Mlh1 is unique among mismatch repair proteins in its ability to promote crossing-over during meiosis

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In eukaryotes, homologs of the bacterial MutS and MutL proteins function in DNA mismatch repair and recombination pathways. The mutL homolog MLH1 is required for nuclear mismatch repair. Previously, cytological analysis of MLH1-deficient mice has implied a role for Mlh1 in crossing-over during meiosis. Here we demonstrate that Saccharomyces cerevisiae diploids containing a deletion of MLH1 have reduced crossing-over in addition to a deficiency in the repair of mismatched DNA during meiosis. Absence of either of the meiosis-specific mutS homologs Msh4 or Msh5 results in a similar reduction in crossing-over. Analysis of an mlhl msh4 double mutant suggests that both genes act in the same pathway to promote crossing-over. All genetic markers analyzed in mlhl mutants display elevated frequencies of non-Mendelian segregation. Most of these events are postmeiotic segregations that represent unrepaired heteroduplex. These data suggest that either restorational repair is frequent or heteroduplex tracts are shorter in wild-type cells. Comparison of mlhl segregation data with that of pms1, msh2, msh3, and msh6 mutants show that the ability to promote crossing-over is unique to MLH1. Taken together these observations indicate that both crossing-over and gene conversion require MutS and MutL functions and that Mlh1 represents an overlap between these two pathways. Models of Mlh1 function are discussed.

[Key Words: MutL homologs; MutS homologs; mismatch repair; meiosis; crossing-over; gene conversion.]

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In eukaryotes, proteins homologous to the MutS and MutL components of the bacterial long-patch mismatch repair system have evolved to perform both DNA repair and recombination functions. In Saccharomyces cerevisiae the MutS homologs Msh2, Msh3, and Msh6 and the MutL homologs Pms1 and Mlh1 are involved in the repair of mismatched DNA formed during replication and recombination (Williamson et al. 1985; Kramer et al. 1989; Reenan and Kolodner 1992b; New et al. 1993; Prolla et al. 1994a; Johnson et al. 1996; Marsischky et al. 1996; for review, see Kolodner 1996; Crouse 1997). Several of these proteins also function to prevent inappropriate genetic exchanges between nonidentical or homeologous DNA sequences (Selva et al. 1995; Chambers et al. 1996; Datta et al. 1996; Hunter et al. 1996; N. Hunter and R. H. Borts unpubl.). Other MutS homologs possess recombination functions that appear to be unrelated to mismatch repair. The S. cerevisiae Msh4 and Msh5 proteins specifically promote crossing-over during meiosis and the Swi4 protein of Schizosaccharomyces pombe is thought to be involved in the termination of DNA synthesis during the copying of the mating-type cassette during switching (Fleck et al. 1992; Ross-Macdonald and Roeder 1994; Hollingsworth et al. 1995).

In this study we examine the activities of the mismatch repair system during meiosis in S. cerevisiae and focus on the role of the mutL homolog MLH1 (Prolla et al. 1994a). In mouse, Mlh1 is required for normal meiotic progression (Baker et al. 1996; Edelmann et al. 1996). In male mlh1 −/− mice, although chromosome synopsis appears normal, pachytene arrest and subsequent apoptosis lead to sterility. Immunolocalization of the Mlh1 protein in normal mice reveals a punctate pattern of staining along the lengths of pachytene chromosomes (Baker et al. 1996). The observations that some foci mark the sites of chiasmata (the cytologically visible manifestation of crossing-over) and that chiasmata are reduced 10- to 100-fold in Mlh1-deficient mice is suggestive of a role for Mlh1 in the process of crossing-over. Meiosis is thought to be disrupted at a similar stage in female mlh1 −/− mice, which are also sterile (Edelmann et al. 1996).

We show that Mlh1-deficient yeast have reduced crossing-over, increased non-Mendelian segregation, and high frequencies of postmeiotic segregation (PMS). These results demonstrate that a MutL homolog is required for both crossover and gene conversion pathways of meiotic recombination.
Results

Frequencies of crossing-over in mismatch repair mutants

Map distances were calculated from tetrad data for four or five genetic intervals in wild type and mhl1, pmsl1, msh2, msh3, and msh6 mismatch repair mutant backgrounds (Table 1). In mhl1 mutants, map distance is reduced significantly in all five intervals \(P < 0.05\) to \(<< 0.001\), with an overall reduction of 33% for the combined genetic distance. In addition, mhl1 msh2 double mutants are indistinguishable from the mhl1 single mutants with respect to crossing-over (Table 1). The one exception is the HIS4-CENIII interval, which has significantly less crossing-over in the mhl1 msh2 double mutant than in the mhl1 single mutant \(P < 0.05\). A reduced frequency of crossing-over is not observed in any of the other mismatch repair mutants examined.

