Role of Mismatch Repair in the Fidelity of *RAD51*- and *RAD59*-Dependent Recombination in *Saccharomyces cerevisiae*

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ABSTRACT

To prevent genome instability, recombination between sequences that contain mismatches (homeologous recombination) is suppressed by the mismatch repair (MMR) pathway. To understand the interactions necessary for this regulation, the genetic requirements for the inhibition of homeologous recombination were examined using mutants in the *RAD52* epistasis group of *Saccharomyces cerevisiae*. The use of a chromosomal inverted-repeat recombination assay to measure spontaneous recombination between 91 and 100% identical sequences demonstrated differences in the fidelity of recombination in pathways defined by their dependence on *RAD51* and *RAD59*. In addition, the regulation of homeologous recombination in *rad51* and *rad59* mutants displayed distinct patterns of inhibition by different members of the MMR pathway. Whereas the requirements for the MutS homolog, *MSH2*, and the MutL homolog, *MLH1*, in the suppression of homeologous recombination were similar in *rad51* strains, the loss of *MSH2* caused a greater loss in homeologous recombination suppression than did the loss of *MLH1* in a *rad59* strain. The nonequivalence of the regulatory patterns in the wild-type and mutant strains suggests an overlap between the roles of the *RAD51* and *RAD59* gene products in potential cooperative recombination mechanisms used in wild-type cells.

GENOMIC instability, a hallmark of aging and carcinogenesis, is manifested as mutation and inappropriate recombination (Bishop and Schiestl 2002; Bohr 2002). An important pathway that promotes genomic stability is the mismatch repair (MMR) pathway, which not only prevents mutation by removing mismatches in newly replicated DNA but also monitors the fidelity of homologous recombination. Although homologous recombination is critical for the repair of DNA double strand breaks (DSBs), it is strictly regulated such that a single mismatch is sufficient to inhibit recombination in the budding yeast *Saccharomyces cerevisiae* (Datta *et al.* 1996). In the absence of MMR, recombination between sequences that are similar but not identical (called homeologous recombination) increases dramatically. Although the role of MMR in the repair of replication errors has been well studied, the mechanism of MMR suppression of homeologous recombination is not yet fully understood.

Eukaryotic MMR proteins are classified by homology to their *Escherichia coli* counterparts, MutS and MutL. In *S. cerevisiae*, nuclear mitotic mismatch recognition is carried out by heterodimers of MutS homologs (Msh2:Msh3 or Msh2:Msh6; Johnson *et al.* 1996; Marsischky *et al.* 1996). Heterodimers of the MutL homologs (Mlh1:Pms1, Mlh1:Mlh2, or Mlh1:Mlh3) then interact with the MutS heterodimer to mediate steps that lead to the removal of the mismatch (Crouse 1998; Wang *et al.* 1999; Harfe *et al.* 2000). Studies of mutator phenotypes and mutation spectra suggest that Msh2:Msh6 heterodimers recognize small insertions/deletions and base-base mismatches, while Msh2:Msh3 heterodimers detect only insertion/deletion loops (Kolodner and Marsischky 1999). The mutator phenotypes of *msh2, msh3 msh6, mlh1*, or *pms1* mutant strains are roughly equivalent, indicating that MMR is primarily carried out by interaction of Mlh1:Pms1 heterodimers with Msh2:Msh3 or Msh2:Msh6 complexes (Flores-Rozas and Kolodner 1998; Harfe and Jinks-Robertson 2000a; Harfe *et al.* 2000). The Msh2:Msh3 complex, together with the Rad1:Rad10 complex, is also involved in the removal of 3′ nonhomologous tails generated during recombination processes (Sugawara *et al.* 1997).

Although loss of the MutS or the MutL complex has similar effects on mutation rates, previous studies suggest that the different MMR complexes perform differently in the regulation of homeologous recombination (Chen and Jinks-Robertson 1999; Nicholson *et al.* 2000). Loss of the MutS homologs, for example, causes a greater increase in homeologous recombination than does the loss of either Mlh1 or Pms1. In addition, the absence of Msh3 causes an increase in recombination between sequences containing only base-base mismatches, despite the conclusions from previous studies that Msh3 has no role in the repair of base-base mismatches (Marsischky *et al.* 1996; Earley and Crouse 1998). The observed differences may arise from unique

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activities or interactions required in postreplication repair *vs.* the maintenance of recombination fidelity. In the repair of replication errors, the binding of the MutS and then the MutL homologs leads to the removal of the newly synthesized strand, resynthesis across the resulting gap, and ligation. In the inhibition of homeologous recombination, binding of MMR proteins to mismatches present in heteroduplex recombination intermediates presumably prevents the completion of recombination by an as-yet-undefined mechanism. Whether this mechanism is by rejection of the invading strand, blocks in Holliday junction migration, or inhibition of the extension of heteroduplex DNA is unknown (Alani *et al.* 1994; Chen and Jinks-Robertson 1998). Characterization of the relationship of MMR-dependent regulation of homeologous recombination and the components of recombination pathways may shed light on the mechanisms by which this regulation takes place.

Genes involved in the DSB repair pathway in *S. cerevisiae* comprise the *RAD52* epistasis group. Numerous genetic and biochemical analyses of the members of this epistasis group have clarified their roles in DSB repair (Paques and Haber 1999; Sung *et al.* 2000; Symington 2002). The Mre11, Rad50, and Xrs2 proteins form the Mre11-Rad50-Xrs2 (MRX) complex, which is thought to have an end-processing role in the creation of the 3′ single-stranded tails used to invade duplex DNA and a structural role in promoting recombination between sister chromatids. Replication protein A (RPA) binds the 3′ single-stranded tails that result from DSB processing, preventing the formation of secondary structure. Rad52 promotes the replacement of RPA by Rad51, the yeast homolog of the bacterial RecA protein, and the resulting Rad51 nucleoprotein filament is stabilized by the Rad55:Rad57 complex. Rad54 has homology to the Swi/Snf family of chromatin-remodeling proteins and facilitates the invasion of a homologous duplex by the nucleoprotein filament. Following strand invasion, repair synthesis is primed from the 3′ end of the invading strand. If the newly extended invading strand is unwound from the invaded duplex, it can reanneal with the other end of the broken molecule in a process called synthesis-dependent strand annealing (SDSA), resulting in a noncrossover event. If, on the other hand, the strand displaced from the duplex by the strand invasion process is captured by the 3′ tail of the other end of the broken molecule, a double Holliday junction will be formed that can be resolved as either a crossover or a noncrossover (gene conversion) event. The proteins that regulate the steps subsequent to strand invasion have not yet been fully characterized.

