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Quantitative Analysis of the Mutagenic Potential of 1-Aminopyrene-DNA Adduct Bypass Catalyzed by Y-Family DNA Polymerases

Shanen M. Sherrer^{a,b}, David J. Taggart^a, Lindsey R. Pack^a, Chanchal K. Malik^c, Ashis K. Basu^c, and Zucai Suo^{a,b,*}

^aDepartment of Chemistry and Biochemistry, The Ohio State University, Columbus, OH 43210, USA

^bThe Ohio State Biochemistry Program, The Ohio State University, Columbus, OH 43210, USA

^cDepartment of Chemistry, University of Connecticut, Storrs, CT 06269, USA

Abstract

N-(deoxyguanosin-8-yl)-1-aminopyrene (dG^{AP}) is the predominant nitro polyaromatic hydrocarbon product generated from the air pollutant 1-nitropyrene reacting with DNA. Previous studies have shown that dG^{AP} induces genetic mutations in bacterial and mammalian cells. One potential source of these mutations is the error-prone bypass of dG^{AP} lesions catalyzed by the low-fidelity Y-family DNA polymerases. To provide a comparative analysis of the mutagenic potential of the translesion DNA synthesis (TLS) of dG^{AP}, we employed short oligonucleotide sequencing assays (SOSAs) with the model Y-family DNA polymerase from *Sulfolobus solfataricus*, DNA Polymerase IV (Dpo4), and the human Y-family DNA polymerases eta (hPol η), kappa (hPol κ), and iota (hPol ι). Relative to undamaged DNA, all four enzymes generated far more mutations (base deletions, insertions, and substitutions) with a DNA template containing a site-specifically placed dG^{AP}. Opposite dG^{AP} and at an immediate downstream template position, the most frequent mutations made by the three human enzymes were base deletions and the most frequent base substitutions were dAs for all enzymes. Based on the SOSA data, Dpo4 was the least error-prone Y-family DNA polymerase among the four enzymes during the TLS of dG^{AP}. Among the three human Y-family enzymes, hPol κ made the fewest mutations at all template positions except opposite the lesion site. hPol κ was significantly less error-prone than hPol ι and hPol η during the extension of dG^{AP} bypass products. Interestingly, the most frequent mutations created by hPol ι at all template positions were base deletions. Although hRev1, the fourth human Y-family enzyme, could not extend dG^{AP} bypass products in our standing start assays, it preferentially incorporated dCTP opposite the bulky lesion. Collectively, these mutagenic profiles suggest that hPol κ and hRev1 are the most suitable human Y-family DNA polymerases to perform TLS of dG^{AP} in humans.

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*Corresponding author: Zucai Suo, 880 Biological Sciences, 484 West 12th Ave., Columbus, OH 43210; Tel: 614-688-3706; Fax: 614-292-6773; suo.3@osu.edu.

6. SUPPORTING INFORMATION AVAILABLE

The DNA ligation method, SOSA sequences and table of SOSA comparisons to pre-steady state kinetics are available as supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Keywords

SOSA; Y-family DNA polymerases; mutagenic analysis; translesion DNA synthesis; 1-nitropyrene

1. INTRODUCTION

DNA lesions, derived from both endogenous and exogenous sources, often cause DNA polymerases to stall during genomic replication [1–3]. To rescue stalled replication machinery, cells often switch to a Y-family DNA polymerase that is specialized to function in DNA lesion bypass, a process known as translesion DNA synthesis (TLS). These Y-family enzymes have been shown to catalyze both error-free and error-prone TLS [4–6]. A model Y-family DNA polymerase, *Sulfolobus solfataricus* DNA Polymerase IV (Dpo4), has been extensively studied *in vitro* [7–14]. As it is the lone Y-family enzyme in the thermophilic archaeon *S. solfataricus* [15], it is no surprise that Dpo4 is capable of bypassing a myriad of DNA lesions including apurinic/apyrimidinic (abasic) sites [11,16], *cis-syn* cyclobutane thymidine (*cis-syn* TT) dimers [8,17], benzo[a]pyrene diol epoxide on deoxyguanosine (BPDE-dG) [18] and *N*-2-acetyl-aminofluorene (AAF) on deoxyguanosine (AAF-dG) [8].

In comparison, humans encode four Y-family DNA polymerases designated as DNA polymerases eta (hPol η), kappa (hPol κ), iota (hPol ι), and Rev1 (hRev1). *In vivo* and *in vitro*, hPol η is known to catalyze the error-free bypass of *cis-syn* TT dimers derived from UV exposure [19–21]. Inactivation of hPol η via genetic mutations leads to the Xeroderma Pigmentosum variant (XPV) disease [19,22], which predisposes individuals to UV-induced skin cancer. Notably, both hPol η and hPol κ have been shown to bypass DNA lesions including AAF-dG [23–25], BPDE-dG [25,26], and abasic sites [23,26,27]. hPol κ also efficiently elongates mispaired primer termini [28]. *In vitro*, hPol ι has been shown to bypass abasic sites [27], *cis-syn* TT dimers [29] and AAF-dG [30], while hRev1 preferentially incorporates dCTP opposite any undamaged template base [31] and DNA lesions, including abasic sites [27] and BPDE-dG [32]. In spite of intense studies of lesion bypass *in vitro*, it is still unclear which human Y-family DNA polymerase bypasses which DNA lesion(s) *in vivo* and this problem is partly due to overlapping lesion-bypass abilities of the four human enzymes.

