Report

Evidence that Errors Made by DNA Polymerase α are Corrected by DNA Polymerase δ

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Summary

Eukaryotic replication [1, 2] begins at origins and on the lagging strand with RNA-primed DNA synthesis of a few nucleotides by polymerase α, which lacks proofreading activity. A polymerase switch then allows chain elongation by proofreading-proficient pol δ and pol ε. Pol δ and pol ε are essential, but their roles in replication are not yet completely defined [3]. Here, we investigate their roles by using yeast pol α with a Leu868Met substitution [4]. L868M pol α copies DNA in vitro with normal activity and processivity but with reduced fidelity. In vivo, the *pol1-L868M* allele confers a mutator phenotype. This mutator phenotype is strongly increased upon inactivation of the 3′ exonuclease of pol δ but not that of pol ε. Several nonexclusive explanations are considered, including the hypothesis that the 3′ exonuclease of pol δ proofreads errors generated by pol α during initiation of Okazaki fragments. Given that eukaryotes encode specialized, proofreading-deficient polymerases with even lower fidelity than pol α [5], such intermolecular proofreading could be relevant to several DNA transactions that control genome stability.

Results and Discussion

L868M Pol α Has Normal Catalytic Efficiency and Processivity but Reduced Fidelity

Were pol α to synthesize five nucleotides of each ∼250 nucleotide Okazaki fragment on the lagging strand, it would synthesize 1% of the human genome. Given a pol α base substitution error rate of ∼10^{-4} [6], this would generate 6000 mismatches during each replication cycle. To test the hypothesis that these mismatches

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might be proofread by yeast pol δ and/or pol ε, we used a mutator allele of yeast pol α in which methionine was substituted for Leu868 at the polymerase active site [4]. The fidelity of DNA synthesis in vitro by the purified catalytic subunit of L868M pol α was compared to that of wild-type pol α by using an M13-based fidelity assay [7]. Error rates with wild-type yeast pol α were similar to those reported earlier [8]. In comparison, the error rate for single-base substitutions, representing an average value for all 12 possible base-base mismatches in numerous sequence contexts, was 5.3-fold higher for L868M pol α (Table 1). The rate for single-base insertion/deletion errors, representing an average value for errors in numerous repetitive and nonrepetitive sequences, was 6.6-fold higher for L868M pol α than for wild-type pol α (Table 1). Thus, L868M pol α synthesizes DNA in vitro less accurately than does wild-type pol α. To determine whether L868M pol α has altered ability to extend terminal mismatches generated by misinsertion, we measured steady-state kinetic parameters for extending a matched terminus versus one containing a T•G mismatch (Table 2). Each enzyme extended the mismatched terminus less efficiently than the matched terminus. However, compared to wild-type pol α, L868M pol α extended the T•G mismatch with higher relative efficiency, i.e., L868M was more promiscuous for mismatch extension. Promiscuity was also (qualitatively) observed for extension of a terminal A•A mismatch (data not shown). When all four dNTPs are included in extension reactions containing a matched primer-template in excess, to assure that each DNA product reflects processive polymerization, wild-type and L868M pol α were observed to have similar processivity (Figure 1). Moreover, equivalent amounts of wild-type and L868M pol α extended similar amounts of primers via a similar number of polymerization cycles (see legend to Figure 1). These results and the similar kinetic parameters for extension of correct termini (Table 2) both support the observation [4] that L868M pol α has a catalytic activity similar to wild-type pol α. They further suggest that L868M pol α is relatively normal with respect to initial enzyme binding and dissociation upon termination of processive synthesis.

The Exonuclease of Pol δ but Not Pol ε Modulates the L868M Pol α Mutator Effects

To test the hypothesis that pol δ and/or pol ε might proofread errors made by pol α, we measured spontaneous mutation rates in haploid yeast strains with single or double mutations in the *POL1*, *POL3*, and *POL2* genes encoding the catalytic subunits of pol α, pol δ, and pol ε, respectively. Rates were measured for mutations that inactivate the *CAN1* gene, for base substitutions that revert the *trp1-289* mutation to Trp+, and for frameshifts (largely +1 events in a run of seven A-T base pairs) that revert the *his7-2* mutation to His+. Compared to the wild-type strain (Table 3, line 1), the single mutant *pol1-L868M* strain had slightly elevated mutation rates at *CAN1* and *trp1-289* and a wild-type mutation rate at

