

Participation of DNA Polymerase ζ in Replication of Undamaged DNA in *Saccharomyces cerevisiae*

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Manuscript received July 17, 2009
Accepted for publication October 12, 2009

ABSTRACT

Translesion synthesis DNA polymerases contribute to DNA damage tolerance by mediating replication of damaged templates. Due to the low fidelity of these enzymes, lesion bypass is often mutagenic. We have previously shown that, in *Saccharomyces cerevisiae*, the contribution of the error-prone DNA polymerase ζ (Pol ζ) to replication and mutagenesis is greatly enhanced if the normal replisome is defective due to mutations in replication genes. Here we present evidence that this defective-replisome-induced mutagenesis (DRIM) results from the participation of Pol ζ in the copying of undamaged DNA rather than from mutagenic lesion bypass. First, DRIM is not elevated in strains that have a high level of endogenous DNA lesions due to defects in nucleotide excision repair or base excision repair pathways. Second, DRIM remains unchanged when the level of endogenous oxidative DNA damage is decreased by using anaerobic growth conditions. Third, analysis of the spectrum of mutations occurring during DRIM reveals the characteristic error signature seen during replication of undamaged DNA by Pol ζ *in vitro*. These results extend earlier findings in *Escherichia coli* indicating that Y-family DNA polymerases can contribute to the copying of undamaged DNA. We also show that exposure of wild-type yeast cells to the replication inhibitor hydroxyurea causes a Pol ζ -dependent increase in mutagenesis. This suggests that DRIM represents a response to replication impediment per se rather than to specific defects in the replisome components.

TRANSLESION synthesis (TLS) DNA polymerases have the ability to bypass lesions in template DNA that block DNA synthesis by replicative enzymes (PRAKASH *et al.* 2005). TLS polymerases are present in all domains of life and include eukaryotic polymerase ζ (Pol ζ), Pol η , Pol ι , Pol κ , and Rev1. Because of the lower selectivity of their active sites, TLS polymerases often introduce errors when bypassing lesions or copying undamaged DNA *in vitro* (SHCHERBAKOVA and FIJALKOWSKA 2006). In yeast and human cells, DNA synthesis by Pol ζ is responsible for nearly all mutagenesis induced by exogenous genotoxicants (WATERS *et al.* 2009). Pol ζ has been isolated from *Saccharomyces cerevisiae* as a complex of two subunits encoded by the *REV3* and *REV7* genes. This complex can synthesize, with a low efficiency, past several types of DNA lesions (NELSON *et al.* 1996; GUO *et al.* 2001). At the same time, Pol ζ is very efficient in extending primers containing a mismatched terminal nucleotide (JOHNSON *et al.* 2000; GUO *et al.* 2001;

HARACSKA *et al.* 2001; SIMHADRI *et al.* 2002). In accordance with these properties, the main function of Pol ζ in TLS is proposed to be the extension from nucleotides incorporated opposite DNA lesions by other DNA polymerases (LAWRENCE and MAHER 2001; BRESSON and FUCHS 2002; PRAKASH and PRAKASH 2002). The mismatch extension and TLS activity of Pol ζ is stimulated by the Rev1 protein (ACHARYA *et al.* 2006). Rev1 is a deoxycytidyl transferase involved in multiple protein-protein interactions with other DNA polymerases, and its essential function in TLS is thought to be structural rather than catalytic (WATERS *et al.* 2009). The TLS activity of Pol ζ is also stimulated by proliferating cell nuclear antigen (PCNA), the DNA polymerase processivity factor (GARG *et al.* 2005; NORTHAM *et al.* 2006).

In addition to the mutagenesis induced by environmental agents, Pol ζ is required for the vast majority of mutations provoked by endogenous DNA damage. For example, Pol ζ is responsible for the increase in mutation rate caused by defects in nucleotide excision repair (NER) (ROCHE *et al.* 1994; HARFE and JINKS-ROBERTSON 2000), base excision repair (BER) (XIAO *et al.* 2001), post-replicative DNA repair (ROCHE *et al.* 1995; BROOMFIELD *et al.* 1998; XIAO *et al.* 1999), homologous recombination (ROCHE *et al.* 1995; HARFE and JINKS-ROBERTSON 2000), the overproduction of 3-methyladenine DNA glycosylase (GLASSNER *et al.* 1998), and the

Supporting information is available online at <http://www.genetics.org/cgi/content/full/genetics.109.107482/DC1>.

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expression of altered uracil-DNA glycosylases that remove undamaged cytosines and thymines in a BER-defective strain (AUERBACH *et al.* 2005). All these defects increase the level of endogenous replication-blocking lesions, and the Pol ζ -dependent mutagenesis likely reflects the function of Pol ζ in the error-prone bypass of these lesions.

There are also multiple reports documenting Pol ζ -dependent mutagenesis in situations in which cells are not expected to accumulate excessive DNA damage. The Pol ζ /Rev1-dependent processes are responsible for 50–70% of spontaneous mutations in wild-type *S. cerevisiae* strains (CASSIER *et al.* 1980; QUAH *et al.* 1980). Pol ζ also contributes to an increased mutation rate associated with high levels of transcription (DATTA and JINKS-ROBERTSON 1995) or double-strand break repair (HOLBECK and STRATHERN 1997). In addition, we and others have shown that Pol ζ participation in replication and mutagenesis is promoted by a variety of replication machinery defects (SHCHERBAKOVA *et al.* 1996; PAVLOV *et al.* 2001b; KAI and WANG 2003; NORTHAM *et al.* 2006). For example, defects in *S. cerevisiae* replicative polymerases Pol δ and Pol ϵ that are thought to affect replication fork progression or the replisome integrity lead to a mutator phenotype. Eighty to 90% of spontaneous mutations in these strains are mediated by Pol ζ (SHCHERBAKOVA *et al.* 1996; PAVLOV *et al.* 2001b; NORTHAM *et al.* 2006). It is not known whether Pol ζ -dependent mutagenesis in the absence of abnormally high levels of DNA damage results from error-prone copying of undamaged DNA or from Pol ζ -mediated bypass of endogenous lesions present at physiological levels. The Pol ζ error rate during the copying of undamaged DNA *in vitro* is several orders of magnitude higher than that for the replicative polymerases (ZHONG *et al.* 2006), so its contribution to the genome replication would be expected to result in increased mutagenesis. In *Escherichia coli*, the high spontaneous mutation rate in strains with constitutive expression of the SOS system has been shown to result from the copying of undamaged DNA by error-prone DNA polymerases (FIJALKOWSKA *et al.* 1997). On the other hand, the mechanism mediating the recruitment of Pol ζ in response to replication defects shares some features with the mechanism of polymerase switching during TLS, such as the requirement for monoubiquitination of PCNA and for the physical interaction of PCNA with Pol ζ (NORTHAM *et al.* 2006). It is possible that the defective replisome may stall more frequently at sites of endogenous lesions, resulting in more need for the error-prone TLS. In this study, we addressed whether defective-replisome-induced mutagenesis (DRIM) reflects the mutagenic bypass of endogenous damage or error-prone copying of the undamaged DNA by Pol ζ . The results argue against the endogenous damage as a key mediator of the mutagenic response and thus provide evidence for Pol ζ participation in the copying of undamaged DNA.

MATERIALS AND METHODS

***S. cerevisiae* strains:** The wild-type haploid strain E134 (*MAT α* *ade5-1 lys2::InsE_{A14} trp1-289 his7-2 leu2-3,112 ura3-52*) and the construction of its *pol3-Y708A*, *rev3 Δ* and *pol3-Y708A rev3 Δ* derivatives have been described (SHCHERBAKOVA and KUNKEL 1999; NORTHAM *et al.* 2006). The *RAD14* and *APN2* genes were disrupted in E134 and in the *pol3-Y708A* mutant by a selectable *hygB* (*rad14*) or *kanMX* (*apn2*) cassette as described (WACH *et al.* 1994). The *APN1* gene was disrupted by transformation with a fragment from plasmid pSCP108 (POPOFF *et al.* 1990) containing the *hisG::URA3::hisG* cassette followed by selection for the loss of the *URA3* marker on medium containing 5-fluoroorotic acid (FOA; BOEKE *et al.* 1984). The disruptions were confirmed by PCR and by sensitivity of the mutants to UV irradiation (*rad14*) or hydrogen peroxide (*apn1 apn2*). The quintuple DNA glycosylase mutant DGD39 (*MAT α* *leu2-3,112 trp1-289 his7-2 ura3-52 lys1-1 ungl Δ ntg1 Δ ntg2 Δ ::kanMX6 ogg1 Δ ::URA3 mag1 Δ ::hphMX4*) and the isogenic wild-type strain FF18733 (KOZMIN *et al.* 2005) were kindly provided by E. Sage (Institut Curie, Orsay, France). A spontaneous *ura3* mutant of DGD39 was obtained by plating the strain onto FOA-containing medium. The wild-type chromosomal *POL3* and *POL2* genes of FF18733 and the *Ura⁻* derivative of DGD39 were then replaced with the *pol3-Y708A* and *pol2-1* alleles as described (SHCHERBAKOVA *et al.* 1996; PAVLOV *et al.* 2001b). The strain $\Delta(1-2)1-7B$ -YUNI300 (*MAT α* *ade2-1 lys2 Δ GG2899-2900 trp1-289 his7-2 leu2 Δ ::kanMX ura3 Δ*) and its *ogg1 Δ* mutant (PAVLOV *et al.* 2002) were obtained from Y. I. Pavlov (University of Nebraska Medical Center).

