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Exonuclease 1 Preferentially Repairs Mismatches Generated by DNA Polymerase α

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Abstract

The *Saccharomyces cerevisiaeEXO1* gene encodes a 5′ exonuclease that participates in mismatch repair (MMR) of DNA replication errors. Deleting *EXO1* was previously shown to increase mutation rates to a greater extent when combined with a mutator variant (*pol3-L612M*) of the lagging strand replicase, DNA polymerase δ (Pol δ), than when combined with a mutator variant (*pol2-M644G*) of the leading strand replicase, DNA polymerase ε (Pol ε). Here we confirm that result, and extend the approach to examine the effect of deleting *EXO1* in a mutator variant (*pol1-L868M*) of Pol α, the proofreading-deficient and least accurate of the three nuclear replicases that is responsible for initiating Okazaki fragment synthesis. We find that deleting *EXO1* increases the mutation rate in the Pol α mutator strain to a significantly greater extent than in the Pol δ or Pol ε mutator strains, thereby preferentially reducing the efficiency of MMR of replication errors generated by Pol α. Because these mismatches are closer to the 5′ ends of Okazaki fragments than are mismatches made by Pol δ or Pol ε, the results not only support the previous suggestion that Exo1 preferentially excises lagging strand replication errors during mismatch repair, they further imply that the 5′ ends serve as entry points for 5′ excision of replication errors made by Pol α, and possibly as strand discrimination signals for MMR. Nonetheless, mutation rates in the Pol α mutator strain are 5- to 25-fold lower in an *exo1Δ* strain as compared to an *msh2*Δ strain completely lacking MMR, indicating that in the absence of Exo1, most replication errors made by Pol α can still be removed in an Msh2-dependent manner by other nucleases and/or by strand displacement.

Keywords

Exo1; mismatch repair; genome stability; replication fidelity

1. Introduction

Because the two strands of duplex DNA are anti-parallel and DNA polymerases only copy DNA in the 5′ to 3′ direction, coordinated replication of the eukaryotic nuclear genome is intrinsically asymmetric, with a leading strand replicated first and a lagging strand replicated slightly thereafter as a series of ~200 base Okazaki fragments (reviewed in [1]). We are interested in understanding relationships between this asymmetry and the fidelity of DNA replication. To investigate this, we are using *Saccharomyces cerevisiae* strains containing mutant alleles of the *POL1* (Pol α), *POL2* (Pol ε) and *POL3* (Pol δ) genes. These alleles,

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pol1-L868M [2, 3], *pol2-M644G* [4] and *pol3-L612M* ([5] and references therein), encode replicases with single amino acid replacements at the polymerase active site that retain high replicative capacity but have reduced fidelity. Strains harboring these alleles have elevated spontaneous mutation rates and a specific error bias signature, thereby identifying the polymerases responsible for generating the majority of replication errors *in vivo*. The patterns of mutagenesis in these strains [4–7] and the strand-specific incorporation of ribonucleotides during replication by Pol ε [6, 7], suggest that under normal circumstances, Pol ε is the primary leading strand replicase, while Pol δ primarily participates in lagging strand replication after Pol α-primase initiates the synthesis of Okazaki fragments.

DNA replication fidelity is determined by the nucleotide selectivity of pol α, δ and ε, by the 3′ exonucleolytic proofreading activities intrinsic to Pols δ and ε (but not Pol α), and by mismatch repair (MMR) of replication errors that escape proofreading. MMR begins when a mismatch generated during nuclear DNA replication is recognized either by Msh2-Msh6 (MutSα), which recognizes single base-base and small insertion-deletion (indel) mismatches, or by Msh2-Msh3 (MutSβ), which recognizes indel mismatches with a specificity that is partially redundant with Msh2-Msh6 ([8–10] and reviewed in [11]). Mismatch recognition initiates a series of downstream steps that ultimately remove the replication error from the nascent strand and allow new DNA to be correctly synthesized. In addition to the mismatch, the MMR machinery requires a signal to direct excision to the newly synthesized strand containing the replication error. Studies *in vitro* show that the signal can be a nick or gap located either 3′ or 5′ to the mismatch, with the protein requirements for MMR differing somewhat depending on the location of the DNA ends relative to the mismatch [12–14]. The origin and exact nature of the strand discrimination signal used for MMR *in vivo* remains uncertain. Attractive possibilities for this signal include the 3′ ends of growing chains at the replication fork and/or the 5′ ends of Okazaki fragments that are transiently present during lagging strand replication.