Small but significant increases in map distance are observed in some intervals for the other mismatch repair mutants [see Table 1, footnote]. Most notable is the interval TRP5-CEN VII, which is expanded by 41% and 33% in msh2 and msh3 mutants, respectively \(P < 0.001\). It should be noted that, although all diploids used in this study are isogenic, the two haploid strains that constitute every diploid are congenic with each other. The increases in map distance may be a reflection of this congenicity [see Discussion section].

Genetic epistasis of MLH1 with MSH4

The crossover deficit of mhl1 is similar to that observed in yeast msh4 and msh5 mutants [Ross-Macdonald and Roeder 1994; Hollingsworth et al. 1995]. This prompted us to investigate the relationship between the MLH1 and MSH4 genes. Because of the extremely poor spore viability in msh4 mutants (Fig. 1; see Table 3 below; Ross-Macdonald and Roeder 1994), random spore analysis was used to examine recombination in the two single mutants and the mhl1 msh4 double mutant. These studies suggest that an epistatic relationship exists between MLH1 and MSH4 (Table 2). As expected, all three strains have reduced frequencies of recombination relative to wild type \(P < 0.001\). Also, nonmating colonies, indicating nondisjunction of chromosome III, are observed in these mutant strains. The msh4 single mutant has a more severe phenotype than mhl1, having 1.4-fold less recombination and five times as many nonmating colonies \(P < 0.05\). However, the mhl1 msh4 double mutant is indistinguishable from the msh4 single mutant \(P > 0.8\), a result not expected if the two genes were acting independently to promote crossing-over. The size of the data set presented in Table 2 is sufficient to detect an additive effect of these mutants.

Spore viability in mismatch repair mutants

Several studies have shown that mismatch repair mu-
tants suffer from significant reductions in spore viability, which has been attributed to their mutator phenotypes (Williamson et al. 1985; Kramer et al. 1989; Reenan and Kolodner 1992b; New et al. 1993; Prolla et al. 1994a). Spore viabilities for wild-type and mismatch repair mutant strains are shown in Table 3. Our standard sporulation media contains a small amount of glucose, which allows diploids to complete a final mitotic division before meiosis, thereby improving the efficiency of sporulation (see Materials and Methods). When mlhl, pms1, msh2, and msh3 diploids are sporulated under these conditions the mlhl strains have 16% fewer viable spores than the least viable of the other three mutant strains, pms1 \((P < 0.001)\), pms1 and mlhl mutants have indistinguishable mutator phenotypes (Prolla et al. 1994a), suggesting that the additional 16% spore death is not exclusively attributable to the accumulation of haplolethal mutations during vegetative growth as a diploid. Sporulation on media without glucose prevents any mitotic growth as a diploid and alleviates 2.5% spore death in a msh2 diploid and 12% spore death in a mlhl strain. The same sporulation regime results in essentially wild-type spore viability for msh6 strains. The data for mlhl and msh2 mutants are compared to both wild-type and msh4 strains in Figure 1. Wild-type diploids are highly viable, with a random distribution of dead spores. All mutants have a highly nonrandom pattern of spore death \((P<<0.005)\). The spectra of spore death are similar between mlhl and msh2, except that mlhl strains produce 7% fewer viable spores \((1609 \text{ of } 1920 \text{ and } 1359 \text{ of } 1500; P<<0.001)\). The additional spore death of mlhl mutants appears to be confined to tetrads with two and zero viable spores \(\text{mlhl}, 68 \text{ of } 480 \text{ tetrads had two or zero viable spores; msh2, 26 of } 375; P<0.001\). Tetrads with two and zero viable spores are also the major classes of tetrads in msh4 mutants [Fig. 1; Ross-Macdonald and Roeder 1994]. These classes of tetrad are expected when one or more pairs of homologous chromosome missegregate at the first meiotic division. The results from random spore analysis suggest an increase in nondisjunction of chromosomes in mlhl mutants [see above, Table 2]. Meiosis I nondisjunction was assessed further by both genetic and physical methods [see Materials and Methods]. Of 480 tetrads from a mlhl diploid, 46 produced two viable spores. Three of these formed two nonmating colonies, suggesting disomy for chromosome III and corresponding to a nondisjunction rate of \(-0.6 \times 10^{-2}\) per meiosis. This is consistent with the data from analysis of random spores [Table 2]. Chromosome nondisjunction in wild-type cells occurs at a rate of \(-1.4 \times 10^{-4}\) to \(1.05 \times 10^{-2}\) per meiosis (Goldway et al. 1993; F.E. Pryde and E.J. Louis, pers. comm.), but was not determined in the strain background used in this study. The chromosome III disomes were confirmed physically when 41 of the 46 tetrads with two viable spores were karyotyped successfully by contour-clamped homogenous electric field (CHEF) gel analysis [Hunter et al. 1996]. Nondisjunctions of chromosomes I and X were also detected [three and two events, respectively; not shown]. Although not a rigorous examination of chromosome missegregation, this analysis suggests that there is an increase in the frequency of meiosis I nondisjunction in mlhl mutants.

