MLH1 Mutations Differentially Affect Meiotic Functions in *Saccharomyces cerevisiae*

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ABSTRACT

To test whether missense mutations in the cancer susceptibility gene *MLH1* adversely affect meiosis, we examined 14 yeast *MLH1* mutations for effects on meiotic DNA transactions and gamete viability in the yeast *Saccharomyces cerevisiae*. Mutations analogous to those associated with *h*ereditary *n*on*p*olyposis *c*olorectal *c*ancer (HNPCC) or those that reduce Mlh1p interactions with ATP or DNA all impair replicative mismatch repair as measured by increased mutation rates. However, their effects on meiotic heteroduplex repair, crossing over, chromosome segregation, and gametogenesis vary from complete loss of meiotic functions to no meiotic defect, and mutants defective in one meiotic process are not necessarily defective in others. DNA binding and ATP binding but not ATP hydrolysis are required for meiotic crossing over. The results reveal clear separation of different Mlh1p functions in mitosis and meiosis, and they suggest that some, but not all, *MLH1* mutations may be a source of human infertility.

THE mismatch repair system plays a number of roles in maintaining genome stability. During mitosis it primarily ensures avoidance of mutations and inappropriate recombination events (reviewed in Harfe and Jinks-Robertson 2000) while during meiosis it is involved in heteroduplex repair, crossing over, chromosome segregation, and avoidance of inappropriate recombination (reviewed in Borts *et al.* 2000). Mismatch repair proteins function as dimers. MutS and MutL in bacteria form homodimers while their eukaryotic homologs form heterodimers. There are six *M*ut*S h*omologs, *MSH1–6*, and four *M*ut*L h*omologs, *MLH1–3* and *PMS1* (PMS2 in humans). Mutation avoidance is accomplished by mispair recognition by Msh2p/Msh6p (MutSα) or Msh2p/Msh3p (MutSβ) and transduction of a signal by a heterodimer of Mlh1p/Pms1p (MutLα) or Mlh1p/Mlh3p (reviewed in Harfe and Jinks-Robertson 2000) to effector molecules. The exonuclease encoded by *EXO1* has been implicated in mismatch repair. However, the mutation rate is increased only moderately by deletion of the gene, indicating that other proteins are involved in mismatch removal (Tishkoff *et al.* 1997; Sokolsky and Alani 2000; Amin *et al.* 2001; Tran *et al.* 2001). In higher organisms mutation accumulation due to deficiency in mismatch repair is associated with carcinogenesis. Specifically, defects in *hMLH1* and *hMSH2* are found in sporadic tumors and a familial cancer syndrome, hereditary nonpolyposis colorectal cancer (HNPCC; reviewed in Peltomaki 2001). Germline mutations in *hEXO1* have also been reported to be associated with HNPCC (Wu *et al.* 2001). In addition to the role of mismatch repair genes in mutation avoidance MutSα and MutLα are responsible for the majority of repair of mismatches in heteroduplex DNA formed during meiotic recombination (Williamson *et al.* 1985; Reenan and Kolodner 1992; Alani *et al.* 1994; Prolla *et al.* 1994; Hunter and Borts 1997; Wang *et al.* 1999). Exo1p plays little or no role in the removal of this type of mismatch (Khazanehdari and Borts 2000; Kirkpatrick *et al.* 2000).

The importance of the role(s) that mismatch repair proteins play in meiosis is illustrated by the infertility found in model organisms deficient in some mismatch repair genes (reviewed in Borts *et al.* 2000; Cohen and Pollard 2001). In yeast, loss of Mlh1p, Mlh3p, Exo1p, and the meiosis-specific Msh4p and Msh5p causes defects in reciprocal recombination and chromosome segregation (Ross-Macdonald and Roeder 1994; Hollingsworth *et al.* 1995; Hunter and Borts 1997; Wang *et al.* 1999; Borts *et al.* 2000; Khazanehdari and Borts 2000; Kirkpatrick *et al.* 2000; Novak *et al.* 2001; Abdullah 2002; Argueso *et al.* 2002). Although the phenotypes of the individual mutants are not identical, in none of the cases studied does the double mutant display a more extreme crossover defect than that of the most severe of the single mutants, Δ*msh4*, suggesting that they all operate in the same crossover pathway (Hollingsworth *et al.* 1995; Hunter and Borts 1997; Borts *et al.* 2000; Khazanehdari and Borts 2000; Abdullah 2002). Mice that are mutant in *MLH1*, *MLH3*, *MSH4*, and *MSH5* have chromosome segrega-

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tion or chromosome pairing abnormalities and are both male and female sterile (Baker *et al.* 1996; Edelmann *et al.* 1996, 1999; Kneitz *et al.* 2000; Cohen and Pollard 2001; Lipkin *et al.* 2002). Cytological studies have indicated that the timing, number, and distribution of MLH1 foci in both humans and mice correlate well with that of late recombination nodules and of chiasmata, the cytological manifestations of crossing over (Barlow and Hulten 1998; Anderson *et al.* 1999). The *Mlh3*$^{-/-}$ mouse has been shown to be deficient in late recombination nodules and fails to form MLH1 foci, suggesting that Mlh3p may recruit Mlh1p (Lipkin *et al.* 2002). Neither the *Mlh1*$^{-/-}$ nor the *Mlh3*$^{-/-}$ mouse has functional chiasmata at diplonema (Baker *et al.* 1996; Lipkin *et al.* 2002). Cytological studies have also indicated that MSH4 foci appear first and are then followed by MLH1 foci (Santucci-Darmanin *et al.* 2000). Physical studies have suggested that mammalian MSH4 protein interacts with both the MLH1 and the MLH3 proteins (Santucci-Darmanin *et al.* 2000, 2002). The cytological data combined with the genetic data from yeast suggest a late role for Mlh1p/Mlh3p in ensuring crossover outcome that is separable from that of the Msh4p/Msh5p complex.

How the Mlh1p/Mlh3p heterodimer exerts its function(s) is not clear. However, by analogy with *Escherichia coli* MutL, it is thought to act by coordinating downstream "effector" molecules such as helicases (Hall *et al.* 1998) and nucleases (Ban and Yang 1998b; Spampinato and Modrich 2000). Among the possible effector proteins known to interact with Mlh1p are ReqQ helicases (yeast Sgs1p and human BLM protein; Langland *et al.* 2001; Pedrazzi *et al.* 2001) and Exo1p (Tran *et al.* 2001). Interestingly neither Sgs1p nor the Bloom's syndrome protein has been implicated in mismatch repair, suggesting a role other than mismatch correction for the interaction of these proteins with Mlh1p. That this role is in the resolution of recombination structures has been suggested by the isolation of a complex containing Top3p, Sgs1p, Mlh1p, and Mlh3p from extracts of meiotic cells (Wang and Kung 2002).

