

Translesion synthesis of acetylaminofluorene-dG adducts by DNA polymerase ζ is stimulated by yeast Rev1 protein

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ABSTRACT

Translesion synthesis is an important mechanism in response to unrepaired DNA lesions during replication. The DNA polymerase ζ (Pol ζ) mutagenesis pathway is a major error-prone translesion synthesis mechanism requiring Pol ζ and Rev1. In addition to its dCMP transferase, a non-catalytic function of Rev1 is suspected in cellular response to certain types of DNA lesions. However, it is not well understood about the non-catalytic function of Rev1 in translesion synthesis. We have analyzed the role of Rev1 in translesion synthesis of an acetylaminofluorene (AAF)-dG DNA adduct. Purified yeast Rev1 was essentially unresponsive to a template AAF-dG DNA adduct, in contrast to its efficient C insertion opposite a template 1,*N*⁶-etheno adenine adduct. Purified yeast Pol ζ was very inefficient in the bypass of the AAF-dG adduct. Combining Rev1 and Pol ζ , however, led to a synergistic effect on translesion synthesis. Rev1 protein enhanced Pol ζ -catalyzed nucleotide insertion opposite the AAF-dG adduct and strongly stimulated Pol ζ -catalyzed extension from opposite the lesion. Rev1 also stimulated the deficient synthesis by Pol ζ at the very end of undamaged DNA templates. Deleting the C-terminal 205 aa of Rev1 did not affect its dCMP transferase activity, but abolished its stimulatory activity on Pol ζ -catalyzed extension from opposite the AAF-dG adduct. These results suggest that translesion synthesis of AAF-dG adducts by Pol ζ is stimulated by Rev1 protein in yeast. Consistent with the *in vitro* results, both Pol ζ and Rev1 were found to be equally important for error-prone translesion synthesis across from AAF-dG DNA adducts in yeast cells.

INTRODUCTION

Translesion synthesis is an important mechanism of damage tolerance in response to unrepaired DNA lesions during replication. Translesion synthesis leads to replication of the damaged regions of the genome. It requires a specialized DNA

polymerase (Pol) to directly copy the damaged site on the DNA template. Since DNA damage can change the coding property of the bases, alter DNA structure or render the site non-coding, an incorrect nucleotide is often inserted opposite the lesion during translesion synthesis. This is referred to as error-prone translesion synthesis. It is also possible that the correct nucleotide is inserted opposite the lesion, resulting in error-free translesion synthesis.

The major translesion synthesis polymerases are Pol ζ and the Y family DNA polymerases in eukaryotes. In addition, Pol μ has also been shown to possess robust translesion synthesis activity *in vitro* (1,2). Whether Pol μ contributes significantly to translesion synthesis as a damage tolerance mechanism *in vivo*, however, remains to be determined. In humans, the Y family of DNA polymerases consists of Pol η , Pol ι , Pol κ and REV1 (3). In the yeast *Saccharomyces cerevisiae*, Pol ι and Pol κ are not found. Genetic studies in yeast have indicated that Pol ζ (the Rev3–Rev7 complex) operates in a biochemical pathway that involves additional proteins including Rev1 (reviewed in refs 4,5), which is also referred to as the Pol ζ mutagenesis pathway. Unlike other members in the Y family of DNA polymerases, Rev1 is a dCMP transferase, rather than a typical DNA polymerase. Rev1 is able to insert a C opposite a template G and several types of DNA lesions, including apurinic/apyrimidinic (AP) site, 8-oxoguanine, (+)-*trans-anti*-benzo[*a*]pyrene-*N*²-dG, (–)-*trans-anti*-benzo[*a*]pyrene-*N*²-dG and 1,*N*⁶-etheno adenine (6–9). At least in the case of cellular response to AP sites in DNA, the Rev1 dCMP transferase directly participates in nucleotide insertion opposite the lesion during translesion synthesis *in vivo* (10).

