Redundancy of *Saccharomyces cerevisiae* MSH3 and MSH6 in MSH2-dependent mismatch repair

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*Saccharomyces cerevisiae* encodes six genes, *MSH1–6*, which encode proteins related to the bacterial MutS protein. In this study the role of *MSH2, MSH3*, and *MSH6* in mismatch repair has been examined by measuring the rate of accumulating mutations and mutation spectrum in strains containing different combinations of *msh2, msh3*, and *msh6* mutations and by studying the physical interaction between the *MSH2* protein and the *MSH3* and *MSH6* proteins. The results indicate that *S. cerevisiae* has two pathways of *MSH2*-dependent mismatch repair: one that recognizes single-base mispairs and requires *MSH2* and *MSH6*, and a second that recognizes insertion/deletion mispairs and requires a combination of either *MSH2* and *MSH6* or *MSH2* and *MSH3*. The redundancy of *MSH3* and *MSH6* explains the greater prevalence of *bnsh2* mutations in HNPCC families and suggests how the role of *bnsh3* and *hmsh6* mutations in cancer susceptibility could be analyzed.

[Key Words: Cancer; mutagenesis; mismatch repair; *mutS*, *MSH2*, *MSH3*, *MSH6*, *Saccharomyces cerevisiae*]

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DNA mismatch repair plays a number of roles in the cell including the repair of mispaired bases produced as a result of DNA replication errors, chemical damage to DNA and DNA precursors, processing of recombination intermediates, and suppression or regulation of recombination between divergent DNA sequences (for review, see Modrich 1991, 1994; Kolodner 1995). DNA mismatch repair is best understood in bacterial systems; however a series of genetic and biochemical studies has shown that eukaryotes contain a mismatch repair system that is similar to the bacterial MutHLS system indicating evolutionary conservation of at least some of the components of mismatch repair (Bishop et al. 1987, 1989; W. Kramer et al. 1989; Reenan and Kolodner 1992a, 1992b; Fishel et al. 1993; Leach et al. 1993; Bronner et al. 1994; Nicolaides et al. 1994; Papadopoulos et al. 1994; Li and Modrich 1995). The recent observations that inherited mutations in mismatch repair genes cause a common human cancer susceptibility syndrome (Fishel et al. 1993; Leach et al. 1993; Bronner et al. 1994; Kolodner et al. 1994, 1995; Liu et al. 1994; Nicolaides et al. 1994; Papadopoulos et al. 1994), that acquired mismatch repair defective mutations occur in sporadic colon tumors (Borresen et al. 1995), and that many human tumor cell lines are mismatch repair defective (Parsons et al. 1993; Umar et al. 1994; Boyer et al. 1995; Liu et al. 1995) have underscored the importance of understanding mismatch repair in detail.

The yeast *Saccharomyces cerevisiae* provides an ideal system for use in understanding mismatch repair because of the availability of both genetic and biochemical methods for analyzing mismatch repair and the complete *S. cerevisiae* genome sequence, soon to be available. Analysis of *S. cerevisiae* has led to the understanding of at least three components of a bacterial MutHLS-like mismatch repair system. *MSH2* is highly related to the bacterial MutS family of proteins, and as predicted by this homology, *MSH2* protein can bind to mispaired bases, albeit with a higher affinity for insertion/deletion mispair than for single-base mispairs (Reenan and Kolodner 1992a, 1992b; Alani et al. 1995). PMS1 and MLH1 are each homologs of MutL, and these two proteins form a complex that can bind to *MSH2* when *MSH2* is bound to a mispaired base, similar to the interaction between *Escherichia coli* MutL and MutS (Grilley et al. 1989; Kramer et al. 1989; Prolla et al. 1994a, b). Human cells contain homologs of the *S. cerevisiae* MSH2 (*hMSH2*), PMS1 (*hPMS2*), and MLH1 (*hMLH1*) proteins, and these proteins appear to play roles that are similar to those of their *S. cerevisiae* counterparts (Fishel et al. 1993; Leach et al. 1993; Bronner et al. 1994; Fishel et al. 1994; Nicolaides et al. 1994; Papadopoulos et al. 1994; Li and Modrich 1995). There is also evidence that the human mispair recognition complex contains a second subunit in addi-
tion to MSH2, GTBP/p160, which is also a MutS homolog (Drummond et al. 1995, Palombo et al. 1995). S. cerevisiae also contains a number of other MSH genes whose function is generally less understood. MSH1 encodes a mispair binding protein that is imported into mitochondria and appears to function in mitochondrial mismatch repair (Reenan and Kolodner 1992a, b; Chi and Kolodner 1994). Mutations in MSH3 confer a weak nuclear mutator phenotype in some mutator assays (New et al. 1993; Alani et al. 1994; Strand et al. 1995). However, the magnitude of these effects is substantially less than those caused by mutations in MSH2, PMS1, or MLH1, suggesting that MSH3 may play a relatively minor role in mismatch repair (Williamson et al. 1985; Reenan and Kolodner 1992a, b; Chi and Kolodner, unpubl.) were provided to J. Jiricny, who found that they exactly matched regions of the human GTBP amino acid sequence. When larger amounts of the human GTBP amino acid sequence became available (Palombo et al. 1995), sequence alignments [Fig. 1B] demonstrated that S. cerevisiae MSH6 and human GTBP were related more closely to each other than to any other MutS homolog (62.6% amino acid identity).

**Results**

**Identification of MSH**

To identify additional MSH genes, the S. cerevisiae Genome Database was searched for protein sequences showing homology to the most conserved region of MSH2 (Reenan and Kolodner 1992b; Fishel et al. 1993). One new gene encoding such a protein was identified and called MSH6. This gene was predicted to encode a 139,992 molecular weight protein, showed homology along its entire length with E. coli MutS and S. cerevisiae MSH2 [Fig. 1A], and had an amino acid identity of 18.5% and 18.1% with these two proteins, respectively.

