

Research Article

Characterization of a Y-Family DNA Polymerase η from the Eukaryotic Thermophile *Alvinella pompejana*

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Human DNA polymerase η (HsPol η) plays an important role in translesion synthesis (TLS), which allows for replication past DNA damage such as UV-induced *cis-syn* cyclobutane pyrimidine dimers (CPDs). Here, we characterized ApPol η from the thermophilic worm *Alvinella pompejana*, which inhabits deep-sea hydrothermal vent chimneys. ApPol η shares sequence homology with HsPol η and contains domains for binding ubiquitin and proliferating cell nuclear antigen. Sun-induced UV does not penetrate *Alvinella*'s environment; however, this novel DNA polymerase catalyzed efficient and accurate TLS past CPD, as well as 7,8-dihydro-8-oxoguanine and isomers of thymine glycol induced by reactive oxygen species. In addition, we found that ApPol η is more thermostable than HsPol η , as expected from its habitat temperature. Moreover, the activity of this enzyme was retained in the presence of a higher concentration of organic solvents. Therefore, ApPol η provides a robust, human-like Pol η that is more active after exposure to high temperatures and organic solvents.

1. Introduction

Although genomic DNA contains genetic information that should be error-free for proper cellular function, it is continually subjected to ubiquitous DNA-damaging agents of both environmental and endogenous origins, such as UV and ionizing radiation and reactive oxygen species (ROS) [1–3]. To maintain genomic integrity, cells possess several repair pathways, including nucleotide excision repair (NER), base excision repair (BER), and recombination repair, to cope with the various resulting lesions. However, some lesions are not repaired and are encountered by the replication machinery. During DNA replication, these lesions block high-fidelity replicative DNA polymerases, and if not processed correctly, eventually lead to mutagenesis, carcinogenesis, or

cell death. To avoid such catastrophic consequences, cells have a translesion DNA synthesis (TLS) mechanism that allows efficient and accurate DNA synthesis past lesions [4–6].

The importance of TLS in humans has been indicated by studies of an inherited human disease called xeroderma pigmentosum variant (XP-V), which is characterized by the hypersensitivity of skin to sunlight and the high incidence of sunlight-induced skin cancer [7, 8]. The *XPV* gene encodes human DNA polymerase η (HsPol η), a 713 amino acid protein that is a member of the Y-family DNA polymerases that includes human DNA polymerases ι , κ , and Rev1 [6, 9, 10]. Cells from XP-V patients are defective in the replication of damaged DNA and show hypermutability after exposure to UV radiation or DNA-damaging agents. These results

indicate that HsPol η is at least involved in the accurate translesion pathway to avoid mutations by UV-induced lesions *in vivo*. Consistent with these observations, biochemical studies revealed that HsPol η , unlike the replicative Pol δ and Pol ϵ , was able to catalyze efficient and accurate TLS past a UV-induced *cis-syn* cyclobutane pyrimidine dimer (CPD). In contrast, DNA containing other UV-induced lesions, such as the pyrimidine(6–4)pyrimidone photoproduct (6–4 pp) and its Dewar valence isomer (Dewar), which are efficiently repaired by NER *in vivo* [11, 12], is not replicated by HsPol η [13, 42].

Furthermore, this enzyme was reportedly able to bypass not only UV-induced lesions but also various other lesions induced by both environmental and endogenous reactive oxygen species. For instance, HsPol η bypasses 7,8-dihydro-8-oxoguanine (8-oxoG), 5R-thymine glycol (5R-Tg), and 5S-thymine glycol (5S-Tg), but not an apurinic/aprimidinic (AP) site [13–19], although these lesions, which have small alterations in their chemical structures, are mainly repaired by BER *in vivo*. Thus, these findings indicate that HsPol η also plays an important function in the replication of DNA containing ROS-induced lesions *in vivo*.

In addition to its role in TLS, HsPol η can extend DNA synthesis from D-loop recombination intermediates, and its activity is stimulated by Rad51 recombinase [20]. Moreover, Pol η -disrupted DT40 cells show significant decreases in the frequencies of both immunoglobulin-variable gene conversion and double-strand break-induced homologous recombination [21]. Taken together, these reports indicate that Pol η is involved in homologous recombination repair, and thus, Pol η seems to be important for DNA damage tolerance in living cells.

Alvinella pompejana is a polychaetous annelid that inhabits active deep-sea hydrothermal vent sites along the East Pacific Rise, where it colonizes the walls of actively venting high-temperature chimneys. The average temperature is about 68°C, with spikes up to 81°C, and thus exceeds the predicted 55°C limit for the survival of eukaryotes [22, 23]. The ability of this worm to survive in such an extreme environment suggests that *A. pompejana* may contain highly stable proteins that can be used for biology, biotechnology, and industry. Several proteins have been characterized from *A. pompejana* and the analyses have revealed the proteins have enhanced thermostability [24–26], which include *A. pompejana* superoxide dismutase (SOD) and U2AF65, which are more stable than their human homologs.

In this study, we cloned an *A. pompejana* gene with homology to the HsPol η gene. The encoded full-length recombinant protein was produced in *Escherichia coli*. Characterization of ApPol η revealed that it had the ability to bypass CPD, 8-oxoG, 5R-Tg, and 5S-Tg, but did not bypass either 6–4 pp, Dewar, or an AP site analog. This substrate specificity is similar to that of the HsPol η , yet ApPol η is comparatively more thermostable than HsPol η and retains activity in the presence of organic solvents. Therefore, ApPol η is a robust human-like Pol η that is useful to complement and expand ideas on TLS mechanisms by virtue of its eukaryotic origin and enhanced stability.

2. Materials and Methods

2.1. Materials. Recombinant HsPol η tagged with (His)₆ at its C-terminal end was produced in Sf9 insect cells using the baculovirus expression system and was purified by sequential column chromatography on HiTrap Q, Ni-NTA agarose, and MonoS, as described previously [17]. Klenow fragment 3′–5′ exonuclease minus (KF) and T7 DNA polymerase were purchased from New England Biolabs and USB, respectively. Oligonucleotides containing CPD [27], 6–4 pp [28], Dewar [29], 5R-Tg [30], and 5S-Tg [31] were synthesized on an Applied Biosystems 3400 DNA synthesizer, as described, and those containing 8-oxoG and the AP site analog (dSpacer) were synthesized using phosphoramidite building blocks purchased from Glen Research.