### Elevated frequencies of non-Mendelian segregation and PMS in mlhl mutants

The frequencies of non-Mendelian segregation were examined in wild-type and mutant strains by tetrad dissection. In Table 4 tetrad data for three defined alleles of the HIS4 gene are presented. In mlhl mutants, the frequencies of non-Mendelian segregation at these three alleles

### Table 2. Analysis of random spores

| Relevant genotype | Percentage recombinants<sup>a</sup> | Percent nonmating colonies<sup>b</sup> |
|-------------------|--------------------------------------|-------------------------------------|
|                   | HIS4-MAT | TRP5-CYH2 |                               |
| Wild type         | [81/200] | [89/200] | 0.0                             |
| mlhl              | [73/280] | [62/280] | 1.1                             |
| msh4              | [41/200] | [27/200] | (11/200)                        |
| mlhl msh4         | [49/240] | [34/240] | (13/240)                        |

<sup>a</sup>For random spores, the map distance in cM is approximately equivalent to the frequency of recombinants. The mlhl single mutant differs from both msh4 and mlhl msh4 in the TRP5-CYH2 interval \((P < 0.05)\) but not at HIS4-MAT. With the size of the current data set the frequency of nonmating haploids produced by the mlhl diploid is not different from the wild-type strain \((P = 0.08)\). (See text for details.)

<sup>b</sup>Nonmating colonies represent nondisjunction of chromosome III. A nondisjunction rate of \(1.05 \times 10^{-3}\) has been determined for wild-type strains (Goldway et al. 1993).

### Table 3. Spore viability

| Genotype [strain] | Percentage spore viability |
|-------------------|-----------------------------|
| Wild type         | 97.69 (1249/1295)           |
| mlhl<sup>a</sup>  | 83.80 (1609/1920)           |
| mlhl              | 71.16 (2354/3308)           |
| pms1              | 87.12 (3349/3844)           |
| msh2<sup>a</sup>  | 90.60 (1359/1500)           |
| msh2              | 88.13 (4262/4836)           |
| msh3              | 90.93 (2506/2756)           |
| msh4              | 25.30 (511/2020)            |
| msh6<sup>a</sup>  | 96.68 (1543/1596)           |

<sup>a</sup>Diploids were sporulated under conditions that prevented any growth as a diploid.
Tetrads producing four viable spores were analyzed for non-Mendelian segregation of the three alleles of HIS4. Segregation patterns were classified according to nomenclature taken from eight spored fungi. Sectored colonies (PMSs) were detected using the plate dissection method (Materials and Methods; Fogel et al. 1979). 5:3 and 3:5 tetrads have a single His+/His- colony; aberrant 4:4 (Ab4:4), aberrant 6:2 (Ab6:2), and aberrant 2:6 (Ab2:6) have two sectored colonies; deviant 5:3 (D5:3) and deviant 3:5 (D3:5) have three sectored colonies.

a These diploids contain the MAT-URA3-MAT interval on chromosome III. The MAT-URA3-MAT interval comprises a 9-kb, nontandem duplication of the mating-type locus flanking pBR322 and URA3 sequences (Borts and Haber 1987), which undergoes high frequencies of crossing-over during meiosis (Table 1). Interestingly, in wild-type strains, the frequency of non-Mendelian segregation at his4-C1 is increased 1.7-fold by the presence of this structure (P < 0.001), although the two loci are located some 132 kb away, on opposite sides of the centromere. The mhl1 diploids have a higher frequency of non-Mendelian segregation than all other equivalent (P < 0.05 to << 0.001), excluding the msh2 mhl1 double mutant strains (NHD133 and NHD155). The proportion of events displaying PMS at his4-C1 is significantly greater in mhl1 strains than in the equivalent msh2, msh3, and msh6 strains (P << 0.001). (Also see text and Fig. 2.)

b These data sets represent pools of diploids that contain different versions of the MAT-URA3-MAT interval but are otherwise isogenic.