To probe the enzymology of MMR *in vivo* and gain insight into the nature of the strand discrimination signal, we are examining the extent to which MMR specificity and efficiency varies as a function of several variables, most especially the polymerase that generates the error. This focus is motivated by a specific prediction that emerges from several related observations. Early studies in *E. coli* demonstrate that MMR most efficiently corrects those mismatches generated at the highest rates during replication, i.e., transition and indel mismatches [15–17]. If reciprocity between production of mismatches and their repair is evolutionarily conserved in eukaryotes, then MMR should not only more efficiently correct transition and indel mismatches, it may also more efficiently correct mismatches made by Pol α as compared to those made by Pol δ, because Pol α is naturally exonuclease deficient and generates mismatches at substantially higher rates than does proofreading-proficient Pol δ [18]. Early studies also showed that the efficiency of MMR in *E. coli* is inversely proportional to the distance between the mismatch and the strand discrimination signal [19]. In the initial study showing that PCNA was required for eukaryotic MMR at a step prior to mismatch excision, we proposed [20] that PCNA could physically link MMR and replication in a manner that would allow DNA ends associated with replication to serve as strand discrimination signals. That study was followed by another [21] which led to the more specific hypothesis that the 5′ ends of Okazaki fragments could signal for MMR of replication errors in the nascent lagging strand. If the relationship between distance and bacterial MMR efficiency is conserved in eukaryotes, and if the 5′ ends of Okazaki fragments can serve as strand discrimination signals, this too predicts that MMR might more efficiently correct mismatches made by Pol α as compared to mismatches made by Pol δ. This is because mismatches made as Pol α initiates Okazaki framents will be closer to DNA 5′ termini than would more internal mismatches made by Pol δ. We initially tested this prediction by comparing mutation rates in L612M Pol δ and L868M Pol α strains that were

either MMR proficient or deleted for *MSH2* (*msh2Δ*) and therefore lacked both Msh2-Msh6-dependent and Msh2-Msh3-dependent MMR activity. Pairwise comparisons of specific single base mutation rates in these strains provided an apparent MMR efficiency for errors made by each polymerase. We found that mismatches made by Pol δ are repaired efficiently, but the equivalent single base-base mismatches made by Pol α appear to be corrected even more efficiently [22]. Possible explantions for this higher efficiency include use of the 5′ ends of Okazaki fragments to initiate mismatch removal, either by strand displacement [23] or by a 5′ exonuclease.

Based on genetic evidence [22], we previously proposed that one candidate for an exonuclease to perform mismatch excision initiated at the 5′ end of an Okazaki fragment is exonuclease 1 (Exo1), which is well known to participate in eukaryotic MMR [24, 25]. In support of this idea, Kolodner and colleagues [26] recently reported that deletion of *EXO1* increases the mutation rate of the *pol3-L612M* mutator strain to a greater extent than for the *pol2-M644G* mutator strain. Here we extend this effort by examining the mutagenic consequences of deleting *EXO1* (*exo1Δ*) in all three strains, *pol1-L868M*, *pol2-M644G* and *pol3-L612M*. The hypothesis that the 5′ DNA ends of Okazaki fragments are used for MMR predicts that deleting Exo1 should reduce the efficiency of MMR of errors made by Pol α even more than for errors made by Pol δ. This is exactly what we observe. We also compare the results to those seen upon complete loss of all Msh2-dependent MMR in the same polymerase mutator strains [6, 22]. The results lead to three interpretations. (I) Exo1 has a much greater role in repairing lagging strand replication errors as compared to leading strand replication errors, supporting a similar conclusion by Kolodner and colleagues [26] and extending it to the second essential lagging strand replicase. (II) The 5′ ends of Okazaki fragments are entry points for 5′ to 3′ excision of mismatches by Exo1 during MMR *in vivo*, and they may also serve as strand discrimination signals. (III) Like DNA replication, MMR enzymology *in vivo* can differ on the lagging and leading strands.

