

Role of DNA polymerase η in the bypass of abasic sites in yeast cells

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ABSTRACT

Abasic (AP) sites are major DNA lesions and are highly mutagenic. AP site-induced mutagenesis largely depends on translesion synthesis. We have examined the role of DNA polymerase η (Pol η) in translesion synthesis of AP sites by replicating a plasmid containing a site-specific AP site in yeast cells. In wild-type cells, AP site bypass resulted in preferred C insertion (62%) over A insertion (21%), as well as –1 deletion (3%), and complex event (14%) containing multiple mutations. In cells lacking Pol η (*rad30*), Rev1, Pol ζ (*rev3*), and both Pol η and Pol ζ , translesion synthesis was reduced to 30%, 30%, 15% and 3% of the wild-type level, respectively. C insertion opposite the AP site was reduced in *rad30* mutant cells and was abolished in cells lacking Rev1 or Pol ζ , but significant A insertion was still detected in these mutant cells. While purified yeast Pol α effectively inserted an A opposite the AP site *in vitro*, purified yeast Pol δ was much less effective in A insertion opposite the lesion due to its 3'→5' proofreading exonuclease activity. Purified yeast Pol η performed extension synthesis from the primer 3' A opposite the lesion. These results show that Pol η is involved in translesion synthesis of AP sites in yeast cells, and suggest that an important role of Pol η is to catalyze extension following A insertion opposite the lesion. Consistent with these conclusions, *rad30* mutant cells were sensitive to methyl methanesulfonate (MMS), and *rev1 rad30* or *rev3 rad30* double mutant cells were synergistically more sensitive to MMS than the respective single mutant strains.

INTRODUCTION

Apurinic/aprimidinic (AP) sites, also referred to as abasic sites, are a major type of spontaneous DNA lesions. Some environmental agents, such as methyl methanesulfonate (MMS), can also induce AP sites in DNA. AP sites are non-coding. Therefore, copying an AP site by any DNA polymerase is error prone. Consequently, AP sites are highly mutagenic. In *Escherichia coli*, translesion synthesis of an AP site

results in preferential insertion of an A opposite the lesion, leading to the 'A rule' hypothesis (1). Such a strong bias opposite an AP site does not appear to be the case in mammals. Using plasmid mutagenesis systems, insertions of A, C, T and G opposite AP sites have all been reported in cultured mammalian cells (2–7). In yeast cells, whereas Lawrence and colleagues (8) observed predominant C insertion opposite AP sites, Haracska *et al.* (9) reported predominant A insertion.

Translesion synthesis, also referred to as lesion bypass, is the cellular process that directly copies damaged sites of the template during DNA synthesis. It consists of nucleotide insertion opposite the lesion and extension synthesis from opposite the lesion. According to this definition, a DNA polymerase that performs the insertion step, the extension step, or both, qualifies as a translesion polymerase. Extensive *in vitro* and some *in vivo* studies have indicated that Pol ζ and the Y family polymerases are important translesion polymerases in eukaryotes [reviewed in (10–14)]. Pol ζ belongs to the same B family of DNA polymerases as the replicative Pol α , Pol δ , and Pol ϵ (15,16). In the yeast *Saccharomyces cerevisiae*, the Y family consists of Pol η and Rev1 (16). Mammals contain two additional members of the Y family polymerases: Polk and Polt (10,16).

Rev1 possesses a dCMP transferase and is efficient in inserting a C opposite an AP site *in vitro*, but it cannot catalyze extension synthesis from opposite the lesion (17,18). The combination of Rev1 and Pol ζ , however, results in bypass of the AP site *in vitro* (17). Genetic experiments have shown that both Rev1 and Pol ζ are required for AP site-induced mutagenesis in yeast cells (9,19,20). Pol ζ is believed to function in extension synthesis during AP site bypass (9,17,21). The role of Rev1 in AP site bypass, however, is controversial. Whereas Lawrence and colleagues (19) concluded that Rev1 acts catalytically by inserting C opposite AP site, Haracska *et al.* (9) concluded that Rev1 predominantly plays a non-catalytic structural role.

Pol η was originally discovered as an important translesion polymerase in response to UV-induced TT dimers in an error-free manner (22,23). However, subsequent studies showed that yeast Pol η is also responsive to template AP sites by catalysis of G and less frequently A insertions opposite the lesion *in vitro* (21). Translesion synthesis of AP sites was later extended to the human Pol η *in vitro*, although it prefers A to G for insertion opposite the lesion (24,25). Furthermore, we observed that sequential actions of Pol η -catalyzed nucleotide insertion and Pol ζ -catalyzed extension resulted in AP site bypass

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in vitro (21). Thus, we proposed that Pol η may be involved in error-prone translesion synthesis of some other DNA lesions such as AP sites (21). Indeed, we found recently that Pol η is involved in error-prone translesion synthesis of benzo[*a*]pyrene DNA adducts in yeast cells (26).

To better understand mechanisms of translesion synthesis across AP sites in eukaryotes, we have performed *in vitro* biochemical and *in vivo* genetic experiments to examine the role of Pol η in AP site bypass in yeast cells. In this report, we (i) describe a site-specific translesion synthesis assay in yeast cells; (ii) demonstrate that yeast Pol η is involved in translesion synthesis of AP sites *in vivo*; and (iii) present evidence suggesting that an important role of Pol η in AP site bypass is to catalyze extension synthesis following A insertion opposite the lesion.

MATERIALS AND METHODS

Materials

T4 DNA ligase, the T4 gene 32 protein, T4 polynucleotide kinase and yeast Pol η were obtained from Enzymax (Lexington, KY). MMS was purchased from Sigma (St Louis, MO). Yeast Pol ζ , and the catalytic subunits of yeast Pol α and Pol δ were purified as previously described (27–29). A 17mer DNA oligonucleotide containing a site-specific tetrahydrofuran (AP site analog) was synthesized by automated DNA phosphoramidite methods by Operon (Alameda, CA). Its sequence is 5'-CGACTXGAAG-GATCCGC-3', where X designates the AP site analog.

Other damaged and undamaged DNA oligonucleotides as indicated were synthesized by Integrated DNA Technologies (Coralville, IA).

Yeast strains. The yeast strains used are the wild-type BY4741 (*MATa his3 leu2 met15 ura3*) and its isogenic BY4741 Δ rad30 (*rad30* deletion mutant), BY4741 Δ rev1 (*rev1* deletion mutant), BY4741 Δ rev3 (*rev3* deletion mutant), BY4741 Δ rev1 Δ rad30 (*rev1 rad30* double deletion mutant) and BY4741 Δ rev3 Δ rad30 (*rev3 rad30* double deletion mutant). BY4741 was purchased from ATCC (Manassas, VA). BY4741 Δ rad30 (lacking Pol η) was purchased from Research Genetics (Huntsville, AL). BY4741 Δ rev3 (lacking Pol ζ) and BY4741 Δ rev3 Δ rad30 were constructed as previously described (26). BY4741 Δ rev1 (lacking Rev1) was constructed by transforming BY4741 cells with a linearized *rev1* deletion plasmid construct. The *rev1* deletion clone was confirmed by a functional assay demonstrating reduced UV-resistance and loss of UV-induced mutagenesis. The *rev1* deletion strain was further tested for complementation of UV resistance and UV-induced mutagenesis by a plasmid carrying the wild-type *REV1* gene. BY4741 Δ rev1 Δ rad30 was similarly constructed by transforming BY4741 Δ rad30 cells with the linearized *rev1* deletion plasmid construct and similarly confirmed for its *rev1* phenotype as described above.

