The Prevention of Repeat-Associated Deletions in *Saccharomyces cerevisiae* by Mismatch Repair Depends on Size and Origin of Deletions

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ABSTRACT

We have investigated the effects of mismatch repair on 1- to 61-bp deletions in the yeast *Saccharomyces cerevisiae*. The deletions are likely to involve unpaired loop intermediates resulting from DNA polymerase slippage. The mutator effects of mutations in the DNA polymerase δ (*POL3*) gene and the recombinational repair *RAD52* gene were studied in combination with mismatch repair defects. The *pol3-t* mutation increased up to 1000-fold the rate of extended (7–61 bp) but not of 1-bp deletions. In a *rad52* null mutant only the 1-bp deletions were increased (12-fold). The mismatch repair mutations *pms1*, *msh2* and *msh3* did not affect 31- and 61-bp deletions in the *pol3-t* but increased the rates of 7- and 1-bp deletions. We propose that loops less than or equal to seven bases generated during replication are subject to mismatch repair by the *PMS1*, *MSH2*, *MSH3* system and that it cannot act on loops ≥31 bases. In contrast to the *pol3-t*, the enhancement of 1-bp deletions in a *rad52* mutant is not altered by a *pms1* mutation. Thus, mismatch repair appears to be specific to errors of DNA synthesis generated during semiconservative replication.

FREQUENTLY represented among mutations are deletions and insertions associated with short DNA repeats. Included among the repeats are multiply reiterated 1- to 4-bp sequences referred to as microsatellites or simple repeats (BECKMAN and WEBER 1992), short homonucleotide runs, and 3- to 10-bp repeats that are separated by unique DNA. Mutations involving such repeats have been associated with several inherited diseases and cancers (see refs. in reviews: KRAWCZAK and COOPER 1991; CASKEY *et al.* 1992; SINDEN and WELLS 1992; THIBODEAU *et al.* 1993; ESHLEMAN and MARKOWITZ 1995). The cause of repeat-associated mutation is generally considered to be due to slippage of the nascent strand in the forward or backward direction along the template during DNA synthesis resulting in an unpaired loop in the template or nascent strand, respectively (STREISINGER *et al.* 1966; also see review by RIPLEY 1990). Support for this mechanism is the dependence of several types of spontaneous and induced repeat-associated deletions on the direction of replication fork movement during semiconservative replication in both prokaryotes (TRINH and SINDEN 1991; VEAUTE and FUCHS 1993; KANG *et al.* 1995) and eukaryotes (TRAN *et al.* 1995). This also implies that there is a strand preference (leading or lagging) in the production of such mutations.

Mismatch repair (MMR) is an important component of genome stability that removes many replication errors including unpaired loops. In this paper a chromosome-based mutation system we developed in yeast (TRAN *et al.* 1995) is used to investigate the limitations on MMR in preventing repeat-associated mutations arising via unpaired loops. We found that yeast MMR is limited by the size of a potential loop and that it cannot repair even small loops occurring during DNA synthesis that are not associated with genome duplication.

Mismatch-recognizing proteins from bacteria (MutS) and eukaryotes (Msh2 and hMsh2) can bind unpaired DNA loops *in vitro* (PARKER and MARINUS 1992; FISHEL *et al.* 1994; ALANI *et al.* 1995), and correction of unpaired loops has been characterized in cell extracts from bacterial and mammalian cells (MUSTER-NASSAL and KOLODNER 1986; LEARN and GRAFSTROM 1989; PARSONS *et al.* 1993; UMAR *et al.* 1994a). *In vivo* correction of unpaired loops has been directly demonstrated in DNAs introduced into bacteria or yeast by transformation or transfection (BISHOP and KOLODNER 1986; DOHET *et al.* 1986; FISHEL *et al.* 1986; KRAMER *et al.* 1989; PARKER and MARINUS 1992). The efficiencies of binding as well as correction differ with the size of loop and between species (see DISCUSSION). Mutations in *Escherichia coli* MMR genes *mutL* and *mutS* and their homologues in yeast, human, and mouse cells result in high rates of deletions and insertions in simple repeats (LEVINSON and GUTMAN 1987; FISHEL *et al.* 1993; LEACH *et al.* 1993; PARSONS *et al.* 1993; STRAND *et al.* 1993; UMAR *et al.* 1994b; BAKER *et al.* 1995; DE WIND *et al.* 1995). In yeast deletions/insertions of one to two dinucleotide units within runs of simple repeats are greatly increased in MMR mutants (STRAND *et al.* 1993). The rate of larger

