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Mutator alleles of yeast DNA polymerase ζ

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Abstract

The yeast *REV3* gene encodes the catalytic subunit of DNA polymerase zeta (pol ζ), a B family polymerase that performs mutagenic DNA synthesis in cells. To probe pol ζ mutagenic functions, we generated six mutator alleles of *REV3* with amino acid replacements for Leu979, a highly conserved residue inferred to be at the pol ζ active site. Replacing Leu979 with Gly, Val, Asn, Lys, Met or Phe resulted in yeast strains with elevated UV-induced mutant frequencies. While four of these strains had reduced survival following UV irradiation, the *rev3-L979F* and *rev3-L979M* strains had normal survival, suggesting retention of pol ζ catalytic activity. UV mutagenesis in the *rev3-L979F* background was increased when photoproduct bypass by pol η was eliminated by deletion of *RAD30*. The *rev3-L979F* mutation had little to no effect on mutagenesis in an *ogg1 Δ* background, which cannot repair 8-oxo-guanine in DNA. UV-induced *can1* mutants from *rev3-L979F* and *rad30 Δ rev3-L979F* strains primarily contained base substitutions and complex mutations, suggesting error-prone bypass of UV photoproducts by L979F pol ζ . Spontaneous mutation rates in *rev3-L979F* and *rev3-L979M* strains are elevated by about 2-fold overall and by 2- to 8-fold for C to G transversions and complex mutations, both of which are known to be generated by wild-type pol ζ *in vitro*. These results indicate that Rev3p-Leu979 replacements reduce the fidelity of DNA synthesis by yeast pol ζ *in vivo*. In conjunction with earlier studies, the data establish that the conserved amino acid at the active site location occupied by Leu979 is critical for the fidelity of all four yeast B family polymerases. Reduced fidelity with retention of robust polymerase activity suggests that the homologous *rev3-L979F* allele may be useful for analyzing pol ζ functions in mammals, where *REV3* deletion is lethal.

Keywords

pol ζ ; translesion synthesis; mutagenesis; yeast

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1. INTRODUCTION

DNA polymerase ζ (pol ζ) is one of many eukaryotic DNA polymerases [1-3], and it has a particularly important role in mutagenesis [4]. Pol ζ consists of an accessory subunit encoded by the *REV7* gene and a polymerase catalytic subunit encoded by the *REV3* gene [5]. Cells disrupted for *REV3* have reduced levels of DNA damage-induced mutagenesis [4], thereby implicating pol ζ in mutagenic translesion DNA synthesis. *REV3* has many functions [4,6,7]. It is needed for the majority of spontaneous mutagenesis [4,8-13], and it contributes to mutagenesis associated with impairment of the major replicative polymerases [11], with defective DNA repair [14-19], with double-strand break repair [20-22], with transcription [23], and with somatic hypermutation of immunoglobulin genes [24,25]. Consistent with these multiple mutagenic functions, pol ζ lacks an intrinsic proofreading exonuclease [5] and it is promiscuous in extending mismatched primer termini [26-29]. Consistent with its role in mutagenic synthesis *in vivo*, purified yeast pol ζ synthesizes DNA *in vitro* with lower fidelity than the major replicative polymerases, pol α , pol δ and pol ϵ [30].

As a member of the B family of DNA polymerases, pol ζ shares homology with pol α , pol δ and pol ϵ , as well as with enzymes that replicate bacteriophage genomes, such as T4 pol and RB69 pol. Residues in motifs A, B and C that form the polymerase active site are particularly conserved. In the crystal structure of RB69 pol [31], an invariant tyrosine in motif A interacts with the ribose of the incoming dNTP. Adjacent to this tyrosine is a hydrophobic residue, methionine in pol ϵ or leucine in other Family B members, e.g., Leu979 in pol ζ . In the major replicative polymerases, replacing this residue with certain other amino acids reduces DNA synthesis fidelity *in vitro* [32-36], due to increased dNTP misinsertion efficiency, increased mismatch extension efficiency and/or decreased proofreading efficiency [see also 37,38]. Consistent with these biochemical properties, strains encoding these mutant polymerases have elevated spontaneous mutation rates that likely reflect reduced DNA replication fidelity *in vivo* [32-37,39,40]. Based on these studies of other Family B polymerases, we hypothesized that Leu979 in Rev3p may have an important role in determining the fidelity of DNA synthesis by pol ζ . To test this, and ultimately to obtain a more thorough understanding of multiple pol ζ functions in mutagenic DNA transactions *in vivo*, here we have undertaken a search for amino acid replacements in Rev3p that are likely to retain pol ζ activity yet result in elevated spontaneous and damage-induced mutagenesis in yeast. This approach to study *rev3* mutator alleles is intended to add to studies in which pol ζ function has been inferred from *rev3* disruption, a strategy has been highly informative in yeast [4] but less informative in mice because *rev3* disruption results in embryonic lethality [41-43].