Table 1. Fidelity of L868M pol α In Vitro

	Wild-Type pol α	L868M pol α
lacZ mutant frequency	0.02 (0.015)[a]	0.07 (0.13)[b]
lacZ mutants sequenced	23	109
Base substitutions	11	80
Error rate ($\times 10^{-4}$)	1.3	6.9 (5.3×)
Single-base insertions/ deletions	4	35
Error rate ($\times 10^{-4}$)	0.29	1.9 (6.6×)

Purification of pol α and its L868M derivative was as described [4]. The fidelity assay and the analysis of the *lacZ* mutants was performed as described [7]. Many of the *lacZ* mutants recovered from reactions with L868M pol α contained more than one mutation, which is why the total number of substitutions and insertions/deletions exceeds the number of *lacZ* mutants sequenced. Only those changes known to result in a mutant plaque phenotype were used to calculate error rates. Error rates are expressed as errors per phenotypically detectable nucleotide polymerized [7]. Eight mutations from reactions with wild-type pol α were other than single-base changes that were similar to those previously described [8].
[a] The value in parentheses is the average of five experiments, from [8].
[b] The value in parentheses is from [4].

his7-2 (line 2). Strains with single *pol3-5DV* [9] and *pol2-4* [10] alleles that inactivate the 3′ exonuclease activity of pol δ and pol ε, respectively, had mutation rates that were also elevated (lines 3 and 7). The double-mutant *pol1-L868M pol2-4* strain (line 8) had mutation rates that were similar to those of the *pol2-4* single mutant (line 7). In contrast, in the double-mutant *pol1-L868M pol3-5DV* strain, mutation rates at all three reporter loci (line 4) were elevated much more than observed with either single mutant alone. These elevated rates were suppressed to the rates observed with single-mutant strains by introducing plasmid vectors encoding wild-type pol α (*POL1*, line 5) or wild-type pol δ (*POL3*, line 6). These data are consistent with the hypothesis that the 3′ exonuclease activity of pol δ modulates the *pol1-L868M*-dependent mutator phenotype. The 3′ exonuclease

Table 2. Extension Efficiency for Matched and Mismatched Primer Termini

Primer Terminus Enzyme	K$_m$ (μM)	k$_{cat}$ (sec^{-1})	k$_{cat}$/K$_m$	f_{ext}	L868M:WT
T-A					
WT	0.17	0.60	3.5	1	
L868M	0.16	0.55	3.4	1	0.97
T-G					
WT	76.0	0.61	0.008	0.0023	
L868M	3.2	0.81	0.25	0.074	32

Oligonucleotides used to prepare substrates were a primer strand 5′-GTAACGCCAGGGTTTTCTCA/G (matched/mismatched) and a template strand 5′-ACGTCGTGAC**T**GAGAAAACCCTGGCGTTACCCA. Reactions (10 μl) were performed with 100 nM radiolabeled primer-template in a buffer containing 20 mM Tris (pH 8.0, 200 μg/ml BSA, 2 mM DTT, and 10 mM MgCl$_2$). Each experiment tested at least six different concentrations of the complementary next nucleotide (dGTP), with the concentration of polymerase and reaction time varied to obtain steady-state conditions. Reaction products were separated on a 12% denaturing polyacrylamide gel, and radiolabeled products were detected and quantified with a PhosphorImager and Image-QuaNT software. K$_m$ and k$_{cat}$ values were calculated as described [29].

activity of pol ε does not appear to do so in these experiments, although it cannot be excluded that pol ε might have an effect under different circumstances, e.g., for mutagenesis at origins or other locations in the genome.

