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Requirement for Msh6, but Not for Swi4 (Msh3), in Msh2-Dependent Repair of Base-Base Mismatches and Mononucleotide Loops in *Schizosaccharomyces pombe*

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ABSTRACT

The *msh6* mismatch repair gene of *Schizosaccharomyces pombe* was cloned, sequenced, and inactivated. Strains bearing all combinations of inactivated *msh6*, *msh2*, and *swi4* (the *S. pombe MSH3* ortholog) alleles were tested for their defects in mitotic and meiotic mismatch repair. Mitotic mutation rates were similarly increased in *msh6* and *msh2* mutants, both for reversion of a base-base substitution as well as of an insertion of one nucleotide in a mononucleotide run. Tetrad analysis and intragenic two-factor crosses revealed that meiotic mismatch repair was affected in *msh6* to the same extent as in *msh2* background. In contrast, loss of Swi4 likely did not cause a defect in mismatch repair, but rather resulted in reduced recombination frequency. Consistently, a mutated *swi4* caused a two- to threefold reduction of recombinants in intergenic crosses, while *msh2* and *msh6* mutants were not significantly different from wild type. In summary, our study showed that Msh6 plays the same important role as Msh2 in the major mismatch repair pathway of *S. pombe*, while Swi4 rather functions in recombination.

IN spite of the high fidelity of DNA polymerase δ and its 3′ exonuclease activity during DNA replication, some base-base mispairs and insertion-deletion loops (IDL) remain (Umar and Kunkel 1996; Jiricny 1998). In *Escherichia coli*, such uncorrected replication errors are mainly removed by the MutHLS system. The first step of the repair process consists of mismatch recognition by the MutS homodimeric protein (Modrich 1991; Kolodner 1996; Allen *et al.* 1997). In eukaryotes, several MutS and MutL homologues are involved in mismatch repair. Proteins homologous to MutS are generally called Msh (*M*ut*S h*omologue) and proteins homologous to MutL are termed Mlh (*M*ut*L h*omologue) or Pms (*p*ost*m*eiotic *s*egregation). PMS reflects the frequent failure to repair mismatches formed during meiotic recombination and was originally described in *Saccharomyces cerevisiae pms1* mutants (Williamson *et al.* 1985). The binding to mismatches appears to be mediated by heterodimers of two Msh proteins (Acharya *et al.* 1996; Marsischky *et al.* 1996; Fishel and Wilson 1997; Gradia *et al.* 1997; Jiricny 1998; Buermeyer *et al.* 1999; Kolodner and Marsischky 1999; Marra and Schär 1999). From *S. cerevisiae* to human, up to six Msh proteins (Msh1–6) were discovered, but only three of them (Msh2, Msh3, and Msh6) are involved in nuclear mismatch repair (Reenan and Kolodner 1992; New *et al.* 1993; Marsischky *et al.* 1996; Fishel and Wilson 1997). Mutations in several human genes homologous to MutS (*MSH2* and *MSH6*) and to MutL (*MLH1*, *PMS1*, and *PMS2*) were found to be responsible for hereditary nonpolyposis colon cancer (HNPCC; Leach *et al.* 1993; Miyaki *et al.* 1997; Prolla 1998; Buermeyer *et al.* 1999; Planck *et al.* 1999).

In *S. cerevisiae*, *msh2* single mutants show a strong mutator phenotype, while the *msh3* and *msh6* single mutants have much weaker effects on mutation rates. However, the *msh3 msh6* double mutant revealed a mutator phenotype that is as strong as that of *msh2*. These observations led Marsischky *et al.* (1996) to propose that two types of heterodimers are involved in mismatch recognition in *S. cerevisiae*: Msh2-Msh3 and Msh2-Msh6. Further studies showed that Msh2-Msh3 heterodimers bind specifically to IDLs, while Msh2-Msh6 heterodimers can bind to small IDLs of one or two unpaired nucleotides and to single-base mispairs (Acharya *et al.* 1996; Johnson *et al.* 1996; Kolodner 1996; Fishel and Wilson 1997; Buermeyer *et al.* 1999; Kolodner and Marsischky 1999).

So far, in the fission yeast *Schizosaccharomyces pombe*, *swi4*$^+$, *pms1*$^+$, and *msh2*$^+$ were cloned, inactivated, and studied (Fleck *et al.* 1992; Schär *et al.* 1997; Rudolph *et al.* 1999). The *msh2*$^+$ gene product is implicated in mismatch repair and mating-type switching (Fleck *et al.* 1994; Rudolph *et al.* 1999). The *swi4*$^+$ gene encodes an amino acid sequence mostly homologous to the proteins of the Msh3 subfamily and is, like *msh2*$^+$, involved in mating-type switching (Fleck *et al.* 1992; New *et al.*

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1993). Its inactivation produces no or only a weak mutator phenotype, as is the case for *msh3* mutants in budding yeast (New *et al.* 1993; Marsischky *et al.* 1996; Earley and Crouse 1998). In this context it was interesting to isolate the *msh6*$^+$ ortholog of *S. pombe*, to study its function in mismatch repair, and to compare the defects caused by mutated *msh2*, *msh6*, and/or *swi4*. We determined reversion rates of a base substitution allele and of a thymine insertion in a homonucleotide run of five thymines. In addition, meiotic mismatch repair was tested by intragenic two-factor crosses and tetrad analysis. The results are discussed in comparison with the situation in *S. cerevisiae* and other species.

MATERIALS AND METHODS

Strains and media: All strains containing the *ade6* mutations *51*, *421*, *485*, *687*, *M387*, and *M26* are from the *S. pombe* collection in Bern. The molecular nature of the mutations was determined by Szankasi *et al.* (1988) and Schär and Kohli (1993). Strains bearing the disrupted *msh6* gene were derived from CT-1 (*h*$^+$ *msh6::arg3*$^+$ *arg3-D4 his3-D1 ura4-D18*, see below). Strains with disrupted *msh2* were derived from crosses with strain Ru39 (*h*$^-$ *msh2::his3*$^+$ *his3-D1*; Rudolph *et al.* 1999). Strains with disrupted *swi4* were derived from strain Ru106 (*h*$^-$ *swi4::ura4*$^+$ *ura4-D18*), which originated from LH110 (Fleck *et al.* 1992). *S. pombe* media YEA (yeast extract agar), YEL (yeast extract liquid), MMA (minimal medium agar), and MEA (malt extract agar, sporulation medium) were prepared according to Gutz *et al.* (1974) and Schär *et al.* (1997). Where required, supplements (adenine, histidine, uracil, leucine, arginine, and lysine) were added (0.01% w/v).