2. MATERIALS AND METHODS

2.1. Media and growth conditions

Yeast strains were grown nonselectively on YPDA media (1% yeast extract, 2% Bacto-peptone, 2% dextrose, 10 mg/L adenine). Synthetic, selective media contained 2% dextrose and appropriate amino acids. Canavanine resistant mutants were identified by growth on synthetic media supplemented with 60 ug/mL canavanine. All growth was at 30°.

2.2. Construction of yeast strains

All strains used in this study were derived from haploid 8C-YUNI101 (MATa his7-2 leu2-3,112 ura3 Δ bik1::ura3-29RL trp1-1_{UAG} ade 2-1_{UAA}) [44]. The *rev3::LEU2* mutant was described previously [44]. All other mutants were constructed by standard one-step (OST) or two-step transplacement (TST) and all strains were verified by PCR and/or sequencing. *RAD30* and *OGG1* deletions were generated via OST with PCR fragments amplified from pFA6-kanMX4 [45] using primers disRAD30-L and disRAD30-R, or disOGG1-L and

disOGG1-R, respectively (for primer sequences see Supplementary Table 1). *rev3-L979X* alleles were created by site-directed mutagenesis of pREV3Cav2 [44] using the QuickChange Kit (Stratagene). *rev3-L979X* yeast strains were made by TST with *Sna*BI-cut mutagenized derivatives of pREV3Cav2. Each of the *rev3-L979X* yeast strains were verified by sequencing using primers designated REV3seq in Supplementary Table 1.

2.3. Measurements of mutation rates

Spontaneous mutation rates were determined by the method of median [46], using at least 22 independent cultures per experiment. Ultraviolet light (UV)-induced mutation frequencies were measured using 2-4 independent cultures. Cultures were grown to saturation in YPDA liquid, then diluted appropriately and plated onto synthetic media supplemented with canavanine and non-selective synthetic media. Plates were immediately exposed to UVC at the indicated dose and were then incubated at 30°C in the dark. Mutation frequencies were calculated as the average of the number of colonies on canavanine plates divided by the number of colonies on non-selective plates multiplied by the dilution factor.

2.4. Sequence analysis of *can1* mutants

UV-induced *can1* mutations were isolated following exposure to 20 J/m² under the conditions described above. At least 24 canavanine resistant isolates were sequenced per strain. A 2.0 kb region covering the 3' end of *CAN1* gene was amplified with primers *can1-F* and *can1-R* (Supplementary Table 1). The 5' end of *CAN1* gene was amplified using primers *can1-F2* and *can1-R* (Supplementary Table 1). The PCR fragments were subsequently sequenced on both strands using sequencing primers *can1-SF1* to *can1-SR3* (Supplementary Table 1).

2.5. Statistical analysis

We first estimated the number of *can1* mutants expected to be due to an individual type of mutation in each strain by multiplying the proportion of that mutation observed in the mutation spectrum (Table 1) by the total number of *can1* survivors obtained after irradiation with 20 J/m² UV. We then estimated mutation frequencies of individual mutation types as the estimated number of *can1* mutants of each type divided by the total number of *can1* survivors. The mutation frequencies of an individual type in each strain were then compared with the wild-type strain using an independent samples t-test for equality of proportions. The variance of this test was adjusted to reflect the number of mutants sequenced. A complete explanation of the two independent samples test for equality of mutation frequencies can be found in the Supplementary Information.

