DOI: 10.1534/genetics.104.035204

Distinct Roles for the *Saccharomyces cerevisiae* Mismatch Repair Proteins in Heteroduplex Rejection, Mismatch Repair and Nonhomologous Tail Removal

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Manuscript received August 20, 2004
Accepted for publication October 19, 2004

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, 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 that displayed separation-of-function phenotypes with respect to nonhomologous tail removal during SSA and heteroduplex rejection were characterized. These studies suggest that nonhomologous 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 nonhomologous tail removal further illustrates that subsets of MMR proteins collaborate with factors in different DNA repair pathways to maintain genome stability.

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 (Pâques and Haber 1999; reviewed in Evans and Alani 2000; 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; Zahrt and Maloy 1997; Stambuk and Radman 1998; Fabisiewicz and Worth 2001). 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 during DNA replication 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 toward 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 *Mut**S** h*omologs (Msh 1–6 proteins) and four *Mut**L** h*omologs (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 postreplicative MMR, Msh2p-Msh3p is involved in facilitating single-strand annealing

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(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 <1 kb in length (Sugawara *et al.* 1997) and when the nonhomologous tails are >30 nucleotides in length (Pâques and Haber 1997). On the basis of 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-nonhomology 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 on the basis of 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 *Saccharomyces cerevisiae*, Datta *et al.* (1996) showed that the MutS (Msh2p, Msh3p, and Msh6p) and MutL (Mlh1p and Pms1p) homologs displayed antirecombination 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 postreplicative MMR, prevention of homeologous recombination involves redundancy at several steps. Alternatively, different sets of interactions may be involved between the MutS homologs 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; Spell and Jinks-Robertson 2004; Sugawara *et al.* 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 nondisjunction, hyper-recombination, and defects in DNA replication (reviewed in Cobb *et al.* 2002; Hickson 2003). 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 nonhomologous 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 nonhomologous 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

***S. cerevisiae* strains tested in the SSA assay:** EAY1141 [*mat::leu2::hisG hmr*Δ*3 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 λDNA (described in Sugawara *et al.* 2004). These strains contain the *GAL10-HO* construct integrated into the *ADE3* locus. EAY1141 is designated as "A-A" because it contains identical repeats of the *URA3* repeat sequence. EAY1143 is designated as "F-A" because one of the *URA3* repeat sequences contains seven single-site mutations. Mutant derivatives of the EAY1141 (A-A) and 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 *MSH2* (*MSH2-HA$_4$::LEU2*, *Aat*II, *Pvu*II digestion of pEAI118), *MSH6* (*MSH6::KANMX*, *Kpn*I, *Bst*EII digestion of pEAI186), *SGS1* (*SGS1::KANMX*, *Xho*I, *Bam*HI digestion of pEAI195), 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 EAY1141 include EAY1400 (*msh2Δ::TRP1*), EAY1309 (*msh2-K564E-HA$_4$::LEU2*), EAY1377 (*msh2Δ1-HA$_4$::LEU2*), EAY1225 (*msh2-S656P-HA$_4$::LEU2*), EAY1314 (*msh2-R730W-HA$_4$::LEU2*), EAY1387 (*msh6Δ::KANMX*), EAY1350 (*msh6-F337A::KANMX*), EAY1347 (*msh6-G987D::KANMX*), EAY1392 (*sgs1Δ::KANMX*), EAY1381 (*sgs1-hd::KANMX*), EAY1343 (*sgs1ΔN644::KANMX*), and EAY 1333 (*sgs1ΔC795::KANMX*). Derivatives of EAY1143 include EAY1401 (*msh2Δ::TRP1*), EAY1227 (*msh2-K564E-HA$_4$::LEU2*), EAY1260 (*msh2Δ1-HA$_4$::LEU2*), EAY1267 (*msh2-S656P-HA$_4$::LEU2*), EAY1265 (*msh2-R730W-HA$_4$::LEU2*), EAY1388 (*msh6Δ::KANMX*), EAY1352 (*msh6-F337A::KANMX*), EAY1297 (*msh6-G987D::KANMX*), EAY1354 (*sgs1Δ::KANMX*), EAY1326 (*sgs1-hd::KANMX*), EAY1345 (*sgs1ΔN644::KANMX*), and EAY1336 (*sgs1ΔC795::KANMX*).

