Distinct roles for the *Saccharomyces cerevisiae* mismatch repair proteins in heteroduplex rejection, mismatch repair, and non-homologous tail removal.

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Running Head: Distinct roles for mismatch repair proteins

Key words: non-homologous tail removal, heteroduplex rejection, mismatch repair

Abbreviations: MMR, mismatch repair; DSB, double-strand break, SSA, single-strand annealing.

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ABSTRACT

The *Saccharomyces cerevisiae* mismatch repair (MMR) protein *MSH6* and the *SGS1* helicase were recently shown to play similarly important roles in preventing recombination between divergent DNA sequences in a single-strand annealing (SSA) assay. In contrast, MMR factors such as Mlh1p, Pms1p, and Exo1p were shown to not be required, or to play only minimal roles. In this study we tested mutations that disrupt Sgs1p helicase activity and Msh2p-Msh6p mismatch recognition and ATP binding and hydrolysis activities for their effect on preventing recombination between divergent DNA sequences (heteroduplex rejection) during SSA. The results support a model in which the Msh proteins act with Sgs1p to unwind DNA recombination intermediates containing mismatches. Importantly, *msh2* mutants were characterized that displayed separation of function phenotypes with respect to non-homologous tail removal during SSA and heteroduplex rejection. These studies suggest that non-homologous tail removal is a separate function of Msh proteins that is likely to involve a distinct DNA binding activity. The involvement of Sgs1p in heteroduplex rejection but not non-homologous tail removal further illustrates that subsets of MMR proteins collaborate with factors in different DNA repair pathways to maintain genome stability.
INTRODUCTION

Recombination between identical or nearly identical DNA sequences scattered throughout a genome can result in potentially lethal chromosomal rearrangements including deletions, insertions, inversions, and translocations (SCHMID 1996). Studies in bacteria, yeast, and humans have identified factors that act to promote and prevent such types of recombination events (reviewed in EVANS and ALANI 2000; PÂQUES and HABER 1999; SURTEES et al. 2004). In *Escherichia coli*, the RecA strand exchange and RuvAB branch migration enzymes are capable of promoting recombination between DNA sequences that display up to 10% sequence divergence. In contrast, the MutS and MutL mismatch repair (MMR) proteins and the RecBCD nuclease act to suppress recombination between slightly divergent, or homeologous, DNA sequences (RAYSSIGUIER et al. 1989; SHEN and HUANG 1989; WORTH et al. 1994; FABISIEWICZ and WORTH 2001; ZAHRT and MALOY 1997; STAMBUK and RADMAN 1998). These studies suggest that recombination events between divergent DNA sequences reflect a balance between those that generate genetic diversity and those that promote genome stability.

MMR proteins are highly conserved and their biochemical activities have been well characterized. In *E. coli*, DNA mismatches and insertion-deletion loops generated both during DNA replication and genetic recombination are recognized by MutS. MutS binding to mismatched DNA results in the recruitment of MutL followed by activation of the MutH endonuclease. This leads to nicking of the newly synthesized, unmethylated, DNA strand. These interactions promote unwinding by UvrD helicase of the newly replicated strand towards the mismatch, which is followed by excision of the mismatch site by single-strand exonucleases. Resynthesis of the gapped DNA results in repair of the mismatch using the parental strand as a template (MODRICH and LAHUE 1996; SCHOFIELD and HSIEH 2003).

Eukaryotes contain six MutS homologues (Msh 1-6 proteins), and four MutL homologues (Mlh1-3 proteins, Pms1p). These proteins are present as Msh and Mlh heterodimers *in vivo*. Msh2p-Msh6p recognizes base-base mismatches and single nucleotide insertion-deletions, while
Msh2p-Msh3p displays specificity for insertion-deletion loops up to 12 nucleotides in length (reviewed in KOLODNER and MARSISCHKY 1999). The Mlh1p-Pms1p complex acts as the major Mlh heterodimer in MMR and is thought to coordinate Msh-DNA binding with downstream repair factors. Recent studies have suggested that such factors include Exo1, a 5′-3’ exonuclease that has been implicated in excision steps, the clamp loader RFC, and the processivity clamp PCNA (SCHOFIELD and HSIEH 2003; DZANTIEV et al. 2004).

In addition to its role in post-replicative MMR, Msh2p-Msh3p is involved in facilitating single-strand annealing (SSA) events when a double-strand break (DSB) occurs in a region flanked by direct repeats (SUGAWARA et al. 1997). SSA is a major repair pathway in many organisms, including mammals, and appears to be the predominant pathway for the repair of breaks occurring between repeated DNA sequences (LIANG et al. 1998). In SSA, a DSB is processed by a 5′-3’ exonuclease activity to expose complementary sequences. Annealing of the sequences results in an intermediate that contains 3’ single-strand tails that must be removed before DNA resynthesis and ligation steps can occur (Figure 1A). Msh2p-Msh3p plays an important role in this process when the complementary regions are less than 1 kb in length (SUGAWARA et al. 1997), and when the non-homologous tails are greater than 30 nucleotides in length (PÂQUES and HABER 1997). Based on these findings, Msh2p-Msh3p has been hypothesized to act during SSA by stabilizing the annealed region, and/or by recruiting the Rad1p-Rad10p endonuclease to the homology-non-homology junction (SUGAWARA et al. 1997). Consistent with these activities, in vitro experiments have shown that Rad1p-Rad10p cleaves DNA substrates containing 3’ single-stranded tails (SUNG et al. 1993; TOMKINSON et al. 1993; BARDWELL et al. 1994), and physical interactions between Msh2p-Msh3p and Rad1p-Rad10p have been observed (BERTRAND et al. 1998). No other components of the nucleotide excision repair or MMR pathways are required in this process (SUGAWARA et al. 1997).

Models to explain how the MutS and MutL family proteins prevent homeologous recombination, also known as heteroduplex rejection, have been developed based on biochemical
and genetic studies (reviewed in EVANS and ALANI 2000). These studies suggest that MMR proteins act to prevent homeologous recombination by transmitting mismatch recognition signals to factors that act in early recombination steps. *In vitro* strand exchange studies involving homeologous DNA substrates and the *E. coli* RecA, MutS, and MutL proteins suggested that MutS and MutL block homeologous strand exchange by interacting with both RecA and the DNA mismatches formed in heteroduplex DNA (WORTH et al. 1994; FABISIEWICZ and WORTH 2001). In *S. cerevisiae*, DATTA et al. (1996) showed that the MutS (Msh2p, Msh3p, Msh6p) and MutL (Mlh1p, Pms1p) homologs displayed anti-recombination activities in an intron-based recombination assay involving inverted repeat sequences. They found that the Msh proteins were required to prevent homeologous recombination when sequences were slightly divergent, but had little effect on highly divergent DNA where strand transfer based on Watson-Crick base pairing was expected to be severely impaired. Mutations in the *MSH* genes conferred the strongest derepression of homeologous recombination whereas mutations in the *MLH* genes, *EXO1*, and *RAD1* caused more modest effects (DATTA et al. 1996; CHEN and JINKS-ROBERTSON 1999; NICHOLSON et al. 2000; SUGAWARA et al. 2004). At present, it is unclear why there is a differential requirement for such factors. One possibility is that like post-replicative MMR, prevention of homeologous recombination involves redundancy at several steps. Alternatively, different sets of interactions may be involved between the MutS homologues and downstream factors during MMR and heteroduplex rejection.