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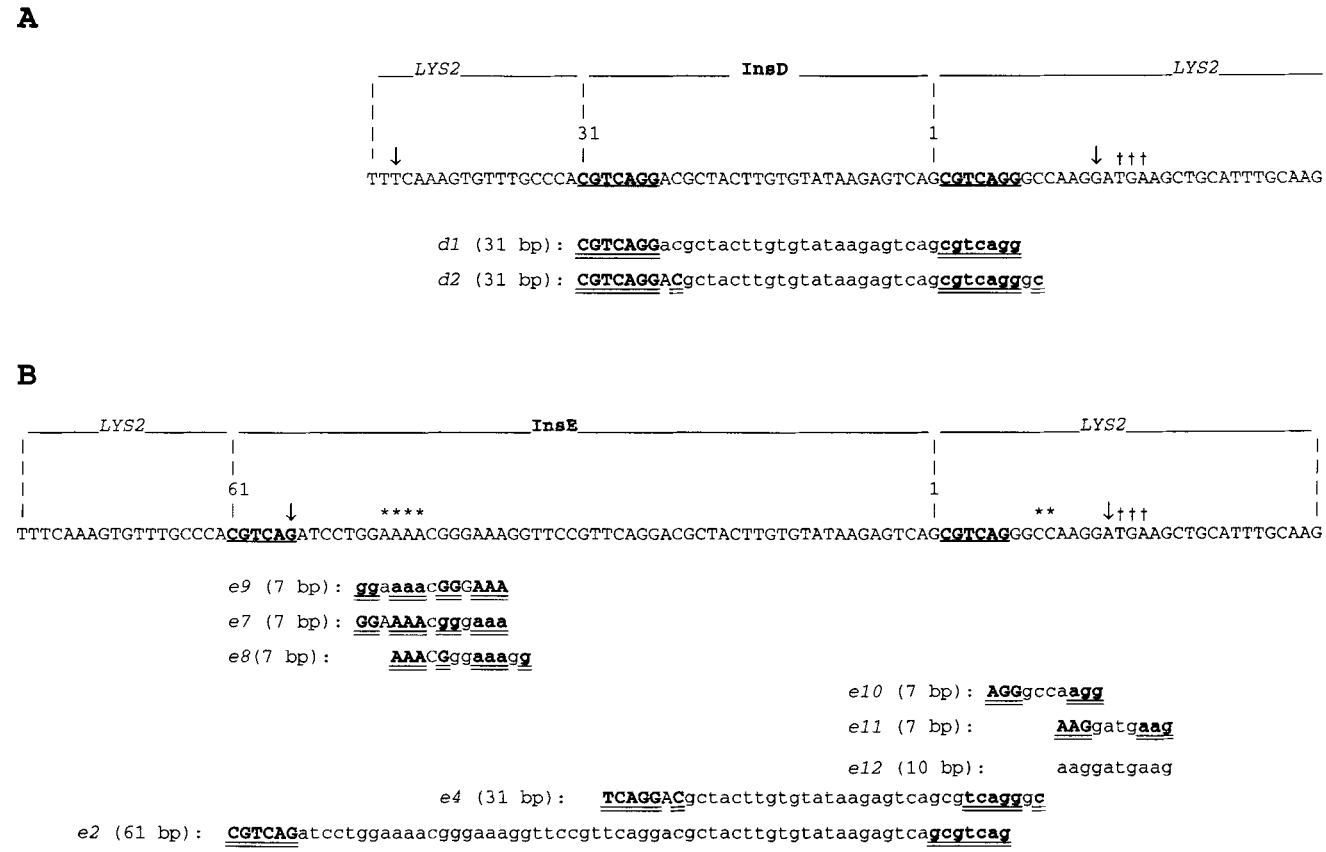

FIGURE 1.—Deletions leading to reversion of the InsD (A) and InsE (B) inserts. The InsD and InsE inserts in the *LYS2* gene and their reversions have been described (GORDENIN *et al.* 1993; TRAN *et al.* 1995). The InsD and InsE sequences are 31- or 61-bp remnants of an original Tn*5* insert, respectively. The inserts are flanked by either 7- or 6-bp short direct repeats (identified by a single underline), one of which belongs to the insert and the other to the *LYS2* gene. Both inserts cause a +1 shift of the *LYS2* reading frame generating a TGA stop codon (marked by † † †) downstream from the insert. Reversions can occur via extended (>1 bp) complete or incomplete deletions that restore the original *LYS2* reading frame. Each deletion, except the rare category e12, involves short (3–7 bp) direct repeats that are either perfect or contain a mismatch. One repeat is removed along with a deleted sequence; the other is retained. DNA sequences that are removed by extended deletions are shown in lowercase beneath the sequences of InsD and InsE. Bases of a repeat remaining after deletion are shown in uppercase. Both repeats (the deleted and the remaining) are indicated by a double underline. Deletions d1, d2, e2, e4, e7, e8, and e9 have the same numbers as previously described in TRAN *et al.* (1995). The rare categories e1 (76-bp deletion), e3 (31-bp deletion), e5 and e6 (16-bp deletions) were not detected in the present experiments, while additional deletion categories e10, e11 and e12 were observed. [Note: the e12 deletion has no repeats at the ends. It was found only once among 66 deletions of the NDL class sequenced in this and previous (TRAN *et al.* 1995) studies.] The other class of reversion events was 1-bp deletions. Two hotspots of 1-bp deletions are indicated by * * * * or **. The limits of the regions over which 1-bp deletions were detected in this and in previous study (TRAN *et al.* 1995) are indicated by (↓).

deletions and insertions associated with such runs is low in wild-type and MMR mutant strains. In *E. coli,* the *mutH, L,* and *S* mutations increase the rate of small (1- to 2-bp) frameshifts in short homonucleotide runs within the *lacI* gene. No increase in rates of extended (6–544 bp) deletions between separated short (3–8 bp) direct repeats has been observed (SCHAAPER and DUNN 1987, 1991). The above results indicate that the known MMR systems in *E. coli* do not act *in vivo* on loops greater than four to five bases. Larger loops may not be subject to repair or, alternatively, they may be acted upon by other repair systems.