Error-prone translesion synthesis is largely responsible for DNA damage-induced mutagenesis. In yeast, the vast majority of mutations induced by UV radiation, acetylaminofluorene (AAF), AP sites and benzo[*a*]pyrene diol epoxide are generated by the Pol ζ mutagenesis pathway (10–14). The Pol ζ mutagenesis pathway also exists in humans (7,15–17) and is shown to be the major mechanism for UV- and benzo[*a*]pyrene diol epoxide-induced mutagenesis in cultured human cells (15,17,18). Thus, it appears that the Pol ζ pathway constitutes a major mechanism of error-prone translesion synthesis and DNA damage-induced mutagenesis in eukaryotes. Although Rev1 is required for the Pol ζ mutagenesis pathway, very little is known about the role of Rev1 in the mutagenesis process. The dCMP transferase of Rev1 is

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unable to respond to a template TT dimer and a template TT (6–4) photoproduct *in vitro* (6,8,10), two major DNA lesions of UV radiation. Yet Rev1 is required for UV-induced mutagenesis (10,19,20). Thus, it has been proposed that Rev1 may play another non-catalytic function in the Pol ζ mutagenesis pathway (10,20). However, it is not well understood about the non-catalytic function of the Rev1 protein in translesion synthesis.

To better understand Rev1 functions, we have analyzed the role of this protein in translesion synthesis of an AAF-dG DNA adduct. Whereas the dCMP transferase of purified yeast Rev1 was essentially unresponsive to a template AAF-dG DNA adduct, this protein strongly stimulated the Pol ζ -catalyzed translesion synthesis of AAF-dG DNA adducts. These results suggest a non-catalytic function of Rev1 in the bypass of AAF-dG adducts. Consistent with the *in vitro* results, we found that both Pol ζ and Rev1 are equally important for error-prone translesion synthesis across from AAF-dG DNA adducts in yeast cells.

MATERIALS AND METHODS

Materials

A mouse monoclonal antibody against the His₆ tag and the *Escherichia coli* expression vector pQE80 were purchased from Qiagen (Valencia, CA). The plasmid vector pQE80C was constructed by inserting a 90mer duplex DNA encoding a calmodulin binding peptide (21) into the BamHI site of pQE80. The coding sequence of the duplex DNA is 5'-GATCCAAGCGACGATGGAAAAAGAATTCATAGCCGTCTCAGCAGCCAACCGCTTTAAGAAAATCTCATCC-TCCGGGGCACTTAGATCTA-3'. The *E.coli* strain BL21 (DE3 Gold) was obtained from Invitrogen (Carlsbad, CA). Calmodulin affinity resin was purchased from Stratagene (La Jolla, CA). Alkaline phosphatase conjugated anti-mouse IgG was from Sigma Chemicals (St Louis, MO). All oligonucleotides were synthesized by Operon (Alameda, CA). Yeast Pol ζ (the Rev3–Rev7 complex) and human REV1 were purified to near homogeneity as described previously (8,22). *N*-Acetoxy-*N*-2-acetylaminofluorene (AAAF, the activated form of AAF) was obtained from the Midwest Research Institute (Kansas City, MO). A 30mer DNA template containing a site-specific AAF-dG adduct was prepared as described previously (23). Its sequence is 5'-CCTTCTTCA-TTCGAACATACTTCTTCTTCC-3', where the modified guanine is underlined. A 36mer template containing a site-specific tetrahydrofuran (AP site analogue) was synthesized by Operon. Its sequence is 5'-GAAGGGATCCTTAAGACTX-TAACCGGTCTTCGCGCG-3', where X designates the AP site.

Yeast strains

Yeast strains used are BY4741 Δ rad14 (proficient in mutagenesis) (*MATa his3 leu2 met15 ura3 rad14::HIS3*) and its isogenic BY4741 Δ rad14 Δ rev1 (*rev1* deletion mutant), and BY4741 Δ rad14 Δ rev3 (*rev3* deletion mutant). BY4741 was purchased from ATCC (Manassas, VA). BY4741 Δ rev3 (lacking Pol ζ) was constructed as described previously (14). 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. Finally, the *RAD14* gene of BY4741, BY4741 Δ rev1 and BY4741 Δ rev3 was deleted by transforming the respective strains with a *rad14* deletion plasmid construct and the *rad14* deletion phenotype confirmed as described previously (14).