3. RESULTS

3.1. Choice of *rev3* missense alleles to investigate

Mutator phenotypes had been previously observed for yeast strains containing the following replacements for homologous residues in the three major yeast replicative DNA polymerases: Val, Lys and Met replacements for Leu868 in pol α [32,33], Val, Lys, Met, Phe, Gly and Asn replacements for Leu612 in pol δ [34,39,40] and Phe replacement for Met644 in pol ϵ [35]. Here we chose to test whether the same replacements for Rev3p-Leu979 might affect the accuracy of DNA synthesis by yeast pol ζ and thereby confer mutator phenotypes characteristic of mutagenic DNA synthesis activity by pol ζ *in vivo*. Thus, we began this study by constructing yeast strains with Gly, Val, Asn, Lys, Met or Phe replacements for Rev3p-Leu979.

3.2. UV-induced killing and mutagenesis

3.2.1. UV sensitivity and mutagenesis of the six *rev3-L979X* strains—Because pol ζ has a well-established role in UV-induced survival and mutagenesis, we first examined

whether the *rev3* missense mutations affected these two parameters. As expected, the *rev3Δ* strain was more sensitive to UV irradiation than was wild-type yeast (Fig. 1A). The *rev3-L979K*, *rev3-L979G*, *rev3-L979N* and *rev3-L979V* strains all had intermediate sensitivity, suggesting partial loss of pol ζ activity. In contrast, the *rev3-L979F* and *rev3-L979M* strains had wild-type sensitivity to UV irradiation, suggesting greater retention of pol ζ catalytic activity. When UV-induced mutant frequencies at the *CAN1* locus were measured, the *rev3Δ* strain was immutable (Fig. 1B). In contrast, all strains with Rev3p-Leu979 replacements were more UV mutable than wild-type yeast (Fig. 1B), suggesting that these replacements reduce the fidelity of pol ζ as it participates in translesion DNA synthesis (TLS) of UV photoproducts.

3.2.2. UV sensitivity and mutagenesis in the *rad30Δrev3-L979F* double mutant strain—In wild-type yeast, the majority of UV photoproduct bypass is conducted by pol η, the product of the *RAD30* gene. However, in a *rad30Δ* strain, pol ζ has been implicated in mutagenic bypass [47,48]. Therefore, we next examined UV-induced sensitivity and mutagenesis in a *rad30Δrev3-L979F* double mutant strain. As expected based on earlier studies [47,49,50], deletion of *RAD30* (*rad30Δ*) slightly reduces survival (Fig. 1C). However, the *rad30Δrev3-L979F* double mutant strain survives exposure to UV radiation at levels comparable to those for the *rad30Δ* single mutant (Fig. 1C). This again suggests that L979F pol ζ retains high pol ζ catalytic activity. In addition, the *rad30Δrev3-L979F* double mutant strain is several-fold more UV mutable than are the single mutant *rev3-L979F* or *rad30Δ* strains, and it is much more mutable than the single *rev3Δ* mutant or the double *rad30Δ revΔ* mutant strain (Fig. 1B and 1D). These results are consistent with participation of L979F pol ζ in mutagenic TLS of UV photoproducts when pol η is absent.

3.2.3. Specificity of UV-induced mutations in *rev3-L979F* mutant strains—In order to determine if the spectrum of mutations resulting from *in vivo* TLS by L979F pol ζ is similar to that of wild-type pol ζ, we determined the types of UV-induced mutations that arose at *CAN1* in wild-type and *rev3-L979F* strains. We also sequenced *can1* isolates from isogenic *rad30Δ* and *rev3-L979F rad30Δ* strains, which lack TLS by pol η. Yeast cells were irradiated with 20 J/m² of UVC, a dose that induces mutants in all four strains examined at frequencies that are much higher than the spontaneous frequencies (Fig. 1 and see below).

The UV-induced mutation spectrum in the wild-type strain contained primarily single base substitutions (76%), all of which occurred at dipyrimidine sequences. Of these, eight are consistent with misincorporation opposite the 3' base of a photodimer and two are consistent with misincorporation opposite the 5' base of a photodimer. An additional nine single base substitutions arose at tripyrimidine sequences and are thus ambiguous with respect to site of the misincorporation. Among transitions, G-C to A-T substitutions predominated, and among transversions, A-T to T-A changes predominated (Table 1). This specificity is consistent with earlier studies [see also 49 and references therein], and suggests that UV light induces mutations predominantly via C•A and T•T mispairs that preferentially arise at the 3' base of photodimers. Additionally, the frequencies of UV-induced insertion/deletion (indel) and complex mutations are at least 100-fold higher than the spontaneous mutation rates for these events (compare Tables 1 and 2), suggesting that these mutations also result from mutagenic bypass of UV photoproducts.

