Control of GT Repeat Stability in *Schizosaccharomyces pombe* by Mismatch Repair Factors

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ABSTRACT

The mismatch repair (MMR) system ensures genome integrity by removing mispaired and unpaired bases that originate during replication. A major source of mutational changes is strand slippage in repetitive DNA sequences without concomitant repair. We established a genetic assay that allows measuring the stability of GT repeats in the *ade6* gene of *Schizosaccharomyces pombe*. In repair-proficient strains most of the repeat variations were insertions, with addition of two nucleotides being the most frequent event. GT repeats were highly destabilized in strains defective in *msh2* or *pms1*. In these backgrounds, mainly 2-bp insertions and 2-bp deletions occurred. Surprisingly, essentially the same high mutation rate was found with mutants defective in *msh6*. In contrast, a defect in *swi4* (a homologue of Msh3) caused only slight effects, and instability was not further increased in *msh6 swi4* double mutants. Also inactivation of *exo1*, which encodes an exonuclease that has an MMR-dependent function in repair of base-base mismatches, caused only slightly increased repeat instability. We conclude that Msh2, Msh6, and Pms1 have an important role in preventing tract length variations in dinucleotide repeats. Exo1 and Swi4 have a minor function, which is at least partially independent of MMR.

MISMATCHED and unpaired bases arise during replication by misincorporation of nucleotides and strand slippage, respectively. The major system directed to repair base-base mismatches and loops with one or a few unpaired nucleotides is the bacterial *mutHLS* pathway and related but more complex systems in eukaryotes (Modrich 1991; Jiricny 1998, 2000; Kolodner and Marsischky 1999; Marra and Schär 1999). In eukaryotes, three MutS homologues, Msh2, Msh3, and Msh6, and three MutL homologues, termed Pms2, Mlh1, and Mlh3 in human (Pms1, Mlh1, and Mlh3 in *Saccharomyces cerevisiae*), are thought to be components of the nuclear mismatch repair (MMR) system.

Two heterodimers, Msh2-Msh6 (MutSα) and Msh2-Msh3 (MutSβ), are involved in the recognition step. After binding of either MutSα or MutSβ to the DNA heterology, a MutL heterodimer is recruited to the MutS-DNA complex. Mlh1-Pms2 is involved in MutSα- and MutSβ-mediated repair of mismatches and loops (Umar and Kunkel 1996; Buermeyer *et al.* 1999; Kolodner and Marsischky 1999; Marra and Schär 1999; Jiricny 2000). Mlh1-Mlh3 plays a minor role in repair of loops bound by MutSβ and might rather have its main function in recombination (Flores-Rozas and Kolodner 1998; Nakagawa *et al.* 1999; Wang *et al.* 1999).

Mutations in the human MMR genes *MSH2* and *MLH1* cause predisposition to hereditary nonpolyposis colon cancer and other types of cancer. In contrast, mutations in *PMS2*, *MSH3*, and *MSH6* are rarely found to be correlated with cancer (Umar and Kunkel 1996; Buermeyer *et al.* 1999). The role of *MLH3* in cancer development remains to be analyzed in more detail, as this gene was just recently discovered (Lipkin *et al.* 2000). A defect in *MSH2*, *PMS2*, or *MLH1* results in increased mutation rates and high instability of microsatellites (Umar and Kunkel 1996; Buermeyer *et al.* 1999). Microsatellites are repetitive sequences consisting of one to several nucleotides per repeat unit and are common motifs in genomes of higher eukaryotes. Instability of repetitive sequences is thought to be caused by frequent strand slippage during replication without subsequent repair. When the slipped and unpaired bases are in the newly synthesized strand, additions of repeat units are the consequence, whereas slippage of the template strand results in deletions.

Microsatellite stability in *S. cerevisiae* is also maintained by the MMR system with Msh2, Pms1, and Mlh1 as key players (Strand *et al.* 1993; Johnson *et al.* 1996; Greene and Jinks-Robertson 1997; Sia *et al.* 1997). A defect in any of the respective genes causes greatly increased rates of length variations. Loss of Msh6 has little effect on repeats with one or two nucleotides per unit. Such

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repeats are moderately affected in *msh3* mutants; *i.e.*, tract alterations occur more often than in *msh6* mutants but clearly not as frequently as in *msh2* mutants. Nevertheless, a *msh3 msh6* double mutant shows essentially the same high instability as the *msh2* mutant. The genetic data originally led to the conception that MutSα and MutSβ can redundantly repair small loops with one or two unpaired nucleotides. However, current studies on human MMR indicate that MutSα is the major recognition factor for base-base mismatches and small loops while MutSβ rather serves as a backup system (Jiricny 1998; Kolodner and Marsischky 1999). This idea is based on studies with human MutSα and MutSβ purified from HeLa cells and is strengthened by the observations that the cellular level of MutSα is much higher than that of MutSβ (Drummond *et al.* 1997; Genschel *et al.* 1998; Marra *et al.* 1998). Furthermore, microsatellite instability is not increased in *MSH3*-deficient human cell lines, while microsatellites of mononucleotide runs are affected in *MSH6*-mutated cell lines (Inokuchi *et al.* 1995; Papadopoulos *et al.* 1995).