Construction of plasmid containing a site-specific AP site

The plasmid vector used is pELUf1 (Figure 1A). The strategy for constructing pELUf1 containing a site-specific AP site is

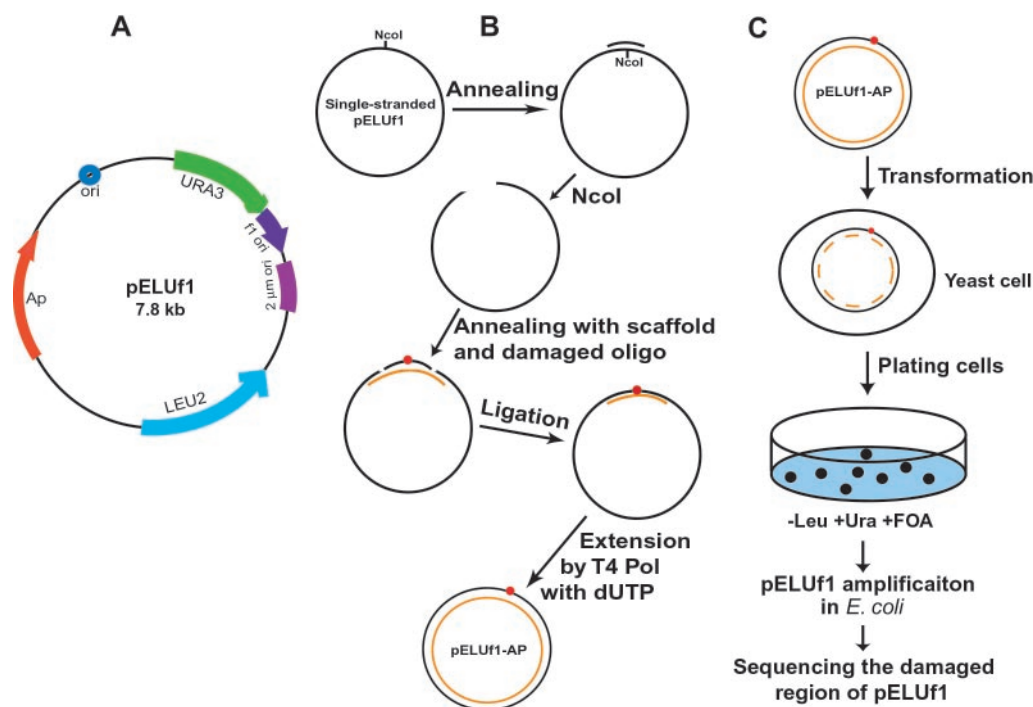


Figure 1. Schematic presentation of an *in vivo* translesion synthesis assay in yeast. (A) The plasmid used for construction of site-specifically damaged DNA vector. (B) Construction of pELUf1-AP containing a site-specific AP site. The AP site (indicated by a red dot) was carried on a 17mer oligonucleotide. Insertion of the damaged oligonucleotide into the unique NcoI site of the vector inactivated the *URA3* gene, thus, allowing yeast cells containing replicated pELUf1-AP to grow on plates containing 5-FOA. The undamaged strand (shown in orange) of pELUf1-AP contained U in place of T. (C) *In vivo* translesion synthesis assay using the site-specifically damaged pELUf1-AP plasmid. Transformation efficiency was measured in the same experiment by using undamaged pELUf1 and plates lacking both leucine and 5-FOA.

shown schematically in Figure 1B. Briefly, *E. coli* DH5 α F'IQ cells containing plasmid pELUf1 were infected with the helper phage M13KO7, and a single-stranded pELUf1 was prepared. The single-stranded pELUf1 was annealed to a 20mer DNA oligonucleotide, 5'-GTGCCCTCCATGGAAAAATC-3', at its unique NcoI restriction site within the vector *URA3* gene, and digested with the NcoI restriction endonuclease. Then, the linearized single-stranded pELUf1 was annealed with a 46mer DNA scaffold, 5'-CTGUGCCCUCCAUGGCGGAU-TCUTCUAGUCGGAAAAAUCAGTCAAG-3', and the 5'-phosphorylated 17mer oligonucleotide containing a site-specific AP site. While the mid-region of the scaffold is complementary to the damaged oligonucleotide, its ends are complementary to the single-stranded pELUf1 ends. Ligation of the damaged oligonucleotide into the pELUf1 vector was performed with T4 DNA ligase at 16°C for 20 h, and DNA was precipitated in ethanol. Finally, the complementary strand of pELUf1 was synthesized with T4 DNA polymerase in the presence of T4 gene 32 protein and 0.5 mM each of dATP, dCTP, dGTP and dUTP, using the scaffold as the primer. The resulting construct, pELUf1-AP, was a double-stranded plasmid containing a site-specific AP site, in which the undamaged strand contained U in place of T. Furthermore, insertion of the damaged oligonucleotide inactivated the vector *URA3* gene. Formation of double-stranded plasmid pELUf1-AP was confirmed by electrophoresis on a 1% agarose gel. Construction of pELUf1-AP was performed by Enzymax.

***In vivo* translesion synthesis assays in yeast**

Undamaged pELUf1 or site-specifically damaged pELUf1-AP plasmid (2 μ g) was transformed into yeast cells of various strains by the lithium acetate method essentially as described in (30). Immediately after transformation, yeast cells were collected by centrifugation (20 s at 5000 r.p.m.) in a micro-centrifuge. Cells transformed with the undamaged pELUf1 were resuspended in 1 ml of sterile water. Aliquots of 0.5–1 μ l were diluted and plated onto YNB minimal agar plates (0.17% yeast nitrogen base, 0.49% ammonium sulfate, 2% glucose and 2% agar) lacking leucine to score for transformation efficiency (colonies containing replicated pELUf1). Cells transformed with the site-specifically damaged pELUf1-AP were resuspended in 400 μ l of sterile water and were directly plated onto two YNB minimal agar plates lacking leucine but supplemented with 5 mM 5-fluoroorotic acid (5-FOA), 150 μ M methionine, and 380 μ M uracil to score for colonies containing replicated pELUf1-AP. Cells transformed by the undesired background plasmid pELUf1 without the damaged oligonucleotide insert remained the *URA3* wild type and thus cannot grow on plates containing 5-FOA. After incubation at 30°C for 3–4 days, yeast colonies were counted. Translesion synthesis was calculated as transformants per microgram of the damaged plasmid per 10^6 transformable cells with the undamaged plasmid (i.e. transformants per microgram of the damaged plasmid $\times 10^6$ /transformation efficiency expressed as transformants per microgram of the undamaged plasmid). Relative translesion synthesis was obtained by comparing translesion synthesis in various mutant strains to that in the wild-type cells.

Replicated pELUf1-AP plasmid clones were individually recovered from yeast colonies on the 5-FOA plates by a

zymolyase method essentially as described in (31) and amplified in *E. coli* DH5 α cells. Each pELUf1-AP plasmid clone was analyzed by digestion with the BamHI restriction endonuclease. A BamHI restriction site was designed into the damaged 17mer oligonucleotide during construction of pELUf1-AP. The BamHI restriction analysis further eliminated undesired background transformants by the undamaged pELUf1. These background transformants escaped selection by the 5-FOA plates because they contained mutations somewhere in the vector *URA3* gene. Plasmid clones that did not contain this added BamHI restriction site were excluded from further analysis and calculation. Finally, the precise specificity of translesion synthesis opposite the site-specific AP site was determined by DNA sequencing.

DNA polymerase assays

A standard DNA polymerase reaction mixture (10 μ l) contained 25 mM KH₂PO₄ (pH 7.0), 5 mM MgCl₂, 5 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 10% glycerol, 50 μ M of dNTPs (dATP, dCTP, dTTP and dGTP individually or together as indicated), 50 fmol of an indicated DNA substrate containing a ³²P-labeled primer and a purified DNA polymerase as indicated. After incubation at 30°C for 10 min, reactions were terminated with 7 μ l of a stop solution (20 mM EDTA, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol). The reaction products were resolved on a 20% polyacrylamide gel containing 8 M urea and visualized by autoradiography. DNA synthesis products were quantitated by scanning densitometry using the Sigma-Gel software (Sigma) for analysis.

