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Mitotic Crossovers between Diverged Sequences Are Regulated by Mismatch Repair Proteins in *Saccharomyces cerevisiae*

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Mismatch repair systems correct replication- and recombination-associated mispaired bases and influence the stability of simple repeats. These systems thus serve multiple roles in maintaining genetic stability in eukaryotes, and human mismatch repair defects have been associated with hereditary predisposition to cancer. In prokaryotes, mismatch repair systems also have been shown to limit recombination between diverged (homeologous) sequences. We have developed a unique intron-based assay system to examine the effects of yeast mismatch repair genes (*PMS1*, *MSH2*, and *MSH3*) on crossovers between homeologous sequences. We find that the apparent antirecombination effects of mismatch repair proteins in mitosis are related to the degree of substrate divergence. Defects in mismatch repair can elevate homeologous recombination between 91% homologous substrates as much as 100-fold while having only modest effects on recombination between 77% homologous substrates. These observations have implications for genome stability and general mechanisms of recombination in eukaryotes.

Much of our knowledge concerning the mechanism of homologous recombination in eukaryotes has come from studies done with the yeast *Saccharomyces cerevisiae* (for a review, see reference 40), in which two basic types of recombination occur: reciprocal recombination (crossing-over) and nonreciprocal recombination (gene conversion). Reciprocal recombination events involve the physical exchange of information between chromosomes and hence alter the genetic linkages of markers on either side of the crossover event. Gene conversion events involve the unidirectional transfer of information from one chromosome to another and are nonrandomly associated with crossing-over. The association between gene conversion and crossing-over has been central to developing molecular models of recombination in which conversion events reflect the formation of a heteroduplex intermediate that can be resolved in either a crossover or noncrossover mode (16, 30, 67). Homologous recombination is usually thought of as occurring between sequences at identical positions on homologous chromosomes (allelic recombination), but it also can involve similar sequences at nonallelic (ectopic) locations (for reviews, see references 20 and 39). A large number of repeated sequences are found dispersed throughout eukaryotic genomes and can serve as substrates for ectopic recombination events. Such repeats exhibit various degrees of homology at the DNA level and are referred to as being homeologous to reflect their partial homology. Ectopic gene conversion between homeologous sequences can lead to the homogenization of repeats and is likely to be an important mechanism for the concerted evolution of multigene families (12). It also can generate novel sequence combinations within a gene family and has been implicated as a possible source of diversity at immunoglobulin loci (6, 28). Crossing-over between dispersed repeats results in genome rearrangements (deletions, duplications, inversions, and translocations) and thus could be one source of the genomic instability frequently found in tumor cells.

In yeast cells, mitotic recombination between artificially constructed ectopic repeats can occur as efficiently as allelic recombination (26), which raises the question of how eukaryotic organisms containing large amounts of repetitive DNA maintain an acceptable level of genome stability. There are, in principle, two mechanisms for limiting recombination between naturally occurring repeated sequences: (i) shrinking the repeats so that the size is below a minimal length that efficiently supports recombination and (ii) diverging the repeats at the DNA sequence level (46). There is experimental evidence that each of these mechanisms effectively can reduce recombination rates (1, 9, 15, 19, 27, 32, 48, 58, 69). In addition, the well-characterized bacterial mismatch repair systems (Mut systems of *Escherichia coli* and *Salmonella typhimurium* and Hex system of *Streptococcus pneumoniae*), which function primarily to edit the products of DNA replication, also regulate the process of homologous recombination (45).

The Mut system of *E. coli* has four key proteins: MutS, MutH, MutL, and UvrD. MutS binds directly to mismatched bases; MutH has an endonuclease activity that cleaves the unmethylated DNA strand at hemimethylated *dam* sites; MutL is proposed to be a molecular matchmaker that promotes interaction between MutS and MutH, thereby coupling mismatch recognition to strand incision; and UvrD is a helicase that ejects the nicked strand containing the mismatch (34, 55). Bacterial mismatch repair systems have been shown to limit recombination either between endogenous homeologous sequences (41) or between endogenous sequences and those introduced by conjugation, transduction, or transformation (13, 29, 41, 48, 59, 74). Prokaryotic mismatch repair systems presumably promote genome stability by preventing chromosomal rearrangements via recombination between naturally occurring homeologous repeats and function as an important species barrier by preventing the stable incorporation of exogenous sequences into the genome. Although the precise step(s) at which the mismatch repair system detects mismatches and exerts its antirecombination effect is not known, it could inter-

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fere with strand assimilation, branch migration, or resolution. It also could melt or multiply nick a heteroduplex intermediate or product that contains numerous mismatches and thereby effectively abort or destroy the recombination event. In vitro studies have demonstrated that MutS can block RecA-promoted strand exchange, indicating that mismatch repair may interfere with recombination at an early step (72); results of genetic studies of *E. coli* are consistent with this view (29).

Much recent attention has focused on MutS- and MutL-homologous eukaryotic mismatch repair proteins because of their association with hereditary nonpolyposis colon cancer (8, 14, 25). In *S. cerevisiae*, multiple MutS and MutL homologs have been identified, with five MutS homologs (Msh1p to Msh5p) and three MutL homologs (Pms1p, Mlh1p, and Mlh2p) having been described to date (17, 22, 36, 43, 49, 52, 62). Msh2p, Msh3p, Pms1p, and Mlh1p are important for correcting mismatches arising during nuclear DNA replication and recombination (21, 36, 43, 50, 62, 63, 70). Mutations in *MSH2*, *PMS1*, and *MLH1* result in strong mutator phenotypes, increase postmeiotic segregation of heterozygous markers, and greatly destabilize simple repeats. Mutations in *MSH3* have relatively modest effects on mutation rates, postmeiotic segregation, and simple repeat stability. Msh1p appears to be involved in the maintenance of stable mitochondrial DNA (50), while Msh4p and Msh5p appear to be meiosis-specific proteins with a role in resolving recombination intermediates rather than a role in mismatch correction of heteroduplex recombination intermediates (17, 52). The sequence of an additional MutS homolog has been found in the yeast genome sequencing project and appears to be the homolog of human p160/GTBP, which, when defective, leads to genetically unstable cells (11, 37, 38).

