Spontaneous Frameshift Mutations in *Saccharomyces cerevisiae*: Accumulation During DNA Replication and Removal by Proofreading and Mismatch Repair Activities

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ABSTRACT

The accumulation of frameshift mutations during DNA synthesis is determined by the rate at which frameshift intermediates are generated during DNA polymerization and the efficiency with which frameshift intermediates are removed by DNA polymerase-associated exonucleolytic proofreading activity and/or the postreplicative mismatch repair machinery. To examine the relative contributions of these factors to replication fidelity in *Saccharomyces cerevisiae*, we determined the reversion rates and spectra of the *lys2ΔBgl* +1 frameshift allele. Wild-type and homozygous mutant diploid strains with all possible combinations of defects in the exonuclease activities of DNA polymerases δ and ε (conferred by the *pol3-01* and *pol2-4* alleles, respectively) and in mismatch repair (deletion of *MSH2*) were analyzed. Although there was no direct correlation between homopolymer run length and frameshift accumulation in the wild-type strain, such a correlation was evident in the triple mutant strain lacking all repair capacity. Furthermore, examination of strains defective in one or two repair activities revealed distinct biases in the removal of the corresponding frameshift intermediates by exonucleolytic proofreading and/or mismatch repair. Finally, these analyses suggest that the mismatch repair machinery may be important for generating some classes of frameshift mutations in yeast.

DNA replication is a highly accurate process with an overall *in vivo* error rate of less than one mutation per 10^9 bases replicated per cell division (Drake *et al.* 1998). The fidelity of DNA synthesis in bacterial and eukaryotic cells is determined at three sequential levels (Kunkel 1992; Schaaper 1993). First, the greatest contribution to replication fidelity is conferred by the inherent base selectivity of the DNA polymerase during nucleotide polymerization. Second, errors made by DNA polymerase can be corrected by a polymerase-associated exonucleolytic proofreading activity that removes terminal nucleotides that are incorrectly base paired with the template. Finally, a postreplicative mismatch repair (MMR) system removes replication errors that escape proofreading. Loss of the MMR system in human cells is associated with tumor formation (reviewed in Buermeyer *et al.* 1999), thus underscoring the importance of efficiently removing DNA replication errors.

In the yeast *Saccharomyces cerevisiae*, three DNA polymerases (Pol α, Pol δ, and Pol ε) are important for the replication of genomic DNA (reviewed in Sugino 1995). Pol α has no associated 3′ → 5′ exonuclease activity and appears to be involved only in primer synthesis. Pols δ and ε each have an associated 3′ → 5′ exonucleolytic proofreading activity, and examination of mutation spectra in strains defective for either the exonuclease activity of Pol δ (*pol3-01* mutants) or Pol ε (*pol2-4* mutants) suggests that one polymerase is leading-strand specific and the other lagging-strand specific (Morrison and Sugino 1994; Shcherbakova and Pavlov 1996; Karthikeyan *et al.* 2000). It also has been demonstrated, however, that DNA replication can be completed in the absence of Pol ε catalytic activity, suggesting that Pol δ is at least capable of replicating both the leading and the lagging strands (Kesti *et al.* 1999). A combination of the *pol2-4* and *pol3-01* alleles is synthetically lethal in haploids and results in a synergistic increase in mutation rate (Morrison and Sugino 1994), suggesting that the exonuclease activities of Pols δ and ε are partially redundant and compete for a common substrate(s). In addition to their roles in proofreading, the exonuclease activities of Pols δ and ε may be involved in MMR, where their associated 3′ → 5′ exonuclease activities have been proposed to act in concert with the 5′ → 3′ exonuclease activity of Exo1p to remove mismatches (Tran *et al.* 1999). Finally, recent work has demonstrated that presence of the *pol3-01* allele is associated with checkpoint-dependent delays in entering and transiting S phase (Datta *et al.* 2000). Surprisingly, the *pol3-01*-associated mutator phenotype is partially depen-

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dent on the S-phase checkpoint, because mutation rates decrease in checkpoint-defective mutants (Datta *et al.* 2000).

The postreplicative MMR system is responsible for correcting polymerization errors that escape proofreading. The best-understood MMR system is the methyl-directed MutHLS system of *Escherichia coli*, where MutL couples MutS mismatch recognition to the downstream processing steps (reviewed in Modrich and Lahue 1996). In yeast, there are six MutS homologs (Msh1p–Msh6p) and four MutL homologs (Pms1p and Mlh1–Mlh3p; reviewed in Harfe and Jinks-Robertson 2000). Msh2p is required for all nuclear mismatch repair, and mismatch recognition is effected by heterodimers of Msh2p with either Msh3p or Msh6p (Johnson *et al.* 1996; Marsischky *et al.* 1996). Although Mlh1p forms heterodimers with Pms1p, Mlh2p, and Mlh3p (Wang *et al.* 1999), most MMR involves the Mlh1p/Pms1p heterodimer. A combination of proofreading-defective Pol δ (*pol3-01* allele) with either a *pms1*Δ or *msh2*Δ allele results in synthetic lethality in haploids, but homozygous diploid strains are viable (Morrison *et al.* 1993; Tran *et al.* 1999). In contrast to the situation with the *pol3-01* allele, haploid strains with a proofreading-defective Pol ε (*pol2-4* allele) and either a *pms1*Δ or *msh2*Δ allele have been isolated (Morrison *et al.* 1993; Tran *et al.* 1997, 1999). Mutation-rate measurements in double-mutant haploid and diploid strains are consistent with the notion that the yeast proofreading and MMR activities act in a series to correct replication errors (Morrison *et al.* 1993).