Measurement of the spontaneous mutation rate and hydroxyurea-induced mutant frequency: To measure the rate of spontaneous mutation, at least nine 7- to 9-ml cultures were started for each strain from single colonies and grown to the stationary phase in liquid yeast extract peptone dextrose medium supplemented with 60 mg/liter adenine and 60 mg/liter uracil (YPDAU). Cells were plated after appropriate dilutions onto synthetic complete medium containing L-canavanine (60 mg/liter) and lacking arginine (SC + CAN) for *Can^r* mutant count and onto synthetic complete (SC) medium for viable count. Yeast extract peptone dextrose and SC media were prepared as described elsewhere (ROSE *et al.* 1990). *Can^r* mutant frequency was calculated by dividing the *Can^r* mutant count by the viable cell count. Mutation rate was calculated from mutant frequency by using the Drake equation (DRAKE 1991). The median value of the mutation rate was used to compare spontaneous mutagenesis in different strains. The 95% confidence limits for the median were determined as described (DIXON and MASSEY 1969). Hydroxyurea (HU)-induced mutagenesis was measured using a similar procedure, but the cultures were started from $\sim 10^4$ -cells inoculum rather than from single colonies and grown in YPDAU medium containing the indicated concentration of HU. The median frequency of *Can^r* mutants was used to compare HU-induced mutagenesis in different strains. To measure the rate of spontaneous mutation under anaerobic conditions, the cultures were grown in a GasPak anaerobic chamber (BD) prior to plating on SC + CAN and SC media for the mutant and viable count. Anaerobic conditions were monitored using GasPak dry anaerobic indicator strips (BD).

Mutational spectra determination: Independent colonies of the *pol3-Y708A* and *pol3-Y708A rev3 Δ* derivatives of E134 were streaked on YPDAU plates, grown for 2 days at 30°, and replica-plated onto SC + CAN medium to select for *can1* mutants. One *Can^r* colony was picked from each patch, and the *CAN1* gene was amplified by PCR and sequenced as described (KOZMIN *et al.* 2003). The sequencing covered the

entire open reading frame with the exception of nucleotides 551–601.

RESULTS

A genetic system for the study of DRIM: We observed previously that many mutations affecting the replisome components result in a Pol ζ -dependent spontaneous mutator phenotype (SHCHERBAKOVA *et al.* 1996; PAVLOV *et al.* 2001b; NORTHAM *et al.* 2006). Here we use two mutations, *pol3-Y708A* and *pol2-1*, that confer the strongest Pol ζ -dependent increase in mutagenesis. The *pol3-Y708A* mutation (PAVLOV *et al.* 2001b) results in a single amino acid change in the polymerase active site of Pol δ (SWAN *et al.* 2009a). The *pol2-1* mutation is an insertion of the *URA3* gene in the middle of the coding region of the *POL2* gene encoding the catalytic subunit of Pol ϵ . The insertion results in a loss of interaction of the catalytic subunit with the other subunits of the Pol ϵ holoenzyme (MORRISON *et al.* 1990). DNA replication is impaired in both mutants as manifested by their slow growth and, in the case of *pol3-Y708A*, sensitivity to HU. Our previous studies support a model in which the defect in the replication fork progression in these mutants provides a signal for constitutive monoubiquitination of PCNA and the recruitment of low-fidelity polymerases, particularly Pol ζ , for DNA replication (NORTHAM *et al.* 2006). Those studies indicated that 80–90% of spontaneous mutations occurring in the *pol3-Y708A* and *pol2-1* strains require the presence of Pol ζ . In this work, we use this clearly detectable Pol ζ -dependent mutator phenotype as a measure of Pol ζ involvement in chromosomal DNA replication.

DRIM is not affected by an increased level of endogenous DNA damage: We reasoned that if the bypass of endogenous damage was a major source of DRIM, the mutator phenotype of replication-deficient strains would be stronger when the level of endogenous damage is elevated. To achieve this, we inactivated the two major damage repair pathways, NER and BER, in DNA replication mutants. The types of lesions that could potentially contribute to DRIM are unknown. Therefore, we studied the effects of both repair pathways to determine if any of the wide spectrum of lesions repaired by NER and BER could be responsible for the increased mutagenesis. A similar approach has been used previously to study the role of endogenous DNA lesions in the SOS mutator activity in *Escherichia coli* (FIJALKOWSKA *et al.* 1997). We first inactivated the NER pathway, which removes a variety of helix-distorting DNA lesions, by disrupting the *RAD14* gene. The Rad14 protein is involved in damage recognition during NER (GUZDER *et al.* 1993). The deletion of the *RAD14* gene results in a weak, but clearly measurable, spontaneous mutator phenotype (Figure 1A, Table S1), consistent with previous reports (BERTRAND *et al.* 1998; HARFE and JINKS-ROBERTSON 2000; SCHELLER *et al.* 2000; YU *et al.* 2003;

GUO *et al.* 2005). This indicates that the *rad14* strain indeed accrues potentially mutagenic DNA damage. The mutator effect of the double *pol3-Y708A rad14* mutation is equal to the sum of the mutator effects of the single mutations (Figure 1A, Table S1), suggesting that the spontaneous mutations arise through different, nonoverlapping pathways in the replication- and repair-deficient strains. We therefore concluded that the spontaneous damage that can be repaired by NER plays no significant role in DRIM.

Next we investigated whether the abasic sites (AP sites), which are known to present an obstacle for the replicative DNA polymerases, contribute to DRIM. We simultaneously disrupted the two genes that encode the apurinic endonucleases essential for the abasic site repair, *APN1* and *APN2*, in the *pol3-Y708A* strain. As reported earlier (JOHNSON *et al.* 1998; BENNETT 1999; XIAO *et al.* 2001), the double *apn1 apn2* mutation itself caused increased spontaneous mutagenesis (Figure 1B, Table S1), consistent with the expected mutagenicity of AP sites that accumulate at high levels in these strains. As in the case of *rad14*, the interaction of the *apn1 apn2* double mutation with the *pol3-Y708A* mutation was strictly additive (Figure 1B, Table S1). This indicates that mutagenesis resulting from the AP site bypass and DRIM represent distinct, nonoverlapping pathways. Thus, the polymerase stalling at abasic sites does not appear to make a substantial contribution to DRIM.

In addition to the AP sites, the BER pathway repairs a variety of other lesions, such as those produced by reactive oxygen species, alkylation, or deamination. To determine if any of these lesions are responsible for DRIM, we studied DRIM in a strain lacking all five known yeast DNA glycosylases (Ogg1, Ntg1, Ntg2, Ung1, and Mag1). The glycosylases are responsible for the recognition and removal of damaged bases, so the quintuple mutant is expected to be defective in BER of all lesions except the AP sites. This mutant showed a 9-fold increase in the rate of spontaneous mutation to canavanine resistance (Can^r), which is likely a consequence of the mutagenic bypass of unrepaired base damage (Figure 1, C and D; Table S2). When the *pol3-Y708A* or *pol2-1* mutations were introduced into the glycosylase-deficient strain, the resulting spontaneous mutation rate was close to that expected in the case of the additive interaction of the BER and DNA polymerase defects (Figure 1, C and D; Table S2). A small (~20–30%) but reproducible increase in the mutation rate value in the *pol3-Y708A BER⁻* and *pol2-1 BER⁻* over the expected additive value was observed. This indicates that a minor fraction of DRIM could be mediated by DNA damage bypass, at least in the situation in which the damage is excessive. However, if the BER-repaired base damage was a major source of DRIM, a multiplicative interaction of the mutator effects would have been observed (a 110- and 270-fold increase over the wild-type mutation rate for the *pol3-Y708A BER⁻* and *pol2-1 BER⁻* strains, re-