Numerous studies of *S. cerevisiae* have been done to address the effect of sequence heterogeneity on mitotic recombination rates (5, 15, 23, 31, 32, 42, 51, 56), and several of these have investigated the possible involvement of the yeast mismatch repair machinery in regulating homeologous interactions (4, 5, 42, 56). These studies used substrates with less than 85% homology, and while all found at least a 15-fold effect of sequence divergence on recombination rates in wild-type strains, the magnitude of the effect was quite variable. This variability likely reflects the very diverse natures of the assay systems used, some of which were chromosomal and some of which were extrachromosomal. Mutations in *PMS1* have not been observed to increase homeologous recombination rates (relative to the control homologous recombination rates) in any of the systems examined thus far (5, 56). The *MSH2* gene, and to a lesser extent the *MSH3* gene, was found to affect chromosomal recombination rates between homeologous direct repeats flanked by perfect sequence homology (56) but had no effect on the rate of homeologous recombination in a transformation-based assay (4).

The degree of sequence heterology could be an important factor when one is assessing the antirecombination activity of mismatch repair proteins in *S. cerevisiae*. One would predict a large effect of mismatch repair proteins on homeologous recombination only if the substrates can be efficiently acted on by the basic recombination machinery. If substrates are too diverged to recombine, eliminating mismatch repair would not substantially improve their recombination. The goal of experiments reported here was to examine the role of mismatch repair proteins in regulating crossovers between substrates of different homologies in *S. cerevisiae*. For these studies, we have developed a unique, function-independent assay system that allows the sequence contents of the substrates to be systematically varied. The recombination substrates were set up as inverted repeats (IRs) within the intron of a chromosomal selectable gene so that crossing-over between them generates a full-length, spliceable marker. Seventy-seven percent homologous substrates (TATA-binding protein genes from *S. cerevisiae* and *Schizosaccharomyces pombe*) and 91% homologous substrates (chicken β-tubulin [cβ] cDNAs) (hereafter referred to as 77 and 91% substrates), as well as the corresponding 100% control substrates, were examined in this system. A direct correlation between the rate of recombination and the degree of substrate sequence homology was observed in wild-type strains. While mutations in *PMS1*, *MSH2*, and *MSH3* had little effect on the rate of crossing-over between the 77% substrates, they had a pronounced stimulatory effect on the 91% substrates. These results are discussed in terms of their importance for aspects of genome stability and general mechanisms of recombination in eukaryotes.

MATERIALS AND METHODS

Media and growth conditions. *S. cerevisiae* strains were grown at 30°C, and bacterial strains were grown at 37°C. YEP medium (1% yeast extract, 2% Bacto Peptone; 2.5% agar for plates) supplemented with 2% glycerol and 4% galactose (YEPGG) or 2% dextrose (YEPD) was used for nonselective growth of yeast strains. Synthetic complete medium (60) supplemented with 2% glycerol and 4% galactose but deficient in histidine (SGG-his) was used to select for yeast prototrophs in the His^+ rate measurement experiments. 5-Fluoroorotic acid (5-FOA) medium containing 1 g of 5-FOA per liter was used to select for Ura^- yeast segregants (7). LB medium (1% yeast extract, 0.5% Bacto Tryptone, 1% NaCl; 1.5% agar for plates) was used for growth of *E. coli* strains. Ampicillin (100 μg/ml) was added to LB for growth of plasmid-containing strains.

Plasmid constructions. All plasmid manipulations were done in *E. coli* TB1 [F^- *ara*Δ(*lac-proAB*) *rpsL* ϕ80d*lacZ*ΔM15 *hsdR17*] or DH5α [F^- ϕ80d*lacZ*ΔM15 *endA1 recA1 hsdR17 supE44 thi-1* λ^- *gyrA96 relA1* Δ(*lacZYA-argF*)U169], using standard molecular biological techniques (54). The homologous and homeologous IR recombination constructs were built as shown schematically in Fig. 1B. Plasmid pSR266 contains the basic *GAL* promoter (*pGAL*)-*HIS3*::intron construct and was constructed as follows. First, a 2-kb *pGAL-HIS3 Bam*HI fragment from YIp-Sc3309 (64) was treated with Klenow enzyme and inserted into a filled-in (with Klenow enzyme) *Bam*HI site within the polylinker region of the *CEN-URA3* vector pRS316 (61), yielding plasmid pSR242. The *Sna*BI-*Pvu*II artificial intron (AI)-containing fragment from pUC-AI (73) was then inserted at the unique *Msc*I site within the *HIS3* coding sequence of pSR242, resulting in plasmid pSR245. pSR245 contains the AI in the *pGAL-HIS3*::intron construct in the correct orientation and reading frame as determined by a functional assay. pSR266 was subsequently generated by substituting the 2.1-kb *Pvu*II fragment containing the *pGAL-HIS3*::intron construct (from pSR245) for the *Pvu*II polylinker fragment of the *URA3*-marked integrating vector pRS306 (61).