SSA time courses: Stationary phase cultures of the above strains were diluted into YP (Rose *et al.* 1990) medium supplemented with lactate (2% w/v final concentration) and grown until midlog phase (1–2 × 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, *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 μm; Sigma, St. Louis) and 0.4 ml phenol:chloroform (1:1). Samples were vortexed for 5 min at 4°, followed by phenol:chloroform extraction. DNA was precipitated by adding 30 μl 3 m 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 hr at 37°, followed by phenol:chloroform extraction and DNA precipitation as described above. DNA was resusupended in 50 μl TE.

Southern blot analysis: DNA samples from the above time courses were digested with *Bgl*II (New England Biolabs, Beverly, MA) and run on 1% agarose gels with 1× TAE buffer. Southern blot transfer and hybridizations were performed essentially as described by the manufacturer (Amersham, Arlington Heights, IL) and Sugawara *et al.* (2004). Blots were visualized using the Phosphor Imaging system and quantified using the Imagequant program (Molecular Dynamics, Sunnyvale, CA). The probe used to visualize SSA products was an 880-bp *Hin*dIII-*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 ^{32}P-dCTP as directed by the manufacturer (New England Biolabs), and were purified using Bio-Spin P30 columns as suggested by the manufacturer (Bio-Rad, Richmond, CA). 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 the 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 pregrown in YP-lactate medium (2% w/v final concentration) to midlog 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 were 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 (*MAT***a**, *HMRa, Δhml::ADE1, Δho, ade1-100, leu2-3, 112, lys5, trp1::hisG, ura3-52, ade3::GAL-HO, MSH2-HA$_4$::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 midlog cultures of EAY1143 (*MSH2*), EAY1257 (*MSH2-HA$_4$::LEU2*), and EAY1378 (*msh2Δ1-HA$_4$::LEU2*) were separated on 8% SDS-PAGE gels and transferred to nitrocellulose membrane (Bio-Rad) using a semi-dry electrophoretic transfer system (Bio-Rad). Western blot analysis was carried out with primary antibody specific to HA (12CA5, Roche, Indianapolis) and secondary anti-mouse IgG antibody at 1:2000 and 1:5000 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 an SSA assay using two 205-bp sequences that either were 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