Replication errors typically can be classified as base substitution events or as insertion/deletion events involving a small number of nucleotides. An insertion/deletion that is not a multiple of 3 bp alters the reading frame of the corresponding gene and almost always eliminates gene function. Because of the highly deleterious nature of frameshift mutations, it is important to understand the mechanisms for generating insertions/deletions during DNA replication as well as the editing functions that prevent fixation of such replication errors. The most widely recognized model of frameshift mutagenesis is the "direct slippage" model, in which DNA polymerase slippage within a tandemly repeated sequence leads to the deletion or addition of one or more repeat units (Streisinger *et al.* 1966). This model is supported by numerous *in vivo* studies, which have demonstrated that tandemly repeated sequences such as mono- and dinucleotide repeats are hotspots for frameshift mutations (see Jinks-Robertson *et al.* 1998 for a review of yeast studies). The other two models of frameshift mutagenesis are based strictly on *in vitro* studies. In the "misincorporation-realignment" model, a misincorporation by DNA polymerase initiates the slippage event, which restores correct base pairing between the 3′ end of the nascent strand and the template (Kunkel and Soni 1988). In the "dNTP-mediated misalignment" model, the misalignment occurs before, rather than after, the incorporation of an incorrect nucleotide, which is specified by the next template base and therefore is not random (Bloom *et al.* 1997). Because the latter two models do not necessarily involve tandemly repeated sequences, they may account for *in vivo* frameshift mutations that involve noniterated sequences.

We and others have used the yeast *lys2*Δ*Bgl* frameshift reversion assay to obtain frameshift mutation rates and spectra in wild-type cells, in cells defective in various MMR components (Marsischky *et al.* 1996; Greene and Jinks-Robertson 1997; Flores-Rozas and Kolodner 1998) or in cells defective in the proofreading activity of Pol δ (Datta *et al.* 2000). In the current study, these analyses are extended by examining *lys2*Δ*Bgl* reversion rates and spectra in wild-type and in completely MMR-defective strains that are deficient in the individual proofreading activities of Pols δ and ε or that are simultaneously deficient in both proofreading activities. These analyses reveal the most frequent frameshift errors made during nucleotide polymerization and provide novel insight into the relative contributions of individual proofreading activities and MMR to the overall stability of the yeast genome.

MATERIALS AND METHODS

Media and growth conditions: Yeast strains were grown in standard media (Sherman 1991) at 30°. Cells were grown nonselectively in YEP (1% yeast extract, 2% Bacto peptone, and 2.5% agar for plates) supplemented with 2% glycerol/2% ethanol (YEPGE) or 2% dextrose (YPD). Selective growth was in synthetic complete (SC) medium containing 2% dextrose and lacking the appropriate amino acid. Ura$^-$ yeast segregants were selected on medium containing 5-fluoroorotic acid (5-FOA; Boeke *et al.* 1987).

Strain constructions: All diploid strains used in this study were derived by mating isogenic derivatives of haploid strains SJR335 and SJR357. A complete list of the haploid strains is given in Table 1. Mutant alleles were introduced into SJR335 and SJR357 by standard transformation procedures (Gietz *et al.* 1992). The *msh2*Δ allele was introduced using *Aat*II/*Xba*I-digested GC1914 (Greene and Jinks-Robertson 1997) and confirmed by PCR. The *pol2-4* and *pol3-01* alleles were introduced by two-step gene replacement using the *URA3*-containing plasmids YIpJB1 (Morrison *et al.* 1991) and YIpAM26 (Morrison *et al.* 1993), respectively. Presence of the *pol2-4* allele was confirmed by allele-specific PCR (Morrison and Sugino 1994); presence of the *pol3-01* allele was confirmed using an associated restriction site polymorphism (Morrison *et al.* 1993). The *leu2-R* allele was introduced into SJR357 derivatives by two-step gene replacement using pJH189 (Lichten *et al.* 1987). Because combinations of most repair-deficient mutant alleles are lethal in haploids, one mutant allele was introduced, followed by a complementing plasmid and then the second mutant allele. Haploids were then mated and diploids that had lost the complementing plasmid were identified. Two independent isolates of each diploid strain, which were derived by mating independent haploid isolates, were used for measurements of reversion rates and determination of mutation spectra.

Strains SJR823 and SJR824 were mated to create the *msh2*Δ/

TABLE 1

Yeast strains

Strain	Description
SJR335	*MAT***a** *ade2-101$_{oc}$ his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R trp1Δ1*
SJR357	*MAT*α *ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl*
SJR480	SJR357 *msh2Δ::hisG*
SJR561	SJR357 *pol2-4*
SJR562	SJR357 *pol3-01*
SJR685	SJR335 *msh2Δ::hisG*
SJR721	SJR335 *pol2-4*
SJR722	SJR335 *pol3-01*
SJR823	SJR357 *msh2Δ::hisG pol2-4* with *MSH2* plasmid pSR578
SJR824	SJR335 *msh2Δ::hisG pol2-4* with *MSH2* plasmid pSR578
SJR882	SJR357 *leu2-R pol3-01*
SJR890	SJR357 *leu2-R pol3-01* with *POL3* plasmid HL1
SJR918	SJR357 *leu2-R pol3-01 msh2Δ::hisG* with *POL3* plasmid HL1
SJR919	SJR335 *pol3-01 msh2Δ::hisG* with *MSH2* plasmid pSR578
SJR920	SJR357 *leu2-R pol2-4 pol3-01* with *POL3* plasmid HL1
SJR921	SJR335 *pol2-4 pol3-01* with *POL3* plasmid HL1
SJR1179	SJR357 *leu2-R pol2-4 pol3-01 msh2Δ::hisG* with *MSH2* plasmid pSR578 and *POL3* plasmid HL1
SJR1180	SJR335 *pol2-4 pol3-01 msh2Δ::hisG* with *MSH2* plasmid pSR578 and *POL3* plasmid HL1

msh2Δ pol2-4/pol2-4 diploid. To construct SJR823, the *msh2Δ* strain SJR480 was first transformed with pSR578 (*MSH2-HIS3-CEN* plasmid; our laboratory collection) and then the *pol2-4* allele was introduced to create SJR823. SJR824 was constructed using the same approach, but starting with *msh2Δ* strain SJR685. SJR824 was transformed with plasmid GC1913 (*MSH2-URA3-CEN* plasmid; obtained from G. F. Crouse) and then was mated to SJR823; the resulting diploids were grown nonselectively to allow loss of pSR578 (His^- segregants). Following loss of pSR578, diploids were plated on 5-FOA to selectively identify loss of plasmid GC1913.