pSR406 is the 100% homologous IR construct that contains cβ2 recombination substrates. pSR406 was made as follows. A 350-bp segment of the cβ2 cDNA sequence was PCR amplified from plasmid pSR257 (66), using primers 5′-CGGTCGACAGATCTGGCCACCATGAGCGGCGTGA-3′ (forward) and 5′-GGGGATCCACTCCACAAAGTAG-3′ (reverse). Both primers have restriction endonuclease sites near the 5′ ends; the forward primer adds *Sal*I and *Bgl*II sites to the amplified cDNA, and the reverse primer contains a cβ2 *Bam*HI site. The amplified product was digested with *Sal*I and *Bam*HI and was inserted into *Sal*I-*Bam*HI-digested pBluescript SK, generating plasmid pSR402. The *Bam*HI-*Spe*I fragment from pSR266 was replaced with the *Bgl*II-*Spe*I cβ2 fragment from pSR402, resulting in plasmid pSR404. pSR404 is a 5′ cβ2 IR cassette containing the 5′ end of the *HIS3* coding sequence, the 5′ end of the AI, and the 350-bp cβ2 cDNA. The 3′ cβ2 IR cassette plasmid, pSR400, was made by substituting the *Sal*I-*Bam*HI cβ2 fragment of pSR402 for the *Sal*I-*Bam*HI fragment of pSR266. Finally, the *Sma*I-*Spe*I 5′ cβ2 IR cassette of pSR404 was purified and ligated to the *Spe*I-*Nae*I-digested 3′ cβ2 IR cassette plasmid (pSR400) to generate pSR406.

pSR407 is the homeologous IR construct containing 91% homologous cβ2 and cβ7 sequences (Fig. 2) and was constructed as follows. A 350-bp segment of the cβ7 cDNA sequence was PCR amplified from plasmid pSR272 (35), using primers 5′-CGGTCGACAGATCTGGCCACCATGAGCGGCGTC-3′ (forward) and 5′-GGGGATCCACTCGACGAAGTAG-3′ (reverse). As described above for the cβ2-specific primers, the cβ7-specific primers contained *Sal*I and *Bgl*II (forward) or *Bam*HI (reverse) restriction sites at the 5′ ends. The amplified product was digested with *Sal*I and *Bam*HI and ligated to *Sal*I-*Bam*HI-digested pBluescript SK to generate plasmid pSR403. pSR401, which contains a 3′ cβ7 IR cassette, was made by substituting the *Sal*I-*Bam*HI cβ7 fragment from pSR403 for the *Sal*I-*Bam*HI fragment of pSR266. pSR407 was then made by ligating the *Sma*I-*Spe*I 5′ cβ2 IR cassette of pSR404 to *Spe*I-*Nae*I-digested pSR401. The amplified cβ segments comprising the 100 and 91% homologous IR constructs were sequenced to confirm the homologies.

