precludes a complete analysis of this issue.

In *pol3-01* strains, although the efficiency of MMR is not decreased, the rates of aberrant segregation for both types of *his4* alleles were significantly reduced (Tables 2.3 and 2.5). Maloisel *et al.* (2004) recently reported that another *pol3* mutation (*pol3-ct*) reduces aberrant segregation and crossovers, without having an effect on mitotic DNA replication. The authors suggest that DNA synthesis by DNA polymerase δ is required for both the elongation of the strand-exchange intermediate, as well as the decision to resolve recombination intermediates as crossovers. In addition to these possibilities, the *pol3-01* mutation could increase

the relative frequency of restoration-type repair compared to conversion-type repair or alter the level of DSBs. Although we observe no effect of *pol3-01* on crossovers (arguing against the last explanation), the level of crossovers is not necessarily directly related to the frequency of DSBs(Henderson & Keeney 2004).

5. Reduced meiotic crossovers in *msh4*, *msh5, mlh1*, *mlh3,* and *exo1* mutants: We measured crossovers for all strains in the study in three genetic intervals on chromosome III (Table 2.6). Most of the mutants had no significant effect on crossing-over in these intervals. Based on genetic and physical studies, it has been suggested that meiotic crossovers are promoted by Msh4p, Msh5p, Mlh1p, and Mlh3p (Hunter & Borts 1997, Wang *et al.* 1999, Kirkpatrick *et al.* 2000, Argueso *et al.* 2004; Börner *et al.* 2004). In support of this conclusion, we found a significant reduction in crossovers in the *CEN3-MAT* interval for strains with mutations in the genes encoding these four proteins (Table 2.6). In addition, we found that crossovers were reduced in the *exo1* mutant strains, as expected from previous studies (reviewed by Hoffmann & Borts 2004).

As increased levels of nondisjunction are often associated with reductions in crossovers, we monitored meiosis I nondisjunction genetically (as described in Materials and Methods). Data from both strains of each genotype were pooled together to determine the ratio of tetrads with nondisjunction of chromosome III to total tetrads: wild-type, 0/1665; *msh4*, 3/464; *msh2 msh4*, 2/569; *msh5*, 10/403; *mlh1*, 2/1585; *mlh3*, 1/683; *mlh1 mlh3*, 3/906; *mlh1 pms1*, 8/575; *exo1*, 2/621. No nondisjunction events were observed in any of the other strains used in this study. Of the eight strains with nondisjunction events, only the *msh4*, *msh5*, and *mlh1*

pms1 strains had a significant increase in nondisjunction compared to the wild type. The statistical analysis involved one-tailed Chi-square tests with $\underline{p}$ values corrected by the Benjamini-Hochberg method; $\underline{p}$ values less that 0.019 were considered significant. The relatively low numbers of nondisjunction events involving chromosome III found in our study, compared to other studies (Hunter & Borts 1997; Wang *et al.* 1999), may be due to the presence of the very strong *HIS4* hotspot on chromosome III in our genetic background.

6. Conversion-type and restoration-type MMR for the A/A, T/T mismatch: As discussed above, DNA mismatches can be repaired to generate a gene conversion event or to restore Mendelian segregation. We have previously argued that an efficiently-repaired base-base mismatch located near the 5' end of *HIS4* (and, therefore, near the DSB site that initiates recombination) is usually repaired to yield a gene conversion event (Detloff *et al.* 1992). This conclusion was based on the observation that the aberrant segregation frequency of an efficiently-repaired mismatch located near the 5' end of the gene was approximately the same as that of an inefficiently-repaired mismatch. The argument that led to this conclusion is best explained by an example. If an efficiently-repaired mismatch occurs in 50% of the tetrads and it is repaired equally frequently by conversion-type and restoration-type repair, then the observed frequency of aberrant segregation for this mismatch will be 25%. If an inefficiently-repaired mismatch at the same location occurs in 50% of the tetrads, one should observe 50% aberrant segregation. Thus, if a difference in the frequency of aberrant segregation is observed for efficiently- and inefficiently-

repaired mismatches at the same position in the gene, one can infer restoration-type repair; a lack of a difference suggests a lack of restoration-type repair.

From the data shown in Table 2.3, we can make a similar argument. If we denote the frequency of heteroduplex formation generating a mismatch by H, the frequency of failure to repair the mismatch by NR, the frequency of conversion-type repair as CR, and the frequency of restoration-type repair as RR, then H = NR + CR + RR. At the *HIS4* locus in the wild-type strain PD83, since 10% of the tetrads were PMS (NR tetrads) and 48% were conversions (CR tetrads), H(wt) = 10% + 48% + RR(wt). In the *msh2* strain, the comparable equation is: H(*msh2*) = 58% + 6% + RR(*msh2*). Since heteroduplex frequency is the same in the wild-type and *msh2* strains (Vedel & Nicolas 1999), H(wt) = H(*msh2*). Thus, RR(wt) = [(58% + 6% + RR(*msh2*)) – (10% + 48%)]. If we assume the frequency of restoration-type repair in the absence of *msh2* is negligible, we estimate the percentage of restoration-type repair as 6%, a value much lower than the measured rate of conversion-type repair in the same strain (48%). This result is shown graphically in Fig. 2.1A. In contrast, in isogenic strains, the same calculations indicate that the *arg4-17* marker has a level of restoration-type repair equal to the level of conversion-type repair in the wild-type strain. The frequency of conversion-type repair is 7% (Table 2.4) and the calculated frequency of restoration-type repair is 9% (Fig. 2.1B).

One difference between the *HIS4* and *ARG4* loci is the strength of the nearby double-strand breaks that initiate recombination. In our strain background, the DSB site located upstream of *HIS4* is one of the strongest hotspots in the genome (ranked second of all 6000 ORFs), whereas the *ARG4* recombination hotspot is

FIGURE 2.1. Comparison of segregation patterns in strains with varying levels of DSB hotspot activity. The observed percentages of total aberrant segregation, gene conversion, and post-meiotic segregation events are indicated as Total Ab., GC, and PMS, respectively. As described in the Results section, we calculated restoration events (Rest.) by comparing the frequencies of aberrant segregation in wild-type and *msh2* strains.

A. Strains with wild-type DSB frequency at the *HIS4* hotspot.

B. Strains with wild-type DSB frequencies at the *ARG4* and *DED81* hotspots.

C. Strains homozygous for an insertion of telomeric sequence upstream of *ARG4* (*arg4-tel*) that increases the DSB frequency at the *ARG4* hotspot and decreases the DSB frequency at the *DED81* hotspot.

D. Strains homozygous for the *bas1* deletion, a deletion that eliminates the DSB located immediately upstream of *HIS4.*

E. Strains homozygous for the *his4-51* mutation. This mutation eliminates the Rap1p binding site upstream of *HIS4*, and eliminates the *HIS4*-associated DSB.

A. *his4-AAG/HIS4*

Wild-type (PD83)

msh2 (No. 5)

B. *arg4-17/ARG4*

Wild-type (MW103)

msh2 (JSY240/JSY343)
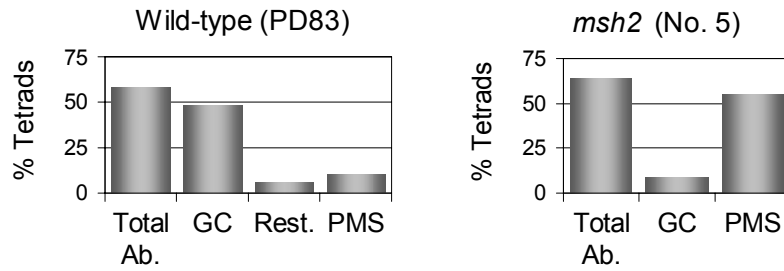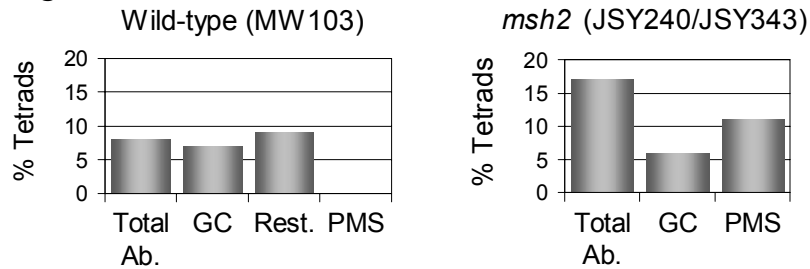
C. *arg4-tel/arg4-tel arg4-17/ARG4*

Wild-type (MW160)

msh2 (JSY282)

D. *bas1/bas1 his4-AAG/HIS4*

Wild-type (JSY320)

msh2 (JSY321)
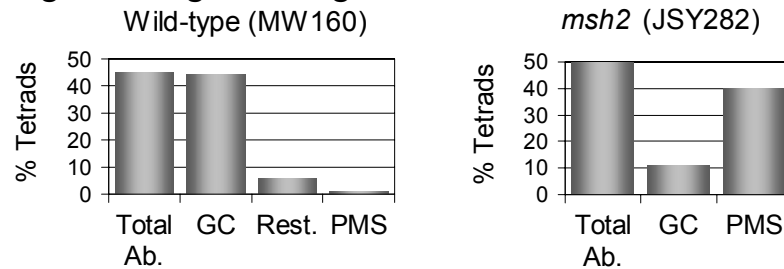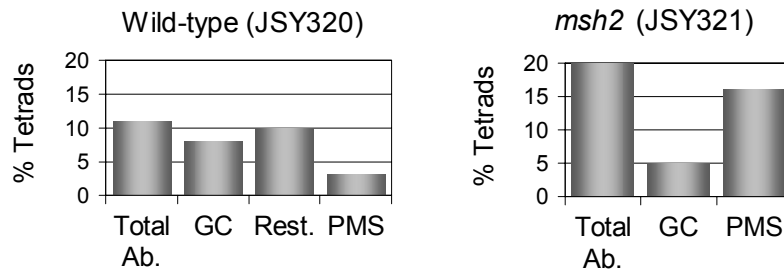
E. *his4-51/his4-51 his4-AAG/HIS4*

Wild-type (JSY338)

msh2 (JSY343)
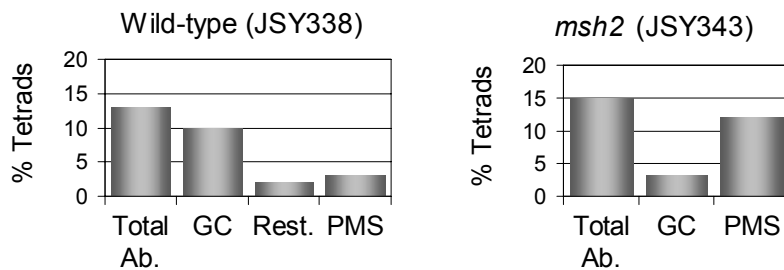
ranked 134th (Gerton *et al.* 2000). To determine whether the frequency of a local

DSB would affect the conversion/restoration ratio, we examined strains in which the

rate of DSB formation upstream of *ARG4* was elevated and other strains in which

the strength of DSB formation upstream of *HIS4* was decreased.

7. **The strength of the *ARG4* hotspot affects the conversion-type/restoration-type repair ratio:** We previously showed (White *et al.* 1993, Fan

et al. 1995) that a short (63 bp) insertion of telomeric DNA located upstream of the

ARG4 gene (*arg4-tel*) significantly elevated the frequency of aberrant segregation

and the frequency of DSB formation at the *ARG4* hotspot; the position of the DSB in

the *arg4-tel* strain, however, is approximately the same as the DSB in the wild-type

strain (Fan *et al.* 1995). We examined the frequency of aberrant segregation of the

arg4-17 mismatch in strains homozygous for *arg4-tel* insertion that are either

proficient or deficient (*msh2*) for MMR. As expected from our previous study, the

frequency of aberrant segregation was very significantly elevated by the *arg4-tel*

insertion, from about 8% to about 50%. In addition, the repair of the mismatch

involving the *arg4-17* allele was strongly biased toward conversion (Fig. 2.1C).

Thus, by increasing local DSB formation at the *ARG4* hotspot, we were able to alter

the ratio of conversion-type to restoration-type MMR to one that is similar to that

observed at *HIS4*.

8. **Effects of reducing *HIS4* hotspot activity on the ratio of conversion- to restoration-type repair:** White *et al.* (1993) previously showed that the activity of

the *HIS4* recombination hotspot required the binding of the transcription factors

Bas1p, Bas2p, and Rap1p. When the gene encoding Bas1p is deleted, aberrant

segregation at *HIS4* is significantly reduced, but not completely eliminated. A similar effect is observed when the Rap1p binding site upstream of *HIS4* is disrupted by the *his4-51* mutation (White *et al.* 1991). We constructed isogenic diploids homozygous for the *bas1* deletion or the *his4-51* mutation, heterozygous for *his4-AAG* and *arg4-17*, and proficient or deficient (as a consequence of the *msh2* mutation) for MMR. We found that the *bas1* deletion (strain JSY320) and the *his4-51* mutation (strain JSY338) reduced aberrant segregation of *his4-AAG* by approximately 75% (Table 2.3) without affecting the frequency of aberrant segregation of *arg4-17* (Table 2.4). Based on our observations of the *ARG4* locus described above, we expected that reduction in the activity of the *HIS4* hotspot would reduce conversion-type repair and increase restoration-type repair. Data from the *bas1* strains (JSY320 and JSY321) were consistent with this expectation (Table 2.3 and Fig. 2.1D) with conversion-type repair and restoration-type repair representing 8% and 10% of the tetrads, respectively.

The data from the *his4-51* strains (JSY338 and JSY343), however, were different from those of the *bas1* strains (Table 2.3 and Fig. 2.1E). The comparison between the *his4-51* and *his4-51 msh2* strains indicates that there was little restoration-type repair in the *his4-51* strain; conversion-type repair and restoration-type repair represent 10% and 2% of the *his4-51* tetrads, respectively (Table 2.3 and Fig. 2.1E). The difference in the results obtained with the *bas1* strains and the *his4-51* strains will be discussed in detail below.

D. Discussion

Most of our understanding of the MMR machinery is based on studies of the correction of misincorporation errors of DNA polymerase (the spellchecker function). In the present study, we examine the effects of the known MutS and MutL homologues, as well various nucleases implicated in MMR, on the repair of mismatches (base-base or a four-base loop) formed during meiotic recombination. In addition to confirming the importance of the Msh2/Msh6/Mlh1/Pms1 complex in the repair of base-base mismatches and the Msh2/Msh3/Mlh1/Pms1 complex in the repair of a four-base loop, we demonstrated the existence of a loop-repair complex that is Mlh1p-independent but dependent on Mlh3p. We showed that the nuclease activities of Exo1p, Rad1p, Rad27p, and DNA polymerase δ have no significant role in meiotic MMR, unless their functions are redundant. We also found that the ratio of conversion-type repair relative to restoration-type repair can be altered by changing the level of local DSBs.

1. **Repair of a base-base mismatch (A/A, T/T) and a four-base loop:** In agreement with the conclusions of others (reviewed by Surtees *et al.*, 2004), most base-base repair was equally dependent on Msh2p, Msh6p, Mlh1p, and Pms1p, as expected if these proteins function in MMR together (Fig. 2.2). None of the nuclease-encoding genes that we tested had a significant effect on the efficiency of mismatch repair. Since it is clear the Exo1p has a role in the excision step of mismatches generated by DNA polymerase misincorporation, our results indicate either that the role of Exo1p is functionally redundant with some other nuclease

during meiotic MMR or that meiosis-specific MMR is accomplished using an untested nuclease.

We found that at least two types of complexes are involved in the meiotic repair of a four-base loop. The strong effects of the *msh2, msh3, pms1,* and *mlh1* mutations argue that the most repair events involve a complex of Msh2p/Msh3p/Mlh1p/Pms1p. Since the *mlh1* and *pms1* mutations have significantly less effect than *msh2*, however, some MMR is Msh2p-dependent, but Mlh1p-independent. Since the *mlh3* mutation has a significant effect on the efficiency of repair of the four-base loop, one obvious candidate for such a complex involves a dimer of Mlh3p, either a heterodimer with a MutL homologue (other than Mlh1p) or as a homodimer (Fig. 2.2).

In a previous study, based on two-hybrid and co-immunoprecipitation analysis, Wang *et al.* (1999) argued that all MutL heterodimers in yeast contained Mlh1p. One explanation of this discrepancy with our data is that the stability of the MutL complexes or the levels of the MutL proteins may be different in vegetative and meiotic cells. Indeed, microarray data indicates that 2-5 hours after induction of sporulation the expression levels of *MLH3*, *PMS1*, and *MSH2* are elevated two-fold over vegetative expression levels, while levels of *MLH1*, *MLH2*, *MSH3*, and *MSH6* remain constant (Chu *et al.* 1998). A second possible explanation is that the stability of the dimers formed between MutL homologues is affected by their interaction with the MutS homologues, an effect that would not be seen in the physical studies. Finally, we cannot exclude the possibility that certain hetero- or homodimers involving the MutL homologues are found in *mlh1* strains, but not in wild-type strains.

Previously, Wang *et al.* (1999) found no effect of the *mlh3* mutation on the efficiency of repair of a four-base loop. Since this issue was examined in a strain with very low levels of aberrant segregation (1.3%) and since the effect of the *mlh3* mutation is smaller than those of *mlh1* or *pms1*, these data are not in significant disagreement with ours. In addition, Harfe and Jinks-Robertson (2000) showed that the *mlh3* mutation decreased the stability of a mononucleotide tract (10 Gs) about 20-fold relative to wild-type, whereas the *msh2* mutation decreased the tract stability about 10000-fold. This result argues that Mlh3p has a minor role in the correction of a one-base loop, the substrate expected from DNA polymerase slippage of a mononucleotide tract. Based on our results, we predict that the *mlh3* mutation might have a substantially stronger effect on the mitotic stability of a microsatellite in which the repeating unit is four bp.

2. Residual gene conversion in MMR-deficient strains: It has been clear since the discovery of mutations that affect MMR (Williamson *et al.* 1985) that none of these mutations eliminates gene conversion, demonstrating that some mismatches are corrected independently of the canonical MMR system. There is evidence for a short-patch repair system in *S. cerevisiae* (Coic *et al.* 2000; E. Hoffman, J. Meadows and R. Borts, personal communication) that is independent of the nucleotide excision repair proteins, although no other proteins involved in this repair process have been identified. Alternatively, some fraction of gene conversion events may occur through a process that does not involve MMR. Merker *et al.*
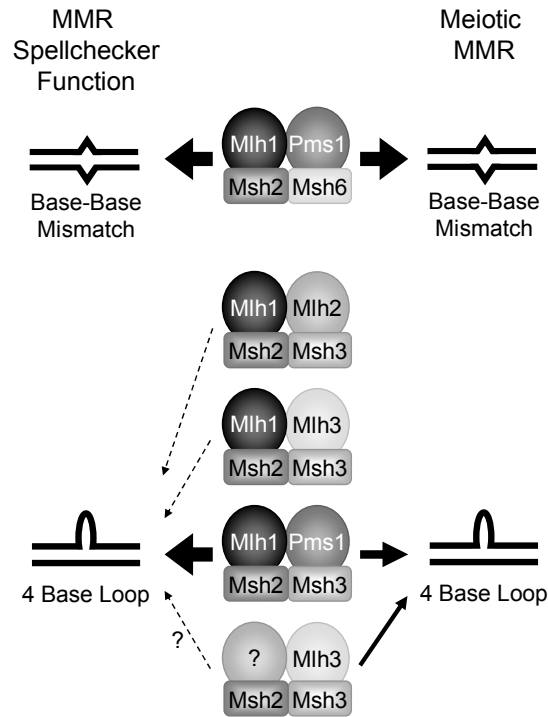
FIGURE 2.2. Comparison of the MutL and MutS homologues involved in the MMR spellchecker function (repair of errors introduced by DNA polymerase) and the MMR of mismatches formed during meiotic recombination. The relative contributions of each complex to the repair of base-base mismatches and four-base loop substrates are indicated by the arrow size. Msh2p/Msh6p/Mlh1p/Pms1p is involved in MMR of base-base mismatches in both contexts. Msh2p/Msh3p/Mlh1p/Pms1p initiates the majority of repair of four-base loops for the spellchecker function, with Msh2p/Msh3p/Mlh1p/Mlh2p and Msh2p/Msh3p/Mlh1p/Mlh3p making very small contributions (Wang *et al.* 1999; Harfe & Jinks-Robertson 2000). The meiotic repair of four-base loops is initiated primarily by Msh2p/Msh3p/Mlh1p/Pms1p, but an Mlh1-independent, Mlh3p-dependent complex (possibly Msh2p/Msh3p/Mlh3p/Mlh3p) is responsible for about one-third of the repair events.

(2003) found that a small fraction (about 5%) of conversion events at the *HIS4* locus appeared to reflect break-induced replication (BIR). In BIR events, a broken end from one chromosome invades and replicates another chromosome, resulting in a gene conversion event for a large chromosomal region that does not involve MMR.

3. **Context-specific efficiency of MMR:** We noted previously that the same base-base mismatch is repaired with nearly 100% efficiency at the *ARG4* locus, but is repaired with only 80-90% efficiency at the *HIS4* locus (Welz-Voegele *et al.* 2002). This result is confirmed in the present study. This difference may reflect an effect of the local sequence context on the repair of a mismatch. Alternatively, different chromosomal regions may have different efficiencies of DNA mismatch repair. An argument in favor of the latter possibility is that the rate of microsatellite alterations of the same reporter gene placed in a number of genomic locations varies by more than ten-fold in a MMR-proficient strain, but only varies two-fold in a MMR-deficient strain (Hawk *et al.* 2005).

4. **Genetic regulation of the rate of crossovers and aberrant segregation:** Several mutations (*msh4, msh5, mlh1, mlh3, exo1,* and *his4-51*) reduced the frequency of crossovers for one or more intervals on chromosome III. From previous studies, *mlh1, mlh3, msh4, msh5,* and *exo1* were expected to reduce crossovers (reviewed by Hoffmann & Borts 2004), although the mechanistic roles of these proteins in crossing over is not yet clear. As already discussed in the Results section, we found that *msh4, msh5, exo1,* and *pol3-01* also decreased aberrant segregation of the *his4-AAG* allele (Table 2.3). There was also a significant decrease in the aberrant segregation frequency in the *mlh2* strain. This effect of the

mlh2 mutation may be strain-specific, since Abdullah *et al.* (2004) reported that *mlh2* mutants had <u>elevated</u> levels of aberrant segregation at a variety of loci, including *HIS4*. Alternatively, the effect of the *mlh2* mutation may reflect the distance between the initiating DSB and the mismatch. In the Abdullah *et al.* study, elevated levels of aberrant segregation were observed only for markers in *HIS4* located more than 500 bp from the initiating lesion.

   5. The conversion- to restoration-type repair ratio: The meiotic repair of a mismatch can lead either to a gene conversion event or can restore Mendelian segregation. Although restoration events are difficult to measure directly, the frequency of such events can be estimated by subtracting the frequency of aberrant segregation in a MMR-proficient strain from the frequency of aberrant segregation in a MMR-deficient strain (details discussed in Results section). In confirmation of our previous results, we found that the base-base mismatch represented by the *his4-AAG* allele was primarily corrected by conversion-type repair (Fig. 2.1A). One explanation of such a bias is that the nick resulting from the DSB that initiates recombination (which we will call the I [Initiating] nick)) usually directs the excision of mismatches, if the mismatch is located near the DSB site (Porter *et al.* 1993); this repair would yield exclusively conversion-type repair (Fig. 2.3A, steps 3-5). This type of repair has also been termed "early repair" (Foss *et al.* 1999).

   Mismatches located in the *HIS4* gene further from the initiating lesion have both restoration-type repair and conversion-type repair (Detloff *et al.* 1992). Foss *et al.* (1999) suggested that restoration-type repair (termed "late repair") could be directed by the nick resulting from resolution of the Holliday junction. We label this nick "R"

(Resolution) in Fig. 2.3A (steps 3'-6'). However, if the R nick is not close enough to the mismatch to direct the excision event and/or repair has not occurred by the time the DNA is ligated following HJ resolution, the repair event could be undirected (50% conversion-type and 50% restoration-type repair). Thus, the ratio of conversion- to restoration-type repair is likely to be related to the distance of the mismatch from the two types of nicks that can direct the excision event. Since the *his4-AAG*-associated mismatch is located near the DNA lesion that initiates the heteroduplex, almost all repair would be directed toward conversion (Porter *et al.*, 1993).

Unlike the mismatch involving *his4-AAG,* the mismatch related to the *arg4-17* substitution had approximately equal frequencies of conversion-type and restoration-type MMR (Fig. 2.1B). In the context of the model shown in Fig. 2.3A, this result is consistent with the *arg4-17*-associated mismatch being equidistant from I and R nicks. This interpretation is inconsistent, however, with the observation that the distance between *his4-AAG* and the recombination-initiating DSB (250 bp; Fan *et al.* 1995, Xu & Petes 1996) is approximately the same as the distance between *arg4-17* and its recombination-initiating DSB (about 330 bp; de Massy & Nicolas 1993). Our alternative interpretation is shown in Fig. 2.3B. We argue that the heteroduplexes that produce mismatches involving the *arg4-17* substitution are initiated at two different sites. Events initiated at the *ARG4* hotspot result in an I nick near the mismatch and, consequently, would usually result in conversion-type repair. We suggest that heteroduplexes initiated as a strong DSB site located upstream of the neighboring gene (*DED81*) also contribute to mismatches involving the *arg4-17* allele (Fig. 2.3B). For this class of events, we suggest that the repair of the

mismatch is usually directed by an R nick, leading to restoration. Alternatively, the mismatch initiated at *DED81* might be corrected by a mechanism that is not nick directed and yields an equal frequency of conversion-type and restoration-type repair.

The hypothesis that the segregation frequency of the *arg4-17* marker is affected by DSBs initiated at more than one site is supported by a number of arguments. First, in our genetic background, the DSB site located upstream of *DED81* is considerably stronger than that located upstream of *ARG4* (Fan *et al.* 1995). Second, in the same genetic background, heteroduplexes initiated upstream of *HIS4* often extend to distances greater than 2.5 kb (Detloff *et al.* 1992, Merker *et al.* 2003), a distance less than that between the *DED81*-associated DSB and the *arg4-17* substitution. Third, recombination events at the *HIS4* locus are initiated at multiple DSB sites (Merker *et al.* 2003). Fourth, the model shown in Fig. 2.3B is consistent with our observation that increasing the strength of the *ARG4*-associated DSB (*arg4-tel* insertion) results in an increase in conversion-type repair relative to restoration-type repair (Fig. 2.1C).

This model also predicts that reduction in the strength of the *HIS4*-associated DSB might alter the ratio of conversion-type to restoration-type repair, since the frequency of repair events directed by the I nick would be reduced. The *HIS4*-associated DSB hotspot requires the binding of the transcription factors Rap1p, Bas1p, and Bas2p, and strains with either a *bas1* deletion or a mutated Rap1p binding site (*his4-51)* eliminate the DSB located upstream of *HIS4* (White *et al.* 1993, Fan *et al.* 1995). In the wild-type strain, the calculated ratio of conversion-type

FIGURE 2.3. Nick-directed mismatch repair.

A. Mismatch repair directed by the nick that initiates DSB formation (Detloff *et al.* 1992; Porter *et al.* 1993) or by the nick that is involved in resolving the recombination intermediate (Foss et al. 1999; "late repair"). Recombination is initiated by a DSB (indicated by large arrowhead) on the wild-type (blue) chromosome, and the broken ends are resected (step 1). After strand invasion, DNA synthesis extends the D-loop, resulting in a mismatch within the heteroduplex DNA (step 2). Steps 3-5 illustrate repair that is directed by the nick remaining at the initiation site (termed the "I" nick). In step 3, DNA from the I nick through the mismatch is excised. DNA synthesis subsequently fills in the gap, duplicating the mutant information (step 4). I nick-directed repair results in a gene conversion event (step 5). Steps 3' to 6' depict repair that is directed by nicks involved in resolving the Holliday junctions (the "R" nicks). In step 3' and 4', the DNA strands that have been involved in the exchange are nicked (shown by small arrowheads). In step 5', DNA is excised from the R nick through the mismatch. DNA synthesis then replicates the wild-type information (step 6'), resulting in a restoration event.

B. Repair directed by DSBs initiated in a neighboring gene. Southern analysis demonstrates that there are two preferred sites for DSB formation near *ARG4*, one immediately upstream of ARG4 and a second upstream of the neighboring *DED81* gene (Nicolas *et al.* 1989, Fan *et al.* 1995). In wild-type strains, more DSBs are generated at *DED81* than at *ARG4*, as indicated in the figure by the size of the arrows. We suggest that the majority of restoration-type repair of the *arg4-17* mismatch are the result of processing of an "R" nick of a heteroduplex initiated by the *DED81* DSB. A second source of restoration events is excision from an "R" nick of a heteroduplex initiated by the *ARG4* DSB, as shown in (A), steps 3'-6'.

A.

B.

DED81   *ARG4*
DSB   DSB

Gene Conversion

Restoration
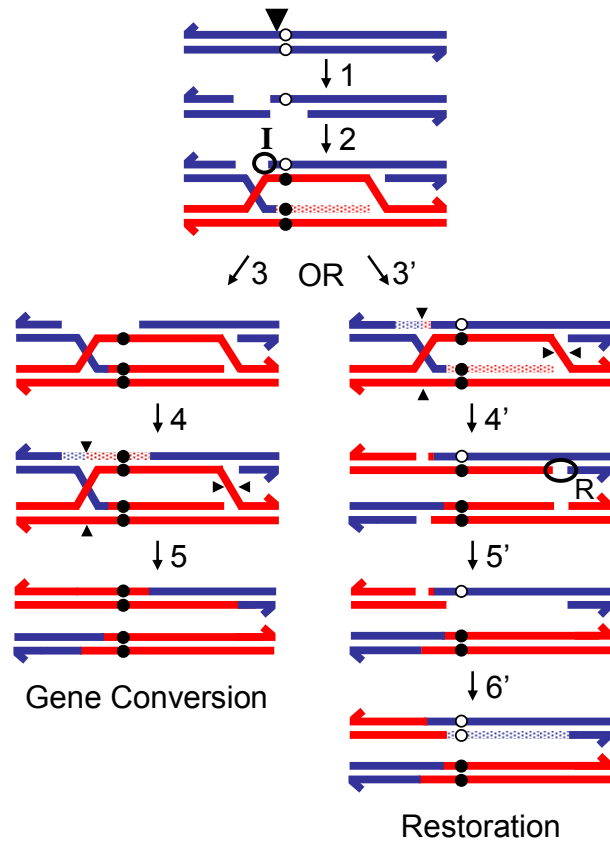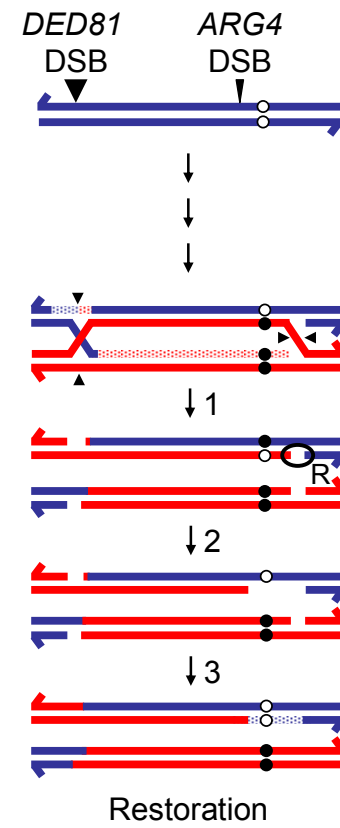
Restoration

repair to restoration-type repair is 8:1; this ratio is reduced to about 1:1 by the *bas1* deletion (Fig. 2.1D). In contrast, in the strain with the *his4-51* mutation, the calculated ratio of conversion-type to restoration-type repair was not significantly reduced.