PCR gene cloning and physical mapping: Cloning of the *S. pombe msh6*$^+$ gene was started by PCR using degenerate primers derived from amino acid sequences highly conserved in the MutS family: TGPNM and DELGR. From a set of primers, the use of G1, 5′-CTGGATCCACNGGNCCN(A/C)ATATG-3′, and G6, 5′-CTGGATCCCTN(T/G)NCC(T/C)A(A/G)(C/T)T(C/A)GTC-3′, allowed successful amplification. We initiated the program by a 4-min denaturation at 94°, 5 cycles of 30 sec at 94°, 30 sec at 45°, 30 sec at 72°, followed by 25 cycles of 20 sec at 94°, 30 sec at 50°, 30 sec at 72°. PCR products were blunt ended by treatment with the large fragment of *E. coli* DNA polymerase I and cloned into the *Sma*I site of pBluescript(+). Cloned PCR products were sequenced using a sequencing kit (Amersham, Pharmacia, Dübendorf, Switzerland).

Isolation of the *msh6*$^+$ gene: A putative 270-bp-long *msh6* PCR product was used to screen an ordered *S. pombe* cosmid library by hybridization (Hoheisel *et al.* 1993). Fourteen cosmids were isolated that all mapped on the right arm of chromosome III between the *adh1* and *ade5* loci. Southern blot analysis allowed us to draw a restriction map and to choose one of the cosmids (ICRFc60B039D) for sequencing. Sequencing of this cosmid was performed with PRISM Ready Reaction AmpliTaqFs DyeDeoxyterminator (Perkin Elmer, Norwalk, CT) and sequencing reactions were run on an AbiPRISM sequencer. Primers were first chosen in the 270-bp starting fragment and then 11 forward and 11 reverse primers were designed to complete the 4248-bp sequence containing the entire *msh6*$^+$ gene (accession no. AF207839).

***msh6* gene disruption:** A 2.1-kb fragment of the *msh6*$^+$ coding region was amplified by PCR with primers G7fwd (5′-GCATCTCCCACAAAATCGAA-3′, nucleotides (nt) 1261–1280) and G2rev (5′-GCTTGAACTGGCTGAAAAAGC-3′, nt 3215–3236) and cloned in pUAg (R&D Systems, Abingdon, UK) to give pUAmsh6. The *S. pombe arg3*$^+$ marker (Waddell and Jenkins 1995) was introduced as a blunt-ended fragment into the *Hpa*I site of pUAmsh6, thus interrupting the *msh6* gene at nt 1595 (start codon at nt 225). The *S. pombe* strain *h*$^+$ *arg3-D4 his3-D1 ura4-D18* was transformed (Ito *et al.* 1983) with gel-purified 3.9-kb *Bam*HI-*Xba*I fragment derived from pUAmsh6 and Arg$^+$ transformants were selected on MMA supplemented with histidine and uracil. Subsequently, several clones were screened by PCR (Figure 1) with primers 3′arg, 5′- CAACACT CATAAGGAAAAAGC-3′ and G1rev2, 5′-ATACAAGGATGTC TCAACTCC-3′. 3′arg derived from the 3′ end of the *arg3*$^+$ gene (nt 1791–1811 according to the sequence deposited in the EMBL database, accession no. X63577), and G1rev2 derived from *msh6*$^+$, outside the transformed fragment (nt 3309–3329). Positive clones were tested by Southern hybridization (data not shown) and one was chosen for further studies (CT-1). The structure of the interrupted *msh6* gene is shown in Figure 1.

Genetic tests for mismatch repair: Fluctuation tests and tetrad analysis were performed according to Rudolph *et al.* (1999) and Schär *et al.* (1997), respectively. Tetrads were dissected from the cross *msh6 ade6-M26* × *msh6 ade6*$^+$. This cross included strains homozygous for the *msh6* disruption and heterozygous for the recombination hot spot *ade6-M26* (Gutz 1971; Szankasi *et al.* 1988; Schuchert *et al.* 1991; Schär and Kohli 1994). G/A and T/C mismatches are formed when the mutated site is included in heteroduplex DNA. Whole chromatid conversions (WCC) and PMS were counted by examination of the white and red colony color of the spore-derived colonies. *ade6* mutants accumulate a red pigment when grown on YEA containing a limited amount of adenine, while *ade6*$^+$ strains form white colonies. Intragenic two-factor crosses with *ade6* mutants were performed to study meiotic mismatch repair as described (Schär and Kohli 1993; Rudolph *et al.* 1998). We included the crosses *421* × *51*, *485* × *51*, and *485* × *M387* (Figure 3). All types of crosses were performed at least three times.

Intergenic two-factor crosses: Recombination rates were measured between the intervals *leu2-120* × *lys7-2* (chromosome I) and *ade1-40* × *lys4-95* (chromosome II). Parental strains were mixed on MEA and incubated for 2–3 days at 25°. Vegetative cells were killed by treatment with a 1:500 (v/v) dilution of snail enzyme (*Helix pomatia* juice, Biosepra, Villeneuve-la-Garenne, France) and spore suspensions were plated on nonselective YEA medium. After 4–5 days of growth at 30°, colonies were checked for their genotypes by replica plating on different types of MMA with respect to the included supplements.

Iodine staining: Colonies grown on MEA at 25° were treated with iodine vapor, which stains spores but not vegetative cells. Colonies from homothallic strains turned homogeneously brown, while heterothallic (nonsporulating) strains are iodine negative. Homothallic strains with a defect in mating-type switching have reduced switching efficiency and form mottled colonies (Egel *et al.* 1984).