Recently, *sgs1* null mutants of *S. cerevisiae* were shown in mitotic gene conversion and SSA assays to be defective in suppressing homeologous recombination (MYUNG et al. 2001; SUGAWARA et al. 2004; SPELL and JINKS-ROBERTSON 2004). Sgs1p, a homolog of the *E. coli* RecQ protein, is a 3'-5' helicase that can unwind duplex and partially duplex DNA. *In vitro* studies have shown that Sgs1p can also extend DNA pairing and disrupt joint molecules formed by aberrant recombination (HARMON and KOWALCZYKOWSKI 1998). *sgs1* mutants display genomic instability phenotypes including increased sister chromatid exchange, chromosome non-disjunction, hyper-recombination, and defects in DNA replication (reviewed in COBB et al.)
An attractive model to explain the role of Sgs1p in preventing homeologous recombination is that it is recruited by Msh proteins to unwind heteroduplex DNA containing mismatches. This would allow the unwound DNA to participate in another homology search (SUGAWARA et al. 2004). Consistent with this model is the finding that a human homolog of Sgs1p, BLM, interacts with human Msh6p (PEDRAZZI et al. 2003).

We conducted genetic and physical analyses of MSH2, MSH6, and SGS1 alleles with the goal of understanding how these factors participate in preventing homeologous recombination. Specific mutations known to disrupt biochemical activities of Msh2p, Msh6p and Sgs1p (mismatch binding, ATP binding, and helicase) were constructed and strains bearing these mutations were examined in a recently developed SSA assay involving homeologous repeat sequences. This assay is of special interest because of the factors known to be important in preventing homeologous recombination, only the Msh and Sgs1 proteins appear to play critical roles. Our results support the idea that the Msh proteins interact with Sgs1p to unwind DNA recombination intermediates containing mismatches. Importantly, we found that msh2 and msh6 mutants defective in MMR were also defective in heteroduplex rejection. We also identified an msh2 mutant defective in non-homologous tail removal but functional in MMR and heteroduplex rejection. These studies suggest that the mode of DNA binding during MMR and homeologous rejection in this assay is likely to be distinct from that required for non-homologous tail removal. It also indicates that subsets of the MMR proteins act to maintain genome stability by collaborating with factors belonging to different DNA repair pathways.

MATERIALS AND METHODS

Saccharomyces cerevisiae strains tested in the SSA assay. EAY1141 [mat::leu2::hisG hmrΔ3 thr4 leu2 trp1 THR4-ura3-A(205bp)-HOcs-URA3-A ade3::GAL10-HO::NAT] and EAY1143 [mat::leu2::hisG hmrΔ3 thr4 leu2 trp1 THR4-ura3-F(205bp)-HOcs-URA3-A ade3::GAL10-HO::NAT] were the parental strains tested in the SSA assay. Both strains contain two 205 bp repeats of URA3 sequence separated by 2.6 kb of DNA containing pUC9 DNA, the HO recognition sequence, and lambda DNA (described in SUGAWARA et al. 2004). These
strains contain the \textit{GAL10-HO} construct integrated into the \textit{ADE3} locus. \textit{EAY1141} is designated as “A-A” because it contains identical repeats of the \textit{URA3} repeat sequence. \textit{EAY1143} is designated as “F-A” because one of the \textit{URA3} repeat sequences contains seven single site mutations. Mutant derivatives of the \textit{EAY1141} (A-A) and \textit{EAY1143} (F-A) parental strains were constructed by single step gene replacement using the lithium acetate method (GEITZ and SCHIESTL 1991). Single step integration vectors for \textit{MSH2} (\textit{MSH2-HA4::LEU2, AatII, PvuII} digestion of p\textit{EAI118}), \textit{MSH6} (\textit{MSH6::KANMX, KpnI, BstEII} digestion of p\textit{EAI186}), \textit{SGS1} (\textit{SGS1::KANMX, XhoI, BamHI} digestion of p\textit{EAI195}), and mutant derivatives were constructed such that the indicated selectable markers were inserted downstream of the open reading frame but within homologous sequence, allowing for targeted gene replacement. The insertion of the selectable marker downstream of the open reading did not disrupt wild type gene function.

Derivatives of \textit{EAY1141} include \textit{EAY1400 (msh2\textDelta::TRP1)}, \textit{EAY1309 (msh2-K564E-HA4::LEU2)}, \textit{EAY1377 (msh2\Delta1-HA4::LEU2)}, \textit{EAY1225 (msh2-S656P-HA4::LEU2)}, \textit{EAY1314 (msh2-R730W-HA4::LEU2)}, \textit{EAY1387 (msh6\Delta::KANMX)}, \textit{EAY1350 (msh6-F337A::KANMX)}, \textit{EAY1347 (msh6-G987D::KANMX)}, \textit{EAY1392 (sgs1\Delta::KANMX)}, \textit{EAY1381 (sgs1-hd::KANMX)}, \textit{EAY1343 (sgs1\Delta644::KANMX)}, and \textit{EAY1333 (sgs1\DeltaC795::KANMX)}.

Derivatives of \textit{EAY1143} include \textit{EAY1401 (msh2\Delta::TRP1)}, \textit{EAY1227 (msh2-K564E-HA4::LEU2)}, \textit{EAY1260 (msh2\Delta1-HA4::LEU2)}, \textit{EAY1267 (msh2-S656P-HA4::LEU2)}, \textit{EAY1265 (msh2-R730W-HA4::LEU2)}, \textit{EAY1388 (msh6\Delta::KANMX)}, \textit{EAY1352 (msh6-F337A::KANMX)}, \textit{EAY1297 (msh6-G987D::KANMX)}, \textit{EAY1354 (sgs1\Delta::KANMX)}, \textit{EAY1326 (sgs1-hd::KANMX)}, \textit{EAY1345 (sgs1\Delta644::KANMX)}, and \textit{EAY1336 (sgs1\DeltaC795::KANMX)}.