2.2. Cloning of the ApPol η Gene. Full-length ApPol η cDNA was isolated by RACE PCR, using cDNA prepared with the GeneRacer kit (Invitrogen) from total RNA isolated from frozen whole worm samples. A 2.5 cm frozen section of the posterior end of *A. pompejana* collected with the DSV Alvin submersible [23] was ground using a mortar and pestle in liquid nitrogen. The ground tissue was aliquoted into eppendorf tubes, and after the addition of Trizol (Invitrogen) (1.4 mL), the mixture was incubated at room temperature for 10 minutes. After centrifugation at 12,000 ×g for 10 minutes, the supernatant (1 mL) was mixed with chloroform (250 μ l), and was incubated at room temperature for 2 minutes. The samples were then centrifuged at 12,000 ×g for 15 minutes to separate the total RNA into the aqueous layer. The RNA was then precipitated by adding isopropanol (500 μ l) to the aqueous solution (600 μ l), and was incubated at 4°C for 1.5 hours. After centrifugation at 12,000 ×g for 10 minutes at 4°C, the pellet was washed with 75% ethanol (1.4 mL) and then stored in 75% ethanol (1 mL) at –80°C. The stored RNA was pelleted by centrifugation and resuspended in diethylpyrocarbonate-treated water (20 μ l), and the concentration was determined by measuring the absorbance at 280 nm. A 5 μ g portion of the total RNA was used to prepare cDNA using the GeneRacer kit (Invitrogen), according to the manufacturer’s instructions. In brief, total RNA was dephosphorylated using calf intestinal alkaline phosphatase, extracted with phenol-chloroform, and precipitated with ethanol. The RNA was decapped using tobacco acid pyrophosphatase, extracted, and precipitated, and then the GeneRacer 5′ oligo was ligated to the 5′ ends. Finally, the RNA was subjected to the reverse transcription reaction using a poly-T primer containing the GeneRacer 3′ oligo sequence, to generate a single-stranded cDNA library with known 5′ and 3′ sequences.

The amino acid sequence of HsPol η was used for an in-house TBLASTN search of a translated *A. pompejana* expressed sequence tag (EST) database comprised of sequences derived from collaborations with the Joint Genome Institute (Walnut Creek, CA) and Genome Therapeutics (Waltham, MA). A positive hit at the 5′ end of the gene was identified on EST CAGA3172. The 3′ sequence was determined from the prepared *A. pompejana* GeneRacer cDNA library by 3′ RACE PCR, using a gene specific primer

(AlviXPVF1 5'GG GAC TGG TCT GTG CTG GCC GTG GAA AT 3') and the GeneRacer 3' oligo primer, together with the touchdown PCR strategy and KOD polymerase (Novagen). The PCR product was TOPO cloned into the pCR2.1 vector (Invitrogen) and was analyzed by restriction enzyme digestion and sequencing. The full-length ApPol η gene was amplified from the cDNA library, using two gene-specific primers, AlviXPVF1 (the above sequence) and AlviXPVR2 (5' GGG ATG ATT CTA CAC TTT GCT GAA AAA TCT ATC CA 3'), and KOD polymerase under the standard PCR conditions. The 2.2 kb product was gel-purified and TOPO cloned into the pCR4 vector (Invitrogen) and was analyzed by restriction enzyme digestion and sequencing. Restriction sites were added to the ends by a subsequent round of PCR and TOPO cloning. The full-length ApPol η cDNA was then subcloned into pET21a (Novagen), using the *Nde* I and *Eco* RI restriction nucleases and T4 DNA ligase, and was analyzed by restriction enzyme digestion and sequencing.

2.3. Expression and Purification of the ApPol η Protein. Recombinant ApPol η gene was expressed in *E. coli* Rosetta2 (DE3) (Novagen). The cells were grown at 37°C to an absorbance at 600 nm of 0.6. The cultures were placed in an ice slurry for 20 minutes, and then isopropyl- β -D-thiogalactopyranoside was added to a final concentration of 0.5 mM. After the cultures were incubated at 12°C for 40 hours, the cells were collected by centrifugation, rinsed with buffer A containing 10 mM sodium phosphate (pH 7.4), 10% glycerol, and 300 mM NaCl, and resuspended in buffer A containing *p*-amidinophenylmethanesulfonyl fluoride hydrochloride and a protease inhibitor cocktail (complete, EDTA free (Roche)). The lysate mixtures were mixed with powdered lysozyme at a final concentration of 2 mg/mL for 10 minutes on ice, and then were subjected to ultrasonication. The lysates were centrifuged, and the supernatants were mixed with Talon resin (Clontech) (50% w/v, equilibrated with lysis buffer containing 10 mM sodium phosphate (pH 7.4), 10% glycerol, 300 mM NaCl, and 5 mM imidazole). The resin was washed with the same buffer, and the bound proteins were eluted with eluate buffer (lysis buffer containing 300 mM imidazole). The eluate was diluted and applied to a HiTrap Heparin HP column (GE Healthcare) equilibrated with buffer B (50 mM sodium phosphate (pH 7.4), 1 mM EDTA, 1 mM β -mercaptoethanol, and 0.01% Triton X-100) containing 0.1 M NaCl. Elution was performed by a stepwise gradient of 0.2 M to 0.6 M NaCl in buffer B. The peak fractions containing ApPol η eluted at 0.5 M NaCl, and were stored at -80°C.

2.4. TLS Reactions. The 5'-³²P-labeled primer-template was prepared by mixing a 16-mer primer labeled at its 5' end with each of the 30-mer templates, at a molar ratio of 1 : 1. Standard reaction mixtures (10 μ l), containing 40 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 100 mM dNTPs, 10 mM DTT, 0.24 mg/mL BSA, 60 mM KCl, 2.5% glycerol, 40 nM 5'-³²P-labeled primer-template, and purified ApPol η , were incubated at 37°C. The reaction times and the enzyme amounts

are indicated in the figure legends. The reactions were terminated by the addition of stop solution (8 μ l), containing 95% formamide, 20 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol. The protein was denatured by boiling, and the products were separated by electrophoresis on a denaturing 16% polyacrylamide gel. Dried gels were analyzed on a FUJIFILM BAS 1800 bioimage analyzer.

2.5. Thermostability of ApPol η . The relative thermostabilities of ApPol η and HsPol η were compared by heating each enzyme to temperatures ranging between 37°C and 58°C for 5 minutes. An aliquot of each enzyme was then used for the standard 20 minutes TLS reaction at 37°C to measure the incorporation of a single nucleotide, dCMP, opposite the template G residue (see the TLS reactions described above). The enzyme concentrations used in the reactions were initially determined by making serial dilutions of each enzyme and identifying the concentration necessary to give the same extension level of the labeled primer. These amounts were 1.8 and 5.9 fmol for HsPol η and ApPol η , respectively. The ability of each enzyme to extend the primer was quantified with MultiGauge software (FUJIFILM), and the specific activity, compared to the activity of each enzyme heated to 37°C, was calculated. The specific activity was plotted as a function of the temperature to which the enzyme was exposed.

3. Results

3.1. Amino Acid Sequence of ApPol η . A cDNA sequence with homology to that of HsPol η was cloned from an *A. pompejana* cDNA library by RACE PCR. The open reading frame encoded a predicted product of 745 amino acid residues, with a calculated molecular mass of 83 kDa. Alignment of the amino acid sequence against HsPol η revealed 38.1% identity and 57.6% similarity overall (identities = 283/708 (39%), positives = 414/708 (58%), gaps = 68/708 (9%)). The alignment (Figure 1(a)) also indicated the high conservation within the N-terminal region and the presence of the four structural domains (finger, palm, thumb, and little finger) that are the hallmarks of the Y-family translesion DNA polymerases [32, 33]. Ubiquitin (Ub)-binding domains [34–36] and a proliferating cell nuclear antigen (PCNA)-binding domain [37, 38], located in the C-terminal region of Pol η , were identified in the ApPol η amino acid sequence, suggesting that Ub and/or PCNA regulate this protein.