Purification of yeast Rev1

The yeast *REV1* gene was obtained by PCR using *S.cerevisiae* DNA as the template and the primers 5'-GAAGATCTATGGGTGAACATGGTGGTCTTG-3' and 5'-ACGCGTCGACAGGTATTGTT-GCAATGC-3'. The 3-kb *REV1* DNA fragment was then cloned into the BglII and SalI sites of the vector pQE80C, yielding pQE80C-yREV1. *Escherichia coli* strain BL21 (DE3 Gold) containing pQE80C-yREV1 was grown at 37°C to an OD₆₀₀ of 0.8. Rev1 expression was induced by addition of IPTG to a final concentration of 1 mM and growth for 14 h at 16°C. Cells were collected by centrifugation and resuspended in an extraction buffer containing 50 mM Tris-HCl pH 7.5, 10% sucrose, 20% glycerol, 5 mM β -mercaptoethanol and protease inhibitors (24). Cells were then homogenized by a sonicator, using four pulses of 30 s each with a 2 min pause on ice between pulses. After centrifugation at 18 000 *g* for 20 min, the clarified sample was loaded onto a 1 ml HiTrap chelating column charged with NiSO₄ (Amersham Pharmacia Biotech, Piscataway, NJ), followed by washing the column sequentially with 15 ml of Ni buffer A (50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 10% glycerol, 5 mM β -mercaptoethanol and protease inhibitors) containing 10 mM imidazole and 25 ml of Ni buffer A containing 35 mM imidazole. Bound proteins were eluted with a linear gradient (25 ml) of 35–500 mM imidazole in Ni buffer A. An equal volume of calmodulin binding buffer (50 mM Tris-HCl pH 8.0, 0.3 M NaCl, 4 mM CaCl₂ and 10 mM β -mercaptoethanol) was added to the pooled Rev1 fractions. After centrifugation at 15 000 *g* for 20 min, the sample was loaded onto a 2 ml calmodulin affinity column. The column was washed with 15 ml of the calmodulin column buffer (50 mM Tris-HCl pH 8.0, 0.3 M NaCl and 10 mM β -mercaptoethanol). Finally, the column was eluted with three EGTA step-gradients in calmodulin column buffer containing 1, 2 and 4 mM EGTA, respectively.

To construct the *REV1 Δ C* gene coding for a truncated Rev1 lacking the C-terminal 205 aa, PCR was performed using the plasmid pQE80C-yREV1 as the template and two primers, 5'-GAAGATCTATGGGTGAACATGGTGGTCTTG-3' and 5'-ACGCGTCGACTAATAATTTCTTTTCTCGAACTCGTTG. The resulting 2.3 kb DNA fragment was cloned into the BglII and SalI sites of the vector pQE80C, yielding pQE80C-yREV1 Δ C. Expression and purification of the C-terminal truncated Rev1 Δ C (94 kDa) were similarly carried out as described above for the full-length Rev1 protein.

DNA polymerase assays

Identical conditions were used for dCMP transferase assays and 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 µg/ml bovine serum albumin, 10% glycerol, 50 µM 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 purified Polζ, Rev1 or both as indicated. After incubation at 30°C for 30 min, reactions were terminated with 7 µl 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% denaturing polyacrylamide gel and visualized by autoradiography. DNA synthesis products were quantitated by scanning densitometry using the SigmaGel software (Sigma, St Louis, MO) for analysis.

Preparation of AAF-damaged plasmid DNA

Plasmid pCLU (50 µg) was incubated with 10 µM AAF in a 500-µl reaction mixture containing 20% ethanol and TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) for 3 h in the dark at 37°C. The modified DNA was then purified by 5–20% sucrose gradient centrifugation at 28 000 r.p.m. for 17 h at 4°C in a Beckman SW41Ti rotor. Fractions of 0.5 ml each were collected from the bottom of the gradient and 5-µl aliquots were analyzed by electrophoresis on a 1% agarose gel to locate the DNA. Fractions containing supercoiled DNA were pooled, precipitated in ethanol and dissolved in TE buffer.