Not much is known about proteins acting further downstream in the MMR system. One factor is Exo1, a 5′ to 3′ exonuclease, which was identified in *Schizosaccharomyces pombe*, *S. cerevisiae*, mouse, and human (Szankasi and Smith 1995; Tishkoff *et al.* 1997, 1998; Schmutte *et al.* 1998; Wilson *et al.* 1998). A mutated *exo1* causes increased mutation rates in epistatic relationship to *msh2* (Tishkoff *et al.* 1997; Rudolph *et al.* 1998). These studies and the ability of Exo1 to interact with Msh2 (Tishkoff *et al.* 1997) suggest a function in the MMR system. However, it should be noted that Exo1 is also involved in recombinational processes (Fiorentini *et al.* 1997).

In *S. pombe*, the MutS homologues Swi4 (most closely related to the Msh3 subgroup), Msh2, and Msh6, and the MutL homologue Pms1 have been identified and characterized (Fleck *et al.* 1992; Schär *et al.* 1997; Rudolph *et al.* 1999; Tornier *et al.* 2001, accompanying article). Msh2, Msh6, and Pms1 turned out to be important components of MMR in repair of base-base mismatches and one-nucleotide loops, while no significant defects in mismatch repair were found in *swi4* mutants. Swi4 has a function in the termination step of mating-type switching, likely in conjunction with Msh2 (Fleck *et al.* 1992; Rudolph *et al.* 1999).

The aim of the work presented here was to analyze the role of Msh2, Msh6, Swi4, Pms1, and Exo1 of *S. pombe* in stability of GT repeats. We constructed strains with insertion of GT repeats in the *ade6* marker gene and measured mutation rates in wild type and in mutants defective in one or more of the MMR genes. To obtain information about the relative contribution of MutSα and MutSβ in repair of dinucleotide loops in *S. pombe*, we were particularly interested in determining the effects caused by loss of Msh2, Msh6, and Swi4.

MATERIALS AND METHODS

General yeast genetic methods and media: The *S. pombe* media YEA (yeast extract agar), YEL (yeast extract liquid), MEA (malt extract agar), and MMA (minimal medium agar) and general genetic methods were used as described elsewhere (Gutz *et al.* 1974; Schär *et al.* 1997).

Construction of *S. pombe* strains with GT repeats in the *ade6* marker gene: pAN-T, a pUC18 derivative, contains a *Xho*I-*Eco*RI fragment of the *ade6* gene of *S. pombe*, in which an internal 340-bp *Dra*III-*Hin*dIII fragment was replaced by the *Escherichia coli* tetracycline resistance gene. The tetracycline resistance gene was then replaced by the previously deleted *Dra*III-*Hin*dIII fragment derived from *ade6*, but additionally containing the $(GT)_8$ repeat close to the *Dra*III site. This fragment was obtained by restriction digestion of a PCR product obtained with primers ade6-GT8, 5′-AAGCACTTGGTGAT CGTGTGTGTGTGTGTGTCCGCTTTATGTTGAAAAA GTT-3′ and ade6-H, 5′-GGGCAAGCTTCAATGGTGTA-3′ and the *ade6*-containing vector pCG162 (Grimm *et al.* 1994) as template. Plasmids were isolated from *E. coli* transformants, which lost resistance to tetracycline and were checked for the presence of the desired insert by restriction digestions and DNA sequencing.

The $(GT)_8$ repeat was then introduced into the *S. pombe ade6* gene by homologous recombination of the 1.7-kb *Xho*I-*Eco*RI fragment transformed into the *S. pombe* strain AM1 (*h*$^-$ *ade6::ura4*$^+$ *his3-D1 leu1-32 ura4-D18*), giving rise to AM3 (*h*$^-$ *ade6-[(GT)*$_8$*-1397] his3-D1 leu1-32 ura4-D18*). AM1 is a derivative of strain PS5, in which the 0.8-kb *Bam*HI-*Hin*dIII fragment of *ade6* is replaced by the *ura4*$^+$ gene (Schuchert *et al.* 1991). Transformants were grown on fluoroorotic acid plates to select for loss of the *ura4*$^+$ marker as described previously (Grimm *et al.* 1988). Correct integration was then proved by PCR and sequencing (see below). All strains with a $(GT)_8$ repeat were obtained from crosses with AM3. During fluctuation tests, the strain OL539 (*h*$^-$ *msh2::his3*$^+$ *ade6-[(GT)*$_{10}$*-1397] his3-D1 ura4-D18*) containing a $(GT)_{10}$ repeat and the strain OL540 (*h*$^-$ *msh2::his3*$^+$ *ade6-[(GT)*$_9$*-1397] his3-D1 ura4-D18*) with a $(GT)_9$ repeat were isolated and identified by sequencing. The repeats were combined by crosses with the various marker-disrupted repair genes, which were studied in this work: *msh2::his3*$^+$ (Rudolph *et al.* 1999), *msh6::arg3*$^+$ (Tornier *et al.* 2001, accompanying article), *swi4::ura4*$^+$ (Fleck *et al.* 1992), *pms1::ura4*$^+$ (Schär *et al.* 1997), *pms1::his3*$^+$ (strain collection of Bern), and *exo1::ura4*$^+$ (Szankasi and Smith 1995; Rudolph *et al.* 1998).