We previously have examined the repair of small and large loops using insertion mutations in the *LYS2* gene of the yeast *Saccharomyces cerevisiae* that enable selection of revertants resulting from 1- to 61-bp deletions. Several observations support the idea that deletions occurred via unpaired loops generated by replication slippage (TRAN *et al.* 1995). All extended (>1 bp) deletions involved 3- to 7-bp repeats sequences flanking the deleted region (Figure 1). The spectrum of extended deletions depended on the orientation relative to a replication origin, supporting a role for replication slippage. Furthermore, a temperature-sensitive mutation (*pol3-t*) in the DNA polymerase δ, which is required for semiconservative replication, exhibited as much as a 1000-fold increase in the rate of extended deletions. Most of the 1-bp deletions occurred in 2- to 4-bp homonucleotide runs suggesting a role for replication slippage. However, their rate was not increased in the *pol3-t* mu-

TABLE 1

Effects of *pol3-t*, mismatch repair, and *rad52* mutations on reversion of *LYS2::InsE* via extended and 1-bp deletions

Strains	Reversion rate × 10^{8a}				
	Total[b]	−61 bp[c] (e2)	−31 bp[c] (e4)	−7 bp[d] (e8)	−1 bp[d]
Wild type	0.4 (0.3–0.5)	0.02 (1)	ND[e]	ND	0.4 (20)
pms1	4.2 (3.1–4.5)	ND	ND	ND	4.2 (25)
msh2	1.7 (1.3–2.8)	ND	ND	ND	1.7 (5/20)
msh3	1.2 (0.96–1.6)	ND	ND	ND	1.2 (6/18)
pms1 msh2	1.5 (1.0–4.5)	ND	ND	ND	1.5 (5/20)
pms1 msh3	4.0 (2.1–8.2)	ND	ND	ND	4.0 (6/20)
msh2 msh3	2.9 (2.3–5.0)	ND	ND	ND	2.9 (7/20)
pms1 msh2 msh3	3.8 (2.1–6.5)	ND	ND	ND	3.8 (6/20)
pol3-t	30 (21–49)	20 (88)	9 (40)	0.9 (4)	0.45 (2)
pol3-t pms1	63 (36–128)	28 (84)	6.7 (20)	6.3 (18)	22 (62)
pol3-t msh2	55 (38–100)	14 (51)	3.3 (12)	4.7 (15)	33 (104)
pol3-t msh3	47 (27–89)	30 (103)	6.0 (21)	4.4 (15)	6.8 (23)
rad52	4.9 (3.9–9.1)	ND	ND	ND	4.9 (45)
rad52 pms1	13 (7.4–18)	ND	ND	ND	13 (35)

[a] The subdivision of the total rate into rates for various categories of mutations (Figure 1) is based on PCR and sequencing as described in MATERIALS AND METHODS. The only or the predominant category of extended deletions is given in each column. Sequencing of PCR products from revertants determined that 61-bp deletions belonged to the e2 category (19 deletions were sequenced) and 31-bp deletions belonged to the e4 category (20 deletions sequenced). Based on sequencing, most of 7-bp deletions (45 of 52) belonged to the category e8. Other categories of 7-bp deletions were uncommon: one e7 deletion in the *pol3-tmsh3* strain, one e9 deletion in the *pol3-t* strain, two e10 deletions in the *pol3-t msh2* and one e10 deletion in the *pol3-tmsh3* strain, one e11 deletion in the *pol3-tmsh2* and one e11 deletion in the *pol3-t msh3* strain. One 10bp deletion (category e12) occurred in the *pol3-tmsh3* strain. The e12 deletion was not included in the calculation of deletion rates. Among the NDL revertants (no detectable loss; see MATERIALS AND METHODS and TRAN *et al.* 1995) both 7- and 1-bp deletions were found. All revertants from the *pol3t* and *pol3-tmsh3* strains identified as NDL by PCR were then sequenced to distinguish between 7- and 1-bp deletions. Only 85–95% of revertants in the *pol3-t pms1* and *pol3-t msh2* strains were sequenced. For these two genotypes the rate of all NDL events was calculated based on PCR data and then proportionally subdivided according to the ratio of 7- and 1-bp deletions in the sequenced group of revertants.

[b] 95% confidence intervals for total rates are given in parentheses.

[c] Number of revertants where the size of deletion was identified by PCR and then, for a sample of PCR products, confirmed by sequencing (see MATERIALS AND METHODS) is given in parentheses.

[d] Presented in parentheses are the number of revertants characterized by sequencing (the only number in parenthesis or the numerator) and by PCR (the denominator). Two 2-bp insertion mutations that occurred in the *pol3-tpms1* and in *pol3-t msh2* strains are also included in this category.

[e] ND, not detected.

tant. To explain this we proposed that one nucleotide replication slippage is also enhanced in the *pol3-t* mutant, but the resulting single base loops are subsequently removed by the MMR system, while bigger loops are not a subject to MMR.

