1200–1218 *Nucleic Acids Research, 2017, Vol. 45, No. 3* *Published online 28 November 2016*
doi: 10.1093/nar/gkw1149

Yeast DNA polymerase ζ maintains consistent activity and mutagenicity across a wide range of physiological dNTP concentrations

Olga V. Kochenova[1,†], Rachel Bezalel-Buch[2,†], Phong Tran[3], Alena V. Makarova[2], Andrei Chabes[3], Peter M. J. Burgers[2] and Polina V. Shcherbakova[1,*]

[1]Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198, USA, [2]Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110, USA and [3]Department of Medical Biochemistry and Biophysics and Laboratory for Molecular Infection Medicine Sweden (MIMS), Umeå University, SE 90187 Umeå, Sweden

Received September 20, 2016; Revised October 31, 2016; Editorial Decision November 01, 2016; Accepted November 02, 2016

ABSTRACT

In yeast, dNTP pools expand drastically during DNA damage response. We show that similar dNTP elevation occurs in strains, in which intrinsic replisome defects promote the participation of error-prone DNA polymerase ζ (Polζ) in replication of undamaged DNA. To understand the significance of dNTP pools increase for Polζ function, we studied the activity and fidelity of four-subunit Polζ (Polζ$_4$) and Polζ$_4$-Rev1 (Polζ$_5$) complexes *in vitro* at 'normal S-phase' and 'damage-response' dNTP concentrations. The presence of Rev1 inhibited the activity of Polζ and greatly increased the rate of all three 'X-dCTP' mispairs, which Polζ$_4$ alone made extremely inefficiently. Both Polζ$_4$ and Polζ$_5$ were most promiscuous at G nucleotides and frequently generated multiple closely spaced sequence changes. Surprisingly, the shift from 'S-phase' to 'damage-response' dNTP levels only minimally affected the activity, fidelity and error specificity of Polζ complexes. Moreover, Polζ-dependent mutagenesis triggered by replisome defects or UV irradiation *in vivo* was not decreased when dNTP synthesis was suppressed by hydroxyurea, indicating that Polζ function does not require high dNTP levels. The results support a model wherein dNTP elevation is needed to facilitate non-mutagenic tolerance pathways, while Polζ synthesis represents a unique mechanism of rescuing stalled replication when dNTP supply is low.

INTRODUCTION

Balanced deoxynucleoside triphosphate (dNTP) pools are critical for maintaining the fidelity of DNA replication. In yeast, the size of dNTP pools is strictly controlled during the cell cycle and expands just enough in the S-phase to allow efficient DNA replication (1). This is achieved through a tight regulation of the expression and activity of ribonucleotide reductase (RNR), the enzyme that catalyzes the rate-limiting step in *de novo* synthesis of dNTPs (2–4). Imbalanced, constantly high or low dNTP concentrations promote genome instability by affecting either the fidelity of DNA polymerases or by slowing down fork progression (5–13). On the other hand, the levels of dNTPs rise approximately 6- to 8-fold after treatment with DNA-damaging agents, such as ultraviolet (UV) light, methyl methanesulfonate and 4-nitroquinoline 1-oxide (4). In response to DNA damage, Mec1/Rad53/Dun1-mediated damage checkpoint activates RNR via degradation of its inhibitor Sml1 and by inducing the expression of genes encoding the RNR subunits (3,14–15). The expansion of dNTP pools is essential for cell survival during DNA damage (4), and it is thought to facilitate lesion bypass by replicative DNA polymerases, as well as specialized translesion synthesis (TLS) DNA polymerases (4,16). In agreement with this view, higher dNTP concentrations improve the efficiency of nucleotide insertion opposite lesions and extension of the resulting aberrant primer termini by various DNA polymerases *in vitro* (16–21). While facilitating lesion bypass, high dNTP levels could conceivably further reduce the fidelity of TLS DNA polymerases leading to accumulation of more mutations in the genome.

*To whom correspondence should be addressed. Tel: +1 402 559 7694; Email: pshcherb@unmc.edu
†These authors contributed equally to this work as the first authors.
Present addresses:
Olga V. Kochenova, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA
Alena V. Makarova, Institute of Molecular Genetics, Russian Academy of Sciences, Kurchatov Sq. 2, Moscow 123182, Russia.

DNA polymerase ζ (Polζ) is a key player in mutagenic TLS in eukaryotic cells. Yeast Polζ is comprised of four subunits encoded by the *REV3*, *REV7*, *POL31* and *POL32* genes (22,23). Despite being a member of the B family DNA polymerases (24,25), Polζ lacks exonuclease activity and is at least two orders of magnitude less accurate than the replicative polymerases Polε and Polδ (26). It is essential for the bypass of most lesions acting predominately as an extender of aberrant primer termini formed at the lesion site (19–20,27). Polζ-deficient cells are unable to undergo DNA damage-induced mutagenesis and show substantially reduced spontaneous mutagenesis (28,29). In addition to its four subunits, the function of Polζ in TLS also requires Rev1, a protein that interacts with both replicative and TLS polymerases (30–36) and possesses deoxycytidyl transferase activity (37). The essential role of Rev1 is structural and likely involves recruiting Polζ to the lesion site and enhancing its lesion bypass capability (38). The catalytic activity of Rev1, although not important for the overall efficiency of TLS, is utilized during the bypass of some lesions and helps shape the mutagenic specificity of bypass (39–44). For example, the Rev1 deoxycytidyl transferase is responsible for the high frequency of C incorporation observed *in vivo* during the bypass of abasic sites, one of the most common DNA lesions (40,44).

In addition to the important role in TLS, Polζ and Rev1 contribute to copying of undamaged cellular DNA in a variety of circumstances. They are recruited to undamaged templates when the normal replisome malfunctions because of a mutation affecting one of the replication proteins (45–47). We have shown that this recruitment is triggered by the replicative polymerase stalling at short hairpin DNA structures, which Rev1 and Polζ help to bypass (46). Because of the low fidelity of Polζ, its increased participation in the replication of undamaged DNA elevates the rate of spontaneous mutation leading to a phenomenon called defective-replisome-induced mutagenesis (DRIM). While originally discovered as a response to mutations in the replicative DNA polymerases α, δ and ε (45,48–49), DRIM can also be promoted by defects in non-catalytic replisome components (45,50–54) or replication-coupled chromatin remodelling (55), as well as by exposure of wild-type cells to the replication inhibitor hydroxyurea (HU) (47). Most recently, DRIM has been observed in yeast strains, in which replication deficiency was caused by a replacement of the catalytic domain of Polδ with that of bacteriophage RB69 DNA polymerase (56), providing further evidence that the recruitment of Polζ is a general response to replication impediment. Like DNA damage-induced mutagenesis, the DRIM phenotype is completely dependent on monoubiquitylation of proliferating cell nuclear antigen (PCNA) by Rad6/Rad18 (45), suggesting that the recruitment of Polζ-Rev1 to undamaged DNA is regulated similarly to the DNA damage response. However, it remained unknown whether replication defects that trigger DRIM also induce the expansion of dNTP pools, and, if so, whether this expansion is needed to facilitate Polζ-dependent mutagenesis.

We and others have recently shown that the mutagenic potential of many replicative DNA polymerase variants is greatly affected by changes in the intracellular dNTP levels (5,8). Inspired by this finding, we set out to determine how natural increases in dNTP levels, such as those occurring during DNA damage response, affect the mutagenic properties of Polζ. We found that yeast strains showing the DRIM phenotype have expanded dNTP pools, in accord with the view that TLS enzymes must function at high dNTP levels. Surprisingly, the activity, fidelity or error specificity of purified Polζ$_4$ and Polζ$_5$ complexes *in vitro* were not greatly affected by the switch from 'normal S-phase' to 'damage-response' dNTP concentrations. Furthermore, we provide evidence that Polζ-dependent lesion bypass and Polζ-dependent mutagenesis during copying of undamaged DNA *in vivo* do not require high dNTP levels. These results argue that Polζ is less sensitive to fluctuations in the size of dNTP pools than the replicative DNA polymerases and, thus, Polζ may be uniquely capable of bypassing lesions or other impediments when dNTP pools are low. This finding explains why Polζ is involved in the generation of spontaneous mutations (57,58), which presumably arise during the normal S-phase in cells with unexpanded dNTP pools.

MATERIALS AND METHODS

Saccharomyces cerevisiae strains and plasmids

The haploid *S. cerevisiae* strain E134 (*MATα ade5-1 lys2::InsE$_{A14}$ trp1-289 his7-2 leu2-3,112 ura3-52*) and its isogenic derivative PS446 (same, but *rev3Δ::LEU2*) used for *in vivo* mutagenesis studies have been described previously (45,59). The *pol3-Y708A* mutants were constructed by using HpaI-cut p170 plasmid as described earlier (48). The presence of the mutation was confirmed by sensitivity to 100 mM HU. The haploid strain PY330 (*MATa can1 his3 leu2 trp1 ura3 pep4::HIS3GAL nam7Δ::KanMX4 rev1Δ::HYG*) was used to overproduce Polζ$_4$ and Polζ$_5$. The plasmids used for the overproduction were pBL818 (same as pB813 (22) but the GST tag on *REV3* was replaced with the IgG binding domain ZZ tag), pBL347 (22) and pBL825 (*TRP1, GAL1-GST-REV1*).

Proteins

Preparations of *S. cerevisiae* PCNA and replication protein A (RPA) used in the fidelity assays have been described previously (8). *S. cerevisiae* replication factor C (RFC), as well as PCNA and RPA used in the replication assays, were overproduced and purified from *Escherichia coli* as described (45,60–63). Rev1 was produced in yeast and purified as described (64). To produce Polζ$_4$, the *REV3*, *REV7*, *POL31* and *POL32* genes were overexpressed from galactose-inducible promoters as described previously (22) except that an IgG-purification cassette (in plasmid pBL818) was used instead of the GST tag. Strains for overproduction of Polζ$_5$ also contained plasmid pBL825. Strains were grown, galactose induction was carried out for 16 h and extracts were made through the ammonium sulfate precipitation step as described previously (22). Polζ$_4$ and Polζ$_5$ were purified from approximately 100 g of cells. Argon de-gassed buffers were used throughout the purification procedure. Ammonium sulfate pellets were resuspended in buffer A1 (50 mM Hepes (pH 8.0), 500 mM NaCl, 30 mM Na_2HPO_4/NaH_2PO_4 (pH 8.0), 8% glycerol,

0.05% Tween 20, 0.01% E10C12, 5 mM 2-mercaptoethanol, 10 μM pepstatin A, 10 μM leupeptin, 2.5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride) and gently agitated with 2 ml of IgG sepharose beads (GE Healthcare) for 2 h. The beads were packed into a disposable 20-ml BioRad column and washed with 20 bed volumes of buffer A1, followed by 50 bed volumes of A2 (A1 plus 5 mM $MgCl_2$ and 1 mM ATP). The beads were washed with additional 20 bed volumes of buffer A1, re-suspended in four bed volumes of buffer A1 and digested overnight at 4°C with PreScission protease by gentle rotation of the capped column. After collection of the eluent, the beads were washed with additional four bed volumes of buffer A1. Fractions were combined and agitated with 0.5 ml Ni-NTA beads (QIAGEN) for 1 h to enrich for stoichiometric complexes containing Pol31-His_7Pol32 in addition to Rev3-Rev7. The beads were packed into a disposable BioRad column and washed with 40 bed volumes of buffer B1 (50 mM Hepes (pH 8.0), 500 mM NaCl, 30 mM Na_2HPO_4/NaH_2PO_4 (pH 8.0), 8% glycerol, 0.05% Tween, 0.01% E10C12, 5 mM 2-mercaptoethanol, 20 mM imidiazole, 10 μM pepstatin A, 10 μM leupeptin, 2.5 mM benzamidine and 0.5 mM phenylmethylsulfonyl fluoride). The proteins were eluted with three bed volumes of buffer B2 (50 mM Hepes (pH 8.0), 500 mM NaCl, 30 mM Na_2HPO_4/NaH_2PO_4 (pH 8.0), 8% glycerol, 0.01% E10C12, 5 mM 2-mercaptoethanol, 300 mM imidiazole, 10 μM pepstatin A, 10 μM leupeptin, 2.5 mM benzamidine and 0.5 mM phenylmethylsulfonyl fluoride). All final preparations were dialyzed against buffer D (30 mM Hepes (pH 8.0), 200 mM NaCl, 8% glycerol, 0.01% E10C12 and 5 mM 2-mercaptoethanol).

Measurement of intracellular dNTP levels

Yeast cells were cultured in either YPDA (1% yeast extract, 2% bacto-peptone, 2% glucose and 0.002% adenine) or YPDA with 20 mM HU at 30°C and 180 rpm. The dNTP pools were measured in asynchronous cultures at OD_{600} of 0.3 or indicated time points as described in (65). Briefly, 3.7×10^7 cells were harvested by filtration, and nucleotides were extracted with trichloroacetic acid and $MgCl_2$, followed by neutralization with a Freon-trioctylamine mix. The dNTPs were separated from NTPs using boronate columns and analyzed by high pressure (or high performance) liquid chromatography. Flow cytometry analysis was performed as described in (16).

