Roles of yeast DNA polymerases δ and ζ and of Rev1 in the bypass of abasic sites

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Abasic (AP) sites are one of the most frequently formed lesions in DNA, and they present a strong block to continued synthesis by the replicative DNA machinery. Here we show efficient bypass of an AP site by the combined action of yeast DNA polymerases δ and ζ. In this reaction, Polδ inserts an A nucleotide opposite the AP site, and Polζ subsequently extends from the inserted nucleotide. Consistent with these observations, sequence analyses of mutations in the yeast *CAN1*^{s} gene indicate that A is the nucleotide inserted most often opposite AP sites. The nucleotides C, G, and T are also incorporated, but much less frequently. Enzymes such as Rev1 and Polη may contribute to the insertion of these other nucleotides; the predominant role of Rev1 in AP bypass, however, is likely to be structural. Steady-state kinetic analyses show that Polζ is highly inefficient in incorporating nucleotides opposite the AP site, but it efficiently extends from nucleotides, particularly an A, inserted opposite this lesion. Thus, in eukaryotes, bypass of an AP site requires the sequential action of two DNA polymerases, wherein the extension step depends solely upon Polζ, but the insertion step can be quite varied, involving not only the predominant action of the replicative DNA polymerase, Polδ, but also the less prominent role of various translesion synthesis polymerases.

[*Key Words*: Abasic sites; mutagenic bypass; yeast; DNA polymerase δ; DNA polymerase ζ]

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Abasic (AP) sites represent one of the most frequently formed DNA lesions in eukaryotes, and it has been estimated that a human cell loses as many as 10^4 purines per day from its genome (Lindahl and Nyberg 1972). In *Saccharomyces cerevisiae*, AP sites are efficiently repaired by the AP endonucleases encoded by the *APN1* and *APN2* genes. *APN1* and *APN2* provide alternate pathways for the removal of AP sites, and consequently, simultaneous inactivation of both the genes results in a dramatic decline in the efficiency to repair AP sites (Johnson et al. 1998).

If the AP sites are not removed by excision repair processes, they present a block to the replication machinery. During replication, AP sites can be bypassed either by a specialized mutagenic DNA polymerase, or by error-free mechanisms such as recombination or a copy-choice type of DNA synthesis. In *S. cerevisiae*, genes in the *RAD6* epistasis group promote replication through DNA lesions (Prakash 1981). The *REV1*, *REV3*, and *REV7* genes of this group are essential for UV-induced mutagenesis (Lawrence and Hinkle 1996), and these genes are also indispensable for mutagenesis induced by AP sites (Johnson et al. 1998). *REV1* encodes a deoxycytidyl transferase activity (Nelson et al. 1996a), and the *REV3*- and *REV7*-encoded proteins together form DNA polymerase ζ (Nelson et al. 1996b).

Although Polζ is absolutely required for damage-induced mutagenesis, and therefore for the mutagenic bypass of a variety of DNA lesions, our recent studies have indicated that on its own, Polζ bypasses UV lesions very inefficiently (Johnson et al. 2000a). This is because Polζ is very inefficient in inserting nucleotides opposite the 3′ T of a *cis–syn* thymine–thymine (T–T) dimer or a (6–4) T–T photoproduct. Polζ, however, efficiently extends from nucleotides placed opposite the 3′ T of these lesions by another DNA polymerase (Johnson et al. 2000a). Because Rev1 can insert a C opposite the AP site (Nelson et al. 1996a), and because Rev1 is essential for mutagenesis induced by AP sites (Johnson et al. 1998), in principle, mutagenic bypass of AP sites could be subsumed by the combined action of Rev1 and Polζ. However, we find that mutational inactivation of the Rev1 C-transferase activity has little effect on AP mutagenesis, and sequence analyses of mutations resulting from AP bypass indicate that in vivo, C is inserted rather infrequently opposite AP sites. These observations suggest that the C-transferase activity of Rev1 is not likely to have a predominant role in AP bypass.

In addition to the requirement of Rev1 and Polζ, genetic studies in yeast have indicated a requirement of Polδ for the mutagenic bypass of DNA lesions. A muta-

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tion, *pol3-13*, in the catalytic subunit of Polδ confers a deficiency in UV mutagenesis (Giot et al. 1997), and deletion of *POL32*, which encodes a nonessential subunit of Polδ, also lowers UV mutagenesis (Gerik et al. 1998). Because Polδ is essential for the replication of both DNA strands and will be the first polymerase to encounter the DNA lesion, it could promote damage bypass by inserting a nucleotide opposite the lesion, which could then be extended by Polζ. Here we provide evidence supporting such a role for Polδ in AP bypass. From the pattern of mutations induced by AP sites in yeast, we infer that in vivo, A is the residue inserted most frequently opposite AP sites, and our biochemical studies indicate that Polδ primarily inserts an A opposite the AP site. Efficient bypass of this lesion occurs when Polδ is combined with Polζ.

Results

Spectrum of CAN1^s *to* can1^r *mutations resulting from AP bypass*

To determine the mutational specificity of AP sites in vivo, we sequenced mutations resulting from their bypass in yeast cells treated with the alkylating agent methyl methanesulfonate (MMS). MMS alkylates the bases in DNA, particularly adenine at the N3 position (3MeA) and guanine at the N7 position (7MeG). The removal of alkylated bases by an *N*-methyl purine DNA glycosylase (Roy et al. 1994; Bjoras et al. 1995) results in an AP site that can be acted upon by the AP endonuclease activity of Apn1 or Apn2 proteins. We examined MMS-induced *CAN1^s* to *can1^r* forward mutations in the *apn1*Δ *apn2*Δ strain because the repair of AP sites is severely inhibited in this strain. Sequence analysis of a number of independent *can1^r* mutations formed in the *apn1*Δ *apn2*Δ strain after MMS treatment indicated that ~70% of these were base substitutions and ~30% were +1 or –1 frameshift mutations (Table 1). The most frequent base substitutions were G : C to T : A transversions (40%). Considering that the majority of AP sites arise from the loss of A or G residues, we calculate that A, C, G, and T nucleotides were inserted opposite AP sites with relative frequencies of 64%, 14%, 11%, and 11%, respectively. These observations indicate that, in vivo, incorporation of an A residue opposite the AP site is the major base substitution caused by this DNA lesion.

Table 1. *MMS induced* can1^r *mutations in the* apn1Δ apn2Δ *strain*

Mutations	Number (frequency)
A:T → T:A	2 (5%)
G:C → T:A	16 (40%)
G:C → C:G	3 (7.5%)
A:T → G:C	4 (10%)
G:C → A:T	3 (7.5%)
+1 frameshifts	7 (17.5%)
–1 frameshifts	5 (12.5%)

Yeast cells were suspended in 0.05 M KPO_4 (pH 7) buffer, and treated with 0.08% MMS for 20 min at 30°C. Following termination of the reaction by the addition of an equal volume of 10% sodium thiosulfate, cells were spun down, resuspended in water, and plated on synthetic complete medium lacking arginine and supplemented with canavanine.