\textbf{SSA time courses.} Stationary phase cultures of the above strains were diluted into YP (ROSE \textit{et al}. 1990) medium supplemented with lactate (2\% w/v final concentration) and grown until mid-log phase (1-2 x 10^7 cells/ml). The cultures were then induced with galactose (2\% w/v final concentration; US Biological) and 45 ml samples were collected at various time points. Thirty minutes after induction, \textit{HO} expression was suppressed by the addition of glucose (2\% w/v final concentration). After centrifugation, each sample was washed with 1 ml ddH_2O,
resuspended in 0.4 ml DNA extraction buffer (2% SDS, 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0), and then added to tubes containing 0.4 ml glass beads (425-600 micron; Sigma) and 0.4 ml phenol:chloroform (1:1). Samples were vortexed for 5 minutes at 4 °C, followed by phenol:chloroform extraction. DNA was precipitated by adding 30 µl 3M Na Acetate (pH 5.2) and 600 µl isopropanol, and washed with 1 ml 75% ethanol. Samples were RNase treated in 0.4 ml of RNase buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 25 µg/ml RNase (Sigma)) for 1 hour at 37 °C, followed by phenol:chloroform extraction and DNA precipitation as described above. DNA was resuspended in 50 µl TE.

**Southern blot analysis.** DNA samples from the above time courses were digested with *Bgl*II (New England Biolabs) and run on 1% agarose gels with 1X TAE buffer. Southern blot transfer and hybridizations were performed essentially as described by the manufacturer (Amersham) and SUGAWARA et al. (2004). Blots were visualized using the Phosphor Imaging system and quantified using the Imagequant program (Molecular Dynamics). The probe used to visualize SSA products was an 880 bp *Hind*III-*Bam*HI fragment downstream of *URA3* obtained by digesting plasmid pNSU151 with *EcoRI* (SUGAWARA et al. 2004). The loading control probe consisted of a 600 bp PCR generated *RAD10* fragment amplified using primers TP30 (5’GGTCACAGCAAGATTTTCATC) and AO641 (5’TAAGGGCTGCATTCTCCTAGAG). Probes were synthesized using an NEBlot kit with 32P-dCTP as directed by the manufacturer (New England Biolabs), and were purified using Bio-Spin P30 columns as suggested by the manufacturer (Bio-Rad). To measure product formation, the intensity of the product band 5 hr after *HO* induction was divided by the intensity of the 0 hr uncut band. Both the 5 hr and 0 hr bands were normalized to the *RAD10* loading control band. For each strain, average product formation and standard deviation were calculated from three to six independent experiments.

**Cell viability analysis.** Cell survival during SSA with both homologous and homeologous recombination was determined as described previously (SUGAWARA et al. 2004). Briefly, cells were pre-grown in YP-lactate medium (2% w/v final concentration) to mid-log phase and plated on YP plates containing either glucose (2% w/v final concentration) or
galactose (2% w/v final concentration). The efficiency of SSA was measured by determining the number of cells growing on the galactose plates compared to those on the glucose plates. Cell viability data was determined by calculating the average and standard deviation of three to seven independent experiments for each strain.

**Determination of mutation rates.** The rate per generation of forward mutation to canavanine resistance was calculated from the median mutation frequency using the method of LEA and COULSON (1949). The forward mutation rate to canavanine resistance (REENAN and KOLODNER 1992) was measured in EAY745 (MATa, HMRa, Δhml::ADE1, Δho, ade1-100, leu2-3, 112, lys5, trpl::hisG, ura3-52, ade3::GAL-HO, MSH2-HA4::LEU2) and msh2Δ (EAY969), msh3Δ (EAY854), msh6Δ (EAY855), and msh2Δ1 (EAY1398) mutant derivatives. The mutation rate and 95% confidence interval were determined from 19 independent measurements for each strain.

**Western blot analysis.** Cell lysates derived from mid-log cultures of EAY1143 (MSH2), EAY1257 (MSH2-HA4::LEU2), and EAY1378 (msh2Δ1-HA4::LEU2) were separated on 8% SDS-PAGE gels and transferred to nitrocellulose membrane (BioRad) using a semi-dry electrophoretic transfer system (BioRad). Western blot analysis was carried out with primary antibody specific to HA (12CA5, Roche) and secondary anti-mouse IgG antibody at 1:2,000 and 1:5,000 dilutions, respectively. The loading control was detected using primary antibody to glucose 6-phosphate dehydrogenase (Sigma) and secondary anti-rabbit IgG antibody at 1:20,000 and 1:10,000 dilutions, respectively. Detection was carried out using ECLPlus (Amersham) according to the manufacturer’s instructions.

**RESULTS**

**Mismatch binding by both Msh2p and Msh6p is required for rejection of SSA between homeologous substrates.** SUGAWARA et al. (2004) developed a SSA assay using two 205 bp sequences that were either identical in sequence, or contained 3% sequence divergence, to identify factors that suppress recombination between divergent DNA sequences (Figure 1A). In the homeologous recombination substrate, one copy of a URA3-containing
sequence (“F”) contains seven substitutions (six single base pair substitutions and one base pair insertion/deletion) relative to the “A” sequence. Heteroduplex DNA formed between the A and F sequences is predicted to contain mismatches that are recognized by Msh2p-Msh6p (SUGAWARA et al. 2004). Southern blot and cell viability analyses were used to measure SSA product levels following induction of a DSB by the HO endonuclease in both the A-A and F-A strains (Figure 1B, C). Rejection of homeologous recombination results in the failure to repair the DSB, resulting in the loss of cell viability. As shown in Table 1, the ratios of product formation and cell viability for A-A versus F-A strains provide a consistent measure of heteroduplex rejection. For wild type strains, these values were 3.5 and 3.4 for product formation and cell viability, respectively.

Substrate competition and viability studies have suggested that the prevention of homeologous recombination, termed heteroduplex rejection, occurs by a mechanism in which mismatch recognition by the Msh proteins results in the recruitment of proteins that facilitate unwinding of the heteroduplex DNA (SUGAWARA et al. 2004). To identify activities in Msh2p-Msh6p required for rejection, we tested the effects of point mutations in both MSH2 and MSH6 using Southern blot analysis and cell viability assays (Figures 1-4, Table 1). This work was guided by biochemical analyses of mutant Msh2p-Msh6p complexes defective in DNA binding and ATP hydrolysis (KIJAS et al. 2003). The requirement for the Msh2p-Msh3p complex in SSA precluded us from looking at msh2 and msh3 null alleles. However, we were able to analyze msh2 separation-of-function mutations that confer strong defects in MMR but are proficient in SSA (STUDAMIRE et al. 1999).