DNA polymerase activity assays

Oligonucleotides SKII-682 (5′-TATCGATAAG CTTGATATCGAATTCC-3′), pr100mer (5′-Cy3-GGTATCGATAAGCTTGATATCGAATT-3′) and 100mer (5′-AACAAAAGCTGGAGCTCCACCGCGGT GGCGGCCGCTCTAGAACTAGTGGATCCCCCGG GCTGCAGGAATTCGATATCAAGCTTATCGATA CCGTCGACCT-3′) were obtained from Integrated DNA Technologies and purified by either PAGE or high-pressure liquid chromatography before use. SKII-682 annealed to the 3-kb circular Bluescript ssSKII DNA. The 100mer was circularized using a bridging primer and T4 ligase (NEB) and purified on urea-PAGE, and the pr100mer Cy3-labeled primer was annealed. All standard 10-μl assays contained 40 mM Tris-HCl pH 7.8, 1 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 8 mM $MgAc_2$, 125 mM NaCl, 0.5 mM ATP and either S-phase or damage-response concentrations of dNTPs (39 μM dCTP, 66 μM dTTP, 22 μM dATP and 11 μM dGTP for S-phase or 195 μM dCTP, 383 μM dTTP, 194 μM dATP and 49.5 μM dGTP for damage-response concentrations (4,16)). Primed templates were coated with RPA, PCNA was loaded by RFC for 30 s at 30°C, and replication reactions were initiated by the addition of the indicated DNA polymerases. The complex of Polζ$_4$ and Rev1 was pre-formed on ice for 30 min. Replication assays on the 3-kb circular DNA contained 2 nM primed ssDNA template, 200 nM RPA, 30 nM PCNA, 6 nM RFC and 30 nM of the indicated polymerase(s). The α-^{32}P-dGTP was added as the radioactive tracer, and reactions were incubated at 30°C for 30 and 60 min. Reactions were stopped with 50 mM ethylenediaminetetraacetic acid (EDTA) and 0.2% sodium dodecyl sulphate and analyzed on a 1.2% alkaline agarose gel. Primer extension assays on the 100-mer ssDNA contained 10 nM 5′-Cy3-labeled DNA substrate, 40 nM RPA, 30 nM PCNA, 6 nM RFC and 30 nM of the indicated polymerase(s). Reactions were incubated at 30°C for 0.5, 1 and 2 min. Reactions were stopped with 50% formamide, 10 mM EDTA and 0.1% sodium dodecyl sulphate and analyzed on 12% polyacrylamide-7 M urea gel. Quantification was done by either phosphorimaging of the dried gel (^{32}P) or fluorescence imaging on a Typhoon system.

Measurement of DNA polymerase fidelity *in vitro*

M13mp2 gapped substrate was prepared and gel-purified as described previously (8,66). DNA synthesis reactions (25 μl) contained 40 mM Tris-HCl (pH 7.8), 60 mM NaCl, 8 mM $MgAc_2$, 0.5 mM ATP, 1 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 20 nM PCNA, 8 nM RFC, 200 nM RPA, 1 nM gapped substrate and 40 nM Polζ$_4$ or 50 nM Polζ$_5$. The reactions were performed at either equimolar dNTP concentrations (100 μM each) or at the intracellular concentrations (S-phase or damage-response). The reactions were incubated at 30°C for 1 h and stopped by placing the reactions on ice and adding 1.5 μl of 0.5 M EDTA. The efficiency of gap filling was determined by agarose gel electrophoresis (Supplementary Figure S1). Aliquots of the reactions were used for transformation of *E. coli* to determine the frequency of mutant plaques. The purification of mutant M13mp2 plaques and isolation of ssDNA were performed as described previously (66). Error rates for individual types of mutations were calculated by using the following equation: $ER = [(N_i/N) \times MF]/(D \times 0.6)$ where N_i – the number of mutations of a specific type, N – the total number of analyzed mutant M13mp2 plaques, MF – frequency of mutant M13mp2 plaques, D – the number of sites in the *lacZ* reporter gene where this type of mutation can be detected and 0.6 is the probability that a mutant allele of the *lacZ* gene will be expressed in *E. coli* (66). Multiple mutations in a single mutant *lacZ* sequence were considered independent events and included separately in the error rate calculations if the distance between mutations was >10 nucleotides. Multiple mutations separated by ten

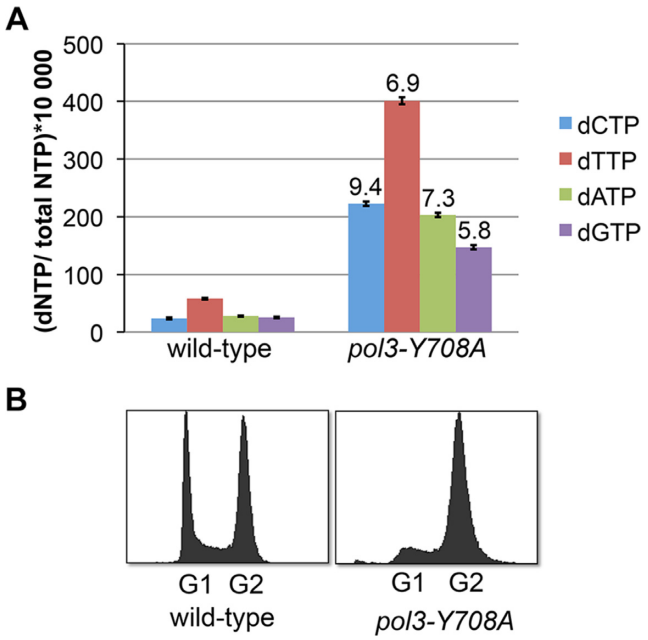


Figure 1. Analysis of deoxynucleoside triphosphate (dNTP) pools and cell cycle in a DNA replication mutant that displays constitutively elevated Polζ-dependent mutagenesis. (**A**) Intracellular dNTP levels normalized to total NTP in wild-type and *pol3-Y708A* strains. Data are presented as mean ± SD (n = 3 for wild-type strains and n = 4 for *pol3-Y708A* mutants) with the numbers above the bars indicating the fold increase compared to the wild-type strain. (**B**) Fluorescence-activated cell sorter (FACS) analysis of asynchronous logarithmically growing wild-type and *pol3-Y708A* cultures that were used for dNTP pool measurements in (A).

or fewer nucleotides were classified as complex mutations and excluded from the calculation of error rates for individual mispairs. The frequency of complex mutations, as well as the frequency of deletions of more than one nucleotide and large rearrangements, was calculated as the total number of these types of mutations divided by the total number of detectable mutations. All data are based on analysis of *lacZ* mutants from at least two independent gap-filling reactions. The statistical significance of differences in the rate of individual errors was assessed by Fisher's exact test by comparing proportions of plaques with a specific nucleotide change among all plaques (mutant and non-mutant) analyzed for that reaction. For example, in order to evaluate the significance of the increase in the rate of C-dCTP mispair in $Pol\zeta_5$ versus $Pol\zeta_4$ reactions at damage-response dNTPs, seven plaques with the C→G substitution found among the total of 7562 plaques analyzed for $Pol\zeta_5$ reaction were compared to one mutant plaque found among 11120 plaques analyzed for $Pol\zeta_4$ reaction ($P = 0.0092$, Supplementary Figure S2B).

Measurement of mutant frequency *in vivo*

To determine the effect of HU treatment on DRIM, at least nine independent cultures were started for each strain (wild-type, *pol3-Y708A* and *pol3-Y708A rev3*Δ) from single colonies and grown overnight at 30°C in liquid YPDAU medium (1% yeast extract, 2% bacto-peptone, 2% glucose, 0.006% adenine, 0.00625% uracil) containing HU at concentrations indicated in Figure 5A. Appropriate dilutions of the overnight cultures were plated onto synthetic complete medium containing L-canavanine (60 mg/l) and lacking arginine (SC –CAN) for selection of Can^r colonies and onto synthetic complete (SC) medium for viability count. Can^r mutant frequency was calculated by dividing the number of Can^r mutants by the number of colonies on SC medium. The median frequency of Can^r mutants was used to compare mutagenesis in different strains and at different HU doses. The significance of the differences between mutation frequencies was estimated by using the Wilcoxon–Mann–Whitney non-parametric criterion.

To determine the effect of HU treatment on UV-induced mutagenesis, appropriate dilutions of overnight cultures of E134 and PS446 strains were plated onto SC and SC –CAN media containing HU at the concentrations indicated in Figure 5E and Supplementary Figure S3A. The cells were irradiated with 10 J/m^2 of 254-nm UV light within 15 min after plating and incubated at 30°C. The mutant frequency was calculated as described above. To study the effect of HU pre-treatment on UV-induced mutagenesis, overnight cultures of E134 strain were diluted 10-fold and grown for 4 h in the presence of 100 mM HU. Appropriate dilutions of the logarithmic cultures were plated onto SC and SC –CAN media containing 100 mM HU and irradiated with UV light at the doses indicated in Supplementary Figure S3B. The mutant frequency was calculated as described above.

RESULTS

A replisome defect that triggers DRIM also induces the expansion of dNTP pools

Various defects in the catalytic and accessory subunits of yeast replicative DNA polymerases impede the progression of the replication fork and cause DRIM (45,48–50,52,54,56). Among these, the *pol3-Y708A* mutation has been used most commonly for the mechanistic studies of DRIM (45–47) because of its rather strong mutator phenotype that is almost entirely Polζ-dependent. The mutation leads to an alanine substitution for Tyr708 at the active site of Polδ (48). It causes a moderate replication deficiency (as manifested by a reduced growth rate and HU sensitivity) and constitutive PCNA monoubiquitylation, a prerequisite for the TLS polymerase recruitment. Here, we use the *pol3-Y708A* mutant to test the hypothesis that replication stalling in mutants experiencing DRIM leads to an increase in dNTP levels. Measurement of the size of dNTP pools in logarithmically growing wild-type and *pol3-Y708A* strains showed a 7-fold increase in the total dNTP level in the *pol3-Y708A* mutant (Figure 1A). The increases for individual dNTPs ranged from approximately 6- to 9-fold and were similar to those observed during DNA damage response (4). Flow cytometry analysis of the logarithmically growing wild-type and *pol3-Y708A* cultures revealed that the *pol3-Y708A* strain had an abnormal cell cycle distribution, with a larger proportion of cells in the G2/M phase (Figure 1B). The prolonged G2/M phase may be a sign of checkpoint activation, which is likely responsible for the expansion of dNTP pools.

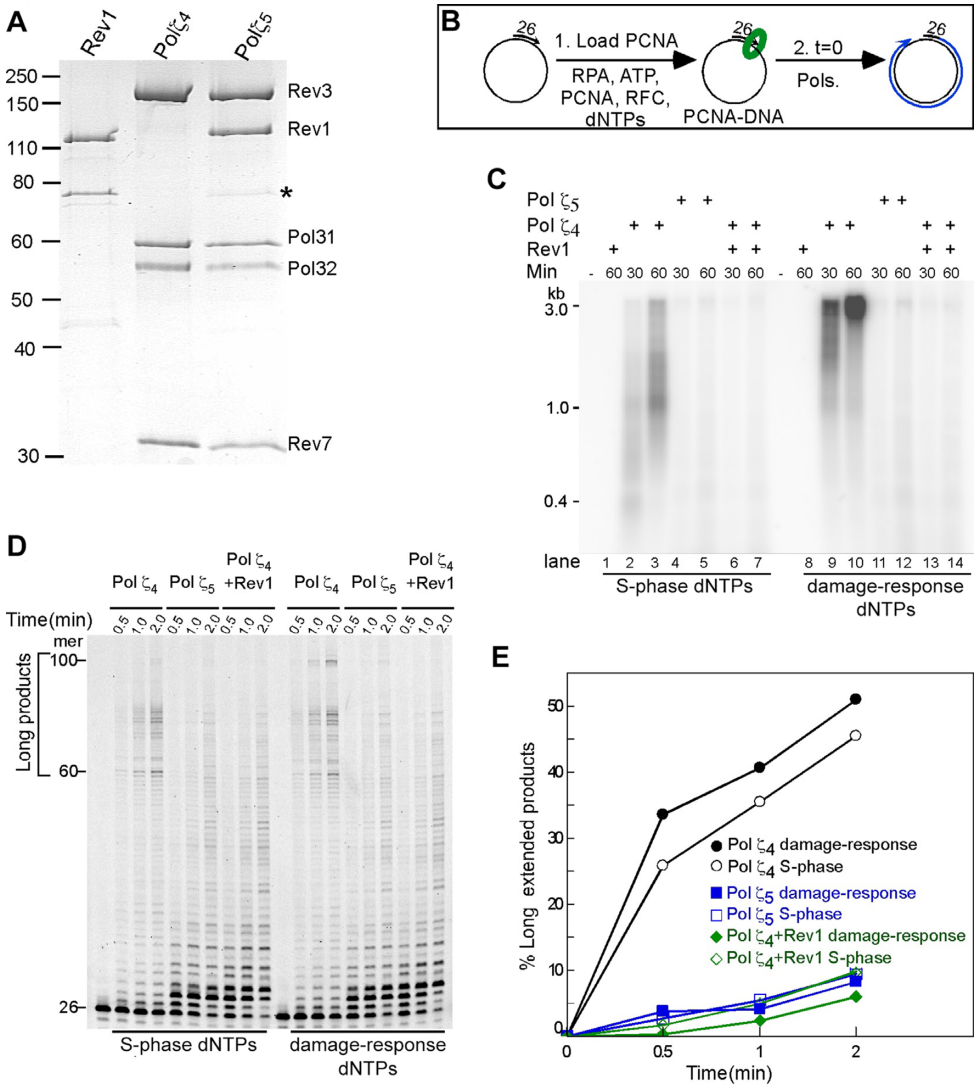


Figure 2. Polζ-dependent DNA synthesis at S-phase and damage-response dNTP concentrations. (**A**) Analysis of Rev1, Polζ$_4$ and Polζ$_5$ by electrophoresis in 10% sodium dodecyl sulphate-polyacrylamide gel. *, Ssa1 chaperone co-purifies with overproduced Rev1 alone. (**B**) Schematic of the replication assays in (C) and (D), as described in Materials and Methods. (**C**) Alkaline agarose gel (1.2%) electrophoresis of replication products on primed Bluescript SKII ssDNA with the indicated DNA polymerases, and either S-phase or damage-response dNTPs. The tracer was [α-^{32}P]-dGTP. (**D**) Urea-PAGE (12%) analysis of primer extension reactions on a 100-mer circular ssDNA with either S-phase or damage-response dNTPs. The 26-mer primer was labeled with Cy3**.** (**E**) Quantification of data in (D)**.** The percentage of long products (≥60 nt) among all extension products is plotted.