MMS sensitivity

Yeast cells grown at 30°C to stationary phase in minimum media were harvested by centrifugation, washed with sterile water and resuspended in 100 mM potassium phosphate (pH 7.0) to 4 OD₆₀₀ cells per milliliter. Cells were then treated with various doses of MMS as indicated at 30°C for 30 min with shaking, followed by centrifugation and washing with sterile water. After dilution, cells were plated in minimum medium plates. Surviving colonies were counted after incubation at 30°C for 2–4 days. Cell survival of each strain was expressed relative to that of untreated cells of the corresponding strain.

RESULTS

An *in vivo* translesion synthesis assay in yeast

To investigate mechanisms of AP site bypass in cells, we established a plasmid-based *in vivo* translesion synthesis assay in the yeast *S. cerevisiae* model system. The vector pELUf1 contains the yeast *LEU2* gene for plasmid selection, the yeast *URA3* gene for accepting a site-specifically damaged oligonucleotide and the f1 replication origin for production of single-stranded phagemid (Figure 1A). A 17mer oligonucleotide containing a site-specific AP site was ligated into the unique NcoI site of the single-stranded pELUf1 vector, with the help of a scaffold oligonucleotide. The resulting pELUf1-AP was then converted into double-stranded plasmid with T4 DNA polymerase, using the scaffold as the primer and dUTP

instead of dTTP. The resulting undamaged second strand of pELUf1-AP contained U in place of T (Figure 1B).

The site-specifically damaged pELUf1-AP was transformed into yeast cells (Figure 1C). Using double-stranded DNA ensured efficient transformation. High levels of U in DNA will lead to extensive DNA fragmentation initiated by uracil-DNA glycosylase (UDG) (32–34). Thus, the U-containing DNA strand was quickly destroyed by the combined actions of UDG and AP endonucleases as the plasmid entered into cells, converting the double-stranded plasmid back into single-stranded DNA. Consequently, plasmid propagation in cells could only be achieved by replicating the damaged DNA strand involving translesion synthesis. Transformed cells were plated on minimal media plates containing uracil and 5-FOA but lacking leucine. While plates without leucine allowed selection for the replicated plasmid, 5-FOA was used to select for *ura3* mutant cells. Since insertion of the damaged oligonucleotide had inactivated the *URA3* gene, cells containing plasmid pELUf1-AP from translesion synthesis were able to grow on the 5-FOA plate. In contrast, cells transformed by the undesired intact pELUf1 without the oligonucleotide insert remained the *URA3* wild type and were therefore unable to grow on the 5-FOA plate. Transformation efficiency was determined by using undamaged and double-stranded pELUf1 plasmid in the same experiment. After correcting for differences in transformation efficiency, translesion synthesis efficiency in various cells relative to that in the wild-type cells was calculated. Replicated plasmid clones were individually isolated from yeast colonies and subsequently amplified in *E.coli*. To further ensure that the plasmid clones were indeed derived from translesion synthesis in yeast cells, a BamHI site was designed into the damaged oligonucleotide, whereas its complementary scaffold DNA contained a mismatch eliminating this restriction site on the undamaged strand. After amplification in *E.coli*, each plasmid clone was digested with BamHI. Clones without this BamHI site were excluded from further analysis. Each clone was sequenced to identify the nucleotide inserted opposite the AP site (Figure 1C).

Pol η is involved in translesion synthesis of AP sites in yeast cells

To determine whether Pol η is involved in AP site bypass in cells, we performed *in vivo* translesion synthesis assays in yeast strains proficient or deficient in Pol η . Since Pol ζ and Rev1 are known to be required for translesion synthesis of AP sites in cells (9,19,20), we also performed *in vivo* translesion synthesis assays in mutant cells lacking Pol ζ (*rev3* deletion mutant) or Rev1 for comparison. As expected, translesion synthesis of the site-specific AP site was reduced to 30 and 15% of the wild-type level in *rev1* and *rev3* mutant cells, respectively (Figure 2). In *rad30* mutant cells lacking Pol η , translesion synthesis was reduced to 30% of the wild-type level (Figure 2). These results show that Pol η is involved in translesion synthesis of AP sites in yeast.

To gain insights into the genetic relationship between Pol η and Rev1 or Pol ζ in AP site bypass, we performed translesion synthesis assays in yeast cells lacking both Pol η and Rev1 or both Pol η and Pol ζ . If Pol η functions in the same bypass pathway with Rev1 and Pol ζ , translesion synthesis in the double mutant would not be further reduced as compared

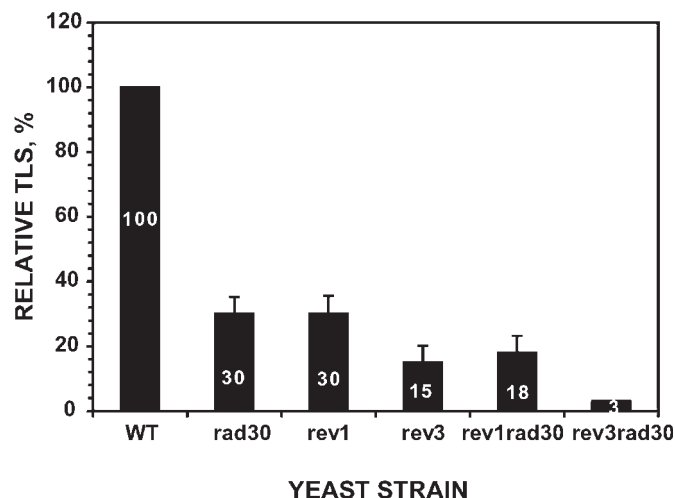


Figure 2. Relative frequencies of AP site translesion synthesis (TLS) in various yeast strains. Using plasmid pELUf1-AP containing a site-specific AP site, *in vivo* translesion synthesis assays were performed as described in Materials and methods. Relative TLS was obtained by comparing translesion synthesis in various mutant strains to that in the wild-type cells. Slightly different transformation efficiencies as determined with the undamaged pELUf1 have been corrected in the calculation. Standard deviations are shown as error bars. WT, wild type; *rad30*, lacking Pol η ; *rev1*, lacking Rev1; *rev3*, lacking Pol ζ ; *rev1 rad30*, lacking both Rev1 and Pol η ; *rev3rad30*, lacking both Pol ζ and Pol η .

with the single mutants. If the Pol η function in AP site bypass involves another mechanism different from that of Rev1 and Pol ζ , translesion synthesis would be more deficient in the double mutant than in the respective single mutants. As shown in Figure 2, translesion synthesis in the *rev1 rad30* double mutant cells was reduced further, as compared to the respective single mutant strains. In the *rev3 rad30* double mutant cells, translesion synthesis was severely deficient, retaining only 3% of that in wild-type cells (Figure 2). These results suggest that the Pol η function in AP site bypass involves another mechanism different from the bypass mechanism mediated by the Pol ζ mutagenesis pathway.

Specificity of *in vivo* translesion synthesis opposite AP sites

To further understand the role of Pol η in AP site bypass, we recovered the replicated plasmids from yeast clones and individually amplified them in *E.coli* for DNA sequencing. Three types of translesion products were observed from various yeast strains: (i) simple translesion synthesis in that 1 nt was inserted, or a -1 deletion was produced, opposite the AP site; (ii) following translesion synthesis opposite the AP site, another mutation was generated downstream of the lesion; and (iii) complex translesion synthesis in that multiple mutations were generated on both sides of the AP site (Figure 3). Translesion synthesis with downstream mutations was rare. Only 2 such clones out of 60 from cells lacking Pol η , 1 out of 29 clones from wild-type cells and 1 out of 27 clones from the *rev1* mutant cells were recovered.

