The 3′→5′ Exonucleases of DNA Polymerases δ and ε and the 5′→3′ Exonuclease Exo1 Have Major Roles in Postreplication Mutation Avoidance in *Saccharomyces cerevisiae*

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Replication fidelity is controlled by DNA polymerase proofreading and postreplication mismatch repair. We have genetically characterized the roles of the 5′→3′ Exo1 and the 3′→5′ DNA polymerase exonucleases in mismatch repair in the yeast *Saccharomyces cerevisiae* by using various genetic backgrounds and highly sensitive mutation detection systems that are based on long and short homonucleotide runs. Genetic interactions were examined among DNA polymerase ε (*pol2-4*) and δ (*pol3-01*) mutants defective in 3′→5′ proofreading exonuclease, mutants defective in the 5′→3′ exonuclease Exo1, and mismatch repair mutants (*msh2, msh3*, or *msh6*). These three exonucleases play an important role in mutation avoidance. Surprisingly, the mutation rate in an *exo1 pol3-01* mutant was comparable to that in an *msh2 pol3-01* mutant, suggesting that they participate directly in postreplication mismatch repair as well as in other DNA metabolic processes.

Chromosome replication fidelity is generally considered to be determined by a combination of base selection and error correction activities of DNA polymerases along with postreplication mismatch repair (MMR). The combined effect in *Escherichia coli* results in an overall error rate that is as low as 10^{-10} errors per replicated nucleotide (27). In *E. coli*, the α polymerase subunit of DNA polymerase III holoenzyme, encoded by the *dnaE* gene, determines base selection and the ε subunit (*dnaQ*) provides proofreading. These subunits are tightly bound together with the θ subunit to form the polymerase III core (16). The errors left by the polymerase III holoenzyme are corrected by the MutHLS MMR system (28).

In eukaryotes the three polymerases required for chromosome replication are polymerase α, δ, and ε, encoded by the *POL1*, *POL3*, and *POL2* genes, respectively. Polymerase α (Polα) is responsible for the synthesis of primers for Okazaki fragments in the lagging strand, and the δ and ε polymerases, it has been proposed, are responsible for lagging and leading DNA strand replication (35), although their specific roles have not been established. Unlike Polα, the δ and ε polymerases also have a 3′→5′ proofreading exonuclease activity in their N-terminal regions (14, 22, 32). In the yeast *Saccharomyces cerevisiae*, the point mutations *pol3-01* and *pol2-4*, which eliminate the proofreading activities of the δ and ε polymerases, respectively, result in a frameshift and base substitution mutator phenotype (22, 23).

The postreplication MMR system is responsible for the correction of errors generated during replication. Based on in vitro analysis with purified proteins, the steps in *E. coli* MMR include mismatch recognition by MutS and MutL proteins, methyl-directed strand discrimination, incision by the MutH endonuclease in the unmethylated nascent strand at sites opposite methylated GATC, degradation from a nick towards the mismatched site by exonucleases (ExoI, ExoVII, or RecJ), and gap filling by polymerase III holoenzyme followed by DNA ligation (21).

Except for mismatch recognition, relatively little is known about the MMR mechanism in eukaryotes. Many genes homologous to *E. coli mutS* and *mutL* have been cloned and studied (21). Strand-specific MMR was demonstrated in human cell extracts when a single-strand nick was introduced on either side of a mismatch (7, 37), suggesting that both 5′→3′ and 3′→5′ exonucleases may be involved. The 5′→3′ exonuclease Exo1 of the yeast *S. cerevisiae* has been implicated in MMR. The *EXO1* gene, which is homologous to the *Schizosaccharomyces pombe EXO1* gene (36), was isolated in a two-hybrid interaction screen with yeast *MSH2* (38). Tishkoff et al. (38) concluded that *EXO1* and *MSH2* are in one epistasis group; however, other interpretations are possible. In an *exo1*-deficient *S. cerevisiae* strain, the canavanine resistance (Can^r) forward and *hom3-10* reverse mutation (−1 frameshifts) rates are increased only eight- and sixfold, respectively, in comparison with a wild-type strain. This is much lower than the corresponding 25- and 850-fold increases observed in *msh2* mutants (38), and the *msh2 exo1* double mutant exhibits rates comparable to that of the single *msh2* mutant. However, it cannot be ascertained from these data whether *MSH2* and *EXO1* have an epistatic or additive interaction (i.e., single versus separate pathways), since the rate in the *msh2* mutant differs little from the sum of the rates in the *msh2* and *exo1* mutants. Since both Exo1 and Msh2 are involved in homologous recombination (9, 26, 34), their physical interaction could be related to this process rather than MMR.

We have examined the role of various exonucleases in MMR as part of our efforts to screen and characterize MMR genes in the yeast *S. cerevisiae*. For example, the small mutator effect of an *exo1* mutation as compared to that of an *msh2* mutation suggests that there are additional exonucleases involved in MMR. A synergistic mutator effect was found for defects in the Exo1 nuclease and the Polε 3′→5′ proofreading exonuclease, suggesting that these activities participate in postreplication

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steps. Cells deficient in both DNA Polδ 3′→5′ proofreading exonuclease and either Exo1, Msh2, or DNA Polε 3′→5′ proofreading exonuclease (24) were inviable, implying a strong synergistic interaction. On the other hand, diploid strains defective in both DNA Polδ 3′→5′ proofreading exonuclease and Exo1 or Msh2 were viable. Surprisingly they exhibit comparable levels of hypermutability. We propose that the Polδ and Polε proofreading exonucleases as well as Exo1 may play a major role in mutation prevention.

MATERIALS AND METHODS

General genetic and molecular methods. Yeast standard media (30) and yeast-extract-peptone-dextrose (YPD) medium with G418 (45) have been described previously. Yeast cells were grown at 30°C. Yeast transformations were performed according to the methods of Gietz and Schiestl (10). The preparation of bacterial media and general molecular methods have been described previously (25).