The deoxycytidyl transferase activity of Rev1 is dispensable for mutagenesis induced by AP sites

To assess the in vivo role of the deoxycytidyl transferase activity of Rev1 in mutagenic bypass of AP sites, we examined the effect of inactivation of this activity on mutagenesis induced by AP sites. For this purpose, we altered the Asp 467 and Glu 468 residues present in the highly conserved motif III consisting of serine, isoleucine, aspartate, and glutamate residues (SIDE) in Rev1 to alanines (Johnson et al. 1999). The analogous mutation in Rad30 inactivates the DNA polymerase activity (Johnson et al. 1999), and similarly, the *rev1 Ala^{467}–Ala^{468}* mutation completely inactivated the deoxycytidyl transferase activity of Rev1 (data not shown). To determine whether Rev1 deoxycytidyl transferase activity was required for AP mutagenesis, the ability of the *rev1 Ala^{467}–Ala^{468}* mutation to complement the *rev1*Δ mutation was examined. The wild-type or the mutant gene was expressed in yeast from the native *REV1* promoter on a low-copy *CEN* plasmid. Yeast cells were treated with various concentrations of MMS, and the rates of forward mutations at the *CAN1^s* locus were examined. Whereas MMS-induced *can1^r* mutations are completely abolished in the *apn1*Δ *apn2*Δ *rev1*Δ strain (Johnson et al. 1998), introduction of the *rev1 Ala^{467}–Ala^{468}* mutant gene into this strain restored MMS-induced *can1^r* mutations to the level seen in the *apn1*Δ *apn2*Δ strain (data not shown). Thus, although the Rev1 protein is absolutely required for damage-induced mutagenesis, its deoxycytidyl transferase activity is dispensable for mutagenesis induced by AP sites.

*Requirement of Pol*δ *for mutagenic bypass of AP sites*

Yeast Polδ is comprised of three subunits of 125, 58, and 55 kD, which are encoded by the *POL3*, *POL31*, and *POL32* genes, respectively (Gerik et al. 1998). The 125-kD catalytic subunit and the 58-kD subunit are essential for viability, but the 55-kD subunit encoded by the *POL32* gene is not essential (Gerik et al. 1998). A mutation in the catalytic subunit of Polδ, *pol3-13*, confers UV sensitivity and a deficiency in UV mutagenesis (Giot et al. 1997). The *pol32*Δ mutant is also UV-sensitive and deficient in UV mutagenesis (Gerik et al. 1998). To determine the role of Polδ in mutagenesis induced by AP sites, we examined the effect of the *pol32*Δ mutation on MMS-induced *CAN1^s* to *can1^r* mutations in the *apn1*Δ *apn2*Δ strain. The *pol32*Δ mutant exhibits somewhat enhanced sensitivity to MMS, and MMS-induced mutations do not occur in this strain (Fig. 1; Gerik et al. 1998). For example, treatment with 0.4% MMS produced ~1000 *can1^r* mutants per 10^7 viable cells in the wild-type strain, whereas MMS-induced mutagenesis was abolished in the *pol32*Δ single mutant (Fig. 1B). These obser-

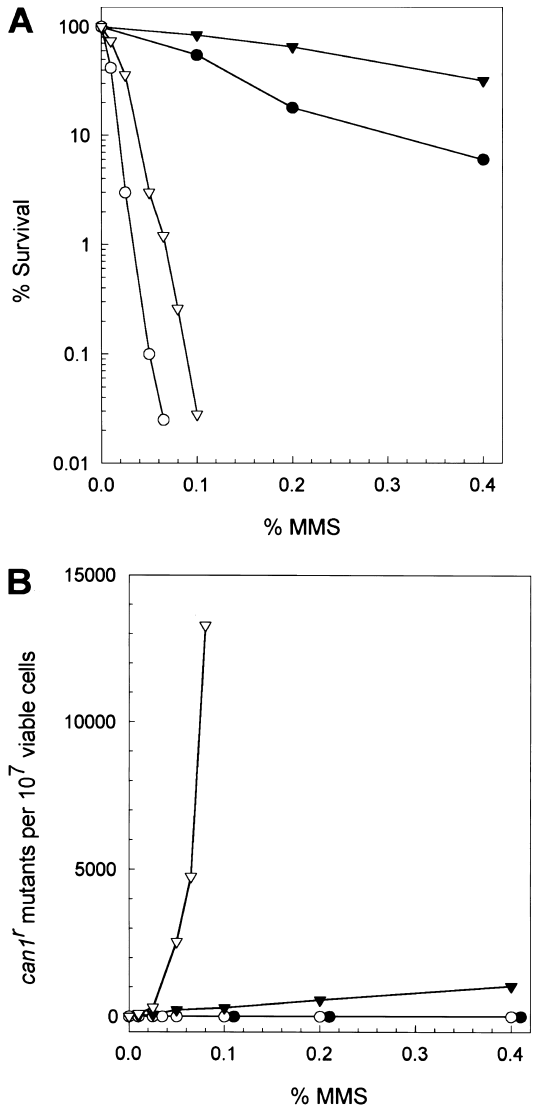

Figure 1. Effect of the *pol32*Δ mutation on AP site induced mutagenesis. (*A*) MMS sensitivity of *pol32*Δ strains. Cells were grown overnight in YPD and treated with MMS at the indicated doses for 20 min. Appropriate dilutions were plated on YPD. All data points represent the average of at least three experiments. (*Filled inverted triangles*) EMY74.7, wild type; (*filled circles*) YPO69, *pol32*Δ; (*open inverted triangles*) YRP269, *apn1*Δ *apn2*Δ; (*open circles*) YPO71, *apn1*Δ *apn2*Δ *pol32*Δ. (*B*) MMS-induced mutagenesis in *pol32*Δ strains. Cells were grown overnight and treated with MMS at the indicated doses for 20 min. Appropriate dilutions were plated on either YPD for viability or on synthetic medium lacking arginine but containing canavanine for measuring the frequency of *can1*^{r} induced mutations. All data points represent of the average of at least three experiments. (*Filled inverted triangles*) EMY74.7, wild type; (*filled circles*) YPO69, *pol32*Δ; (*open inverted triangles*) YRP269, *apn1*Δ *apn2*Δ; (*open circles*) YPO71, *apn1*Δ *apn2*Δ *pol32*Δ.

vations indicate a role for the Pol32 subunit of Polδ in the mutagenic bypass of MMS-induced base damage. The *apn1*Δ *apn2*Δ strain displays a very high incidence of MMS-induced mutations, ~13,000 per 10^7 cells at 0.08% MMS, owing to the presence of unrepaired AP sites. By contrast, MMS-induced mutagenesis was abolished in the *apn1*Δ *apn2*Δ *pol32*Δ strain (Fig. 1B). The *apn1*Δ *apn2*Δ *pol32*Δ strain also exhibits a greater sensitivity to MMS than the *apn1*Δ *apn2*Δ strain does (Fig. 1A). These results indicate a requirement of the Pol32 subunit of Polδ for the mutagenic bypass of AP sites.

