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The A-Rule and Deletion Formation During Abasic and Oxidized Abasic Site Bypass by DNA Polymerase θ

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Abstract

DNA polymerase θ (Pol θ) is implicated in various cellular processes including double-strand break repair and apurinic/aprimidinic site bypass. Because Pol θ expression correlates with poor cancer prognosis, the ability of Pol θ to bypass the C4'-oxidized abasic site (C4-AP) and 2-deoxyribonolactone (L), which are generated by cytotoxic agents, is of interest. Translesion synthesis and subsequent extension by Pol θ past C4-AP or L and an abasic site (AP) or its tetrahydrofuran analogue (F) was examined. Pol θ conducts translesion synthesis on templates containing AP and F with similar efficiencies and follows the "A-rule," inserting nucleotides in the order A > G > T. Translesion synthesis on templates containing C4-AP and L is less efficient than AP and F, and the preference for A insertion is reduced for L and absent for C4-AP. Extension past all abasic lesions (AP, F, C4-AP, and L) was significantly less efficient than translesion synthesis and yielded deletions caused by the base one or two nucleotides downstream from the lesion being used as a template, with the latter being favored. These results suggest that bypass of abasic lesions by Pol θ is highly mutagenic.

Graphical abstract

Mammalian cells contain at least 15 DNA polymerases, more than half of which play roles in repair and damage tolerance. DNA polymerase θ (Pol θ), an A-family polymerase, is a particularly interesting example because of its diverse range of activities and its potential as

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchem-bio.7b00211. Experimental procedures, mass spectra of oligonucleotides containing oxidized abasic site precursors, representative PAGE of single nucleotide insertion products and full-length extension experiments, representative active site titration of Pol θ (PDF)

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Notes

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a cancer drug target.¹ Overexpression of Pol θ in some types of cancer cells is associated with poor patient outcomes, and cancer cells that are deficient in homologous recombination are dependent upon Pol θ for survival.^{2,3} Pol θ is an essential component of double-strand break repair within eukaryotic cells by alternative end joining and has been proposed to play a role in additional cellular processes, such as mammalian base excision repair (BER) and translesion synthesis.^{4–8} Pol θ bypasses apurinic/aprimidinic (AP) sites and thymine glycol (Tg).^{9–12} The latter lesion is bypassed in human cells in a mutagenic fashion.⁹ The ability of Pol θ to tolerate oxidized abasic sites formed by cytotoxic agents such as bleomycin and ionizing radiation, which the enzyme confers resistance to, is unknown.¹³ Consequently, we examined how Pol θ bypasses oxidized abasic sites (C4-AP, L), as well as AP lesions and a model abasic site (F) for comparison.

Approximately 10 000 AP sites are produced per day in a single cell, making them the most prevalent DNA lesion.¹⁴ Oxidized abasic sites (e.g., C4-AP, L) are produced by a variety of DNA oxidants, including those that are used to treat cancer.^{15–17} Abasic sites are potentially cytotoxic, a desirable property when γ -radiolysis or chemotherapeutic agents are purposefully used to damage cellular DNA. Abasic sites also have high promutagenic potential due to the absence of a Watson–Crick base and are excised by repair enzymes.^{18–21} A- and B-family polymerases generally follow the A-rule, preferentially inserting dA opposite AP, while X- and Y-family polymerases often deviate from this rule, and in some cases use an adjacent nucleotide or an active site amino acid to direct incorporation.^{21–24} Extension past AP is less efficient than translesion insertion for most polymerases, although Pol ζ , which has primarily been studied in yeast, is substantially more efficient at extension.²⁵ Pol θ , η , and κ are the only human polymerases known to conduct appreciable extension beyond the model abasic site, F.^{10,21}

Human Pol θ is a 2590 residue protein containing a C-terminal polymerase domain, a large central domain, and an N-terminal helicase-like domain.¹⁰ It is classified as an A-family polymerase due to sequence homology, but its high error rate and lack of proofreading exonuclease activity are more similar to Y-family polymerases.²⁶ A fragment of the protein (residues 1792–2590) containing the polymerase domain and a portion of the central domain bypasses F.^{12,13} Pol θ also bypasses the bulkier Tg lesion present in synthetic templates and in cells.^{9,11,12} Full-length Pol θ selectively incorporated dA opposite AP.¹⁰ Incorporation of dA opposite AP is only ~4-fold less efficient than opposite thymidine by Pol θ , an unusual property, and the difference is largely attributed to differences in $V_{\max}$. The next most likely

nucleotide incorporated opposite AP, dG, is introduced almost 10-fold less efficiently, and pyrimidines are incorporated at least another 10-fold less efficiently. Pol θ discriminates between the four native nucleotides mostly based upon K_m . Translesion synthesis products containing dA opposite AP are also the most efficiently extended nucleotide-abasic site base pair, and extension was reported to be equally as efficient as translesion insertion.