The UV-induced mutation spectrum of the *rev3-L979F* single mutant strain (Table 1 and Fig. 2) differed from that of wild-type yeast in several ways. Foremost was the presence of eight complex mutations (32% of *can1* mutations; for sequences see Supplementary Table 2), which contain multiple, closely spaced base substitutions and/or indel mutations. The frequency of complex mutations was significantly higher in the *rev3-L979F* mutant compared to the wild-type strain (9-fold, *p* = 0.0046). This increase is consistent with mutagenic TLS of UV photoproducts by L979F pol ζ that is less accurate than TLS by wild-type pol ζ. The frequencies

of tandem double base substitutions and G-C to T-A transversions were also elevated 5-7 fold in the *rev3-L979F* mutant. Because only 25 clones were sequenced, the number of each type of event is small, such that these increases are not statistically significant. All seven of single base substitutions from the *rev3-L979F* mutant strain that occurred at dipyrimidines are consistent with misincorporation opposite the 3' base of the dipyrimidine. This specificity suggests that although L979F pol ζ is less accurate than wild-type pol ζ , it participates in a bypass reaction that retains the preference for misincorporation opposite the 3' base of a photodimer.

The *rad30 Δ* single mutant (Table 1 and Fig. 2) had a significantly elevated frequency of UV-induced G-C to A-T transitions compared to the wild-type strain (3-fold, $p = 0.019$). Also slightly elevated were G-C to T-A transversions (4-fold) and complex mutations containing multiple, closely-spaced nucleotide changes (7-fold). Twenty of 21 single base substitutions from the *rad30 Δ* strain occurred at dipyrimidine or tripyrimidine sequences. Of these, 11 are consistent with misincorporation opposite the 3' base of a photodimer, two are consistent with misincorporation opposite the 5' base of a photodimer and seven are ambiguous, as they occurred at tripyrimidine sites. Since pol ζ is present in the *rad30 Δ* single mutant strain, these data are consistent with a contribution of pol ζ to mutagenic TLS of photoproducts that yields single base mispairs preferentially located at the 3' base of photodimers, as well as complex errors (Table 1, Fig. 2). The complex mutations observed when pol ζ is present and pol η is absent are reminiscent of those generated by purified yeast pol ζ as it copies undamaged DNA *in vitro* [30].

The UV-induced mutation spectrum of the *rad30 Δ rev3-L979F* double mutant showed combined characteristics of the spectra for the *rad30 Δ* and *rev3-L979F* single mutant strains (Table 1, Fig. 2). The frequencies of three types of UV-induced mutations were significantly elevated in the *rad30 Δ rev3-L979F* double mutant compared than those of wild-type: G-C to A-T transitions (8-fold, $p = 0.009$), G-C to T-A transversions (26-fold, $p = 0.04$), and tandem, double base substitutions (26-fold, $p = 0.04$). UV-induced complex mutations were also elevated 9-fold, although this elevation was not statistically significant. Of the sixteen base substitution errors in the *rad30 Δ rev3-L979F* double mutant, eleven occurred at dipyrimidines and four at tripyrimidines. All of the mutations that occurred at dipyrimidines are consistent with misincorporation opposite the 3' base, again implicating misinsertions opposite photodimers as the mutagenic event in *rev3-L979F* strains. This suggests that the lesions responsible for most of these substitutions in the double mutant strain are photodimers that are normally copied by pol η , such that in its absence (*rad30 Δ*), L979F pol ζ participates in mutagenic TLS. The situation may be slightly different for indels and complex mutations, whose mutant frequencies are similar in the *rad30 Δ rev3-L979F* double mutant and the *rev3-L979F* single mutant (Table 1, Fig. 2).