Exposure to fine particulate air pollution containing toxic chemicals such as 1-nitropyrene (1-NP) has been linked to increased risk of lung cancer and cardiopulmonary mortality [33,34]. The metabolites of 1-NP, one of the most abundant polycyclic aromatic hydrocarbons (PAH) [35,36], react with DNA to predominantly form *N*-(deoxyguanosin-8-yl)-1-aminopyrene (dG^{AP}) [37]. Due to high dG^{AP} induced DNA mutagenesis in both bacterial and mammalian cells, 1-NP is classified as a class 2B carcinogen [38,39]. Previously, we have shown that dG^{AP} alters the nucleotide incorporation kinetics in the immediate vicinity of the lesion site when catalyzed by Dpo4 and by each human Y-family polymerase [14,40]. However, the full mutagenic potential of dG^{AP} during TLS catalyzed by each of the five Y-family enzymes remains unknown. In this study, we quantitatively evaluated the dG^{AP} bypass abilities of Dpo4 and the human Y-family DNA polymerases using short oligonucleotide sequencing assays (SOSAs) in combination with standing start assays. Our SOSA data demonstrate how Dpo4 aids relatively high genomic stability of *S. solfataricus* [3,15,41] in the presence of DNA damaging reagents. Moreover, the mutagenic spectra of dG^{AP} bypass generated from our investigation illustrate the mutagenic potential of environmental pollutant 1-NP and provide insight into which human enzyme(s) likely perform(s) TLS of dG^{AP} *in vivo*.

2. MATERIAL AND METHODS

2.1. Materials

Reagents were purchased from the following companies: OptiKinase from USB Corporation, [γ - ^{32}P]ATP from MP Biomedicals, and dNTPs from GE Healthcare. *S. solfataricus* Dpo4 with a C-terminal His₆ tag, human Pol η with a C-terminal His₆ tag, human Pol ν with an N-terminal GST tag, human Rev1 with an N-terminal His₆ tag and human Pol κ with an N-terminal His₆ tag were expressed in *E. coli* and purified as previously described [7,27].

2.2. DNA Substrates

The DNA templates 73-merAP and 73-mer (Table 1) were generated by ligation of the 26-mer-dG^{AP} or the 26-mer (Table 1), respectively, with a 27-mer and 20-mer as shown in Supplementary Figure 1. All other DNA substrates (Table 1), purchased from Integrated DNA Technologies, were purified, radiolabeled, and annealed as previously described [13,14].

2.3. Reaction buffers

All standing start assays and SOSAs were performed in reaction buffer R (50 mM HEPES, pH 7.5 at 37 °C, 5 mM MgCl₂, 50 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 10 % glycerol, and 0.1 mg/ml BSA). All reported concentrations are final after mixing the reaction solutions.

2.4. Standing Start Assay

A solution of hRev1 (1 μM) and either 5'-[^{32}P]-labeled 20-mer/26-mer (100 nM) or 5'-[^{32}P]-labeled 20-mer/26-mer-dG^{AP} (100 nM) in reaction buffer R at 37 °C was rapidly mixed with only dATP (200 μM), only dCTP (200 μM), only dGTP (200 μM), only dTTP (200 μM), or all four dNTPs (200 μM each). The reaction mixtures were stopped after 10 min by the addition of 0.37 M EDTA. The products were resolved via denaturing PAGE, and quantified by using a Typhoon Trio (GE Healthcare).

2.5. Short Oligonucleotide Sequencing Assay

SOSAs were performed as previously described [42] with the following modifications. The damaged DNA substrate (17-merS/73-merAP, Table 1) contained a dG^{AP} site, which was engineered to be 41 nucleotides from the 3'-end of the DNA template. The control DNA substrate (17-merS/73-mer, Table 1) contained a dG in place of dG^{AP}. To differentiate the newly synthesized DNA products from the templates, the 17-merS primer was designed to anneal 13 bp from the 3'-end of the 73-merAP and 73-mer DNA templates. After incubation with each Y-family DNA polymerase, the newly synthesized DNA products were effectively purified from the templates by using denaturing PAGE. The PAGE purified DNA products were then PCR amplified by using the primers 17-merS and 18-merS (Table 1) and subsequently ligated into vector pCR4-TOPO by using a TOPO TA cloning kit (Invitrogen). Plasmids containing the full-length DNA products were then sequenced from bacterial colonies (Genewiz, Inc.). This method is summarized in Scheme 1. The total relative error rates of the SOSA products generated with control or damaged DNA substrates were compared at each template position by using Student unpaired *t*-tests. All *p*-values less than 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

In previous studies [14,40], our pre-steady state kinetic analysis indicated that Dpo4, hPol η , hPol ι , hPol κ , and hRev1 bypassed and extended from a site-specifically placed dG^{AP} lesion with strikingly different catalytic efficiencies. Here, we employed standing start assays and SOSAs to visualize and quantify the mutagenic potential of TLS of dG^{AP} catalyzed by Dpo4 and by each of the four human Y-family DNA polymerases. Our studies expanded our kinetic understanding of dG^{AP} bypass [14,40], including uncovering the effects that influence the nucleotide incorporation tendencies of each Y-family enzyme both upstream and downstream of a site-specifically placed dG^{AP} lesion. Our studies also provided information about which human Y-family DNA polymerase(s) is likely involved in TLS of dG^{AP} *in vivo*.