Why does the *bas1* deletion affect the ratio of conversion-type to restoration-type repair differently than the *his4-51* mutation? As discussed above, in a MMR-deficient strain, the frequency of aberrant segregation is primarily a reflection of the frequency of heteroduplex formation at the position of the heterozygous marker, whereas, in a MMR-proficient strain, the frequency of aberrant segregation reflects the frequency of heteroduplex formation and the ratio of conversion-type to restoration-type repair. Since both the *bas1* and *his4-51* strains lack the DSB located upstream of *HIS4* that is responsible for most recombination at this locus (Fan *et al.*, 1995), the heteroduplexes that result in aberrant segregation of *his4-AAG* are a consequence of DSBs located at other sites (as shown previously in Merker *et al.* 2003). We suggest that the rate of DSB formation at these other sites and/or the distribution of I and R nicks are different in *bas1* and *his4-51* strains. Although this suggestion is somewhat *ad hoc*, the effects of Bas1p and Rap1 on local chromatin structure at the *HIS4* locus are likely to be different, since Rap1p binding is required for binding of Bas1p upstream of *HIS4*, but Bas1p binding is not required for the binding of Rap1p (Morse 2000). Given these uncertainties, the experiment in which we altered the DSB frequency upstream of *ARG4* is the more definitive study.

6. Conclusions: We found that a complex of Msh2p/Msh6p/Mlh1p/Pms1p is required for repair of base-base mismatches generated during meiotic recombination. At least two complexes initiate repair of the four-base loop: Msh2p/Msh3p/Mlh1p/Pms1p is responsible for about two-thirds of MMR and the remaining repair events are initiated by an Mlh1p-independent, Mlh3p-dependant complex. In addition, our results indicate that the rate of aberrant segregation for a single marker and the direction in which a mismatch is repaired are often influenced by DSBs located at several positions. This conclusion may be relevant to the variability in the effects of mutations catalyzing MMR observed by different researchers, working in different genetic backgrounds analyzing different loci.

TABLE 2.1. Haploid yeast strains

Strain	Relevant genotype[a]	Construction details or reference[b]
AS4-derived haploids		
DTK225	rad1::ura3	Kirkpatrick & Petes (1997)
HMY104	msh2::kanMX4	Kearney et al. (2001)
HMY105	mlh1::kanMX4	Kearney et al. (2001)
HMY106	pms1::kanMX4	Kearney et al. (2001)
HMY110	msh3::kanMX4	Kearney et al. (2001)
HMY118	exo1::kanMX4	Kearney et al. (2001)
HMY123	rad27::hisG	Kearney et al. (2001)
HMY134	msh6::kanMX4	Kearney et al. (2001)
HMY158	mlh3::kanMX4	Kearney et al. (2001)
HMY161	mlh2::kanMX4	Kearney et al. (2001)
HMY195	mlh1::hygB his4::U1.1a	Kearney et al. (2001)
HMY223	msh4::kanMX4	Kearney et al. (2001)
JSY119	his4::ura3 mlh1::hygB	5FOA[R] isolate of HMY195 (Kearney et al. 2001)

Strain	Genotype	Source
JSY125	a + pDJ173 (plasmid-borne *STP22 URA3*)	Spore colony from MC156
JSY129	a *mlh1::hygB* + pDJ173	Spore colony from JSY119 X JSY125 cross
JSY149	*mlh1::hygB mlh3::kanMX4*	Spore colony from HMY158 X JSY129 cross
JSY181	*mlh1::hygB mlh2::kanMX4*	Spore colony from HMY161 X JSY129 cross
JSY218	a *msh2::kanMX4* +pDJ173	Spore colony from HMY104 X JSY125 cross
JSY269	*arg4-tel pms1::hygB*	OST in MW81 with *pms1::hygB*, PCR primers f pms1-Δ and r pms1-Δ (Kearney *et al.* 2001), pA632 template (Goldstein & McCusker 1999)
JSY274	*mlh1::hygB pms1::kanMX4*	Spore colony from HMY106 X JSY129 cross
JSY279	*arg4-tel msh2::kanMX4*	OST in MW81 with *msh2::kanMX4*, PCR primers f msh2-Δ and r msh2-Δ (Kearney *et al.* 2001), template pFA6-*kanMX4* (Wach *et al.* 1994)
JSY293	*msh5::hygB*	OST in AS4 with *msh5::hygB*, PCR primers OL65-f*MSH5*+kan (5'ACAACTCATTCAAAATAACTTACTCATTCATTCATATACTGCCACC

Strain	Genotype	Source/Construction
JSY307	pol3-01	AAATGGAATcgtacgctgcaggtcgac) and OL66-rMSH5+kan (5'TTATTAACTTAAATATGTTACAAGTAAGCGTTTTTTATTCTT TGATATAatcgatgaattcgagctcg), pA632 template TST in AS4 with BamHI-cut YIpAM26 (Morrison et al. 1993)
JSY316	bas1::hygB	OST in AS4 with bas1::hygB, PCR primers bas1-del-F (5' CCGGTTTGCTGATTGAATCGTTCTCTGCAACAATTGTTGATC and bas1-del-R (5' TCTAGTGGGTcgtacgctgcaggtcgac) and bas1-del-R (5' GCGAGTCATGAAACTACACGTTTTTCAGAGAATATTATCCA TCTTGTGCTatcgatgaattcgagctcg), pA632 template
JSY318	bas1::hygB msh2::Tn10LUK7-7	OST in RKY1721 using primers and template described for JSY316
JSY336	his4-51 msh2::kanMX4	Spore colony from MW30 X JSY218 cross
JSY347	msh2::kanMX4 msh4::kanMX4	Spore colony from JSY218 X HMY223 cross
MW30	his4-51	White et al. (1991)
MW81	arg4-tel	White et al. (1993)
RKY1721	msh2::Tn10LUK7-7	Alani et al. (1994)

AS13-derived haploids:

Strain	Genotype	Source
HMY91	*mlh1::kanMX4 his4-AAG*	Welz-Voegele *et al.* (2002)
HMY92	*pms1::kanMX4 his4-AAG*	Welz-Voegele *et al.* (2002)
HMY131	*α*	Kearney *et al.* (2001)
JSY127	*α his4-AAG*	Spore colony from PD73 x HMY131 (Kearney *et al.* 2001) cross
JSY133	*exo1::kanMX his4-AAG*	Spore colony from HMY119 (Kearney *et al.* 2001) X JSY127 cross
JSY135	*α exo1::kanMX his4::U1.1a*	Spore colony from HMY119 (Kearney *et al.* 2001) x JSY127 cross
JSY137	*mlh3::kanMX4 his4-AAG*	Spore colony from HMY159 (Kearney *et al.* 2001) X JSY127 cross
JSY139	*mlh2::kanMX4 his4-AAG*	Spore colony from HMY162 (Kearney *et al.* 2001) X JSY127 cross
JSY143	*msh3::kanMX4 his4-AAG*	Spore colony from HMY111 (Kearney *et al.* 2001) X JSY127 cross
JSY144	*msh6::kanMX4 his4-AAG*	Spore colony from HMY135 (Kearney *et al.* 2001) X JSY127 cross
JSY151	*msh4::kanMX4 his4-AAG*	Spore colony from HMY242 (Kearney *et al.* 2001) X JSY127 cross
JSY177	*α HIS4 msh3::hygB*	Spore colony from HMY131 (Kearney *et al.* 2001) X HMY220 (Kearney *et al.* 2001) cross
JSY183	*α his4-Sal*	Spore colony from HMY131 (Kearney *et al.* 2001) X MW1 cross
JSY184	*msh3::hygB his4-Sal*	Spore colony from MW1 X JSY177 cross

Strain	Genotype	Source
JSY186	*exo1::kanMX4 his4-Sal*	Spore colony from MW1 X JSY135 cross
JSY193	*msh6::kanMX4 his4-Sal*	Spore colony from HMY135 (Kearney *et al.* 2001) X JSY183 cross
JSY195	*mlh1::hygB his4-Sal*	Spore colony from HMY196 (Kearney *et al.* 2001) X JSY183 cross
JSY196	*α mlh1::hygB his4-Sal*	Spore colony from HMY196 (Kearney *et al.* 2001) X JSY183 cross
JSY197	*mlh2::kanMX4 his4-Sal*	Spore colony from HMY162 (Kearney *et al.* 2001) X JSY183 cross
JSY206	*msh2::kanMX4 his4-Sal*	Spore colony from HMY101 (Kearney *et al.* 2001) X JSY183 cross
JSY211	*mlh3::kanMX4 his4-Sal*	Spore colony from HMY159 (Kearney *et al.* 2001) X JSY183 cross
JSY213	*α mlh3::kanMX4 his4-U1.1a*	Spore colony from HMY159 (Kearney *et al.* 2001) X JSY183 cross
JSY214	*α msh2::kanMX4 his4-AAG*	Spore colony from HMY101 (Kearney *et al.* 2001) X JSY127 cross
JSY231	*pms1::kanMX4 his4-Sal*	Spore colony from HMY103 (Kearney *et al.* 2001) X JSY183 cross
JSY234	*rad27::hisG his4-Sal*	Spore colony from HMY145 (Kearney *et al.* 2001) X JSY183 cross
JSY249	*msh4::kanMX4 his4-Sal*	Spore colony from HMY242 (Kearney *et al.* 2001) X JSY183 cross
JSY252	*rad1:ura3 his4-Sal*	Spore colony from HMY35 (Kearney *et al.* 2001) X JSY183 cross
JSY259	*mlh1::hygB mlh2::kanMX4 his4-Sal*	Spore colony from JSY197 X JSY196 cross
JSY261	*mlh1::hygB mlh3::kanMX4 his4-Sal*	Spore colony from JSY196 X JSY211 cross
JSY267	*rad27::hisG his4-Sal*	Spore colony from JSY234 X JSY213 cross

JSY270	*arg4-tel pms1::hygB*	OST in MW79 with *pms1::hygB*, PCR primers f pms1-Δ and r pms1-Δ (Kearney *et al.* 2001), pA632 template
JSY273	*mlh1::hygB pms1::kanMX4 his4-Sal*	Spore colony from JSY196 X JSY231 cross
JSY278	*arg4-tel msh2::kanMX4*	OST in MW79 with *msh2::kanMX4*, PCR primers f msh2-Δ and r msh2-Δ (Kearney *et al.* 2001), template pFA6-*kanMX4*
JSY294	*msh5::hygB his4-AAG*	OST in PD73 with *msh5::hygB*, PCR primers OL65-f*MSH5*+kan (5'ACAACTCATTCAAAATAACTTACTCATTCATATACTGCCACC AAATGGAATcgtacgctgcaggtcgac) and OL66-rMSH5+kan (5'TTATTAACTTAAATATGTTACAAGTAAGCGTTTTTTTATTCTT TGATATAatcgatgaattcgagctcg), pA632 template
JSY295	*msh5::hygB his4-Sal*	OST in MW1 with *msh5::hygB*, PCR primers OL65-f*MSH5*+kan (5'ACAACTCATTCAAAATAACTTACTCATTCATATACTGCCACC AAATGGAATcgtacgctgcaggtcgac) and OL666-rMSH5+kan (5'TTATTAACTTAAATATGTTACAAGTAAGCGTTTTTTTATTCTT

Strain	Genotype	Construction/Source
JSY308	pol3-01 his4-Sal	TST in MW1 with BamHI-cut YIpAM26 (Morrison et al. 1993)
JSY312	pol3-01 his4-AAG	TST in PD73 with BamHI-cut YIpAM26 (Morrison et al. 1993)
JSY317	bas1::hygB his4-AAG	OST in PD73 with bas1::hygB, PCR primers bas1-del-F (5' CCGGTTTGCTGATTGAATCGTTCTCTGCAACAATTGTTGATC TCTAGTGGGGTcgtacgctgcaggtcgac) and bas1-del-R (5' GCGAGTCATGAAACTACACGTTTTTTCAGAGAATATTATCCA TCTTGTGCTatcgatgaattcgagctcg), pA632 template
JSY319	bas1::hygB msh2::Tn10LUK7-7 his4-AAG	OST in RKY1452 with bas1::hygB, PCR primers bas1-del-F (5' CCGGTTTGCTGATTGAATCGTTCTCTGCAACAATTGTTGATC TCTAGTGGGGTcgtacgctgcaggtcgac) and bas1-del-R (5' GCGAGTCATGAAACTACACGTTTTTTCAGAGAATATTATCCA TCTTGTGCTatcgatgaattcgagctcg), pA632 template
JSY337	his4-51 his4-AAG	TST of PD73 with BsrGI-cut pMW35 (White et al. 1991)
JSY342	msh2::kanMX4 his4-51 his4-AAG	Spore colony from JSY214 X JSY337 cross
JSY348	msh2::kanMX4 msh4::kanMX4 his4-AAG	Spore colony from JSY214 X JSY151 cross

AAG

MW1	*his4-Sal*	Nag, White, and Petes (1989)
MW79	*arg4-tel*	White *et al.* (1993)
PD73	*his4-AAG*	Detloff *et al.* (1991)
RKY1452	*msh2::Tn10LUK7-7 his4-AAG*	Alani *et al.* (1994)

Mating-type testers:

| A364a | a *ade1 ade2 his5 lys2 ura1* | Petes & Botstein (1977) |
| 2262 | α *ade1 his5 leu2 lys1 ura1* | Petes & Botstein (1977) |

---

[a] All strains, except for mating-type testers, were derived from the haploid strains AS4 (α *arg4-17 trp1 tyr7-1 ade6 ura3*) and AS13 (a *leu2 ade6 ura3 rme1*) (Stapleton & Petes 1991) by transformation or by crosses with isogenic strains. Only those markers that are different from the haploid progenitor strains are shown.

[b] Some strains were made by one-step transplacement (OST) or two-step transplacements (TST). Strains constructed by one-step transplacements, were made using PCR fragments containing a selectable gene flanked by sequences of the gene to be deleted (Wach *et al.* 1994). For each such construction, either the primers or a reference for the primers are indicated as well as the template plasmid. Within the primer sequences, capital letters indicate genomic sequences identical to the disrupted gene and lower case letters indicate sequences identical to the selectable gene.

TABLE 2.2. Diploid yeast strains

Strain	Relevant homozygous mutations	Cross[a]
Strains heterozygous for *his4-AAG*:		
DTK224	*rad1::ura3*	TP1011 X TP1010 (Kirkpatrick & Petes 1997)
HMY95	*mlh1::kanMX4*	HMY105 X HMY91 (Welz-Voegele *et al.* 2002)
HMY96	*pms1::kanMX4*	DNY95 X HMY92 (Welz-Voegele *et al.* 2002)
JSY136	*exo1::kanMX4*	HMY118 X JSY133
JSY163	*msh3::kanMX4*	HMY110 X JSY143
JSY164	*msh6::kanMX4*	HMY134 X JSY144
JSY165	*mlh2::kanMX4*	HMY161 X JSY139
JSY178	*mlh3::kanMX4*	HMY158 X JSY137
JSY291	*msh4::kanMX4*	HMY223 X JSY151
JSY297	*msh5::hygB*	JSY293 X JSY294
JSY314	*pol3-01*	JSY307 X JSY312

JSY320	*bas1::hygB*	JSY316 X JSY317
JSY321	*bas1::hygB msh2::Tn10LUK7-7*	JSY318 X JSY319
JSY338	*his4-51*	MW30 X JSY337
JSY343	*his4-51 msh2::kanMX4*	JSY336 X JSY342
JSY349	*msh2 ::kanMX4 msh4 ::kanMX4*	JSY347 X JSY348
No. 5	*msh2::Tn10LUK7-7*	RJK1721 X RJK 1452 (Alani *et al.* 1994)
PD83	*wild-type*	AS4 X PD73 (Detloff *et. al.* 1991)

Strains heterozygous for *his4-Sal*:

JSY188	*msh3::hygB/kanMX4*	HMY110 X JSY184
JSY210	*mlh2::kanMX4*	HMY161 X JSY197
JSY216	*mlh3::kanMX4*	HMY158 X JSY211
JSY217	*msh6::kanMX4*	HMY134 X JSY193
JSY230	*mlh1::hygB/kanMX4*	HMY105 X JSY195
JSY233	*pms1::kanMX4*	HMY106 X JSY231
JSY239	*rad27::hisG*	HMY123 X JSY267

JSY240	*msh2::kanMX4*	HMY104 X JSY206
JSY255	*rad1:ura3*	DTK225 X JSY252
JSY277	*mlh1::hygB pms1::kanMX4*	JSY274 X JSY273
JSY283	*mlh1::hygB mlh3::kanMX4*	JSY149 X JSY261
JSY284	*exo1::kanMX4*	HMY118 X JSY186
JSY285	*msh4::kanMX4*	HMY223 X JSY249
JSY292	*mlh1::hygB mlh2::kanMX4*	JSY181 X JSY259
JSY296	*msh5::hygB*	JSY293 X JSY295
JSY313	*pol3-01*	JSY307 X JSY308
MW103	wild-type	AS4 X MW1 (Nag *et al.* 1989)

Other diploids:

JSY276	*arg4-tel pms1::hygB*	JSY269 x JSY270
JSY282	*arg4-tel msh2::kanMX4*	JSY279 X JSY278
MW160	*arg4-tel*	MW81 X MW79 (White *et al.* 1993)
MC153	wild-type	AS4 X AS4 (created by inducing *HO* expression on

the plasmid p*GAL-HO* (Herskowitz & Jensen 1991)

in AS4 and then selecting for a diploid that had lost

the plasmid)

MC156   wild-type +pDJ173, plasmid-borne   MW153, carrying pDJ173 (Li *et al.* 1999)

STP22 URA3

[a] Genotypes of the haploids used in the constructions are given in Table 1. In crosses, AS4- and AS13-

derived strains are shown on the left and the right of the X, respectively.

TABLE 2.3. Meiotic segregation patterns for the *his4-AAG* marker

Strain	Relevant genotype	# Tetrads	Ab. seg.[a] (%)	PMS[b] (%)	PMS/ab. events[c] (%)	% of total tetrads				Other
						6:2	2:6	5:3	3:5	ab.[d]
PD83[e]	wild-type	482	58	10	18	15	22	5	4	11
No. 5[f]	*msh2Δ*	111	64	55	90[i]	5	3	14	23	19
JSY163	*msh3Δ*	253	59	10	18	18	18	4	3	17
JSY291	*msh4Δ*	95	41[g]	7	21	19	12	2	3	5
JSY297	*msh5Δ*	119	47[g]	9	18	14	20	2	8	3
JSY164	*msh6Δ*	208	57	46	85[i]	5	4	16	16	16
JSY349	*msh2Δ msh4Δ*	198	45[g]	40	91[i]	4	1	16	13	12
HMY95[e]	*mlh1Δ*	465	57	49	90[i]	4	3	18	15	17
JSY165	*mlh2Δ*	194	42[g]	8	23	12	18	4	3	6
JSY178	*mlh3Δ*	155	65	11	18	17	22	8	3	16
HMY96[e]	*pms1Δ*	641	59	43	78[i]	5	6	15	16	16

Strain	Genotype								
JSY136	*exo1Δ*	209	30[g]	9	30	9	10	3	5	3
JSY314	*pol3-01*	245	38[g]	2	9[i]	17	15	1	1	4
DTK224[f]	*rad1Δ*	220	64[g]	13	21	19	22	6	5	12
JSY320	*bas1Δ*	285	11[g]	3	26	4	5	2	1	0
JSY321	*bas1Δ msh2Δ*	220	21[g, h]	16	77[i]	3	2	7	7	2
JSY338	*his4-51*	321	13[g]	3	26	3	7	1	1	1
JSY343	*his4-51 msh2Δ*	316	15[g]	12	81[i]	1	2	5	5	2

All diploids were made by batch mating (described in Materials and Methods) except DTK224, which is a purified diploid. All strains were sporulated at 18°.

[a] Percentage of total tetrads with aberrant (non-4:4) segregation.

[b] Percentage of total tetrads with one or more PMS events, excluding tetrads with one gene conversion event and one PMS event (7:1, 1:7).

[c] Percentage of aberrant events that are PMS events, calculated as described in the text.

[d] This class includes tetrads with two or more PMS (ab4:4, ab6:2, ab2:6, dev5:3, dev3:5, dev4:4) events, one PMS event and one conversion event (7:1, 1:7), and two conversion events (8:0, 0:8).

[e] Data from Welz-Voegle et al. (2002).

[f] Data from Kirkpatrick and Petes (1997).

[g] Frequency of aberrant segregation significantly different from wild-type (PD83). The p values were determined using two-tailed Fisher exact tests. The p values for each of the 17 comparisons were ranked and the Benjamini & Hochberg (1995) procedure was applied using an initial α=0.05. p values less than 0.032 were considered significant.

[h] Frequency of aberrant segregation in JSY321 (bas1Δ msh2Δ) significantly increased compared to the bas1Δ strain (JSY320). A one-tailed Fisher exact test was used to determine the p value of 0.0041.

[i] Significant difference in PMS events relative to gene conversion events compared to the wild-type strain (PD83). The p values were determined using two-tailed Fisher exact tests. The p values for each of the 17 comparisons were ranked and the Benjamini & Hochberg (1995) procedure was applied using an initial α=0.05. p values less than 0.024 were considered significant.

TABLE 2.4. Meiotic segregation patterns for the *arg4-17* marker

Strain(s)	Relevant genotype	# Tetrads	Ab. seg. (%)	PMS (%)	PMS/ab. events (%)	% of total tetrads				Other ab.
						6:2	2:6	5:3	3:5	
MW103 / JSY338[a]	wild-type	742	8	0	2	4	3	0	0	1
JSY240 / JSY343[a]	*msh2Δ*	502	16[d]	11	67[e]	4	1	5	4	2
JSY188	*msh3Δ*	167	8	0	0	5	4	0	0	0
JSY285	*msh4Δ*	224	5	0	0	4	2	0	0	0
JSY296	*msh5Δ*	155	6	0	0	3	3	0	0	0
JSY217	*msh6Δ*	182	17[d]	5	61[e]	5	1	7	2	2
JSY349	*msh2Δ msh4Δ*	198	17[d]	13	83[e]	4	1	4	8	2
JSY230	*mlh1Δ*	193	13[d]	7	50[e]	4	2	3	4	1
JSY210	*mlh2Δ*	190	8	0	0	5	3	0	0	0
JSY216	*mlh3Δ*	219	10	0	0	7	2	0	0	0
JSY233	*pms1Δ*	159	9	4	43[e]	3	3	3	1	0

82

Strain	Genotype									
JSY277	*mlh1Δ pms1Δ*	383	14[d]	8	54[e]	4	3	3	4	1
JSY292	*mlh1Δ mlh2Δ*	181	12	4	29[e]	2	6	2	1	1
JSY283	*mlh1Δ mlh3Δ*	388	15[d]	9	63[e]	4	2	3	6	0
JSY284	*exo1Δ*	196	4	0	0	1	3	0	0	0
JSY313	*pol3-01*	173	9	0	0	5	2	0	0	1
JSY255	*rad1Δ*	202	11	1	4	5	3	0	0	1
JSY239	*rad27Δ*	242	8	0	0	3	5	0	0	0
MW160	*arg4-tel*[b]	291	45[d]	1	1	24	18	0	1	3
JSY282	*arg4-tel*[b] *msh2Δ*	276	51[d]	40	81[e]	8	2	16	17	8
JSY276	*arg4-tel*[b] *pms1Δ*	252	46[d]	28	64[e]	12	5	13	12	5
JSY320	*bas1Δ*	285	6	0	0	3	3	0	0	0
JSY321	*bas1Δ msh2Δ*	220	23[d]	14	57[e]	6	2	8	5	1

All diploids were made by batch mating and were sporulated at 18°. The column headings have the same definitions as those of Table 3.

[a] Data from these two strains were pooled. The two strains are isogenic except for the *his4-51* mutation, which has no significant effect on segregation of the *arg4-17* marker.

[c] *arg4-tel* is a homozygous insertion of 60bp of telomeric sequence upstream of *ARG4*.

[d] Frequency of aberrant segregation significantly different from the wild-type strains MW103 and JSY338. The p values were determined using two-tailed Fisher exact tests. The p values for each of the 22 comparisons were ranked and the Benjamini & Hochberg (1995) procedure was applied using an initial α=0.05. p values less than 0.023 were considered significant.

[e] Significant increase in PMS events relative to gene conversion events compared to wild-type MW103 and JSY338. The p values were determined using one-tailed Fisher exact tests. The p values for each of the 22 comparisons were ranked and the Benjamini & Hochberg (1995) procedure was applied using an initial α=0.05. p values less than 0.025 were considered significant.

TABLE 2.5. Meiotic segregation patterns for the *his4-Sal* marker

Strain	Relevant genotype	# Tetrads	Ab. seg. (%)	PMS (%)	PMS/ab. events (%)	% of total tetrads				Other ab.
						6:2	2:6	5:3	3:5	
MW103	wild-type	421	25	0	0	10	13	0	0	3
JSY240	msh2Δ	186	37	30	84[b]	4	3	12	11	6
JSY188	msh3Δ	167	47[a]	34	76[b]	9	2	16	11	9
JSY285	msh4Δ	224	17[a]	0	0	8	9	0	0	0
JSY296	msh5Δ	155	22	0	0	7	15	0	0	1
JSY217	msh6Δ	182	26	0	0	12	12	0	0	2
JSY230	mlh1Δ	193	37	19	54[b,c]	14	2	10	6	5
JSY210	mlh2Δ	190	23	0	0	7	13	0	0	3
JSY216	mlh3Δ	219	27	7	28[b,c,d]	11	7	3	4	2
JSY233	pms1Δ	159	31	18	59[b,c]	7	4	8	8	3
JSY277	mlh1Δ pms1Δ	383	25	16	67[b]	6	3	9	6	2

JSY292	*mlh1Δ mlh2Δ*	181	28	15	55[b]	9	3	7	7	1
JSY283	*mlh1Δ mlh3Δ*	388	30	21	70[b, e]	6	2	11	8	3
JSY284	*exo1Δ*	196	19[a]	1	3	10	8	0	1	1
JSY313	*pol3-01*	173	17[a]	0	0	7	9	0	0	1
JSY255	*rad1Δ*	202	23	0	0	8	15	0	0	1
JSY239	*rad27Δ*	242	21[a]	0	0	10	11	0	0	0

All diploids were made by batch mating and were sporulated at 18°. The column headings have the same definitions as those of Table 3.

[a] Frequency of aberrant segregation significantly different from wild-type (MW103). The p values were determined using two-tailed Fisher exact tests. The p values for each of the 16 comparisons were ranked and the Benjamini & Hochberg (1995) procedure was applied using an initial $\alpha=0.05$. p values less than 0.0125 were considered significant.

[b] Significant increase in PMS events relative to gene conversion events compared to the wild-type strain (MW103). The p values were determined using one-tailed Fisher exact tests. The p values for each of the 16 comparisons were ranked and the Benjamini & Hochberg (1995) procedure was applied using an initial $\alpha=0.05$. p values less than 0.025 were considered significant.

[c] Significant decrease in PMS events relative to gene conversion events compared to the *msh2Δ* strain (JSY240). The p values were determined using one-tailed Fisher exact tests. The p values for three comparisons (JSY240 versus JSY230, JSY216, and JSY233) were ranked and the Benjamini & Hochberg (1995) procedure was applied using an initial α=0.05. p values less than 0.05 were considered significant.

[d] Significant decrease in PMS events relative to gene conversion events compared to the *mlh1Δ* strain (JSY230) and to the *pms1Δ* (JSY233) strain. The p values were determined using one-tailed Fisher exact tests. The p values for two comparisons were ranked and the Benjamini & Hochberg (1995) procedure was applied using an initial α=0.05. p values less than 0.05 were considered significant.

[e] Significant increase in PMS events relative to gene conversion events compared to the *mlh1Δ* strain (JSY230). These events were compared by one-tailed Fisher exact tests using data from the strains JSY277 (*mlh1Δ pms1Δ*), JSY292 (*mlh1Δ mlh2Δ*), and JSY283 (*mlh1Δ mlh3Δ*). The p values for the resulting three comparisons were ranked and the Benjamini & Hochberg (1995) procedure was applied using an initial α=0.05. p values less than 0.017 were considered significant.

87

TABLE 2.6. Genetic distances in three genetic intervals on chromosome III

Strain(s)[a]	Relevant Genotype	HIS4-LEU2				LEU2-CEN3				CEN3-MAT			
		P	N	T	CM[b]	P	N	T	CM[c]	P	N	T	CM[c]
PD83 / MW103	wild-type	286	8	168	24	358	338	165	10	209	185	364	24
JSY240	msh2Δ	73	3	37	24	70	74	33	9	40	45	82	25
JSY163 / JSY188	msh3Δ	104	4	81	28	157	162	78	10	91	95	168	24
JSY291 / JSY285	msh4Δ	172	2	49	14[d]	129	113	47	8	107	112	88	14[f]
JSY297 / JSY296	msh5Δ	141	0	31	9[d]	112	106	40	8	106	99	55	11[f]
JSY164 / JSY216	msh6Δ	120	3	87	25	132	161	78	11	70	82	154	25
JSY349	msh2Δ msh4Δ	90	1	17	11[d]	81	83	33	8	76	71	40	11[f]
HMY95 / JSY230	mlh1Δ	220	3	77	16[d]	261	258	125	10	142	155	121	14[f]
JSY165 / JSY210	mlh2Δ	155	5	87	24	138	168	61	8	97	108	162	22
JSY178 / JSY216	mlh3Δ	133	4	81	24	168	155	50	7	148	123	140	17[f]
HMY96 / JSY233	pms1Δ	226	1	128	19	325	336	117	8	136	116	192	22
JSY277	mlh1Δ pms1Δ	210	0	70	13[d]	159	147	62	8	127	102	108	16[f]

Strain	Genotype											
JSY292	*mlh1Δ mlh2Δ*	99	0	25	10[d]	60	75	33	10	53	75	46	13[f]
JSY283	*mlh1Δ mlh3Δ*	180	3	74	18	130	163	70	10	120	142	117	15[f]
JSY136 / JSY284	*exo1Δ*	223	2	69	14[d]	170	166	30	4[e]	132	139	84	12[f]
JSY314 / JSY313	*pol3-01*	85	5	43	27	55	63	38	12	35	43	77	25
JSY255	*rad1Δ*	82	7	56	34	85	91	23	6	74	46	79	20
JSY239	*rad27Δ*	127	2	54	18	96	93	39	9	36	36	77	26
JSY320	*bas1Δ*	163	1	63	15	93	95	54	11	67	75	110	22
JSY321	*bas1Δ msh2Δ*	108	1	45	17	77	78	41	11	55	47	86	23
JSY338	*his4-51*	195	5	65	18[d]	130	109	53	9	101	69	135	22
JSY343	*his4-51 msh2Δ*	184	11	102	29	131	100	74	12	78	70	151	25

P, N, and T indicate parental ditype, nonparental ditype, and tetratype tetrads, respectively. We included only those tetrads in which both flanking markers had Mendelian segregation.

[a] For some intervals, data from multiple strains were pooled. These strains were isogenic, except for the specific mutations at the *HIS4* locus.

[b] Calculated by the equations of Perkins (1949).

[c] Calculated as the percentage of second-division segregation (tetratype tetrads) divided by two. First-division

segregation (FDS) and second-division segregation (SDS) tetrads were determined using the heterozygous centromere-linked *trp1* marker.

[d] Significant difference compared to the wild-type strain (PD83) in the numbers of PD:NPD:T tetrads. The p values were determined using Chi-square analysis. The p values for each of the 21 comparisons were ranked and the Benjamini & Hochberg (1995) procedure was applied using an initial α=0.05. p values less than 0.019 were considered significant.

[e] Significant difference compared to the wild-type strain (PD83) in the numbers of FDS: SDS tetrads for the *LEU2-CEN3* interval. The p values were determined using two-tailed Fisher exact tests. The p values for 21 comparisons were ranked and the Benjamini & Hochberg (1995) procedure was applied using an initial α=0.05. p values less than 0.002 were considered significant.