The effect of dNTP levels on the catalytic activities of Polζ$_4$ and Polζ$_5$

We have previously shown that Rev1 associates with the four-subunit form of Polζ (22). In order to obtain a stoichiometric Polζ$_5$ complex, we overproduced all five subunits (Rev3-Rev7-Pol31-Pol32-Rev1) in yeast and purified the complex by tandem affinity chromatography (Figure 2A). As controls, Polζ$_4$ was purified from a *rev1*Δ strain and the single Rev1 protein was also purified from yeast.

The activities of the various complexes were tested on two primed circular ssDNA substrates, one 3 kb in length (SKII, Figure 2C), and the other 100 nt in length (100mer, Figure 2D and E), in the presence of dNTP concentrations that mimic intracellular S-phase or damage-response levels. The S-phase concentrations were 39 μM dCTP, 66 μM dTTP, 22 μM dATP and 11 μM dGTP, and damage-response concentrations were 195 μM dCTP, 383 μM dTTP, 194 μM dATP and 49.5 μM dGTP, as described elsewhere (16). These concentrations were calculated based on the reported amount of dNTPs per cell in logarithmically growing yeast cultures or in cultures treated with 0.2 mg/l 4-nitroquinoline 1-oxide for 150 min (4) using a haploid yeast cell volume estimate of 45 μm^3. Both SKII and 100mer DNA substrates allow stable loading of PCNA, which is an essential processivity factor for Polζ (67). PCNA was loaded by RFC, and replication of either template was initiated by the addition of the relevant DNA polymerases (Figure 2B). Analysis of replication of the 3-kb template showed that the activity of Polζ$_4$ was inhibited upon the addition of Rev1 (Figure 2C, compare lanes 6, 7 with 2, 3 and 13, 14 with 9, 10). Furthermore, Polζ$_5$ purified as a complex rather than reconstituted from Polζ$_4$ and Rev1

showed a similar low activity (lanes 4, 5 and 11, 12). The increase in dNTP concentrations from the S-phase levels to the damage-response levels resulted in a significant increase in activity, which was only noticeable in reactions with Polζ$_4$.

In the replication assay on the 3-kb template, metabolic labeling with ^{32}P-dNTPs results in longer products being more radioactive. This amplifies the actual differences in the polymerase activity and complicates quantitative comparison. In order to obtain a more accurate assessment of the activities of Polζ complexes and the effects of dNTP levels, we carried out DNA synthesis assays using the 100mer template and a Cy3-labeled primer, and analyzed the replication products by urea-PAGE (Figure 2D). As a measure of replication activity, we determined the ratio of long extension products (60–100 nt) to short extension products (27–59 nt) (Figure 2E). Analogous to the results in Figure 2C, Rev1 inhibited DNA synthesis by Polζ$_4$, and the isolated Polζ$_5$ complex had a low activity similar to the complex reconstituted from Polζ$_4$ and Rev1. The several-fold increase in dNTP concentrations (from S-phase to damage-response) resulted in only a minor increase in Polζ$_4$ activity and no detectable increase in Polζ$_5$ activity. It is worth noting that the effect of increasing the dNTP levels on Polζ$_4$ was more pronounced on the 3-kb template (Figure 2C) compared to the 100mer template (Figure 2E), even after we take into account the amplification of differences due to the metabolic labeling in the former assay. One possible explanation is that a subtle increase in processivity at higher dNTP concentrations reduces the number of cycles of dissociation and re-association required to fully replicate the 3-kb template, thus resulting in a greater apparent increase in activity.

The fidelity and error specificity of Polζ$_4$ and Polζ$_5$ at S-phase and damage-response dNTP levels

Next, we aimed to understand the effects of DNA damage-induced expansion of dNTP pools on the fidelity and error specificity of Polζ. To this end, we performed the M13mp2 *lacZ* forward mutation assay (66) with purified Polζ$_4$ and Polζ$_5$ using the S-phase and damage-response dNTP concentrations. In this assay, a 407-nt single-stranded gap in a double-stranded M13mp2 DNA is filled by DNA polymerases *in vitro* and nucleotide changes introduced during the gap-filling synthesis are detected by genetic selection in *E. coli*. All reactions were performed in the presence of the polymerase accessory proteins PCNA, RFC and RPA. Analysis of the reaction products by agarose gel electrophoresis showed that, under the conditions used (see Materials and Methods), the 407-nucleotide gap was filled completely by Polζ$_4$ (Supplementary Figure S1). Consistent with the inhibitory effect of Rev1 described previously ((64) and Figure 2), synthesis by Polζ$_5$ was less efficient. Nevertheless, using a higher concentration of the five-subunit complex (50 nM instead of 40 nM), we were able to achieve a nearly complete gap filling (Supplementary Figure S1).

The frequency of *lacZ* mutants obtained upon transfecting *E. coli* with Polζ$_4$ gap-filling reactions was only slightly elevated (1.3-fold) when damage-response dNTP concentrations were used instead of the S-phase dNTPs (Table 1). As suggested by this minimal change, the increase in dNTP concentrations also did not greatly affect the overall error rate, and, notably, did not affect the error specificity of Polζ$_4$ (Table 1 and Figure 3A). The mutational spectra of Polζ$_4$ at both dNTP levels were dominated by single-base substitutions that occurred at rates of 7.5×10^{-4} and 9.2×10^{-4} at S-phase and damage-response dNTPs, respectively (Table 1). The rate of single-base insertions/deletions (indels) was relatively low (1.7×10^{-5} and 2.9×10^{-5} for S-phase and damage-response dNTPs, respectively; Table 1). Polζ$_4$ was predominantly promiscuous at G template nucleotides at both dNTP levels. Interestingly, Polζ$_4$ was very inefficient at generating all three types of X-dCTP mismatches, with the C-dCTP mismatch being the least frequent among all 12 possible mispairs ($<0.54 \times 10^{-5}$ at S-phase dNTPs and 0.89×10^{-5} at damage-response dNTPs; Table 1 and Figure 3A). At both dNTP levels, Polζ$_4$ showed notable propensity to create multiple sequence changes (Tables 1 and 2). Their frequency was unaffected by the increase in dNTP concentrations. Approximately 5% of *lacZ* mutants contained multiple changes within short (≤10 nucleotides) stretches of DNA, which we classified as complex mutations and which Polζ is notorious for generating during TLS and copying of undamaged DNA *in vivo* (47,68). An additional 10% contained multiple mutations separated by larger distances (Table 2).

Because Rev1 is indispensable for Polζ-dependent mutagenesis *in vivo*, we examined how the presence of Rev1 modulates the fidelity of Polζ. We found that the five-subunit complex was slightly more error-prone than Polζ$_4$. The frequencies of *lacZ* mutants determined upon transfecting *E. coli* with the products of Polζ$_5$ gap-filling reactions were increased approximately 1.5-fold at both S-phase and damage-response dNTP concentrations in comparison to reactions with Polζ$_4$ (Table 1). As in the case of Polζ$_4$, the switch from S-phase to damage-response dNTP concentrations did not greatly affect the *lacZ* mutant frequency, the overall error rate or the error specificity of Polζ$_5$ complex (Table 1 and Figure 3B). Like Polζ$_4$, the five-subunit complex was the most promiscuous at G nucleotides, with G-dATP being the most frequently generated mispair. Generally, the error spectra produced by Polζ$_5$ were remarkably similar to those of Polζ$_4$, with one important exception: the presence of Rev1 significantly increased the rates of all three X-dCTP mispairs (Table 1, Figure 3B and Supplementary Figure S2B). This increase accounted for most of the difference in the overall error rate between Polζ$_4$ and Polζ$_5$. The dCTP misincorporation is likely due to the deoxycytidyl transferase activity of Rev1, and it indicates that Polζ and Rev1 can exchange at the primer terminus during DNA synthesis *in vitro*. In comparison to Polζ$_4$, a somewhat higher proportion of *lacZ* mutations from Polζ$_5$ reactions constituted complex changes (9% and 15% at S-phase and damage-response dNTP concentrations, respectively). This is consistent with the important role of Rev1 in the generation of Polζ-dependent complex mutations *in vivo* (46). An additional 7% and 15% of *lacZ* mutants from reactions with S-phase and damage-response dNTPs, respectively, contained multiple mutations separated by more than 10 nucleotides (Table 3). Interestingly, Polζ$_5$ reactions produced a new class of large rearrangements, which involved substitutions of a large stretch of DNA (>30 nucleotides)

Table 1. Fidelity of *in vitro* DNA synthesis by Polζ$_4$ and Polζ$_5$ at cellular dNTP concentrations

	Polζ$_4$[a]						Polζ$_5$[a]					
	S–phase dNTPs			Damage-response dNTPs			S-phase dNTPs			Damage-response dNTPs		
	Detectable	Non-detectable	ER ($\times10^{-5}$)[b]	Detectable	Non-detectable	ER ($\times10^{-5}$)[b]	Detectable	Non-detectable	ER ($\times10^{-5}$)[b]	Detectable	Non-detectable	ER ($\times10^{-5}$)[b]
Base substitutions (mispair)	237	18	75	182	9	92	166	9	125	147	24	135
A → G (A·dCTP)	10	1	2.9	2	1	0.97	10		6.6	9	2	7.5
A → T (A·dATP)	8	2	2.0	12		4.9	13		7.4	13	2	9.1
A → C (A·dGTP)	10		3.8	4		2.5	7		6.1	8	1	8.6
T → C (T·dGTP)	20	1	5.2	16		6.9	10		6.0	6		4.4
T → A (T·dTTP)	23	5	10.5	10		7.5	6		6.3	7	1	9.1
T → G (T·dCTP)	12		4.4	2		1.2	10		8.4	9	1	9.3
G → A (G·dTTP)	20	1	6.3	29	3	15	12	1	8.7	10	1	9.8
G → C (G·dGTP)	39	3	12	31		16	19		14	16	2	14
G → T (G·dATP)	46	3	14	44	4	22	36	4	25	37	5	32
C → T (C·dATP)	20		5.7	18	1	8.5	11	1	7.2	13	4	11
C → G (C·dCTP)			<0.54	1		0.89	14	1	17	7	4	11
C → A (C·dTTP)	29	2	8.2	13		6.1	18	2	12	11	1	8.9
Single-base indels	38		1.7	39		2.9	33		3.5	27	1	3.4
-1 run	12		0.54	17		1.3	18		1.9	8		1.0
+1 run	6		0.27	9		0.67	2		0.21	8	1	1.0
-1 non-run	20		0.90	12		0.89	13		1.3	8		1.0
+1 non-run			<0.04	1		0.074	1		0.10	3		0.38
Complex[c]	13		4.5%	12		5.2%	19		8.6%	34		15%
Other[d]	3		1.0%			<0.4%	3		1.4%	14		6.3%
Total[e]	291			233			221			222		
lacZ mutant frequency		0.015			0.02			0.025			0.027	

[a] All reactions were performed in the presence of PCNA, RFC and RPA. The background mutation frequency for unfilled M13mp2 gapped substrate was 0.0009.
[b] Error rates (ER) for individual mutation types were calculated as described in Materials and Methods. Only detectable mutations were included in the error rate calculation. Percent of the total number of detectable mutations is shown for complex and 'other' types of mutations.
[c] Complex mutations are multiple changes within short DNA stretches (≤10 nucleotides; see Tables 2 and 3).
[d] 'Other' mutations include deletions of more than one nucleotide and large rearrangements (see Figure 4, Tables 2 and 3).
[e] Data for Polζ$_4$ at S-phase and damage-response dNTPs are based on the analysis of 280 and 220 mutant plaques, respectively. Data for Polζ$_5$ at S-phase and damage-response dNTPs are based on the analysis of 210 and 207 mutant plaques, respectively. Some of the plaques contained multiple detectable mutations. The numbers show the total number of detectable mutations found in the plaques analyzed.

Table 2. Complex and multiple mutations induced by Polζ$_4$*in vitro*

dNTPs	Mutation type	Sequence change	Location in *lacZ*
S-phase	Complex	TC → CA	80–81
		CCC → TC	−45 to −43
		GTG → TTT	82–84
		TCG → TTCA	139–141
		TGGCC → GGC (2X)	61–65
		TAATAG → CAATAA	152–157
		GTTTTAC → TTTTTA	69–75
		CCCTTCCCA → TCCTTCCCT	179– 87
		ATTACGAATTCACTG → CGAATTCAC	48–62
		ATTACGAATTCACTGGCC → CGAATTCACGC	48–65
	Multiple	A → G; T → G	91; 103
		C → A; T → A	134; 147
		G →T; ΔG	102; 123
		G → C; ΔG	148; 169
		A → G; T → G	48; 70
		G → T; C → A	53; 81
		G → T; T → C	−68; −36
		C → A; G → C	81; 118
		T → A; +T	98; 139
		ΔC; ΔC	143; 189
		C → A; G → T	37; 88
		G → T; A → G	102; 153
		T → C; ΔG	104; 159
		C → A; A → T	−55; 1
		T → A; T → C	67; 138
		C → T; G → T	58; 148
		C → A; T → A	−16; 87
		T → C; T → A	−58; 49
		T → A; C → T	−50; 58
		T → A; T → C	−2; 121
		G → A; A → T	−66; 59
		G → A; A → C	9; 171
		G → T; G → T	−68; 102
		G → T; +T	−38; 139
		G → C; G → T	−84; 102
		T → C; G → C	−22; 169
		T → A; GTAA → GTTTT	−54; 151–154
		G → T; G → C	−84; 148
		GA → TG; G → T	−66 to −67; 149
		T → A; G → C; ΔG	−67; 100; 126
Damage-response	Complex	GTG → TTTG	−6 to −4
		GTG → TTT	82–84
		TGC → CC	122–124
		CGCAC → T	168–172
		TGGCC → GGC (2X)	61–65
		AGCTGC → TGCGCA	190–195
		CGTCGTG → GTCGTT	78–84
		TCCCCCTTT → ACCCCCTTTT (4X)	131–139
	Multiple	G → A; T → C	99; 112
		A → G; G → T	130; 145
		G → C; A → C	99; 130
		G → T; G → T	53; 84
		G → T; G → A	118; 157
		T → C; G → T	−36; 11
		G → C; C → T	118; 180
		G → T; G → A	−1; 66
		G → T; A → C	123; 190
		G → T; A → C	−66; 28
		C → A; A → T	−55; 39
		G → T; A → G	88; 188
		T → C; G → T	−21; 84
		G → T; ΔG	12; 123
		T → A; G → C	56; 141
		G → T; G → C	53; 169
		A → T; ΔA	−26; 94
		T → G; T → C	−63; 61
		G → C; G → C	−68; 79
		G → A; G → T	−68; 84
		G → A; +T	−77; 139
		G → A; +T	−84; 139
		ΔG; G → C	−47; 178

Sequence changes are listed in the order of increasing distance between two nucleotide changes. Mutations with the distance between them of 10 nucleotides or fewer were considered complex mutations and counted as a single event. All other detectable nucleotide changes were included into calculation of error-rates for individual mutation types in Table 1. Δ, deletion; +, insertion.