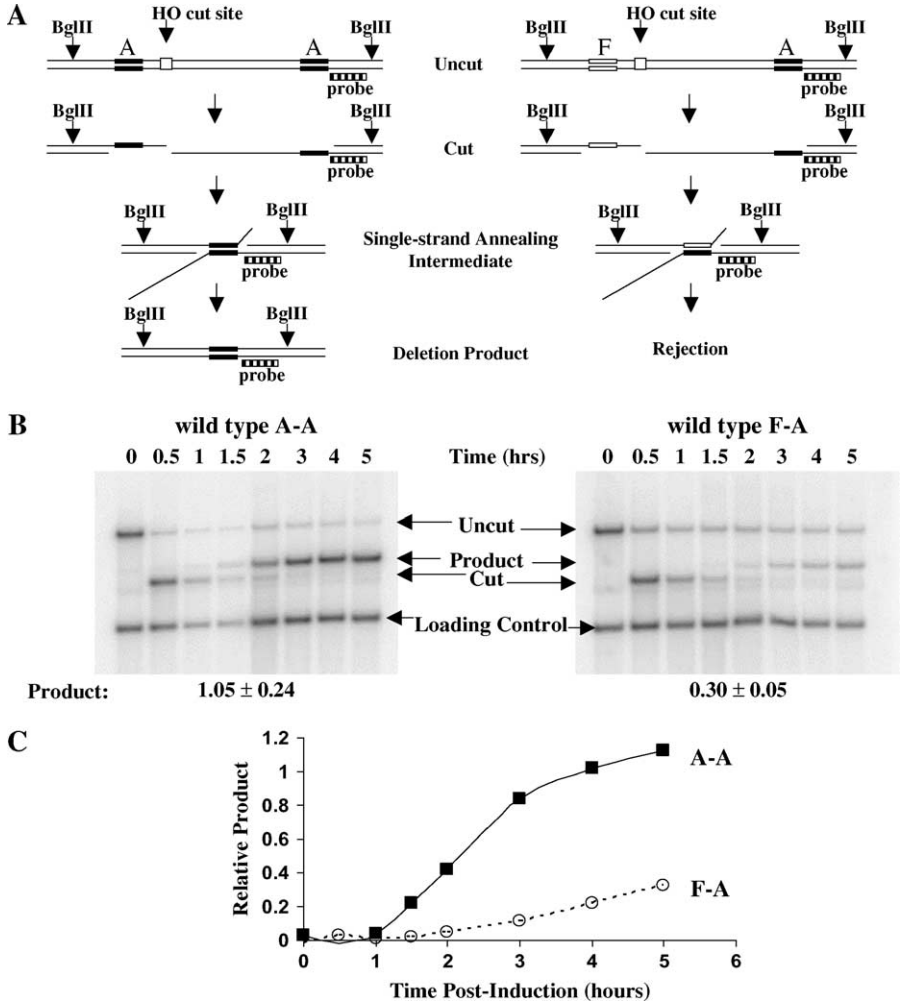

FIGURE 1.—Overview of the SSA assay involving homologous and divergent DNA substrates. (A) A DSB is induced at an HO cut site flanked by *URA3* homologous (A-A, left) or divergent (F-A, right) 205-bp repeat sequences. Repair of the DSB by SSA, followed by Msh2p-Msh3p- and Rad1p-Rad10p-mediated nonhomologous tail removal and subsequent repair synthesis, results in a deletion between the *URA3* repeats. (B) The kinetics of the SSA repair reaction was monitored at times after *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 B.

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 the F-A strains (Figure 1, B and 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 *vs.* 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 (LAMERS *et al.* 2000; OBMOLOVA *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 (LAMERS *et al.* 2000; OBMOLOVA *et al.* 2000). Residues within the antiparallel 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

TABLE 1

Product formation and cell survival following *HO* induction in *wild-type*, *msh2*, *msh6*, and *sgs1* A-A and F-A strains

Relevant genotype	Southern blot product formation			Cell survival		
	A-A	F-A	A-A/F-A ratio	F-A	A-A	A-A/F-A ratio
wild type	1.05 ± 0.24	0.30 ± 0.05	3.5	0.61 ± 0.12	0.19 ± 0.03	3.4
*msh2*Δ	ND	ND		0.04 ± 0.01	0.04 ± 0.03	1.0
*msh2*Δ*1*	0.11 ± 0.02	0.04 ± 0.01	2.9	0.11 ± 0.05	0.04 ± 0.01	2.8
msh2-K564E	0.89 ± 0.09	0.57 ± 0.07	1.6	0.63 ± 0.09	0.31 ± 0.05	2.0
msh2-R730W	0.81 ± 0.12	0.62 ± 0.06	1.3	0.60 ± 0.12	0.42 ± 0.09	1.4
msh2-S656P	0.47 ± 0.08	0.35 ± 0.05	1.3	0.39 ± 0.02	0.23 ± 0.07	1.7
*msh6*Δ	1.12 ± 0.26	0.86 ± 0.07	1.3	0.87 ± 0.02	0.61 ± 0.06	1.4
msh6-F337A	1.06 ± 0.20	0.74 ± 0.14	1.4	0.77 ± 0.06	0.57 ± 0.05	1.4
msh6-G987D	1.05 ± 0.14	0.86 ± 0.15	1.2	0.65 ± 0.13	0.64 ± 0.04	1.0
*sgs1*Δ	0.91 ± 0.12	0.73 ± 0.04	1.2	0.79 ± 0.16	0.75 ± 0.18	1.1
sgs1-hd	0.87 ± 0.10	0.88 ± 0.11	1.0	0.89 ± 0.13	0.68 ± 0.04	1.3
*sgs1*Δ*C795*	1.21 ± 0.18	0.91 ± 0.11	1.3	0.90 ± 0.08	0.69 ± 0.13	1.3
*sgs1*Δ*N644*	1.15 ± 0.09	0.90 ± 0.17	1.3	1.00 ± 0.02	0.75 ± 0.20	1.4