An alternative recombination pathway that can occur in the absence of *RAD51* but that requires *RAD59* was identified in a screen for mutants defective in inverted repeat recombination in a *rad51* background (Bai and Symington 1996). This *RAD59*-dependent alternative pathway involves break-induced replication (BIR) with single-strand annealing (SSA). BIR occurs when DNA synthesis primed from the 3′ invading tail of a broken chromosome proceeds down the entire arm of the intact chromosome (Malkova *et al.* 1996). SSA takes place between direct repeats after resection of the ends of a DSB reveals complementary single-stranded tails that can base pair, resulting in the deletion of the region between the repeats as well as one of the repeats. Rad59 has partial homology to Rad52 and has some inherent strand-annealing activity. It has been suggested that the functions of Rad52 and Rad59 may overlap to provide strand invasion capability in the absence of Rad51 (Bai *et al.* 1999).

We have utilized an intron-based inverted-repeat assay system (Figure 1) to explore the regulation of homeologous recombination. This system detects recombination events that reorient the region between homologous or homeologous inverted repeats, leading to the expression of a selectable marker. To elucidate potential mechanisms for the inhibition of recombination between homeologous substrates, we examined the effects of the loss of individual proteins involved in the yeast recombination pathways. In this study, the mutants fall into two classes defined by *rad51* and *rad59* mutant phenotypes with respect to their effect on homeologous recombination relative to homologous recombination. The difference in the phenotypes suggests that the regulation of homeologous recombination is different in recombination pathways dependent on *RAD51* or *RAD59*. To dissect the role of the MMR machinery in that regulation, we examined the phenotypes of strains defective in MMR and in either *RAD51* or *RAD59*. The effect of MMR defects on the corresponding pathways suggests that the regulation of homeologous recombination in different pathways is distinct and is affected by separate activities of MMR complexes.

MATERIALS AND METHODS

Media and growth conditions: Yeast strains were grown nonselectively in YEP medium (1% yeast extract, 2% Bacto-peptone, 250 mg/liter adenine; 2% agar for plates) supplemented with either 2% dextrose (YEPD) or 2% glycerol and 2% ethanol (YEPGE). Selective growth was done on synthetic complete (SC) media lacking the appropriate nutrient (Sherman 1991) and supplemented with 2% dextrose (SCD) or with 2% galactose, 2% glycerol, and 2% ethanol (SCGGE). Ura$^-$ derivatives were isolated on SCD-uracil plates containing 1 g/liter 5-fluoroorotic acid and 12 mg/liter uracil (Ausubel *et al.* 1994). Geneticin- and hygromycin-resistant transformants were isolated on YEPD supplemented with 200 mg/liter geneticin (G418) or 300 mg/liter hygromycin B, respectively. Methyl methanesulfonate (MMS) sensitivity was tested on YEPD containing 0.016% MMS (Kodak). Mutator phenotype was assessed by forward mutation at *CAN1* on SC-arginine containing 60 mg/liter canavanine. All incubations were done at 30°.

Strain construction: All strains used in this study (Table 1) were derived from the congenic strains SJR1486 and SJR1487, which contain 100 or 91% identical inverted repeats, respectively (Welz-Voegele *et al.* 2002). The repeats are 783 bp in

TABLE 1

***S. cerevisiae* strains used in this study**

Relevant genotype	Strain	
	100% homology	91% homology
Wild type	SJR1486	SJR1487
rad50Δ::kan	SJR1773	SJR1550
mre11Δ::kan	SJR1980	SJR1982
rad51Δ::URA3	SJR1551	SJR1552
rad52Δ::URA3	SJR1548	SJR1549
rad54Δ::URA3	SJR1559	SJR1560
rad55Δ::kan	SJR1669	SJR1553
rad57Δ::kan	SJR1555	SJR1556
rad59Δ::kan	SJR1670	SJR1554
rad59Δ::kan, rad51Δ::URA3	SJR1914	SJR1825
msh2Δ::hisG	SJR1652	SJR1653
mlh1Δ::hyg	SJR1946	SJR1947
msh6Δ::hisG	SJR1922	SJR1875
msh3Δ::hisG	SJR2031	SJR2032
rad1Δ::hisG	SJR2033	SJR2034
msh3Δ::hisG, msh6Δ::hisG	SJR2076	SJR2077
msh2Δ::hisG, rad51Δ::URA3	SJR1774	SJR1775
msh2Δ::hisG, rad59Δ::kan	SJR1772	SJR1767
mlh1Δ::hyg, rad51Δ::URA3	SJR1956	SJR1957
mlh1Δ::hyg, rad59Δ::kan	SJR1958	SJR1959
msh6Δ::hisG, rad51Δ::URA3	SJR1932	SJR1927
msh6Δ::hisG, rad59Δ::kan	SJR1933	SJR1936
msh3Δ::hisG, rad51Δ::URA3	SJR2039	SJR2040
msh3Δ::hisG, rad59Δ::kan	SJR2042	SJR2043
rad1Δ::hisG, rad51Δ::URA3	SJR2046	SJR2041
rad1Δ::hisG, rad59Δ::kan	SJR2053	SJR2044
msh3Δ::hisG, msh6Δ::hisG, rad59Δ::kan	SJR2078	SJR2079

All strains were derived for this study from the congenic strains, "100% homologous" SJR1486 (*MATα ade2-101$_{oc}$ his3Δ200 ura3(Nhe)-HIS3::intron::*cβ2/cβ2 inverted repeats-*ura3 lys2ΔRV::hisG leu2-K::lys2Δ5′-lys2Δ3′-LEU2*) and "91% homologous" SJR1487 (*MATα ade2-101$_{oc}$ his3Δ200 ura3(Nhe)-HIS3::intron::*cβ2/cβ7 inverted repeats-*ura3 lys2ΔRV::hisG leu2-K::lys2Δ5′-lys2Δ3′-LEU2*).

length, and the sequence differences in the inverted repeats of SJR1487 are exclusively base substitutions.

Disruptions of the genes of the mismatch repair and recombination pathways were created in both parent strains using standard genetic techniques. To disrupt *RAD50*, *MRE11*, *RAD55*, *RAD57*, and *RAD59*, homology to the targeted genes was added to the kanMX2 cassette by PCR, and transformants were selected on YEPD containing G418 (Wach *et al.* 1994). *RAD51*, *RAD52*, and *RAD54* were disrupted using restriction fragments containing *rad51::URA3*, *rad52::hisG-URA3-hisG*, and *rad54::URA3* alleles, respectively (Freedman and Jinks-Robertson 2002). *MSH2* was deleted by transformation with *Aat*II-*Xba*I-digested pΔ*msh2* (Earley and Crouse 1998); *MSH3* was disrupted by transformation with *Eco*RI-digested pEN33 (Datta *et al.* 1996); *RAD1* was deleted using *Sal*I-*Eco*RI-digested pR1.6 (Higgins *et al.* 1983); and *MSH6* was disrupted by transformation with *Eco*RI-*Sac*I-digested Msh6pHUH (Kramer *et al.* 1996). *MLH1* was disrupted using a PCR-generated hygMX2 cassette (Goldstein and McCusker 1999). Double mutants defective in both MMR and recombination were created by first disrupting the relevant MMR gene and then the relevant recombination gene. The presence of each targeted disruption was inferred using phenotypic tests (MMS sensitivity and mutator phenotype for recombination-defective and MMR-defective strains, respectively) and confirmed by PCR. Primer sequences are available upon request.