Strains SJR918 and SJR919 were mated to create the *msh2Δ/msh2Δ pol3-01/pol3-01* diploid. To construct SJR918, the *pol3-01* and *leu2-R* alleles were introduced into SJR357, creating SJR882. SJR882 was transformed with the *POL3*-containing plasmid HL1 (*POL3-LEU2-CEN*; Gordenin *et al.* 1992), creating SJR890, and then the *msh2Δ* allele was introduced into SJR890 to give SJR918. SJR919 was constructed by introducing *pol3-01* into a *msh2Δ* strain (SJR685) containing an *MSH2*-complementing plasmid (pSR578). SJR919 was transformed with GC1913 (*MSH2-URA3-CEN* plasmid) prior to mating with SJR918. The resulting diploid was grown nonselectively to allow loss of HL1 and pSR578 (Leu^- and His^- segregants, respectively) and dilutions were then plated on 5-FOA to select loss of plasmid GC1913.

Strains SJR920 and SJR921 were mated to create the *pol2-4/pol2-4 pol3-01/pol3-01* diploid. SJR920 was constructed by introducing the *pol2-4* allele into SJR890, a *pol3-01* strain containing the *POL3*-complementing plasmid HLI. SJR921 was similarly constructed starting with the *pol3-01* strain SJR722 containing plasmid HL1. SJR921 was transformed with pBL304 (*POL3-URA3-CEN*; Gordenin *et al.* 1992) and mated with SJR920. Diploid cells were grown nonselectively to allow loss of HL1 (Leu^- segregants) and dilutions were then plated on 5-FOA to select for loss of the plasmid pBL304.

Strains SJR1179 and SJR1180 were mated to create the *pol2-4/pol2-4 pol3-01/pol3-01 msh2Δ/msh2Δ* triple-mutant diploid. SJR1179 and SJR1180 were constructed by introducing the *pol2-4* allele into *pol3-01 msh2Δ* haploid strains (SJR918 and SJR919, respectively), which had previously been transformed with *MSH2*- and *POL3*-complementing plasmids (pSR578 and HL1, respectively). SJR1179 and SJR1180 were mated, and diploids were transformed with the plasmid pBL304 (*POL3-URA3-CEN*). The diploids were grown nonselectively to allow loss of the plasmids pSR578 and HL1 (His^- and Leu^- segregants, respectively), and dilutions were then plated on 5-FOA to select for loss of plasmid pBL304.

Reversion rates and spectra: For rate determinations, 2-day-old colonies were taken from YEPD plates, inoculated into 5 ml YEPGE liquid medium, and grown for 2 days on a roller drum. Cells were harvested by centrifugation, washed once with sterile H_2O, and resuspended in 1 ml of H_2O. Aliquots (100 μl) of appropriate dilutions were plated on SC-Lys to select Lys^+ revertants and on YEPD to determine viable cell numbers. Lys^+ colonies were counted on day 3 after selective plating. Because of slow growth, the *pol2-4 pol3-01 msh2Δ* triple mutant was grown 3 days in YEPGE and Lys^+ revertants were counted on day 5 after selective plating. Reversion rates were determined by the method of the median (Lea and Coulson 1949), using data from 10 to 20 cultures of each strain. The 95% confidence intervals for the rates were calculated as described by Dixon and Massey (1969).

To isolate independent Lys^+ revertants for DNA sequence analysis, 1-ml YEPGE cultures were grown as described above and a single aliquot was plated on SC-Lys. One revertant from each culture was purified for subsequent molecular analysis. Standard dideoxy DNA sequencing of revertants was performed as described by Greene and Jinks-Robertson (1997). Pairwise comparisons of mutation spectra were done using an algorithm developed by Adams and Skopek (see Cariello *et al.* 1994). All spectral comparisons yielded highly significant *P* values.

RESULTS

The *lys2ΔBgl* allele is the result of a GATC insertion into the *Bgl*II site in the N-terminal portion of *LYS2* (Greene and Jinks-Robertson 1997), and reversion of this allele was used to assess frameshift mutagenesis. The *lys2ΔBgl* allele reverts by compensatory $3N - 1$

frameshift events, which are constrained by stop codons in alternative reading frames to occur within an ~150-bp "reversion window" surrounding the *lys2ΔBgl* mutation. The reversion window contains several mononucleotide runs as well as extensive stretches of nonrepetitive sequence, thus allowing the identification of a wide variety of frameshift mutations. To assess the relative roles of proofreading and MMR in replication fidelity, the yeast MMR machinery was inactivated by deletion of *MSH2* and the proofreading activities of Pols δ and ε were inactivated using appropriate exonuclease-deficient alleles (*pol3-01* and *pol2-4*, respectively). Diploid strains were used in all experiments because of the documented synthetic lethality between *pol3-01* and *pms1*Δ or *msh2*Δ alleles (Morrison *et al.* 1993; Tran *et al.* 1999) and between *pol2-4* and *pol3-01* alleles (Morrison and Sugino 1994) in haploid yeast strains. Although disruption of *MSH2* in a *pol2-4* haploid strain has been reported (Tran *et al.* 1999), our repeated attempts to similarly disrupt *MSH2* in our *pol2-4* strains were unsuccessful. In addition, *pol2-4* haploid strains containing a *MSH2*-complementing plasmid were unable to lose the plasmid after disruption of *MSH2*. A combination of *msh2*Δ with *pol2-4* thus is synthetically lethal in our haploid strain backgrounds.

***lys2ΔBgl* reversion rates:** The reversion rates of the *lys2ΔBgl* allele in wild-type and various single-, double-, and triple-mutant strains are given in Table 2. The increases in reversion rate of *lys2ΔBgl* in the *pol2-4* mutant (a 13-fold increase), the *pol3-01* mutant (a 300-fold increase), and the *msh2*Δ mutant (a 200-fold increase) are consistent with previously reported reversion rate increases for the *his7-2* frameshift allele (Morrison and Sugino 1994). The reversion rate increases in the *pol2-4 msh2*Δ and *pol3-01 msh2*Δ strains relative to the single mutant strains are approximately multiplicative (2300-fold and 16,000-fold, respectively), a behavior that is consistent with exonucleolytic proofreading and MMR acting sequentially on the same frameshift intermediates (Morrison *et al.* 1993). Combination of *pol2-4* and *pol3-01* results in a synergistic, 11,000-fold increase in the *lys2ΔBgl* reversion rate, which is in agreement with similar measurements of *his7-2* reversion rate (Morrison and Sugino 1994). Surprisingly, the *pol2-4 pol3-01 msh2*Δ triple mutant exhibits no significant change in the *lys2ΔBgl* reversion rate relative to the *pol2-4 pol3-01* double mutant. As has been argued for *E. coli* (Fijalkowska and Schaaper 1996), the lack of an increase in mutation rate in the triple mutant could simply be due to a saturation of the mismatch repair system in the *pol2-1 pol3-01* double mutant. Examination of the corresponding mutation spectra, however, suggests that this explanation does not adequately account for the rate results in yeast (see discussion). It should be noted that a *pol2-4 pol3-01 msh2*Δ triple-mutant strain defective in all repair capacity has not been previously described. In addition to having a highly elevated mutation rate, the triple-mutant strain grew relatively slowly in liquid culture, had a low plating efficiency (only 50% of cells produced colonies), and produced colonies of variable size when plated (data not shown).

