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Genetics

The role of mismatch repair in the prevention of base pair mutations in *Saccharomyces cerevisiae*

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ABSTRACT **In most organisms, the mismatch repair (MMR) system plays an important role in substantially lowering mutation rates and blocking recombination between nonidentical sequences. In *Saccharomyces cerevisiae*, the products of three genes homologous to *Escherichia coli mutS*—*MSH2*, *MSH3*, and *MSH6*—function in MMR by recognizing mispaired bases. To determine the effect of MMR on single-base pair mismatches, we have measured reversion rates of specific point mutations in the *CYC1* gene in both wild-type and MMR-deficient strains. The reversion rates of all of the point mutations are similar in wild-type cells. However, we find that in the absence of *MSH2* or *MSH6*, but not *MSH3*, reversion rates of some mutations are increased by up to 60,000-fold, whereas reversion rates of other mutations are essentially unchanged. When cells are grown anaerobically, the reversion rates in MMR-deficient strains are decreased by as much as a factor of 60. We suggest that the high reversion rates observed in these MMR-deficient strains are caused by misincorporations opposite oxidatively damaged bases and that MMR normally prevents these mutations. We further suggest that recognition of mispairs opposite damaged bases may be a more important role for MMR in yeast than correction of errors opposite normal bases.**

The DNA mismatch repair (MMR) system is conserved from bacteria to humans. The methyl-directed DNA MMR system of *Escherichia coli* is one of the best-studied systems (1). In repairing replication errors, the MutS protein recognizes and binds to a mismatch (2) and MutH binds a hemimethylated GATC site on one side of the mismatch (3). The endonuclease activity of MutH is stimulated in the presence of MutL and ATP and nicks the newly synthesized strand (4). The nicked strand is digested beyond the site of the mismatch and is resynthesized by using the methylated single-strand template (3).

In *Saccharomyces cerevisiae*, there are six *mutS* homologues, *MSH1-6* (5–10), and four *mutL* homologues, *MLH1-3* and *PMS1* (11–13), but not all function in nuclear-DNA MMR. Different combinations of these genes have roles in repair of noncomplementary base pairs generated during recombination and replication and in additional aspects of recombination in both mitotic and meiotic cells (13). The current model for nuclear DNA MMR in yeast proposes that a Msh2p/Msh6p heterodimer recognizes and stimulates repair of single-base pair mismatches or small loops, whereas a Msh2p/Msh3p heterodimer recognizes loops but not single-base pair mismatches (9, 10). The next step in repair involves association with a Pms1p/Mlh1p heterodimer (10, 14–16). It is still unclear how the mismatch repair machinery is targeted to the newly replicated strand. Recently, there has been some evidence that the mismatch repair machinery is linked to proliferating-cell nuclear antigen, suggesting that the MMR system tracks along the replication fork (17, 18).

The types of mutations prevented by MMR can be determined by examining the excess mutations created in its absence. Although the traditional view of MMR has been that it exists as a backup system to correct base-pair misincorporations during replication, various assays have shown that a large number of the mutations in MMR-defective strains are frameshift mutations, presumably caused by slipped mispairing in homopolymeric runs of nucleotides (13). For example, 85% of mutations to canavanine resistance in *msh2* cells were caused by frameshift mutations in such runs (10), and about 40% of mutations found in *SUP4-o* in MMR-defective strains were insertions or deletions (19). We wanted to determine the effect of MMR on preventing base-substitution mutations. The problem with previously used assays in *S. cerevisiae* was that they could examine only a mix of insertion/deletion events and base-pair substitutions; hence, DNA sequencing was necessary to determine the nature of each mutation (10, 19). We therefore decided to use a *cyc1* reversion assay originally developed by Hampsey (20) to test the specificity of various mutagens. Hampsey created six isogenic *cyc1* strains, each with a different point mutation in the Cys-22 codon of *CYC1*. Only true revertants can give a Cyc^+ phenotype. By determining the *cyc1* reversion rate for each mutation in both wild-type and MMR-defective strains, we have determined the frequency with which specific mismatches are recognized and corrected by the MMR system. We have found that some strains have reversion rates several orders of magnitude higher than others in the absence, but not the presence, of MMR. We suggest that these reversion rates result, at least in part, from mispairs opposite oxidatively damaged bases and that these mispairs are ordinarily recognized by the MMR system.