are elevated 1.4- to 2.2-fold above wild type (P < 0.001; Table 4; Fig. 2). In contrast, the total frequencies of non-Mendelian events at the his4-X and his4-Cl alleles are not increased significantly by mutation of MSH2 [NHD161 and NHD162, his4-RI was not tested]. Also, with the exception of msh6 [see below], the other mismatch repair mutants do not alter significantly the total frequency of events at his4-Cl. In wild-type cells the

| his4 allele | Genotype [strain] | Tetrad class | Percent total events | Percent PMS/total |
|------------|------------------|--------------|----------------------|-------------------|
|            |                  | 6:2 | 2:6 | 5:3 | 3:5 | Ab4:4 | other |                  |                  |
| his4-Cl    | wild type        | 228 | 258 | 0   | 0   | 6   | 8:0   | 14.9 | 0.0             |
|            | mlh1             | 7   | 12  | 76  | 95  | 15  | 1:7   | 21.0 | 90.9            |
|            | pms1             | 8   | 29  | 33  | 33  | 2   | 0:8   | 13.2 | 63.9            |
|            | msh2             | 27  | 19  | 47  | 54  | 2   | 0:8   | 15.3 | 68.9            |
|            | msh3             | 70  | 54  | 19  | 11  | 1   | 0:8   | 17.3 | 20.7            |
| msh6      |                  | 26  | 24  | 1   | 2   | 0   | 1:0   | 7.9  | 5.7             |
| msh2 mlh1  | [NHD133]         | 2   | 2   | 16  | 14  | 4   | 0:4   | 18.6 | 89.7            |
| his4-X     | wild type        | 15  | 18  | 0   | 0   | 0   | 0:0   | 11.1 | 0.0             |
|            | mlh1             | 24  | 2   | 24  | 15  | 4   | 0:4   | 24.7 | 62.3            |
|            | msh2             | 1   | 2   | 8   | 8   | 1   | 0:0   | 11.7 | 85.0            |
| msh1 mlh1  | [NHD155]         | 10  | 2   | 17  | 10  | 2   | 1:0   | 21.6 | 71.4            |
| his4-Cl    | wild type        | 33  | 25  | 0   | 0   | 0   | 1:0   | 8.7  | 57.1            |
|            | mlh1             | 2   | 4   | 23  | 31  | 4   | 0:0   | 15.9 | 89.2            |
| msh2 mlh1  | [NHD131,132]     | 4   | 1   | 5   | 3   | 0   | 1:0   | 9.6  | 57.1            |
|            | mlh1             | 1   | 1   | 14  | 10  | 3   | 0:0   | 26.6 | 93.1            |
|            | msh1 mlh1        | 9   | 10  | 0   | 0   | 0   | 0:0   | 8.3  | 0.0             |
| his4-RI    | wild type        | 10  | 2   | 7   | 11  | 3   | 1:0   | 16.9 | 61.7            |

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b These data sets represent pools of diploids that contain different versions of the MAT-URA3-MAT interval but are otherwise isogenic.
his4-X, his4-Cl, and his4-RI alleles were never observed to undergo PMS and therefore, represent efficiently repaired mismatches. Tetrads with one or more colonies displaying PMS account for 62–91% of all non-Mendelian segregations in mlhl mutants. At his4-Cl the proportion of events displaying PMS is significantly greater for mlhl strains than for the other mismatch repair mutants \( P < 0.001 \). At the his4-X allele, the msh2 mutation does not increase the total frequency of non-Mendelian segregation \( \text{unlike mlhl} \), but a significantly greater proportion of these events are PMS when compared to his4-X in mlhl strains \( 17 \text{ of } 20 \text{ and } 43 \text{ of } 69 \), respectively, \( P < 0.05 \).

We also noted that the his4-Cl allele \( \text{a } 2\text{-bp duplication} \) is repaired nearly four times less frequently than either the his4-X or his4-RI alleles \( \text{4-bp duplications} \) in mlhl strains. The latter also show disparities in the direction of gene conversion, with a 5- to 12-fold bias toward 6:2 events \( P < 0.01 \text{ to } < 0.001 \), indicating preferential excision of the mutant allele.

The HIS4 gene conversion polarity gradient

Studies by other investigators have demonstrated that in wild-type cells the HIS4 gene displays a 5’ to 3’ gradient in the frequency of non-Mendelian segregation of well-repaired alleles \( \text{White et al. 1992; Alani et al. 1994} \). This gradient is disrupted in msh2 mutants \( \text{[Reenan and Kolodner 1992b; Alani et al. 1994]} \) and when palindromic loop mismatches \( \text{that frequently escape mismatch repair} \) are examined \( \text{[Detloff et al. 1992; White et al. 1992]} \).