3.3. Minimal effect of the *rev3-L979F* allele on *ogg1*-dependent mutagenesis

To determine if the *rev3-L979F* allele has an effect on mutagenesis involving a spontaneously arising oxidative lesion, we examined mutation rates in *ogg1 Δ* mutants, which are deficient for the glycosylase that removes 8-oxo-guanine from DNA. In *REV3* strains, *ogg1 Δ* results in an elevated mutation rate at *CAN1*, and this rate is further elevated in an *ogg1 Δ rad30 Δ* double mutant (Fig. 3A). These results are consistent with earlier studies that revealed the roles of Ogg1 and pol η in preventing G-C to T-A substitutions resulting from spontaneous oxidative stress [51,52]. Interestingly, rates are not further elevated in *ogg1 Δ* or *ogg1 Δ rad30 Δ* mutant strains that harbor the *rev3-L979F* allele (Fig. 3A). Similar results were obtained for reversion of the *ura3-29* reporter (Fig. 3B), which in an *ogg1 Δ* background is a highly sensitive marker for G-C to T-A transversions [53]. These results imply that, unlike the situation for UV photoproducts, L979F pol ζ has little if any role in mutagenic bypass of spontaneously arising

8-oxo-guanine in DNA *in vivo*. This is consistent with an earlier study indicating that wild-type pol ζ does not have a major role in mutagenesis in an *ogg1 Δ* strain [51].

3.4. Spontaneous mutation rates in *rev3-L979F* and *rev3-L979M* strains

In the above experiments, the spontaneous mutation rate at *CAN1* for the *rev3-L979F* strain (3.1×10^{-7} , 95% CI = 2.6 – 4.2) was 1.8-fold higher than for the wild-type strain (1.7×10^{-7} , 95% CI = 1.1 – 2.2). In a second experiment, the *rev3-L979F* and *rev3-L979M* strains had spontaneous mutation rates that were 1.6-fold and 1.8-fold higher, respectively, than in a wild-type strain (Table 2), and 2.1-fold and 2.3-fold higher, respectively, than in the *rev3 Δ* strain. Thus, the *rev3-L979F* and *rev3-L979M* alleles confer a weak spontaneous mutator phenotype to yeast. To determine if this effect was specific to any particular types of mutations, independent *can1* mutants were sequenced. The results (Table 2) indicate that the rates of G-C to C-G transversions and of complex mutations involving multiple base changes in the *rev3-L979F* and *rev3-L979M* strains were higher than in the wild-type or *rev3 Δ* strains. The *rev3-L979M* strain also had a higher rate of indels, all but one of which were single base deletions, and all of which occurred in non-iterated sequences or sequences with only two repeat units.

4. DISCUSSION

Our current understanding of the cellular functions of DNA polymerase ζ largely derives from comparing the phenotypes of normal cells to those in which the *REV3* gene is disrupted or its expression is reduced. These approaches typically reduce both spontaneous and DNA damage-induced mutagenesis, indicating that DNA polymerase ζ contributes to mutagenic DNA synthesis in cells. Here we have taken a different and complementary approach by demonstrating that yeast cells expressing *REV3* derivatives with amino acid replacements in the pol ζ active site have mutator phenotypes. The results are informative when compared to studies of other Family B polymerases, and also regarding the role of pol ζ in mutagenic bypass of UV photoproducts and in spontaneous mutagenesis.

4.1. Inferred activity of pol ζ L979X mutants

The reduced survival of *rev3-L979V*, *rev3-L979N*, *rev3-L979G* and *rev3-L979K* mutant strains following UV irradiation (Fig. 1A) suggests that pol ζ harboring any of these four amino acid replacements is less active than wild-type pol ζ . The inferred reduction in polymerase activity resulting from these replacements is consistent with the observation that yeast strains containing homologous L612N, L612G and L612K replacements in pol δ [40] progress more slowly through S phase and have increased sensitivity to DNA damaging agents. Nonetheless, the *rev3-L979V*, *rev3-L979N*, *rev3-L979G* and *rev3-L979K* mutant strains apparently retain some pol ζ activity, because their survival (Fig. 1A) and mutant frequencies (Fig. 1B) following UV irradiation are greater than those of a *rev3 Δ* strain. An even higher level of pol ζ activity is apparently retained by the *rev3-L979F* and *rev3-L979M* strains, since their survival remains relatively normal following UV radiation (Fig. 1A), even when pol η -dependant bypass of UV-induced cyclobutane pyrimidine dimers is eliminated by deletion of *RAD30* (Fig. 1C). Retention of robust polymerase activity by pol ζ L979F and L979M mutants is consistent with previous studies showing nearly wild-type polymerase activity *in vitro* for pol α L868F and L868M mutants [32,33], a pol δ L612M mutant [34] and a pol ϵ M644F mutant [35].

