Characterization of Insertion Mutations in the *Saccharomyces cerevisiae* *MSH1* and *MSH2* Genes: Evidence for Separate Mitochondrial and Nuclear Functions

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ABSTRACT

The *MSH1* and *MSH2* genes of *Saccharomyces cerevisiae* are predicted to encode proteins that are homologous to the *Escherichia coli* MutS and *Streptococcus pneumoniae* HexA proteins and their homologs. Disruption of the *MSH1* gene caused a petite phenotype which was established rapidly. A functional *MSH1* gene present on a single-copy centromere plasmid was incapable of rescuing the established *msh1* petite phenotype. Analysis of *msh1* strains demonstrated that mutagenesis and large-scale rearrangement of mitochondrial DNA had occurred. 4′,6-Diamidino-2-phenylindole (DAPI) staining of *msh1* yeast revealed an aberrant distribution of mtDNA. Haploid *msh2* mutants displayed an increase of 85-fold in the rate of spontaneous mutation to canavanine resistance. Sporulation of homozygous *msh2/msh2* diploids gave rise to a high level of lethality which was compounded during increased vegetative growth prior to sporulation. *msh2* mutations also affected gene conversion of two *HIS4* alleles. The *his4x* mutation, lying near the 5′ end of the gene, was converted with equal frequency in both wild-type and *msh2* strains. However, many of the events in the *msh2* background were post-meiotic segregation (PMS) events (46.4%) while none (<0.25%) of the aberrant segregations in wild type were PMS events. The *his4b* allele, lying 1.6 kb downstream of *his4x*, was converted at a 10-fold higher frequency in the *msh2* background than in the corresponding wild-type strain. Like the *his4x* allele, *his4b* showed a high level of PMS (30%) in the *msh2* background compared to the corresponding wild-type strain where no (<0.26%) PMS events were observed. These results indicate that *MSH1* plays a role in repair or stability of mtDNA and *MSH2* plays a role in repair of 4-bp insertion/deletion mispairs in the nucleus.

MISMATCH repair has been detected in a wide variety of organisms from procaryotes to mammalian systems. Two sets of studies suggest that the components and mechanism of mismatch repair have been highly conserved throughout evolution. First, homologs of proteins encoded by the *Escherichia coli* *mutHLS*, *uvrD* mismatch repair genes, whose products have been characterized in the greatest biochemical detail, have been identified in a broad range of organisms. Gram-negative bacteria, gram-positive bacteria, mouse and human homologs of the *E. coli* MutS protein have been identified (FUJII and SHIMADA 1989; HABER *et al.* 1988; LINTON *et al.* 1989; PRIEBE *et al.* 1988) and homologs of the *mutL* and *uvrD* genes are known in gram-negative bacteria, gram-positive bacteria and yeast (ABOUSSEKHRA *et al.* 1989; KRAMER *et al.* 1989b; PRUDHOMME *et al.* 1989). Secondly, mutations in some of the genes encoding these homologs have proven to affect mismatch repair in other organisms besides *E. coli*. Mutations of the *hexA* and *hexB* genes of *Streptococcus pneumoniae* (homologs of *E. coli* *mutS* and *mutL* genes) drastically affect mismatch repair in this organism (LACKS 1970; TIRABY and SICARD 1973; LACKS, DUNN and GREENBERG 1982; GASC, SICARD and CLAVERYS 1989). Mutations in the *PMS1* gene of yeast (the yeast *mutL* homolog) similarly affect mismatch repair (BISHOP *et al.* 1987; BISHOP, ANDERSON and KOLODNER 1989; WILLIAMSON, GAME and FOGEL 1985; KRAMER *et al.* 1989a), and several less well characterized mutations cause phenotypes that could possibly be explained by an effect on mismatch repair (WILLIAMSON, GAME and FOGEL 1985).

The identification of a *Saccharomyces cerevisiae* homolog of *mutL/hexB* that functions in mismatch repair provided the rational for searching for *mutS* homologs and suggested that such homologs may function in mismatch repair. In a companion study (REENAN and KOLODNER 1992), we reported the cloning and sequence analysis of two yeast homologs of *mutS/hexA*, *MSH1* and *MSH2*. In this report, we describe the isolation of mutations in the *MSH1* and *MSH2* genes and a preliminary genetic analysis to determine the role that *MSH1* and *MSH2* play in repair. The results suggest that *MSH1* and *MSH2* are involved in mismatch repair in the mitochondrion and nucleus, respectively.

MATERIALS AND METHODS

Enzymes and chemicals: Chemicals, enzymes and oligonucleotides are as described in a companion study (REENAN and KOLODNER 1992).

Strains and media: The *S. cerevisiae* strains used in this study are derived from SK1 and were the gift of NANCY KLECKNER (Harvard University, Cambridge, Massachusetts). Methods for the construction and manipulation of these strains have been described elsewhere (TISHKOFF, JOHNSON and KOLODNER 1991; CAO, ALANI and KLECKNER 1990). The two strain combinations NK859: *MATa ho::LYS2 lys2 ura3 leu2::hisG his4x* and NK860: *MATα ho::LYS2 lys2 ura3 leu2::hisG his4b* or NK858: *MATα ho::LYS2 lys2 ura3 leu2::hisG his4x* and NK861: *MATa ho::LYS2 lys2 ura3 leu2::hisG his4b* were crossed to construct the diploids used for all *MSH* gene disruptions. Haploid strains bearing the *MSH* gene insertion mutations in combination with a particular *HIS4* allele were generated as needed from the disruption heterozygotes and used for phenotypic characterization or constructing diploids homozygous for the insertion mutations. This was done as a precaution, assuming the disruption mutants might be mutators. The *his4b* and *his4x* alleles used in these studies are four base insertion mutations (CAO, ALANI and KLECKNER 1990). Wild-type *HIS4* alleles were generated from the above mentioned strains by selection on media lacking histidine. All strains described in this work are derived from these starting strains by transformation and are therefore isogenic. Canavanine plates lacked arginine and contained 30 μg/ml canavanine. The nonfermentable carbon source plates used here were both YPAcetate (YPAc) and YPGlycerol (YPgly) formulated as described by SHERMAN, FINK and HICKS (1986). Other yeast and *E. coli* media were as described in a companion study (REENAN and KOLODNER 1992). The *E. coli* strain RK1400 (SYMINGTON, FOGERTY and KOLODNER 1983) was used for all plasmid constructions. Strains used for transposon mutagenesis are described below.