Determination of recombination rates: Yeast colonies grown 2 days on YEPD were inoculated into 5 ml YEPGE and grown for 3 days on a roller drum. Two or more isolates of each strain were tested, using a minimum of six colonies per isolate. The cultures were washed with 5 ml sterile H_2O and resuspended in 1 ml sterile H_2O. Appropriate dilutions were plated on YEPD and SCGGE-His to calculate the total number of viable cells and number of recombinants per culture. Colonies on YEPD and SCGGE-His were counted after 2 and 5 days growth, respectively. Recombination rates (recombination per cell per generation) were determined by the method of the median or the method of p_0 (Lea and Coulson 1949; Spell and Jinks-Robertson 2004). To calculate the 95% confidence interval for each recombination rate, the numbers of recombinants per culture were first ranked in ascending order. Table B11 of Altman (1991) was then used to identify which ranked cultures should provide the number of recombinants for calculation of the upper and lower rate limits that define the confidence interval.

RESULTS

The regulation of homeologous recombination is affected by mutations in *RAD52* epistasis group genes: To

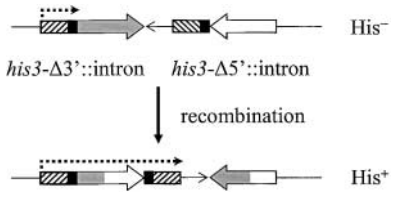

FIGURE 1.—Intron-based inverted-repeat recombination assay. Inverted repeats (shaded and open arrows) were fused to intron splice sites (solid boxes) and placed next to the 5′ and 3′ halves of the coding sequence for *HIS3* (hatched boxes). The direction of transcription of *HIS3* is indicated by dashed arrows. Recombination between the repeats that leads to the inversion of the sequence between the repeats (invertible segment) allows expression of the full-length *HIS3* gene and growth on plates lacking histidine (see Figure 4 for recombination models of the reorientation process). To measure homeologous recombination, repeats of 91% identity were used; to measure homologous recombination, 100% identical repeats were used.

determine the genetic dependence of the regulation of homeologous recombination on the members of the *RAD52* epistasis group, we examined the recombination rates in null mutants of *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57,* and *RAD59.* The levels of spontaneous recombination between homeologous and homologous substrates were assayed using inverted repeats of 91 or 100% identity, respectively, contained within introns fused to the coding sequence of a selectable marker, *HIS3.* Recombination between the repeats reorients the region between them such that *HIS3* expression is restored (Figure 1).

The recombination rates in wild-type and recombination-defective strains are given in Table 2. In wild-type cells, the rate of homeologous recombination is suppressed ~40-fold relative to homologous recombination (4.2×10^{-8} and 1.7×10^{-6}, respectively), and as expected, homeologous recombination and homologous recombination rates are severely reduced in strains lacking the *RAD52* gene that defines the epistasis group. Recombination is elevated in *rad50* mutants, with the rate of homeologous recombination being elevated more than that of homologous recombination (4.3-fold *vs.* 1.9-fold, respectively). A similar increase is seen in mutants of another member of the MRX complex, *MRE11* (4.6-fold increase in homeologous recombination rates *vs.* 3.2-fold increase in homologous recombination). Recombination is generally decreased in the other recombination mutants tested, but the degree of the decrease varies, depending on whether the substrates are homologous or homeologous.

The differential effect of a given *rad* mutation on homeologous and homologous recombination is evident in the graph of recombination levels relative to wild type in Figure 2 and as a change in the relative homeologous to homologous recombination ratio in Table 2. In Figure 2, the relative homeologous recombination rate is greater than the relative homologous recombination rate for each of the *rad* strains tested except the *rad59* mutant. Compared to wild type, the relative homeologous to homologous recombination ratio is elevated in strains defective in *RAD50, RAD51, RAD54, RAD55,* or *RAD57* (Table 2). This increase in levels of homeologous recombination relative to homologous recombination levels suggests that either the suppression of homeologous recombination is dependent on these gene products or the loss of the gene products shifts recombination into a pathway that is more tolerant of mismatches. In contrast, strains defective in *RAD59* have a greater reduction in homeologous recombination than in homologous recombination, indicating that either the mechanism of homeologous recombination is dependent on *RAD59* or loss of *RAD59* shifts recombination into a more stringent pathway.

Homeologous recombination requires either *RAD51* or *RAD59*: Previous studies have shown that strains defective in either *RAD51* or *RAD59* have small decreases in recombination, whereas strains defective in both suffer severe decreases (BAI and SYMINGTON 1996). To confirm that these two pathways account for the bulk of the homeologous recombination measured in our inverted-repeat assay, double-mutant strains defective in both genes were constructed. The levels of homeologous recombination and homologous recombination in the *rad51rad59* double mutant approach those of a *rad52* mutant (Table 2 and Figure 2), and the residual recombination levels seen in the double mutant resemble those seen in other studies (BAI and SYMINGTON 1996). The synergistic phenotype of the double mutant generally has been interpreted as a competition or functional redundancy between distinct pathways requiring either *RAD51* or *RAD59*, with one pathway compensating for the loss of the other (BAI and SYMINGTON 1996). It should be noted, however, that the level of recombination provided by the remaining pathway in the single mutants may not necessarily reflect the levels performed by that pathway in a wild-type cell.