The Y-family DNA polymerases ι (Pol ι) and κ (Pol κ) and the B-family DNA polymerase ζ (Pol ζ) are known to share sequence homology with the Y-family HsPol η . To analyze the relationship between this *A. pompejana* protein and other translesion DNA polymerases, a phylogenetic tree was produced on the basis of the amino acid sequences of these DNA polymerases, by the unweighted pair group method with arithmetic mean (UPGMA). The tree revealed that this protein was not related to Pol κ , Pol ι , or Pol ζ , but was more closely related to Pol η (Figure 1(b)). Therefore, we concluded that this protein is a member of the DNA polymerase η

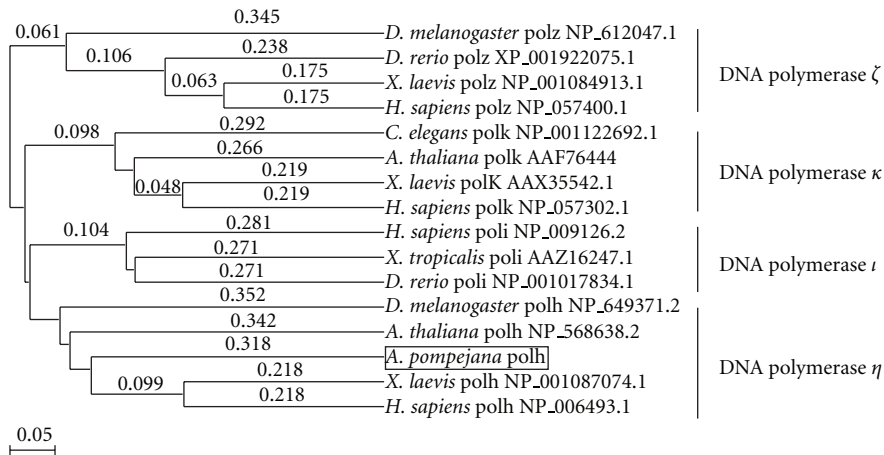
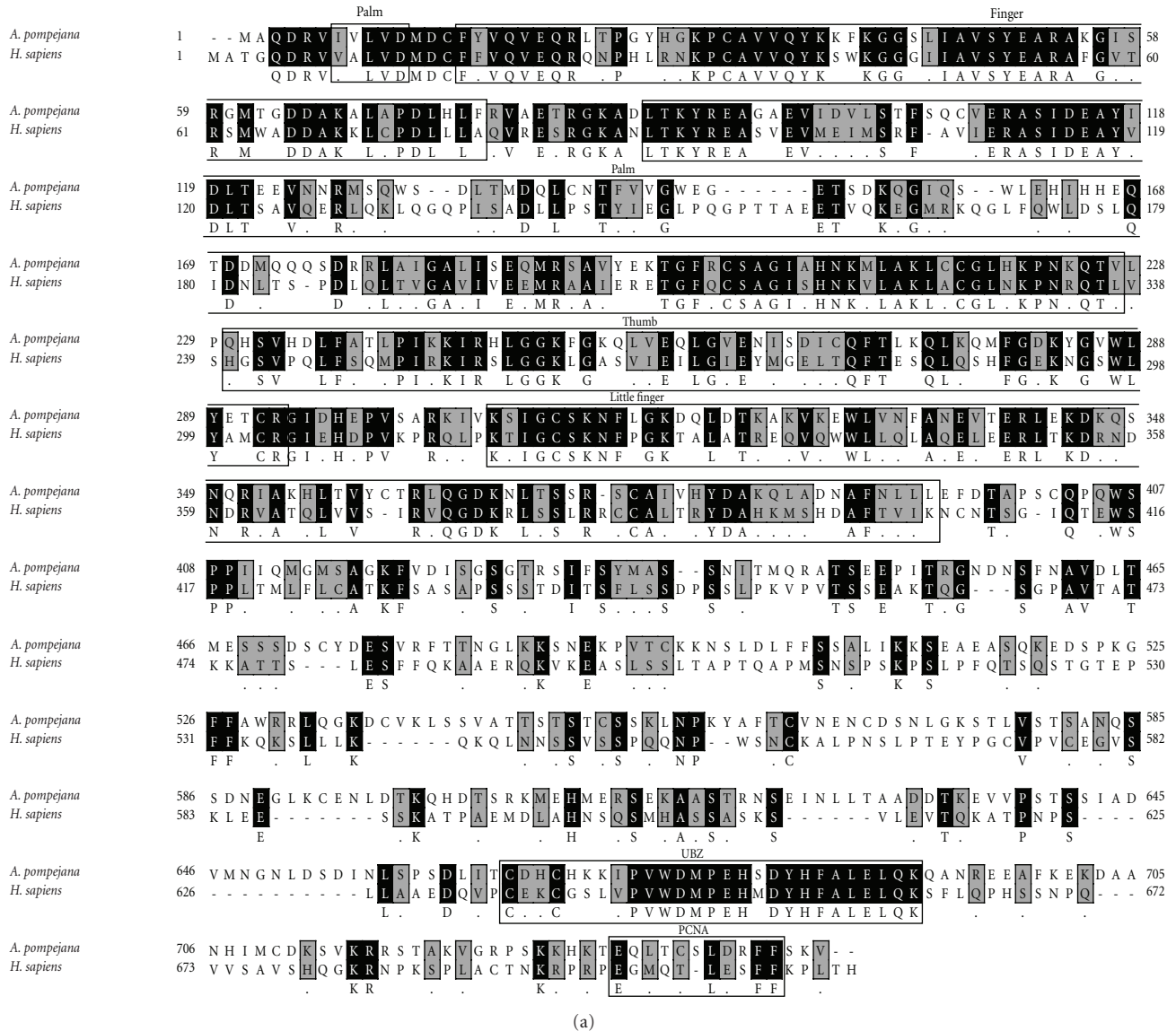


FIGURE 1: (a) Alignment of the amino acid sequences of ApPol η and HsPol η (NP_006493.1). Identical and similar residues between the two enzymes are indicated in black and grey, respectively. (b) Phylogenetic analysis of TLS polymerases. A phylogenetic tree was constructed by the UPGMA method, based on the amino acid sequences of the ApPol η and other Y- and B-family DNA polymerases.