Strains and plasmids. A series of isogenic strains were constructed from the original CG379 (*MATα ade5-1 his7-2 leu2-3,112 trp1-289 ura3-52*) (22) and *pol2-4* and *pol3-01* derivatives (40). The *pol2-4* and *pol3-01* mutations are point mutations in the exonuclease domain of the *POL2* and *POL3* genes, respectively, resulting in the loss of 3′→5′ proofreading exonuclease activities in the corresponding polymerases (22–24). These strains contain modified *insE* inserts in the chromosomal *LYS2* gene, where the A_4 run was changed to A_5, A_{12}, and A_{14} (40). Mutations of the following DNA metabolism genes were introduced into these strains: MMR genes *MSH2*, *MSH3*, and *MSH6* and the 5′→3′ exonuclease gene *EXO1*.

Strains containing combinations of *pol2-4 lys2::insE-A₁₂*, *pol2-4 lys2::insE-A₁₄*, *pol3-01 lys2::insE-A₁₂*, and *pol3-01 lys2::insE-A₁₄* were constructed by transferring *lys2::insE-A₁₂* and *lys2::insE-A₁₄* inserts from the Pol⁺ strains (40) to strains with *pol2-4* or *pol3-01* mutations by plasmid gap-filling and allele replacement techniques as described previously (40, 41).

A mismatch repair gene tester strain (*MATa pms1::LEU2 msh2::ADE2 mlh1::URA3*) was derived from our strain 1036 (*lys2-BX MATa ade2-1 arg4-8 leu2-3,112 lys2-BX thr1-4 trp1-1 ura3-52 cup1-1*) *lys2-BX* is a deletion of the *Bam*HI-*Xho*I region covering the *insE* inserts in the *LYS2* gene). The 1036 *lys2-BX exo1::kanMX* strain was obtained from 1036 (*lys2-BX*) by deleting the *EXO1* gene by PCR disruption with a *kanMX* cassette (see below).

The replicative pBL304 plasmid containing the *POL3* and *URA3* markers was constructed by P. Burgers and has been described previously (24).

Mutator screening with a disruption library. Plasmid DNA of a gene disruption library, kindly provided by M. Snyder (4), was digested with *Not*I to release yeast genomic DNA fragments containing Tn*3::lacZ-LEU2* inserts and transformed into Pol⁺ *lys2::insE-A₁₄* or *pol2-4 lys2::insE-A₁₄* strains, and Leu⁺ transformants were selected. The transforming DNA fragments randomly knock out different nonessential genes through homologous recombination. The Leu⁺ transformants were replicated to complete medium without lysine in order to identify clones that could yield Lys⁺ papillae. These potential mutators were verified and crossed with tester strain 1036 (*lys2-BX pms1 msh2 mlh1*) to identify possible MMR mutators. The nature of the disrupted genes in the remaining clones was determined by using an inverse PCR technique instead of the rescue plasmid technique described by Burns et al. (4). First, genomic DNA from the clones was isolated and digested with the frequently cutting *Hpa*II or *Taq*I endonucleases. These enzymes will cut inside the Tn*3-lacZ-LEU2* insert as well as nearby regions. Digested DNA fragments were circularized by DNA ligation. After circularization, the junction region between the yeast genomic DNA and Tn*3-lacZ-LEU2* insert was amplified with the following primers: lacZ, 5′-GCG GGCCTCTTCGCTATTACG-3′, and lacZ-2, 5′-TGAATGGCGAATGGCGC TTTG-3′. The PCR products were sequenced with the lacZ-1 primer, 5′-GTC ACGACGTTGTAAAACGACG-3′.

DNA and mutation analysis. An ABI sequencer was used for DNA sequencing. Mutation rates were determined by a fluctuation test by the method of the median (19) with at least 12 independent cultures. The nature of the Lys⁺ revertants was identified by sequencing the reversion window of the *lys2::insE* insert as described previously (41). The DNA regions sequenced between the Tn*3-lacZ-LEU2* inserts and the yeast genome were identified by using the *S. cerevisiae* DNA database (26a), and Swissprot was used to search the protein database (24a).

Gene replacement and disruption. The following genes were disrupted: *MSH2*, *MSH3*, *MSH6*, and *EXO1*. For *MSH2* disruption we used a *Sac*I-*Pst*I *msh2::LEU2* fragment from p203 (39). The *Bam*HI-*Aat*II fragment from pmsh3::LEU2 (29) was used to disrupt the *MSH3* gene.

The entire open reading frames of the *EXO1* and *MSH6* genes were deleted by the PCR disruption technique with the *kanMX* module (45) and primers described below. Lowercase letters indicate nucleotide sequences that belong to the *kanMX* cassette; DNA sequences belonging to the genes are written in uppercase. For the *EXO1* gene we amplified the *kanMX* cassette with EXO1-kanMX-3′ (5′-TTGGCTTGACTTAGTAGTTTCGATGTCCCTTTTCTTACT Tatcgatgaattcgagctcg-3′) and EXO1-kanMX-5′ (5′-AGGTATGAAGGAGAAG

TGTTAGCCATTGATGGCTATGCATcgtacgctgcaggtcgac-3′). For the *MSH6* gene the following primers were used: MSH6-kanMX-5′ (5′-CTACCCCTAA AACTTCTAAGACTGCACACTTCGAAAATGGatcgatgaattcgagctcg-3′) and MSH6-kanMX-3′ (5′-GTCCATCTCCGTACGCAATTCGAACGAAATCACT TTGTAAcgtacgctgcaggtcgac-3′). YPD medium with G418 was used for the selection of transformants (45). To verify the disruption of the *EXO1* gene we used the following pair of primers for the *EXO1* gene: EXO1-test-3 (5′-ATTG GGAAAGCAAGGAGATAG-3′) and EXO1-test-5 (5′-TCTTCTTCCTCAGT TAAAGC-3′). The disruption of the *MSH6* gene was verified by PCR with primers MSH6-test-5 (5′-CAGCTACCCCTAAAACTTC-3′) and MSH6-test-3 (5′-TTCCAATCATAGTTCAAGACCCC-3′). The *EXO1* gene was also disrupted with the *Hin*dIII-*Kpn*I fragment (*exo1::URA3*) from plasmid p244. The p244 plasmid was constructed by first generating a PCR product of the chromosome *EXO1* gene with primers EXO1-test-3 and EXO1-test-5. A *Bgl*II-*Nsi*I fragment from the PCR product was cloned into the *Bam*HI-*Pst*I sites of the pUC19 plasmid, resulting in p243. The *URA3* gene *Bgl*II-*Bgl*II fragment from pFL34 (3) was cloned into the *Bam*HI site inside the *EXO1* gene of p243, resulting in plasmid p244. To verify the disruption of *MSH2* and *MSH3* genes, PCR primers and conditions were used as described previously (42).