Loss of *MSH2* has a differential effect on homeologous recombination occurring by the *RAD51*- and *RAD59*-dependent pathways: Previous studies have shown that most of the suppression of homeologous recombination results from the activity of the MMR machinery (DATTA *et al.* 1996; CHEN and JINKS-ROBERTSON 1999; NICHOLSON *et al.* 2000). In agreement with these studies, homeologous recombination and homologous recombination rates are statistically equivalent in strains defective in the major MMR gene, *MSH2* (Table 3). To determine the role of MMR in the suppression of homeologous recombination in the *RAD51 vs. RAD59* recombination pathways, one recombination pathway was eliminated, and the MMR-dependent regulation of the other pathway was assessed. For example, in a *rad51* mutant where only the *RAD59*-dependent pathway functions, the role of MMR proteins in the regulation of *RAD59*-dependent

TABLE 2

Homeologous and homologous recombination rates in *rad* mutants

Relevant genotype	Homeologous recombination		Homologous recombination		Relative HER/HR ratio
	Rate × 10^{-8}	Relative rate	Rate × 10^{-8}	Relative rate	
Wild type	4.2 (4.0–4.7)	1.0	170 (150–210)	1.0	1.0
*rad50*Δ	18 (15–19)	4.3	320 (270–390)	1.9	2.3
*mre11*Δ	19 (17–22)	4.6	540 (500–560)	3.2	1.4
*rad51*Δ	5.0 (4.4–5.4)	1.2	53 (46–62)	0.31	3.8
*rad52*Δ[a]	0.09 (NA)	0.02	1.2 (0.68–1.9)	0.0071	3
*rad54*Δ	1.5 (1.3–1.8)	0.36	20 (16–22)	0.12	3.0
*rad55*Δ	2.6 (2.1–2.9)	0.62	64 (58–75)	0.38	1.6
*rad57*Δ	2.2 (2.0–2.9)	0.52	30 (25–36)	0.18	3.0
*rad59*Δ	1.1 (0.91–1.3)	0.26	76 (64–85)	0.45	0.59
*rad51*Δ *rad59*Δ	0.3 (0.2–0.3)	0.07	3.2 (2.4–4.2)	0.019	4

Recombination rates were determined by the method of the median for substrates of 91% identity (homeologous) or 100% identity (homologous; Lea and Coulson 1949). Confidence intervals of 95% are indicated within parentheses. The relative ratio of homeologous recombination to homologous recombination was calculated by dividing the relative homeologous recombination rate by the relative homologous recombination rate. HER, homeologous recombination; HR, homologous recombination; NA, not applicable.

[a] The homeologous recombination rate for the *rad52*Δ strain was calculated using the fraction of cultures with no recombinants (p_0; Lea and Coulson 1949). Confidence intervals cannot be calculated for homeologous recombination in this mutant.

recombination becomes apparent. The recombination rates in the relevant single- and double-mutant strains are given in Table 3. Homeologous recombination catalyzed by the *RAD59*-dependent pathway increases 3.6-fold in an MMR-defective strain (*msh2 rad51*) relative to an MMR-proficient strain (*rad51*; Figure 3). In contrast to this small increase, loss of MMR in a *msh2 rad59* strain in which only the *RAD51*-dependent pathway is intact shows a much larger, 27-fold increase relative to the *rad59* single mutant. These differences demonstrate that Msh2 has a stronger suppressive effect on *RAD51*-mediated homeologous recombination than on *RAD59*-mediated homeologous recombination.

Mutations in *MLH1* and *MSH6* have similar effects on homeologous recombination occurring by the *RAD51*- and *RAD59*-dependent pathways: It has been shown that mutations in the MutS homolog *MSH2* and in the MutL homologs *MLH1* or *PMS1* affect homeologous recombination differently, with *msh2* mutants exhibiting higher levels of homeologous recombination than *mlh1* or *pms1* strains exhibit (Chen and Jinks-Robertson 1999; Nicholson *et al.* 2000). To test the role of *MLH1* in regulating homeologous recombination in the *RAD51*- and *RAD59*-dependent pathways, homeologous recombination rates were measured in *mlh1*, *rad51 mlh1*, and *rad59 mlh1* mutants (Table 3). It should be noted that the complete deletion of the *MLH1* locus used here (*mlh1*Δ::*hyg*) causes slightly higher increases in the homeologous to homologous recombination ratio in a *RAD* background than that seen in studies using the *mlh1*Δ::*URA3* allele (R. Spell and S. Jinks-Robertson, unpublished results). Because the *mlh1*Δ::*URA3* allele deletes only the amino terminus of the protein (Prolla *et al.* 1994; Nicholson *et al.* 2000; Welz-Voegele *et al.* 2002), it is possible that the carboxy terminus alone or as part of a fusion protein may confer residual antirecombination activity. However, the stronger phenotype caused by the complete loss of *MLH1* is still

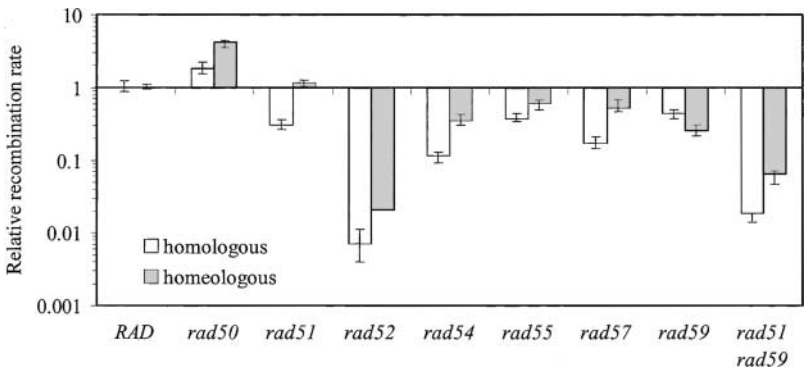

Figure 2.—The recombination rate of homeologous and homologous substrates in recombination-defective strains relative to wild-type strains. Recombination rates were determined for wild-type and *rad* mutant strains for homologous substrates (open bars) and homeologous substrates (shaded bars). The relative rate was determined by dividing the recombination rate of mutant strains by that of the same substrates in the wild-type strain. Error bars based on the confidence intervals for the rates are indicated.

TABLE 3

Homeologous and homologous recombination rates in MMR-defective *rad* mutants

Relevant genotype	Homeologous recombination		Homologous recombination		Relative HER/HR ratio
	Rate × 10^{-8}	Relative rate	Rate × 10^{-8}	Relative rate	
RAD	4.2 (4.0–4.7)	1.0	170 (150–210)	1	1.0
*RAD msh2*Δ	270 (240–320)	64	320 (220–470)	1.9	34
*RAD mlh1*Δ	120 (110–130)	29	250 (190–290)	1.5	19
*RAD msh6*Δ	18 (12–40)	4.3	130 (110–150)	0.8	5.6
*RAD msh3*Δ	13 (9.1–18)	3.1	550 (380–650)	3.2	1.0
*RAD rad1*Δ	21 (16–29)	5.0	600 (570–690)	3.5	1.4
*RAD msh3*Δ *msh6*Δ	290 (180–630)	69	540 (450–810)	3.2	22
*rad51*Δ	5.0 (4.4–5.4)	1.0	53 (46–62)	1.0	1.0
*rad51*Δ *msh2*Δ	18 (15–23)	3.6	77 (45–81)	1.5	2.5
*rad51*Δ *mlh1*Δ	13 (11–15)	2.6	71 (61–140)	1.3	1.9
*rad51*Δ *msh6*Δ	15 (10–16)	3.0	57 (53–61)	1.1	2.8
*rad51*Δ *msh3*Δ	6.1 (5.2–7.7)	1.2	56 (47–78)	1.1	1.2
*rad51*Δ *rad1*Δ	5.8 (4.8–7.8)	1.2	110 (84–170)	2.1	0.56
*rad59*Δ	1.1 (0.91–1.3)	1.0	76 (64–85)	1.0	1.0
*rad59*Δ *msh2*Δ	30 (25–37)	27	64 (57–110)	0.8	32
*rad59*Δ *mlh1*Δ	4.2 (2.7–5.0)	3.8	92 (77–140)	1.2	3.2
*rad59*Δ *msh6*Δ	5.3 (4.3–6.0)	4.8	72 (62–93)	0.9	5.1
*rad59*Δ *msh3*Δ	1.5 (0.97–2.3)	1.4	70 (62–78)	0.9	1.5
*rad59*Δ *rad1*Δ	1.2 (0.76–1.9)	1.1	53 (41–60)	0.7	1.6
*rad59*Δ *msh3*Δ *msh6*Δ	48 (46–70)	44	61 (54–75)	0.8	54