In vivo mutagenesis assay in yeast

A plasmid-based mutagenesis assay in yeast cells was performed as we reported recently (14). Briefly, damaged or undamaged plasmid pCLU DNA (2 µg) was transformed into yeast cells. Immediately after transformation, cells were collected by centrifugation (20 s at 5000 r.p.m.) in a microcentrifuge, and resuspended in 1 ml of sterile water. An aliquot of 999 µl cell suspension was plated onto three YNB minimal agar plates lacking leucine but supplemented with 5 mM 5-fluoroorotic acid (5-FOA), 150 µM methionine and 380 µM uracil to score for colonies containing *ura3* mutant pCLU. Another aliquot of 1 µl cell suspension was diluted and plated onto YNB minimal agar plates lacking leucine to score for colonies containing replicated pCLU (plasmid survival). Yeast colonies were counted after incubation at 30°C for 3–4 days. Mutation frequency was calculated by dividing the number of the *ura3* mutant colonies by the number of colonies containing replicated pCLU.

RESULTS

The dCMP transferase of purified yeast Rev1 is essentially unresponsive to a template AAF-dG adduct

To understand the role of Rev1 in translesion synthesis of AAF-dG DNA adducts, we first determined whether the dCMP transferase of yeast Rev1 is able to insert a C opposite the lesion *in vitro*. To avoid potential contamination by yeast proteins, we purified yeast Rev1 from *E. coli* cells following its expression from a plasmid construct. The Rev1 preparation contained two bands of ~120 and 90 kDa, respectively (Fig. 1A). Western blot analysis using a monoclonal antibody against the N-terminal His₆ tag confirmed that both bands are yeast Rev1 protein (Fig. 1B). While the larger band is consistent with the calculated molecular mass of yeast Rev1 (112 kDa), thus representing the full-length protein, the

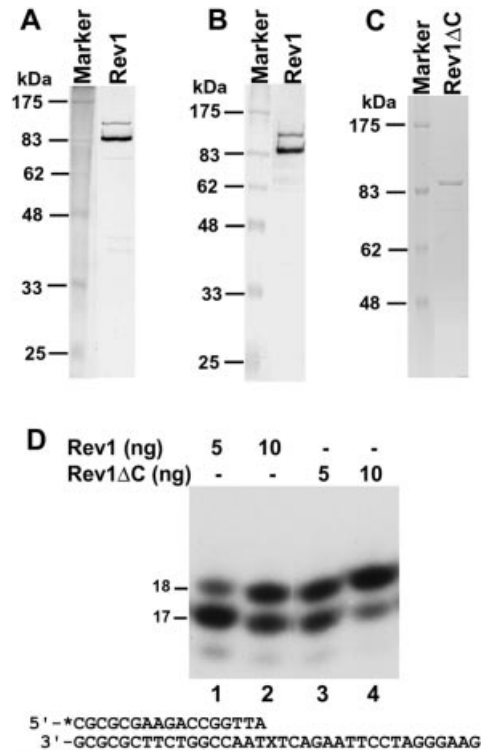


Figure 1. Analyses of purified yeast Rev1. (A) The purified yeast Rev1 protein (300 ng) was analyzed by electrophoresis on a 10% SDS-polyacrylamide gel and visualized by silver staining of the gel. Protein size markers are indicated on the left. (B) The purified yeast Rev1 protein (300 ng) was analyzed by a western blot using a mouse monoclonal antibody against the N-terminal His₆ tag. (C) The purified Rev1ΔC protein (300 ng) lacking the C-terminal 205 aa of yeast Rev1 was analyzed by 10% SDS-polyacrylamide gel and visualized by staining with Coomassie blue. The identity of the protein band that migrated at the ~90 kDa position on the gel was confirmed by western blot analysis using a mouse monoclonal antibody against the N-terminal His₆ tag. (D) A 5'-³²P-labeled 17mer primer was annealed to a damaged 36mer template, terminating right before the AP site as shown at the bottom. Then, DNA polymerase assays were performed using 5 or 10 ng of purified yeast Rev1 or Rev1ΔC protein as indicated. DNA size markers in nucleotides are indicated on the left. X, the AP site.

smaller band is probably a C-terminal truncated product of Rev1. The eukaryotic Y family DNA polymerases, including Rev1, are relatively unstable and protein truncation is a common feature among them. To determine whether the truncated Rev1 protein retains activity, we deleted 205 aa from its C-terminus, yielding Rev1ΔC (94 kDa). Rev1ΔC was similarly expressed in *E. coli* and purified (Fig. 1C), yielding a protein of approximately the size of the ~90 kDa band in Figure 1A. As shown in Figure 1D, both Rev1 and Rev1ΔC preparations were similarly active in dCMP transferase in response to a template AP site. Thus, the C-terminal truncated protein of ~90 kDa retains the Rev1 dCMP transferase activity.