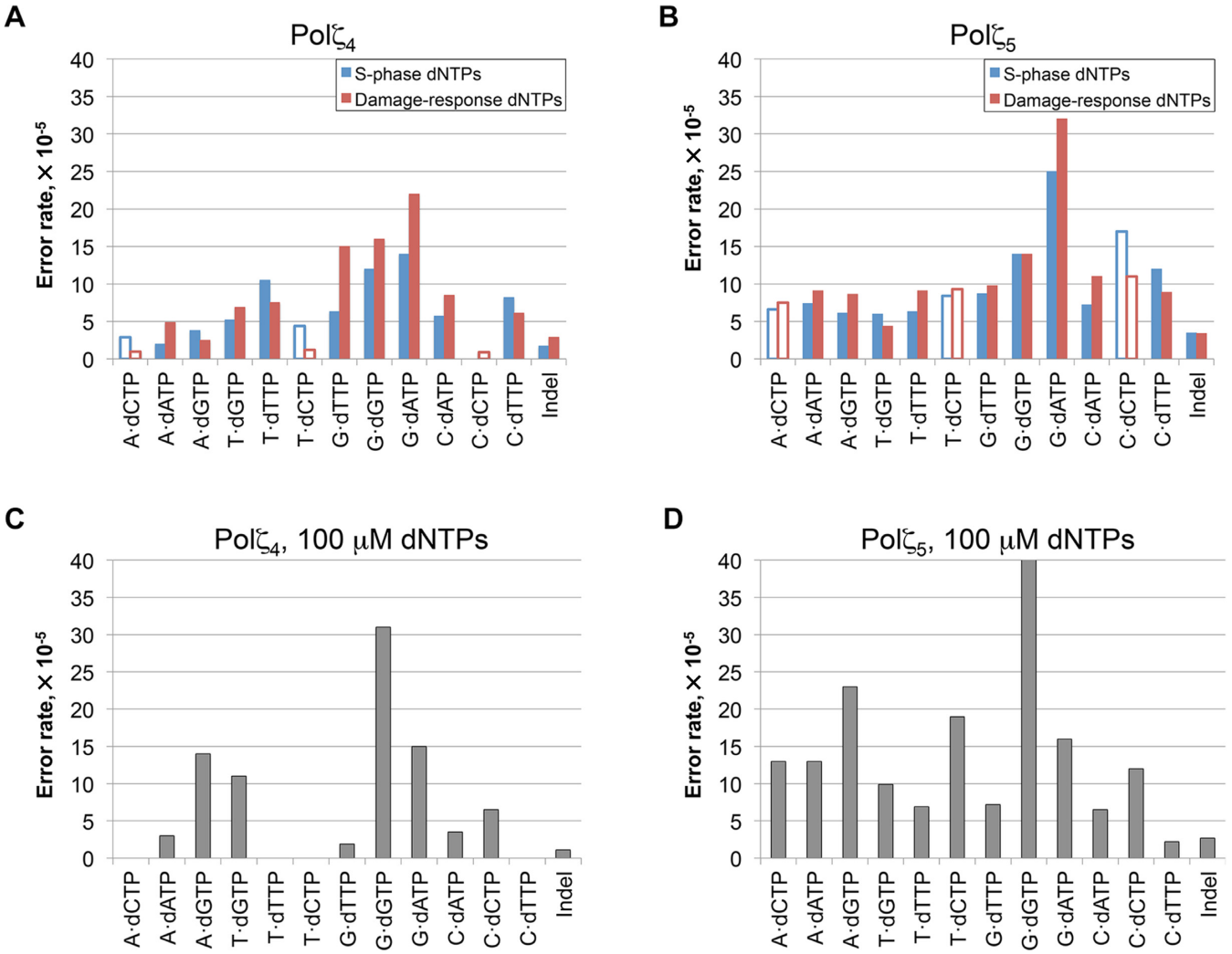


Figure 3. Rates of individual single-base errors generated by Polζ *in vitro* at intracellular and equimolar dNTP concentrations. The diagrams show rates of single-base mispairs and insertion/deletion mismatches observed in reactions with (**A** and **C**) Polζ$_4$ and (**B** and **D**) Polζ$_5$ at (**A** and **B**) S-phase and damage-response dNTP concentrations and at (**C** and **D**) standard 100 μM dNTPs. Data in panels (A) and (B) are from Table 1. Data for Polζ$_4$ and Polζ$_5$ at 100 μM dNTP are based on the analysis of 53 and 80 mutant plaques, respectively. In panels (A) and (B), 'X·dCTP' mispairs are shown as open bars.

with a different, typically much shorter, sequence (Table 3). At damage-response dNTP concentrations, these large rearrangements were observed in 5% of *lacZ* mutants. Unlike complex mutations affecting short stretches of DNA, such large rearrangements are not usually seen in the spectra of Polζ-dependent mutations *in vivo*. It is possible that they result from the inhibitory effect of Rev1 on Polζ-dependent synthesis *in vitro* and may not be relevant to *in vivo* situations.

Prior to this work, the error specificity of Polζ has been studied using equimolar (100 μM) dNTP concentrations and enzyme preparations containing mostly Rev3–Rev7 subassembly (26). Although the mutational spectrum observed in that earlier study similarly showed a predominance of base substitutions and a high frequency of complex mutations, the spectrum of base substitutions was drastically different from the one shown in Figure 3A. To determine if the proper dNTP balance was the key in shaping the error signature of Polζ, we performed gap-filling reactions with Polζ$_4$ and Polζ$_5$ using 100 μM concentration of each dNTP. The average *lacZ* mutant frequency for Polζ$_4$ reactions (0.018) and the overall error rate for single-nucleotide changes (8.7×10^{-4}) were similar to those observed at the intracellular dNTP levels. However, the error specificity of Polζ$_4$ in reactions with equimolar dNTPs was profoundly different (Figure 3C). The dGTP misincorporation became the predominant source of mutations, with the G-dGTP mispair being the single most frequent error. The use of equimolar dNTP concentrations also elevated the rate of C-dCTP mispair more than 7-fold in comparison to reactions with intracellular dNTPs (Figure 3A and C and Supplementary Figure S2C). At the same time, the use of 100 μM dNTPs significantly lowered the ability of Polζ$_4$ to misincorporate dTTP: the rates of all three possible X-dTTP mispairs were drastically decreased (Figure 3A and C and Supplementary Figure S2C). The changes in the base substitution pattern were consistent with the dNTP imbalance introduced by the use of equimolar con-

Table 3. Complex mutations, multiple mutations and large rearrangements induced by Polζ $_{5}$*in vitro*

dNTPs	Mutation type	Sequence change	Location in *lacZ*
S-phase	Complex	TA → G	−50 to −49
		GC →T	−38 to −37
		TA → AGC	38–39
		TA → AG	38–39
		GG → TC	89–90
		GC → AT	145–146
		ATG → TTT	−11 to −9
		GTG → CTT	82–84
		GCG → TCC	100–102
		CTG → ATT	146–148
		GCA → CCC	169–171
		TGCA → G	122–125
		GCTG → CCTA	145–148
		AATAG → AT	153–157
		GTAATAG → T	151–157
		TTAATGT → ATAAAGA	−73 to −67
		AAGAGGCCC → GGGGGCC	160–168
	Multiple	C → G; ΔC	146; 158
		ΔT; C → A	113; 129
		ΔA; C → A	−45; −23
		ΔA; C → A	94; 146
		T → C; G → T	−58; 7
		C → T; CCCCC → TCCCT	68; 132–136
		ΔG; G → T; G → T	−38; 41; 53
		C → G; T → G; G → T	−55; 3; 47
		G → T; ΔT	12; 122
		ΔC; A → T; T → C	10; 31; 121
		G → T; G → T; G → C	63; 149; 178
		C → A; C → G	−59; 60
		del(90); T → C; G → A	−167 to −77; −34; 84
		T → C; G → A	−10; 191
	Large rearrangements	TGTGTGGAATTGTGAGCGGATAACAATTTCAC → CGT	−7 to 25
Damage-response	Complex	TA → CT	38 – 39
		TG → CT	87–88
		GG → TC	88–89
		GC → CT	149–150
		GG → CT	148–149
		CCC → GCCT	134–136
		TCG → CA	176–178
		GCAC → TC	−47 to −44
		CGTG → TGTA	81–84
		AAAA → GAAC	91–94
		TTA → GTC	103–105
		ATGTT → TAGTTT	−11 to −7
		TCGTG → GTGTA	80–84
		TCGTG → GGGGGG	80–84
		CGCAC → GGCA	168–172
		CCGTCG → ACGTCC	64–69
		GCACCG → CCACCT	169–174
		TCCCAA → AT	183–188
		ATCCCCC → TTCCCCT	130–136
		AGAGGC → GGAGGGGG	161–167
		GTGTGGAAT → TTTGAAAG	−6 to 3
		TCCCCCTTT → CCCCCCTTTTAT	131–140
		AGCACATCCCCC → TCC	125–136
		ACCCTGGCGTTA → CCCCTGGCGTTC	94–105
		ATTACGAATTCACTGG → CGAATTCACTG	48–63
	Multiple	CCCAGGCTTTACAC → Δ; C → T	−43 to −30; −20
		G → T; C → G	89; 101
		TG → AA; ΔG	–69 to −68; 47
		C → T; A → T	−14; 24
		+T; C → G	139; 177
		G → A; A → G	149; 188
		A → T; ΔC	128; 168
		G → T; A → T	88; 130
		A → T; C → A	24; 68
		C → T; G → T; G → C	134; 151; 178
		G → T; A → G	84; 130
		A → C; TCCCCC → TTCCCCT	94; 131–136
		T → G; GCG → CCT	104; 149–151
		G → T; C → A	84; 136
		T → A; +T	80; 139
		GTCGTTTTACAACG → TTTTTTAA; TCCCCC → TTCCCCT	66–76; 131–136
		G → C; A → G	79; 161
		C → A; C → G	65; 146
		A → C; GGC → AGG	85; 164–166
		G → T; AACAATTT → GACAATTTT; CGTTTTACAACG → TGTTTTACAACA	−4; 15–23; 68 – 79
		G → C; G → C; G → T	−9; 11; 88
		G → T; G → C	−18; 88

Table 3. Continued

dNTPs	Mutation type	Sequence change	Location in *lacZ*
		C → G; C → G	10; 142
		G → T; G → T	−47; 88
		C → A; A → T	10; 160
		C → T; +T	−30; 139
		+T; GGCGTTA → TTCGGTC	−71; 99–105
		G → A; ΔA; G → T	−24; 94; 157
		T → A; C → T	−2; 189
		T → C; G → T; G → A	−36; 149; 164
		A → T; C → T	−74; 136
	Large rearrangements	GTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTC → TTTTA	102–140
		GTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAG GA → AC	−9 to 31
		CGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCA CACAGG → TGTATT	−14 to 31
		GACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAA CGCAATTAATGTGAGTTAGCTCACTCA → TTTTTTT	−150 to −52
		CAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCA GTGAGCGCAACGCAATTAATGTG → ATTAGTA	−127 to −66
		CTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATA ACAATTTCACACAGGAAACAGCTATGAC → AA	−23 to 43
		GTTGTGTGGAATTGTGAGCGGATAACAATTTCACAC → A; A→C	−9 to 27; 59
		TGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACA GG → AG	−10 to 30
		GACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGT GAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTT ATG → TTTT	−105 to −24
		CTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTT CACACAGGAAACAGCTATGACCATGATTACGAATTCAC TGGCCGTCGTTTT → TCTGGTTCGCTTTGAAGCTCGAATTAAAACGCGATATTTG AAGTCTTTCGGGCTTCCTCTTAATCTT	−16 to 73

See Table 2 legend for detailed explanation of symbol and data representation.