Reversion spectra in wild-type and triple mutant strains: The relative efficiencies of the proofreading and MMR systems in removing different types of frameshift intermediates can be inferred by comparing the reversion spectra derived from mutant *vs.* wild-type strains. If a given repair system removes all intermediates with the same efficiency, then the mutant spectrum should resemble the wild-type spectrum. If, however, some frameshift intermediates are removed with greater efficiency than are other intermediates, then the spectra of wild-type *vs.* mutant strains will be different. Specifically, those frameshift intermediates that are corrected most efficiently by a given repair system will comprise a greater proportion of the mutation spectrum in the mutant than in the wild-type strain.

The reversion events observed in the wild-type diploid (Figure 1A) presumably reflect polymerization errors that are corrected neither by proofreading nor by the postreplicative MMR system, and the spectrum is very similar to that reported previously with one of the haploid parental strains (Greene and Jinks-Robertson 1997). Single base deletions account for 95% (97/102) of the reversion events in the diploid; the remaining events are composed of four 2-bp insertions and a single complex event in which a base substitution accompanies a 1-bp deletion. In an earlier analysis of *lys2ΔBgl* reversion, homopolymer runs $>3N$ were hotspots for frameshift events, as they accumulated frameshift events more often than would be predicted for noniterated sequences of the same length (Greene and Jinks-Robertson 1997). In the current study, 58% (59/102) of the 1-bp deletions in the wild-type diploid strain occur in the four monotonic runs that are $>3N$: the 6A, 5T, 4C, and 4A runs beginning at nucleotides (nt) 664, 720, 697, and 727, respectively, and accounting for 13% of the reversion window. The 6A and 4C runs are particularly prone to accumulating −1 frameshifts and account for 49% (50/102) of the total reversion events.

Examination of the mutation spectrum in a strain simultaneously defective for MMR and for the proofreading activities of both Pol δ and Pol ε should, in principle, reflect the frameshift errors that occur during DNA replication. The mutation spectrum from the *pol2-4 pol3-01 msh2*Δ triple mutant demonstrates that frameshift mutations accumulate primarily in homopolymer runs, with 83% (81/98) of events occurring in runs $>3N$ (Figure 1B). In contrast to the spectrum obtained from the wild-type background, however, the frequency of events in the runs is directly proportional to the run length. The 6A run thus accounts for 50% (49/98; 48 1-nt deletions, one 2-nt insertion) of the total events, followed by 19% (19/98) of the events in the 5T run and ~7% of the events in each of the $4N$ runs

TABLE 2

Rates and distributions of *lys2ΔBgl* reversion events

Genotype	Lys$^+$ rate[a] ($\times 10^{-9}$)	Lys$^+$ Rate Relative to wild type	Rates of 1-nt deletions in *N*-length runs in mutant strains relative to the wild-type strain[b]							
						4*N*				
			1*N*	2*N*	3*N*	4A	4C	5*N*	6*N*	Other
Wild type	1.7 (1.5–2.0)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
pol2-4	22 (15–38)	13	6.1	2.5	2.7	—[c]	37	6.4	11	5.6
pol3-01	510 (300–900)	300	720	370	710	68	230	160	170	68
msh2Δ	350 (270–470)	200	—[c]	—[c]	180	—[c]	350	—[c]	430	—[c]
pol2-4 msh2Δ	3,900 (3,000–4,200)	2,300	270	130	1,200	2,500	260	1,400	6,900	610
pol3-01 msh2Δ	28,000 (20,000–38,000)	16,000	15,000	13,000	25,000	7,500	30,000	47,000	5,700	—[c]
pol2-4 pol3-01	18,000 (5,300–28,000)	11,000	60,000	9,000	15,000	2,500	2,700	5,700	1,700	—[c]
pol2-4 pol3-01 msh2Δ	8,900 (3,100–23,000)	5,200	—[c]	1,200	12,000	9,100	1,500	24,000	940	1,100

[a] Numbers in parentheses under the rates correspond to the 95% confidence intervals.

[b] The rate of −1 events in a given category was obtained by multiplying the total Lys$^+$ rate by the proportion of total events in the category (see spectra in Figures 1 and 2). The relative rate given is the rate in the mutant strain divided by the corresponding rate in the wild-type strain.

[c] No events were observed in the mutation spectra (Figures 1 and 2), so no rate could be calculated.