In a strain background that has extremely high frequencies of aberrant segregation at HIS4, an approximately twofold gradient is observed over the entire gene. The gradient is just 1.5-fold over the region we have examined \( \text{[Detloff et al. 1992; White et al. 1992]} \). Given such a shallow gradient and the fact that the wild-type strains used in this study have relatively low frequencies of aberrant segregation at HIS4, we could not expect to see a statistically significant polarity gradient unless the data sets were extremely large. However, the gradient of aberrant events between his4-X and his4-RI is significant in mlhl strains \( P < 0.05 \), Table 4, Fig. 2). This suggests that mutation of MLH1 does not disrupt the gene conversion polarity gradient at HIS4. Analysis of mlhl msh2 double mutants supports this proposal. At his4-X, the frequency of non-Mendelian events in a mlhl msh2 double mutant \( \text{Table 4, strain NHD155} \) is not significantly different to the equivalent mlhl single mutant. However, at his4-Cl \( \text{Table 4, strain RHB2644} \), the frequency of events in the double mutant is 1.7-fold higher than in a mlhl single mutant \( P < 0.01 \), demonstrating the dependence of the HIS4 polarity gradient on MSH2 function.

Discussion

A unique role for Mlh1 during meiosis

Among the five MutL and MutS homologs with known mismatch repair activities \( \text{Pms1, Mlh1, Msh2, Msh3, and Msh6} \) only Mlh1 has a role in promoting crossing-over during meiosis. Although small changes in the frequencies of crossing-over are observed in mismatch repair mutants other than mlhl, these are almost exclusively increases in recombination frequency. With respect to Pms1 and Msh2 in particular, this is consistent with the antirecombination activity that has been demonstrated for these proteins \( \text{[Selva et al. 1995; Chambers et al. 1996; Datta et al. 1996; Hunter et al. 1996; N. Hunter and R. H. Borts, unpubl.]} \). This activity functions to prevent genetic exchange between diverged DNA sequences. In yeast diploids with a truly isogenic background \( \text{i.e., perfectly homologous chromosomes, pms1 and msh2 mutants do not have significantly altered frequencies of exchange [Hunter et al. 1996]} \). Therefore, it seems likely that the small increases in recombination observed for pms1 and msh2 are attributable to the low levels of sequence divergence present between the congenic parental haploid strains and the absence of an antirecombination activity.

There are two other prominent features of the meiotic phenotype of mlhl mutants: (1) a general increase in the frequency of non-Mendelian segregation and (2) a very high incidence of PMS events.

The crossover function of Mlh1

The crossover deficit observed in mlhl diploids is simi-
lar to that of msh4 and msh5 yeast mutants. A 1.4- to 3.4-fold and a 1.9- to 4.0-fold reduction in crossing-over are observed in msh4 and msh5 strains, respectively (Ross-Macdonald and Roeder 1994; Hollingsworth et al. 1995). Studies of the double mutant indicate that the Msh4 and Msh5 proteins function in the same epistasis group to promote crossing-over (Hollingsworth et al. 1995). This process is independent of the presence of mismatches and Msh4 and Msh5 are not involved in mismatch repair. Mlh1 also appears to promote crossing-over independently of heterologies. Although the haploid strain backgrounds used in this study are congenic (and, therefore, may be very slightly diverged), the artificial MAT-URA3-MAT intervals are perfectly homologous and display the greatest reduction in exchange [2.3-fold] of all the intervals examined in mlh1 mutants. Also, if the crossover function of Mlh1 was dependent on the recognition of mismatches, lack of the major mismatch-binding protein Msh2 might be expected to affect the mlh1 crossover phenotype. The data for a mlh1 msh2 double mutant indicate that the crossover function of Mlh1 is not Msh2 dependent. Analysis of a mlh1 msh4 double mutant suggests that the Mlh1 and Msh4 [and presumably Msh5] proteins function in the same pathway to promote reciprocal exchange. We note that msh4 mutants and the mlh1 msh4 double mutant have a generally more severe defect with less crossing-over, more chromosome nondisjunction, and lower spore viability than mlh1 mutants (this study, Ross-Macdonald and Roeder 1994; Hollingsworth et al. 1995). Therefore, we tentatively propose that Mlh1 is required at a step after the action of Msh4 and Msh5.

The gene conversion function of Mlh1

Our results indicate that most meiotic gene conversions proceed through a heteroduplex intermediate and the Msh/Mlh system is the major heteroduplex repair pathway. Mlh1 appears to be central to this pathway.