Information on the translesion bypass of oxidized abasic sites by eukaryotic polymerases is limited. The only report concerns the L bypass in *Saccharomyces cerevisiae*, which gives rise to greater dA incorporation than does AP.²⁷ Replication of templates containing AP, F, L, and C4-AP in *E. coli* gives rise to very different outcomes, despite an inability of these lesions to form Watson–Crick base pairs.^{28–30} Only bypass of AP and its stable analogue, F, results in preferential translesion incorporation of dA (the A-rule) in *E. coli*.³¹ C4-AP yields large amounts of three-nucleotide deletions. Replication experiments in *E. coli* and kinetic measurements using L and analogues suggest that higher levels of dG incorporation result from DNA polymerase V recognition of the carbonyl oxygen, raising questions about whether abasic lesions are non-instructive.^{32,33} Replication of such disparate behavior when interacting with mammalian enzymes could be physiologically significant. The prevalence of Pol θ in cancer cells, its mutagenic bypass of Tg in human cells, and the association of oxidized abasic sites with antitumor agents provided the impetus to examine the ability of this polymerase to bypass C4-AP and L and place this in context with that of AP (and analogue F).

RESULTS AND DISCUSSION

Full-Length Extension of Primer-Template Complexes by Pol θ and the Formation of 1- and 2-Nucleotide Deletions

Qualitative examination of oxidized abasic site bypass by Pol θ (23.8 nM) was carried out using primer template complexes **1a–1e** (100 nM; Figure 1). Reactions were carried out at high concentrations (500 μ M) of the four native dNTPs to maximize the probability that reactions proceeded at maximum velocities. The control reaction involving the dU (**1a**) primer-template complex yielded complete formation of a mixture of full-length products containing one and two additional nucleotides resulting from blunt end addition. The latter is consistent with template independent Pol θ activity.¹⁰ Extension of primer-template complexes containing abasic lesions (**1b–1e**) was qualitatively less efficient and gave rise to products that were two nucleotides shorter than full-length products. In the case of AP (**1b**) and C4-AP (**1c**), significant amounts of shorter products were formed, while smaller amounts of these shorter products were formed from primer-template complexes containing L (**1d**) or F (**1e**). In some instances, (e.g., AP (**1b**), Figure 1) minor amounts of products whose migration in the denaturing PAGE gel was consistent with $N-1$ and $N+1$ products were also observed. While the $N+1$ product could result from nontemplate addition, it was not possible from this experiment to determine whether the $N-1$ length product corresponds to a one-nucleotide deletion or nontemplate addition to a two-nucleotide deletion. Pol β , Pol η , and Pol κ yield one-nucleotide deletions during F bypass *via* a misalignment–misinsertion mechanism.^{22,34,35} Extension past AP by Pol α and the Klenow fragment of Pol I inefficiently yields one- and two-nucleotide deletions *via* the same

mechanism.³⁶ The misalignment–misinsertion mechanism, where the abasic site is displaced from the template (“looping out”), has been structurally characterized in two bacterial polymerases.^{37,38}

Suspecting that a similar mechanism was responsible for a putative two-nucleotide deletion by Pol θ , primer extension experiments were conducted on primer templates containing dA opposite AP with either 3′-GTA (**4a**) or 3′-GAA (**2**) flanking the lesion (Figure S1). Two nucleotide deletion products were observed for **4a** but not **2**. Incorporation of the correct nucleotide, dC, was observed for **4a** and **2**, while incorporation of T was observed only for **4a**. These observations are consistent with a misalignment–misinsertion mechanism dependent upon formation of a Watson–Crick base pair between the nucleotide opposite the lesion and a downstream nucleotide present in the template. (The mechanism is illustrated in Scheme 1 for a 3′-GTA flanking sequence containing dA opposite AP (**4a**).) Misalignment–misinsertion was also operative when AP was flanked by 3′-CTA, and the product distribution observed for this sequence depended upon whether the lesion was opposed by dA (**11b**) or dG (**8**; Figure S2). AP opposed by dA (**11b**) yielded full-length and two-nucleotide deletion products but only one-nucleotide deletions when dG was opposite the lesion. Consistent with these results and with the proposed mechanism for deletion formation, dC and T insertion was detected for **11b**, while dA was inserted for **8** and T was not (see Supporting Information). To our knowledge, single nucleotide deletion formation upon Pol θ bypass of abasic sites has not been reported. The formation of two-nucleotide or greater deletions is in general even more unusual.^{28,36} In addition, there are no reports of oxidized abasic lesion (L, C4-AP) bypass by any mammalian translesion polymerase. A more quantitative examination of these activities was prompted by the novelty of the results, and to confirm that the observations were not the result of nontemplate nucleotide addition by Pol θ .