The crystal structure of the E. coli and Thermus aquaticus (Taq) MutS-mismatch complexes revealed that MutS acts as an asymmetric homodimer, with each subunit making distinct interactions with the DNA (OBMOLOVA et al. 2000; LAMERS et al. 2000). MutS subunit A is thought to be equivalent to Msh6p, while subunit B corresponds to Msh2p. Conserved residues in domain I of subunit A and domain IV of subunit B are thought to be critical for mismatch binding. The phenylalanine from residue F39 of subunit A in the Taq MutS
structure is thought to intercalate with the DNA and base stack with the mismatch (OBMOLOVA et al. 2000; LAMERS et al. 2000). Residues within the anti-parallel β-sheet structure in domain IV, which includes K471, are thought to form hydrogen bonds with the sugar-phosphate backbone surrounding the mismatch. Mapping of *S. cerevisiae* Msh2p-Msh6p onto the *Taq* MutS structure revealed that F337 of Msh6p and K564 of Msh2p correspond to positions F39 and K471 of *Taq* MutS, respectively (KIJAS et al. 2003). Biochemical analyses of msh6-F337A and msh2-K564E indicated that these mutations cause defects in mismatch binding within the context of the Msh2p-Msh6p complex (BOWERS et al. 1999; KIJAS et al. 2003). However, weak DNA binding activity by msh2-K564Ep-Msh6p could still be observed in gel shift and DNA bending assays (KIJAS et al. 2003). As shown previously (STUDAMIRE et al. 1999) and in Figure 2 and Table 1, this mutation did not affect SSA, since product levels observed for completely homologous substrates (A-A) did not differ from those observed in wild type.

As shown in Figure 2 and Table 1, msh2-K564E was defective in heteroduplex rejection in the F-A assay, displaying product and cell viability ratios, 1.6, 2.0, respectively, that approached the levels seen in the msh6Δ strain (1.3, 1.4). The msh6-F337A mutation conferred a defect in heteroduplex rejection that resembled the msh6Δ mutation (A-A/F-A ratios of 1.4 for product, 1.4 for cell viability), suggesting a direct correlation between mismatch recognition by Msh2p-Msh6p and heteroduplex rejection. The finding that the msh2-K564E strain displayed a somewhat less severe defect than the msh6 strains suggests that the residual msh2p-Msh6p binding activity observed in this mutant (KIJAS et al. 2003) may be sufficient to promote a low level of heteroduplex rejection.

**A msh2 DNA binding domain I deletion mutant that is functional in MMR and heteroduplex rejection, but defective in non-homologous tail removal.** We used the *Taq* MutS crystal structure as a guide to make deletions of DNA binding domains I (amino acids 2-133, designated as *msh2ΔI*) and IV (amino acids 497-606, designated as *msh2ΔIV*) in Msh2p (OBMOLOVA et al. 2000). We were interested in testing the domain I deletion in Msh2p
because this domain makes very few contacts with the DNA mismatch substrate within the corresponding subunit in the MutS crystal structure (OBMOLOVA et al. 2000). As described above, the DNA binding domain IV of MSH2 contains the K564 residue that was shown to be important for mismatch recognition (KIJAS et al. 2003). The msh2Δ4 mutation conferred null-like phenotypes in MMR and non-homologous tail removal assays (data not shown). Surprisingly, a complete deletion of DNA binding domain I (amino acid 2-133) conferred only a weak defect in MMR as measured in the canavanine resistance assay (Figure 3A). This assay measures the forward mutation rate in the CAN1 gene, and was shown previously to be specific to DNA lesions recognized by Msh2p-Msh6p (MARSISCHKY et al. 1996).

Western blot analysis indicated that the msh2Δ1p was expressed at wild type levels (Figure 3B). However, the msh2Δ1 mutation conferred a severe defect in non-homologous tail removal in the Southern blot assay (Figure 3C, Table 1). Cell viability analysis indicated that cell survival in the A-A strain background was nearly as low in the msh2Δ1 strain (0.11 ± 0.05) as in the msh2Δ strain (0.04 ± 0.01). Consistent with a somewhat functional Msh2p-Msh6p complex, the A-A/F-A product formation and cell viability ratios for the msh2Δ1 strain (2.9, 2.8) were similar to that seen in wild type (3.5, 3.4), indicating that heteroduplex rejection was still functional (Table 1; Figure 3C). These data suggest that domain I in Msh2p plays an important functional role in non-homologous tail removal when acting within the Msh2p-Msh3p complex. It is important to note that the strong defect in non-homologous tail removal in msh2Δ1 strains made it difficult to accurately assess heteroduplex rejection (Table 1). The fact that msh2Δ1 strains displayed A-A/F-A ratios similar to wild type in both assays and that the mutant strain appeared functional for Msh2p-Msh6p mediated MMR support our conclusion. However, it will be important to test the effect of the msh2Δ1 mutation in other homeologous recombination assays that do not involve non-homologous tail removal (e.g. NICHOLSON et al. 2000).

ATP binding and hydrolysis by the MutS homologues is required for rejection. Genetic and biochemical studies have shown that the ATP binding domain in each subunit of Msh2p-Msh6p is required for MMR (OBMOLOVA et al. 2000; JUNOP et al. 2001;
Analysis of the Msh2p-Msh6p complex has led to a model where the two Msh subunits hydrolyze ATP sequentially, with the Msh6p ATPase activity acting as a mismatch sensor (Kijas et al. 2003; Studamire et al. 1998; Iaccarino et al. 1998). The role of ATP binding and hydrolysis in the rejection of homeologous recombination was tested by studying the effects of both Msh2p and Msh6p ATPase mutants in the SSA assay. The msh6-G987D mutation contains a substitution at the Walker A motif in Msh6p that is predicted to disrupt ATP binding. Previous work indicated that this mutant is capable of recognizing and binding mismatches, but is unable to signal mismatch recognition to activate downstream repair factors (Studamire et al. 1998; Kijas et al. 2003). The equivalent mutation in Msh2p (msh2-G693D) could not be studied in this assay because it causes a complete defect in non-homologous tail removal (Studamire et al. 1999). However, two msh2 separation-of-function mutations, msh2-R730W and msh2-S656P, were isolated that are functional in non-homologous tail removal yet defective in ATP binding and/or hydrolysis (Studamire et al. 1998; Kijas et al. 2003).

The msh2-R730Wp-Msh6p complex is functional for ATP-dependent recruitment of Mlh1p-Pms1p but is hypothesized to be defective in a late step in MMR, perhaps in the recycling of MMR components or the recruitment of downstream factors (Kijas et al. 2003). While this complex appears proficient in ATP binding and Mlh1p-Pms1p recruitment, it displays a significant defect in ATP hydrolysis (Kijas et al. 2003). The msh2-R730W mutation maps to a region on the Taq MutS crystal structure near residues thought to be important for γ-phosphate binding of the ATP molecule in the adjacent subunit (Kijas et al. 2003). The msh2-S656P mutation maps to a region on the Taq MutS crystal structure that is approximately 7 Å from the bound ATP, and could affect the structure of the ATP binding pocket (Kijas et al. 2003). Biochemical studies showed that msh2p-Msh6p complexes containing the msh2-S656P mutation display defects in both ATP binding and hydrolysis and in interacting with Mlh1p-Pms1p on a DNA mismatch substrate (Kijas et al. 2003). It is important to note that all of the ATP binding mutant Msh2p-Msh6p complexes display similar mismatch binding activities in the absence of
ATP in gel shift assays (KIJAS et al. 2003).