AP bypass by the concerted action of Polδ and Polζ

AP bypass was examined with a running-start and a standing-start DNA substrate that were constructed using a linear 75-nt template DNA containing a single AP site, or, as a control, containing a C residue instead of the AP site, and annealed to a 5′-^{32}P-labeled 29-nt or a 44-nt primer, respectively. DNA synthesis reactions with these substrates were carried out in the presence of Polδ and Polζ. We also included Rev1 in these experiments (Fig. 2). With the running-start DNA substrate, none of the individual Polδ, Polζ, or Rev1 enzymes were able to bypass the AP site (Fig. 2A, lanes 10–12). The AP site is a strong block to synthesis by Polζ, and Polζ was unable to insert a nucleotide opposite this lesion (Fig. 2A, lane 10). By contrast, Polδ catalyzed nucleotide incorporation opposite the AP site but did not extend the resulting primer end (Fig. 2A, lane 11). In the control reactions, both polymerases carried out efficient synthesis to the end of the unmodified template (Fig. 2A, lanes 2,3). Efficient bypass of the AP site, however, could be achieved when Polδ was combined with Polζ (Fig. 2A, lane 13). Compared to the synthesis on undamaged DNA (Fig. 2A, lane 5), Polδ and Polζ together replicated through 55% of the AP sites (Fig. 2A, lane 13). In agreement with the previously published ability of Rev1 to promote AP bypass in combination with Polζ (Nelson et al. 1996a), Rev1 and Polζ together replicated through 32% of the AP sites (Fig. 2A, cf. lanes 6 and 14). However, Rev1 did not stimulate AP bypass when it was combined with Polδ (Fig. 2A, lane 15), nor did it increase the bypass activity of the Polδ/Polζ combination (Fig. 2A, cf. lanes 13 and 16). The key observation here is that the two DNA polymerases, Polδ and Polζ, together carry out efficient AP bypass, and in this reaction, Polδ inserts the nucleotide opposite the AP site and Polζ then extends from that nucleotide.

With the standing-start substrate, essentially similar results were observed (Fig. 2B). Thus, the combination of Polδ and Polζ bypassed the AP site about 60% as efficiently as the replication of undamaged DNA (Fig. 2B, cf. lanes 5 and 13).

Nucleotide incorporated opposite the AP site by Polδ

To identify the deoxynucleotide inserted by Polδ opposite the AP site, we used an 18-nt template having an AP site at position 13 from the 3′ end and primed with a standing-start 12-nt primer (Fig. 3). As markers we used 13-nt oligonucleotides containing the 12-nt primer with an additional C, A, T, or G residue at position 13; these were distinguished by their relative electrophoretic mo-

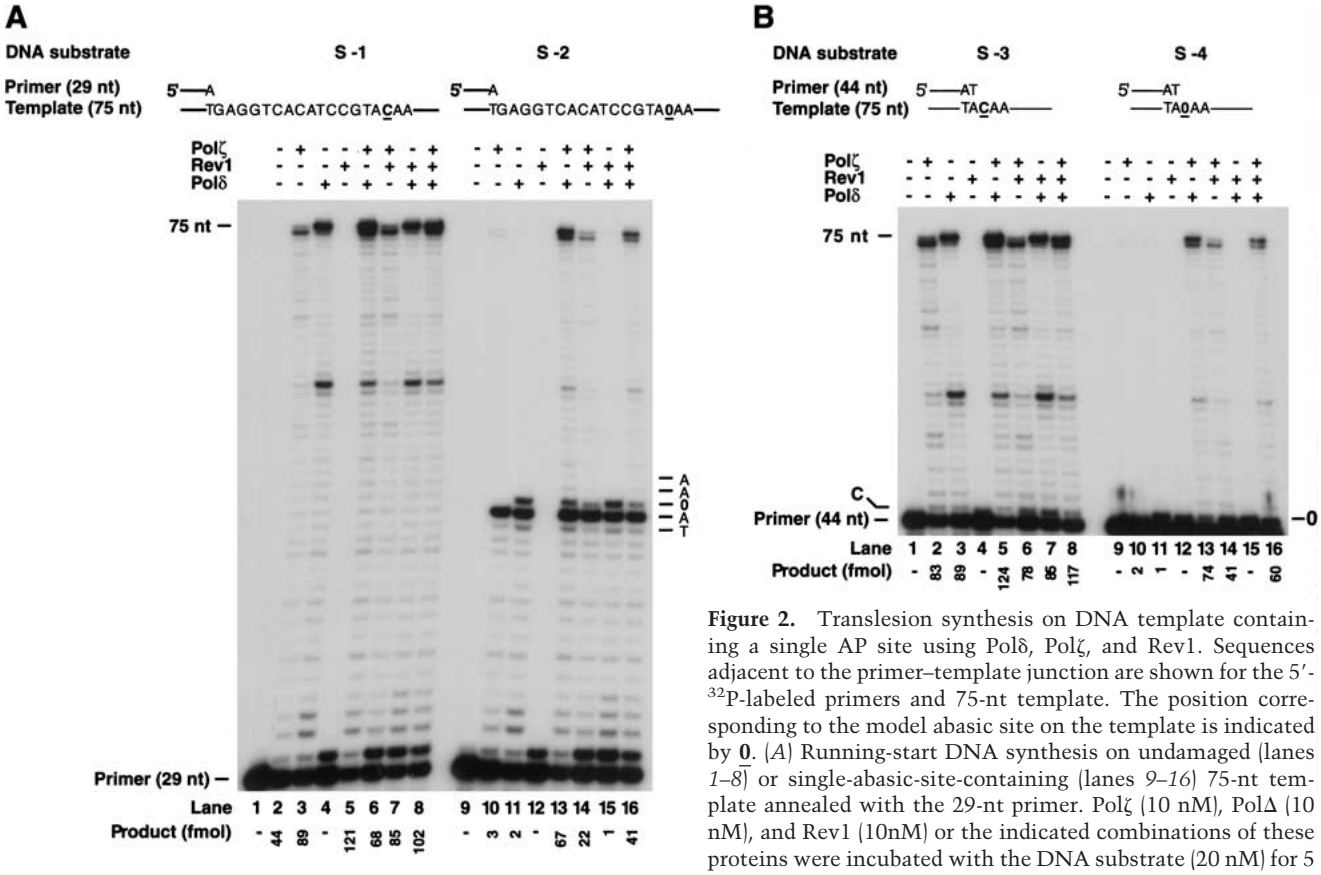

Figure 2. Translesion synthesis on DNA template containing a single AP site using Polδ, Polζ, and Rev1. Sequences adjacent to the primer–template junction are shown for the 5′-^{32}P-labeled primers and 75-nt template. The position corresponding to the model abasic site on the template is indicated by **0**. (*A*) Running-start DNA synthesis on undamaged (lanes *1–8*) or single-abasic-site-containing (lanes *9–16*) 75-nt template annealed with the 29-nt primer. Polζ (10 nM), PolΔ (10 nM), and Rev1 (10nM) or the indicated combinations of these proteins were incubated with the DNA substrate (20 nM) for 5 min at 30°C in the presence of 100 μM of each of four dNTPs. The reaction products were resolved on 10% denaturing polyacrylamide gel and visualized by autoradiography. The gel was analyzed by PhosphorImager and the fraction of total radioactivity present as 46- to 75-nt products was used to calculate femtomoles of product. (*B*) Standing-start DNA synthesis on an undamaged (lanes *1–8*) or a single-AP-site-containing (lanes *9–16*) 75-nt template annealed with the 44-nt primer. The reactions and the analysis were carried out as in *A*.