[f] Significant difference compared to the wild-type strain (PD83) in the numbers of FDS:SDS tetrads for the *CEN3-MAT* interval. The p values were determined using two-tailed Fisher exact tests. The p values for 21 comparisons were ranked and the Benjamini & Hochberg (1995) procedure was applied using an initial α=0.05. p values less than 0.021 were considered significant.

III. INVOLVEMENT OF PCNA IN MISMATCH REPAIR

A. Introduction

The mismatch repair (MMR) proteins play a number of roles that are critical for maintaining genome stability. First, MMR corrects DNA mismatches, which can be created by DNA replication errors (reviewed by Modrich 1991) or by recombination between sequences that are not completely identical (reviewed by Petes *et al.* 1991, Borts *et al.* 2000, Surtees *et al.* 2004). MMR proteins are also involved in preventing recombination between diverged sequences; this is known as the "anti-recombination" function (reviewed by Harfe & Jinks-Robertson 2000b). A few MMR-related proteins are involved in the processing of meiotic recombination intermediates that resolve as crossovers (reviewed by Hoffmann & Borts 2004). Finally, MMR has been linked to DNA damage checkpoint regulation in mammals (reviewed by Stojic *et al.* 2004); however, a similar function has not been described in yeast.

The repair of replication errors (the MMR "spellchecker" function) in *E. coli* and eukaryotes consists of the following steps: 1) recognition of the mismatch, 2) identification of the newly-synthesized strand that contains the mismatch (strand discrimination), 3) creation or recognition of nick on the newly synthesized strand, 4) unwinding and excision of DNA from the nick through the mispaired DNA, and 5) DNA synthesis and ligation to fill in the gap (reviewed by Modrich 1991). In *S.*

cerevisiae, repair of base-base mismatches is initiated by Msh2p/Msh6p and

Mlh1p/Pms1p heterodimers, while repair of 1-14bp loops is initiated by

Msh2p/Msh3p and Mlh1p/Pms1p heterodimers (reviewed by Harfe & Jinks-

Robertson 2000a). Strand discrimination and subsequent processing steps are not

as well understood in yeast and higher eukaryotes as in *E. coli*, although nick-

directed MMR has recently been reconstituted *in vitro* from extracts of human cells

(Dzantiev *et al.* 2004, Constantin *et al.* 2005, Zhang *et al.* 2005). Excision directed

by a strand break located 5' of a mismatch requires hMsh2p/hMsh6p, RPA, and the

hExo1p nuclease, while excision from a 3' strand break requires the addition of

hMlh1p/hPms2p and loading of replication protein PCNA (proliferating cell nuclear

antigen) by RFC (replication factor C) (Dzantiev *et al.* 2004, Constantin *et al.* 2005).

These data support proposals that interactions between MMR proteins and

replication machinery may facilitate strand discrimination and coordinate

downstream processes.

  PCNA is a replication factor that forms a homotrimeric ring around DNA (Krishna

et al. 1994). PCNA serves as a loading dock for DNA polymerase δ, and

subsequently provides the stability required for processive DNA replication

(reviewed by Johnson & O'Donnell 2005). Defects in processivity cause the

polymerase to slip off the template frequently and this slippage can result in insertion

or deletion errors in simple repetitive DNA sequences (microsatellites) because the

newly-synthesized DNA can misalign during the reannealing of the primer to the

template. In addition to its role in replication, PCNA coordinates interactions

between the replication machinery and a number of other cellular processes that

affect cell growth and genome stability, including MMR, nucleotide excision repair, base excision repair, translesion DNA synthesis, cell cycle regulation, and chromatin remodeling (reviewed by Maga & Hubscher 2003).

PCNA was originally identified as an MMR-associated protein in a yeast two-hybrid screen for proteins that interact with Mlh1p and Msh2p (Umar *et al.* 1996). *In vitro* studies indicated that PCNA has a role in MMR both before and during DNA re-synthesis (Umar *et al.* 1996). These data led to the proposal that PCNA may serve to couple replication and MMR and that this coupling may facilitate the strand discrimination step of MMR. It has since been suggested that PCNA also has a role in mismatch recognition, as the mismatch-binding capacity of Msh2p/Msh6p is enhanced by PCNA (Flores-Rozas *et al.* 2000) and PCNA can transfer Msh2p/Msh6p to mismatched DNA (Lau & Kolodner 2003). The primary interaction between PCNA and MMR machinery occurs via PCNA-binding motifs located at the N-termini of Msh3p and Msh6p (Clark *et al.* 2000). Mutations in these PCNA-interacting motifs result in weak mutator phenotypes and eliminate the physical interactions of these proteins with PCNA (Clark *et al.* 2000). Mutations in *POL30*, which encodes PCNA in *S. cerevisiae*, cause moderate to strong mutator phenotypes (Ayyagari *et al.* 1995, Umar *et al.* 1996, Chen *et al.* 1999, Lau *et al.* 2002). The *pol30-52* mutation destabilized microsatellites with small (1-7 bp) and large (≥8bp) repeat units, indicating a MMR defect (which leads to increased instability of microsatellites with small repeats) and increased DNA polymerase slippage (which leads to increased instability of microsatellites with larger repeats)

(Kokoska *et al.* 1999). The two effects are separable since *pol30* mutants have been identified that affect MMR, but not replication (Lau *et al.* 2002).

In the study summarized in chapter II, I found that repair of meiotic base-base mismatches and four-base loops are similar to the repair of replication errors in terms of the MutS and MutL homologues required for the repair of each substrate. Since PCNA is thought to have a role in the repair of replication errors at a step before re-synthesis of the gap resulting from excision of the mismatch (Umar *et al.* 1996), PCNA also may be required for recombination-associated MMR processes. I have examined two *pol30* mutants, *pol30-52* and *pol30-201*, for repair of base-base mismatches created during meiotic recombination (termed "meiotic MMR"). In order to assay the anti-recombination function of MMR, our collaborators examined the effects of the *pol30* mutants on homologous and homeologous mitotic recombination substrates. Our work suggests that PCNA is involved in both meiotic MMR and the anti-recombination function of MMR. In addition, I examined the effect of lowering the levels of DNA polymerase δ on the length of the gene conversion track.

B. Materials and Methods

1. Yeast strains. Diploid strains used in meiotic recombination studies were constructed by mating isogenic haploid derivatives of AS4 (*MATα arg4-17 trp1 tyr7 ade6 ura3*) and AS13 (*MATa leu2 ade6 ura3*) (Stapleton & Petes 1991). Genotypes and references for each strain can be found in Table 3.1. Strains were constructed by transformation or by crosses with isogenic strains. All transformants were confirmed by PCR analysis and all *pol30-201* mutations were confirmed by

sequencing. *pol30-52* strains were made by two-step transplacement with *Eco*RI-cut pBL241-52 (Ayyagari *et al.* 1995). *pol30-201.LEU2* strains were made by one-step transplacement with the *POL30*-containing *Sac*I fragment of pRDK925 (Lau *et al.* 2002). *ARG4* derivatives of AS4-derived strains were made by one-step transplacement with a 2.4kb *Sal*I fragment of pMW52 (White *et al.* 1993).

R. J. Kokoska created the *kanMX6-GALI1-(3xHA)-POL3* strains by one-step transplacement with the pFA6a-kanMX6-PGAL1-3HA cassette, as described previously (Kokoska *et al.* 2000). *(3xHA)-POL3* strains were made by one-step transplacement with a DNA fragment which was generated by amplification of genomic DNA from RJK394 (Kokoska *et al.* 2000) with primers POL3r+578 (5'-GAATCAATAGCGTGGTCAATT) and POL3f-286 (5'-GCAGTTCGATCTGGTGTTCTCC).

2. Genetic Techniques. Standard growth conditions and genetic techniques were used (Sherman *et al.* 1982), except where indicated. Some of the mutations used in our study are known to have a mutator phenotype that causes high levels of spore inviability. To maximize the number of viable spores in such strains, we constructed diploids by mating haploid strains overnight at 30° C and transferring the resulting diploids to sporulation medium without purifying the diploids. Strains were sporulated at either 18° C or 30° C, as indicated in the tables. Following tetrad dissection onto places containing rich growth medium (YPD), spore colonies were replica plated to various omission media (SD) to check the segregation of various markers. Spore colonies on histidine omission media and arginine omission media were examined microscopically. Spore colonies were checked for mating type by

95

replica-plating to *a* or α tester strains (A364a and 2262, respectively) on YPD plates. After incubation overnight, YPD plates were replica plated to adenine omission media to select for diploids. Since A364a and 2262 have *ade1* and *ade2* mutations and AS4 and AS13 have an *ade6* mutation, diploids formed between haploid strains derived from these two genotypes can grow in the absence of adenine.

Strains containing *POL3* under the control of the *GAL1/10* promoter were maintained and dissected on YPR medium (1% Bacto Yeast Extract, 1% Bacto Peptone, 3% raffinose) containing 0.05% galactose. These strains were sporulated on plates containing 1% potassium acetate, 0.0005% adenine, and 0.005% galactose plus 0.045% raffinose, 0.05% galactose, or 0.5% galactose. Omission media used to check spore colonies contained 3% raffinose and 0.05% galactose instead of glucose.

3. Statistical Analysis. All comparisons were performed by the Fisher exact test or by Chi-square analysis (for comparisons of more than two measurements or for comparisons with numbers too high for the Fisher exact test). Mutation rates and mitotic recombination rates were calculated using the method of median (Lea & Coulson 1949).

C. Results

This section is divided into the following sections: 1) the effect of the *pol30-52* mutation on the mutation rate and spectrum of mutations at the *CAN1* locus, 2) the effect of the *pol30-201* mutation on the mutation rate at the *CAN1* locus, 3) the effect of the *pol30* mutations on the anti-recombination MMR function, 4) a description of

the meiotic recombination system, 5) the effect of the *pol30* mutations on meiotic

MMR, 6) the effect of the *pol30* mutations on the frequency of crossovers, and 7) the

effect of the level of DNA polymerase δ on meiotic recombination.

1. *pol30-52* results in a very strong mutator phenotype. Previous studies

showed that the *pol30-52* allele results in a strong mutator phenotype and elevates

the instability of minisatellites and microsatellites (Umar *et al.* 1996, Kokoska *et al.*

1999, Chen *et al.* 1999).   In these studies, the effect of *pol30-5*2 was generally

similar or slightly less than that observed with *msh2*. In my studies, however, I found

that In AS4- and AS13-derived haploids, *CAN1* mutation rates for *pol30-52* mutants

were three-fold to six-fold higher than those observed for *msh2* mutants (Table 3.2).

As *msh2* strains should be completely devoid of MMR activity, these data

demonstrate that *pol30-52* is affecting another pathway that can generate *can1*

mutations. In order to test this hypothesis, I constructed an AS13-derived *pol30-52*

msh2 double mutant strain. The *pol30-52 msh2* strain had a *CAN1* mutation rate

that was four-fold higher than that of the *pol30-52* mutant. Thus, the *pol30-52*

elevates mutation rates in two ways, by reducing the efficiency of MMR and by

elevating mutation rates by an MMR-independent mechanism.

The most likely MMR-independent mechanism to generate elevated mutation

rates in *pol30* mutants is a partial defect in DNA replication. Such a possibility has

been previously suggested based on the observation that *pol30-52* mutants are

sensitive to hydroxyurea (which inhibits DNA replication), and DNA damaging

agents, such as ultraviolet light and methyl-methanesulfanate (Ayyagari *et al.* 1995,

Lau *et al.* 2002). An elevated rate of replication errors would probably result in

97

increased rates of base substitutions or small deletions/additions within short homopolymeric tracts within the *CAN1* gene. However, since our mutation rate assay detects all types of mutations that affect the expression of the *CAN1* gene, it is also possible that strong mutator phenotype observed in our *pol30-52* strains is a consequence of gross chromosomal rearrangements (deletions or translocations). In order to determine the types of changes associated with the *pol30-52* mutation, Adam Cheely, a rotating graduate student in the lab, sequenced 17 canavanine-resistant isolates derived from an AS13 *pol30-52* mutant. Of the *can1* mutations he identified, most were one-base insertions or deletions in homopolymeric runs and a few were base substitutions (Table 3.3). This result is similar to the *can1* mutation spectras observed for both *pol30-52* and *msh2* mutants in the S288C strain background (Tishkoff *et al.* 1997b, Chen *et al.* 1999). These data are consistent with the hypothesis that *pol30-52* causes an increase in DNA polymerase slippage events, resulting in insertions and deletions in repetitive sequences.

While the *pol30-52* mutation has been previously suggested to cause a replication defect in addition to an MMR defect (Chen *et al.* 1999, Lau *et al.* 2002), the replication defect appears to be much more severe in the AS4/AS13 genetic background than in other genetic backgrounds. This increase in polymerase slippage could be due to strain-specific differences in PCNA or other replication-related proteins. Sequencing of the *POL30* ORF from both AS4 and AS13 strains revealed only silent mutations compared with the *POL30* sequence from the S288C background. Specifically, AS4 has two silent polymorphisms in *POL30*: C171T and

A267G (R. Gealy and S. Jinks-Robertson, unpublished data). We have not
sequenced other components of the replication machinery.

    2. *pol30-201* has a weaker mutator phenotype than *pol30-52.* Lau *et al.*
(2002) identified *pol30-201* as a mutant that is defective for MMR, but does not
affect DNA replication. Interestingly, the spectrum of mutation observed in a *pol30-
201* strain was closer to that found in wild-type strains than in MMR-deficient strains;
pol30-201 causes a slight bias for base-substitution errors over frameshifts (Lau *et
al.* 2002). In an AS13-derived haploid, *pol30-201* resulted in a mutation rate that is
slightly less than that of an *msh2* mutant (Table 3.2). We found that the mutation
rate in a *pol30-201 msh2* double mutant is additive relative to the *msh2* and *pol30-
201* single mutants. These data contradict the previous report that the double
mutant does not have an increased mutation rate relative to the *msh2* single mutant
in the S288C background (Lau *et al.* 2002). The difference between the two genetic
backgrounds is surprising because AS13 is closely related to S288C (Stapleton &
Petes 1991).

    3. The "anti-recombination" MMR function is reduced by *pol30* mutants.
Our collaborators, Regan Gealy and Sue Jinks-Robertson, have assayed *pol30*
mutants for their effects on the anti-recombination function of MMR. The system
used to assay the anti-recombination function is illustrated in Figure 3.1 and has
been previously described (Nicholson *et al.* 2000). In a wild-type cell, the MMR anti-
recombination function prevents mitotic recombination between homeologous
(related but non-identical) sequences but allows mitotic recombination between
identical sequences. Mutations that affect the recognition of mismatches increase

99
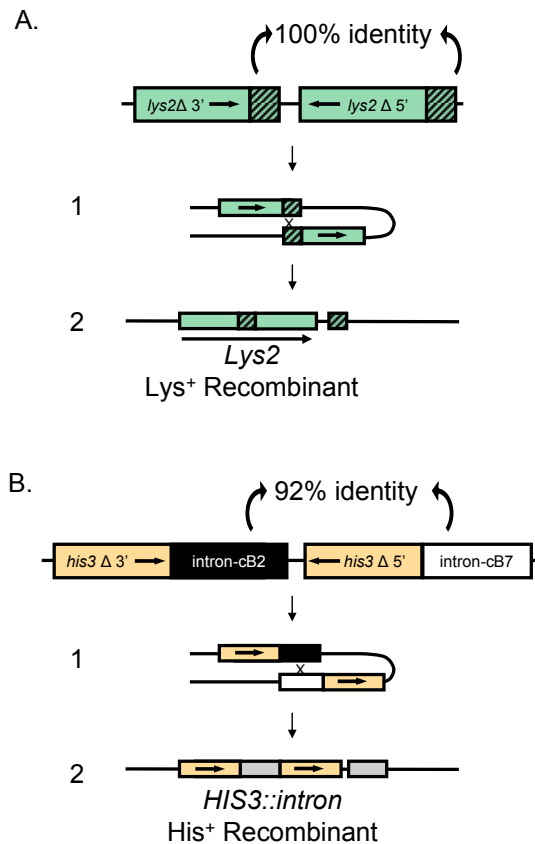
FIGURE 3.1. Mitotic recombination system. (A) The homologous *LYS2::intron* substrate has inverted repeats with 100% sequence identity. (B) The homeologous *HIS3::intron* substrate has inverted repeats with 92% sequence identity (and thus will contain 8% mismatches if recombination occurs). In step 1, mitotic recombination occurs the between the inverted repeats. In step 2, mitotic recombination has re-oriented the sequences between the repeats and this re-orientation restores the function of the intervening auxotrophic marker. Colonies in which mitotic recombination has occurred can be selected using the appropriate medium.

the recombination rate for the homeologous substrate, which contains 8% base-base mismatches, but have no effect on the recombination of the homologous substrate, which contains no mismatches. Thus, the efficiency of the anti-recombination function of MMR can be measured by examining the ratio of the homologous to homeologous recombination rates. Finally, it should be noted that the strains used in the anti-recombination study are derived from a genetic background that is different than the one used in my mutation rate and meiotic recombination assays.

When the rate of homeologous recombination is normalized to the rate of homologous recombination, homeologous recombination is elevated two-fold and three-fold in *pol30-52* and *pol30-201* strains, respectively (Table 3.4). These effects are minimal when compared to the 17-fold elevation observed in the *msh3 msh6* double mutant, which is completely MMR-deficient. These data suggest that PCNA is involved in the anti-recombination function of MMR, but that requirement for PCNA is not absolute.

4. Genetic system used to examine meiotic MMR. Meiotic recombination is initiated by a double-strand break (DSB) and subsequent formation of heteroduplex DNA creates mismatches if the DNA sequences of the homologues are not completely identical. To examine the effects of *pol30* mutants on the repair of mismatches created during meiotic recombination, we used isogenic AS4/AS13 diploid strains heterozygous for both *his4-AAG* and *arg4-17*. The *his4-AAG* mutation is a T-to-A substitution at the second position of the *HIS4* start codon (Detloff *et al.* 1991). If heteroduplexes are formed between DNA strands with the

wild-type and *his4-AAG* sequences, an A/A or a T/T mismatch is created (Fig. 3.2).

Similarly, the *arg4-17* mutation is a T-to-A substitution at position +127 of the *ARG4*

coding sequence and also results in A/A or T/T mismatches in heteroduplex DNA

(White *et al.* 1985).

The A/A mismatch shown on the left side of Fig. 3.2 can be repaired in one of

two ways. First, the mutant strand can be used as a template for repair, resulting in

a gene conversion event with one His$^+$ spores and three His$^-$ spores (2:6, using the

nomenclature for eight-spored fungi). Alternatively, the wild-type strand can be used

as a template for repair, resulting in a restoration of Mendelian segregation. If an

A/A mismatch persists through meiosis without being repaired, one spore will

receive both mutant and wild-type information. Such a spore would form a sectored

His$^+$/His$^-$ spore colony, which reflects a post-meiotic segregation (PMS). A tetrad

that results in one His$^+$ spore colony, two His$^-$ spore colonies, and one sectored

His$^+$/His$^-$ spore colony is termed a 3:5 event. Alternatively, if the recombination

event is initiated by a DSB upstream of the *his4-AAG* allele, a T/T mismatch is

created. Repair of this T/T mismatch will result in either a 6:2 gene conversion or

restoration of Mendelian segregation. Failure to repair the T/T mismatch would lead

to a 3:5 PMS event.

Microarray technology has been used to study the levels of nearby meiotic DSBs

for each of the 6000 *S. cerevisiae* ORFs in our genetic background. The DSB

hotspot immediately upstream of *HIS4* was found to be the second hottest DSB site

in the entire yeast genome, while the hotspot upstream of *ARG4* was found to be the

134th hottest (Gerton *et al.* 2000). Thus, while the *HIS4* and *ARG4* loci both

FIGURE 3.2. Segregation patterns resulting from repair or lack of repair of a meiotic mismatch. This figure shows a modified form of the double-strand break (DSB) repair model (Szostak *et al.* 1983, Sun *et al.* 1991) in which recombination is initiated by a DSB (indicated by the black triangle) on the wild-type chromosome (left side of figure) or the mutant chromosome (right side of figure). 3' ends of chromosomes are flagged. Open and closed circles represent wild-type and mutant alleles, respectively. The specific mutation is a T-to-A substitution. After DSB formation (step 1), the broken ends are resected leaving 3' ended single-stranded tails (step 2). A limited amount of strand invasion occurs, followed by new DNA synthesis (indicated with stippled lines) that displaces a "D loop" (step 3). The heteroduplex DNA formed when the D loop is displaced contains a mismatch, as indicated by the pairing of open and closed circles. Failure to repair the mismatch results in a 3:5 PMS event (if the initiating DSB occurred on the wild-type chromosome) or a 5:3 PMS event (if the initiating DSB occurred on the mutant chromosome). Repair of this mismatch can result in a 2:6 gene conversion (if the initiating DSB occurred on the wild-type chromosome), a 6:2 gene conversion (if the initiating DSB occurred on the mutant chromosome), or restoration of Mendelian segregation (4:4).

1

2

3

No repair   Repair   Repair   No repair

Post-meiotic segregation (3:5)   **Gene conversion (2:6)**   **Restoration of Mendelian segregation (4:4)**   **Gene conversion (6:2)**   **Post-meiotic segregation (5:3)**
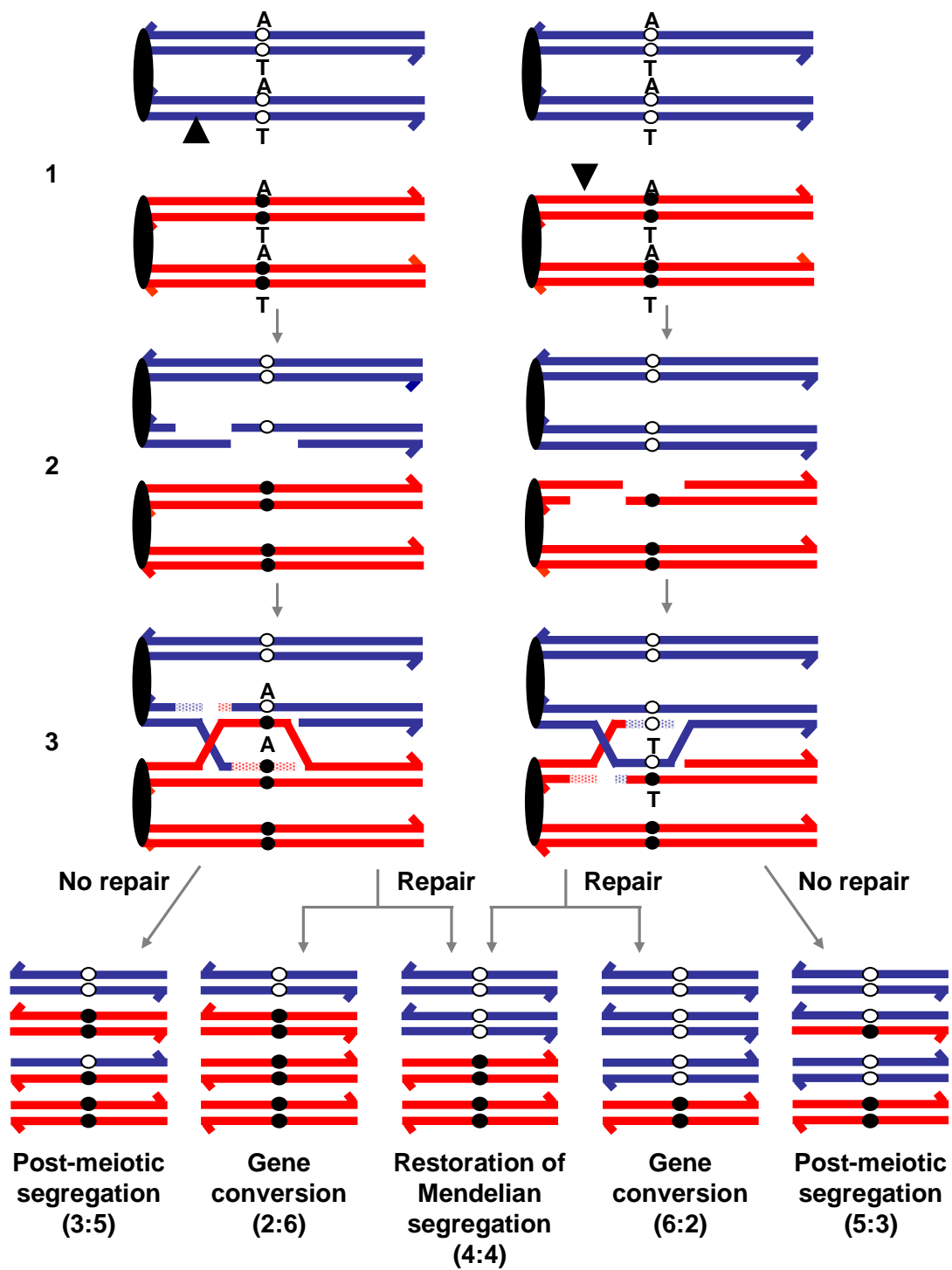
have levels of meiotic recombination greater than the genome average, there is much more recombination at *HIS4* than at *ARG4*. As described below, by examining the patterns of meiotic segregation of the *his4-AAG* and *arg4-17* alleles in strains with mutations in *pol30*, I was able to demonstrate a role of PCNA in meiotic MMR.

5. *pol30-52* and *pol30-201* strains have a meiotic MMR defect and reduced aberrant segregation. In the AS4/AS13 strain background, sporulation at 18° C results in elevated rates of meiotic recombination at the *HIS4* locus (Nag & Petes 1993, Fan *et al.* 1995). The *pol30-52* mutants, however, are cold-sensitive (Ayyagari *et al.* 1995), and *pol30-52* diploids do not sporulate efficiently at either 18° C or 25° C. Sporulation of the *pol30-52* diploid is most efficient at 30° C, although spore viability remains low (42%). MMR-proficient (wild-type) and MMR-deficient (*msh2*) strains were sporulated at 30° C for comparison. Sporulation of the wild-type strain at 30° C resulted in a three-fold decrease in aberrant segregation of *his4-AAG* relative to sporulation at 18° C (compare Tables 3.5 and 3.6), but did not affect the frequency of aberrant segregation of *arg4-17* (Table 3.7). Compared to the wild-type strain, the *pol30-52* allele resulted in a significant decrease in the aberrant segregation rate of arg*4-17* and a slight (not statistically significant) decrease in the rate of aberrant segregation of *his4-AAG*.

As PMS events represent spores that received an unrepaired mismatch, the relative abundance of PMS events can be used as a measure of meiotic MMR efficiency. The efficiency of meiotic MMR is reflected by the ratio of number of PMS tetrads divided by the total number of aberrant tetrads (PMS + gene conversion tetrads). We previously reported that there is a low level of PMS for the *his4-AAG*

105

mismatch in the wild-type strain (Detloff *et al.* 1991, Stone & Petes 2006); the percent of PMS/aberrant tetrads is 18% (Table 3.6). At 30° C, this ratio is even lower (6%, Table 3.5). In the strain with the *pol30-52* mutant, 30% of the aberrant segregants were PMS tetrads (Table 3.5), suggesting that *pol30-52* reduces the efficiency of meiotic MMR. The PMS/aberrant ratio observed for the *pol30-52* strain is only about half of the ratio found in MMR-deficient *msh2* strain, indicating that the *pol30-52* mutation does not completely eliminate meiotic MMR.

Since only 10% of tetrads derived from the *pol30-52* strain had four viable spores (Fig. 3.3A), we also monitored the frequency of sectored spore colonies in tetrads with less than four viable spores. The level of sectored spore colonies observed was significantly increased from 0.3% in the wild-type strain to 1.0% in the *pol30-52* strain. The level of sectored colonies in the *pol3-52* strain, however, was still significantly less than the 3.7% observed for the *msh2* strain (Table 3.5).

Unlike the *pol30-52* allele, the *pol30-201* allele does not confer cold sensitivity. We analyzed the *pol30-201* diploid after sporulation at 18° C. The *pol30-201* diploid had a low level of aberrant segregation events at *HIS4* relative to the wild-type strain sporulated at 18° C (Table 3.6). The rate of aberrant segregation at the *ARG4* locus was not affected by *pol30-201* (Table 3.7). Although the efficiency of MMR, as measured by the PMS/aberrant ratio, for both the *his4-AAG* and *arg4-17* mismatches was reduced by *pol30-201*, the effect on the *his4-AAG* mismatch was more severe (Tables 3.6 and 3.7). For the *his4-AAG* mismatch, the PMS/aberrant ratio was about 60% of that observed in the *msh2* strain.

If there are any restoration-type repair events in the *pol30* strains, the PMS/aberrant ratio would underestimate the frequency of meiotic MMR. Although the rate of restoration-type repair events cannot be measured directly, this rate can be estimated from comparisons of the meiotic segregation patterns with that of an *msh2* strain (Stone & Petes 2006). For example, the aberrant segregation rate of *his4-AAG* in an *msh2* strain is 64% (Table 3.6). If we assume that this rate represents the total rate of heteroduplex formation at the *his4-AAG* position and that the rate of heteroduplex formation is not altered in the wild-type strain, we calculate that the wild-type strain has 6% restoration tetrads (64% minus 58%, Fig. 3.5). In order to determine if there was any restoration-type repair in the *pol30-201* strain, I created a *pol30-201 msh2* double mutant. The level of aberrant segregation for the *his4-AAG* marker in the *pol30-201 msh2* double mutant strain was similar to that of the *pol30-201* strain, indicating that there is no restoration-type repair in the *pol30-201* strain. These data support the argument that meiotic MMR is decreased, but not completely eliminated, in *pol30* mutants. The possible reasons for this effect will be discussed below.

6. *pol30* mutations decrease crossovers, but have no effect on chromosome III disjunction. In addition to decreasing the rate of aberrant segregation, as discussed above, both the *pol30-52* and *pol30-201* mutations decreased crossovers by 20-40% over each of the three genetic intervals examined (Table 3.8). Mutations that decrease crossing over often result in increased levels of chromosome nondisjunction (Wang *et al.* 1999). Meiosis I nondisjunction events result in tetrads with two viable and two-dead spores. Compared to wild-type, both

pol30-52 and *msh2* diploids had increased numbers of tetrads with two viable and two dead spores, while *pol30-201* diploids did not (Fig. 3.3). Nondisjunction of chromosome III often leads to spores that fail to mate because they have two copies of chromosome III, one of each mating type (Wang *et al.* 1999, Stone & Petes 2006). I found no non-mating spores (number of tetrads examined in parentheses) in the wild-type (766), *pol30-52* (915), or *msh2* (827) strains, all sporulated at 30° C. Similarly, I found no non-mating spores in the wild-type (675), *pol30-52* (649), or *msh2* (712) strains, all sporulated at 18° C. In contrast, deletion of *MSH5* decreases crossovers and results in chromosome III nondisjunction events in 2.5% of tetrads (Stone & Petes 2006). The lack of nondisjunction events in the *pol30* mutants suggests that the tetrads containing only two viable spores represent either non-disjunction of chromosomes other than III or a heterozygous lethal mutation in the diploid parent. The latter class of events has been observed previously for MMR-deficient mutants such as *msh2* (Reenan & Kolodner 1992, Hunter & Borts 1997).