3.1. dG^{AP} bypass catalyzed by hRev1

The bypass of DNA lesions is essential for cell survival under environmental stress. To determine if human Y-family DNA polymerases are capable of bypassing dG^{AP} *in vitro*, we previously performed running start assays for each enzyme in the presence and absence of dG^{AP} under identical reaction conditions [40]. Because hRev1 failed to synthesize full-length products over the templates 26-mer and 26-mer-dG^{AP} (Table 1) within 12 hrs [40], we performed standing start assays to directly evaluate its activity and fidelity opposite dG^{AP} (Figure 1). As expected, hRev1 preferentially incorporated correct dCTP opposite dG^{AP}, which is consistent with the function of hRev1 as a dCTP transferase *in vitro* [31,43,44]. Structurally, among natural dNTPs, only dCTP can form two hydrogen bonds with the active site residue Arg357 of hRev1 while the template base is evicted from the active site by Leu358 and is thus not involved in base pairing with an incoming nucleotide [44]. Interestingly, although error-prone, hRev1 incorporated fewer incorrect nucleotides opposite the bulky lesion (Figure 1B) than opposite undamaged dG (Figure 1A). Consistently, our kinetic studies showed that the fidelity of hRev1 is higher with the templating base dG^{AP} (10^{-4} – 10^{-4}) than undamaged dG (10^{-1} – 10^{-4}) [40]. Soon after dG^{AP} bypass, primer elongation ceased even in the presence of all dNTPs, indicating that hRev1 failed to extend the lesion bypass product. Based on the ternary crystal structure of hRev1 [44], we hypothesize that the bulky 1-AP adduct sterically clashes with either the N-digit or the polymerase-associated domain (PAD) within the active site of hRev1 and thereby blocks DNA translocation after dG^{AP} bypass, leading to earlier termination of DNA synthesis with the template 26-mer-dG^{AP} than 26-mer (Figure 1). Therefore, hRev1 cannot catalyze TLS of dG^{AP} alone *in vivo*. Instead, hRev1 may function in conjunction with another polymerase that proficiently extends the lesion bypass product, likely hPol κ (Section 3.3). Alternatively, hRev1 may merely serve as a protein scaffold in TLS of dG^{AP}, as Rev1 specifically interacts with eukaryotic Pol η , Pol κ , and Pol ι , but not with other DNA polymerases such as Pol β or Pol μ [45,46]. Because hRev1 failed to synthesize full-length TLS products, we could not quantitatively analyze the mutagenic profile of dG^{AP} bypass by hRev1 via SOSA.

3.2. Quantitative analysis of the mutational spectra derived from TLS of dG^{AP}

To analyze the mutagenic profiles of TLS of dG^{AP} catalyzed by Dpo4, hPol η , hPol κ and hPol ι , we modified our previously published SOSA method [27,42] by using a template 73-merAP (Table 1) which was easily separated from the 13-nucleotide shorter TLS products through denaturing PAGE (Scheme 1). The synthesis of the long DNA templates, 73-mer and 73-merAP (Table 1, Supplementary Figure 1) was performed via ligation of three oligomers including the 26-mer or 26-mer-dG^{AP} used in our kinetic studies [14,40]. Based on the kinetic statistics of the dNTP misincorporation for Dpo4 and human Y-family DNA polymerases [7,40], we expected to observe at least one base substitution per 1,000 dNTP incorporations with an undamaged DNA template. In order to limit sequencing cost, we

chose to analyze at least 40 individual colonies with a sequence window of at least 25 bases by using SOSA.

3.2.1. Dpo4—For SOSA with Dpo4 and 17-merS/73-merAP (Table 1), we sequenced 52 colonies, which are summarized in Supplementary Figure 2A. Dpo4 incorporated either dCTP (7.7%), or no dNTP (1.9%) opposite dG^{AP} (Figure 2A). Thus, Dpo4 was mostly error-free while incorporating an incoming dNTP opposite dG^{AP}. This finding is in line with the results of our previous kinetic investigation of dG^{AP} bypass catalyzed by Dpo4 [14]. To quantitatively compare mutation frequencies at the DNA lesion site with other DNA template positions, we plotted relative error% as a function of DNA template positions (Figure 3A) within the sequencing window of SOSA. The sequencing window of SOSA was defined as the DNA template positions located between the upstream and downstream PCR primers (Supplementary Figure 2). Interestingly, nucleotide incorporation was most significantly affected at the DNA lesion site (Position X) and one nucleotide downstream from dG^{AP} (Position +1) as evidenced by the highest relative error frequency (~9.6%) within the sequencing window (Figure 3A).