The Pms1 and Mlh1 proteins are believed to function as a heterodimer during mismatch correction [Prolla et al. 1994b; Li and Modrich 1995]. Previous genetic data suggested an equal requirement for both gene products in yeast meiosis [Prolla et al. 1994a]. However, the crossover function is unique to Mlh1. This indicates that the Pms1/Mlh1 heterodimer is not the exclusive functional form of these proteins. In addition, our observations indicate a greater role for Mlh1 in at least some heteroduplex correction reactions. The 2-bp duplication mismatch created at the his4-Cl allele is repaired almost exclusively by a Mlh1-dependent pathway. In contrast, 38% of heteroduplexes created at the his4-X and his4-R1 alleles [4-bp duplications] are repaired by a Mlh1-independent mechanism. This pathway produces a 5- to 12-fold bias toward removal of the mutant allele, indicating preferential excision of the longer, mutant strand. The formation of heterodimers of Pms1 or Mlh1, with one of the two uncharacterized mutL homologs MLH2 and MLH3 (Prolla et al. 1994a; Saccharomyces genome database), could account for this pathway and explain the phenotypic differences between pms1 and mlh1 mutants.

Another unique feature of mlh1 mutants is the increase in total non-Mendelian segregation. A number of possible explanations can account for this phenotype. First, absence of Mlh1 could lead to an increase in the number of recombinational interactions that are initiated. This proposal predicts a role for Mlh1 at an early stage in the recombination pathway, and would represent a novel activity for MutL proteins. Second, the additional PMS events detected in mlh1 mutants may represent mismatches that are normally repaired to restore the parental ratio of alleles [4:4 segregation]. However, if all mismatch correction during meiosis [conversions and restorations] were dependent on mismatch recognition by the Msh2 protein, then the frequency of aberrant events in msh2 strains should be equivalent to, or greater than, that observed for mlh1 and other mismatch repair mutants. The examination of alleles of the HIS4 gene in this study indicates that this is not the case. About half of the events observed in mlh1 strains occur independently of the other mismatch repair proteins. Therefore, if frequent restoration does occur in wild-type cells, it is not Msh2 dependent. Moreover, disruption of restorational repair might be expected to disrupt gene conversion polarity [see below]. These caveats make the restoration hypothesis less attractive to us. A third possibility is that Mlh1 promotes the formation of a class of recombination intermediates that have short heteroduplex tracts, perhaps by restricting DNA synthesis or eliminating the products of over-replication [see model in Fig. 3 and below]. In mlh1 mutants these events may mature by a different pathway producing intermediates with longer heteroduplexes that are generally resolved as non-crossovers. Alleles will have a greater chance of being incorporated into these longer heteroduplex tracts and an apparently new set of non-Mendelian events will result. Finally, two studies have demonstrated that non-Mendelian segregation is asymmetric with respect to recombination initiation sites [i.e., non-Mendelian segregation occurs on only one side of the initiation site; Porter et al. 1993; Gilbertson and Stahl 1996]. If Mlh1 were required to generate this asymmetry, an elevated frequency of non-Mendelian segregations might result in mlh1 mutants. These last two explanations are not mutually exclusive as both result in the formation of more heteroduplex.

Interestingly, small increases in non-Mendelian segregation are also observed in msh4 and msh5 mutants yet, unlike mlh1 mutants, they are not mismatch repair defective. In the study of Ross-Macdonald and Roeder [1994], non-Mendelian segregation was increased by 1.4- to 2.3-fold at four of the six alleles examined. Similarly, Hollingsworth et al. [1995] observed 1.3- to 6.1-fold increases at the three loci they examined. However, given the size of the data sets presented, a significant increase was only recorded for a single locus in each of these studies. In this respect the phenotypes of msh4 and msh5 mutants may represent a moderate mlh1-like defect in a mismatch repair proficient context.

The HIS4 gene conversion polarity gradient is not
Figure 3. Model for Mlh1 function. Only the two chromatids involved in the recombination event are shown. Symbols represent heterozygous alleles. (A) Double-strand cleavage and 5' to 3' exonucleolytic resection produces long 3' single-stranded tails. (B) Strand invasion primes DNA synthesis, which displaces the non-template strand, allowing the formation of heteroduplex DNA. The crossed strand junctions are bound and stabilized by Msh4 and Msh5. Mlh1 may also promote this transition. (C) In wild-type cells heteroduplex DNA is repaired to produce a gene conversion. A covalently closed double Holliday junction is then formed. Although these structures are likely to be the precursors of crossovers (Schwacha and Kleckner 1995) they are not essential for the formation of noncrossovers (gene conversions without an associated crossover; Resnick 1976; Hastings 1988; Schwacha and Kleckner 1995). Noncrossovers may bypass the double Holliday junction stage by unwinding the structure shown in B. (D) The double Holliday junction is resolved to give crossover or noncrossover recombinants. (E) In the absence of Mlh1, Msh4, or Msh5 instability of one or both junctions may lead to over-replication of recombination intermediates resulting in longer heteroduplex DNA. If such an intermediate is going to produce a crossover the cross-junctions must be reestablished to allow double Holliday junction formation. (F) In mlh1 mutant cells recombination intermediates cannot progress efficiently to the double Holliday junction stage and, therefore, the frequency of crossing-over is reduced. The structure is broken down by the action of a helicase, topoisomerase, or junction-specific nuclease. One possible outcome is shown. (G) Primarily noncrossover products are produced. Because Mlh1 is required for mismatch correction, heteroduplex is not repaired resulting in PMS. Note that the heteroduplex tract is longer than the intermediate shown in B. (See text for further discussion.)