4.2. Mutator effect of pol ζ L979X mutants compared to other Family B polymerases

All six amino acid replacements for Leu979 in yeast pol ζ result in increased UV-induced mutagenesis (Fig 1B) and the L979F and L979M replacements increase spontaneous mutagenesis (Table 2). Spontaneous mutator phenotypes have also been reported in yeast strains with similar replacements for the homologous amino acids in active site motif A of yeast pol α Leu868 [32,33], pol δ Leu612 [34,37,40]), and pol ϵ Met644 [35,36]. Those studies

further revealed reduced DNA synthesis fidelity by the mutant polymerases, leading us to infer that this is likely to be the case for the pol ζ mutants studied here. If so, the identity of the side chain at this location is a determinant of the fidelity of DNA synthesis *in vivo* by each of the four eukaryotic Family B DNA polymerases. In the crystal structure of prokaryotic Family B member RB69 pol [31], Leu415 at this position interacts with an adjacent, invariant tyrosine (Tyr416), which in turn interacts with the ribose of the incoming dNTP. Given this location, amino acid replacements for Leu979 in pol ζ , or for homologous residues in pols α , δ and ϵ , may alter the geometry of the nascent base pair binding pocket and/or the chemistry of the reaction. This in turn may reduce selectivity against incorrect dNTP insertion and/or increase the probability of extending mismatched termini. Indeed, both parameters are affected by replacements for Leu868 in yeast pol α [32,33], L614 in yeast pol δ [34] and Met644 in yeast pol ϵ [35,36]. Attempts are currently underway to express and purify yeast pol ζ with the L979F replacement, in order to examine its properties in comparison to the wild-type pol ζ .

4.3. Mutagenic bypass of UV photoproducts by pol ζ L979X mutants

The spontaneous mutator phenotypes of the pol α , δ and ϵ mutant strains reported earlier revealed the importance of this conserved residue in the fidelity of replication of undamaged DNA. The UV-induced mutator phenotype of the pol ζ L979X mutants (Fig. 1) extends this by indicating that Leu979 is a determinant of the fidelity of pol ζ dependent translesion DNA synthesis *in vivo*. The mutational specificity data imply that the majority of the lesions responsible for UV-induced mutagenesis are dipyrimidine photoproducts. In the wild-type and *rev3-L979F* strains, where cyclobutane pyrimidine dimers (CPDs) can be efficiently bypassed by pol η , the mutagenic lesions may primarily be 6-4 photoproducts. In the *rad30 Δ* and *rad30 Δ rev3-L979F* strains, which lack pol η , a greater contribution of CPDs to pol ζ -dependent mutagenesis seems likely. In all four strains, there is a bias for UV-induced base substitutions at the 3' base of dipyrimidines. Given the increased frequency of UV-induced base substitutions in *rev3-L979F* strains (Table 1, Fig. 2) compared to *REV3* strains, synthesis by L979F pol ζ is inferred to contribute to mutagenesis opposite the 3' base during photodimer bypass *in vivo*. Whether this reflects L979F pol ζ misinsertions opposite the damaged bases, extension of mismatched primers, or both, awaits biochemical analysis. However, this *in vivo* specificity is consistent with kinetic data showing that wild-type yeast pol ζ preferentially misinserts nucleotides opposite the 3' T as compared to the 5' T of a *cis-syn* cyclobutane TT dimer [28, 54]. This same bias is shared by human pol η [55] and may reflect differences in base-base hydrogen bonding potential for the 3' versus 5' base of a photodimer bound in the polymerase active site, as has been observed for *Sulfolobus sulfataricus* Dpo4 [56].

