

## Roles of Rev1, Pol $\zeta$ , Pol32 and Pol $\eta$ in the bypass of chromosomal abasic sites in *Saccharomyces cerevisiae*

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**Translesion synthesis (TLS) on DNA is a process by which potentially cytotoxic replication-blocking lesions are bypassed, but at the risk of increased mutagenesis. The exact *in vivo* role of the individual TLS enzymes in *Saccharomyces cerevisiae* has been difficult to determine from previous studies due to differing results from the variety of systems used. We have generated a series of *S.cerevisiae* strains in which each of the TLS-related genes *REV1*, *REV3*, *REV7*, *RAD30* and *POL32* was deleted, and in which chromosomal apyrimidinic sites were generated during normal cell growth by the activity of altered forms of human uracil-DNA glycosylase that remove undamaged cytosines or thymines. Deletion of *REV1*, *REV3* or *REV7* resulted in slower growth dependent on (*rev3* $\Delta$  and *rev7* $\Delta$ ) or enhanced by (*rev1* $\Delta$ ) expression of the mutator glycosylases and a nearly complete abolition of glycosylase-induced mutagenesis. Deletion of *POL32* resulted in cell death when the mutator glycosylases were expressed and, in their absence, diminished spontaneous mutagenesis. *RAD30* appeared to be unnecessary for mutagenesis in response to abasic sites, as deleting this gene caused no significant change in either the mutation rates or the mutational spectra due to glycosylase expression.**

### Introduction

DNA is at risk of damage from sources that are both endogenous and exogenous to the cell. In order to protect the genome, organisms have evolved a number of repair processes that effectively remove DNA lesions and maintain the integrity of genetic information. Unrepaired DNA lesions can block the progress of high-fidelity replicative polymerases (1, 2), requiring the activation of pathways to bypass the damage in a process known as translesion synthesis (TLS). TLS makes use of lower fidelity polymerases, and while these processes allow replication to continue, it can be at the cost of increased mutagenesis [see (3) for review]. Of the wide variety of DNA lesions, abasic (apurinic/apyrimidinic or AP) sites have perhaps the most mutagenic potential, due to their frequency of formation (4, 5) and their inherently non-coding nature (6).

In the budding yeast *Saccharomyces cerevisiae*, the enzymes that seem to have a role in TLS are Rev1, Pol  $\zeta$ , Pol  $\eta$  and Pol  $\delta$  (through the Pol32 subunit). *REV1* was originally identified during a screen for genes required for damage-induced mutagenesis (7). Rev1 protein possesses a deoxycytidyl

transferase activity (8) that specifically inserts dCMP residues opposite an AP lesion or (less efficiently) undamaged guanine residues in the template DNA (9). Rev1 is also necessary for the bypass of some types of ultraviolet (UV) photoadducts *in vivo* (10), and the protein is an important structural component for TLS in general (11, 12). Neither of these latter functions requires the Rev1 dCMP transferase function.

Pol  $\zeta$  is a heterodimer encoded by the genes *REV3* (7) and *REV7* (13), and the enzyme is necessary for multiple types of lesion bypass. Pol  $\zeta$  replicates past thymine cyclobutane dimers in an error-prone fashion (14), but bypasses thymine glycol lesions error-free (with the insertion of dAMP) (15). More importantly for general TLS, and specifically for the bypass of AP sites, Pol  $\zeta$  is able to extend the terminal mismatches that result from nucleotide incorporation opposite an AP site or a damaged nucleotide (16, 17). Pol  $\zeta$  is weakly processive, with most molecules dissociating from the template after inserting only a few nucleotides (14). Rev1 directly interacts with both Rev7 (18, 19) and Rev3 (20), and the presence of proliferating cell nuclear antigen protein (PCNA) stimulates TLS by Pol  $\zeta$ , but as Pol  $\zeta$  lacks a known PCNA-binding domain, it is unclear how this interaction occurs (21).

Pol  $\eta$  [*RAD30* (22)] is important for the error-free bypass of cis-syn thymine cyclobutane dimers in both yeast (23) and humans (24) (where mutations in the gene are responsible for the xeroderma pigmentosum variant syndrome). The *S.cerevisiae* Pol  $\eta$  is also capable of error-free bypass of 8-oxoG (25) and *O*<sup>6</sup>-meG (26). Bypass of (6-4) pyrimidine dimers by Pol  $\eta$  is error-prone and requires Pol  $\zeta$  for extension (27). The role of Pol  $\eta$  in the bypass of AP sites is less clear, with *in vitro* data showing incorporation of dGMP and dAMP opposite an AP site (28), but with recent *in vivo* data suggesting that AP site bypass may not be the major function of Pol  $\eta$  (29, 30).