abolished in mlh1 mutants. However, the gradient is disrupted by mutation of MSH2, even in the absence of MLH1 function. As mlh1 mutants are severely repair deficient, this observation suggests that the HIS4 polarity gradient may depend on the recognition, but not the correction, of mismatches. This interpretation supports the proposal that Msh2 blocks the extension of heteroduplex when a mismatch is encountered (Alani et al. 1994). An alternative explanation of polarity is that a gradient in the direction of mismatch correction extends from an initiation site for heteroduplex formation (Detloff et al. 1992). In this model, repair toward the conversion of an allele predominates close to the initiation site, whereas restorative repair is more frequent further along a heteroduplex tract. In the light of the data presented for mlh1 mutants this model is difficult to accommodate as the HIS4 polarity gradient occurs in the absence of mismatch correction.
Models for Mlh1 function

Ross-Macdonald and Roeder (1994) have suggested that the Msh4 protein may promote reciprocal exchange by stabilizing junction structures, thereby counteracting the forces that could destroy early recombination intermediates. This stabilization activity may be a common feature of several MutS homologs. For example, the Msh2 and Msh3 proteins are components of the Rad1/10 pathway of mitotic recombination [Saparbaev et al. 1996]. More specifically, Msh2 and Msh3 are required for single-strand annealing reactions [N. Sugawara, F. Pâques, M. Colaïacovo, and J.E. Haber, pers. comm.] where they may stabilize the junctions between double-stranded and 3' single-stranded tails and then recruit the Rad1/10 endonuclease to cleave the 3' tails [Ivanov and Haber 1995]. The S. pombe MutS homolog Swi4 may also be involved in junction recognition (see below). In the context of a stabilization mechanism, we propose that Mlh1 acts after Msh4 and Msh5 to reinforce the stabilization and promote progression to crossover resolution. The fact that Mlh1 does not appear to be required for mitotic recombination [Saparbaev et al. 1996] indicates that this is a meiosis-specific specialization.

To account for longer heteroduplex in mlh1 mutants we propose that Mlh1 is required to prevent the over-replication of recombination intermediates or to trim the products of over-replication, thereby allowing the formation of a double Holliday junction [see Fig. 3]. Interaction with an endonuclease may be required to execute this step. This may be analogous to the way that MutL is thought to function as an interface between the mismatch binding and excision and resynthesis reactions of mismatch repair [for review, see Friedberg et al. 1995]. The S. pombe MutS homolog Swi4 may be a component of a similar pathway during mating-type switching.

Materials and methods

Plasmids

The LEU2 gene was inserted into a SnBlI site in the wild-type MSH2 gene contained in plasmid pII-2 [Reenan and Kolodner 1992a] to produce pRBH113 (msh2::LEU2). PWK4-mps1 is a deletion of almost the entire PST1 open reading frame [ORF] [Kramer et al. 1989]. The deletion was marked by inserting the LEU2 gene into an adjacent Xbol site to create the plasmid pRHB197. pmlh1ΔLEU2 is a 530-bp deletion of the 5' coding region and upstream sequences of MLH1 marked with the LEU2 gene [Prolla et al. 1994a] and was kindly provided by Dr. M. Liskay [Oregon Health Sciences University, Portland]. pSAL1 [+]/ΔCHLEU2 is a disruption/deletion of MSH3 created by replacing a ClaI–HpaI fragment of MSH3 with the LEU2 gene [a kind gift of G. Carignani, Università degli Studi di Padova, Italy]. A 4-kb PCR fragment containing the MSH6 gene was cloned into the SphI site of PCRScript [Stratagene]. A PvuI–EcorV fragment containing the kanMX4 module [Wach et al. 1994] was then used to replace a SnBlI–SpeI fragment of the MSH6 ORF to produce the plasmid pSRC9 [msh6Δ::kanMX4], kindly provided by S.R. Chambers, Oxford University, UK. pRH811, pRHB12, and pRHB13 contain Xhol, EcoRI, and ClaI restriction site fill-in mutations of the HIS4 gene.