Recombination rates were determined by the method of the median for substrates of 91% identity (homeologous) or 100% identity (homologous; Lea and Coulson 1949). Confidence intervals of 95% are indicated within parentheses. The relative rate was calculated by dividing the MMR$^-$ mutant rate by the relevant *RAD/rad* rate. The relative ratio of homeologous recombination to homologous recombination was calculated by dividing the relative homeologous recombination rate by the relative homologous recombination rate. HER, homeologous recombination; HR, homologous recombination.

below that of a *msh2* mutant (Nicholson *et al.* 2000; Welz-Voegele *et al.* 2002). In a *rad51* background, the deletion of *MLH1* has the same effect as the deletion of *MSH2* (Figure 3). This suggests that the MMR-dependent regulation in the *RAD59*-dependent pathway requires both Mlh1 and Msh2. In contrast, in a *rad59* background, elimination of *MLH1* elevates homeologous recombination much less (4-fold *vs.* 27-fold) than the deletion of *MSH2* does (Figure 3). Therefore, most of the homeologous recombination inhibition in the *RAD51* pathway is effected by *MSH2* and does not require *MLH1*.

The effect of the loss of Msh6, Msh3, and Rad1 on the regulation of homeologous recombination: The difference in the suppression of homeologous recombination by Msh2 and Mlh1 in the *RAD51*-dependent pathway could be due to different roles of MutS and MutL homolog proteins in MMR-related processes or could be due to functions the proteins play in other processes. In addition to its role in mismatch recognition, for example, Msh2 together with Msh3 and Rad1:Rad10 are also involved in the removal of 3′ nonhomologous tails in recombination intermediates (Sugawara *et al.* 1997). To determine if the differential effect of loss of *MSH2* and *MLH1* in the *RAD51*-dependent pathway was attributable to the role of Msh2 in MMR or in nonhomologous tail removal, we also examined the role of the proteins that form the clippase complex and the role of a MutS homolog not required for the clippase activity, Msh6 (Table 3). Because all of the sequence nonidentities in the homeologous recombination substrates are base substitutions, we would predict that all potential mismatches in the recombination intermediates should be recognized by Msh2:Msh6. The increase in the homeologous recombination rate in the *msh6 rad59* double mutant relative to the *rad59* single mutant demonstrates that the loss of Msh6 has a much smaller effect on homeologous recombination than does the loss of Msh2 and is comparable to the effect of Mlh1 loss (Figure 3). Although this result would be consistent with clippase activity causing the 10-fold difference in the relative homeologous to homologous recombination ratio in *rad59 msh2 vs. rad59 mlh1* mutants, neither loss of Msh3 nor loss of Rad1 in a *rad59* background leads to signifi-