RESULTS

Identification of the *S. pombe msh6*$^+$ gene: The aim of this work was to study the function of the *S. pombe msh6*$^+$ gene in mismatch repair. We were particularly interested to learn about the roles of *S. pombe msh6*$^+$ and *swi4*$^+$ (the *S. pombe MSH3* ortholog) in mismatch repair and whether their functions are similar or different to those devoted to *MSH6* and *MSH3* of other organ-

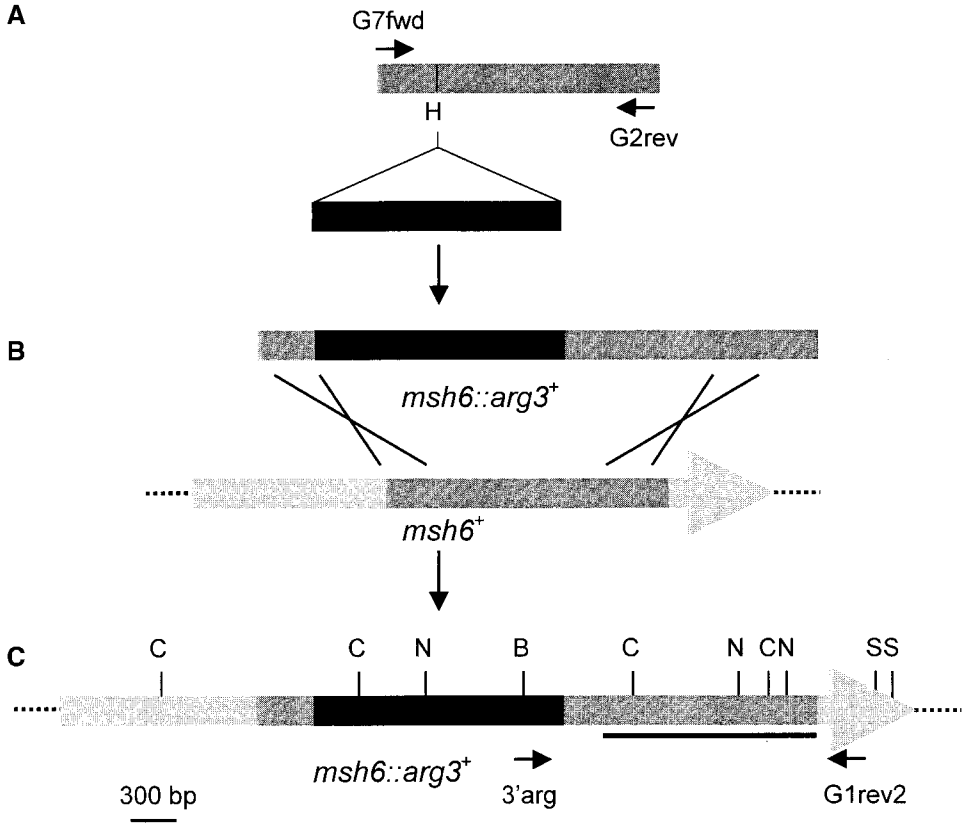

FIGURE 1.—Construction of the *msh6::arg3*$^+$ disruption strain. (A) The *arg3*$^+$ gene (solid box) was cloned into the *Hpa*I site of plasmid pUAgmsh6, containing a fragment of *msh6* (shaded box), previously amplified by PCR with the primers G7fwd and G2rev. (B) The resulting *msh6::arg3*$^+$ cassette was obtained by digestion with *Bam*HI/*Xba*I, transformed into *S. pombe*, and integrated via homologous recombination at the *msh6*$^+$ locus. (C) Structure of the resulting *msh6::arg3*$^+$ locus and restriction map. Arg$^+$ transformants were checked for correct disruption by PCR with primers 3′arg and G1rev2 and by Southern blot analysis using restriction enzymes *Bgl*II, *Cla*I, *Nru*I, and *Sca*I. The probe used for hybridization derived from the 3′ region of *msh6* (black bar). Restriction sites are as follows: B, *Bgl*II; C, *Cla*I; H, *Hpa*I; N, *Nru*I; S, *Sca*I.

isms. *swi4* is known to be involved in mating-type switching, a special mitotic recombination event (FLECK *et al.* 1992), but an involvement in mismatch repair could not be demonstrated so far. Conservation of the MutLS system among species and the discovery of two members of the MSH family in *S. pombe*, namely Msh2 and Swi4, suggested that a Msh6 ortholog exists in *S. pombe* too. We cloned the *msh6*$^+$ gene as described in MATERIALS AND METHODS. It is contained in cosmid ICRFc60B039D, which maps between *adh1* and *ade5* on chromosome III (HOHEISEL *et al.* 1993). Sequencing revealed a short open reading frame (ORF) starting at nt 225 and ending at nt 818 and a long ORF starting at nt 935 and ending at nt 4101 (the 4248-bp sequence containing *msh6*$^+$ has been deposited in the EMBL database under accession no. AF207839). The following features suggested the existence of a 117-nt-long intron between the two ORFs (nt 819 to 934). First, 5′ and 3′ splice sites (GTATGG and TAG, respectively) at the ends of the putative intron and a branch site (CTAAC) close to the 3′ splice site are present, which correspond to respective consensus sequences (PRABHALA *et al.* 1992). Second, the peptide sequences deduced from the nucleotide sequences 5′ and 3′ to the intron show homology to both bacterial MutS and eukaryotic MSH proteins. The deduced amino acid sequence of *S. pombe msh6*$^+$ is 35–42%, identical with MSH6 orthologs of other organisms (highest homology to *S. cerevisiae* Msh6). It is ~26% identical with MSH4 and MSH5 proteins, ~28% identical with MSH2 proteins, and 27–32% identical with Msh3 proteins. The fission yeast Msh6 amino acid sequence contains the general MSH motifs (Figure 2). It has an extended N-terminal region, which is common with Msh6 proteins of other organisms. In addition, Msh6 contains several motifs, which are apparently specific to the MSH6 subgroup. Computer analysis of the *msh6*$^+$ sequence revealed a putative GAL1-TATA site starting at nt 121 (ATG at nt 225) and a CTCATGTT sequence at nt 145, which might serve as a transcription initiation site (Sigscan program, GCG software package, University of Wisconsin).