The IR constructs with the *SPT15* sequences were generated in a manner

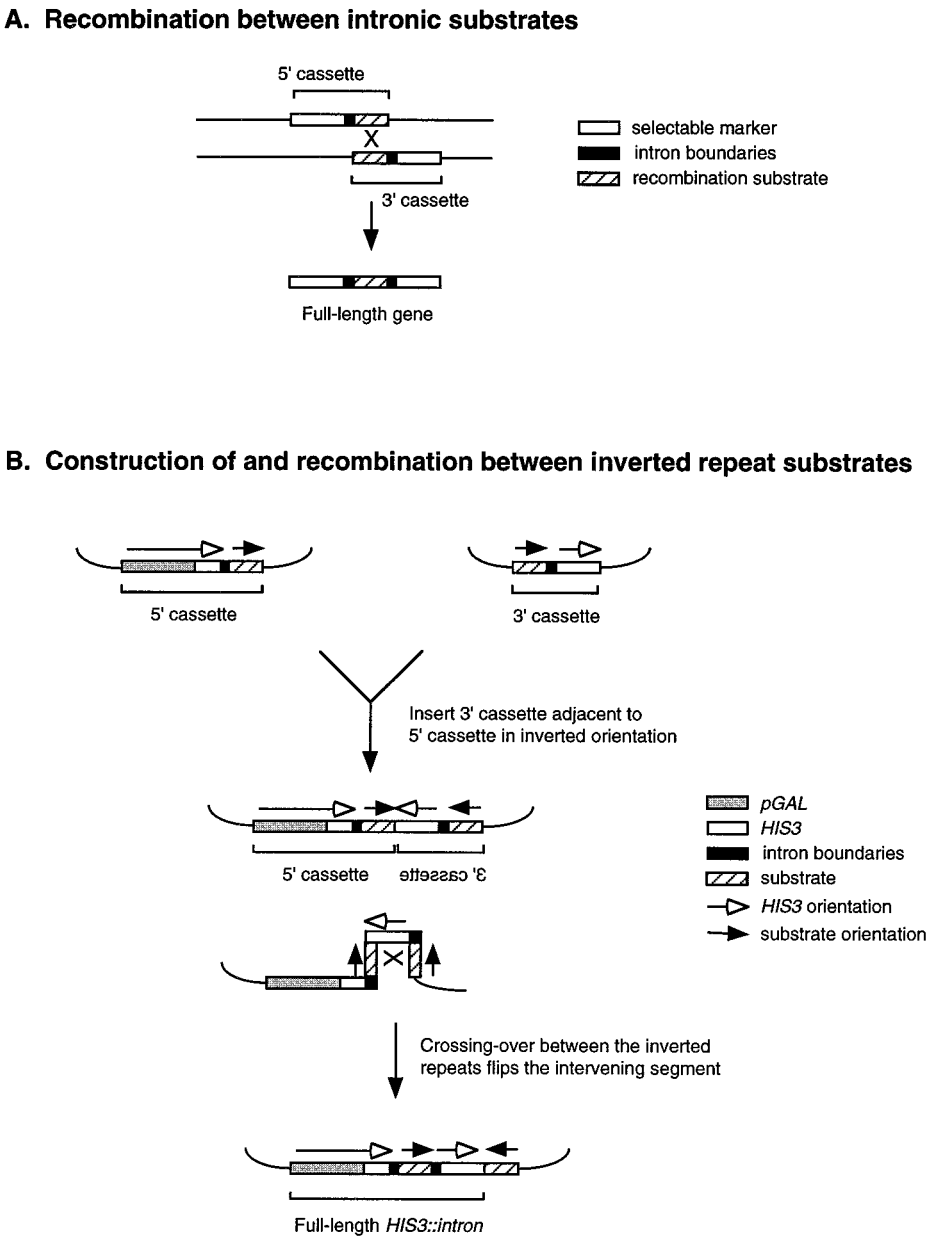

FIG. 1. The intron-based recombination system for assaying crossing-over between homeologous sequences. A 5′ cassette contains the 5′ part of a selectable marker, the 5′ part of the AI (5′ donor site), and a full-length recombination substrate; a 3′ cassette contains a full-length recombination substrate, the 3′ part of the AI (TACTAAC box and 3′ acceptor site), and the 3′ end of the selectable marker. (A) How recombination between intronic substrates yields a full-length, spliceable gene. (B) How the 5′ and 3′ cassettes were combined as IRs. Crossing-over between the homeologous IRs flips the region between the repeats so that the 3′ end of *HIS3* is in the same orientation as the 5′ end, and the 3′ end of the AI is in the correct orientation for splicing to occur.

similar to that described above for the cβ sequences. Two 360-bp fragments, one (designated C) from the *S. cerevisiae SPT15* gene (p306RB [56]) and the other (designated P) from the *S. pombe* homolog (pYIS5 [56]), were PCR amplified with primers 5′-GGGATCCATCTCTTTACCTCAGG-3′ (forward) and 5′-GTCTACTCCTTCCCCATCACA-3′ (reverse). The amplified fragments were digested with *Bam*HI and *Hin*dIII and subcloned into *Bam*HI-*Hin*dIII-digested pBluescript KS, yielding pBluescriptC (*S. cerevisiae* sequences) and pBluescriptP (*S. pombe* sequences). The *Bam*HI-*Sal*I fragment of pSR266 containing the 5′ portion of the *pGAL-HIS3*::intron construct was replaced with the *SPT15 Bam*HI-*Sal*I fragments from pBluescriptC and pBluescriptP to make the 3′ *SPT15* cassette plasmids p2663C and p2663P, respectively. 5′ IR cassette plasmids p2665C and p2665P were generated by replacing the *Bam*HI-*Sst*I segment of pSR266 containing the 3′ half of the *pGAL-HIS3*::intron allele with the *SPT15 Hin*cII-*Sst*I fragments from pBluescriptC and pBluescriptP, respectively, after filling in of the *Bam*HI site in the vector. Finally, the *Sma*I-*Sst*I 5′ IR cassette fragment from p2665C was inserted into either *Sst*I-*Nae*I-digested p2663C or p2663P to make p266C/C (100% homologous C/C inverted repeats) or p266C/P (77% homologous C/P inverted repeats), respectively. p266P/P (100% homologous P/P inverted repeats) was made by inserting the *Sma*I-*Sst*I 5′ IR cassette fragment from p2665P into *Sst*I-*Nae*I-digested p2663P.

pSR211 and p306msRID are plasmids for introducing the *pms1*Δ and *msh2*Δ alleles, respectively, and contain *URA3* as a selectable marker. Plasmid pSR211 (*pms1*Δ; obtained from D. Maloney) was constructed by inserting a 5.2-kb *Kpn*I fragment containing *PMS1* into YIp5 and then deleting a *PMS1*-internal 2.7-kb *Mlu*I-*Sst*I fragment. Plasmid p306m2RID was constructed by inserting a 4.2-kb *Stu*I-*Xba*I *MSH2*-containing fragment (from plasmid p11-2, obtained from R. Kolodner) into pRS306 (61) and then deleting an *MSH2*-internal 2.4-kb *Pvu*II-*Xba*I fragment. pEN33 (obtained from R. Kolodner) contains an *msh3*Δ::*hisG-URA3-hisG* allele and was generated by replacing the *URA3* marker in plasmid *msh3*Δ::*URA3* (36) with the *hisG-URA3-hisG* cassette (2).