The Pol32 subunit of Pol  $\delta$  has a more complicated and poorly understood role in the cell. Pol32 is non-essential for growth, but deletion mutants are sensitive to cold temperatures and to DNA-damaging agents (31). This subunit also interacts with Pol  $\alpha$  and stimulates progression of the cell cycle from G2 to M phase (32). DNA synthesis by Pol  $\delta$  lacking Pol32 is inefficient and results in frequent pausing (33). Lesion bypass studies have shown that not only is Pol32 essential for mutagenesis but it may also modulate dAMP insertion opposite an AP site (12) and prevent deletions at lesion sites (34). Pol32-mediated mutagenesis is dependent upon the PCNA-binding domain in this protein (35). Further, Pol32 interacts with Rev3, and deletion of *POL32* results in increased homologous recombination and decreased genomic stability (36).

The present study examines the contribution of each TLS gene in the tolerance of and mutagenesis by AP sites generated in yeast chromosomal DNA. Deletions were made for each

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TLS gene in an *apn1-Δ1* background to preserve AP sites through replication by preventing their processing by base excision repair, and the AP sites were generated by expressing modified versions of human uracil-DNA glycosylase that slowly remove either normal cytosines (CDG enzyme) or normal thymines (TDG enzyme) from yeast nuclear DNA (37). Changes in the mutation rate and the spectrum of base substitutions for each TLS deletion strain were compared to those for TLS-competent yeast to determine the *in vivo* contribution of each enzyme to mutagenesis by AP sites.

## Materials and methods

### Plasmids and strains

The expression cassettes for the CDG and TDG glycosylases equipped with the conjugated SV40 nuclear localization sequence were excised from the original pYES2-based vectors (38) by digestion with KpnI. Plasmid pYES3 (containing the *TRP1* selectable marker) was purchased from Invitrogen (Carlsbad, CA, USA), digested with KpnI and dephosphorylated with Antarctic Phosphatase (New England Biolabs, Ipswich, MA, USA). The glycosylase fragments were ligated to the linearized pYES3 (Quick Ligation Kit; New England Biolabs) to produce pYES3-CDG (expressing the CDG glycosylase) and pYES3-TDG (expressing the TDG glycosylase). Correct orientation of the inserted genes was confirmed by BamHI digestion and sequencing. Glycosylase expression was confirmed by the *GAL1* promoter, as previously described (38). The unmodified pYES3 vector was used as a control in all mutation rate experiments in this study.

The *S. cerevisiae* strains used in this study are listed in Table I. All strains were derived from LB20a, the construction of which was previously described (38). The construction of strains PAY01a and PAY07a was also described previously (38). The other TLS knockout strains were constructed in analogous fashion, using the protocols of the *Saccharomyces* Genome Deletion Project ([http://sequence-www.stanford.edu/group/yeast\\_deletion\\_project/](http://sequence-www.stanford.edu/group/yeast_deletion_project/)). Briefly, BY4741 yeast strains with the appropriate locus replaced with the G418 (geneticin) resistance cassette *kanMX4* were purchased (American Type Culture Collection, Manassas, VA, USA), and the cassettes with flanking genomic sequences were amplified by polymerase chain reaction (PCR; see Primers and oligonucleotides). The resulting PCR fragments were then used to transform strain LB20a. Putative transformants were screened by G418 resistance and increased sensitivity to killing by 254-nm UV light (7) and gene replacement was confirmed by PCR. The resulting *rev3::kanMX4* strain was designated as PAY03a, the *rad30::kanMX4* strain as PAY030a and the *pol32::kanMX4* strain as PAY032a.

### Determination of mutation rates and sequencing of *ura3* mutants

Mutation rates were determined using the method of the median (39). Putative *ura3* mutants were isolated and analyzed as previously described (38). Briefly, after induction of the mutator glycosylases (see Determination of growth rates, below), putative *ura3* mutants were selected on medium containing glucose and 5-fluoro-orotic acid (5-FOA, CAS registry number 703-95-7; Research Products International Corp., Mt Prospect, IL, USA) (40). 5-FOA-resistant (5-FOA<sup>R</sup>) colonies were then re-streaked onto plates containing complete minimal medium lacking uracil (CM-ura) to confirm the expected uracil auxotrophy. 5-FOA<sup>R</sup> colonies unable to grow on CM-ura plates were inoculated into 1 ml CM-trp plus glucose. After the cultures reached saturating density (~2 days), genomic DNA was extracted from each culture (Epicentre, Madison, WI, USA), and the *ADP1::URA3* locus was amplified by PCR. The PCR conditions were an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 0.5 min, 56°C for 0.5 min and 72°C for 2.5 min and a final extension step at 72°C for 7 min. PCR fragments were then purified (QIAGEN Sciences, Valencia, CA, USA), sequenced and the results catalogued. DNA

sequencing was performed at the Dana Farber/Harvard Cancer Center High-Throughput DNA Sequencing Facility.