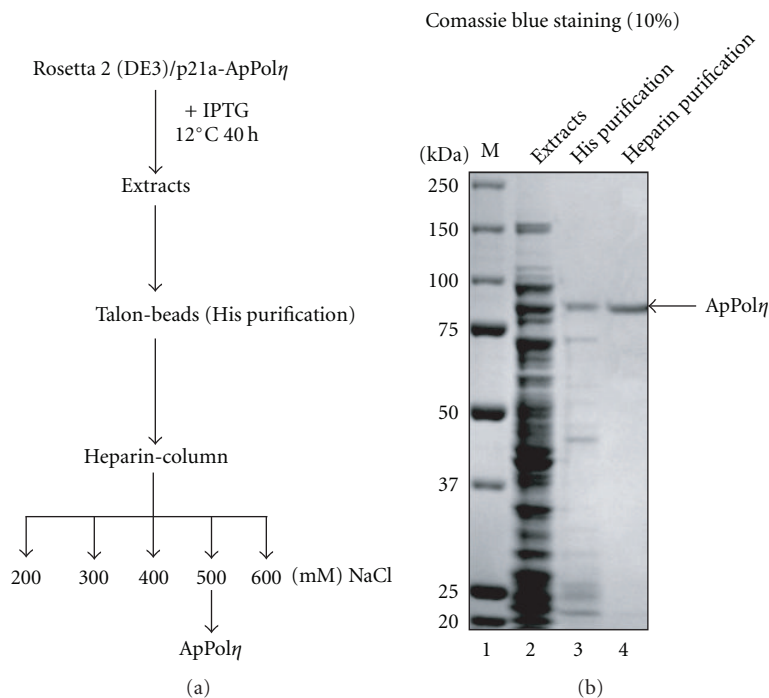


FIGURE 2: Purification of the recombinant ApPol η protein. Aliquots from various steps of the purification were subjected to 10% SDS-PAGE analysis, and the proteins were visualized by staining with Coomassie Brilliant Blue. ApPol η has a molecular mass of 85 kDa. Lane 1, markers; lane 2, cell extract; lane 3, Talon eluate; lane 4, HiTrap Heparin HP eluate.

group, and thus referred to the protein as *A. pompejana* DNA polymerase η .

3.2. Purification of ApPol η . To examine the biological activities of the protein encoded by the ApPol η cDNA, we prepared the recombinant ApPol η protein with a C-terminal (His) $_6$ tag in *E. coli*. The extract was first fractionated on Talon beads, and then purified on a Heparin column. The recombinant protein was eluted by a step gradient of NaCl (Figure 2(a)). As expected from the calculated relative molecular mass, the purified recombinant ApPol η bearing the (His) $_6$ tag migrated as a single band of approximately 85 kDa in the SDS-PAGE analysis (Figure 2(b)), and exhibited DNA polymerase activity (Figure 3(a)). The recombinant human Y-family polymerases, for example, Pol η , Pol ι , and Pol κ , are usually prepared from baculovirus-infected insect cells, as reported previously [13, 17, 39, 40]. In the present study, the incubation of the cultures at a low temperature after induction enabled the production of full-length ApPol η in *E. coli*, as was also reported by Hoffman et al. [41] for Pol η from other species. The yield of the purified protein was 15.5 μ g per liter of the culture.

3.3. TLS Past UV-Induced Photoproducts by ApPol η . UV irradiation induces the formation of pyrimidine dimers, namely, CPD and 6–4 pp. The latter is isomerized to Dewar by exposure to UVA/B. HsPol η catalyzed efficient and accurate TLS past CPD [13], but not 6–4 pp [13] and Dewar (unpublished results). To examine whether ApPol η possessed TLS activity for UV-induced lesions, 30-mer oligonucleotide

templates containing a single lesion were hybridized to a 5'- 32 P-labeled 16-mer oligonucleotide primer, and these duplexes were employed for the TLS reactions (Figure 3). Klenow fragment (KF) inserted one nucleotide opposite CPD, but did not elongate the primer (Figure 3(b), lane 1), whereas ApPol η could efficiently elongate the primer up to the end of the 30-mer, in an enzyme concentration-dependent manner (Figure 3(b), lanes 3–7). Similar experiments were performed with other UV-induced lesions, 6–4 pp and Dewar. ApPol η could insert one nucleotide opposite these lesions, but elongation of the primers was not observed (Figures 3(c) and 3(d)), in a manner similar to HsPol η [13, 42].

To examine the preference for nucleotide incorporation opposite the lesion by ApPol η , the polymerization reactions were performed in the presence of only a single deoxyribonucleoside triphosphate (Figure 4). Although HsPol η replicates undamaged DNA with low fidelity [43, 44], it can accurately incorporate dATP opposite the TT sequence of CPD [13]. On the undamaged template, ApPol η preferred to incorporate dATP correctly, and dGTP was incorporated less frequently (Figure 4(a)), indicating that ApPol η , like HsPol η , replicates undamaged DNA with low fidelity. When the template contained CPD, ApPol η incorporated dATP exclusively (Figure 4(b), lane 4), indicating that ApPol η , like HsPol η , can accurately perform TLS past CPD. For both 6–4 pp and Dewar, primers that were elongated with dAMP and dGMP by ApPol η were detected, although the elongation efficiency was very low (Figures 4(c) and 4(d)). While we are able to test these activities biochemically, establishing the