The increases in mutation rates in the *pol1-L868M pol3-5DV* strain were higher (e.g., Table 2, line 4, 120-fold increase at *CAN1*) than the multiplicative increase in mutation rate expected (e.g., 2.4-fold × 9.1-fold = 22-fold at *CAN1*) if the nucleotide selectivity of L868M pol α and the proofreading activity of pol δ act in series. Several possibilities for this observation can be considered. Pol δ may correct a relatively larger proportion of errors in the *pol1-L868M* strain than in the strain with wild-type pol α. Some errors made by exonuclease-deficient pol δ may be extended by L868M pol α via its more promiscuous mismatch-extension capacity (Table 2). Two facts argue against this idea. First, spontaneous mutagenesis in a strain defective in pol δ 3′ exonuclease is largely independent of pol ζ [11, 12]. Pol ζ is an exonuclease-deficient B family polymerase that is naturally highly promiscuous for mismatch extension and known to be responsible for a considerable amount of spontaneous mutagenesis [13]. Second, inhibition of mismatch repair did not yield a multiplicative increase in spontaneous mutagenesis in a *pol1-L868M pol3-5DV* mutant strain (see further explanation below). Nonetheless, this possibility is worth further investigation in the future, especially because L868M pol α is the first eukaryotic replicative DNA polymerase demonstrated to be promiscuous for mismatch extension. It is also worth noting that the hypothesis of L868M pol α extension of some mismatches made by exonuclease-deficient pol δ does not exclude the proofreading hypothesis, mentioned above, that motivated this study.

Hypothetically, some of the mutagenesis observed in the *pol1-L868M pol3-5DV* mutant may reflect partial loss of mismatch repair (MMR). This might result either from saturation due to a high level of mismatches generated in the *pol1-L868M pol3-5DV* strain or from loss of mismatch excision by the 3′ exonuclease activity of pol δ, which has been implicated in excision of the nascent strand [14]. Defective excision seems less likely because the 3′ exonuclease activity of pol ε has also been implicated in mismatch excision [14] yet the *pol2-4* mutation had no effect on the mutability of the *pol1-L868M* strain (Table 3). In any case, we tested whether MMR was operative in the *pol1-L868M pol3-5DV* strain in two ways. First, we sequenced *can1* mutants to determine whether the mutational signature at the *CAN1* locus is consistent with a MMR defect. In a strain that is wild-type for pol α, inactivating MMR via an *msh2* mutation yields 85%–90% frameshift mutations in the *CAN1* gene (Table 4, lines 2 and 3). In contrast, when MMR is inactivated in the *pol1-L868M* strain, more than 95% of mutations are base substitutions (Table 4, line 6). When 38 Can^r mutants from the *pol1-L868M pol3-5DV* strain were sequenced, the ratio of base substitution to frameshift mutations was 3:1 (Table 4, line 7). This ratio is similar to the 4:1 ratio resulting from loss of pol δ proofreading in the *pol3-5DV* strain (line 3), but it is very different from the ~1:6 ratio resulting from loss of MMR in an *msh2* strain (lines 2 and 3) or from the 20:1 ratio in the *pol1-L868M msh2* strain (line 6). These data suggest that the majority of the increases in mutation rates in the *pol1-L868M*
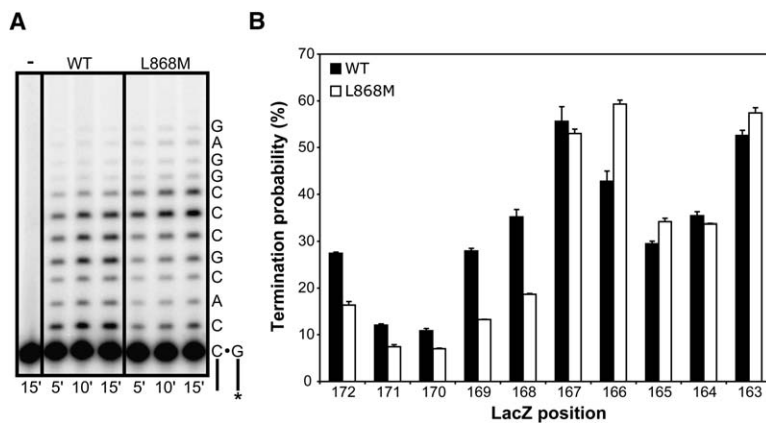
A **B**

Figure 1. Processivity of Wild-Type and L868M pol α

The analysis was performed as described in [28]. Briefly, 0.5 fmol of either wild-type or L868M pol α was incubated with 150 fmol template-primer (template-primer present at 300-fold excess over enzyme), and reaction aliquots were stopped after 5, 10, and 15 min of incubation at 37°C.

(A) Products of primer extension by wild-type (left) and L868M (right) pol α. Reaction time is noted at bottom, and template sequence is shown on the right.