To better understand the role of *MLH1* in meiosis we have assessed meiotic phenotypes conferred by a number of missense mutations that all result in defective mismatch repair (Pang *et al.* 1997; Shcherbakova and Kunkel 1999; Hall *et al.* 2002; M. Hall, P. Shcherbakova and T. Kunkel, unpublished data). Many of the known mutations map to the highly conserved amino-terminal domain of Mlh1p (Figure 1), which has been shown to have ATPase and DNA-binding activities that are essential for repair of replication errors (Tran and Liskay 2000; Hall *et al.* 2002; M. Hall, P. Scherbakova and T. Kunkel, unpublished data). Seven mutations (yP25L/hP28L, yM32R/hM35R, yA41F/hS44F, yG64R/hG67R, yI65N/hI68N, yT114M/hT117M, and yG243D/hG244D) are analogues of human HNPCC mutations. Six of these (yP25L/hP28L, yM32R/hM35R, yG64R/hG67R, yI65N/hI68N, yA41F/hS44F, and yT114M/hT117M) are inferred to reduce the ATPase activity of Mlh1p (Ban and Yang 1998a; Ban *et al.* 1999). Four changes (F96A, R97A, G98A, and G98V) reside in the highly conserved "GFRGEAL" box that composes the "lid" of the ATP-binding pocket (Ban and Yang 1998a; Ban *et al.* 1999; Guarne *et al.* 2001) and are also inferred to interfere with ATP binding or hydrolysis. Each has individually been shown to confer reduced mismatch repair (Pang *et al.* 1997). Replacement of Asn35 with alanine (N35A) results in an N-terminal domain with no ATP-binding or hydrolysis capacity, and replacement of Glu31 with alanine (E31A) results in an N-terminal domain that binds ATP but very inefficiently hydrolyzes it (Hall *et al.* 2002) and is partially repair defective (Tran and Liskay 2000; Hall *et al.* 2002). A double replacement, R273E-R274E, reduces DNA binding by the Mlh1p/Pms1p heterodimer and also confers a mismatch repair defect (M. Hall, P. Shcherbakova, J. Fortune and T. Kunkel, unpublished data). The final substitution studied, G243D, maps to the interface of two domains identified in the crystal structure (Ban and Yang 1998a). The observation that the bacterial protein with this substitution is insoluble suggests that this amino acid change causes the protein to misfold (Ban *et al.* 1999). Fourteen strains, each bearing one of these mutations, were analyzed by tetrad dissection for their effects on meiotic heteroduplex repair, crossing over, chromosome segregation, and gamete viability.

MATERIALS AND METHODS

Plasmids, strains, and sporulation: *MLH1* point mutations were constructed using site-directed mutagenesis (Erdeniz *et al.* 1997; Shcherbakova and Kunkel 1999) and were then introduced into *Saccharomyces cerevisiae* Y55 haploid strains with the following genotypes: Y55-2834 (*MAT*α *HIS4 LEU2 ADE1 trp5-1 cyh2 met13-2 lys2-c ura3-1*) and Y55-2835 (*MAT***a** *his4-r leu2-r ade1-1 TRP5 CYH2 MET13 lys2::InsE-A*$_{14}$ *ura3-1*). The presence of the mutations was confirmed by DNA sequencing. *his4-r* is a 4-bp insertion mutation (Borts and Haber 1989). *met13-2* has a stop codon at position 278 (C → A; Abdullah 2002). The *lys2::InsE-A*$_{14}$ allele contains a homopolymeric A insertion in *LYS2* (Tran *et al.* 1997; Shcherbakova and Kunkel 1999). *MLH1* deleted strains (Δ*mlh1*) were generated using a PCR-based gene disruption method (Wach *et al.* 1994). The diploid strains used are listed in Table 1. Mating, sporulation, and tetrad dissection have been described previously (Hunter and Borts 1997; Abdullah and Borts 2001).

Genetic analysis and statistical methods: Genetic markers were analyzed by direct replication of dissected spore colonies to omission media as described previously (Hunter and Borts 1997; Abdullah and Borts 2001). Non-Mendelian segregation (NMS; 6:2/2:6 conversions and 5:3/3:5 postmeiotic segregation) and reciprocal crossing over were scored only in tetrads containing four viable spores. Map distance in centimorgans was calculated according to the formula cM = 1/2 (TT + 6NPD)/(NPD + PD + TT) (Perkins 1949), where PD, NPD, and TT refer to parental ditype, nonparental ditype, and tetratype segregation patterns. Statistical comparisons were carried out as follows. All of the data were compared to the wild-type and Δ*mlh1* strains. The distribution of tetrad classes with respect to the crossover and viability data were compared using a *G*-test of heterogeneity (Sokal and Rohlf