After the studies described here had been partially completed, we became aware of the identification of the human GTBP gene by J. Jiricny (Instituto Di Richerche Di Biologia Molecolare P. Angeletti, Rome, Italy) and collaborators. Regions of protein sequence identity between S. cerevisiae and mouse MSH6 (G. Crouse and R. Kolodner, unpubl.) were provided to J. Jiricny, who found that they exactly matched regions of the human GTBP amino acid sequence. When larger amounts of the human GTBP amino acid sequence became available (Palombo et al. 1995), sequence alignments [Fig. 1B] demonstrated that S. cerevisiae MSH6 and human GTBP were related more closely to each other than to any other MutS homolog (26.6% amino acid identity).

**MSH6 is involved in DNA repair**

To determine whether MSH6 is involved in DNA repair, MSH6 was disrupted in a diploid strain, which was then sporulated and analyzed by tetrad analysis. In all cases [27 spore clones analyzed] msh6 mutations cosegregated with a mutator phenotype as assessed in patch tests that

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**Figure 1.** Sequence analysis of the S. cerevisiae MSH6 gene product. [A] Alignment of the amino acid sequences of E. coli MutS and S. cerevisiae MSH2 and MSH6. The amino acid identities with E. coli MutS are indicated by shaded boxes. [B] Phylogenetic tree of MutS-related proteins. The first 21 amino acids of the S. cerevisiae MSH1 sequence, encoding the mitochondrial targeting sequence, were not included in the analysis. All sequences were retrieved from GenBank except for the mouse MSH6 sequence (G. Crouse and R. Kolodner, unpubl.).
detected the production of canavanine-resistant mutants [Can\(^r\)]. To further analyze this mutator phenotype, a series of isogenic strains was constructed containing the hom3-10 and lys2-Bgl alleles [+1 and +4 base frameshift mutations, respectively] and either msh6, msh2 or msh6, and msh2 mutations. Patch tests [Fig. 2; data not shown] demonstrated that in all cases analyzed, the msh6 mutation caused an increase in the rate of accumulation of Can\(^r\) mutations, but only a very small increase in reversion of hom3-10 or lys2-Bgl (7 isolates analyzed) compared to that seen in a msh2 mutant. This was in contrast to the msh2 (13 isolates analyzed) and msh2, msh6 (5 isolates analyzed) strains that had a similar increase in both the rate of accumulation of Can\(^r\) mutations and reversion of hom3-10 and lys2-Bgl.

Fluctuation analysis [described in Materials and methods] demonstrated that the rate of accumulation of Can\(^r\) mutations was 18-fold higher in a msh6 mutant compared with wild type; however, there was a very small increase in the rate of reversion of hom3-10 and lys2-Bgl in a msh6 mutant compared with wild-type strains (Table 1). This was in contrast to msh2 mutations, which caused increases in the rate of accumulation of Can\(^r\) mutations and reversion of hom3-10 and lys2-Bgl by 40-, 662- and 55-fold, respectively, compared with the wild-type control strain. Note that whereas the rate of reversion of hom3-10 and lys2-Bgl observed in the msh6 mutant was significantly higher than the wild-type rate, it was at best 1% of the rate of reversion of hom3-10 and lys2-Bgl observed in msh2 mutants. The rate of accumulation of mutations in the msh2, msh6 strain was similar to that of the msh2 strain, consistent with MSH2 being epistatic to MSH6.

**Analysis of the spectrum of mutations that accumulate in msh2 and msh6 mutants**

To gain insight into the repair events in which MSH2 and MSH6 participate, the sequence of the Can\(^r\) mutations arising in msh2 and msh6 mutants and the sequence of the Hom\(^+\) and lys2-Bgl mutations arising in msh2 mutants were determined. The sequences of only a small number of the Hom\(^+\) and lys2-Bgl mutations arising in msh6 mutants were determined because hom3-10 and lys2-Bgl only revert at a low rate above background, making it difficult to isolate such mutations or perform a definitive analysis in this case.

To determine the sequence of the Hom\(^+\) revertants, it was first necessary to determine the sequence of the hom3-10 allele. The hom3-10 allele present in several different *S. cerevisiae* strains [RKY2575 constructed in this laboratory by gene replacement using pK8; MW3069-15A and MW3070-6C constructed in the laboratory of S. Fogel and supplied by A. Jeyaprakash, University of Florida, Gainesville] and the hom3-10 allele that had been rescued onto a plasmid were sequenced. Analysis of these sequence data demonstrated that hom3-10 was a +1 frameshift mutation caused by the insertion of a single T in a run of 6 T's constituting nucleotides 646–651 of the wild-type gene [Fig. 3].

The most probable mechanism by which frameshift mutations such as hom3-10 and lys2-Bgl revert is either through direct reversion to the wild-type allele or by mutation events resulting in the insertion/deletion of nucleotides between the relevant upstream or downstream stop codons and the original mutation such that the correct reading frame is restored. Analysis of 20 or more each Hom\(^+\) and lys2-Bgl revertants arising in a msh2 mutant showed that all reversion events involved the deletion of one nucleotide to restore the correct open reading frame. All of the deletion events were the deletion of a single nucleotide in short mononucleotide repeat sequences, similar to the prevalence of deletions seen during instability of dinucleotide repeat sequences used to study repeat instability in human tumor cells in some cases [Shibata et al. 1994; Papadopoulos et al. 1995]. These data (Table 2; Fig. 3) support the idea that insertion/deletion mis-
Table 1. Mutation rate analysis