A major role of the *S. pombe msh6*$^+$ gene in mitotic mismatch repair: To study the phenotypes caused by mutated *msh6* in *S. pombe*, we inactivated this gene by gene targeting using the *arg3*$^+$ gene as selection marker (Figure 1). A mutator phenotype of *msh6* mutants was noted by mere visual inspection. *ade6* mutants form red colonies on YEA medium (GUTZ *et al.* 1974; RUDOLPH *et al.* 1999). In mismatch repair-proficient *ade6* strains the red color is quite homogeneous. In contrast, *msh6::arg3*$^+$ *ade6* colonies developed white sectors within 1–2 weeks of growth, indicative of the occurrence of mutations in *ade* genes acting upstream of *ade6* in the purine biosynthesis pathway. A similar observation had been made with *msh2* mutants but not with *swi4* mutants (FLECK *et al.* 1994; RUDOLPH *et al.* 1999).

To obtain quantitative values of mutation rates we measured reversions to Ade$^+$ of two defined *ade6* muta-

FIGURE 2.—Comparison of human (h), *S. cerevisiae* (Sc), and *S. pombe* (Sp) homologues belonging to the eukaryotic Msh6 subfamily of bacterial MutS. Sequences were retrieved from GenBank and were aligned using the Pile-Up program (GCG software package, version 10.1, University of Wisconsin) and the Boxshade program (http://www.ch.embnet.org/software/BOX_form.html). Amino acids at a given position are shown in black when at least two residues are identical and in gray when at least two residues are similar. The position of the intron in the *S. pombe* Msh6 encoding gene between residues K^{198} and P^{199} is marked by an arrow. The *S. pombe msh6* sequence has been deposited in the EMBL database under accession no. AF207839.

tions: *ade6-51* (a C to T transition at nt 2141) and *ade6-687* (a T insertion in a run of 5 thymines at nt 1216). These mutations were representatively used to study repair of two main types of replicative errors processed by the mismatch repair (MMR) system: base-base mispairs mainly caused by misincorporation and insertion-deletion mismatches produced by strand slippage in repetitive DNA (STRAND *et al.* 1993; UMAR and KUNKEL 1996; MARRA and SCHÄR 1999). In all strain backgrounds, reversion rates were ~10 times lower for the base substitution *ade6-51* ($\sim 5 \times 10^{-10}$ in wild type) than for the insertion *ade6-687* ($\sim 5 \times 10^{-9}$ in wild type). In *msh6* strains, reversion rates of both types of *ade6* mutations were ~15 times increased as compared to wild type and were in the same range as those found in *msh2* strains (Table 1). The *msh2 msh6* double mutant showed reversion rates similar to *msh6* and *msh2* single mutants. In contrast the *swi4* mutation either alone or in combination with *msh2* and/or *msh6* did not significantly alter mutation rates.

TABLE 1

Mitotic reversion rates

Relevant genotype	*ade6-51*[a]		*ade6-687*[b]	
	Mutation rate[c]	Fold increase[d]	Mutation rate	Fold increase
msh$^+$	$5.3 \pm 0.3 \times 10^{-10}$	1	$5.0 \pm 0.4 \times 10^{-9}$	1
msh6	$9.2 \pm 2.6 \times 10^{-9}$	17	$7.0 \pm 0.8 \times 10^{-8}$	14
msh2	$8.0 \pm 2.0 \times 10^{-9}$	15	$6.7 \pm 0.1 \times 10^{-8}$	13
swi4	$9.9 \pm 4.4 \times 10^{-10}$	1.9	$3.8 \pm 0.9 \times 10^{-9}$	0.8
msh6 msh2	$1.4 \pm 0.1 \times 10^{-8}$	26	$7.5 \pm 0.3 \times 10^{-8}$	15
msh6 swi4	$1.3 \pm 0.1 \times 10^{-8}$	25	$5.1 \pm 1.0 \times 10^{-8}$	10
msh2 swi4	$1.1 \pm 0.0 \times 10^{-8}$	21	$3.8 \pm 0.9 \times 10^{-8}$	7.6
msh6 msh2 swi4	$9.0 \pm 1.4 \times 10^{-9}$	17	$9.5 \pm 0.8 \times 10^{-8}$	19

Spontaneous mitotic reversion rates were obtained from at least two independent experiments.
[a] *ade6-51* is a C to T transition.
[b] *ade6-687* is a T insertion in a run of five thymines.
[c] Numbers represent mean values with standard deviations.
[d] Fold increase of mutation rates as compared to wild type.

Elevated PMS frequency caused by a mutated *msh6*: Tetrad analysis is a direct approach to obtaining information on mismatch repair during meiotic recombination. Aberrant tetrads in one-factor crosses are mainly of the types $6^+:2^-$ and $2^+:6^-$ (WCC) or $5^+:3^-$ and $3^+:5^-$ (PMS). Repair of a mismatch in the heteroduplex leads to either $6^+:2^-$, $2^+:6^-$, or $4^+:4^-$ segregation. The latter type represents restoration events, which are not distinguishable from normal $4^+:4^-$ tetrads. The failure to repair a mismatch in heteroduplex DNA results in PMS events. We dissected tetrads from the cross *msh6 ade6-M26* × *msh6 ade6*$^+$. In the wild-type cross, 5.1% WCC and no PMS were found among 1018 tetrads (Gutz 1971). In the *msh6* mutant cross, the WCC frequency decreased to 1.1%, while PMS increased to 3.4% among 796 tetrads (Table 2). Among the aberrant events, the PMS frequency increased from <1.9% in wild type to 75% in *msh6* background. In the same type of cross, 85% PMS events were found for *msh2* (Rudolph *et al.* 1999).

Tetrad analysis also allows determining viability of spores derived from four-spored asci. In the *msh6* mutant we observed a spore viability of 90%. A spore viability of 86% was found for *msh2* (Rudolph *et al.* 1999) and >92% for wild type (Schär *et al.* 1997). Thus, like mutated *msh2*, inactivation of *msh6* has only a weak effect on spore viability.