In the current study we examine the potential for replication slippage mutations in the *pol3-t* strain and the role of the MMR system (*PMS1, MSH2* and *MSH3*) genes in correcting such slippage mutations. The MMR system prevents the mutator effect of *pol3-t* for 1-bp deletions and reduces the incidence of 7-bp deletions. However, MMR did not affect the two other categories of extended deletions (31 and 61 bp) that could be identified in this study, indicating that the limit of MMR for unpaired loops in yeast is between 7 and 31 bp.

Another source of deletion mutations could be polymerase slippage during DNA repair synthesis. This may account for the frameshift mutator phenotype of the *rad18* (DATTA and JINKS-ROBERTSON 1995) and *rad52* (DATTA and JINKS-ROBERTSON 1995; TRAN *et al.* 1995) yeast mutants. The 1-bp deletions that appear in the *rad52* mutator background were not subject to MMR, whereas in a *pol3-t* strain MMR greatly reduced the potential for these mutations. Thus, MMR of unpaired loops is limited not only by the size of loop but also by the origin of the loop.

MATERIALS AND METHODS

Yeast strains: All strains were isogenic and were derived from *pol3-t*-DM (GORDENIN *et al.* 1992). All the strains were *MATα leu2-2 trp1-Δ1 ura3-x* with various combinations of mutant or wild-type *POL3, PMS1, MSH2, MSH3* genes and the inserts InsD, InsE. The Pol$^+$ and *pol3-t* strains with inserts InsD (31 bp) and InsE (61 bp) have been described (GORDENIN *et al.* 1993; TRAN *et al.* 1995). These inserts (Figure 1) were derived from imprecise excision of Tn*5-13::URA3* in the chromosomal *LYS2* gene. InsD and InsE are flanked by two short direct repeats (7 and 6 bp, respectively). In each case one repeat belongs to the insert and the other belongs to *LYS2*. For the strain *pol3-t* DM *lys2::TRP1 pms1::LEU2* the *Bgl*II-*Bam*HI 5′-region of the *LYS2* gene was replaced by the 850-bp *Bgl*II fragment containing the *TRP1* gene.

Disruption and deletion mutations in the MMR genes: Fragments of the following plasmids were used: *MluI-ApaI* (*pms1::LEU2*) from pAM58 to replace the *PMS1* gene (MORRISON *et al.* 1993) (almost the entire reading frame of the *PMS1* gene was replaced by the *LEU2* gene), *SpeI* from pmsh2::Tn*10*LUK (REENAN and KOLODNER 1992) to disrupt the *MSH2* at 183 nt after ATG start codon, *Bam*HI-*Eco*RI from pmsh3::*TRP1* (SELVA *et al.* 1995) to replace the *MSH3* gene with *TRP1*. Disruptions or replacements were verified by PCR using the following pairs of primers flanking the disrupted regions: for *PMS1*-pms1-1 (5′-CTG GAC AAG TTA TCA CCG ACT-3′) and pms1-2 (5′-CCA GCA CCA CAA GTT CAT CAA TG 3′), for *MSH2*-msh2-1 (5′ATT CTC TGA TGT ATC AGA GG 3′) and msh2-2 (5′-GCT CAT TAA CTT GCT CAA TG-3′), for *MSH3*-msh3-1 (5′-ATT AGA GTA GGC TAC AAG TAC -3′) and msh3-2 (5′-AAC ATA CGT ACC ATC CGC ATC-3′). Amplification was for 35 cycles (30 sec at 96°, 1 min at 55°, and 6 min at 68°) using Taq polymerase (Perkin Elmer Cetus).

Other genetic and molecular procedures: Genetic and molecular procedures were previously described (GORDENIN *et al.* 1991, 1992, 1993). Precise or in-frame imprecise deletions of the insertion mutations (*lys2::InsD* and *lys2::InsE*) were selected as Lys$^+$ revertants. Reversion rates were determined by fluctuation analysis using 11–24 independent cultures as previously described (GORDENIN *et al.* 1992). Strains were grown at 25°.

Genomic DNAs from independent Lys$^+$ revertants were classified using PCR method as described (TRAN *et al.* 1995). Three classes of PCR products were identified: loss of 61 bp, loss of 31 bp and no detectable loss (NDL). Sequencing (described in TRAN *et al.* 1995) of 126 PCR products from the first two classes confirmed the PCR classifications (a rare category of 16-bp deletions among InsE revertants was not observed in the present experiments). Most of the PCR products from the NDL class were sequenced to distinguish the 1- and 7-bp deletions. The contribution of each deletion type (−61, −31, −7 and -1 bp) to the total reversion rate was determined based on sequencing and PCR results.

RESULTS

Effects of DNA polymerase δ and MMR defects on rates of extended and 1-bp deletions: The impact of DNA polymerase and MMR defects on the appearance of deletions was examined with our previously described system based on reversion of *lys2* mutations. The *lys2* mutants were the result of 31- (InsD) or 61-bp (InsE) insertions resulting in +1 frameshifts. Revertants arise by 1-bp deletions or extended (7–61 bp) deletions (Figure 1) (TRAN *et al.* 1995 and this study). We previously reported that while the rate of extended deletions was increased several hundred-fold in a *pol3-t* mutant as compared to a Pol$^+$ strain, there was no change in the rate of 1-bp deletions. To determine if MMR played a role in short and/or extended deletions, we determined the incidence and spectrum of InsE and InsD reversions in Pol$^+$ and *pol3-t* strains with null mutations in at least one of the mismatch repair genes *PMS1*, *MSH2* or *MSH3* (Tables 1 and 2).