Plasmids: Plasmids were constructed using the materials and standard procedures outlined in a companion study (REENAN and KOLODNER 1992). The plasmid pNK1206 was obtained from NANCY KLECKNER (HUISMAN and KLECKNER 1987). The Tn*10*LLK construct was made as follows. Yep13 DNA (BROACH, STRATHERN and HICKS 1979) was digested with *Bgl*II and the 2.6-kb fragment harboring the *LEU2* gene was isolated. This fragment was then inserted into the *Bam*HI site located between the *lac*Z and *kan*^{R} sequences of Tn*10*LK of pNK1206 to yield pTN*10*LLK (*L*ac *L*eu *K*an). The orientation of the *Bgl*II fragment in the *Bam*HI site has not been determined. In order to transform yeast and replace the *URA3* marker of the Tn*10*LUK insertion by recombination with TN*10*LLK containing a *LEU2* marker, pTn *10* LLK was digested with *Bcl*I and *Nru*I and the DNA used directly in LiCl transformation (ITO *et al.* 1983). *Bcl*I and *Nru*I cleave pTN*10*LLK at sites in the *lac*Z and *kan*^{R} sequences, respectively.

Transposon mutagenesis: Plasmids pI-A5 and pII-2 (REENAN and KOLODNER 1992) were transformed into NK5830/pNK629 (HUISMAN and KLECKNER 1987) selecting for ampicillin (pI-A5 and pII-2) and tetracycline (pNK629) resistance and then mutagenized with Tn*10*LUK by infection with phage λ 1224 following a method similar to HUISMAN and KLECKNER (1987). The resulting pools of mutagenized plasmid DNA were used to transform NK8017 (HUISMAN and KLECKNER 1987) and plasmid DNA was isolated from individual transformants (HOLMES and QUIGLEY 1981). An individual mutant plasmid DNA was isolated from each pool to assure independence of insertions. Insertions into the desired fragments were then identified by restriction mapping. These insertion mutations were then introduced into their homologous location in the yeast genome using the one step transplacement method (ROTHSTEIN 1991). The allele number for each mutation obtained is listed in Figure 1.

Polymerase chain reaction (PCR) techniques: Primers used in PCR were the sequencing primers as described in REENAN and KOLODNER (1992). For PCR analysis of the presence of insertion mutations, DNA was isolated from individual transformants and PCR was performed as described in REENAN and KOLODNER (1992) except that 5 pmol of each primer and 1 μg of yeast DNA were used as substrate in each 50-μl reaction. The primers used are listed in the legend to Figure 2.

Growth protocols for *MSH2/MSH2* viability experiments: *Minimal vegetative growth regimen:* Two wild-type or *msh2*::Tn*10*LUK haploids were mated and single colonies (≥3 mm) were isolated on rich medium (YPD). These diploid colonies were used to inoculate 5 ml of presporulation medium (YPAc) at low cell density and growth was allowed to proceed to saturation. The culture was then washed with sporulation medium and then incubated for 24 hr in sporulation medium.

Zero growth regimen: Haploid strains were patched onto rich medium (YPD) directly from frozen stocks and allowed to grow overnight. Haploids of opposite mating-type were suspended in liquid YPD, mixed and plated back onto a YPD plate. The mating was allowed to proceed for 4 hr on rich medium and then the mating mixture was transferred directly to sporulation medium, allowing no vegetative growth. Sporulation was allowed to proceed for 24 hr.

Determination of mutation and recombination rates: Mutation rates were determined by a fluctuation test and two or three independent experiments were performed for each strain tested (LEA and COULSEN 1949). Strains to be tested were plated for single colonies at 30° on YPD plates. Eleven single colonies (>3 mm) were excised from the plate and resuspended in sterile water. Appropriate dilutions were then plated to determine the number of viable cells and canavanine resistant cells per culture and these data were analyzed by the method of LEA and COULSEN (1949). Using this method, $r_O = M(1.24 + \ln M)$ where r_O is the median number of canavanine-resistant colony-forming units per culture among the 11 platings and M is the average number of canavanine-resistant mutations per culture. M was solved by interpolation and then used to calculate the mutation or recombination rate, $r = M/N$ where N is the final average number of viable cells per plating.

Meiotic recombination was measured by determining the frequency of His$^+$ cells present before and after sporulation of individual cultures of cells. Strains were grown to an OD_{600} of 0.5 in YPD and then washed with presporulation medium (YPAc) twice. These cells were resuspended at low density in YPAc (OD_{600} of 0.0025) and growth was continued until an OD_{600} of 1.0 was reached. The cells were then washed twice in sporulation medium and resuspended in sporulation medium. These cells were the 0 time point and were sonically disrupted and plated on plates lacking histidine and minimal complete plates to determine the frequency of recombinants. The remaining cells were allowed to sporulate for 20 hr and analyzed as described above. The frequency of His$^+$ cells before and after induction of meiosis is given.

RESULTS

Isolation of *MSH1* and *MSH2* disruptions: To ascertain the role of the *MSH1* and *MSH2* genes in