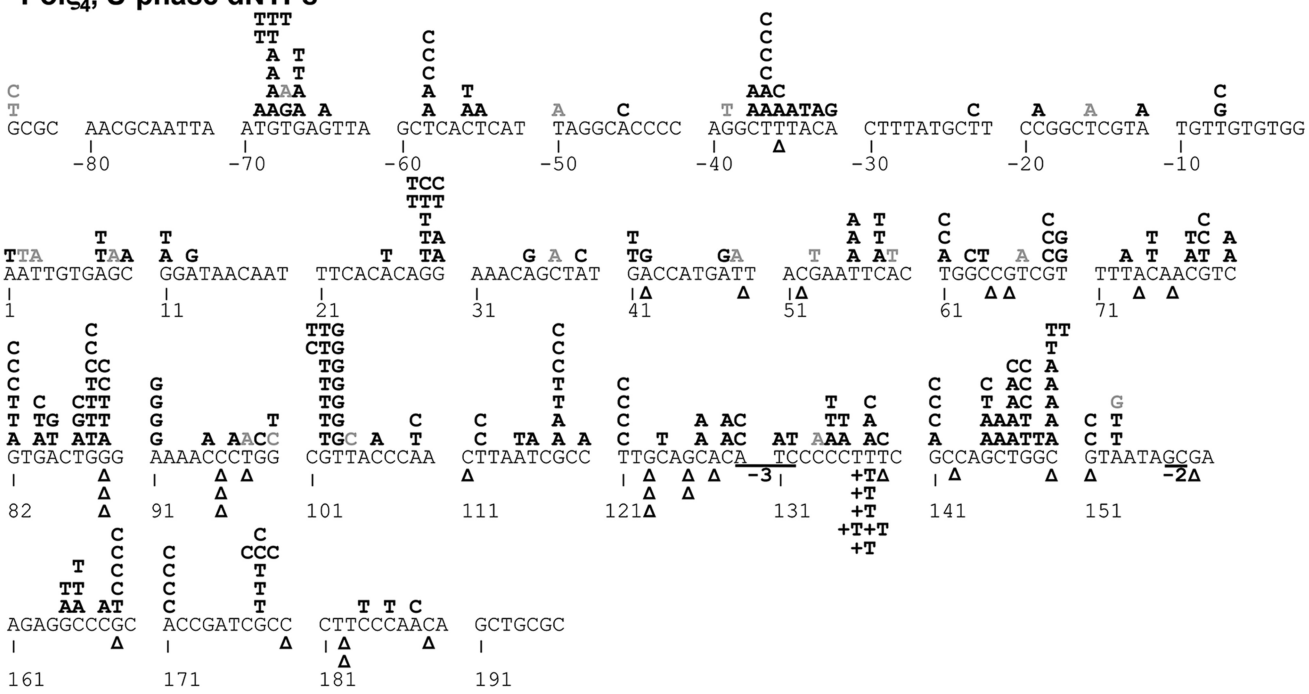
centrations (relatively higher dGTP and dCTP levels, and a lower dTTP level). Interestingly, the percentage of *lacZ* mutants resulting from complex mutations was greater at 100 μM dNTPs and constituted 13% (compared to 5% with intracellular dNTPs). Similar results were observed with Polζ$_5$: its overall error rate at 100 μM dNTPs (1.7×10^{-3}) was comparable to that at the intracellular dNTPs (Table 1), but the spectrum of single-base changes was dramatically different (Figure 3B and D and Supplementary Figure S2D). As in the case of Polζ$_4$, the majority of mutations produced by Polζ$_5$ at 100 μM dNTPs resulted from dGTP and dCTP incorporation, with the G-dGTP being the single most frequent error (Figure 3D). Again, this is consistent with the non-physiological high levels of dGTP and dCTP in the reactions with equimolar dNTPs. Taken together, these data provide evidence that, although the shift from S-phase to damage-response dNTP concentrations does not affect the fidelity and error specificity of Polζ$_4$ or Polζ$_5$, a non-physiological dNTP ratio, as in the case of 100 μM dNTPs, can dramatically change the error signature of these polymerases.

Figure 4 shows the distribution in the *lacZ* sequence of single-nucleotide changes made by Polζ$_4$ and Polζ$_5$ at the intracellular dNTP concentrations. The overall distribution of mutations appears to be quite uniform in all four spectra, with the exception of several mild hotspots. The strongest hotspot was observed in the Polζ$_4$ spectra for a +1 frameshift in the TTT homonucleotide run at position 137–139 where almost all +1 frameshifts occurred (Figure 4A and B). Although we could not discern any specific nucleotide context for generating particular types of mutations by Polζ$_4$, it could be noted that most of the sites with frequent G misincorporation are followed by a template C, such as at positions −36, 121, 169, 171, 178 in the 'S-phase' mutational spectrum and 79, 121, 141 in the 'damage-response' mutational spectrum. This might point to primer-template misalignment as a possible mechanism for generating these types of mutations at these particular sites. The presence of Rev1 in the complex with Polζ$_4$ did not change the distribution of mutations (Figure 4C and D), suggesting that Rev1 does not stimulate misincorporation of nucleotides at any particular sequence context.

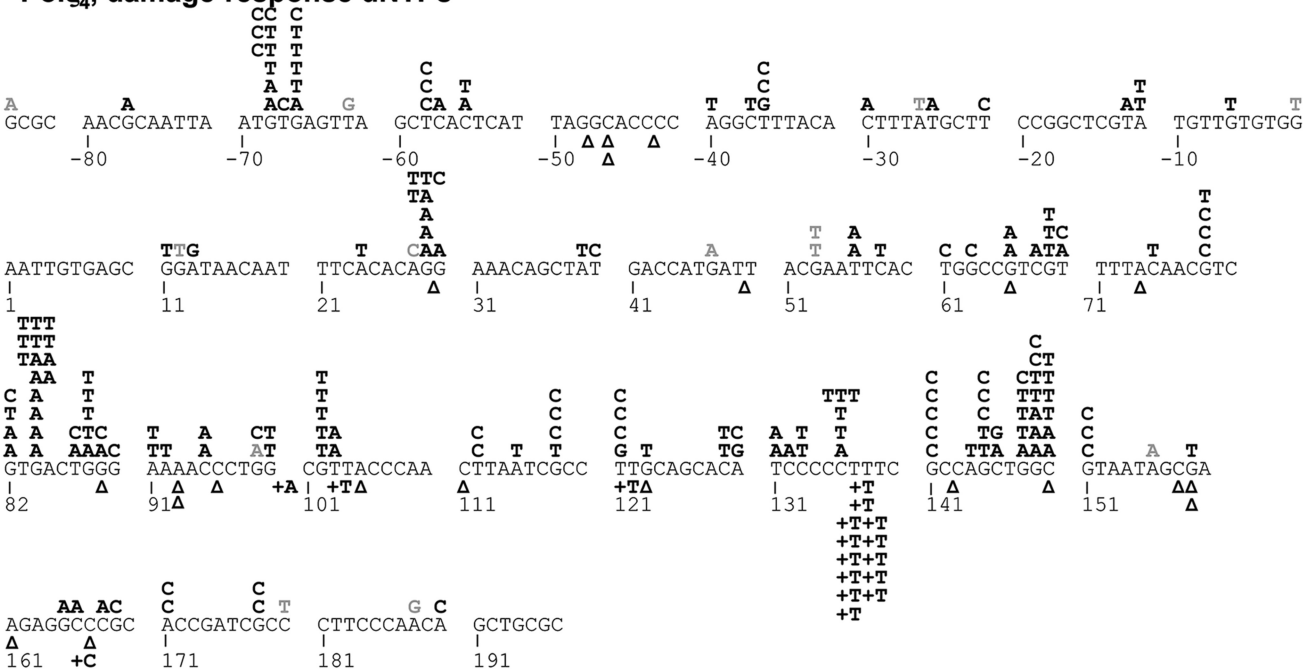
Polζ-dependent mutagenesis *in vivo* does not require high dNTP levels

The *in vitro* data described in the previous subsections indicate that the switch from the S-phase to the damage-response dNTP concentrations could facilitate copying of long DNA stretches by Polζ. At the same time, Polζ activity on shorter templates, fidelity and error specificity are only minimally affected by dNTP levels. In yeast cells, Polζ-dependent mutagenesis is mostly observed when dNTP pools are expanded. We, therefore, aimed to determine whether high dNTP levels are essential for Polζ function *in vivo*. We first tested whether depletion of dNTP pools by treatment with HU, an inhibitor of RNR (69), would decrease Polζ-dependent spontaneous mutagenesis in the *pol3-Y708A* strain. Because the *pol3-Y708A* mutant cannot tolerate high HU concentrations (48), we used a range of lower concentrations (10–20 mM) that did not cause growth arrest in this strain. At 20 mM HU, dNTP pools in the *pol3-Y708A* mutant were reproducibly decreased by ap-

A **Polζ$_{4}$, S-phase dNTPs**



B **Polζ$_{4}$, damage-response dNTPs**



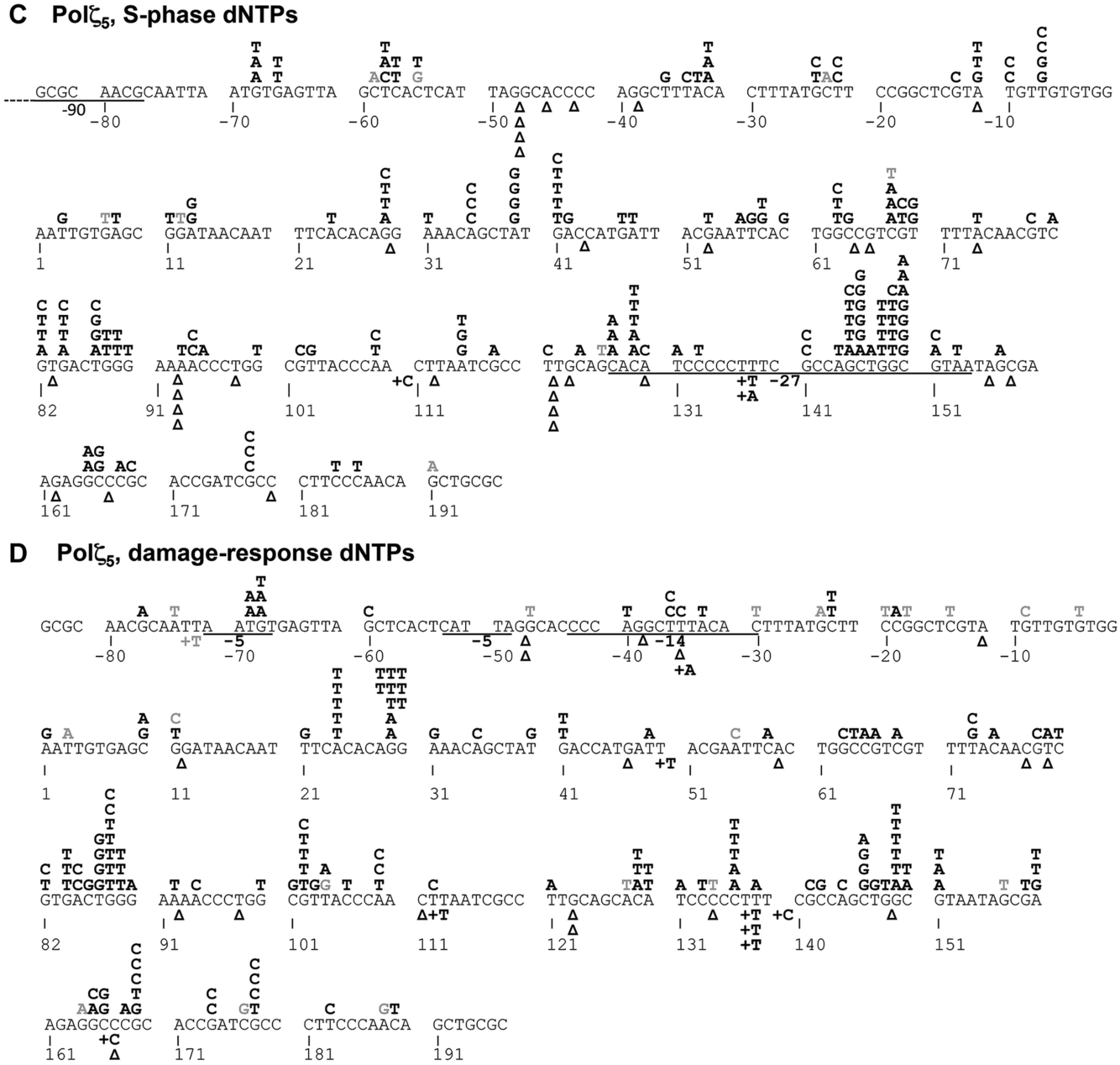


Figure 4. Spectra of single-base substitutions and insertion/deletion mutations generated by Polζ complexes in the *lacZ* gene at cellular dNTP concentrations. (**A**) Polζ$_4$, S-phase dNTPs. (**B**) Polζ$_4$, damage-response dNTPs. (**C**) Polζ$_5$, S-phase dNTPs. (**D**) Polζ$_5$, damage-response dNTPs. In addition to the mutations shown, one *lacZ* mutant contained a large deletion spanning nucleotides −119 to 150. Base substitutions are displayed above the *lacZ* sequence, insertions and deletions are below the *lacZ* sequence. Single-base deletions and insertions are shown as triangles and letters with a '+' symbol, respectively. Deletions of more than one nucleotide are indicated by a line below the sequence with a number of deleted nucleotides next to it. Detectable mutations are in black, bold text. Silent mutations are in grey. Data are summarized in Table 1 and Figure 3.

proximately 25% within 2 h after the addition of the drug to logarithmically growing cultures (Figure 5B). An isogenic wild-type strain also showed decreased average dNTP levels in the first 30 min of treatment with 20 mM HU (Figure 5B), although at least some of it could be attributed to the changing cell cycle distribution. Particularly, the proportion of G1 cells, which have approximately 2-fold lower dNTP pools (4), varied between the time points (Figure 5C). In contrast, the cell cycle distribution in the *pol3-Y708A* strain did not change significantly during the 2 h in 20 mM HU (Figure 5C), so the dNTP measurements shown in Figure 5B reflect the actual decrease in intracellular levels. Remarkably, the frequency of mutation to canavanine resistance (Can^r) in the *pol3-Y708A* strain was not reduced in the presence of HU, but was in fact slightly elevated (up to 2-fold at 20 mM HU; Figure 5A). The mutator effect of *pol3-Y708A* in the presence of HU remained completely dependent on Polζ: the mutant frequency in the *pol3-Y708A rev3*Δ strain was

similar to that in the wild-type strain. These data indicate that the participation of Polζ in replication of undamaged DNA *in vivo* does not depend on high dNTP levels, and that it is stimulated rather than suppressed by the decrease in dNTP pools. It is, therefore, likely that the high dNTP pools in the *pol3-Y708A* strain are required for efficient replication by Polδ rather than Polζ. In support of this idea, we found that the moderate decrease in dNTP concentrations induced by low doses of HU in our experiments led to a dramatic reduction in the survival of the *pol3-Y708A* strain (Figure 5D). Replication problems caused by the Polδ defect are likely exacerbated by dNTP depletion, increasing the need for the recruitment Polζ, whose function is unaffected by the reduced dNTP levels.