Single or multiple MMR mutations increased the overall reversion rate of the *lys2::InsE* mutation in a Pol$^+$ background between three- to 10-fold (Table 1). The reversion rates for individual *msh2* and *msh3* mutants were comparable. This differs from results for forward mutations to canavanine resistance and for reversions of the frameshift mutations *hom3-10* and *lys2-Bgl*, where *msh3* was clearly a weaker mutator than *msh2* (ALANI *et al.* 1994; MARSISCHKI *et al.* 1996). Similar to those results, there was no statistically significant difference between the mutation rates for strains with multiple MMR defects and the single mutant with the highest rate, supporting the hypothesis that the MMR genes act in a concerted fashion in the repair of a mismatch. (Note: Because of data variations we cannot exclude additivity of rates in the *msh2 msh3* double mutant.) Based on PCR analysis of 18–25 Lys$^+$ revertants and subsequent sequencing of PCR products from five to 25 revertants from each strain, all Lys$^+$ reversions of the InsE mutation in the Pol$^+$ strains were due to 1-bp deletions. Nearly all reversions occurred at homonucleotide runs of two or more bases (see below). Since there were essentially no extended deletions in the MMR proficient (Mmr$^+$) or in the MMR deficient (Mmr$^-$) strains, the low rate of extended deletions in the Pol$^+$ strain cannot be attributed to MMR.

As previously reported (TRAN *et al.* 1995) the rates of reversion of both the InsE and InsD mutations are greatly increased (nearly 100-fold) in a *pol3-t* mutant (Tables 1 and 2). The enhancement was not due to increased 1-bp deletions but was due only to extended deletions. However, in a Mmr$^-$ background the *pol3-t* mutation appeared to be a strong mutator for 1-bp deletions. There was a five- to 19-fold increase in the rates of these mutations over the rates in a Pol$^+$ Mmr$^-$ strain. Of the three categories of extended deletions that can be identified in this system, only the 7-bp deletions were increased (five- to sevenfold) in the *pol3-t* Mmr$^-$-deficient strains. Thus, MMR appears to be able to act on loops of at least seven bases, but not on loops that are 31 bases or larger.

TABLE 2

Effects of *pol3-t* and mismatch repair mutations on reversion of *LYS2::InsD* via extended and 1-bp deletions

Strain	Reversion rate × 10^{8a} Total[b]	−31 bp[c] (d1, d2)	−1 bp[c]
Wild type	0.5 (0.2–0.8)	0.19 (30)	0.31 (50)
pol3-t	53 (13–98)	53 (60)	ND[e]
pol3-t pms1	128 (64–220)	116 (70)	12 (7)
pol3-t msh2	108 (65–272)	72 (64)	24 (32)

[a] Subdivision is based on PCR and sequencng as described in MATERIALS AND METHODS.

[b] 95% confidence intervals for total rates are given in parentheses.

[c] Number of revertants characterized by PCR or by sequencing is given in parentheses. Sequencing data for extended deletions are summarized in Table 3.

[e] ND, not detected.

TABLE 3

Effect of the *pms1* and *msh2* mutations on the appearance of extended deletions (31 bp) between identical repeats and between repeats containing a mismatch

Strains	Repeats at the breakpoints of InsD deletions		
	Total sequenced[a]	Identical repeats[b]	Repeats with mismatch[c]
Wild type	18	18	0
pol3-t	23	11	12
pol3-t pms1	22	11	11
pol3-t msh2	25	20	5

[a] Data for wild-type and *pol3-t* strains are taken from (TRAN *et al.* 1995). Revertants in the wild-type strain are summarized from results obtained at three different temperatures (20°, 25° and 30°). Revertants in the other three strains were obtained at 25°.

[b] Deletions of the d1 type (see Figure 1).

[c] Deletions of the d2 type (see Figure 1).

Previously, we showed that many of the extended deletions in the *pol3-t* strains occurred between short direct repeats containing a mismatch (TRAN *et al.* 1995). The MMR system was proposed to play an important role in one of the models accounting for these deletions. Similar to our previous study, the rate of extended (>1 bp) deletions was elevated >100-fold in *pol3-t* strains. Sequencing of 20 revertants of the InsE that occurred *via* 31-bp deletions in Mmr^+ and Mmr^- strains (five revertants for each strain) identified them all as e4 type of deletion that involves mismatched repeats. For extended deletions leading to reversion of the InsD mutation in the *pol3-t* strain, about half the revertants involved mismatched direct repeats (Table 3). Since there was no increase of the mismatched repeats category in the *pol3-t pms1* or *pol3-t msh2* strains compared to the *pol3-t* strain, the MMR system does not appear to affect the interaction between mismatched direct repeats.

Effects of the *rad52* and *pms1* mutations on 1-bp deletions: Loss of *RAD52* function results in a mutator phenotype for small frameshift mutations (1-bp deletions in our system) (DATTA and JINKS-ROBERTSON 1995; TRAN *et al.* 1995). We have examined whether MMR influences this mutator phenotype.