### Determination of growth rates

Starter cultures were grown in CM-trp plus raffinose to saturation (~2 days) at 30°C for yeast strains carrying pYES3 (control), pYES3-CDG or pYES3-TDG (Table I). Cell growth was monitored by measuring the optical density of the cultures at 600 nm (OD<sub>600</sub>) using a spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Cells were transferred to CM-trp medium containing galactose at a starting OD<sub>600</sub> of ~0.1 and incubated with shaking at 30°C. Absorbance readings (performed in triplicate) were taken at 2-hour intervals starting at 12 hours of incubation through 24 hours of incubation. For cell density determinations at OD<sub>600</sub> ≥ 1, 10-fold dilutions in growth medium were made and the optical density of the diluted sample was measured. Final absorbance readings were taken after 48 hours of incubation for each strain. Growth rates, expressed as the time required for cultures to double in optical density (doubling time,  $T_d$ ), were calculated by comparing the optical density at multiple time points during the linear growth phase and using the following formula:  $T_d = (t_2 - t_1)/[\log_2(OD_2/OD_1)]$ .

### Cell viability assays

Cell viability was determined by exclusion of the vital dye trypan blue, based on previously published protocols (41). Starter cultures of LB20a and PAY01a carrying either pYES3 or pYES3-TDG were grown overnight in CM-trp with glucose at 30°C. The OD<sub>600</sub> of each saturated culture was determined and fresh cultures in CM-trp with glucose were seeded at densities that would result in an OD<sub>600</sub> ~0.6 after 16 hours of growth (based on the  $T_d$  as calculated above). Cultures were centrifuged at 1000 g for 5 min at room temperature, washed three times with sterile H<sub>2</sub>O, re-suspended in CM-trp with galactose and incubated with aeration at 30°C. Samples of each culture were taken at the time of initial galactose induction (0 hour) and at 4, 8 and 24 hours of induction. Samples were mixed with equal volumes of 0.4% trypan blue in phosphate-buffered saline (GIBCO; Invitrogen) and incubated at room temperature for 30 min. The numbers of dead (blue) and live (no colour) cells were determined by counting with a haemocytometer.

### Primers and oligonucleotides

All primers are listed 5' to 3'. The primers used to amplify each TLS G418-resistance knockout cassette were as follows: *REV1* knockout—forward, CTGCGTGTACTGTGCTGAAATGTTTTTTTTTTAATTCA and reverse, TCTCAAAAATAAATCGATACTGCAATTCATTCTAGGCATATCCAGCCATG; *REV3* knockout—forward, AAATAACTACTCATCATTTTGGGAGACATA-TCTGTGTCTAGATTA and reverse, CAATACAAAACACTACAAGTTGTGGCGAAATAAAAATGTTGGAAATG; *REV7* knockout—forward, ACATTTAATTTTAAATTCATTCTTCAAATTTTCATTTTTGCACITTA and reverse, ATCCAAGAAGAAAAAATAGTAATCGTTGCGTCAGCTTTATG; *RAD30* knockout—forward, GTTTTAGTTGCTGAAGCCATATAATTGTC-TATTTGGAATAG and reverse, GCCTGCTCATTTTTGAACGGCTTTGATAAACAAAGACAAAG; *POL32* knockout—forward, CATCAACAATTAG-TAATGGAAAGTGTGGAAAAAAGAAG and reverse, TAACAAC-CAGAAATAGGCTTATGTTAACTCAATCGGTAATT. The primers internal to the *kanMX4* gene used for PCR confirmation of gene replacement were forward, TGATTTTGATGACGAGCGTAAT and reverse, CTGCAGCGAG-GAGCCGTAAT. The primers external to each knockout cassette used in conjunction with the *kanMX4* internal primers for PCR confirmation of gene replacement were as follows: *REV1* knockout—forward, ATATTACAGG-TAATGTTTCGC and reverse, AGAAGTAACGAGTTGACAGA; *REV3* knockout—forward, GATACCGTCTATCTCTTCGT and reverse, GCCATCTCTG-TAATAGAAG; *REV7* knockout—forward, TTAGTGGTTTCACGATACCC and reverse, TATACGTTTTTGGCAGCACT; *RAD30* knockout—forward, CAACAAAACCTGGCGCCCGTGAAT and reverse, AAGTGTGCTATTG-TCCCGTTCAGG; *POL32* knockout—forward, GCTGCGCTATTGCT-TTTGCTGATTGAA and reverse, CGCAGAAGTTCGTTACATCGCAAT-CAGA. The primers used to amplify the *ADP1::URA3* locus were forward, GAGAGTGGGCTCATCTTG and reverse, AGTAGCACCTCGCCTTCC. The primers used to sequence *URA3* were forward, CGCATATGTAGTGTGAAG and reverse, TTCCCGGGTAATAACTGATA. The sequencing primers used to confirm the orientation of the glycosylase genes in pYES3-CDG and pYES3-TDG were forward, GGGAAAGAAAGCGAAAGGAGCGG and reverse, GGAGGGCGTGAATGTAAGCGTGA.