Yeast strain constructions. All yeast strains used in this study are listed in Table 1 and, with the exception of SJR381, were derived from SJR231 (*MAT*α

FIG. 2. DNA sequence alignments of the homeologous substrates. DNA sequences were aligned by using the PILEUP program of the Genetics Computer Group (10). Identical bases are highlighted in black. The *SPT15* sequences from *S. cerevisiae* (spt15c) and *S. pombe* (spt15p) are 77% homologous, with the longest stretch of uninterrupted perfect homology being 26 bp. The GC contents are 39 and 40% for spt15c and spt15p, respectively. The cβ2 and cβ7 sequences are 91% homologous, with the longest stretch of uninterrupted identity being 60 bp. The GC contents are 65 and 67% for cβ2 and cβ7, respectively.

ade2-101$_{oc}$ his3Δ200 ura3-Nhe) by transformation using a lithium acetate procedure (18). SJR381 was derived by transforming SJR328 (*MAT*α *ade2-101 his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R*), an isogenic derivative of SJR231 containing two additional auxotrophic markers. *pms1*Δ and *msh2*Δ strains were constructed by a standard two-step gene transplacement method (53), using the *URA3*-containing plasmids pSR211 and p306m2RID, respectively. pSR211 and p306m2RID were linearized with *Bst*XI to target to the *PMS1* and *MSH2* loci, respectively, and Ura$^+$ transformants were selected. Plasmid pop-out events were selected on 5-FOA medium and screened for a mutator phenotype. *msh3*Δ::*hisG* strains were constructed by one-step gene disruption with *Eco*RI-

TABLE 1. Recombination rates of homologous and homeologous sequences in mismatch repair-defective strains

Substrate (% homology)	Strain	Relevant genotype	Rate of His$^+$ recombinants	Rate relative to wild-type rate
cβ2/cβ2 (100)	SJR381	Wild type	7.6×10^{-7}	1.0
	SJR385	*pms1*Δ	10×10^{-7}	1.3
	SJR387	*msh2*Δ	20×10^{-7}	2.7
	SJR389	*msh3*Δ	18×10^{-7}	2.4
	SJR393	*msh2*Δ *msh3*Δ	21×10^{-7}	2.8
cβ2/cβ7 (91)	SJR382	Wild type	1.8×10^{-8}	1.0
	SJR386	*pms1*Δ	19×10^{-8}	11
	SJR388	*msh2*Δ	130×10^{-8}	71
	SJR390	*msh3*Δ	16×10^{-8}	8.9
	SJR391	*msh2*Δ *msh3*Δ	190×10^{-8}	100
C/C (100)	GCY104	Wild type	4.9×10^{-6}	1.0
	GCY111	*pms1*Δ	6.2×10^{-6}	1.3
	GCY117	*msh2*Δ	4.8×10^{-6}	1.0
	GCY119	*msh3*Δ	4.0×10^{-6}	0.8
	GCY122	*msh2*Δ *msh3*Δ	4.3×10^{-6}	0.9
P/P (100)	GCY262	Wild type	5.1×10^{-6}	1.0
	GCY261	*pms1*Δ	15×10^{-6}	2.9
	GCY260	*msh2*Δ	3.5×10^{-6}	0.7
	GCY263	*msh3*Δ	3.6×10^{-6}	0.7
	GCY259	*msh2*Δ *msh3*Δ	3.4×10^{-6}	0.7
C/P (77)	GCY103	Wild type	2.3×10^{-9}	1.0
	GCY110	*pms1*Δ	4.7×10^{-9}	2.0
	GCY118	*msh2*Δ	13×10^{-9}	5.9
	GCY120	*msh3*Δ	10×10^{-9}	4.4
	GCY123	*msh2*Δ *msh3*Δ	17×10^{-9}	7.5

digested pEN33, followed by 5-FOA selection to identify loss of the *URA3* marker via recombination between the flanking *hisG* direct repeats. The *msh2*Δ *msh3*Δ double-mutant strains were generated by disrupting *MSH2* in an *msh3*Δ mutant strain. All gene disruptions were confirmed by Southern blot or PCR analysis.

Plasmids containing the homologous or homeologous IR constructs were targeted to integrate at the *URA3* locus by digesting DNA with *Stu*I prior to transformation. Ura^+ transformants were checked by Southern blot analysis, and only those containing a single copy of plasmid integrated at *URA3* were used for measuring recombination rates.

Measurement of recombination rates. Recombination rates were determined by the method of the median (24) as follows. Two-day-old colonies were excised from YEPD plates, inoculated into 5 ml of YEPGG medium, and grown for 2 days on a roller drum at 30°C. Cells were harvested and washed once with sterile H_2O and resuspended in 1 ml of H_2O. One hundred-microliter aliquots of cells were plated on SGG-his selective medium. When the recombinant yield was low, 10 aliquots of 100 μl each were plated onto each of 10 SGG-his plates. When the recombinant yield was high, 100-μl aliquots of appropriately diluted cells were plated together with 100-μl aliquots of undiluted nonreverting cells (SJR231) in order to achieve uniform cell densities on all plates. His^+ colonies were counted on day 4 after selective plating and were used to determine the total number of recombinants per culture. All strains containing a given substrate were grown and plated at the same time, with three cultures of each strain being plated on 3 separate days. The median number of His^+ colonies per culture was determined by using these nine independent cultures. The experimentally determined median was used to calculate the recombination rate (number of recombinants per generation).

The time necessary for His^+ colony appearance in the wild-type and mismatch repair-defective strains was determined by reconstruction experiments. His^+ recombinants were purified and grown in YEPGG medium as in the actual fluctuation experiments. The cultures were harvested and washed, and approximately 100 cells were plated onto SGG-his plates in the presence of nonreverting parental cells (SJR231). The number of His^+ colonies appearing daily was recorded. In all strains, >90% of the His^+ colonies were evident by day 4 postplating.