Standing Start Steady-State Analysis for Translesion Synthesis

Qualitative experiments indicated that Pol θ (475 pM) incorporated significant levels of dA and dG opposite L (**1d**) and C4-AP (**1c**) when the DNA primer-template complexes were in excess (50 nM) and high concentrations of the corresponding dNTPs (500 μ M) were present (data not shown). Significant T incorporation was observed opposite C4-AP but not L, while no 2′-deoxycytidine was incorporated opposite any of the lesions under these conditions. Standing start kinetic experiments were carried out for dA and dG incorporation opposite the oxidized abasic sites (**1c**, **1d**), AP (**1b**), and F (**1e**; Table 1).³⁹ Thymidine incorporation opposite AP and C4-AP was also measured. Previously reported translesion synthesis using a template containing the AP analogue F was repeated to benchmark the results with the oxidized abasic sites in our hands using the same flanking sequence.¹⁰ The original work utilized the full-length protein (~290 kDa), whereas the data presented here were obtained using the truncated protein (residues 1792–2590, 98 kDa) employed in recent crystallography experiments.¹¹ The corresponding K_m and k_{cat} values for dA or dG incorporation were remarkably similar whether AP (**1b**) or F (**1e**) was present in the template (Table 1). In addition, the K_m values were very similar to those reported by Wood in a different local sequence.¹⁰ Although it is not possible to directly compare the k_{cat} values measured in this study, the preference (F_{ins}) for inserting dA versus dG opposite F (~7-fold,

Table 1) was only slightly less than that previously reported (~9-fold), suggesting that k_{cat} values are comparable in the different investigations. The significantly less efficient incorporation of T opposite AP was consistent with the aforementioned qualitative experiments.

Nucleotide incorporation opposite any of the lesions was at least 30-fold less efficient than dA incorporation opposite dU (**1a**, Table 1). Translesion synthesis by Pol θ was most efficient on a template containing AP (**1b**), with incorporation of dA opposite AP being 10-times more efficient than opposite C4-AP (**1c**) and L (**1d**). The selectivity for dA insertion over dG was also greatest opposite AP (>7-fold), while it was slightly lower for L (6-fold) and nonexistent for C4-AP, opposite which dA and dG were inserted with equal efficiency. Insertion of T was considerably less efficient than both dA and dG for all lesions, although Pol θ showed a greater preference for dA over T opposite AP (60-fold) than for C4-AP (12-fold).

Abasic lesions are often thought of as noninstructive lesions during replication, because they lack Watson–Crick bases.³¹ Interestingly, the selectivity for dA over dG (and T when measured) by Pol θ is governed more by differences in K_m than k_{cat} (Table 1). The differences in K_m are greatest when the primer-template complexes contain AP or F and smallest opposite C4-AP. However, the latter primer-template shows no preference for nucleotide incorporation. Overall, translesion synthesis selectivities opposite the abasic sites indicate that differences in K_m contribute to incorporation selectivity, contrary to what is expected for noninstructive lesions.

Pol θ is only the second bypass polymerase whose ability to carry out translesion synthesis on templates containing oxidized abasic sites was examined quantitatively. DNA polymerase V from *E. coli* (Pol V) preferentially incorporates dG over dA opposite L in a process that is controlled by a lower K_m for the former, consistent with shuttle vector experiments in *E. coli* that supported the involvement of the lactone carbonyl as a hydrogen bond acceptor capable of forming hydrogen bonds with the N1 and N2 amino groups on dG.^{29,32,33} The only kinetic data reported for translesion synthesis involving C4-AP utilized the Klenow exo^- fragment of DNA polymerase I from *E. coli*. Klenow exo^- treated a template containing C4-AP in a very similar manner to that containing AP.⁴⁰ The selectivity for dA versus dG incorporation opposite each lesion was remarkably similar, as were the absolute values of the respective specificity constants.