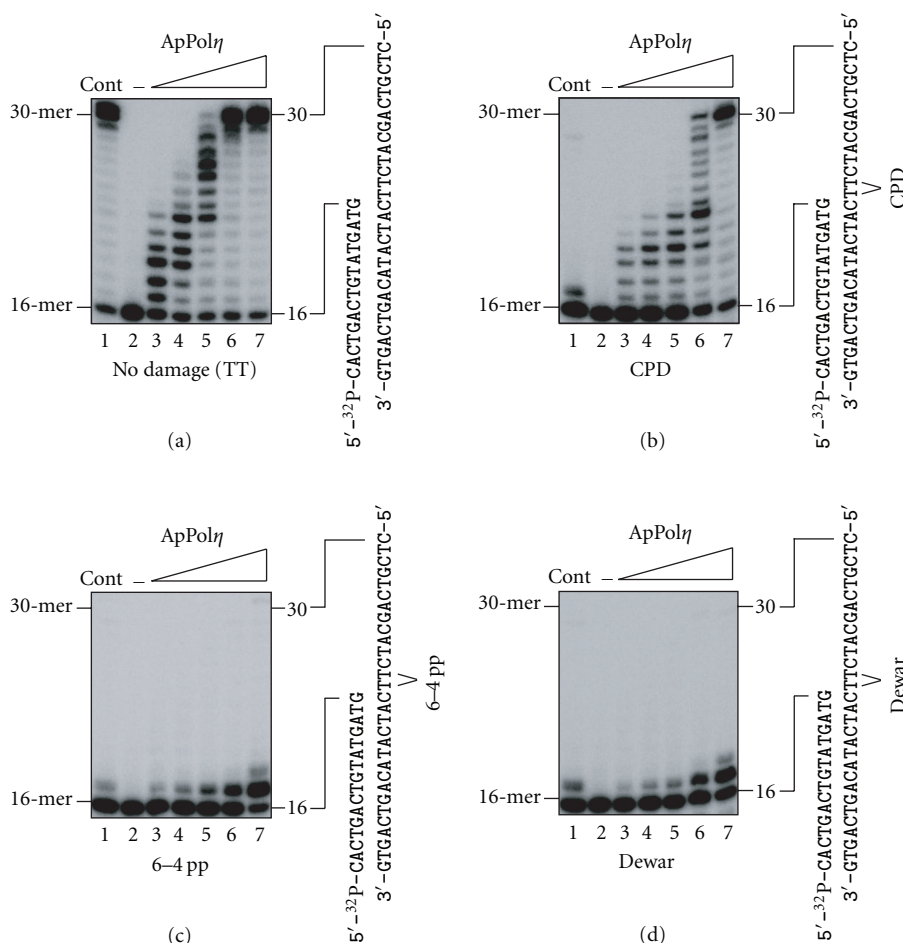


FIGURE 3: TLS by ApPol η past CPD, 6-4 pp, and Dewar. Increasing amounts of ApPol η (12, 24, 59, 118, and 588 fmol in lanes 3-7, resp.) were incubated with 40 nM of the primer-templates indicated beside each panel. The templates contained undamaged TT (a), CPD (b), 6-4 pp (c), and Dewar (d). The samples for lanes 1 and 2 contained 0.1 U KF and no enzyme, respectively. The reactions were incubated at 37°C for 20 minutes, and then were subjected to 15% polyacrylamide/7.5 M urea gel electrophoresis. The autoradiograms of the gels are shown.

biological roles of ApPol η would require additional assays such as genetic complementation, however these results were also similar to those obtained for HsPol η .

3.4. TLS Past Oxidative Lesions by ApPol η . Since *A. pompejana* inhabits deep sea thermal vent regions that are in excess of 1.5 km under the ocean surface where UV-rays from the sun do not penetrate [45, 46], we thought that CPD containing DNA may not be a major template for ApPol η . Other candidates were assumed to be oxidative lesions, because the high temperature and the solutes at the hydrothermal vent sites might induce oxidative stress. Tg is one of the major DNA lesions produced by ROS, and two isomeric forms, 5R-Tg and 5S-Tg, are produced in DNA. Tg often blocks DNA replication by replicative DNA polymerases [40], but rarely induces mutation [41]. However, HsPol η can catalyze efficient and accurate TLS past each Tg isomer [17].

Using the 30-mer oligonucleotides containing a single lesion at the 17th nucleotide from its 3' end as templates, we examined the ability of ApPol η to replicate DNA past

5R-Tg and 5S-Tg. At both lesions, the replicative T7 DNA polymerase was blocked completely (Figures 5(a) and 5(b), lane 1), but ApPol η efficiently catalyzed the replication reaction (Figures 5(a) and 5(b), lanes 3-7). To examine the preference of nucleotide incorporation opposite each Tg isomer, the polymerization reactions were tested in the presence of only a single deoxyribonucleoside triphosphate (Figures 6(a) and 6(b)). ApPol η preferred to incorporate dATP (Figures 6(a) and 6(b), lane 4). Although dGTP was incorporated in the absence of the other nucleotides (lane 6), this product, which exhibited slightly slower migration in the PAGE analysis, was not detected when the four nucleotides were added (lane 3). These results indicated that ApPol η had the ability to catalyze TLS accurately past both 5R-Tg and 5S-Tg.

Another major oxidative DNA lesion induced by ROS is 8-oxoG, which is an important promutagen *in vivo* and *in vitro* [1]. Similar experiments were performed with a template containing 8-oxoG, instead of Tg. No insertion of any nucleotide opposite the lesion by T7 DNA polymerase was observed (Figure 5(d), lane 1), whereas ApPol η could

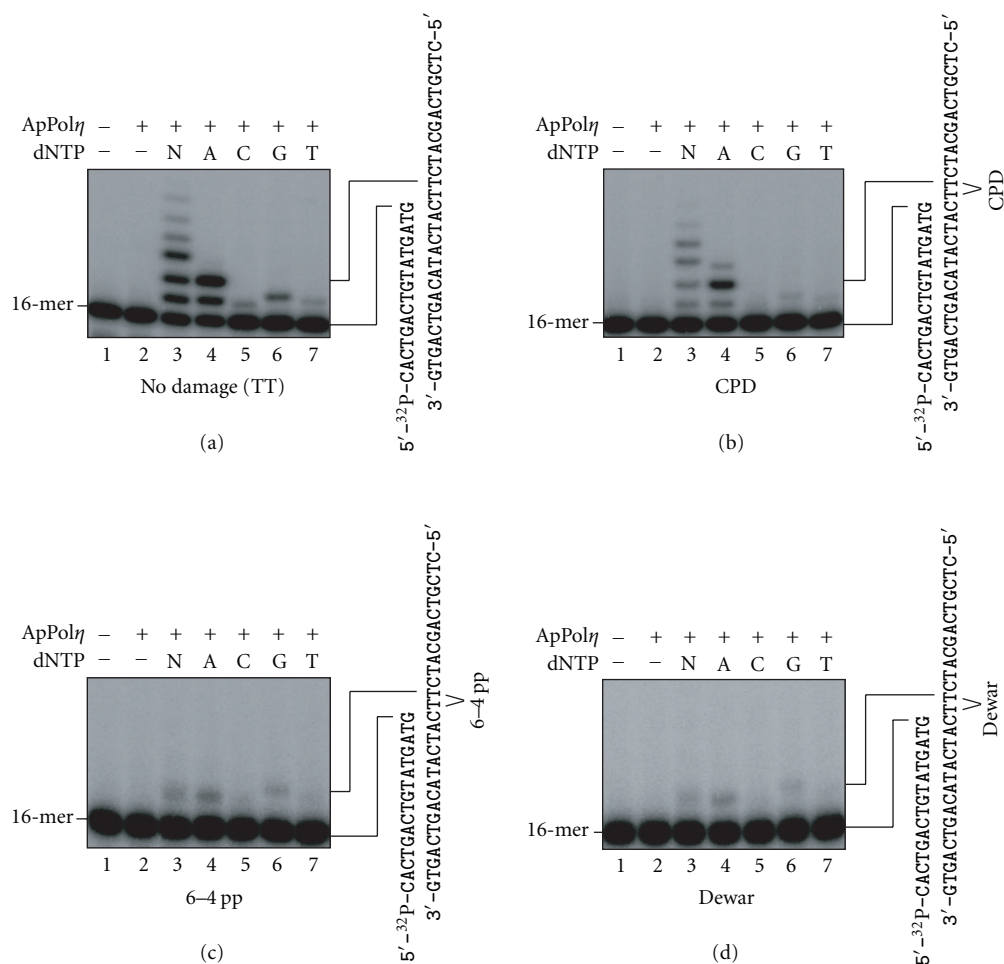


FIGURE 4: Selectivity of nucleotide incorporation by ApPol η opposite undamaged TT (a), CPD (b), 6-4 pp (c), and Dewar (d). ApPol η (12 fmol in lanes 2-7) was incubated with each primer-template in the presence of the four dNTPs (lane 3), the indicated dNTP (lanes 4-7), or the absence of dNTP (lane 2). Lane 1 contained no enzyme. The reactions were incubated at 37°C for 5 minutes, and then were subjected to 15% polyacrylamide/7.5 M urea gel electrophoresis. The autoradiograms of the gels are shown.