RESULTS

The intron-based homeologous recombination assay. A chromosomal assay system in which the recombination substrates are located within the intron of a selectable marker was developed in order to avoid the functional constraints normally placed on the products of a recombination event. As illustrated in Fig. 1A, recombination between the intronic substrates generates a full-length, spliceable gene whose product can be selected for on appropriate medium. The yeast *HIS3* gene was used as the selectable marker in the intron-based homeologous recombination system. Since this gene does not contain an intron, the AI of Yoshimatsu and Nagawa (73) was inserted near the 5′ end of the coding sequence. The AI is 150 bp long and contains the three essential elements of yeast introns: a 5′ donor splice site, a TACTAAC box at the branch point, and a 3′ acceptor site (reviewed in reference 71). Additionally, a multiple cloning site is present between the 5′ donor site and the TACTAAC box, a region that is highly variable in size in naturally occurring yeast introns. The AI was positioned within a *HIS3* gene under transcriptional control of the galactose-inducible *GAL1-10* promoter (64). A highly transcribed *pGAL-HIS3* gene was used in order to compensate for possible inefficiencies in splicing. The recombination substrates were cloned into the multiple cloning site of the intron, and the resulting *pGAL-HIS3*::intron::substrate constructs were split into 5′ and 3′ cassettes. A 5′ cassette contains the 5′ part of *pGAL-HIS3*, the 5′ part of the intron (5′ donor site), and a full-length recombination substrate; a 3′ cassette contains a full-length recombination substrate, the 3′ part of the intron (TACTAAC box and 3′ acceptor site), and the 3′ end of the *HIS3* coding sequence. The 5′ and 3′ cassettes were combined as IRs (Fig. 1B) on an integrative yeast vector containing *URA3* as a selectable marker. The IR constructs were integrated at the *URA3* locus on chromosome V in a yeast strain deleted for most of the *HIS3* locus on chromosome XV. As shown in Fig. 1B, crossovers between the substrates invert the region between them, generating a full-length, intron-containing *HIS3* gene. The rate of crossing-over between the substrates, therefore, can be assessed by measuring the rate of His^+ prototroph formation. It should be noted that there are no extraneous flanking homologous sequences near the recombination substrates, and thus these substrates provide an appropriate model for examining the recombination efficiency between dispersed homeologous sequences. During the course of developing the system described here, a similar intron-based system was developed independently for use in mammalian cells (57).

Effect of sequence divergence on recombination in wild-type strains. The only limitation placed on the substrates in the intron-based homeologous recombination system is that they be of a size that allows the intron to be spliced efficiently. Initial characterization of the system indicated that substrates sizes of up to 400 bp can be positioned within the AI without affecting the splicing efficiency (8a). With larger substrates, splicing was observed to be inefficient and the growth rate of recombinants was quite variable on histidine-deficient selective medium. We thus chose to use 350-bp sequences as recombination substrates. Two types of sequences were used to construct substrates of variable homology: (i) cβ cDNA sequences and (ii) portions of the TATA-binding protein genes from *S. cerevisiae* (*SPT15*) and *S. pombe*. Ninety-one percent substrates (cβ2/cβ7) were constructed by using cβ2 and cβ7 sequences, while 77% substrates (C/P) were constructed from the *S. cerevisiae* and *S. pombe* sequences. Figure 2 presents a sequence alignment of the 91 and 77% substrates. For both pairs of homeologous substrates, the mismatches are distributed in an apparently random manner across the sequences. The longest stretch of continuous homology in the 91% substrates is 60 bp, while the longest stretch of uninterrupted homology in the 77% substrates is only 26 bp. In addition to the homeologous substrates, three types of 100% substrates were constructed: cβ2/cβ2, C/C, and P/P.

The rates of His^+ prototroph formation were measured by fluctuation analysis in wild-type yeast strains containing the three different 100% substrates, the 91% substrates (cβ2/cβ7), and the 77% substrates (C/P). The rate data are presented in Table 1. All three 100% substrates recombined at a much higher rate than the homeologous substrates, and the 91% substrates recombined much better than the 77% substrates. There is thus a clear relationship between the degree of sequence divergence and the rate of recombination in yeast strains. Although of virtually identical size, is it somewhat surprising that the three 100% recombination substrates recombined at rates that differ by as much as sevenfold. The reason for this is unclear but may be related to the different GC contents of the recombination substrates; the 91% substrates are 65% GC, while the 77% substrates are 40% GC.

Effect of sequence divergence on recombination in mismatch repair-defective strains. The effects of mutations in several well-characterized mismatch repair genes on the rates of recombination between the 100, 91, and 77% homologous substrates were examined. For these experiments, strains containing deletions of the *PMS1*, *MSH2*, or *MSH3* gene, as well as an *msh2*Δ *msh3*Δ double-mutant strain, were constructed. *PMS1* encodes a MutL homolog, while *MSH2* and *MSH3* encode MutS homologs. The various intron-based recombination constructs (100% cβ2/cβ2, 91% cβ2/cβ7, 100% C/C, 100% P/P, and 77% C/P) were then introduced individually into each mutant strain. The rate of His^+ recombinants was determined for each strain by fluctuation analysis, and these rates are presented in Table 1.

Mutations in the MutS homologs resulted in a small (two- to

TABLE 2. Comparison of homeologous and homologous recombination in wild-type and mutant strains[a]

Substrate (% homology)	Genotype	Recombination rate relative to that of the 100% substrate
cβ2/cβ7 (91)	Wild type	0.02
	*pms1*Δ	0.20
	*msh2*Δ	0.62
	*msh3*Δ	0.09
	*msh2*Δ *msh3*Δ	0.88
C/P (77)	Wild type	0.0005
	*pms1*Δ	0.0004
	*msh2*Δ	0.0033
	*msh3*Δ	0.0027
	*msh2*Δ *msh3*Δ	0.0045

[a] The cβ2/cβ7 relative rates were calculated by normalizing each cβ2/cβ7 rate to the cβ2/cβ2 rate in the strain of the same genotype. The C/P relative rates were calculated by normalizing each C/P rate to an average of the sum of the C/C and P/P rates in the strains of the same genotype. All rates are given in Table 1.

threefold) but reproducible stimulatory effect on recombination between the cβ2/cβ2 100% substrates. In contrast, mutations in *MHS2* or *MSH3* had no effect on either the C/C or P/P 100% substrate. Mutation of the MutL homolog had no effect on either the cβ2/cβ2 or C/C substrate but had a small stimulatory effect on the P/P substrate. The reason for the differential effects of a given mutation on the cβ2/cβ2 substrate and the C/C and P/P substrates is not clear but again could be related to the very different GC contents of these substrates or the potential of different substrates for intrastrand base pairing.