To further evaluate the effect of dG^{AP} on upstream and downstream nucleotide incorporations catalyzed by Dpo4, we performed SOSA using a control DNA substrate 17-merS/73-mer which has a template base dG (Position 0, Table 1) at the corresponding position of dG^{AP} in 73-merAP. Based on the sequences of 52 full-length products (Supplementary Figure 2B), Dpo4 incorporated mostly correct dCTP (98.1%) and occasionally did not incorporate a dNTP (1.9%) opposite dG at Position 0 (Figure 2B). When comparing the mutations generated within the vicinity of Position X/0 in damaged versus undamaged templates, we found that the mutations changed only in frequency and consisted primarily of single-base deletions and single-base substitutions (Figures 3A and 3B). Comparing the total error rate at each template position between the damaged and control DNA substrates (Supplementary Table 1), only the difference in total error rate at Position +1 was found to be statistically significant ($p < 0.03$), confirming the relatively low error-prone tendencies of Dpo4 while bypassing dG^{AP}. Overall, the base substitution frequency of Dpo4 was calculated to be 1.2×10^{-2} with 17-merS/73-merAP and 3.1×10^{-3} with 17-merS/73-mer (Table 2). These values are comparable to the nucleotide incorporation fidelities of 1.4×10^{-2} and 3.7×10^{-3} opposite the templating base dG^{AP} and dG, respectively, measured through pre-steady state kinetic analysis [14]. Notably, differences in the mutagenic data generated with the damaged or undamaged templates suggest that the increased frequencies of base deletions and substitutions observed with 17-mer/73-merAP (Table 1) were most likely caused by the presence of dG^{AP}. The 1.9% relative deletion error frequencies at Positions -5 and -3 of the damaged template (Figure 3A) hinted that dG^{AP} amplified the error rate of Dpo4 upstream from the damage site. This observation suggests that Dpo4 was able to detect the presence of dG^{AP} before the bulky DNA lesion even entered its active site.

3.2.2. hPol η —The sequences of 44 TLS products synthesized by hPol η with the damaged DNA substrate 17-merS/73-merAP (Table 1) were determined via SOSA and are summarized in Supplementary Figure 3A. These sequence data indicate that hPol η preferred to create a -1 frameshift opposite dG^{AP} (45.5%). hPol η also incorporated correct dCTP (36.4%), incorrect dATP (11.4%), incorrect dGTP (2.3%), and incorrect dTTP (4.5%) opposite dG^{AP} (Figure 2A). Interestingly, the percentage of G→T mutations derived from 11.4% dATP misincorporation opposite dG^{AP} was comparable to the measured G→T transversion percentage (6.2%) within kidney cell lines in the presence of dG^{AP} [47]. Furthermore, the total percentage of deletion and base substitution mutations at the dG^{AP} site (64%) indicates that hPol η was highly error-prone during the lesion bypass step.

To determine the effect of dG^{AP} on nucleotide selection by hPol η at other positions of 73-merAP (Table 1), we plotted relative error% as a function of template position (Figure 4A). This figure shows that hPol η made errors at a frequency of 64% at Position X and 38.6% at Position +1. Markedly, hPol η preferred to create deletions at Positions X and +1. At positions flanking the dG^{AP} site, hPol η generated a substantial number of base substitutions and deletions, including an 8-nucleotide deletion (Supplementary Figure 3A). Such a large deletion mutation caused by dG^{AP} has been observed within *E. coli* cells defective for the *dnaQ* gene, which encodes the ϵ subunit (possessing a 3'→5' exonuclease activity) of *E. coli* replicative DNA polymerase III [39]. If not repaired, such an hPol η -catalyzed deletion event is expected to lead to severe mutagenic outcomes *in vivo*.

The base insertion, deletion, and base substitution frequencies varied at all positions of the template 73-merAP (Figure 4A) and the average values were calculated to be 1.9×10^{-3} , 4.9×10^{-2} , and 8.0×10^{-2} , respectively (Table 2). Moreover, hPol η made more deletion and insertion errors at downstream positions than at upstream positions from the dG^{AP} lesion site while the average substitution error frequencies were similar upstream and downstream of Position X (Table 2). Notably, the total relative error frequency at each position along the damaged 73-merAP template appeared to oscillate every 3 to 4 base positions (Figure 4A), likely due to the presence of the bulky 1-AP adduct as the DNA helix turned. Such a cyclic pattern has also been observed kinetically as Dpo4 bypassed a double-base lesion, cisplatin-dGpG [9], which is known to structurally distort the axis of the DNA helix.

For further evaluation of the dG^{AP} effect on upstream and downstream nucleotide incorporations catalyzed by hPol η , we performed SOSA using the control DNA substrate 17-merS/73-mer (Table 1) and obtained the DNA sequences of 51 products displayed in Supplementary Figure 3B. Overall, the base substitution frequency was calculated to be 6.1×10^{-2} with 17-merS/73-mer (Table 2), which is comparable to the error rate of 7.5×10^{-2} calculated previously by using SOSA with a different undamaged DNA template [27], and to the nucleotide incorporation fidelity (2.0×10^{-3} to 2.1×10^{-2}) with undamaged DNA substrates measured by pre-steady state kinetic methods [40].

With control 17-merS/73-mer, hPol η incorporated only correct dCTP opposite dG at Position 0 (Figure 2B) and preferentially incorporated correct dGTP opposite dC at Position +1 (Figure 2D). Along the template 73-mer, the mutations created were mostly single base substitutions although base deletions and insertions were also generated (Figure 4B). The average error frequency of 4.7% with control 17-merS/73-mer (Figure 4B) was ~2.5-fold lower than that (11.9%) with 17-merS/73-merAP (Figure 4A), revealing the mutagenic effect of a single dG^{AP} lesion on DNA synthesis catalyzed by hPol η . The most prominent type of mutation induced by the bulky lesion was base deletions as the damaged template had a 3.1-fold higher average base deletion frequency than undamaged DNA (Table 2). Furthermore, the impact of dG^{AP} was larger at downstream template positions (2.8-fold) than at upstream template positions (1.5-fold) from the lesion site (Table 2). The observed increases in base deletions from Position -8 to Position -1 suggest that hPol η , like Dpo4, detected the presence of dG^{AP} before the lesion encountered the active site of hPol η .