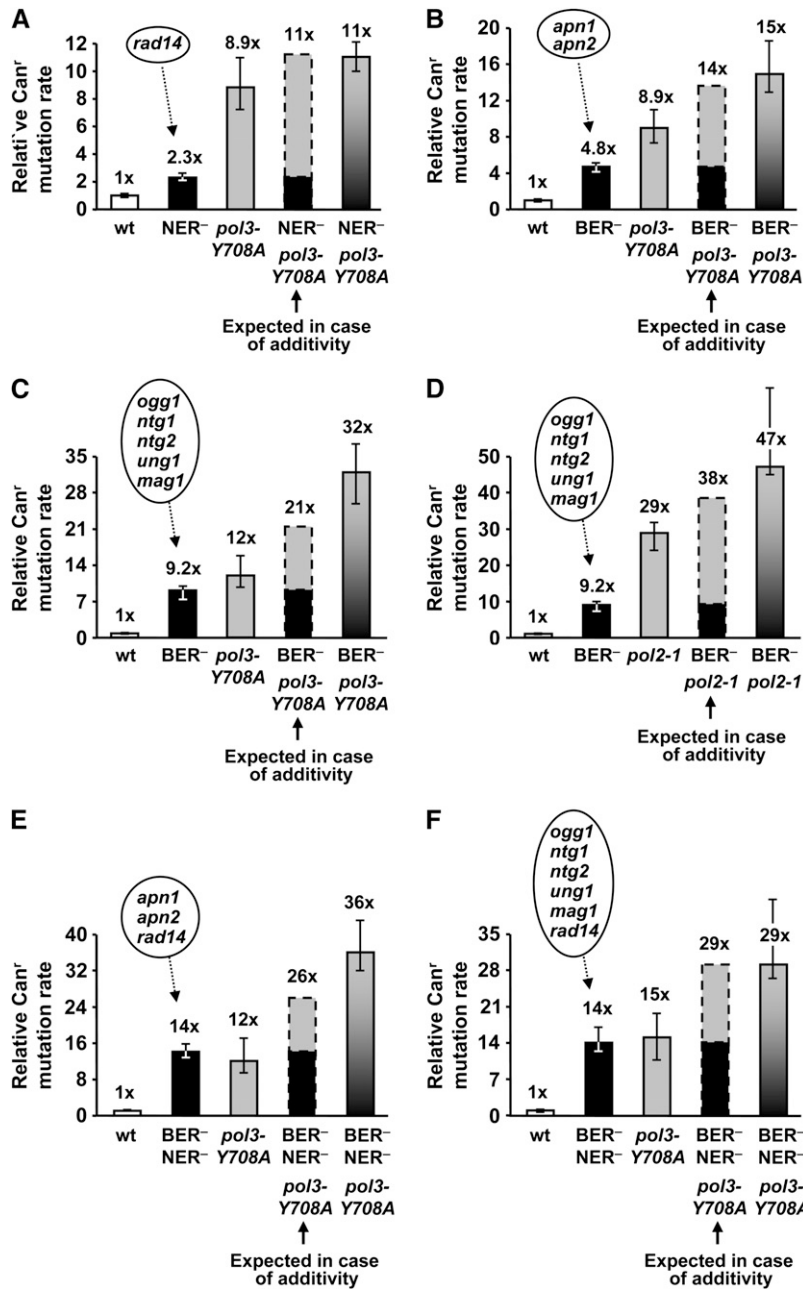


FIGURE 1.—Effect of DNA repair deficiencies on the spontaneous mutator phenotype of *pol3-Y708A* and *pol2-1* mutants. (A) Interaction of the mutator effects of *pol3-Y708A* and *rad14Δ* alleles. (B) Interaction of the mutator effects of *pol3-Y708A* and the double *apn1Δ apn2Δ* mutation. (C and D) Interaction of the mutator effects of *pol3-Y708A* and *pol2-1*, respectively, with the effect of quintuple DNA glycosylase mutation *ogg1Δ ntg1Δ ntg2Δ ung1Δ mag1Δ*. (E and F) Interaction of the mutator effects of *pol3-Y708A* and the double NER⁻ BER⁻ deficiencies, *apn1Δ apn2Δ rad14Δ* and *ogg1Δ ntg1Δ ntg2Δ ung1Δ mag1Δ rad14Δ*, respectively. The DNA repair mutations designated as NER⁻ and BER⁻ are indicated in ovals. Rate of Can⁺ mutation relative to the wild type is shown. The data are from Table S1 (A and B), Table S2 (C and D), Table S3 (E), and Table S4 (F) and are medians and 95% confidence intervals for at least nine cultures. Rates expected in the case of additivity were calculated as a sum of the mutation rates in the strains with the corresponding single defects. This approach may slightly overestimate the expected additive rate value (by a maximum of 9%, 7%, 4.8%, and 2.6% for the experiments in A–D, respectively) because some of the mutations occurring spontaneously in the wild-type strain could still occur in the replication and repair mutants and thus would be counted twice in this calculation.

spectively). The actual mutator effects of the *pol3-Y708A* BER⁻ and *pol2-1* BER⁻ combinations are 32- and 47-fold, respectively, which is far lower than the mutator effects expected for the multiplicative interaction. Overall, the data suggest that the bypass of lesions that are normally a subject of BER does not make a major contribution to DRIM.

Several studies indicated that NER and BER have overlapping specificity in respect to the spectrum of lesions repaired (SWANSON *et al.* 1999; TORRES-RAMOS *et al.* 2000; GELLON *et al.* 2001). If the bypass of lesions that could be efficiently repaired by both NER and BER were a major source of DRIM, the experiments shown in Figure 1, A–D, would fail to detect this. Therefore, we also measured DRIM in strains defective simultaneously

in NER and BER. Similar to the results obtained with strains defective in a single repair pathway, the interaction of the mutator effects caused by the *pol3-Y708A* allele and the double NER⁻ BER⁻ deficiency was nearly additive (Figure 1, E and F; Table S3; Table S4). This further supports our conclusion that DRIM is unrelated to the bypass of endogenously generated lesions, including the ones that could be processed by both NER and BER.

DRIM is not affected by a reduced level of oxidative DNA damage: Reactive oxygen species are produced in cells during normal aerobic metabolism. Oxidative damage to DNA is thought to account for the majority of endogenously generated DNA lesions. These lesions are primarily repaired by BER (D'ERRICO *et al.* 2008).

The fact that we did not detect a significant effect of BER on DRIM (Figure 1) suggests that oxidative lesions are unlikely to play a major role in this mutagenesis pathway. To provide additional evidence for this, we asked whether the mutator phenotype of the *pol3-Y708A* strain would be affected by anaerobic growth conditions in which the oxidative damage to DNA is greatly reduced. To demonstrate that the level of mutagenic endogenous damage was indeed reduced in our anaerobic experiments, we measured the spontaneous mutation rate in *ogg1* mutants obtained in three different genetic backgrounds. The spontaneous mutations in *ogg1* strains result mainly from the accumulation of unrepaired 8-oxoguanine residues in DNA. The mutator effect of the *ogg1* mutation was decreased under anaerobic conditions (Figure 2; Table S5). Remarkably, the mutator phenotype of *pol3-Y708A* was essentially the same during aerobic and anaerobic growth (Figure 2; Table S5). We therefore concluded that the spontaneous oxidative DNA damage plays no significant role in DRIM.

The spectrum of spontaneous mutations in the *pol3-Y708A* strain shows a characteristic Pol ζ signature: To gain further insight into the mechanism of DRIM, we compared the spectrum of spontaneous mutations generated in the *pol3-Y708A* strain in the presence and in the absence of Pol ζ (Table 1, Figure 3 and Figure 4). We analyzed 178 and 214 independent Can^r mutants of the *pol3-Y708A* and *pol3-Y708A rev3 Δ* strains, respectively. The *pol3-Y708A* spectrum was composed predominantly of base substitutions (70%) followed by complex mutations (13%). The latter represented multiple changes within a stretch of no more than six nucleotides (Figure 5B). We define these as complex mutations type I to distinguish them from other types of complex changes that were seen at a low frequency in the Pol ζ ⁻ derivative of *pol3-Y708A* (see below). The other mutations detected in the *pol3-Y708A* strain were small (mostly single nucleotide) deletions or insertions (8% of all sequenced mutations) and larger deletions (8%). All larger deletions affected sequences flanked by short (three to eight nucleotides) direct repeats (Figure 3).

Deletion of *REV3* in the *pol3-Y708A* strain decreased the rate of Can^r mutation rate 6-fold (from 300×10^{-8} to 50×10^{-8} , NORTHAM *et al.* 2006). The spectrum of mutations in the *rev3 Δ* derivative of *pol3-Y708A* is shown in Table 1 and Figure 4. To provide an informative comparison of the *pol3-Y708A* and *pol3-Y708A rev3 Δ* mutational spectra, we calculated the rates of each individual type of mutation in the two strains as described in Table 1. The overall reduction in the mutation rate in the *rev3 Δ* derivative was largely due to a large decrease in base substitution mutations, particularly GC \rightarrow CG transversions. The GC \rightarrow CG changes constituted nearly half of all base substitution mutations in the *pol3-Y708A* strain (Table 1, Figure 5A). No GC \rightarrow CG transversions were found in the *pol3-Y708A rev3 Δ* strain, which translates into a >400-fold reduction in the rate of

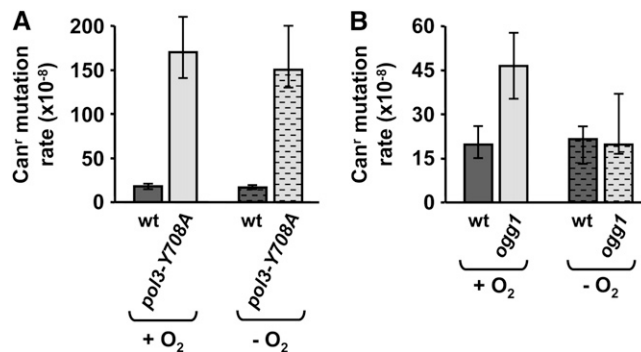


FIGURE 2.—Effect of anaerobic growth conditions on the spontaneous mutator phenotype of *pol3-Y708A* (A) and *ogg1 Δ* (B) strains. The bar graphs show the rate of Can^r mutation in E134 strain and its *pol3-Y708A* derivative and in the $\Delta l(-2)l-7B-YUNI300$ strain and its *ogg1 Δ* derivative. All data are from Table S5 and are medians and 95% confidence intervals for at least nine cultures.

these events upon the inactivation of Pol ζ . Decreases in the rate of other base substitutions were also observed. In addition, the rate of type I complex mutations was reduced >170-fold when Pol ζ was inactivated. Although five complex changes were found in the *pol3-Y708A rev3 Δ* strain, four of them were different from the type I complex mutations and involved DNA stretches longer than six nucleotides. We define these larger changes as complex mutations type II (see Figure 4 for a detailed description of these mutations).