| Genotype           | Can'     | Hom'     | Lys'     |
|--------------------|----------|----------|----------|
| Wild type          | $8.0 \times 10^{-8}$ | $1.5 \times 10^{-8}$ | $2.9 \times 10^{-8}$ |
| $msh2$             | $1.2 \times 10^{-7}$ (1) | $1.0 \times 10^{-8}$ [1] | $1.4 \times 10^{-8}$ [1] |
| $msh2$             | $4.3 \times 10^{-8}$ | $5.2 \times 10^{-6}$ | $1.7 \times 10^{-6}$ |
| $msh2$             | $3.7 \times 10^{-6}$ [40] | $1.2 \times 10^{-5}$ [662] | $7.3 \times 10^{-7}$ [55] |
| $msh2$             | $9.9 \times 10^{-8}$ | $7.4 \times 10^{-8}$ | $7.4 \times 10^{-8}$ |
| $msh2$             | $1.1 \times 10^{-7}$ (1) | $3.4 \times 10^{-8}$ [4] | $1.7 \times 10^{-8}$ [2] |
| $msh2$             | $1.8 \times 10^{-6}$ (18) | $1.0 \times 10^{-7}$ | $5.2 \times 10^{-8}$ |
| $msh2$             | $9.1 \times 10^{-6}$ (55) | $2.6 \times 10^{-6}$ (300) | $2.2 \times 10^{-6}$ [91] |
| $msh2$             | $3.7 \times 10^{-6}$ (29) | $6.7 \times 10^{-6}$ | $6.8 \times 10^{-7}$ [31] |
| $msh2$             | $3.3 \times 10^{-6}$ (37) | $6.6 \times 10^{-6}$ | $3.8 \times 10^{-6}$ |
| $msh2$             | $4.0 \times 10^{-6}$ (37) | $8.6 \times 10^{-6}$ (585) | $2.0 \times 10^{-6}$ [123] |
| $msh2$             | $3.1 \times 10^{-6}$ (30) | $6.0 \times 10^{-6}$ | $6.1 \times 10^{-7}$ |
| $msh2$             | $2.5 \times 10^{-6}$ (28) | $5.1 \times 10^{-6}$ (431) | $6.4 \times 10^{-7}$ (29) |

The data from two independent experiments are presented. The number in parenthesis is the fold induction relative to the wild-type value based on the average of the data from both experiments. The strains tested were RKY 2575 (wild type), RKY 2558 (msh2), RKY 2582 (msh3), RKY 2580 (msh6), RKY 2581 (msh2 msh6), RKY 2561 (msh2 msh3), RKY 2567 (msh3 msh6), and RKY 2571 (msh2 msh3 msh6).

pairs produced by DNA polymerase errors are not corrected in msh2 mutants. The small number of Hom' and Lys' revertants we were able to obtain from msh6 mutants were all -1 frameshift mutations of the type seen in msh2 mutants [Table 2]. The limited reversion of horn3-10 and lys2-Bgl seen in msh6 mutants (~1% of the rate seen in a msh2 mutant) and the type of mutations obtained suggest that msh6 mutants have at best a small defect in recognition of insertion/deletion mispairs, including 1-base insertion/deletion mispairs.

To gain further insight into MSH2- and MSH6-dependent repair, the Can' mutations [any mutation inactivating the arginine permease gene] arising in msh2 and msh6 mutants were sequenced. This assay is particularly useful because unlike the horn3-10 and lys2-Bgl reversion assays, the Can' mutation assay is unbiased and can detect any type of inactivating mutation that can occur in the 1.8-kb arginine permease gene. The data [Table 3] demonstrate that 86% of the Can' mutations in the msh6 mutant were single-base substitutions, whereas the Can' mutations arising in the msh2 mutant were primarily [85%] single-base insertion/deletion muta-

Figure 3. DNA sequence analysis of Hom' and Lys' revertants. Nucleotides 565–788 of the HOM3 gene and nucleotides 270–583 of the LYS2 gene were determined as described in Materials and methods; however, only the sequencing chromatogram of the coding strand of the region containing either the horn3-10 or lys2-Bgl allele is shown. (A) Sequence chromatogram from a wild-type strain showing the 6T wild-type HOM3 allele; (B) sequence chromatogram from a horn3-10 mutant strain showing the 7T mutant allele; (C) sequence chromatogram from a Hom' revertant showing reversion to the 6T wild-type allele; (D) sequence chromatogram from a wild-type strain showing the 6T wild-type LYS2 allele; (E) sequence chromatogram from a lys2-Bgl mutant strain showing the GATC duplication caused by the lys2-Bgl allele; (F) sequence chromatogram from a Lys' revertant showing the 4 C's to 3 C's - 1 frameshift, which restores the correct reading frame in the revertant. The site of the BglII site in the LYS2 gene and the run of 4 C's in the LYS2 gene where the -1 C deletion occurred in the Lys' revertant are indicated by overlining in D.
DNA mispair recognition

Table 2.  Mutation spectrum of Horn+ and Lys+ revertants

| Genotype | Hom+ | Lys+ |
|----------|------|------|
|          | mutation | occurrence | mutation | occurrence |
| msh2    | \( \Delta T \) | \( T_6 \rightarrow T_5 \) | 22/22 | \( \Delta A \) | \( A_6 \rightarrow A_5 \) | 9/21 |
|          | \( \Delta C \) | 11/21 |
| msh6    | \( \Delta T \) | \( T_6 \rightarrow T_5 \) | 6/6 | \( \Delta A \) | \( A_6 \rightarrow A_5 \) | 1/4 |
|          | \( \Delta T \) | 3/4 |

The strains tested were RKY 2558 (msh2) and RKY 2580 (msh6). All of the Horn+ revertants involved the direct reversion of the 7T hom3 10 allele to the 6T HOM3 allele, whereas a variety of mutations at LYS2 were observed. Only a summary of the mutations observed is presented. A detailed mutation spectrum is available on request.