7. Aberrant segregation of a marker located far from the *HIS4* DSB site is greatly decreased in a *pol30-201* strain. Previously, Maloisel *et al.* (2004) found evidence that a mutation in DNA polymerase δ (*pol3-ct*) resulted in shortened meiotic heteroduplexes. The decreased levels of crossovers and aberrant segregation observed in the *pol30* mutants suggest that *pol30* mutations may also affect the extension of meiotic heteroduplex DNA. Shortening of meiotic heteroduplexes can be detected genetically, as the rate of aberrant segregation of a marker which is located far from the DSB site will be greatly reduced compared to wild-type, while there will be less of an effect on segregation of markers located

A. 30° C Sporulations
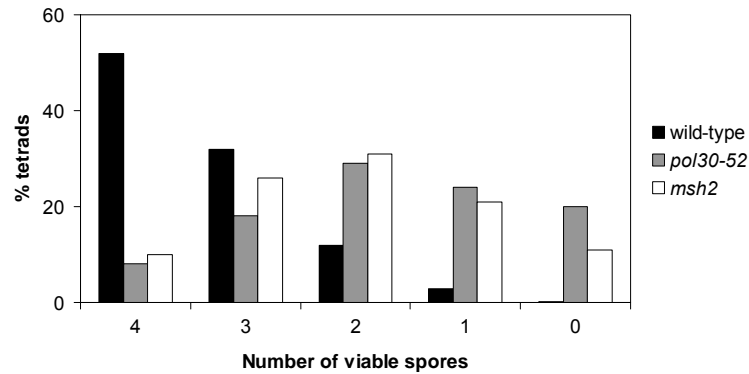
B. 18° C Sporulations
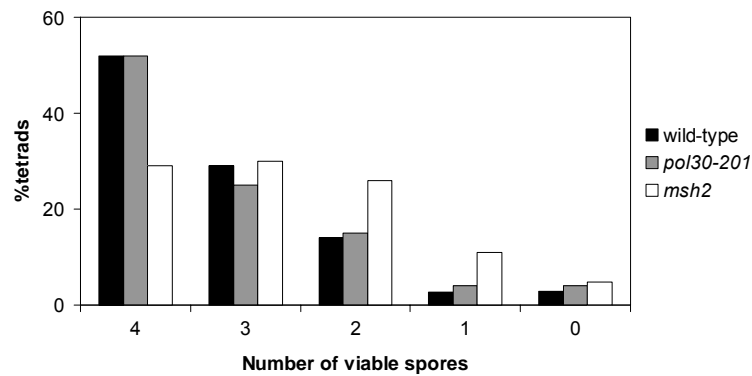
FIGURE 3.3. Spore viability patterns. The proportion of tetrads in each class is shown for (A) stains sporulated at 30° C and (B) strains sporulated at 18° C. The number of tetrads analyzed for each strain is as follows: (A) wild-type, 766; *pol30-52*, 247; *msh2*, 827; (B) wild-type, 932; *pol30-201*, 649; *msh2*, 645.

close to the DSB site. For this reason I chose to examine the effect of the *pol30-201*

mutation on segregation of the *his4-3133* marker. *his4-3133* is a poorly repaired

marker that is located more than 2 kb further from the *HIS4* DSB site than the *his4-*

AAG marker. I found that there was 9.4% aberrant segregation for the *his4-3133*

marker in a *pol30-201* mutant, which is 75% reduction relative to the wild-type level

(Table 3.9). In comparison, the level of aberrant segregation of the *his4-AAG*

marker was only reduced by 40% in the *pol30-201* strain (Table 3.6). These data

are consistent with the idea that the average length of meiotic heteroduplexes is

shortened in *pol30-201* strains.

   8. Effects of low levels of DNA polymerase δ on meiotic recombination. As

a mutation in DNA polymerase δ (*pol3-ct*) was previously found to result in

shortened meiotic heteroduplexes (Maloisel *et al.* 2004), we decided to determine

whether lower levels of DNA polymerase δ would have the same effect. If

heteroduplexes are shortened, then markers near the beginning of the DSB site

would have normal levels of aberrant segregation, whereas those further from the

initiating DSB would have reduced levels of aberrant segregation. To test the effect

of DNA polymerase levels on heteroduplex length, I used a galactose-inducible

promoter fused to *POL3*, the gene encoding the catalytic subunit of DNA polymerase

δ. Diploids used in this study were heterozygous for either *his4-IR9* or *his4-3133*.

These *his4* alleles were chosen because they are 26 bp palindromic insertions

located near the initiating DSB (*his4-IR9*; Nag & Petes 1991) or distant from the

initiating DSB (*his4-3133*; Detloff *et al.* 1992). Short palindromic sequences, when

located within heteroduplexes, are resistant to MMR, even in a wild-type strain (Nag *et al.* 1989, Nag & Petes 1991). Thus, these insertions result in high levels of PMS.

In addition to the *GAL1/10* promoter, a 3xHA tag was inserted at the N-terminus of Pol3p to facilitate protein detection as monitored by Western blots. Kokoska et al. (2000) found that when *GAL1-(3xHA)-POL3* haploid strains of a different genetic background from the one used in our meiotic studies were grown vegetatively in medium containing 0.5%, 0.05%, or 0.005% galactose, the levels of 3xHA-Pol3p were 27-fold, 8-fold, and 0.08-fold relative to 3xHA-Pol3p expressed from its native promoter. Preliminary Western blots indicated that there were similar levels of (3xHA)-Pol3p in AS4/AS13-derived *GAL1-(3xHA)-POL3* diploids grown vegetatively under the same conditions (data not shown). Western blots were also performed on cell extracts from AS4/AS13 diploids that had been incubated at 18° C for 48 hours on sporulation medium containing various levels of galactose. However, the results were inconclusive because 3xHA-Pol3p appears to be unstable in meiotic cell extracts.

In order to monitor the effects of varying the level of Pol3p on recombination, we sporulated the AS4/AS13-derived *GAL1-(3xHA)-POL3* diploids on medium containing 0.5%, 0.05% or 0.005% galactose. These strains were also heterozygous for either *his4-IR9* (JSY61) or *his4-3133* (RJK493). We found that sporulation on 0.005% galactose-containing medium resulted in a significant two-fold decrease in aberrant segregation for both *his4-IR9* and *his4-3133* relative to sporulation on 0.5% galactose-containing medium (Table 3.10 and Fig 3.4). The levels of aberrant segregation at the *his4-IR9* and *his4-3133* were reduced by low

111

DNA polymerase δ to approximately the same extent. Thus, the effects of low DNA polymerase δ are not on the extent of heteroduplex formation. We also monitored the effects of low DNA polymerase on the rate of aberrant segregation of *arg4-17*. No significant effects were found (data not shown).

In order to determine if the concentration of the carbon source alone might affect segregation of *his4* markers, I also sporulated strains that did not contain the *GAL1-(3xHA)-POL3* alteration on media containing varying levels of galactose and glucose. I found that aberrant segregation was significantly higher in *POL3* strains sporulated in the presence of 0.5% galactose than in the strains sporulated in 0.05% galactose or 0.005% galactose (Table 3.10 and Fig 3.4). I also found that sporulation of these *POL3* strains on medium containing 0.5% glucose did not result in any differences in the segregation of *his4-IR* or *his4-3133* relative to the those observed originally involving sporulation medium containing 0.05% glucose (Nag & Petes 1991, Detloff *et al.* 1992).

I also examined the rates of aberrant segregation of markers in strains (JSY83 and JSY82) in which *POL3* had the 3xHA tag, but not the *GAL1/10* promoter. In these strains sporulated in medium with 0.5% galactose, the aberration segregation frequencies of both mutant *his4* alleles were reduced (Table 3.10). These data indicate that the presence of the 3xHA tag at the N-terminus of (3xHA)-POL3p interferes with meiotic recombination events initiated at the *HIS4* DSB site and that this effect is constant across the *HIS4* locus.

In summary, my studies of *POL3* support several conclusions. First, the 3xHA tag on DNA polymerase δ reduces that rate of aberrant segregation at the *HIS4*
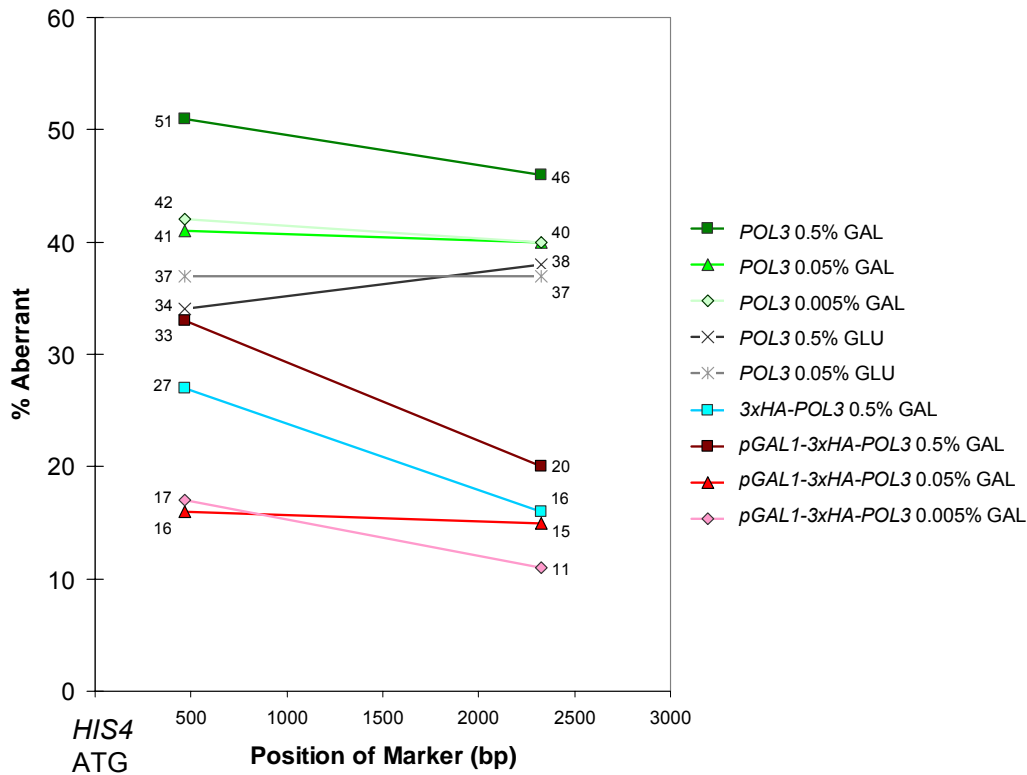
FIGURE 3.4. Effects of varying the expression of *POL3* on the gradient of aberrant segregation at the *HIS4* locus. Expression of *POL3* was controlled either by the native promoter (*POL3* and *(3xHA)-POL3* strains) or by the galactose-inducible *GAL1/10* promoter (*GAL1-(3xHA)-POL3* strains). Strains were sporulated on medium containing varying concentrations of galactose (GAL) or glucose (GLU), as indicated. Strains were heterozygous for either *his4-IR9*, located at position +497, or *his4-3133*, located at position +2327. When included in heteroduplex DNA, both *his4-IR9* and *his4-3133* generate palindromic loops, which are inefficiently repaired.

113

locus. This reduction does not appear to affect the extent of heteroduplex formation, but some other parameter (DSB production, conversion/restoration ratio, etc.). Second, the rate of aberrant segregation at the *HIS4* locus is also reduced by low levels of DNA polymerase δ (JSY61 and RJK493 data in Table 3.10).

D. Discussion

Previous studies have implicated PCNA in several of the steps required for the spellchecker function of MMR, including strand-specific recognition of the mismatch and re-synthesis of the DNA strand excised during removal of the mismatch. We studied two *pol30* alleles that were previously known to affect the MMR spellchecker function and found that both elevate mutations by a mechanism independent of their effects on MMR. We found that these *pol30* mutations also had several other effects including: (1) increased mitotic recombination between homeologous sequences, (2) decreased repair of base-base mismatches generated during meiotic recombination, (3) decreased levels of non-Mendelian segregation, and (4) decreased meiotic crossovers. Together these data suggest that PCNA is involved in the recognition and repair of mismatches generated by recombination between non-identical sequences.

1. The role of PCNA in meiotic mismatch repair. My experiments demonstrate that PCNA is required for optimal levels of MMR during meiotic recombination. The effect of the mutant *po30* alleles, however, is less than the effect observed in strains with a null mutation in *MSH2*. This observation suggests that there may be a pathway of meiotic MMR that is dependent on Msh2p, but is

114

independent of PCNA. Alternatively, since the *pol30* alleles analyzed in our study are hypomorphic, it is possible that all MMR is dependent on PCNA. The *in vitro* studies of human nick-directed MMR are consistent with the second possibility (Dzantiev *et al.* 2004, Constantin *et al.* 2005).

2. Links between replication and processing of recombination intermediates. The decreased levels of meiotic crossovers and aberrant segregation in *pol30-52* and *pol30-201* strains cannot be accounted for by MMR defects. For this reason, we suggest that *pol30-52* and *pol30-201* may cause additional defects in the processing of the meiotic recombination intermediates. Maloisel *et al.* (2004) previously proposed a model in which DNA replication is linked to gene conversion track length and crossing-over. These authors found that a mutation in the catalytic subunit of DNA polymerase δ (*pol3-ct*) shortened meiotic heteroduplexes and reduced the levels of crossovers. I have presented evidence that that meiotic heteroduplexes are also shortened in *pol30-201* strains, suggesting that the *pol30-201* and *pol3-ct* mutations may have similar effects on the processing of meiotic heteroduplexes.

Maloisel *et al.* (2004) proposed that the DNA repair synthesis primed by strand invasion is less processive in *pol3-ct* mutants than in wild-type strains. If the DNA synthesis were to stop prior to completely filling in the single-strand gaps left by resection of the DSB ends, the recombination intermediate might be destabilized, facilitating strand displacement (Fig. 3.5B). If this displacement occurs frequently, there could be an increase in processing of recombination intermediates through the synthesis-dependant strand-annealing (SDSA) pathway (Fig 3.5B), relative to the

115

DSB repair pathway (Fig 3.5A). As SDSA events result in noncrossovers, an increase in SDSA relative to DSB repair could account for the decrease in crossovers in the *pol30-52* and *pol30-201* strains. When recombination events do proceed through the DSB repair pathway, then heteroduplex extension depends directly on the length of DNA synthesis primed by strand invasion. Thus, the shortened heteroduplex track length observed in *pol3-ct* and *pol30-201* strains could also result from less processive DNA synthesis.

   3. Conclusions. This study is the first to demonstrate that PCNA contributes to recombination-associated MMR processes. I have presented evidence from our collaborators that the MMR anti-recombination function is compromised when interactions between MMR machinery and PCNA are disrupted, which indicates that PCNA has a role in recognition of mismatches generated during recombination events. I found that PCNA is also involved in the repair of base-base mismatches generated during meiotic recombination. Finally, I have presented a model in which an increased level of SDSA relative to DSB repair can account for decreases in aberrant segregation and crossovers observed in *pol30* mutants.

FIGURE 3.5. *pol30* **mutations could result in an increase in meiotic recombination events resolved via SDSA compared to DSB repair.** The *pol30* mutations might decrease the relative frequency of DSB repair (A) relative to SDSA (B). In each of these models, the early steps of meiotic recombination are the same: a DSB initiates recombination, followed by resection of the DSB ends (step 1) and single-strand invasion (step 2), which primes repair DNA synthesis (step 3). In (A), following repair synthesis (step 4A) and HJ resolution (step 5A and 5A'), the recombination event can result in a crossover or a noncrossover. In (B), repair synthesis stops before gaps resulting from resection of the DSB ends are filled in (step 3B). As a result, the recombination intermediate is destabilized and the single-strand invasion is reversed (step 4B). Consequently, the recombination event is resolved without crossing-over (step 5B).

A. DSB Repair

B. SDSA

DSB

↓ 1

↓ 2

↓ 3

a

c ► ◄ d ►

b

4

cut at a & b

cut at c & d

↓ 5

↓ 5'

Crossover

Noncrossover

DSB

↓ 1

↓ 2

↓ 3

↓ 4

↓ 5

Noncrossover
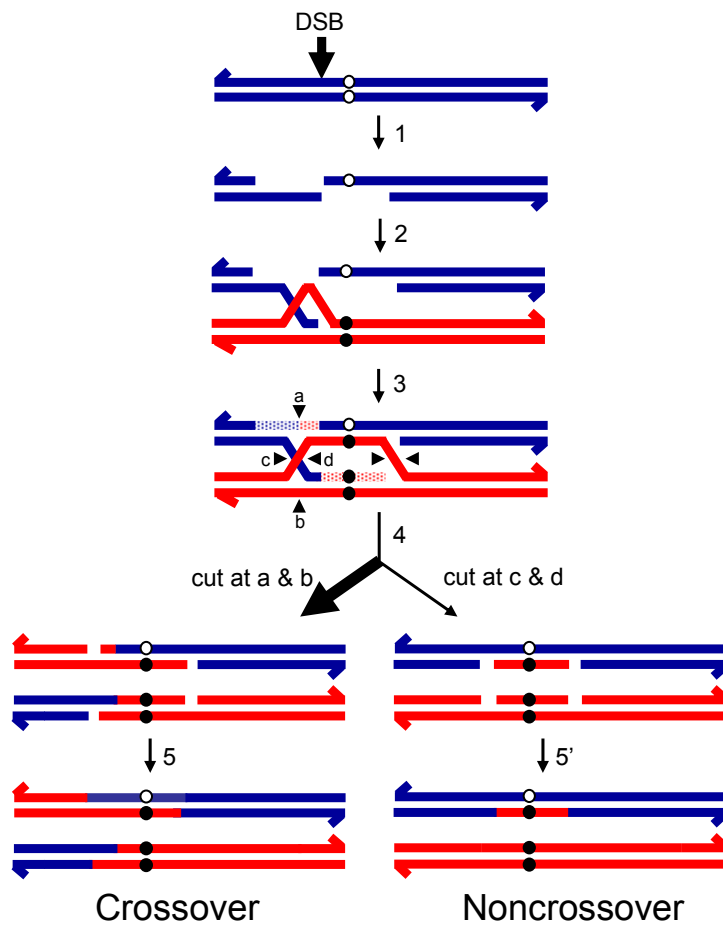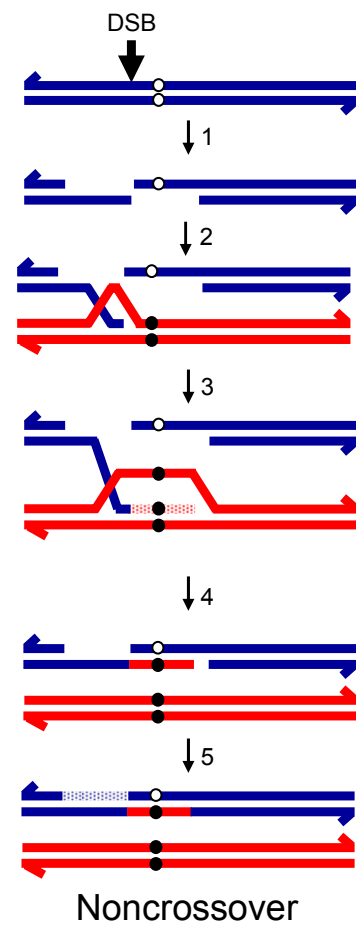
TABLE 3.1. Yeast strains

Strain	Genotype	Construction details or reference
Mating type testers:		
2262	*α ade1 his5 leu2 lys1 ura1*	Petes & Botstein (1977)
A364a	*a ade1 ade2 his5 lys2 ura1*	Petes & Botstein (1977)
Strains used for *POL30* experiments:		
AS4	*α arg4-17 trp1-1 tyr7-1 ade6 ura3*	Stapleton & Petes (1991)
AS13	*a leu2-Bst ade6 ura3 rme1*	Stapleton & Petes (1991)
HMY104	AS4 *msh2::kanMX4*	Kearney *et al.* (2001)
JSY162	PD73 *pol30-52*	This study
JSY173	AS4 *pol30-52*	This study
JSY175	JSY173 X JSY162	This study
JSY203	RJK1721 *ARG4*	This study
JSY206	MW1 *msh2::kanMX4*	Stone & Petes (2006)
JSY208	AS4 *ARG4*	This study
JSY209	JSY173 *ARG4*	This study
JSY222	JSY162 *msh2::kanMX4*	spore colony from JSY162 X JSY214 (Stone & Petes 2006)
JSY240	HMY104 X JSY206	Stone & Petes (2006)
JSY332	SJR2202 *msh2::Hyg*	OST of SJR2202 with *msh2::Hyg* cassette (Stone & Petes 2006)
JSY336	MW30 *msh2::kanMX4*	Stone & Petes (2006)

JSY337	PD73 *his4-51*	Stone & Petes (2006)
JSY338	MW30 X JSY337	Stone & Petes (2006)
JSY342	JSY206 *his4-51*	Stone & Petes (2006)
JSY343	JSY336 X JSY342	Stone & Petes (2006)
JSY344	SJR2203 *leu2-Bst*	spore colony from SJR2203 X JSY127 (Stone & Petes 2006)
JSY345	SJR2202 *LEU2*	spore colony from SJR2202 X JSY125 (Stone & Petes 2006)
JSY346	JSY345 X JSY344	This study
JSY350	JSY332 *leu2-Bst*	This study
JSY351	JSY332 *HIS4 leu2-Bst*	This study
JSY352	JSY345 *msh2::kanMX4*	This study
JSY354	JSY352 X JSY350	This study
JSY355	JSY351 *his4-3133*	This study
JSY356	JSY345 X JSY355	This study
No. 5	RJK1721 X RJK1452	Alani *et al.* (1994)
PD73	PD73 *his4-AAG*	Detloff *et al.* (1991)
PD83	AS4 X PD73	Detloff *et al.* (1991)
RKY1452	PD73 *msh2::Tn10LUK7-7*	Alani *et al.* (1994)
RKY1721	AS4 *msh2::Tn10LUK7-7*	Alani *et al.* (1994)
SJR2183	AS4 *leu2::kanMX4*	This study
SJR2184	PD73 *leu2::kanMX4*	This study
SJR2203	SJR2184 *pol30-201.LEU2*	This study

SJR2202	SJR2183 *pol30-201.LEU2*	This study
MW1	AS13 *his4-Sal*	Nag *et al.* (1989)
MW30	AS4 *his4-51*	White *et al.* (1991)
MW103	AS4 X MW1	Nag *et al.* (1989)

Strains used for *POL3* experiments:

DNY47	AS13 *his4-IR9*	Nag & Petes (1991)
DNY48	AS4 X DNY47	Nag & Petes (1991)
JSY50	RJK487 *α HIS4*	Spore colony from RJK487 X HMY131 (Kearney *et al.* 2001)
JSY58	DNY47 *kanMX6-GAL1-(3xHA)-POL3*	Spore colony from JSY50 X DNY47
JSY60	DNY47 *α*	Spore colony from JSY50 X DNY47
JSY61	RJK448 X JSY58	This study
JSY62	AS4 X JSY60	This study
JSY69	RJK487 (*3xHA*)-POL3	OST of RJK487 as described in Materials and Methods
JSY70	RJK488 (*3xHA*)-POL3	OST of RJK488 as described in Materials and Methods
JSY71	JSY58 RJK487 (*3xHA*)-POL3	OST of JSY58 as described in Materials and Methods
JSY82	JSY70 X JSY69	This study
JSY83	JSY70 X JSY71	This study

PD98	AS13 *his4-3133*	Detloff *et al*. (1992)
PD99	AS4 X PD98	Detloff *et al*. (1992)
RJK415	PD98 Gal⁺	Selection for improved growth on YPR + Galactose medium
RJK430	AS4 X RJK415	This study
RJK487	RJK415 *kanMX6-GAL1-(3xHA)-POL3*	OST of RJK415 as described in Materials and Methods
RJK488	AS4 *kanMX6-GAL1-(3xHA)-POL3*	OST of AS4 as described in Materials and Methods
RJK493	RJK488 X RJK487	This study

TABLE 3.2. *CAN1* mutation rates for AS4- and AS13-derived haploids

Strain	Relevant genotype	*CAN1* mutation rate x 10^{-7} (95% CI)	Fold increase relative to wild-type
AS13 derivatives:			
PD73	wild-type	1.41 (1.16 – 1.62)	1.0
RKY1452	*msh2Δ*	25.2 (23.4 – 28.2)	18
JSY162	*pol30-52*	90.8 (70.7 – 139)	64
JSY222	*msh2Δ pol30-52*	412 (240 – 526)	292
SJR2203	*pol30-201*	17.3 (14.9 – 19.5)	12
JSY332	*pol30-201 msh2Δ*	34.2 (32.5 – 39.4)	24
AS4 derivatives:			
JSY208	wild-type	1.17 (1.10 – 1.65)	1.2
JSY203	*msh2Δ*	19.3 (17.9 – 20.9)	14
JSY209	*pol30-52*	129 (85.7 – 183)	91

CI designates confidence interval.

Experiments performed at 30° C.

TABLE 3.3. *CAN1* mutation spectrum for an AS13-derived *pol30-52* mutant[a]

Type of event	Frequency	Mutation
Base substitutions	4/18 (22%)	
	1/18	A→G[b]
	1/18	A→T
	1/18	G→T
	1/18	T→C
Frameshifts	12/18 (67%)	
	4/18	A6→A5
	1/18	T6→T7[b]
	5/18	T6→T5
	1/18	T5→T6
	2/18	T5→T4
Other mutations	1/18 (6%)	
	1/18	2 bp deletion (TC$_2$→TC$_1$)

[a] Sequencing was done by rotation student Adam Cheely.

[b] These two mutations were found in the same *can1* isolate.

TABLE 3.4. Mitotic Recombination Rates[a]

Strain	Genotype	Recombination Rate (x10^{-8})	95% confidence intervals	Fold increase over wild-type	Homeologous/ homologous rate	Homeologous/ homologous Fold increase over wild-type
cB2cB2 – 100% substrate[b]:						
GCY313	wild type	135	119 - 158	1.0X		
SJR2083	pol30-52	1020	758 - 1490	7.6X		
SJR1906	pol30-201	225	174 - 364	1.7X		
GCY420	msh3Δ msh6Δ	251	206 - 283	1.9X		
cB2/cB2 – ns substrate[c]:						
GCY623	wild type	8.2	7.1 - 8.7	1.0X	0.061	1.0X
SJR2081	pol30-52	131	115 - 305	16X	0.129	2.1X
SJR1899	pol30-201	42.3	36.3 - 52.2	5.2X	0.188	3.1X
GCY834	msh3Δ msh6Δ	266	227 - 290	32X	1.059	17X

a Data provided by R. Gealy and S. Jinks-Robertson. Strain construction details available upon request.

b This recombination substrate has been described previously (Nicholson *et al.* 2000). The inverted repeats in this substrate are 100% homologous.

c This recombination substrate has been described previously (Nicholson *et al.* 2000). The inverted repeats in this substrate are 92% homologous.

TABLE 3.5. Meiotic segregation patterns of the *his4-AAG* marker in wild-type, *msh2*, and *pol30-52* strains sporulated at 30° C

Strain	Relevant genotype	# Tetrads	% Ab. seg.[a]	% PMS events[b]	% PMS/Ab. events[c]	% Sectored spore colonies (sectored/total)[d]	% Segregation events				Other
							6:2	2:6	5:3	3:5	Ab.[e]
PD83	wild-type	335	19	1.2	6.1	0.3 (7/2598)	8	10	1	0	0
JSY200	*msh2Δ*	79	20	14	69	3.7 (64/1739)	2	3	4	5	2
JSY175	*pol30-52*	74	13	4.1	30*	1.0 (16/1581)[†]	4	2	1	2	0

[a] Percentage of total tetrads with aberrant (non-4:4) segregation.

[b] Percentage of total tetrads with one or more PMS events, excluding tetrads with one gene conversion event and one PMS event (7:1, 1:7).

[c] Percentage of aberrant events that are PMS events, calculated as described by Stone & Petes (2006).

[d] Percentage of spore colonies that were sectored (indicating a PMS event), including spores from tetrads in which one or more spore were inviable. Actual numbers are given in parentheses.

[e] This class includes tetrads with two or more PMS (ab4:4, ab6:2, ab2:6, dev5:3, dev3:5, dev4:4) events, one PMS event and one conversion event (7:1, 1:7), and two conversion events (8:0, 0:8).

* Significant increase from PD83 and significant decrease from JSY200 in the number of spores with a PMS event versus the number of spores with a gene conversion event. One-tailed Fisher exact tests were used to determine p values and p values were considered significant when $p<0.05$.

† Chi-square tests were used to determine that there is a significant increase from PD83 and a significant decrease from JSY200, when comparing the number spore colonies which were sectored to the total number of spore colonies which were not sectored. p values were considered significant when $p<0.05$.

TABLE 3.6. Meiotic segregation patterns of the *his4-AAG* marker in wild-type, *msh2*, and *pol30-201* strains sporulated at 18° C[a]

Strain	Relevant genotype	# Tetrads	% Ab. seg.	% PMS events	% PMS/Ab. events	% Segregation 6:2	2:6	5:3	3:5	Other Ab.
PD83[b]	wild-type	482	58	10	18	15	22	5	4	11
No. 5[c]	*msh2Δ*	111	64	55	90	5	3	14	23	19
JSY346	*pol30-201*	336	34*	16[†]	52[‡]	7	8	6	8	4
JSY354	*pol30-201 msh2*	184	35*	26	80	4	2	8	13	7

[a] The column headings have the same definitions as those of Table 4. One-tailed Fisher exact tests were used to determine p values, except were noted. p values were considered significant when p<0.05.

[b] Data from Welz-Voegle *et al.* (2002).

[c] Data from Kirkpatrick and Petes (1997). Diploid strains No. 5 and JSY200 were created from the same haploid parents on different dates.

* Significant difference from PD83 in the numbers of 4:4 tetrads versus the number of aberrant tetrads.

129

[†] Significant increase from PD83 and significant decrease from No. 5 in the number of tetrads with a PMS event

(excluding 7:1 and 1:7) versus all other tetrads.

[‡] Significant increase from PD83 and significant decrease from No. 5 in the number of spores with a PMS event versus

the number of spores with a gene conversion event.

TABLE 3.7. **Meiotic segregation patterns for *arg4-17* in wild-type, *msh2*, *pol30-52*, and *pol30-201* strains** [a]

Temp	Strain(s)	Relevant genotype	# Tetrads	% Ab. seg.	% PMS events	% PMS/Ab. events	% Segregation 6:2	2:6	5:3	3:5	Other Ab.
30° C	PD83	wild-type	335	9.6	0	0	5	4	0	0	1
	JSY200	*msh2Δ*	79	8.9	5	57	4	0	4	1	0
	JSY175	*pol30-52*	74	1.4*	1.4	100[†]	0	0	0	1	0
18° C	MW103/JSY338[b]	wild-type	742	7.5	0	2	4	3	0	0	1
	JSY240/JSY343[c]	*msh2Δ*	502	16	11	67	4	1	5	4	2
	JSY346	*pol30-201*	336	8.6	1.5[§]	16[‡]	4	2	1	0	1
	JSY354	*pol30-201 msh2*	184	11	3.8	35	4	3	2	2	0

[a] The column headings have the same definitions as those of Table 4. One-tailed Fisher exact tests were used to determine p values, except were noted. p values were considered significant when p<0.05.

[b] Data from Stone & Petes (2006). MW103 and JSY338 are isogenic to PD83, except for mutations at the *HIS4* locus, which have no significant effect on segregation of the *arg4-17* marker. Data from MW103 and JSY33 are shown

131

because spore colonies from these strains were examined microscopically for small sectors on medium lacking arginine, while spore colonies from 18° C sporulations of PD83 were not.

[c] Data from Stone & Petes (2006). JSY240 and JSY346 are isogenic to JSY200, except for mutations at the *HIS4* locus, which have no significant effect on segregation of the *arg4-17* marker. Data from JSY240 and JSY346 are shown because spore colonies from these strains were examined microscopically for small sectors on medium lacking arginine, while spore colonies from 18° C sporulations JSY200 were not.

* Significant decrease from PD83 in the numbers of 4:4 tetrads versus the number of aberrant tetrads.

[†] Significant increase from PD83 in the number of spores with a PMS event versus the number of spores with a gene conversion event. There significant difference between JSY175 and JSY200 in the number of spores with a PMS event versus the number of spores with a gene conversion event.

[‡] Significant increase from MW103/ JSY338 and significant decrease from JSY240/JSY346 in the number of spores with a PMS event versus the number of spores with a gene conversion event.