Next, we examined whether high dNTP pools are required for Polζ-dependent mutagenesis during lesion bypass. While Polζ is predominately an extender polymerase during TLS across from most DNA lesions, previous studies showed that it might be a major polymerase involved in the bypass of UV-induced lesions at low doses of UVC light (70,71). To study whether Polζ-dependent mutagenesis at low UV doses is affected by changes in dNTP pools, we measured UV-induced Can^r mutant frequency in the presence of HU, such that the bypass of UV lesions would happen in cells with reduced dNTP levels. Overnight cultures were plated on complete and selective media containing HU at the concentrations indicated in Figure 5E and irradiated with 10 J/m^2 of 254 nm UV light within 15 min after plating. These experiments were done with wild-type yeast strains, so we could use higher HU concentrations (up to 100 mM), which deplete dNTP pools more efficiently. UV-induced mutagenesis was only marginally decreased (approximately 1.5-fold) at the highest dose of HU, while still remaining an order of magnitude higher than the level of spontaneous mutagenesis (Figure 5E). Notably, UV-induced mutagenesis observed in the presence of HU was completely dependent on Polζ: no induced mutagenesis was seen in the *rev3Δ* strain with or without HU (Figure 5E). These data indicate that, like copying of undamaged DNA, lesion bypass by Polζ *in vivo* does not require high dNTP pools. Mutagenesis at higher UV doses, however, was significantly suppressed by the HU treatment (Supplementary Figure S3A), consistent with the idea that high dNTP levels are required for the activity of other DNA polymerases that become important for TLS at these doses.

To strengthen the conclusion that Polζ function in TLS and damage-induced mutagenesis does not require high dNTP pools, we measured UV-induced Can^r mutant frequency in cells that were pre-treated with 100 mM HU for 4 h before UV irradiation. We reasoned that dNTP pools in this case could be more severely reduced by the time DNA replication machinery encounters lesions. We found that the frequency of mutation induced by lower doses of UV (up to 30 J/m^2) was, in fact, significantly elevated in HU-treated cells in comparison to cells not treated with HU (Supplementary Figure S3B). Similar to the experiment shown in Supplementary Figure S3A, mutagenesis at higher UV doses was reduced in cells pre-treated with HU. The increase in Polζ mutagenesis at lower UV doses could potentially result from the inhibition of error-free mechanisms of lesion bypass under conditions of severely reduced dNTP pools, or from altered fidelity of nucleotide incorporation opposite lesions by Polζ. In either case, the results clearly demonstrate that the capacity of Polζ to bypass lesions *in vivo* does not require expanded dNTP pools. High dNTP levels, however, might be essential for lesion bypass by other DNA polymerases and for repair under DNA-damaging conditions.

DISCUSSION

Accumulating *in vivo* and *in vitro* data suggest that intracellular dNTP levels play an important role in determining the fidelity of DNA replication, and mimicking physiologically relevant dNTP concentrations is important for deducing DNA polymerase signatures from *in vitro* experiments (5,7–8,13,72). High or imbalanced dNTP pools increase the probability of nucleotide misinsertion, mismatched primer extension and strand misalignment by replicative DNA polymerases. For example, mutations in the yeast *RNR1* gene encoding a subunit of RNR lead to alterations in dNTP pools and, as a result, to a dramatic increase in genome instability (6). The mutational specificity observed in these strains correlates well with misincorporation of nucleotides that are in excess. Higher dNTP concentrations may reduce proofreading by replicative DNA polymerases, contributing to the increased error rate (6,9,13,72). Recent work by Mertz *et al*. demonstrated that the use of equimolar dNTP concentrations to determine error specificity of a mutator Polδ variant in the M13mp2 assay drastically underestimated the actual error rates for individual mispairs and significantly altered the mutational signature (8). Increasing dNTP concentrations also decreased the fidelity of exonuclease-deficient Polε in the M13mp2 assay and altered the specificity of nucleotide misincorporation (73). While the expansion of cellular dNTP pools is an integral part of DNA damage response, the effects of dNTP levels on the function of TLS polymerases are poorly understood. A previous study suggested that high dNTP levels stimulate TLS in *E. coli* by attenuating the proofreading activity of replicative DNA polymerase III (13). However, it has not been established whether elevated dNTP pools also stimulate the activity of TLS polymerases. In this work, we determined how the physiological shift from the S-phase to the damage-response dNTP concentrations affects the activity, fidelity and error specificity of yeast Polζ. We provide evidence that, unlike replicative DNA polymerases, Polζ is remarkably insensitive to proportional increases and decreases in dNTP concentrations and does not require high dNTP levels for its *in vivo* functions. Thus, Polζ-dependent synthesis might represent a unique cellular mechanism for tolerating low dNTP levels. The ability of Polζ to work at low dNTP levels also explains the long-known involvement of this polymerase in the generation of spontaneous mutations (57,58), which presumably arise during the normal S-phase when checkpoints are not activated and dNTP pools are not expanded.

One of the important insights from this work is that the error signature of Polζ at the physiological dNTP levels (Figure 3A) is drastically different from its previously reported signature observed at equimolar (100 μM) dNTP concentrations (26). This finding further emphasizes the

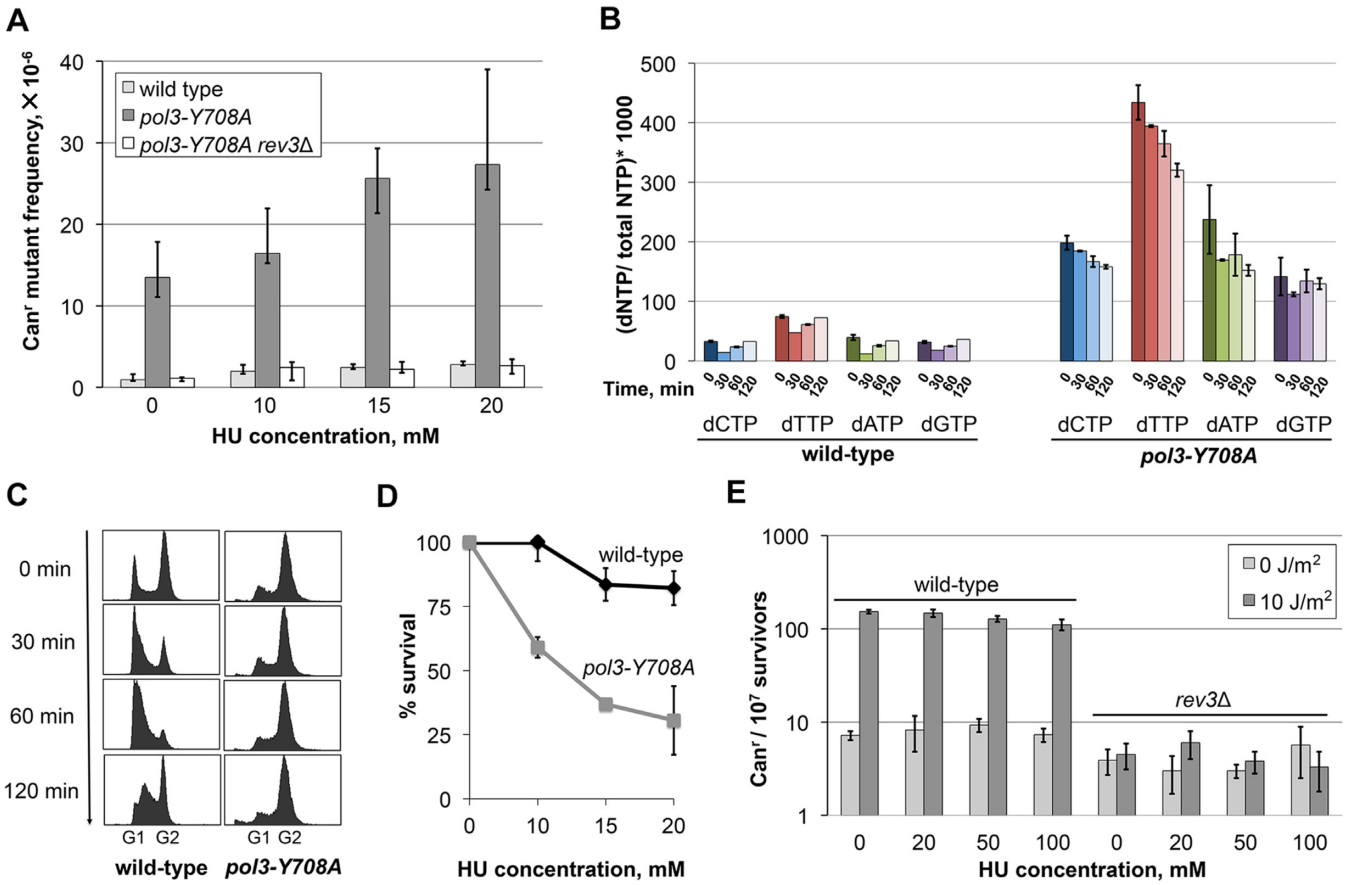


Figure 5. Polζ-dependent mutagenesis *in vivo* does not require high dNTP levels. (**A**) The effect of hydroxyurea (HU) treatment on the Polζ-dependent mutator phenotype of the *pol3-Y708A* yeast strain. Wild-type, *pol3-Y708A* and *pol3-Y708A rev3*Δ strains were grown overnight in the presence of indicated HU concentrations and then plated onto selective and complete media. Mutant frequencies are medians and 95% confidence intervals for at least 18 independent cultures. (**B**) Time course analysis of intracellular dNTP levels in wild-type and *pol3-Y708A* strains treated with 20 mM HU. Time after the addition of HU is indicated on the X-axis. The dNTP levels are normalized to total NTPs. Data are presented as the mean for two independent measurements. Error bars represent the range of values. (**C**) FACS analysis of HU-treated cultures of wild-type and *pol3-Y708A* strains that were used for dNTP pool measurements in *B*. Time after the addition of HU is indicated on the left. (**D**) The effect of HU treatment on survival of the *pol3-Y708A* and wild-type strains. The strains were grown overnight in the presence of indicated HU concentrations, and appropriate dilutions were then plated on SC medium. Survival was determined by dividing the number of colonies from HU-treated cultures by the number of colonies from untreated cultures. Data are means for 18 independent cultures. Standard errors are shown unless the size of the error bar is smaller than the size of the plot symbol. (**E**) The effect of HU treatment on Polζ-dependent mutagenesis induced by 10 J/m^2 UV irradiation. Overnight cultures of the wild-type and *rev3*Δ strains were plated onto selective and complete media with indicated HU concentrations and then irradiated with 10 J/m^2 of UV light. Data are the average frequencies and standard errors for three independent determinations.

need to mimic absolute and relative *in vivo* dNTP levels in order to deduce DNA polymerase signatures from *in vitro* studies. It is also interesting that, in addition to using a non-physiological dNTP ratio, the study by Zhong *et al*. was performed at a time when Polζ was thought to be a two-subunit enzyme. Although low levels of four-subunit enzyme in those Polζ preparations are now thought to be predominately responsible for the observed polymerase activity (22), the abundance of two-subunit Rev3-Rev7 complex and the variable content of Pol31–Pol32 subunits could have contributed to the differences in error signature. Curiously, while we could not recapitulate the error spectrum reported by Zhong *et al*. even when we used 100 μM dNTPs with Polζ$_4$, we saw a rather close similarity when we used Polζ$_5$ and 100 μM dNTPs (see Figure 3D and the error specificity of Polζ in the presence of accessory proteins in (26). The only major difference was a higher rate of A-dCTP errors in the study by Zhong *et al*. that might have resulted from a bias introduced by strong hotspots that we did not observe. This profound spectra similarity suggests that the error spectrum reported by Zhong *et al*. might have, in fact, resulted from the activity of Polζ$_5$.

In line with the previous report (64), we observed that Rev1 drastically inhibits the activity of Polζ$_4$ *in vitro* (Figure 2C–E). Although the mechanism of this inhibition remains enigmatic and requires further investigation, we speculate that the competition of Rev1 and Polζ for the primer terminus could negatively affect the rate of DNA synthesis. The strong increase in C misincorporation in Polζ$_5$ reactions in comparison to Polζ$_4$ (Table 1, Figure 3A and B and Supplementary Figure S2A and B) is consistent with Polζ and Rev1 switching at the primer terminus. However, presumably lower processivity and a slower rate of dNTP incorporation by Rev1, as well as the delays associated with the

physical exchange of the polymerases, could decrease the overall rate of synthesis. While it is an intriguing possibility that Rev1 restricts the activity of Polζ on undamaged DNA, thus preventing excessive mutagenesis, it is also possible that the inhibitory effect of Rev1 is related to a missing regulatory component in our *in vitro* assays. In particular, monoubiquitination of PCNA or phosphorylation of Rev1 might be necessary for the stimulation of Polζ by Rev1. In agreement with the latter idea, we have recently identified a mutant form of Rev1 with a deletion of the highly conserved M1 motif (Rev1-(135–150)). This Rev1 variant enhances TLS and processive replication by Polζ and possibly phenocopies a required post-translational modification of Rev1 (64).