As shown in Figure 4 and Table 1, the msh6-G987D, msh2-R730W and msh2-S656P mutations all caused severe defects in heteroduplex rejection as seen in both Southern blot analysis (A-A/F-A ratios of 1.2-1.3) and cell viability (A-A/F-A ratios of 1.0-1.7) assays. The defect in homeologous rejection conferred by the msh2-R730W mutation was similar to that reported previously using a plasmid-based GAL10-HO induction system (SUGAWARA et al. 2004). Product formation and cell viability were unaffected by the msh6-G987D and msh2-R730W mutations in the A-A assay but were reduced in the msh2-S656P mutant, indicating that non-homologous tail removal was somewhat compromised in the msh2-S656P mutant, but not in the other two ATPase mutants (Figure 4, Table 1). It is important to note that while ATP binding by Msh2p-Msh6p is required for the formation of a complex containing a DNA mismatch substrate, Msh2p-Msh6p, and Mlh1p-Pms1p in MMR, the Mlh homologs were shown to have little to no effect on rejection in the SSA pathway (SUGAWARA et al. 2004). The finding that ATP binding and hydrolysis are required for heteroduplex rejection independent of a requirement for the MutL homologs suggests that the role of ATP binding and hydrolysis during the rejection of homeologous recombination is not likely to involve the formation of a ternary complex with Mlh1p-Pms1p. This indicates that the requirement for ATP binding and hydrolysis by the Msh proteins in heteroduplex rejection is likely to be distinct from that observed during MMR. One possible explanation of the results is that mismatch binding by Msh2p-Msh6p is not sufficient for rejection and that conformational changes induced by ATP binding and/or interactions with downstream factors are likely to be required for heteroduplex rejection.

**The helicase domain of Sgs1 is required for rejection of homeologous recombination.** The 1447 amino acid Sgs1p is a 3’-5’ helicase that contains an acidic amino terminal region and a conserved helicase motif (MULLEN et al. 2000). Because SUGAWARA et al. (2004) hypothesized that homeologous rejection occurs by an unwinding mechanism, we investigated the effect of sgs1 helicase mutations on heteroduplex rejection. The sgs1-hd allele contains a lysine to alanine substitution at position 706 that affects the ATP binding domain and
disrupts Sgs1p helicase activity (LU et al. 1996). Previous studies have indicated that sgs1ΔC795, an allele that contains a deletion of the C-terminal 795 amino acids, including the entire helicase domain, confers a phenotype that is less severe than the helicase point mutant in a subset of assays including MMS sensitivity, hyper-recombination, and growth in the presence of the top1 mutation (MULLEN et al. 2000). Based on this and other work, MULLEN et al. (2000) proposed that the amino terminal region of Sgs1p contains a functional domain that is somehow inhibited by the sgs1-hd point mutation. We also tested the sgs1ΔN644 mutation, an allele that contains a deletion of the first 644 amino acids of Sgs1p but retains the helicase domain (MULLEN et al. 2000). The sgs1ΔN644 mutation conferred a more severe phenotype than the sgs1-hd mutation in a subset of the assays listed above. As shown in Figure 5 and Table 1, all three alleles conferred a heteroduplex rejection phenotype that was indistinguishable from the sgs1Δ mutation. It is important to note that cell survival appeared to be higher in the A-A assay in the sgs1 strains (0.79-1.0) compared to wild type (0.61, Table 1). One way to explain this difference is that the hyper-recombination phenotype observed in sgs1 strains is able to overcome a block to recombination that is observed during SSA.

DISCUSSION

SSA represents a major homologous recombination pathway for the repair of double-strand breaks that occur between repeat sequences. Because factors involved in conservative recombination events such as Rad51, 54, 55, and 57 proteins are not required for SSA (IVANOV et al. 1996), this system represents a simplified, yet biologically relevant model to study the requirements for heteroduplex rejection. It is likely, however, that heteroduplex rejection during conservative recombination events (e.g. strand exchange during inter-homolog recombination) will involve mechanistic steps that are distinct from those that function during SSA. Mutations in the human homologues of some of the MMR genes and Sgs1p have been correlated with human diseases that are associated with genome instability. These consist of hereditary non-polyposis colorectal cancer for the MMR genes and Bloom’s, Werner’s and Rothmund-Thompson syndromes for three of the SGS1 homologs (HICKSON 2003; SCHOFIELD and
An analysis of these proteins in genetic recombination should provide a better understanding of how defects in these factors lead to disease susceptibility. The fact that Sgs1p acts in heteroduplex rejection but not non-homologous tail removal illustrates how subsets of MMR proteins collaborate with factors belonging to different genome stability pathways. An example of such interactions is shown for the SSA reaction (Figure 6). During heteroduplex rejection, Msh factors are thought to recognize mismatches in SSA intermediates and recruit the Sgs1p helicase to unwind the annealed region. In the absence of heteroduplex rejection, the SSA intermediate is thought to be repaired through a non-homologous tail removal pathway involving Msh2p-Msh3p and the Rad1p-Rad10p endonuclease. The identification of msh2 mutants proficient in one pathway but not the other (e.g. msh2Δ1, msh2-K564E, msh2-R730W) strengthens the idea that the pathways are distinct.

Studies in bacterial and eukaryotic systems indicated that msh null mutations confer the largest stimulation of homeologous recombination (CHEN and JINKS-ROBERTSON 1999; NICHOLSON et al. 2000; JUNOP et al. 2003; SUGAWARA et al. 2004). In E. coli, mismatch binding and ATP binding and hydrolysis by MutS are required for suppressing recombination between homeologous substrates (WORTH et al. 1998; FABISIEWICZ et al., 2001; JUNOP et al., 2003). In a comprehensive study, JUNOP et al. (2003) found that all of the mutS mutations that disrupted mismatch repair also conferred a defect in rejecting homeologous recombination. In this study we examined the effect of site-specific mutations in each subunit of the Msh2p-Msh6p heterodimer, with the goal of identifying the contributions made by each subunit in rejecting homeologous recombination. Like JUNOP et al. (2003), we found that mutations that disrupted MMR also caused defects in preventing homeologous recombination.