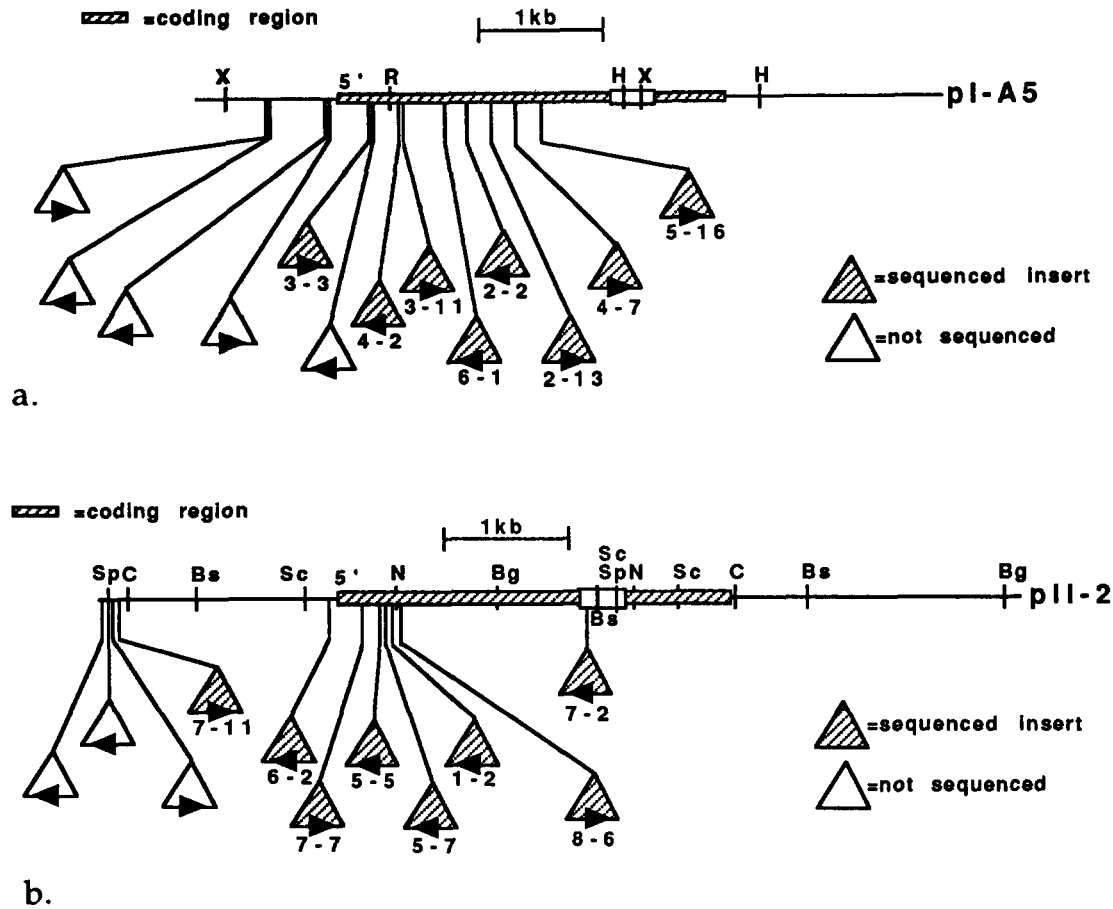

FIGURE 1.—Map positions of Tn*10*LUK insertions in the *MSH1* and *MSH2* genes. The positions of Tn*10*LUK insertions into plasmids pI-A5 (*MSH1*) and pII-2 (*MSH2*) are indicated on maps of the plasmids. The conserved 351-bp region is indicated by an open box. Insertions indicated by open triangles were mapped by restriction mapping only. Cross hatched triangles indicate insertions which were subjected to sequence analysis and the exact insertion site is known. The number below each insertion (x-y) is its isolation number and specific insertion mutations will be referred to as *msh(n)*::Tn*10*LUK(x-y). The nucleotide position [see Figure 4 in REENAN and KOLODNER (1992)] of the base of chromosomal DNA adjacent to the *lacZ* end of the sequenced Tn*10*LUK inserts is: *msh1-3-3*, 477; *msh1-4-2*, 818; *msh1-3-11*, 827; *msh1-6-1*, 1205; *msh1-2-2*, 1376; *msh1-2-13*, 1620; *msh1-4-7*, 1841; *msh1-5-16*, 2113; *msh2-7-11*, −1184; *msh2-6-2*, −75; *msh2-7-7*, 185; *msh2-5-5*, 368; *msh2-5-7*, 498; *msh2-1-2*, 792; *msh2-8-6*, 801; *msh2-7-2*, 2133.

DNA repair, genetic analysis of insertion mutations was undertaken. The positions of the insertion mutations isolated in both plasmids pI-A5 and pII-2 are shown in Figure 1, a and b. The insertion mutations were moved into the yeast chromosome by recombination using a diploid SK1 strain as host. Disruption heterozygotes were verified by a PCR assay to insure that they were located in the gene of interest rather than at a nonhomologous location. This was accomplished through the use of three different oligonucleotide primers (see Figure 2). Two of the primers were complementry to the yeast sequences flanking the insertion point and were oriented to prime DNA synthesis toward the Tn*10*LUK insertion point. The third primer was complementary to the sequences very near the ends of the Tn*10* inverted repeats and primed DNA synthesis out of the ends of the insert into flanking DNA. Thus, the wild-type locus would yield a large amplification product representing the wild-type sequences located between the two primers. A disrupted copy of the *MSH* gene would give rise to two smaller products due to amplification of the segment of DNA between the ends of the transposon and the two original primer sites located in the gene. Conditions were such that amplification through the Tn*10*LUK containing (6 kb) insert by the two outside primers did not occur. PCR-verified disruption heterozygotes were then sporulated and each spore product subjected to PCR analysis. An example of the results from the PCR analysis of a disruption heterozygote containing the *msh1*::Tn*10*LUK4-2 insertion is shown in Figure 2. Disruptions always segregated with mutant phenotypes for the six disruptions analyzed. Also, the mutant phenotypes caused by all of the disruptions in each gene were identical, regardless of the position or orientation of the insertion mutation. The procedure described above was performed on every insertion characterized genetically.

Disruptions of *MSH1*: Sporulation of diploids heterozygous for the *msh1*::Tn*10* LUK4-2 insertion

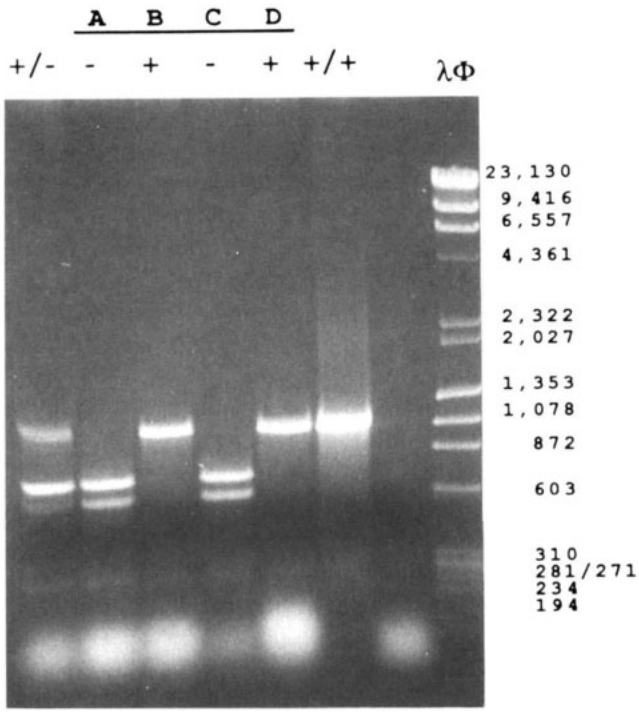

FIGURE 2.—PCR analysis of the meiotic segregation of *msh1* insertion mutations. Genomic DNA was prepared from the *msh1*::Tn *10* LUK4-2/*MSH1* disruption heterozygote (+/−), a *MSH1*/*MSH1* wild-type diploid (+/+) and the four spore clones, A to D (− indicates *msh1*::Tn*10*LUK4-2, + indicates *MSH1*), of a representative tetrad obtained by sporulation of the heterozygote. The complete genotype of these strains, in addition to the indicated *MSH1* alleles, is *MATα/MATa ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 leu2::hisG/leu2::hisG his4x/his4b*. These genomic DNAs were used in standard PCR reactions as described in MATERIALS AND METHODS. Oligonucleotide primers 2909 (5′-GCTTTCATCAACGACGTG-3′, 5′ coordinate 1471) and 3438 (5′-GGTTTTAACA-CAAATGGG-3′, 5′ coordinate 290) were used as well as a primer homologous to the inverted repeats of Tn*10* (primer 2917, 5′-GACAAGATGTGTATCCACC-3′. The 5′ end is 62 bp from the ends of IS10). Thirty reaction cycles were performed as follows: 1, *denaturation* −1 min, 94°; 2, *annealing* −2 min, 52°; 3, *polymerization* −4.5 min, 72°. The predicted sizes of the amplification products were: wild type, 1181 bp; insertion mutant, 528 bp and 653 bp.

showed 2:2 segregation for a small scalloped colony phenotype when tetrads were dissected onto rich medium (YPD) (Figure 3). This phenotype was found to be associated with a petite phenotype, as all such colonies failed to grow when they were replica plated to plates containing the nonfermentable carbon sources glycerol (YPgly) or acetate (YPAc). The petite phenotype associated with the *msh1*::Tn*10*LUK4-2 mutation was recessive. The initial disruption heterozygotes were not petite, and subsequent matings of petite haploid *msh1*::Tn*10*LUK4-2 mutants to wild-type yielded diploids that could grow on YPgly plates and could be streaked to yield single colonies on YPgly plates. The behavior of *msh1* petites in crosses with wild-type strains under nonselective conditions will be discussed below.