The *pms1* mutation greatly enhances the mutator effect of DNA polymerase δ and ϵ mutants that are deficient in proofreading exonuclease activity (MORRISON *et al.* 1993; MORRISON and SUGINO 1994). We also found this type of synergistic interaction for the appearance of 1-bp deletions in the *pol3-t* Mmr^- double mutants (Tables 1 and 2). We investigated whether the rate of 1-bp deletions would be similarly increased if the *pms1* and *rad52* mutations were combined (Table 1). The mutation rates in a *pms1 rad52* strain were increased relative to the rates in the single mutants. However, the increase was additive rather than synergistic (to account for statistical variation see 95% confidence intervals), suggesting that these mutator activities are independent.

Distribution of 1-bp deletions: If slippage is the predominant mechanism of 1-bp deletions, then most of them should occur in runs of at least two identical nucleotides. This was confirmed through sequencing of nearly 300 InsE revertants (summarized in Table 4; complete sequencing data are available on request). Consistent with our previous report (TRAN *et al.* 1995), all the 1-bp deletions were confined to changes within or near the InsE region. Regardless of the genetic background, over 84% of the 1-bp deletions occurred in homonucleotide runs. Greater than 27% of the deletions were located in a single run AAAA (see Figure 1), even though this run represented only 6% (4/71) of the total nucleotides in the region where revertants have been obtained. An additional hotspot located at a CC run accounted for approximately one-quarter of the events in the *rad52* mutant. This hotspot was also present in the double mutant *rad52 pms1*, which further supports the view that the *pms1* and *rad52* mutator activities are independent. The distributions of 1-bp deletions among the rest of the strains were comparable, regardless of polymerase mutation or MMR defect. The 1-bp deletion hotspot at the AAAA run was not affected by replication direction [*pol3 pms1* (p93) and *pol3 pms1* (p95) in Table 4], although replication direction affects the production of extended deletions (TRAN *et al.* 1995).

DISCUSSION

Using our reversion system based on InsE and InsD mutations, we investigated the role of replication slippage and MMR in the production and distribution of reversion mutations. Since several categories of repeat-associated deletions are not affected by MMR, our approach will help to identify and better understand factors other than MMR that can influence genetic instability in yeast and in higher eukaryotes.

The following observations (TRAN *et al.* 1995 and this study) support the view that reversion events occur primarily through DNA polymerase slippage during replication: (1) all extended deletions are associated with short, direct repeats, (2) the deletion spectrum is orientation dependent (relative to the replication origin), (3) most 1-bp deletions occur within homonucleotide runs, and (4) deletion rates are increased by the *pol3-t* mutation. We, therefore, assume in the following discussion that the main source of deletions associated with short repeats and homonucleotide runs is replication and that they result from DNA polymerase slippage between repeats. The template strand contains the initial sequence in an unpaired loop and the newly synthesized slipped strand lacks the information contained in the loop.

TABLE 4

Association of 1-bp deletion revertants of InsE with homonucleotide runs

Strains	No. of 1 bp deletions located[a] in various positions					
	4 bp run (1 run: AAAA)	3 bp runs (3 runs)	2 bp runs (11 runs)	CC hotspot	Not in runs	Total
Wild type[b]	8 (40)	3 (15)	7 (35)	0 (0)	2 (10)	20
pms1	17 (68)	4 (16)	1 (4)	0 (0)	3 (12)	25
rad52	12 (27)	9 (20)	6 (13)	11 (24)	7 (16)	45
rad52 pms1	17 (49)	2 (6)	2 (6)	10 (29)	4 (12)	35
pol3-t	9 (75)	1 (8)	2 (17)	0 (0)	0 (0)	12
pol3-t pms1	34 (55)	10 (16)	11 (18)	5 (8)	2 (3)	62
pol3-t msh2	48 (45)	22 (21)	23 (22)	4 (4)	7 (7)	104
pol3-t msh3	12 (52)	2 (9)	3 (13)	3 (13)	3 (13)	23
pol3-t pms1 (p93)[c]	16 (67)	4 (17)	2 (8)	1 (4)	1 (4)	24
pol3-t pms1 (p95)[c]	22 (74)	4 (13)	1 (3)	0 (0)	3 (10)	30

[a] The locations of the AAAA and CC hotspots are indicated on Figure 1 as **** and **, respectively. Other 2 and 3 bp runs are located in various positions in the 71-bp region where 1-bp deletions can lead to reversion. Complete information about localization of mutation is available upon request. Only two 2-bp insertion were detected among revertants. Values in parentheses are percentages.

[b] Summarized data for mutations obtained at 20°, 25° and 30° (TRAN *et al.* 1995). Mutations in all other strains were obtained at 25°.

[c] Reversion of the *LYS2::InsE* mutation which is present in orientation A in plasmid p93 and the opposite orientation B in plasmid p95 (TRAN *et al.* 1995).