We further calculated the rate of mutations that could be specifically attributed to Pol ζ by subtracting the background mutation rate seen in the absence of Pol ζ (*pol3-Y708A rev3 Δ*) from the mutation rate seen in the presence of Pol ζ (*pol3-Y708A*) (Table 1). The Pol ζ -dependent mutations were almost exclusively base substitutions and complex mutations. A few small deletions or insertions, mostly -1 frameshifts, were also apparently generated by Pol ζ . The rates shown in the last column of Table 1 imply that $\sim 75\%$ of Pol ζ -dependent mutations are base substitutions, $\sim 16\%$ are complex mutations, and $\sim 7\%$ are small deletions or insertions. Interestingly, the rate of large deletions was not significantly different in the *pol3-Y708A* and *pol3-Y708A rev3 Δ* strains. This indicates that these deletions are not generated by Pol ζ , but are likely a result of faulty replication by the defective Pol δ itself. Mutations in the *POL3* gene have been previously reported to increase the rate of deletions between short direct repeats (VON BORSTEL *et al.* 1993; TRAN *et al.* 1995). The length of the repeats flanking the deleted sequences was similar in the Pol ζ ⁺ and Pol ζ ⁻ strains (three to eight nucleotides, Figures 3 and 4). Interestingly, however, a substantial difference was seen in the size of the deletions (Figure 6). While only 1 of 15 deletions found in the Pol ζ ⁺ strain was larger than 40 nucleotides, sequences longer than 40 nucleotides (up to 646) were deleted in $\sim 42\%$ of all deletion cases in the Pol ζ ⁻ strain. This represents a

TABLE 1
Spontaneous *can1* mutations in the *pol3-Y708A* strain and its *rev3Δ* derivative

Mutation	<i>pol3-Y708A</i>		<i>pol3-Y708A rev3Δ</i>		Rate of Polζ-dependent mutations ($\times 10^{-8}$) ^c
	No.	MR ($\times 10^{-8}$) ^b	No.	MR ($\times 10^{-8}$) ^b	
Base substitutions					
GC → AT	11	18	23	5.4	13
AT → GC	8	13	7	1.6	11
GC → TA	16	27	20	4.7	22
GC → CG	55	92	0	<0.23	92
AT → CG	7	12	0	<0.23	12
AT → TA	29	48	36	8.4	40
Total	126 ^a	210	86	20	190
Small indels					
-1	8	13	4	0.93	12
-2	2	3.3	4	0.93	2.4
-3	0	<1.7	2	0.47	<1.3
-4	0	<1.7	2	0.47	<1.3
-5	0	<1.7	2	0.47	<1.3
+1	4	6.7	14	3.3	3.4
+2	0	<1.7	1	0.23	<1.5
+4	1	1.7	0	<0.23	≤1.7
Total	15	25	29	6.8	18
Large rearrangements (≥8 nucleotides)					
Deletions between short direct repeats	15	25	92	21	4.0
Other deletions	0	<1.7	1	0.23	<1.5
Duplications	0	<1.7	1	0.23	<1.5
Complex mutations type I (≤6 nucleotides) ^d	24	40	1	0.23	40
Complex mutations type II (≥7 nucleotides) ^d	0	<1.7	4	0.93	<0.8
Total	180 ^a	300	214	50	250

^aTwo *can1* mutants of the *pol3-Y708A* strain carried double base substitutions. The mutations were separated by 93 and 476 nucleotides in these mutants. These were counted as four individual base substitutions.

^bRate for each type of mutation was calculated as follows: $MR_i = (M_i/M_T) \times MR$, where M_i is the number of mutations of the particular type, M_T is the total number of mutations, and MR is the rate of *Can*^r mutation in the corresponding strain determined by fluctuation analysis, 3×10^{-6} for the *pol3-Y708A* and 5×10^{-7} for the *pol3-Y708A rev3Δ* strain (NORTHAM *et al.* 2006).

^cRate of Polζ-dependent mutations was calculated by subtracting the rate for the *pol3-Y708A rev3Δ* strain from the rate for the *pol3-Y708A* strain.

^dComplex mutations type I are defined as replacements of one to six adjacent nucleotides with a different sequence no more than six nucleotides long. Complex mutations type II are defined as replacements where the original sequence or the new sequence or both are longer than six nucleotides.

fivefold increase in the rate of large deletions upon Polζ inactivation, suggesting that Polζ could play a role in suppressing the formation of larger deletions.

HU treatment induces a Polζ-dependent mutagenic response: DRIM has been observed in a variety of DNA replication mutants, including those with defects in Polα, Polδ, and Polε (NORTHAM *et al.* 2006). This observation led to a hypothesis that DRIM is a response to the impediment of the replication fork progression rather than to the specific DNA polymerase defects. To test this, we determined whether treatment of the wild-type cells with HU would induce Polζ-dependent mutagenesis. HU is known to trigger replication stalling due to the inhibition of dNTP synthesis while causing no damage to the DNA or replication proteins. HU is also known to induce the PCNA monoubiquitylation at

Lys164 (KANNOUCHE *et al.* 2004; DAVIES *et al.* 2008), and the role of this modification in the recruitment of specialized DNA polymerases during TLS and DRIM is well established (HOEGE *et al.* 2002; NORTHAM *et al.* 2006). In the experiment shown in Figure 7, we measured the frequency of the *Can*^r mutation in yeast cultures grown in the presence of varying concentrations of HU. A concentration of 200 mM causes a nearly complete cell-cycle arrest in most strains, so we used a range of smaller HU doses that do not interfere dramatically with the culture growth. This better mimics the situation in the replicative polymerase mutants that are able to replicate their DNA and divide. While HU was clearly mutagenic in the wild-type strain, the mutant frequency was reduced up to 15-fold in the *rev3* strain lacking Polζ. This indicates that the phenomenon of



FIGURE 3.—Spectrum of spontaneous *can1* mutations in the *pol3-Y708A* strain. The coding sequence of the *CAN1* gene is shown. Letters and triangles above the sequence indicate base substitutions and single base deletions, respectively. Letters with a “+” symbol indicate single base insertions. Red boxes show complex mutations. Deletions of 2–5 nucleotides are designated by a line above the sequence with a number of deleted nucleotides shown above the line. For larger deletions, short direct repeats flanking the deleted region are shown by colored arrows with a deletion identification number inside the arrow. The green box indicates a 4-nucleotide insertion within an 11-nucleotide region.



FIGURE 4.—Spectrum of spontaneous *can1* mutations in the *pol3-Y708A rev3Δ* strain. Dashed colored lines below the *CAN1* sequence show multi-base deletions between imperfect direct repeats. The corresponding imperfect repeats are indicated by the same-color shaded boxes. The non-identical nucleotides within the repeats are in yellow. A deleted region not flanked by short repeats is shown by a purple dashed line below the *CAN1* sequence. A 2-nucleotide addition is shown by a line above the *CAN1*

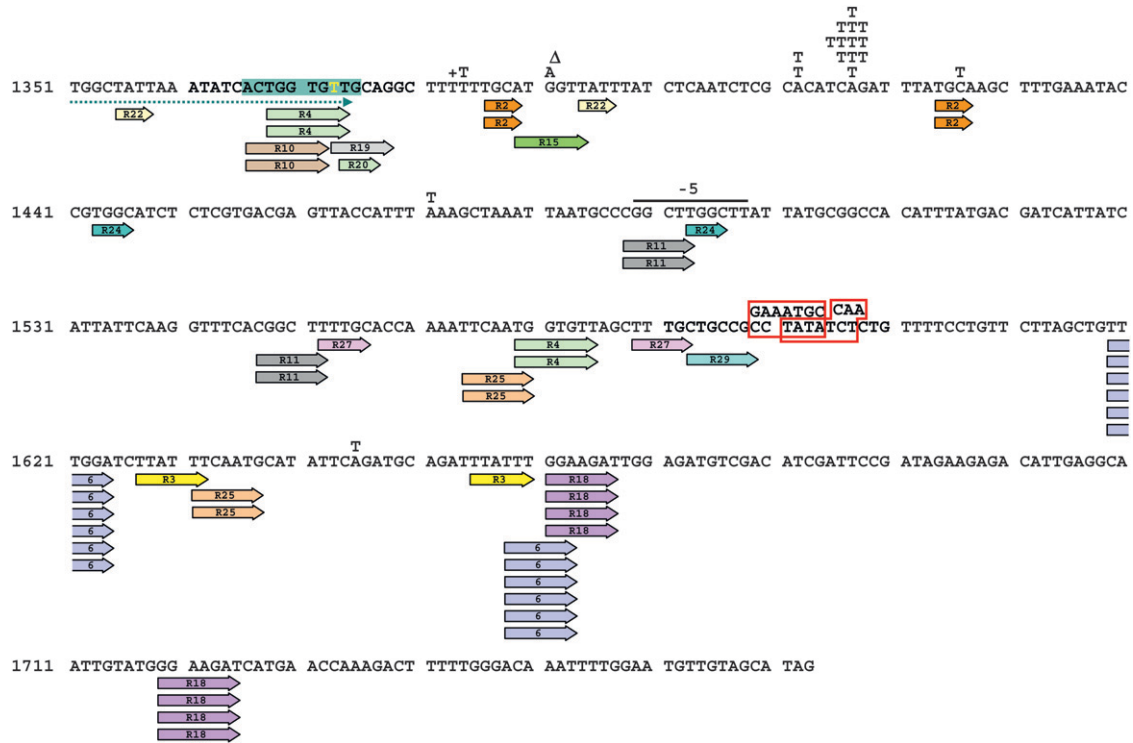


FIGURE 4.—Continued.