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

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 binding 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 and 2.0, respectively, that approached the levels seen in the *msh6*Δ strain (1.3 and 1.4). The *msh6-F337A* mutation conferred a defect in heteroduplex rejection that resembled the *msh6*Δ mutation (A-A/F-A ratios of 1.4 for product and 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.

An *msh2* DNA binding domain I deletion mutant that is functional in MMR and heteroduplex rejection, but defective in nonhomologous 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 *msh2*Δ*1*) and IV (amino acids 497–606, designated as *msh2*Δ*4*) 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

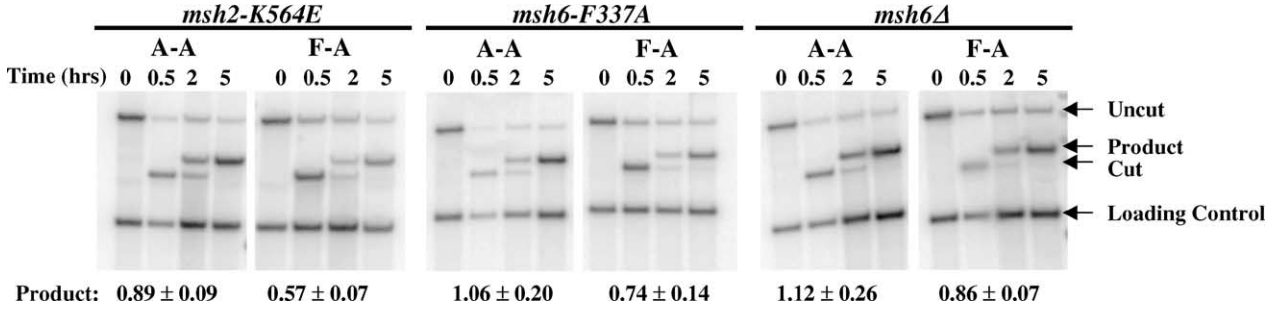

FIGURE 2.—The effect of *msh2* (*-K564E*) and *msh6* (*-F337A*) mismatch binding mutations on SSA in strains bearing the A-A and F-A repeat constructs. See Figure 1 for details.