(B) The termination probabilities at each template position, expressed in percentage as the ratio of products at each site to the products at that site plus all greater-length products. The termination probability is shown for the 10 min time point of the reactions shown in (A). The standard deviation was determined by comparing termination probabilities after 10 min of reactions with template-primer at 250, 300, and 400-fold excess over enzyme. In 15 min, wild-type pol α extended 13.4% of the available primer and cycled 40 times, and L868M pol α extended 14.6% of the available primer and cycled 44 times.

pol3-5DV strain are not simply due to loss of mismatch repair resulting from loss of pol δ exonuclease activity. Moreover, the mutational specificity data in the pol1-L868M pol3-5DV strain are informative regarding the hypothesis of defective excision of DNA flaps during Okazaki fragment processing, because we did not observe duplications characteristic of such a defect [9, 15].

As a second test for MMR capacity, we analyzed mutation rates in strains treated with cadmium, which strongly inhibits Msh2-Msh6-dependent and Msh2-Msh3-dependent MMR in yeast [16]. Because the pol3-5DV mutation is lethal in haploid strains that are unable to perform MMR, we used diploid strains known to rescue this lethality [9]. Spontaneous mutation rates at CAN1 in untreated diploid strains are shown in the upper part of Table 5. Because can1 mutations are recessive, resistance to canavanine requires inactivation of both copies of CAN1 (for details on mechanisms of mutagenesis in diploids, see footnote to Table 5), such that rates are very low in most of the strains examined, with the exception of diploids with the pol1-L868M pol3-5DV combination (Table 5, line 4). In contrast, reversion of the other reporter genes examined occurs by dominant mutations, and their rates are higher in diploids [17]. In these diploid strains, growth in the presence of cadmium

resulted in mutation rates in the pol1-L868M pol3-5DV strain that were increased by about 10-fold, indicating that under normal conditions this strain retains robust MMR capacity.

It is interesting that, in the strain homozygous for pol1-L868M, we observed a 37-fold increase in the rate of can1 mutants and a 120-fold increase of rate of base-pair substitutions leading to Trp+ revertants, but only a 3.4-fold increase in the rate of frameshift mutations (His+ reversion). These data are consistent with the results in Table 4 indicating that in the pol1-L868M mutant, MMR predominantly removes errors leading to base substitutions. Cadmium was strongly mutagenic in the diploid homozygous for pol3-5DV. The increases in mutation rates were 630-fold for Can^r, 520-fold for Trp+, and 84-fold for His+. The effects were further elevated in pol1-L868M pol3-5DV: 1700-, 1200-, and 170-fold, respectively. The cadmium-induced increase in mutation rates in the double mutant was higher than expected for an additive interaction based on rate increases in single mutants, suggesting that the majority of mutations in the double mutant were corrected by MMR. However, the multiplicative effect, expected from action of MMR is series [18], was not observed except for His+ reversion. One explanation is that the spontaneous mutation

Table 3. Mutation Rates in Yeast Strains with Defective pol α, pol δ, and pol ε Alleles

Yeast Strain	Relevant Mutation(s)	Mutation Rate (× 10^−8) (95% Confidence Limits)*					
		Can^r		Trp+		His+	
		Rate	n-Fold Change	Rate	n-Fold Change	Rate	n-Fold Change
CG379	none	23 (10–38)	1	3.1 (1.7–4.4)	1	1.3 (0.9–1.7)	1
L868M	pol1-L868M	59 (39–100)	2.4	4.6 (2.9–12)	1.5	1.3 (1.0–2.2)	1.0
CG379 δ exo−	pol3-5DV	210 (150–280)	9.1	9.5 (6.5–16)	3.0	8.7 (5.6–16)	6.7
L868M δ exo−	pol1-L868M pol3-5DV	2800 (2300–2900)	120	67 (54–120)	22	93 (80–120)	72
+ pCD17-8	+ POL1	110 (70–220)	4.7	7.0 (5–12)	2.2	12 (4.0–21)	9.2
+ pBL304	+ POL3	47 (29–95)	2.0	5.4 (2.9–13)	1.7	2.8 (1.1–5.3)	2.1
CG379 ε exo−	pol2-4	83 (65–180)	3.6	4.1 (2.5–11)	1.3	4.4 (2.4–6.4)	3.4
L868M ε exo−	pol1-L868M pol2-4	88 (59–150)	3.8	4.6 (2.6–7.8)	1.5	3.1 (1.5–6.1)	2.4

The genetic experiments were performed with haploid derivatives of the strain CG379 (MATalpha ade5-1 his7-2 ura3-52 trp1-289) [18] carrying the pol1-L868M mutation [4]. The double mutants combining the pol1-L868M and pol2-4 and pol3-5DV alleles of the POL2 and POL3 genes encoding for exonuclease-defective pol ε and pol δ were constructed by integration-excision [9, 10]. Fluctuation tests were performed as described in [30].