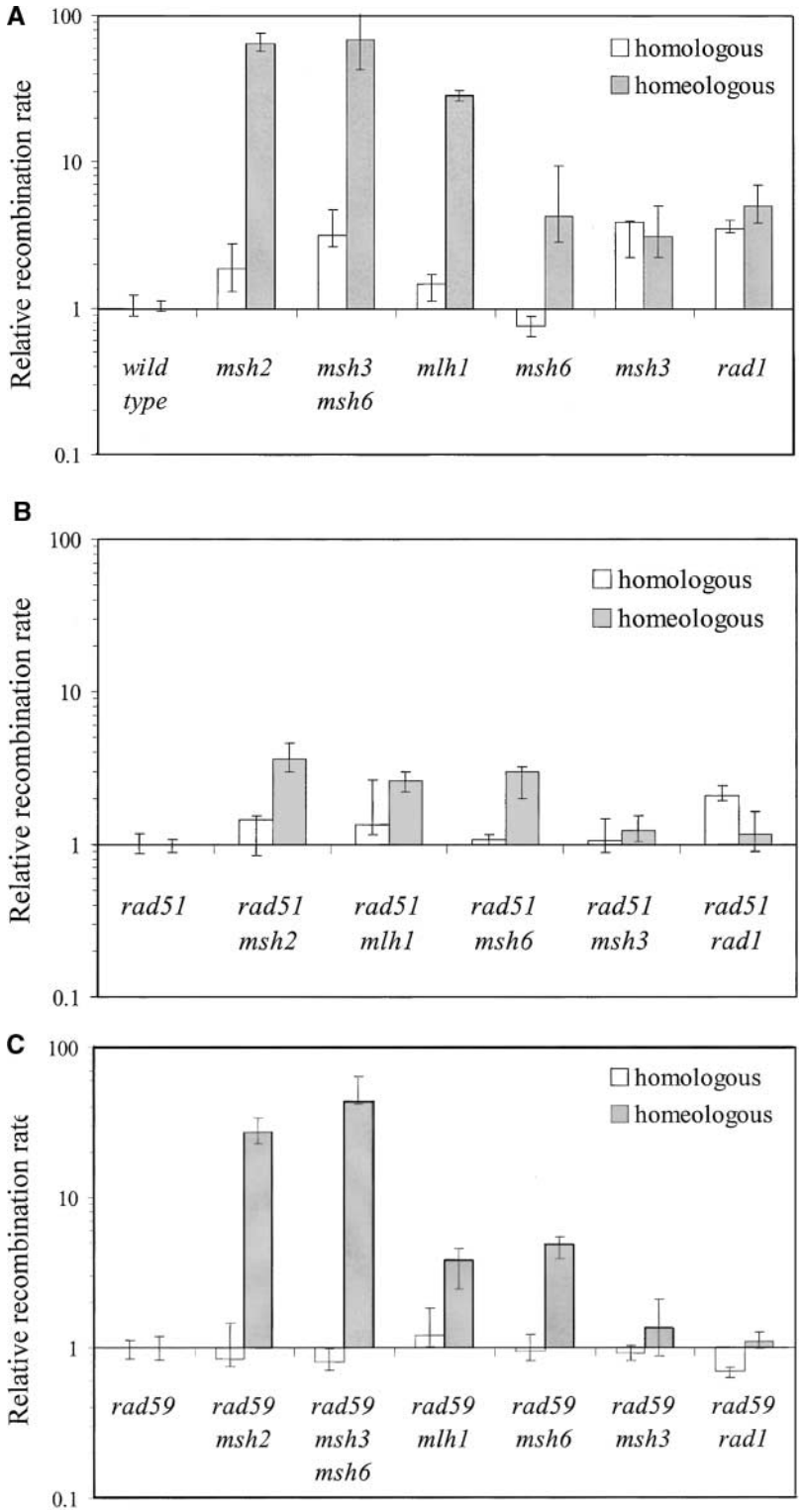

FIGURE 3.—The effect of MMR defects in separate recombination pathways. Homologous (open bars) and homeologous (shaded bars) recombination rates were determined for *RAD*(A), *rad51*(B), and *rad59*(C) mutant strains in MMR-proficient and MMR-defective backgrounds. The relative rate was determined by dividing the recombination rate of double-mutant strains by that of the same substrates in the corresponding *RAD* or *rad* single-mutant strain. Error bars based on the confidence intervals for the rates are indicated.

cant changes in the relative level of homeologous to homologous recombination (Table 3, Figure 3). Together, these results suggest that either the Msh2:Msh3 complex inhibits homeologous recombination in a clippase-independent fashion that is redundant with the Msh2:Msh6 complex or Msh2 acts independently of the Msh2:Msh3 and Msh2:Msh6 complexes to regulate homeologous recombination. These two possibilities can be distinguished by comparing the recombination phenotype of the *rad59 msh2* double mutant to that of the *rad59 msh3 msh6* triple mutant. As shown in Table 3, the relative homeologous to homologous recombination ratio is similar in the double and triple mutants, indicating that the Msh2:Msh6 and Msh2:Msh3 com-

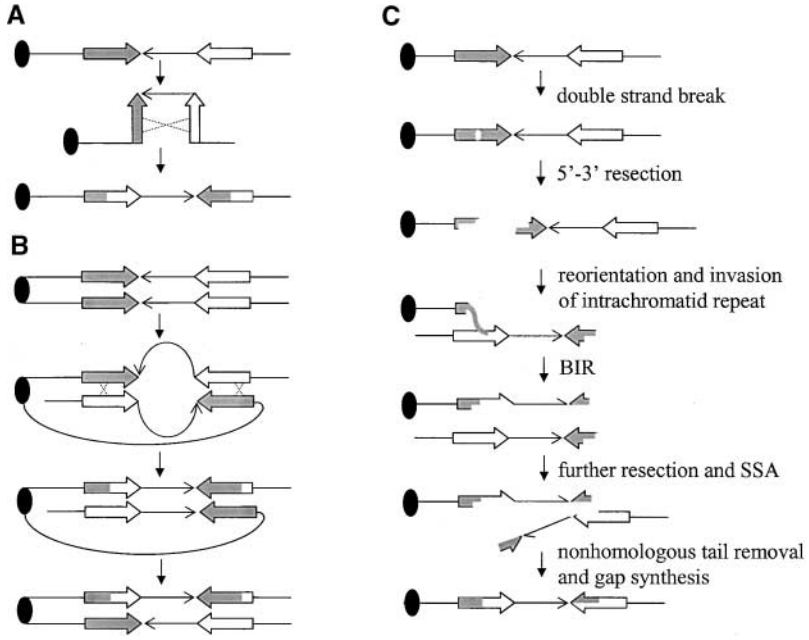

FIGURE 4.—Recombination mechanisms for reorientation of the invertible segment in an inverted repeat assay system. Homeologous inverted repeats (shaded and open arrows) are depicted flanking the invertible segment (solid arrow, see Figure 1). (A) Intrachromatid crossover. Intramolecular crossing over between flanking inverted repeats reorients the *HIS3* sequences to create a His^+ prototroph. (B) Sister-chromatid gene conversion without crossing over. Long conversion tracts between misaligned sister chromatids will lead to inversion of the intervening sequence, allowing expression of the full-length *HIS3* gene. Following alignment of the flanking repeats, the intervening sequence can loop around to create a continuous tract of homology. Conversion of the intervening region will reorient the *HIS3* coding sequence to allow His^+ prototrophy. (C) BIR with SSA. The 3′ tail of a DSB in one repeat invades the intact repeat and primes DNA synthesis to the other end of the break. Reannealing of the single-stranded repeat sequences can occur such that the intervening sequence is inverted 50% of the time, allowing *HIS3* expression.

plexes have overlapping or competing roles in the regulation of homeologous recombination in the *RAD51*-dependent pathway. These roles are only partially dependent on the Mlh1 protein.

DISCUSSION

The importance of homologous recombination for genome stability is indicated by the many levels of regulation and by the disease processes that result from a breakdown of that regulation (HARFE and JINKS-ROBERTSON 2000b; BISHOP and SCHIESTL 2002). A crucial level of regulation is the homology restrictions enforced by the MMR pathway, such that the level of recombination between substrates of 91% identity (homeologous recombination) is reduced 40-fold relative to recombination between 100% identical substrates (homologous recombination) in the assay system used here. To determine if interaction with specific factors in recombination pathways is required for that suppression, the MMR-dependent inhibition of recombination between nonidentical substrates was examined in several recombination mutants. We found that the level of homeologous recombination suppression is distinct in different recombination pathways and dependent on different activities of the MMR proteins.

Potential mechanisms for the recombination detected by the inverted repeat assay used in this study are depicted in Figure 4. First, reorientation of the region between the inverted repeats can occur by intramolecular recombination that requires crossing over between the repeats on a single chromosome (intrachromatid crossing over; Figure 4A). A second method is gene conversion without crossing over between sister chromatids after unequal alignment of the sisters (sister-chromatid gene conversion; Figure 4B). Pairing of the misaligned repeats makes possible a long conversion tract that extends from one set of paired repeats, through the invertible segment, into the other set of paired repeats such that the segment between the inverted repeats is reoriented on the repaired sister (Figure 4B). Because gene conversion can occur via SDSA or via an intermediate that involves Holliday junction formation/resolution (see Introduction), the intermediate steps of this mechanism are not depicted. Both of these processes in Figure 4, A and B, are completely dependent on *RAD51* (PAQUES and HABER 1999).