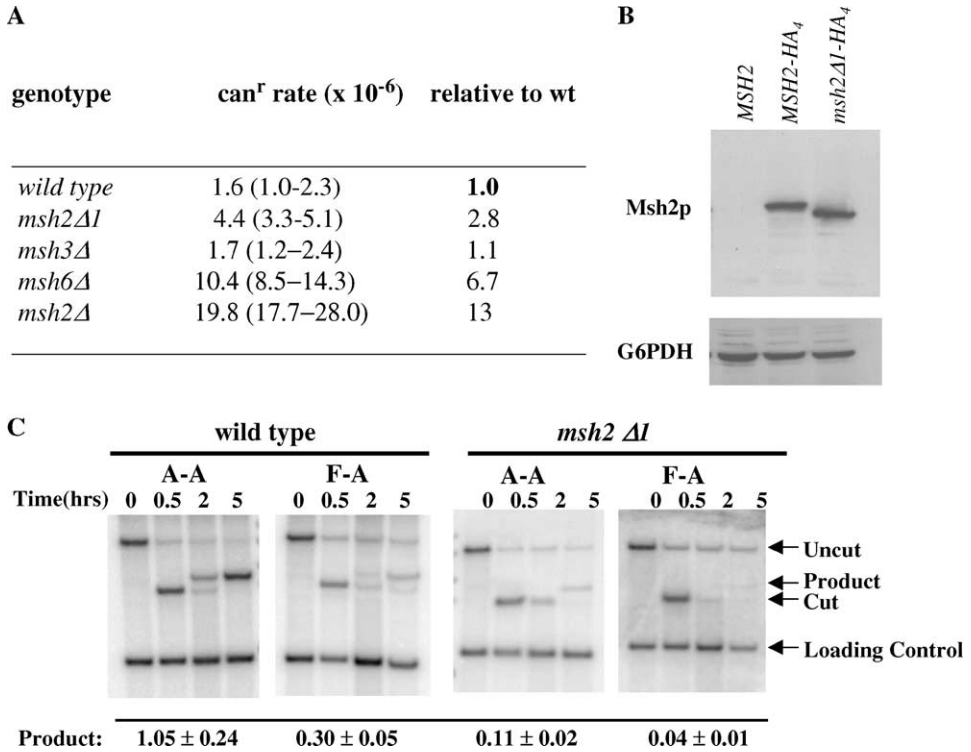

FIGURE 3.—The *msh2Δ1* mutant displays a defect in nonhomologous tail removal during SSA but is somewhat functional in Msh2p-Msh6p-mediated MMR. (A) Canavanine resistance assays were performed with the indicated strains as described in MATERIALS AND METHODS. The rates, actual and relative to the wild-type strain EAY 745, 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 *msh2Δ1* mutation on SSA in strains bearing the A-A and F-A repeat constructs. Southern blots were performed and quantified as described in MATERIALS AND METHODS.

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 nonhomologous tail removal assays (data not shown). Surprisingly, a complete deletion of DNA binding domain I (amino acids 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 nonhomologous 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 and 2.8) were similar to those seen in wild type (3.5 and 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 nonhomologous tail removal when acting within the Msh2p-Msh3p complex. It is important to note that the strong defect in nonhomologous 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 those of wild type in both assays and the mutant strain appeared functional for Msh2p-Msh6p-mediated MMR supports our conclusion. However, it will be important to test the effect of

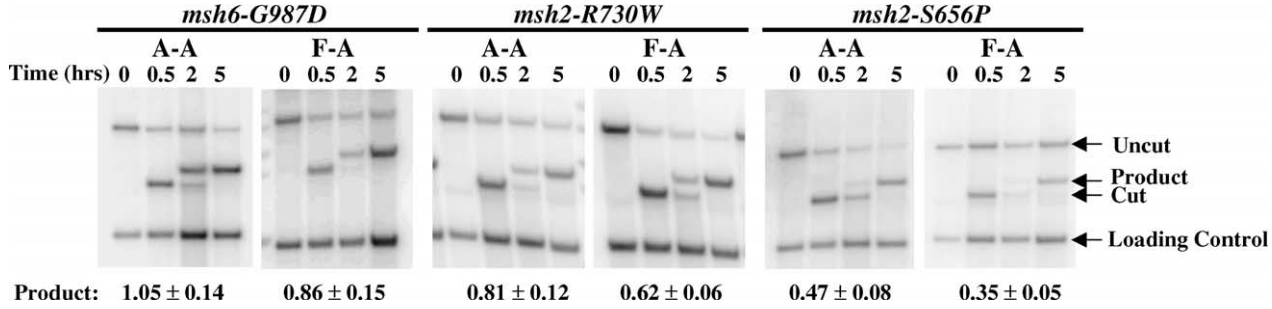

FIGURE 4.—The effect of *msh2* (*-R730W* and *-S656P*) and *msh6* (*-G987D*) ATP binding domain mutations on SSA in strains bearing the A-A and F-A repeat constructs. See Figure 1 for details.

the *msh2Δ1* mutation in other homeologous recombination assays that do not involve nonhomologous tail removal (*e.g.*, Nicholson *et al.* 2000).