Tetrad analysis. The *MATα pol3-01 lys2::insE-A₄* and *MATα pol3-01 lys2::insE-A₅* strains were mated with strain 1036 (*lys2-BX exo1::kanMX*). In the diploid strains the second copy of the *EXO1* gene was disrupted with the *Hin*dIII-*Kpn*I (*exo1::URA3*) fragment from p244. The diploid strains were sporulated, and tetrad analysis was performed. The spore colonies with the wild-type *POL3* gene were distinguished from *pol3-01* mutants by using PCR. Genomic DNA from viable spores was isolated and analyzed by PCR with two primers, p3 (5′-GGAGATACCAAATTACCA-3′) (785-802) and d8 (5′-CTTGTACCATA AGCCTTC-3′) (1512-1495). The PCR product covers the *pol3-01* mutation. The PCR products were digested with *Eco*RV; the *pol3-01* mutation lacks an *Eco*RV site (23).

Construction of homozygous diploid strains. Haploid *leu2* strains were transformed with plasmid YEpHO (a gift from Y. Chernoff) carrying the *LEU2* marker and the *HO* endonuclease gene. The *HO* endonuclease will induce mating type switching in haploid strains from *MATa* to *MATα* or vice versa. Haploid strains with the opposite mating types could form *MATa/MATα* homozygous diploid strains. Transformants with YEpHO were grown on YPD media to allow loss of the plasmid. Single Leu⁻ clones were isolated. Diploid clones were identified as nonmating with both *MATa his3* and *MATα his3* testers as well as giving a low forward mutation rate to Can⁺ due to the presence of two *CAN1* gene copies. For strains in which the *LEU2* marker could not be used we utilized plasmid pGHO-TRP1 (1) containing the *TRP1* marker and the *HO* gene under the *GAL1-10* promoter. Mating type switching was induced in galactose media for 8 h, and diploid clones were identified as described above.

Plasmid loss rate measurement. To establish the requirement for the *POL3* gene in strains with *pol3-01 msh2* or *pol3-01 exo1* combinations, the rate of loss of the plasmid-borne *POL3* gene on pBL304 was determined. By using the *URA3* marker on pBL304, the plasmid loss rate was determined from the median in a fluctuation test of 10 independent clones on 5-FOA (5-fluoro-orotic acid) media (2). For each fluctuation test, 10 5-FOA-resistant clones were analyzed by PCR to determine the loss of the *POL3* gene as described above (see the paragraph on tetrad analysis).

RESULTS

Isolation of mutators for a long A₁₄ homonucleotide run. The MMR process is conserved in *E. coli* and eukaryotes. There are several *E. coli* MutS and MutL homologs that have been identified in yeast (21). While genes involved in mismatch recognition are well characterized, genes involved in later MMR steps such as strand discrimination, mismatch excision, and DNA resynthesis remain to be identified in *S. cerevisiae*. Previously a *lys2::insE-A₁₄* mutation system based on a homonucleotide run of 14 A that is hypersensitive to defects in MMR has been described (40). For example, an *msh2* mutant exhibits a 10,000-fold increase in the Lys⁺ reversion rate over the wild type. Thus, even a relatively small impact of mutators can be detected. Using this sensitive system we tried to identify additional mutators affecting the instability of a long homonucleotide run through a saturation inactivation screen of nonessential genes.

The Pol⁺ *lys2::insE-A₁₄* (S1-A₁₄) or *pol2-4 lys2::insE-A₁₄* (S3-A₁₄) mutator detection strain containing the homonucleotide run A₁₄ in the *LYS2* gene was transformed with a gene disruption library (4), and colonies that exhibited higher Lys⁺ reversion rates were identified. As shown below, the *pol2-4* background increases the magnitude of a mutator effect.

TABLE 1. Relative increases in mutation rates in homonucleotide runs (A_4, A_5, A_{12}, and A_{14} of *lys2::insE* alleles) and *CAN1*

Strain genotype	Relative rate of mutation[a] (mutants vs wild type)[b]				
	A_4 (−1)	A_{14} (−1)	A_5 (+1)	A_{12} (+1)	Can[r]
Wild type					
Absolute (10^9)	0.4	186	1.1	140	240
Relative	1 (4/55)[c]	1 (10/10)[c]	1 (7/21)[c]	1 (8/8)[c]	1
exo1	2.5 (4/34)	97 (8/8)	2 (19/29)	2 (5/5)	6.5
msh2	77.5 (20/30)[c]	9,460 (8/8)	35 (16/30)[c]	1,240 (8/8)	27.4
exo1 msh2	90 (14/22)	8,980 (6/6)	47 (16/21)	544 (5/5)	32.2
pol2-4	6 (7/20)[c]	2 (10/10)	7 (13/27)[c]	1 (10/10)	2.3
pol2-4 exo1	278 (29/38)	925 (6/6)	769 (33/35)	116 (6/6)	37
pol2-4 msh2	2,870 (26/29)[c]	23,390 (10/10)	10,820 (30/30)	2,040 (9/9)	475
pol2-4 exo1 msh2	ND[d]	16,666	ND	3,150	729
msh3	ND	6 (6/6)	ND	2 (6/6)	2.0
pol2-4 msh3	ND	15 (6/6)	ND	2 (6/6)	4.3
msh6	ND	189 (7/7)	ND	32 (7/7)	9.2
pol2-4 msh6	ND	317 (7/7)	ND	60 (7/7)	480
msh3 msh6	ND	18,820 (6/6)	ND	1,670 (6/6)	29.2
pol2-4 msh3 msh6	ND	46,450 (6/6)	ND	3,170 (6/6)	1,440
pol3-01	37.5 (4/37)[c]	54 (10/10)	93 (19/22)[c]	20 (10/10)	44.2

[a] The mutation rate in the run (R_r) was calculated as follows: $R_r = R_t (N_r/N_t)$ where R_t is the total rate of reversion, which was determined from the median in a fluctuation test of more than 12 independent cultures (19). The fold increase in the mutation rate was determined as the ratio of the mutation rate of the mutant strain to the mutation rate of the wild-type strain. The nature of the Can[r] mutants was not determined. The Can[r] mutation rate was determined from the median in a fluctuation test of more than 12 independent cultures.