3.2.3. hPol κ —We sequenced 45 colonies that contained products from TLS of dG^{AP} catalyzed by hPol κ (Supplementary Figure 4A). Of the nucleotide incorporation events opposite dG^{AP} (Figure 2A), 37.8% were correct dCTP incorporations, 26.7% were dATP misincorporations, 2.2% were dTTP misincorporations, and 33.3% were no dNTP incorporations. Thus, hPol κ only slightly favored accurate lesion bypass over generating -1 frameshifts at the dG^{AP} site and was highly error-prone if it did incorporate a nucleotide opposite dG^{AP}. It appears to be abnormal that dGTP misincorporations opposite dG^{AP} did not occur in the SOSA reactions but were observed in our single-nucleotide kinetic assays

with 20-mer/26-mer-dG^{AP} [40]. However, the next template base in template 26-mer-dG^{AP} is dC, suggesting that the dGTP incorporations observed in our kinetic assays [40] may actually occur via the “lesion-looped out” mechanism which allowed the incoming dGTP to base pair with the next template base dC [42]. Thus, SOSA provides additional valuable information when compared to kinetic assays at a template position where a DNA polymerase prefers to use the “lesion-looped out” mechanism to select and incorporate an incoming nucleotide. By examining the whole sequencing window, we observed that the most frequent mutations were base deletions, followed by base substitutions and insertions (Figure 4C). Consistently, the average base deletion, substitution, and insertion error rates were calculated to be 1.9×10^{-2} , 2.9×10^{-2} , and 2.8×10^{-3} , respectively (Table 2).

In addition, our SOSA data with the control DNA substrate 17-merS/73-mer (Supplementary Figure 4B) demonstrate that hPol κ made significantly fewer mutations with undamaged (Figure 4D) than damaged DNA (Figure 4C). Interestingly, the average substitution and insertion error rates were almost unchanged in the presence or absence of dG^{AP} while average base deletion rate (7.4×10^{-3}) increased by 2.6-fold in the presence of the lesion (Table 2). Although hPol κ predominantly generated single-base deletions in the vicinity of the 1-AP adduct (Figure 4C), mostly single-base substitutions were observed with undamaged 17-merS/73-mer (Figure 4D).

3.2.4. hPol ι —To quantitatively analyze the mutagenic profile of dG^{AP} bypass catalyzed by hPol ι , we sequenced 43 full-length lesion bypass products (Supplementary Figure 5A). Similar to the SOSA results for abasic site bypass catalyzed by hPol ι [27], only one out of 43 bypass products generated from TLS of dG^{AP} was correct. These data indicate that hPol ι was more error-prone than Dpo4, hPol η and hPol κ during TLS of dG^{AP}. Opposite the bulky lesion, hPol ι preferred correct dCTP incorporation (48.8%) over base deletion (34.9%), dATP misincorporation (9.3%), dGTP misincorporation (4.7%), and dTTP misincorporation (2.3%) (Figure 2A). The G→T mutation percentage observed with hPol ι , which is equal to the frequency 9.3% of dATP misincorporation opposite dG^{AP}, was also comparable to the measured G→T transversion percentage (6.2%) within kidney cell lines in the presence of dG^{AP} [47].

Strikingly, hPol ι generated a significant number of base deletions and substitutions at DNA template positions located both upstream and downstream from the dG^{AP} site, which led the total relative error% at each template position to vary from 2% to 91% (Figure 4E). Some lesion bypass products contained multi-base deletions, substitutions or insertions (Supplementary Figure 5A). Notably, hPol ι was more error-prone at Position -10 than at any other positions including the lesion site (Figure 4E), suggesting that dNTP incorporation opposite template base dT is more problematic than for other bases. Indeed, the DNA template positions with the highest relative error frequencies (Positions -10, -6, -5, +7, +9, and +12, Figure 4E) were all template dTs. At these positions, hPol ι preferred to either skip or misincorporate dGTP opposite dT (Supplementary Figure 5A). Thus, hPol ι was most error-prone when opposite template dTs near the dG^{AP} lesion. This bias is consistent with the fact that Pol ι preferentially forms the incorrect base pair dGTP:dT over the Watson-Crick base pair dATP:dT [27,48].

The effect of dG^{AP} on DNA synthesis catalyzed by hPol ι was more evident when comparing the sequencing data for the damaged (17-merS/73-merAP) and undamaged (17-merS/73-mer) DNA substrates (Supplementary Figure 5). At Position 0, hPol ι became almost error-free with only 2% frequency of dATP misincorporation (Figure 2B). Moreover, 44 of the 50 products synthesized by hPol ι with undamaged DNA were completely error-free (Supplementary Figure 5B). Overall, the average base deletion, substitution, and insertion error rates with the undamaged template 73-mer were calculated to be 1.5×10^{-2} ,

1.5×10^{-2} , and 1.7×10^{-3} , respectively (Table 2). In the presence of a dG^{AP}, the average base deletion, substitution, and insertion error rates increased by 15.3-, 17.3-, and 9.4-fold, respectively (Table 2). The differences in Figures 4E and 4F indicate that dG^{AP} significantly increased the error rate of hPol η at template positions at and near the lesion, especially opposite template dTs.