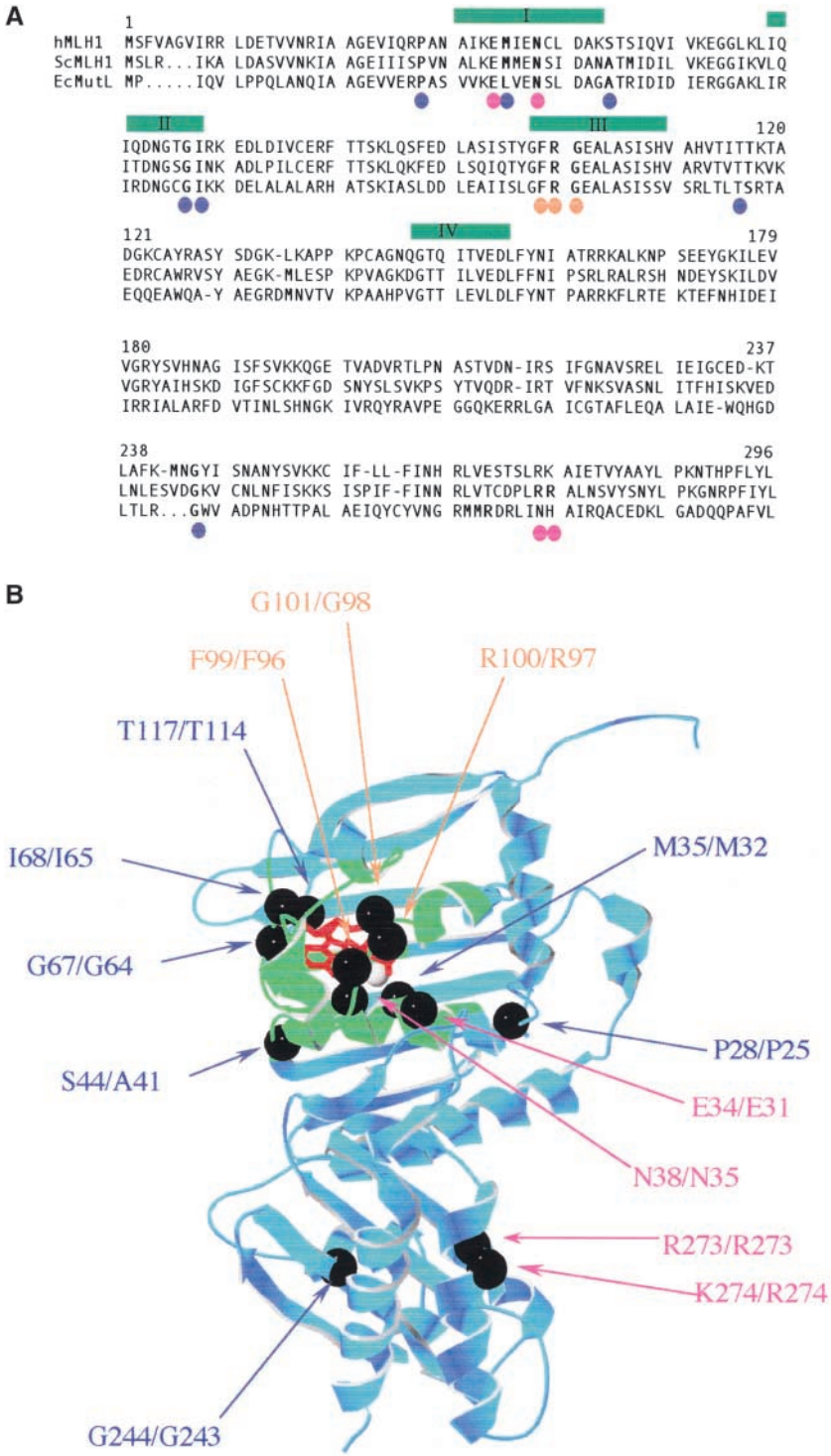

FIGURE 1.—(A) Alignment of the N termini of *E. coli* MutL (Z11831), *S. cerevisiae*, and *hMLH1*. Blue dots represent the HNPCC mutations, green bars highlight the ATPase domain (motifs I–IV), and magenta and orange dots identify the functionally defined mutations and GFRGEAL box mutations, respectively. (B) Crystal structure of MutL, with first the human mutations and then the equivalent yeast residue indicated. The α-carbon of the residue is represented by a black ball. Green indicates the ATP-binding site, ATP is shown in red, and the gray ball is Mg^{2+}.

1969). To compare NMS and the proportion of meiotic repair events, we employed Fisher's exact test, using the one-tailed distribution (http://faculty.vassar.edu/lowry/VassarStats.html). For comparisons of data sets containing >100 tetrads for which the Fisher's exact test cannot be used, we employed a two-sample *z*-test (http://faculty.vassar.edu/lowry/VassarStats.html). In all of the statistical comparisons, we used the Dunn-Sidak correction (SOKAL and ROHLF 1969) for significance testing, which is required when multiple comparisons using the same data sets are made. For example, $\alpha < 0.05$ is normally set as the basis for rejection of the null hypothesis when a single pairwise comparison is made. However, statistical theory necessitates that α be adjusted to reflect multiple comparisons. Thus when a missense mutation was compared to both the wild-type and the Δ*mlh1* strains (*e.g.*, crossover data and meiotic repair data) $P < 0.025$ was considered significant. *P* values <0.017 were considered significant when a given data set was compared to those of the wild-type, Δ*mlh1*, and Δ*msh2* strains. The NPD ratio was calculated using the equation of PAPAZIAN (1952), where an NPD ratio significantly lower than one indicates interference. The method of Stahl and Lande (http://www.groik.com/stahl/) was also used calculate "*m*" where a value of *m* significantly greater than zero is indicative of interference (STAHL and LANDE 1995).

TABLE 1

Strains used in this study

Strain name	Genotype[a]
ERY68	*his4-r leu2-r MAT***a** / *HIS4 LEU2 MAT*α *ade1-1 TRP5 CYH2 MET13* / *ADE1 trp5-1 cyh2 met13-2*
ERY103	*HYG his4-ATC BIK1 NAT leu2-r MAT*α / *HIS4*-1605 *BIK1*-939 *LEU2 MAT***a** *ade1-1 trp5-1 cyh2 MET13* / *ADE1 TRP5 CYH2 met13-2*
ERY12	Δ*mlh1/MLH1*
ERY14	Δ*mlh1*/Δ*mlh1*
ERY102	Δ*msh2*/Δ*msh2* remaining genotype as ERY103
ERY112	Δ*mlh1*/Δ*mlh1* remaining genotype as ERY103
ERY82	*mlh1-P25L/mlh1-P25L*
ERY83	*mlh1-I65N/mlh1-I65N*
ERY87	*mlh1-M32R/mlh1-M32R*
ERY88	*mlh1-G64R/mlh1-G64R*
ERY89	*mlh1-T114M/mlh1-T114M*
ERY90	*mlh1-G243D//mlh1-G243D*
ERY132	*mlh1-A41F/mlh1-A41F*
ERY84	*mlh1-R273E-R274E/mlh1-R273-R274E*
ERY105	*mlh1-N35A/mlh1-N35A*
ERY148	*mlh1-N35A/MLH1*
ERY125	*mlh1-E31A/mlh1-E31A*
ERY32	*mlh1-F96A*/Δ*mlh1*
ERY33	*mlh1-G98V*/Δ*mlh1*
ERY159	*mlh1-G98A*/Δ*mlh1*
ERY123	*mlh1-R97A*/Δ*mlh1*

[a] All strains are isogenic derivatives of ERY68 unless otherwise noted. In addition, all of the strains are *lys2-14A/lys2-c* and *ura3/ura3*, except ERY103 and derivatives thereof, which are *lys2-c/lys2-d* and *ura3/ura3*.