MATERIALS AND METHODS

Media and Chemicals. Yeast strains used in the reversion analysis were grown in yeast extract/peptone/dextrose (YPD) liquid medium and on YPD (21) and yeast extract/peptone/glycerol/dextrose (YPGD) plates (20). Ura^+ transformants were plated on synthetic complete media (SC-Ura) containing 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% glucose, uracil drop-out mix, and 2.5% agar (21). Canavanine sensitivity was determined by plating on SC-Arg medium containing 60 μg/ml canavanine. All media were from Difco. All restriction enzymes and modifying enzymes were from New England Biolabs.

S. cerevisiae* Strains.** All strains used for analysis are isogenic diploids derived by mating strains YMH2–7 (*MATα cyc1–22 cyc7–67 ura3–52 leu2–3,112 cyh2*) and B-7462 (*MATa** *cyc1–1 cyc7–67 ura3–52 his1–1 can1–100*) obtained from M. Hampsey (University of Medicine and Dentistry of New Jersey, Pisca-

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Abbreviations: MMR, mismatch repair; 8-oxodG, 8-hydroxy-2′-deoxyguanosine.
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taway, NJ) (20). The YMH strains differ from each other only by the single base mutation in the *CYC1* gene as indicated in Table 1.

Construction of Mutants. Haploid strains were deleted for *MSH2* by using pΔ*msh2*, a plasmid in which all but 207 base pairs of the *MSH2* coding region has been replaced with a *hisG-URA3-hisG* cassette (22, 23). This plasmid was made by ligating a 3,592-bp *Msp*AI/*Xba*I fragment of *MSH2* from pII-2 (5) to an *Eco*RV/*Xba*I fragment of pBlueScript II KS(+), giving pBS*MSH2*. A *Pvu*II fragment of pHUKH2 was ligated into the blunted *Eco*NI/*Hpa*I fragment of pBS*MSH2*. The 1,240-bp kanamycin gene was removed by digesting with *Pst*I, and the plasmid was religated. A 4,670-bp *Aat*II/*Xba*I, fragment was transformed into yeast and selected on SC-Ura plates. Strains were confirmed by using PCR and by measuring canavanine sensitivity.

Haploid strains were deleted for *MSH3* by using a pΔ*msh3* disruption plasmid that contains the *hisG-URA3-hisG* cassette. This plasmid was constructed by cloning a 3,741-bp *Afl*II/*Nco*I fragment of *MSH3* into the same sites of pMTL20. The *hisG-URA3*-Kan-*hisG* cassette was removed from pHUKH2 as above and ligated to blunted *Mlu*I and *Pml*I sites of *MSH3*/pMTL20, removing 3,079 bp of *MSH3*. The kanamycin gene was removed by digesting with *Pst*I, and the plasmid was religated. A 4,522-bp *Afl*II/*Msc*I fragment from this plasmid was transformed into yeast and selected on SC-Ura plates. Strains were confirmed by using PCR.

Haploid strains were deleted for *MSH6* by using the *msh6*pBUH plasmid from W. Kramer (Georg-August-Universität, Göttingen, Germany) (24). This plasmid contains the *MSH6* gene disrupted by the *hisG-URA3-hisG* cassette. A 5,980-bp *Eco*RI/*Sac*I fragment was transformed into yeast, and transformants were selected on SC-Ura plates. Strains were confirmed by using PCR and by measuring canavanine sensitivity.

Construction of Diploid Strains. Diploid strains were constructed by mating the haploid YMH strains and their derivatives with the appropriate B-7462 strains. Matings were done on YPD plates at 30°C overnight, and diploid strains were selected by replica plating on SC minimal plates supplemented with uracil.

Reversion Analysis. Reversion rates were determined by using fluctuation analysis and the method of Luria and Delbrück (25). Four to five cultures each of at least two independent colonies were tested. Each analysis was done twice. All strains were grown for two days in YPD media at 30°C to $\approx 7 \times 10^7$ cells per ml. Five-milliliter cultures were harvested, washed with sterile distilled, deionized H_2O, and resuspended to 1 ml in ddH_2O. A portion of each culture was diluted in ddH_2O and plated onto two YPD plates. For most cultures, the remainder was plated onto YPGD plates at a density of not more than 9×10^3 cells per mm^2. For some cultures with an increased reversion rate, a 1:10 dilution of the original culture was plated onto two YPGD plates at a density of not more than 7×10^2 cells per mm^2. YPD plates were counted after 2 days at 30°C, and YPGD plates were counted after 7 days at 30°C.