2. Materials and Methods

Strains, mutation rates, and sequencing ura3 mutants

All strains used in this study are isogenic derivatives of strain Δ|(2)|-7B-YUNI300 (*MAT*a *CAN1 his7-2 leu2-Δ::kanMX ura3-Δ trp1-289 ade2-1 lys2-ΔGG2899-2900*) [27]. Mutator alleles; *pol1-L868M*, *pol2-M644G*, and *pol3-L612M* have been described previously [2–5]. Heterozygous *EXO1/exo1*Δ diploids were generated by PCR based targeted gene-disruption. Deletion of *EXO1* was verified by phenotype and by PCR across the disrupted region, and haploids were obtained from tetrad dissection. Measurements of spontaneous mutation rates by fluctuation analysis were as described previously [4, 5]. For each *ura3* mutant sequenced, an independent colony was patched to YPDA and then replica plated to media containing 5-FOA. Genomic DNA from a single 5-FOA-resistant colony from each patch was isolated, and the *URA3* gene was amplified by PCR and sequenced.

Statistical analysis

To determine if differences in mutation rates between strains are significant we used a one-sided Mann-Whitney t-test.

3. Results and Discussion

3.1. The *exo1Δ* mutator effect is greater for Pol α than for Pol δ or Pol ε

Mutation rates for resistance to 5-fluoro-orotic acid (5-FOA) were determined in *EXO1* versus *exo1Δ* strains that encode either wild type DNA polymerases or mutator variants of Pol α (*pol1-L868M*), Pol δ (*pol3-L612M*) or Pol ε (*pol2-M644G*). Compared to the wild

type (*POLEXO1*) strain, the mutation rate of the *exo1Δ* single mutant strain was elevated by 12-fold, and as previously observed, the mutation rates in the *pol1-L868M, pol2-M644G* and *pol3-L612M* [4, 5] single mutant strains were each elevated by a few-fold (Fig. 1A). The mutation rate in the double mutant *pol1-L868M exo1Δ* strain was increased by 140-fold, to 51 $\times 10^{-7}$. This is greater than the 64-fold increase observed in the *pol3-L612M exo1Δ* strain (rate = 23 $\times$ 10^{-7}). The rates in these double mutant strains are much more than the sum of rates in the individual single mutant strains, consistent with the idea that two processes are acting in series, i.e., Exo1-dependent MMR is correcting replication errors that escape the fork. In contrast, the mutation rate in the *pol2-M644G exo1Δ* strain was 5.2 $\times$ 10^{-7}, which is remarkably close to the sum of the increases observed for the *pol2-M644G* and *exo1Δ* single mutants (5.7 $\times$ 10^{-7}). When mutation rates were measured at the *CAN1* locus in these same strains, similar relative differences among the double mutant strains were obtained (Fig 1B). At both *URA3* and *CAN1*, the rates in the *pol1-L868M exo1Δ* strain were significantly higher than the rates in the *pol3-L612M exo1Δ* and *pol2-M644G exo1Δ* strains (p values < 0.05, Fig. 1A).

3.2. Determining the specificity of *exo1Δ* mutator effects

The above interpretation depends on a demonstration that the mutations arising in the *pol1-L868M exo1Δ* and *pol3-L612M exo1Δ* strains are characteristic of partial loss of MMR of DNA replication errors rather than a defect in other DNA transactions in which Exo1 participates [28]. One advantage of the polymerase variants used here is that, in the absence of Msh2-dependent MMR, they have mutational signatures characteristic of common replication errors [22]. To determine if these signatures are observed in the *exo1Δ* strains, we examined the DNA sequence changes in the *URA3* gene responsible for 5-FOA-resistance, in independent mutants collected from the *pol1-L868M exo1Δ* and *pol3-L612M exo1Δ* strains. The majority of mutants did indeed contain single base substitutions and deletions (Table 1), and these were non-randomly distributed throughout the *URA3* open reading frame (Figure 2).