bypass 8-oxoG efficiently (Figure 5(d), lanes 3-7). For the nucleotide preference, ApPol η preferred to incorporate the correct dCTP opposite the undamaged template G (Figure 6(c)), whereas it mainly incorporated the incorrect dATP opposite 8-oxoG (Figure 6(d)). Thus, this enzyme replicates DNA containing 8-oxoG efficiently but incorrectly, leading to frequent G·C to T·A transversions. In addition, we investigated whether ApPol η has TLS activity on a template containing the AP site analog. However, like the other Y-family DNA polymerases, this enzyme was unable to catalyze TLS past this lesion (data not shown).

3.5. In Vitro Stability of ApPol η . As *A. pompejana* colonizes the walls of actively venting high-temperature chimneys [23, 45, 46], we hypothesized that its enzymes may be more thermostable than those found in mesophilic organisms, as shown for other TLS polymerases derived from single cellular hyperthermophilic organisms such as *Sulfolobus solfataricus* DNA polymerase 4 (Dpo4) [47, 48]. To test this possibility, we compared the activity of ApPol η along

with that of HsPol η after heating both enzymes to various temperatures for 5 minutes. After this treatment, we tested the ability of both enzymes to incorporate a single dCMP opposite a template G. As shown in Figure 7, ApPol η retained the activity even after it was heated to 49°C. In contrast, the activity of HsPol η diminished rapidly after heating to temperatures higher than 43°C. Therefore, ApPol η is relatively more thermostable than HsPol η .

3.6. Stability of ApPol η in Organic Solvents. As a further test of ApPol η stability, we tested the ability of ApPol η to incorporate dCMP opposite a template G in the presence of various organic solvents. In these assays, dimethyl sulfoxide (DMSO), ethanol, and isopropanol (IPA) were used at concentrations of 20% (v/v). Similar experiments were performed in a study on TLS past a benzo[*a*]pyrene adduct by another thermostable Y-family DNA polymerase, archaeal Dpo4, although the organic solvents were added to stabilize the benzo[*a*]pyrene moiety in the major groove, rather than to test the protein stability [45]. As shown in Figure 8(a),

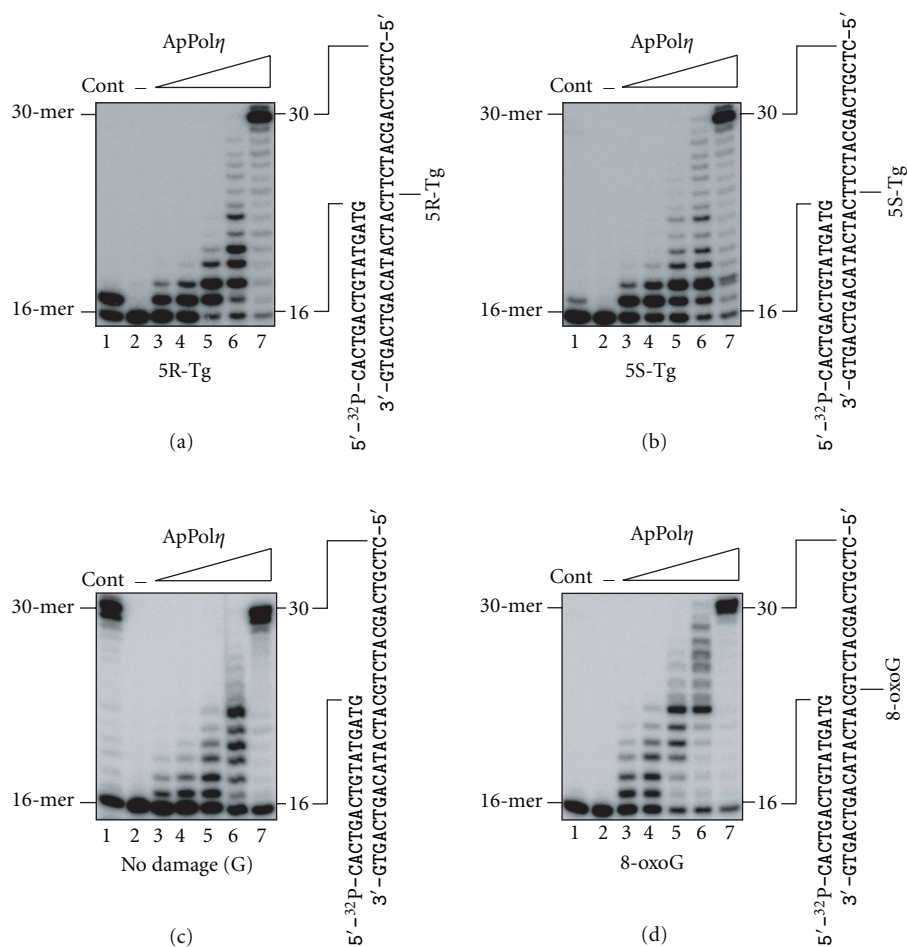


FIGURE 5: TLS by ApPol η past 5R-Tg, 5S-Tg, and 8-oxoG. Increasing amounts of ApPol η (12, 24, 59, 118, 588 fmol in lanes 3–7, resp.) were incubated with 40 nM of the primer-templates indicated beside each panel. The templates contained 5R-Tg (a), 5S-Tg (b), undamaged G (c), and 8-oxoG (d). The samples for lanes 1 and 2 contained 0.1 U T7 DNA polymerase and no enzyme, respectively. The reactions were incubated at 37°C for 20 minutes, and then were subjected to 15% polyacrylamide/7.5 M urea gel electrophoresis. The autoradiograms of the gels are shown.

ApPol η exhibited higher activity than HsPol η in the presence of all of the solvents used in this study, although IPA greatly reduced the enzyme activity. In the presence of DMSO, ApPol η retained the full activity, even when the reaction mixture contained this solvent at a concentration of 20% (Figure 8(b)). These results support our conclusion that the full-length ApPol η protein is more stable than its human homolog.

4. Discussion

4.1. CPD and Tg in TLS by ApPol η . In this study, we cloned the cDNA encoding the novel thermostable DNA polymerase η from *A. pompejana*, which colonizes deep-sea hydrothermal vents. This enzyme was produced in *E. coli*, and was purified as a full-length recombinant protein (85 kDa) bearing a (His)₆ tag. The purified ApPol η protein displayed DNA polymerase activity. This enzyme catalyzed TLS past CPD, 5R-Tg, 5S-Tg, and 8-oxoG, but not past 6–4 pp, Dewar, or the AP site analog, in a similar manner to

TABLE 1: Comparison of nucleotide incorporation into lesion-containing template-primers between HsPol η and ApPol η .