[b] The ratio of the number of revertants with a frameshift mutation in the run (N_r) to the total number of revertants (N_t) analyzed by sequencing is given in parentheses. As observed previously (40) all the frameshift mutations in the A_4 and A_{14} runs that were analyzed were due to −1 nucleotide deletions; all frameshift mutations in the A_5 and A_{12} runs were due to +1 nucleotide insertions.

[c] Data from the work of Tran et al. (40), with permission.

[d] ND, not determined.

Among more than 100,000 transformants of the Pol$^+$ strain and 50,000 transformants of the *pol2-4* mutant, 44 and 25, respectively, exhibited markedly increased Lys$^+$ reversion frequencies. Of these hypermutable transformants, 34 and 19, respectively, carried mutations in the MMR gene *PMS1*, *MSH2*, or *MLH1*. The frequency of repeat mutants indicates that we have efficiently inactivated most nonessential genes that were likely to lead to an increase of the mutation rate in a sensitive system. Included in the remaining 10 hypermutable transformants of the Pol$^+$ strain were 5 *exo1-dhs1* mutants (ORF-YOR033C; the *DHS1* gene also has been referred to as *EXO1* [38]). The Tn3::lacZ-LEU2 inserts were at nucleotide positions 13, 104, 131, 150, and 163 from the *EXO1* start codon. Among six hypermutable transformants of *pol2-4*, two were *exo1* mutants and three were *msh6* mutants.

To establish that the increased reversion rate of the *lys2::insE-A$_{14}$* allele in the *exo1* mutant strains was due to the disruption of the *EXO1* gene and not to additional mutations elsewhere in the genome, the *EXO1* gene was deleted from the S1-14A strain by using a *kanMX* cassette in combination with PCR (reference 45; see Materials and Methods). The disruption of *EXO1* led to a 100-fold increase in the Lys$^+$ reversion rate that was due to mutations in the A$_{14}$ run (Table 1). In the six mutator clones lacking alterations in *EXO1* or MMR genes (five from the Pol$^+$ strain and one from the *pol2-4* strain), disruptions were identified in the *OXA1*, *CSD3*, *PMR2*, *ENA2*, and 25S rRNA genes. Since the direct disruption of these genes (except 25S rRNA) in the S1-14A strain did not lead to a mutator phenotype, the mutator phenotype of these transformant clones is likely to be due to secondary mutator mutations elsewhere in the genome.

Based on the number of repeated isolations of different mutants we propose that we have nearly exhausted the number of single mutants that can lead to small (see below) as well as large increases in the mutation rate in a long homonucleotide run. It is possible that additional mutants might be lost in this screen if growth defects reduce the likelihood of detection and/or if the genes have only small open reading frames so that there is less likelihood of disruption. (These reasons could account for our lack of detection of a *rad27* mutant in this screen although it is known to be a mutator [13]. The role of *RAD27* in MMR and interactions between *rad27*, MMR, and polymerase mutants will be presented elsewhere.)

Mutator activity of an *exo1* mutant. Based on genetic and in vitro observations, exonucleases play an important role in mutation avoidance in *E. coli* (5, 18, 44). We, therefore, examined the role of *EXO1* by using several assays that reveal −1 and +1 frameshift mutations in or near short (A$_4$ and A$_5$) and long (A$_{14}$ and A$_{12}$) homonucleotide runs within the *lys2::insE* sequence (40). Forward mutation to canavanine resistance was also examined in these strains (see reference 30). Many of the *lys2::insE-A$_4$* and *lys2::insE-A$_5$* revertants were not due to frameshift mutations in homonucleotide A$_4$ and A$_5$ runs, respectively (Table 1). Instead, most mutations were frameshifts located elsewhere in the reversion window (i.e., the small region where mutations occur; see references 40 and 42). As expected, all *lys2::insE-A$_{14}$* and *lys2::insE-A$_{12}$* revertants were due to −1 and +1 nucleotide frameshift mutations in the A$_{14}$ and A$_{12}$ runs, respectively (Table 1). Forward mutation rates in the *CAN1* gene were determined, but Can^r mutations were not sequenced.

Inactivation of the *EXO1* gene led to only a weak mutator phenotype for the A$_4$ and A$_5$ homonucleotide runs and a sevenfold increase in the appearance of Can^r forward mutations. However, we observed a strong effect (a 100-fold increase) on a long homonucleotide A$_{14}$ run that was specific for −1 frameshift mutations; there was only a twofold increase in +1 insertions in the A$_{12}$ run (Table 1). The preference of the mutator effect for deletions over insertions is characteristic of defects in MMR genes such as *MSH2*, *MSH3*, and *MSH6*

TABLE 2. Relative mutation rates in short and long homonucleotide runs and the *CAN1* gene in various *pol2-4* mutants

Strain genotype comparison	Relative mutation rate				
	A_4 (−1)	A_{14} (−1)	A_5 (+1)	A_{12} (+1)	Can^r
pol2-4 vs wild type	6	2.2	7	1	2.3
exo1 pol2-4 vs *exo1*	111	10	385	55	5.7
msh2 pol2-4 vs *msh2*	37	2.5	309	1.6^a	17
msh3 pol2-4 vs *msh3*	ND^b	2.6	ND	1.1^a	2.2
msh6 pol2-4 vs *msh6*	ND	1.7	ND	1.9	52
msh3 msh6 pol2-4 vs *msh3 msh6*	ND	1	ND	1	49

^{a} No significant difference; the 95% confidence intervals for the mutation rate in these two strains overlap.
^{b} ND, not determined.

TABLE 3. Spontaneous mutation rates in homozygous diploid strains measured as the rate of reversion of *his7-2* or *lys2::insE-A$_{14}$* to His$^+$ or Lys$^+$, respectively

Strain genotype	Relative reversion rate^a	
	his7-2	*lys2::insE-A$_{14}$*
POL$^+$/POL$^+$		
Absolute (10^9)	13	600
Relative	1	1
pol3-01/pol3-01	454	200
exo1/exo1	10	38
msh2/msh2	85	6,000
pol3-01/pol3-01 exo1/exo1	8,460	4,500
pol3-01/pol3-01 msh2/msh2	21,310	5,670

^{a} Fold increase over the wild type.