Determination of GT repeat instability: All repeats used in this study are insertions at an existing GT dinucleotide in the *ade6* gene at position 1397, with the ATG start codon at position 875 and the stop codon at position 2531 according to the nomenclature of Szankasi *et al.* (1988). $(GT)_8$ and $(GT)_9$ (insertions of 14 and 16 bp, respectively) produce in frame stop codons located 47 and 12 bp downstream of the insertion, respectively, and cause adenine auxotrophy. In contrast, $(GT)_{10}$ represents an in frame insertion of 18 bp, allowing growth on minimal medium without adenine. Instability of GT repeats was measured by fluctuation tests as described previously (Schär *et al.* 1997; Rudolph *et al.* 1998, 1999). In the case of strains with $(GT)_8$ or $(GT)_9$ repeats, seven small colonies were each inoculated in 5-ml YEL and grown to stationary phase. Appropriate amounts were plated on minimal medium for selection of Ade$^+$ revertants and on minimal medium supplemented with adenine for determination of cell titers. A maximum of 5×10^7 cells were streaked out per plate, a density that does not significantly affect growth of Ade$^+$ revertants (data not shown). After 7 days of growth at 30°, mutation rates were calculated from the median number of

Ade$^+$ revertants per total cell number of the culture (Lea and Coulson 1949). In the case of $(GT)_{10}$, one small colony was picked from a minimal medium plate, suspended in 0.5 ml 0.85% NaCl, and diluted 1:100. About 15 1-ml YEL tubes were each inoculated with ~100 cells and grown for 24–36 hr until ~10,000–30,000 cells were present per culture. The total of the cultures was plated out on YEA, allowing growth of all cells. After 4 days of growth at 30°, red colonies (for colony color see below) were counted and tested for adenine auxotrophy. Mutation rates were calculated either from the median or by the zero method from the number of cultures without Ade$^-$ colonies per total number of cultures.

Determination of tract length variations of GT repeats: Variations in the number of GT dinucleotide units were determined by visual inspection of the colony color and by DNA sequencing. *ade6* mutants form red colonies on YEA with a limited amount of adenine and cannot grow on minimal medium without adenine. Strains with a $(GT)_8$ or $(GT)_9$ repeat in *ade6* form red colonies. Among the Ade$^+$ revertants, $(GT)_4$, $(GT)_7$, and $(GT)_{10}$ repeats were identified by sequencing (Table 1), which all restored the reading frame of *ade6*. No other events were detected. Strains with $(GT)_4$ or $(GT)_7$ repeats form white colonies on YEA and MMA plates, like *ade6*$^+$ wild-type strains. In contrast, strains containing a $(GT)_{10}$ repeat form pink colonies on both types of medium, indicating that the activity of the *ade6* gene product is not completely retained. Thus the simple determination of the colony color of Ade$^+$ revertants derived from strains with either a $(GT)_8$ or $(GT)_9$ repeat allows discrimination between deletions [$(GT)_4$ or $(GT)_7$] and insertions [$(GT)_{10}$] of repeat units (Figure 1). The mutational spectra were determined from the sum of pink and of white colonies of all cultures examined.

DNA sequences were determined by direct sequencing of PCR products (Dorit *et al.* 1995). PCR was performed on chromosomal DNA using primers ade6-1275, 5′-GGCCAA GAGTTTGGTTATCC-3′ and ade6-H, 5′-GGGCAAGCTTCAA TGGTGTA-3′ under standard conditions (5 min at 94°; 30 cycles of 45 sec at 94°, 45 sec at 50°, 45 sec at 72°; 10 min at 72°). The PCR product was purified from primers by precipitation with 2.5 M ammonium acetate/1 volume absolute ethanol and used for amplification of a single strand with primer ade6-H (5 min at 94°; 20 cycles of 45 sec at 94°, 45 sec at 48°, 45 sec at 72°; 10 min at 72°). After removal of free primers, sequences were determined with primer ade6-1275 by the dideoxy method using a sequencing kit (Amersham Pharmacia, Dübendorf, Switzerland).