Kinetics of petite formation: Proceeding with the initial assumption that *MSH1* might be involved in repair of mtDNA, we were surprised by the rapidity with which the petite phenotype was established. By the time a *msh1* mutant spore, obtained by tetrad dissection of a heterozygote, had grown less than 20 generations on YPD plates to yield a small colony (2–3 mm), less than 0.01% of the cells in the colony could be plated on media containing nonfermentable carbon sources and give rise to a colony. This result indicated that mitochondrial function had been lost within several generations.

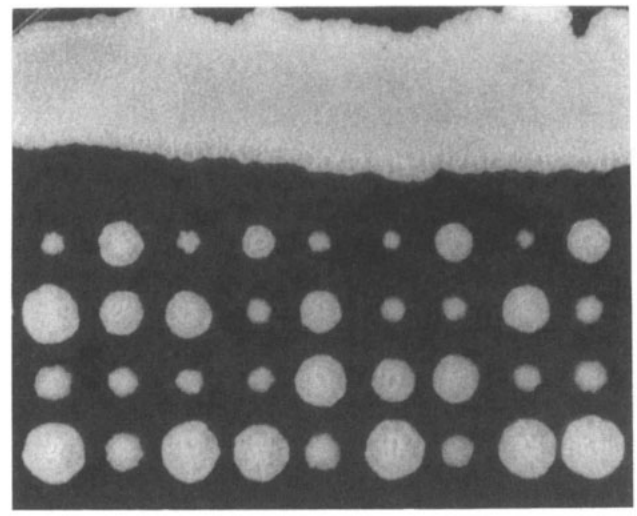

FIGURE 3.—Segregation of the *msh1* petite phenotype after sporulation of a *msh1*::Tn*10*LUK4-2/*MSH1* heterozygote. A *msh1*::Tn*10*LUK4-2/*MSH1* disruption heterozygote was sporulated, tetrad dissected onto rich media (YPD) and allowed to grow for 3.5 days at 30°. The complete genotype of the strain, in addition to the indicated *MSH1* alleles, is *MATα/MATa ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 leu2::hisG/leu2::hisG his4x/his4b*. The petite phenotype always segregated 2:2.

It was of interest to determine whether the *msh1*::Tn*10*LUK4-2 spores were initially, upon sporulation, invested with respiratory capacity. *MSH1*/*msh1*::Tn *10*LUK4-2 disruption heterozygote tetrads were dissected directly onto plates that contained nonfermentable carbon sources, either acetate or glycerol. The viability of the *msh1*::Tn*10*LUK4-2 spores was identical to wild type in their ability to begin growing. However, the colonies derived from *msh1*::Tn*10* LUK4-2 spores never reached more than 5–10% the size of a wild-type colony and the majority of *msh1*::Tn*10*LUK4-2 colonies (>70%) never grew to a size larger than a microcolony of a few hundred to a few thousand cells. These microcolonies grew as monolayers of cells which then stopped growing and became large and swollen. In a minority (<30%) of the mutant colonies, growth continued beyond the microcolony stage. In these colonies, most of the cells had stopped dividing and had a swollen cell monolayer appearance while a small part of the colony continued growing. Subsequent growth was limited to this outgrowth and frequently a second smaller outgrowth

would appear upon the first and so on. These bifurcations of outgrowth eventually ended in single multiply budded cells projecting out from the cell mass.

Rescue of the petite phenotype by the cloned *MSH1* gene: To determine whether the insertion mutations in the *MSH1* gene were actually responsible for the petite phenotype, a *msh1*::Tn*10*LLK4-2/*MSH1* heterozygote strain was transformed to Ura$^+$ with the plasmid pI-A5 that carried the *MSH1* gene. This strain was sporulated and 48 tetrads dissected. When the plasmid (Ura$^+$) segregated with the *msh1*::Tn*10*-LLK4-2 disruption (Leu$^+$), the resulting colony was always found to be wild type. When the disruption segregated away from the plasmid, Ura$^-$ Leu$^+$, a petite colony was obtained in every case. Occasionally, Ura$^+$ Leu$^+$ colonies gave rise to sectors of retarded growth (33% of the tetrads had 1 sectored colony). These sectors were shown to have lost the plasmid and to have acquired the petite phenotype. This experiment showed that the plasmid encoded copy of *MSH1* was able to complement the disruption prior to establishment of the petite phenotype and that loss of the plasmid resulted in acquisition of the petite phenotype. When a haploid petite *msh1*::Tn*10*LLK4-2 strain was transformed with the same *MSH1* plasmid, the established petite phenotype could not be rescued, indicating that the petite phenotype was irreversible once established.

Genetic behavior of *msh1* generated petite mtDNA: The zygotic progeny of crosses between *msh1* generated petites and strains containing wild-type mitochondrial DNA were examined. Fifteen *msh1* haploid petite spore colonies were generated by sporulation of the original *msh1*/*MSH1* disruption heterozygotes. Individual cells from these fifteen spore colonies were mated back to wild-type cells, diploids selected and grown on rich nonselective media. The zygotes thus generated were presumed to initially possess a heteroplasmic or mixed population of petite and wild-type mtDNAs. The resulting colonies of diploid cells were dispersed in liquid media, plated for single colonies on YPD and replica-plated onto YPgly to determine the proportions of petite and wild-type cells in the zygotic progeny of the mating. Five of 15 of the diploids gave rise to mixtures of petite and wild-type colonies suggesting that the *msh1* petite and wild-type mtDNAs had segregated from each other during growth of the single colony, giving rise to homoplasmic populations of cells containing only one mitochondrial genotype. The percentage of petites in these mixed colonies varied from 5 to 30%. A minority of the colonies (<25%) were sectored for growth on YPD, and the slower growing sectors failed to grow upon replica-plating to YPgly. The plasmid loss sectoring seen, as described above, was indistinguishable from this phenotype. This and the above data indicate that the *msh1* background generates classical mtDNA mutations resulting in loss of mitochondrial function.

