Analysis of Conditional Mutations in the *Saccharomyces cerevisiae MLH1* Gene in Mismatch Repair and in Meiotic Crossing Over

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ABSTRACT

In mismatch repair (MMR), members of the *MLH* gene family have been proposed to act as key molecular matchmakers to coordinate mismatch recognition with downstream repair functions that result in mispair excision. Two members of this gene family, *MLH1* and *MLH3*, have also been implicated in meiotic crossing over. These diverse roles suggest that a mutational analysis of *MLH* genes could provide reagents required to identify interactions between gene products and to test whether the different roles ascribed to a subset of these genes can be separated. In this report we show that in *Saccharomyces cerevisiae* the *mlh1*Δ mutation confers inviability in *pol3-01* strain backgrounds that are defective in the Polδ proofreading exonuclease activity. This phenotype was exploited to identify four *mlh1* alleles that each confer a temperature-sensitive phenotype for viability in *pol3-01* strains. In three different mutator assays, strains bearing conditional *mlh1* alleles displayed wild-type or nearly wild-type mutation rates at 26°. At 35°, these strains exhibited mutation rates that approached those observed in *mlh1*Δ mutants. The mutator phenotype exhibited in *mlh1-I296S* strains was partially suppressed at 35° by *EXO1* overexpression. The *mlh1-F228S* and *-I296S* mutations conferred a separation-of-function phenotype in meiosis; both *mlh1-F228S* and *-I296S* strains displayed strong defects in meiotic mismatch repair but showed nearly wild-type levels of crossing over, suggesting that the conditional mutations differentially affected *MLH1* functions. These genetic studies suggest that the conditional *mlh1* mutations can be used to separate the MMR and meiotic crossing-over functions of *MLH1* and to identify interactions between *MLH1* and downstream repair components.

IN prokayotes and eukaryotes, highly conserved mismatch repair (MMR) systems play key roles in mutation avoidance. In *Escherichia coli*, DNA replication errors that result in base-base and insertion/deletion loops are substrates for the MutSLH MMR system (reviewed in Modrich and Lahue 1996). Following mispair recognition by MutS, interactions between the MutS-mispair complex and MutL result in activation of the MutH endonuclease activity. This activity results in cleavage of the unmethylated DNA strand at hemimethylated d(GATC) sites that are transiently present after replication fork passage and provides an entry point for DNA helicase II (UvrD), single-strand binding protein, and single-stranded DNA exonucleases to excise the mispair. The resulting single-strand gap is a substrate for resynthesis steps by DNA polymerase III. This mechanism coordinates MMR with DNA replication, so that newly formed mismatches are repaired using the methylated parental strands as template. MutL has been proposed to act as a key molecular matchmaker to coordinate MutS mismatch binding, MutH endonuclease, and UvrD helicase activities (Au *et al.* 1992; Sancar and Hearst 1993; Ban and Yang 1998; Hall *et al.* 1998; Hall and Matson 1999; Schofield *et al.* 2001).

In *Saccharomyces cerevisiae*, three of the six *M*ut*S h*omologs (Msh), Msh2p, Msh3p, and Msh6p, form heterodimers that recognize mispairs and small insertion/deletion loops (reviewed in Kolodner and Marsischky 1999). The Msh2p-Msh3p heterodimer initiates repair of small loop mismatches while the Msh2p-Msh6p heterodimer initiates repair of both nucleotide substitutions and small loop mismatches. Binding of these complexes to mispairs is thought to signal recruitment of *M*ut*L h*omolog (Mlh) heterodimers (Prolla *et al.* 1994; Habraken *et al.* 1998). Four Mlh proteins (Pms1 and Mlh1, -2, and -3) have been identified in *S. cerevisiae*. An Mlh1p-Pms1p complex functions in both Msh3p- and Msh6p-dependent repair pathways and is likely to be the major Mlh complex in mismatch repair. In addition, an Mlh1p-Mlh3p complex has been implicated in the Msh3p-dependent repair pathway and an Mlh1p-Mlh2p complex has been shown to play a minor role in the removal of frameshift intermediates (Flores-Rozas and Kolodner 1998; Wang *et al.* 1999; Harfe *et al.* 2000). Finally, MMR proteins play important roles in meiotic crossing over. Strains lacking the *MSH4*, *MSH5*, *MLH1*, or *MLH3* gene products display reduced meiotic crossing over

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between homologous chromosomes. For each mutant strain, the defect is accompanied by an increase in meiosis I chromosome nondisjunction and reduced spore viability (Ross-Macdonald and Roeder 1994; Hollingsworth *et al.* 1995; Hunter and Borts 1997; Wang *et al.* 1999).

As described above, *E. coli* MutL acts as a molecular matchmaker between the MutS mismatch recognition factor and downstream MMR components. Do Mlh proteins play an analogous role in eukaryotic MMR? In *S. cerevisiae*, genetic and biochemical studies have resulted in the identification of interactions between Mlh1p and two components, proliferating cell nuclear antigen (PCNA) and Exo1p, that are thought to play downstream roles in MMR. The replication processivity factor PCNA, which has been shown to interact by two-hybrid analysis with Mlh1p, has been implicated in MMR at steps prior to and during strand resynthesis and may play a critical role in strand discrimination by targeting MMR proteins to excise newly synthesized DNA components (Johnson *et al.* 1996; Umar *et al.* 1996; Gu *et al.* 1998; Chen *et al.* 1999).

More recently, Mlh1p has been shown to interact with Exo1p, a nuclease that belongs to the Rad27p/Fen-1p family of double-stranded DNA 5′ to 3′ exonucleases (Szankasi and Smith 1995; Fiorentini *et al.* 1997; Tishkoff *et al.* 1997; Amin *et al.* 2001; Schmutte *et al.* 2001; Tran *et al.* 2001). In addition, like *MLH1, EXO1* plays an important role in meiotic crossing over (Khazanehdari and Borts 2000; Kirkpatrick *et al.* 2000; Tsubouchi and Ogawa 2000). *EXO1* was first linked to MMR in *S. cerevisiae* through a two-hybrid interaction with *MSH2* (Tishkoff *et al.* 1997). While genetic analysis suggests that Exo1p acts in an Msh-Mlh mismatch repair pathway, *exo1*Δ strains exhibit mutation rates that are much lower than those found in other MMR mutants, suggesting that other exonucleases with redundant functions can act in MMR or that Exo1p plays a minor role in MMR (Tishkoff *et al.* 1997). Redundancy of exonuclease activity has been observed in *E. coli* where four exonucleases have been implicated in mismatch repair (Viswanathan *et al.* 2001). In support of the idea that Exo1p plays an important role in MMR, Sokolsky and Alani (2000) found that overexpression of *EXO1* suppressed the conditional inviability of *msh2-L560S pol3-01* strains as well as the mutator phenotype of *msh2-L560S POL3* strains. The *pol3-01* mutation, which causes a defect in polymerase δ proofreading function, has been hypothesized to cause lethality in MMR-defective strains as the result of high mutational load (Morrison *et al.* 1993) or by eliciting an S-phase checkpoint (Datta *et al.* 2000).

The finding that *MLH1* plays roles in both MMR and meiotic crossing over and that Mlh proteins are thought to act as matchmakers to recruit downstream MMR components encouraged us to identify conditional *mlh1* mutations. Conditional mutations have often provided effective tools to identify interactions between gene products and to separate the function of genes required in multiple pathways (Jarvik and Botstein 1975). In this article we describe the isolation of four *mlh1* alleles that conferred a conditional mutator phenotype as well as conditional viability in *pol3-01* strains. Overexpression of *EXO1* partially suppressed the mutator phenotype exhibited in *mlh1-I296S* strains. In meiotic assays, the *mlh1-F228S* and *mlh1-I296S* strains displayed a separation-of-function phenotype with respect to MMR and crossing over. Together, these observations support the idea that conditional mutations in *MLH1* can be used to probe its functions in mismatch repair and in meiotic recombination.