Strains

All haploid strains are isogenic derivatives of either H330 [ura3-1 can1 ade1 lys2-c met13-2 cyh2 trp5-1 leu2-K MATa] or RHB2096-1a [ura3-1 lys2-d met13-4 cyh2 leu2-R MATa]. RHB2096-1a was created by transformation of the ade1 strain H394 with a 1.4-kb XhoI fragment containing the wild-type ADE1 gene and selecting for Ade+ transformants. H330 and H394 are congeneric and have been described previously [Borts and Haber 1989]. Although all diploids are derived from this congenic pair of haploid parents they still constitute an isogenic set. The his4-X, his4-Cl, and his4-RI alleles were introduced by a two-step gene replacement. Combinations of the above markers are segregating in all strains except NHD132, which contains trp1-H3, a restriction-site fill-in mutation. Mismatch-repair mutants were obtained by one-step gene replacement with the appropriate fragments from the plasmids pRHB113 (msh2::LEU2), pRHB197 (pms1Δ::LEU2), pmlh1Δ::LEU2 [Prolla et al. 1994a], pGEM7Zf[+]/ΔCHLEU2 (msh3Δ::LEU2), or pSRC9 (msh6Δ::kanMX4). The msh4 mutation is a complete deletion of the MSH4–coding region obtained using PCR-mediated gene disruption [Wach et al. 1994]. Mutant strains are referred to by their relevant genotypes (e.g., mlh1). Double mutant combinations were obtained by crossing the appropriate isogenic single mutants. The MAT–URA3–MAT interval has been described [Borts and Haber 1987] and was introduced by integrative, site-directed transformation [Orr-Weaver et al. 1983]. Transformations were verified by Southern blot [Southern 1975; Sambrook et al. 1989] using the digoxigenin, nonradioactive system as recommended by the manufacturer [Boehringer Mannheim].

Genetic procedures

Yeast manipulations and media are as described previously [Rose et al. 1990]. Strains were grown at 30°C on YPD medium and synthetic complete media lacking one or more nutritional
supplements. Mismatch repair mutant diploids were made by mixing approximately equal amounts of the parental haploids in 100-200 μl of liquid YPD and then spreading the mixture on solid YPD plates. Mating was allowed to proceed for 6 hr at 30°C or up to 12 hr at room temperature before replicating directly to sporulation medium. Sporulation was performed at room temperature on plates containing 2% potassium acetate, 0.22% yeast extract, 0.05% glucose, 2.5% agar, and 0.09% complete amino acid mixture, or 2% potassium acetate, 2.5% agar, and 0.09% complete amino acid mixture. The latter recipe does not permit vegetative growth and was found to improve the viability of mismatch repair mutant strains. Dissected tetrads were grown for 3-4 days at 30°C. Only spores that formed colonies visible to the naked eye were scored as being viable. Secorted colonies were detected using the plate dissection method [Fogel et al. 1979]. Ambiguous sectored colonies were streaked onto YPD plates to obtain single colonies and then replicated to selective media. A mixture of prototrophic and auxotrophic colonies was scored as a PMS. Random spores were prepared as described [Lichten et al. 1987] and grown on synthetic complete medium lacking arginine and containing cycloheximide (10 mg/liter) and canavanine (40 mg/liter) for 3-4 days at 30°C.

One-step and two-step gene replacements were performed as described [Rose et al. 1990]. Yeast transformation was carried out using a modification of the lithium acetate method [Gietz et al. 1992].

PCR-mediated gene disruption

PCR-mediated disruption of the MS14 gene was performed as described [Wach et al., 1994], using the 64-mer decosyloligodeoxynucleotides AGTTATACGATTGAAATCTGATCGATC- AACCCTAAACTATATGCAGCTACGTCAGGCTGAC and CAGAAATAATGGATTATAGTTTAAAGCTAGGGCGAA- AAGCCAAAATCGATGAATTCGAGCTCG and the pFA6- kanMX4 plasmid [containing the kanr resistance gene [Wach et al. 1994]]. Transformants were selected on YPD media containing 400 mg/liter of geneticin [G148, Boehringer Mannheim].

Karyotyping of segregants

Random spore segregants were karyotyped as described [Nau- mov et al. 1992]. Normally, disomy can be assigned accurately by eye for the ten smallest S. cerevisiae chromosomes [Hunter et al. 1996], however, the comigration of chromosomes V and VIII in the parental strains RHB2096-1a and H330 made assignment of disomy for these chromosomes difficult, and therefore, only eight chromosomes could be analyzed.

Data analysis

Data sets were analyzed using the standard normal and G-tests as described [Sokal and Rohlf 1969]. The G-test is equivalent to the $X^2$ contingency test. Values of $P < 0.05$ were considered significant.

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