Ten of 15 of the zygotes gave rise to colonies in which all of the zygotic progeny were petite. These petite diploid colonies contain a functional copy of the *MSH1* gene, and thus the *msh1* generated mtDNA itself possesses the property that it displaces the wild-type mtDNA. This behavior is characteristic of hypersuppressive petites, which result from large scale rearrangements and deletions of mtDNA that confer a selective advantage upon the mtDNA harboring them (DUJON 1981).

Mitochondrial DNA was prepared from five haploid *msh1* petite spore colonies obtained directly from sporulation of a heterozygote. The petite mtDNAs and a wild-type mtDNA control were digested with *Hin*dIII and analyzed by agarose gel electrophoresis (data not shown). Two of the *msh1* petite mtDNAs gave the same restriction pattern as wild type. In these two cases, the petite phenotype may be due to point mutations or possibly small deletions or rearrangements in the mtDNA that could not be detected in this analysis. The other three petites gave a restriction pattern in which some wild-type fragments were missing and additional novel fragments were present. All three rearranged mtDNA restriction patterns observed were similar. In one case, a petite mutant containing rearranged mtDNA and another petite mutant containing un-rearranged mtDNA were obtained from the same tetrad. The proportion of spore clones obtained containing these large scale mtDNA rearrangements is similar to the proportion of spore clones that were hypersuppressive petites. This is consistent with the observation that the hypersuppressive petites often contain large scale rearrangements of mtDNA (DUJON 1981).

Rate of spontaneous mutation to canavanine resistance in *msh1* mutants: It remained to be ascertained whether *msh1* disruption mutations had an effect on the spontaneous mutation rate of a nuclear gene. The spontaneous mutation rate to canavanine resistance was determined by fluctuation analysis of a haploid *msh1*::Tn*10*LUK3-3 strain and found to be identical to wild type (see MATERIALS AND METHODS). The mutation rate was also determined in the same isogenic petite strain carrying a plasmid copy of the *MSH1* gene with the same result (see Table 1). These results suggest that the *MSH1* gene is not involved in mutation avoidance in the nucleus.

4′,6-Diamidino-2-phenylindole (DAPI) staining of mtDNA in *msh1* mutants: Wild-type and *msh1*::Tn*10*LUK3–3 haploid strains were grown on rich medium (YPD) and subjected to DAPI staining and photographed (Figure 4). In wild type, the mtDNA appeared as small dispersed patches of stain-

TABLE 1

Effect of *msh1*::Tn*10*LUK3-3 and *msh2*::Tn*10*LUK7-7 mutations on spontaneous mutation to canavanine resistance

Strain	Rate	Fold increase
Wild-type	4.0×10^{-7}	
msh2	3.4×10^{-5}	85×
msh1	6.9×10^{-7}	1.7×
msh1, *msh2*	2.6×10^{-5}	65×
msh1 + pIA5	7.0×10^{-7}	1.8×

The mutation rates were calculated using a fluctuation test as described under MATERIALS AND METHODS. Each rate is the average of three determinations, each of which used 11 individual platings. The plates were spread with dilutions of the strain being tested and canavanine resistant colonies were scored on the second day of growth at 30°. Viable cell determinations were determined similarly by plating onto plates without arginine. The complete genotype of the strains other than the indicated *MSH1* and *MSH2* alleles is *MATa ho::LYS2 lys2 ura3 leu2::hisG his4x*.

ing throughout the cytoplasm. In *msh1* mutants the only fluorescence other than that in the nucleus appeared as larger patches, sometimes only one or two per cell and occasionally reaching ~20% the size of the nucleus. This altered mtDNA distribution may be a result of abnormal morphology and distribution of mitochondria in petite mutants rather than an actual reflection of a DNA metabolic defect.

Disruptions of *MSH2*: Disruptions of the *MSH2* gene in the plasmid pII-2 were isolated as described above. The location of the insertions are shown in Figure 1. When necessary, the *msh2*::Tn*10*LUK disruptions were converted to Tn*10*LLK disruptions as described under MATERIALS AND METHODS. Sporulation and subsequent dissection of diploids heterozygous for the *msh2* insertion mutations always yielded four equal sized spore clones indicating that *msh2* mutations did not have an obvious effect on cell growth.

Rate of spontaneous mutation to canavanine resistance in *msh2* mutants: The spontaneous mutation rate to canavanine resistance was determined by fluctuation analysis of the disruption mutant *msh2*::Tn*10*LUK7-7, was elevated 70–100-fold over that of wild type (Table 1). This increased level of spontaneous mutation was easily visualized by patching out spore clones and replica plating to canavanine plates. Using this test to analyze the segregation of both the mutator phenotype and *msh2* mutations indicated that the mutator phenotype always segregated with the *msh2* disruption mutation.

Spore viability of *msh2* homozygous diploids: The viability of spores from *msh2*::Tn*10*LUK7-7 homozygous diploids was significantly lower than from either a *msh2*::Tn*10*LUK7-7 heterozygote or a wild-type diploid. Since diploids are not affected by the occurrence of recessive lethal mutations, it seemed likely that the reduced viability of spores observed was due to accumulated recessive mutations that were uncovered upon sporulation. Assuming that the proportion of mutations accumulated increases with the number of rounds of replication, lethality should increase with the number of generations of vegetative growth allowed between zygote formation and sporulation. To test this, two mating, growth and sporulation regimens were analyzed (see MATERIALS AND METHODS); a minimal vegetative growth regimen and a zero-vegetative growth regimen.

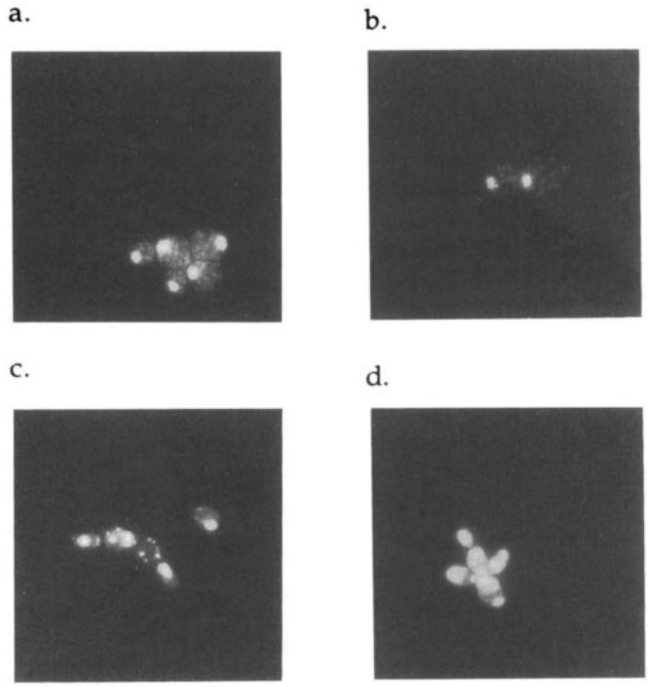

FIGURE 4.—DAPI staining of wild-type and *msh1*::Tn*10*LUK3-3 petite mutants. Haploid cells of the genotype *MSH1* (panels a and b) or *msh1*::Tn*10*LUK3-3 (panels c and d) were grown overnight on rich media (YPD) and washed several times with distilled water. The complete genotype of the strain other than the indicated *MSH1* allele is *MATa ho::LYS2 lys2 ura3 leu2::hisG his4x*. Cells were then fixed in 50% ethanol and sonically disrupted to break up cell clumps. DAPI stain (Sigma, St. Louis, Missouri) was added to a final concentration of 0.1–1.0 μg/ml and the cells were photographed after 5 min. Wild-type cells in panels a and b show a granular distribution of mtDNA molecules with each granule estimated to contain about 1–2 molecules of mtDNA. The mutants in panels c and d show a much less granular distribution of mtDNA staining. The mtDNA staining is much more intense and in fewer regions ranging from 2–5 in panel c to a common intense doublet of staining seen opposite the nucleus in panel d.