MATERIALS AND METHODS

S. cerevisiae* strains:** Strains that were used to examine the conditional mutator phenotype of *mlh1*^{ts} alleles were derived from the FY (S288C) background (Winston *et al.* 1995). The *mlh1*Δ*::hisG* allele contains only the amino-terminal 12 amino acids of the 769-amino-acid *MLH1* coding region. To test *mlh1*Δ *pol3-01* synthetic lethality, EAY312 (*MATa** *ura3-52 leu2*Δ*1 trp1*Δ*63 mlh1*Δ*::hisG*) was mated to EAY575 (*MAT*α *his3*Δ *leu2*Δ*1 ura3-52 pol3-01*). The EAY312/EAY575 diploid was sporulated and resulting tetrads were dissected. The spore clones were then genotyped by PCR (Sokolsky and Alani 2000). Conditional *mlh1* alleles were isolated from EAY644 [*MAT***a** *leu2*Δ *trp1*Δ *ura3-52 pol3-01 mlh1*Δ, pEAA110 (*MLH1 URA3 ARSH4 CEN6*)] transformed with a library of pEAA109 plasmid (*MLH1 LEU2 ARSH4 CEN6*) mutagenized within the *MLH1* gene (Figure 1, and below). Lys$^+$ reversion rates were examined in EAY652 (*MAT*α *leu2*Δ*1 mlh1*Δ*::hisG lys2-Bgl*II *his3*Δ *ura3-52*) containing *MLH1* or *mlh1* derivatives of pEAA109 (Table 2). Forward mutation to canavanine resistance and repeat tract instability were measured in EAY774 (*MAT***a** *mlh1*Δ*::hisG lys2-Bgl*II *leu2*Δ*-1 trp1*Δ*63 ura3-52 his3*Δ; Tables 3 and 4; see below).

Yeast strains were grown in either yeast extract-peptone-dextrose (YPD) or minimal selective media (Rose *et al.* 1990). When required, canavanine (Sigma, St. Louis) was included in minimal selective media at 60 mg/liter and cycloheximide (Sigma) was included in minimal selective media at 3 mg/liter. 5-Fluoroorotic acid (5-FOA; United States Biologicals) plates were prepared as described previously (Rose *et al.* 1990), and sporulation plates and procedures were as described previously (Detloff *et al.* 1991).

PCR mutagenesis of *MLH1*: We took advantage of the error rate of *Taq* DNA polymerase under standard PCR conditions to create a library of mutagenized *MLH1* plasmids. Six PCR reactions were performed, each containing 0.4 μg of pEAA109 (*MLH1 LEU2 ARSH4 CEN6*); 10 pmol AO144 (5′ AGTCAGT GAGCGAGGAAGC); 10 pmol AO324 (5′ ATAGTGTAGGA GGCGCTG); and concentrations of *Taq* DNA polymerase, buffer, and dNTPs recommended by Perkin-Elmer Cetus. PCR reactions were run for 12 cycles using a 30-sec denaturation step at 95°, a 30-sec annealing step at 56°, and a 5-min polymerization step at 72°. Primers AO144 and AO324 amplified a 3.2-kb fragment that spanned the entire *MLH1* open reading frame. This fragment was gel purified, digested with *Nhe*I and *Sac*I restriction enzymes, and then subcloned into corresponding sites in pEAA109. The six subcloned libraries, one derived from each PCR, were amplified prior to transformation into EAY644. Oligonucleotide synthesis and double-stranded DNA sequencing were performed at the Cornell Biotechnology Analytical-Synthesis Facility (Ithaca, NY). To identify the DNA

sequence change in the *mlh1* mutations, the entire subcloned *Nhe*I and *Sac*I fragment was sequenced in the pEAA109 derivatives. All restriction endonucleases and T4 DNA ligase were from New England Biolabs (Beverly, MA) and used according to manufacturer's specifications.

Isolation of *mlh1^{ts}* alleles: EAY644 containing pEAA110 was transformed with the PCR-mutagenized library of pEAA109. Transformants were then replica plated to duplicate 5-FOA containing minimal media to select for loss of pEAA110. After replica plating, one set of the 5-FOA plates was incubated at 26° while the other was incubated at 35°. A total of 12,600 transformants from six individually mutagenized pools were screened. Approximately 10% of these transformants displayed inviability on both the 26° and 35° incubated 5-FOA plates, indicating that loss-of-function mutations were obtained at a high frequency. After recovery of the initial 19 temperature-sensitive candidates and retransformation into EAY644 to confirm the original phenotype, 4 temperature-sensitive alleles (*mlh1-T113A*, *-I147T*, *-F228S*, and *-I296S*) were identified.

Determination of mutation rates: The rate per generation of *lys2-Bgl*II reversion, forward mutation to canavanine resistance, and dinucleotide repeat tract instability was calculated from the median mutation frequency using the method of Lea and Coulson (1949). Reversion of *lys2-Bgl*II to Lys$^+$ was tested in EAY652 strains transformed with pRS415 (*LEU2 ARSH4 CEN6*), pEAA109 (*MLH1 LEU2 ARSH4 CEN6*), or pEAA109 derivatives pEAA127 (*mlh1-T113A*), pEAA128 (*mlh1-I147T*), pEAA126 (*mlh1-F228S*), or pEAA130 (*mlh1-I296S*). The forward mutation rate to canavanine resistance (Reenan and Kolodner 1992) was measured in EAY774 transformed with pEAA109, -126, -127, -128, -130, or pRS415.

Repeat-tract instability rates were determined in EAY774 by measuring frameshift events within the poly(TG) tract of pSH44 [*ARS CEN*, *TRP1*, *(TG)$_{16}$T-URA3*; Henderson and Petes 1992] that resulted in resistance to 5-FOA. To examine the repeat tract instability phenotype of the *mlh1* conditional alleles, EAY774 containing pSH44 was transformed with pRS415, pEAA109, -126, -127, -128, or -130. To test high-copy suppression of the conditional *mlh1* alleles by *EXO1* and *SGS1*, EAY774 was also transformed with pRS423 (*HIS3* 2μ; Christianson *et al.* 1992), pEAM81 (*EXO1 HIS3* 2μ), or pEAM87 (*SGS1 HIS3* 2μ). pEAM87 was created by inserting a 4.8-kb *Bam*HI-*Xho*I *SGS1* fragment from pWJ691 (kindly provided by J. Weinstein and R. Rothstein) into corresponding sites in pRS423.

All steps in the above studies were performed at the indicated temperatures (26° or 35°) with the exception of the repeat tract instability assay, where cells were grown to single colonies at 26° or 35° and plated onto 5-FOA and complete media and then incubated at 26°. The genetic data presented in Table 4 were analyzed using the Mann-Whitney test statistic where P values <0.05 are considered significant (Pfaffenberger and Patterson 1977).

Yeast two-hybrid analysis: Plasmids used in the two-hybrid analysis were generously provided by the Liskay, Kleckner, and Stagljar laboratories. The *mlh1^{ts}* alleles were subcloned into the LexA-Mlh1 vector pBTM-yMLH1 (Pang *et al.* 1997). The L40 strain used for two-hybrid analysis (Vojtek *et al.* 1993) was first transformed with plasmids carrying *GAL4* activation domain fusions to *PMS1*, *EXO1*, *SGS1*, or *MLH3*, followed by transformation with pBTM-yMLH1 and *mlh1^{ts}* derivatives. Expression of the *lacZ* reporter gene was determined by color filter assays as described (Pang *et al.* 1997).