### Statistical analyses

Differences in mutation rates were analyzed for significance using a two-tailed *t*-test, and differences in the frequencies of base substitutions were analyzed for

**Table I.** The *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Reference
LB20a	<i>MATa, his3-Δ, leu2, ura3-52, ADP1::URA3, trp1-Δ1::hisG, apn1-Δ1::HIS3, can1, GAL<sup>+</sup></i>	(38)
PAY01a	LB20a, <i>rev1::kanMX4</i>	(38)
PAY03a	LB20a, <i>rev3::kanMX4</i>	This study
PAY07a	LB20a, <i>rev7::kanMX4</i>	(38)
PAY030a	LB20a, <i>rad30::kanMX4</i>	This study
PAY032a	LB20a, <i>pol32::kanMX4</i>	This study

significance by a comparison of proportions or chi-square analysis. In each case, a 99% confidence interval was used and a  $P$ -value  $<0.01$  indicated a significant difference and a rejection of the null hypothesis (that there were no differences in the mutation rates or frequency of base substitutions).

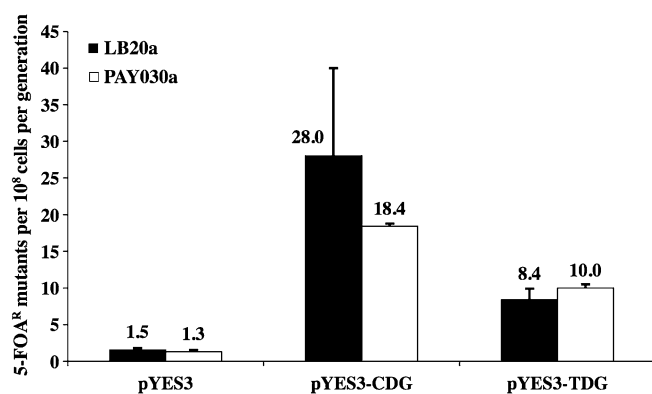
## Results

### Mutagenesis by mutant uracil-DNA glycosylases on plasmid pYES3

We have previously reported a large increase in the mutation rates for *S.cerevisiae* *apn1-Δ1* strains expressing the CDG and TDG glycosylases, compared to the strains containing the empty vector control (38). A significant percentage of the 5-FOA<sup>R</sup> mutants isolated in our experiments were due to gene conversion involving a *ura3*-deletion allele in the glycosylase expression vectors (38). Therefore, we re-cloned the glycosylase expression genes into a new vector lacking this sequence, pYES3 (see Materials and methods). Mutation rates in strain LB20a (*apn1-Δ1*) were calculated for cells transformed with pYES3 (control), pYES3-CDG or pYES3-TDG (Figure 1; black bars). The spontaneous mutation rate was low, whereas the mutation rate with expression of each of the glycosylases was greatly increased. Expression of CDG increased the mutation rate nearly 19-fold over the control vector, whereas expression of TDG increased the mutation rate nearly 6-fold. The lower mutation rates observed here compared to our previous report (38) are consistent with the elimination of the frequent gene conversions found with glycosylase vectors containing the *URA3* fragment. The modestly higher mutation rate with pYES3-CDG compared to pYES3-TDG is consistent with our published observations using the pYES2-based vectors (38). Thus, the general mutator effects are due to the glycosylases themselves, rather than some specific aspect of the expression vector used.