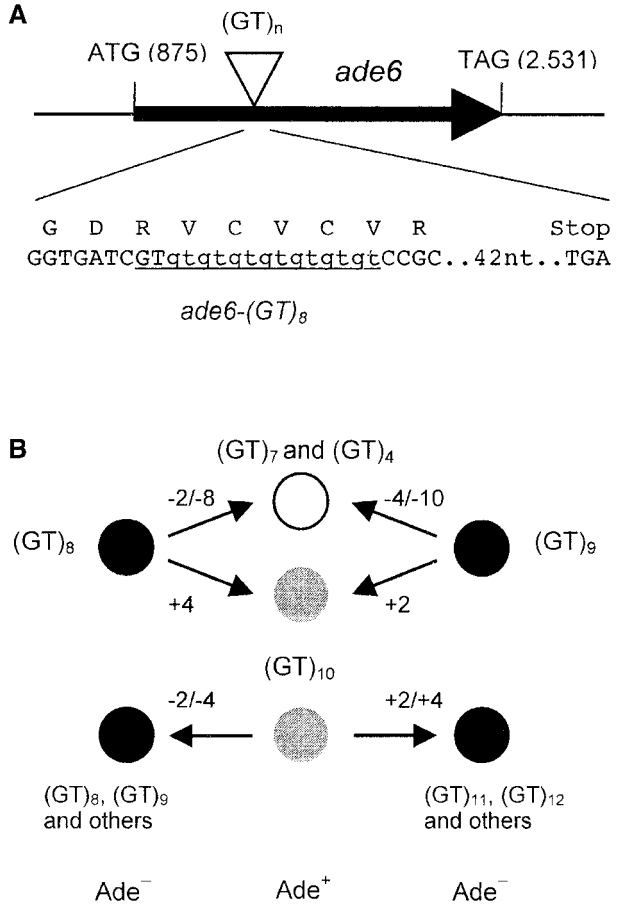

Figure 1.—Test system for GT repeat stability. (A) GT repeats were introduced at position 1397 of the *ade6* gene (the ATG start codon is at position 875). $(GT)_8$ is shown as an example (underlined) and was created by insertion of 7 GT units (letters in lowercase) at an existing GT site. The insertion causes a frame shift and an in frame stop codon downstream. (B) Mutation rates and spectra detectable with the repeats $(GT)_8$, $(GT)_9$, and $(GT)_{10}$. $(GT)_8$- and $(GT)_9$-containing strains are Ade$^-$ and form red colonies on YEA (solid circles). Ade$^+$ revertants occur either by deletions or insertions of repeat units (changes of the nucleotide numbers are indicated). Strains with $(GT)_7$ and $(GT)_4$ form white colonies (open circle), while $(GT)_{10}$ strains form pink colonies (shaded circles). With the $(GT)_{10}$ repeat events are detectable that lead to Ade$^-$ (solid circles).

RESULTS

Test system to measure GT repeat instability in *S. pombe*: In this work we were interested in studying the instability of GT dinucleotide repeats in *S. pombe*. The emphasis was to understand the role of genes with a known function in repair of base-base mismatches, *i.e.*, *msh2*, *pms1*, *msh6*, and *exo1*. We also analyzed *swi4*, which has no detectable function in base-base mismatch repair but might have a function in loop repair, similar to the situation in *S. cerevisiae* (Johnson *et al.* 1996; Greene and Jinks-Robertson 1997; Sia *et al.* 1997). The assays to measure GT repeat instability are illustrated in Figure 1. We introduced GT repeats at an existing GT dinucleotide into the *ade6* gene as described in materials and methods. A region inside the *ade6* gene was chosen, where none of the known mutations is located, in the hope that in frame additions of several nucleotides do not disrupt the function of the *ade6* gene. We tested strains in which seven, eight, or nine GT repeat units were inserted at an existing GT site and thus tested instability of $(GT)_8$, $(GT)_9$, and $(GT)_{10}$ repeats.

$(GT)_8$ and $(GT)_9$ repeats produce frame shifts of +2 and +1 nucleotide, respectively. Strains with $(GT)_8$ and $(GT)_9$ are Ade$^-$ and form red colonies on media with a limited amount of adenine (materials and methods). Strains with either $(GT)_8$ or $(GT)_9$ were used to measure reversions to Ade$^+$; +4-, −2-, and −8-bp changes are detectable with $(GT)_8$ strains, and +2-, −4-, and −10-bp changes with $(GT)_9$ strains. These changes resulted in

TABLE 1

Identification of repeat tract changes in the $(GT)_8$ and $(GT)_9$ repeats by sequencing

	$(GT)_8$			$(GT)_9$		
	White[a]		Pink[a]	White		Pink
Relevant genotype	$(GT)_4$ (−8 bp)	$(GT)_7$ (−2 bp)	$(GT)_{10}$ (+4 bp)	$(GT)_4$ (−10 bp)	$(GT)_7$ (−4 bp)	$(GT)_{10}$ (+2 bp)
Wild type	2	3	5	3	2	5
msh2	0	11	5	0	3	5
msh6	0	5	5	0[b]	0[b]	5
pms1	0	5	5	0	2	5
swi4	0	6	5	2	2	3
exo1	1	10	5	2	1	3

Numbers represent revertants containing the indicated type of repeat as identified by sequencing. From wild type and the various single mutants, 3–5 revertants forming pink colonies were analyzed for both $(GT)_8$ and $(GT)_9$. In all cases such revertants contained a $(GT)_{10}$ repeat. Sequencing of a total of 60 revertants forming white colonies revealed either a $(GT)_4$ or $(GT)_7$ repeat. The sequencing data showed that white revertants originated from deletions of GT units, while pink revertants were caused by insertions of GT units. However, it should be noted that the numbers given for deletions *vs.* insertions do not reflect the relative distribution of such events (compare with Table 3).

[a] Colony color of revertants (see Figure 1).

[b] No white revertants of $(GT)_9$ in *msh6* background found.