DNA lesion	HsPol η	ApPol η
CPD	AA [7–10]	AA
6–4 pp	— ^a [9, 10]	— ^a
Dewar	— ^{a,b}	— ^a
5R-Tg	A [15]	A
5S-Tg	A [15]	A
8-oxoG	C/A [16, 17]	C/A
AP site analog	— ^a [10, 17]	— ^a

^ano incorporation; ^bunpublished result.

HsPol η (Table 1). We also demonstrated that ApPol η was more stable than HsPol η .

Despite the fact that *A. pompejana* inhabits the deep sea, where UV-rays do not penetrate, the UV-induced CPD was found to be an excellent template for ApPol η . Considering

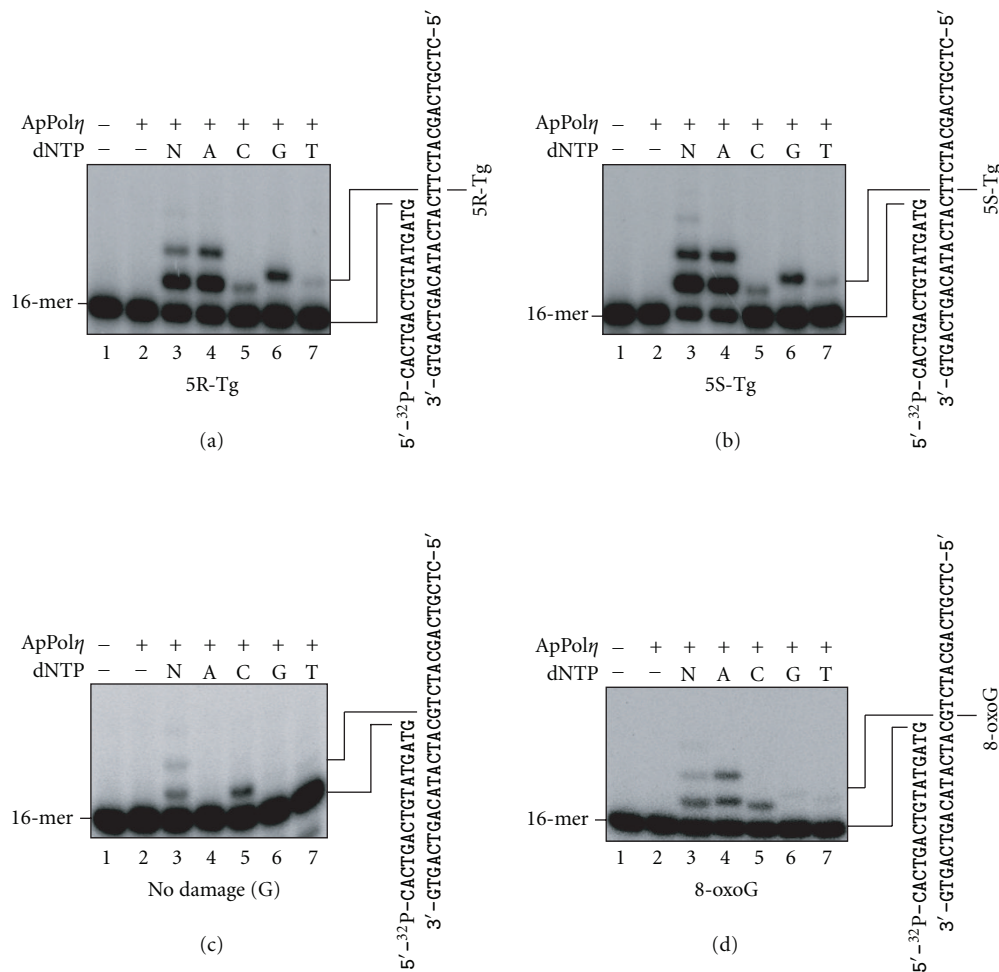


FIGURE 6: Selectivity of nucleotide incorporation by ApPol η opposite 5R-Tg (a), 5S-Tg (b), undamaged G (c), and 8-oxoG (d). ApPol η (12 fmol in lanes 2–7) was incubated with each primer-temple in the presence of the four dNTPs (lane 3), the indicated dNTP (lanes 4–7), or the absence of dNTP (lane 2). Lane 1 contained no enzyme. The reactions were incubated at 37°C for 5 minutes, and then were subjected to 15% polyacrylamide/7.5 M urea gel electrophoresis. The autoradiograms of the gels are shown.

that a genetic defect in the HsPol η gene results in the XP-V syndrome, and that XP-V patients are sensitive to sunlight and highly prone to cancer development, the critical role of HsPol η is thought to be error-free TLS across CPD in human cells. As *A. pompejana*'s environment is significantly less subject to UV radiation produced by the sun, we speculate that the inclusion of the TLS polymerase system within the organism may arise from specific environmental adaptations. Evidence for photosynthetic events at hydrothermal vent sites has been noted [49], where energy for the reactions may arise from vent activity. Moreover, the vent area is sporadically subject to periods of intense volcanic and geothermal activity that may cause production of bulky DNA lesions within these organisms. The presence of ApPol η may also play a role in protecting developing embryos, as embryos pass through a period of developmental arrest when passing from one colony site to another before renewed growth [50]. In these cases, they may experience periods of geothermal light during passage and during their development at their new colonization site. Thus, these Pol η proteins may protect the

embryo until it becomes more self-motile and can build its housing tube.

Here, we also showed that this polymerase catalyzes efficient and accurate TLS past both 5R-Tg and 5S-Tg, which are generated by ROS. Since Tg can block DNA replication by replicative DNA polymerases and also induces cell death, the critical role of ApPol η might be error-free TLS across Tg in *A. pompejana* cells.

4.2. Stability of ApPol η . Since *A. pompejana* inhabits hydrothermal vent sites at temperatures that often measure within the representative classifications for thermophilic (40–60°C) and hyperthermophilic organisms (60°C and higher), we expected that ApPol η would be thermostable. In agreement with these expectations, we found that ApPol η was more thermostable than HsPol η (Figure 7), but less stable than an archaeal DinB-like polymerase from the hyperthermophile *S. solfataricus* P2 [47, 51] which lives at higher temperatures (>75°C) than *A. pompejana*. Posttranslational modification of proteins is sometimes important for