A mutated *swi4*, but not *msh6* or *msh2*, caused reduced recombination frequencies: To measure a possible effect of MMR mutations on recombination frequencies we performed the intergenic crosses *leu2* × *lys7* and *ade1* × *lys4* (Table 3). Compared to wild type, we found an approximately two- to threefold reduction when *swi4* was mutated, while *msh2* or *msh6* did not significantly change the recombination frequencies.

Intragenic two-factor crosses: Crosses involving very close markers were performed to study repair of mismatches produced during meiotic recombination (Table 4 and Figure 3). During meiosis, recombination between homologous but nonidentical sequences creates mismatch-containing heteroduplex DNA. We studied the effect of the various inactivated *msh* genes on intragenic recombination within the *ade6* gene using defined and closely linked mutations. Two independent

TABLE 2

Increase of PMS and decrease of WCC in the *msh6* cross *ade6*$^+$ × *ade6-M26*

Relevant genotype	Number of tetrads	WCC		PMS			PMS/ WCC + PMS[b]
		$6^+:2^-$	$2^+:6^-$	$5^+:3^-$	$3^+:5^-$	$ab4^+:4^-$[a]	
msh$^+$[c]	1018	46	6	0	0	0	<0.02
msh6	796	9	0	23	0	2	0.75

The cross *msh6 ade6*$^+$ × *msh6 ade6-M26* was performed as described (Schär *et al.* 1997). G/A and T/C mismatches can be produced when *M26* is included in heteroduplex DNA. WCC, whole chromatid conversions ($6^+:2^-$ and $2^+:6^-$); PMS, postmeiotic segregation ($5^+:3^-$, $3^+:5^-$, and $ab4^+:4^-$).
[a] Aberrant $4^+:4^-$ ($ab4^+:4^-$) tetrads were counted as two PMS events.
[b] The ratio PMS/WCC + PMS represents PMS events among aberrant tetrads.
[c] Data from Gutz (1971).

TABLE 3

Meiotic intergenic recombination in MMR mutants

	leu2 × lys7		*ade1 × lys4*	
Genotype	Recombinants (%)[a]	Fold reduction[b]	Recombinants (%)	Fold reduction
msh$^+$	14 ± 3.5	1	28 ± 2.7	1
swi4	7.1 ± 1.3	1.9	9.4 ± 3.4	2.9
msh2	11 ± 1.7	1.2	29 ± 2.2	1.0
msh6	12 ± 0.7	1.1	28 ± 2.7	1.0

All types of crosses were performed three times as described in MATERIALS AND METHODS.
[a] Mean values of recombinants in percentage with standard deviations.
[b] Reduction relative to the wild-type crosses.

repair systems are known to operate in *S. pombe*: (1) the long-patch MMR system (MutLS pathway), which efficiently repairs all mismatches except C/C, and (2) a minor short-patch repair system, which repairs C/C mismatches and in the absence of the MMR system also other mismatches (SCHÄR and KOHLI 1993; RUDOLPH *et al.* 1998; FLECK *et al.* 1999). Recently some factors of the nucleotide-excision repair pathway were identified as components of the minor system (FLECK *et al.* 1999). It was estimated that the MMR system has excision-resynthesis tracts of ~100 nucleotides unidirectional from a mismatch, while the minor pathway leads to excision-resynthesis tracts of ~10 nucleotides unidirectionally (SCHÄR and KOHLI 1993).

We measured prototroph frequencies of the crosses *421 × 51*, *485 × 51*, and *485 × M387* (Table 4). In all three crosses, mismatches that arise in heteroduplex DNA are separated by <100 nucleotides (Figure 3). Thus, they are in most cases corepaired on the same strand by the long-patch MMR system, which prevents formation of prototrophic recombinants. One exception is the cross *485 × M387* where two C/C mismatches can be produced in the same heteroduplex. They are not a substrate of MMR, but can be independently repaired by the short-patch repair system, which frequently results in prototrophs. Prototrophic recombinants will be generated when the two mismatches in the same heteroduplex are repaired independently toward wild-type information, *i.e.*, when the bases of the opposite strands are replaced. Prototrophs can also be produced when one of the mismatches is repaired toward wild-type information and the other remains unrepaired. In this case, one of the daughter cells that are formed after replication conserves the mutation, while the other will have inherited the wild-type information and thus is a prototroph. The distance between the two point mutations is so short that recombinants arising by a simple crossing over are considered to be rare.

In the cross *421 × 51* only non-C/C mismatches are generated in heteroduplex DNA at a distance of 90 bp apart. We found a prototroph frequency of 15×10^{-6} for the wild-type cross (Table 4). The frequency is rather low because repair of one mismatch by the MMR system is in most cases accompanied by corepair of the second mismatch on the same strand. When either *msh6* or *msh2* was mutated, the prototroph frequency increased

TABLE 4

Prototroph frequencies in intragenic two-factor crosses

	421 × 51		*485 × 51*		*485 × M387*	
Relevant genotype	Frequency[a]	Fold increase[b]	Frequency	Fold increase	Frequency	Fold increase
msh$^+$	15 ± 3.2	1	15 ± 5.9	1	223 ± 39	1
msh6	85 ± 35	5.7	184 ± 92	12	253 ± 0.0	1.1
msh2	109 ± 41	7.3	254 ± 30	17	263 ± 32	1.2
swi4	4.7 ± 2.0	0.3	6.4 ± 1.2	0.4	94 ± 25	0.4
msh6 msh2	192 ± 95	13	380 ± 42	25	399 ± 11	1.8
msh6 swi4	44 ± 17	2.9	93 ± 12	6.2	145 ± 44	0.7
msh2 swi4	12 ± 5.4	0.8	27 ± 21	1.8	104 ± 14	0.5
msh6 msh2 swi4	26 ± 17	1.7	104 ± 21	6.9	45 ± 25	0.2

Intragenic two-factor crosses were carried out at least three times. The crosses are illustrated in Figure 3.
[a] Numbers represent mean values and standard deviations of prototrophic recombinants per 10^6 colony-forming spores.
[b] Fold increase relative to wild type.