Meiotic analysis of *mlh1^{ts}* strains: S288C-derived MGD strains were used to examine the meiotic phenotypes conferred by the *mlh1^{ts}* mutations (Table 1; Rocco *et al.* 1992; kindly provided by B. deMassy and A. Nicolas). These strains contain markers (*yhr017W::TRP1, yhr020W::URA3*) that flank the *ARG4* locus; strains heterozygous for these markers can be used to detect crossovers between *URA3* and *TRP1*. Similarly, the *ADE2-HIS3* genetic map distance can be measured. The *TRP1* gene was inserted at the *Bgl*II site of *YHR017W* (*YSC83*) and the *URA3* gene was inserted at the *Bam*HI site of *YHR020W* (*DED82*). Because *YHR020W* is essential, the strains used in this study contained a second copy of *YHR020W* inserted at *URA3* (*ura3::YHR020W*). The *mlh1^{ts}* mutations were introduced into MGD strain EAY777 (*MAT***a** *yhr017W::TRP1 ura3::YHR020W leu2-3,112 trp1-289 ade2*) by two-step gene replacement.

Wild type, *mlh1*Δ, and *mlh1^{ts}* derivatives of the MGD strains were sporulated using the zero growth mating protocol (Reenan and Kolodner 1992). Briefly, *MAT***a** and *MAT*α strains were mated for 4 hr at 30° on YPD plates, transferred to sporulation media, and incubated for 3 days at 30°. Tetrads were dissected on YPD plates after zymolyase treatment. After 3 days growth at 30°, spore clones were replica plated onto relevant selective plates and incubated at 30°. Tetrads with aberrant segregations at *ADE2*, *HIS3*, or *CYH* were discarded to eliminate possible false tetrads. Aberrant segregations were scored 1 day after replica plating. Sectored colonies were confirmed by microscopic examination. Genetic map distance was determined by the formula of Perkins (1949). Only four-spore viable asci that displayed Mendelian segregation for relevant markers were scored. All tetrad data were evaluated using a G-test (Sokal and Rohlf 1969).

RESULTS

Isolation of *mlh1* alleles that exhibit conditional lethality in *pol3-01* strain backgrounds: *pol3-01* strains, which are defective in polδ proofreading exonuclease activity, display a strong mutator phenotype (Morrison *et al.* 1993). Previous studies showed that *msh2*Δ *pol3-01*, *msh6*Δ *pol3-01*, and *pms1*Δ *pol3-01* strains were inviable; high mutational load and checkpoint activation models have been developed to explain these observations (Morrison *et al.* 1993; Tran *et al.* 1999; Datta *et al.* 2000; Sokolsky and Alani 2000). Because *MLH1* acts in the same pathway as *MSH2*, we hypothesized that *mlh1*Δ *pol3-01* strains would also be inviable (Strand *et al.* 1993; reviewed in Kolodner and Marsischky 1999). To test this, EAY312 (*mlh1*Δ) was mated to EAY575 (*pol3-01*), and tetrads from the resulting diploids were examined for spore viability and segregation of markers. No spore clones containing both mutations were identified by genotyping tests. Spore clones containing both *mlh1*Δ and *pol3-01* alleles were classified as inviable on the basis of detection of inviable spore segregation patterns consistent with two genes segregating independently (PD = NPD). Using a plasmid shuffle approach, we identified four *mlh1* mutations present on *ARSH4 CEN6* plasmids that conferred temperature-sensitive inviability in the *pol3-01 mlh1*Δ haploid strain EAY644 (Figure 1; materials and methods).

Mapping of the *mlh1* conditional mutations onto the MutL crystal structure: The N termini of MutL homolog family proteins are highly conserved and can be aligned over an ~300-amino-acid region. This alignment allowed us to map the *mlh1* conditional mutations onto the crys-

TABLE 1

Diploid strains used in the meiotic analysis of the *mlh1*^{ts} mutations

Diploid no.	Strain
1	(EAY506) *MAT***a** *yhr020W::URA3 arg4-RV yhr017W::TRP1 ura3::YHR020W leu2-3,112 trp1-289 his3Δ1 ade2*
	(EAY512) *MAT*α *YHR020W ARG4 YHR017W ura3::YHR020W leu2-3,112 trp1-289 HIS3 ADE2*
2	(EAY492) *MAT***a** *yhr020W::URA3 ARG4 yhr017W::TRP1 ura3::YHR020W leu2-3,112 trp1-289 his3Δ1 ade2 cyh*^{r}
	(EAY502) *MAT*α *YHR020W arg4-BglII YHR017W ura3::YHR020W leu2-3,112 trp1-289 HIS3 ADE2 cyh*^{s}
3	(EAY629) *MAT***a** *yhr020W::URA3 arg4-RV yhr017W::TRP1 ura3::YHR020W leu2-3,112 trp1-289 his3Δ1 ade2 mlh1Δ::LEU2*
	(EAY630) *MAT*α *YHR020W ARG4 YHR017W ura3::YHR020W leu2-3,112 trp1-289 HIS3 ADE2 mlh1Δ::LEU2*
4	(EAY627) *MAT***a** *yhr020W::URA3 ARG4 yhr017W::TRP1 ura3::YHR020W leu2-3,112 trp1-289 his3Δ1 ade2 cyh*^{r} *mlh1Δ::LEU2*
	(EAY628) *MAT*α *YHR020W arg4-BglII YHR017W ura3::YHR020W leu2-3,112 trp1-289 HIS3 ADE2 cyh*^{s} *mlh1Δ::LEU2*
5	(EAY778) *MAT*α *yhr020W::URA3 arg4-BglII YHR017W ura3::YHR020W leu2-3,112 trp1-289 his3Δ1 ADE2 cyh*^{r} *MLH1*
	(EAY806) *MAT***a** *YHR020W ARG4 yhr017W::TRP1 ura3::YHR020W leu2-3,112 trp1-289 HIS3 ade2 cyh*^{s} *mlh1Δ::hisG*
6	(EAY805) *MAT*α *yhr020W::URA3 arg4-BglII YHR017W ura3::YHR020W leu2-3,112 trp1-289 his3Δ1 ADE2 cyh*^{r} *mlh1Δ::hisG*
	(EAY806) *MAT***a** *YHR020W ARG4 yhr017W::TRP1 ura3::YHR020W leu2-3,112 trp1-289 HIS3 ade2 cyh*^{s} *mlh1Δ::hisG*
7	(EAY805) *MAT*α *yhr020W::URA3 arg4-BglII YHR017W ura3::YHR020W leu2-3,112 trp1-289 his3Δ1 ADE2 cyh*^{r} *mlh1Δ::hisG*
	(EAY831) *MAT***a** *YHR020W ARG4 yhr017W::TRP1 ura3::YHR020W leu2-3,112 trp1-289 HIS3 ade2 cyh*^{s} *mlh1-T113A*
8	(EAY805) *MAT*α *yhr020W::URA3 arg4-BglII YHR017W ura3::YHR020W leu2-3,112 trp1-289 his3Δ1 ADE2 cyh*^{r} *mlh1Δ::hisG*
	(EAY833) *MAT***a** *YHR020W ARG4 yhr017W::TRP1 ura3::YHR020W leu2-3,112 trp1-289 HIS3 ade2 cyh*^{s} *mlh1-I147T*
9	(EAY805) *MAT*α *yhr020W::URA3 arg4-BglII YHR017W ura3::YHR020W leu2-3,112 trp1-289 his3Δ1 ADE2 cyh*^{r} *mlh1Δ::hisG*
	(EAY829) *MAT***a** *YHR020W ARG4 yhr017W::TRP1 ura3::YHR020W leu2-3,112 trp1-289 HIS3 ade2 cyh*^{s} *mlh1-F228S*
10	(EAY805) *MAT*α *yhr020W::URA3 arg4-BglII YHR017W ura3::YHR020W leu2-3,112 trp1-289 his3Δ1 ADE2 cyh*^{r} *mlh1Δ::hisG*
	(EAY836) *MAT***a** *YHR020W ARG4 yhr017W::TRP1 ura3::YHR020W leu2-3,112 trp1-289 HIS3 ade2 cyh*^{s} *mlh1-I296S*

The above strains were from the MGD strain background (Rocco *et al.* 1992; MATERIALS AND METHODS). The *arg4-Bgl*II marker contains a 4-bp insertion at +1274 in *ARG4*, and the *arg4*-RV marker contains a 2-bp deletion at +260 in *ARG4*. The location of the mutation in the various *ARG4* markers is shown with respect to the first nucleotide in the initiating ATG.

tal structure of a 349-amino-acid N-terminal fragment of MutL (LN40; Ban and Yang 1998). All four of the *mlh1* mutations map to residues that are highly conserved among MutL family members.