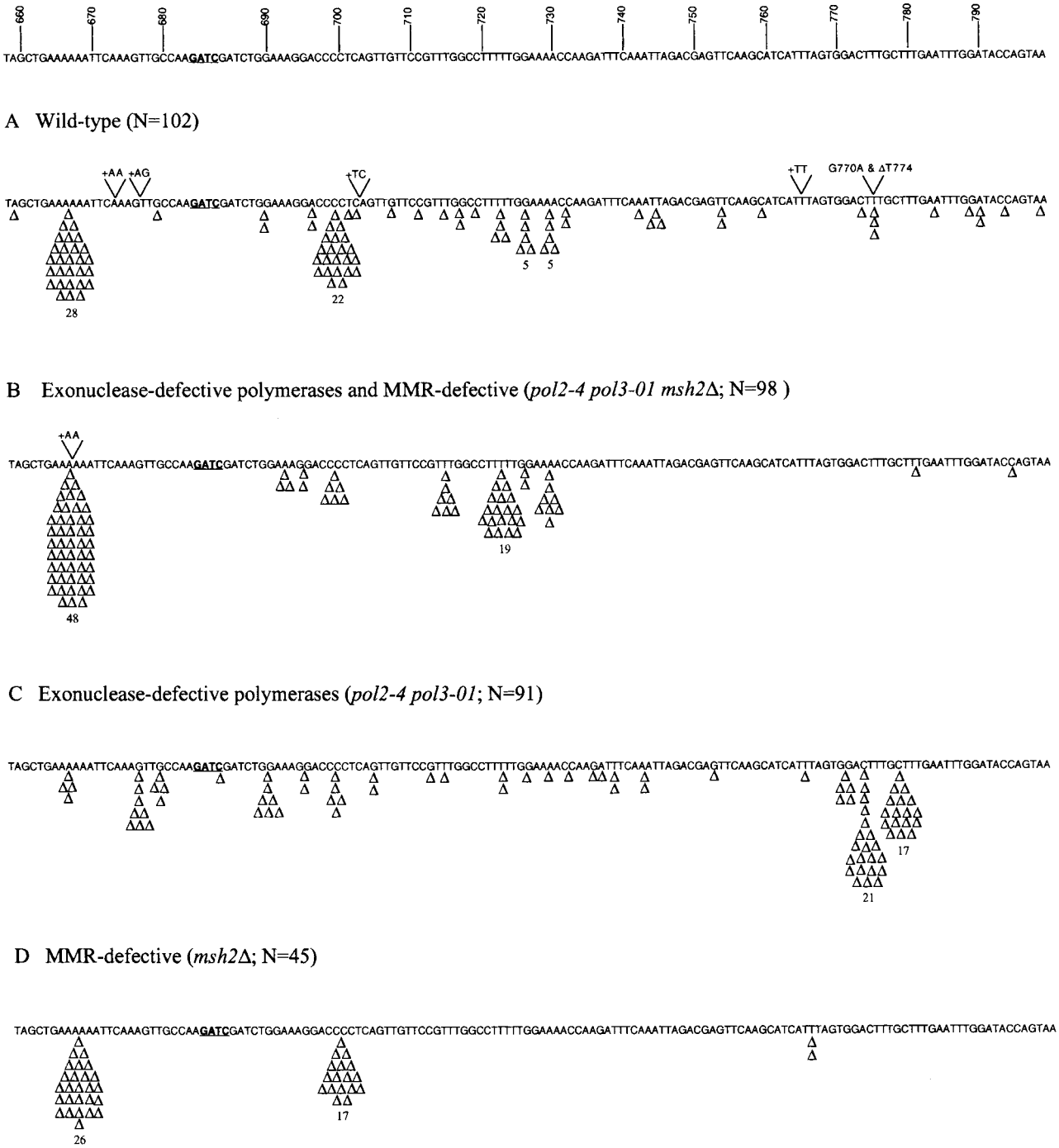

FIGURE 1.—Frameshift reversion spectra for a wild-type strain and for strains defective in proofreading and/or mismatch repair. The +4 insertion that creates the *lys2ΔBgl* allele is underlined and in boldface type. Single base deletions are represented by individual Δ's below the sequence and insertions are indicated above the sequence. *N* is the total number of revertants sequenced to obtain each spectrum. Pairwise comparisons of the wild-type (WT) spectrum with each mutant spectrum yielded highly significant *P* values ($P < 0.01$), with the exception of the WT-*msh2* comparison ($P = 0.076$). This exception is likely related to the relatively small sample size of the *msh2* Lys^+ revertants sequenced ($N = 45$), as pooling the diploid data reported here with the haploid data reported previously (GREENE and JINKS-ROBERTSON 1997) yielded a highly significant *P* value.

(6/98 and 8/98 in the 4C and 4A runs, respectively). Although 3*N* runs are not considered hotspots for frameshift events in this system, the 3T run at position 713 accounts for ~7% (7/98) of the events in the triple mutant. There are nine 3*N* runs in the reversion window, and 7 of the 11 frameshift events that occur in the 3*N* runs are in the nucleotide 713 3T hotspot. This particular run was previously shown to be unique among the 3*N* runs, as it was found to be a novel deletion hotspot in a *msh6*Δ strain (GREENE and JINKS-ROBERT-

son 1997). We speculate that the 3T run at nucleotide 713 may be particularly prone to frameshift events because of sequence context.

Reversion spectra in completely proofreading-defective (*pol2-4 pol3-01* double mutant) or MMR-defective (*msh2*Δ) strains: In contrast to frameshift events in the wild-type and triple mutant strains, the frameshift events in a *pol2-4 pol3-01* double-mutant strain do not occur preferentially in homopolymer runs >3*N* (Figure 1C). Sixty-three percent (57/91) of the 1-bp deletions occur in noniterated sequences and three prominent deletion hotspots account for 51% (46/91) of the total events: G676, C773, and C778. Comparison of these three sites yields the consensus sequence of 5′-CTTTG-3′, with deletion of the cytosine comprising the selected frameshift event. Two other hotspots are at GG dinucleotide repeats at positions 689 and 770, with each accounting for ~6% of the total events (7/91 and 5/91, respectively).

Relative to the wild-type and triple-mutant strains, the reversion spectrum for the *msh2*Δ diploid strain shows a dramatic increase in the proportion of events in the 6A and 4C runs (Figure 1D), which is similar to previous results obtained in haploid strain backgrounds (Marsischky *et al.* 1996; Greene and Jinks-Robertson 1997). The 6A and 4C runs account for 96% (43/45) of the frameshift events, compared to 49% of the events in the wild-type strain and 55% of the events in the triple-mutant strain.

Reversion spectra in strains defective in the exonucleolytic proofreading activity of either Pol δ (*pol3-01*) or Pol ε (*pol2-4*): In addition to obtaining frameshift spectra in either the presence or the absence of the exonuclease activities of both Pol δ and Pol ε, we also analyzed strains defective in the exonuclease activity of only a single polymerase (Figure 2). This analysis was done in both MMR-proficient and MMR-deficient backgrounds. The spectra obtained in the absence of the Pol δ *vs.* the Pol ε exonuclease activity are strikingly different. Elimination of only the Pol ε exonuclease activity (*pol2-4* allele; Figure 2, A and B) results in a clustering of the frameshift events at the 6A and 4C homopolymer runs, which is reminiscent of the pattern obtained in the MMR-deficient background (Figure 1D). In the *pol2-4* single-mutant spectrum, 86% (83/97) of the 1-bp deletions are the 6A or 4C run, with a 3:1 bias for events in the 4C run (Figure 2A). In the *pol2-4 msh2*Δ double mutant, however, almost all −1 events (68/80 = 85%) are in the 6A run and very few events (2/80 = 3%) are in the 4C run (Figure 2B).