In contrast to the results obtained with the 100% substrates, mutations in mismatch repair proteins had very strong stimulatory effects on the rates of recombination between the cβ2/cβ7 91% substrates. The rates were elevated approximately 10-fold in the *pms1*Δ and *msh3*Δ strains, 70-fold in the *msh2*Δ strain, and 100-fold in the *msh2*Δ *msh3*Δ double mutant. As in other types of assays, the phenotype of the *msh2*Δ mutant was stronger than the phenotype of the *msh3*Δ mutant (4, 36, 56). The effects of *msh2*Δ and *msh3*Δ appear to be additive in this assay. Table 2 presents a direct comparison of the effects of mismatch repair proteins on the 91% cβ2/cβ7 and the 100% cβ2/cβ2 substrates. While the 91% rate was 50-fold lower than the 100% rate in a wild-type background, the two rates were essentially the same in an *msh2*Δ *msh3*Δ double mutant. This finding indicates that 91% homologous sequences were inherently able to recombine as well as 100% homologous sequences in *S. cerevisiae* but that recombination between the 91% substrates was prevented by the mismatch repair system.

Examination of the effects of mismatch repair proteins on recombination between the 77% C/P substrates demonstrated that these proteins had very little effect relative to what was observed with the 91% cβ2/cβ7 substrates. The recombination rate between the 77% C/P substrates strain was essentially the same in the *pms1*Δ and wild-type strains. There was a modest 5- to 10-fold increase in the recombination rate between the 77% substrates in the *msh2*Δ, *msh3*Δ, and *msh2*Δ *msh3*Δ strains. As shown in Table 2, even in an *msh2*Δ *msh3*Δ double-mutant strain, the 77% substrates still recombined 100-fold less efficiently than the 100% substrates.

DISCUSSION

Typical yeast chromosomal systems for assaying homeologous recombination make use of diverged auxotrophic markers, with recombinants being identified by selecting for prototrophic colonies (5, 15, 51). Such systems have three limitations: (i) there is no straightforward way to vary the degree of sequence homology, (ii) truly 100% homologous control substrates are not available because, by definition, the auxotrophic substrates used must contain at least one mismatch, and (iii) only those recombination events giving rise to a functional gene product are identified. At least two studies indicate that the functionality constraint might bias the events detected (15, 32). While transformation-based assays that are not subject to the above limitations have been described (32, 42), the relevance of the repair of a linearized plasmid during transformation to events that occur on a chromosome is not clear. Indeed, one recent study indicates that there may be different genetic requirements for plasmid versus chromosomal recombination events (65). Also, studies done with mammalian cells indicate that chromosomal recombination is much more sensitive to sequence divergence than is extrachromosomal recombination (68).

A unique intron-based homeologous recombination assay system which avoids the limitations of heteroallelic systems was developed in order to examine the effects of yeast mismatch repair functions on recombination between substrates with different degrees of sequence homology. The recombination substrates in this assay were positioned as IRs so that crossing-over between them generates a spliceable intron within a full-length prototrophic marker. Since the recombination products either are contained within the intron or are distal to the selectable marker, they do not affect the final gene product. Studies done with *S. cerevisiae* indicate that IRs are an appropriate model for examining general aspects of homologous recombination (47). It should be noted that the intron-based system described here can detect only crossovers; simple gene conversion events that are not associated with exchange do not give rise to prototrophs. Given that crossover events are the important events when one is considering aspects of genome stability, we do not believe that the failure of the system to detect gene conversions is a serious limitation. In fact, looking at a single type of event rather than multiple possible events makes the data interpretation more straightforward. It also should be noted that the events detected with the intron-based system are strictly intrachromatid. While it is possible that intrachromatid events have homology constraints different from those of events between homologous or nonhomologous chromosomes, we think that this is unlikely. Finally, it should be noted the recombination substrates in the intron-based assay are not flanked by regions of perfect homology, which have been present in other assay systems (32, 56). There is evidence from mammalian cells that flanking perfect homology can influence homeologous interactions (69).

We examined the rate of mitotic crossing-over between five different recombination substrates: 77% (C/P) and 91% (cβ2/cβ7) substrates and three control 100% substrates (C/C, P/P, and cβ2/cβ2). In a wild-type background, there was a clear correlation between the degree of sequence divergence and the rate of recombination (Table 1). Recombination between the 91% sequences was reduced 50-fold relative to the 100% cβ2/cβ2 control, and recombination between the 77% sequences was reduced 1,000-fold relative to the C/C and P/P controls. Recombination between sequences in the 90% range has not been reported to occur in *S. cerevisiae*, but several studies using sequences of approximately 75 to 85% identity have been done (5, 15, 32, 56). The reduction in recombination between the 77% sequences relative to the 100% control sequences reported here is generally higher than other studies have found. Although we do not know the reason for this discrepancy, it could be due to use of an extrachromosomal assay system (32),

the presence of perfect flanking homology (56), the presence of mismatches in the 100% substrates (5, 15), or the fact that multiple types of events were being detected in other systems (5, 15, 56). The relationship between sequence divergence and recombination rate reported here is in general agreement with that seen in a bacterial system (58). Experiments using a variety of additional substrates to more systematically examine the nature of the relationship between sequence divergence and recombination rate in *S. cerevisiae* are in progress.