(references 31 and 33; also see Table 1). The pattern of a relatively weak effect on short runs and a strong effect on long runs is typical for an *msh2* defect (reference 40 and Table 1). These observations led us to determine the consequences of combining these two mutations (i.e., *exo1* and *msh2*). Since there was no synergy for the combined *exo1 msh2* mutants as compared to *msh2*, it appears either that *msh2* is epistatic to *exo1* or that their effects are additive (Table 1). These modes of interaction cannot be distinguished because of the relatively small effect of the *exo1* mutant on mutation rates.

Mutator phenotype resulting from a lack of DNA 3′→5′ Polε proofreading and 5′→3′ Exo1 exonucleases. The mutator phenotype of the Exo1 defects suggests that this protein might be involved in MMR. Since DNA polymerase proofreading and postreplication MMR act in series, we examined the mutator phenotype of double mutants defective in DNA Polε proofreading (*pol2-4*) and the 5′→3′ exonuclease Exo1. The double mutant lacking proofreading and Exo1 activities exhibited a synergistic mutator effect for all mutation rates examined (Tables 1 and 2). The *pol2-4* mutant is a weak mutator (less than a sevenfold increase over the wild type; Table 1). In the *pol2-4 exo1* double mutant, mutation rates were increased by as much as 10- to 100-fold over those for either single mutant (Tables 1 and 2).

Previously, the 3′→5′ proofreading exonuclease activity of DNA Polε had been shown to have almost no effect on frameshift mutations in long homonucleotide runs (≥8 nucleotides) during replication (40). The lack of proofreading in long homonucleotide runs accounts for the absence of a multiplicative mutator effect when the proofreading exonuclease mutant is combined with a complete (*msh2*) or partial (*msh3* and *msh6*) defect in the MMR system (Tables 1 and 2). The synergistic effect of *exo1* with the *pol2-4* defect for mutations in long homonucleotide runs suggests that the Polε-exonuclease can prevent errors by a process other than replication proofreading (see Discussion).

Mutation in DNA Polδ proofreading and Exo1 or Msh2 is lethal. The synergism of mutations in *exo1* with *pol2-4* led us to examine the double mutant phenotype for *exo1* with *pol3-01*, a 3′→5′ exonuclease deficient mutation of DNA Polδ. The *pol3-01* mutant exhibited a much stronger mutator effect than the *pol2-4* strain in all assays examined (Table 1). We failed to obtain an *exo1 pol3-01* double mutant by the deletion of the *EXO1* gene in a *pol3-01* strain. To analyze the apparent synthetic lethality of the double mutant, we crossed a *pol3-01* and an *exo1* mutant (see Materials and Methods) and subsequently inactivated the second copy of the *EXO1* gene with an *exo1::URA3* DNA fragment. Among 14 tetrads analyzed, all segregated as 2 viable:2 nonviable. Microscopic analysis after 4

days revealed that the two spores that did not yield colonies underwent several divisions, resulting in microcolonies containing approximately 100 cells. Using PCR and restriction analysis (see Materials and Methods), we established that the viable spores contained the wild-type copy of the *POL3* gene. Thus, *exo1 pol3-01* mutants are inviable.

We transformed the *pol3-01 lys2::insE-A$_{14}$* strain with plasmid pBL304 (24) carrying the *POL3* and the *URA3* genes. The *POL3* gene on the plasmid suppresses the mutator phenotype of the chromosomal *pol3-01* mutation (data not shown). Subsequently the *EXO1* gene was deleted from the *pol3-01* strain carrying the pBL304 plasmid to assess the compatibility of *pol3-01* and *exo1*. Since the *pol3-01* mutation was previously shown to be lethal in combination with the MMR *pms1* mutant (23), we also deleted *MSH2* from the *pol3-01* pBL304 background. While the *pol3-01* pBL304 strain lost the plasmid at a high rate when measured on 5-FOA medium, which selects against the Ura$^+$ phenotype (~10^{-2} loss events per cell/generation), the *pol3-01 msh2* pBL304 or *pol3-01 exo1* pBL304 strain demonstrated an undetectable level of plasmid loss (5-FOA^r colonies formed at a rate lower than 10^{-5} loss events per cell/generation). Based on PCR analysis, the 5-FOA^r clones derived from the *pol3-01 msh2* pBL304 or *pol3-01 exo1* pBL304 strain still retain the wild-type copy of the *POL3* gene. Thus, the *pol3-01 msh2* and *pol3-01 exo1* haploid double mutants are inviable.

Viability of *msh2 pol3-01* and *exo1 pol3-01* diploids. A likely explanation for the inviability of the *pol3-01 msh2* and *pol3-01 exo1* haploid strains is that unedited replication errors generated by the proofreading-deficient DNA Polδ are lethal to a haploid cell. If this were the case, then diploid strains might be viable due to the fact that most mutations are recessive. We constructed diploid strains that contained the pBL304 plasmid and had the following homozygous mutations: *pol3-01/pol3-01 msh2/msh2* and *pol3-01/pol3-01 exo1/exo1* (see Materials and Methods). The plasmid loss rates in the diploid strains were measured on 5-FOA media. Unlike the haploid strain, all diploid strains were able to lose the plasmid rapidly (3 × 10^{-3} loss events/cell/generation).

Synergistic interaction between a DNA Polδ 3′→5′ proofreading defect and *exo1* or *msh2* mutations in diploid strains. The genetic interaction between *pol3-01* and *msh2* or *exo1* in diploid strains was studied by examining the reversion of homozygous *lys2::insE-A$_{14}$* and *his7-2* mutations. (While the *his7-2* molecular defect is not known, it has been used in several studies investigating proofreading and MMR defects [22, 23, 24].) As shown in Table 3, the *pol3-01/pol3-01 msh2/*