In contrast to the clustering of frameshift events in homopolymer runs in the *pol2-4* mutant, events in the *pol3-01* mutant are more variable. In the *pol3-01* single mutant, only 33% (31/95) of the frameshifts are in the 6A or 4C run, and events are distributed evenly between the two runs (Figure 2C). Twenty-five percent (24/95) of the 1-bp deletions involve noniterated sequences and the hotspot at G676 that was seen in the *pol2-4 pol3-01* double mutant is evident, corresponding to 75% (18/24) of the events in noniterated sequences. Interestingly, the other two 1*N* deletion hotspots evident in the *pol2-4 pol3-01* double mutant (Figure 1C) are not distinct hotspots when the exonuclease activity of only one polymerase is defective. In the *pol3-01 msh2*Δ double mutant, 62% (58/93) of the events are in the runs >3*N*, and there is a 4:1 bias for events in the 4C run *vs.* the 6A run.

DISCUSSION

One means of assessing the relative roles of proofreading and MMR in removing mutational intermediates is to compare mutation rates and spectra in wild-type or completely repair-defective cells to those obtained in cells defective for either proofreading or MMR. Such an approach has been successfully applied in *E. coli* using a forward mutation system that detects primarily base substitutions (Schaaper 1993), and we have undertaken a similar strategy in yeast using the frameshift-specific *lys2*Δ*Bgl* reversion assay. To facilitate comparisons of relevant mutation spectra shown in Figure 1, the distributions of *lys2*Δ*Bgl* reversion events are graphically summarized in Figure 3.

Proofreading by DNA polymerases provides the first step for editing potential frameshift intermediates. In principle, eliminating the exonuclease activity of either Pol δ or Pol ε should reveal the proofreading specificity of the corresponding polymerase and reflect the underlying polymerization errors (see Morrison and Sugino 1994 for a discussion). In our frameshift-specific assay, as in other assays (Morrison and Sugino 1994; Shcherbakova and Pavlov 1996; Karthikeyan *et al.* 2000), the mutation spectra generated in the *pol2-4 vs. pol3-01* single mutants were strikingly different, which is consistent with distinct roles of the polymerases during DNA replication. A synergistic increase in mutation rate similar to that observed for the *lys2*Δ*Bgl* allele has been previously documented in *pol2-4 pol3-01* double mutants (Morrison and Sugino 1994), but no corresponding mutation spectra have been reported. In the frameshift-specific assay used here, the rates of frameshift mutations increased in both noniterated (1*N*) sequences and mononucleotide runs (2*N*–6*N*) in the double mutant relative to the wild-type strain, with the largest increase being observed in 1*N* sequences and the smallest increase in the 6*N* sequence (60,000-fold and 1700-fold, respectively; Table 2 and Figure 3). The shift in the distribution of 1-bp deletions in the *pol2-4 pol3-01* double mutant is consistent with the notion that nonrepetitive sequences are more efficient substrates for proofreading than are repetitive sequences (Kunkel and Bebenek 2000). Interestingly, there were two notable 1*N* hotspots (ΔC773 and ΔC778) in the *pol2-4 pol3-01* double-mutant spectrum that were not evident in either single-mutant spectrum. The presence of these hotspots only in the double mutant is consistent with the functional