### Changes in cell proliferation rates in TLS knockout strains

Because the TLS proteins may be required for replication bypass of AP sites, as a measure of the toxicity of unrepaired abasic DNA damage, we examined changes in the growth rates (assessed as the doubling time,  $T_d$ ) of the TLS knockout strains compared to LB20a. Expression of the mutator glycosylases in strain LB20a did not increase the average doubling time



**Fig. 1.** Overall mutation rates in yeast expressing the pYES3-CDG and pYES3-TDG glycosylase vectors, compared to pYES3 (control) for strains LB20a (REV<sup>+</sup>; black bars) and PAY030a (*rad30*; white bars). Mutation rates to *ura3* were estimated by selecting for 5-FOA<sup>R</sup> and checking for Ura auxotrophy as described in the text. The values shown are an average of three independent determinations, with standard deviations indicated by the error bars.

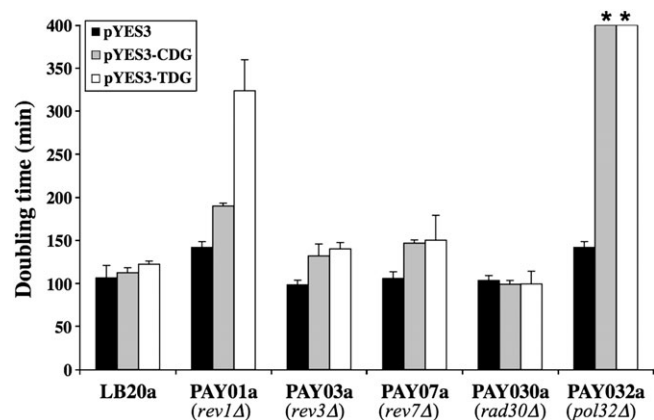
significantly (Figure 2). In strain PAY01a (*rev1Δ*) with the empty vector pYES3,  $T_d$  was elevated  $\sim 34\%$  compared to LB20a (142 versus 106 min), while expression of the mutator glycosylases further increased  $T_d$  to 190 min for CDG and 324 min for TDG (Figure 2). Thus, Rev1 helps maintain the normal cell growth rate under our conditions, and this role becomes very pronounced under a burden of unrepaired AP sites.

Eliminating either subunit of Pol  $\zeta$  (a *rev3* deletion in strain PAY03a or a *rev7* deletion in strain PAY07a) did not significantly affect the growth rate in the absence of mutator glycosylase expression. However, the observed  $T_d$  for PAY03a and PAY07a under expression of the mutator glycosylases was increased 30–40% compared to the strains with pYES3 alone (Figure 2). These results indicate that Pol  $\zeta$  also contributes to the maintenance of normal cell growth when the burden of AP sites is increased.

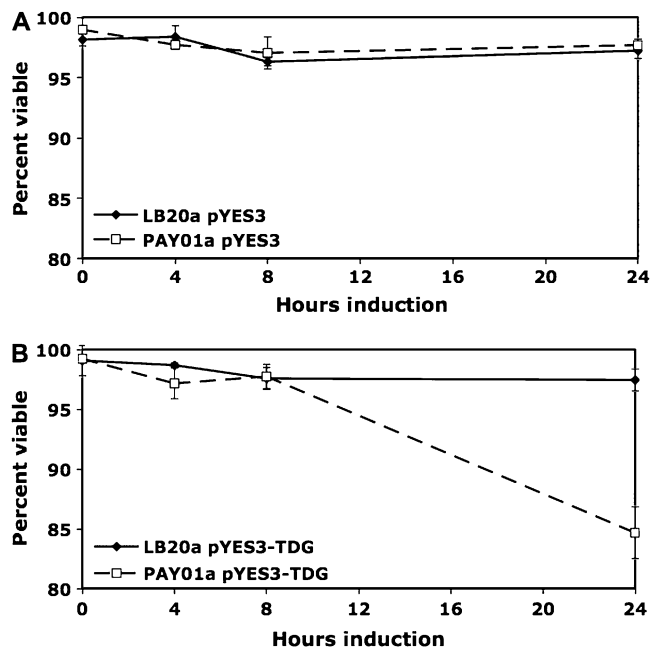
Deleting *RAD30* (Pol  $\eta$ ) did not affect  $T_d$  compared to LB20a, with or without the mutator glycosylases. However, strain PAY032a (*pol32Δ*) showed very striking effects. For PAY032a with pYES3,  $T_d$  was already increased  $\sim 34\%$  compared to LB20a, and expression of either CDG or TDG blocked measurable cell growth completely (Figure 2). Although these measurements do not distinguish between cytostatic and cytotoxic effects, it is clear that Pol32 is required for replication when AP sites are induced *in vivo*.