Also of note are the higher frequencies of UV-induced tandem double base substitutions in the *rev3-L979F* and *rad30 Δ rev3-L979F* mutant strains in comparison to their *REV3* counterparts (Fig. 2). None of these were a CC to TT change that could have resulted from "correct" insertion of two dAMPs opposite uracils created by deamination of cytosine in photodimers. Thus, the L979F replacement renders pol ζ more capable of contributing to mutagenesis that requires an initial misinsertion, a second misinsertion from a mismatched primer terminus, and extension of the resulting doubly mismatched terminus. This remarkable promiscuity extends to an even more diverse array of complex UV-induced mutations containing two or more substitutions, deletions and/or insertions (Table 1, Fig 2). These mutations, which are more frequent in the *rev3-L979F* strains (Table 1, Fig. 2), require formation and extension of aberrant primer-templates having multiple mismatches of different types. The results presented in Table 1 are consistent with the possibility that pol ζ may contribute to the UV-induced triplet mutation formation described in a recent report [57].

4.4. Contribution of pol ζ L979F/M mutants to spontaneous mutagenesis

The remarkable promiscuity of L979F pol ζ revealed in the UV-induced mutation spectra is also apparent during spontaneous mutagenesis, in the form of an increased rate of complex mutations (Table 2). These complex events could result from mutagenic bypass of endogenously arising lesions in DNA. Supporting this possibility is a previous study showing that wild-type pol ζ contributes to formation of spontaneous, complex mutations in a yeast strain defective in nucleotide excision repair [9]. That study provided evidence that the responsible lesion was a modified guanine resulting from spontaneous oxidative stress. This is particularly interesting in relation to our observation that the *rev3-L979F* allele does not contribute strongly to mutagenesis in *ogg1 Δ* strains (Fig. 3). This implies that, unlike the situation for UV photoproducts, L979F pol ζ may have little if any role in mutagenic bypass of spontaneously arising 8-oxo-guanine in DNA *in vivo*. This does not exclude a role for L979F pol ζ in bypass of other oxidative lesions, e.g., those that may be subject to nucleotide excision repair [9] and/or toxic oxidation products of 8-oxo-guanine [58]. Indeed, some secondary oxidation products of 8-oxo-guanine result in GC to C-G transversions [59], which is interesting because G-C to C-G transversions are elevated in *rev3-L979F* and *rev3-L979M* strains (Table 2).

An additional, nonexclusive possibility is that some of the spontaneous complex mutations and G-C to C-G substitutions seen in the *rev3-L979F* and *rev3-L979M* strains result from L979F/M pol ζ -dependent copying of undamaged DNA. This possibility is consistent with the error specificity of wild-type yeast pol ζ as it copies an undamaged DNA template *in vitro* [30]. Compared to the major replicative DNA polymerases, wild type pol ζ has lower fidelity, yet it can perform processive DNA synthesis [60]. Interestingly, wild type pol ζ generates *lacZ* mutants *in vitro* [30] and L979F pol ζ generates *can1* mutants *in vivo* that contain multiple sequence changes separated by several correct base pairs (Table 2; including a mutant with three substitutions in a stretch of 18 base pairs). This combination of inaccurate but processive synthesis is shared only by DNA polymerase κ [61], making these two TLS polymerases prime candidates for generating mutational “showers” in genomes [62]. Such showers of multiple, dispersed sequence changes within a single mutant clone have important implications for the evolutionary fitness of organisms and for the origins of diseases [63].

4.5. Utility of pol ζ mutator alleles

As mentioned in the Introduction, inactivation of the *REV3* gene in mice is lethal [41-43,64]. Thus, the present search for mutator alleles of yeast pol ζ that retain robust pol ζ catalytic activity was partly motivated by the desire to study the function of mammalian pol ζ using homologous mouse *rev3* missense mutants that are predicted to be viable. The genetic results presented here suggest that mutations equivalent to yeast *rev3-L979F* and *rev3-L979M* may be ideal, in that polymerases with these changes appear to retain high polymerase activity but they appear to have lower fidelity. If the equivalent mouse pol ζ mutants are viable and confer mutator effects, then given the multiple functions of pol ζ mentioned in the Introduction, it will be interesting to determine if mice harboring these *rev3* alleles have altered sensitivity to environmental stress, altered rates of spontaneous and/or DNA damage induced mutagenesis, altered susceptibility to environmental diseases (e.g., cancer), or altered somatic hypermutation of immunoglobulin genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. UV-induced killing and mutagenesis in yeast strains harboring *rev3-L979X* alleles
 A. Survival following UV irradiation. B. UV-induced mutagenesis at the *CAN1* locus. C. Survival following UV irradiation in *rad30Δ* strains. D. UV-induced mutagenesis at the *CAN1* locus in *rad30Δ* strains. Error bars represent one standard deviation.