$(GT)_{10}$, $(GT)_7$, and $(GT)_4$ repeats, respectively (Figure 1). During the fluctuation tests, we noticed that Ade^+ revertants formed either white or pink colonies. DNA sequencing of 56 pink colonies derived from the various strain backgrounds exclusively revealed $(GT)_{10}$ repeats and sequencing of 60 white colonies, either $(GT)_7$ or $(GT)_4$ repeats (Table 1). No Ade^+ revertants with other variations in repeat tract length were found. Thus, starting with *ade6*-mutated strains containing either a $(GT)_8$ or $(GT)_9$ repeat, Ade^+ revertants with additions of repeat units can be easily distinguished by their colony color from those with deletions (Figure 1; Table 1). Moreover, the use of $(GT)_8$ and $(GT)_9$ strains allows determination of how frequently specific events occur in the various strain backgrounds. Due to the possibility of selection for Ade^+ revertants, even relatively rare events and small effects can be detected. However, it should be kept in mind that not all events are detectable when either $(GT)_8$ or $(GT)_9$ were assayed. Therefore, tract length variations in a $(GT)_{10}$ repeat were also analyzed, where most of the possible changes are detectable. The $(GT)_{10}$ repeat includes 18 additional nucleotides and thus an in frame insertion. Strains containing a $(GT)_{10}$ repeat were used to measure all events (+4, +2, −2, −4 bp, and others) leading to red colonies (Ade^-).

Requirement for mismatch repair genes in stability of GT dinucleotide repeats: *ade6* mutants containing either a $(GT)_8$ or $(GT)_9$ repeat were used to measure reversion rates to Ade^+ by fluctuation tests as described in MATERIALS AND METHODS. In mismatch repair-proficient wild type, reversions occurred rarely in the case of the $(GT)_8$ repeat, but quite frequently for $(GT)_9$, with a rate ~160 times higher than that for $(GT)_8$ (Table 2). The reversion rates were dramatically increased when *msh2*, *msh6*, or *pms1* were defective. We found rates of $\sim 5 \times 10^{-5}$ for $(GT)_8$, and of $\sim 1.7 \times 10^{-4}$ for $(GT)_9$, which correspond to ~15,000- and 300-fold increases, respectively. In contrast, inactivation of *swi4* caused only a 12-fold increase of reversions of $(GT)_8$ and, surprisingly, a 3-fold decrease of $(GT)_9$ reversions. *msh2 swi4* and *msh6 swi4* double mutants showed the same high reversion rates as *msh2* and *msh6* single mutants in the case of $(GT)_8$, but an ~4-fold reduction in the case of $(GT)_9$, when compared with *msh2* and *msh6*. In addition, the triple mutant *msh2 msh6 swi4* also showed a 10 times lower $(GT)_9$ reversion rate than *msh2 msh6* (Table 2).

When *exo1* was mutated, a nine-fold increase was found for $(GT)_8$ and a two-fold increase for $(GT)_9$ (Table 2). Reversion of $(GT)_8$ in the *swi4 exo1* double mutant was in the same range as in respective single mutants. In contrast, reversion rates of $(GT)_9$ were reduced, which is similar to *swi4* and different from *exo1*. The *msh2 exo1* and *pms1 exo1* mutants showed extreme instability of both $(GT)_8$ and $(GT)_9$. We measured reversion rates in the range of 5×10^{-4}, which is significantly higher than the rates caused by inactivation of either *msh2* or *pms1* alone.

The nature of the reversions in the various strain backgrounds was determined by DNA sequencing and by inspection of the colony color. Sequencing of revertants with pink colonies exclusively revealed a $(GT)_{10}$ repeat and of revertants with white colonies either a $(GT)_7$ or $(GT)_4$ repeat (Table 1). The distributions of deletions and insertions in the $(GT)_8$ and the $(GT)_9$ repeats are summarized in Table 3. In all strain backgrounds reversion of $(GT)_9$ occurred in almost all of

TABLE 2

Reversion rates of $(GT)_8$ and $(GT)_9$ repeats in wild-type and mutant strains

	GT repeat			
	$(GT)_8$		$(GT)_9$	
Relevant genotype	Rate	Fold increase[a]	Rate	Fold increase
Wild type	$3.4 \pm 1.2 \times 10^{-9}$	1	$5.5 \pm 1.7 \times 10^{-7}$	1
msh2	$4.8 \pm 0.8 \times 10^{-5}$	1×10^4	$1.9 \pm 0.3 \times 10^{-4}$	300
msh6	$5.7 \pm 5.1 \times 10^{-5}$	2×10^4	$1.5 \pm 1.2 \times 10^{-4}$	300
pms1	$4.4 \pm 0.3 \times 10^{-5}$	1×10^4	$1.6 \pm 0.1 \times 10^{-4}$	300
swi4	$4.2 \pm 2.4 \times 10^{-8}$	12	$1.7 \pm 1.0 \times 10^{-7}$	0.3
exo1	$3.0 \pm 2.0 \times 10^{-8}$	9	$1.1 \pm 0.3 \times 10^{-6}$	2
msh2 msh6	NT	—	$2.0 \pm 0.3 \times 10^{-4}$	400
msh2 swi4	$4.2 \pm 0.2 \times 10^{-5}$	1×10^4	$5.0 \pm 2.4 \times 10^{-5}$	90
msh6 swi4	$4.7 \pm 2.2 \times 10^{-5}$	1×10^4	$4.0 \pm 2.3 \times 10^{-5}$	70
msh2 msh6 swi4	NT	—	$2.1 \pm 0.2 \times 10^{-5}$	40
swi4 exo1	$3.2 \pm 0.6 \times 10^{-8}$	10	$7.1 \pm 0.4 \times 10^{-8}$	0.1
msh2 exo1	$7.1 \pm 2.8 \times 10^{-4}$	2×10^5	$4.9 \pm 4.1 \times 10^{-4}$	900
pms1 exo1	$2.5 \pm 0.5 \times 10^{-4}$	7×10^4	$4.7 \pm 1.5 \times 10^{-4}$	900

Rates of tract alterations, which are detectable with the $(GT)_8$ or $(GT)_9$ repeats (see Figure 1), are represented as averages with standard deviations. NT, not tested.