This study also reveals that Polζ does not require high dNTP pools for the replication of undamaged DNA or the bypass of DNA lesions *in vivo*. DRIM was not decreased in the *pol3-Y708A* strain, but on the contrary, was even further elevated when dNTP pools were brought down by treatment with HU (Figure 5A). Similarly, mutagenesis induced by low doses of UV light was increased rather than decreased when cells were treated with HU prior to UV irradiation (Supplementary Figure S3B). In line with these observations, using damage-response dNTP concentrations for TLS by Polζ *in vitro* only slightly improved nucleotide incorporation opposite *cis-syn* cyclobutane pyrimidine dimer and (6–4)-photoproduct and the bypass of these lesions (17). Furthermore, we observed only a minor difference in the activity, fidelity and error specificity of $Pol\zeta_4$ and $Pol\zeta_5$ when damage-response dNTP concentrations were used instead of S-phase concentrations (Figure 2, Table 1 and Figure 3A and B). These findings are consistent with an earlier observation that K_m for the insertion of a properly base-paired nucleotide by Polζ is much lower than the calculated S-phase dNTP levels even when a rather inactive two-subunit Polζ without PCNA is used (74). Thus, the rise in dNTP levels in response to DNA damage or replication perturbations may be primarily needed to facilitate other, non-mutagenic tolerance mechanisms. High dNTP levels could improve the activity of replicative DNA polymerases, as well as the TLS capacity of Polη, which, at least in the case of UV-induced lesions, would contribute to mutation avoidance. Expanded dNTP pools could also potentially promote DNA repair and high-fidelity template-switching mechanisms of damage tolerance, where synthesis by replicative DNA polymerases might be required. Indeed, up-regulation of the RNR activity has been shown to promote the rate of fork progression during normal replication and under conditions of replication stress (75). Recent biochemical studies showed that the rate of DNA synthesis by Polδ is not optimal at physiological dNTP concentrations and can be substantially improved by increasing dNTP levels (76). Increased dNTP concentrations are also known to facilitate the bypass of certain lesions by replicative DNA polymerases *in vitro* and *in vivo* (16,77).

Previous studies of the UV sensitivity of yeast strains deficient in TLS revealed a differential involvement of TLS polymerases in the bypass of UV lesions at low and high doses of irradiation. Polζ-deficient strains show higher sensitivity to low doses of UV light than Polη mutants, while Polη-deficient strains are more sensitive to higher doses (>30 J/m^2; (70)). These data imply that the bypass of UV-induced lesions at lower doses relies predominantly on Polζ, while other polymerases become important at higher doses. The lack of effect of HU treatment on UV-induced mutagenesis at the low UV dose and the clear inhibition of mutagenesis by HU at higher UV doses (Figure 5E and Supplementary Figure S3) further proves that, unlike other DNA polymerases, Polζ does not require high dNTP levels for TLS *in vivo*. On the contrary, expanded dNTP pools become vital for lesion bypass at higher doses of UV irradiation when other DNA polymerases must be involved, such as Polη or replicative polymerases. The importance of high dNTP concentrations at high doses of UV light has been noted previously, and it has been suggested that elevated dNTP pools promote lesion bypass by Polδ (77). In addition, upregulation of dNTPs improves DNA damage tolerance of yeast strains deficient in all three TLS polymerases, presumably by stimulating synthesis by replicative polymerases (16). While the extent of dNTP pool expansion in cells treated with low and high UV doses has not been compared, experiments with chemical mutagens showed that lower doses result in a less pronounced increase in dNTP levels (4). It is tempting to speculate that the crucial role of Polζ in damage tolerance at lower UV doses is due to dNTP levels not being high enough at these doses for the other polymerases to bypass lesions.

In summary, it appears possible that Polζ evolved toward decreasing the dependence of its DNA synthesis activity on the levels of intracellular dNTPs, providing cells with a rescue tool when normal DNA replication is perturbed due to low dNTP supply. This hypothesis is further reinforced by our earlier finding that treatment of wild-type yeast strains with HU causes a Polζ-dependent increase in mutagenesis (47). Interestingly, it has been reported that depletion of dNTP pools can contribute to early stages of tumorigenesis by promoting replication stress and genome instability (78–80). The yeast and mammalian Polζ have the same subunit composition, show high amino acid sequence homology, and perform similar functions in TLS and DNA damage-induced mutagenesis (81–87). If human Polζ is similarly insensitive to decreases in dNTP levels, it is likely that the genome instability induced by depletion of dNTP pools in human cells results, at least in part, from error-prone DNA synthesis by Polζ recruited to the stalled replication forks.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank Tony Mertz for the preparations of PCNA and RPA used in the fidelity assays, Tom Kunkel and Kasia Bebenek for *E. coli* CSH50 strain used in these assays, and Elizabeth Moore and Krista Brown for technical assistance.

FUNDING

National Institutes of Health [ES015869 to P.V.S., GM032431 and GM118129 to P.M.B.]; Swedish Cancer Society, the Knut and Alice Wallenberg Foundation

and the Swedish Research Council grants to A.C.; University of Nebraska Medical Center Graduate Studies Assistantship/Fellowship (to O.V.K.). Funding for open access charge: NIH [ES015869].
Conflict of interest statement. None declared.

REFERENCES

1. Labib,K. and De Piccoli,G. (2011) Surviving chromosome replication: the many roles of the S-phase checkpoint pathway. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **366**, 3554–3561.
2. Reichard,P. (1988) Interactions between deoxyribonucleotide and DNA synthesis. *Annu. Rev. Biochem.*, **57**, 349–374.
3. Andreson,B.L., Gupta,A., Georgieva,B.P. and Rothstein,R. (2010) The ribonucleotide reductase inhibitor, Sml1, is sequentially phosphorylated, ubiquitylated and degraded in response to DNA damage. *Nucleic Acids Res.*, **38**, 6490–6501.
4. Chabes,A., Georgieva,B., Domkin,V., Zhao,X., Rothstein,R. and Thelander,L. (2003) Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. *Cell*, **112**, 391–401.
5. Williams,L.N., Marjavaara,L., Knowels,G.M., Schultz,E.M., Fox,E.J., Chabes,A. and Herr,A.J. (2015) dNTP pool levels modulate mutator phenotypes of error-prone DNA polymerase ε variants. *Proc. Natl. Acad. Sci. U.S.A.*, **112**, E2457–E2466.
6. Kumar,D., Abdulovic,A.L., Viberg,J., Nilsson,A.K., Kunkel,T.A. and Chabes,A. (2011) Mechanisms of mutagenesis *in vivo* due to imbalanced dNTP pools. *Nucleic Acids Res.*, **39**, 1360–1371.
7. Kumar,D., Viberg,J., Nilsson,A.K. and Chabes,A. (2010) Highly mutagenic and severely imbalanced dNTP pools can escape detection by the S-phase checkpoint. *Nucleic Acids Res.*, **38**, 3975–3983.
8. Mertz,T.M., Sharma,S., Chabes,A. and Shcherbakova,P.V. (2015) Colon cancer-associated mutator DNA polymerase δ variant causes expansion of dNTP pools increasing its own infidelity. *Proc. Natl. Acad. Sci. U.S.A.*, **112**, E2467–E2476.
9. Watt,D.L., Buckland,R.J., Lujan,S.A., Kunkel,T.A. and Chabes,A. (2016) Genome-wide analysis of the specificity and mechanisms of replication infidelity driven by imbalanced dNTP pools. *Nucleic Acids Res.*, **44**, 1669–1680.
10. Ahluwalia,D. and Schaaper,R.M. (2013) Hypermutability and error catastrophe due to defects in ribonucleotide reductase. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, 18596–18601.
11. Tse,L., Kang,T.M., Yuan,J., Mihora,D., Becket,E., Maslowska,K.H., Schaaper,R.M. and Miller,J.H. (2016) Extreme dNTP pool changes and hypermutability in *dcd ndk* strains. *Mutat. Res.*, **784-785**, 16–24.
12. Schaaper,R.M. and Mathews,C.K. (2013) Mutational consequences of dNTP pool imbalances in *E. coli*. *DNA Repair (Amst)*, **12**, 73–79.
13. Gon,S., Napolitano,R., Rocha,W., Coulon,S. and Fuchs,R.P. (2011) Increase in dNTP pool size during the DNA damage response plays a key role in spontaneous and induced-mutagenesis in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.*, **108**, 19311–19316.
14. Huang,M., Zhou,Z. and Elledge,S.J. (1998) The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. *Cell*, **94**, 595–605.
15. Zhao,X., Chabes,A., Domkin,V., Thelander,L. and Rothstein,R. (2001) The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage. *EMBO J.*, **20**, 3544–3553.
16. Sabouri,N., Viberg,J., Goyal,D.K., Johansson,E. and Chabes,A. (2008) Evidence for lesion bypass by yeast replicative DNA polymerases during DNA damage. *Nucleic Acids Res.*, **36**, 5660–5667.
17. Stone,J.E., Kumar,D., Binz,S.K., Inase,A., Iwai,S., Chabes,A., Burgers,P.M. and Kunkel,T.A. (2011) Lesion bypass by *S. cerevisiae* Pol ζ alone. *DNA Repair*, **10**, 826–834.
18. Haracska,L., Prakash,S. and Prakash,L. (2000) Replication past O(6)-methylguanine by yeast and human DNA polymerase η. *Mol. Cell. Biol.*, **20**, 8001–8007.
19. Haracska,L., Unk,I., Johnson,R.E., Johansson,E., Burgers,P.M., Prakash,S. and Prakash,L. (2001) Roles of yeast DNA polymerases δ and ζ and of Rev1 in the bypass of abasic sites. *Genes Dev.*, **15**, 945–954.
20. Johnson,R.E., Washington,M.T., Haracska,L., Prakash,S. and Prakash,L. (2000) Eukaryotic polymerases ι and ζ act sequentially to bypass DNA lesions. *Nature*, **406**, 1015–1019.
21. Johnson,R.E., Haracska,L., Prakash,S. and Prakash,L. (2001) Role of DNA polymerase η in the bypass of a (6-4) TT photoproduct. *Mol. Cell. Biol.*, **21**, 3558–3563.
22. Makarova,A.V., Stodola,J.L. and Burgers,P.M. (2012) A four-subunit DNA polymerase ζ complex containing Pol δ accessory subunits is essential for PCNA-mediated mutagenesis. *Nucleic Acids Res.*, **40**, 11618–11626.
23. Johnson,R.E., Prakash,L. and Prakash,S. (2012) Pol31 and Pol32 subunits of yeast DNA polymerase δ are also essential subunits of DNA polymerase ζ. *Proc. Natl. Acad. Sci. U.S.A.*, **109**, 12455–12460.
24. Braithwaite,D.K. and Ito,J. (1993) Compilation, alignment, and phylogenetic relationships of DNA polymerases. *Nucleic Acids Res.*, **21**, 787–802.
25. Ito,J. and Braithwaite,D.K. (1991) Compilation and alignment of DNA polymerase sequences. *Nucleic Acids Res.*, **19**, 4045–4057.
26. Zhong,X., Garg,P., Stith,C.M., Nick McElhinny,S.A., Kissling,G.E., Burgers,P.M. and Kunkel,T.A. (2006) The fidelity of DNA synthesis by yeast DNA polymerase ζ alone and with accessory proteins. *Nucleic Acids Res.*, **34**, 4731–4742.
27. Guo,D., Wu,X., Rajpal,D.K., Taylor,J.S. and Wang,Z. (2001) Translesion synthesis by yeast DNA polymerase ζ from templates containing lesions of ultraviolet radiation and acetylaminofluorene. *Nucleic Acids Res.*, **29**, 2875–2883.
28. Lawrence,C.W. (2004) Cellular functions of DNA polymerase ζ and Rev1 protein. *Adv. Protein Chem.*, **69**, 167–203.
29. Makarova,A.V. and Burgers,P.M. (2015) Eukaryotic DNA polymerase ζ. *DNA Repair (Amst)*, **29**, 47–55.
30. D'Souza,S. and Walker,G.C. (2006) Novel role for the C terminus of *Saccharomyces cerevisiae* Rev1 in mediating protein-protein interactions. *Mol. Cell. Biol.*, **26**, 8173–8182.
31. Guo,C., Fischhaber,P.L., Luk-Paszyc,M.J., Masuda,Y., Zhou,J., Kamiya,K., Kisker,C. and Friedberg,E.C. (2003) Mouse Rev1 protein interacts with multiple DNA polymerases involved in translesion DNA synthesis. *EMBO J.*, **22**, 6621–6630.
32. Murakumo,Y., Ogura,Y., Ishii,H., Numata,S., Ichihara,M., Croce,C.M., Fishel,R. and Takahashi,M. (2001) Interactions in the error-prone postreplication repair proteins hREV1, hREV3, and hREV7. *J. Biol. Chem.*, **276**, 35644–35651.
33. Ohashi,E., Murakumo,Y., Kanjo,N., Akagi,J., Masutani,C., Hanaoka,F. and Ohmori,H. (2004) Interaction of hREV1 with three human Y-family DNA polymerases. *Genes Cells*, **9**, 523–531.
34. Acharya,N., Haracska,L., Johnson,R.E., Unk,I., Prakash,S. and Prakash,L. (2005) Complex formation of yeast Rev1 and Rev7 proteins: a novel role for the polymerase-associated domain. *Mol. Cell. Biol.*, **25**, 9734–9740.
35. Acharya,N., Johnson,R.E., Pages,V., Prakash,L. and Prakash,S. (2009) Yeast Rev1 protein promotes complex formation of DNA polymerase ζ with Pol32 subunit of DNA polymerase δ. *Proc. Natl. Acad. Sci. U.S.A.*, **106**, 9631–9636.
36. Tissier,A., Kannouche,P., Reck,M.P., Lehmann,A.R., Fuchs,R.P. and Cordonnier,A. (2004) Co-localization in replication foci and interaction of human Y-family members, DNA polymerase pol η and REV1 protein. *DNA Repair (Amst)*, **3**, 1503–1514.
37. Nelson,J.R., Lawrence,C.W. and Hinkle,D.C. (1996) Deoxycytidyl transferase activity of yeast REV1 protein. *Nature*, **382**, 729–731.
38. Waters,L.S., Minesinger,B.K., Wiltrout,M.E., D'Souza,S., Woodruff,R.V. and Walker,G.C. (2009) Eukaryotic translesion polymerases and their roles and regulation in DNA damage tolerance. *Microbiol. Mol. Biol. Rev.*, **73**, 134–154.
39. Pagès,V., Johnson,R.E., Prakash,L. and Prakash,S. (2008) Mutational specificity and genetic control of replicative bypass of an abasic site in yeast. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 1170–1175.
40. Kim,N., Mudrak,S.V. and Jinks-Robertson,S. (2011) The dCMP transferase activity of yeast Rev1 is biologically relevant during the bypass of endogenously generated AP sites. *DNA Repair (Amst)*, **10**, 1262–1271.
41. Otsuka,C., Kunitomi,N., Iwai,S., Loakes,D. and Negishi,K. (2005) Roles of the polymerase and BRCT domains of Rev1 protein in translesion DNA synthesis in yeast *in vivo*. *Mutat. Res.*, **578**, 79–87.