A third mechanism, originally described for inverted repeats on plasmids, is BIR followed by SSA (see Introduction and Figure 4C; KANG and SYMINGTON 2000). BIR is the one-ended invasion that can prime DNA synthesis down an entire chromosome arm. In the case of a DSB in an inverted repeat, the broken end of one repeat can invade the intact repeat on the same chromatid and prime replication through the intervening sequence to the other end of the break. Thus, BIR not only partially duplicates the repeats but also inverts the region between them. SSA between the repeats can then occur such that *HIS3* can be expressed (Figure 4C). For simplicity, an intramolecular event is depicted, but BIR with SSA between sister chromatids could also lead to His^+ prototrophy, although this would require an interruption in replication of the sister-chromatid arm to allow SSA. Studies of BIR followed by SSA with plasmids containing inverted repeats have shown that such events can occur in the absence of *RAD51* yet are dependent on *RAD59* and *RAD50* (KANG and SYMINGTON 2000; IRA and HABER 2002). *RAD52* is required for both *RAD51*-

and *RAD59*-dependent recombination mechanisms (Bai *et al.* 1999).

In our assay system, recombination between homeologous substrates or homologous substrates is severely reduced in the absence of *RAD52* or of both *RAD51* and *RAD59*. The slightly higher level of homeologous and homologous recombination in the *rad51 rad59* double mutant than in the *rad52* single mutant may reflect the ability of the cells to undertake some BIR coupled with SSA (see Figure 4) or nonhomologous end joining in the absence of *rad59* (Richardson and Jasin 2000; Sugawara *et al.* 2000; Signon *et al.* 2001). The similar genetic dependencies of homologous and homeologous recombination on *RAD52* and on either *RAD51* or *RAD59* suggest that the recombination mechanism is the same for homeologous recombination as for homologous recombination.

Loss of each of the members of the *RAD52* epistasis group, with the exception of *RAD50*, caused decreases in the level of homologous recombination. *RAD50* has long confused geneticists because its mutant phenotype is assay dependent. For example, the level of inverted-repeat recombination on chromosomes and plasmids typically decreases in a *rad50* mutant (Rattray and Symington 1995; Ira and Haber 2002), whereas the level of allelic recombination between homologous chromosomes or ectopic recombination between nonhomologous chromosomes increases (Malone *et al.* 1990; Freedman and Jinks-Robertson 2002). Increases in recombination between different chromosomes in *rad50* strains have led to the suggestion that Rad50 plays a role in fostering sister-chromatid interactions. Because of the nature of the recombination mechanisms proposed for the assay system described here, it was assumed that *rad50* mutants would also display a decrease in recombination. The opposite occurred, however; *rad50* as well as *mre11* mutants displayed a mild hyperrecombination phenotype in this assay. In addition, measurement of the levels of recombination on other chromosomes in *rad50* or *mre11* mutants has demonstrated strong chromosome context effects with our assay system (R. Spell and S. Jinks-Robertson, unpublished results). Our results indicate that the sister-chromatid interaction model for Rad50 may be too simplistic, and this view is supported by the finding that the genetic requirement for *RAD50* differs for spontaneous and DNA-damage-induced recombination (Dong and Fasullo 2003). Context and assay dependence suggests that the type and location of the recombination-initiating lesion may affect the requirement for *RAD50* and other members of the MRX complex.

Strikingly, mutations in the *RAD52* epistasis group genes affected the level of homeologous recombination differently than the level of homologous recombination, as if the regulation of homeologous recombination was altered in the mutants. The mutants fell into two groups, defined by the phenotypes of the *rad51* and *rad59* single mutants. For clarity, we discuss the recombination that occurs in the *rad51* and *rad59* single mutants as *RAD59* dependent (*i.e.*, *RAD51* independent) and *RAD51* dependent (*i.e.*, *RAD59* independent), respectively. In the first class, containing *RAD50, RAD51, RAD54, RAD55*, and *RAD57*, loss of the gene caused a larger decrease in homologous recombination than in homeologous recombination, indicating a loss of mismatch-associated inhibition of recombination. For example, in the *rad51* mutant, homeologous recombination remained at wild-type levels, while homologous recombination decreased to one-third of the wild-type level. The increase in the homeologous to homologous recombination ratio in the *rad50* mutant may result from less efficient end processing, perhaps forcing the use of shorter homologies and thereby lowering the chance of mismatches in heteroduplex DNA. Explanations for the observed increase in the ratio of homeologous to homologous recombination in the *rad51* mutant include the slight possibility that Rad51 is partially required for homologous recombination, while homeologous recombination is completely independent of Rad51. However, the evidence that the mechanism for homeologous recombination and homologous recombination is similar makes that possibility unlikely. Alternatively, the increase in the homeologous recombination to homologous recombination ratio in a *rad51* mutant may indicate that Rad51 is involved in the normal suppression of homeologous recombination. This possibility is made more interesting by the reports of physical interactions between Rad51 and the MMR factor Mlh1, between Rad51 and the BLM helicase homolog Sgs1, and between Mlh1 and Sgs1 (Pedrazzi *et al.* 2001; Wu *et al.* 2001; Ho *et al.* 2002). The potential for the recruitment of Sgs1 to homeologous recombination intermediates by interaction with Rad51 and Mlh1 suggests that one mechanism for rejecting recombination intermediates containing heterology occurs by unwinding heteroduplex DNA. Such a model is consistent with the observation that mutation in *SGS1* causes an increase in homeologous recombination (Myung *et al.* 2001; R. M. Spell and S. Jinks-Robertson, unpublished data). Finally, in the absence of the *RAD51*-dependent pathway, homeologous recombination intermediates may be shunted into a recombination pathway that is less sensitive to mismatches or involves formation of fewer mismatches.

The loss of the *RAD59*-dependent recombination pathway defines the second phenotypic class with regard to the change in the ratio of homeologous to homologous recombination. In the *rad59* mutant, there was a larger decrease in homeologous recombination than in homologous recombination, as if these mutants had lost a pathway that is more tolerant of mismatches. One potential explanation for lower stringency of the *RAD59*-dependent pathway is the ability of *RAD59*-dependent recombination to use shorter lengths of homology, as described by Ira and Haber using an HO-induced in-

traplasmid recombination assay (Ira and Haber 2002). The use of shorter homologies could lower the number of mismatches in the heteroduplex recombination intermediates formed between homeologous substrates, making them a less-efficient target for the antirecombination activity of the MMR machinery.