MutL protein is an ATPase that contains four motifs (I–IV) that are also found in DNA gyrase (NgyrB) and Hsp90; these three proteins together form the GHL superfamily of ATPases (reviewed in Dutta and Inouye 2000). The four motifs coordinate ATP binding by directly interacting with ATP and by providing structural integrity to the ATP-binding pocket. The T113, I147, F228, and I296 amino acid residues in Mlh1p correspond to the T111 (domain I, β sheet 3), L145 (domain I, β sheet 6), I227 (domain II, β sheet 9), and L292 (domain II, β sheet 13) amino acid residues in MutL, respectively. In MutL, the T111 residue lies on the surface of the protein and maps to a region that is predicted to be important for maintaining the structural integrity of the ATP-binding site. The L145 residue in MutL maps close to motif IV (G142, T143), which in both the NgyrB-ADPnP and LN40-ADP complexes is positioned to interact directly with ATP (Wigley *et al.* 1991; Prodromou *et al.* 1997; Stebbins *et al.* 1997; Ban *et al.* 1999). These observations suggest that the *mlh1-T113A* and *mlh1-I147T* mutations are likely to affect ATP binding and/or hydrolysis.

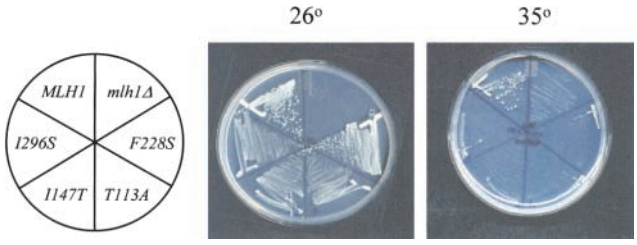

Figure 1.—*mlh1* alleles display temperature-sensitive synthetic lethality with *pol3-01*. Growth phenotypes for *mlh1* temperature-sensitive alleles expressed from *ARS CEN* plasmids in EAY644 [relevant genotype: *mlh1*Δ, *pol3-01*, pEAA110 (*MLH1, URA3*)] at 26° and 35° on 5-FOA containing minimal media.

The *MLH1* residues F228 and I296 align to conserved hydrophobic residues that are part of a second α/β barrel domain (II) in MutL, which form an inner core and are buried inside the MutL structure. These residues may provide general stability to the MutL protein; it is also possible that these residues contribute to the formation of a single-stranded DNA (ssDNA) binding domain that has been proposed in the *MLH* gene family on the basis of genetic and crystallographic analysis of the N-terminal fragment of MutL (Ban *et al.* 1999).

Mutator phenotype exhibited by *mlh1*^{ts} alleles at permissive and nonpermissive temperatures: To determine whether *mlh1* alleles temperature sensitive for synthetic lethality were also temperature sensitive for MMR, the four conditional *mlh1* mutations were tested for their ability to confer mutator phenotypes at 26° and 35° in the *lys2-Bgl*II reversion, *CAN1* forward mutation, and dinucleotide repeat instability assays (Tables 2–4; Henderson and Petes 1992; Marsischky *et al.* 1996; Flores-Rozas and Kolodner 1998). All three assays were

TABLE 2

***lys2-BglII* reversion rates in strains containing conditional *mlh1* mutations**

	26°		35°	
Relevant genotype	Rate relative to wt	Average	Rate relative to wt	Average
Wild type	0.94, 1.1	1.0	0.91, 1.3, 0.82	1.0
*mlh1*Δ	39, 39	39	50, 39, 36	42
*exo1*Δ[a]	3.5, 2.8	3.1	3.5, 3.4	3.5
mlh1-T113A	0.72, 0.88	0.8	71, 24	48
mlh1-I147T	0.69, 2.1	1.4	54, 33	44
mlh1-F228S	1.2, 1.7	1.5	80, 30	55
mlh1-I296S	1.0, 2.2	1.6	67, 39	53

EAY652 (*mlh1*Δ, *lys2-BglII*), was transformed with pEAA109 (*MLH1 ARSH4 CEN6*)-derived plasmids carrying the indicated *mlh1* alleles. Reversion rates per generation were determined as described in MATERIALS AND METHODS and are reported relative to EAY652 transformed with pEAA109 at the respective temperature (1.6×10^{-8} at 26°, 2.2×10^{-8} at 35°). The reversion rate of each independent experiment is presented (median of 6–11 cultures; LEA and COULSON 1949). wt, wild type.

[a] Data from SOKOLSKY and ALANI (2000).

performed because each examines a different mutational spectrum. Previous DNA sequencing analysis indicated that in MMR-defective strains Lys^+ reversions resulted almost exclusively from single-nucleotide deletions in short mononucleotide repeats within the *LYS2* gene. Canavanine resistance resulted primarily from nucleotide misincorporations and single-nucleotide deletions within the *CAN1* gene (MARSISCHKY *et al.* 1996; FLORES-ROZAS and KOLODNER 1998), and dinucleotide repeat instabilities were due primarily to single-repeat insertion/deletions within the TG repeat, with the majority of events consisting of deletions (JOHNSON *et al.* 1996).

In *lys2-BglII*, *CAN1*, and repeat tract instability assays, *mlh1*Δ strains exhibited mutation rates that were ~40-, 17-, and 200-fold higher than that of wild type, respectively (Tables 2–4). All four *mlh1* alleles displayed a tight temperature-sensitive phenotype. At 35°, strains bearing the conditional *mlh1* mutations displayed mutation rates that were similar to that observed in *mlh1*Δ strains. At 26°, these strains displayed wild-type or nearly wild-type mutation rates. Unfortunately, we were unsuccessful in our attempts to measure Mlh1p levels by Western blot analysis in *mlh1*^{ts} cells grown at 26° and 35° because the presence of cross-reactive bands made it difficult to accurately assign Mlh1p-specific bands (data not shown).

***EXO1* overexpression partially suppresses the conditional mutator phenotype observed in *mlh1-I296S* strains:** As outlined in the Introduction, MutL is thought to act as a molecular matchmaker by recruiting downstream repair components such as MutHp, UvrD helicase, and single-strand exonucleases. To test the idea that Mlh1p can recruit exonucleases to mispair sites, we examined whether the conditional mutator phenotype exhibited by *mlh1*^{ts} strains could be suppressed by *EXO1* overexpression. The repeat-tract instability assay was used

TABLE 3

Rates of canavanine resistance in strains bearing conditional *mlh1* mutations

	26°		35°	
Relevant genotype	Rate relative to wt	Average	Rate relative to wt	Average
Wild type	0.74, 1.5, 0.74	1.0	0.51, 0.39, 2.1	1.0
*mlh1*Δ	9.5, 20.2, 20.3	16.7	16.1, 11.3, 26.0	17.8
mlh1-T113A	0.49, 0.79, 0.53	0.61	8.2, 4.4, 15.9	9.5
mlh1-I147T	0.45, 0.44, 1.3	0.72	4.7, 18.0, 15.9	12.8
mlh1-F228S	0.59, 1.0, 1.0	0.89	3.4, 4.4, 9.1	5.6
mlh1-I296S	0.55, 0.76, 1.3	0.85	17.6, 23.1, 28.7	23.1

EAY774 (*mlh1*Δ), was transformed with pEAA109 (*MLH1 ARSH4 CEN6*)-derived plasmids carrying the indicated *mlh1* alleles or pRS415 (*ARSH4 CEN6*) and tested for resistance to canavanine. Forward mutation rates per generation were determined as described in MATERIALS AND METHODS and are reported relative to EAY774 (*mlh1*Δ) transformed with pEAA109 (*MLH1*) at the respective temperature (4.8×10^{-7} at 26°, 2.6×10^{-7} at 35°). The forward mutation rate of each independent experiment is presented (median of seven cultures; LEA and COULSON 1949).