[§] Significant increase from MW103/ JSY338 and significant decrease from JSY240/JSY346 in the number of tetrads with a PMS event (excluding 7:1 and 1:7) versus all other tetrads.

TABLE 3.8. Genetic distances in three intervals on chromosome III in

wild-type, *msh2*, *pol30-52*, and *pol30-201* strains[a]

Temp	Strain	Genotype	HIS4-CEN3				MAT-CEN3				HIS4-MAT			
			P	N	T	cM[b]	P	N	T	cM[b]	P	N	T	cM[c]
30°C	PD83	wild-type	65	74	113	22	88	80	137	23	140	27	249	49
	JSY200	*msh2Δ*	68	46	94	23	73	39	110	25	60	10	124	47
	JSY175	*pol30-52*	19	38	16	13*	27	17	28	19*	90	8	97	37‡
18°C	PD83	wild-type	53	50	91	23	97	85	166	24	47	14	78	58
	JSY240	*msh2Δ*	32	33	58	24	40	45	82	25	42	9	56	51
	JSY346	*pol30-201*	52	62	66	18	52	54	61	18†	52	4	54	35§

[a] P, N, and T indicate parental ditype, nonparental ditype, and tetratype tetrads, respectively. Only those tetrads

in which both flanking markers had Mendelian segregation were included.

[b] The centromere-linked *trp1* marker was used to determine if each tetrads was PD, NPD, or T. The genetic

distances (cM) were calculated as the percentage of T tetrads (i.e. second division segregations) divided by two.

[c] Calculated by the equation of Perkins (1949).

* Significant difference ($p<0.05$) in the numbers of PD+NDP:T when compared to PD83 sporulated at 30° C.

Two-tailed Fisher exact tests were used to determine p values for the *HIS4-CEN3* and *MAT-CEN3* intervals

† Significant difference ($p<0.05$) in the numbers of PD+NDP:T when compared to PD83 sporulated at 18° C.

Two-tailed Fisher exact tests were used to determine p values for the *HIS4-CEN3* and *MAT-CEN3* intervals

‡ Significant difference ($p<0.05$) in the numbers PD:NDP:T when compared to PD83 sporulated at 30° C.   Chi square analysis was used to determine the p value.

§ Significant difference ($p<0.05$) in the numbers PD:NDP:T when compared to PD83 sporulated at 18° C.   Chi square analysis was used to determine the p value.

134

TABLE 3.9. Meiotic segregation patterns of the *his4-3133* marker in wild-type and *pol30-201* strains sporulated at 18° C

| Strain | Relevant genotype | # Tetrads | % Ab. seg. | % PMS events | % PMS/Ab. tetrads[a] | % Segregation | | | | Other |
						6:2	2:6	5:3	3:5	Ab.
PD99[b]	wild-type	344	37	24	67	6	6	10	11	4
JSY356	*pol30-201*	288	9.4*	4.2	44	2	2	2	2	1

[a] Number of tetrads with one or more PMS events (including tetrads with one PMS and one gene conversion) divided by total number of aberrant tetrads (PMS + gene conversion tetrads).

[b] Data from Detloff *et al.* (1992). PD99 is a purified diploid.

* Significant difference from PD83 in the numbers of 4:4 tetrads versus the number of aberrant tetrads. A two-tailed Fisher exact test was used to determine the p value. The p value was considered significant, as p<0.05.

135

TABLE 3.10. Segregation of *his4-IR9* and *his4-3133* in *POL3*, *(3xHA)-POL3*, and *GAL1-(3xHA)-POL3* strains sporulated on media containing various concentrations glucose or galactose[a]

Strain	Heterozygous HIS4 allele	Homozygous POL3 allele	Sporulation medium[b]	# tetrads	Ab. %	PMS/Ab. %	HIS4-LEU2 (cM)
JSY 62[c]	his4-IR9	POL3	0.5% Gal	203	51	84	32
			0.05% Gal	116	41	80	34
			0.005% Gal	150	42	94	28
			0.5% Glu	111	34*	84	33
DNY48[d]	his4-IR9	POL3	0.05% Glu	379	37	78	NA
JSY 83[c]	his4-IR9	(3xHA)-POL3	0.5% Gal	77	27*	71	18
JSY 61[c]	his4-IR9	GAL1-(3xHA)-POL3	0.5% Gal	165	33	80	33
			0.05% Gal	109	16†	53	21
			0.005% Gal	128	17†	46	26
RJK 430[c]	his4-3133	POL3	0.5% Gal	188	46	72	40

Strain	Genotype	POL3	Carbon source[b]				
			0.05% Gal	305	40	72	36
			0.005% Gal	165	40	69	39
			0.5% Glu	123	38	83	39
PD99[e]	his4-3133	POL3	0.05% Glu	344	37	67	29
JSY 82[c]	his4-3133	(3xHA)-POL3	0.5% Gal	226	16‡	67	27
			0.05% Glu	168	16	67	24
RJK 493[c]	his4-3133	GAL1-(3xHA)-POL3	0.5% Gal	364	20	75	31
			0.05% Gal	326	15	47	27
			0.005% Gal	150	11§	47	26

[a] The column headings have the same definitions as those of Table 4. NA indicates data not available.

[b] Concentration of carbon source in the sporulation medium. Gal indicates galactose and Glu indicates glucose.

[c] The AS13-derived haploid parents of these strains were derived from the Gal+ strain RJK415. RJK415 was selected as an isolate of PD98 which has improved growth on galactose-containing medium relative to other AS13-derived strains.

[d] Data from Nag & Petes (1991). NA indicates data not available for reanalysis.

[e] Data from Detloff *et al.* (1992).

* Significant decrease compared to JSY62 sporulated in 0.5% Gal in the numbers of tetrads with aberrant segregation versus the numbers of tetrads with 4:4 segregation.

[†] Significant decrease compared to JSY61 sporulated in 0.5% Gal in the numbers of tetrads with aberrant segregation versus the numbers of tetrads with 4:4 segregation.

[‡] Significant decrease compared to RJK430 sporulated in 0.5% Gal in the numbers of tetrads with aberrant segregation versus the numbers of tetrads with 4:4 segregation.

[§] Significant decrease compared to RJK493 sporulated in 0.5% Gal in the numbers of tetrads with aberrant segregation versus the numbers of tetrads with 4:4 segregation. RJK493 sporulated at 0.05% Gal and 0.005% Gal are not significantly different from each other

138

IV. Alleles of the Yeast *PMS1* Mismatch-Repair Gene That Differentially Affect Recombination- and Replication-Related Processes[2]

A. Introduction

   Mismatch repair (MMR) systems promote genome stability by detecting and dealing with distortions in the DNA double helix (reviewed by Harfe & Jinks-Robertson 2000a). These systems are best known for their role in removing mispaired or extrahelical nucleotides generated during DNA replication ("spellchecker" function), with defects resulting in a strong mutator phenotype. In addition to their replication-editing function, MMR systems also detect mismatches in the heteroduplex recombination intermediates that involve the pairing of single strands derived from different duplex DNA molecules. Detection of recombination-associated mismatches triggers either a repair process that restores perfect base complementarity or an antirecombination activity that prevents the recombination event from going to completion. Finally, MMR systems in some organisms are important for detecting DNA damage and for triggering appropriate cell-cycle arrest or apoptotic responses.

   The MMR system of Escherichia coli contains three dedicated "Mut" proteins and has served as a paradigm for the more complicated MMR systems of eukaryotic organisms (reviewed by Modrich & Lahue 1996). MMR in *E. coli* is initiated when a

---

[2] This chapter has been published previously (Welz-Voegele *et al.* 2002). I contributed data for Figure 4.4 and Tables 4.4, 4.5, and 4.6.

homodimer of the MutS protein binds mismatches. MutL homodimer then couples

the MutS-dependent mismatch recognition to downstream processing steps by

activating the latent endonuclease activity of the MutH protein. MutH specifically

nicks the nascent strand to initiate its removal by a helicase and one or more

exonucleases, and the resulting gap is filled in by DNA polymerase and sealed by

ligase to complete the repair process. In eukaryotes there are multiple MutS and

MutL homologs (Msh and Mlh proteins, respectively) that are involved in MMR

processes, but no known MutH homologs (reviewed by Harfe & Jinks-Robertson

2000a). The active forms of the eukaryotic Msh and Mlh proteins are heterodimers

instead of homodimers, with the heterodimers generally having distinct but

overlapping functions in MMR. The recently solved crystal structures of bacterial

MutS homodimers have revealed that they are, in fact, structural heterodimers

(Lamers *et al.* 2000, Obmolova *et al.* 2000), which can account for the existence of

heterodimers rather than homodimers in eukaryotes. In the yeast *Saccharomyces

cerevisiae*, mismatches in nuclear DNA are recognized by either an Msh2p-Msh3p

or an Msh2p-Msh6p heterodimer (Marsischky *et al.* 1996, Johnson *et al.* 1996b),

which then interacts with a MutL-like heterodimer composed of Mlh1p complexed

with Pms1p, Mlh2p, or Mlh3p (WANG et al. 1999 Down). As the Mlh1p-Mlh2p and

Mlh1p-Mlh3p heterodimers play only minor roles in the repair of replication errors

(Harfe *et al.* 2000, Flores-Rozas *et al.* 2000) and have no reported antirecombination

activity, only the Mlh1p-Pms1p heterodimer will be considered here.

Functionally important regions of the yeast Mlh1p and Pms1p proteins have been

deduced by aligning MutL homologs from diverse organisms (Ban & Yang 1998,

Crouse 1998) and by mutational analyses (Pang *et al.* 1997, Tran & Liskay 2000).

Protein alignments have revealed a highly conserved region at the amino terminus

that contains the four domains characteristic of the GHL (gyrase b, Hsp90, and

MutL) family of ATPases (Dutta & Inouye 2000). Functionally important

conformational changes in the N-terminal regions of GHL family proteins are

associated with ATP binding and hydrolysis, with the N-terminal ends of MutL, and

with gyrase b homodimerizing upon ATP binding (Wigley *et al.* 1991, Ali *et al.* 1993,

Prodromou *et al.* 1997a, Prodromou *et al.* 1997b, Ban & Yang 1998). Studies with

mutant Mlh1p and Pms1p proteins support a comparable amino-terminal

heterodimerization cycle associated with ATP binding and hydrolysis (Tran & Liskay

2000). In addition, genetic studies have revealed an ATP-related asymmetry

between the yeast Mlh1p and Pms1p subunits in terms of their contributions to the

spellchecker function of the complex (Tran & Liskay 2000). Although Mlh1p and

Pms1p share little amino acid similarity outside of the highly conserved N terminus,

the C-terminal 200–300 amino acids of each protein are necessary and sufficient for

ATP-independent heterodimer formation and are required for the spellchecker

function of the complex (Pang *et al.* 1997). Finally, the C-terminal 13 amino acids of

yeast Mlh1p are identical to the C-terminal 13 amino acids of human MLH1, but this

highly conserved motif is not present in Pms1p. This carboxy-terminal homology

(CTH) motif of Mlh1p is not required for interaction with Pms1p in two-hybrid assays,

but is required for spellchecker function (Pang *et al.* 1997).

The repair of mismatches in heteroduplex recombination intermediates can result

in the replacement of one allele with the sequence of another allele ("gene

conversion"), which is manifested in meiosis as the non-Mendelian segregation of allelic sequences. If mismatches in meiotic recombination intermediates are not repaired, segregation of the corresponding alleles at the next round of DNA replication will result in genetically different daughter cells (postmeiotic segregation, or PMS). In yeast, gene conversion is much more common than PMS, indicating that most mismatches are efficiently recognized and repaired by the MMR machinery (Petes *et al.* 1991). Although it is not known what triggers the repair vs. antirecombination activity of MMR systems, the antirecombination activity effectively limits recombination between nonidentical ("homeologous") sequences, thereby reducing genome rearrangements and enforcing species barriers (reviewed by Harfe & Jinks-Robertson 2000a, Harfe & Jinks-Robertson 2000b). In *S. cerevisiae*, mitotic recombination is exquisitely sensitive to potential mismatches, with a single nonidentity between 350-bp recombination substrates being sufficient to reduce the rate of recombination in a MMR-dependent manner (Datta *et al.* 1997). Although elimination of yeast Msh2p, Mlh1p, or Pms1p results in identical mutator phenotypes, the antirecombination activity of Msh2p is consistently greater than that of Pms1p or Mlh1p (Chen & Jinks-Robertson 1998, Nicholson *et al.* 2000). In addition, the Sgs1p helicase appears to be redundant with MMR-associated antirecombination (Myung *et al.* 2001), but has no known role in the repair of DNA mismatches.

The genetic differences between the MMR-associated spellchecker and antirecombination activities in yeast suggest that the Mlh1p-Pms1p-dependent steps downstream of mismatch recognition may be different during DNA replication vs.

recombination. In addition, the ATPase-related functional asymmetry observed in the spellchecker functions of Mlh1p and Pms1p may extend to the recombination-related activities of the proteins as well. These issues are addressed in the current study by (1) identifying "separation-of-function" alleles of *PMS1* that partially uncouple the mitotic spellchecker and antirecombination functions, (2) examining the mitotic antirecombination effects of known mutations in *MLH1* or *PMS1* that compromise ATP binding or hydrolysis, and (3) examining the effects of eliminating Mlh1p or Pms1p ATP hydrolysis activity on the repair of mismatches in meiotic recombination intermediates.

B. Materials and Methods

1. Media and Growth Conditions: Strains were grown vegetatively at 30° C and sporulated at 18° C; a complete list of yeast strains is given in Table 4.1. Standard media and genetic techniques were used for mitotic growth, sporulation, and tetrad dissection (Sherman *et al.* 1982), except as noted below. Strains were grown nonselectively in YEP medium containing either 2% glycerol and 2% ethanol (YEPGE) or 2% dextrose (YEPD). Each liter of YEPGE and YEPD was supplemented with 500 mg adenine hemi-sulfate (Sigma, St. Louis) to avoid adenine limitation during nonselective growth. For selection of yeast transformants that had incorporated the kan marker, each liter of YEPD was supplemented with 200 mg of Geneticin (Sigma).

Synthetic dextrose (SD) medium was supplemented with all but the one amino acid or base needed for selective growth (e.g., SD-His is deficient in histidine).

143

Additional tryptophan (30 µg/ml) was added to the SD media as well as to the YEP media for growth of strains containing the trp5Δ allele (i.e., SJR1392 and its derivatives). Canavanine-resistant (Can-R) mutants were selected on SD-Arg medium supplemented with L-canavanine sulfate to a concentration of 60 µg/ml (SD-Arg + Can). Ura- segregants were selected on SD plates supplemented with required amino acids and containing 0.1% 5-fluoroorotic acid (5-FOA; BOEKE et al. 1984 Down). For the selection of His+ mitotic recombinants, dextrose in the SD medium was replaced with 2% glycerol, 2% ethanol, and 2% galactose (SGGE-His).

Sporulation of diploid cells and tetrad dissection were performed as described by FAN et al. 1995 Down. For meiotic analyses, purified diploids were not used, since diploids homozygous for pms1 or mlh1 rapidly accumulate recessive lethal mutations. Instead, haploid parents were mated overnight on YEPD plates and then were immediately transferred to sporulation plates. Tetrads were dissected on YEPD medium and the resulting spore clones were directly replica plated to appropriate selective media. Sectored His+/His- colonies were scored by light microscopy.

2. Yeast strains used for mitotic studies: Strain SJR1294 was used as a host to identify plasmid-encoded *pms1* alleles conferring mutator and/or hyperrecombination phenotypes. The mutator phenotype was assessed by forward mutation to canavanine resistance, while the recombination phenotype was assessed using 94%-identical *HIS3::intron::cß2* inverted-repeat (IR) substrates (see Datta *et al.* 1997). In preliminary experiments, we found that the high chromosomal mutation rate of a *pms1Δ* host strain interfered with the efficient detection of

144

plasmid-encoded pms1 alleles. To circumvent this problem, the endogenous *PMS1*

promoter was replaced with the *GAL1* promoter by transforming cells with a PCR

fragment generated using plasmid pFA6a-kanMX6-PGAL1(Longtine *et al.* 1998) as

a template. The presence of the resulting galactose-regulated *PMS1* allele (pGAL-

PMS1) resulted in strong mutator and hyper-recombination phenotypes only when

cells were grown in the absence of galactose.

Strain SJR1392 contains both homeologous (92% identical) and homologous

(100% identical) IR recombination substrates. This strain was constructed by

targeting plasmids containing homeologous *HIS3::intron::cß2/cß7* substrates

(pSR303) and homologous *LYS2* substrates (pRS304) to the *URA3* and *LEU2* loci,

respectively. Transformants containing a single copy of each plasmid were

identified by Southern analysis. Ura⁻ segregants were selected on 5-FOA medium,

and retention of the homeologous recombination substrates was confirmed by the

ability to produce His⁺ recombinants.

An *mlh1Δ::URA3* allele was introduced into SJR1392 by transformation with

*Sac*I/*Bam*HI-digested *ymlh1::URA3* (Prolla *et al.* 1994). *Bst*XI-digested pJH523

(Kramer *et al.* 1989b) was used in a two-step allele replacement procedure to

introduce a *pms1Δ* allele. All *mlh1Δ* and *pms1Δ* strains were verified by PCR or

Southern analysis. Derivatives containing point mutations in *PMS1* or *MLH1* were

constructed by two-step allele replacement, and the presence of the mutation of

interest was confirmed by genomic DNA sequencing. Plasmids pYI-mlh1-31, pYI-

mlh1-98, pYI-pms1-61 TV II, and pYI-pms1-128 TV II were used to introduce the

mlh1-E31A, *mlh1-G98A*, *pms1-E61A*, and *pms1-G128A* alleles, respectively (Tran &

Liskay 2000). The *pms1-L124S*, *pms1-I854M*, and *mlh1-757stop* alleles were

introduced using plasmids pSR759, pSR760, and pSR746, respectively (see below).

3. Strains used for meiotic recombination studies: Diploid strains used for

meiotic recombination experiments were constructed by mating isogenic derivatives

of the *HIS4* strain AS4 (STAPLETON and PETES 1991) and the *his4-AAG* strain PD73

(Detloff *et al.* 1991). All diploids thus are heterozygous for the *his4-AAG* mutant

allele, which has a single-base-pair change at the second position of the *HIS4* start

codon. Haploid derivatives containing an *mlh1::URA*, *pms1∆*, *mlh1-E31A*, or *pms1-

E61A* allele were constructed by transformation as described above for SJR1392.

The *mlh1∆::kanMX4* and *pms1∆::kanMX4* alleles were introduced by transformation

with PCR deletion cassettes generated using pFA6-*kanMX4* (WACH *et al.* 1994) as a

template. To determine forward mutation rates at the *CAN1* locus, *ARG4* derivatives

of haploid strains with the AS4 genetic background were constructed by

transformation with *Age*I-digested pMW52 (White *et al.* 1993).

4. Plasmids: Plasmid pSR303 contains the *HIS3::intron::c*ß*2/c*ß*7* homeologous

recombination substrates and was constructed by combining 5' cß2 and 3' cß7

recombination cassettes as inverted repeats (Fig 4.1A). Plasmid pSR266 contains a

full-length *HIS3::intron* gene, with a unique *Bam*HI site within the intron, and was

used to generate both the 5' and 3' cassettes (Datta *et al.* 1996). Plasmids pSR273

and pSR301 were constructed by inserting an 800-bp *Bam*HI/*Bgl*II cß2 and a 783-bp

*Bam*HI/*Bgl*II cß7 fragment, respectively, into the *Bam*HI site of plasmid pSR266. The

cß7 3' cassette plasmid pSR302 was derived from pSR301 by deleting the *Sal*I

fragment upstream of the cß7 sequences (*i.e.,* the 5' portion of *HIS3* and the 5' part

146

of the intron). A *Sma*I fragment containing the 5' cß2 cassette (from pSR273) was then inserted into the filled-in *Spe*I site of pSR302 in reverse orientation relative to the 3' cß7 cassette.

Plasmid pSR304 contains the *lys2∆5'* and *lys2∆3'* homologous recombination substrates oriented as IRs (Fig 4.1B) and was constructed using *LYS2* sequences derived from pDP6 (Fleig *et al.* 1986). First, a 2.7-kb *Hinc*II/*Hin*dIII fragment containing the 3' end of *LYS2* (*lys2∆5'* allele) was directionally cloned into *Sma*I/*Hin*dIII-digested pRS305 (Sikorski & Hieter 1989), yielding plasmid pSR300. A 3-kb *Xba*I/*Stu*I fragment containing the 5' end of *LYS2* (*lys2∆3'* allele) was then inserted into *Xba*I/*Sst*I(blunt)-digested pSR300, with the resulting plasmid (pSR304) containing the *lys2∆5'* allele downstream of and in inverted orientation relative to the *lys2∆3'* allele. The region of overlap between the *lys2∆5'* and *lys2∆3'* alleles is ~900 bp.

Plasmid pSR758 contains the 2715-bp *PMS1* open reading frame and was constructed by cloning a 4-kb chromosomal *Bgl*II/*Sal*I fragment (from YIp5-PMS1; obtained from D. Maloney) into *Bam*HI/*Sal*I-digested pRS315 (LEU2-CEN vector; Sikorski and Hieter 1989). pSR758 was the source for the *PMS1* fragments that comprise the three deletion plasmids used in gap-repair experiments. pRS315-PMS1∆1 (pSR764) has a deletion of the first 590 bp of the *PMS1* coding sequence between the *Mlu*I and *Hin*P1I sites at -33 and +591, respectively, relative to the start codon; pRS315-PMS1∆2 (pSR765) has a centrally located 1090-bp deletion extending from the *Eco*0109I site at +387 to the *Fok*I site at +1477; and pRS315-PMS1∆3 (pSR766) has a 927-bp deletion encompassing the C-terminal region of

PMS1, extending from the *Bsp*HI site at +1713 to the *Nco*I site at +2640. Each of the three deletion plasmids contains a unique *Bam*HI site between the *PMS1* fragments that flank the deleted segment.

pSR761 contains the *MLH1* locus and was derived by inserting a 7-kb chromosomal *Sac*I fragment (from YEp24-MLH1; Prolla *et al.* 1994) into pRS306 (Sikorski & Hieter 1989). The *mlh1-757stop* allele (pSR746) was constructed by replacing codon 757 of the *MLH1* coding sequence with a TAG stop codon using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The resulting mutation was confirmed by DNA sequencing.

5. Random mutagenesis of the *PMS1* coding sequence and incorporation of mutations by gap repair: Appropriate *PMS1* fragments were generated by mutagenic PCR and were then recombined *in vivo* into plasmids pRS315-PMS1Δ1, pRS315-PMS1Δ2, or pRS315-PMS1Δ3 using a standard yeast gap-repair procedure (Muhlrad *et al.* 1992). *Taq* DNA polymerase was used for the PCR mutagenesis; the error frequency of the enzyme was increased by doubling the concentration of dATP or dGTP relative to the other dNTP's and by increasing the $MgCl_2$ concentration to 3 mM. Plasmids for the *in vivo* gap repair were prepared by digestion with *Bam*HI, followed by treatment with shrimp alkaline phosphatase. Gap repair of pRS315-PMS1Δ1 was accomplished using a 687-bp PCR fragment extending from -61 to +626 of the *PMS1* sequence; gap repair of plasmid pRS315-PMS1Δ2 was effected using an 1167-bp PCR fragment extending from +349 to +1516 of the *PMS1* sequence; and gap repair of pRS315-PMS1Δ3 was done using a 1029-bp PCR fragment extending from +1668 to +2697 of the *PMS1* sequence.

Strain SJR1294 was cotransformed with 1 μg of purified PCR fragment and 0.1 μg of gapped vector, and transformants were selected on SD-Leu medium. Control experiments with gapped vector only indicated a gap-repair efficiency of >95%. Approximately 1000 transformants derived from each of the three gap-repair reactions were selectively purified. Transformants were patched onto SD-Arg + Can or SGGE-His medium to score mutation or homeologous recombination frequency, respectively. Approximately 30% of the transformants exhibited phenotypes characteristic of a *pms1Δ* strain and were assumed to contain plasmid-encoded null alleles. Plasmid DNA was isolated from those transformants that consistently exhibited a separation-of-function phenotype (either a mutator or a hyper-rec phenotype, but not both phenotypes) and was used to retransform SJR1294. Following the confirmation of a separation-of-function phenotype, the relevant portion of the mutagenized *pms1* allele was sequenced. The separation-of-function alleles *pms1-L124S* and *pms1-I854M* were identified in this manner. For integration into the yeast genome, the *pms1-L124S* and *pms1-I854M* alleles were transferred to the integrating vector pRS306 (Sikorski & Hieter 1989) as *Sac*II/*Kpn*I fragments, yielding plasmids pSR759 and pSR760, respectively.

6. Two-hybrid assays: Plasmids used in two-hybrid assays were constructed by inserting the coding sequences of wild-type Pms1p and Mlh1p into vectors pGAD424 (Bartel *et al.* 1993) and pBTM116 (Vojtek *et al.* 1993), respectively. pGAD424 and pBTM116 contain the Gal4p activation and the LexA DNA-binding domains, respectively. The pGAD-*PMS1* construct was mutagenized to yield pGAD-*pms1-I854M* using the QuikChange site-directed mutagenesis kit (Stratagene).

Interactions of proteins were assessed by cotransforming pGAD and pBT derivatives into yeast strain L40, which contains both *lacZ* and *HIS3* reporter constructs (Vojtek *et al.* 1993). ß-Galactosidase activity was measured in liquid assays as described previously (Pang *et al.* 1997).

7. Rate measurements and statistical analyses: The method of the median (Lea & Coulson 1949) was used to calculate mutation and recombination rates. Data from at least 16 independent cultures (typically 8 cultures from each of two independent isolates) were used for each rate determination. For the experimentally derived medians, 95% confidence intervals (CIs) were determined (Dixon & Massey 1969) and these were then used to calculate 95% CIs for the corresponding rates. For rate determinations using SJR1392 and its derivatives, individual colonies were inoculated into 5 ml YEPGE medium and grown for 3 days on a roller drum. Cells were harvested by centrifugation, washed with H_2O, and resuspended in 1 ml H_2O. Aliquots of appropriate dilutions were plated on SD-Arg + Can to select Can-R mutants, on SD-His to select His$^+$ (homeologous) recombinants, on SD-Lys to select Lys$^+$ (homologous) recombinants, and on YEPD to determine the number of viable cells. Plates were incubated for 2 days (YEPD, SD-Arg + Can, SD-Lys) or 4 days (SGGE-His) before counting colonies. Mutation rates to Can-R in AS4 and PD73 and in their derivatives were similarly determined, except that cultures were grown overnight in YEPD before selective plating, total viable cells were determined by plating on SD-Arg, and colonies arising on SD-Arg + Can were counted after 3 days. Comparisons of the distributions of meiotic spore classes derived from different

diploids were made by Fisher's exact test with two-tailed P values. Results were considered significant if $P < 0.05$.

C. Results

1. Mutagenesis of *PMS1* and identification of mitotic separation-of-function mutations: A *PMS1* gene contained on a *CEN* vector was randomly mutagenized and the resulting alleles were screened for a mitotic separation-of-function phenotype in a strain devoid of the wild-type Pms1 protein (see MATERIALS AND METHODS for details of the mutagenesis). Specifically, transformants containing the mutagenized plasmids were screened for an associated increase in either the spontaneous mutation or the homeologous recombination frequency, but not both. The mutator phenotype was assessed by replica plating transformants to canavanine medium, which selectively identifies forward mutations at the *CAN1* locus (Can-R mutants). The level of homeologous recombination was assessed by replica plating transformants to histidine-deficient medium, which selects for inversion events that reconstitute a full-length *HIS3::intron* gene (Fig 4.1A). All candidate separation-of-function plasmids identified in the screen conferred little or no mutator phenotype, but resulted in a clearly elevated level of homeologous recombination. No candidates with the opposite phenotype were identified.

To confirm that the elevated recombination conferred by the putative *pms1* separation-of-function alleles was specific for homeologous substrates, the plasmid-encoded alleles were introduced into the *PMS1* locus of strain SJR1392, which

FIGURE 4.1. Homeologous and homologous inverted-repeat recombination substrates. (A) Homeologous substrates. The 5' cß2 cassette consists of the 5' portion of the *HIS3* coding sequence, the 5' portion of the intron, and the cß2 cDNA sequences, whereas the 3' cassette is composed of cß7 cDNA sequences, the 3' portion of the intron, and the 3' portion of the *HIS3* coding sequence. Open boxes correspond to *HIS3* coding sequences, solid boxes to intron sequences, and the cross-hatched boxes to the cß2 and cß7 recombination substrates (~800 bp of homology with 92% sequence identity). (B) Homologous substrates. The hatched box within the *LYS2* gene corresponds to the ~900 bp of perfect identity between the *lys2Δ5'* and *lys2Δ3'* recombination substrates. In both A and B, selection of prototrophic colonies identifies recombination events that reorient the region between the inverted-repeat recombination substrates. Reorientation can occur by a crossover event, by unequal sister chromatid gene conversion (Chen & Jinks-Robertson 1998), or by a combination of break-induced replication and single-strand annealing (Bartsch *et al.* 2000).

A

HIS3::intron-cβ2   HIS3::intron-cβ7

5' cassette   3' cassette

Combine as inverted repeats

Recombination

HIS3::intron
His+ recombinant

B

LYS2

lys2Δ3'   lys2Δ5'

Combine as inverted repeats

lys2Δ3'   lys2Δ5'

Recombination

LYS2
Lys+ recombinant
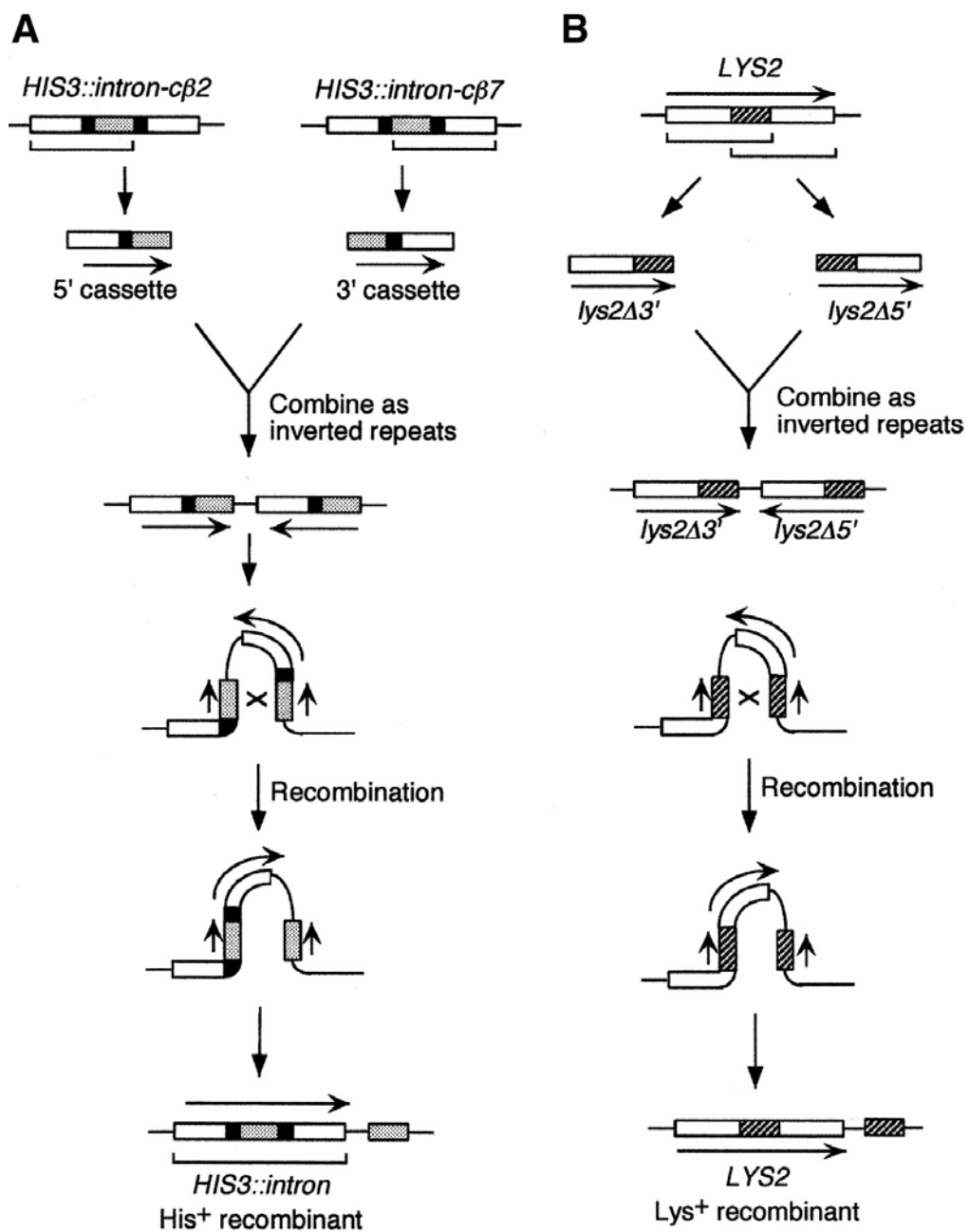
contains identical ("homologous") *LYS2* recombination substrates as well as the homeologous *HIS3::intron* substrates. The *LYS2*-based homologous system (Fig 4.1B) is composed of inverted repeats and thus is comparable in structure to the homeologous *HIS3::intron* system. As with the *HIS3::intron* system, replication between the *lys2* inverted repeats reorients the region between them, resulting in a full-length *LYS2* gene whose presence can be identified on lysine-deficient medium.