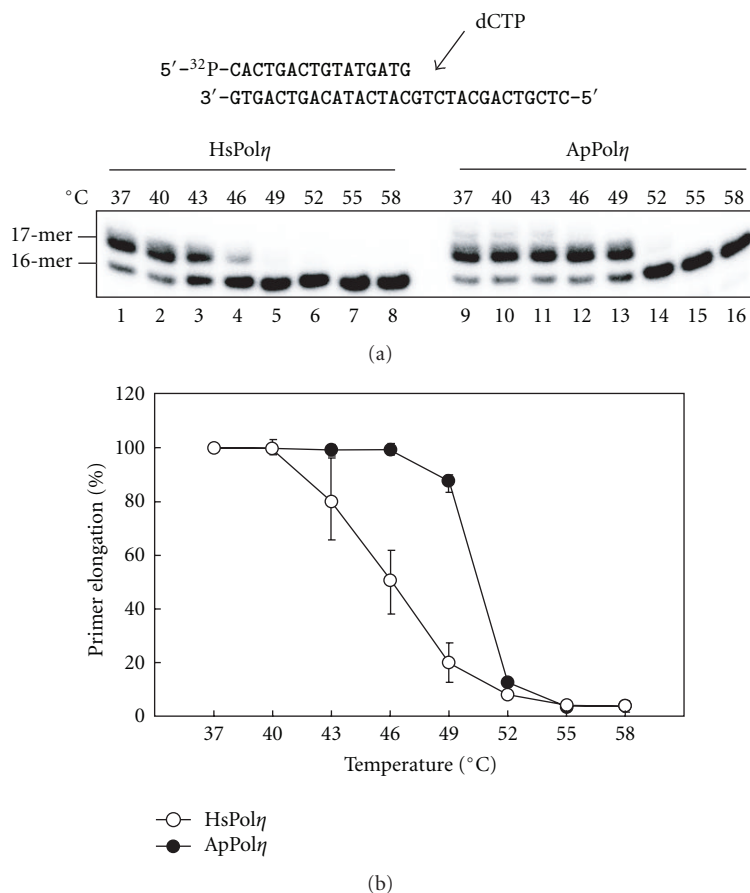


FIGURE 7: Thermostability of ApPol η . (a) Aliquots of ApPol η (closed circles) and HsPol η (open circles) were heated to the indicated temperature for 5 minutes. The effect of this treatment on the polymerase activity was subsequently determined by analyzing the respective enzyme's ability to extend a radiolabeled primer by incorporating dCMP opposite the template G. Replication assays were performed at 37°C for 20 minutes. (b) The ability of each enzyme to extend the primer was quantified, and the specific activity compared to that of each enzyme heated at 37°C was calculated and subsequently plotted as a function of the temperature to which the enzyme was initially exposed.

their structure and function. Since the ApPol η used in this study was produced in *E. coli*, the measured thermostability was that without eukaryotic post-translational modifications. We expected that this enzyme may be modified in insect cells, which are more closely related to *A. pompejana* cells than to *E. coli* cells, and thus might gain higher thermostability. Therefore, we prepared ApPol η in Sf9 insect cells using the baculovirus expression system. The ApPol η protein produced in Sf9 cells could catalyze DNA synthesis at 37°C, but lacked translesion activity after it was heated to 55°C (data not shown).

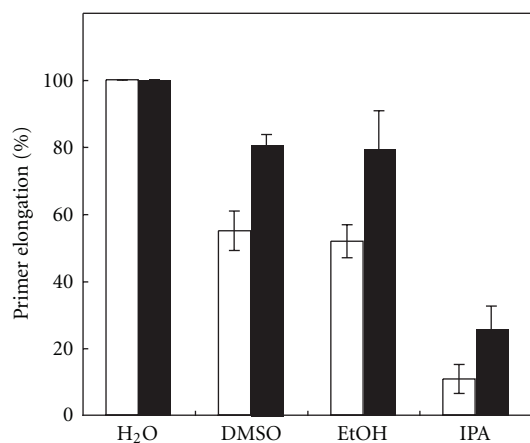
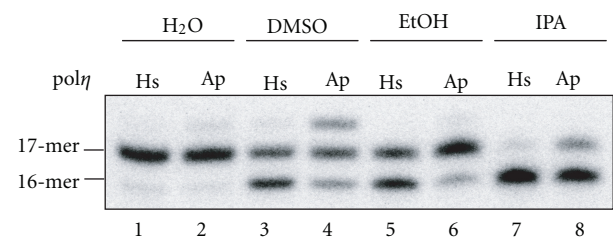
There might be several reasons why ApPol η is not even more thermostable. (1) It has been reported that Pol η functions in a replication complex during TLS [37, 38, 52]. Thus, it is possible that ApPol η becomes more heat resistant with the help of other thermostable replication proteins in *A. pompejana* cells. In fact, ApPol η has a PCNA binding motif (Figure 1(a)), indicating that ApPol η may exist in a replication complex with PCNA. (2) Y-family DNA polymerases replicate undamaged DNA in an error-prone manner [34–36, 38]. Hence, to maintain genetic integrity, it might not be favorable for cells to have an extremely stable

mutator polymerase. The present study is the starting point for the elucidation of the temperature preference of each *A. pompejana* enzyme and its biological significance, which will further our understanding of the ecology of this unique living organism.

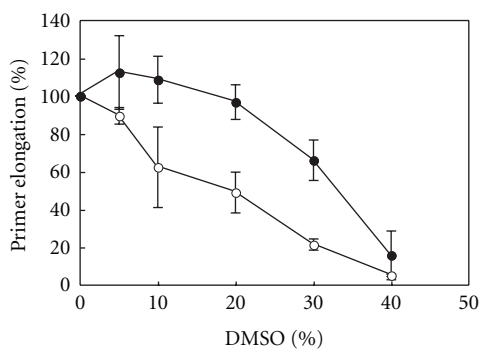
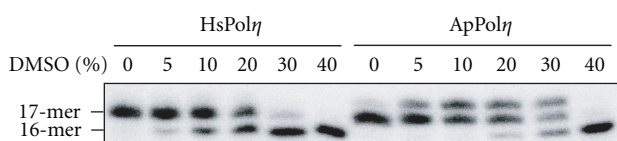
ApPol η could be useful for *in vitro* studies as a new paradigm system to understand TLS mechanisms and also could be a starting material for the development of a more stable eukaryotic-like TLS polymerase by directed evolution. Such an enzyme will enable us to amplify heavily damaged DNA, such as ancient DNA, by PCR-like methods.

5. Conclusions

In this study, we cloned an *A. pompejana* gene with homology to the HsPol η gene. The primary structure of the protein indicated the conservation of the PCNA binding domain and the ubiquitin-binding zinc finger motif. The encoded full-length recombinant protein, ApPol η , was readily produced in *Escherichia coli* and purified. We demonstrated that ApPol η could bypass CPD, 8-oxoG, 5R-Tg, and 5S-Tg, but not



(a)



(b)

FIGURE 8: Activity of ApPol η in organic solvent-containing solutions. (a) Incorporation of dCMP in the presence of DMSO, ethanol, and IPA at a concentration of 20% (v/v) was analyzed, using the primer-template shown in Figure 7. Replication assays were performed at 30°C for 10 minutes. (b) The same assays were performed with various concentrations of DMSO.

6–4 pp, Dewar, or any AP site analog revealing a similar spectrum of activities to those of the human homolog. ApPol η 's properties of higher stability with human-like activities offer advantages over other known eukaryotic TLS polymerases for future basic and practical applications.

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