[a] Fold increase relative to wild type.

the cases (93–100%) by insertion of two nucleotides. In wild type, 83% of Ade^+ revertants of $(GT)_8$ repeats produced pink colonies. Thus, $(GT)_8$ reverted mainly by insertion of four nucleotides. Among the 17% Ade^+ with white colonies, both 2-bp and 8-bp deletions were detected (Table 1). No big difference in the pattern was observed for *exo1* mutants. A total of 73% of $(GT)_8$ revertants and 99% of $(GT)_9$ revertants formed pink colonies and thus derived from insertion of 4 and 2 bp, respectively, both resulting in $(GT)_{10}$ repeats. Among the white $(GT)_8$ revertants sequenced, 10 with a $(GT)_7$ repeat and 1 with a $(GT)_4$ repeat were identified (Table 1).

When either *msh2*, *msh6*, or *pms1* was mutated, almost all of the $(GT)_8$ revertants formed white colonies (Table 3). All white revertants from *msh2*, *msh6*, or *pms1* that were analyzed by sequencing contained a $(GT)_7$ repeat (Table 1). Most of the $(GT)_9$ revertants formed pink colonies and thus were caused by insertion of two nucleotides. Pink colonies derived from $(GT)_8$ and white colonies derived from $(GT)_9$ occurred with low frequencies (Table 3). Sequencing of white revertants derived from $(GT)_9$ revealed only $(GT)_7$ repeats (Table 1). We conclude that in *msh2*, *msh6*, and *pms1* genetic backgrounds most $(GT)_8$ reversions were due to deletions of two

TABLE 3

Distribution of deletions and insertions in the $(GT)_8$ and $(GT)_9$ repeats

	$(GT)_8$		$(GT)_9$	
Relevant genotye	% deletions (−2 or −8 bp)	% insertions (+4 bp)	% deletions (−4 or −10 bp)	% insertions (+2 bp)
Wild type	17	83	1	99
msh2	98	2	3	97
msh6	98	2	0	100
pms1	99	1	1	99
swi4	78	22	3	97
exo1	27	73	1	99
msh2 swi4	>99	<1	<1	>99
msh6 swi4	95	5	0	100
msh2 exo1	>99	<1	1	99
pms1 exo1	99	1	1	99
swi4 exo1	69	31	7	93

Numbers represent the percentages of deletions and insertions found with the $(GT)_8$ and $(GT)_9$ repeats. Calculation is based on the occurrence of white and pink colonies as described in MATERIALS AND METHODS.

TABLE 4

Mutation rates of the $(GT)_{10}$ repeat

Relevant genotype	Average no. of cells per culture $\times 10^3$	Cultures without Ade^- per total no. of cultures[a]	Median no. of Ade^- per culture[b]	Rate	Average rate	Fold increase[c]
Wild type	22.5	58/60	—	1.5×10^{-6}		1
swi4	19.4	70/70	—	$<7.4 \times 10^{-7}$		<0.5
exo1	15.9	81/89	—	5.9×10^{-6}		4
msh2	29.8	—	5	8.0×10^{-5}	9.0×10^{-5}	60
	9.2	—	1	9.7×10^{-5}		
	18.6	—	3	9.2×10^{-5}		
msh6	7.9	—	4	2.3×10^{-4}	1.8×10^{-4}	120
	7.0	—	2	1.3×10^{-4}		
msh2 exo1	14.3	—	8	2.3×10^{-4}	2.2×10^{-4}	150
	10.2	—	4	2.0×10^{-4}		

[a] Mutation rates from $(GT)_{10}$ to Ade^- were calculated on the basis of the proportion of cultures without Ade^-, when rates were low (wild type, *swi4*, and *exo1*). In these cases, data of all cultures were pooled.

[b] In *msh2*, *msh6*, and *msh2 exo1* strains virtually all cultures contained Ade^-, allowing calculation of mutation rates by the method of the median (Lea and Coulson 1949). In these cases, data of individual experiments, each with 15 cultures, are shown together with the average.

[c] Fold increase relative to wild type.

nucleotides, and most $(GT)_9$ reversions were due to insertions of two nucleotides. Insertion or deletion of four nucleotides was relatively rare (Table 3), and larger deletions were not detected (Table 1). Insertions of more than four nucleotides probably do not give a functional *ade6* gene and therefore cannot be detected with the $(GT)_8$ or $(GT)_9$ assays.