To examine the response of yeast Rev1 to an AAF-dG adduct, we annealed a ³²P-labeled 17mer primer to the damaged template with the primer 3' end terminating right before the lesion (Fig. 2A), and performed standard dCMP transferase assays. As shown in Figure 2A, purified yeast Rev1 was unable to effectively catalyze nucleotide insertion opposite the AAF-dG adduct. In contrast, it efficiently inserted

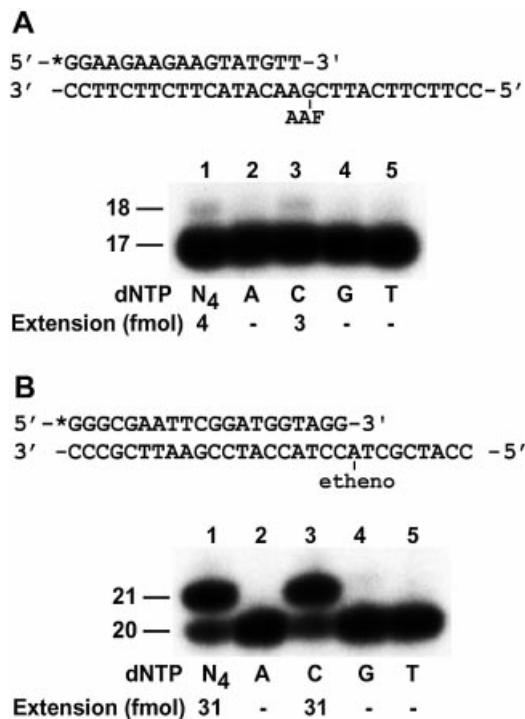


Figure 2. Response of the yeast Rev1 dCMP transferase to AAF-dG and 1,*N*⁶-ethenoadenine DNA adducts. DNA polymerase assays were performed with 5 ng (45 fmol) of purified yeast Rev1 in the presence of all four dNTPs (N₄), or a single deoxyribonucleotide triphosphate, dATP (A), dCTP (C), dGTP (G), or dTTP (T). DNA size markers in nucleotides are indicated on the left. Quantitation of extended primers is shown below the gel. (A) Assays with DNA templates containing a site-specific AAF-dG adduct. A 17mer primer was labeled with ³²P at its 5' end and annealed to the damaged template with the primer 3' end terminating right before the lesion as shown on the top. (B) Assays with DNA templates containing a site-specific 1,*N*⁶-ethenoadenine adduct. A 20mer primer was labeled with ³²P at its 5' end and annealed to the damaged template with the primer 3' end terminating right before the lesion as shown on the top.

a C opposite a template 1,*N*⁶-ethenoadenine adduct (Fig. 2B). These results show that the yeast Rev1 dCMP transferase is essentially unresponsive to a template AAF-dG adduct.

Synergistic effect of Polζ and Rev1 combination during bypass of a template AAF-dG adduct *in vitro*

Since purified yeast Polζ alone is inefficient in performing translesion synthesis across from a template AAF-dG adduct (22), we suspected that accessory factor(s) might be required to stimulate lesion bypass by Polζ *in vivo*. To test whether Rev1 could play such a role, we performed DNA synthesis reactions with the AAF-damaged template by combining purified yeast Polζ and Rev1 together. In the reaction, a 13mer ³²P-labeled primer was annealed to the damaged template, terminating 4 nt before the lesion (Fig. 3). Consistent with our previous finding (22), the AAF-dG adduct strongly blocked DNA synthesis by yeast Polζ right before the lesion, as evidenced by the strong 17mer DNA band (Fig. 3, lane 3). Only very small amounts of the damaged templates were bypassed (longer than 18mer DNA bands), and the 30mer full-length product or near full-length products were not detected (Fig. 3, lane 3). When purified yeast Rev1 protein was added to the reaction, much higher levels of the damaged templates