Table 3.  Mutation spectrum of Can+ mutants

| Genotype | Insertion/deletion | Base change |
|----------|-------------------|-------------|
|          | mutation | occurrence | mutation | occurrence |
| msh2    | \( \Delta A \) | \( A_6 \rightarrow A_5 \) | 7/20 | \( C \rightarrow A \) | 1/20 |
|          | \( \Delta T \) | \( T_6 \rightarrow T_5 \) | 2/20 | \( G \rightarrow T \) | 1/20 |
|          | \( T_5 \rightarrow T_4 \) | 2/20 |
|          | \( T_4 \rightarrow T_3 \) | 4/20 |
|          | \( T_3 \rightarrow T_2 \) | 1/20 |
|          | \( T_2 \rightarrow T_1 \) | 1/20 |
|          | \( +T \) | \( T_6 \rightarrow T_7 \) | 17/20 (85%) | \( 3/20 (15\%) \) |
| msh6    | \( \Delta T \) | \( T_6 \rightarrow T_5 \) | 1/21 | \( C \rightarrow A \) | 1/21 |
|          | \( T_5 \rightarrow T_4 \) | 1/21 |
|          | \( \Delta C \) | \( C_3 \rightarrow C_2 \) | 1/21 | \( G \rightarrow T \) | 4/21 |
|          | \( T \rightarrow C \) | 2/21 |
|          | \( T \rightarrow G \) | 1/21 |
|          | \( 3/21 (14\%) \) | 18/21 (86%) |
| msh3 msh6 | \( \Delta A \) | \( A_6 \rightarrow A_5 \) | 4/22 | \( A \rightarrow G \) | 1/22 |
|          | \( \Delta T \) | \( T_6 \rightarrow T_5 \) | 7/22 | \( C \rightarrow A \) | 2/22 |
|          | \( T_5 \rightarrow T_4 \) | 2/22 |
|          | \( \Delta G \) | \( G_1 \rightarrow G_0 \) | 1/22 | \( G \rightarrow A \) | 1/22 |
|          | \( +T \) | \( T_6 \rightarrow T_7 \) | 1/22 | \( T \rightarrow C \) | 1/22 |
|          | \( 15/22 (68\%) \) | 7/22 (32%) |

The strains tested were RKY 2558 [msh2], RKY 2580 [msh6], and RKY 2567 [msh3 msh6]. Only a summary of the mutations observed is presented. A detailed mutation spectrum is available on request.

The pms3-1 mutation is an allele of MSH6

One of the original pms mutations, pms3-1, causes phenotypes that are similar to those caused by msh6 mutations. pms3-1 mutations cause an increased rate of accumulation of Can+ mutations but do not cause an increase in the rate of reversion of hom3-10 [Williamson et al. 1985; Jeyaprakash et al. 1994]. In addition, it has been shown that pms3-1 mutations cause a complete defect in the repair of heteroduplex plasmid DNAs containing each of the eight possible single-base substitution mispairs, similar to that caused by mutations in PMS1 and PMS2 [MLH1]. However, in contrast to mutations in PMS1 and PMS2 (the original PMS2 gene is identical to MLH1; Jeyaprakash et al. 1996), pms3-1 mutations do not cause a defect in repair of heteroduplex plasmid DNAs containing either a + A or a + T single-base insertion mispair [B. Kramer et al. 1989]. To determine whether pms3-1 is a allele of MSH6, we transformed a
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pms3-1 mutant strain with either a plasmid containing the wild-type MSH6 gene or the cloning vector as a control and tested eight independent transformants each for complementation of the pms3-1 mutator phenotype using patch tests on canavanine plates as described in Fig. 2. In all cases, complete complementation was observed with the MSH6 plasmid but not with the vector control, consistent with the idea that pms3-1 is an allele of MSH6.

To confirm that pms3-1 mutant strains contain a mutation in MSH6, the MSH6 gene was amplified from both a pms3-1 mutant and a wild-type parental strain and sequenced. One difference from the wild-type MSH6 sequence was observed (Fig. 4). This change was a G → A change at nucleotide 1262 (codon 421), which results in a substitution of Asp for Gly, a significant amino acid change. This Gly is a highly conserved amino acid located in a conserved region of the known MutS homologs, except S. cerevisiae MSH4 and MSH5, which are highly divergent in this region (most amino acids in this region are not even present in MSH4 and MSH5). Gly-421 is perfectly conserved in a subset of MutS homologs: the MutS subfamily, the MSH2 subfamily, the MSH6 subfamily, and MSH1. In the MSH3 subfamily, this residue is either Gly, Ser, or Ala, the latter two amino acids being conservative substitutions. These data, combined with the observation that MSH6 complements pms3-1, indicate that pms3-1 is a missense mutation in MSH6. We propose that the MSH6 gene continue be called

Figure 4. DNA sequence analysis of the pms3-1 allele. The entire MSH6 gene was sequenced from congenic wild-type and pms3-1 strains as described in Materials and methods. (A) Sequence chromatogram of nucleotides 1252–1270 of the MSH6 gene and the pms3-1 allele. The G → A change at nucleotide 1262 is overlined. (B) DNA sequence of nucleotides 1249–1275 of the MSH6 gene with the translated amino acid sequence listed below and the effect of the pms3-1 mutation on the DNA and amino acid sequence indicated above. (C) Alignment of the relevant region of amino acid sequence from different MutS homologs and the pms3-1 allele of S. cerevisiae MSH6. S. cerevisiae MSH4 and MSH5 are not shown as they are highly divergent in this region. Most amino acids in this region are not even present in MSH4 and MSH5. The conserved Gly-421 and equivalent residues in other MutS homologs are indicated in bold.
MSH6 because this designation provides information about the function of the gene, in keeping with this, pms3-1 should be renamed msh6-1.

**Analysis of the role of MSH3 in mismatch repair**

The low rate of accumulation of insertion/deletion mutations in msh6 mutants is reminiscent of msh3 mutants, which show low but increased rates of reversion of hom3-10 and lys2–Bgl but essentially no increased rate of accumulation of Can' mutants [New et al. 1993; Alani et al. 1994]. One explanation for the low rate of accumulation of insertion/deletion mutations in msh6 and msh3 single mutants is that there are two mismatch repair pathways: a MSH2, MSH6 pathway, which recognizes single-base mispairs and insertion/deletion mispairs, and a MSH2, MSH3 pathway, which only recognizes insertion/deletion mispairs. To test this possibility, a msh3, msh6 double mutant was constructed and analyzed to determine the effect of this double mutation combination on the accumulation of mutations.