T insertion opposite the AP site was not observed among all yeast strains examined. In wild-type cells (Table 1), the majority of translesion synthesis (62%) resulted from C insertion opposite the AP site. Less frequently (21%) A was inserted

opposite the lesion. A small fraction (14%) of the bypassed products were derived from complex translesion synthesis, while -1 deletion occurred only as a very minor event (3%). G was not recovered opposite the AP site. In *rad30*, *rev1*, *rev3*, *rev1 rad30* and *rev3 rad30* mutant cells, the spectra of translesion synthesis were significantly altered (Table 1). In cells lacking either Rev1 or Pol ζ , C insertion opposite the AP site was not detected (Table 1). In *rad30* mutant cells lacking Pol η , A insertion was favored over C insertion opposite the AP site (Table 1). Furthermore, G insertion opposite the lesion became detectable in *rev3* (lacking Pol ζ) or *rad30* mutant cells (Table 1).

When the altered bypass in the mutant strains was directly compared to AP site bypass in wild-type cells, expressed as translesion synthesis relative to that in wild-type cells, the effects of Pol η , Rev1 and Pol ζ became more clear (Table 2). For AP site bypass with C incorporation, while Rev1 and Pol ζ were indispensable, Pol η had a strong stimulatory effect (Table 2). In contrast, AP site bypass with A incorporation remained as the most frequent mechanism in cells lacking Pol η , Rev1 or Pol ζ , although such bypass

frequency was reduced by approximately 2-fold in these mutant cells (Table 2). This type of translesion synthesis became largely abolished only when both the Pol η and Rev1 or both the Pol η and Pol ζ activities were eliminated (Table 2). AP site bypass with -1 deletion was not significantly affected in cells lacking Pol η , Rev1 or Pol ζ , but was abolished in cells lacking both Pol η and Pol ζ (Table 2). The complex translesion product was significantly reduced in cells lacking Pol ζ and abolished in cells lacking both Pol ζ and Pol η (Table 2). These results suggest that Pol η is generally involved in AP site bypass in yeast cells but it acts in a mechanism(s) different from the Pol ζ mutagenesis pathway that involves Rev1 and Pol ζ .

In vitro activities of Pol η in response to a template AP site

Our genetic assay is an endpoint measurement that includes the two distinct steps of *in vivo* translesion synthesis: nucleotide insertion and extension. Hence, the genetic assay cannot assign the observed effect to either the insertion or extension step, or both. To better define the roles of Pol ζ and Pol η in AP site bypass, we examined *in vitro* activities of both polymerases in response to an AP site. As shown in Figure 4A, the AP site strongly blocked yeast Pol ζ *in vitro* (Figure 4A, lane 1). Nevertheless, weak insertion activity was detected with purified Pol ζ , which inserted a G opposite the AP site (Figure 4A, lane 5). In contrast, purified yeast Pol η effectively inserted a G (Figure 4B, lane 5) and less frequently an A (Figure 4B, lane 2) opposite the AP site in the same sequence context as that used for the *in vivo* translesion synthesis assays (Figure 4B). Subsequent extension synthesis, however, was inefficient (Figure 4B, lane 1). The Pol η results are similar to our earlier observation with another AP site in a different sequence context where the template base immediately 5' to the lesion was an A (21). Since G insertion opposite the AP site was not detected during *in vivo* translesion synthesis in wild-type cells (Table 1), we conclude that neither Pol η nor Pol ζ is significantly involved in catalyzing nucleotide insertion opposite the AP site in yeast cells. Apparently, the intrinsic G insertion activity of Pol η was not employed to a significant extent for *in vivo* bypass of AP sites even in the absence of Rev1 or Pol ζ (Tables 1 and 2).

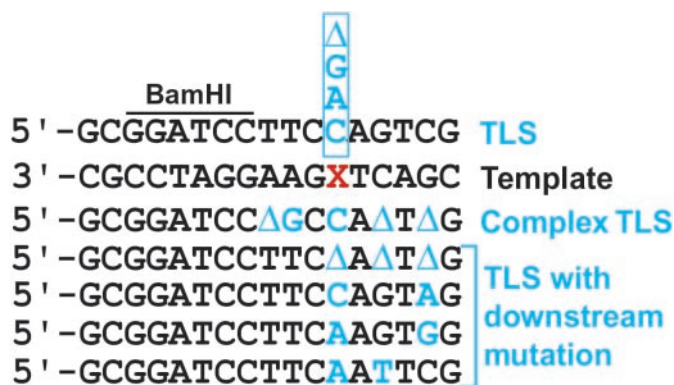


Figure 3. Types of AP site bypass products recovered from various yeast strains. The damaged 17mer oligonucleotide contained in plasmid pELUf1-AP is shown as the template for translesion synthesis, with the AP site designated by an X. Simple translesion synthesis with C, A or G insertion, or -1 deletion opposite the AP site are indicated above the damaged template. Complex TLS and TLS with downstream mutations (rare bypass events) are indicated below the damaged template.

Table 1. Specificity of translesion synthesis opposite the AP site in various yeast strains

Strain ^a	Clones sequenced ^b	TLS ^c				Complex TLS ^e
		A	C	G	Δ ^d	
WT	29	6 (21%)	18 (62%)	—	1 (3%)	4 (14%)
<i>rad30</i>	60	25 (42%)	18 (30%)	1 (2%)	3 (5%)	13 (22%)
<i>rev1</i>	27	13 (48%)	—	—	5 (19%)	9 (33%)
<i>rev3</i>	34	22 (65%)	—	1 (3%)	4 (12%)	7 (21%)
<i>rev1 rad30</i>	22	4 (18%)	—	—	3 (14%)	15 (68%)
<i>rev3 rad30</i>	2	2 (100%)	—	—	—	—

^aWT, wild type; *rad30*, lacking Pol η ; *rev1*, lacking Rev1; *rev3*, lacking Pol ζ ; *rev1 rad30*, lacking both Rev1 and Pol η ; *rev3 rad30*, lacking both Pol ζ and Pol η .

^bNumber of independent clones sequenced following *in vivo* translesion synthesis assays using pELUf1-AP plasmid containing a site-specific AP site. Only two clones were recovered from the *rev3 rad30* double mutant cells.

^cNumber of various translesion synthesis products are shown. The percentage of a particular type of TLS among all of the bypassed products in a strain is shown in the parenthesis.

^dThe -1 deletion.

^eOnly one type of complex TLS was observed, which contained mutations on both sides of the AP site. The precise sequence of the complex TLS product is shown in Figure 3.

Table 2. Changes in translesion synthesis specificity in mutant cells relative to that in wild-type cells

Strain ^a	TLS ^b		G	Δ^c	Complex TLS ^d	Total
	A	C				
WT	0.21	0.62	—	0.03	0.14	1
<i>rad30</i>	0.13	0.09	0.006	0.02	0.07	0.30
<i>rev1</i>	0.14	—	—	0.06	0.10	0.30
<i>rev3</i>	0.10	—	0.005	0.02	0.03	0.15
<i>rev1 rad30</i>	0.03	—	—	0.03	0.12	0.18
<i>rev3 rad30</i>	0.03	—	—	—	—	0.03

^aWT, wild type; *rad30*, lacking Pol η ; *rev1*, lacking Rev1; *rev3*, lacking Pol ζ ; *rev1 rad30*, lacking both Rev1 and Pol η ; *rev3 rad30*, lacking both Pol ζ and Pol η .

^bTranslesion synthesis in various mutant strains is expressed relative to that in the wild-type cells. Calculation was based on Figure 2 and Table 1.

^cThe -1 deletion.

^dOnly one type of complex TLS was observed, which contained mutations on both sides of the AP site. The precise sequence of the complex TLS product is shown in Figure 3.