As shown in Table 4.2, elimination of Pms1p in the SJR1392 strain background resulted in a 60-fold increase in the rate of Can-R mutants. When normalized to the homologous recombination rate, the increase in the homeologous recombination rate was 11-fold in the *pms1Δ* mutant relative to the *PMS1* strain; similarly, normalized rates are used when describing homeologous recombination in *pms1* (or *mlh1*) missense mutants. Quantitation of recombination and mutation rates in the *pms1* mutants identified in the screen confirmed only two separation-of-function *pms1* alleles. Each allele resulted in a significant (3- to 4-fold) increase in the homeologous recombination rate, but no significant increase in the forward mutation rate at the *CAN1* locus (Table 4.2). DNA sequence analysis of the separation-of-function alleles revealed a mutation resulting in a leucine-to-serine change at amino acid 124 in one mutant (*pms1-L124S* allele) and a mutation causing an isoleucine-to-methionine change at amino acid 854 in the other mutant (*pms1-I854M* allele).

2. Pms1p-I854M interacts normally with Mlh1p in two-hybrid assays: The *pms1-I854M* allele alters a single amino acid in the C-terminal region of Pms1p, a region that is essential for interaction with Mlh1p in two-hybrid assays (Fig 4.2; Pang *et al.* 1997). One possible explanation for the separation-of-function phenotype
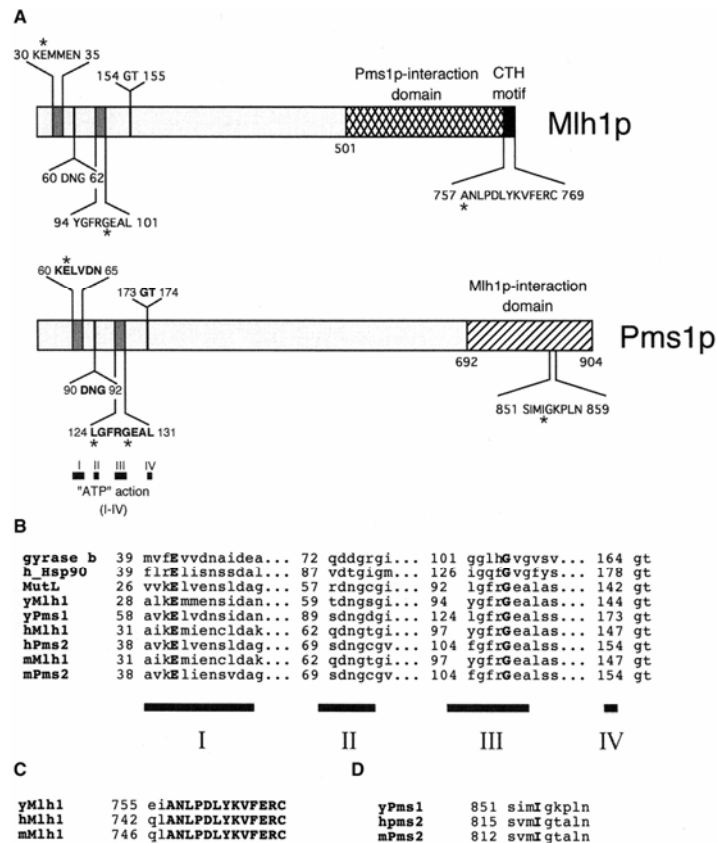
FIGURE 4.2. Domains of Mlh1p and Pms1p. (A) Schematic representations of yeast Mlh1p and Pms1p are shown with specific motif sequences highlighted above or below each protein. Numbers correspond to the amino acid position in the protein. Asterisks denote residues that were mutated and examined as detailed in the text. (B) NH$_2$-terminal ATPase domains of GHL ATPases. ATPase motifs I–IV are designated by solid boxes and aligned sequences are shown above the motif boxes. Numbers correspond to the amino acid position of the first residue in the relevant motif. Conserved residues that were mutated in Mlh1p and/or Pms1p are shown in boldface type. (C) The CTH motifs of the yeast, human, and mouse Mlh1p orthologs are aligned and highlighted by boldface uppercase letters. (D) The COOH-terminal motif of yeast Pms1p that contains the *pms1-I854M* separation-of-function mutation. The yeast Pms1p I854 residue and the surrounding motif are present in the human and mouse orthologs.

155

conferred by the *pms1-I854M* allele is that more of the Mlh1p-Pms1p complex is needed to regulate mitotic recombination than is needed to correct DNA replication errors. A decrease in the amount/stability of the complex would thus be expected to elevate homeologous recombination rates to a greater extent than mutation rates. To determine whether the I854M change significantly affects the level of the Pms1p-Mlh1p complex *in vivo*, we used two-hybrid assays to compare the interaction of Mlh1p with the wild-type *vs.* the I854M mutant Pms1 protein. As shown in Fig 4.3, the interactions were indistinguishable in a qualitative phenotypic assay as well as in a quantitative ß-galactosidase assay, suggesting that the I854M change affects neither the stability of Pms1p nor its interaction with Mlh1p.

3. Role of Pms1p ATP binding/hydrolysis in mitotic MMR functions: The L124S change is immediately adjacent to conserved motif III of the GHL family of ATPases, which is important in ATP binding and/or associated conformational changes (Ban *et al.* 1999; see Fig 4.2). The identification of the *pms1-L124S* allele in the separation-of-function screen suggested that ATP binding/hydrolysis by Pms1p might be more important for its antirecombination activity than for its spellchecker function. To pursue this further, we introduced the *pms1-G128A* and *pms1-E61A* alleles, which are predicted to compromise ATP binding (and/or associated conformational changes) and hydrolysis, respectively, into the SJR1392 strain background. These alleles were previously reported to have little, if any, effect on the spellchecker functions of the corresponding proteins in the *CAN1* mutation assay (Tran & Liskay 2000). The results obtained with the *pms1-E61A* and *pms1-G128A* alleles were indistinguishable from those obtained with the *psm1-L124S* allele. As shown in Table 4.2, there was no significant increase in the rate of Can-R
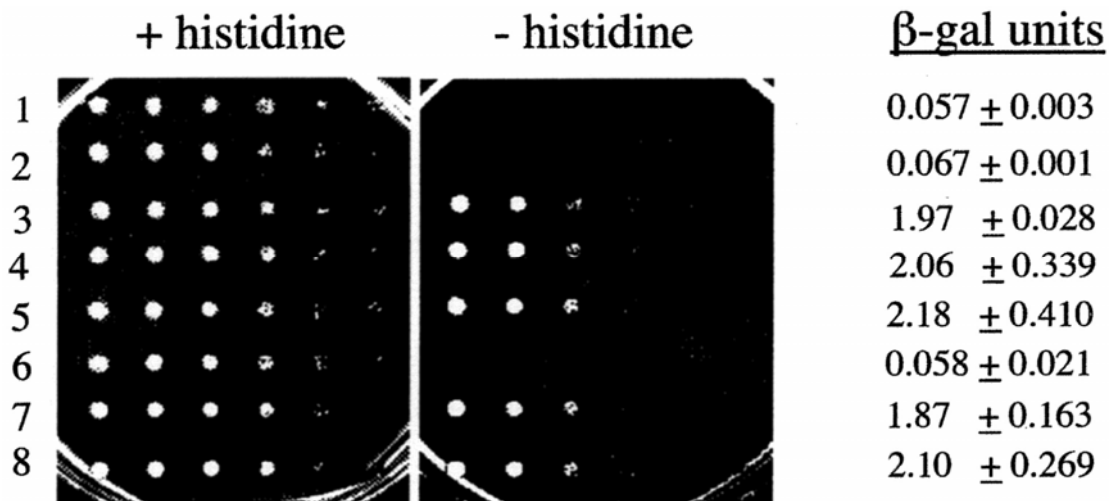
FIGURE 4.3. Pms1-I854Mp interacts normally with Mlh1p in two-hybrid assays.

Semiquantitative *HIS3* reporter assays were performed by serially diluting overnight

cultures 1:5 and spotting cells using a 48-prong replicator onto minimal medium

containing or lacking histidine. ß-Glactosidase assays were performed two times

with each strain; mean activity units and standard deviations are given. Row 1, pBT

(vector) and pGAD-*PMS1*; row 2, pBT-*MLH1* and pGAD (vector); row 3, pBT-*MLH1*

and pGAD-*PMS1*; rows 4–8, pBT-*MLH1* and pGAD-*pms1-I854M* clones 1–5. The

pGAD-*pms1-I854M* clone in row 6 was found to have a nonsense mutation that

truncated the protein after amino acid 865. On the basis of previous two-hybrid

analyses (Pang *et al.* 1997), such a truncated protein would not be expected to

interact with Mlh1p.

colonies, but there was a significant (three- to fourfold) increase in the rate of homeologous recombination in the *pms1-E61A* and *pms1-G128A* strains.

The separation-of-function phenotype conferred by mutations in the N-terminal ATP binding/hydrolysis domains of Pms1p was very similar to that associated with the C-terminal *pms1-I854M* allele. To determine whether the C- and N-terminal mutations affect Pms1p in fundamentally different ways, we constructed strains containing the double-mutant *pms1-E61A,I854M* or *pms1-G128A,I854M* allele. Both double-mutant strains exhibited significantly higher mutation and homeologous recombination rates than those observed with the corresponding single-mutant strains (Table 4.2), suggesting that the individual mutations have functionally distinct consequences. The mutator phenotype of the double mutants was very weak, however, with the mutation rates being 10-fold lower than that of an isogenic *pms1Δ* strain. In contrast, the ratio of homeologous to homologous recombination in the double mutants was similar to that in a *pms1Δ* strain. The double-mutant proteins thus retain most of their spellchecker activity, but appear to be completely defective for the mitotic antirecombination activity.

4. Role of Mlh1p ATP binding/hydrolysis in mitotic MMR functions: A functional asymmetry in the ATPase activities of Pms1p and Mlh1p has been demonstrated previously, with disruption of Mlh1p ATP binding/hydrolysis resulting in stronger mutator phenotypes than those resulting from comparable changes in Pms1p (Tran & Liskay 2000). We therefore examined the effects of the *mlh1-E31A* and *mlh1-G98A* alleles (direct counterparts of the *pms1-E61A* and *pms1-G128A* alleles, respectively; see Fig 4.2) on the mitotic spellchecker and antirecombination

158

functions of the encoded mutant proteins (Table 4.3). In the SJR1392 strain

background, deletion of *MLH1* resulted in a 53-fold elevation in the rate of forward

mutation at *CAN1* and in a 9.7-fold increase in the rate of homeologous (relative to

homologous) recombination. These effects are statistically the same as those

observed in the *pms1Δ* mutant. Both the *mlh1-E31A* and *mlh1-G98A* alleles were

indistinguishable from the *mlh1Δ* allele in terms of the rate of His$^+$ recombinants,

indicating that both ATP binding and hydrolysis are essential for the

antirecombination activity of Mlh1p. In terms of the spellchecker function, the *mlh1-*

G98A allele resulted in a 48-fold increase in the rate of Can-R colonies while the

mlh1-E31A allele resulted in a lesser, 20-fold increase. In agreement with an earlier

study (Tran & Liskay 2000), it thus appears that Mlh1p retains residual spellchecker

activity when ATP hydrolysis, but not binding, is compromised. Finally, we

constructed an *mlh1-G98A pms1-G128A* double-mutant strain. The spellchecker

and antirecombination phenotypes of the double mutant were indistinguishable from

those of an *mlh1Δ* or *pms1Δ* mutant.

    5. Role of the Mlh1p CTH domain in mitotic MMR functions: The final 13

amino acids of yeast Mlh1p and human MLH1 are identical and constitute the CTH

domain. This domain is not required for interaction between yeast Mlh1p and Pms1p

in two-hybrid assays, but is required for the spellchecker function of the complex

(Pang *et al.* 1997). To examine the role of the CTH domain in mitotic

antirecombination, we replaced the codon specifying the first amino acid of the CTH

domain with a stop codon (*mlh1-757stop* allele; see Fig 4.2). As shown in Table 4.3,

the *mlh1-757stop* allele produced mutator and recombination phenotypes that were indistinguishable from those of a null (*mlh1Δ*) mutant.

6. Roles of Pms1p and Mlh1p ATP hydrolysis in the repair of mismatched meiotic recombination intermediates: In addition to the mitotic spellchecker and antirecombination activities, the yeast MMR system also detects and repairs the mismatch formed when a heterozygous marker (*e.g.*, alleles *A* and *a*) is included in a heteroduplex recombination intermediate. Efficient MMR is associated with high levels of gene conversion and low levels of PMS for heterozygous markers, and inefficient MMR results in low levels of conversion and high levels of PMS. PMS tetrads with two *A* spore colonies, one *a* spore colony, and one sectored *A/a* spore colony are called "5*A*:3*a*" tetrads whereas those with one *A* spore colony, two *a* spore colonies, and one sectored *A/a* colony are called "3*A*:5*a*" tetrads. Using this nomenclature (derived from eight-spored fungi), we define Mendelian segregation as 4*A*:4*a* and gene conversion events as 6*A*:2*a* or 2*A*:6*a*.

Diploid strains heterozygous for the *his4-AAG* mutation in the *HIS4* start codon were used to analyze the effects of defects in Pms1p- or Mlh1p-associated ATP hydrolysis (*pms1-E61A* and *mlh1-E31A* alleles, respectively) on the repair of mismatches in heteroduplex recombination intermediates. The PD83 strain background was used in these experiments because of the very high level of meiotic recombination at *HIS4* (Nag & Petes 1993), which occurs as a consequence of a high frequency of meiosis-specific double-strand breaks near the 5' end of the gene (Fan *et al.* 1995). Depending on which DNA strand is transferred during heteroduplex formation, a heteroduplex composed of a wild-type strand and a strand

with the *his4-AAG* substitution will contain either an A-A or a T-T mismatch (Detloff *et al.* 1991). As shown in Table 4.4, a strain with normal MMR (PD83) repaired both mismatches efficiently (Detloff *et al.* 1991), resulting in high levels of gene conversion and low levels of PMS.

In our previous studies, the efficiency of mismatch repair was determined by dividing the number of tetrads in which one or more spores exhibit PMS by the total number of tetrads with non-Mendelian (aberrant) segregation. This approach has two inherent problems. First, tetrads with multiple PMS or multiple conversion events are counted as equivalent to tetrads with a single PMS or conversion event. Second, it is not clear how to count a tetrad that contains both a conversion event *and* a PMS event. Consequently, here we used a different method to measure the efficiency of meiotic heteroduplex repair, which is based on counting the number of individual PMS and gene conversion spore *colonies* rather than *tetrads*. Using the definitions of tetrad classes given in Detloff *et al.* (1991), the number of PMS spore colonies (indicated in parentheses) counted in each class is the following: normal 4:4 (0), 6:2 (0), 2:6 (0), 5:3 (1), 3:5 (1), aberrant 4:4 (2), aberrant 6:2 (2), aberrant 2:6 (2), deviant 5:3 (3), deviant 3:5 (3), deviant 4:4 (4), 7:1 (1), 1:7 (1), 8:0 (0), and 0:8 (0). The number of gene conversion spore colonies counted for each class of tetrad is the following: normal 4:4 (0), 6:2 (1), 2:6 (1), 5:3 (0), 3:5 (0), aberrant 4:4 (0), aberrant 6:2 (0), aberrant 2:6 (0), deviant 5:3 (0), deviant 3:5 (0), deviant 4:4 (0), 7:1 (1), 1:7 (1), 8:0 (2), and 0:8 (2).

The tetrad/spore data for PD83 and mutant derivatives are presented in Table 4.4. The levels of aberrant segregation tetrads in all strains were similar, varying

161

between 57 and 60%. As expected from previous studies ((Williamson *et al.* 1985,

Prolla *et al.* 1994), diploids homozygous for *mlh1Δ* or *pms1Δ* exhibited an increase in

the relative frequencies of PMS spore colonies from 18% in wild type to 89 or 78%,

respectively, indicating inefficient meiotic mismatch repair. Statistical comparison of

the relative number of PMS *vs.* gene conversion spore colonies indicates that *mlh1Δ*

strains had significantly less mismatch repair than the *pms1Δ* strains ($P < 0.0001$).

Although the *mlh1-E31A* strain had significantly less MMR than the wild-type strain

($P < 0.0001$), it had significantly more repair than the *mlh1Δ* strain ($P < 0.0001$).

Similarly, the *pms1-E61A* strain has less mismatch repair than the wild-type strain (P

< 0.002), but more repair than the *pms1Δ* strain ($P < 0.0001$). Finally, relative to the

corresponding null allele, the *pms1-E61A* allele did not appear to confer as severe a

defect in meiotic MMR as the *mlh1-E31A* allele.

The frequency of PMS events at *HIS4* in the wild-type strain PD83 was higher

than that observed in most studies involving different mutant alleles in other genetic

backgrounds. Although one interpretation of this finding is that PD83 has a less

efficient MMR system than that of other wild-type strains, we prefer a different

explanation: that the efficiency of MMR is context dependent. One argument in

support of this conclusion is based on an analysis of aberrant segregation of the

heterozygous *arg4-17* allele in PD83. In 482 tetrads, we found 37 conversion events

and no PMS events, a significant difference ($P < 0.002$) in the relative number of

conversion and PMS tetrads compared to that observed for the *his4-AAG* marker.

Since a heteroduplex formed between *arg4-17* and *ARG4* would contain either an

A/A or a T/T mismatch (the same type of mismatch as expected for the *his4-AAG*

162

marker), these results argue that the efficiency of meiotic MMR is affected by the context of the mismatch. In a previous study, Fogel *et al.* (1979) found that A/G or C/T mismatches failed to get repaired in a wild-type strain at frequencies (expressed as the percentage of PMS tetrads divided by total aberrant tetrads) of 12, 4, and 0% for mismatches located at the *HIS4*, *ARG4*, and *TRP1* loci, respectively.

7. Meiotic crossovers and spore viability in *pms1* and *mlh1* mutants: It has been observed previously that deletion of *MLH1*, but not *PMS1*, reduces crossovers in a variety of intervals (Hunter & Borts 1997, Wang *et al.* 1999). We calculated map distances for several genetic intervals on chromosome *III* in wild-type and mutant strains using only tetrads in which both markers for each interval underwent Mendelian segregation (Table 4.5). This analysis confirmed the previous observation that the *mlh1Δ* mutation significantly reduces crossovers in most genetic intervals examined (Hunter & Borts 1997, Wang *et al.* 1999). In contrast, the *mlh1-E31A* mutation significantly reduced crossovers only in the *HIS4-CEN3* interval. The difference in tetrad classes for the *mlh1Δ* compared to the *mlh1-E31A* strain was significant only for the *MAT-CEN3* interval. Neither the *pms1Δ* nor the *pms1-E61A* mutation significantly affected crossovers in any of the intervals examined.

Diploid strains with mutations in *MLH1* or *PMS1* have reduced spore viability compared to wild-type strains, with *mlh1* alleles having a stronger effect than *pms1* alleles (Prolla *et al.* 1994, Hunter & Borts 1997, Wang *et al.* 1999). Compared to the wild-type strain (84% spore viability), we found significant ($P < 0.0001$) decreases in total spore viability with the *mlh1Δ*, *pms1Δ*, and *mlh1-E31A* strains (69, 77, and 79% spore viabilities, respectively), but not with the *pms1-E61A* strain (84% spore

viability; $P = 0.2$). There also were significant ($P < 0.01$) elevations in the proportion

of tetrads with two viable and two inviable spores for the *mlh1Δ*, *pms1Δ*, and *mlh1-*

E31A strains but not for the *pms1-E61A* strain; none of the MMR-deficient strains

had a significant elevation in the proportion of tetrads with three viable spores (Fig

4.4). The *mlh1Δ* and *mlh1-E31A* strains were significantly different from each other

in all spore viability classes except the class with three viable spores.

The specific increase in the proportion of tetrads with two live:two dead spores is

consistent with either meiosis I nondisjunction resulting from reduced crossing over

or segregation of a heterozygous recessive lethal mutation. Meiosis I nondisjunction

involving chromosome *III* can be readily assessed, with the two surviving spores

predicted to be nonmaters because of heterozygosity at *MAT*. Wang *et al.* (1999)

reported that 20 of 1632 tetrads in an *mlh1Δ* deletion strain had this segregation

pattern, whereas no such tetrads were observed in wild-type or *pms1Δ* strains.

Among the 1936 tetrads derived from the *mlh1Δ* and *mlh1-E31A* homozygous strains

examined here, we found only 3 with the pattern of two nonmating spores and two

dead spores. PCR analysis with *MATa*- and *MATα*-specific primers indicated that

only 1 of the tetrads contained spores disomic for chromosome *III* (data not shown).

In samples of 1074, 975, and 696 tetrads from wild-type, *pms1Δ*, and *pms1-E61A*

strains, respectively, none had the segregation pattern characteristic of meiosis I

nondisjunction of chromosome *III*. These results suggest that, although the *mlh1Δ*

mutation clearly reduces the frequency of crossing over on chromosome *III*, this

reduction is not sufficient to result in elevated meiosis I nondisjunction. The

difference between our results and those of Wang *et al.* (1999)
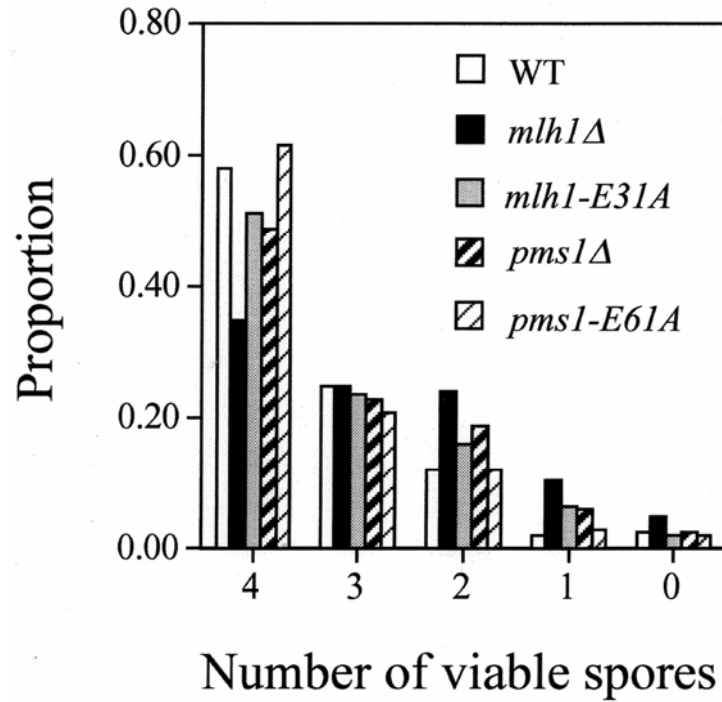
FIGURE 4.4. Spore viability patterns in wild-type and mutant strains. The proportions of tetrad classes are based on the analysis of 1074 (wild type), 1005 (*mlh1Δ*), 931 (*mlh1-E31A*), 975 (*pms1Δ*), and 696 (*pms1-E61A*) tetrads. All diploids were sporulated without prior purification (see MATERIALS AND METHODS).

may reflect the different genetic backgrounds used in the two studies; in our genetic background, the *HIS4* locus (on chromosome *III*) is an extraordinarily strong recombination hotspot (White *et al.* 1993). In addition, in our experiments, the strains were sporulated at 18° C rather than at room temperature.

8. **Different efficiencies of mismatch repair in different strain backgrounds:** As described above, the *pms1-E61A* allele had no significant effect on mutation rates at the *CAN1* locus in the haploid strain SJR1392 (Table 4.2), while the *mlh1-E31A* allele resulted in a mutator phenotype intermediate between those of wild-type and *mlh1Δ* strains (Table 4.3). Because these mutator assays were done in a strain background unrelated to the strains used in the meiotic experiments, we repeated the *CAN1* mutator assay in derivatives of the haploid parental strains used to construct the diploids (Table 4.6). In agreement with earlier studies (Tran & Liskay 2000)and the results reported here with the SJR1392 derivatives, the effect of the *mlh1-E31A* allele is about one-half that observed in the *mlh1Δ* mutant, whereas the effect of *pms1-E61A* on mutation rate is very subtle. Although the mutator phenotypes reported here are qualitatively similar in different strain backgrounds, the absolute effects of null mutations in *MLH1* and *PMS1* on the forward mutation rate at the *CAN1* locus vary considerably between strains. Relative to the isogenic wild-type strain, deletion of *MLH1* or *PMS1* elevates the *CAN1* mutation rate ~60-fold in the SJR1392 background (Table 4.2 and Table 4.3), 30-fold in the AS4 background, and only 15-fold in the PD73 background (Table 4.6). Although rate differences of this sort generally are attributed to minor variations in the mutator assay as performed in different labs at different times, the mutator phenotypes for AS4 and

PD73 were determined simultaneously. In addition, we performed side-by-side

CAN1 mutation rate measurements for SJR1392 and PD73 and confirmed that the

mutation rates are elevated to different extents in *mlh1*Δ mutants (data not shown).

The strain-to-strain differences in mutation rates documented here likely reflect

strain-dependent differences in the fidelity of DNA polymerase and/or differences in

the efficiency of MMR.

D. Discussion

Although the precise roles of MutL homologs are not known, it is generally

assumed that heterodimers of these proteins serve as "matchmakers" to couple

MutS-dependent mismatch recognition to the appropriate processing steps (see

Harfe & Jinks-Robertson 2000a). The nature of the downstream processing steps is

not clear, however, nor is it known whether these steps are identical in all MMR-

related processes. For example, the repair of mismatch-containing replication

intermediates must incorporate a strand discrimination step, which may not be

relevant to the recognition/repair of mismatches in recombination intermediates. To

address possible dissimilarities between MMR mechanisms in replication *vs.*

recombination, we randomly mutagenized the *PMS1* gene and screened for alleles

that differentially affected the mitotic spellchecker and antirecombination activities of

the corresponding proteins. Two such separation-of-function alleles were identified

in the screen (*pms1-L124S* and *pms1-I854M*), each of which resulted in a reduction

in the antirecombination activity of Pms1p, but had no significant effect on the

spellchecker function. When the *pms1-I854M* mutation was combined in the same

gene with mutations similar to the *pms1-L124S* mutation (*pms1-E61A,I854M* and *pms1-G128A,I854M* double-mutant alleles; Table 4.2), the mutations were not epistatic and, therefore, likely affect the function of the Mlh1p-Pms1p complex in fundamentally different ways.

The alteration conferred by the *pms1-I854M* allele is within the C-terminal 200 amino acids of Pms1p, a region that is highly conserved with the human PMS2 protein, but is not represented in the yeast and human Mlh1 proteins or in the bacterial MutL protein (Crouse 1998). Because the conserved C-terminal region of Pms1p is required for ATP-independent heterodimer formation with Mlh1p (Pang *et al.* 1997), the separation-of-function phenotype associated with the *pms1-I854M* allele might simply reflect a reduction in the amount of the Mlh1p-Pms1p complex, with full antirecombination activity requiring more of the complex than the spellchecker function. Our two-hybrid analysis (Fig 4.3) indicates, however, that the I854M amino acid change does not significantly affect the level of the Mlh1p-Pms1p interaction. An alternative to a stability-related explanation for the *pms1-I854M* separation-of-function phenotype derives from studies of the C-terminal region of the bacterial MutL protein, which not only is important for analogous homodimer formation (Drotschmann *et al.* 1998), but also is required for interaction of MutL with the MutH endonuclease and with the UvrD helicase in two-hybrid assays (Hall *et al.* 1998, Hall & Matson 1999). It thus is intriguing to speculate that the *pms1-I854M* mutation alters an interaction with a protein that is important in mitotic antirecombination, but that plays little, if any, role in mutation avoidance. Candidates for such a protein might include members of the *RAD52* epistasis group of

168

recombination proteins (Sung *et al.* 2000) if mismatches are sensed during the strand invasion process or might include proteins involved in disrupting or destroying a recombination intermediate (*e.g.*, helicases or nucleases) if mismatches are detected after formation of stable heteroduplex DNA.

Although it is unclear how the *pms1-I854M* mutation impacts protein function, the *pms1-L124S* allele changes an amino acid that is immediately adjacent to conserved motif III of the GLH superfamily of ATPases (Fig 4.2). Motif III is part of the "ATP lid," which undergoes dramatic conformational change when the N-terminal fragment of MutL (LN40) binds ATP (BAN *et al.* 1999). This ATP-dependent conformational change is accompanied by dimerization of LN40, which is required for subsequent ATP hydrolysis and is speculated to be critical for the interaction of MutL with proteins that participate in the downstream steps of MMR. The weak ATPase activity of MutL presumably returns the protein to its starting conformation so that it can participate in another round of MMR. The situation is more complex in eukaryotes than in bacteria, with the active forms of the comparable MutL-like complexes being heterodimers. A functional asymmetry in the yeast Mlh1p-Pms1p heterodimer has been observed in genetic studies in which targeted mutations were introduced into the ATP binding/hydrolysis domains of the individual subunits (Tran & Liskay 2000). In these studies, mutations that impacted ATP binding or the associated conformational changes (*mlh1-G98A* and *pms1-G128*) resulted in a stronger mutator phenotype than that of the ATP hydrolysis mutations (*mlh1-E31A* and *pms1-E61A*), and the mutations in Mlh1p resulted in a stronger mutator phenotype than that of the comparable mutations in Pms1p (Tran & Liskay 2000). On the basis of these results,

it was suggested that ATP binding by the individual subunits in the Mlh1p-Pms1p complex may be sequential, with ATP binding by Mlh1p preceding and perhaps facilitating ATP binding/hydrolysis by Pms1p. In support of this model, recent biochemical studies have shown that an N-terminal fragment of yeast Mlh1p binds ATP with 10-fold higher affinity than does a comparable N-terminal fragment of Pms1p (Hall *et al.* 2002, see also Tomer *et al.* 2002). The asymmetry between Mlh1p and Pms1p may not be limited only to ATP binding, but may also extend to ATP hydrolysis, as each N-terminal fragment has inherent ATPase activity (Guarne *et al.* 2001, Hall *et al.* 2002). This is in contrast to an N-terminal fragment of the bacterial MutL protein, where only the homodimer exhibits ATPase activity (Ban *et al.* 1999).