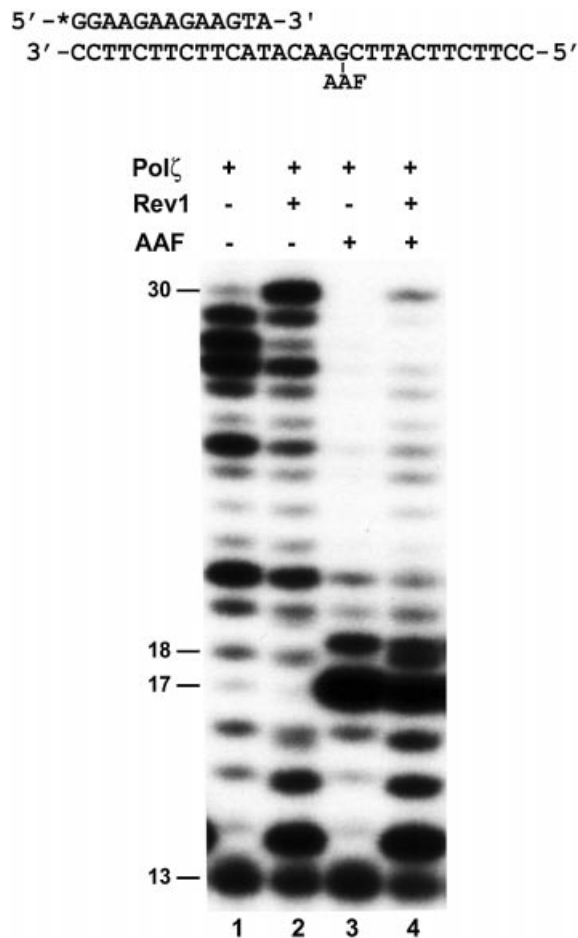


Figure 3. Effect of Rev1 protein on Polζ-catalyzed bypass of the template AAF-dG adduct. A 5'-³²P-labeled 13mer primer was annealed to a damaged 30mer template with the primer 3' end terminating 4 nt before the AAF-dG adduct as shown on the top. As the control, the ³²P-labeled primer was separately annealed to the undamaged template with identical sequence. DNA polymerase assays were then performed with 10 ng (50 fmol) of yeast Polζ in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of purified yeast Rev1 protein (5 ng, 45 fmol). Lanes 1 and 2, undamaged DNA template; lanes 3 and 4, damaged DNA template. DNA size markers in nucleotides are indicated on the left.

were bypassed and the 30mer full-length product was readily detected (Fig. 3, lane 4). These results show that combination of yeast Polζ and Rev1 has a synergistic effect on the bypass of AAF-dG DNA adducts.

We consistently observed that the last template base was inefficiently copied by Polζ even from undamaged templates (22) (Fig. 3, lane 1). Surprisingly, addition of yeast Rev1 protein greatly stimulated DNA synthesis at the end of the undamaged template (Fig. 3, lane 2). The 30mer full-length product migrated at the same position in the absence or presence of Rev1 (Fig. 3, compare lanes 1 and 2). Therefore, the last nucleotide copied at the very end of the template is consistent with Polζ-catalyzed G addition, rather than C addition catalyzed by the Rev1 dCMP transferase. C addition would have resulted in a 30mer product that migrated significantly faster than the 30mer DNA band in lane 1 of Figure 3. These results show that Rev1 stimulates the inefficient DNA synthesis of Polζ at the end of the template.

translesion synthesis of AP sites (10). It is of great interest to determine in the future whether such an involvement of the Rev1 dCMP transferase is only limited to cellular response to AP sites or constitutes a more general mechanism in the Pol ζ mutagenesis pathway. Regardless of the extent to which the catalytic dCMP transferase function is employed during *in vivo* translesion synthesis of various types of DNA lesions, a non-catalytic role appears to be an important and may well be the predominant function of Rev1 in the Pol ζ mutagenesis pathway. Our studies suggest one such non-catalytic function for Rev1 in the translesion synthesis of AAF-dG DNA adducts. Extended studies into other types of DNA lesions could bring in more insights into our understanding of this fascinating member of the Y family DNA polymerases in translesion synthesis.

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