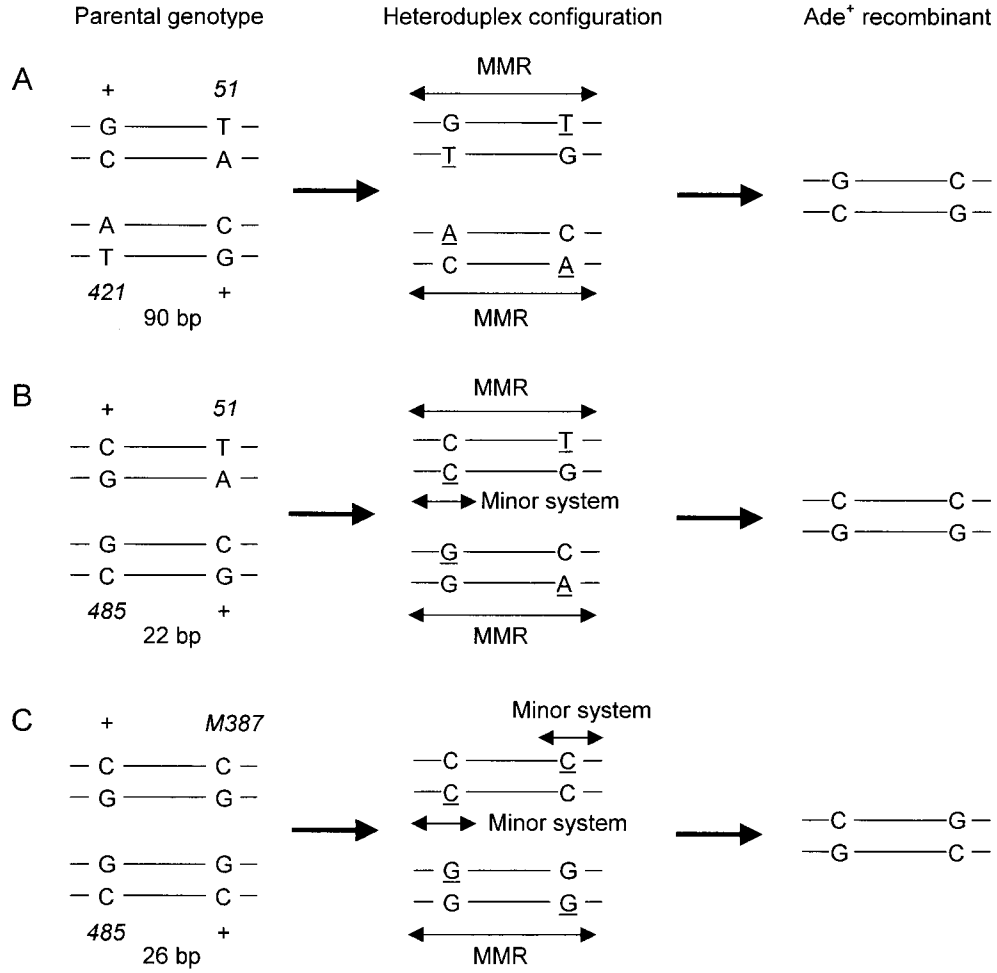

FIGURE 3.—Formation and repair of mismatches in intragenic two-factor crosses. The *ade6* mutants that were crossed are shown on the left. When both mutated sites are included in heteroduplex DNA during recombination, two mismatches are formed in the same heteroduplex. Two mismatch repair systems can correct these mismatches. The MMR system recognizes all mismatches except C/C and has long excision tracts, leading to frequent cocorrection of both mismatches (SCHÄR and KOHLI 1993; RUDOLPH *et al.* 1998). The minor system, requiring nucleotide-excision repair factors, corrects C/C mismatches and has short excision tracts (FLECK *et al.* 1999). MMR-mediated repair is indicated by long double arrows. Repair by the minor system, which is accompanied by short excision tracts, is indicated by short double arrows (only processing of C/C mismatches is shown). Prototrophic recombinants (right side) can arise when the underlined bases are replaced. (A) Cross *421* × *51*. Only non-C/C mismatches can be formed (distance of 90 bp). These are frequently corepaired by the MMR system. (B) Cross *485* × *51*. Heteroduplexes with either a C/C and a T/G, or a G/G and a C/A can be produced (distance of 22 bp). The C/C can be independently repaired by the minor system or is corepaired with the T/G mismatch by the MMR pathway. (C) Cross *485* × *M387*. Heteroduplexes can contain either two C/C or two G/G mismatches (distance of 26 bp). The C/C mismatches are not a substrate of MMR but can be often independently repaired by the minor system.

~6- to 7-fold, and a 13-fold increase was found with the *msh2 msh6* double mutant. The increase is likely caused by more frequent independent repair of the mismatches by the short-patch repair system due to the absence of functional MMR.

In the *485* × *51* cross, one heteroduplex contains a C/C and a T/G mismatch, and the other heteroduplex contains a G/G and a C/A mismatch (Figure 3). Prototrophic recombinants can be formed in the same way as in the cross *421* × *51*. However, one difference is that the short-patch repair system can repair the C/C mismatch, which is not a substrate of MMR. When the MMR system acts before the minor system, the long-patch excision-resynthesis will frequently corepair the C/C mismatch with the G/T mismatch on the same strand, thus preventing formation of prototrophs. In contrast, when the first event is processing of the C/C mismatch by the short-patch repair system, corepair of the G/T mismatch will be rather rare. Thus, although the mismatches are separated by only 22 bp, a relatively high frequency of prototrophs is expected in wild type. In fact, we found a frequency of 15×10^{-6}, which is the same as in the cross *421* × *51*, where the distance of the mismatches is 90 bp. The prototroph frequencies of *msh2*, *msh6*, and *msh2 msh6* mutant crosses were 17-, 12-, and 25-fold increased, respectively. Thus, as in the cross *421* × *51*, independent repair of the mismatches occurred more frequently when the MMR system was defective.