Anaerobic Reversion Analysis. Anaerobic reversion rates were determined by using fluctuation analysis and the method of Luria and Delbrück (25). The appropriate diploid strains were streaked onto YPD plates and grown at 30°C for 2 days. Eight to ten single colonies from each strain were patched onto YPD agar containing Oxyrase (Oxyrase, Mansfield, Ohio), a commercial enzyme additive of *E. coli* membrane fractions that contains the cytochrome-based electron transport system that reduces free oxygen to water by transferring hydrogen from donors in the medium. The plates were placed in an airtight jar containing a disposable hydrogen- and carbon dioxide-generating envelope (BBL GasPak Plus) and grown anaerobically at 30°C for 3 days. The cells were scraped off the plates and resuspended in sterile ddH_2O, washed once in sterile ddH_2O, and resuspended in 1 ml of sterile ddH_2O. A portion of each culture was diluted in ddH_2O and plated on two YPD plates. The remainder of the culture was plated onto three YPGD plates at a density of not more than 5.2×10^3 cells per mm^2. YPD plates were counted after 2 days at 30°C, and YPGD plates were counted after 7 days at 30°C.

RESULTS

The Reversion Rate of Single Point Mutations in the Presence and Absence of MMR. The *cyc1* reversion assay utilizes six strains with different point mutations in codon 22. This cysteine residue is essential for activity of iso-1-cytochrome *c* (the gene product of the *CYC1* gene) because it and Cys-19 form a covalent thioether linkage to a heme prosthetic group that is required for activity (20). Strains with mutations at Cys-22 cannot grow on a nonfermentable carbon source such as glycerol. The advantage of this assay is that only true revertants will give a Cyc$^+$ phenotype (20). We have tested our revertants by using a PCR assay that will amplify only if the wild-type sequence is present; all tested revertants, picked at random, were true revertants (data not shown).

Table 1 shows the reversion rates of the six different *cyc1* Cys-22 point mutations in both isogenic wild-type and MMR-defective strains. We found, as did Hampsey (20), that it was necessary to use diploid strains for all reversion assays. Two strains, YMH53 and YMH56, have reversion rates that are at or below the level of sensitivity of the assay. A third strain, YMH55, showed a low but similar reversion rate in both MMR-proficient and -defective strains. However, the remaining three strains showed large increases in reversion rates in strains deficient in Msh2p or Msh6p, but not Msh3p. We did not anticipate any effect of *MSH3* on single-base pair mismatches, as the evidence suggests that Msh2p and Msh3p form a complex that recognizes small insertion/deletion mispairs of 1 to 4 nucleotides, whereas Msh2p and Msh6p form a complex that can recognize single-base pair mismatches as well as insertion/deletion mispairs of 1 to 2 nucleotides (9, 10).

Table 1. Reversion rates of wild-type and MMR-deficient strains

		Mismatch required, template/nascent		Reversion rate, $\times 10^{-6}$			
Strain	Codon 22	Top strand	Bottom strand	Wild type	*msh2*	*msh3*	*msh6*
YMH52	CGC	C/A	G/T	0.0024	1.7	0.0033	1.5
YMH53	AGC	A/A	T/T	<0.0001	<0.0001	<0.0001	<0.0001
YMH54	GGC	G/A	C/T	0.0024	5.6	0.0027	6.3
YMH55	TCC	C/C	G/G	0.0011	0.0012	0.0019	0.0012
YMH56	TTC	T/C	A/G	0.0001	0.0002	0.0001	0.0013
YMH57	TAC	A/C	T/G	0.0001	0.31	<0.0001	0.33

This table shows the reversion rates of *CYC*1 codon 22 to the wild-type codon (TGC) and the mismatches that would have to be tolerated for this reversion event to occur during replication.

The relative reversion rates of the strains are compared in Table 2. The overall replication fidelity of the various point mutations is roughly equivalent as can be seen by the similar wild-type reversion rates of all of the strains. The rate at which various mispairs are generated by DNA polymerase and escape proofreading can be observed in the MMR-deficient strains, and it is clear that different mispairs arise at very different rates. The reversion rates of the mutations in YMH53, YMH56, and YMH55 are not measurably affected by the presence or absence of MMR, whereas the mutations in YMH57, YMH52, and YMH54 show an increase in reversion rate of up to 60,0000-fold in the absence of MMR.