msh2 double mutant exhibits a His$^+$ reversion rate 47-fold higher than that of the *pol3-01/pol3-01* strain and 250-fold higher than that of the *msh2/msh2* diploid. The rate of Lys$^+$ reversion in the A$_{14}$ homonucleotide run in the *pol3-01/pol3-01 msh2/msh2* strain is similar to that in an *msh2/msh2* strain (Table 3). The data are consistent with previous suggestions based on in vitro and in vivo data (17, 40) that DNA polymerase exonucleolytic proofreading becomes ineffective during replication of long repetitive sequences (e.g., ≥8 nucleotides in a homonucleotide run). The strong synergistic interaction between *exo1* and *pol3-01* was observed for Lys$^+$ as well as His$^+$ reversion (Table 3); the reversion rates were multiplicative for the double versus single mutants. The rate of Lys$^+$ reversion for the A$_{14}$ homonucleotide run in the *pol3-01/pol3-01 exo1/exo1* strain was 23-fold higher than the rate in the *pol3-01/pol3-01* strain and 120-fold higher than the rate in an *exo1/exo1* strain. The reversion rates to His$^+$ and Lys$^+$ in the *pol3-01/pol3-01 exo1/exo1* strain are approximately the same as those found in the *pol3-01/pol3-01 msh2/msh2* double mutant (Table 3). In contrast, in a Pol$^+$ strain the *exo1* deficiency increases the mutation rate in the A$_{14}$ run 100-fold less than an *msh2* deficiency (Table 1). Since DNA Polδ exonucleolytic proofreading is inefficient during replication of a long homonucleotide run (A$_{14}$), the synergistic effect between *exo1* and *pol3-01* for mutations in long homonucleotide runs suggests that the Polδ-exonuclease can prevent errors by processes other than replication proofreading.

DISCUSSION

We have investigated mutator effects due to the inactivation of the 5′→3′ Exo1 and 3′→5′ DNA polymerase exonuclease activities in yeast. To investigate possible genetic networks of mutation avoidance, we combined *exo1* with mutations in MMR genes and/or deficiencies in the 3′→5′ proofreading activities of the DNA polymerase genes. Based on the phenotypes of the mutants and double or triple mutants, we propose that the exonuclease activities of Exo1, Polδ, and Polε participate directly in MMR.

Participation of the Exo1 5′→3′ exonuclease in MMR. It has been proposed that Exo1 may function in MMR (38). The *exo1* defect caused a nearly 100-fold increase in −1 frameshift mutations within the A$_{14}$ homonucleotide run but had a smaller effect in other assays (two- to threefold increases for A$_4$, A$_5$, and A$_{12}$ runs and a 6.5-fold increase for the Can^r forward mutation; see Table 1). In all cases, the mutation rate in the *exo1 msh2* mutant was comparable to the rate in the *msh2* mutant (Table 1 and reference 38). This suggests that *msh2* is epistatic to *exo1* and that Exo1 is involved in the *MSH2*-dependent MMR pathway. (However, separate pathways resulting in the additivity of mutation rates cannot be excluded because the single *exo1* mutant exhibits a low mutation rate.)

The conclusion that Exo1 and Msh2 are epistatic is consistent with the following properties of the *exo1* mutant: (i) Msh2 interacts physically with the C-terminal region of Exo1 (38); (ii) defects in MMR or in Exo1 lead to a stronger mutator effect in long versus short homonucleotide runs (Table 1; references 11 and 40); and (iii) proofreading interacts with the MMR system based on the synergy between the double mutant *pol2-4 pms1* (24) and *pol2-4 msh2* (40) strains. The observed synergy between *exo1* and the proofreading defect (*pol2-4*) for all mutation detection systems examined (Table 1) is also consistent with a role for Exo1 in MMR. In addition, as reported previously for *pms1* (23) and for *msh2* (this work), the combination of *exo1* with the DNA Polδ 3′→5′ proofreading exonuclease defect (*pol3-01*) results in lethality for haploid strains,

which is likely due to excessive errors. Diploid *pol3-01 msh2* and *pol3-01 exo1* strains are viable and exhibit synergistic mutator effects (Table 3).

Participation of 3′→5′ exonucleases of Polε and Polδ as well as the 5′→3′ exonuclease Exo1 in error avoidance. Proofreading decreases with the increased size of the homonucleotide run that is replicated (17, 40). Previously it has been shown that both MMR and DNA Polε proofreading are efficient at preventing mutations in short runs (synergy had been shown for *pol2-4* and *pms1* or *msh2* double mutants [24, 40], while only MMR prevents frameshift mutations in homonucleotide runs larger than 7 nucleotides [40]). It has been proposed that frameshift intermediates generated in long runs escape Polε proofreading but are correctable by MMR. This would explain the lack of synergy for mutations in long homonucleotide runs when proofreading (*pol2-4*) and *msh2*, *msh3*, or *msh6* mutants are combined (Table 2 and reference 40).

Unlike the situation with the double mutant *pol2-4 msh2*, *pol2-4 msh3*, and *pol2-4 msh6* strains, synergy was clearly observed for mutations in long homonucleotide runs when *pol2-4* was combined with an *exo1* mutation (Table 2). The combination of *pol2-4* and *exo1* led to an increase in the mutation rate of up to 55-fold over that found for either single mutant, consistent with the finding that MMR in eukaryotes may be bidirectional (7, 37). Since Polε-exonuclease proofreading is absent in long homonucleotide runs, we propose that this synergy is a manifestation of both gene products (Exo1 and Polε) participating in error avoidance. While other mechanisms might be involved we suggest that the Exo1 and Polε exonucleases function in the MMR of long homonucleotide runs and compete for substrates when mismatch recognition components are present (i.e., Msh2, Msh3, and Msh6). Another possibility is that the MMR system is saturated in a *pol2-4* mutant due to the accumulation of mismatches. Thus, a partial defect in MMR due to loss of Exo1 might lead to a synergistic increase in the instability of long homonucleotide runs. However, this contradicts the lack of synergy for mutation of long homonucleotide runs in double mutants between *pol2-4* and either *msh3* or *msh6*, which are partially defective in MMR (Table 2).

The proposed role for the Exo1 and Polε exonucleases could occur if the two types of exonucleases participate in the excision of mismatches in a manner similar to that found for the RecJ and ExoVII 5′→3′ exonucleases and the Exo1 3′→5′ exonuclease in *E. coli* MMR (21). However, in *E. coli* the *recJ xseA* double mutant defective in RecJ and ExoVII is not hypermutable, implying that the system functions efficiently even when restricted to a unidirectional mode of action (5). Recently, Viswanathan and Lovett (44) reported that frameshift mutations were stimulated in a RecJ$^-$ Exo1$^-$ ExoVII$^-$ triple mutant, although base substitution mutations were not increased in several assays. This mutator effect was primarily due to a synergistic interaction between the Exo1$^-$ and ExoVII$^-$ mutations.