In *swi4* background, 78% of $(GT)_8$ revertants showed white colonies (Table 3). Sequencing of six white $(GT)_8$ revertants revealed that all contained $(GT)_7$ (Table 1). The rate of 4-bp insertions was only slightly increased and events detectable with the $(GT)_9$ assay (+2, −4, −10) seem to be rather reduced (Table 2). Thus, the majority of events caused by a defect in *swi4* were deletions of two nucleotides. The distributions of *msh2 swi4*, *msh6 swi4*, *msh2 exo1*, and *pms1 exo1* double mutants were similar to those of strains mutated in *msh2*, *msh6*, or *pms1* (Table 3), obviously due to the high mutation rates caused by these mutations in comparison to the weak effects caused by *swi4* or *exo1*. In the *swi4 exo1* double mutant, 69% of the reversion events in the $(GT)_8$ repeat were deletions (Table 3). This pattern is different from that of the respective single mutants, but more similar to that of *swi4*.

Our analyses of GT repeat instability were extended by testing the $(GT)_{10}$ repeat. $(GT)_{10}$ is an insertion of nine GT units at an existing GT dinucleotide and thus an in frame insertion of 18 bp. The $(GT)_{10}$ assay can be used to measure all changes in the repeat that lead to frame shifts (Figure 1). Fluctuation tests were performed with wild type and the single mutants *msh2*, *msh6*, *swi4*, and *exo1*, as well as with the double mutant *msh2 exo1* (Table 4). The repair-proficient wild type showed a rate of 1.5×10^{-6}. Among the two Ade^- isolated from 60 cultures, we found one 2 and one 4-bp insertion. In *msh2* and *msh6* mutants, the mutation rate was increased 60- and 120-fold, respectively. With one exception (a 4-bp insertion in *msh6* background), all of the events were either 2-bp insertions or 2-bp deletions, both of which occurred with similar frequencies (Table 5). A 4-fold increase was measured with *exo1* (Table 4). The mutation rate of the *msh2 exo1* mutant was increased ~150-fold, which is higher than that of *msh2*, although not very different from that measured with *msh6* (Table 4). No Ade^- colonies were found among 70 cultures of the *swi4* mutant. Thus the mutation rate seems to be even lower than that in wild type, although the data are not significantly different ($\chi^2 = 2.37$, $\chi^2_{0.05} = 3.84$). However, a *swi4*-dependent reduction was also found with the $(GT)_9$ assay (Table 2).

DISCUSSION

Mainly insertions occurred in GT repeats of wild-type cells: We have established an assay in *S. pombe* that allows

TABLE 5

Alterations in the $(GT)_{10}$ repeat in *msh2* and *msh6* mutants

Relevant genotype	Alteration			
	Deletions		Insertions	
	−4 bp	−2 bp	+2 bp	+4 bp
msh2	0	9	7	0
msh6	0	9	9	1

Numbers represent the frequencies of deletions (−4 or −2) and insertions (+2 or +4) of nucleotides within the $(GT)_{10}$ repeat. All Ade^- analyzed here by DNA sequencing derived from independent cultures.

measurement of the stability of GT repeats, a frequent type of microsatellites in DNA. We tested GT repeats with a length of 8, 9, or 10 units, which enabled us to detect different types of insertions and deletions (Figure 1). In repair-proficient cells the majority of the detectable changes are insertions of two nucleotides (1 GT unit). In addition, 4-nucleotide insertions occur more frequently than 2 deletions (Table 3). The bias toward insertions can be explained by more frequent slippage of the newly synthesized strand and/or less frequent repair of loops in the newly synthesized strand. Since similar frequencies of insertions and deletions were found in *msh2* and *msh6* mutants (Table 5), it is rather likely that slippage of the template occurs as frequently as slippage of the nascent strand. Thus, loops in the newly synthesized strand quite frequently escape repair in wild-type cells. In wild type, quite low reversion rates were found for the $(GT)_8$ repeat, in contrast to the $(GT)_9$ repeat, which reverted ~160 times more frequently to Ade^+. This bias is likely due to the selection for events restoring the open reading frame of *ade6*$^+$. Thus, 2-bp insertions for example, likely occur with similar frequencies in $(GT)_8$ and $(GT)_9$ repeats, but are only detectable in the latter case.

Control of GT repeat stability by MMR factors: A defect in the mismatch repair genes *msh2*, *msh6*, or *pms1* caused highly increased destabilization of GT repeats (Table 2). The major events when either one of these genes was defective were insertions and deletions of two nucleotides (1 GT unit). Reversions from $(GT)_9$ mainly occurred by 2-bp insertions, while most of the reversions of $(GT)_8$ were 2-bp deletions (Tables 1 and 3). Although the rates were about three to four times higher for $(GT)_9$, +2 and −2 events were equally produced in the $(GT)_{10}$ repeat in *msh2* and *msh6* mutants (Table 5). Thus the increased reversion rates of $(GT)_9$ compared to $(GT)_8$ were rather due to the presence of 1 additional repeat unit, which might cause more frequent strand slippage. In *S. cerevisiae*, instability of GT repeats concomitantly increased with increasing tract sizes, both in wild type and in *msh2* mutant cells (Wierdl *et al.* 1997).