The minimal growth regimen resulted in 92% 4-spore-viable tetrads from a wild-type diploid, with 95% of the spore colonies giving rise to a normal-appearing colony (see Table 2). When this experiment was performed using a *msh2*::Tn*10*LUK7-7 homozygous diploid, only 37% 4-spore-viable tetrads resulted and only 23% of surviving spores gave rise to normal, large colonies. Also, a large proportion of 2-spore-viable colonies were observed (33%) and a significant number of the 4-spore-viable tetrads segregated a growth defect (small colony size) 2:2. When the zero-vegetative growth regimen was tested, wild-type dip-

TABLE 2

Effect of *msh2*::Tn*10*LUK7-7 mutations on spore viability

Presporulation growth regimen	Relevant genotype	Viable:dead (%) 4:0	3:1	2:2	1:3	0:4	n
Minimal	*MSH2/MSH2*	92	2	3	2	1	192
	msh2/msh2	37	10	33	14	6	192
Zero	*MSH2/MSH2*	88	4	6	1	1	232
	msh2/msh2	63	14	17	4	2	240
Presporulation growth regimen	Relevant genotype	Colony size (%) L	M	S	VS	D	
Minimal	*MSH2/MSH2*	95	0.5	0.5	0	6	
	msh2/msh2	23	26	10	4	37	
Zero	*MSH2/MSH2*	93	0.5	0.5	0.5	3.5	
	msh2/msh2	70	9	3	2	16	

Two different mating and growth regimens were used to study the viability of spores obtained from wild-type and *msh2*::Tn*10*LUK7-7/*msh2*::Tn*10*LUK7-7 homozygous diploids as described under MATERIALS AND METHODS. Tetrads were dissected onto YPD plates and scored for viable vs. dead spore ratios and colony size after incubation of the tetrad dissection plates for 48 h at 30°. Large colonies (L), >4 mm; medium colonies (M), colonies between 1.5 and 4 mm; small colonies (S), colonies between 0.5 and 1.5 mm; very small colonies (VS), just visible to the naked eye; and dead (D), spores which failed to germinate or were undetectable to the naked eye. The total numbers of tetrads dissected is listed (n). The complete genotype of the strains used, in addition to the indicated *MSH2* alleles is *MATa/MATα ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 leu2::hisG/leu2::hisG his4x/his4b*.

loids gave rise to 88% 4-spore-viable tetrads with 93% of the surviving spores exhibiting a normal, large colony phenotype. The *msh2*::Tn*10* LUK7-7 homozygous diploids gave rise to 63% 4-spore-viable tetrads, a significant increase over the vegetative growth regimen, and of the surviving spores, 70% had a normal, large colony phenotype. These results suggest that the inviability phenotype of spores from a *msh2*::Tn*10*LUK7-7/*msh2*::Tn*10*LUK7-7 strain is mutagenic in origin and is exacerbated by continued vegetative growth as a diploid. The failure of the zero-vegetative growth regimen to eliminate all of the spore inviability suggests that some meiosis-specific process may also be contributing to the increase in spore inviability seen in *msh2* mutants. Similar results have been seen with *pms1* mutants (KRAMER *et al.* 1989a).

Meiotic recombination in *msh2* homozygous diploids: To measure the rate of recombination in homozygous *msh2* diploids, wild-type and *msh2/msh2* diploids were constructed which contained the *his4x* and *his4b* heteroalleles. These heteroalleles are restriction site ablations of the *Xho*I and *Bgl*II sites, respectively, of the *HIS4* gene, causing 4-bp insertions that are separated by 1.6 kb. The meiotic induction of recombination at *HIS4* was unaffected by the presence of the *msh2* disruption mutations. Both wild-type and *msh2/msh2* diploids exhibited the same level of induction of His^+ recombinants (50–60-fold) and the same final frequency of His^+ recombinants ($1.1–1.2 \times 10^{-2}$). Meiotic recombination in the interval between *HIS4* and the *MAT* locus was also unaffected or only slightly increased (data not shown).

TABLE 3

The effect of *msh2*::Tn*10*LUK7-7 mutations on gene conversion

Relevant genotype	4:4	6:2	2:6	5:3	3:5	ab4:4	*n*	BCF	%PMS
MSH2/MSH2 his4x/HIS4	373	14	17				404	7.7%	
msh2/msh2 his4x/HIS4	307	6	9	6	6	1	335	8.4%	46.4
MSH2/MSH2 his4b/HIS4	385	2	1				338	0.8%	
msh2/msh2 his4b/HIS4	287	8	6	4	2		307	6.5%	30.0

The indicated strains were sporulated using the zero growth protocol as described under MATERIALS AND METHODS and the resulting tetrads were dissected onto YPD plates. After growth for 48–72 hr at 30°, the His phenotype of each spore clone was determined by replica plating onto medium not containing histidine. *n* is the total number of tetrads dissected. BCF is the basic conversion frequency and equals the total number of aberrant segregations (6:2 + 2:6 + 5:3 + 3:5 + ab4:4) divided by the total number of tetrads analyzed (*n*). %PMS, the percentage of aberrant segregations which are due to post-meiotic segregation, is equal to the number of PMS events (5:3 + 3:5 + ab4:4) divided by the total number of aberrant segregations. The complete genotype of the strains used, other than the indicated *MSH2* alleles and *HIS4* alleles (*HIS4*, *his4x*, *his4b*) is *MATa/MATα ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 leu2::hisG/leu2::hisG*.