In the *485* × *M387* cross, one heteroduplex contains two C/C and the other heteroduplex two G/G mismatches. The MMR system frequently corepairs the two G/G, preventing formation of prototrophs. The minor system can frequently repair the two C/C mismatches independently and without competition by MMR. This explains the high number of prototrophs (223×10^{-6}) generated in wild type. In contrast to the other crosses, *msh6*, *msh2*, and *msh6 msh2* mutants did not significantly alter the frequency of prototroph formation. These data confirm that *msh2* is not involved in the short-patch C/C correcting pathway (RUDOLPH *et al.* 1998; FLECK *et al.* 1999) and show that the same is true for *msh6*.

All three types of crosses including *swi4* mutants showed a reduction of prototroph frequencies (Table 4). In addition, the crosses with double and triple mutants additionally mutated in *msh2* and/or *msh6* gave fewer prototrophs than respective crosses with an intact *swi4*$^+$ wild-type gene. The *swi4*-dependent reduction was in about the same range as that measured with the intergenic crosses (Table 3). One explanation for the decrease of prototroph frequencies is that mutated *swi4* causes a general reduction in meiotic recombination frequencies.

Msh6 is not involved in mating-type switching: Swi4 and Msh2 (originally identified as Swi8) are both involved in the termination step of mating-type switching (Egel *et al.* 1984; Fleck *et al.* 1992, 1994; Rudolph *et al.* 1999). Homothallic strains defective in either *swi4* or *msh2* form mottled colonies on sporulation medium when stained with iodine vapor, while colonies of switching-proficient strains are homogeneously brown. The mottled phenotype is caused by a reduced frequency of correct switching. In addition, heterothallic colonies frequently segregate as iodine negative. The segregants show arrangements in the mating-type region that are caused by incorrect termination of the switching process (Egel *et al.* 1984; Fleck *et al.* 1990, 1992, 1994). We tested whether Msh6 is also implicated in mating-type switching. Cells from a homothallic *msh6* strain were grown to colonies on sporulation medium and subsequently treated with iodine vapors. All colonies showed a homogeneously stained brown color, like switching-proficient strains. Thus, mating-type switching does not require *msh6*.

DISCUSSION

The MSH family is characterized by a conserved region located between two almost invariable motifs (TGPNM and DELGR) in the C-terminal part of the amino acid sequences. We performed PCR with degenerate primers directed to these motifs and identified the *S. pombe msh6*$^+$ gene. To study the function of Msh6 *in vivo*, the *msh6* gene was disrupted and analyzed as a single mutation and in combination with *msh2* and *swi4* mutations. Mitotic mutation rates caused by *msh6* were equivalent to those seen in *msh2*, not only in repair of base-base mispairs but also of mononucleotide loops. In contrast, the mutation rates of *swi4* strains were found to be as low as that of wild type in both types of reversion assays. Thus, repair of base-base mispairs mediated by the MMR system seems to be similar in *S. pombe* and *S. cerevisiae*, while they likely differ in repair of mononucleotide loops. *msh6* mutants of *S. cerevisiae* are severely affected in repair of base-base mispairs but only slightly in repair of insertion-deletion mismatches (Marsischky *et al.* 1996; Greene and Jinks-Robertson 1997; Sia *et al.* 1997; Earley and Crouse 1998). The latter was explained by a partial compensation by the functional Msh3 protein. Consistently, in *S. cerevisiae*, repair of small loops is similarly affected in a *msh3 msh6* double mutant as in a *msh2* mutant. Thus, *S. cerevisiae* has two MMR pathways depending on either Msh2-Msh3 or Msh2-Msh6, while *S. pombe* seems to have only one. The hypothesis of a single Msh2-Msh6 pathway for both types of mutations in *S. pombe* is strengthened by the finding that all double mutants as well as the triple mutant exhibited mutation rates similar to those of *msh6* and *msh2* single mutants. In addition, we found that inactivation of *msh6* caused a strong increase in GT repeat instability similar to that of *msh2* and *pms1*, while *swi4* had nearly no effect (Mansour *et al.* 2001, accompanying article). Thus, repair of both base-base mispairs and small loops with one or two unpaired nucleotides requires Msh2-Msh6, while Swi4 plays no or only a minor role.

Tetrad analysis and determination of PMS frequencies allows us to estimate repair efficiencies of mismatches formed during meiotic recombination. A repair event is represented by WCC, while unrepaired mismatches cause PMS. We dissected tetrads from the cross *msh6::arg3*$^+$ *ade6-M26* × *msh6::arg3*$^+$ *ade6*$^+$, where G/A and T/C can be produced, and compared the data with those of a repair-proficient wild-type cross (Gutz 1971). We found that *msh6* inactivation increased the frequency of PMS events and concomitantly decreased the WCC frequency (Table 2). A similar effect was observed for *msh2* and *pms1* mutants in *S. cerevisiae* and *S. pombe* (Alani *et al.* 1994; Schär *et al.* 1997; Fleck *et al.* 1999; Rudolph *et al.* 1999). The frequency of aberrant events (PMS + WCC) in the cross *ade6*$^+$ × *ade6-M26* is not significantly different among wild type (5.1 ± 0.7%), *msh2* (6.1 ± 1.1%; Rudolph *et al.* 1999), *pms1* (4.4 ± 1.1%; Schär *et al.* 1997), and *msh6* (4.5 ± 0.7%). These results imply that the MMR system preferentially repairs mismatches at *ade6-M26* toward gene conversion. Repair toward restoration would result in undetectable 4$^+$:4$^-$ events, while the failure of restoration-type repair would cause additional PMS events and thus increase the frequency of aberrant events in MMR mutants.