### Decreased cell viability due to expression of a mutator glycosylase

To determine whether the increased  $T_d$  of TLS knockout strains were the result of cytostasis or cell death, we examined changes in cell viability by a vital dye exclusion assay. For this study, we compared the *rev1Δ* strain PAY01a/pYES3-TDG to LB20a/pYES3-TDG because of the large difference in  $T_d$  but still compatible with cell proliferation (Figure 2). The complete cessation of proliferation in the *pol32Δ* strain PAY032a upon expression of mutator glycosylases (Figure 2) prevented its use for this experiment. Without glycosylase induction (controls using the pYES3 vector), viability did not decrease over time



**Fig. 2.** Effects of CDG and TDG on the growth rates of LB20a and TLS-deficient yeast strains. Cells were transferred to galactose-containing medium and the density measured at various points thereafter. The doubling times were calculated from data obtained over 48 hours as described in Materials and methods. The asterisk indicates that no growth was detected during the course of the incubation (maximum 48 hours). The values shown are an average of three independent determinations, with standard deviations indicated by the error bars.



**Fig. 3.** Cell viability of LB20a (*REV1*) and PAY01a (*rev1*) strains without expression of TDG (A) and with expression of TDG (B). Cells were transferred to medium containing galactose to induce expression from pYES3-TDG and sampled at various times after for viability as determined by the exclusion of the vital dye trypan blue, as described in Materials and methods. The values shown are the means of three independent determinations, with standard deviations indicated by the error bars.

when cells were grown in galactose-containing medium (Figure 3A).

With expression of TDG, neither strain showed a significant decrease in cell viability at early time points (0, 4, and 8 hours induction; Figure 3B). At 24 hours of glycosylase induction, however, PAY01a showed a significant decrease in viability compared to LB20a: ~98% for LB20a versus ~85% for PAY01a (Figure 3B) (*t*-test,  $P = 0.0007$ ). This result indicates that the increase in  $T_d$  for PAY01a observed during mutator glycosylases expression results initially from a prolonged cell cycle due to the decreased efficiency of bypassing AP sites in the absence of Rev1, a deficiency that eventually results in cytotoxicity.

#### Requirement of *REV1*, *REV3* and *REV7* for mutagenesis

To determine the mutational capacity of strains lacking the main enzymes involved in TLS in yeast, Rev1 and Pol  $\zeta$  (Rev3/Rev7), we analyzed spontaneous and AP-induced mutagenesis in strains deficient for these activities. All three strains showed a severe defect in mutagenesis compared to strain LB20a. Only a few 5-FOA<sup>R</sup> mutants were isolated per experiment using the TLS-deficient strains, even with the expression of the mutator glycosylases, which precluded accurate determinations of the mutation rates in these backgrounds. Attempts to overcome this limitation by seeding the cultures with a larger number of cells, growing more cultures, growing the cultures for longer times or plating larger culture volumes did not solve this problem (data not shown). Similarly, low mutation rates also characterized the *rev1* $\Delta$  and *rev7* $\Delta$  strains expressing glycosylases from the pYES2-based vectors, as reported previously (38). Consistent with the known biochemistry of Pol  $\zeta$ , both the Rev3 and the Rev7 subunit are thus required for mutagenesis at AP sites.

The small number of *ura3* mutations isolated in PAY01a (*rev1* $\Delta$ ) were subjected to DNA sequence analysis. Of the three CDG-induced mutants isolated in PAY01a, all were GC>TA transversions; of the 16 TDG-induced mutants isolated, five were GC>TA transversions, five were AT>TA transversions, five were GC>AT transitions and one was an AT>GC transition. In the case of *REV1*<sup>+</sup> strains, the specificity of TDG produced a strong bias toward mutations at AT base pairs (38), in contrast to the current observations with *rev1* $\Delta$ . There may be a low level of glycosylase-induced mutagenesis in a Rev1-independent manner, but the few available sequenced *ura3* mutants made it difficult to draw any clear conclusions about the mutational specificity of AP sites in the absence of Rev1.

#### Requirement of *POL32* for AP site tolerance and mutagenesis

The exact mutational contribution of the Pol  $\delta$  subunit Pol32 is unclear, and we were unable to isolate any 5-FOA<sup>R</sup> mutants in the *pol32* $\Delta$  yeast strain (PAY032a) carrying pYES3 or the mutant glycosylase vectors. As noted earlier, this strain exhibited a heightened sensitivity to the expression of the mutator glycosylases (Figure 2). Expression of CDG or TDG in the PAY032a background was at least cytostatic, with few isolates able to grow significantly, if at all, in galactose cultures (Figure 2). The few isolates that did grow yielded no 5-FOA<sup>R</sup> mutants. PAY032a carrying the control pYES3 vector was able to grow in galactose, but generated no spontaneous 5-FOA<sup>R</sup> mutants. This suggests that the growth defect for PAY032a expressing CDG and TDG is specifically due to the activity of the glycosylases and not from any intrinsic inability to grow in galactose. These data also indicate that Pol32 is required for both spontaneous and AP site-induced mutagenesis, as well as survival when these lesions accumulate.