The results (Table 1, Fig. 2) demonstrated that the rate of accumulation of Can' mutations and the rate of reversion of hom3-10 and lys2–Bgl in the msh3, msh6 double mutant (8 isolates analyzed) was increased synergistically in comparison with either the msh3 or msh6 single mutants (5 and 7 isolates analyzed, respectively) and was essentially indistinguishable from that observed in a msh2 single mutant (13 isolates analyzed). Both the msh2, msh3 double mutant strain (two isolates analyzed) and the msh2, msh3, msh6 triple mutant strain (two isolates analyzed) had the same phenotype as the msh2 single mutant, as assessed in both patch tests (Fig. 2) and quantitative rate tests (Table 1), suggesting that MSH2 is epistatic to MSH3 and MSH6. There are some small differences in mutation rates seen in the strains containing multiple combinations of msh2, msh3 and msh6 mutations relative to msh2 mutant strains; however, these differences are no more than about two-fold, which is the level of variation seen in these types of experiments.

Sequence analysis of the Can' mutations that occurred in the msh3, msh6 double mutant demonstrates that like the msh2 single mutant, single-base insertion/deletion mutations [primarily deletions] in short mononucleotide repeat sequences predominate over single-base substitution mutations in the msh3, msh6 double mutant [Table 3]. The mutation spectrum of Can' mutations in the msh3 single mutant was not analyzed because the rate of accumulation of mutations in msh3 mutants is essentially the same as in wild-type control strains. Within the error caused by the sample size used, the mutation spectrum in the msh3, msh6 double mutant is essentially the same as in the msh2 single mutant, both with regard to the preponderance of single-base insertion/deletion mutations and the type of insertion/deletion mutations seen. This result indicates that the synergistic effect of msh3 and msh6 mutations is primarily confined to the rate of accumulation of insertion/deletion mutations and that the rate of accumulation of single-base substitution mutations is similar to that observed in either msh2 or msh6 single mutants. These data are consistent with the idea that a combination of mutations in MSH3 and MSH6 inactivates mismatch repair in a way that is similar to that caused by mutations in MSH2. However, our data do not rule out the possibility that there are small differences in mutagenesis in the msh3, msh6 double mutant compared with the msh2 single mutant.

**MSH2 interacts with MSH3 and MSH6**

The observation that human MSH2 interacts with the human homolog of MSH6, GTBP/p160, suggests that MSH2 might interact with both MSH3 and MSH6. To test this possibility, MSH3, hexa-His-tagged MSH3, MSH6, hexa-His-tagged MSH6, MSH2, and 12CA5-epitope-tagged MSH2 [Alani et al. 1995] were transcribed and translated in vitro to determine whether MSH2 could interact with MSH3 or MSH6. The DNA templates used in these studies were produced by PCR and contained only the MSH3, hexa-His-tagged MSH3, MSH6, hexa-His-tagged MSH6, MSH2, and 12CA5 epitope-tagged MSH2 coding sequences, as needed to ensure that only these proteins were synthesized. Control experiments verified that for each of these proteins, full-length protein could be synthesized under the in vitro transcription and translation conditions used.

To detect interactions between MSH2 and MSH6, different combinations of MSH6 or hexa-His-tagged MSH6 plus MSH2 or 12CA5-epitope-tagged MSH2 were cotranslated and translated in the presence of [%]methionine. These proteins were then analyzed using a two-step procedure in which the proteins were first captured and eluted from Ni-beads and then immunoprecipitated with anti-12CA5 antibody (Fig. 5A). A 140-kD polypeptide the size of MSH6 and a 109-kD polypeptide the size of MSH2 were coimmunoprecipitated only when MSH6 contained the hexa-His tag and MSH2 contained the 12CA5 epitope. The recovery of this complex was dependent on the presence of the 12CA5 MSH2 epitope, the hexa-His MSH6 tag, the MSH6 coding region [Fig. 5A], and the MSH2 coding region [data not shown], indicating that the observed complex was caused by a specific interaction between MSH2 and MSH6. An identical series of experiments was performed to determine whether MSH3 would interact with MSH2 except that the MSH3 gene sequence was substituted for the MSH6 gene sequence [data not shown]. These experiments similarly demonstrated that a 120-kD polypeptide the size of MSH3 and a 109-kD polypeptide the size of MSH2 protein were communoprecipitated only when MSH3 contained the hexa-His tag and MSH2 contained the 12CA5 epitope. The recovery of this complex was dependent on the presence of the 12CA5 MSH2 epitope, the hexa-His MSH3 tag, the MSH3 coding region, and the MSH2 coding region [data not shown], indicating that the observed complex was attributable to a specific interaction between MSH2 and MSH3. In these experiments, the MSH2–MSH3 and
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MSH2–MSH6 complexes could be quantitatively recovered by immunoprecipitation in the presence of 1% Triton X-100 and 0.5 M NaCl, indicating that they were the result of a strong interaction between the proteins.

A second series of experiments was performed in which individual proteins were separately transcribed and translated in vitro and then mixed together to allow complex formation to occur. These experiments [Fig. 5B] demonstrated that a 120-kD polypeptide, which is the predicted size of MSH3, could be immunoprecipitated by anti-12CA5 antibody if translated 35S-labeled MSH3 was mixed with epitope-tagged MSH2. The 120-kD MSH3 polypeptide was not immunoprecipitated if the MSH3 gene was not included in the transcription–translation mixes or if the translated MSH2 did not contain the 12CA5 epitope. These experiments indicated that the 120-kD MSH3 polypeptide was immunoprecipitated only in the presence of 12CA5 epitope-tagged MSH2, indicating a specific interaction between MSH3 and MSH2. An identical series of experiments was also performed to determine whether MSH6 would interact with MSH2, except that the MSH6 gene was substituted for the MSH3 gene [data not shown]. These experiments similarly demonstrated that the 140-kD polypeptide encoded by the MSH6 gene specifically interacted with the MSH2 protein and could be immunoprecipitated through a specific interaction with MSH2. In these experiments, the MSH2–MSH3 and MSH2–MSH6 complexes could be quantitatively recovered by immunoprecipitation in the presence of 1% Triton X-100 and 0.5 M NaCl, indicating that they were the result of a strong interaction between the proteins.