* indicates the median from three independent experiments with nine cultures, statistically tested for homogeneity.

Table 4. Rates of Single-Base Mutations at *CAN1* Locus in Various Yeast Strains

	Substitutions		Insertions/Deletions	
Yeast Strain	Mutants Total	Rate[a] (*n*-fold change)	Mutants Total	Rate[a] (*n*-fold change)
wild-type[b]	26/35	17 (1)	9/35	5.9 (1)
msh2[c]	3/20	60 (3.5)	17/20	340 (57)
msh2[d]	4/24	110 (6.3)	20/24	540 (91)
pol3-5DV[e]	16/20	170 (10)	4/20	42 (7)
pol1-L868M	16/16	59 (3.4)	0/16	<0.4 (<1.1)
pol1-L868M msh2[f]	20/21	9500 (560)	1/21	480 (81)
pol1-L868M pol3-5DV	29/38	2100 (125)	9/38	660 (112)

[a] Unless otherwise noted, rates are all $\times 10^{-8}$, calculated with the rates in Table 1.
[b] The cumulative sequencing results are from [4, 15].
[c] Sequencing results and the average mutation rate of 400×10^{-8} are from [31].
[d] The sequencing results and the average mutation rate of 650×10^{-8} for the CG379 strain background are from the present study and [14].
[e] Sequencing results are from [9], with the same strain (CG379) used here.
[f] Sequencing results and the average mutation rate of 1×10^{-4} are from the present study.

rate in the double mutant (line 4) is the sum of the rates of uncorrected errors made by exo⁻ pol δ during the synthesis of the major portion of Okazaki fragments that is unrelated to pol α (e.g., note the strong mutator effect

of the *pol3-5DV* mutation, line 9) plus errors made by pol α during synthesis of shorter patches of DNA. This would render inaccurate the fold-increases estimates. This lack of multiplicity argues against the hypothesis that pol α L868M extends mismatches generated by exonuclease-defective pol δ (see above). In contrast to results with the *pol1-L868M pol3-5DV* mutant strain, the increase in mutation rates in the double *pol1-L868M pol2-4* mutant was additive, suggesting that lack of interaction of the *pol1-L868M* and *pol2-4* mutations is also observed when MMR is defective. This provides additional support for the hypothesis that the exonuclease activity of Pol δ is correcting errors made by pol α, whereas the exonuclease activity of pol ε is not.

Conclusions

The results presented here are consistent with accumulating evidence that pol δ participates in replication of the lagging strand. That interpretation was derived from earlier studies indicating that pol δ also contributes to maturation of Okazaki fragments [9, 19, 20] and to telomere addition [21]. Our results are consistent with the possibility that lagging-strand replication errors made by pol α in vivo are removed by the exonuclease activity of pol δ. Given that the 3′ exonuclease of pol δ has been implicated in processing flaps during maturation of Okazaki fragments, in MMR and in S phase checkpoint control, at least some of the hypermutability in the *pol1-L868M pol3-5DV* strain could be due to a defect in one or more of these processes, or even to other putative