Physical analysis of disomy: Tetrads with two or three viable spores were analyzed for chromosomal aneuploidy using clamped homogeneous electric field (CHEF) gel analysis (Khazanehdari and Borts 2000). Rates of disomy were calculated by dividing the observed number of two-viable-spore asci containing disomes by the number of tetrads that it took to obtain the number of two-viable-spored tetrads that were analyzed. Of the 16 yeast chromosomes, only 10 can be assayed by intensity of the chromosome band. Thus the value obtained is an underestimate of the frequency of aneuploidy.

Alignment and protein modeling: The *E. coli* MutL, *S. cerevisiae MLH1*, and human *MLH1* were aligned using MegAlign (DNA Star) by the Jotun Hein method. Molecular representation of the MutL crystal structure (accession no. 1B63.pdb in the Brookhaven protein database) was made using Swiss-PdbViewer.

RESULTS

***MLH1* is dominant and haplosufficient:** All of the HNPCC and the mlh1p-N35A, E31A, and R273E-R274E mutations were studied as homozygotes (*e.g.*, *mlh1-E31A/mlh1-E31A*). However, the GFRGEAL box mutations were studied in heterozygous diploid strains (*e.g.*, *mlh1-F96A/*Δ*mlh1)*. To confirm that this would not interfere with comparisons between strains we analyzed *MLH1/*Δ*mlh1*. The *MLH1/*Δ*mlh1* strain was indistinguishable from wild type with respect to all meiotic phenotypes (Tables 2, 3, 4, and 5), indicating that a single wild-type gene is sufficient to ensure normal levels of crossing over, gene conversion, nondisjunction, and chromosome segregation. We also analyzed *mlh1-N35A/MLH1* because *mlh1-N35A* has been suggested to be dominant negative with respect to mitotic mismatch repair (Hall *et al.* 2002). This does not appear to be the case for meiotic functions as the heterozygous diploid is indistinguishable from both of the wild-type diploids analyzed (Tables 2–5).

Crossing over is affected only in a subset of mutants: The *MLH1* missense mutations fell into two groups when meiotic crossing over in four genetic intervals was determined (Tables 2 and 6). Strains bearing group I mutations (*mlh1-P25L*, *mlh1-E31A*, *mlh1-I65N*, *mlh1-T114M*, *mlh1-F96A*, *mlh1-R97A*, and *mlh1-G98A*) had normal levels of crossing over and had crossover frequencies significantly greater than those of Δ*mlh1* ($P < 0.05$, *G*-test of homogeneity). In contrast, the group II strains (*mlh1-M32R*, *mlh1-N35A*, *mlh1-A41F*, *mlh1-G64R*, *mlh1-G98V*, *mlh1-G243D*, and *mlh1-R273E-R274E*) exhibited reduced crossing over in all four intervals relative to the wild type ($P < 0.05$). Crossing over was reduced to a level that was indistinguishable from that observed in the Δ*mlh1* strain. The observation that the mlh1-R273E-R274E protein, which displays reduced binding of DNA,

TABLE 2

Map distances in the *MLH1* mutant strains

	Interval[a]															
	HIS4-LEU2				*LEU2-MAT*				*MET13-CYH2*				*CYH2-TRP5*			
Genotype	PD	NPD	TT	cM	PD	NPD	TT	cM	PD	NPD	TT	cM	PD	NPD	TT	cM
Group I: crossover proficient[b]																
MLH1/MLH1	207	3	107	19.7	191	4	166	26.3	240	2	104	16.8	88	26	247	55.8
MLH1/Δmlh1	216	2	95	17.1	204	3	125	21.5	241	1	96	15.1	77	21	246	54.1
MLH1/mlh1-N35A	59	0	27	15.7	51	2	42	28.4	61	0	30	16.5	24	4	68	47.9
mlh1-P25L/mlh1-P25L	130	2	50	17.0	104	4	82	27.9	118	0	70	18.6	44	26	134	71.1
mlh1-E31A/mlh1-E31A	124	2	73	21.4	119	8	94	32.1	143	1	71	17.9	65	18	144	55.5
mlh1-165N/mlh1-I65N	250	1	103	15.4	214	5	179	26.3	275	0	113	14.6	102	27	275	54.1
mlh1-F96A/Δmlh1	95	3	50	23.0	101	4	60	25.5	116	0	44	13.8	54	11	102	50.3
mlh1-R97A/Δmlh1	156	4	74	20.9	112	14	133	41.9	156	1	89	19.3	47	24	183	64.4
mlh1-G98A/Δmlh1	129	1	56	16.7	108	5	89	29.5	145	2	45	14.8	58	26	118	67.8
mlh1-T114M/mlh1-T114M	109	2	55	20.2	109	6	71	28.8	119	1	63	18.9	43	15	127	58.6
Group II: crossover deficient[c]																
Δmlh1/Δmlh1	341	2	80	10.9	318	5	138	18.2	670	9	112	10.5	424	14	409	29.1
mlh1-M32R/mlh1-M32R	225	3	59	13.4	200	3	97	19.2	237	0	53	9.1	156	6	139	29.1
mlh1-N35A/mlh1-N35A	111	0	24	8.9	90	1	31	15.2	105	0	26	9.9	79	2	58	25.2
mlh1-A41F/mlh1-A41F	157	1	40	11.6	156	2	59	16.4	171	0	39	9.3	110	3	99	27.6
mlh1-G64R/mlh1-G64R	196	0	34	7.4	197	2	44	11.5[d]	192	1	42	10.2	123	7	115	32.0
mlh1-G98V/Δmlh1	114	1	27	11.6	95	0	42	15.3	132	0	26	8.2	83	1	83	26.6
mlh1-G243D/mlh1-G243D	199	2	63	14.2	206	6	68	18.6	218	1	45	9.7	154	7	121	28.9
mlh1-R273E-R274E/ mlh1-R273E-R274E	241	1	65	11.6	231	5	91	18.5	264	2	55	10.4	169	9	158	31.5

[a] The *HIS4-LEU2* and *LEU2-MAT* intervals are located on chromosome III, whereas the *MET13-CYH2* and *CYH2-TRP5* intervals are on chromosome VII. PD, parental ditype; NPD, nonparental ditype; TT, tetratype.