bility on a 20% polyacrylamide gel (Fig. 3, lanes 9–12). We found that Polδ mostly inserts an A (~95%) and some G (~5%) opposite the AP site (Fig. 3, lane 3), and Polζ extends from the inserted nucleotide (Fig. 3, lane 5). We also examined the ability of Rev1 and Polζ to insert nucleotides opposite the AP site and to extend therefrom on this template. As expected, Rev1 incorporates only a C across from the lesion (Fig. 3, lane 4), and Polζ extends from this nucleotide (Fig. 3, lane 6). When Polδ and Rev1 are combined, both A and C are inserted opposite the AP site, but there is no extension (Fig. 3, lane 7); extension, however, occurs upon the addition of Polζ (Fig. 3, lane 8).

Steady-state kinetic analyses of nucleotide insertion opposite the AP site and of subsequent extension by Polζ

Next, we measured the kinetic parameters of nucleotide insertion opposite the AP site and of subsequent extension by Polζ. The kinetics of insertion of a single deoxynucleotide opposite the AP site, and as a control opposite an undamaged C or G residue, and the kinetics of addition of the next correct nucleotide to various 3′-primer termini situated across from the AP site or opposite undamaged C or G residues were determined as a function of deoxynucleotide concentration under steady-state conditions. The patterns of insertion of nucleotides opposite the AP site and of extension from the G, A, T, or C 3′-primer termini situated across from the AP site are shown in Figure 4. From the kinetics of deoxynucleotide incorporation, the apparent K_m and V_{max} values were determined, and the frequency of deoxynucleotide insertion (f_{inc}) and extension (f^0_{ext}) were calculated (Mendelman et al. 1990; Goodman et al. 1993; Creighton et al. 1995). As shown in Table 2, Polζ misincorporates nucleotides opposite a template C or G with a frequency of ~10^{-4}, and it inserts nucleotides opposite the AP site with a frequency of 10^{-4} to 10^{-5}. However, Polζ extends from the primer end situated opposite the abasic site very efficiently. Compared to the extension from a G opposite C in the template, Polζ extended from an A, a G, a C, and a T residue opposite the AP site with frequencies of ~3×10^{-1}, 1×10^{-1}, 2×10^{-2}, and 7×10^{-3}, respectively (Table 3). Thus, Polζ extends from an A or a G opposite the AP site about 3- and 10-fold less efficiently than it extends from a G opposite template C, whereas C opposite the AP site is extended about 50-fold less efficiently than is G opposite C. Polζ also extends

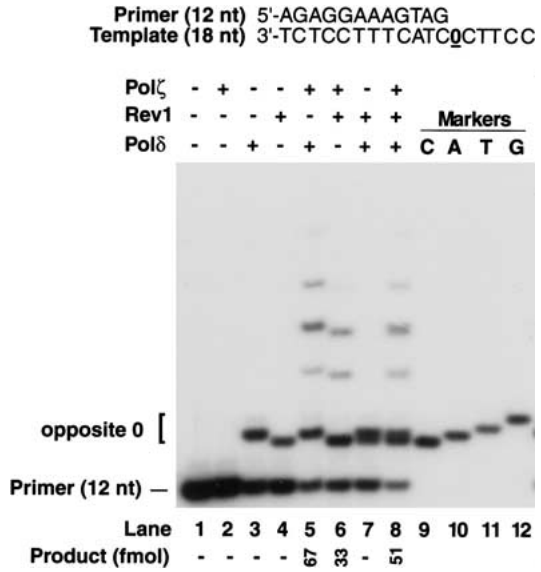

Figure 3. Nucleotide incorporation opposite AP site and extension from the ensuing primer end. Nucleotide incorporation opposite an AP site in reactions catalyzed by different combinations of Polζ, Polδ, and Rev1. Standing-start primer extension reactions were carried out on an AP-site-containing 18-nt template primed with the 5′-^{32}P-labeled 12-nt oligonucleotide. The position of the AP site on the template is indicated by **0**. The following enzyme concentrations were used: Polζ (20 nM), Polδ (20 nM), and Rev1 (20 nM). Electrophoretic mobilities of reaction products were compared with those of 13-nt oligonucleotide standards containing a C (lane *9*), an A (lane *10*), a T (lane *11*), or a G (lane *12*) residue opposite the AP site at position 13.

from 3′-terminal mispaired ends quite readily. For example, an A opposite template C is extended about 25% as efficiently as a G opposite template C. Overall, Polζ extends from base mispairs with frequencies of 10^{-1} to 10^{-2} (Table 3).

Polζ extension of primer end situated opposite the AP site

Next, we examined the ability of Polζ to extend from various 3′ termini situated across from the AP site in the 18-nt template in the presence of 100 μM of each of the four dNTPs (Fig. 5). Although Polζ extended each of the C, A, T, or G 3′-primer ends situated opposite the AP site (Fig. 5, lanes 2–5), extension from A was ~5-fold more efficient than from the C primer end, and the order and the frequency of extension from various 3′-terminal deoxynucleotides were A : G : C : T = 9.8 : 6.7 : 1.7 : 1. Thus, even under saturating dNTP concentrations, Polζ shows a preference for extending from an A opposite the AP site.

Discussion

To determine the mutational specificity of AP sites in eukaryotes, we analyzed the sequence of *can1*^{r} mutations obtained following MMS treatment of the *apn1*Δ *apn2*Δ yeast strain. Our results indicate that A is inserted most frequently opposite the AP site, and that C, G, and T together are inserted at about 50% the frequency of A. Previously, in one study done in yeast with plasmids containing an AP site, C was found to be inserted preferentially (~80%) opposite this lesion (Gibbs and Lawrence 1995), whereas in another study with the *SUP4* gene carried on a plasmid, the frequency of spontaneous A · T → C · G events increased in the *apn1*Δ strain (Kunz et al. 1994), suggesting an insertion of G opposite the AP site resulting from the loss of an A. Thus, the mutagenic consequences of AP sites in yeast may differ depending on whether the lesion is on a plasmid or in the genome. Also, we note that mutagenesis at the *SUP4* locus differs from mutagenesis at the majority of loci (Drake 1991).

NMR structural studies have shown that A fits into the double helix opposite the AP site without conferring any distortion. Such DNA retains all aspects of B-form DNA, in which the A residue and the abasic residue lie inside the double helix, and the melting temperature of the A · AP site is the same as that of the A · T base pair (Cuniasse et al. 1987, 1990; Kalnik et al. 1988). Thus, the geometry of the A residue opposite the AP site is very similar to that of the A · T base pair. G opposite the AP site is less stable, but at low temperatures this residue is also predominantly intrahelical (Cuniasse et al. 1990). By contrast, a pyrimidine opposite the AP site is not stable, and in this case, both the pyrimidine and the abasic sugar are extrahelical (Cuniasse et al. 1990). In *Escherichia coli*, synthesis past an AP site is accompanied by the preferential incorporation of an A opposite this lesion, a phenomenon known as the "A-rule" (Strauss 1991). Polδ may act in a similar fashion, as it predominantly inserts an A opposite the AP site. Although the geometry of an A · AP pair resembles that of the A · T base pair, Polδ nonetheless can distinguish between these pairs, since it does not extend from the A residue of an A · AP pair.