Discussion

In this communication we describe a sixth S. cerevisiae MSH gene, MSH6. The MSH6 protein is related most closely to GTBP/p160, a protein identified because it interacts with human MSH2 and thus appears to be a second subunit of a human mispair recognition complex [Drummond et al. 1995; Palombo et al. 1995]. Mutations in MSH6 cause a partial mutator phenotype consistent with a defect in recognition of single-base mispairs similar to that observed in a msh2 mutant, but only a small defect [−1%] in the recognition of insertion/deletion mispairs compared with that observed in msh2 mutants. The prior demonstration that pms3-1 mutations cause a direct defect in the repair of single-base substitution
mispairs and not single-base insertion mispairs [B. Kramer et al. 1989], combined with the observation presented here that *pms3-1* mutations are alleles of *MSH6*, confirm that *msh6* mutations cause a specific defect in the repair of single-base substitution mispairs but not single-base insertion mispairs. In contrast, *msh2* mutations cause defects that are consistent with a defect in recognition of both single-base and insertion/deletion mispairs during mismatch repair. The analysis of *msh3* mutations presented here confirms previously published results that *msh3* mutations cause only limited defects in mismatch repair [New et al. 1993; Alani et al. 1994]. Surprisingly, *msh3, msh6* double mutant strains had essentially the same mutator phenotype and mutation spectrum as *msh2* mutant strains. The ~100-fold synergistic effect of *msh3* and *msh6* mutations on the rate of accumulation of mutations is confined to the accumulation of insertion/deletion mutations, whereas the accumulation of single-base substitution mutations is *msh6* dependent and *msh3* independent. Epistasis analysis of *MSH2, MSH3* and *MSH6* is consistent with the idea that *MSH3* and *MSH6* encode redundant functions that act in *MSH2*-dependent mismatch repair of insertion/deletion mispairs, whereas *MSH6* appears to also be required for *MSH2*-dependent mismatch repair of single-base substitution mispairs.

Fig. 6 illustrates a model that is consistent with the results presented above. Based on the observations that human GTBP/p160 (MSH6) forms a heterodimer with *MSH2*, which recognizes mispaired bases [Drummond et al. 1995; Palombo et al. 1995], and that *S. cerevisiae* MSH2 forms complexes with both *MSH3* and *MSH6*, we propose that there are two mispair recognition complexes: an MSH2–MSH6 complex that recognizes both single-base mispairs and insertion/deletion mispairs, and a MSH2–MSH3 complex that recognizes insertion/deletion mispairs and does not recognize single-base mispairs or has only very low affinity for them. Given that MSH2 can recognize mispaired bases by itself [Fishel et al. 1994; Alani et al. 1995], MSH3 and MSH6 could either modify the specificity of MSH2 or could participate directly in mismatch recognition. The proposed relative affinity of each complex for different mispairs and the redundancy of MSH3 and MSH6 with regard to recognition of insertion/deletion mispairs provides an explanation for the following observations: (1) the strong mutator phenotype caused by *msh2* mutations; (2) the accumulation of single-base substitution mutations in *msh6* mutants; (3) the lack of effect of *msh3* and *msh6* mutations on accumulation of insertion/deletion mutations; (4) the observation that *msh6* (*pms3-1*) mutations cause a direct defect in the repair of single-base substitution mispairs but not single-base insertion mispairs [B. Kramer et al. 1989]; and (5) the synergistic effect of *msh3* and *msh6* mutations on the accumulation of insertion/deletion mutations. Whereas the proposed model is consistent with both the genetic data and the protein–protein interaction data presented here, a complete test of this model will require purification of each complex and analysis of its mispair binding properties, which is in progress. Furthermore, whereas our data are consistent with the idea that MSH2–MSH3 and MSH2–MSH6 complexes direct the majority of mismatch repair, it remains possible that any one of these proteins can form complexes with itself and that such complexes can direct a limited amount of mismatch repair. This may explain how MSH2 recognizes mispaired bases by itself.

In human tumor cell lines *gtbp* mutations cause a strong in vitro defect on repair of single-base substitution and single-base insertion/deletion mispairs and a variable partial defect in repair of larger insertion/deletion mispairs [Drummond et al. 1995]. *GTBP* mutant human tumor cell lines have been reported to have microsatellite instability in vivo, at both mononucleotide and dinucleotide repeat loci, although apparently at lower rates than in tumor cell lines having mutations in *hMSH2* or *hMLH1* [Parsons et al. 1993; Bhattacharyya et al. 1994; Shibata et al. 1994; Umar et al. 1994; Papadopoulos et al. 1995]. Our results with *msh6* mutants most closely resemble the results of microsatellite instability.

**Figure 6.** Model for mismatch recognition in *S. cerevisiae*. The various postulated complexes between *MSH2* and either *MSH3* or *MSH6* are illustrated interacting with either a single-base substitution mispair or an insertion/deletion mispair; exactly which of the proteins in these complexes, *MSH2, MSH3*, or *MSH6* actually interacts with the mispaired base is not known. Also indicated is the previously described MLH1–PMS1 complex which interacts with the mispair recognition complex [Prolla et al. 1994]. The *S. cerevisiae* protein names are given as primary names, the human protein names are the same except for PMS1, which is called PMS2 in humans, and MSH6, which has been called GTBP or p160 in humans.
analysis of gtbp mutant human tumor cell lines [Shibata et al. 1994; Papadopoulos et al. 1995]. We do not understand the basis for the differences in results obtained with in vivo and in vitro measurements of the effect of msh6/gtbp mutations. It is possible these differences could be due to methodological differences, possible instability of selected proteins in mutant cells [Drummond et al. 1995] or the interaction between mismatch repair and DNA replication that occurs in vivo but not in vitro. Mutations in GTBP [MSH6] have not been found in HNPPC families and have only been found in a small proportion of tumor cell lines [Papadopoulos et al. 1995].