TABLE 4

Rates of repeat-tract instability in strains containing conditional *mlh1* mutations

	26°		35°	
Relevant genotype	Rate relative to wt	Average	Rate relative to wt	Average
Wild type	1.3, 1.9, 0.3, 0.6	1.0	1.3, 1.7, 0.7, 0.8, 0.9, 0.9, 0.5, 1.3	1.0
Wild type + *pEXO1, 2μ*	0.6, 0.8, 0.4	0.5	1.1, 1.3, 0.4, 1.0, 0.8, 2.0, 1.0, 0.4	1.0
Wild type + *pSGS1, 2μ*	NT		4.0, 2.1, 1.9	2.7
mlh1Δ	225, 183, 145, 1140	423	373, 392, 478, 555, 234, 497, 384, 492, 781	465
mlh1Δ + pEXO1, 2μ	310, 230, 83, 230	213	506, 784, 279, 284, 2409, 350, 285, 530, 413	649
mlh1Δ + pSGS1, 2μ	NT		365, 370, 622	452
mlh1-T113A	2.5, 1.0, 1.0, 1.2	1.4	143, 155, 213, 184, 126, 370, 136, 121	181
mlh1-T113A + pEXO1, 2μ	0.6, 0.8, 0.4, 0.7, 0.7	0.7	164, 160, 145, 52, 180, 256, 253, 85, 93	154
mlh1-I147T	1.0, 2.7, 1.7, 1.3, 1.4	1.6	242, 87, 186, 112, 200, 88	153
mlh1-I147T + pEXO1, 2μ	0.6, 0.8, 0.6, 1.0, 1.0	0.8	279, 84, 101, 57, 122	129
mlh1-I147T + pSGS1, 2μ	NT		99, 154	126
mlh-F228S	2.2, 2.0, 0.8, 2.2	1.8	361, 177, 52, 180, 182, 265, 180, 172, 90	184
mlh1-F228S + pEXO1, 2μ	1.4, 1.4, 0.6, 0.8	1.1	321, 232, 44, 99, 65, 98, 160, 90, 49	129
mlh1-I296S	5.7, 6.9, 2.8,1.3, 1.9	3.7	237, 172, 317, 116, 364, 214, 208, 44, 170, 226, 110, 277, 80, 225, 146	194
mlh1-I296S + pEXO1, 2μ	1.3, 2.0, 2.5,1.5, 0.4	1.5	53, 98, 105, 84, 47, 80, 22, 93, 91, 69, 61, 42	70
mlh1-I296S + pSGS1, 2μ	NT		146, 229, 387, 176	235

EAY774 (*mlh1Δ*) was transformed with pEAA109 (*MLH1 ARSH4 CEN6*)-derived plasmids carrying the indicated *mlh1* alleles, pSH44, and one of the following three plasmids: pEAM81 (*EXO1, 2μ*), pEAM87 (*SGS1, 2μ*), or pRS423 (*2μ*). Rates per generation for repeat-tract instability were determined using the method of Lea and Coulson (1949) and are reported relative to EAY774 (*mlh1Δ*) transformed with pEAA109 and pRS423 at the respective temperature (1.34×10^{-5} at 26°, 1.15×10^{-5} at 35°). The repeat-tract instability for each independent experiment is presented (median of seven cultures). NT, not tested.

to examine suppression because it showed the greatest range (~300-fold) between wild-type and *mlh1Δ* mutants of the three assays presented in this article. As shown in Table 4, *EXO1* overexpression partially suppressed the mutator phenotype exhibited in *mlh1-I296S* strains at 35° in the repeat tract instability assay (Table 4, $P = 0.0003$) but did not significantly suppress the mutator phenotypes exhibited in the three other *mlh1^{ts}* strains.

Recently, Langland *et al.* (2001) and Pedrazzi *et al.* (2001) showed in two-hybrid and coimmunoprecipitation studies that the Bloom syndrome gene product (BLM) helicase, defects of which are associated with chromosome instability in Bloom's syndrome patients, interacts with Mlh1p. Pedrazzi *et al.* (2001) also showed that the *S. cerevisiae* homolog of BLM, Sgs1p, interacts with Mlh1p. Cell extracts defective in the BLM helicase, however, were proficient in MMR, suggesting either that the BLM helicase was not required for MMR or that its activity was redundant with another MMR activity. These observations encouraged us to test whether overexpression of *SGS1*, a yeast homolog of BLM that has been hypothesized to repair stalled replication forks (Gangloff *et al.* 1994; Chakraverty and Hickson 1999), could suppress the mutator phenotype exhibited in *mlh1* strains in the repeat tract instability assay. *SGS1* overexpression did not suppress the mutator phenotype observed in *mlh1-I296S* and *mlh1-I147T* strains at 35° (Table 4).

Analysis of *mlh1^{ts}* strains in meiotic MMR and crossing over: MMR proteins repair mispairs in heteroduplex DNA that form during genetic recombination (White *et al.* 1985; Detloff *et al.* 1991; Alani *et al.* 1994). This function can be easily observed at loci such as *ARG4*, *HIS4*, *HIS2*, and *CYS3* that display high levels of meiotically induced double-strand breaks (DSBs) and undergo high levels of meiotic recombination. At these loci, high frequencies of non-Mendelian segregation (aberrant events) are observed in strains heterozygous for genetic markers located near meiotically induced DSB sites and lower levels are observed for strains heterozygous for markers located farther away (Lissouba *et al.* 1962; Fogel *et al.* 1981; Rossignol *et al.* 1984; Nicolas *et al.* 1989; Detloff *et al.* 1992; Vedel and Nicolas 1999). This phenomenon is referred to as a conversion gradient. In wild-type cells, the vast majority of aberrant events are gene conversions; each spore clone contains markers derived from only one parent. In contrast, a large percentage (~30–80%) of the aberrant events in *msh2/msh2* and *pms1/pms1* strains heterozygous for a genetic marker appear as postmeiotic segregations (PMS). Instead of showing a uniform marker phenotype, a PMS spore forms a sectored colony that displays both parental markers. The presence of these PMS events is consistent with genetic recombination proceeding through a heteroduplex DNA intermediate; the failure of MMR proteins to repair mispairs in the heteroduplex

DNA thus results in a PMS event. Two recent studies by Allers and Lichten (2001a,b) provide direct support for these ideas; using physical methods, they identified heteroduplex DNA in a recombination intermediate, termed joint molecules, that contain two Holliday junctions.

In addition to displaying a high frequency of PMS events at loci that undergo high levels of genetic recombination, *mlh1/mlh1* strains displayed a reduced frequency of meiotic crossing over (Hunter and Borts 1997; Wang *et al.* 1999). This was revealed by an ~40% reduction in genetic map distances as compared to wild-type cells. Because conditional mutations can result from defects in specific protein domains, we tested whether the *mlh1*^{ts} alleles displayed different phenotypes for mismatch repair and meiotic crossing over. As shown below, *mlh1-F228S* and *mlh1-I296S* mutants displayed phenotypes indicating that the vegetative MMR, meiotic MMR, and crossover functions of Mlh1p could be separated genetically.