3.3. General patterns of mutagenic events during TLS of dG^{AP}

Analysis of the nucleotide incorporation profiles during TLS of dG^{AP} catalyzed by Dpo4, hPol η , hPol κ , and hPol ι revealed several general trends: **i)** at all DNA template positions other than Position X, all four Y-family enzymes had higher base deletion and/or insertion error rates with the damaged template 73-merAP than with the control template 73-mer (Table 2, Figures 3 and 4); **ii)** among the four enzymes, hPol ι possessed the highest mutation frequency at all template positions other than at Position X in 73-merAP (Figure 4); **iii)** Dpo4, hPol η and hPol κ displayed higher mutation frequencies at Positions X and +1 than at any other position on template 73-merAP (Figures 3 and 4); **iv)** all three human enzymes generated more base deletions than base substitutions when bypassing dG^{AP}, and none of the Y-family enzymes made a base insertion (Figure 2); **v)** among the base substitutions at Position X, all four enzymes were more likely to misincorporate dATP than dGTP and dTTP opposite dG^{AP}, which led to G→T transversions during the next round of DNA replication. These mutations described in **iv)** and **v)** have *in vivo* relevance as G→T transversions and -1 frameshifts are the most frequently observed mutations induced by dG^{AP} in cell-based assays [39,47]; **vi)** opposite dG^{AP}, all four enzymes generated frameshift mutations with the following order of relative base deletion%: hPol η > hPol ι > hPol κ > Dpo4 (Figure 2A); **vii)** the base deletion error rates of the three human Y-family enzymes significantly increased after they encountered dG^{AP} (Table 2, Figure 4); and **viii)** For Positions +1 to +14, hPol κ generated fewer mutations than hPol η and hPol ι (Figure 4). Moreover, only Positions X and +3 were significantly different in total error rate from the corresponding control template position in our statistical analysis, which suggests hPol κ was more error-prone opposite the bulky lesion than Position +1 (Figure 4C, Supplementary Table 1). Thus, hPol κ is the best suited human Y-family DNA polymerase to extend dG^{AP} bypass products in order to maintain genetic stability in human beings. This conclusion is in line with previous observations that Pol κ can efficiently extend mispaired or abnormal primer termini [28,49].

In addition, the base deletions and insertions at the upstream template positions from dG^{AP} increased considerably with all four enzymes, especially with Dpo4, hPol η and hPol ι (Figures 3 and 4). Such an increase in mutagenic potential suggests a sensing mechanism by which each enzyme detected the presence of dG^{AP} several template positions prior to the bulky DNA lesion entering the active site. This phenomenon has also been observed during TLS of an abasic site catalyzed by hPol η , hPol κ and hPol ι [27]. Although the sensing mechanism is not mechanistically understood, we hypothesize that it arises from protein-DNA interactions as the 5' single-stranded portion of the DNA template is threaded through the interface between the Finger and PAD (or Little Finger) domains of each Y-family DNA polymerase. Based on published crystal structures of the Y-family DNA polymerases [18,44,50–52], the presence of a bulky DNA lesion will potentially alter these protein-DNA interactions, thereby permitting the enzyme to sense the upstream lesion. Furthermore, we hypothesize that the increased relative error frequency at the downstream template positions from dG^{AP} is due to local DNA structural distortions induced by the dG^{AP}. More specifically, we predict that the bulky lesion structurally interferes with the translocation of each of the Y-family DNA polymerases along the damaged DNA template. Clearly, these hypotheses need to be verified by experimental evidence.

3.4. Comparison of error frequencies measured by using SOSAs and pre-steady state kinetic assays

As predicted by the fidelity values measured kinetically in our previous studies [14,40], our SOSA analysis confirmed that the error rates for nucleotide incorporations opposite both dG^{AP} and the downstream template base dC catalyzed by Dpo4, hPol η , hPol κ , and hPol ι were all significantly higher than those obtained with undamaged DNA (Figure 2, Supplementary Table 2). However, Supplementary Table 2 shows that the error rates at these two template sites calculated based on our SOSA results (Supplementary Figures 2–5), including or excluding the base deletion events, were not always comparable to the corresponding pre-steady state kinetic probabilities [14,40]. These disparities can be explained by the following experimental and methodology differences between pre-steady state kinetic assays and SOSAs: i) SOSAs were performed with all four dNTPs present while pre-steady state kinetic assays were performed with a single dNTP present; ii) SOSA analysis was restricted by the ability of each DNA polymerase to synthesize full-length products whereas the DNA products from the extension of a primer by one or more nucleotides are all counted in pre-steady state kinetic analysis. Thus, shorter than full-length products in SOSAs were not counted when calculating error rates; iii) SOSA analysis accounted for consecutive dNTP incorporation events to form each full-length dG^{AP} bypass product and thus, each dNTP incorporation was dependent upon preceding incorporation events. In contrast, pre-steady state kinetic analysis assumes each dNTP incorporation was opposite a specific template base are independent of preceding events; iv) SOSA analysis identified both complex and multi-base mutations within a single full-length product (Supplementary Figures 2–5). Although an incorrect nucleotide was occasionally incorporated multiple times, these extra misincorporations opposite a specific template position were not counted during the fidelity measurements via pre-steady state kinetic analysis; v) SOSA analysis identifies all mutagenic events (base substitutions, deletions, and insertions) whereas pre-steady state kinetic analysis assumes each dNTP misincorporation as a base substitution. Thus, the fidelity derived from pre-steady state kinetic analysis was inaccurate for positions on the templates where deletions dominated the total mutagenic events (Figures 2A, 2C, and 4). The observed deletions and insertions are likely due to either the looping out of primer or template base(s) caused by a lesion [42], or DNA polymerase slippage at GC-rich regions or repeated sequences. If these non-canonical mechanisms are involved in nucleotide incorporation, pre-steady state kinetic analysis is not suitable for aforementioned reasons.