3.3. Exo1 preferentially participates in MMR of Pol α errors

Focused on the hypothesis that deleting Exo1 would reduce the efficiency of MMR of lagging strand replication errors made by Pol α more than for errors made by Pol δ, the data in Figures 1 and 2 were used to calculate specific mutation rates (Table 1 and Fig. 3A) and MMR correction factors (Fig. 3B) for three classes of mutations that are signatures of a complete loss of MMR in *pol1-L868Mmsh2Δ* strains [22]. These classes are (i) total single base errors (base substitutions and indels), (ii) total G to A transitions, which comprise 74% of all single base errors in *pol1-L868Mmsh2Δ* and *pol3-L612M msh2Δ* strains and are generated by misincorporation of dTTP opposite template G during lagging strand replication by Pol α (*pol1-L868M*) or Pol δ (*pol3-L612M*) and (iii) G to A transitions at characteristic hotspots in the *URA3* reporter gene (Figure 2 and 3B) [22]. Mutation rates for these events are shown on a log scale in Figure 3A, where rates for MMR-proficient strains are depicted as open bars, rates for *exo1Δ* strains are represented by open + grey bars, and rates for *msh2Δ* strains are represented by open + grey + black bars. Dividing the rates in the *exo1Δ* strains by the rates in the MMR-proficient strains provides the correction factor (CF values in Fig. 3B) for Exo1-dependent MMR of each type of error. Correction efficiencies for these mismatches in the *pol1-L868M* strain range from 43 to 490. These values are consistently higher than the 9- to 15-fold correction efficiencies for the same classes of mismatches generated in the *pol3-L612M* strain (Fig. 3B), thus supporting the hypothesis.

3.4. The role of Exo1 in MMR of lagging strand errors is important, but redundant

Dividing the mutation rates in the *pol1-L868M exo1Δ* strain by the rates in the *pol1-L868M msh2Δ* strain that is completely defective in MMR reveals yields values of 4% to 19% (Fig. 3C). Thus efficient MMR still occurs in the absence of Exo1.

4. Discussion

About 10% of Okazaki fragment synthesis is catalyzed by Pol α. This presents a strong challenge to nuclear genome stability because Pol α is a naturally proofreading-deficient replicase that is substantially less accurate than is proofreading-proficient Pol δ. An early idea for eliminating errors made by Pol α was that the mismatches it generates are removed by strand displacement synthesis during normal Okazaki fragment maturation, a process that historically has not invoked participation of MMR proteins [29]. However, canonical Okazaki fragment maturation proteins alone are insufficient to remove many Pol α errors, as clearly illustrated by the fact that the mutation rate of the *pol1-L868M* strain is very strongly elevated by deleting Msh2 (see [2, 5] and Fig. 3). Now, in addition to Msh2, the present study demonstrates the involvement of Exo1 in MMR of Pol α errors. That Exo1 is indeed correcting Pol α replication errors, rather than affecting mutation rates via another DNA transaction, is clearly illustrated by the increase in single base mutations characteristic of loss of MMR in the Pol α mutator strain (Figs. 2 and 3). Because mismatches generated by Pol α are closer to the 5′ ends of Okazaki fragments than are mismatches made by Pol δ, the stronger mutator effect of deleting Exo1 in the *pol1-L868M* strain strongly support the hypothesis that the 5′ ends of Okazaki fragments are entry points for Exo1-dependent excision of a mismatch, and may possibly serve as strand discrimination signals for MMR. The fact that deleting *EXO1* is much less mutagenic than deleting *MSH2* indicates that Pol α errors can also be corrected by an Exo1-independent MMR process. This repair may involve partially redundant nucleases, and/or a strand displacement mechanism to remove mismatches [13, 14, 23, 30–32]. The possibility that Exo1 is redundant with other exonucleases has been suggested before [11, 32–34], and our data extend this idea to include replication errors made by both lagging strand replicases. Finally, the fact that Exo1 influences MMR of errors made by the two lagging strand replicases to a greater extent than the leading strand replicase illustrates that, like DNA replication, eukaryotic DNA mismatch repair is enzymologically asymmetric.