Genetic studies in yeast have indicated the absolute requirement of Polζ for mutagenesis induced by AP sites (Johnson et al. 1998). However, on its own, Polζ bypasses this lesion very poorly. This is because Polζ is highly inefficient in inserting deoxynucleotides opposite the AP site, but it efficiently extends from the nucleotide inserted opposite the AP site by another DNA polymerase. Steady-state kinetic analyses further demonstrate that Polζ extends most efficiently from an A opposite the AP site. Relative to the extension from G opposite template C, Polζ extends from an A opposite the AP site with a frequency of 3×10^{-1}, and it extends from a G opposite this lesion with a frequency of ~10^{-1}. Thus, the insertion of an A opposite the AP site by Polδ, followed by the efficient extension of this primer end by Polζ, explains our in vivo mutagenesis results of the preferential incorporation of an A opposite this DNA lesion.

In addition to the insertion of an A, we also observe the insertion of C, G, and T residues opposite the AP site. Although our studies on mutational inactivation of Rev1 C-transferase activity have provided no evidence for the requirement of this activity in the mutagenic

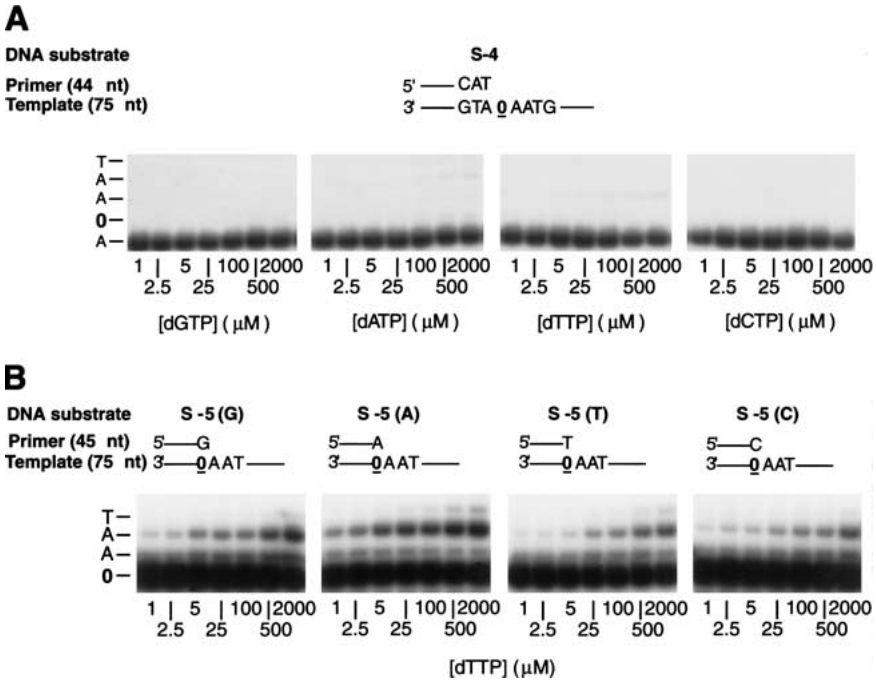

Figure 4. Steady-state deoxynucleotide incorporation and primer-extension reactions catalyzed by Polζ on an AP-site-containing DNA template. (*A*) Deoxynucleotide incorporation opposite a template AP site. Polζ (5 nM) was incubated for 1 min at 30°C with the S-4 primer–template DNA substrate (20 nM) and increasing concentrations (0–2000 μM) of a single deoxynucleotide (dGTP, dATP, dTTP, or dCTP) in the standard reaction buffer. The quenched samples were analyzed by 10% denaturing polyacrylamide gel electrophoresis. (*B*) Extension of primers containing a G, an A, a T, or a C residue opposite the AP site by Polζ. Primers differing only in the last nucleotide at the 3′ end were annealed separately to an AP-site-containing DNA template as shown on the top. Reactions were carried out in the presence of increasing dTTP concentration (0–2000 μM) as in *A*. The position of the AP site in the template is indicated by 0.

bypass of AP sites, they do not rule out a minor contribution of this activity to AP bypass. Since only ~10% of *can1*^{r} mutations are expected to derive from the insertion of C opposite the AP site, it would be difficult to ascertain such a limited involvement of Rev1 C-transferase activity in AP bypass from the analyses of mutation frequencies. Furthermore, there is the possibility that enzymes other than Rev1 also insert a C opposite the AP site. Thus, although the Rev1 protein is essential for mutagenesis induced by AP sites, its C-transferase activity is not prominently involved in the bypass of this lesion. Presumably, the indispensability of the Rev1 protein for AP mutagenesis derives from its structural role during Polζ-dependent bypass of this lesion (see Fig. 6). Since Polδ would be the first polymerase to arrive at the AP site, we surmise that its ability to insert an A opposite this lesion supercedes the insertion of C by Rev1, and also, since Polζ extends from an A opposite the AP site about 15-fold more efficiently than from C (Table 3), we expect A to be incorporated much more frequently during AP bypass than is C. Unlike the efficient bypass of a *cis–syn* T–T dimer (Johnson et al. 2000b; Washington et al. 2000), or an 8-oxoguanine lesion (Haracska et al. 2000), Polη does not bypass an AP site; however, Polη may contribute to the insertion of a G opposite the AP site (Haracska et al. 2001). In humans, Polι, a very low fidelity enzyme, would additionally function in the insertion of nucleotides opposite the AP sites (Johnson et al. 2000a). Therefore, AP bypass in eukaryotes is mediated by the sequential action of two DNA polymerases,

Table 2. *Kinetic parameters of nucleotide insertion reactions catalyzed by yPolζ*

Site	dNTP added	K_m (μM)	V_{max} (%/min)	V_{max}/K_m	f_{inc}
Insertion opposite abasic site					
	dGTP	543 ± 42	1.4 ± 0.19	0.003	2.1×10^{-5}
5′----CAT	dATP	81 ± 2.9	4.8 ± 0.07	0.059	4.2×10^{-4}
----GTA**0** AA--	dTTP	125 ± 8.5	3.3 ± 0.09	0.026	1.8×10^{-4}
	dCTP	113 ± 14	1.4 ± 0.12	0.012	8.6×10^{-5}
Insertion opposite C					
	dGTP	0.11 ± 0.008	15.4 ± 2.0	140	1
5′----CAT	dATP	267 ± 18	2.1 ± 0.14	0.0078	5.5×10^{-5}
----GTA**C** AA--	dTTP	552 ± 96	3.8 ± 1.1	0.0069	4.9×10^{-5}
	dCTP	321 ± 14	2.9 ± 0.32	0.009	6.4×10^{-5}
Insertion opposite G					
	dGTP	344 ± 24	4.3 ± 0.12	0.0125	1×10^{-4}
5′----CAT	dATP	118 ± 5	1.4 ± 0.33	0.012	1×10^{-4}
----GTA**G** AA--	dTTP	428 ± 54	3.6 ± 0.28	0.0084	7×10^{-5}
	dCTP	0.14 ± 0.03	16.7 ± 1.1	119.3	1