[b] All of the mutants belonging to group I were indistinguishable from the wild type at all intervals ($P > 0.05$, using the *G*-test comparing the distribution of PD, NPD, and TT) and were different from *Δmlh1* at all of the intervals tested ($P < 0.05$).

[c] All of the mutants belonging to group II had map distances similar to *Δmlh1* at all intervals tested ($P > 0.05$, *G*-test) and were different from wild type at all intervals ($P < 0.05$, *G*-test).

[d] This value was significantly lower than *Δmlh1* ($P < 0.01$, *G*-test).

TABLE 3

Repair of mismatches in meiotic heteroduplex DNA

Relevant genotype	Locus: *his4-r* % repair[a]	*his4-r* % NMS[b]	*met13-2* % repair[a]	*met13-2* % NMS[b]	Total tetrads
		Group I: crossover proficient			
Mismatch repair proficient					
MLH1/MLH1	100 (45/45)	12.0	100 (17/17)	4.6	366
MLH1/Δmlh1	100 (31/31)	8.3	100 (15/15)	4.0	341
mlh1-P25L/mlh1-P25L	100 (24/24)	8.3	100 (8/8)[c]	2.8	289
mlh1-E31A/mlh1-E31A	93 (25/27)	12.0	87 (13/15)	6.5	232
mlh1-R97A/Δmlh1	94 (34/36)	11.0	94 (17/18)	5.2	349
Intermediate mismatch repair					
mlh1-I65N/mlh1-I65N	90 (47/52)	13.0	56 (10/18)**	4.4	407
Mismatch repair deficient					
mlh1-F96A/Δmlh1	43 (9/21)*	12.0	22 (2/9)[c]	5.3	170
mlh1-G98A/Δmlh1	67 (10/15)*	5.7	25 (3/12)*	5.8	206
mlh1-T114M/mlh1-T114M	86 (19/23)*	12.0	50 (1/2)[c]	1.0	192
		Group II: crossover deficient			
Mismatch repair deficient					
Δmlh1/Δmlh1	71 (24/34)	8.0	19 (4/21)	4.9	427
mlh1-M32R/mlh1-M32R	47 (8/17)*	5.5	19 (3/16)*	5.1	311
mlh1-N35A/mlh1-N35A	35 (6/17)**	6.8	55 (6/11)*	7.7	143
mlh1-A41F/mlh1-A41F	55 (11/20)*	9.0	83 (5/6)[c]	2.7	222
mlh1-G64R/mlh1-G64R	53 (8/15)*	6.0	13 (2/15)*	6.0	251
mlh1-G98V/Δmlh1	62 (16/26)*	10.0	15 (2/13)*	5.0	261
mlh1-G243D/mlh1-G243D	47 (9/19)*	6.5	13 (3/23)*	7.9	291
mlh1-R273E-R274E/ mlh1-R273E-R274E	62 (18/29)*	8.5	36 (6/17)*	5.0	341

*Proportions statistically different from the wild-type ($P < 0.025$, Fisher's exact test) but not from the Δ*mlh1* strain ($P > 0.025$, Fisher's exact test). **Proportions statistically different from both the wild-type and Δ*mlh1* strains ($P < 0.025$, Fisher's exact test).

[a] Percentage of repair is calculated as the no. of gene conversions/total non-Mendelian segregation. The observed values are given in parentheses.

[b] Percentage of non-Mendelian segregation is the no. of gene conversions plus the no. of postmeiotic segregations observed divided by the total tetrads.

[c] Total no. of NMS events are too low to compare statistically to the wild-type and Δ*mlh1* strains.

is deficient for crossing over suggests that DNA binding may be important for crossing over during meiosis. Group II also includes mlh1p-N35A, whose N-terminal domain does not bind ATP, suggesting that ATP binding may also be important for meiotic crossing over. In contrast, ATP hydrolysis may be less critical, since the *mlh1-E31A* mutant strain has normal crossing over yet it encodes an N-terminal domain that binds but does not efficiently hydrolyze ATP.

Meiotic mismatch repair efficiencies: The effect of each mutation on mismatch repair efficiency during meiosis was determined by assessing the frequency of postmeiotic segregation events (phenotypic sectoring of the genetic marker) that result from failure to repair heteroduplex DNA (Williamson *et al.* 1985). Repair of a 4-bp insertion at *HIS4* and a mispair at *MET13* were measured. The missense mutations yielded three general phenotypes with respect to efficiency of repair of meiotic heteroduplex (Tables 3 and 6). Nine mutations (*mlh1-M32R*, *mlh1-N35A*, *mlh1-G64R*, *mlh1-F96A*, *mlh1-G98A*, *mlh1-G98V*, *mlh1-T114M*, *mlh1-G243D*, and *mlh1-R273E-R274E*) resulted in loss of all Mlh1p-dependent meiotic heteroduplex repair (Fisher's exact test, $P < 0.025$ with respect to *MLH1* and $P > 0.025$ with respect to Δ*mlh1*). The *mlh1-A41F* strain was clearly defective for meiotic mismatch repair of the *his4-r* allele whereas the data for the *met13-2* allele were ambiguous. Strains with the *mlh1-E31A*, *mlh1-P25L*, and *mlh1-R97A* mutations displayed wild-type or near wild-type levels of repair at both loci tested ($P > 0.025$). Consistent with this, these three mutations have the lowest published mitotic mutation rates of the mutations analyzed (Pang *et al.* 1997; Shcherbakova and Kunkel 1999; Hall *et al.* 2002). In contrast, the *mlh1-I65N* strain displayed allele-

TABLE 4

Frequency of nondisjunction in *MLH1*-defective strains

Genotype	Pairs of disomes[a]	Total tetrads	Frequency (%)
	Group I: crossover proficient		
MLH1/MLH1	1	970	0.1
mlh1-P25L/mlh1-P25L	0	292[b]	0.0
mlh1-I65N/mlh1-I65N	0	183	0.0
mlh1-T114M/mlh1-T114M	0	176	0.0
mlh1-E31A/mlh1-E31A	0	232	0.0
*mlh1-R97A/*Δ*mlh1*	0	183	0.0
*mlh1-G98A/*Δ*mlh1*	Not done		
*mlh1-F96A/*Δ*mlh1*	Not done		
	Group II: crossover deficient		
Δ*mlh1/*Δ*mlh1*	5	131	3.8
mlh1-M32R/mlh1-M32R	2	165	1.2
mlh1-A41F/mlh1-A41F	1	115	0.8
mlh1-G64R/ mlh1-G64R	1	210	0.5
mlh1-G243D/mlh1-G243D	2	172	1.2
mlh1-N35A/mlh1-N35A	2	111	1.8
*mlh1-G98V/*Δ*mlh1*	Not done		
mlh1-R273E-R274E/mlh1-R273E-R274E	2	135	1.5

[a] Tetrads with two surviving spores were analyzed for the presence of disomes by CHEF analysis. A total of 10–25 two-viable-spore tetrads were analyzed for each mutant and the nondisjunction rate was determined as the no. of paired disomic chromosomes observed divided by the total no. of asci.