To extend the analysis on meiotic mismatch repair, intragenic two-factor crosses were also performed. Such crosses with closely situated mutations in the *ade6* gene originally led to the discovery of two pathways acting on mismatches during meiosis (Schär and Kohli 1993). The long-patch repair pathway, efficiently correcting most types of mismatches except C/C, was found to be equivalent to the MMR systems of other organisms. The short-patch system repairs C/C mismatches and, with low efficiency, other types of mismatches and requires components of nucleotide-excision repair (Schär and Kohli 1993; Schär *et al.* 1997; Rudolph *et al.* 1998, 1999; Fleck *et al.* 1999). With the intragenic two-factor crosses prototrophic recombinants can be determined. The types and distances of the mismatches that can be produced influence the frequency of prototroph formation, which directly depends on substrate specificity and repair efficiency of the two pathways. However, as forma-

tion of mismatches in heteroduplex DNA requires recombination, also the crossover frequency and the extension of heteroduplex tracts can modulate prototroph frequencies. We performed the crosses *421* × *51*, *485* × *51*, and *485* × *M387*. In all three crosses, the MMR system often corepairs the two mismatches in the same heteroduplex, with the exception of the two C/C produced in the cross *485* × *M387* (Figure 3). Thus when MMR is defective, a strong increase of prototroph frequencies is expected for the crosses *421* × *51* and *485* × *51*, but not for *485* × *M387*. Consistently, and in agreement with previous work, inactivation of *msh2* caused increased prototroph frequencies in the crosses *421* × *51* and *485* × *51*, involving no or only one C/C mismatch, but not in the cross *485* × *M387*. We found that prototroph frequencies in *msh6* mutants were in the same range as those of the *msh2* crosses (Table 4). Thus, loss of Msh6 uncovers meiotic short-patch repair of C/C and other types of base-base mismatches to an extent similar to that of inactivation of Msh2. In addition, Msh6, like Msh2, has no function in the short-patch repair system.

Concerning *swi4*, we found that crosses with strains bearing a *swi4* defect resulted in a decrease of prototroph frequencies. The decrease was similar in all types of crosses and thus independent from the distances and the types of mismatches that can be produced. Fewer prototrophs were also found with double and triple mutants additionally defective for *msh2* and/or *msh6* when compared to respective crosses with the *swi4*$^{+}$ wild-type gene (Table 4). Thus, the reduction in prototroph frequencies is likely not due to a defect in either the long-patch or short-patch repair pathway. As mentioned above, the frequency of prototrophs derived from intragenic two-factor crosses also depends on recombination. We performed intergenic two-factor crosses and found that *swi4*, but not *msh2* or *msh6*, caused a decrease in recombinants (Table 3). Similar to the intragenic crosses, a two- to threefold reduction to wild-type crosses was observed. We propose that Swi4 is involved in meiotic recombination but not in repair of base-base mismatches arising during either meiosis or vegetative growth.

Swi4 and Msh2 are both involved in the termination step of mating-type switching in conjunction with Swi10 and Rad16, which form a heterodimeric 5′ endonuclease, also implicated in nucleotide-excision repair (Egel *et al.* 1984; Fleck *et al.* 1992, 1994; Rödel *et al.* 1992; Carr *et al.* 1994). It was proposed that correct termination requires binding of Msh2-Msh3 to a stem-loop structure either by recognizing DNA loops or by branched DNA, which is then processed by Rad16-Swi10 (Rudolph *et al.* 1999). In this study, we found that Msh6 likely has no function in mating-type switching. In budding yeast, Msh2-Msh3 and Rad1-Rad10, the homologues of Rad16-Swi10, but not Msh6, act in the same pathway of mitotic recombination (Saparbaev *et al.* 1996; Sugawara *et al.* 1997). It was suggested that branched structures, which can be formed during recombination by strand invasion or single-strand annealing, are bound by Msh2-Msh3 and subsequently processed by Rad1-Rad10 (Sugawara *et al.* 1997). In this respect, the mechanisms of mating-type switching are similar in *S. cerevisiae* and *S. pombe*. However, Swi4 in *S. pombe* seems to have also a Msh2-independent role in meiotic recombination, as crossover frequencies appeared to be reduced in intergenic two-factor crosses (Table 3). In *S. cerevisiae*, such a function was described for the meiosis-specific MutS homologues Msh4 and Msh5 (Ross-Macdonald and Roeder 1994; Hollingsworth *et al.* 1995). Interestingly, homologues of Msh4 or Msh5 were not identified in *S. pombe* so far, although ~95% of the genome was already sequenced (http://www.sanger.ac.uk/Projects/S_pombe/).

Our studies revealed that *msh6* mutants and *msh2* mutants were similarly affected, both in repair of base-base mismatches and of loops with one or two unpaired nucleotides (this work; Mansour *et al.* 2001, accompanying article). In contrast, inactivated *swi4* caused no or only slight effects. As already discussed, the relative contribution of Msh3 and Msh6 in Msh2-dependent repair clearly differs from the *S. cerevisiae* MMR system. Also in mammals, specific roles in MMR were assigned to the various MSH proteins. Mice deficient for *MSH2* or *MSH6*, but not for *MSH3*, present a predisposition to cancer (De Wind *et al.* 1995, 1999; Reitmair *et al.* 1995; Edelmann *et al.* 1997). Inactivation of both *MSH3* and *MSH6* does not significantly increase the development of cancer compared to *MSH6*$^{-/-}$ mice, although such mice developed more intestinal tumors, which were considered to be HNPCC-like (De Wind *et al.* 1999). HNPCC in humans is frequently correlated with mutated *MSH2*, less frequently with mutated *MSH6*, and so far not found to be associated with a mutated *MSH3* gene (Fishel and Wilson 1997; Miyaki *et al.* 1997; Prolla 1998). However, some sporadic types of tumors were also found in patients with *MSH3* mutations. Repetitive DNA of one or several nucleotides per repeat unit is drastically destabilized in MSH2 deficient cell lines but not when MSH3 is inactivated (Inokuchi *et al.* 1995). Loss of MSH6 causes instability of mononucleotide repeats but not of other repeats (Papadopoulos *et al.* 1995). A recent study revealed that MSH2-MSH6 mediates repair not only of base-base mismatches but also of loops with up to eight unpaired nucleotides (Genschel *et al.* 1998). The studies on mice and humans implicate MSH2-MSH6 as the major recognition complex, while MSH2-MSH3 rather has a secondary function in mismatch repair. In this respect, the *S. pombe* MMR system seems to be closer to mammalian MMR than to that of *S. cerevisiae*.

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