Steady-State Analysis for Primer Extension Past the dA•Abasic Site Base Pair

The buildup of single nucleotide insertion product in qualitative replication experiments (Figure 1) suggested that translesion incorporation was far more efficient than subsequent primer extension. Consequently, a ~ 2-fold excess of primer-templates **4a–4c** through **6a–6c** was utilized for qualitative extension experiments instead of the greater than 100-fold excess employed in translesion synthesis experiments. Significant extension was observed when either dCTP or dTTP (500 μM) was present, but only when dA was opposite the abasic lesion.

Therefore, extension was quantitatively examined under steady-state conditions only when dA was opposite AP, F, L, and C4-AP (**4a–7a**; Table 2). Extension efficiency past an abasic site•dA base pair using the correct nucleotide triphosphate (dCTP) was >7000 less efficient than for a primer-template complex containing a dU•dA base pair (**3a**). The k_{cat} for dC incorporation followed the order L (**6a**) > AP (**4a**) ~ F (**7a**) $\gg$ C4-AP (**5a**) and encompassed an almost 20-fold range. The respective K_{m} values were greater than 175 μM and varied less than 2-fold. With the exception of L (**1d**), K_{m} values for extension were significantly greater than for translesional dA incorporation (Table 1), resulting in significantly lower specificity constants for the former. These data are consistent with the buildup of translesion synthesis product in the qualitative full-length extension experiments (Figure 1).

Overall, the kinetics for thymidine incorporation relative to that of dC are also consistent with the full-length primer extension experiment (Figure 1). Most importantly, thymidine incorporation is consistent with two-nucleotide deletion formation in **1b–1e**, presumably *via* a misalignment– misinsertion mechanism (Scheme 1). Thymidine incorporation was no more than 3-fold less efficient than dC incorporation when using primer templates containing L (**6a**), AP (**4a**), and F (**7a**) and was twice as efficient when C4-AP (**5a**) was present in the template. The relatively high k_{cat} for dC incorporation when 2-deoxyribonolactone (L) was opposite dA (**6a**) and other kinetic parameters for T and dC incorporation explain why relatively small amounts of two-nucleotide deletion products are observed upon bypass of this oxidized abasic site when the respective dNTPs are present at 500 μM (Figure 1). The respective k_{cat} and K_{m} values for T and dC incorporation in primer-template complexes containing dA opposite AP (**4a**) and C4-AP (**5a**) also correlate with the relative amounts of full-length product and two-nucleotide deletion in each instance at high dNTP concentrations (500 μM).

Other Primer-Template Sequence Effects on Deletion Product Formation

The scope of deletion formation was initially explored by replacing the downstream dG (**4a**) with T (**9b**). While a downstream 3'-TTA sequence could give rise to one- and two-nucleotide deletions, this sequence was less likely to lead to T misincorporation without misalignment. T incorporation efficiency in a template containing AP increased more than 17-fold (Table 3) compared to when the flanking sequence was 3'-GTA (Table 2). The large increase in T incorporation rate was attributable to more favorable k_{cat} and K_{m} values compared to the related primer-template complex **4a**. Correct nucleotide incorporation (dA) efficiency increased more modestly, resulting in a reversal from **4a** and >2-fold preference for incorporating a nucleotide that could give rise to a deletion when AP is in the template. This experiment does not distinguish between dA incorporation opposite the 5'-adjacent thymidine or the pyrimidine further downstream that is accessible by misalignment. The latter could ultimately yield one-nucleotide deletion, or full-length product following realignment. dA and T incorporation kinetics are consistent with the qualitative full-length extension experiment (see Supporting Information) in which two-nucleotide deletion is favored over full-length product by ~4:1. The large increase in extension efficiency when the downstream sequence was changed from 3'-GTA to 3'-TTA was also observed when C4-AP (**9c**) was present in the template, although in this instance the efficiency for correct nucleotide incorporation (dA) was 30-times greater than when dG was the 5'-adjacent

nucleotide (**5a**, Table 2). Thymidine incorporation, which would give rise to deletion product(s), increased more modestly but was still >8-fold more efficient than in **5a**.