Table 5. Mutation Rates in Diploid Yeast Strains with and without Cadmium

		Mutation Rate ($\times 10^{-8}$) (95% Confidence Limits)					
		Can[r]		Trp[+]		His[+]	
Yeast Strain	Relevant Mutation(s)	Rate	*n*-Fold Change	Rate	*n*-Fold Change	Rate	*n*-Fold Change
Spontaneous Mutation Rates							
YPOM143	none	<0.2		2.1 (1.2–2.8)	1	1.8 (1.2–2.4)	1
YPOM148	*pol1-L868M/pol1-L868M*	<0.5		6.7 (4.1–12)	3.2	1.5 (1.2–2.5)	0.8
YPOM153	*pol3-5DV/pol3-5DV*	1 (0.5–3)	>5	20 (17–39)	9.5	22 (5–40)	12
YPOM158	*pol1-L868M pol3-5DV/ pol1-L868M pol3-5DV*	140 (54–450)	>700	580 (210–1400)	280	800 (310–1100)	440
YPOM165	*pol2-4/pol2-4*	<0.3		2.8 (1.7–3.8)	1.3	7.6 (6.1–9.2)	4.2
YPOM171	*pol2-4 pol1-L868M/ pol2-4 pol1-L868M/*	<0.4		8 (3–21)	3.8	8.0 (2.9–14)	4.4
Mutation Rates in the Presence of 5 μM CdCl₂							
YPOM143	none	1 (0.2–7)	1	4 (0.8–7.5)	1	51 (43–58)	1
YPOM148	*pol1-L868M/pol1-L868M*	37 (28–59)	37	465 (150–850)	120	175 (150–430)	3.4
YPOM153	*pol3-5DV/pol3-5DV*	630 (480–810)	630	2100 (1730–2700)	520	4300 (3800–5600)	84
YPOM158	*pol1-L868M pol3-5DV/ pol1-L868M pol3-5DV*	1700 (1400–2400)	1700	5000 (3700–7900)	1200	8800 (7000–11000)	170
YPOM165	*pol2-4/pol2-4*	24 (16–90)	24	61 (30–163)	15	3500 (2500–5100)	68
YPOM171	*pol2-4 pol1-L868M/ pol2-4 pol1-L868M/*	64 (51–180)	64	440 (320–780)	110	3600 (2600–5000)	70

Experiments were performed with diploids resulting from crosses of derivatives of strain CG379 (*MATalpha ade5-1 his7-2 ura3-52 trp1-289*) [4] with derivatives of strain I(-2)I-7B-YUNI300 (*MATa CAN1 his7-2 leu2-Δ::kanMX ura3-Δ trp1-289 ade2-1 lys2-Δ GG2899-2900*). Single and double mutants were constructed by integration-excision [4, 9, 10, 30]. Growth in the presence of CdCl₂ was performed as described [16]. The appearance of *can*[r] mutants in diploids requires either a mutation in both alleles or mitotic homozygotization of the recessive allele by recombination or chromosome loss, as discussed in detail elsewhere [17, 32, 33].

biological roles of pol α [22, 23]. Nonetheless, the data presented here are largely consistent with our hypothesis that the 3′ exonuclease of pol δ can perform intermolecular proofreading of L868M pol α errors made during lagging-strand replication. This idea is further supported by three different biochemical studies. The 3′ exonuclease activity of calf-thymus pol δ can excise mismatches generated by calf-thymus pol α during DNA synthesis in vitro [24]. During SV40 origin-dependent replication of undamaged duplex DNA in vitro by the human replication machinery [2], errors generated by highly inaccurate DNA polymerase η, which lacks an intrinsic 3′ exonuclease activity, are proofread [25]. Finally, errors generated by DNA polymerase η during bypass of a cyclobutane pyrimidine dimer in vitro can be proofread [26].

If correct, the hypothesis of proofreading of pol α errors by pol δ has several implications. It would imply that replication errors made by either pol α or pol δ could be responsible for mutations that underlie the increased cancer susceptibility observed in mice lacking the proofreading activity of pol δ [27]. The observation that the exonuclease activity of pol ε does not modulate the mutator phenotype of the *pol1-L868M* strain (Table 3) implies that the lagging-strand replication machinery is organized in a manner that carefully regulates which polymerase has access to primer termini. Regulating access to primer termini at the replication fork is likely to be relevant to controlling mismatch extension by pol ζ (also a B family enzyme) and translesion synthesis by Y family polymerases such as REV1p, pol η, pol ι, and pol κ. These polymerases, and indeed the majority of eukaryotic DNA polymerases [5], lack intrinsic 3′ exonuclease activity. Thus, proofreading activity intrinsic to a DNA polymerase is the exception, not the rule. Moreover, many other exonuclease-deficient eukaryotic DNA polymerases have lower fidelity than pol α, sometimes much lower [6]. Because these polymerases operate in DNA transactions that are critical for maintaining genome stability in the face of DNA damage [5], the ability of a separate exonuclease to proofread their mistakes could be generally important.

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