The Reversion Rates of Strains Grown Anaerobically. There is a strand bias in the generation of mispairs; both YMH56 and YMH54 can revert via G/A or C/T mismatches, but with very different rates. When the A and T are on the template strands, as in YMH56, the reversion rate is very low in all strains; when the G and C are on the template strands (as in YMH54), *msh2* and *msh6* reversion rates are three orders of magnitude higher than in YMH56. YMH54, which has the highest reversion rate of all six strains, reverts via GC→TA transversions. Such transversions are a common product of an oxidatively damaged guanine, 8-hydroxy-2′-deoxyguanosine (8-oxodG), which can mispair with an adenine (26). To test whether oxidative damage was playing a role in the reversion rate of any of the highly reverting strains, we grew the strains with high reversion rates in an oxygen-deprived atmosphere in an attempt to reduce oxidative damage in the cells. Because the *CYC1* gene is expressed only under aerobic conditions, cells grown anaerobically were plated on selective plates under aerobic conditions, thus limiting to some extent the protective effect of anaerobic growth. Control experiments showed that the plating efficiency of Cyc^+ and Cyc^+ *msh2* cells on selective plates was not affected by the change in growth conditions (results not shown). The reversion rate was decreased in all three strains grown under anaerobic conditions (Table 3).

Table 3. Comparison of aerobic reversion rate versus anaerobic reversion rate

Strain	Reversion rate, $\times 10^{-6}$		Anaerobic suppression	Mutation
	Aerobic	Anaerobic		
YMH57/*msh2*	0.31	0.025	12	T → C
YMH57/*msh6*	0.33	0.0099	33	
YMH52/*msh2*	1.7	0.038	45	C → T
YMH52/*msh6*	1.5	0.051	29	
YMH54/*msh2*	5.6	0.12	47	G → T
YMH54/*msh6*	6.3	0.10	63	

Anaerobic suppression is the ratio of aerobic to anaerobic reversion rates. The mutation shown is the one most likely to occur due to oxidative damage (e.g., G → T rather than C → A).

DISCUSSION

A Reversion Assay for Specific Point Mutations. By using the *cyc1* reversion assay, we have been able to determine the rate at which various mismatches are generated. In the presence of MMR, there is less than a 30-fold difference in the rate at which different mismatches escape MMR and lead to reversion events. However, in the absence of MMR, some mismatches occur at a 60,000-fold greater rate than other mismatches. What could account for the large variation in the rate at which mismatches occur in the absence of MMR? One possibility is that some mismatches are more easily incorporated by polymerase and are ineffectively proofread. Although DNA polymerases and proofreading are not equally accurate for all base–base combinations, there is no *in vitro* evidence for such wide differences in polymerase and proofreading efficiencies (27, 28). The realization that the highest reversion rates were those most likely to result from oxidative damage to the DNA led to attempts to reduce spontaneous oxidative damage in the cells. We found that growing the cells under anaerobic conditions reduced the reversion rates of the three highly reverting strains, suggesting that oxidative damage to the DNA is an important contributor to the high reversion rates observed.

MMR and Damaged DNA. Although the standard textbook view of MMR portrays its role as preventing mutation caused by base–base mismatches between normal DNA bases, there is much evidence concerning MMR and damaged bases (29). MMR-defective cells of many organisms display tolerance to alkylation damage; alkylation-induced killing is most commonly explained by continued MMR-driven excision of bases added opposite alkylated bases (29). Cell lines selected for resistance to alkylating agents have been found to be defective in MMR (30). This finding has clinical relevance because some chemotherapeutic agents are alkylating agents and many cancers are caused by MMR defects (31). MMR-defective cells are also resistant to ionizing radiation (32) and to treatment with cisplatin and adriamycin, both common antitumor drugs (33–35). It is clear from these results that MMR plays a significant role in recognition of exogenously damaged DNA. However, the role that MMR may play in recognition of spontaneously damaged DNA has received little attention. It has been estimated that oxidative damage is the most common form of spontaneous DNA damage; in steady-state rat cells there are approximately 24,000 8-oxodG lesions per cell (36). There are repair mechanisms for oxidatively damaged DNA, but the high level of these lesions suggests that repair lags behind damage. Additionally, it appears that 8-oxodG/A mispairs are slowly repaired in nonreplicating mammalian cells, whereas 8-oxodG/C mispairs are efficiently repaired (37). There is little direct data pertaining to the interaction of MMR and oxidatively damaged bases in eukaryotic cells. However, it has recently been found that transcription-coupled repair of thymine glycols depends on the MMR system in yeast (38), so it appears that MMR can recognize at least some forms of oxidative damage. The dramatic lowering of the reversion rates when three of the MMR-defective strains are grown anaero-

Table 2. Comparison of relative reversion rates

Strain	Relative reversion rate		Suppression by mismatch repair *msh2*	Relative reversion rate *msh6*	Suppression by mismatch repair *msh6*
	Wild-type	*msh2*			
YMH53 (A/A;T/T)	1	1	1	1	1
YMH56 (T/C;A/G)	1	2	2	13	13
YMH57 (A/C;T/G)	1	3,100	3,100	3,300	3,300
YMH55 (C/C;G/G)	11	12	1.1	12	1.1
YMH52 (C/A;G/T)	24	17,000	710	15,000	630
YMH54 (G/A;C/T)	24	56,000	2,300	63,000	2,600