The significant increase in incorporation efficiency favoring two-nucleotide deletion formation using **9b,c** compared to **4a** and **5a** led us to explore whether thymidine substitution for dG in the template also induces one-nucleotide deletion formation. If a misalignment–misinsertion mechanism is operative, primer-template complex **10b**, in which an AP containing template is flanked by 3′-TAA (Table 4), can only yield full-length product when the correct nucleotide (dA) is incorporated or one-nucleotide deletion upon thymidine incorporation. Thymidine incorporation in **10b** (Table 4) was more than 5-fold less efficient than in **9b** (Table 3) but more than 2-fold faster than when the flanking sequence was 3′-GTA (**4a**, Table 2). Because dA incorporation was considerably more compromised, T incorporation and the corresponding single nucleotide deletion product were preferred by almost 14-fold.

The extension of primer-templates containing dG opposite AP (**11b**, **12b**) and C4-AP (**11c**, **12c**) was also examined (Table 5). Although dG insertion is less efficient opposite AP (**1b**) than is dA insertion (Table 1), Pol θ inserts both nucleotides with equal efficiency opposite C4-AP (**1c**). Therefore, the ability of Pol θ to extend past G opposite template C4-AP was of particular interest. One pair of primer-template complexes (**11**) was designed such that misalignment–insertion yields one-nucleotide deletions, while the other (**12**) would yield two-nucleotide deletions. Correct nucleotide insertion was not detected for any of these primer-template complexes (data not shown). This was not an indication of overall recalcitrance of the primer-template complexes to undergo extension, because templates containing either AP (**12b**) or C4-AP (**12c**) designed to yield two-nucleotide deletions did so as efficiently as when the lesions were flanked by 3′-TTA (**9**, Table 3), and even more so when C4-AP (**12c**) was in the template. Furthermore, a greater efficiency for two-nucleotide deletion formation compared to one-nucleotide deletion was revealed in these primer-template complexes.

Verification of Deletion Formation

The single nucleotide incorporation kinetic experiments described above suggest that Pol θ will yield one- and two-nucleotide deletions upon bypassing AP sites and that the distribution of these will depend upon flanking sequence. The kinetic data are also consistent with the apparent deletion products observed by gel electrophoresis (Figure 1, see Supporting Information). Conclusive evidence for deletion product formation was obtained by sequencing the bypass products of **13a** and **13b**.⁴¹ Briefly, the extended primer was isolated using magnetic beads, amplified by PCR, and subcloned into a plasmid, which was transformed into *E. coli* and then sequenced. The sequence was chosen such that one- or two-nucleotide deletion products could be formed from a misalignment–misinsertion mechanism (Scheme 2). As expected, only dA was inserted during extension of the dU-containing primer-template **13a**, and no deletions were observed (Table 6). Bypass products for AP-containing primer-template **13b** contained only dA in the position opposite the lesion. This is consistent with a preference for dA insertion opposite AP and with the negligible extension of primers containing dG opposite AP in instances where the template

sequence does not allow misalignment– misinsertion. Strikingly, 95% of extension products contained two-nucleotide deletion, and analysis of the sequence indicated that all of these are consistent with a misalignment-misinsertion mechanism.

Conclusions

AP is the most commonly formed DNA lesion, and oxidized abasic lesions are produced by a variety of DNA damaging agents. All are potentially cytotoxic if not repaired, are blocks for replicative polymerases, and are often mutagenic when they are bypassed. There is little information available concerning the effects of oxidized abasic sites on mammalian translesion polymerases. Of several error-prone polymerases that can potentially bypass abasic sites, Pol θ is an interesting example because of its increased expression in some tumors and its ability to tolerate AP and Tg in DNA templates. Templates containing AP or its stable analogue F were examined to calibrate observations on the Pol θ bypass of oxidized abasic sites L and C4-AP but yielded results that were interesting in their own right. For instance, we observed that translesion synthesis opposite AP and F (**1b**, **1e**) was >30-fold slower than nucleotide incorporation opposite Watson–Crick base containing nucleotides (**1a**, **2a**), as opposed to <5-fold less efficient.¹⁰ In addition, we observed that extension of nascent primers opposite AP, F, as well as oxidized abasic sites (L, C4-AP) was >100-fold less efficient than translesion synthesis, and in some instances >1,000-fold slower. C4-AP also does not adhere to the A-rule, although translesion synthesis opposite AP, F, and L does. Moreover, the selectivity for dA incorporation is largely governed by K_m .