4. CONCLUSION

Our SOSA studies serve to define the precise mutagenic profiles of three human Y-family DNA polymerases and Dpo4, the lone Y-family enzyme of *S. solfataricus*, in response to a single dG^{AP} lesion. We also calculated the error rates of dG^{AP} bypass for each of these enzymes and compared these values to those of normal DNA synthesis (Table 2). Under the reaction conditions in this paper, our data suggest that the relatively faithful and efficient *in vivo* dG^{AP} bypass pathway in humans involves hPol κ , due to i) the lowest error frequency of the extension of dG^{AP} bypass products catalyzed by hPol κ (Figure 4), ii) the preferential creation of small frameshift mutations by hPol κ opposite the dG^{AP} lesion (Figure 2), and iii) the 2nd highest efficiency of dG^{AP} bypass as determined by pre-steady state kinetic analysis [40]. The second human Y-family enzyme which likely participates in dG^{AP} bypass *in vivo* is hRev1. This is because when bypassing dG^{AP}, hRev1 is most accurate (Figure 1 and reference [40]) and has relatively high dCTP incorporation efficiency [40]. Thus, the best dG^{AP} bypass scenario involving Y-family DNA polymerases would be for hRev1 (Figure 1) to insert correct dCTP opposite the bulky lesion and for hPol κ to extend the lesion bypass product.

Relative to hPol η , hPol κ , and hPol ι , Dpo4 generated significantly fewer mutations during TLS of dG^{AP}. A higher TLS fidelity of Dpo4 is presumably required to compensate for the lack of multiple Y-family DNA polymerases present in *S. solfataricus* in order to maintain archaeal genomic stability which is higher than other DNA-based organisms including human beings [15,41,53,54]. Markedly, the bulky dG^{AP} lesion increased the relative error frequencies of all four Y-family enzymes upstream and/or downstream from the lesion site, possibly by interfering with polymerase translocation along the damaged template during DNA synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AAF-dG	<i>N</i> -acetyl-2-aminofluorene adduct at the C8 position of deoxyguanosine
1-AP	1-aminopyrene
BPDE-dG	benzo[a]pyrene 7,8-diol 9,10-epoxide-derived adduct at the N2 position of 2'-deoxyguanosine
BSA	bovine serum albumin
dG^{AP}	<i>N</i> -(deoxyguanosin-8-yl)-1-aminopyrene
dNTP	2'-deoxynucleoside 5'-triphosphate
Dpo4	<i>Sulfolobus solfataricus</i> DNA Polymerase IV
PAD	polymerase-associated domain
SOSA	short oligonucleotide sequencing assay
TLS	translesion DNA synthesis

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HIGHLIGHTS

1. Frameshifts observed with the SOSA method may lead to genetic mutations *in vivo*.
2. *S. solfataricus* Dpo4 is less error-prone than human Y-family DNA polymerases.
3. Frameshift error frequency caused by Y-family enzymes increases in the presence of dG^{AP}.
4. hRev1 is the most suitable human Y-family enzyme to faithfully bypass dG^{AP}.
5. hPol κ is the most suitable human Y-family enzyme to faithfully extend dG^{AP} bypass products.

5' -AACGACGGCCAGTGAATTCG-3'
 3' -TTGCTGCCGGTCACTTAAGC**G**CGCCC-5'

Figure 1.

Standing start assay for hRev1 at 37 °C. The sequence of damaged DNA substrate is shown above the gel image with the location of dG^{AP} (21st position) is colored in red and in bold type. Each lane is a 10-min reaction. “0”, “A”, “C”, “G”, “T” and “4” denote the addition of no dNTPs, dATP (200 μM), dCTP (200 μM), dGTP (200 μM), dTTP (200 μM), and all four dNTPs (200 μM each), respectively. DNA substrates used were (A) 20-mer/26-mer and (B) 20-mer/26-mer-dG^{AP}.

Figure 2.

Comparison of preferred actions by Dpo4, hPol η , hPol κ , and hPol ι opposite dG^{AP} and an immediate downstream template position in the DNA substrate 17-merS/73-merAP or the corresponding template bases dG and dC in the control DNA substrate 17-merS/73-mer. The relative frequencies (%) of nucleotide misincorporations or deletions were calculated at template Position X (A) or 0 (B) and Position +1 (C and D) in the presence of dG^{AP} (A and C) or dG (B and D) for Dpo4 (black bar), hPol η (white bar), hPol κ (striped bar), and hPol ι (light grey bar).

Figure 3.

Histogram of relative error% produced by Dpo4 as a function of DNA template position. At each position along the DNA template, the relative base substitution% (white bar) and deletion% (black bar) are shown to reveal the total relative error% and the contribution of each type of mutations simultaneously. The dG^{AP} site is indicated as an “X” along the X-axis (A), and the corresponding template base dG is denoted as “0” along the X-axis (B).

Figure 4.

Histogram of relative error% as a function of DNA template position for human Y-family DNA polymerases. At each position along the DNA template, the relative base insertion% (striped bar), substitution% (white bar) and deletion% (black bar) are shown to reveal the total relative error% and the contribution of each type of mutations simultaneously. The dG^{AP} site is indicated as an “X” along the X-axis, and the corresponding template base dG is denoted as “0” along the X-axis. The dG^{AP} bypass analysis is shown for hPol η (A), hPol κ (C), and hPol ι (E). DNA synthesis with the control 17-merS/73-mer was also analyzed for hPol η (B), hPol κ (D), and hPol ι (F). Statistically significant differences ($p < 0.05$) of the relative total error rates between the SOSA products generated from the damaged and control DNA substrates at each template position are indicated by *.