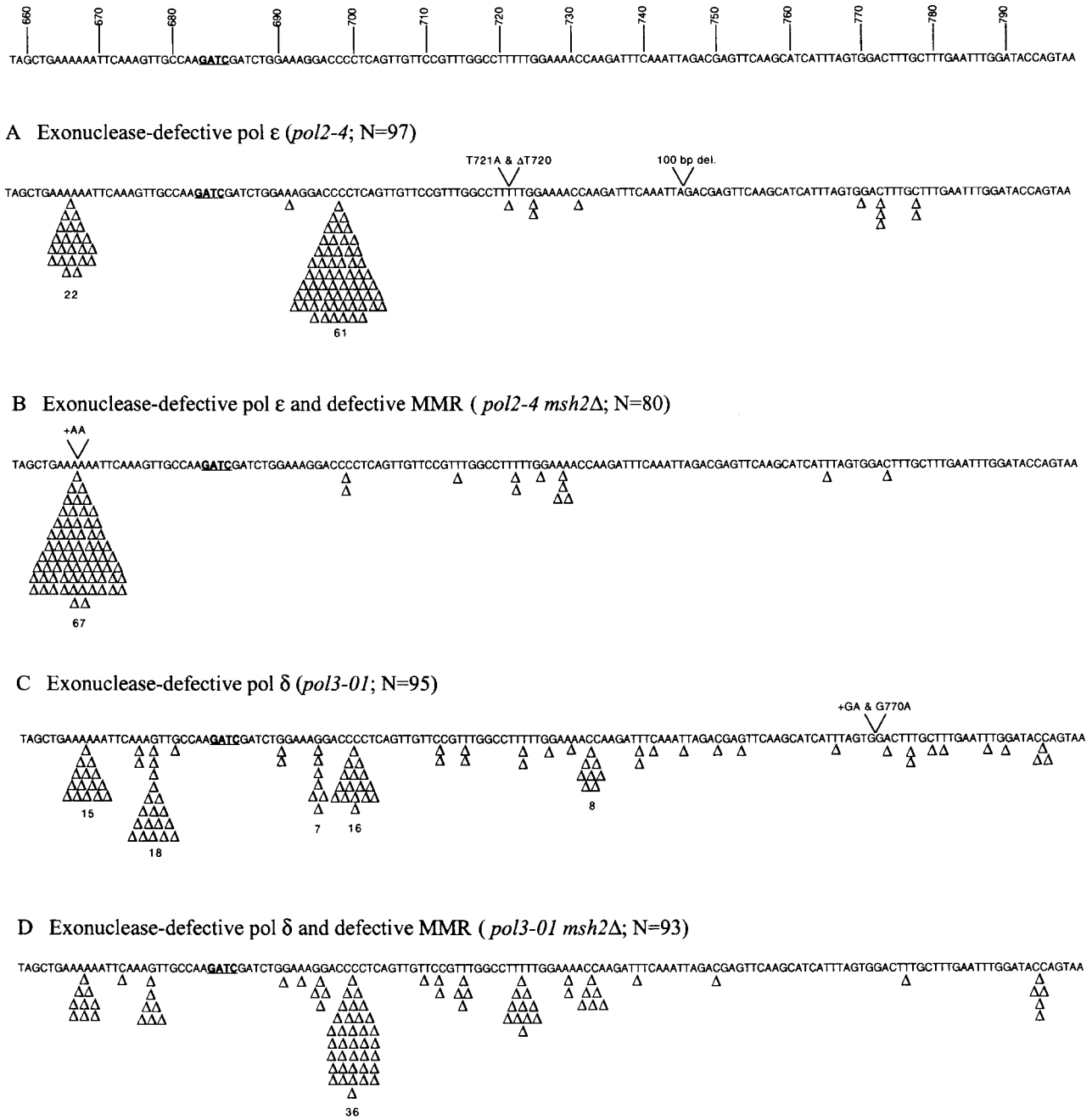

FIGURE 2.—Frameshift reversion spectra for strains deficient in the exonuclease activities of individual polymerases. See Figure 1 for further explanation.

redundancy between the Pol δ and Pol ε exonuclease activities as deduced from mutation rate measurements. It has been suggested that the synergism may reflect either the ability of one polymerase to proofread the mistakes of the other (MORRISON and SUGINO 1994) or roles of the exonuclease activities in MMR processes as well as in proofreading (TRAN *et al.* 1999). It should be noted that the spectrum of mutations in the *pol2-4 pol3-01* double mutant is distinctly different from that of an MMR-defective (*msh2*Δ) strain, an observation that does not support a concomitant defect in MMR.

Although useful information concerning polymerase fidelity can be obtained by comparing exonuclease-proficient and exonuclease-deficient strains that are otherwise wild type, an alternative way to assess the role of proofreading in mutation avoidance is to make the comparison in strains that are MMR defective (*i.e.*, *msh2*Δ *vs. pol2-4 po3-01 msh2*Δ). This latter type of comparison not only eliminates the replication-editing function of the MMR machinery, which can greatly impact mutation rates and spectra, but also eliminates any potential contributions that polymerase-associated exonuclease activ-

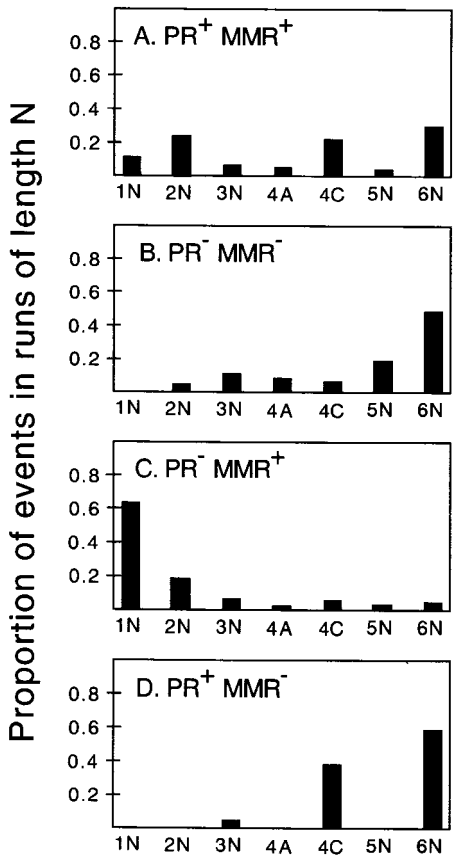

FIGURE 3.—Proportion of deletion events in noniterated sequences and in homopolymer runs of various length (*N*). Because of the difference in the behaviors of the 4C and 4A runs, these are treated separately. (A) Wild-type strain. (B) *pol2-4 pol3-01 msh2*Δ strain. (C) *pol2-4 pol3-01 MSH2* strain. (D) *POL2 POL3 msh2*Δ strain.

ities might have to MMR. The *lys2*Δ*Bgl* reversion rate was elevated 25-fold in the *pol2-4 po3-01 msh2*Δ strain relative to the *msh2*Δ strain (Table 2) and there was a notable shift in the distribution of frameshift events within runs >3*N* (compare Figure 3, B and D). Specifically, the data suggest that proofreading is more efficient in the 4A and 5T runs than in the 4C and 6A runs. The relatively inefficient proofreading of slippage events in the 6A run is most likely due to the length of the run, as it is near the proofreading threshold deduced from *in vitro* and *in vivo* experiments (KROUTIL *et al.* 1996; TRAN *et al.* 1997). We suggest that the relatively inefficient removal of frameshift intermediates in the 4C run is related to sequence composition, although sequence context also may be important. Studies using bacteriophage T4 indeed have implicated base composition as an important determinant of proofreading efficiency, and it has been suggested that stable (*i.e.*, GC-rich) regions of DNA are less likely to have hydrogen bonding disrupted and should, therefore, be less available to exonucleolytic proofreading activity (BESSMAN and REHA-KRANTZ 1977; GOODMAN and FYGENSON 1998).

The MMR pathway represents the last step for editing DNA polymerization, and its contribution to mutation avoidance was estimated by comparing the *lys2*Δ*Bgl* reversion rates and spectra in wild-type *vs. msh2*Δ strains. Loss of MMR activity was accompanied by a 200-fold increase in reversion rate, and there was a striking shift in the distribution of frameshift mutations (Table 2 and Figure 3). One interpretation of the run-associated clustering of mutations upon loss of Msh2p is that the yeast MMR system removes frameshift intermediates in runs >3*N* more efficiently than those in shorter runs or noniterated sequence. It is difficult, however, to imagine a mechanism whereby the MMR system might recognize and repair an extrahelical base in a run better than that in a noniterated sequence. It seems more likely that the frameshifts that are underrepresented in the *msh2*Δ spectrum might arise out of the context of normal DNA replication (*e.g.*, during DNA repair or bypass), where they either may not be sensed by the MMR machinery or may not be subject to the strand bias normally associated with MMR. Alternatively, it is formally possible that a functional MMR system is required to generate some classes of frameshift mutations (see below).