Gene conversion in *msh2* homozygous diploids: Gene conversion events in wild-type yeast lead almost exclusively to 6:2 and 2:6 events. The rarer 5:3, 3:5 and ab4:4 type events are indicative of the presence of heteroduplex DNA intermediates in the spores which subsequently undergo DNA replication prior to the first cell division after the spore germinates (PETES, MALONE and SYMINGTON 1991). Wild-type and *msh2* homozygous diploids heterozygous for either the *his4x* or *his4b* allele were constructed, and the zero growth mating and sporulation regimen was used to generate tetrads that had high spore viability in order to detect aberrant segregations at the *HIS4* locus (see Table 3). The mating type of the spore colonies was also determined to follow aberrant segregations at *MAT* and to eliminate any possible false tetrads from the analysis. The *his4x* allele showed a high frequency of aberrant segregations, or a high basic conversion frequency (BCF; the frequency of all aberrant segregations), in both the wild-type and *msh2* backgrounds. However, the frequency of 3:5, 5:3 and ab4:4 events, indicative of post-meiotic segregation (PMS) of an unrepaired recombination intermediate, rose from <0.25% in the wild-type background to 46% in the *msh2* background. One ab4:4 event in 642 tetrads was observed in the *msh2* strain. This latter type of event is extremely rare in yeast (PETES, MALONE and SYMINGTON 1991). The increase in PMS

was accompanied by a concomitant decrease in 6:2 and 2:6 segregations. These data suggest that the *MSH2* gene product is involved in the repair of the *his4x* 4-bp insertion/deletion mispair. The *his4b* mutation, located 1.6 kb downstream of *his4x*, showed low levels of conversion in the wild-type background and <0.26% PMS events. However, in the *msh2* background the BCF was almost 10-fold higher and 30% of the aberrant events were PMS events. Thus, *MSH2* is also likely involved in the repair of heteroduplex DNA containing a *his4b* insertion/deletion mispair. The significance of the overall increase of events at *his4b* in *msh2* mutants is discussed further below.

DISCUSSION

In a companion study we described the cloning and sequence analysis of the *MSH1* and *MSH2* genes of yeast (REENAN and KOLODNER 1992). This analysis suggested that *MSH1* functioned in the mitochondria and that *MSH2* functioned in the nucleus. In this study, we present the analysis of mutations introduced into these two genes. *msh1* disruptions rapidly bestowed a mitochondrial petite phenotype upon cells harboring the mutation. The behavior of the petite mtDNA in crosses, preliminary characterization of the petite mtDNA and characterization of the mtDNA distribution in the petites suggest that an ongoing process of mtDNA mutagenesis leading to large scale rearrangements and deletions occurs in *msh1* mutants. We suggest that the *MSH1* gene is either involved in repair of damage that accumulates in mtDNA or in the maintenance of mtDNA. Mutations in the *MSH2* gene lead to a nuclear mutator phenotype as evidenced by an 85-fold increase in the rate of accumulation of canavanine resistant mutants. Furthermore, homozygous *msh2* diploids had a high level of lethality upon sporulation which was presumably due to the accumulation of recessive lethal mutations. The effect of *msh2* mutations on meiotic gene conversion events at the site of 4-bp insertion mutations in genetic crosses suggests that the *MSH2* gene product is involved in the repair of mispaired bases present in recombination intermediates. All of the phenotypes caused by *msh2* mutations are similar to the phenotypes caused by mutations in the *PMS1* gene of yeast, the yeast *mutL/hexB* homolog, which is known to be involved in mismatch repair (BISHOP *et al.* 1987; BISHOP, ANDERSON and KOLODNER 1989; KRAMER *et al.* 1989a,b).

***MSH1* functions in the repair or maintenance of mitochondrial DNA:** The data presented in this report suggest that the *MSH1* gene product functions in the repair or structural maintenance of mtDNA because mtDNA maintained in the *msh1* background rapidly undergoes genetic changes that render it unable to support mitochondrial function. The mating and zygotic behavior of the *msh1* mutants, when mated to wild-type strains, suggests that two types of alterations of mtDNA can occur in *msh1* mutants. Normally when one mates a cytoplasmic petite with a wild-type strain, the mtDNA population exists transiently as a heteroplasm containing the two types of genomes. For most petites, the mitochondrial genomes will randomly homogenize, or become homoplasmic, over the next several generations to give rise to a mixture of cells containing both wild-type and petite cells. However, in the case of hypersuppressive petites the mutant mtDNA can outcompete wild-type DNA synthesis and displace the wild-type genomes (DUJON 1981). These hypersuppressive petites are always associated with large scale DNA rearrangements and/or duplications of the mitochondrial genome. The results of crossing *msh1*-generated petites to a wild-type *MSH1* strain showed that hypersuppressive petite diploids were generated in a majority of crosses (67%), suggesting that large scale mtDNA rearrangements can be caused by passage through a *msh1* background. On the other hand, some *msh1/MSH1* zygotic clones (33%) gave rise to a mixture of petite and wild-type phenotypes (ranging from 5 to 30% petite). These data suggest that in these cases the petite mtDNA, which has been through a *msh1* background, can behave as if it contains mutations which destroy mitochondrial function while leaving mtDNA segregation normal. In support of this, preliminary analysis of mtDNA obtained from different randomly selected petites demonstrated the formation of petites containing mtDNA indistinguishable from wild-type mtDNA (2/5) and petites containing mtDNA with rearrangements and deletions (3/5) in proportion to the number of hypersuppressive (67%) and non-hypersuppressive (33%) petites observed.

It is unclear what type of process gives rise to the mutagenic events observed. However, the lack of some type of post-replication repair in *msh1* mutants is one possibility given the homology between *MSH1* and *mutS/hexA*. Mismatch repair has not been demonstrated in mitochondria. The mitochondrial DNA polymerase lacks a 3′ to 5′ proofreading exonuclease and could therefore have a high *in vivo* misincorporation rate (DUJON 1981). This suggests that a mismatch repair system may be required in the mitochondria to increase the fidelity of DNA replication. The phenotypes of *msh1*::Tn*10*LUK mutants suggest a repair defect, particularly since plasmids carrying the wild type *MSH1* gene are unable to rescue the petite phenotype once it is established. The rapidity of onset of the petite phenotype suggests that the mutation rate in the absence of *MSH1* function must be very high in the mitochondria.

There are two nuclear genes in which mutations cause changes in mitochondrial function that are rem-

iniscent of the phenotypes caused by *msh1* mutations. The *PIF1* gene encodes a mitochondrial helicase and is homologous to the *E. coli uvrD* gene, which is known to be required for mismatch repair in *E. coli* (FOURY and VAN DYCK 1985; FOURY and LAHAYE 1987; LAHAYE *et al.* 1991; MODRICH 1987). *pif1* strains are not themselves petite but give rise to an increased frequency of petites and are UV sensitive. The fact that the *pif1* mutants are not as dysfunctional as *msh1* mutants suggests that *PIF1* may not be completely deficient for repair. At present, the evidence suggests that *PIF1* plays a role in UV damage repair or recombination rather than a major role in mismatch repair. The nuclear *uvsp72* mutation causes a petite phenotype that is accompanied with an abnormal cellular mtDNA distribution similar to that observed in *msh1* strains (DUJON 1981). The *uvsp72* mutation causes enhanced susceptibility to petite induction by UV and other agents suggesting a repair defect (DUJON 1981). However, this defect of *uvsp72* mutations is different from that caused by *msh1* mutations. *uvsp72* mutants also appear to be defective in mitochondrial biogenesis. At present, it is unclear if these three genes are required for the same or different mitochondrial processes.