The effect of the *mlh1*Δ mutation on meiotic MMR and crossing over at *ARG4*: We tested the effect of the *mlh1*Δ and *mlh1*^{ts} mutations in meiotic MMR and crossing over in diploid strains heterozygous for one of two restriction site mutations (*arg4*-RV, *arg4-Bgl*II) in the *ARG4* gene (Table 1 legend, Nicolas *et al.* 1989). The *ARG4* locus undergoes high levels of meiotic gene conversion and displays a meiosis-specific double-strand break within the *ARG4* promoter (Nicolas *et al.* 1989; Sun *et al.* 1989). The *arg4*-RV and *arg4-Bgl*II markers are located at the high and low ends, respectively, of the *ARG4* conversion gradient. Conversion gradients have been hypothesized to form as the result of repairing mispairs located near the DSB to gene conversions and repairing mispairs located far from the DSB to restorations (Detloff *et al.* 1992). Other models to explain conversion gradient formation, including by heteroduplex rejection, have been presented (see Nicolas *et al.* 1989; Alani *et al.* 1994; Hillers and Stahl 1999). Crossing over was also examined in these strains by measuring genetic map distances between *URA3* and *TRP1* markers that were inserted on opposite sides of the *ARG4* locus (chromosome VIII).

In wild-type strains, the frequency of aberrant events involving *arg4* alleles was 5.0% at *Eco*RV and 1.0% at *Bgl*II (diploids 1 and 2, Table 5). All of the aberrant events were gene conversions (6:2 and 2:6 tetrads). The difference in the frequency of these events was statistically significant ($P = 3.3 \times 10^{-8}$). In contrast, in *mlh1*Δ/*mlh1*Δ strains, the percentage of aberrant segregations was 8.3% at *Eco*RV and 5.9% at *Bgl*II (diploid 3 *vs.* 4, $P = 0.14$). For each *arg4* marker the increase in aberrant segregations in the *mlh1*Δ strain was significant (*arg4*-RV, diploid 1 *vs.* 3, 5.0 *vs.* 8.3%, $P = 0.02$; *arg4-Bgl*II, diploid 2 *vs.* 4, 1.0 *vs.* 5.9%, $P = 5.3 \times 10^{-9}$). These observations are consistent with the *mlh1*Δ mutation causing a disruption of the *ARG4* conversion gradient; a similar disruption of the *ARG4* conversion gradient was observed in *msh2*Δ and *pms1*Δ strains (Alani *et al.* 1994). Previously, Hunter and Borts (1997) reported that the *mlh1* null mutation did not disrupt the *HIS4* polarity gradient but raised aberrant event frequencies across *HIS4* (see discussion).

*mlh1*Δ diploids also displayed an increase in the frequency of PMS events (~50% 5:3 and 3:5 tetrads) as was expected for strains defective in mismatch repair (Table 5; White *et al.* 1985; Detloff *et al.* 1991; Alani *et al.* 1994). In crosses involving the *arg4-Eco*RV allele, we did not observe a significant difference in the directionality of aberrant events (parity) from wild type to mutant, as the ratio of (6:2 + 5:3)/(2:6 + 3:5) tetrads was ~1 (data not shown). For the *arg4-Bgl*II allele, the *mlh1*Δ strains showed a ratio of (6:2 + 5.3)/(2:6 + 3:5) tetrads that was 4.1 ($P = 0.003$), while in wild type (diploid 2) this ratio was ~1 (four 6:2 and six 2:6 gene conversions). Deviation from parity has been observed in cases where two recombination substrates show differences in the frequency of initiating double-strand breaks (Allers and Lichten 2001a,b). Disparities could also result if each substrate initiates recombination events at a similar frequency but restoration type repair occurs more frequently when a recombination event is initiated by one partner compared to the other. An understanding of how the absence of *MLH1* could cause disparity for one, but not both *arg4* markers, will require further investigation.

In *mlh1*Δ strains the frequency of meiotic crossover events involving *ARG4* flanking markers was ~60% of the wild-type frequency (Table 5). This reduction was significant and of similar magnitude in both *arg4*-RV/*ARG4* and *arg4-Bgl*II/*ARG4* strains (diploid 1 *vs.* 3, $P = 0.02$, and diploid 2 *vs.* 4, $P = 0.01$). The spore viability of *mlh1*Δ strains was also reduced compared to wild type (Table 5) and this reduction was similar to that observed previously (Hunter and Borts 1997).

The effect of the *mlh1*^{ts} mutations on MMR and crossing over: To study the meiotic MMR and crossing-over phenotypes conferred by the conditional *mlh1* mutations, each *mlh1*^{ts} allele was introduced into the *MLH1* locus in EAY777 by two-step gene replacement (Table 1; materials and methods). All four strains displayed a conditional mutator phenotype in the vegetative growth canavanine assay that was indistinguishable from that observed in EAY652 and EAY774 strains transformed with *mlh1*^{ts} *ARS-CEN* plasmids (data not shown). Because our strains did not sporulate at 35°, all meiotic studies were performed at 30°. In canavanine mutator patch assays performed at 30°, the *mlh1*^{ts} derivatives of EAY777 displayed a phenotype that was indistinguishable from wild type (Table 6).

The meiotic phenotype conferred by the *mlh1*^{ts} mutations was examined in *mlh1*^{ts}/*mlh1*Δ strains. We used this approach because the *MLH1/mlh1*Δ strain (diploid 5, Table 1) was indistinguishable from the homozygous

TABLE 5

Tetrad analysis of *MLH1/MLH1* and *mlh1*Δ*/mlh1*Δ diploids sporulated and germinated at 30°

	Marker at *ARG4*			
	*ARG4/arg4-Eco*RV		*ARG4/arg4-Bgl*II	
MMR genotype:	*MLH1/MLH1*	*mlh1*Δ*/mlh1*Δ	*MLH1/MLH1*	*mlh1*Δ*/mlh1*Δ
Diploid:	1	3	2	4
Tetrads scored	1088	496	1041	714
Spore viability (%)	95	72	95	72
ARG4 aberrants (%)	5.0	8.3	1.0	5.9
PMS/*ARG4* aberrants	0/54	19/41	0/10	26/42
URA3-TRP1 genetic distance (cM)	4.4	2.7	4.4	3.1

The indicated diploid strains (Table 1) were analyzed for spore viability, *ARG4* aberrants, and genetic map distance as described in MATERIALS AND METHODS. Aberrant events include all tetrads that deviated from 4:4 segregation (FOGEL *et al.* 1978); with rare exceptions, gene conversions consisted of the 6:2 and 2:6 class of tetrads and postmeiotic segregation (PMS) the 5:3 and 3:5 class of tetrads. PMS/*ARG4* aberrants represents the proportion of aberrant *ARG4* events that displayed PMS.

wild type (diploid 2). As shown in Table 7, all four *mlh1*^{ts} strains displayed an intermediate level of spore viability. However, two strains, *mlh1-T113A/mlh1*Δ (diploid 7) and *mlh1-I147T/mlh1*Δ (diploid 8), were indistinguishable from wild type for meiotic MMR and for crossing over at both the *URA3-TRP1* and *ADE2-HIS3* (chromosome XV) intervals tested. The other two strains, *mlh1-F228S/mlh1*Δ (diploid 9) and *mlh1-I296S/mlh1*Δ (diploid 10), displayed levels of aberrant segregation at *arg4-Bgl*II that resembled the *mlh1*Δ*/mlh1*Δ levels and were significantly different from that found in the *MLH1/mlh1*Δ control (diploid 5 *vs.* 9, 1.6 *vs.* 5.2%, $P = 0.0012$; diploid 5 *vs.* 10, 1.6 *vs.* 5.1%, $P = 0.0014$). In addition, the frequency of PMS events in these two *mlh1*^{ts}*/mlh1*Δ strains was similar to that observed in the *mlh1*Δ*/mlh1*Δ strain (diploid 6). These results indicated that the *mlh1-F228S* and *mlh1-I296S* alleles conferred a severe defect in meiotic MMR at 30°.