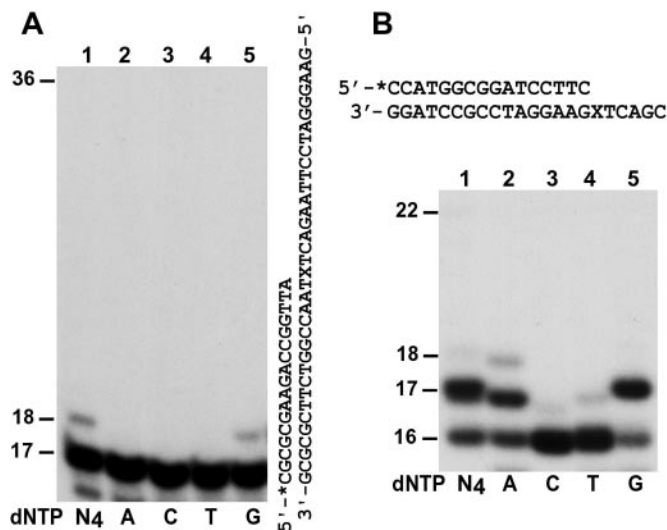


Figure 4. Nucleotide insertion opposite a template AP site by purified yeast Pol η and Pol ζ . (A) A ³²P-labeled 17mer primer was annealed to the damaged template with the primer 3' end terminating right before the AP site as shown on the right. DNA synthesis assays were performed in the presence of a single dATP (A), dCTP (C), dTTP (T) or dGTP (G), or all four dNTPs (N₄), using 14 ng (69 fmol) of purified yeast Pol ζ . Products of DNA synthesis were separated by 20% denaturing polyacrylamide gel and visualized by autoradiography of the gel. DNA size markers in nucleotides are indicated on the left. (B) A ³²P-labeled 16mer primer was annealed to the damaged template with the primer 3' end terminating right before the AP site as shown on the top. DNA synthesis assays were performed with 10 ng (140 fmol) of purified yeast Pol η . X, the AP site.

To examine a possible role of Pol η in extension synthesis, we separately annealed four ³²P-labeled 17mer primers to the damaged template, terminating with an A, C, T or G, respectively, opposite the lesion (Figure 5A). DNA synthesis assays were then performed with purified yeast Pol η . For comparison, similar experiments were also performed with an undamaged template G opposite the primer 3' end (Figure 5A). As expected, Pol η was capable of extension synthesis from matched and mismatched primer 3' end with varying efficiencies (Figure 5A, lanes 1 to 4). The full-length extension products (22mer DNA band) were generated from the T–G

(primer–template) and G–G mismatches (Figure 5A, lanes 3 and 4). However, extension of the A–G mismatch yielded predominantly 1 nt shorter products (21mer DNA band) (Figure 5A, lane 1), indicating that extension was mainly mediated by a -1 deletion mechanism through Pol η -catalyzed realignment of the primer 3' A with the next template T prior to extension synthesis. In response to a template AP site, extension synthesis was observed with Pol η from the primer 3' A opposite the lesion, generating both full-length and -1 deletion products in similar quantities (Figure 5A, lane 5). Extension was ineffective from the primer 3' G opposite the AP site (Figure 5A, lane 8), and was not detected from the primer 3' C or T (Figure 5A, lanes 6 and 7). To determine whether extension from A opposite the AP site requires a template T immediately 5' to the lesion, we performed the extension assay again with another DNA substrate containing a template G immediately 5' to the AP site. As shown in Figure 5B (lanes 3 to 7), extension by Pol η was also observed from A opposite the AP site, generating predominantly full-length products. Furthermore, the extension activity of Pol η was comparable to that of purified yeast Pol ζ (Figure 5B, lane 2). Since Pol ζ is inefficient in copying the last template base even from undamaged templates (27,29), the 35mer extension product of Pol ζ (Figure 5B, lane 2) most likely resulted from a normal extension mechanism rather than a -1 deletion mechanism. These results show that Pol η possesses a significant biochemical activity of extension synthesis from a primer 3' A opposite an AP site.

Response of the Pol δ proofreading exonuclease activity to the primer 3' A opposite an AP site

Pol ζ and Rev1 are unable to insert A opposite an AP site (Figure 4) (17,18,35), and cells lacking Pol η could still significantly insert A opposite the lesion (Tables 1 and 2). Thus, it is possible that a replicative polymerase such as Pol α or Pol δ may be important for A insertion opposite AP sites in yeast cells. To test this possibility, we annealed a 16mer primer to the damaged template, terminating right before the AP site (Figure 6). DNA synthesis assays were then performed using the purified catalytic subunits of yeast Pol α and Pol δ . As shown in Figure 6 (lanes 2 to 6), Pol α efficiently inserted an A opposite the AP site, but was unable to extend it. In contrast, Pol δ was very inefficient in A insertion opposite the AP site (Figure 6, lanes 8 to 12), and extension from opposite the lesion was not detected.

Nucleotide insertion by Pol δ opposite the AP site was further reduced in the presence of dATP alone (Figure 6, compare lanes 8 and 9). Moreover, nuclease activities were apparent when the translesion synthesis reactions were performed using dATP, dTTP or dGTP alone (Figure 6, lanes 9, 11 and 12). Therefore, it is possible that A insertion opposite the AP site may be greatly suppressed by the 3'→5' proofreading exonuclease activity of Pol δ . To test this possibility, we determined whether A opposite the AP site is subject to removal by the proofreading exonuclease activity of Pol δ . We labeled a 17mer primer with ³²P at its 5' end and annealed it to the damaged template with the primer 3'A terminating opposite the AP site (Figure 7). Purified yeast Pol δ was then incubated with this DNA substrate in the absence of deoxyribonucleoside triphosphates. DNA substrates