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Highlights

- Deleting *EXO1* increases the mutation rate in the Pol α mutator strain to a significantly greater extent than in the Pol δ or Pol ε mutator strains.
- Exo1 preferentially excises lagging strand replication errors during mismatch repair
- Transient lagging strand 5′ ends serve as entry points for 5′ excision of replication errors made by Pol α, and possibly as strand discrimination signals for MMR.
- In the absence of Exo1, most replication errors made by Pol α can still be removed in an Msh2-dependent manner.

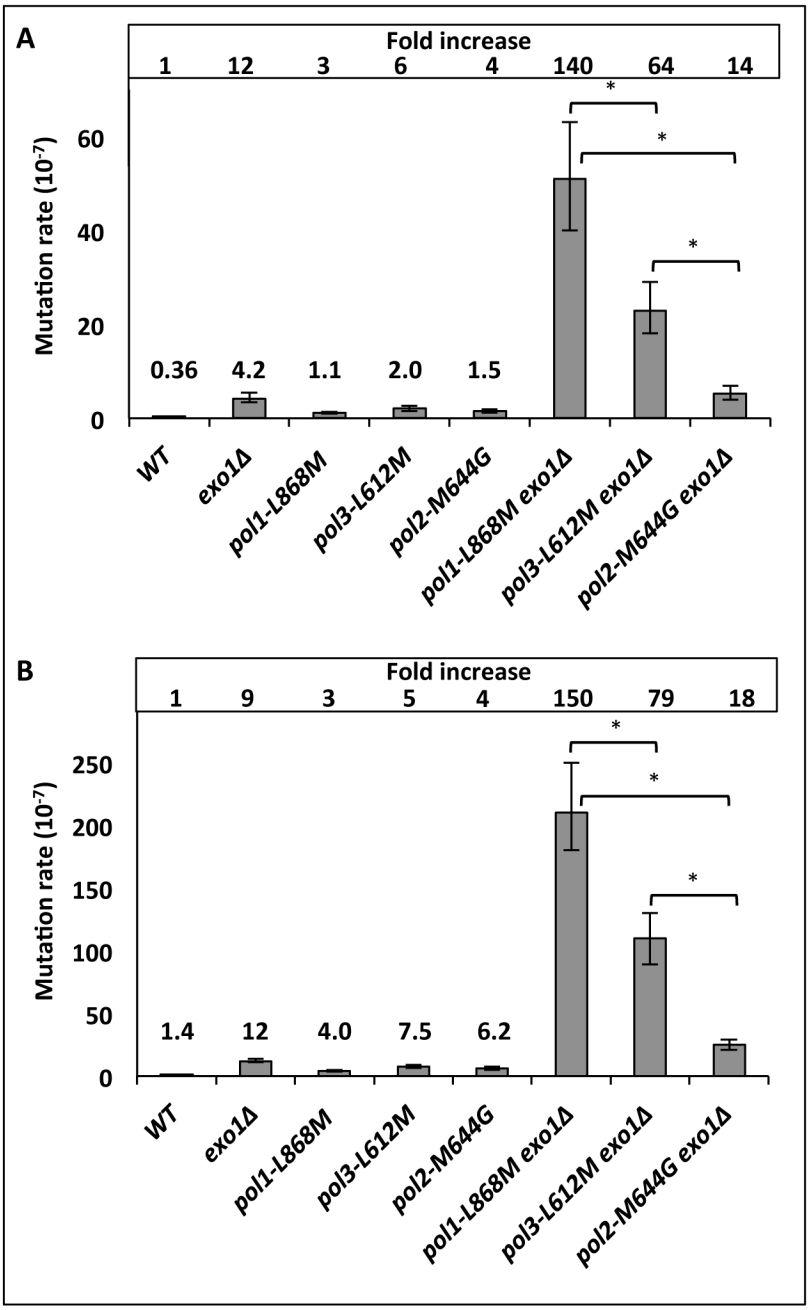

Figure 1. Polymerase-specific mutator effects in *exo1*Δ strains

Spontaneous mutation rates (with 95% confidence intervals) to **(A)** 5-FOA resistance and **(B)** canavanine resistance. Differences in mutation rates between the *pol1-L868M exo1Δ* and other two double mutant strains are statistically significant (p <0.05), as indicated by asterisks. Deleting *EXO1* results in a 46-fold and a 12-fold increase in mutation rates to 5-FOA resistance compared to the corresponding single pol mutants; *pol1-L868M* and *pol3-L612M*, respectively. For canavanine resistance, deletion of *EXO1* in a pol mutator background causes a mutation rate increase of 52-fold and 14-fold, compared to *pol1-L868M* and *pol3-L612M*, respectively.