Pol ζ -dependent mutagenesis is not a unique feature of the replicative polymerase mutants that we study, but rather represents a general response to replication fork stalling. Additional, Pol ζ -independent pathways apparently contribute to the HU-induced mutagenesis as well because the *rev3* strain still showed an elevated frequency of Can^r mutation in the presence of HU, particularly at higher HU doses (Figure 7; Table S6).

DISCUSSION

In this study, we provide several lines of evidence that DRIM is unrelated to the role of Pol ζ in mutagenic TLS and represents Pol ζ -dependent error-prone copying of undamaged DNA. First, epistatic analysis of mutation rates in DNA replication and repair mutants indicated that mutagenesis induced by endogenous lesions and DRIM represent separate, nonoverlapping pathways. Second, DRIM remains unchanged when spontaneous oxidative damage is eliminated. Third, the spectrum of mutations occurring during DRIM shows similarities to the error specificity of purified Pol ζ on undamaged DNA *in vitro*. This demonstrates that Pol ζ can be recruited to perform DNA synthesis on undamaged DNA templates. This could potentially involve processive error-prone DNA synthesis by Pol ζ , extension of mis-

matches generated by the defective replicative DNA polymerase, or both. The induction of a Pol ζ -dependent mutagenic response with HU illustrates that DRIM does not require the presence of a defective replicative DNA polymerase that could generate mismatches due to relaxed nucleotide selectivity. Thus, the studies of DRIM using the *pol3-Y708A* mutant as a model are applicable to a variety of situations where the fork progression is impeded.

The data presented in Table 1 and Figure 5 indicate that two types of events, complex mutations and GC \rightarrow CG transversions, can be considered the hallmarks of the Pol ζ mutational signature in the *pol3-Y708A* strain. Importantly, these two types of mutations were also the unique features of the spectrum of errors generated by purified yeast Pol ζ during replication of undamaged DNA *in vitro* (ZHONG *et al.* 2006). The G-G and C-C mispairs were among the three most frequent errors made by Pol ζ *in vitro*. This predicts that a high rate of GC \rightarrow CG transversion would result if Pol ζ were to contribute to DNA synthesis *in vivo*. The purified Pol ζ created complex mutations at an average frequency of 12%, which is similar to the frequency of complex mutations in the spectrum of Pol ζ -dependent mutations in the *pol3-Y708A* strain (15%). The overall representation of the various mutation classes was also similar in the

sequence with the inserted nucleotides above the line. A 22-nucleotide duplication is indicated by a line above the sequence. All other symbols are as in Figure 3.

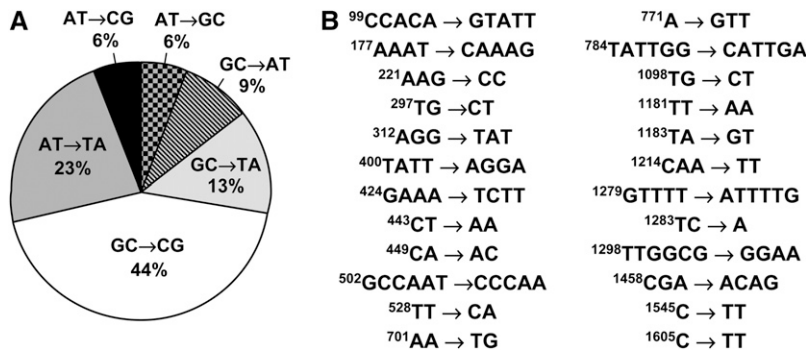


FIGURE 5.—GC → CG transversions and complex mutations are the two characteristic features of spontaneous Polζ-dependent mutagenesis in the *pol3-Y708A* strain. (A) The spectrum of base substitutions in the *CAN1* gene of the *pol3-Y708A* strain. The proportions of individual base substitution were calculated from the data shown in Table 1. (B) Complex mutations found in the *CAN1* gene of the *pol3-Y708A* strain. The supercript numbers indicate nucleotide position.

in vitro and *in vivo* spectra, including the predominance of base substitutions, the same proportion of complex mutations, and a low frequency of frameshifts. The only major difference between the two spectra was the very high rate of A-C mispairs during *in vitro* DNA synthesis by Polζ and the relatively low rate of the corresponding AT → GC transitions in the *pol3-Y708A* strain. This could be caused by differences in the reporter genes used, contribution of other replication proteins, and/or correction of these mispairs by the DNA mismatch repair system.

There is a remarkable similarity between the types of complex mutations generated by Polζ on an undamaged DNA template *in vitro* and in the *pol3-Y708A* strain *in vivo*. In both cases, these are multiple changes within no more than six or seven bases, and about half are accompanied by a frameshift. The ones that involve 5- to 7-nucleotide stretches are typically two base substitutions or a base substitution and a single base deletion/addition separated by several unchanged nucleotides (Figure 5B and ZHONG *et al.* 2006). In contrast, complex mutations of this type either are not seen or are seen at a much lower frequency during Polζ-dependent TLS. During the bypass of site-specific model lesions in yeast, complex mutations constituted <3% of all TLS-associated mutations and typically involved sequence replacements of no more than 3 adjacent nucleotides (GIBBS *et al.* 1995; GIBBS and LAWRENCE 1995; GIBBS *et al.* 2005). Simultaneous changes of >3 nucleotides were not seen in the spectrum of UV-induced mutations in the chromosomal *URA3* gene (LEE *et al.* 1988). In one TLS assay system used by the Z. Wang laboratory, multiple changes in larger stretches of DNA (up to 12 nucleotides) are seen at a high frequency during the bypass of various lesions (ZHAO *et al.* 2004; XIE *et al.* 2005; ZHAO *et al.* 2006). These, however, are seen in *rev3* mutant strains as well, so they are apparently not generated by Polζ. Complex mutations have been shown to represent a characteristic signature of Polζ-mediated bypass of endogenous DNA damage *in vivo* (HARFE and JINKS-ROBERTSON 2000). In that study, only mutations leading to a +1 frameshift were analyzed, and the vast majority of these mutations occurred in TTT homonucleotide runs. In contrast, among the frameshift-associ-

ated complex mutations found in the *pol3-Y708A* strain, the majority did not involve homonucleotide runs, despite ample possibility for generating a Can^r mutation via a frameshift in a mononucleotide repeat. This provides an additional indication that mutagenesis mediated by DNA damage bypass and DRIM occurs via different mechanisms, supporting the view that DRIM results from copying of undamaged DNA by Polζ.

A recent report suggested that a strong Polζ-dependent increase in mutagenesis could result from the accumulation of extended stretches of single-stranded DNA (ssDNA) in yeast, presumably due to the fact that damage in ssDNA cannot be repaired (YANG *et al.* 2008). The accumulation of ssDNA at stalled replication forks has been suggested to serve as a signal for the Rad6/Rad18-dependent monoubiquitylation of PCNA, which, in turn, is required for the recruitment of TLS polymerases to sites of DNA damage (DAVIES *et al.* 2008). We have previously reported that the *pol3-Y708A* strains, as well as other replication mutants that are Polζ-dependent mutators, show a robust constitutive monoubiquitylation of PCNA, which is required for DRIM (NORTHAM *et al.* 2006). This is consistent with the idea that the replication mutants may have an excessive amount of ssDNA at the replication forks. However, three observations argue against the major role in DRIM of endogenous lesions present in single-stranded regions. First, even a nearly complete replication block, such as the one resulting from HU treatment, increases the amount of ssDNA at the replication forks no more than twofold (SOGO *et al.* 2002; DAVIES *et al.* 2008). This increase is likely to be even smaller in the replication mutants, such as *pol3-Y708A*, which are quite capable of DNA replication. The mutator effect due to the excessive DNA damage in the ssDNA thus is expected to be less than twofold. Second, anaerobic growth conditions, which presumably reduce the damage in ssDNA, had no effect on DRIM (Figure 2, Table S5). Third, the spectrum of mutations that was suggested to result from the Polζ-dependent bypass of endogenous lesions accumulating in ssDNA (YANG *et al.* 2008) is drastically different from the mutational signature of DRIM (Table 1). Particularly, the ssDNA-stimulated mutagenesis showed no complex changes involving more