Table 3. *Kinetic parameters of extension reactions catalyzed by yPolζ*

Site	dNTP added	K_m (μM)	V_{max} (%/min)	V_{max}/K_m	f^0_{ext}
Extension from G, A, T, or C opposite abasic site					
5′----CATG ----GTA**0** AA--	dTTP	6.5 ± 0.55	12 ± 2.2	1.85	9.5×10^{-2}
5′----CATA ----GTA**0** AA--	dTTP	2.3 ± 0.17	13 ± 1.5	5.65	2.9×10^{-1}
5′----CATT ----GTA**0** AA--	dTTP	49 ± 3.7	6.8 ± 0.24	0.14	7.2×10^{-3}
5′----CATC ----GTA**0** AA--	dTTP	21 ± 1.2	8.4 ± 1.3	0.4	2.1×10^{-2}
Extension from G, A, T or C opposite C					
5′----CATG -----GTA**C** AA--	dTTP	0.35 ± 0.02	6.8 ± 0.56	19.4	1
5′----CATA -----GTA**C** AA--	dTTP	2.3 ± 0.22	12 ± 0.89	5.22	2.6×10^{-1}
5′----CATT ----GTA**C** AA--	dTTP	15 ± 0.9	8.9 ± 1.3	0.59	3.0×10^{-2}
5′----CATC -----GTA**C** AA--	dTTP	26 ± 2.8	3.9 ± 0.08	0.15	7.7×10^{-3}
Extension from G, A, T or C opposite G					
5′----CATG ----GTA**G** AA--	dTTP	6.9 ± 0.2	10.7 ± 1.8	1.55	3.6×10^{-2}
5′----CATA -----GTA**G** AA--	dTTP	2.1 ± 0.14	8.4 ± 0.77	4.0	9.3×10^{-2}
5′----CATT -----GTA**G** AA--	dTTP	28.8 ± 4.6	14.1 ± 1.7	0.48	1.1×10^{-2}
5′----CATC -----GTA**G** AA--	dTTP	0.23 ± 0.018	9.9 ± 1.6	43	1

wherein the extension step depends solely on Polζ, but the insertion step can be quite varied, involving not only the predominant action of the replicative DNA polymerase, Polδ, but also the less prominent role of various translesion synthesis polymerases.

The absolute requirement of Polζ for the extension step explains its indispensability for AP mutagenesis. Our proposal that Polδ acts primarily in the insertion of an A, whereas Rev1 and the other translesion synthesis enzymes catalyze the insertion of C and of other nucleotides opposite the AP site, however, fails to explain the absolute requirement of the Pol32 subunit of Polδ and of Rev1 in AP mutagenesis. It is possible that the requirement of Pol32 reflects the need for this Polδ subunit in the assembly of Polζ and Rev1 at the lesion site (Fig. 6B). Because Polδ is involved in the replication of both the leading and lagging DNA strands and binds the two strands as a dimer, following the incorporation of a nucleotide opposite the AP site, Polδ may be displaced from the damage-containing template upon ubiquitin conjugation by the Rad6–Rad18 complex (Bailly et al. 1997), which is essential for damage bypass (Fig. 6A). If, however, Polδ is unable to insert a nucleotide opposite the AP site, or if the inserted nucleotide is removed by its proofreading exonuclease activity, then Rad6–Rad18-mediated displacement of Polδ may occur before the insertion step, whereupon any of the translesion synthesis enzymes such as Rev1, Polη, and additionally, Polι in

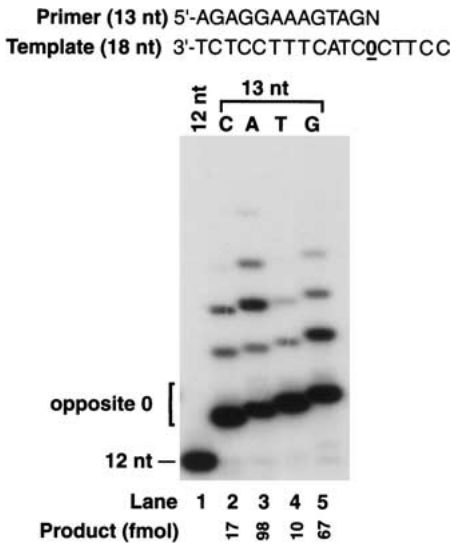

Figure 5. Extension of primers with various 3′-terminal primer dNMPs opposite the template AP site by Polζ. Four different 5′-^{32}P-labeled 13-nt primers, differing only in their 3′-terminal nucleotide, were annealed to an 18-nt template containing an AP site. In the template–primer shown, N designates the position of the variable terminal primer dNMP and **0** designates the position of the AP site. Lane *1* has a 12-nt primer and lacks the 13th nucleotide indicated by N. DNA substrate (20 nM) was assayed with 20 nM Polζ (lanes *1–5*) in the presence of each of the four dNTPs (100 μM).