According to the model described above, the Mlh1p-Pms1p complex would be expected to retain some function if the ATPase activity of Pms1p is compromised, but would be expected to retain little, if any, function if the ATPase activity of Mlh1p is eliminated. This prediction not only is consistent with spellchecker phenotypes reported previously (Tran & Liskay 2000), but also is supported by the recombination analyses reported here, with mutations in the ATPase domain of Mlh1p resulting in a more severe *in vivo* phenotype than those in the ATPase domain of Pms1p. The functional asymmetry observed for the ATPase domains of Mlh1p and Pms1p in correcting DNA replication errors (Tran & Liskay 2000) thus extends to the mitotic antirecombination function of the MMR system as well as to the repair of mismatches in meiotic recombination intermediates.

In addition to the asymmetry between Mlh1p and Pms1p observed in both the spellchecker and recombination assays, the results reported here indicate that disruption of the ATPase activity of Pms1p impacts the recombination-related functions of the Mlh1p-Pms1p complex more than the replication-related spellchecker function. The differential effect was evident when examining mitotic recombination between homeologous substrates (Table 4.2) and when assessing the repair of mismatches in meiotic recombination intermediates (Table 4.4). These results suggest that the cycles of conformational changes induced by ATP binding/hydrolysis by Pms1p are more important in the recognition or processing of DNA mismatches in recombination intermediates than in the recognition or processing of DNA mismatches resulting from DNA replication errors. Although the purpose of these conformational changes is not clear, it is likely that they are important in interactions of the Mlh1p-Pms1p heterodimer with other proteins involved in MMR-related functions. The proteins required for processing mismatch-containing recombination *vs.* replication intermediates may be different or the proteins simply could be present at different levels in recombination *vs.* replication intermediates. For example, proliferating cell nuclear antigen (PCNA), which is known to interact with MMR proteins (Umar *et al.* 1996, Johnson *et al.* 1996a, Flores-Rozas *et al.* 2000), would be expected to be found at high concentrations at DNA replication forks, but may not necessarily be present at high levels in a heteroduplex recombination intermediate. Thus, the Pms1p ATPase motifs might be necessary for formation and/or stability of a functional mismatch-repair complex in the absence of PCNA. Alternatively, the ATPase activity of Pms1p may be required

171

to direct the activity of downstream factors in the absence of a replication-associated signal that determines which strand is to be repaired.

Although we favor the explanation that the *pms1-E61A*, *pms1-L124S*, and *pms1-G128A* mutations partially separate the functions of the yeast Mlh1p-Pms1p complex in replication and recombination, there are several caveats to this conclusion. First, since the assays used to monitor recombination-related functions of the MMR system were quite different from those used to assess the spellchecker function, we cannot rule out the possibility of DNA sequence-specific or chromosome context-specific effects on MMR activity. In addition, there may be competing systems of repair that operate differently on recombination *vs.* replication intermediates. Finally, recombination-related processes may be more sensitive to the concentration of the Mlh1p-Pms1p complex than are replication-related processes. It is formally possible that the separation-of-function mutations in *PMS1* decrease the overall stability of the protein and thereby reduce the concentration of the Mlh1p-Pms1p complex. Such a stability explanation has been invoked to explain *MLH1* separation-of-function alleles that affect the repair of mismatches in meiotic recombination intermediates more than meiotic crossing over (Argueso *et al.* 2002). Although we have not directly examined the stabilities of the mutant Pms1p proteins, as reported here for the *pms1-I854M* allele, introduction of the *PMS1* or *MLH1* ATP binding/hydrolysis mutations does not affect the stability of two-hybrid fusion proteins (Tran & Liskay 2000).

In summary, the results presented here demonstrate that it is possible to mutationally separate the replication *vs.* recombination roles of the yeast Pms1p

protein. The identification of *pms1* separation-of-function alleles is consistent with the notion that the Mlh1p-Pms1p complex couples mismatch recognition to the appropriate downstream processing steps and suggests that the downstream steps may differ, depending on the context of the mismatch. A major goal of future MMR studies in yeast will be to define the relevant downstream steps in replication *vs.* recombination processes.

TABLE 4.1. Yeast Strains

Strain[a]	Description	Source of construction
SJR1294	Used to screen for separation-of-function *pms1* alleles	This study
	Contains homologous (*LYS2*) and homeologous (*HIS3*) recombination	
SJR1392	substrates	This study
SJR1470	SJR1392 *mlh1Δ::URA3*	This study
SJR1471	SJR1392 *pms1Δ*	This study
SJR1528	SJR1392 *mlh1-E31A*	This study
SJR1529	SJR1392 *mlh1-G98A*	This study
SJR1530	SJR1392 *pms1-E61A*	This study
SJR1531	SJR1392 *pms1-G128A*	This study
SJR1532	SJR1392 *pms1-L124S*	This study
SJR1533	SJR1392 *pms1-I854M*	This study
SJR1561	SJR1392 *mlh1-G98A pms1-G128A*	This study
SJR1578	SJR1392 *mlh1-757stop*	This study
SJR1789	SJR1392 *pms1-E61A,I854M*	This study
SJR1790	SJR1392 *pms1-G128A,I854M*	This study
PD73	*his4-AAG* haploid used to construct diploids	Detloff *et al.* (1991)

for meiotic analyses

HMY91	PD73 *mlh1Δ::kanMX4*	This study
HM94	PD73 *mlh1Δ::URA3*	This study
HM92	PD73 *pms1Δ::kanMX4*	This study
DB10	PD73 *pms1Δ*	This study
JSY88	PD73 *mlh1-E31A*	This study
JSY116	PD73 *pms1-E61A*	This study
AS4	*HIS4* haploid used to construct diploids for meiotic analyses	Stapelton & Petes (1991)
DTK318	AS4 *mlh1Δ::URA3*	This study
DNY95	AS4 *pms1Δ*	This study
JSY89	AS4 *mlh1-E31A*	This study
JSY117	AS4 *pms1-E61A*	This study
PD83	*his4-AAG/HIS4*	PD73 x AS4 (Detloff *et al.* 1992)
HMY95	*his4-AAG/HIS4 mlh1Δ::kanMX4/mlh1Δ::URA3*	HMY91 x DTK318
DB101	*his4-AAG/HIS4 mlh1Δ::URA3/mlh1Δ::URA3*	HMY94 x DTK318
HMY96	*his4-AAG/HIS4 pms1Δ::kanMX4/pms1Δ*	HMY92 x DNY95
DB100	*his4-AAG/HIS4 pms1Δ/pms1Δ*	DB10 x DNY95
JSY76	*his4-AAG/HIS4 mlh1-E31A/mlh1-E31A*	JSY88 x JSY89

JSY80	*his4-AAG/HIS4 pms1-E61A/pms1-E61A*	JSY116 x JSY117
	ARG4 derivative of AS4 for measuring Can-R	
JSY106	rate	This study
JSY108	AS4 *ARG4 mlh1Δ::URA3*	This study
JSY104	AS4 *ARG4 mlh1-E31A*	This study
JSY107	AS4 *ARG4 pms1Δ*	This study
JSY105	AS4 *ARG4 pms1-E61A*	This study

[a] SJR1294, *MATα ade2-101$_{oc}$ his3Δ200 ura3(Nhe)-[HIS3::intron::cß2(94%)]-ura3(Nhe) lys2ΔRV::hisG leu2-R pGAL-PMS1*; SJR1392, *MATα ade2-101$_{oc}$ his3Δ200 lys2ΔRV::hisG trp5Δ::kan ura3(Nhe)-[HIS3::intron::cß2/cß7(91%)]-ura3(Nhe) leu2(K)-[lys2Δ3'-lys2Δ5']-LEU2*; AS4, *MATα trp1 arg4 tyr7 ade6 ura3*; PD73, *MATa leu2 ade6 ura3 his4-AAG*.

176

TABLE 4.2. Mutation and recombination rates in wild-type and *pms1* strains

Genotype[a]	Mutation rate (x10^-7)[b]	Mutation rate relative to WT	Recombination rate (x10^-6)[b] Homologous (Lys+)	Homeologous (His+)	His+/Lys+ rate[c]	His+/Lys+ rate relative to WT
Wild type	1.19 (1.12–1.56)	1.0	3.32 (2.66–4.33)	0.0576 (0.0542–0.0742)	0.017	1.0
pms1Δ	71.1* (52.6–87.2)	60	3.85 (3.07–5.25)	0.725* (0.549–0.885)	0.19	11
pms1-L124S	1.55 (1.25–2.25)	1.3	2.33 (1.75–2.83)	0.171* (0.142–0.228)	0.073	4.2
pms1-I854M	1.48 (1.24–1.68)	1.2	2.96 (2.29–3.49)	0.132* (0.103–0.164)	0.046	2.7
pms1-E61A	1.65 (1.43–2.25)	1.4	3.54 (3.09–4.58)	0.172* (0.129–0.244)	0.049	2.8
pms1-G128A	1.29 (1.18–1.63)	1.1	2.62 (2.29–3.57)	0.174* (0.146–0.227)	0.066	3.8
pms1-E61A,I854M	5.47* (4.87–7.15)	4.6	2.53 (1.87–2.94)	0.337* (0.298–0.429)	0.16	9.4
pms1-G128A,I854M	7.18* (5.82–8.28)	6.0	2.03* (1.72–2.37)	0.318* (0.260–0.403)	0.19	11

[a] All strains are isogenic derivatives of SJR1392. WT, wild type.

[b] Ninety-five percent confidence intervals are indicated in parentheses.

177

[c] The His+/Lys+ rate corresponds to the average rate ratio for the independent cultures examined. The His+/Lys+ rate ratio for each independent culture was obtained by dividing the His+ rate by the Lys+ rate for that particular culture. The His+ or Lys+ rate calculation for an individual culture was determined by using the number of prototrophic colonies as the median (Lea & Coulson 1949).

* Significantly different from the rate in the wild-type control strain.

TABLE 4.3. Mutation and recombination rates in wild-type and *mlh1* strains

Genotype[a]	Mutation rate (x10^{-7})[b]	Mutation rate relative to WT	Recombination rate (x10^{-6})[b] Homologous (Lys$^+$)	Homeologous (His$^+$)	His$^+$/Lys$^+$ rate[c]	His$^+$/Lys$^+$ rate relative to WT
Wild type	1.19 (1.12–1.56)	1.0	3.32 (2.66–4.33)	0.0576 (0.0542–0.0742)	0.017	1.0
mlh1Δ	63.0* (52.6–98.1)	53	3.17 (2.71–5.61)	0.532* (0.348–0.775)	0.17	9.7
mlh1-E31A	23.5* (14.0–26.7)	20	3.35 (2.42–6.09)	0.528* (0.363–0.621)	0.16	9.1
mlh1-G98A	56.8* (41.2–79.6)	48	3.65 (2.85–4.86)	0.673* (0.498–0.815)	0.18	11
mlh1-G98A, pms1-G128A	71.5* (53.9–87.5)	60	4.14 (2.98–5.35)	0.815* (0.247–1.07)	0.20	11
mlh1-757stop	69.2* (49.9–82.0)	58	4.43 (2.72–6.63)	0.815* (0.565–0.905)	0.18	11

* Significantly different from the rate in the wild-type control strain.

[a] All strains are isogenic derivatives of SJR1392.

[b] Ninety-five percent confidence intervals are indicated in parentheses.

179

[c] The His^+/Lys^+ rate corresponds to the average rate ratio for the independent cultures examined. The His^+/Lys^+ rate ratio for each independent culture was obtained by dividing the His^+ rate by the Lys^+ rate for that particular culture. The His^+ or Lys^+ rate calculation for an individual culture was determined by using the number of prototrophic colonies as the median (Lea & Coulson 1949).

TABLE 4.4. Rates of aberrant segregation at the *HIS4* locus in wild-type, *pms1*, and *mlh1* strains

| Strain | Relevant genotype | Total tetrads | No. of tetrads/class | | | | | | % ab. | % PMS/ab. tetrad[b] | % PMS/ab. spore[c] | Relative increase in PMS[d] |
			4:4	6:2	2:6	5:3	3:5	Other ab.[a]				
PD83[e]	Wild type	482	202	73	108	22	21	56	58	18	18	1
HMY95/DB101[f]	*mlh1Δ*	465	199	18	16	83	70	79	57	85	89	4.9
JSY76	*mlh1-E31A*	388	154	24	42	52	45	71	60	62	69	3.8
HMY96/DB100[f]	*pms1Δ*	641	266	32	38	99	103	103	59	73	78	4.3
JSY80	*pms1-E61A*	358	150	45	42	33	29	59	58	35	40	2.2

[a] This class includes tetrads with two or more PMS and conversion events. The number of events (shown in

parentheses for each class for each genotype): wild type: ab. 4:4 (1), ab. 2:6 (1), deviant (dev.) 5:3 (1), 7:1 (3), 8:0 (11),

0:8 (16); *mlh1Δ*: ab. 4:4 (13), ab. 6:2 (5), ab. 2:6 (4), dev. 5:3 (7), dev. 4:4 (1), 7:1 (3), 8:0 (1); *mlh1-E31A*: ab.

4:4 (12), ab. 6:2 (3), ab. 2:6 (1), dev. 3:5 (2), 7:1 (4), 1:7 (4), 8:0 (1), 0:8 (3); *pms1Δ*: ab. 4:4 (24), ab. 6:2 (5),

ab. 2:6 (8), dev. 5:3 (5), dev. 3:5 (1), 7:1 (7), 1:7 (7), 8:0 (3), 0:8 (2); *pms1-E61A*: ab. 4:4 (2), ab. 6:2 (2), ab. 2:6 (1), 7:1 (13), 1:7 (7), 8:0 (8), 0:8 (10).

[b] These percentages were calculated by dividing the total number of aberrant tetrads by the number of tetrads with one or more PMS event, excluding those that had both a PMS and a gene conversion event.

[c] These percentages were calculated (as described in the text) by dividing the number of sectored (PMS) spore colonies by the total number of PMS plus gene-conversion spore colonies.

[d] The percentage of PMS/aberrant spore colony values for each mutant strain were divided by the percentage of PMS/aberrant spore colony values observed in wild type.

[e] Although this strain was examined previously with purified diploids (Detloff *et al.* 1991), the data shown here include only diploids sporulated soon after mating.

[f] Data from these pairs of strains were not significantly different and were pooled.

182

TABLE 4.5. Genetic distances for intervals on chromosome *III* in wild-type and mutant strains

Strain	Genotype	HIS4-LEU2				LEU2-CEN3				HIS4-CEN3				MAT-CEN3				HIS4-MAT			
		P	N	T	cM	P	N	T	cM	P	N	T	cM	P	N	T	cM	P	N	T	cM
PD83	Wild type	118	1	73	21	204	182	82	9	53	50	91	23	97	85	166	24	47	14	78	58
HMY95	*mlh1Δ*	140	3	43	16*	191	187	79	9	58	67	72	18*	87	82	68	14*	56	4	42	32*
JSY76	*mlh1-E31A*	104	4	43	22	165	163	62	8	49	52	51	17*	87	73	112	21	45	6	56	43
HMY96	*pms1Δ*	161	1	89	19	264	270	93	7	70	69	100	21	93	75	124	21	47	8	80	47
JSY80	*pms1-E61A*	95	1	50	20	139	144	64	9	42	41	63	22	71	61	110	23	39	5	52	43

The numbers of tetrads in each class is shown, with P, N, and T indicating parental ditype, nonparental ditype, and tetratype tetrads, respectively. Only tetrads in which the flanking markers underwent Mendelian (4:4) segregation are included. Map distances for the *HIS4-LEU2* and *HIS4-MAT* intervals were calculated using the Perkins equation (Perkins 1949). Gene-centromere distances were calculated using the heterozygous centromere-linked *TRP1* marker as described by Sherman & Wakem (1991). For the gene-centromere intervals, the number of first-division segregation tetrads (P and N) and second-division segregation tetrads (T) were compared by Fisher's exact test. For the gene-gene intervals, the number of P, N, and T tetrads was compared using a 3 x 2 contingency chi-square test.

* Significantly reduced (*P* < 0.05) map distance relative to the wild-type strain.

Table 4.6. Forward mutation rate at the *CAN1* locus in AS4- and PD73-related haploids

Strain	Relevant genotype	*CAN1* mutation rate (x10^{-7})[a]	Fold increase in rate[b]
PD73 derivatives			
PD73	Wild type	1.2 (1.1–1.7)	1
HMY91	*mlh1Δ::kanMX4*	17 (15–24)	14
JSY88	*mlh1-E31A*	11 (8–17)	9
HMY92	*pms1Δ::kanMX4*	16 (13–23)	13
JSY116	*pms1-E61A*	2.3 (2.1–2.7)	2
AS4 derivatives			
JSY106	Wild type	0.9 (0.6–1.1)	1
JSY108	*mlh1Δ::URA3*	20 (17–25)	23
JSY104	*mlh1-E31A*	21 (19–28)	25
JSY107	*pms1Δ*	35 (28–48)	41
JSY105	*pms1-E61A*	2 (1.1–5.8)	2

[a] Rates were calculated by fluctuation analysis as described in MATERIALS AND METHODS. Numbers in parentheses indicate 95% confidence intervals.

[b] The rates observed for the mutant strains were divided by the wild-type rate.

CHAPTER V. CONCLUSIONS AND FUTURE DIRECTIONS

The data presented in this dissertation provide several new insights into the fields of DNA mismatch repair (MMR) and meiotic recombination. The MMR machinery can recognize and correct DNA mismatches that result from misincorporated bases during DNA replication (known as the "spellchecker" function), as well as DNA mismatches that form during recombination events between strands that are not completely complementary. The work described here demonstrates that many of the proteins required for the repair of replication errors are also required for the efficient repair of mismatches in heteroduplex DNA formed during meiotic recombination. The latter type of repair, known as "meiotic MMR", can result in a gene conversion event (conversion-type repair) or can restore Mendelian segregation (restoration-type repair). My data suggest that the ratio of conversion-type repair to restoration-type repair is affected by both the strength and location of the local recombination-initiating double-strand breaks (DSBs).

A. MutS and MutL homologues involved in the repair of base-base mismatches and four-base loops: In Chapter 2, I examined the involvement of MutS and MutL homologues in the repair of two types of mismatches created during meiotic recombination. These studies demonstrated that the substrate specificities of the MutS and MutL homologues involved in meiotic MMR were the same as those previously identified for the spellchecker function. Specifically, I found that the repair

of base-base mismatches in meiotic heteroduplexes requires Msh2p/Msh6p and Mlh1p/Pms1p heterodimers. At least two different complexes can repair four-base loops in meiotic heteroduplexes: (1) Msh2p/Msh3p and Mlh1p/Pms1p heterodimers initiate about two-thirds of the repair events, and (2) a novel Mlh1-independent, Mlh3-dependent complex initiates the remaining one-third of the repair events.

Mlh1p-independent, Mlh3p-dependent MMR has not yet been demonstrated in vegetative cells. Previous physical studies by Wang *et al.* (1999) found that all detectable heterodimers of the MutL homologues in vegetative cells include Mlh1p, a result that contradicts the possibility of Mlh1p-independent MMR in vegetative cells. It is possible, however, that Mlh1p-independent, Mlh3p-dependent complexes were not detected in these studies because they occur at very low frequencies relative to Mlh1-dependent complexes or because the interaction between Mlh3p and its partner(s) is unstable. Alternatively, it is possible that Mlh1-independent, Mlh3-dependent complexes only form and/or function during meiosis because of differential expression patterns of MMR components. In support of this possibility, microarray analysis showed that the expression of *MLH3*, *PMS1*, and *MSH2* in cells sporulated for 2-5 hours is two-fold elevated relative to that found in vegetatively growing cells. The expression patterns of *MLH1*, *MLH2*, *MSH3*, and *MSH6* were similar in meiotically and vegetatively growing cells (Chu *et al.* 1998).

If Mlh1p-independent, Mlh3p-dependent MMR does indeed function in vegetative cells, I predict that it could be detected genetically as an increase in the instability of a microsatellite with a four-base repeat, in an *mlh3* mutant. However, four-base repeats are uncommon in the *S. cerevisiae* genome. A more biologically relevant

assay would be to asses the effect of the *mlh3* mutation on microsatellites with repeats of one or two bases, which the most common types of repetitive elements the yeast. Frameshifts in mononucleotide runs were previously found to be elevated in *mlh3* mutants, although this effect was very subtle compared to *msh2* mutants (Harfe *et al.* 2000). It would be interesting to determine if microsatellites composed of two-base repeats are also destabilized in *mlh3* mutants. If maintaining stability of this type of microsatellite does depend on Mlh3p, it would suggest a reason why yeast maintains both Mlh1p-independent and Mlh1p-dependent MMR complexes capable of repairing small loops.

B. Effects of mutations in nuclease-encoding genes on meiotic mismatch repair: Previous studies have been interpreted as indicating that there a number of nucleases which are functionally redundant in the excision step of MMR (Tishkoff *et al.* 1997a, Tishkoff *et al.* 1997b, Kokoska *et al.* 1998, Kokoska *et al.* 2000, Tran *et al.* 2001, Tran *et al.* 2004). In the studies presented here, I examined only the nuclease activities which had been found to result in a mutator phenotype when absent. I found that the meiotic repair of either base-base mismatches or four-base loops was unaffected by the absence of Exo1p, Rad1p, Rad27p, or the proofreading exonuclease activity of DNA polymerase δ. Further studies aimed at identifying the nuclease(s) that function in MMR have been hindered because the deletion of more than one of the nucleases often results in synthetic lethality in yeast, an effect likely due to a rapid accumulation of mutations. It is possible that these synthetic lethalities could be mitigated by using a regulatable promoter to reduce the expression of one or more of the nuclease-encoding genes.

As described in Chapter 1, hPms2p was recently shown to have latent endonuclease activity (Kadyrov *et al.* 2006). A mutation predicted to disrupt this endonuclease activity in yeast Pms1p confers a mutation rate similar to that observed for *pms1* null strains (T. Kunkel, personal communication). Mlh3p, but not Mlh1p or Mlh2p, has a similar endonuclease-like domain, suggesting that Mlh3p may also have latent endonuclease activity. It would be interesting to analyze the effect(s) of endonuclease-defective alleles of *PMS1* and *MLH3* on meiotic MMR. Based on studies of *in vitro* nick-directed MMR (Dzantiev *et al.* 2004, Constantin *et al.* 2005), it was suggested that, when a mismatch is located 3' of a nick, the Pms1p endonuclease activity is required to make a single-strand incision 5' to the mismatch. The 5' to 3' exonuclease Exo1p can then excise the DNA between this Pms1p-mediated incision and the mismatch. Since conversion-type repair is believed to involve a similar nick-directed, 3' to 5' excision of a mismatch (see Fig 2.3), endonuclease-defective alleles of *PMS1* or *MLH3* might alter meiotic MMR such that conversion-type repair is decreased relative to restoration-type repair. Based on my studies, the endonuclease-defective *pms1* allele would be expected to affect the repair of both base-base mismatches and four-base loops, while the endonuclease-defective *mlh3* allele would be expected to affect only the repair of four-base loops.

C. Mismatch repair proteins involved in crossover regulation: Previous studies indicated that Mlh1p, Mlh3p, Msh4p, Msh5p, and possibly Exo1p, act in single pathway for the processing of meiotic recombination intermediates that result in crossovers (reviewed by Hoffmann & Borts 2004). As expected from the previous studies of others, I found that meiotic crossovers were decreased in *mlh1*, *mlh3*,

188

msh4, *msh5*, and *exo1* mutants. Aberrant segregation was also reduced in *msh4* and *msh5* strains. This reduction in aberrant segregation was due to a reduction in the level of heteroduplex DNA at 5' end of *HIS4* rather than due to an elevation in the level of restoration-type repair. These data support a model proposed by Börner *et al.* (2004), in which Msh4p and Msh5p are involved in determining whether a recombination-initiating DSB is processed to create a crossover or a noncrossover. Based on physical studies of recombination intermediates, this decision must be made at an early step in the recombination process, preceding the formation of single-end invasion intermediates (Börner *et al.* 2004). Subsequently, Msh4p and Msh5p are required to stabilize crossover-designated single-end invasion intermediates. Thus, the majority of recombination intermediates formed in the absence of Msh4p or Msh5p are resolved as noncrossovers, and those that do form single-end invasion intermediates are unstable.

D. Conversion-type and restoration type repair of base-base mismatches at different loci: My results showed that the repair of a mismatch at the *HIS4* locus is biased toward conversion-type repair, while a mismatch at the *ARG4* locus is unbiased (equal frequencies of conversion-type and restoration-type repair). To explain this finding, I suggest that, at the *HIS4* locus, almost all of the mismatches are the result of heteroduplexes initiated at the *HIS4* DSB site. Due to the close proximity of these mismatches and the *HIS4* DSB site, repair is directed to conversion-type repair by a nick located near to the site of the DSB (defined as "early" repair in the model of Foss *et al.* [1999]). In contrast, at the *ARG4* locus, the mismatches are the result of heteroduplexes initiated at two different sites. Those

mismatches resulting from heteroduplexes initiated at the *ARG4* DSB site are directed to conversion-type repair, whereas those mismatches resulting from heteroduplexes initiated at the neighboring *DED81* DSB hotspot are directed to restoration-type repair. In support of this model, I found that increasing the activity of the *ARG4* DSB biases the repair to conversion-type repair. Conversely, eliminating the activity of the *HIS4* DSB site, which increases the frequencies of DSBs in regions flanking *HIS4*, relieves the bias for conversion-type repair of *HIS4*-associated mismatches. Thus, both the rate of aberrant segregation and the ratio of conversion-type to restoration-type repair for a single marker can be influenced by the relative strength and position of more than one DSB. This conclusion may explain some of the variable observations made with various MMR mutants at different loci in different genetic backgrounds.

E. PCNA is involved in recombination-associated mismatch repair processes: *In vitro* studies implicated the replication factor PCNA (<u>p</u>roliferating <u>c</u>ell <u>n</u>uclear <u>a</u>ntigen) in MMR at a step preceding the DNA synthesis that is required to fill in the gap resulting from the excision of a mismatch (Umar *et al.* 1996, Lau & Kolodner 2003). In genetic studies, mutations in *POL30*, the gene that encodes yeast PCNA, have been shown to impair both MMR and DNA replication, thereby inducing mutator phenotypes (Ayyagari *et al.* 1995, Umar *et al.* 1996, Kokoska *et al.* 1999, Chen *et al.* 1999, Lau *et al.* 2002). Our collaborators in the Jinks-Robertson lab demonstrated that two *pol30* alleles caused a slight increase in mitotic recombination between sequences with 92% homology, a finding that suggests that mismatch recognition may be compromised by these mutations. Using these same

two *pol30* alleles, I observed that the repair of base-base mismatches formed during meiotic recombination was reduced, but not eliminated by these mutations. Therefore, there may be both PCNA-dependent and PCNA-independent pathways for recombination-associated MMR. Alternatively, since these *pol30* mutations are hypomorphic, they may not completely eliminate the function of PCNA in MMR. To determine whether PCNA is essential for the recognition of mismatches in meiotic heteroduplexes, alleles of *MSH3* and *MSH6* that are deficient in PCNA-binding should be examined.

In addition to meiotic MMR defects, the *pol30* strains were found to have decreased levels of gene conversion and meiotic crossovers. Moreover, the level of aberrant segregation of a marker located far from the *HIS4* DSB site was decreased more than that of a marker located close to the *HIS4* DSB site in *pol30* strains. These data suggest that the *pol30* mutations may cause a decrease in the processivity of the DNA synthesis that is primed by the strand invasion step of meiotic recombination. This effect could both decrease the length of the heteroduplex DNA and increase the frequency of SDSA, resulting in a decrease in the rate of aberrant segregation and in a decrease in crossovers.

F. ***pms1* and *mlh1* separation-of-function mutants:** In Chapter 4, I presented studies that showed that mutations in the ATPase domain of *PMS1* impaired both the mitotic anti-recombination function of MMR and meiotic MMR. In contrast, these mutations had very little effect on the spellchecker function. A similar separation-of-function phenotype was observed for some temperature-sensitive *mlh1* alleles (Argueso *et al.* 2002). Mutations in the ATPase domain of *MLH1*, however, impair

MMR in all three contexts. Interestingly, certain *mlh1* alleles confer a separation-of-function phenotype opposite to the effect observed for the *pms1* ATPase mutants; they cause spellchecker defects but have no effect on meiotic MMR (Argueso *et al.* 2003, Hoffmann *et al.* 2003). The detection of *pms1* and *mlh1* separation-of-function mutations supports the possibility that the Mlh1p/Pms1p heterodimer couples mismatch recognition with the downstream processing steps of MMR. These types of mutations may be very useful in determining how the structural differences between replication forks and recombination intermediates alter the mechanisms of MMR.

G. Conclusions: In summary, I characterized the yeast proteins required for meiotic MMR and demonstrated that the efficiency of MMR is affected by the chromosome context. The repair of base-base mismatches in meiotic heteroduplex DNA requires Msh2p/Msh6p and Mlh1p/Pms1p heterodimers. PCNA is also involved in the repair of base-base mismatches, as well as the processing of recombination intermediates. In contrast, the efficient repair of four-base loops in meiotic heteroduplex DNA requires Msh2p/Msh3p and Mlh1p/Pms1p heterodimers; however, a novel Mlh1p-independent, Mlh3p-dependent complex competes with Mlh1p-dependent complexes for the repair of four-base loops. In agreement with previous studies, I also found that meiotic crossovers and aberrant segregation are reduced in the absence of various MMR-associated proteins. Finally, I found that the location and strength of local recombination-inducing DSBs affect the relative levels of conversion-type and restoration-type repair. While these studies were performed in *S. cerevisiae*, the highly conserved nature of MMR and the meiotic

recombination machinery suggests that these findings will be applicable to higher

eukaryotes, including humans.

LITERATURE CITED

Abdullah, M. F., E. R. Hoffmann, V. E. Cotton, and R. H. Borts, 2004 A role for the MutL homologue *MLH2* in controlling heteroduplex formation and in regulating between two different crossover pathways in budding yeast. Cytogenet.Genome Res. **107**: 180-190.

Alani, E., 1996 The Saccharomyces cerevisiae Msh2 and Msh6 proteins form a complex that specifically binds to duplex oligonucleotides containing mismatched DNA base pairs. Mol.Cell Biol. **16**: 5604-5615.

Alani, E., R. A. Reenan, and R. D. Kolodner, 1994 Interaction between mismatch repair and genetic recombination in *Saccharomyces cerevisiae*. Genetics **137**: 19-39.

Ali, J. A., A. P. Jackson, A. J. Howells, and A. Maxwell, 1993 The 43-kilodalton N-terminal fragment of the DNA gyrase B protein hydrolyzes ATP and binds coumarin drugs. Biochemistry **32**: 2717-2724.

Allers, T., and M. Lichten, 2001 Differential timing and control of noncrossover and crossover recombination during meiosis. Cell **106**: 47-57.

Argueso, J. L., A. W. Kijas, S. Sarin, J. Heck, M. Waase *et al.* 2003 Systematic mutagenesis of the *Saccharomyces cerevisiae MLH1* gene reveals distinct roles for Mlh1p in meiotic crossing over and in vegetative and meiotic mismatch repair. Mol.Cell Biol. **23**: 873-886.

Argueso, J. L., D. Smith, J. Yi, M. Waase, S. Sarin *et al.* 2002 Analysis of conditional mutations in the *Saccharomyces cerevisiae MLH1* gene in mismatch repair and in meiotic crossing over. Genetics **160**: 909-921.

Argueso, J. L., J. Wanat, Z. Gemici, and E. Alani, 2004 Competing crossover pathways act during meiosis in *Saccharomyces cerevisiae*. Genetics **168**: 1805-1816.

Ayyagari, R., K. J. Impellizzeri, B. L. Yoder, S. L. Gary, and P. M. Burgers, 1995 A mutational analysis of the yeast proliferating cell nuclear antigen indicates distinct roles in DNA replication and DNA repair. Mol.Cell Biol. **15**: 4420-4429.