A striking observation involving AP, F, L, and C4-AP was that high levels of deletion products formed in a manner consistent with a misalignment–misinsertion mechanism (Scheme 2) were produced. The viability of such a deletion forming process was validated using full-length extension experiments, steady-state kinetic analyses, and *via* sequencing of extension products for one sequence containing AP. One- and two-nucleotide deletions were formed in a sequence-dependent manner. In the one sequence examined (**13b**, Table 6) where both sizes of deletions were possible, 95% of the products from extension were two-nucleotide deletions. The preference for two-nucleotide over one-nucleotide deletion formation from this limited series of flanking sequences is different than that reported for the prokaryotic Klenow fragment and calf thymus Pol α , which preferentially yielded the latter when interacting with AP containing templates.³⁶

Pol θ is the first mammalian error-prone polymerase to be examined on templates containing oxidized abasic lesions. Although its selectivity for translesion nucleotide incorporation is significantly lower opposite C4-AP than L, AP, or F, its most remarkable property is the formation of high levels of one- and two-nucleotide deletions upon encountering any of these abasic lesions. Although Pol θ has been shown to bypass Tg in cells, it is not known how its presence affects the abasic site (of any type) bypass in a cellular environment and/or how this would contribute to a cell's ability to cope with these nucleic acid modifications. These experiments indicate that the (oxidized) abasic site bypass in cells by Pol θ will be highly mutagenic.

METHODS

Materials and General Methods

Oligonucleotides were prepared on an Applied Biosystems Inc. 394 DNA synthesizer using reagents from Glen Research. The oligonucleotide used to prepare **1c** and **5** was previously reported.⁴² Oligonucleotides containing the photolabile L and C4-AP precursors used to prepare **1d**, **2b**, **6**, **9c**, **11c**, and **12c** were synthesized as previously described.^{43–46} All others were synthesized and deprotected using standard protocols. Oligonucleotides containing (oxidized) abasic site precursors were characterized by ESI-MS. Preparation of substrates containing AP was accomplished by treating the corresponding dU-containing duplex with uracil DNA glycosylase (5 units, 37 °C, 30 min). Preparation of substrates containing C4-AP was accomplished by photolysis of the orthonitroveratryl-protected precursor (350 nm, 10 min), while L was generated by photolyzing the 7-nitroindole-protected precursor (350 nm, 20 min). In control experiments in which the dU-containing strand was radiolabeled, 100% cleavage was observed upon alkaline treatment following UDG treatment, indicating that the enzyme reaction was complete under these conditions. Similar analysis of complexes containing photolabile precursors to C4-AP or L indicated that the photochemical conversion was 95%. DNA substrates used in this study are presented in Chart 1. The nucleotide removal kit was from Qiagen. Dynabeads M-270 Streptavidin and DH5 α cells were from Invitrogen. The Quick Ligase Kit, Phusion polymerase, NEB Buffer 3.1, T4 polynucleotide kinase, Acc6rI, *Eco*RI, and UDG were obtained from New England Biolabs. DNA Pol θ catalytic fragment (residues 1792–2590) was expressed and purified as previously described.¹² ³²P-Labeled nucleotides were obtained from PerkinElmer and MP Biochemical. Analysis of radiolabeled oligonucleotides was carried out using a Storm 860 Phosphorimager and ImageQuant 7.0 TL software. Pre-steady-state kinetics were carried out using a RQF-3 rapid quench instrument (Kintek).

Pol θ Translesion Synthesis and Extension

Pol θ (23.8 nM) was incubated with **1a–1e** (100 nM) at RT with all four dNTPs (500 μ M) in a reaction buffer (20 mM Tris-HCl at pH 8.0, 25 mM KCl, 10 mM MgCl₂, 1 mM BME). In a typical experiment, a 5 $\times$ solution of primer-template complex (4 μ L) was added to 10 $\times$ reaction buffer (200 mM Tris-HCl, 250 mM KCl, 100 mM MgCl₂, 10 mM BME (2 μ L), H₂O (10 μ L)) and 10 $\times$ dNTP mix (5 mM, 2 μ L). A 10 $\times$ solution of Pol θ (238 nM, 2 μ L) was added, and aliquots (4 μ L) were removed at the indicated times, usually 1 and 30 min, and quenched with a solution of 95% formamide with 25 mM EDTA (6 μ L) containing trace bromophenol blue and xylene cyanol. Samples were heated (95 °C, 1 min) and then immediately placed on ice prior to loading (5 μ L) on 20% denaturing PAGE run at 55 W.