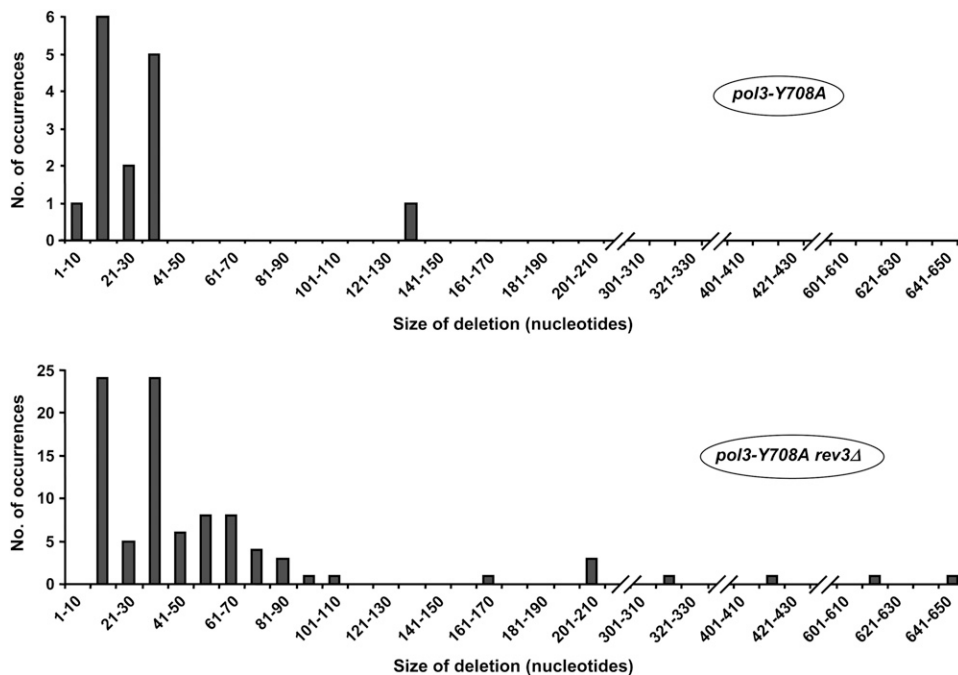


FIGURE 6.—Size of deletions between short direct repeats in the *CAN1* gene of the *pol3-Y708A* strain and its *rev3Δ* derivative. The exact locations of the deletion breakpoints are given in Figures 3 and 4.

than two adjacent nucleotides (a frequency of <3%), a significantly higher proportion of small indels (21% *vs.* 7% during DRIM), and the predominance of transitions rather than transversions among base substitutions. Taken together, these observations indicate that DRIM is unrelated to the bypass of spontaneous lesions formed in double- or single-stranded DNA.

It is interesting to compare the mutational signature of Pol ζ seen in the *pol3-Y708A* strain with the specificity of spontaneous mutagenesis in other situations in which a substantial contribution of Pol ζ may be expected. Pol ζ is responsible for 50–70% of spontaneous mutations in wild-type yeast strains (CASSIER *et al.* 1980; QUAH *et al.* 1980). Multiple changes within stretches of up to seven nucleotides were reported to occur at ~10% frequency in the *CAN1* gene (TISHKOFF *et al.* 1997; NI *et al.* 1999). Such changes, however, were not seen by other laboratories in spontaneous *Can^r* mutants (TRAN *et al.* 2001; HUANG *et al.* 2002; RATTRAY *et al.* 2002) or in other reporter systems (LEE *et al.* 1988; KANG *et al.* 1992; VON BORSTEL *et al.* 1993; SCHELLER *et al.* 2000). It is possible that the contribution of Pol ζ to mutagenesis could vary depending on the genetic background and/or growth conditions. Remarkably, a mutational spectrum apparently showing the Pol ζ signature was seen in the strain carrying another mutant allele of *POL3*, *mut7-1* (VON BORSTEL *et al.* 1993). As in the case of *pol3-Y708A*, the *mut7-1* spectrum shows the predominance of base substitutions, almost half of which are GC \rightarrow CG transversions, a smaller number of frameshifts, and a sizable proportion of multiple changes within stretches of up to six nucleotides. This is consistent with our earlier observation that many mutations in the repli-

some components could lead to the recruitment of Pol ζ to the primer terminus (NORTHAM *et al.* 2006).

The high frequency of complex changes during Pol ζ -dependent DNA synthesis is intriguing. It is clear that the capacity to make complex mutations lies in Pol ζ itself, as it generates these mutations at the same frequency *in vitro* in the absence of any other proteins (ZHONG *et al.* 2006). The mechanism could potentially involve multiple rounds of misinsertion and extension of the aberrant primer terminus, as suggested previously (ZHONG *et al.* 2006). Most DNA polymerases are very inefficient in extending primers with even a single mismatched nucleotide at the 3'-end (PAVLOV *et al.* 2006). Pol ζ is a notable exception; it is highly capable of utilizing primers with a mismatched terminal nucleotide. The ability of Pol ζ to extend primers containing multiple mismatches, however, was not investigated. Alternatively, the complex changes could arise through a relocation of the primer, copying a short DNA sequence elsewhere in the genome, and the realignment of the primer at the original location. A “misincorporation slippage” model has been proposed to explain the generation of complex mutations by Pol ζ during the endogenous DNA damage bypass (HARFE and JINKS-ROBERTSON 2000). In this model, misincorporation of a nucleotide opposite a lesion in the template yields an unstable 3' terminus, which then promotes a slippage in the adjacent homopolymeric run, resulting in a base substitution accompanied by a frameshift. By analogy to this model, a destabilization of the 3' terminus could result from the frequent stalling of the defective replicative polymerase in our case, which could provide an opportunity for a temporary relocation of the primer.

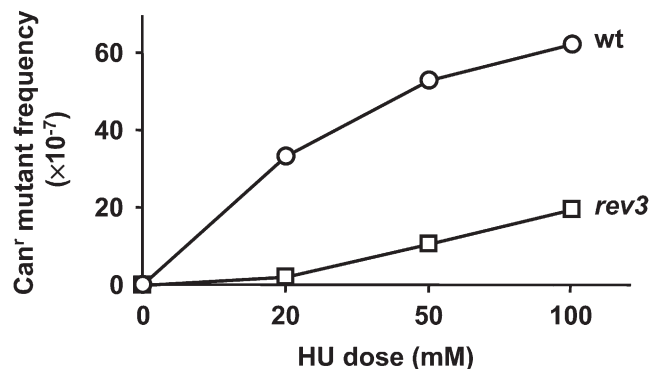


FIGURE 7.—Mutagenesis induced by HU in the wild-type strain E134 and its *rev3* derivative. The data are from Table S6 and are median mutant frequencies for at least 11 cultures. The HU-induced mutant frequency shown on the graph was calculated by subtracting the mutant frequency in the absence of HU from the mutant frequency in the HU-treated cultures. The difference between the two strains is statistically significant at all HU doses, as indicated by nonoverlapping 95% confidence intervals (Table S6).

Both misinsertion–extension and relocation mechanisms could potentially contribute to the generation of complex mutations. Among 12 complex changes in the *pol3-Y708A* strain that involved the replacement of three or more nucleotides, two could apparently be templated by a sequence in the vicinity of the mutation site (Figure S1). The others could occur by different mechanisms or involve primer relocation to a more distant site.

The analysis of the DNA sequence context of Polζ-dependent base substitutions suggests that the contribution of Polζ to DNA synthesis is not the same during copying of the two opposite DNA strands. For the largest class of mutations, GC → CG transversions, the sequence context was significantly different depending on whether the “G” or “C” was originally present in the coding or noncoding strand of the *CAN1* gene (Table S7). In 29 of 30 cases (97%) when “G” was the original coding strand base, the mutations occurred at sequences containing between two and eight consecutive G-C pairs. In contrast, when “C” was the original coding strand base, the surrounding sequence was generally A-T-rich, with 17 of 25 mutations (68%) at sites having A-T pairs both 3′ and 5′ to the “C.” In fact, 40% of all coding strand “C” substitutions occurred at five distinct TCAA sites (the mutated “C” is underlined), suggesting that it may be the preferable sequence context for this orientation of the G-C pair. The seemingly opposite context preferences for G → C *vs.* C → G changes may result from a combination of several factors: There could be different sequence context requirements for the formation of G-G *vs.* C-C mispairs. There could also be differences in the frequency at which Polζ is recruited to copy the two opposite strands. Finally, the sequence context preferences for the same Polζ-dependent replication error could be different on the two DNA strands due to, for example, different composition

of the replicating complexes. Further studies using a system that would distinguish between the replication errors made during copying of the opposite strands would be required to distinguish between these possibilities.