Minor roles of Exo1 and Swi4: Loss of *exo1* function caused only slightly increased instability of GT repeats, in contrast to *msh2*, *msh6*, and *pms1*. However, mutation rates were further increased in *msh2 exo1* and *pms1 exo1* double mutants when compared with respective single mutants (Table 2). Thus, *exo1* might have a function in GT loop repair, which is independent of the MMR pathway. Our data also suggest that the contribution of *exo1* is different in GT repeat stability and in repair of base-base mismatches. In reversion assays of defined point mutations, *exo1* mutants showed an increase of mutation rates that is ~50% of that of *msh2* and *pms1* mutants (Szankasi and Smith 1995; Schär *et al.* 1997; Rudolph *et al.* 1998). In addition, it was shown that *exo1* acts in the *msh2*-dependent pathway for correction of base-base mismatches (Rudolph *et al.* 1998). Epistasis analysis revealed that a defect of *exo1* is synergistic with *msh2* and *pms1* mutations in GT loop repair (Table 2). However, we cannot exclude that *exo1* has an additional minor function in the MMR pathway of loop repair.

A defect in *swi4* caused only weak effects on GT repeat instability. This finding is in sharp contrast to the situation in *S. cerevisiae*: GT repeats are more unstable in *msh3* mutants than in *msh6* mutants, while the high instability observed with *msh2* is achieved only in the *msh3 msh6* double mutant (Johnson *et al.* 1996; Sia *et al.* 1997). These and further studies with *S. cerevisiae* suggest that small loops are redundantly repaired by the heterodimers Msh2-Msh3 and Msh2-Msh6 (Alani 1996; Habraken *et al.* 1996; Iaccarino *et al.* 1996; Marsischky *et al.* 1996). Our analysis about GT repeat stability in *S. pombe* showed that Msh6 has the same important function as Msh2, while Swi4 has only a minor and probably different function. The reversion rate of $(GT)_8$ was not elevated in *msh6 swi4* as compared to *msh6*. Moreover, the mutation rate of $(GT)_9$ and presumably also of $(GT)_{10}$ was rather reduced in *swi4* mutants. This *swi4*-dependent reduction was also observed in mutants additionally defective in *msh2* and/or *msh6* as well as in *swi4 exo1* double mutants. Determination of the mutational spectra of reversions in a *swi4* background revealed that the main effects were decreased rates of 2-bp insertions and increased rates of 2-bp deletions.

Msh6 is as important in mismatch repair as Msh2: Our recent study revealed that *swi4* was not significantly different from wild type in repair of base-base mismatches and of one-nucleotide loops, while *msh6* showed essentially the same increased mutation rates as *msh2* mutants (Tornier *et al.* 2001, accompanying article). Although we found some minor effects on GT repeat stability, the role of Swi4 in repair of small loops is clearly different from that of Msh3 of *S. cerevisiae*. Another difference is the contribution of Msh6, which is as important as Msh2 in repair of two unpaired nucleotides (this study). In this respect, it would be interesting to study the function of Swi4 and Msh6 in repair of larger loops. However, the results of the GT repeat assays already indicate that repair of four unpaired nucleotides is reduced in *msh6* mutants to an extent similar to that in *msh2* mutants, but is only marginally affected in *swi4* mutants. Consistent with our genetic data is a previous study that showed that purified human MutSα is able to bind to loops with up to eight unpaired nucleotides (Genschel *et al.* 1998). In addition, MutSα is much more abundant than MutSβ (Drummond *et al.* 1997). These findings suggest that MutSα is the major MMR-dependent recognition complex for mismatches and loops in human cells. We propose that MutSβ in *S. pombe* and in human rather serves as a backup system and that it might have its main function in other cellular processes like recombination.

Microsatellites in *S. pombe*: A number of genetic human disorders are caused by or associated with microsatellite instability (McMurray 1995; Debrauwere *et*

al. 1997; Sidransky 1997). Repeat length variation in genes results in loss of function and in many cases results in diseases. However, the human genome mainly consists of noncoding DNA with a frequent occurrence of microsatellites. Consequently, changes in such repetitive regions do not result in loss of functions. In contrast, the genome of *S. pombe* is quite compact with a high density of genes. Microsatellites in genes represent high risk factors for losing cellular functions by repeat length variations and are even more critical in haploid organisms like *S. pombe*. We searched the *S. pombe* genome released by the *S. pombe* Sequencing Project (http://www.sanger.ac.uk/Projects/S_pombe/) for the presence of repetitive DNA with units of one or two nucleotides when ~83% of the genome sequence was available. We found six mononucleotide runs with unit numbers between 12 and 39 and an enormous number of smaller mononucleotide repeats. Among the larger dinucleotide repeats (>10 units), four GT repeats (the largest consists of 18 units), two AG repeats, and three AT repeats are present in the known part of the genome. Neither one of these microsatellites (the smaller mononucleotides were not analyzed) nor two other repetitive regions containing arrays of dinucleotide repeats, disrupted by other nucleotides, are situated in an open reading frame. Thus, none of the identified microsatellites in *S. pombe* seems to be a critical factor for loss of gene functions. During evolution such sequences were likely eliminated by mutational changes in *S. pombe*, while they can be accumulated in a diploid and multicellular organism like us.

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