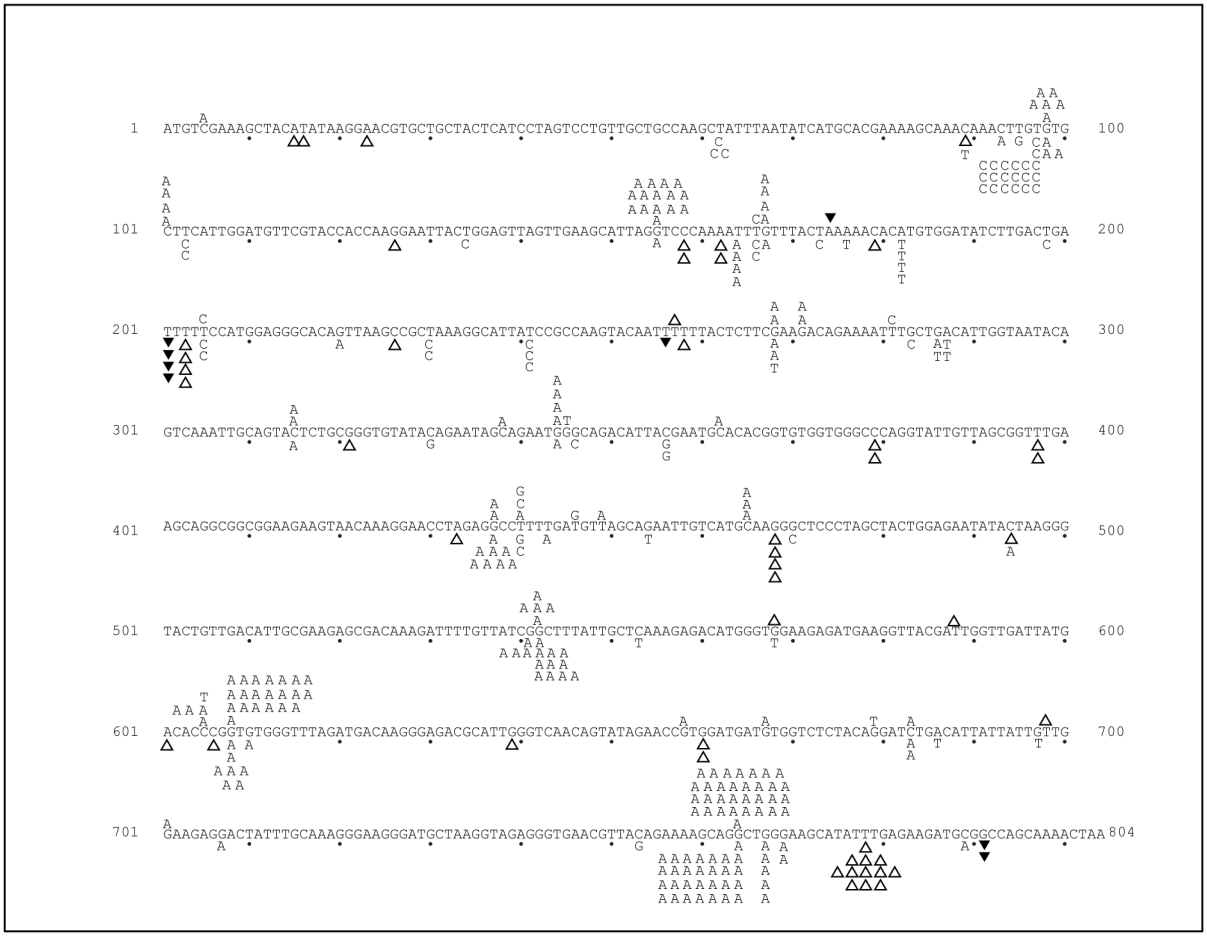

Figure 2. Spectra of *ura3* mutations in *exo1Δ* strains

The coding strand of the *URA3* ORF is shown, with every tenth base indicated by a closed dot. Single letters represent base substitutions, open triangles represent single-base deletions, and closed triangles represent single base insertions. Spectra for the *pol1-L868Mexo1* Δ and *pol3-L612Mexo1* Δ strains are shown above and below the *URA3* ORF, respectively.