Studies of the mechanism of DNA polymerase switching during TLS led to two models that are not necessarily mutually exclusive (reviewed recently in WATERS *et al.* 2009). According to the first model, stalling of the replicative polymerase at the site of the lesion leads to the recruitment of TLS polymerases that then act in the context of the replication fork to bypass the lesion. Resumption of the accurate processive replication requires a switch back to the replicative DNA polymerase, which becomes possible once the lesion is bypassed. In the second model, once the fork encounters a lesion, a quick restart of replication occurs downstream of the lesion, leaving a gap between the site of the lesion and the site of the restart. TLS polymerases then bypass the lesion and, possibly, fill the remaining gap, thus working outside of the replication fork. In yeast, the electron microscopy and two-dimensional gel analysis of the *in vivo* replication intermediates provided support for the second idea (LOPES *et al.* 2006). Similar to the mechanism of TLS, Polζ-dependent DNA synthesis in response to intrinsic replisome defects could occur in the context of the replication fork or as a part of the post-replicative gap filling process. Errors made during ongoing DNA replication are expected to be corrected by the DNA mismatch repair (MMR) system. Indeed, we previously observed that Polζ-dependent base substitution errors in the *pol3-Y708A* background are corrected by MMR. The rate of Polζ-dependent base substitutions increases ~30-fold when MMR is inactivated (PAVLOV *et al.* 2001b). It, however, cannot be excluded that errors made during the gap-filling synthesis could be processed by MMR. In addition, it is possible that Polζ could contribute to DNA synthesis both at the fork and in post-replicative gaps. In this case, the effect of MMR on Polζ-dependent mutagenesis would be seen even if the errors made during the gap-filling synthesis are not corrected. Further experiments would be required to distinguish between these possibilities. Additionally, it is possible that the replisome defects lead to the accumulation of double-strand breaks (DSB), and the processing of these breaks could contribute to the increased Polζ-dependent mutagenesis (HOLBECK and STRATHERN 1997). However, the types of mutations seen during DSB-repair-associated mutagenesis [a large proportion of frameshifts and very few GC → CG transversions and complex mutations (RATTRAY *et al.* 2002)] is significantly different from the mutational spectrum generated during Polζ-dependent DRIM. This argues against a major contribution of the DSB repair to DRIM.

The observation that an error-prone TLS polymerase can contribute to the copying of undamaged DNA is highly important given the possible devastating effects of mutagenic DNA synthesis on genome stability. In-

terestingly, the other yeast TLS polymerase, Pol η , is apparently not able to access the primer terminus in the situation in which the progression of the replication fork is impeded due to the intrinsic replisome defects. Pol η has an extremely low fidelity (MATSUDA *et al.* 2000), so any contribution of this polymerase to the copying of undamaged DNA would be expected to be highly mutagenic. Unlike Pol ζ , Pol η did not appreciably contribute to DRIM (PAVLOV *et al.* 2001b). This suggests that the participation of Pol η in replication is more strictly regulated. In agreement with this idea, overproduction of Pol η did not increase the rate of spontaneous mutation in yeast or in human cells (PAVLOV *et al.* 2001a; KING *et al.* 2005). An interesting possibility is that Pol ζ has specifically evolved as a polymerase capable of rescuing replication forks stalled for any reason, which is not necessarily related to damage in the template DNA. This would be consistent with the high promiscuity of Pol ζ for the extension of primer termini that could not be efficiently used by other DNA polymerases. In this case, it would be reasonable to expect that the control over the access of Pol ζ to the primer terminus could be more relaxed, which would allow it to respond to a wide variety of situations that slow replication fork progression.

In addition to the *pol3-Y708A* and *pol2-1* strains discussed in this study, the recruitment of Pol ζ has been shown to be responsible for a significant proportion of spontaneous mutations in several other DNA replication mutants. These include *pol3-t* mutants with a temperature-sensitive Pol δ , *dpb3* mutants with the deletion of an accessory subunit of Pol ϵ , and the *pol1-1* mutant defective in the interaction between the catalytic and the primase subunits of Pol α (NORTHAM *et al.* 2006). As discussed previously, the spectrum of spontaneous mutations in the *mut7-1* (VON BORSTEL *et al.* 1993) is also indicative of the involvement of Pol ζ . Although it appears to be a common mechanism of mutagenesis, the recruitment of Pol ζ is clearly not the only way by which a replicative DNA polymerase mutation can produce a mutator phenotype. In recent years, mutations were identified that affect the nucleotide selectivity, but not the catalytic activity of Pol α , Pol δ and Pol ϵ (NIIMI *et al.* 2004; LI *et al.* 2005; VENKATESAN *et al.* 2006; NICK McELHINNY *et al.* 2007; PURSELL *et al.* 2007). Thus, these mutations are not expected to cause defects in the replication fork progression that could trigger the recruitment of specialized DNA polymerases. The vast majority of spontaneous mutations in the corresponding mutants does not require Pol ζ (SUZUKI *et al.* 2009; Y. I. PAVLOV, personal communication) and is thought to result from inaccurate DNA synthesis by the replicative DNA polymerases themselves. Similarly, the mutator phenotype of strains defective in the proofreading activity of Pol δ and Pol ϵ (MORRISON *et al.* 1991; SIMON *et al.* 1991) apparently results from the low-fidelity synthesis by the replicative polymerases (SHCHERBAKOVA

et al. 2003; FORTUNE *et al.* 2005) and is not affected by the inactivation of Pol ζ (SHCHERBAKOVA *et al.* 1996). It has been reported recently that the temperature-sensitive *pol3-t* mutants grown at the permissive temperature of 23° display a mutator phenotype that is independent of Pol ζ (MITO *et al.* 2008). This is in contrast to our earlier observation that nearly two-thirds of spontaneous mutations in the *pol3-t* strains require Pol ζ when the cells are grown at 30° (NORTHAM *et al.* 2006), the condition at which *pol3-t* strains show much more severe growth and genome stability defects (GORDENIN *et al.* 1991, 1993; TRAN *et al.* 1995). While these differences could be due to a different strain background as suggested by MITO *et al.* (2008), it is likely that the effect of the *pol3t* mutation on the replication fork progression is not the same at 23° and at 30°. It is possible that the replication intermediates that lead to the recruitment of Pol ζ are not accumulated in sufficient amounts at 23°, where *pol3t* strains have no detectable growth defect. Further studies will be needed to determine the exact nature and/or level of the aberrant replication intermediates necessary for the activation of one or another mutagenesis pathway.

Interestingly, the mutator phenotype of the *pol3t* strains observed by MITO *et al.* (2008) required another TLS polymerase, Rev1. While the catalytic activity of Rev1 can operate during TLS, particularly during abasic site bypass, the essential role of Rev1 in most forms of TLS is structural (WATERS *et al.* 2009). Yeast Rev1 and its mammalian homologs are involved in multiple physical interactions with other TLS polymerases and the Pol32 subunit of Pol δ (ACHARYA *et al.* 2009; WATERS *et al.* 2009). This led to the idea that Rev1 could provide a docking site to help exchange different DNA polymerases at the replication fork. Binding of Rev1 to Pol ζ also stimulates the mismatch extension activity of Pol ζ (ACHARYA *et al.* 2006). The TLS function of yeast and mammalian Rev1, in turn, is regulated by monoubiquitylation of the Lys164 residue of PCNA (GARG and BURGERS 2005; GUO *et al.* 2006; WOOD *et al.* 2007). Since we previously established that PCNA ubiquitylation at Lys164 is required for Pol ζ -dependent DRIM (NORTHAM *et al.* 2006), it is plausible that Rev1 could play an organizing role in this mutagenesis pathway as well. On the other hand, the structural basis for nucleotide insertion by Rev1 (NAIR *et al.* 2005; SWAN *et al.* 2009b) is such that the only reaction that it can efficiently perform on undamaged DNA is the insertion of C opposite template G. Thus, the catalytic activity of Rev1 cannot be responsible for the wide spectrum of base substitutions and complex mutations observed during DRIM. Experiments are in progress to investigate the role of Rev1 and other factors involved in TLS, such as Pol32 or cell-cycle checkpoint proteins (KOREN 2007), in Pol ζ -dependent DRIM.

In addition to Pol ζ -dependent DNA synthesis, the cells presumably have other ways of tolerating the replication defects. In fact, the inactivation of Pol ζ in

DNA replication mutants does not confer any additional growth defect beyond that conferred by the replication mutation itself (our unpublished data). This suggests that the replication can proceed in these strains without the genome-destabilizing mutagenic DNA synthesis by Pol ζ . Our results suggest, however, that Pol ζ plays an essential role in preventing larger genome rearrangements in this situation, such as large deletions between repeated sequences. There is evidence that deletions between short direct repeats are mediated by DNA polymerase slippage (TRAN *et al.* 1995). The dramatic increase in the size of such deletions in the Pol ζ^- strain (Figure 6) is consistent with a hypothesis that longer stretches of ssDNA could form in the absence of Pol ζ , which would permit replication slippage over longer distances. Mammalian cells lacking Pol ζ show an elevated rate of DSB and large chromosomal rearrangements, particularly translocations (VAN SLOUN *et al.* 2002; WITTSCHIEBEN *et al.* 2006). It would be interesting to determine whether the increase in the size of deletions in the yeast Pol ζ^- cells and the genomic instability in the mammalian Pol ζ^- cells reveal the same role of Pol ζ in the control of genome stability.