The crossing-over frequency observed at two intervals in the *mlh1-F228S/mlh1*Δ and *mlh1-I296S/mlh1*Δ strains was similar to that observed in the *MLH1/mlh1*Δ strain and significantly different from the *mlh1*Δ*/mlh1*Δ strain. In these strains, the genetic distance between *URA3* and *TRP1* was significantly higher than that observed in the *mlh1*Δ*/mlh1*Δ strain (diploid 6 *vs.* 9, 2.9 *vs.* 5.8 cM, $P = 0.016$; diploid 6 *vs.* 10, 2.9 *vs.* 4.6 cM, $P = 0.026$). For the *ADE2-HIS3* interval, both the *mlh1-F228S/mlh1*Δ and *mlh1-I296S/mlh1*Δ strains exhibited map distances (32.3 and 34.2 cM, respectively) that, while lower than that of the *MLH1/mlh1*Δ strain (39 cM), were significantly higher than that observed in the *mlh1*Δ*/mlh1*Δ strain (diploid 6 *vs.* 9, 23.0 *vs.* 32.3 cM, $P = 0.0001$; diploid 6 *vs.* 10, 23.0 *vs.* 34.2 cM, $P = 3 \times 10^{-7}$). When the *mlh1-I296S/mlh1*Δ strain was sporulated and germinated at 26°, a completely wild-type phenotype was observed in meiotic MMR and crossing-over assays (Table 7).

Interactions in the two-hybrid system between the *mlh1*^{ts} alleles and known *MLH1* partners: To determine whether the phenotypes observed in *mlh1*^{ts} strains were due to the disruption of specific protein-protein interactions, we subcloned the *mlh1*^{ts} mutations into a *lexA-MLH1* bait construct and tested for two-hybrid interactions with *GAL4* activation domain fusions of *PMS1* (PANG *et al.* 1997), *MLH3* (WANG *et al.* 1999), a truncated

TABLE 6

Canavanine patch assay of *mlh1*^{ts} strains grown at 30°

Strain	Genotype	Median no. of canavanine-resistant papillations for each experiment	Average fold increase in canavanine papillation relative to *MLH1*
EAY777	*MLH1*	4, 2, 15, 7, 7, 6, 5	1
EAY806	*mlh1*Δ*::hisG*	125, 101, 123, 144, 139	19.2
EAY831	*mlh1-T113A*	4, 6, 4, 3, 13, 11	1.0
EAY833	*mlh1-I147T*	8, 6, 8, 6, 5, 5	1.0
EAY829	*mlh1-F228S*	3, 6, 9, 7, 6, 15, 8, 12	1.3
EAY836	*mlh1-I296S*	9, 7, 6, 4, 5, 3	0.9

Single colonies of the indicated strains were grown on YPD for 3 days at 30°, after which 11 independent colonies (2 mm in diameter) were patched onto canavanine plates. In each experiment, the median number of canavanine papillations scored after a 3-day incubation at 30° is presented.

TABLE 7

Tetrad analysis of *mlh1^{ts}*/*mlh1Δ* diploids sporulated and germinated at 30°

Marker at *ARG4*:	*ARG4/arg4-Bgl*III						
MMR genotype:	*MLH1*	*mlh1Δ*	*mlh1-T113A*	*mlh1-I147T*	*mlh1-F228S*	*mlh1-I296S*	*mlh1-I296S* at 26°
Diploid:	5	6	7	8	9	10	10
Tetrads scored	511	712	474	534	540	545	352
Spore viability (%)	90	68	81	84	81	79	95
ARG4 aberrants (%)	1.6	5.6	2.3	1.3	5.2	5.1	0.9
PMS/*ARG4* aberrants	0/8	13/40	0/11	0/7	9/28	6/28	0/3
URA3-TRP1 genetic distance (cM)	5.1	2.9	4.9	4.4	5.8	4.6	6.0
PD:TT:NPD[a]	444:50:0	630:39:0	418:45:0	474:46:0	461:47:2	473:48:0	300:41:0
ADE2-HIS3 genetic distance (cM)	39.0	23.0	37.5	37.4	32.3	34.2	38.1
PD:TT:NPD[a]	187:309:15	409:298:5	178:283:12	175:268:12	251:277:12	232:300:12	129:214:9

The indicated diploid strains (Table 1) were analyzed as described in Table 5.
[a] Only tetrads that contained Mendelian segregations for the markers located at the indicated intervals were used to measure genetic distances.

FIGURE 2.—Two-hybrid interactions between *lexA-mlh1^{ts}* and *GAL4-PMS1*, *-EXO1*, and *-SGS1* fusion constructs. Plates overlaid with paper filters were incubated at 26°, 30°, and 35° and expression of the *lacZ* reporter gene was determined (MATERIALS AND METHODS).

version of *EXO1* (TRAN *et al.* 2001), and *SGS1* (PEDRAZZI *et al.* 2001; Figure 2). For the *mlh1^{ts}* baits, all interactions were similar to the wild-type bait at 26°; however, for all four *mlh1^{ts}* baits, the strength of the interactions decreased with increasing temperature. Interactions involving the *mlh1-T113A* and *-I147T* constructs were more resistant, compared to the *mlh1-F228S* and *-I296S* constructs, to elevated temperature. As shown in Figure 2, interactions between the *mlh1-T113A* and *-I147T* baits and the *PMS1* target construct were still observed at 35°. It is important to note that the two-hybrid interaction between Mlh1p and Mlh3p was very weak, making it difficult to assess the effect of the *mlh1^{ts}* mutations (data not shown). Together, these studies indicated that none of the *mlh1^{ts}* mutations disrupted a specific Mlh1p interaction; instead, they suggest that the mlh1^{ts} mutant proteins were unstable at high temperature.

DISCUSSION

In this study four *mlh1^{ts}* mutations were identified that conferred conditional synthetic lethality with the *pol3-01* mutation as well as a conditional mutator phenotype. The conditional mutator phenotype conferred by one mutation, *mlh1-I296S*, was partially suppressed by *EXO1* overexpression. For diploid strains sporulated at 30°, the *mlh1-F228S* and *-I296S* mutations conferred a defect in meiotic MMR, but displayed nearly wild-type levels of meiotic crossing over.

Two-hybrid analysis suggests that the *mlh1^{ts}* mutations confer protein instability at 35°: In two-hybrid analysis all four *mlh1^{ts}* alleles displayed a pattern of interaction with *EXO1*, *PMS1*, and *SGS1* that was similar to *MLH1* at 26° but was lower or undetectable at 35°. Specific *mlh1^{ts}* two-hybrid interaction defects were not observed at 35° despite deletion analyses suggesting that Mlh1p interacts with Exo1p, Pms1p, and Sgs1p each in a distinct manner and that all of the *mlh1^{ts}* mutations mapped outside of the region required for Pms1p interaction (PANG *et al.* 1997; PEDRAZZI *et al.* 2001; SCHMUTTE *et al.* 2001; TRAN *et al.* 2001). These results, and the finding

that all of the *mlh1*^{ts} strains displayed null phenotypes at 35°, suggested that the *mlh1*^{ts} mutations disrupted the stability of Mlh1p. Unfortunately, we were unable to test this hypothesis directly at the protein level because Mlh1p could not be specifically identified by Western blot analysis.