In addition to comparing wild-type and MMR-defective strains, we also examined MMR specificity under conditions where proofreading was not contributing to error avoidance. Our expectation was that the *pol2-4 pol3-01 msh2*Δ triple mutant would exhibit a greatly elevated mutation rate relative to the *pol2-4 pol3-01* double mutant, but surprisingly, the *lys2*Δ*Bgl* reversion rates were not statistically different in the two strains. Although the simplest explanation for the lack of a further increase in mutation rate in the triple mutant is that the MMR system was already saturated in the *pol2-4 pol3-01* double mutant (see FIJALKOWSKA and SCHAAPER 1996), this explanation cannot account for the dramatic shift in the mutation spectrum observed in the triple mutant. Almost 60% (57/91) of the frameshift events in the double mutant were in 1*N* sequences, for example, and yet there were no events in 1*N* sequences among 98 revertants sequenced from the triple mutant. In addition, there was a sevenfold decrease in the rate of events in 2*N* runs in the triple mutant relative to the double mutant. This spectral shift suggests that a functional MMR system may be required to generate specific classes of frameshift intermediates, most notably those in nonrun (1*N* and 2*N*) sequences (see below for further discussion).

The majority of frameshift events are assumed to arise in runs of repeated sequence, where the potential number of correct base pairs that can stabilize a slippage-generated frameshift intermediate, as well as the total number of potential intermediates, increases as the run length increases (KUNKEL and BEBENEK 2000). This leads to the prediction that the slippage rate should increase as the length of the run increases and this

prediction has been confirmed in *in vitro* DNA replication assays (Kroutil *et al.* 1996) and *in vivo* yeast studies (Tran *et al.* 1997). As expected, the majority (58%) of deletion events in the wild-type spectrum occurred in mononucleotide runs longer than 3*N*, which together comprise only 13% of the *lys2ΔBgl* reversion window. The distribution of events, however, was not consistent with run length being the primary determinant of frameshift accumulation (Figure 3A and Greene and Jinks-Robertson 1997). Specifically, the proportions of deletions in the two 4*N* runs were not equal (5/102 and 22/102 events in the 4A and 4C runs, respectively), and the 5T run was no more likely to accumulate frameshift mutations than random sequence of the same length.

Because the *pol2-4 pol3-01 msh2*Δ triple-mutant strain lacks both proofreading and MMR, the corresponding frameshift spectrum should provide an accurate reflection of the errors made during replicative DNA synthesis. Not only was the mutation rate elevated 5200-fold in the triple mutant relative to the wild-type strain (Table 2), but there also was a shift in the mutation spectrum. There was a larger proportion of deletion events in mononucleotide runs >3*N* in the triple mutant than in the wild-type strain (83% *vs.* 58%) and the distributions of events between the runs >3*N* differed significantly in two strains ($P < 0.01$ by contingency chi-square). Most notably the distribution of 1-bp deletion events was correlated with increasing mononucleotide run length in the triple mutant, with the 6*N* run accounting for the majority of events, followed by the 5*N* run and then the two 4*N* runs (Figure 3B). As there was a direct correlation between run length and frameshift distribution in the triple mutant, the apparent run specificity for deletions observed in the wild-type strain can be attributed to differential repair of frameshift intermediates rather than to preferential polymerase errors.

As discussed above, the data obtained with the wild-type, *pol2-4 pol3-01* double-mutant, and *pol2-4 pol3-01 msh2*Δ triple-mutant strains fit the general predictions that slippage frequency should be directly proportional and proofreading efficiency should be inversely proportional to run length (Kunkel and Bebenek 2000). Two implicit assumptions were made, however, in interpreting the data. First, we assumed that the elimination of polymerase-associated exonuclease activity affects only the proofreading of DNA replication errors, and second, we assumed that elimination of Msh2p impacts only postreplicative MMR. If the first assumption is indeed true, then the frameshift mutations observed in the *pol2-4 pol3-01* strain should directly reflect the primary slippage errors generated by the corresponding wild-type polymerases. It is possible, however, that the nature of the primary polymerization errors might be altered in the absence of proofreading capacity. In the absence of proofreading, for example, a base substitution intermediate might have a much higher probability of being converted into a frameshift intermediate by slippage between the nascent and template strands (see Bebenek and Kunkel 1990). Such slippage would restore base pairing between the 3′ end of the nascent strand and the template, thus allowing a polymerase that cannot go backward to proceed forward. Mispair-promoted slippage could occur either during normal replicative DNA synthesis or in association with a checkpoint response to a mispaired 3′ end, in which case it might involve a translesion polymerase (see Datta *et al.* 2000). It follows that the types and proportions of frameshift intermediates generated by a proofreading-defective polymerase might be different from those generated by a more processive, proofreading-proficient polymerase.

The second assumption inherent in our analyses is that the only mutation-related process affected by removal of Msh2p is postreplicative MMR. In mammalian cells, however, there is evidence that MSH2 also is involved in triggering apoptosis in response to DNA-damaging agents, and it has been suggested that the MSH2 may function as a general damage sensor (Gong *et al.* 1999; Toft *et al.* 1999). Although yeast cells do not undergo apoptosis in response to DNA damage, they do delay cell-cycle progression (Foiani *et al.* 2000). One intriguing possibility is that yeast Msh2p is involved in recognizing DNA damage (or perhaps aberrant replication intermediates) and may thereby be important in triggering a checkpoint response that leads to novel types of frame-shift intermediates. Such a scenario could account for the observation that elimination of Msh2p in either a wild-type or a *pol2-4 pol3-01* background was accompanied by a proportional decrease of frameshift events in nonrun sequences.

In summary, the data reported here provide a comprehensive analysis of frameshift mutagenesis in yeast strains that are singly, doubly, or triply defective in the MMR and the proofreading activities of Pols δ and ε. These analyses indicate very different specificities for Pol δ and Pol ε in the generation and/or removal of frameshift intermediates, even though loss of the exonuclease activity of one polymerase can be partially compensated for by that of the other polymerase. In addition, comparisons of frameshift spectra in MMR-proficient and MMR-deficient strains suggest either that the efficiency with which the MMR system removes extrahelical bases is greatly influenced by sequence context or that the generation of some classes of frameshifts is actually dependent on the presence of the MMR system. These results affirm the complexities and the highly interconnected natures of the pathways that generate and remove −1 frameshift intermediates, and one can expect similar complexities to emerge in analyses of other types of mutational intermediates.

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