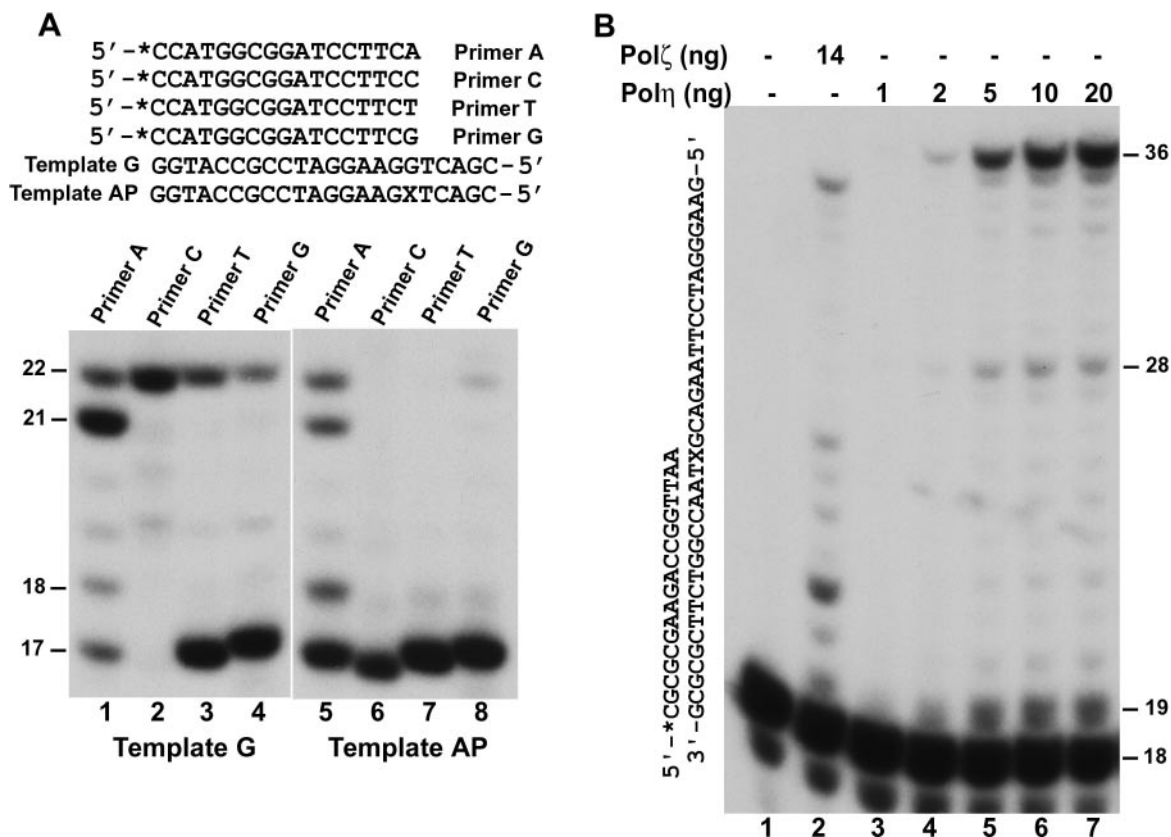


Figure 5. Extension synthesis from opposite the AP site by yeast Pol η . (A) Four 32 P-labeled 17mer primers were separately annealed to the damaged template (Template AP) or an undamaged template (Template G) with the primer 3' A, C, T or G, respectively, terminating opposite the AP site or a template G as shown on the top. DNA synthesis assays were performed with 2.5 ng (35 fmol) (lanes 1 to 4) or 10 ng (140 fmol) (lanes 5 to 8) of purified yeast Pol η . (B) A 32 P-labeled 18mer primers were annealed to the damaged template with the primer 3' A terminating opposite the AP site as shown on the right. DNA synthesis assays were performed with 14 ng (69 fmol) of purified yeast Pol ζ (lane 2) or increasing concentrations of purified yeast Pol η (lanes 3 to 7). Products of DNA synthesis were separated by 20% denaturing polyacrylamide gel and visualized by autoradiography of the gel. DNA size markers in nucleotides are indicated on the sides. X, the AP site.

containing a C–G (primer–template) base pair or an A–G mismatch at the primer 3' end were used as controls. As shown in Figure 7, the 3'→5' proofreading exonuclease of Pol δ was active in removing the A–G mismatch (60% removal), but inactive against the C–G base pair (compare lanes 7 and 8) at the primer 3' end. The primer 3' A opposite the AP site was also actively removed by the 3'→5' proofreading exonuclease of Pol δ (68% removal) to a similar extent as the A–G mismatch (Figure 7, compare lanes 2 and 6). The primer 3' C opposite the AP site, however, was more resistant to the 3'→5' proofreading exonuclease of Pol δ (38% removal) (Figure 7, lane 4). These results show that, while Pol α is capable of efficiently inserting an A opposite the AP site, Pol δ is much less effective in A insertion opposite the lesion due to its 3'→5' proofreading exonuclease activity.

Cellular resistance to MMS conferred by Pol η , Pol ζ and Rev1

Genetic studies have demonstrated that defects in translesion synthesis result in cellular sensitivity to DNA-damaging agents. For example, Pol η , Pol ζ and Rev1 play major roles in translesion synthesis in response to UV radiation (22,23,36,37). Consequently, their corresponding mutant cells are hypersensitive to UV radiation (36–40). Based on

our results that Pol η plays a significant role in the bypass of AP sites in yeast cells, we predicted that *rad30* mutant cells lacking Pol η would be sensitive to AP site-inducing agents. Even though there is not a known agent that specifically induces AP site in DNA, MMS is probably the most effective agent for AP site induction. As is the case of UV radiation, MMS induces multiple types of DNA lesions. Nevertheless, AP sites are induced by MMS as a major type of lesion, since it reacts with DNA forming mainly unstable *N*³-methyladenine and *N*⁷-methyl guanine adducts, which readily depurinate to AP sites (41,42). To test our prediction, we determined cellular sensitivity to MMS treatment in the presence or absence of Pol η . For comparison, *rev1* and *rev3* mutant cells were also included in these experiments. As expected, *Rev1* and *rev3* mutant cells were sensitive to MMS treatment as compared to the wild-type cells (Figure 8). Furthermore, the survival curve of *rev1* and *rev3* mutant cells were essentially identical (Figure 8), consistent with the notion that Rev1 and Pol ζ function in the same Pol ζ mutagenesis pathway for AP site bypass. Similarly, *rad30* mutant cells were also sensitive to MMS treatment (Figure 8). In the absence of both Rev1 and Pol η , the *rev1 rad30* double mutant cells became much more sensitive to MMS than either of the single mutant strains (Figure 8). In the absence of both Pol ζ and Pol η , the *rev3 rad30* double mutant cells were much more sensitive to MMS

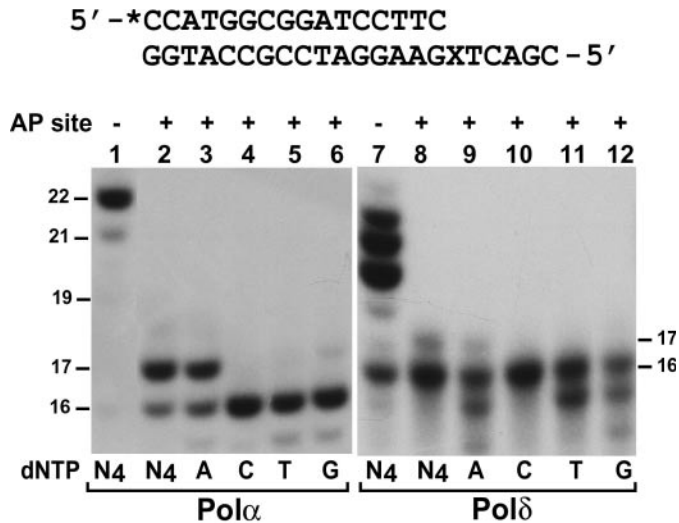


Figure 6. Response of yeast Pol α and Pol δ to a template AP site. A 32 P-labeled 16mer primer was annealed to the damaged template with the primer 3' end terminating right before the AP site as shown on the top. DNA synthesis assays were performed in the presence of a single dATP (A), dCTP (C), dTTP (T) or dGTP (G) or all four dNTPs (N₄) with 12 ng (72 fmol) of the purified catalytic subunit of yeast Pol α (lanes 2 to 6) or 15 ng (120 fmol) of the purified catalytic subunit of yeast Pol δ (lanes 8 to 12) using the damaged template (AP site, +) as indicated. Control experiments were also performed with the undamaged template containing a G in place of the AP site (AP site, -) (lanes 1 and 7). Products of DNA synthesis were separated by 20% denaturing polyacrylamide gel and visualized by autoradiography of the gel. DNA size markers in nucleotides are indicated on the sides. X, the AP site.

than either of the single mutant strains, and exhibited more sensitivity than the *rev1 rad30* double mutant (Figure 8). These results show that Pol η , like Pol ζ and Rev1, confers cellular resistance to MMS and that the function of Pol η in response to MMS does not completely overlap with the Pol ζ mutagenesis pathway. These results further support our conclusion that Pol η plays a significant role in AP site bypass in yeast cells.