[b] Only two two-viable-spored asci were obtained.

specific levels of repair. The *mlh1-I65N* strain had wild-type levels of repair at *his4-r* but was significantly different from both wild type ($P < 0.025$) and Δ*mlh1* ($P < 0.025$) for repair at *met13-2*. The effect of the missense mutations on total frequency of non-Mendelian segregation varied with no obvious pattern (Table 3).

The crossover defect does not predict the degree of aneuploidy: The crossover defect of Δ*mlh1* has previously been shown to be associated with a moderate amount of nondisjunction (Hunter and Borts 1997). To determine what the contribution of nondisjunction was to meiotic inviability in the strains with missense mutations we measured disomy rates by CHEF gel analysis (Tables 4 and 6). Because the sample sizes for the missense mutation strains are individually too small to allow statistical analysis, we pooled the data from all of the mutant strains exhibiting crossover frequencies indistinguishable from those of the Δ*mlh1* strain. These strains have a disomy rate of 1.1% (10/908). This is significantly lower ($P < 0.05$, *z*-test) than that found in Δ*mlh1* (5/131, 3.8%). In contrast, the crossover-proficient strains were indistinguishable from the wild-type strain (0/1066 *vs.* 1/970).

Nondisjunction contributes to gamete death: Gamete death in Δ*mlh1* strains is due to at least two factors whose relative contributions are unknown, aneuploidy and the accumulation of haplolethal mutations (including synthetic lethal mutations) that are uncovered by meiosis (Hunter and Borts 1997). To assess the relative contributions of the mitotic mutator phenotype and nondisjunction to gamete viability, we compared the spore viability of strains with the crossover-defective missense mutations to that of Δ*msh2* and Δ*mlh1* (Tables 5 and 6). Since the Δ*msh2* and Δ*mlh1* strains have equivalent mitotic mutation rates, but Δ*msh2* strains have no crossover or segregation defects (Hunter and Borts 1997), gamete viability in the Δ*msh2* strain provides an estimate of the contribution of mitotically acquired haplolethals and meiotic repair deficiency to gamete death. Consistent with the previous report, the Δ*msh2* mutant strain had viability intermediate between wild-type and Δ*mlh1* strains. As might be predicted, the three mutant strains, *mlh1-R97A*, *-E31A*, and *-P25L*, reported to have moderate mutation rates (Pang *et al.* 1997; Shcherbakova and Kunkel 1999; Hall *et al.* 2002) and without a crossover defect had wild-type or intermediate levels of spore viability. In addition, all of the missense mutant strains with repair defects had significantly poorer viability than that of the wild type. Furthermore, the mismatch repair-defective, crossover-proficient missense mutations had the same pattern of spore viability as Δ*msh2*. However, the crossover-deficient strains fell into two classes. They were either Δ*msh2*-like (*mlh1-M32R*, *mlh1-N35A*, and *mlh1-R273E-R274E*) or intermediate between Δ*mlh1* and Δ*msh2*. None were Δ*mlh1*-like except perhaps *mlh1-G98V*, which could not be distinguished from either

TABLE 5

Spore viability patterns in *MLH1* strains (%)

Relevant genotype	Viable spores per tetrad					Spore viability (%)[a]	Total asci
	4	3	2	1	0		
Group I: crossover proficient							
MLH1/MLH1	89	9.0	1.7	0.3	0.0	97	1570
MLH1/Δmlh1	87	8.0	4.0	1.0	0.0	96	366
mlh1-N35A/MLH1	84	10	6.0	0.0	0.0	95	125
mlh1-P25L/mlh1-P25L	93	6.7	0.3	0.0	0.0	98	313
mlh1-E31A/mlh1-E31A	93	5.0	2.0	0.0	0.0	98	255
*mlh1-R97A/Δmlh1***	80	14	5.0	1.0	0.0	93	374
*Δmsh2/Δmsh2**	67	18	11	3.0	1.0	87	919
*mlh1-I65N/mlh1-I65N**	68	16	11	3.0	2.0	91	777
*mlh1-F96A/Δmlh1**	58	20	18	3.0	1.0	83	415
*mlh1-G98A/Δmlh1**	65	19	12	3.0	1.0	86	395
*mlh1-T114M/mlh1-T114M**	76	14	8.0	1.0	1.0	91	282
Group II: crossover deficient							
Δmlh1/Δmlh1	60	16	15	5.0	4.0	81	1685
*mlh1-M32R/mlh1-M32R**	66	16	14	3.0	1.0	86	302
*mlh1-N35A/mlh1-N35A**	61	16	20	2.0	1.0	84	258
*mlh1-R273E-R274E/ mlh1-R273E-R274E**	70	16	11	2.0	1.0	88	522
*mlh1-A41F/mlh1-A41F***	71	11	11	4.0	3.0	86	323
*mlh1-G64R/mlh1-G64R***	66	16	11	2.0	5.0	86	405
*mlh1-G243D/mlh1-G243D***	69	13	13	5.0	0.0	87	458
*mlh1-G98V/Δmlh1****	58	19	18	4.0	1.0	82	296

*Distribution of classes is the same as the Δ*msh2* strain ($P > 0.017$, *G*-test) and different from both the wild-type and Δ*mlh1* strains; **distribution of classes does not match any of the three control strains ($P < 0.017$, *G*-test); ***distribution of classes is not significantly different from either the Δ*mlh1* strain ($P > 0.017$, *G*-test) or the Δ*msh2* strain but is different from wild type.

[a] Calculated as (4 × the no. of four-viable-spore tetrads + 3 × the no. of three-viable-spore tetrads + 2 × the no. of two-viable-spore tetrads + the no. of one-viable-spore tetrads)/(4 × the total no. of tetrads) × 100.

Δ*msh2* ($P = 0.09$) or Δ*mlh1* ($P = 0.06$). This indicates that apparently equivalent crossover and repair defects do not translate directly into an equivalent defect in viability and suggests that Mlh1p may be playing a role in meiotic viability separable from its role in crossing over.