ATP binding and hydrolysis by the MutS homologs 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 (Studamire *et al.* 1998; 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 (Iaccarino *et al.* 1998; Studamire *et al.* 1998; Kijas *et al.* 2003). 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 nonhomologous tail removal (Studamire *et al.* 1999). However, two *msh2* separation-of-function mutations, *msh2-R730W* and *msh2-S656P*, were isolated that are functional in nonhomologous 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 ~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 nonhomologous 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 that of the helicase point mutant in a subset of assays including MMS sensitivity, hyperrecombination, and growth in the presence of the *top1* mutation (Mullen *et al.* 2000). On the basis of 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 did in a subset of the

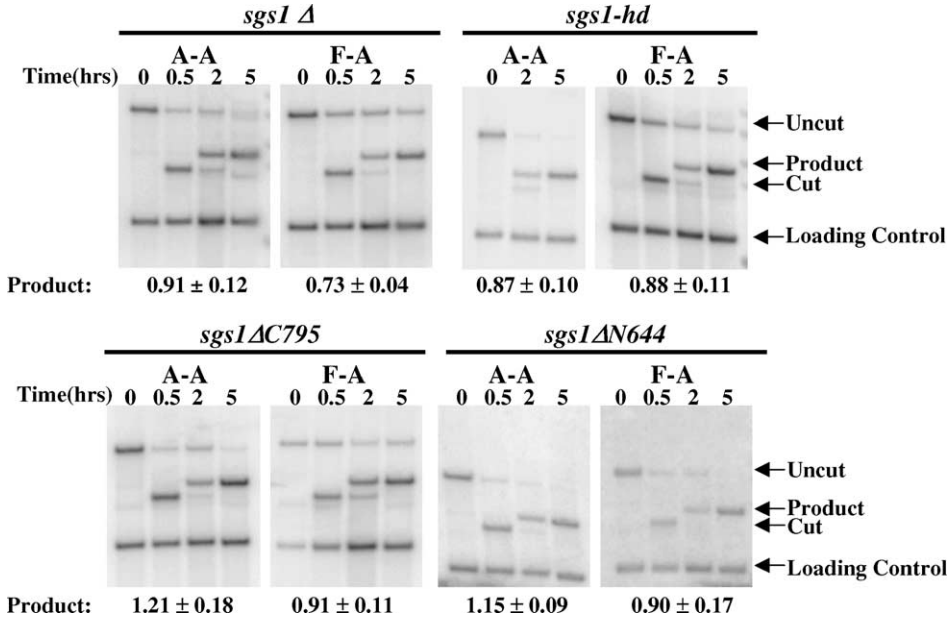

FIGURE 5.—*sgs1* mutants are defective in heteroduplex rejection in the SSA assay. See MATERIALS AND METHODS and the Figure 1 legend for details.

assays listed above. As shown in Figure 5 and Table 1, all three alleles conferred a heteroduplex rejection phenotype that was indistinguishable from the *sgs1*Δ 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 hyperrecombination 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 interhomolog recombination) will involve mechanistic steps that are distinct from those that function during SSA. Mutations in the human homologs of some of the MMR genes and Sgs1p have been correlated with human diseases that are associated with genome instability. These consist of hereditary nonpolyposis 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 HSIEH 2003). 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 nonhomologous 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 nonhomologous 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*, and *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 and WORTH 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*