***MSH2* is part of a nuclear mismatch repair system:** Our analysis of *msh2* mutations indicates that *MSH2* functions in the nucleus. Disruptions of the *MSH2* gene resulted in a mutator phenotype similar to that of *mutS* mutants (COX 1976; GLICKMAN, VAN DEN ELSEN and RADMAN 1978; GLICKMAN and RADMAN 1980; MODRICH 1987). *msh2*::Tn*10*LUK mutations had a significant effect on meiotic gene conversion as measured with two different 4-bp insertion mutations at the *HIS4* locus. Analysis of aberrant segregation events at both *his4x* and *his4b* showed that *msh2* mutations caused a decrease in the ratio of gene conversion events (6:2 and 2:6) to postmeiotic segregation events (5:3, 3:5 and ab4:4). Recently, we have demonstrated that *msh2* mutations have a similar affect on gene conversion when mutations caused by single base changes are analyzed (E. ALANI, N.-W. CHI and R. KOLODNER, unpublished). These effects on both spontaneous mutation rate and gene conversion are similar to results observed with mutations in *PMS1*, a *mutL/hexB* homolog of yeast (KRAMER *et al.* 1989b; WILLIAMSON, GAME and FOGEL 1985). We believe these effects are best explained by a defect in a mismatch repair pathway that is similar to the bacterial mismatch repair pathway dependent on the *mutH,L,S* and *uvrD* genes (MODRICH 1987).

msh2 mutations caused two distinct changes in gene conversion. At *his4x* there was a decrease in 6:2 and 2:6 type of conversion events along with a concomitant increase in PMS events. This can most easily be explained by a decrease in the efficiency in mismatch repair after formation of a heteroduplex recombination intermediate. At *his4b* there was an increase in both gene conversion and PMS events. This is consistent with *msh2* mutations causing two effects; an increase in recombination events that lead to gene conversion at *his4b* and a decrease in the efficiency in mismatch repair. A similar increase in the basic conversion frequency of some alleles has been observed in *PMS1* mutants (WILLIAMSON, GAME and FOGEL 1985).

Recombination at many loci, including *HIS4*, is associated with a gradient of gene conversion (FOGEL and HURST 1967; FOGEL, MORTIMER and LUSNAK 1981; DETLOFF, WHITE and PETES 1992; SCHULTES and SZOSTAK 1990). The high conversion end of the gradient is thought to be close to a recombination initiation site, and the gradient is thought to be due to a decreasing gradient of heteroduplex DNA tracts extending from this initiation site (SCHULTES and SZOSTAK 1990; FOGEL, MORTIMER and LUSNAK 1981; SUN *et al.* 1989). Such a gradient is implied in the *HIS4* gene with conversion of the *his4x* mutation near the 5′ end of the gene defining the high end of the conversion gradient (8%) and the conversion of the *his4b* mutation located 1.6 kb downstream defining the low end of the conversion gradient (0.8%). [Also see DETLOFF, WHITE and PETES (1992) for a discussion of polarity gradients at *HIS4*.]

One interpretation of our results is that mismatch repair is closely coupled to recombination and that the defect in mismatch repair increases the persistence or length of heteroduplex DNA farther away from the initiation site. According to current models of recombination, alleles like *his4x* near the initiation site have a high probability of being included in heteroduplex DNA (SZOSTAK *et al.* 1983; RADDING 1982; LICHTEN *et al.* 1990; SUN, TRECO and SZOSTAK 1991; PETES, MALONE and SYMINGTON 1991). Assuming that mismatch repair does not influence initiation of recombination, then the effect of a repair defect on alleles near the initiation site should reveal itself only as a channeling of gene conversion events (repair) to PMS (failure to repair) events as observed for *his4x*. In the case of alleles at the low end of the conversion gradient, both the total number of conversion events and the proportion exhibiting PMS should increase as observed for *his4b* in *msh2* mutants. This idea is similar to the proposal that mismatch repair interacts with the recombination process to reject potential recombination intermediates and which has been termed the anti-recombinogenic role of mismatch repair (RAYSSIGUIER, THALER and RADMAN 1989; PETIT *et al.* 1991).

There is a second possible explanation for the effects of *msh2* mutations on gene conversion at *HIS4*. It is possible that *MSH2* functions in a restoration

(HASTINGS 1984) type of mismatch repair pathway and that a second mismatch repair pathway exists but is masked by the restoration pathway. The restoration pathway would be responsible for post-replication mutation avoidance and would also convert mispairs formed during recombination back to the wild type allele. The second mismatch repair pathway would act on mispairs that escape the restoration pathway and would be responsible for the residual 6:2 and 2:6 segregations and some of the mutation events observed in *msh2* mutants. In this case, an explanation for polarity gradients is that the restoration pathway would have to initiate excision at the end of the heteroduplex tract that is farthest from the recombination initiation site which would preferentially reduce apparent gene conversion at that end of the gene. Such a role for the ends of nascent DNA strands in mismatch repair has been postulated in the *S. pneumoniae* hex system (MANNARELLI *et al.* 1985; CLAVERYS and LACKS 1986).

Our proposals on the interaction between mismatch repair and gene conversion are based on a small data set and additional experiments on gene conversion at other loci will be required to firmly establish this point and determine if it is a general feature of gene conversion. However, recent studies on gene conversion at *HIS4* using mutations that act as if they lead to the formation of mispairs that escape mismatch repair have shown that the absence of mismatch repair leads to the reduction of polarity gradients (DETLOFF, WHITE and PETES 1992). These observations are similar to those presented here and support the idea of a possible interaction between the formation of heteroduplex DNA and a mismatch repair system.

The strong mutator phenotype caused by *msh2* mutations suggests that *MSH2*-dependent mismatch repair is highly efficient. This seems in contrast to the fact that a significant number of residual 6:2 and 2:6 segregations occur in *msh2* mutants. One possible explanation is that not all gene conversion events involve a heteroduplex intermediate and occur by another mechanism such as gap repair (SZOSTAK *et al.* 1983). However, recent analysis of the formation and repair of heteroduplex intermediates at *ARG4* can be interpreted as suggesting that a large proportion of conversion events involve the formation and subsequent repair or failure to repair of a heteroduplex intermediate (LICHTEN *et al.* 1990). An alternative explanation is that some of the 4-bp insertion/deletion mismatches that lead to 6:2 and 2:6 segregations are repaired by a second mismatch repair system. With regards to this latter possibility, L. NEW, K. LIU and G. F. CROUSE (personal communication) have recently identified a third *MSH* gene, called *MSH3*, in yeast. We are now determining if *MSH3* functions in a mismatch repair pathway that is responsible for the residual 6:2 and 2:6 segregations that occur in *msh2* mutants.

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