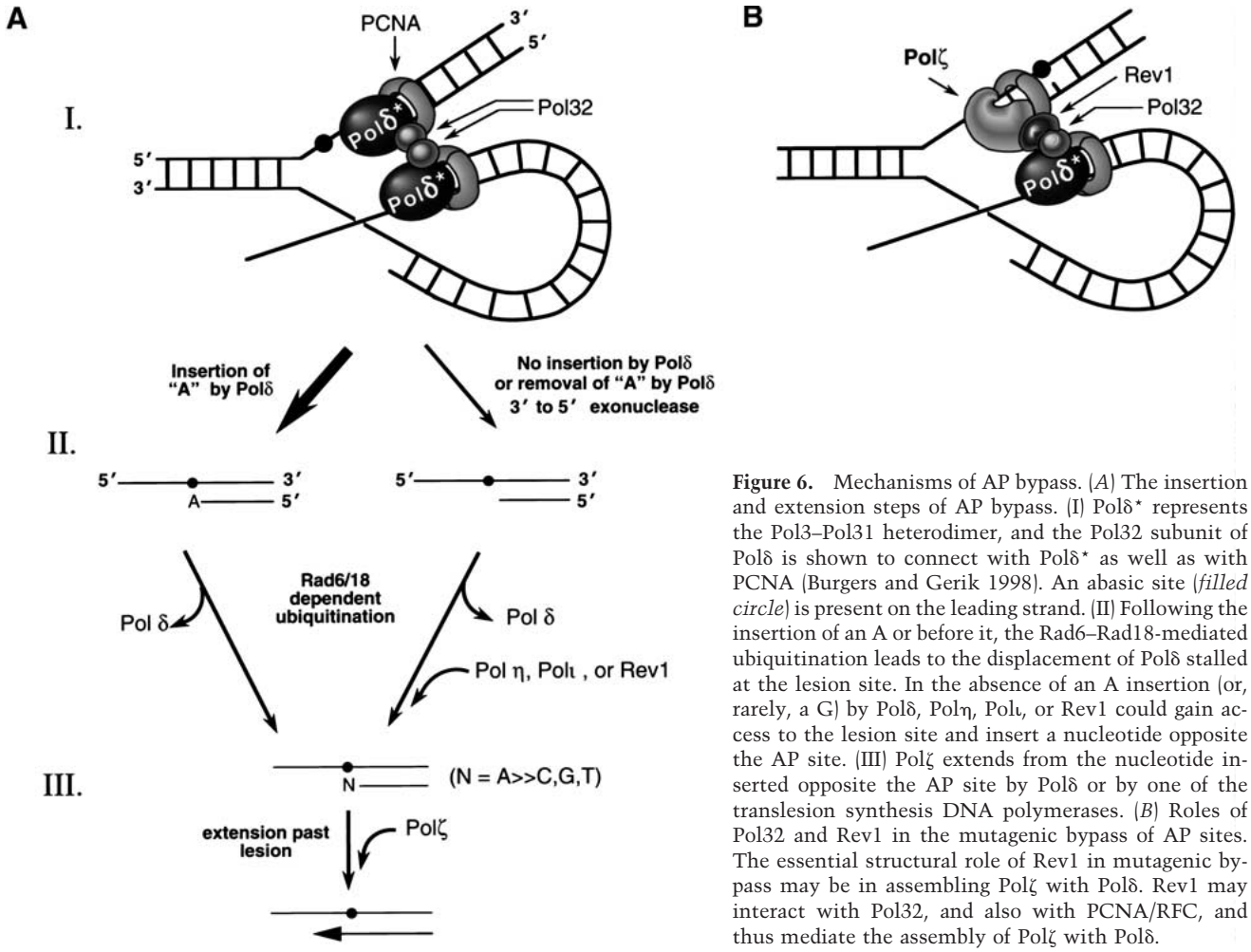

Figure 6. Mechanisms of AP bypass. (*A*) The insertion and extension steps of AP bypass. (I) Polδ* represents the Pol3–Pol31 heterodimer, and the Pol32 subunit of Polδ is shown to connect with Polδ* as well as with PCNA (Burgers and Gerik 1998). An abasic site (*filled circle*) is present on the leading strand. (II) Following the insertion of an A or before it, the Rad6–Rad18-mediated ubiquitination leads to the displacement of Polδ stalled at the lesion site. In the absence of an A insertion (or, rarely, a G) by Polδ, Polη, Polι, or Rev1 could gain access to the lesion site and insert a nucleotide opposite the AP site. (III) Polζ extends from the nucleotide inserted opposite the AP site by Polδ or by one of the translesion synthesis DNA polymerases. (*B*) Roles of Pol32 and Rev1 in the mutagenic bypass of AP sites. The essential structural role of Rev1 in mutagenic bypass may be in assembling Polζ with Polδ. Rev1 may interact with Pol32, and also with PCNA/RFC, and thus mediate the assembly of Polζ with Polδ.

humans, may gain access to the lesion site and insert a nucleotide opposite the AP site (Fig. 6A). Following the insertion of a nucleotide by Polδ or by one of the translesion synthesis polymerases, Polζ may be targeted to the lesion site via its interaction with Rev1, which in turn may bind the Pol32 subunit of Polδ remaining bound to the undamaged DNA strand (Fig. 6B). The nonenzymatic requirement of Rev1 may therefore reflect the need for this protein in the assembly of Polζ with Polδ via its Pol32 subunit. Rev1 may also facilitate interactions of Polζ with the other components of the replication machinery such as PCNA and RFC (Fig. 6B). Accordingly, the requirement of Pol32 as well as Rev1 for AP bypass may derive from their respective roles in providing access of Polζ to the lesion site.

The insertion of an A by Polδ opposite the AP site raises the possibility that Polδ is an A-rule polymerase, able to insert an A opposite various other DNA lesions as well (Strauss 1991). For example, in addition to the formation of cyclobutane dimers at TT sequences, UV induces lesions at the 5′-TC-3′ and 5′-CC-3′ sequences. The 3′-C in both sequences is highly mutagenic, and in both yeast and humans, UV-induced mutations occur predominantly by a 3′-C → T transition that results from the insertion of an A opposite the 3′ damaged C during DNA replication (Armstrong and Kunz 1990; Brash 1997; Canella and Seidman 2000). Genetic studies in yeast have shown that Polζ is responsible for the 3′-C → T mutagenesis resulting from the replicative bypass of UV lesions at TC and CC sites (Yu et al. 2001). However, because Polζ functions at the step of extending from the nucleotide incorporated opposite the 3′ residue of UV lesions by another DNA polymerase (Johnson et al. 2000a), we suggest that Polδ is the enzyme responsible for inserting an A opposite the 3′-C of UV lesions formed at TC and CC sites. Hence, by inserting an A opposite DNA lesions, Polδ could be a major contributor to DNA damage-induced mutagenesis in eukaryotes.

Materials and methods

Generation of yeast null mutations

The strains used in the genetic studies were EMY74.7 (*MAT*α *his3*Δ*-100*, *leu2-3*, *112*, *trp1*Δ, *ura3-52*) and its derivatives. To construct the *pol32*Δ-generating plasmid, the 1.2- and 1-kb PCR products corresponding to the 5′- and 3′-flanking regions of the *POL32* gene, respectively, were directionally cloned into pUC19. The *URA3* geneblaster fragment containing the yeast *URA3* gene flanked by the duplicated *Salmonella typhimurium hisG* gene (Alani et al. 1987) was then inserted between the two

PCR products. The resulting *pol32*Δ-generating plasmid was digested with *Pvu*II, which releases a 6-kb fragment, introduction of which into yeast deletes nucleotides from +6 to +960 of the 1053-nt *POL32* open reading frame. The presence of the *pol32*Δ mutation in the various yeast strains was confirmed by PCR of genomic DNA. Loss of the *URA3* gene was selected for by plating strains on medium containing 5-fluoro-orotic acid.