DISCUSSION

In this study, we have examined the role of Pol η in translesion synthesis of AP sites in yeast cells. *In vivo* translesion synthesis was directly examined through replication of a plasmid containing a site-specific AP site. A similar assay in yeast cells was originally developed by Lawrence and colleagues (43) using single-stranded plasmid DNA containing a site-specific lesion. Owing to the extremely low transformation efficiency of single-stranded plasmid in yeast cells, the *in vivo* assay was later modified by using a double-stranded plasmid containing a site-specific lesion located in a small gap (8). This *in vivo* translesion synthesis assay essentially measures a gap filling reaction in yeast. Nevertheless, the gap filling reaction did reflect translesion synthesis as indicated by a genetic requirement for Pol ζ and Rev1 (19). In our assay system, a significant modification was made in that the site-specifically damaged and single-stranded plasmid was converted into the double-stranded form prior to transformation, replacing T with U during *in vitro* synthesis of the complementary strand. Upon entering into cells, the complementary strand was degraded as a result of replacement of T by U, thus, converting

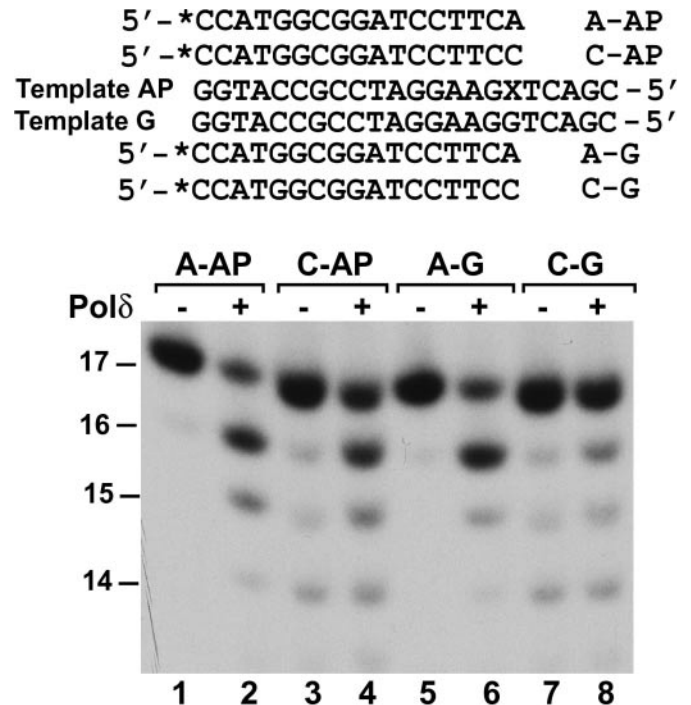


Figure 7. Proofreading exonuclease activity of Pol δ against an A opposite the AP site. Two 32 P-labeled 17mer primers were separately annealed to the damaged template (Template AP) with the primer 3' A or C, respectively, terminating opposite the AP site as shown on the top. As controls, the two primers were also separately annealed to an undamaged template (Template G), forming an A-G mismatch or a C-G base pair, respectively, at the primer 3' end. The four types of DNA substrates as indicated were incubated with 15 ng (120 fmol) of the purified catalytic subunit of yeast Pol δ (lanes 2, 4, 6 and 8) at 30°C for 30 min under the DNA polymerase assay conditions in the absence of dNTPs. Lanes 1, 3, 5 and 7, reactions without Pol δ . Reaction products were separated by 20% denaturing polyacrylamide gel and visualized by autoradiography of the gel. DNA size markers in nucleotides are indicated on the left. X, the AP site.

the plasmid DNA back into single-stranded form containing a site-specific lesion. An advantage of our system is that replication of the damaged plasmid specifically reflects translesion synthesis without the interference by DNA repair, a template switching mechanism, recombination or selective replication of the undamaged complementary strand. Our experiments were designed such that if the complementary strand were replicated, a T would have been inserted at the position corresponding to the opposite of the original AP site. This was not observed, thus, supporting our expectation that the complementary strand cannot be replicated due to its degradation inside cells. Like earlier systems (19,44), our *in vivo* translesion synthesis assays also reflected a genetic requirement for Pol ζ and Rev1 that are known to be required for translesion synthesis of AP sites in cells.

Using our *in vivo* translesion synthesis assay, we found that Pol η makes a significant contribution to AP site bypass in yeast cells. Without Pol η , translesion synthesis of AP sites is significantly impaired. Furthermore, the specificity of nucleotide insertion opposite the AP site is altered in *rad30* mutant cells lacking Pol η . Yeast Pol η is capable of inserting a G and less frequently an A opposite an AP site *in vitro*, regardless of whether a template T or a template A is located immediately 5' to the lesion (21) (Figure 4B). Thus, if Pol η

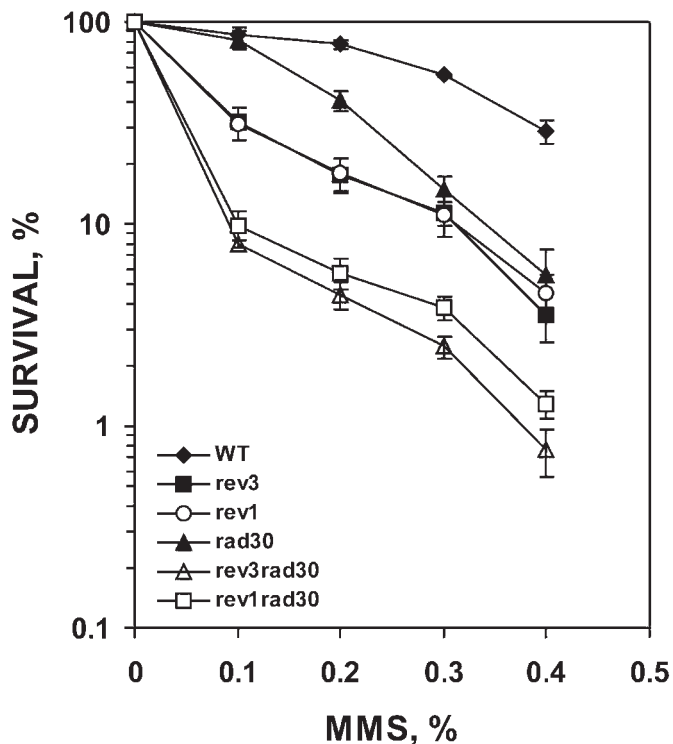


Figure 8. MMS sensitivity of various yeast strains. Yeast cells of various strains were treated with increasing concentrations of MMS as indicated at 30°C for 30 min and the cell survival determined as described in Materials and methods. Survival rates, with the standard deviations shown, are expressed relative to those of untreated cells. Results are the average of three experiments. WT, wild type (closed diamond); rev3, lacking Polζ (closed square); rev1, lacking Rev1 (open circle); rad30, lacking Polη (closed triangle); rev3 rad30, lacking both Polζ and Polη (open triangle); rev1 rad30, lacking both Rev1 and Polη (open square).

participates in AP site bypass by catalyzing nucleotide insertion opposite the lesion, then, significant G insertion opposite the AP site should be recovered from the replicated plasmids in wild-type cells. This was not the case. Only C and A insertions were detected among the bypassed products in wild-type cells, accounting for 62 and 21%, respectively, of the total bypassed products. Even in the *rev1* or *rev3* mutant cells that lack the Polζ mutagenesis pathway, G was still not significantly inserted opposite the AP site. Similarly, G insertion opposite the AP site was not observed in a study by Nelson *et al.* (19). Therefore, although Polη possesses an intrinsic biochemical activity of G insertion opposite AP sites, this activity is not significantly recruited for translesion synthesis of AP sites *in vivo*. This underscores the importance of performing *in vivo* genetic experiments to validate the *in vitro* biochemical results obtained from the various translesion polymerases. Nevertheless, it is possible that Polη may play a minor role in catalyzing G and A insertions opposite AP sites, but G was less effectively extended than A by Polζ (9). In such a case, Polη would make a small contribution to the detected A insertion during AP site bypass in yeast cells.

Then, what is the major role of Polη in translesion synthesis of AP sites in yeast cells? Our genetic and biochemical results together suggest that an important role of Polη in AP site bypass is to catalyze extension synthesis from the primer 3' A opposite the lesion. This activity was detected by *in vitro*

biochemistry, and was supported by *in vivo* results that A insertion opposite the AP site was reduced approximately 2-fold in *rad30* mutant cells as compared to the wild-type cells (Table 2). We found that extension from opposite the AP site by Polη was most efficient from the primer 3' A, much less efficient from the primer 3' G, and not detected from the primer 3' C or T (Figure 5A). These biochemical properties are also observed with the human Polη (45). Additionally, our genetic experiments suggest that Polη facilitates AP site bypass with C insertion in yeast cells (Table 2). Since Polη is unable to perform effective C insertion or extension synthesis from C opposite the AP site, this polymerase may somehow facilitate Rev1-catalyzed C insertion opposite the lesion. Most recently, it was reported that mouse Polη physically interacts with mouse Rev1 at its C-terminal region (46). Whether there exists a similar interaction between the yeast proteins and whether such an interaction is involved in the putative stimulatory action of Polη on the Rev1 dCMP transferase remain to be determined.