Based on in vitro data, Longley et al. (20) have suggested that DNA Polδ participates in MMR at the resynthesis step. Our genetic data suggest that DNA Polδ proofreading exonuclease may also be directly involved in the excision step of MMR. Similar to the DNA Polε proofreading exonuclease, DNA Polδ-exonuclease also appears inefficient during replication of the homonucleotide run A$_{14}$, so there is no synergistic interaction between proofreading defects and *msh2* (Tables 2 and 3). While the haploid *pol3-01 exo1* strain is inviable, the homozygous diploid strain is viable and exhibits a strong synergistic mutator interaction both for the instability of the A$_{14}$ run and the reversion of *his7-2* (Table 3). The synergism between *exo1* and *pol3-01* for mutations in the A$_{14}$ run suggests
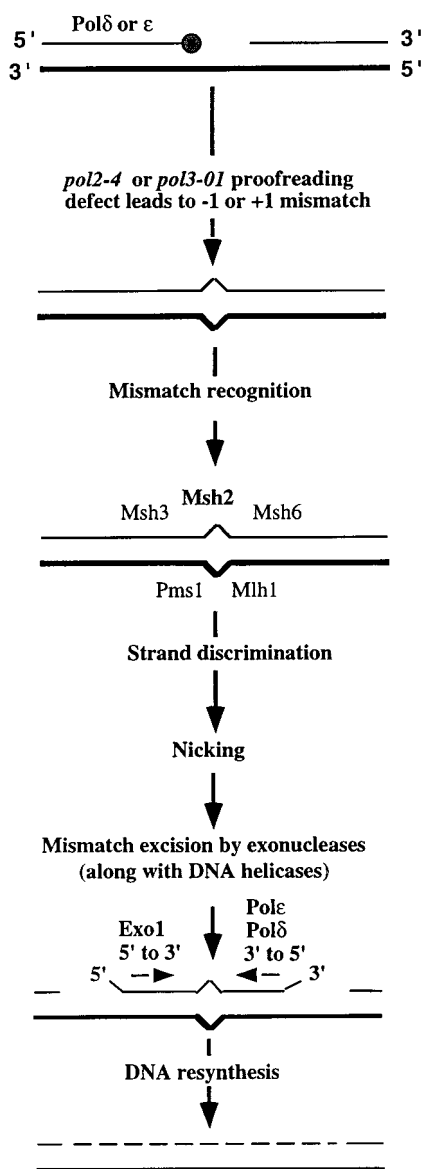
FIG. 1. Model of interaction between $3' \rightarrow 5'$ and $5' \rightarrow 3'$ nucleases in the appearance and prevention of frameshift mutations. The small shaded circle represents DNA polymerase. As discussed in the text, frameshift intermediates escape proofreading in long homonucleotide runs. The DNA Polε and DNA Polδ $3' \rightarrow 5'$ exonucleases as well as the Exo1 $5' \rightarrow 3'$ exonuclease, in conjunction with a DNA helicase similar to the helicase involved in *E. coli* MMR (5, 18), could participate in mismatch removal in opposite directions, so that multiple enzyme deficiencies cause synergistic mutator effects for frameshift mutations.

that the Polδ-exonuclease prevents errors by processes other than proofreading. Since Exo1 is likely to be involved in MMR (reference 38 and this work) we propose that the mutator synergy between *exo1* and *pol3-01* is due to these exonucleases being able to act on the same substrate. Therefore, an essential step in MMR (such as removal of the mismatch) could not occur if both exonuclease functions were inactivated. This view is supported by the observation that the rate of reversion to Lys[+] and His[+] in the *pol3-01/pol3-01 exo1/exo1* double mutant is comparable to the rate in the *pol3-01/pol3-01 msh2/msh2* double mutant (Table 3).

Presented in Fig. 1 is a model describing how Exo1 along with DNA Polδ and/or DNA Polε could act to reduce frame-

shift mutations in homonucleotide runs. The DNA Polδ and Polε $3' \rightarrow 5'$ proofreading exonuclease reduces the incidence of frameshift intermediates at the time of replication. Frameshift intermediates that escape proofreading are subsequently corrected by MMR. The synergy between the *pol2-4* (or *pol3-01*) and the *exo1* mutation in short homonucleotide runs (or in the *his7-2* reversion assay) can be explained by the participation of Exo1 in the removal of mismatches generated during replication by the proofreading-deficient Polε- or Polδ-exonuclease (i.e., acting in series with proofreading). Although the proofreading exonucleases of Polδ and Polε have no function in long homonucleotide runs, we propose that they function along with the $5' \rightarrow 3'$ exonuclease of Exo1 to excise mismatches in the MMR pathway. The degradation process would be facilitated by DNA helicases, since Exo1, Polε, and Polδ nucleases degrade single-stranded DNA efficiently (9, 22, 32). (In an *E. coli* reconstituted MMR system, both a DNA helicase and exonucleases are required for mismatch removal [5, 18].) In the framework of this model, the stronger synergy of the *pol2-4* or the *pol3-01* mutation with the *msh2* defect in MMR for mutations in short as compared to long homonucleotide runs can be due to *pol2-4* or *pol3-01* affecting both proofreading and MMR in short homonucleotide runs, but only MMR in the longer runs.

The importance of the combination of Polδ $3' \rightarrow 5'$ exonuclease with Exo1 in error prevention is apparent for the long homonucleotide run and provides strong support for our model, in which these nucleases are involved in a late step of strand-specific mismatch removal. For the long homonucleotide runs A_{12} and A_{14}, in which Polε-exonuclease proofreading is inefficient, a defect in Exo1, Polε-exonuclease, or Polδ-exonuclease alone results in a mutation rate that is <1% of that for an *msh2* strain. The *pol2-4 exo1* double mutant exhibits mutation rates that are 10% of those seen with the *pol2-4 msh2* strains (Table 1). However the combination of *pol3-01* with *exo1* in a diploid strain (the haploid strain is inviable) has an even more profound effect, resulting in mutation rates that are comparable to the most mutation-prone genetic combination, *pol3-01 msh2* (mutation rates of the *pol3-01 exo1* mutant are from 40 to 80% of those seen in the *pol3-01 msh2* mutant; Table 3). Assuming that their impact on the mutation rate is directly due to a role in MMR, our genetic results suggest that Polδ-exonuclease and Exo1 may be responsible for much of the mismatch excision during MMR. The synergistic interaction between Polδ-exonuclease and Exo1 in MMR is similar to that found for Msh3 and Msh6. While the single mutant *msh3* and *msh6* strains have a small effect on the mutation rate, the *msh3 msh6* double mutant exhibits a mutator phenotype comparable to that of the *msh2* mutant (Table 1). We therefore suggest that Exo1 and Polδ-exonucleases are major exonucleases involved in the mismatch excision step.