Repair of loops during replication: We have found that one base loops are efficiently repaired by the MMR system. The rates of 1-bp deletions are the same low level in Pol^+ and *pol3-t* strains that are Mmr^+. In a Mmr^- background the *pol3-t* causes up to a 20-fold increase in the 1-bp deletion rate (see Tables 1 and 2). We conclude that the *pol3-t* strains are highly mutation prone for 1-bp deletions but MMR efficiently protects against these mutations. The low background level of 1-bp deletions in the Mmr^+ background may be due in part to incomplete MMR and/or they may arise in MMR-independent pathways (see discussion about *rad52* and *pms1* interactions). The lower rate of 1-bp deletions in the *pol3-t msh3* strain as compared to the *pol3-t msh2* and *pol3-t pms1* strains is consistent with the idea that the corresponding genes may have different roles in MMR (NEW *et al.* 1993; STRAND *et al.* 1995; MARSISCHKY *et al.* 1996). The efficiency of repair for 1-bp loops appears comparable over the range of 2-, 3- and 4-bp homonucleotide runs (Table 4).

The *pol3-t* allele results in up to a 1000-fold increase in extended 7-, 31- and 61-bp deletions (Tables 1 and 2). Of these mutations only the 7-bp deletions are subject to MMR identified with the *PMS1, MSH2* and *MSH3* genes, although repair is less efficient than for 1-bp deletions. The MMR system removes all potential 1-bp deletions that could result from the defective polymerase δ, while 15–20% of the 7-bp deletions are not removed. Based on these results, we conclude there is a capacity in yeast for repair of unpaired loops that decreases with increase in loop size. The limit in the loop size that is a subject to MMR appears to be between seven and 31 bases.

MMR in yeast has been proposed to act on meiotic heteroduplexes containing large unpaired loops since various deletion mutations showed mostly conversion rather than postmeiotic segregation (PMS) (FOGEL *et al.* 1981; WHITE *et al.* 1988). Only one deletion (*ade8-18*) showed an abnormally high frequency of PMS and this was assigned to sequence specificity of correction in meiotic heteroduplex. An alternative explanation is that conversion of deletions with low PMS occurs *via* gap repair without formation of a loop heteroduplex. Based on the strong increase in PMS of four base insertions in a *msh2* mutant, MMR can repair four base loops in meiotic heteroduplexes (REENAN and KOLODNER 1992). There was no systematic examination of the MMR role for meiotic conversion of larger deletions.

Previous studies with various *in vitro* and *in vivo* bacterial and yeast systems have not directly addressed the capability of MMR to act on loops generated during replication. The MutS protein of *Salmonella* exhibits binding only to loops less than five bases (PARKER and MARINUS 1992). The Msh2 homologues of the bacterial MutS protein in yeast and in humans are capable of most efficient binding *in vitro* to unpaired loops up to 14 bases; the efficiency of binding decreases as the loop size is decreased to one base (FISHEL *et al.* 1994; ALANI *et al.* 1995). Recent results (DRUMMOND *et al.* 1995; PALOMBO *et al.* 1995; MARSISCHKI *et al.* 1996) indicate that eukaryotic Msh2 acts as a complex with other proteins (Msh3 or Msh6 in yeast or GTBP in human cells). Therefore binding of Msh2 alone to loops may not be relevant to *in vivo* MMR. Efficient MMR of base-base mismatches and unpaired loops up to 16 bases was directly demonstrated in human cell extracts (UMAR *et al.*

1994a). In *E. coli* one- to three-base loops in transforming DNA are efficiently repaired while there is no repair of loops that are five and more bases (PARKER and MARINUS 1992; CARRAWAY and MARINUS 1993). Yeast can repair much larger loops, eight and 12 bases in transforming DNA and four and seven base loops in cell extracts (correction of smaller loops was not examined in these experiments) (BISHOP and KOLODNER 1986; MUSTER-NASSAL and KOLODNER 1986). A 38-base loop in transforming DNA escaped MMR in yeast, although one-base loops and base-base mismatches in the same experiments were subject to efficient *PMS1*-dependent repair (KRAMER *et al.* 1989), which is consistent with results in the present study.

All of the studies cited above involved the use of static (*i.e.,* preformed) loops and did not address MMR of unpaired loops that might occur during replication where MMR is known to prevent mutations. We have specifically examined the impact of MMR in protecting the genome from various kinds of mutations that are proposed to arise *in vivo* during replication as a result of slippage and have shown that it can prevent deletions of 1 and 7 bp. There are several explanations for the reduction in efficiency of MMR on 7- as compared to 1-bp deletions and the absence of an effect for deletions ≥31 bp. Possibly there is reduced processing by proteins involved with MMR of unpaired DNA loops with increased loop size. Another possibility is that there is reduced strand preference, *i.e.,* there becomes less strand discrimination for MMR as the loop is increased. This latter explanation, however, is not consistent with the 31- and 61-bp deletion results. According to this hypothesis, a twofold increase in deletions would be expected in a MMR mutant, since half of the unpaired loops would be repaired to wild-type sequence and half of the loops would be excised resulting in a deletion. However, except for InsD reversion, there were at most only small increases of 31- or 61-bp deletions in *pol3-t* MMR deficient as compared to repair proficient strains (Tables 1 and 2). Regardless, the present results suggest that the MMR pathway involving *PMS1, MSH2* and *MSH3* cannot correct unpaired loops that result in 31- or 61-bp deletions in our system. Our results do not exclude the possibility of a low level of *PMS1*-, *MSH2*- and *MSH3*-associated MMR or of another type of MMR that acts on large loops and is saturated by a large increase in slippage events in the *pol3-t* background. Based on the dramatic increase in extended deletions caused by the *pol3-t* mutation, we conclude that in Pol^+ strains the rarity of such deletions is due to accurate replication and not to MMR.