Pol θ Translesion Synthesis Kinetics

Polymerase reactions were conducted with Pol θ (475 pM), primer-template complex (**1a–1e**; 50 nM), and various concentrations of the indicated dNTP at RT in the above reaction buffer. In a typical experiment, a 2 $\times$ DNA-enzyme solution was prepared by mixing primer-template (500 nM, 30 μ L), 10 $\times$ reaction buffer (30 μ L), Pol θ (4.75 nM, 30 μ L), and H₂O (60 μ L). The 2 $\times$ DNA-enzyme solution (3 μ L) was added to the appropriate 2 $\times$ dNTP solution (3 μ L). The reaction was conducted for a fixed time at RT and then quenched with

95% formamide loading buffer containing 25 mM EDTA (8 μ L). Samples were heated (95 °C, 1 min) and then immediately placed on ice prior to loading (5 μ L) on a 20% denaturing PAGE. The dNTP concentration range and reaction time varied for each primer-template complex (see Supporting Information).

Pol θ Extension Kinetics

Polymerase extension reactions were conducted with Pol θ (475 pM for reactions with native templates and 11.9 nM for reactions with lesion-containing templates), primer-template complexes **3–7** and **9–12** (50 nM for native templates and 25 nM for lesion-containing templates), and various concentrations of the indicated dNTP at RT in reaction buffer. The experimental setup was the same as for translesion synthesis kinetics (above) with the exception of the difference in Pol θ and primer-template concentrations. The concentration range of dNTP and the reaction time varied for each lesion (see Supporting Information).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Qualitative primer extension by Pol θ using DNA complexes **1a–1e**. Reactions were conducted with Pol θ (23.8 nM), primer-template (100 nM), and dNTPs (500 μ M). Please note that the reaction involving the template containing F is from a different experiment and is pasted here for comparison.

Scheme 1. Competition between Full-Length and 2-Nucleotide Deletion Product Formation

Scheme 2. Competition between 1- and 2-Nucleotide Deletion Product Formation

Chart 1. Primer-Template Complexes Used in This Study

Table 1

Steady-State Analysis of Nucleotide Insertion Opposite Abasic Lesions^a

5'-ACA					
3'-TGTXGTA					
X	dNTP	k _{cat} (min ⁻¹)	K _m (μM)	k _{cat} /K _m (min ⁻¹ ·μM ⁻¹)	F _{ins} ^b
dU (1a)	A	70.7 ± 0.3	1.5 ± 0.3	4.7 × 10 ¹	
AP (1b)	A	18.8 ± 0.4	12.4 ± 0.5	1.5	1
AP (1b)	G	9.6 ± 0.4	49.0 ± 8.8	2.0 × 10 ⁻¹	0.13
AP (1b)	T	14.2 ± 1.3	529.0 ± 14.4	2.7 × 10 ⁻²	1.7 × 10 ⁻²
C4-AP (1c)	A	10.3 ± 0.1	84.0 ± 3.0	1.2 × 10 ⁻¹	1
C4-AP (1c)	G	15.8 ± 0.1	130.5 ± 30.6	1.2 × 10 ⁻¹	1
C4-AP (1c)	T	4.7 ± 0.4	537.3 ± 28.7	8.7 × 10 ⁻³	0.08
L (1d)	A	25.7 ± 4.0	201.5 ± 20.8	1.3 × 10 ⁻¹	1
L (1d)	G	11.4 ± 0.9	436.5 ± 56.9	2.6 × 10 ⁻²	0.17
F (1e)	A	16.6 ± 0.5	13.0 ± 0.4	1.3	1
F (1e)	G	9.7 ± 0.3	55.3 ± 5.0	1.8 × 10 ⁻¹	0.14

^aData are the average ± std. dev. of experiments, each consisting of three replicates.

^bF_{ins} = (V_{max}/K_m)dNTP/(V_{max}/K_m)dATP (both opposite the same lesion).

Table 2

Steady-State Analysis of Extension Past Abasic Lesions Flanked by 3'-GTA^a

5'-ACAA					
3'-TGTXGTA					
X	dNTP	$k_{\text{cat}}(\text{min}^{-1})$	$K_m(\mu\text{M})$	$k_{\text{cat}}/K_m(\text{min}^{-1}\mu\text{M}^{-1})$	F_{ins}^b
dU (3a)	C	31.0 ± 1.0	1.5 ± 0.3	20.7	
AP (4a)	C	0.19 ± 0.02	219.3 ± 32.4	8.6×10^{-4}	1
AP (4a)	T	0.16 ± 0.02	323.1 ± 19.4	5.0×10^{-4}	0.6
C4-AP (5a)	C	0.03 ± 0.001	183.6 ± 19.1	1.6×10^{-4}	1
C4-AP (5a)	T	0.11 ± 0.004	298.7 ± 15.1	4.0×10^{-4}	2.3
L (6a)	C	0.56 ± 0.04	213.6 ± 3.6	2.7×10^{-3}	1
L (6a)	T	0.08 ± 0.02	98.8 ± 14.7	8.1×10^{-4}	0.31
F (7a)	C	0.15 ± 0.001	290.8 ± 18.5	5.1×10^{-4}	1
F (7a)	T	0.10 ± 0.001	408.6 ± 28.4	2.4×10^{-4}	0.5