Each parental reversion rate is compared to the lowest measurable rate of 1×10^{-10}. Relative reversion rates are compared to the wild type. Note that the reversion rates shown for YHM53 are too low to be measured accurately and may be lower than indicated in the table.

bically is consistent with high reversion rates being caused by oxidative damage. Although we cannot rule out alternative explanations for the lowered reversion rates under anaerobic growth conditions, such as more efficient repair systems in anaerobic cells, such explanations fail to explain the large differences we see with aerobic growth conditions. It should be noted that although growing the strains anaerobically may be expected to reduce oxidative damage, there are still many necessary internal oxygen-generating processes at work, especially because functioning mitochondria are required for growth of the strains on selective plates. The three mutations that are increased in MMR-defective strains, G → T, C → T, and T → C, are those frequently observed in a human leukemia cell line subjected to increased oxidative damage (39). The G → T transversions observed in strain YMH54 are likely the result of misinsertion of A opposite 8-oxodG. The exact lesions responsible for mutation in the other strains are not clear, although it is likely that oxidized cytosines are responsible for the C→T transitions (40). Thymine glycol is one of the major products of oxidative damage, but it has not been found to be very mutagenic, at least in MMR-proficient cells (41). However, both thymine glycol and urea (a hydrolytic product of thymine glycol) have the potential to miscode, with the misinserted base being a G, which would create T → C transitions (42). It may be that the mutagenic potential of these compounds is masked in MMR-proficient cells.

We suggest two alternative possibilities for the response of MMR to oxidative damage in the template strand of DNA. One possibility is that, for example, MMR recognizes an 8-oxodG/A pair as incorrect but accepts an 8-oxodG/C pair; the A in the 8-oxodG/A mispair would be removed, and during resynthesis, it would likely be replaced by a C. The other possibility is that MMR would not accept any base opposite an oxidatively damaged base. In both cases, there then would be an opportunity for *OGG1* (43, 44) to repair the 8-oxodG in a resynthesized 8-oxodG/C pair before the initiation of another round of MMR. This second possibility is consistent with the greater repair efficiency of 8-oxodG/C compared with 8-oxodG/A that is observed in mammalian cells (37). MMR may be particularly important for this type of mispair in yeast because there is no sequence homologue of MutY (see below) and no equivalent repair activity has been found. In either case, MMR would prevent mutation but would not repair the oxidative damage.

The suggestion that the most important role of MMR in preventing point mutations in our strains is its recognition of mispairs involving damaged bases provides an interesting parallel with the role of MutY, which was thought originally to be specific for A/G mispairs (45) but was later found to have an 8-oxodG/A mispair as its primary substrate, removing the mispaired A (46). This finding does not negate the role of MMR in repair of mismatches involving normal bases. It may be that in other organisms or in some cellular circumstances, oxidative damage occurs less often or is repaired more efficiently, with the result that the role of MMR in normal base MMR would be more important. In *E. coli*, the 8-oxodG repair pathway very efficiently removes oxidative damage (47) and there seems to be little effect of the MMR system on oxidative damage (48, 49). In line with this observation, a deficiency in MMR in *E. coli* results in very few GC→TA transversions (50). Yeast may lack some of the genes involved in oxidative-damage repair that are found in *E. coli* and thus may have an increased reliance on MMR to prevent mutations caused by oxidative damage.

There are relatively few data for yeast with which to compare our results. Sequencing of mutations in the *CAN1* gene in MMR-defective cells revealed a pattern consistent with these results, with 26/28 sequenced mutations in one of the three classes of highly reverting mutations and a distribution of those mutations similar to those observed here (10). The majority of mutations observed with the *SUP4-o* assay system in MMR-defective strains also were the same as we observed, although there were substantial numbers of other mutations (19). Unlike the other assay systems, the *SUP4-o* gene is carried on a plasmid, and it may be that error rates in plasmid replication are different from chromosomal genes.

Implications of MMR and Damage Recognition. Although defects in MMR are recognized to be a major cause of hereditary nonpolyposis colon carcinoma (51, 52), there are a number of other cancer types that are associated with defects in MMR (31). The distribution of these cancers has been puzzling—MMR proteins are ubiquitously distributed, but there are certain tissue types that seem to be particularly vulnerable to loss of MMR. One explanation may be that certain tissues are likely to suffer more spontaneous damage.

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