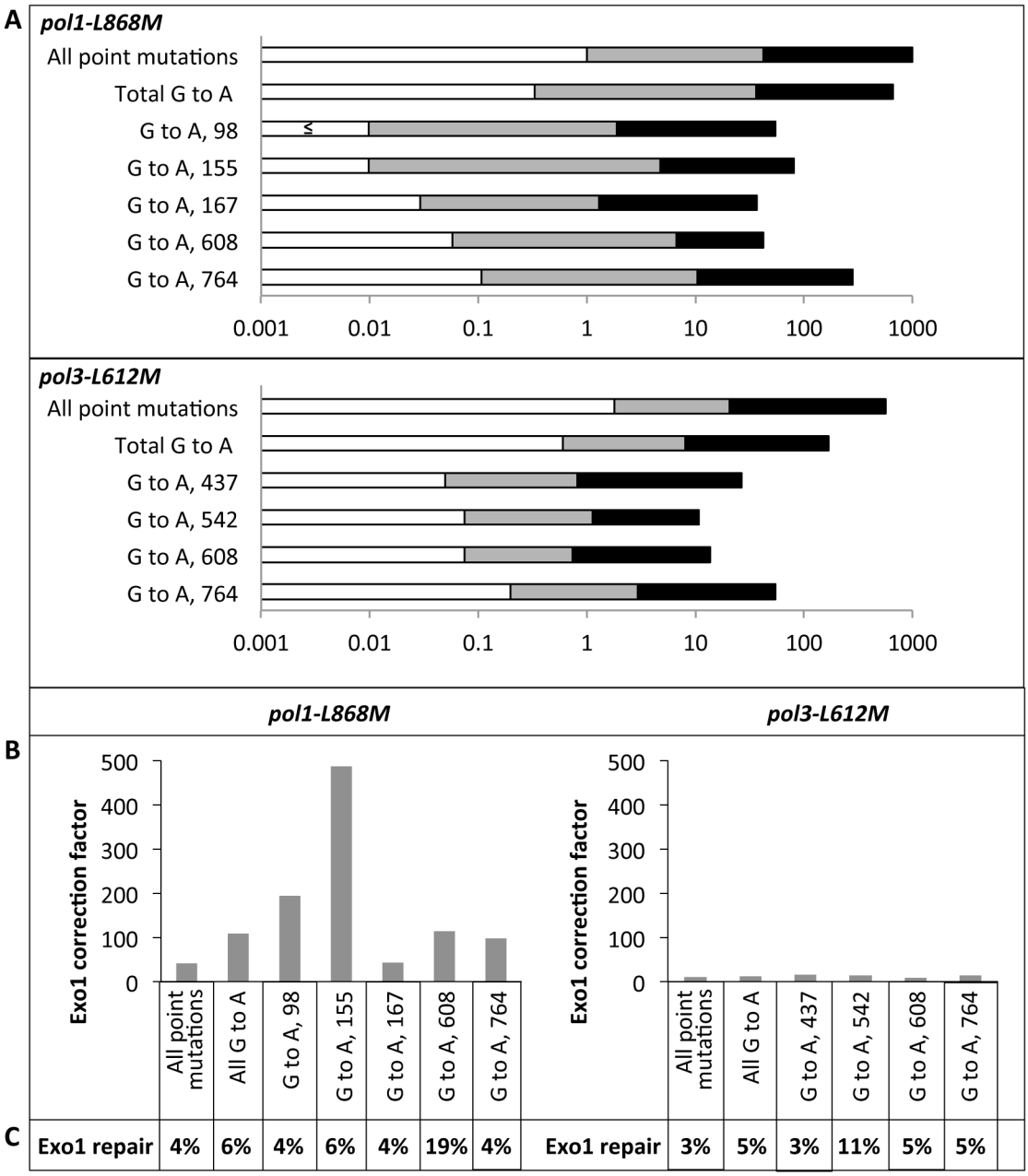

Figure 3. Rates and Exo1-dependent MMR correction factors for single base mutations in *pol1-L868M* and *pol3-L612M* strains

(A) The base substitution and its position in *URA3* are indicated on the left. Mutation rates ($\times 10^{-7}$) for specific base substitutions in *EXO1 MSH2* (open bars), *exo1Δ* (open + grey bars), and *msh2Δ* strains (open + grey + black bars) are shown on a log scale (x-axis). In cases where no occurrences were observed (see Table 1), the rate is estimated as (≤).**(B)** Exo1-dependent MMR correction factors are the rate in *exo1Δ* strains divided by the rate in *EXO1* strains. **(C)** The ratios of Exo1-dependent repair compared to Msh2-dependent repair, shown as percentages.

Table 1

Specific mutation rates for strains used in this study

	pol1-L868M			*po13-L612M*		
	MMR*	***msh2Δ****	***exo1Δ***	***MMR****	***msh2Δ****	***exo1Δ***
Total rate 95 % CI	1.7 (1.2–4.9)	1000 (960–2100)	51 (40–63)	2.7 (1.9–7.3)	560 (500–750)	23 (18–29)
***ura3* mutants sequenced**	175	180	162	217	174	241
All point mutations	100(1.0)	172 (960)	129(41)	144 (1.8)	172 (550)	201 (19)