Figure 2. Relative frequencies of UV-induced mutagenesis for various classes of mutations White, hatched, grey and solid bars correspond to wild-type, *rev3-L979F*, *rad30 Δ* and *rev3-L979F rad30 Δ* strains, respectively. Mutation frequencies (and one standard deviation) for each class were calculated by multiplying the proportion of the specific type of event in the corresponding mutation spectrum (Table 1) by the total mutation frequency. These data were collected after a dose of 20 J/m². As the data from the *rad30 Δ* mutant were more variable than those from the other strains, the error bars for the *rad30 Δ* mutant are comparatively large.

A. *can1* **B. *ura3-29***

Figure 3. Spontaneous mutation rates in *ogg1*, *rad30* and *rev3* single, double and triple mutant strains

Mutation rates were measured for (A) the *can1* forward and (b) *ura3-29* reversion reporters. Mutation rates shown were normalized relative to the values observed in the wild-type strain, which were 1.72×10^{-7} (95% confidence limits: $1.10 - 2.19 \times 10^{-7}$) for *can1* and 6.57×10^{-9} (95% confidence limits: $4.34 - 8.04 \times 10^{-9}$) for *ura3-29*. Error bars represent 95% confidence limits.

Table 1

UV-induced mutations in wild-type, *rad30* and *rev3* yeast strains.

Freq. ($\times 10^{-6}$)	wild-type		<i>rev3-L979F</i>		<i>rad30Δ</i>		<i>rad30Δ rev3-L979F</i>	
	88 [59 - 120]		200 [50 - 350]		200 [170 - 230]		540 [470 - 590]	
	Events	Freq.	Events	Freq.	Events	Freq.	Events	Freq.
A-T to G-C	4	14	0	0	2	16	0	0
G-C to A-T	8	28	6	48	12	92 ^c	10	220 ^c
A-T to T-A	6	21	2	16	4	32	2	43
A-T to C-G	0	0	0	0	1	8	0	0
G-C to T-A	1	4	2	16	2	16	4	86 ^c
Tandem bps	1	4	3	24	0	0	4	86 ^c
Indels ^a	3	11	4	32	0	0	2	43
Complex ^b	2	7	8	64 ^c	5	48	3	65

Cells were exposed to 20 J/m² of UVC.

Freq. represents mutation frequency. All mutation frequencies are ($\times 10^{-6}$). Numbers in brackets represent one standard deviation.

^aOne and two-base insertions and deletions were categorized as indels.

^bMultiple, closely spaced base substitutions and/or indels were categorized as complex mutations. One of the *can1* isolates from the *rad30Δ* strain contained three single base changes within 30 base-pairs.

^cSignificant difference ($p < 0.05$) from the *REV3* strain. *P*-values were determined as described in the materials and methods.

Table 2

Spontaneous mutations in wild-type and *rev3* yeast strains.

Rate ($\times 10^{-8}$)	wild-type		<i>rev3-L979F</i>		<i>rev3-L979M</i>		<i>rev3Δ</i>	
	Events	Rate	Events	Rate	Events	Rate	Events	Rate
A-T to G-C	1	1	1	2	1	2	2	2
G-C to A-T	6	6	8	13	5	10	10	8
A-T to T-A	3	3	0	≤ 2	2	4	1	1
A-T to C-G	2	2	0	≤ 2	1	2	1	1
G-C to T-A	5	5	6	10	2	4	1	1
G-C to C-G	1	1	5	8	3	6	3	2
Tandem bps	1	1	0	≤ 2	1	2	0	≤ 1
Indels ^a	5	5	0	≤ 2	8	15	5	4
Duplications	1	1	1	2	0	≤ 2	2	2
Complex ^b	0	≤ 1	4	7	1	2	0	≤ 1

All rates are ($\times 10^{-8}$). Numbers in brackets are 95% confidence intervals.

^aOne to twelve-base insertions and deletions were categorized as indels.

^bMultiple, closely spaced base substitutions and/or indels were categorized as complex mutations.