DISCUSSION

Meiotic mismatch repair generally reflects mitotic repair efficiency: The efficiency of repair of meiotic heteroduplex DNA by the strains with missense substitutions is for the most part consistent with the published mutation rates. However, *mlh1-I65N* displays wild-type repair at one of the alleles studied despite high mitotic mutation rates. This allele-specific effect could reflect different functional requirements for repair of a single mispair compared to a four-base insertion or could reflect the position of the marker relative to the double-strand break, which initiates meiotic recombination. Current models for the repair of mismatches in meiotic heteroduplex envisage distinctly different fates for alleles close to the double-strand break and those far away (reviewed in Borts *et al.* 2000). Thus mutations in *MLH1* might differentially affect the processing of mismatched heteroduplex in a context-specific manner, *i.e.*, if it is coupled to strand invasion *vs.* being directed by Holliday junction resolution (Alani *et al.* 1994; Gilbertson and Stahl 1996). The observation that total levels of non-Mendelian segregation vary with no apparent pattern may be an indication of the complexity of the interrelationship between the repair of meiotic heteroduplex and crossing over. Another possibility is that these differential repair defects reflect different levels of the various mutant proteins that are partially or even fully active but are limiting in different contexts. This explanation has been proposed to account for the phenotype of the temperature-sensitive *MLH1* mutants found by Argueso *et al.* (2002). In a systematic site-directed mutagenesis of *MLH1*, a mutation (*mlh1-2*) that is partially defective for both meiotic repair and meiotic crossing over and abolishes the gene conversion gradient at *ARG4* was identified (Argueso *et al.* 2003). Such a mutant might reflect an absence of crossover resolution-directed repair.

Structure function relationships revealed by the missense mutations: The results with strains bearing mutant proteins that have known biochemical defects (mlh1p-E31A, mlh1p-N35A, and mlh1p-R273E-R274E) begin to

TABLE 6

Summary of meiotic phenotypes

Genotype	Crossing over[a]	Meiotic mismatch repair[a]	Aneuploidy[a]	Gamete viability[b]
	Group I			
MLH1/MLH1	+	+	+	+
MLH1/Δmlh1	+	+	ND	+
mlh1-P25L/mlh1-P25L	+	+	+	+
mlh1-E31A/mlh1-E31A	+	+	+	+
mlh1-R97A/Δmlh1	+	+	ND	±
mlh1-I65N/mlh1-I65N	+	±	+	±
Δmsh2/Δmsh2	+	−	+	±
mlh1-F96A/Δmlh1	+	−	ND	±
mlh1-G98A/Δmlh1	+	−	ND	±
mlh1-T114M/mlh1-T114M	+	−	+	±
	Group II			
mlh1-M32R/mlh1-M32R	−	−	±	±
mlh1-N35A/mlh1-N35A	−	−	±	±
mlh1-R273E-R274E/mlh1-R273E-R274E	−	−	±	±
mlh1-A41F/mlh1-A41F	−	−	±	∓
mlh1-G64R/mlh1-G64R	−	−	±	∓
mlh1-G243D/mlh1-G243D	−	−	±	∓
mlh1-G98V/Δmlh1	−	−	ND	NS
Δmlh1/Δmlh1	−	−	−	−

[a] + indicates a wild-type phenotype; − indicates rates similar to Δ*mlh1*; ± indicates mutants that have an intermediate phenotype. ND, not determined.

[b] ± signifies values between the wild-type and Δ*msh2* strains; ∓ signifies values between the Δ*msh2* and Δ*mlh1* strains; − signifies Δ*mlh1* strain values. NS, not significantly different from either the Δ*msh2* or Δ*mlh1* strains but different from wild type.

offer some insights into the importance of ATP binding, ATP hydrolysis, and DNA binding by Mlh1p for different meiotic functions. The observations that the N-terminal domains of the mlh1p-N35A and mlh1p-R273E-R274E substituted proteins have reduced binding of ATP and DNA, respectively, and the corresponding mutants are defective for crossing over and heteroduplex repair suggests that both substrate-binding properties of Mlh1p are important for these meiotic functions. From a comparison of the meiotic phenotypes of *mlh1-E31A* and *mlh1-N35A* and the observation that mlh1p-E31A is capable of binding but not hydrolyzing ATP while mlh1p-N35A does neither, we conclude that ATP binding is sufficient for executing the crossover functions of Mlh1p. This conclusion is supported by data from a similar study where it was shown that a mutation of E31 to lysine is recombination defective (Argueso *et al.* 2003). In *E. coli*, a change in a nearby conserved glutamic acid (E32, E34 in yeast) to lysine reduces ATP binding and the interaction of MutL with MutH (Spampinato and Modrich 2000), suggesting that a lysine substitution at E31 also abolishes ATP binding. However, the relationship between ATP interactions and crossing over is complex. This is indicated by the fact that both groups I and II contain substitutions for highly conserved residues that, on the basis of the crystal structures of *E. coli* MutL (Ban and Yang 1998a; Ban *et al.* 1999) and human PMS2 (Guarne *et al.* 2001), should alter ATP interactions. Perhaps this distinction in phenotype can be used to infer how some of the amino acid substitutions influence protein function. For example, G64R and I65N substitutions both result in completely defective mismatch repair in mitotic cells and are predicted to interfere with ATP binding and/or hydrolysis. However, only G64R affects crossing over, suggesting that perhaps only the G64R substitution interferes with ATP binding whereas the I65N substitution affects only hydrolysis. These predictions can be supported only by biochemical studies.

Other structural or functional inferences can be drawn from the phenotypic data. A comparison of our observation that the *mlh1-R273E-R274E* strain is crossover deficient with the observation that when the adjacent arginines are replaced with alanines the resultant strain is crossover proficient (Argueso *et al.* 2003) leads us to predict that the alanine substitutions do not impair DNA binding. By analogy with the *E. coli* data on MutL-G238D, which indicate that the protein is insoluble (Ban *et al.* 1999), one might predict that *mlh1-G243D* would be phenotypically identical to Δ*mlh1*. This is not the case, as it falls into the class of mutants that have better viability and better disjunction than the deletion.

As discussed below we interpret the improved disjunction and viability with respect to the deletion to mean that Mlh1p has a structural role in segregation. This inferred structural role seems to be fulfilled by the mutant protein encoded by *mlh1-G243D*.