We thank Yuri Pavlov for yeast strains and for critically reading the manuscript, Evelyne Sage and Stas Kozmin for providing DNA glycosylase mutants, Victoria Liston and Corinn Grabow for technical assistance, and Jan Drake and Dmitry Gordenin for advice on mutation rate measurement. This work was supported in part by National Institutes of Health grants ES011644 and ES015869 and by a Nebraska Department of Health and Human Services grant LB506 to P.V.S. and the Blanche Widaman Fellowship to M.R.N.

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Communicating editor: D. LEACH

GENETICS

Supporting Information

<http://www.genetics.org/cgi/content/full/genetics.109.107482/DC1>

Participation of DNA Polymerase ζ in Replication of Undamaged DNA in *Saccharomyces cerevisiae*

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DOI: 10.1534/genetics.109.107482

TABLE S1
Effect of *RAD14*, *APN1* and *APN2* inactivation on the spontaneous mutator phenotype of the *pol3-Y708A* mutant

Strain ^a	Can ^r mutation	
	Mutation rate ($\times 10^{-8}$) ^b	Fold increase
Wild type	19 (16-22)	1
<i>pol3-Y708A</i>	170 (140-210)	8.9
<i>rad14</i> Δ	43 (39-48)	2.3
<i>rad14</i> Δ <i>pol3-Y708A</i>	200 (180-220)	11
<i>apn1</i> Δ <i>apn2</i> Δ	91 (79-100)	4.8
<i>apn1</i> Δ <i>apn2</i> Δ <i>pol3-Y708A</i>	290 (250-360)	15

^a All strains are isogenic to E134.

^b Mutation rates are given as medians for at least 36 cultures. The 95% confidence limits are shown in parentheses.

TABLE S2
Effect of DNA glycosylase deficiency on the spontaneous mutator phenotype of
the *pol3-Y708A* and *pol2-1* mutants

Strain ^a	Can ^r mutation	
	Mutation rate (x10 ⁻⁸) ^b	Fold increase
Wild type	13 (11-15)	1
<i>ogg1 ntg1 ntg2 ung1 mag1</i>	120 (98-130)	9.2
<i>pol3-Y708A</i>	160 (130-210)	12
<i>ogg1 ntg1 ntg2 ung1 mag1 pol3-Y708A</i>	420 (340-490)	32
<i>pol2-1</i>	380 (320-420)	29
<i>ogg1 ntg1 ntg2 ung1 mag1 pol2-1</i>	610 (580-890)	47

^a All strains are isogenic to FF18733.

^b Mutation rates are given as medians for at least 18 cultures. The 95% confidence limits are shown in parentheses.

TABLE S3

Effect of simultaneous inactivation of *APN1*, *APN2* and *RAD14* on the spontaneous mutator phenotype of the *pol3-Y708A* mutant

Strain ^a	Can ^r mutation	
	Mutation rate (x10 ⁻⁸) ^b	Fold increase
Wild type	14 (13-17)	1
<i>pol3-Y708A</i>	170 (130-240)	12
<i>apn1Δ apn2Δ rad14Δ</i>	200 (180-220)	14
<i>apn1Δ apn2Δ rad14Δ pol3-Y708A</i>	500 (440-600)	36

^a All strains are isogenic to E134.

^b Mutation rates are given as medians for 18 cultures. The 95% confidence limits are shown in parentheses.

TABLE S4

Effect of simultaneous inactivation of *OGG1*, *NTG1*, *NTG2*, *UNG1*, *MAG1* and *RAD14* on the spontaneous mutator phenotype of the *pol3-Y708A* mutant

Strain ^a	Can ^r mutation	
	Mutation rate (x10 ⁻⁸) ^b	Fold increase
Wild type	11 (7.7-14)	1
<i>pol3-Y708A</i>	170 (120-220)	15
<i>ogg1 ntg1 ntg2 ung1 mag1 rad14</i>	150 (130-180)	14
<i>ogg1 ntg1 ntg2 ung1 mag1 rad14 pol3-Y708A</i>	320 (290-460)	29

^a All strains are isogenic to FF18733.

^b Mutation rates are given as medians for at least 17 cultures. The 95% confidence limits are shown in parentheses.

TABLE S5**Effect of anaerobic growth conditions on the spontaneous mutator phenotype of *pol3-Y708A* and *ogg1Δ* strain**

Growth conditions	Strain ^a	Can ^r mutation	
		Mutation rate (x10 ⁻⁸) ^b	Fold increase
Aerobic	E134 ^c	19 (16–22)	1
	E134 <i>pol3-Y708A</i> ^c	170 (140-210)	8.9
Anaerobic	E134	17 (16–20)	1
	E134 <i>pol3-Y708A</i>	150 (130-200)	8.8
Aerobic	Δ(-2)l-7B-YUNI300	20 (15–26)	1
	Δ(-2)l-7B-YUNI300 <i>ogg1Δ</i>	46 (35-58)	2.3
Anaerobic	Δ(-2)l-7B-YUNI300	22 (13–26)	1
	Δ(-2)l-7B-YUNI300 <i>ogg1Δ</i>	20 (16-37)	0.9
Aerobic	8C-YUNI101	23 (21–43)	1
	8C-YUNI101 <i>ogg1Δ</i>	55 (47-77)	2.4
Anaerobic	8C-YUNI101	29 (25–42)	1
	8C-YUNI101 <i>ogg1Δ</i>	33 (25-47)	1.1
Aerobic	FF18733	12 (8.7–15)	1
	FF18733 <i>ogg1Δ</i>	82 (61-160)	6.8
	FF18733 <i>pol3-Y708A</i>	120 (80-170)	10
Anaerobic	FF18733	30 (20–49)	1
	FF18733 <i>ogg1Δ</i>	31 (17-54)	1

FF18733 pol3-Y708A

110

9.2

(75-220)

^a 8C-YUNI101 (*MAT_a ade2-1 trp1-1 his7-2 leu2-3,112 ura3 Δ bik1::*

ura3-29RL) was described previously (PAVLOV *et al.* 2001b). Its *ogg1 Δ* mutant was

provided by Dr. Y.I. Pavlov (University of Nebraska Medical Center).

The *ogg1 Δ* mutant of FF18733 was a gift from Dr. E. Sage (Institut

Curie, Orsay, France). Other strains are described in Materials and

Methods.

^b Mutation rates are given as medians for at least nine cultures.

The 95% confidence limits are shown in parentheses.

^c Data for E134 and E134 pol3-Y708A under aerobic conditions are from

supplemental Table 1.

TABLE S6**Effect of hydroxyurea treatment on mutagenesis in the wild-type strain E134 and its *rev3* derivative**

Strain	Frequency of Can ^r mutants ($\times 10^{-7}$) ^a			
	no HU	20 mM HU	50 mM HU	100 mM HU
Wild type	9.7 (6.8-13)	43 (33-54)	62 (49-91)	72 (52-110)
<i>rev3</i> Δ	5.4 (3.7-9.8)	7.5 (6.1-9.5)	16 (11-19)	25 (16-40)

^a Mutant frequencies are given as medians for at least 11 cultures. The 95% confidence limits are shown in parentheses.

TABLE S7**DNA sequence context of GC → CG transversions in the *pol3-Y708A* strain**

Substitution in the coding DNA strand	
G → C	C → G
TGGG <u>GC</u> TCTT (5)	AAATT <u>TCAA</u> ATA (3)
ACGCC <u>GG</u> CCCA (4)	AAATT <u>TCAA</u> AAG (2)
GGGT <u>GC</u> TTCC (4)	GTTCC <u>C</u> GTATT (2)
TTCC <u>CG</u> TATTT (3)	TCTAT <u>TCAA</u> AGA (2)
ACGTT <u>GG</u> TTCC (2)	CCTGT <u>TCAA</u> AGGA (2)
TGGT <u>GT</u> ACTA	TGGGT <u>TCAA</u> TAC
CTTT <u>GC</u> ATAT	GAGAG <u>C</u> TTAAG
AAAT <u>GG</u> TACA	AAAGA <u>C</u> ATATT
CATTT <u>GG</u> TGCG	TGGTA <u>C</u> AGGTC
TTCTT <u>GGG</u> CAA	CGGCC <u>C</u> AGTGG
CCCT <u>GG</u> AACTT	ATGAA <u>C</u> TTGTT
TTCGA <u>G</u> TTCTG	TGAAG <u>C</u> TGCAA
TTTTA <u>GCC</u> ATT	CTTAA <u>C</u> AACCA
AGTT <u>GT</u> TATCA	TATTT <u>C</u> TGCCG
AAGCT <u>G</u> CAAAC	TTCTG <u>C</u> CGCAA
TTT <u>CCG</u> TATCT	GTGTT <u>C</u> CATAC
TGCAA <u>G</u> CTTTG	GCTTA <u>C</u> ATGGA
	TATGA <u>C</u> GATCA
	GAGTT <u>C</u> TGGGT

The mutated nucleotide is bold and underlined. For the G → C substitutions, the stretches of several consecutive G•C pairs at the mutation site are underlined. For the C → G substitutions, the “hotspot” TCAA sequences at the site of mutation are underlined. Number of occurrences is shown in parentheses.