The genetic analysis presented in this article adds to the literature indicating that Exo1p-Mlh1p interactions are important for MMR (Tran and Liskay 2000; Amin *et al.* 2001; Schmutte *et al.* 2001; Tran *et al.* 2001). The finding that overexpression of *EXO1* had only a weak effect on suppressing the mutator phenotype of *mlh1-I296S* alleles was not surprising considering the two-hybrid data presented above. High-copy suppression between interacting gene products is thought to be most effective in situations where a mutant protein is stable but compromised for interactions with its partner. In this scenario, increased expression of a wild-type partner would favor the formation of a functional complex through mass action (Jarvik and Botstein 1975; Guarente 1993). High-copy suppression could also occur if excessive partner protein acts to stabilize the mutant protein. Recent genetic studies by Amin *et al.* (2001) suggested that Exo1p plays a structural role by stabilizing complexes that contain multiple MMR proteins; the finding that Exo1p can interact with both Msh2p and Mlh1p (Tran and Liskay 2000; Amin *et al.* 2001; Schmutte *et al.* 2001; Tran *et al.* 2001) provides support for this idea and suggests that the mild suppression of the *mlh1-I296S* mutator phenotype by Exo1p overexpression was due to weak stabilization of mlh1-I296Sp.

As described in results, all four *mlh1*^{ts} mutations conferred a conditional mutator phenotype in *POL3* strains and conditional viability in *pol3-01* strains. Interestingly, these phenotypes do not mirror those observed with six *msh2*^{ts} mutations that were also identified on the basis of conditional viability in *pol3-01* strains (Sokolsky and Alani 2000). In the canavanine resistance assay, all of the *msh2*^{ts} alleles conferred a strong mutator phenotype at both permissive (26°) and nonpermissive (35°) temperatures. Sokolsky and Alani (2000) hypothesized that synthetic lethality in the *msh2*^{ts} *pol3-01* strains was caused by defects in DNA metabolism in *pol3-01* strains that were unrelated to Msh2p mismatch repair functions but involved Msh2p in some other capacity, such as in the processing of Okazaki fragments or in recognizing other types of DNA lesions. A key difference between these analyses was that five of the six *msh2*^{ts} strains showed wild-type Msh2p levels at 35° while two-hybrid analysis suggested that all of the mlh1^{ts} proteins were unstable at this temperature. We are currently studying a large set of *mlh1* mutations and plan to examine whether any of the MMR-defective *mlh1* alleles maintain viability in a *pol3-01* strain background. Such an analysis will likely require us to test whether any MMR-defective *msh2* or *mlh1* alleles that maintain viability in *pol3-01* strains display a mutation spectrum that is different from the null.

Two *mlh1*^{ts} mutations confer a separation-of-function phenotype in meiosis: In meiotic assays, the *mlh1-F228S* and *mlh1-I296S* mutations conferred a separation-of-function phenotype with respect to MMR and crossing over. These observations suggest that Mlh1 protein's role in crossing over does not require its MMR activities. In addition, these studies argue that the vegetative and meiotic MMR functions can be genetically separated, although the interpretation of these results is complicated by the fact that the vegetative MMR assays were performed in haploid strains and the meiotic MMR assays were performed in *mlh1*^{ts}/*mlh1*Δ diploids.

The two-hybrid studies, which suggested that the *mlh1*^{ts} alleles are unstable at high temperature, allow us to speculate why the *mlh1-F228S* and *-I296S* mutations conferred a meiotic mismatch repair defect at 30° but showed nearly wild-type levels of meiotic crossing over. In vegetative MMR assays all four *mlh1*^{ts} strains displayed wild-type function at 26° and 30°; however, the *mlh1-F228S* and *-I296S* mutants could be distinguished from the other two strains because they displayed weaker two-hybrid interactions with Pms1p at 35°. One way to explain the difference in meiotic phenotypes between the two sets of *mlh1*^{ts} strains is that the meiotic MMR defect observed in *mlh1-F228S* and *-I296S* strains at 30° was due to a reduced abundance or stability of Mlh1p. Increased proteolysis has been reported in meiosis (Hopper *et al.* 1974; Zubenko and Jones 1981); such a change in protein turnover could cause an already compromised interaction with Pms1p in the *mlh1-I296S* and *-F228S* strains to be reduced to a level below that required for efficient MMR. The crossover data for the *ADE2-HIS3* interval is also consistent with this idea. While all of the *mlh1*^{ts} strains displayed levels of meiotic crossing over that were higher than the null, only the *mlh1-T113A* and *-I147T* strains displayed the full wild-type phenotype. The *mlh1-F228S* and *-I296S* strains displayed intermediate levels of crossing over that correlated with stronger defects in the two-hybrid assay. It is important to note that *mlh1-I296S* strains did not show a meiotic mismatch repair defect at 26°, suggesting that the distinction between vegetative and meiotic MMR activities occurs only at temperatures where mlh1-I296Sp would be predicted to be unstable.

If the meiotic MMR defect of *mlh1-F228S* and *-I296S* strains at 30° is caused by reduced abundance or stability of Mlh1p, why are these strains still functional in crossing over? One possibility is that the meiotic crossing over functions of Mlh1p can tolerate increased protein turnover or instability because Mlh1p is not playing a critical enzymatic role in this process that requires concerted interactions with other MMR proteins. Such a model predicts that the MMR functions would be easier to disrupt than crossover functions. In support of this idea we found that in an analysis of a large set of site-

specific mutations in the *MLH1* gene, MMR was consistently more easily disrupted than meiotic crossing over (J. L. Argueso and E. Alani, unpublished observations). Alternatively, instability of the mlh1-F228S and -I296S proteins could lead to a greater decrease in the concentration of Mlh1p-Pms1p complexes compared to Mlh1p-Mlh3p complexes because in this model the Mlh1p-Mlh3p complex displays higher binding affinity. In such a model the residual Mlh1p-Mlh3p complex is still able to perform its role in meiotic crossing over (Wang *et al.* 1999) while the even lower level of Mlh1p-Pms1p complex does not allow MMR to proceed. Unfortunately, the weak Mlh1p-Mlh3p signal in the two-hybrid assay made it impossible to assess this interaction with the mutant *mlh1* alleles. We cannot exclude the possibility that the defects observed in the *mlh1-F228S* and *-I296S* strains are not due to protein instability but are instead due to mutations in the proposed ssDNA binding domain of Mlh1p that causes a greater defect in MMR *vs.* crossing over. Both of these mutations map to a domain implicated in ssDNA binding in MutL (Ban *et al.* 1999).

Our experiments showed that the *mlh1*Δ mutation disrupted the *ARG4* conversion gradient to an extent that was similar to that reported for the *msh2*Δ and *pms1*Δ mutations (Alani *et al.* 1994). Recently, Abdullah and Borts (2001) showed that the nutritional state of a cell (*e.g.*, Leu^+ *vs.* Leu^-) can affect the initiation of recombination at the *HIS4* hotspot. They also proposed that the elevated frequency of aberrant events at *HIS4* in *mlh1*Δ*::LEU2* strains (Hunter and Borts 1997) was the result of a change in the metabolic state of the cell rather than the absence of Mlh1p. We believe that such a nutritional marker effect is not occurring in our strains at *ARG4* because we did not detect differences in aberrant events or crossing over for the *arg4-Bgl*II marker between homozygous *mlh1*Δ*::LEU2/mlh1*Δ*::LEU2* (diploid 4, Table 5) and *mlh1*Δ*::hisG/mlh1*Δ*::hisG* (diploid 6 marked with a bacterial sequence, Table 7) diploids. Also, *LEU2/LEU2* derivatives of diploids 1 and 2 did not change the *ARG4* conversion gradient presented in Table 5 (J. L. Argueso, unpublished observations).

We have recently identified several *mlh1* alleles that showed null phenotypes in vegetative mismatch repair but are functional in meiotic crossing over. Similar separation-of-function *mlh1* mutations have been identified independently (E. R. Hoffmann and R. Borts, personal communication). A genetic analysis of these mutations in conjunction with the conditional mutations should allow us to obtain a better understanding of how MMR proteins can function in a crossover pathway that does not appear to involve the specific recognition of DNA mispairs.

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