#### Role of *RAD30* in AP site-induced mutagenesis

To determine if Pol  $\eta$  (the *RAD30* gene product) has a significant role in mutagenesis caused by AP sites, we constructed a *rad30* $\Delta$  yeast strain (PAY030a). Pol  $\eta$  deficiency showed no marked effect on the overall mutation rates compared to the control strain LB20a (Figure 1; black bars). Even for CDG expression, the difference between the *rad30* $\Delta$  strain and LB20a (Figure 2) was not statistically significant (*t*-test,  $P = 0.449$ ).

With no significant change in the overall basal mutation rates for *rad30* $\Delta$  mutants compared to *RAD30* cells, we analyzed the spectrum of single-base-pair substitutions in PAY030a to gain additional information on the possible role of Pol  $\eta$  in mutagenesis by AP sites. As shown in Table II, the CDG-induced mutants isolated in both the *RAD30* and the *rad30* $\Delta$  backgrounds were predominantly GC>CG transversions (52 and 64%, respectively, for LB20a and PAY030a), with the next most abundant types as GC>AT transitions (24 and 18.5%) and GC>TA transversions (19 and 14%). Similarly, TDG-induced mutants in both backgrounds were predominantly AT>CG transversions (73% for LB20a and 83% for PAY030a), followed by AT>TA transversions (15% for LB20a and 7% for PAY030a) (Table II). As there was no significant difference between these spectra (chi-square,  $P > 0.01$ ), it is likely that Pol  $\eta$  does not significantly contribute to the mutagenic bypass of AP sites *in vivo*. Likewise, the lack of effect of CDG or TDG on the growth rate of PAY030a (Figure 2) indicates that Rad30 does not

**Table II.** Mutation spectrum for base substitutions in cells expressing the CDG or TDG glycosylases

	Transitions			Transversions				
	Total	AT>GC	GC>AT	Total	AT>TA	AT>CG	GC>TA	GC>CG
LB20a CDG <sup>a</sup>	68	2 (0.7%)	66 (24%)	212	4 (1.3%)	9 (3%)	53 (19%)	146 (52%)
PAY030a CDG	<i>15</i>	0	15 (18.5%)	<i>66</i>	2 (2.5%)	1 (1%)	11 (14%)	52 (64%)
LB20a TDG <sup>a</sup>	8	6 (2%)	2 (1%)	244	37 (15%)	185 (73%)	18 (7%)	4 (2%)
PAY030a TDG	5	1 (1%)	4 (5%)	77	6 (7%)	68 (83%)	3 (4%)	0

PAY030a is a *rad30A* derivative of LB20a. The percentages given for each type of base substitution were calculated from the number of that mutation relative to the total number of base substitutions isolated for that strain. The numbers in italics indicate the total transitions or transversions scored for that strain.

<sup>a</sup>Combined data from this study and a previous report (38).

contribute significantly to tolerance or non-mutagenic bypass of AP sites.

## Discussion

Using a system that generates AP sites endogenously in *S.cerevisiae* chromosomal DNA and in a controlled fashion (37), we have studied the individual contributions of TLS enzymes in the tolerance of AP sites and the mutations they provoke. Studies of TLS enzyme roles by using transforming plasmids containing site-specific AP sites (29, 30, 42) have provided informative data, but it is uncertain to what extent AP site repair, DNA replication and mutagenesis are affected by the transformation process compared to chromosomal DNA. Furthermore, studies that have relied on the use of tetrahydrofuran as an AP site analogue (42) should not be considered representative of real AP sites, given the observed differences in the mutagenic specificity of each type of lesion (43).

Our results demonstrate the relative importance of individual TLS enzymes in both the AP site mutagenesis and the maintenance of cell proliferation in the face of excess AP sites. That both Rev1 and Pol  $\zeta$  were required for detectable damage-induced mutagenesis was consistent with both our previous results (38) and numerous other studies in yeast (7, 13, 30, 44). However, the essentially absolute Rev1/3/7 dependence of AP mutagenesis in our experiments contrasts with the partial dependence reported in at least one study using a DNA transformation approach (29). The general requirement for Rev1 and Pol  $\zeta$  for AP site mutagenesis in different systems highlights the central role of these gene products in facilitating bypass of abasic lesions. Interestingly, while CDG or TDG expression resulted in similar decreases in growth rates in the Pol  $\zeta$ -deficient strains, in the Rev1-deficient strain, expression of TDG had a stronger influence on the doubling time than did CDG (Figure 2). This result could indicate that Rev1 is more important for facilitating the bypass of thymine-derived AP sites or that AP sites opposite adenines are more prone to be processed in a manner that relies heavily on Rev1 for replication. Such a difference between the bypass of thymine-derived AP sites and cytosine-derived AP sites could provide some insight into our previously reported strand bias observed for TDG-induced mutants (38).