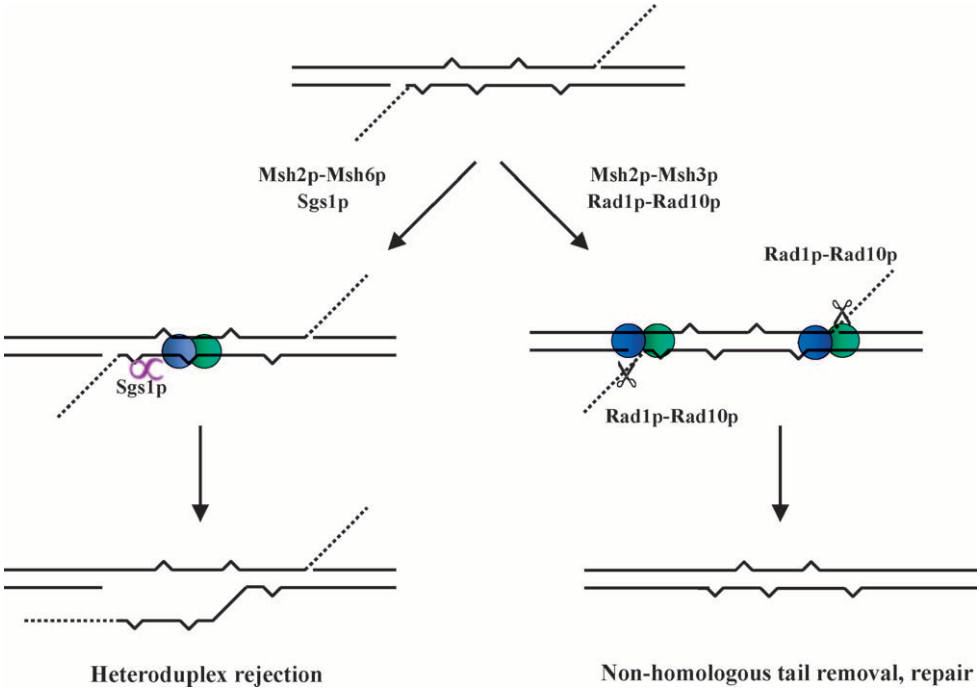

FIGURE 6.—A model showing heteroduplex rejection and nonhomologous tail removal pathways acting on SSA intermediates. In one pathway, Msh2p-Msh6p recognizes mismatches in the SSA intermediate and recruits the Sgs1p helicase to unwind the annealed region. In the absence of heteroduplex rejection, the SSA intermediate is repaired through nonhomologous 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 nonhomologous tail removal but conferred defects in Msh2p-Msh6p-mediated heteroduplex rejection. In contrast the *msh2Δ1* mutation conferred severe defects in nonhomologous tail removal but did not appear to disrupt heteroduplex rejection.

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 antirecombination; 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 (*msh2Δ1*) 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 (LAMERS *et al.* 2000; OBMOLOVA *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 (GRADIA *et al.* 1999; JUNOP *et al.* 2003). 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 (LAMERS *et al.* 2000; OBMOLOVA *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 (LAMERS *et al.* 2000; OBMOLOVA *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 in *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 *msh2Δ1* mutant displayed a DNA slippage phenotype similar to the

*msh3*Δ mutant (E. Alani and T. Goldfarb, 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 to 20 nucleotides will be important in determining whether the defect in nonhomologous 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 (Iaccarino *et al.* 1998; Studamire *et al.* 1998; Bjornson *et al.* 2000; Antony and Hingorani 2003; Kijas *et al.* 2003). As shown in Figure 4, the *msh6-G987D* allele conferred a defect in heteroduplex rejection that was similar to the *msh6*Δ mutation. Although *msh2-S656P* strains displayed a defect in nonhomologous 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; Spell and Jinks-Robertson 2004; Sugawara *et al.* 2004). Null mutations in *SGS1* display a variety of phenotypes including MMS sensitivity, synthetic lethality with *SLX1-4*, hyperrecombination, suppression of the *top3* slow growth phenotype, and slow growth in the presence of the *top1*Δ 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 as 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* did in MMS sensitivity assays, hyperrecombination, 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.

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 Postgraduate Scholarship award and E.A. was supported by National Institutes of Health grant GM-53085.

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Communicating editor: S. T. Lovett