Mutations that disrupted mismatch binding in both MSH2 (msh2-K564E) and MSH6 (msh6-F337A) conferred defects in homeologous rejection, with the msh6-F337A mutation conferring a more severe defect that was indistinguishable from the msh6Δ mutation. The different phenotypes seen for the msh2-K564E and msh6-F337A mutants appear specific to anti-recombination; previous genetic studies showed that the msh2-K564E and msh6-F337A mutants
are similarly defective in MMR (STUDAMIRE et al. 1999). One explanation for the different phenotypes in the two assays is that there may be a different kinetic requirement for the Msh proteins during DNA replication, where MMR is thought to be coordinated with fork movement, and heteroduplex rejection, which occurs within the context of a relatively slow DNA repair event (see Figure 1). Thus the residual DNA binding activity observed for the msh2-K564Ep-Msh6p complex may be sufficient to reject homeologous recombination at some level (KIJAS et al. 2003; SCHOFIELD and HSIEH 2003).

In contrast to the subunit A domain I (msh6-F337A) and subunit B domain IV (msh2-K564E) DNA binding mutants, a complete deletion of DNA binding domain I of subunit B (msh2ΔI) caused only a weak defect in Msh2p-Msh6p specific MMR. A deletion of domain I is likely to enlarge the channel that is present in the MutS crystal structure (OBMOLOVA et al. 2000; LAMERS et al. 2000; Figure 3A). At present, it is not clear whether this channel plays any role in MMR or in the formation of the Msh diffusible clamp that is hypothesized to form in the presence of ATP (JUNOP et al. 2003; GRADIA et al. 1999). The Taq and E. coli MutS crystal structures indicated that the phenylalanine residue at position 39 and 36 of subunit A, respectively, intercalate with the mismatch, making direct contact with DNA (OBMOLOVA et al. 2000; LAMERS et al. 2000). Analogous substitutions in Msh2p-Msh6p showed that the msh6-F337A caused a dramatic defect in MMR while the msh2-Y42A mutation appeared silent (BOWERS et al. 1999; DUFNER et al. 2000). These results are consistent with the Taq and E. coli MutS crystal structure diagrams indicating that domain I of subunit B (the “Msh2p” subunit) does not make direct contact with the DNA (OBMOLOVA et al. 2000; LAMERS et al. 2000). An amino acid alignment analysis indicates that a lysine residue in Msh3p (amino acid 187) is located where a phenylalanine is present in Msh6p and E. coli and Taq MutS. Together, these observations provide additional evidence that Msh2p-Msh3p and Msh2p-Msh6p bind DNA lesions in distinct ways. Further support for this idea was obtained in a dinucleotide repeat instability assay (HENDERSON and PETES 1992), where we found that the msh2ΔI mutant displayed a DNA slippage phenotype similar to the msh3Δ mutant (E. A. and T. G., unpublished...
observations). A systematic investigation of the effect of the msh2Δ1 mutation, alone and in combination with other MMR mutations, on the repair of loop mismatches varying in size from 1-20 nucleotides will be important in determining whether the defect in non-homologous tail removal observed in msh2Δ1 strains extends to other Msh2p-Msh3p-dependent repair processes (HENDERSON and PETES 1992; SIA et al. 1997).

We investigated the effects of ATP binding mutations in the Msh2p and Msh6p subunits (msh2-S656P and msh6-G987D, respectively) on heteroduplex rejection. These experiments were performed to test whether mismatch binding alone by Msh2p-Msh6p was sufficient to elicit rejection. If this were the case, mutant Msh complexes defective in ATP binding/hydrolysis but proficient in mismatch binding would be functional in preventing homeologous recombination. Mutations predicted to disrupt ATP binding/hydrolysis in both subunits were tested because studies indicated that the two subunits of the heterodimer bind and hydrolyze ATP with different affinities and at different rates (STUDAMIRE et al. 1998; IACCARINO et al. 1998; BJORNSON et al. 2000; KIJAS et al. 2003; ANTONY and HINGORANI 2003). As shown in Figure 4, the msh6-G987D allele conferred a defect in heteroduplex rejection that was similar to the msh6Δ mutation. Although msh2-S656P strains displayed a defect in non-homologous tail removal, a comparison of product formation in the A-A and F-A assays clearly showed that this mutation conferred defects in heteroduplex rejection. Finally, the msh2-R730W strain showed a defect in homeologous rejection that was observed previously (SUGAWARA et al. 2004).

These data indicate that mismatch binding alone is not sufficient for rejection of homeologous recombination, and that the conformational changes in the Msh proteins induced by ATP binding and hydrolysis (e.g. KIJAS et al. 2003) are likely to be important during heteroduplex rejection processes to recruit downstream factors, such as the Sgs1 helicase protein (Figure 6).

Recent genetic and physical studies have implicated the Sgs1p helicase in rejecting homeologous recombination (MYUNG et al. 2001; SUGAWARA et al. 2004; SPELL and JINKS-ROBERTSON 2004). Null mutations in SGS1 display a variety of phenotypes including MMS sensitivity, synthetic lethality with SLX1-4, hyper-recombination, suppression of the top3
slow growth phenotype, and slow growth in the presence of the top1Δ mutation (GANGLOFF et al. 1994; MULLEN et al. 2000, 2001). Physical interactions in mammalian cell lines between hMSH6 and BLM suggest that these proteins could work in the same pathway (PEDRAZZI et al. 2003). We analyzed previously characterized alleles in Sgs1p (MULLEN et al. 2000) to determine the activities required for heteroduplex rejection in the SSA assay. Previous work with these alleles indicated that Sgs1p contains a bipartite structure consisting of a helicase domain and an amino terminal region. It was proposed that this amino terminal region could contain an activity such as a nuclease function like that found in WRN, or could be required for interactions with other proteins (MULLEN et al. 2000). Previous work demonstrated that protein levels from these alleles are as high or higher than those observed for the wild type protein, indicating that loss-of-function is not a result of unstable protein (MULLEN et al. 2000).

We found that that a helicase point mutant (sgs1-hd), an N-terminal truncation (sgs1ΔN644), and a C-terminal truncation (sgs1ΔC795) all showed defects in rejecting homeologous recombination (Figure 5). The null phenotype observed for sgs1-hd in our assay supports a model in which the helicase activity of Sgs1p is required to unwind intermediates formed during heteroduplex rejection. This is in agreement with previous data suggesting that heteroduplex rejection occurs by an unwinding mechanism (SUGAWARA et al. 2004; Figure 6). We were somewhat surprised that sgs1ΔC795 and sgs1-hd strains displayed indistinguishable defects in heteroduplex rejection because a previous analysis showed that the sgs1ΔC795 mutation conferred a less severe phenotype than sgs1-hd in MMS sensitivity assays, hyper-recombination, and sgs1 top1 complementation assays (MULLEN et al. 2000). One way to explain this difference is that the sgs1ΔC795 mutation disrupts interactions between Sgs1p and Msh6p or other factors involved in the rejection of homeologous recombination. Alternatively, the limited range of the heteroduplex rejection assay may prevent the detection of subtle differences in Sgs1p function. Experiments to test these ideas are planned.
ACKNOWLEDGEMENTS

We thank James Haber, Neal Sugawara, Steve Brill and the Alani lab for reagents, helpful discussions, and comments on the manuscript, and Ann Bernard and Miltiadis Kininis for initiating some of the experiments presented. T.G. was supported by a Natural Sciences and Engineering Research Council of Canada PGSB Award and E.A. by NIH grant GM53085.