Rev1 efficiently inserts a C opposite a template AP site *in vitro* (17,18,35). *In vivo*, C insertion opposite the AP site was abolished in *rev1* mutant cells. Therefore, in wild-type cells, C insertion opposite the AP site is catalyzed by the Rev1 dCMP transferase. The subsequent extension synthesis, however, requires another polymerase. *In vitro*, Polζ is capable of extension synthesis from opposite an AP site (9,17,21). Thus, it is believed that Polζ is an important extension polymerase during translesion synthesis (9,17,21). Consistent with this notion, C insertion was also abolished in *rev3* mutant cells, and A insertion was reduced by approximately 2-fold opposite the AP site. In cells lacking Polζ, the significant residual extension activity from 3' A opposite the AP site probably resulted from Polη activity. Supporting this interpretation, AP site bypass with A insertion opposite the lesion was nearly abolished in cells lacking both Polζ and Polη.

In mutant cells lacking Polη, Polζ or Rev1, A was still significantly inserted opposite the AP site. Thus, a replicative polymerase is likely responsible for the A insertion opposite the AP site in yeast cells. Indeed, opposite an AP site, Polα possesses a significant A insertion activity and Polδ has a weak A insertion capacity *in vitro* (Figure 7) (9). Since A insertion by Polδ is greatly limited by its 3'→5' proofreading exonuclease activity, Polα likely plays a significant role in A insertion opposite the AP site in yeast cells. The rare G insertions in the *rad30* and *rev3* mutant cells (Table 2) were likely catalyzed by Polζ and Polη, respectively. The precise mechanism of complex translesion synthesis involving base substitutions and deletions on both sides of the AP site is not clear. Nevertheless, Polζ and Polη may be involved, as indicated by a reduction of this type of bypass products in the respective mutant strains (Table 2).

Taken together both *in vitro* and *in vivo* results, we propose a model for translesion synthesis of AP sites in yeast cells as shown in Figure 9. In our model, most AP sites in DNA block the replicative polymerases before the lesion. Thus, a major mechanism of translesion synthesis is mediated by C insertion opposite the AP site catalyzed by Rev1 and somehow facilitated by Polη. Then, translesion synthesis is completed by Polζ-catalyzed extension (Figure 9). There is another parallel mechanism for translesion synthesis of AP sites, in which Polα and Polδ insert A opposite the lesion. Polη may also

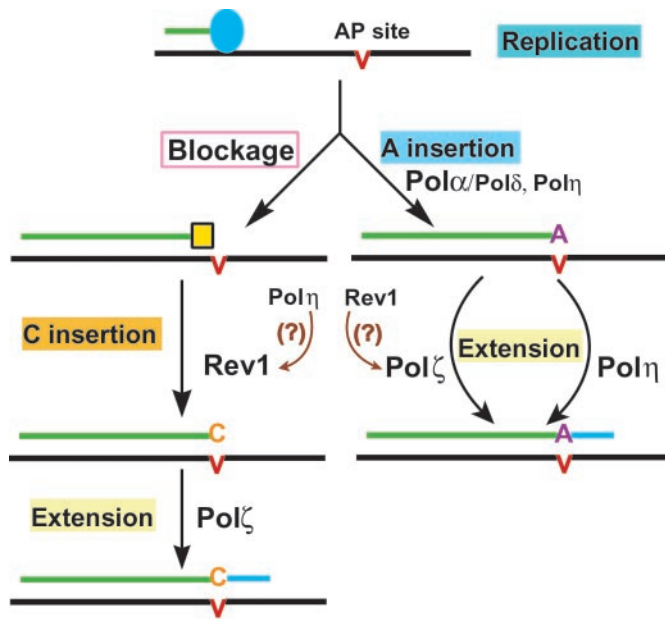


Figure 9. A mechanistic model of AP site bypass in yeast cells. When the replication complex (represented by the filled blue oval) encounters AP sites in the DNA template, two parallel bypass mechanisms can occur. In one mechanism (scheme on the left side), the replicative polymerase is blocked by the lesion and subsequently replaced by Rev1. C insertion is catalyzed by Rev1 and stimulated by Pol η . Bypass is completed by Pol ζ -catalyzed extension from opposite the lesion. In the other mechanism (scheme on the right), A is inserted opposite the AP site by Pol α and Pol δ . Pol η may also catalyze A insertion to a limited extent. Extension is then catalyzed independently by Pol ζ or Pol η . Rev1-catalyzed C insertion may be facilitated by Pol η , and Pol ζ -catalyzed extension may be facilitated by Rev1, although the precise nature of such facilitation is not clear yet.

contribute to a small fraction of A insertions opposite the lesion. Subsequent extension synthesis is then catalyzed independently by Pol ζ and Pol η (Figure 9). Rev1 probably plays an accessory role for Pol ζ -catalyzed extension from the primer 3' A opposite the AP site (Figure 9). The precise role of Rev1, however, is not known. Supporting a role of Rev1 in Pol ζ -catalyzed extension, A insertion was reduced by approximately 2-fold in the *rev1* mutant cells and was largely abolished in *rev1 rad30* double mutant cells (Table 2), suggesting a synergistic effect of Rev1 and Pol η in extension synthesis from A-terminated primers opposite AP sites. In the *rev3 rad30* double mutant cells, extensions in the two paralleled bypass mechanisms are completely blocked, leading to the most severe defect in AP site bypass, as indicated by results from both the *in vivo* translesion synthesis experiments and the cellular sensitivity to MMS.

Lawrence and colleagues (8,19) originally reported that Rev1-catalyzed C insertion opposite the lesion was the predominant mechanism of AP site bypass in yeast cells. However, Haracska *et al.* (9) recently concluded that Pol δ -catalyzed A insertion was the major mechanism of AP site bypass. Our model of AP site bypass could provide an explanation for these conflicting reports. Our studies and those of Lawrence and colleagues with wild-type cells were performed under normal growth conditions, thus yielding similar results of AP site bypass. Haracska *et al.* (9), however, performed their experiments with the *anp1 anp2* double mutant strain under MMS treatment conditions that killed nearly all cells

(<0.3% cell survival). The double mutant cells are unable to repair AP sites in DNA. Hence, MMS treatment of these cells would yield excessive unrepaired AP sites in the genome during replication. Excessive AP sites may lead to saturation of the Rev1-catalyzed C insertion mechanism (Figure 9). Consequently, the majority of AP sites would have to be bypassed by the Pol α -catalyzed A insertion mechanism (Figure 9), resulting in predominant A insertion opposite AP sites under the experimental conditions of Haracska *et al.* (9).

As indicated by our results and our model (Figure 9), AP site bypass in yeast cells is a complex cellular process involving different mechanisms and multiple polymerases. Such complexity offers some levels of functional redundancy. Indeed, some residual levels of AP site bypass can still take place in the absence of any single translesion polymerase. In higher eukaryotes, AP site bypass is further complicated by the presence of two more Y family translesion polymerases: Polk and Polt, which efficiently and predominantly insert an A and a G, respectively, opposite a template AP site *in vitro* (47–49). Thus, these two polymerases may participate in nucleotide insertion during AP site bypass in mammalian cells. Furthermore, Polk is able to perform efficient extension synthesis by a –1 deletion mechanism in certain sequence contexts *in vitro* (47,49), raising the possibility that it may also participate in extension synthesis to some extent from the primer 3' A opposite the AP site. A better understanding of AP site bypass in higher eukaryotes should be facilitated by genetic analyses as we have performed and the conceptual model we have generated here in the yeast model system.

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