Both in yeast (GORDENIN *et al.* 1992, 1993; VON BORSTEL *et al.* 1993; TRAN *et al.* 1995) and in *E. coli* (MO *et al.* 1991), DNA polymerase mutations can enhance rates of deletions associated with short repeats. The largest increase in *pol3-t* strains was in the extended deletion category (TRAN *et al.* 1995 and this study). These observations may be relevant to the mutation basis of some diseases. Polymorphism of DNA polymerase δ alleles has been found in human cell lines prone to simple repeat instability and to colon cancer (DA COSTA *et al.* 1995). Altered DNA polymerase molecules may be a source of mutations that could lead to some cancers or inherited diseases. The impact would be even greater if the mutations are not subject to MMR, as for the case of extended deletions in the present study.

Unpaired DNA loops generated in a *rad52* mutator strain are not subject to *PMS1*-associated MMR: Based on mutation rates (Table 1) and the distribution of mutations (Table 4), *pms1* and the *rad52* mutator effects for 1-bp deletions are additive. The observed rate of 1-bp deletions in the double *rad52 pms1* mutant is comparable to the expected rate for additive interaction. There was only 1.4-fold difference between the observed and expected rates, which is a good agreement considering 95% confidence intervals. MMR is inefficient at preventing 1-bp deletions that occur due to the *rad52* defect compared to MMR prevention of 1-bp deletions occurring in the DNA polymerase δ mutant (*pol3-t*). In the *pol3-t* strain the MMR appears capable of preventing all 1-bp deletions that could occur due to the DNA polymerase mutation [*pol3-t* is not a mutator for 1-bp deletions in the MMR^+ background (Table 1)]. In contrast the *rad52* is a mutator for 1-bp deletions in the MMR^+ strain. If MMR would be capable of preventing mutations resulting from the *rad52* defect as efficiently as it acts in the *pol3-t* background, the rate of 1-bp deletions in the *rad52 pms1* double mutant would be expected to be much higher than in the *pol3-t pms1* double mutant. The observed relation between the mutation rates is opposite to this expectation (Table 1). Synergism would be expected if mutator activity of the *rad52* for 1-bp deletions was directly associated with semiconservative replication. For example, we have demonstrated a genetic interaction between the recombinational repair system and the DNA replication machinery based in part on the observation that *rad52* and the *rad50* mutations reduce the high rate of extended deletions in a *pol3-t* mutant (GORDENIN *et al.* 1992; TRAN *et al.* 1995). Synergistic interactions would be also expected if small loops occurred in the course of error-prone DNA repair synthesis in the *rad52* mutant and were subsequently corrected by *PMS1* associated MMR. A similar scenario was proposed for the synergistic interaction between *mutH, L, S* MMR mutants and the SOS mutator system in *E. coli* (CAILLET-FAUQUET *et al.* 1984; CAILLET-FAUQUET and MAENHAUT-MICHEL 1988).

Possibly the lack of a *pms1* effect on 1-bp deletions generated in the *rad52* mutator strain is due to the inability of MMR to correct a unique class of mismatches (or mismatches in certain contexts). This could account for the hotspot at the CC sequence, observed only in the *rad52* and the *rad52 pms1* mutants but not in the single *pms1* mutant. However, there are

no other significant differences between the *rad52*, the *pms1* and the *rad52 pms1* spectra of 1-bp deletions (Table 4), including the hotspot in the AAAA run. Therefore, sequence context specificity or mismatch specificity do not account for the observed additive interaction between the *rad52* and *pms1*. Recently, the *rad52* mutator effect was demonstrated to be dependent on the *REV3* gene that encodes the putative error-prone DNA polymerase (ROCHE *et al.* 1995). Various error-prone DNA polymerases deficient in proofreading exonuclease activity were recently demonstrated to enhance the level of −1 and +1 frameshifts in short homonucleotide runs (KROUTIL *et al.* 1996). We propose that the additional one-base unpaired DNA loops in the *rad52* strain do not occur during semiconservative DNA replication but instead arise during error-prone repair synthesis and that MMR cannot correct such loops toward the initial sequence. This could be due either to inability of MMR to act on 1-bp loops generated separately from genome duplication or due to the lack of strand preference in repair of such loops.

DNA synthesis associated with DNA repair could be highly mutagenic. This was demonstrated, for example, for the repair of a site-specific double strand break in yeast (STRATHERN *et al.* 1995). The results with the *rad52* mutant have important implications for mutations *via* pathways not subject to MMR. The rate of mutation per nucleotide synthesized must be much higher for the error-prone repair operating in the *rad52* mutant *vs.* the semiconservative replication since the amount of DNA synthesis associated with repair in the *LYS2* region must be much less than the DNA synthesis associated with genome duplication. The results with the *rad52* mutation demonstrate that homonucleotide runs may be susceptible to mutation through MMR independent as well as dependent pathways. As shown for various genes, homonucleotide regions are often hotspots of spontaneous mutability (see review by RIPLEY 1990) and have been found to be associated with commonly occurring cancer (MARKOWITZ *et al.* 1995).

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