LITERATURE CITED


Mitotic crossovers between diverged sequences are regulated by mismatch repair proteins

DUFNER, P., G. MARRA, M. RASCHLE, and J. JIRICNY, 2000 Mismatch recognition and
dNA-dependent stimulation of the ATPase Activity of hMutSα Is abolished by a single

DZANTIEV L., N. CONSTANTIN, J. GENSCHEL, R. R. IYER, P. M. BURGERS, and
P. MODRICH, 2004 A defined human system that supports bidirectional mismatch-

EVANS, E., and E. ALANI, 2000 Roles for mismatch repair factors in regulating genetic

FABISIEWICZ, A., and L. WORTH JR., 2001 *Escherichia coli* MutS,L modulate RuvAB-
dependent branch migration between diverged DNA. J. Biol. Chem. **276**: 9413-9420.

GANGLOFF, S., J. P. MCDONALD, C. BENDIXEN, L. ARTHUR, and R. ROTHSTEIN, 1994
The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog: a

GEITZ, R. D. and R. H. SCHIESTL, 1991 Applications of high efficiency lithium acetate
transformation of intact yeast cells using single-stranded nucleic acids as carrier. Yeast
**7**: 253-263.

GRADIA, S., D. SUBRAMANIAN, T. WILSON, S. ACHARYA, A. MAKHOV, J. GRIFFITH,
and R. FISHEL, 1999 hMSH2-hMSH6 forms a hydrolysis-independent sliding clamp on

HARMON, F. G., and S. C. KOWALCZYKOWSKI, 1998 RecQ helicase, in concert with RecA
and SSB proteins, initiates and disrupts DNA recombination. Genes Dev. **12**: 1134-1144.

HENDERSON, S. T., and T. D. PETES, 1992 Instability of simple sequence DNA in

IACCARINO I., G. MARRA, F. PALOMBO, and J. JIRICNY, 1998  hMSH2 and hMSH6 play distinct roles in mismatch binding and contribute differently to the ATPase activity of hMutSa. EMBO J. 17: 2677-2686.


MARSISCHKY, G. T., N. FILOSIS, M. F. KANE, and R. KOLODNER, 1996  Redundancy of
Saccharomyces cerevisiae MSH3 and MSH6 in MSH2-dependent mismatch repair.


RAYSSIGUIER, C., D. S. THALER, and M. RADMAN, 1989 The barrier to recombination between Escherichia coli and Salmonella typhimurium is disrupted in mismatch-repair

REENAN, R. A., and R. D. KOLODNER, 1992 Characterization of insertion mutations in the
Saccharomyces cerevisiae MSH1 and MSH2 genes: evidence for separate mitochondrial

ROSE, M. D., F. WINSTON, and P. HIETER, 1990 Methods in yeast genetics. Cold Spring
Harbor Laboratory Press, Cold Spring Harbor, N.Y.

SCHMID, C. W., 1996 Alu: structure, origin, evolution, significance and function of one-

SCHOFIELD M. J., and P. HSIEH, 2003 DNA mismatch repair: molecular mechanisms

SHEN, P. and H. HUANG, 1989 Effect of base pair mismatches on recombination via

instability in yeast: dependence on repeat unit size and DNA mismatch repair genes. Mol.

SPELL, R. M., and S. JINKS-ROBERTSON Roles of Sgs1 and Srs2 helicases in the
enforcement of recombination fidelity in Saccharomyces cerevisiae. Submitted.

STAMBUK, S., and M. RADMAN, 1998 Mechanism and control of interspecies
recombination in Escherichia coli. I. Mismatch repair, methylation, recombination and

STUDAMIRE, B., T. QUACH, and E. ALANI, 1998 Saccharomyces cerevisiae Msh2p and
Msh6p ATPase activities are both required during mismatch recognition. Mol. Cell. Biol.
18: 7590-7601.

STUDAMIRE, B., G. PRICE, N. SUGAWARA, J. E. HABER, and E. ALANI, 1999
Separation of function mutations in Saccharomyces cerevisiae MSH2 that confer
mismatch repair defects but do not affect non-homologous-tail removal during


FIGURE LEGENDS

FIGURE 1. Overview of the SSA assay involving homologous and divergent DNA substrates. A. A DSB is induced at an HO cut site flanked by \textit{URA3} homologous (A-A, left panel) or divergent (F-A, right panel) 205 bp repeat sequences. Repair of the DSB by SSA, followed by Msh2p-Msh3p and Rad1p-Rad10p mediated non-homologous tail removal and subsequent repair synthesis, results in a deletion between the \textit{URA3} repeats. B. The kinetics of the SSA repair reaction was monitored at times after \textit{HO} induction by Southern blot hybridization using the indicated probe in the A-A (EAY1141) and F-A (EAY1143) strains. The uncut, product, and cut bands are 8.3, 5.5, and 4.8 kb, respectively. Southern blot product formation is presented for each strain (MATERIALS and METHODS). C. Densitometric analysis of the data presented in Panel B.

FIGURE 2. The effect of \textit{msh2 (-K564E)} and \textit{msh6 (-F337A)} mismatch binding mutations on SSA in strains bearing the A-A and F-A repeat constructs. See Figure 1 for details.

FIGURE 3. The \textit{msh2Δ1} mutant displays a defect in non-homologous tail removal during SSA but is somewhat functional in Msh2p-Msh6p-mediated MMR. A. Canavanine resistance assays were performed with the indicated strains as described in the MATERIALS and METHODS. The rates, actual and relative to the wild type strain EAY745, are presented with 95% confidence intervals in parentheses. B. Western blot analysis of yeast strains bearing HA-tagged Msh2p and msh2Δ1p. Msh2-HA protein was detected with the 12CA5 anti-HA antibody. An antibody specific to glucose 6-phosphate dehydrogenase was used as a loading control. C. Effect of the \textit{msh2Δ1} mutation on SSA in strains bearing the A-A and F-A repeat constructs. Southern blots were performed and quantified as described in the MATERIALS and METHODS.