To our knowledge, no systematic search has been made for mutations in hMSH3 [DUC and MRP1 are other names for human MSH3] in HNPPC families. Such mutations must be rare in microsatellite unstable, mismatch repair-defective human tumor cell lines since the majority of such cell lines can be explained by the presence of either msh2 or mlh1 mutations [Boyer et al. 1995]. Our results have important implications for the analysis of mismatch repair defective mutations and their association with cancer susceptibility. First, the redundancy of MSH3 and MSH6 compared with the apparently universal requirement for MSH2 in mismatch repair provides an explanation for the high prevalence of msh2 mutations in HNPPC families and human tumors [Fishel et al. 1993; Leach et al. 1993; Kolodner et al. 1994; Liu et al. 1994; Nystrom-Lahti et al. 1994; Borresen et al. 1995; Boyer et al. 1995]. This is because independent mutations in both MSH3 and MSH6, which seem unlikely, would be required to produce the same mismatch repair defect as that caused by mutations in MSH2. It is also possible that the lack of another entirely MSH2-dependent process rather than the loss of mismatch repair is the underlying cause of cancer susceptibility. Second, by selecting for tumors and syndromes [HNPPC] associated with high degrees of microsatellite instability [Aaltonen et al. 1993; Ionov et al. 1993; Peltonaki et al. 1993a,b; Bhattacharyya et al. 1994; Honchel et al. 1994; Orth et al. 1994; Shibata et al. 1994; Eshleman and Markowitz 1995], one may select against cancers caused by msh6 or msh3 mutations. In contrast, one might expect to find msh6 mutations associated with tumors that have increased rates of accumulating single-base substitution mutations but have little if any microsatellite instability [Eshelman et al. 1995]. By analogy, one might only expect to find msh3 mutations associated with cancers showing low level microsatellite instability but lacking other mutator phenotypes. Third, it is possible that the relatively low mutator phenotype caused by msh3 or msh6 mutations compared with msh2 mutations is insufficient to cause cancer initiation or progression, or that such mutations have lower penetrance than msh2 mutations. Finally, the mutational spectra presented here suggest that in human tumor cells having complete inactivation of MSH2-dependent mismatch repair, the most likely mutations to occur within gene coding sequences are frameshift mutations within short mononucleotide repeat sequences which can be quite prevalent within coding sequences.

**Materials and methods**

**Strains, plasmids and media**

All of the *S. cerevisiae* strains used in this study for the analysis of mutations in MSH2, MSH3, and MSH6 were isogenic strains derived from a MGD strain provided by A. Nicolas [Rocco et al. 1992] and are listed in Table 4. The wild-type and pms3-1 mutant strains used to analyze the pms3-1 mutation were MW3070-6C and MW2649-24C, respectively, and were kindly provided by A. Jeyaprakash [Jeyaprakash et al. 1994]. Disruption mutations in MSH2, MSH3 and MSH6 as well as the *hom3-10* and *lys2-Bgl* mutations were introduced by standard lithium acetate transformation based gene disruption techniques and, in some cases, by crossing mutant derivatives to isogenic derivatives of MGD. The presence of mutations in MSH2, MSH3 and MSH6 was always verified using PCR amplification of the mutant and wild-type alleles, whereas the presence of *hom3-10* and *lys2-Bgl* was verified by PCR amplification followed by DNA sequence analysis (see below). Derivatives of strains in which the *URA3* gene had been excised by recombination between *hisG* repeats were selected on minimal dropout plates containing 5-FOA. The MSH6 gene [S51246, YD8557.04c] was disrupted using a PCR-based method to generate a DNA fragment consisting of the *URA3* gene flanked by DNA from the 5' and 3' ends of the *MSH6* gene. DNA from the 5' end of *MSH6* was amplified with

| Table 4. *S. cerevisiae* strains used in this study |
|-------------------------------|------------------|
| **Strain** | **Genotype** |
| RKY 2583 | *MATα*, ade2/ADE2, *ura3·52*/*ura3·52*, *trpl·289*/*trpl·289*, *leu2·3·112*/*leu2·3·112*, *HIS3/his3Δ1*, CYH2/cyh2 |
| RKY 2558 | *MATα*, ade2/*ura3·52*, *leu2·3·112*, *trpl·289*, *his3Δ1*, *lys2-Bgl*, *hom3·10*, *msh2·hisG* |
| RKY 2569 | *MATα*, ade2/*ura3·52*, *leu2·3·112*, *trp1·289*, *his3Δ1*, *lys2-Bgl*, *hom3·10*, *msh2·hisG*, *msh3·hisG–URA3·hisG* |
| RKY 2567 | *MATα*, ade2/*ura3·52*, *leu2·3·112*, *trp1·289*, *his3Δ1*, *lys2-Bgl*, *hom3·10*, *msh2·hisG–URA3·hisG*, *msh6::URA3* |
| RKY 2571 | *MATα*, ade2/*ura3·52*, *leu2·3·112*, *trp1·289*, *his3Δ1*, *lys2-Bgl*, *hom3·10*, *msh2·hisG*, *msh3·hisG–URA3·hisG*, *msh6::URA3* |
| RKY 2575 | *MATα*, ade2/*ura3·52*, *leu2·3·112*, *trp1·289*, *hom3·10*, *msh2·hisG* |
| RKY 2580 | *MATα*, ade2/*ura3·52*, *leu2·3·112*, *trp1·289*, *his3Δ1*, *lys2-Bgl*, *hom3·10*, *msh6::URA3* |
| RKY 2581 | *MATα*, ade2/*ura3·52*, *leu2·3·112*, *trp1·289*, *his3Δ1*, *lys2-Bgl*, *hom3·10*, *msh2·hisG*, *msh6::URA3* |
| RKY 2582 | *MATα*, ade2/*ura3·52*, *leu2·3·112*, *trp1·289*, *his3Δ1*, *lys2-Bgl*, *hom3·10*, *msh2·hisG–URA3·hisG* |
| RKY 2584 | *MATα*, ade2/*ura3·52*, *leu2·3·112*, *trp1·289*, *his3Δ1*, *lys2-Bgl*, *hom3·10*, *msh2·hisG–URA3·hisG*, *msh6::URA3* |

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was grown to a sufficient cell density so that mutant cells were isolated. In these fluctuation tests, five independent testers, and Lys\(^+\) revertants were isolated by first screening cultures for each experiment and each culture plate lacking lysine.