The 100, 91, and 77% substrates were used to examine the effects of mutations in well-characterized yeast mismatch repair genes (*PMS1*, *MSH2*, and *MSH3*) on recombination between sequences of different homologies (Tables 1 and 2). As observed in previous studies (5, 56), deletion of *PMS1* had no effect on recombination between the 77% substrates (relative to the 100% control substrates). In the *msh2*Δ, *msh3*Δ, and *msh2*Δ *msh3*Δ strains, the rates of recombination between the 77% C/P substrates were 5- to 10-fold higher than the rate in the wild-type control strain; it is not clear whether the slight increase in the *msh2*Δ *msh3*Δ double mutant represents an additive effect. The 10-fold elevation in the recombination rate between the 77% C/P substrates in the *msh2*Δ *msh3*Δ double mutant corresponds to only 1% of the C/C or P/P rate. Therefore, for the 77% substrates, the major factor limiting recombination appears to be sequence divergence rather than the mismatch repair system. We note that our results with the 77% C/P substrates are in general agreement with those obtained by Selva et al. (56) using essentially the same C/P *SPT15* substrates.

In contrast to the results obtained with the 77% C/P substrates, the rate of recombination between the 91% cβ2/cβ7 substrates was elevated 10-fold in the *pms1*Δ strain. This represents the first report of a clear effect of *PMS1* on homeologous recombination in any system. In addition, the stimulation of homeologous recombination in the *msh2*Δ mutant was more pronounced for the 91% substrates (70-fold) than for the 77% substrates (6-fold), while deletion of *MSH3* had a small but comparable stimulatory effect (5-fold) on both the 91 and 77% substrates. As with the 77% substrates, the rate of recombination between the 91% substrates in the *msh2*Δ *msh3*Δ double mutant is suggestive of an additive effect. In contrast to the results obtained with the 77% substrates, elimination of both Msh2p and Msh3p increased the recombination rate between the 91% cβ2/cβ7 substrates 100-fold, making the rate comparable to that observed with the 100% cβ2/cβ2 substrates. This finding indicates that the 91% substrates can be as efficiently recombined as the 100% substrates but that the recombination rate is regulated solely by mismatch repair functions. It is clear that with the system used here, the apparent effect of the yeast mismatch repair system on homeologous recombination is related to the degree of sequence divergence. If recombination is initiated successfully, as appears to happen with the 91% substrates, then the mismatch repair system very efficiently edits the intermediates formed and prevents the reaction from going to completion. We believe that the 77% sequences are simply too diverged to be acted on efficiently by the yeast recombination machinery and that the relatively small effect of mismatch repair functions reflects elimination of the few recombination intermediates that can form. Using 89 and 100% substrates in a bacterial system, Shen and Huang (59) also concluded that both the degree of sequence divergence and the mismatch repair system limit homeologous interactions.

One can imagine two distinct steps at which the mismatch repair machinery might edit the process of homologous recombination. First, mismatch repair proteins could regulate the process of strand assimilation and/or branch migration. Worth et al. (72) demonstrated that MutS alone can inhibit strand transfer between diverged DNAs in a purified in vitro system, and addition of MutL potentiated the effect of MutS. In addition to these in vitro studies, Alani et al. (4) obtained genetic data indicating that Msh2p might block branch migration when mismatched bases are encountered during meiotic recombination in yeast cells. They further proposed that this blockage might lead to "heteroduplex rejection," thereby preventing crossing-over, but not necessarily gene conversion, between diverged sequences. The experiments reported here demonstrate clearly that the yeast mismatch repair system can prevent mitotic crossing-over between mismatched sequences. In addition to regulating an early step of the recombination process, mismatch repair proteins also could destroy a recombination intermediate or product by multiply nicking heteroduplex DNA (45) or unwinding the invading strand. As in bacterial systems, yeast MutS homologs have been shown to bind to mismatches bases (3, 33, 44); the precise role of the yeast MutL homologs in mismatch repair is not clear, but they presumably act at a step subsequent to mismatch recognition. The consistently greater stimulation of homeologous recombination in *msh2*Δ *msh3*Δ double mutants than in *pms1*Δ mutants indicates that the initial recognition of mismatches by MutS homologs during intermediate formation is sufficient to block most recombination between mismatched substrates. The more subtle effect of Pms1p on this process may reflect elimination of intermediates at a later step or could reflect a potentiating role similar to that seen in the in vitro strand exchange system (72).

In summary, our results demonstrate that the effect of the yeast mismatch repair machinery on homeologous recombination during mitosis is related to the degree of substrate sequence divergence. Since it is unlikely that mismatch repair proteins would function as better recombination editors on substrates with relatively few mismatches, we suggest that the differential effects on the 77 and 91% substrates reflect limitations of the general recombination machinery. Whereas strand exchange between the 91% substrates can be initiated efficiently by the recombination machinery, strand exchange between the 77% substrates cannot. In terms of genome stability in higher eukaryotes, our results indicate that defects in the mismatch repair machinery will lead to rearrangements involving relatively recently diverged sequences. It will be of particular interest to examine additional recombination substrates in the 90 to 100% range, since this will indicate whether one or several mismatches are sufficient for the mismatch repair machinery to exert its antirecombination role.

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