42. Wiltrout,M.E. and Walker,G.C. (2011) The DNA polymerase activity of *Saccharomyces cerevisiae* Rev1 is biologically significant. *Genetics*, **187**, 21–35.
43. Zhou,Y., Wang,J., Zhang,Y. and Wang,Z. (2010) The catalytic function of the Rev1 dCMP transferase is required in a lesion-specific manner for translesion synthesis and base damage-induced mutagenesis. *Nucleic Acids Res.*, **38**, 5036–5046.
44. Chan,K., Resnick,M.A. and Gordenin,D.A. (2013) The choice of nucleotide inserted opposite abasic sites formed within chromosomal DNA reveals the polymerase activities participating in translesion DNA synthesis. *DNA Repair (Amst)*, **12**, 878–889.
45. Northam,M.R., Garg,P., Baitin,D.M., Burgers,P.M. and Shcherbakova,P.V. (2006) A novel function of DNA polymerase ζ regulated by PCNA. *EMBO J.*, **25**, 4316–4325.
46. Northam,M.R., Moore,E.A., Mertz,T.M., Binz,S.K., Stith,C.M., Stepchenkova,E.I., Wendt,K.L., Burgers,P.M. and Shcherbakova,P.V. (2014) DNA polymerases ζ and Rev1 mediate error-prone bypass of non-B DNA structures. *Nucleic Acids Res.*, **42**, 290–306.
47. Northam,M.R., Robinson,H.A., Kochenova,O.V. and Shcherbakova,P.V. (2010) Participation of DNA polymerase ζ in replication of undamaged DNA in *Saccharomyces cerevisiae*. *Genetics*, **184**, 27–42.
48. Pavlov,Y.I., Shcherbakova,P.V. and Kunkel,T.A. (2001) *In vivo* consequences of putative active site mutations in yeast DNA polymerases α, ε, δ, and ζ. *Genetics*, **159**, 47–64.
49. Shcherbakova,P.V., Noskov,V.N., Pshenichnov,M.R. and Pavlov,Y.I. (1996) Base analog 6-*N*-hydroxylaminopurine mutagenesis in the yeast *S. cerevisiae* is controlled by replicative DNA polymerases. *Mutat. Res.*, **369**, 33–44.
50. Aksenova,A., Volkov,K., Maceluch,J., Pursell,Z.F., Rogozin,I.B., Kunkel,T.A., Pavlov,Y.I. and Johansson,E. (2010) Mismatch repair-independent increase in spontaneous mutagenesis in yeast lacking non-essential subunits of DNA polymerase ε. *PLoS Genet.*, **6**, e1001209.
51. Becker,J.R., Nguyen,H.D., Wang,X. and Bielinsky,A.K. (2014) Mcm10 deficiency causes defective-replisome-induced mutagenesis and a dependency on error-free postreplicative repair. *Cell Cycle*, **13**, 1737–1748.
52. Garbacz,M., Araki,H., Flis,K., Bebenek,A., Zawada,A.E., Jonczyk,P., Makiela-Dzbenska,K. and Fijalkowska,I.J. (2015) Fidelity consequences of the impaired interaction between DNA polymerase ε and the GINS complex. *DNA Repair (Amst)*, **29**, 23–35.
53. Grabowska,E., Wronska,U., Denkiewicz,M., Jaszczur,M., Respondek,A., Alabrudzinska,M., Suski,C., Makiela-Dzbenska,K., Jonczyk,P. and Fijalkowska,I.J. (2014) Proper functioning of the GINS complex is important for the fidelity of DNA replication in yeast. *Mol. Microbiol.*, **92**, 659–680.
54. Kraszewska,J., Garbacz,M., Jonczyk,P., Fijalkowska,I.J. and Jaszczur,M. (2012) Defect of Dpb2p, a noncatalytic subunit of DNA polymerase ε, promotes error prone replication of undamaged chromosomal DNA in *Saccharomyces cerevisiae*. *Mutat. Res.*, **737**, 34–42.
55. Kadyrova,L.Y., Mertz,T.M., Zhang,Y., Northam,M.R., Sheng,Z., Lobachev,K.S., Shcherbakova,P.V. and Kadyrov,F.A. (2013) A reversible histone H3 acetylation cooperates with mismatch repair and replicative polymerases in maintaining genome stability. *PLoS Genet.*, **9**, e1003899.
56. Stodola,J.L., Stith,C.M. and Burgers,P.M. (2016) Proficient replication of the yeast genome by a viral DNA polymerase. *J. Biol. Chem.*, **291**, 11698–11705.
57. Cassier,C., Chanet,R., Henriques,J.A. and Moustacchi,E. (1980) The effects of three *PSO* genes on induced mutagenesis : a novel class of mutationally defective yeast. *Genetics*, **96**, 841–857.
58. Quah,S.K., von Borstel,R.C. and Hastings,P.J. (1980) The origin of spontaneous mutation in *Saccharomyces cerevisiae*. *Genetics*, **96**, 819–839.
59. Shcherbakova,P.V. and Kunkel,T.A. (1999) Mutator phenotypes conferred by *MLH1* overexpression and by heterozygosity for *mlh1* mutations. *Mol. Cell. Biol.*, **19**, 3177–3183.
60. Henricksen,L.A., Umbricht,C.B. and Wold,M.S. (1994) Recombinant replication protein A: expression, complex formation, and functional characterization. *J. Biol. Chem.*, **269**, 11121–11132.
61. Eissenberg,J.C., Ayyagari,R., Gomes,X.V. and Burgers,P.M. (1997) Mutations in yeast proliferating cell nuclear antigen define distinct sites for interaction with DNA polymerase δ and DNA polymerase ε. *Mol. Cell. Biol.*, **17**, 6367–6378.
62. Fortune,J.M., Stith,C.M., Kissling,G.E., Burgers,P.M. and Kunkel,T.A. (2006) RPA and PCNA suppress formation of large deletion errors by yeast DNA polymerase δ. *Nucleic Acids Res.*, **34**, 4335–4341.
63. Gomes,X.V., Gary,S.L. and Burgers,P.M. (2000) Overproduction in *Escherichia coli* and characterization of yeast replication factor C lacking the ligase homology domain. *J. Biol. Chem.*, **275**, 14541–14549.
64. Makarova,A.V., Nick McElhinny,S.A., Watts,B.E., Kunkel,T.A. and Burgers,P.M. (2014) Ribonucleotide incorporation by yeast DNA polymerase ζ. *DNA Repair (Amst)*, **18**, 63–67.
65. Jia,S., Marjavaara,L., Buckland,R., Sharma,S. and Chabes,A. (2015) Determination of deoxyribonucleoside triphosphate concentrations in yeast cells by strong anion-exchange high-performance liquid chromatography coupled with ultraviolet detection. *Methods Mol. Biol.*, **1300**, 113–121.
66. Bebenek,K. and Kunkel,T.A. (1995) Analyzing fidelity of DNA polymerases. *Methods Enzymol.*, **262**, 217–232.
67. Garg,P., Stith,C.M., Majka,J. and Burgers,P.M. (2005) Proliferating cell nuclear antigen promotes translesion synthesis by DNA polymerase ζ. *J. Biol. Chem.*, **280**, 23446–23450.
68. Harfe,B.D. and Jinks-Robertson,S. (2000) DNA polymerase ζ introduces multiple mutations when bypassing spontaneous DNA damage in *Saccharomyces cerevisiae*. *Mol. Cell*, **6**, 1491–1499.
69. Krakoff,I.H., Brown,N.C. and Reichard,P. (1968) Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. *Cancer Res.*, **28**, 1559–1565.
70. Abdulovic,A.L. and Jinks-Robertson,S. (2006) The *in vivo* characterization of translesion synthesis across UV-induced lesions in *Saccharomyces cerevisiae*: insights into Pol ζ- and Pol η-dependent frameshift mutagenesis. *Genetics*, **172**, 1487–1498.
71. Sharma,N.M., Kochenova,O.V. and Shcherbakova,P.V. (2011) The non-canonical protein binding site at the monomer-monomer interface of yeast proliferating cell nuclear antigen (PCNA) regulates the Rev1-PCNA interaction and Polζ/Rev1-dependent translesion DNA synthesis. *J. Biol. Chem.*, **286**, 33557–33566.
72. Buckland,R.J., Watt,D.L., Chittoor,B., Nilsson,A.K., Kunkel,T.A. and Chabes,A. (2014) Increased and imbalanced dNTP pools symmetrically promote both leading and lagging strand replication infidelity. *PLoS Genet.*, **10**, e1004846.
73. Shcherbakova,P.V., Pavlov,Y.I., Chilkova,O., Rogozin,I.B., Johansson,E. and Kunkel,T.A. (2003) Unique error signature of the four-subunit yeast DNA polymerase ε. *J. Biol. Chem.*, **278**, 43770–43780.
74. Haracska,L., Prakash,S. and Prakash,L. (2003) Yeast DNA polymerase ζ is an efficient extender of primer ends opposite from 7,8-dihydro-8-Oxoguanine and O^6-methylguanine. *Mol. Cell. Biol.*, **23**, 1453–1459.
75. Poli,J., Tsaponina,O., Crabbe,L., Keszthelyi,A., Pantesco,V., Chabes,A., Lengronne,A. and Pasero,P. (2012) dNTP pools determine fork progression and origin usage under replication stress. *EMBO J.*, **31**, 883–894.
76. Stodola,J.L. and Burgers,P.M. (2016) Resolving individual steps of Okazaki-fragment maturation at a millisecond timescale. *Nat. Struct. Mol. Biol.*, **23**, 402–408.
77. Lis,E.T., O'Neill,B.M., Gil-Lamaignere,C., Chin,J.K. and Romesberg,F.E. (2008) Identification of pathways controlling DNA damage induced mutation in *Saccharomyces cerevisiae*. *DNA Repair (Amst)*, **7**, 801–810.
78. Bester,A.C., Roniger,M., Oren,Y.S., Im,M.M., Sarni,D., Chaoat,M., Bensimon,A., Zamir,G., Shewach,D.S. and Kerem,B. (2011) Nucleotide deficiency promotes genomic instability in early stages of cancer development. *Cell*, **145**, 435–446.
79. Gorrini,C., Squatrito,M., Luise,C., Syed,N., Perna,D., Wark,L., Martinato,F., Sardella,D., Verrecchia,A., Bennett,S. *et al.* (2007) Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. *Nature*, **448**, 1063–1067.
80. Niida,H., Shimada,M., Murakami,H. and Nakanishi,M. (2010) Mechanisms of dNTP supply that play an essential role in maintaining genome integrity in eukaryotic cells. *Cancer Sci.*, **101**, 2505–2509.

81. Baranovskiy,A.G., Lada,A.G., Siebler,H.M., Zhang,Y., Pavlov,Y.I. and Tahirov,T.H. (2012) DNA polymerase δ and ζ switch by sharing accessory subunits of DNA polymerase δ. *J. Biol. Chem.*, **287**, 17281–17287.
82. Van Sloun,P.P., Varlet,I., Sonneveld,E., Boei,J.J., Romeijn,R.J., Eeken,J.C. and De Wind,N. (2002) Involvement of mouse Rev3 in tolerance of endogenous and exogenous DNA damage. *Mol. Cell. Biol.*, **22**, 2159–2169.
83. Gibbs,P.E., McGregor,W.G., Maher,V.M., Nisson,P. and Lawrence,C.W. (1998) A human homolog of the Saccharomyces cerevisiae *REV3* gene, which encodes the catalytic subunit of DNA polymerase ζ. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 6876–6880.
84. Van Sloun,P.P., Romeijn,R.J. and Eeken,J.C. (1999) Molecular cloning, expression and chromosomal localisation of the mouse *Rev3l* gene, encoding the catalytic subunit of polymerase ζ. *Mutat. Res.*, **433**, 109–116.
85. Li,Z., Zhang,H., McManus,T.P., McCormick,J.J., Lawrence,C.W. and Maher,V.M. (2002) hREV3 is essential for error-prone translesion synthesis past UV or benzo[a]pyrene diol epoxide-induced DNA lesions in human fibroblasts. *Mutat. Res.*, **510**, 71–80.
86. Wu,F., Lin,X., Okuda,T. and Howell,S.B. (2004) DNA polymerase ζ regulates cisplatin cytotoxicity, mutagenicity, and the rate of development of cisplatin resistance. *Cancer Res.*, **64**, 8029–8035.
87. Lee,Y.S., Gregory,M.T. and Yang,W. (2014) Human Pol ζ purified with accessory subunits is active in translesion DNA synthesis and complements Pol η in cisplatin bypass. *Proc. Natl. Acad. Sci. U.S.A.*, **111**, 2954–2959.