The rate of accumulation of Can\(^r\) mutants, Horn\(^+\) revertants, and Lys\(^+\) revertants in cell populations was determined by fluctuation analysis using the method of the median (Lea and Coulson 1948) as described previously (Reenan and Kolodner 1992a; Alani et al. 1994). Can\(^r\) revertants which contain the wild-type and 12CA5 epitope-tagged MSH6::hisG-URA3-hisG disruption plasmid, pK8, which contains the wild-type and 12CA5 epitope-tagged MSH6 gene, respectively, have been described previously (Alani et al. 1995). The plasmids pEN28, which contain the wild-type MSH2::hisG-URA3-hisG gene, were maintained in S. cerevisiae strains were prepared as described previously (Alani et al. 1995). msh6::URA3 disruption plasmid pSR125 was obtained from S.

The msh6::URA3 chromosomal region by long-range PCR (Barnes et al. 1994; see also DNA sequencing, below) using primers 22604 (5\'-CATCCGTGTAAGCCTCAGTCGCAACAAATGGAATAATCTATTACGTCG-3\') and 22605 (5\'-5\'CATCTCTTCGCCGGATCGAAGACTCGAATTCAAGGAATACAAT-GAAAATCTAATATTAGC-3'). To identify the mutations causing reversion of either horn3-10 or lys2-Bgl, chromosomal DNA preparations were made by preparing glass bead lysates of cells harvested from a single colony using a method developed by D. Tishkoff in this laboratory. In the case of horn3-10, nucleotides 565–788 of the horn3-10 allele were amplified by PCR using primers 21514 (5\'-TTATACGTGCTCTACTCATGTCG-3') and 21515 (5\'-TAAACGTGCTCTACTCATGTCG-3'). The plasmid pK8 DNA containing the horn3-10 allele was prepared by cloning the 0.4-kb EcoRI sites flanking the URA3 gene on the plasmid YEp24. The 3.3-kb disruption fragment was created by amplifying the 0.4-kb 5\' MSH6 and the 0.9 kb 3' MSH6 fragments together with a BamHI/EcoRI digest of YEp24 using the primers 22605 and 21740. This yielded a MSH6 DNA fragment containing 0.4 kb of 5' homology and 0.9 kb of 3' homology in which the DNA sequence encoding amino acids 132–929 of MSH6 had been substituted by URA3. The disruption fragment was then transformed into the diploid MGD strain RKY2583, and disruptants were selected on URA dropout plates. Haploid msh6::URA3 strains were obtained by sporulation. This was done by intercrossing hom3-10 or lys2-Bgl strains to Ura-.
These DNA templates were then transcribed and translated in promoter and lowercase type indicates the gene sequence. 418 GENES & DEVELOPMENT primer 23592 for synthesis of hexa-His-tagged ATACGA CTCACTATAGGGAGACCACATGCATCATCA-
cases uppercase type indicates the sequences containing the T7
transcription were prepared by PCR amplification. PCR reactions were
MSHZ MSH3, 23586 (5'-ggaacaattcaaaaacgagaaagtg]. The primer 5' hexa-His-
CGACTCACTATAGGGAGACCACcatggccccagctacccctaaaa-
fication of taatgaacctaaactgg) was substituted for primer 23597 for synthe-
sis of hexa-His-tagged MSH6. 

To identify the pms3-1 mutation, the Msh6 gene was amplified by PCR from strains MW3070-6C (wild type) and MW3049-24C (pms3-1 mutant) using primers 22604 (5'-CATCGGT-

Protein analysis DNA templates containing the T7 RNA polymerase promoter linked to the coding sequence of MSH2, 12CA5 epitope-tagged MSH2, MSH3, and MSH6 for in vitro transcription and translation were prepared by PCR amplification. PCR reactions were performed essentially as described above for the analysis of Hom* and Lys* revertants and contained 100 pg of pEN11 DNA [MSH2], pEN43 DNA (12CA5 epitope-tagged MSH2), pEN28 DNA [MSH3], or pRDK377 DNA [MSH6] as template as required. The primers used for amplification of MSH2 and 12CA5 epitope-tagged MSH2 were 5'-MSH2-23587 (5'-GG-

For in vitro transcription and translation reactions, 50- to 100-

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ilton, G.M. Petersen, K. W. Kinzler, B. Vogelstein, and A. de

In some experiments, pairs of DNA templates were transcribed and translated together in the presence of [35S]methionine. The reaction mixtures were plated on ice, and 50 ml of 2X Ni binding buffer (1 mm imidazole, 1 M NaCl, 2% Triton X-100, 50 mm Tris-Cl [pH 7.6], 2 mm EDTA, 5 mm 2-mercaptoethanol, 2 mm PMSF) was added to each 50 ml of reaction mixture followed by the addition of 20 ml of Ni-NTA-agarose beads (Qiagen, Chatsworth, CA; as a 50% suspension in 1X Ni binding buffer). After incubation on ice for 1 hr, the Ni-beads were collected by centrifugation in a microcentrifuge, washed four times with 150 ml of Ni binding buffer and the proteins were then eluted by addition of 100 ml of Ni elution buffer [500 mm imidazole, 0.5 M NaCl, 1% Triton X-100, 25 mm Tris-Cl [pH 7.6], 2 mm EDTA, 5 mm 2-mercaptoethanol, 1 mm PMSF], followed by centrifugation to remove the Ni-beads. The eluted proteins were then analyzed by immunoprecipitation in the presence of 1% Triton X-100 and 0.5 M NaCl with anti-12CA5 monoclonal antibody as described above.

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Dna templates were then transcribed and translated in vitro in 50-ml volumes containing 400 ng of the gene required template DNA using TNT kits from Promega (Madison, WI) exactly as described by the manufacturer with or without added 35S-labeled methionine (Amersham, Arlington Heights, IL) as required. In experiments where the DNA templates were transcribed and translated individually, the resulting reaction mixtures were mixed and incubated on ice for 1 hr to allow proteins to interact. The proteins were then analyzed by immunoprecipitation in the presence of 1% Triton X-100 and 0.5 M NaCl with anti-12CA5 monoclonal antibody [Berkeley Antibodies, Richmond, CA] exactly as described previously [Alani et al. 1995] except that the resulting gels were analyzed by either autoradiography or PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

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