Formally, it is possible that there are two separate pathways (polymerase exonucleases plus Exo1 versus Msh2) of error avoidance, so that defects in both pathways might lead to additive increases in mutation rates. Because of the observed high error rates, it would be difficult to distinguish epistasis (same pathway) from additivity (separate pathways). Consistent with a single pathway is the fact that the Lys[+] frameshift reversion spectra in the *msh2*, *msh2 pol2-4*, and *exo1 pol2-4* strains are similar in that they exhibit hotspots in the A_4 and A_5 homonucleotide runs of the *lys2::insE-A$_4$* and *lys2::insE-A$_5$* alleles, respectively (Table 1).

Roles of $5' \rightarrow 3'$ and $3' \rightarrow 5'$ nucleases in DNA metabolism. We and others have found that several combinations of nuclease mutations can lead to lethality for haploid yeast cells (Table 4). The inviability of a double mutant has been attributed

TABLE 4. Effects on viability and mutation rates in haploid strains that are double mutants for deficiency in nuclease and/or MMR activities

Mutation	Effect on viability and mutation rates when combined with:		
	msh2	pol2-4	pol3-01
exo1	Epistasis	Synergy	Inviable[a]
pol3-01	Inviable[b,c]	Inviable[d]	
pol2-4	Synergy[c]		

[a] The *pol3-01 exo1* haploid double mutant is not viable. The diploid strain exhibits a synergistic hypermutable phenotype (Table 3).

[b] The *pol3-01* mutant is also inviable in combination with a strain with another MMR defect, *pms1*; the *pol3-01 pms1* (23) and *pol3-01 msh2* diploid strains are viable and exhibit a synergistic hypermutable phenotype (Table 3).

[c] The synergistic interactions between *msh2* and proofreading defects (*pol2-4* and *pol3-01*) are observed only for short runs and base substitutions but not in the long homonucleotide runs A_{12} and A_{14} for *pol2-4* (Table 2) or in the A_{14} run for *pol3-01* (Table 3).

[d] Morrison and Sugino (24). The *pol2-4 pol3-01* haploid double mutant is not viable. The diploid strain exhibits a synergistic hypermutable phenotype.

to a high mutation rate resulting in error catastrophe (8, 23, 24). In *E. coli* the *dnaQ926* mutation appears to cause lethality as a result of the loss of proofreading and the subsequent saturation of DNA MMR. Consistent with this, *dnaQ926* strains are viable if they carry a *dnaE* antimutator allele or a multicopy plasmid with the *E. coli mutL* gene (8).

The *pol2-4 exo1* double mutant is viable and exhibits a synergistic increase in mutation rate relative to the single mutants. The *pol3-01 msh2* and *pol3-01 exo1* haploid strains are inviable. However, *pol3-01 msh2* and *pol3-01 exo1* double mutants are viable as diploid strains. The inviability of the haploid double mutant *pol3-01 msh2* and *pol3-01 exo1* strains is most likely due to an accumulation of excessive replication errors based on the hypermutability of the homozygous diploid strains (Table 3).

Our results suggest that both DNA Polε-exonuclease and DNA Polδ-exonuclease participate in the removal of mismatches during MMR and that DNA Polδ is associated with MMR (20). It is interesting that both DNA Polδ and DNA Polε interact with PCNA (6), which is also involved in MMR (12, 43). DNA Polδ and DNA Polε might be recruited to the MMR complex by an interaction with PCNA or with other MMR proteins. Thus, mutations in the putative MMR protein interaction domains of DNA Polε and DNA Polδ could lead to a defect in the excision step of MMR. Such mutations might exhibit phenotypes similar to *pol2-4* and *pol3-01* in combination with *exo1* (i.e., instability of long homonucleotide runs but normal proofreading). Recently we identified such a mutation in the *POL2* gene located between the polymerase and checkpoint domains (14a). A search for similar mutations in the *POL3* gene is in progress.

Previously, it was reported that the *pol2-4 pol3-01* haploid double mutant was also inviable, but diploid double mutants are viable and exhibit a mutator synergy (24). It was proposed that even if MMR is functional, a complete deficiency in proofreading of both polymerases can lead to high mutation rates, resulting in error catastrophe. As an alternative, we propose that the double defect in *pol2-4 pol3-01* leads not only to a loss of proofreading but also to a defect in MMR; this reflects the participation of polymerase-associated exonucleases in MMR as described above (Fig. 1).

As summarized in Table 4, this and other studies demonstrate that while defects in the individual Exo1, DNA Polδ, and DNA Polε nucleases may have a small effect, the combination of mutations can have a profound impact on genome stability.

Single mutations in these exonucleases have only a moderate mutator phenotype in comparison with a mutation in *MSH2*. We have shown that double mutations in each pair of these three nuclease genes can lead to strong, synergistic mutator effects or lethality for haploid yeast. The deletion of another 5′→3′ exonuclease gene, *RAD27*, in combination with *pol3-01* or *exo1* mutations results in lethality (15, 38). In recent studies with this flap endonuclease, we have shown that it also exhibits synergy with either *pol2-4* or *msh2*, suggesting a possible role in mismatch excision (unpublished data).

Synergistic interactions affecting genome instability, such as those described in this work, have important implications for the human genome, in which genetic polymorphisms are common. Given the close relationship between yeast and human genes involved in replication and repair, particularly MMR, similar relationships may apply to the equivalent pathways in human cells. For example, synergistic effects might be expected for partially defective gene products that alone cause a weak phenotype. These alleles would then cause a much more serious deficiency and phenotype when combined with other weakly defective proteins that act in overlapping genetic pathways.

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