^aData are the average $\pm$ std. dev. of experiments, each consisting of three replicates.

^b $F_{\text{ins}} = (V_{\text{max}}/k_{\text{m}})(d\text{TTP}/(V_{\text{max}}/k_{\text{m}})d\text{CTP})$ (opposite the same lesion).

Table 3

Steady-State Analysis of Extension Past Abasic Lesions Flanked by 3'-TTA^a

5'-ACAA					
3'-TGTXTTA					
X	dNTP	k_{cat} (min ⁻¹)	K_m (μM)	k_{cat}/K_m (min ⁻¹ μM ⁻¹)	F_{ins}^b
dU (9a)	A	56.9 ± 2.5	4.8 ± 1.6	11.9	
AP (9b)	A	0.22 ± 0.04	60.3 ± 12.4	3.6 × 10 ⁻³	1
AP (9b)	T	0.53 ± 0.07	61.6 ± 1.7	8.6 × 10 ⁻³	2.2
C4-AP (9c)	A	0.45 ± 0.01	88.3 ± 8.8	5.1 × 10 ⁻³	1
C4-AP (9c)	T	0.59 ± 0.03	179.3 ± 5.6	3.3 × 10 ⁻³	0.67

^aData are the average ± std. dev. of experiments, each consisting of three replicates.

^b $F_{\text{ins}} = (V_{\text{max}}/K_m)d\text{TTP}/(V_{\text{max}}/K_m)d\text{ATP}$ (opposite the same lesion).

Table 4

Steady-State Analysis of Extension Past AP Flanked by 3'-TAA^a

5'-ACAA					
3'-TGTXTAA					
X	dNTP	k_{cat} (min ⁻¹)	K_m (μM)	k_{cat}/K_m (min ⁻¹ μM ⁻¹)	F_{ins}^b
dU (10a)	A	35.8 ± 3.9	6.6 ± 0.2	5.4	
AP (10b)	A	0.026 ± 0.003	206.5 ± 4.8	1.3 × 10 ⁻⁴	1
AP (10b)	T	0.52 ± 0.03	300.3 ± 19.5	1.7 × 10 ⁻³	13.8

^aData are the average ± std. dev. of experiments, each consisting of three replicates.

^b $F_{\text{ins}} = (V_{\text{max}}/K_m)(dNTP)/(V_{\text{max}}/K_m)dATP$.

Table 5
Steady-State Analysis of Extension Past dG•dN Base Pairs As a Function of Downstream Flanking Sequence^a

5'-ACAG					
3'-TGTXNNN					
X	3'-NNN	dNTP	k_{cat} (min⁻¹)	K_m (μM)	k_{cat}/K_m (min⁻¹·μM⁻¹)
C (11a)	CTA	G	40.9 ± 5.7	7.5 ± 0.4	5.5
AP (11b)	CTA	A	0.20 ± 0.002	123.7 ± 4.5	1.6 × 10 ⁻³
C4-AP (11c)	CTA	A	0.31 ± 0.02	136.6 ± 21.1	2.2 × 10 ⁻³
C (12a)	TCA	A	52.4 ± 4.2	8.8 ± 0.01	6.0
AP (12b)	TCA	T	0.75 ± 0.06	87.6 ± 8.8	8.6 × 10 ⁻³
C4-AP (12c)	TCA	T	0.66 ± 0.02	93.6 ± 18.0	7.1 × 10 ⁻³

^aData are the average ± std. dev. of experiments, each consisting of three replicates.

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Table 6Fidelity of Pol θ during Bypass of dU or AP Flanked by 3'-TTA

template nucleotide	# colonies sequenced	dA opposite X	error-free, full-length extension	one-nucleotide deletion	two-nucleotide deletion
dU (13a)	11	11 (100%)	11 (100%)	0	0
AP (13b)	39	39 (100%)	1 (2.5%)	1 (2.5%)	37 (95%)