To explore the roles of different MMR proteins in the suppression of homeologous recombination in different potential recombination pathways described above, we examined several MMR-defective backgrounds. Strains lacking Msh2 lack both the Msh2:Msh3 and Msh2:Msh6 mismatch recognition complexes and, in addition, are deficient in the clippase function of Msh2:Msh3 together with Rad1:Rad10. It should be noted that loss of Msh6 specifically removes the recognition of base-base mismatches, the only type of mismatch present in heteroduplex recombination intermediates of the repeats used here. In the absence of Mlh1, mismatch recognition presumably cannot be coupled to the downstream processing events, which may be different for the antimutator *vs.* the antirecombination activity of the MMR machinery (Welz-Voegele *et al.* 2002). It has been shown previously that the homeologous recombination phenotypes of mutants defective in *MSH2, MSH6,* or *MLH1* are not equivalent (Nicholson *et al.* 2000; this study), with loss of *MSH2* causing the greatest increase in homeologous recombination. Despite the fact that only Msh6 should be involved in the recognition of potential mismatches between the recombination substrates used here, the *msh6* phenotype was much weaker than that of *msh2* mutants (Nicholson *et al.* 2000; this study). As reported previously, the ratio of homeologous to homologous recombination in a *msh3 msh6* double mutant was the same as that in a *msh2* single mutant. Because loss of Msh3 alone or of Rad1 did not affect the regulation of homeologous recombination in the current assays, we conclude that the antirecombination activity of the Msh2:Msh3 complex is not related to its clippase function. Finally, the level of homeologous recombination in *mlh1* strains was only approximately one-half of that in a *msh2* mutant. The distinction between phenotypes in *msh2* and *mlh1* backgrounds suggests that mismatch recognition alone can contribute to the inhibition of homeologous recombination.

Analysis of the impact of MMR defects when only *RAD51* or *RAD59* is functional reveals the distinct roles that individual MMR proteins play in the corresponding pathways. In a *rad51* background (*RAD59*-dependent recombination), the pairing of single DNA strands in recombination intermediates would presumably occur via an annealing mechanism, whereas in a *rad59* background (*RAD51*-dependent recombination) pairing would involve invasion of a duplex DNA molecule. In a *rad51* background, loss of Msh2, Msh6, or Mlh1 resulted in an approximately threefold increase in the ratio of homeologous to homologous recombination, while elimination of either Msh3 or Rad1 had no effect. This difference suggests that the MMR-associated suppression of homeologous recombination in the *RAD59*-dependent pathway involves complexes of Msh2 with Msh6 and also requires the Mlh1:Pms1 complex (Figure 5). Despite the increase in the homeologous to homologous recombination ratio observed upon elimination of MMR components in a *rad51* background, the homeologous recombination rate was still fourfold below the homologous recombination rate. Therefore, although homeologous recombination in the *RAD59*-dependent annealing pathway is regulated by the MMR machinery, most of the regulation occurs via an MMR-independent mechanism.

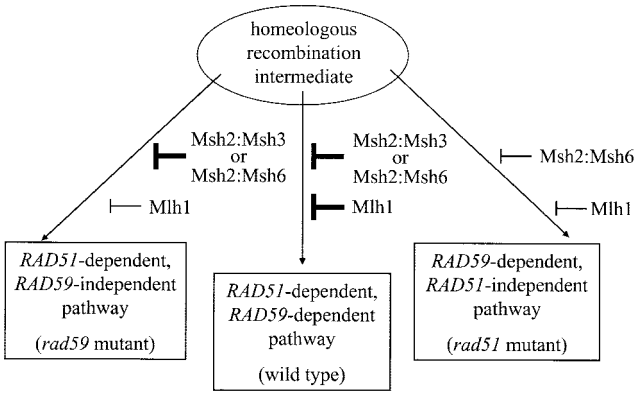

Figure 5.—Relationship between recombination pathways and MMR-dependent regulation of homeologous recombination. A homeologous recombination intermediate can enter pathways dependent on Rad51, Rad59, or both. Depending on the pathway, MMR proteins inhibit homeologous recombination to different degrees. Regulation of homeologous recombination in wild-type cells suggests that recombination intermediates utilize overlapping pathways.

In contrast to the ~3-fold increase in homeologous recombination observed upon loss of Msh2 in a *rad51* background, there was an ~30-fold increase in homeologous recombination associated with loss of Msh2 in a *rad59* background (*RAD51*-dependent pathway). The phenotypes of *msh3, msh6, rad1,* and *msh3 msh6* mutants demonstrate that regulation in the *RAD59*-dependent pathway is mediated by both the Msh2:Msh6 and the Msh2:Msh3 complexes but does not significantly involve clippase activity. Also, in the *rad59* mutant background, the 10-fold difference in homeologous recombination rates in *msh2 vs. mlh1* strains suggests that Mlh1 has relatively little antirecombination activity in the *RAD51*-dependent strand invasion pathway. Therefore, in the *RAD51*-dependent recombination pathway, most suppression of homeologous recombination is effected by a Msh2-containing complex, with a MutL-like complex being largely dispensable (Figure 5). Finally, in contrast to the minor role of Msh2 in regulation of the *RAD59*-dependent pathway, Msh2 plays a major role in the enforcement of recombination fidelity in the *RAD51*-dependent pathway (Figure 5).

In addition to advancing the understanding of the

role of MMR in homeologous recombination regulation, the pattern of regulation revealed in the wild-type, *rad51*, and *rad59* mutant backgrounds suggests that neither pathway remaining in the single mutants accurately represents the mechanism utilized in wild-type cells (Figure 5). This is especially evident when Mlh1 is also absent. Whereas loss of Mlh1 causes a large increase in homeologous recombination in a *RAD* background, the effect of *mlh1* mutation in *rad51* or *rad59* backgrounds is much weaker (29-fold *vs.* 2.6-fold or 3.8-fold, respectively). Thus, the greater effect of the loss of Mlh1 in the presence of both Rad51 and Rad59 suggests that the regulation of recombination in wild type is not fully described by the separate regulation of either the *RAD51*-dependent pathway or the *RAD59*-dependent pathway. Since the phenotype of the *rad51 rad59* mutants suggests that Rad51 and Rad59 promote the bulk of all mitotic recombination, one need not invoke a pathway involving novel proteins to explain these results. Instead, it is much more reasonable to imagine a pathway that involves the activities of both Rad51 and Rad59 (Figure 5). It is possible, for example, that Rad59 may facilitate the reannealing of 3′ ends following the extension of one of those ends during Rad51-mediated SDSA. Alternatively, Rad51 could facilitate strand invasion for BIR that is completed by Rad59-mediated SSA (see Figure 4C). Indeed, the potential overlap of *RAD51*- and *RAD59*-dependent processes has been noted for inverted repeat recombination on plasmids (Bartsch *et al.* 2000; Kang and Symington 2000; Ira and Haber 2002). This study provides further support for such a model of cooperation between Rad51 and Rad59 for spontaneous recombination between repeats in chromosomal DNA.

In summary, the study of spontaneous recombination between homeologous repeats in chromosomal DNA has revealed the fidelity of recombination in different recombination pathways, defined by their dependence on *RAD51* and *RAD59*. Examination of the loss of suppression of inappropriate recombination between mismatched sequences in strains lacking different members of the MMR pathway suggests distinct roles of these proteins in the different pathways. These studies highlight the complexity of the regulatory mechanisms that affect recombination fidelity and genome stability.

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