Total G to A	34 (0.33)	112 (620)	114 (36)	50 (0.6)	49 (160)	77 (7.4)
G to A 98	0 (≤0.01)	9 (50)	6 (1.9)	n.a.	n.a.	n.a.
G to A 155	1 (0.01)	13 (72)	15 (4.7)	n.a.	n.a.	n.a.
G to A 167	3 (0.03)	6 (33)	4 (1.3)	n.a.	n.a.	n.a.
G to A 437	n.a.	n.a.	n.a.	4 (0.05)	8 (26)	8 (0.8)
G to A 542	n.a.	n.a.	n.a.	6 (0.07)	3 (9.7)	11 (1.0)
G to A 608	6 (0.06)	6 (33)	21 (6.6)	6 (0.07)	4 (13)	7 (0.7)
G to A 764	11 (0.1)	51 (280)	32 (10)	16 (0.2)	16 (52)	29 (2.8)

The number of events are shown and the specific rates are displayed in parenthesis

*From Nick McElhinny et al., 2010. Mutation rates and detailed error specificity for the wild type strain has been published in Nick McElhinny et al., 2010.

n.a.: no events or not a significant number of events.

A number of 5-FOA resistant mutants had no sequence change in the 804 base pair *URA3* open reading frame. These mutants were not investigated further, but they may result from epigenetic silencing, they may contain sequence change in the promoter or the 3* untranslated region of *URA3*, or they may contain mutations in other genes that result in 5-FOA resistance.