Scheme 1.

Table 1

DNA substrates.

Primers	
17-merS	5'-CTACCTGAACGACGGCC-3'
18-merS	5'-GGTCAACATTGCACTAGC-3'
20-mer	5'-AACGACGGCCAGTGAATTCG-3'
Templates ^a	
26-mer	3'-TTGCTGCCGGTCACTTAAGCGCGCCC-5'
26-mer-dG ^{AP}	3'-TTGCTGCCGGTCACTTAAGC <u>G</u> CGCCC-5'
73-mer	3'-CTACTCAGCCGTTGATGGACTTGCTGCCGGTCACTTAAGCGCGCCCCTGTCCTGCCGATCACGTTACAACCTGG-5'
73-merAP	3'-CTACTCAGCCGTTGATGGACTTGCTGCCGGTCACTTAAGC <u>G</u> CGCCCCTGTCCTGCCGATCACGTTACAACCTGG-5'

^aG designates dG^{AP}.

Table 2

Error rates of Y-family DNA polymerases determined by SOSA analysis.

Enzyme	DNA	Event	Insertion Error Rate ^a	Insertion Error Ratio ^b	Deletion Error Rate ^a	Deletion Error Ratio ^b	Substitution Error Rate ^a	Substitution Error Ratio ^b
Dpo4	17-merS73-mer	Total ^c	0		8.0×10 ⁻⁴		3.1×10 ⁻³	
		Upstream ^d	0		1.9×10 ⁻³		3.8×10 ⁻³	
		Downstream ^e	0		0		0	
Dpo4	17-merS73-merAP	Total ^c	0	-	3.2×10 ⁻³	4.0	1.4×10 ⁻²	4.5
		Upstream ^d	0	-	3.8×10 ⁻³	2.0	9.6×10 ⁻³	2.5
		Downstream ^e	0	-	2.7×10 ⁻³	-	5.5×10 ⁻³	-
hPolη	17-merS73-mer	Total ^c	2.5×10 ⁻³		1.6×10 ⁻²		6.1×10 ⁻²	
		Upstream ^d	0		5.9×10 ⁻³		4.3×10 ⁻²	
		Downstream ^e	4.2×10 ⁻³		2.2×10 ⁻²		2.2×10 ⁻²	
hPolκ	17-merS73-merAP	Total ^c	1.9×10 ⁻³	0.8	4.9×10 ⁻²	3.1	8.0×10 ⁻²	1.3
		Upstream ^d	0	-	3.2×10 ⁻²	1.5	4.1×10 ⁻²	1.0
		Downstream ^e	3.3×10 ⁻³	0.8	6.2×10 ⁻²	2.8	3.1×10 ⁻²	1.4
hPolκ	17-merS73-mer	Total ^c	2.5×10 ⁻³		7.4×10 ⁻³		2.9×10 ⁻²	
		Upstream ^d	0		7.8×10 ⁻³		2.0×10 ⁻²	
		Downstream ^e	4.2×10 ⁻³		7.0×10 ⁻³		1.1×10 ⁻²	
hPolκ	17-merS73-merAP	Total ^c	2.8×10 ⁻³	1.1	1.9×10 ⁻²	2.6	2.9×10 ⁻²	1.0
		Upstream ^d	0	-	1.1×10 ⁻²	1.4	1.1×10 ⁻²	0.6
		Downstream ^e	4.8×10 ⁻³	1.1	2.4×10 ⁻²	3.3	1.4×10 ⁻²	1.3
hPolh	17-merS73-mer	Total ^c	1.7×10 ⁻³		1.5×10 ⁻²		1.5×10 ⁻²	
		Upstream ^d	4.0×10 ⁻³		8.0×10 ⁻³		1.8×10 ⁻²	
		Downstream ^e	0		2.0×10 ⁻²		0	
hPolh	17-merS73-merAP	Total ^c	1.6×10 ⁻²	9.4	2.3×10 ⁻¹	15.3	2.7×10 ⁻¹	17.3
		Upstream ^d	2.8×10 ⁻²	7.0	1.6×10 ⁻¹	20.0	1.4×10 ⁻¹	7.8
		Downstream ^e	6.6×10 ⁻³	-	2.8×10 ⁻¹	14.0	9.5×10 ⁻²	-

- ^aCalculated using $\Sigma(\text{specific mutation type})/[(\text{number of samples}) \times (\text{number of bases in event})]$.
- ^bCalculated using $\{\Sigma(\text{specific mutation type})/[(\text{number of samples}) \times (\text{number of bases in event})]\}73\text{-merAP}/\{\Sigma(\text{specific mutation type})/[(\text{number of samples}) \times (\text{number of bases in event})]\}73\text{-mer}$.
- ^cTotal events count all events except those that occurred at Position X/0 (the dGAP site) in Supplementary Figures 2–5.
- ^dUpstream events include all events that occurred before an enzyme encountered Position X/0 in Supplementary Figures 2–5.
- ^eDownstream events include all events that occurred after an enzyme traversed Position X/0 in Supplementary Figures 2–5.
- “.” means the ratio cannot be calculated because the denominator is 0.