Ban, C., M. Junop, and W. Yang, 1999 Transformation of MutL by ATP binding and hydrolysis: a switch in DNA mismatch repair. Cell **97**: 85-97.

Ban, C., and W. Yang, 1998 Crystal structure and ATPase activity of MutL: implications for DNA repair and mutagenesis. Cell **95**: 541-552.

Bartel, P., C. T. Chien, R. Sternglanz, and S. Fields, 1993 Elimination of false positives that arise in using the two-hybrid system. Biotechniques **14**: 920-924.

Bartsch, S., L. E. Kang, and L. S. Symington, 2000 *RAD51* is required for the repair of plasmid double-stranded DNA gaps from either plasmid or chromosomal templates. Mol.Cell Biol. **20**: 1194-1205.

Benjamini, Y., and Y. Hochberg, 1995 Controlling the False Discovery Rate - A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B-Methodological **57**: 289-300.

Bertrand, P., D. X. Tishkoff, N. Filosi, R. Dasgupta, and R. D. Kolodner, 1998 Physical interaction between components of DNA mismatch repair and nucleotide excision repair. Proc Natl Acad Sci U S A **95**: 14278-14283.

Börner, G. V., N. Kleckner, and N. Hunter, 2004 Crossover/noncrossover differentiation, synaptonemal complex formation, and regulatory surveillance at the leptotene/zygotene transition of meiosis. Cell **117**: 29-45.

Borts, R. H., S. R. Chambers, and M. F. Abdullah, 2000 The many faces of mismatch repair in meiosis. Mutat.Res. **451**: 129-150.

Chen, C., B. J. Merrill, P. J. Lau, C. Holm, and R. D. Kolodner, 1999 *Saccharomyces cerevisiae pol30* (proliferating cell nuclear antigen) mutations impair replication fidelity and mismatch repair. Mol.Cell Biol. **19**: 7801-7815.

Chen, W., and S. Jinks-Robertson, 1998 Mismatch repair proteins regulate heteroduplex formation during mitotic recombination in yeast. Mol Cell Biol **18**: 6525-37.

Chen, W., and S. Jinks-Robertson, 1999 The role of the mismatch repair machinery in regulating mitotic and meiotic recombination between diverged sequences in yeast. Genetics **151**: 1299-313.

Chu, S., J. DeRisi, M. Eisen, J. Mulholland, D. Botstein *et al.* 1998 The transcriptional program of sporulation in budding yeast. Science **282**: 699-705.

Clark, A. B., F. Valle, K. Drotschmann, R. K. Gary, and T. A. Kunkel, 2000 Functional interaction of proliferating cell nuclear antigen with MSH2-MSH6 and MSH2-MSH3 complexes. J.Biol.Chem. **275**: 36498-36501.

Coic, E., L. Gluck, and F. Fabre, 2000 Evidence for short-patch mismatch repair in *Saccharomyces cerevisiae*. EMBO J. **19**: 3408-3417.

Constantin, N., L. Dzantiev, F. A. Kadyrov, and P. Modrich, 2005 Human mismatch repair: reconstitution of a nick-directed bidirectional reaction. J.Biol.Chem. **280**: 39752-39761.

Crouse, G. F., 1998 Mismatch repair systems in *Saccharomyces cerevisiae*, pp. 411-448 in *DNA Damage and Repair, Volume 1: DNA Repair in Prokaryotes and Lower Eukaryotes*, edited by J. A. Nickoloff, and M. F. Hoekstra. Humana Press, Totowa, NJ.

Datta, A., A. Adjiri, L. New, G. F. Crouse, and R. S. Jinks, 1996 Mitotic crossovers between diverged sequences are regulated by mismatch repair proteins in *Saccaromyces cerevisiae*. Mol.Cell Biol. **16**: 1085-1093.

Datta, A., M. Hendrix, M. Lipsitch, and S. Jinks-Robertson, 1997 Dual roles for DNA sequence identity and the mismatch repair system in the regulation of mitotic crossing-over in yeast. Proc Natl Acad Sci U S A **94**: 9757-62.

de los Santos, T., N. Hunter, C. Lee, B. Larkin, J. Loidl *et al.* 2003 The Mus81/Mms4 endonuclease acts independently of double-Holliday junction resolution to promote a distinct subset of crossovers during meiosis in budding yeast. Genetics **164**: 81-94.

de Massy, B., and A. Nicolas, 1993 The control in cis of the position and the amount of the *ARG4* meiotic double-strand break of *Saccharomyces cerevisiae*. EMBO J. **12**: 1459-1466.

Detloff, P., J. Sieber, and T. D. Petes, 1991 Repair of specific base pair mismatches formed during meiotic recombination in the yeast *Saccharomyces cerevisiae*. Mol Cell Biol **11**: 737-45.

Detloff, P., M. A. White, and T. D. Petes, 1992 Analysis of a gene conversion gradient at the *HIS4* locus in *Saccharomyces cerevisiae*. Genetics **132**: 113-23.

Dixon W. J., and F. J. Massey, 1969 Introduction to Statistical Analysis. McGraw-Hill, New York.

Dohet, C., R. Wagner, and M. Radman, 1985 Repair of defined single base-pair mismatches in *Escherichia coli*. Proc Natl Acad Sci U S A **82**: 503-505.

Drotschmann, K., A. Aronshtam, H. J. Fritz, and M. G. Marinus, 1998 The Escherichia coli MutL protein stimulates binding of Vsr and MutS to heteroduplex DNA. Nucleic Acids Res. **26**: 948-953.

Dutta, R., and M. Inouye, 2000 GHKL, an emergent ATPase/kinase superfamily. Trends Biochem.Sci. **25**: 24-28.

Dzantiev, L., N. Constantin, J. Genschel, R. R. Iyer, P. M. Burgers *et al.* 2004 A defined human system that supports bidirectional mismatch-provoked excision. Mol.Cell **15**: 31-41.

Earley, M. C., and G. F. Crouse, 1998 The role of mismatch repair in the prevention of base pair mutations in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A **95**: 15487-15491.

Fan, Q., F. Xu, and T. D. Petes, 1995 Meiosis-specific double-strand DNA breaks at the *HIS4* recombination hot spot in the yeast *Saccharomyces cerevisiae*: control in cis and trans. Mol.Cell Biol. **15**: 1679-1688.

Fang, W. H., and P. Modrich, 1993 Human strand-specific mismatch repair occurs by a bidirectional mechanism similar to that of the bacterial reaction. J.Biol.Chem. **268**: 11838-11844.

Fleck, O., E. Lehmann, P. Schar, and J. Kohli, 1999 Involvement of nucleotide-excision repair in msh2 pms1-independent mismatch repair. Nat.Genet. **21**: 314-317.

Fleig, U. N., R. D. Pridmore, and P. Philippsen, 1986 Construction of *LYS2* cartridges for use in genetic manipulations of *Saccharomyces cerevisiae*. Gene **46**: 237-245.

Flores-Rozas, H., D. Clark, and R. D. Kolodner, 2000 Proliferating cell nuclear antigen and Msh2p-Msh6p interact to form an active mispair recognition complex. Nat.Genet. **26**: 375-378.

Flores-Rozas, H., and R. D. Kolodner, 1998 The Saccharomyces cerevisiae *MLH3* gene functions in *MSH3*-dependent suppression of frameshift mutations. Proc Natl Acad Sci U S A **95**: 12404-12409.

Fogel, S., R. Mortimer, K. Lusnak, and F. Tavares, 1979 Meiotic gene conversion: a signal of the basic recombination event in yeast. Cold Spring Harb.Symp.Quant.Biol. **43 Pt 2**: 1325-1341.

Fogel, S., R. K. Mortimer, and K. Lusnick, 1981 Mechanisms of meiotic gene conversion, or "Wanderings on a forgein strand", pp. 289-339 in *The Molecular Biology of the Yeast Saccharomyces*, edited by J. Strathern, E. W. Jones, and J. Broach. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory, NY.

Foss, H. M., K. J. Hillers, and F. W. Stahl, 1999 The conversion gradient at *HIS4* of *Saccharomyces cerevisiae*. II. A role for mismatch repair directed by biased resolution of the recombinational intermediate. Genetics **153**: 573-83.

Genschel, J., L. R. Bazemore, and P. Modrich, 2002 Human exonuclease I is required for 5' and 3' mismatch repair. J.Biol.Chem. **277**: 13302-13311.

Gerton, J. L., J. DeRisi, R. Shroff, M. Lichten, P. O. Brown *et al.* 2000 Inaugural article: global mapping of meiotic recombination hotspots and coldspots in the yeast *Saccharomyces cerevisiae*. Proc.Natl.Acad.Sci.U.S.A **97**: 11383-11390.

Goldstein, A. L., and J. H. McCusker, 1999 Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. Yeast **15**: 1541-1553.

Greene, C. N., and S. Jinks-Robertson, 1997 Frameshift intermediates in homopolymer runs are removed efficiently by yeast mismatch repair proteins. Mol.Cell Biol. **17**: 2844-2850.

Gu, L., Y. Hong, S. McCulloch, H. Watanabe, and G. M. Li, 1998 ATP-dependent interaction of human mismatch repair proteins and dual role of PCNA in mismatch repair. Nucleic Acids Res. **26**: 1173-1178.

Guarne, A., M. S. Junop, and W. Yang, 2001 Structure and function of the N-terminal 40 kDa fragment of human PMS2: a monomeric GHL ATPase. EMBO J. **20**: 5521-5531.

Habraken, Y., P. Sung, L. Prakash, and S. Prakash, 1996 Binding of insertion/deletion DNA mismatches by the heterodimer of yeast mismatch repair proteins MSH2 and MSH3. Curr.Biol. **6**: 1185-1187.

Hall, M. C., J. R. Jordan, and S. W. Matson, 1998 Evidence for a physical interaction between the *Escherichia coli* methyl-directed mismatch repair proteins MutL and UvrD. EMBO J. **17**: 1535-1541.

Hall, M. C., and S. W. Matson, 1999 The *Escherichia coli* MutL protein physically interacts with MutH and stimulates the MutH-associated endonuclease activity. J.Biol.Chem. **274**: 1306-1312.

Hall, M. C., P. V. Shcherbakova, and T. A. Kunkel, 2002 Differential ATP binding and intrinsic ATP hydrolysis by amino-terminal domains of the yeast Mlh1 and Pms1 proteins. J.Biol.Chem. **277**: 3673-3679.

Harfe, B. D., and S. Jinks-Robertson, 1999 Removal of frameshift intermediates by mismatch repair proteins in *Saccharomyces cerevisiae*. Mol.Cell Biol. **19**: 4766-4773.

Harfe, B. D., and S. Jinks-Robertson, 2000a DNA mismatch repair and genetic instability. Annu.Rev.Genet. **34**: 359-399.

Harfe, B. D., and S. Jinks-Robertson, 2000b Mismatch repair proteins and mitotic genome stability. Mutat.Res. **451**: 151-167.

Harfe, B. D., B. K. Minesinger, and S. Jinks-Robertson, 2000 Discrete in vivo roles for the MutL homologs Mlh2p and Mlh3p in the removal of frameshift intermediates in budding yeast. Curr.Biol. **10**: 145-148.

Hawk, J. D., L. Stefanovic, J. C. Boyer, T. D. Petes, and R. A. Farber, 2005 Variation in efficiency of DNA mismatch repair at different sites in the yeast genome. Proc Natl Acad Sci U S A **102**: 8639-8643.

Henderson, K. A., and S. Keeney, 2004 Tying synaptonemal complex initiation to the formation and programmed repair of DNA double-strand breaks. Proc Natl Acad Sci U S A **101**: 4519-4524.

Herskowitz, I., and R. E. Jensen, 1991 Putting the HO gene to work: practical uses for mating-type switching. Methods Enzymol. **194**: 132-146.

Hoffmann, E. R., and R. H. Borts, 2004 Meiotic recombination intermediates and mismatch repair proteins. Cytogenet.Genome Res. **107**: 232-248.

Hoffmann, E. R., P. V. Shcherbakova, T. A. Kunkel, and R. H. Borts, 2003 MLH1 mutations differentially affect meiotic functions in Saccharomyces cerevisiae. Genetics **163**: 515-526.

Holliday, R., 1964 A mechanism for gene conversion in fungi. Genet.Res. **5**: 282-304.

Hollingsworth, N. M., L. Ponte, and C. Halsey, 1995 *MSH5*, a novel MutS homolog, facilitates meiotic reciprocal recombination between homologs in *Saccharomyces cerevisiae* but not mismatch repair. Genes Dev. **9**: 1728-1739.

Hunter, N., and R. H. Borts, 1997 Mlh1 is unique among mismatch repair proteins in its ability to promote crossing-over during meiosis. Genes Dev **11**: 1573-82.

Iyer, R. R., A. Pluciennik, V. Burdett, and P. L. Modrich, 2006 DNA mismatch repair: functions and mechanisms. Chem.Rev. **106**: 302-323.

Johnson, A., and M. O'Donnell, 2005 Cellular DNA replicases: components and dynamics at the replication fork. Annu.Rev.Biochem. **74**: 283-315.

Johnson, R. E., G. K. Kovvali, S. N. Guzder, N. S. Amin, C. Holm *et al.* 1996a Evidence for involvement of yeast proliferating cell nuclear antigen in DNA mismatch repair. J.Biol.Chem. **271**: 27987-27990.

Johnson, R. E., G. K. Kovvali, L. Prakash, and S. Prakash, 1996b Requirement of the yeast *MSH3* and *MSH6* genes for *MSH2*-dependent genomic stability. J.Biol.Chem. **271**: 7285-7288.

Kadyrov, F. A., L. Dzantiev, N. Constantin, and P. Modrich, 2006 Endonucleolytic function of MutLalpha in human mismatch repair. Cell **126**: 297-308.

Kearney, H. M., D. T. Kirkpatrick, J. L. Gerton, and T. D. Petes, 2001 Meiotic recombination involving heterozygous large insertions in *Saccharomyces cerevisiae*: formation and repair of large, unpaired DNA loops. Genetics **158**: 1457-1476.

Keeney, S., C. N. Giroux, and N. Kleckner, 1997 Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. Cell **88**: 375-84.

Khazanehdari, K. A., and R. H. Borts, 2000 *EXO1* and *MSH4* differentially affect crossing-over and segregation. Chromosoma **109**: 94-102.

Kirkpatrick, D. T., J. R. Ferguson, T. D. Petes, and L. S. Symington, 2000 Decreased meiotic intergenic recombination and increased meiosis I nondisjunction in *exo1* mutants of *Saccharomyces cerevisiae*. Genetics **156**: 1549-1557.

Kirkpatrick, D. T., and T. D. Petes, 1997 Repair of DNA loops involves DNA-mismatch and nucleotide-excision repair proteins. Nature **387**: 929-31.

Kokoska, R. J., L. Stefanovic, A. B. Buermeyer, R. M. Liskay, and T. D. Petes, 1999 A mutation of the yeast gene encoding PCNA destabilizes both microsatellite and minisatellite DNA sequences. Genetics **151**: 511-519.

Kokoska, R. J., L. Stefanovic, J. DeMai, and T. D. Petes, 2000 Increased rates of genomic deletions generated by mutations in the yeast gene encoding DNA polymerase delta or by decreases in the cellular levels of DNA polymerase delta. Mol.Cell Biol. **20**: 7490-7504.

Kokoska, R. J., L. Stefanovic, H. T. Tran, M. A. Resnick, D. A. Gordenin *et al.* 1998 Destabilization of yeast micro- and minisatellite DNA sequences by mutations affecting a nuclease involved in Okazaki fragment processing (*rad27*) and DNA polymerase delta (*pol3-t*). Mol Cell Biol **18**: 2779-88.

Kramer, B., W. Kramer, M. S. Williamson, and S. Fogel, 1989a Heteroduplex DNA correction in *Saccharomyces cerevisiae* is mismatch specific and requires functional PMS genes. Mol.Cell Biol. **9**: 4432-4440.

Kramer, W., B. Kramer, M. S. Williamson, and S. Fogel, 1989b Cloning and nucleotide sequence of DNA mismatch repair gene *PMS1* from *Saccharomyces cerevisiae*: homology of *PMS1* to procaryotic MutL and HexB. J.Bacteriol. **171**: 5339-5346.

Krishna, T. S., D. Fenyo, X. P. Kong, S. Gary, B. T. Chait *et al.* 1994 Crystallization of proliferating cell nuclear antigen (PCNA) from *Saccharomyces cerevisiae*. J.Mol.Biol. **241**: 265-268.

Kunkel, T. A., 2004 DNA replication fidelity. J.Biol.Chem. **279**: 16895-16898.

Lamers, M. H., A. Perrakis, J. H. Enzlin, H. H. Winterwerp, N. de Wind *et al.* 2000 The crystal structure of DNA mismatch repair protein MutS binding to a G x T mismatch. Nature **407**: 711-717.

Lau, P. J., H. Flores-Rozas, and R. D. Kolodner, 2002 Isolation and characterization of new proliferating cell nuclear antigen (*POL30*) mutator mutants that are defective in DNA mismatch repair. Mol.Cell Biol. **22**: 6669-6680.

Lau, P. J., and R. D. Kolodner, 2003 Transfer of the MSH2.MSH6 complex from proliferating cell nuclear antigen to mispaired bases in DNA. J.Biol.Chem. **278**: 14-17.

Lea, D. E., and C. A. Coulson, 1949 The distribution of the numbers of mutants in bacterial populations. J.Math.Biol. **49**: 264-285.

Levinson, G., and G. A. Gutman, 1987 Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Mol.Biol.Evol. **4**: 203-221.

Li, Y., T. Kane, C. Tipper, P. Spatrick, and D. D. Jenness, 1999 Yeast mutants affecting possible quality control of plasma membrane proteins. Mol.Cell Biol. **19**: 3588-3599.

Liu, Y., H. I. Kao, and R. A. Bambara, 2004 Flap endonuclease 1: a central component of DNA metabolism. Annu.Rev.Biochem. **73**: 589-615.

Longtine, M. S., A. McKenzie, III, D. J. Demarini, N. G. Shah, A. Wach *et al.* 1998 Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast **14**: 953-961.

Maga, G., and U. Hubscher, 2003 Proliferating cell nuclear antigen (PCNA): a dancer with many partners. J.Cell Sci **116**: 3051-3060.

Maloisel, L., J. Bhargava, and G. S. Roeder, 2004 A role for DNA polymerase delta in gene conversion and crossing over during meiosis in *Saccharomyces cerevisiae*. Genetics **167**: 1133-1142.

Marsischky, G. T., N. Filosi, M. F. Kane, and R. Kolodner, 1996 Redundancy of *Saccharomyces cerevisiae MSH3* and *MSH6* in *MSH2*-dependent mismatch repair. Genes Dev. **10**: 407-420.

Marsischky, G. T., and R. D. Kolodner, 1999 Biochemical characterization of the interaction between the *Saccharomyces cerevisiae* MSH2-MSH6 complex and mispaired bases in DNA. J.Biol.Chem. **274**: 26668-26682.

Marti, T. M., and O. Fleck, 2004 DNA repair nucleases. Cell Mol.Life Sci. **61**: 336-354.

Merker, J. D., M. Dominska, and T. D. Petes, 2003 Patterns of heteroduplex formation associated with the initiation of meiotic recombination in the yeast *Saccharomyces cerevisiae*. Genetics **165**: 47-63.

Modrich, P., 1991 Mechanisms and biological effects of mismatch repair. Annu.Rev.Genet. **25**: 229-253.

Modrich, P., and R. Lahue, 1996 Mismatch repair in replication fidelity, genetic recombination, and cancer biology. Annu.Rev.Biochem. **65**: 101-133.

Morrison, A., A. L. Johnson, L. H. Johnston, and A. Sugino, 1993 Pathway correcting DNA replication errors in *Saccharomyces cerevisiae*. EMBO J. **12**: 1467-1473.

Morse, R. H., 2000 RAP, RAP, open up! New wrinkles for RAP1 in yeast. Trends Genet. **16**: 51-53.

Muhlrad, D., R. Hunter, and R. Parker, 1992 A rapid method for localized mutagenesis of yeast genes. Yeast **8**: 79-82.

Myung, K., A. Datta, k. chen, and R. Kolodner, 2001 Chopping and changing. Nat.Genet. **27**: 1-2.

Nag, D. K., and T. D. Petes, 1991 Seven-base-pair inverted repeats in DNA form stable hairpins in vivo in *Saccharomyces cerevisiae*. Genetics **129**: 669-673.

Nag, D. K., and T. D. Petes, 1993 Physical detection of heteroduplexes during meiotic recombination in the yeast *Saccharomyces cerevisiae*. Mol.Cell Biol. **13**: 2324-2331.

Nag, D. K., M. A. White, and T. D. Petes, 1989 Palindromic sequences in heteroduplex DNA inhibit mismatch repair in yeast. Nature **340**: 318-20.

New, L., K. Liu, and G. F. Crouse, 1993 The yeast gene *MSH3* defines a new class of eukaryotic MutS homologues. Mol.Gen.Genet. **239**: 97-108.

Nicholson, A., M. Hendrix, S. Jinks-Robertson, and G. F. Crouse, 2000 Regulation of mitotic homeologous recombination in yeast. Functions of mismatch repair and nucleotide excision repair genes. Genetics **154**: 133-146.

Nicolas, A., D. Treco, N. P. Schultes, and J. W. Szostak, 1989 An initiation site for meiotic gene conversion in the yeast Saccharomyces cerevisiae. Nature **338**: 35-39.

Obmolova, G., C. Ban, P. Hsieh, and W. Yang, 2000 Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA. Nature **407**: 703-710.

Pang, Q., T. A. Prolla, and R. M. Liskay, 1997 Functional domains of the *Saccharomyces cerevisiae* Mlh1p and Pms1p DNA mismatch repair proteins and their relevance to human hereditary nonpolyposis colorectal cancer-associated mutations. Mol.Cell Biol. **17**: 4465-4473.

Paques, F., and J. E. Haber, 1999 Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev **63**: 349-404.

Perkins, D. D., 1949 Biochemical mutants in the smut fungus *Ustilago maydis*. Genetics **34**: 607-626.

Petes, T. D., and D. Botstein, 1977 Simple Mendelian inheritance of the reiterated ribosomal DNA of yeast. Proc Natl Acad Sci U S A **74**: 5091-5095.

Petes, T. D., R. E. Malone, and L. Symington, 1991 Recombination in yeast, pp. 407-521 in *The Molecular and Cellular Biology of the Yeast Saccharomyces*, edited by J. Broach, E. W. Jones, and J. R. Pringle. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Porter, S. E., M. A. White, and T. D. Petes, 1993 Genetic evidence that the meiotic recombination hotspot at the *HIS4* locus of *Saccharomyces cerevisiae* does not represent a site for a symmetrically processed double-strand break. Genetics **134**: 5-19.

Prodromou, C., S. M. Roe, R. O'Brien, J. E. Ladbury, P. W. Piper *et al.* 1997a Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. Cell **90**: 65-75.

Prodromou, C., S. M. Roe, P. W. Piper, and L. H. Pearl, 1997b A molecular clamp in the crystal structure of the N-terminal domain of the yeast Hsp90 chaperone. Nat.Struct.Biol. **4**: 477-482.

Prolla, T. A., D. M. Christie, and R. M. Liskay, 1994 Dual requirement in yeast DNA mismatch repair for *MLH1* and *PMS1*, two homologs of the bacterial mutL gene. Mol.Cell Biol. **14**: 407-415.

Rayssiguier, C., D. S. Thaler, and M. Radman, 1989 The barrier to recombination between *Escherichia coli* and *Salmonella typhimurium* is disrupted in mismatch-repair mutants. Nature **342**: 396-401.

Reenan, R. A., and R. D. Kolodner, 1992 Characterization of insertion mutations in the S*accharomyces cerevisiae MSH1* and *MSH2* genes: evidence for separate mitochondrial and nuclear functions. Genetics **132**: 975-985.

Ross-Macdonald, P., and G. S. Roeder, 1994 Mutation of a meiosis-specific MutS homolog decreases crossing over but not mismatch correction. Cell **79**: 1069-1080.

Sherman F., G. R. Fink, and J. B. Hicks, 1982 Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sherman, F., and P. Wakem, 1991 Mapping yeast genes. Methods Enzymol. **194**: 38-57.

Sia, E. A., S. Jinks-Robertson, and T. D. Petes, 1997a Genetic control of microsatellite stability. Mutat Res **383**: 61-70.

Sia, E. A., and D. T. Kirkpatrick, 2005 The yeast MSH1 gene is not involved in DNA repair or recombination during meiosis. DNA Repair (Amst) **4**: 253-261.

Sia, E. A., R. J. Kokoska, M. Dominska, P. Greenwell, and T. D. Petes, 1997b Microsatellite instability in yeast: dependence on repeat unit size and DNA mismatch repair genes. Mol Cell Biol **17**: 2851-8.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics **122**: 19-27.

Stapleton, A., and T. D. Petes, 1991 The Tn3 beta-lactamase gene acts as a hotspot for meiotic recombination in yeast. Genetics **127**: 39-51.

Stojic, L., R. Brun, and J. Jiricny, 2004 Mismatch repair and DNA damage signalling. DNA Repair (Amst) **3**: 1091-1101.

Stone, J. E., and T. D. Petes, 2006 Analysis of the proteins involved in the *in vivo* repair of base-base mismatches and four-base loops formed during meiotic recombination in the yeast *Saccharomyces cerevisiae*. Genetics **173**: 1223-1239.

Sugawara, N., F. Paques, M. Colaiacovo, and J. E. Haber, 1997 Role of *Saccharomyces cerevisiae* Msh2 and Msh3 repair proteins in double-strand break-induced recombination. Proc Natl Acad Sci U S A **94**: 9214-9.

Sun, H., D. Treco, and J. W. Szostak, 1991 Extensive 3'-overhanging, single-stranded DNA associated with the meiosis-specific double-strand breaks at the *ARG4* recombination initiation site. Cell **64**: 1155-1161.

Sung, P., K. M. Trujillo, and S. Van Komen, 2000 Recombination factors of *Saccharomyces cerevisiae*. Mutat.Res. **451**: 257-275.

Surtees, J. A., J. L. Argueso, and E. Alani, 2004 Mismatch repair proteins: key regulators of genetic recombination. Cytogenet.Genome Res. **107**: 146-159.

Szostak, J. W., T. L. Orr-Weaver, R. J. Rothstein, and F. W. Stahl, 1983 The double-strand-break repair model for recombination. Cell **33**: 25-35.

Tishkoff, D. X., A. L. Boerger, P. Bertrand, N. Filosi, G. M. Gaida *et al.* 1997a Identification and characterization of *Saccharomyces cerevisiae EXO1*, a gene encoding an exonuclease that interacts with MSH2. Proc.Natl.Acad.Sci.U.S.A **94**: 7487-7492.

Tishkoff, D. X., N. Filosi, G. M. Gaida, and R. D. Kolodner, 1997b A novel mutation avoidance mechanism dependent on *S. cerevisiae RAD27* is distinct from DNA mismatch repair. Cell **88**: 253-263.

Tomer, G., A. B. Buermeyer, M. M. Nguyen, and R. M. Liskay, 2002 Contribution of human mlh1 and pms2 ATPase activities to DNA mismatch repair. J.Biol.Chem. **277**: 21801-21809.

Tornier, C., S. Bessone, I. Varlet, C. Rudolph, M. Darmon *et al.* 2001 Requirement for Msh6, but not for Swi4 (Msh3), in Msh2-dependent repair of base-base mismatches and mononucleotide loops in *Schizosaccharomyces pombe*. Genetics **158**: 65-75.

Tran, H. T., D. A. Gordenin, and M. A. Resnick, 1999 The 3'-->5' exonucleases of DNA polymerases delta and epsilon and the 5'-->3' exonuclease Exo1 have major roles in postreplication mutation avoidance in *Saccharomyces cerevisiae*. Mol.Cell Biol. **19**: 2000-2007.

Tran, P. T., N. Erdeniz, L. S. Symington, and R. M. Liskay, 2004 EXO1-A multi-tasking eukaryotic nuclease. DNA Repair (Amst) **3**: 1549-1559.

Tran, P. T., and R. M. Liskay, 2000 Functional studies on the candidate ATPase domains of *Saccharomyces cerevisiae* MutLalpha. Mol.Cell Biol. **20**: 6390-6398.

Tran, P. T., J. A. Simon, and R. M. Liskay, 2001 Interactions of Exo1p with components of MutLalpha in *Saccharomyces cerevisiae*. Proc.Natl.Acad.Sci.U.S.A **98**: 9760-9765.

Trinh, T. Q., and R. R. Sinden, 1991 Preferential DNA secondary structure mutagenesis in the lagging strand of replication in *E. coli*. Nature **352**: 544-547.

Tsubouchi, H., and H. Ogawa, 2000 Exo1 roles for repair of DNA double-strand breaks and meiotic crossing over in Saccharomyces cerevisiae. Mol.Biol.Cell **11**: 2221-2233.

Umar, A., A. B. Buermeyer, J. A. Simon, D. C. Thomas, A. B. Clark *et al.* 1996 Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. Cell **87**: 65-73.

Vedel, M., and A. Nicolas, 1999 *CYS3*, a hotspot of meiotic recombination in *Saccharomyces cerevisiae*. Effects of heterozygosity and mismatch repair functions on gene conversion and recombination intermediates. Genetics **151**: 1245-59.

Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper, 1993 Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74**: 205-214.

Wach, A., A. Brachat, R. Pohlmann, and P. Philippsen, 1994 New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast **10**: 1793-1808.

Wang, T. F., N. Kleckner, and N. Hunter, 1999 Functional specificity of MutL homologs in yeast: evidence for three Mlh1-based heterocomplexes with distinct roles during meiosis in recombination and mismatch correction. Proc Natl Acad Sci U S A **96**: 13914-9.

Welz-Voegele, C., J. E. Stone, P. T. Tran, H. M. Kearney, R. M. Liskay *et al.* 2002 Alleles of the yeast *PMS1* mismatch-repair gene that differentially affect recombination- and replication-related processes. Genetics **162**: 1131-1145.

Westmoreland, J., G. Porter, M. Radman, and M. A. Resnick, 1997 Highly mismatched molecules resembling recombination intermediates efficiently transform mismatch repair proficient *Escherichia coli*. Genetics **145**: 29-38.

White, J. H., K. Lusnak, and S. Fogel, 1985 Mismatch-specific post-meiotic segregation frequency in yeast suggests a heteroduplex recombination intermediate. Nature **315**: 350-352.

White, M. A., M. Dominska, and T. D. Petes, 1993 Transcription factors are required for the meiotic recombination hotspot at the *HIS4* locus in *Saccharomyces cerevisiae*. Proc.Natl.Acad.Sci.U.S.A **90**: 6621-6625.

White, M. A., M. Wierdl, P. Detloff, and T. D. Petes, 1991 DNA-binding protein *RAP1* stimulates meiotic recombination at the *HIS4* locus in yeast. Proc.Natl.Acad.Sci.U.S.A **88**: 9755-9759.

Wigley, D. B., G. J. Davies, E. J. Dodson, A. Maxwell, and G. Dodson, 1991 Crystal structure of an N-terminal fragment of the DNA gyrase B protein. Nature **351**: 624-629.

Williamson, M. S., J. C. Game, and S. Fogel, 1985 Meiotic gene conversion mutants in *Saccharomyces cerevisiae*. I. Isolation and characterization of *pms1-1* and *pms1-2*. Genetics **110**: 609-646.

Xu, F., and T. D. Petes, 1996 Fine-structure mapping of meiosis-specific double-strand DNA breaks at a recombination hotspot associated with an insertion of telomeric sequences upstream of the *HIS4* locus in yeast. Genetics **143**: 1115-25.

Zhang, Y., F. Yuan, S. R. Presnell, K. Tian, Y. Gao *et al.* 2005 Reconstitution of 5'-directed human mismatch repair in a purified system. Cell **122**: 693-705.