The strains bearing mutations in the GFRGEAL box also fall into both groups. Two different substitutions for the same amino acid (*e.g.*, G98A *vs.* G98V) result in proteins with differential effects on crossing over *vs.* meiotic and mitotic mismatch repair. Gly98 is in the GFRGEAL box that not only contacts the nucleotide but also is implicated in dimerization of the N-terminal domain upon ATP binding (Ban and Yang 1998a; Ban *et al.* 1999; Tran and Liskay 2000). The valine substitution alters the interaction of Mlh1p with Pms1p (Tran and Liskay 2000) while the alanine substitution does not. Thus the role Gly98 plays in crossing over can be accomplished when it is replaced by alanine but not when it is replaced by valine, suggesting that the lid interaction with the nucleotide may not be as important for meiotic recombination as it is for mitotic mismatch repair. It has been proposed previously (Ban *et al.* 1999; Tran and Liskay 2000; Hall *et al.* 2002) that ATP binding induces the conformational changes leading to changes in partner binding while the hydrolysis restores the previous conformation. In this context, we suggest that ATP binding is sufficient to ensure that the downstream effector molecules for crossing over are capable of interacting functionally. If, as suggested, the dimerization of Mlh1p with Mlh3p is similar to its dimerization with Pms1p, then the crossover defect in *mlh1-G98V* strains may be attributable to an effect on dimerization with Mlh3p. Due to the difficulty demonstrating the known interaction between Mlh1p and Mlh3p with wild-type proteins (Argueso *et al.* 2002) we have been unable to test this hypothesis.

As discussed above, the conformational change associated with ATP binding is also thought to signal the effector molecules (Ban *et al.* 1999). Among the proteins known to interact with Mlh1p and possible effectors of its meiotic functions are Mlh3p (Wang *et al.* 1999; Borts *et al.* 2000), Msh4p (Santucci-Darmanin *et al.* 2000), Exo1p (Amin *et al.* 2001; Tran *et al.* 2001), and Sgs1p (Langland *et al.* 2001; Pedrazzi *et al.* 2001; Wang and Kung 2002). Three of the severely crossover-defective mutations are known to be (A41F and G98V; Pang *et al.* 1997) or presumed to be (N35A) defective in their N-terminal interaction with Pms1p. If Mlh3p interacts with Mlh1p in a manner similar to that of Pms1p, as suggested by studies of the human proteins (Kondo *et al.* 2001) and MutL (Ban and Yang 1998a,b), these mutations can be predicted to interfere with the Mlh1p-Mlh3p interaction and this may account for their crossover defect. The role of the interactions between Exo1p and Mlh1p in crossing over is unclear. We have shown previously that Δ*exo1* has a defect similar to that of Δ*mlh1* in crossing over and segregation but has no defect in repair of mismatched heteroduplex, although total non-Mendelian segregations are reduced at some loci (Khazanehdari and Borts 2000). The single amino acid change T117M in human *MLH1* is reported to disrupt the interaction with *hEXO1* (Jager *et al.* 2001). However, strains with the corresponding T114M mutation in yeast Mlh1p do not display a defect in crossing over as might have been expected if an interaction between Mlh1p and Exo1p were functionally important for crossing over. Perhaps Mlh1p and Exo1p do not interact via this residue in yeast to exert their crossover function or their respective roles in crossing over do not require them to interact. Alternatively, they may be involved in different types of crossovers, as has been suggested (Khazanehdari and Borts 2000). It has recently been hypothesized that the role of the Mlh1p/Mlh3p heterodimer is to recruit Sgs1p/Top3p to the sites of late recombination intermediates to aid in their resolution as crossovers (Wang and Kung 2002). It will be interesting to determine if any of the crossover-defective mutants interfere with a meiotic Sgs1p/Mlh1p interaction.

A structural role for Mlh1p in segregation? Some of the missense mutations are as defective as the deletion strain for both mismatch repair and crossing over, yet have significantly better viability and less nondisjunction than the deletion strain. There are a number of possible explanations for the poor correlation between crossover defectiveness, nondisjunction, and viability. One possibility is that the intervals studied are not an accurate reflection of the crossing over in the genome as a whole. Possibly, the deletion of *MLH1* is affecting another interval to a greater extent than the missense mutations and that crossing over in this interval is more relevant to segregation. Given recent suggestions that there are at least two types of crossovers in yeast, this is not an unreasonable hypothesis (Ross-Macdonald and Roeder 1994; Zalevsky *et al.* 1999; Khazanehdari and Borts 2000; Abdullah 2002). However, one class of these crossovers, those known to be dependent on Msh4p, display a nonrandom distribution of exchanges indicative of a phenomenon termed interference (Ross-Macdonald and Roeder 1994; Novak *et al.* 2001). If Mlh1p acted in the same complex as Msh4p, then one would predict that its absence should lead to loss of interference. This is not the case as indicated by strong interference detected (NPD ratio of 0.38, $P < 0.05$, $1 < m < 2$ in the *TRP5-CYH2* interval) in the Δ*mlh1* strain and in a previous study (Argueso *et al.* 2002). These data further support separable roles for the MutS and MutL homologs during meiosis. Another possibility for the poor correlation between nondisjunction and viability is that the greater nondisjunction defect in the Δ*mlh1* strain as compared to some of the missense mutations is caused by the loss of the protein that impairs formation of a complex important for chromosome segregation but not exchange at the DNA levels. One possibility is

that it is a component of the proteinaceous structure associated with chiasmata such as a "chiasma binder" suggested by Carpenter (1994).

Implications for human fertility: Our results indicate that Mlh1p has at least three meiotic functions, heteroduplex repair, crossing over, and chromosome segregation, that are separable from each other and from mismatch repair of replication errors in mitotic cells. To date, no infertility has been linked to *MLH1* HNPCC patients, perhaps due to the absence of homozygous individuals or the rarity of loss of heterozygosity in the germline. The results presented here suggest that *MLH1*-dependent aneuploidy leading to reduced fertility would be specific to certain mutations. Hence not all HNPCC carriers would be at equal risk for fertility problems, which may be a reason why it has not been previously noted. Perhaps even polymorphisms in the general population may result in reduced fertility due to impaired crossing over. For example, three single-nucleotide polymorphisms have recently been shown to reduce the interaction between *hMLH1* and *hPMS2* (Yuan *et al.* 2002) *in vitro*. Such "polymorphisms" may confer defects in crossing over and may be a possible source of infertility.

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