FIGURE 4. The effect of \textit{msh2 (-R730W, -S656P)} and \textit{msh6 (-G987D)} ATP binding domain mutations on SSA in strains bearing the A-A and F-A repeat constructs. See Figure 1 for details.
FIGURE 5. *sgs1* mutants are defective in heteroduplex rejection in the SSA assay. See MATERIALS and METHODS and the legend for Figure 1 for details.

FIGURE 6. A model showing heteroduplex rejection and non-homologous tail removal pathways acting on SSA intermediates. In one pathway, Msh2p-Msh6p recognizes mismatches in the SSA intermediate and recruits the Sgs1p helicase to unwind the annealed region. In the absence of heteroduplex rejection, the SSA intermediate is repaired through non-homologous tail removal involving Msh2p-Msh3p and Rad1p-Rad10p, followed by DNA synthesis and ligation steps. As described in the text, two classes of *msh2* mutations were identified. The *msh2-K564E* and *msh2-R730W* mutations did not disrupt Msh2p-Msh3p-mediated non-homologous tail removal but conferred defects in Msh2p-Msh6p-mediated heteroduplex rejection. In contrast the *msh2Δ1* mutation conferred severe defects in non-homologous tail removal but did not appear to disrupt heteroduplex rejection.
Goldfarb Fig. 1

A

Wild type A-A inner

F-A outer

B

Wild type A-A

Product: 1.05 ± 0.24

Wild type F-A

Product: 0.30 ± 0.05

C

Relative Product

Time Post-Induction (hours)
<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>A-A</th>
<th>F-A</th>
<th>A-A</th>
<th>F-A</th>
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<th>F-A</th>
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<td>0</td>
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<td></td>
<td>0.89 ± 0.09</td>
<td>0.57 ± 0.07</td>
<td>1.06 ± 0.20</td>
<td>0.74 ± 0.14</td>
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<td>0.5</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
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*Goldfarb Fig. 2*
A

<table>
<thead>
<tr>
<th>genotype</th>
<th>can^r rate (x 10^-6)</th>
<th>relative to wt</th>
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</thead>
<tbody>
<tr>
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<td>msh2Δ1</td>
<td>4.4 (3.3-5.1)</td>
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<tr>
<td>msh3Δ</td>
<td>1.7 (1.2-2.4)</td>
<td>1.1</td>
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<tr>
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<td>10.4 (8.5-14.3)</td>
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<tr>
<td>msh2Δ</td>
<td>19.8 (17.7-28.0)</td>
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</table>

B

C

<table>
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<tr>
<th>genotype</th>
<th>can^r rate (x 10^-6)</th>
<th>relative to wt</th>
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<tr>
<td>wild type</td>
<td>1.05 ± 0.24</td>
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</tr>
<tr>
<td>msh2Δ1</td>
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Goldfarb Fig. 3
<table>
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<tr>
<th>Time (hrs)</th>
<th>A-A</th>
<th>F-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>0.5</td>
<td>2</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

**msh6-G987D**

Product: 1.05 ± 0.14

**msh2-R730W**

Product: 0.86 ± 0.15

**msh2-S656P**

Product: 0.81 ± 0.12

Product: 0.62 ± 0.06

Product: 0.47 ± 0.08

Product: 0.35 ± 0.05

Uncut

Product

Cut

Loading Control

Goldfarb Fig. 4
### Goldfarb Fig. 5

<table>
<thead>
<tr>
<th></th>
<th>sgs1Δ</th>
<th>sgs1-hd</th>
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</thead>
<tbody>
<tr>
<td><strong>Time (hrs)</strong></td>
<td>A-A 0 0.5 2 5</td>
<td>F-A 0 0.5 2 5</td>
</tr>
<tr>
<td>Product:</td>
<td>0.91 ± 0.12 0.73 ± 0.04</td>
<td>0.87 ± 0.10 0.88 ± 0.11</td>
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</table>

<table>
<thead>
<tr>
<th></th>
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<th>sgs1-hd</th>
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</thead>
<tbody>
<tr>
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<td>A-A 0 0.5 2 5</td>
<td>F-A 0 0.5 2 5</td>
</tr>
<tr>
<td>Product:</td>
<td>1.21 ± 0.18 0.91 ± 0.11</td>
<td>1.15 ± 0.09 0.90 ± 0.17</td>
</tr>
</tbody>
</table>

- Uncut
- Product
- Cut
- Loading Control
Heteroduplex rejection

Non-homologous tail removal, repair

Msh2p-Msh6p
Sgs1p

Msh2p-Msh3p
Rad1p-Rad10p

Rad1p-Rad10p

Goldfarb Fig. 6
Table 1. Product formation and cell survival following HO induction in wild type, msh2, msh6, and sgs1 A-A and F-A strains.

<table>
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<th>Relevant genotype</th>
<th>Southern Blot Product Formation</th>
<th>Cell Survival</th>
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<tbody>
<tr>
<td></td>
<td>A-A/F-A ratio</td>
<td></td>
</tr>
<tr>
<td>wild type</td>
<td>1.05 ± 0.24</td>
<td>0.61 ± 0.12</td>
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<tr>
<td>msh2Δ</td>
<td>ND*</td>
<td>0.04 ± 0.01</td>
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<tr>
<td>msh2ΔI</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.05</td>
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<tr>
<td>msh2-K564E</td>
<td>0.89 ± 0.09</td>
<td>0.63 ± 0.09</td>
</tr>
<tr>
<td>msh2-R730W</td>
<td>0.81 ± 0.12</td>
<td>0.60 ± 0.12</td>
</tr>
<tr>
<td>msh2-S656P</td>
<td>0.47 ± 0.08</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>msh6Δ</td>
<td>1.12 ± 0.26</td>
<td>0.87 ± 0.02</td>
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<tr>
<td>msh6-F337A</td>
<td>1.06 ± 0.20</td>
<td>0.77 ± 0.06</td>
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<tr>
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<td>1.05 ± 0.14</td>
<td>0.65 ± 0.13</td>
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<tr>
<td>sgs1Δ</td>
<td>0.91 ± 0.12</td>
<td>0.79 ± 0.16</td>
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<tr>
<td>sgs1-hd</td>
<td>0.87 ± 0.10</td>
<td>0.89 ± 0.13</td>
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<tr>
<td>sgs1ΔC795</td>
<td>1.21 ± 0.18</td>
<td>0.90 ± 0.08</td>
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<tr>
<td>sgs1ΔN644</td>
<td>1.15 ± 0.09</td>
<td>1.00 ± 0.02</td>
</tr>
</tbody>
</table>

Southern blot product formation (Figures 1-5) and cell viability data are presented as the average of three to seven experiments ± one SD. See MATERIALS and METHODS for experimental details. *ND = not determined.