Both subunits of Pol  $\zeta$  (Rev3 and Rev7) were required in our studies for AP site mutagenesis, with the loss of either protein resulting in a similar strong defect in mutagenesis. In previous studies, where the role of Pol  $\zeta$  has been examined, either only the *REV3* gene encoding the catalytic subunit was mutated (29, 30) or when both the *REV3* and the *REV7* genes were deleted, chemically induced adducts were used for mutagenesis (44). Our results demonstrate that the *REV3* and *REV7* genes are

both required for mutagenesis provoked by endogenously generated chromosomal AP sites. In this context, it is noteworthy that the *rev1Δ*, *rev3Δ* and *rev7Δ* strains used here were able to grow in spite of the extra burden of AP sites, albeit more slowly than the *REV*<sup>+</sup> control (Figure 2) and with decreased viability (Figure 3B).

That the *RAD30* gene (encoding Pol  $\eta$ ) did not appear to play a significant role in the *in vivo* mutagenic bypass of AP sites may not be surprising. Pol  $\eta$  is best characterized for its requirement in the bypass of UV damage independent of Rev1 and Pol  $\zeta$  (22, 45). *In vitro* data suggest that Pol  $\eta$  does not bypass AP sites efficiently (28), even when stimulated by replication factors (46). Our finding that Pol  $\eta$  is not a major player in the mutagenic bypass of AP sites is in agreement with some reports (43), but at odds with others (29), possibly due to the differences in the mutation targets used (transformation with double-stranded plasmid versus a duplex oligonucleotide). One could also question whether even moderate differences in the spectra of base substitutions between *RAD30* and *rad30Δ* previously reported (30) are truly significant. While the statistical test used by these researchers (30) was not discussed, the most likely choice would have been a chi-square test as we used; a *P*-value of 0.02 was given, significant at the 95% confidence level. If the more stringent 99% confidence interval were applied to the published data, *P* = 0.02 would not be significant (see Materials and methods). While the statistical analysis of our data indicated that Pol  $\eta$  is not involved in TLS of chromosomal AP sites, larger data sets and a more refined statistical analyses of published data may be required to identify more subtle differences.

The effects of a *pol32* mutation in the current study were striking, and they also differed from previous reports. Strains deleted for *POL32* are hypersensitive to UV and to DNA-alkylating agents compared to wild-type yeast (12, 47). A deficit in background mutagenesis has also been reported for *pol32Δ* strains (34). Although our results similarly show the requirement of *POL32* for spontaneous mutagenesis, the more severe effects of CDG or TDG expression in *pol32Δ* cells were not expected. While previous studies have demonstrated poor mutagenesis in the *pol32Δ* background (12, 30, 34, 47), mutants were nevertheless isolated at very low frequency. This new observation may reflect greater dependence on *POL32* for mutagenesis by and replication past AP sites than for other lesions. Recent work has shown that Pol32 forms a complex with Rev1 and Pol  $\zeta$  during TLS (48), the lack of which could explain the inability of *pol32Δ* cells to bypass AP sites effectively. A less likely possibility is that the difference could be associated with the particular mutation targets. In the case of replication past a plasmid-based AP site (30), the residual ability of *pol32Δ* strains to effect TLS, albeit at a much lower

frequency than *POL32* strains, might also point to a difference between the *in vivo* bypass of AP sites in transforming plasmids compared to chromosomal DNA.

As demonstrated by our results, AP site bypass in yeast is a complex process involving the coordinated activity of multiple pathways and polymerases. That cell survival can be maintained even in the absence of the main enzymes required for TLS of AP sites suggests a redundant pathway that does not increase mutagenesis or effective mechanisms for tolerating these lesions. Recombinational repair would be an obvious candidate, which is consistent with the strong hypersensitivity of *rad52* strains to the toxicity of alkylating agents (49). Detailed analysis of residual mutagenesis in *rev1* or Pol  $\zeta$ -deficient strains, specifically the frequencies of base substitutions or deletions during bypass, may provide insight as to the identity and effects of such alternate pathways.

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