Construction of the Rev1 C-transferase mutant

A 5.6-kb DNA fragment containing the entire yeast *REV1* gene and including ~1.1 kb of 5′-flanking DNA sequence and 1.4 kb of 3′-flanking DNA sequence, respectively, was cloned into pUC19, generating plasmid pBJ67, which was used as the starting material for constructing the Rev1 C-transferase mutant. This mutant, in which D467 and E468 are each changed to A, was generated by mutagenic PCR using the antisense oligonucleotide N4865 (5′-CACAAACAGCTGCAGCAATAGATA TAGGTAAAATC-3′) and the sense oligonucleotide N4866 (5′-TCTATTGCTGCAGCTGTTTGTGTGAGGATAATCCC-3′), resulting in a PCR fragment containing the D467, E468 → A467, A468 mutation as well as a *Pst*I site at these positions. A 250-bp *Bam*HI–*Pst*I PCR fragment encompassing nucleotides +1158 to +1401 of the *REV1* gene, and a 1560-bp *Pst*I–Asp 718 PCR fragment encompassing nucleotides +1401 to the termination codon at position +2958, were directionally cloned into the *Bam*HI–Asp 718 sites of pUC19, generating plasmid pBJ652. The presence of the A467, A468 mutation located at the *Pst*I site was confirmed by DNA sequencing. Subsequently, the remainder of the REV1 ORF, from nucleotides +1 to +1158 along with the 5′-flanking DNA and 3′-flanking DNA, obtained from pBJ67 was cloned into pBJ652, regenerating the entire 5.6-kb *REV1* DNA fragment, but now containing the *rev1* D467, E468 → A467, A468 mutation. The wild-type and mutant genes were then cloned into YCplac33, a low-copy-number vector that carries the yeast *URA3* gene as a selectable marker, as 5.4-kb *Sph*I–*Bgl*II fragments, generating plasmids pBJ660 and pBJ661, respectively.

MMS sensitivity and MMS-induced mutagenesis

Cells were grown overnight in YPD medium, sonicated to disperse clumps, washed, and resuspended in 0.05 M KPO_4 buffer at pH 7.0. Appropriate dilutions of MMS were added to 1-mL suspensions of cells adjusted to 1.5×10^8 cells/mL. Samples were incubated with vigorous shaking for 20 min at 30°C. Reactions were terminated by the addition of 1 mL of 10% sodium-thiosulfate. Appropiate dilutions were plated on YPD for viability determinations, and on synthetic complete medium lacking arginine but containing canavanine for determining the frequency of *can1*^{r} mutations. Plates were incubated at 30°C, and colonies were counted after 3 d for viability and after 4–5 d for mutagenesis.

For determining the mutational specificity of AP sites, the *apn1*Δ *apn2*Δ strain was treated with 0.08% MMS for 20 min at 30°C. Genomic DNA was isolated from *can1*^{r} mutants resulting from AP bypass and sequenced. Under these experimental conditions, relative to the spontaneous *can1*^{r} mutation frequency, the increase in MMS-induced *can1*^{r} mutations in the *apn1*Δ *apn2*Δ strain was >100-fold.

Purification of enzymes

Yeast Polδ was purified as described (Burgers and Gerik 1998). The purification of yeast Polζ and Rev1 was based on methods published previously, but in both cases the purification procedure included one additional chromatographic step. A GST–Rev3 fusion protein in complex with Rev7 protein (Polζ) was overexpressed in yeast strain Sc334 containing the plasmids pGST–REV3 and pREV7. The purification of GST–Rev3–Rev7 on glutathione-Sepharose 4B chromatography was carried out as described (Nelson et al. 1996b). Proteins eluted with glutathione from the matrix were dialyzed against buffer A (25 mM $NaPO_4$ at pH 7.4, 100 mM NaCl, 10% glycerol, 0.01% NP-40, 5 mM DTT, 0.5 mM EDTA), followed by loading onto a Mini-Q column (Pharmacia). The column was washed with 20 column volumes of buffer A, and the proteins were eluted with a gradient of 10 column volumes of 100–500 mM NaCl. The GST–Rev3–Rev7 peak fractions were pooled and concentrated by dialysis in buffer A containing 200 mM NaCl and 50% glycerol. To overexpress Rev1 protein as a GST fusion protein, plasmid pBJ 392 (PKG : GST–REV1) was introduced in yeast strain LY2 (*Matα gal1 reg1-501 leu2-3,-112 ura3-52 trp1*Δ *pep4-3 prb1-112*). Cells were grown for 12 h in synthetic complete medium lacking leucine. Purification of GST–Rev1 on glutathione-Sepharose 4B was carried out as described for GST–Rev3–Rev7. The eluted protein sample was dialyzed against buffer A, followed by MiniS (Pharmacia) chromatography. GST–Rev1 protein was eluted by 100–500 mM NaCl gradient in buffer A, and pooled fractions were frozen in aliquots under liquid nitrogen and kept at −70°C.

DNA synthesis reactions

Standard DNA polymerase reactions (10 µL) contained 40 mM Tris-HCl at pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, bovine serum albumin (100 µg/mL), 10% glycerol, 100 µM dNTP, and 20 nM 5′-^{32}P-labeled oligonucleotide primer annealed to an oligonucleotide template. Reactions were initiated by adding the enzymes Polζ, Polδ, or Rev1 in the amounts indicated in the figure legends. After incubation for 5 min at 30°C, reactions were terminated by the addition of 40 µL of loading buffer containing 20 mM EDTA, 95% formamide, 0.3% bromphenol blue, and 0.3% cyanol blue. The reaction products were subjected to electrophoresis in 10% or 20% polyacrylamide gels containing 8 M urea and visualized autoradiographically. Quantitation of reaction products was done with a Molecular Dynamics STORM phosphoimager and the ImageQuant software. DNA substrates S-2, S-4, S-5(G), S-5(A), S-5(T), S-5(C) were generated by annealing the 75-nt oligonucleotide template 5′-AGCTACC ATGCCTGCCTCAAGAGTTCGTAA**0**ATGCCTACACTGGA GTACCGGAGCATCGTCGTGACTGGGAAAAC-3′, which contained a model abasic site (a tetrahydrofuran moiety, purchased from Midland Co.) at the underlined position, to the 29-nt, 44-nt, and four different 45-nt 5′-^{32}P-labeled oligonucleotide primers, N4577: 5′-GTTTTCCCAGTCACGACGATGCT CCGGTA-3′, N4309: 5′-GTTTTCCCAGTCACGACGATGCT CCGGTACTCCAGTGTAGGCAT-3′, or oligonucleotides that contain N4309 with one additional G,A,T, or C residue at its 3′ end, respectively. In the control nondamaged substrate S-1 or S-3, the 75-nt template oligonucleotide with a C residue instead of the AP site at position 45 was annealed to N4577 and N4309, respectively. The sequences of DNA substrates containing 18-nt template oligonucleotides annealed to 12-nt primer DNA are shown in the figures.

Steady-state kinetic analyses

Analysis of kinetic parameters for deoxynucleotide incorporation opposite the AP site or primer extension from nucleotides opposite this lesion was done as described (Mendelman et al. 1990; Goodman et al. 1993; Creighton et al. 1995). Briefly, Polζ was incubated with increasing concentrations of a single deoxynucleotide (0–2000 µM) for 1 min under standard reaction conditions. Gel band intensities of the substrates and products were

quantitated by PhosphorImager. The percentage of primer extended was plotted as a function of dNTP concentration, and the data were fit by nonlinear regression using SigmaPlot 5.0 to the Michaelis–Menten equation describing a hyperbola, $v = (V_{max} \times [dNTP])/(K_m + [dNTP])$. Apparent K_m and V_{max} steady-state parameters were obtained from the fit and used to calculate the frequency of deoxynucleotide incorporation (f_{inc}) and the frequency of extension (f_{ext}^0) using the following equation: $f_{inc\ or\ ext} = (V_{max}/K_m)_{incorrect\ pair}/(V_{max}/K_m)_{correct\ pair}$.

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