Sloppy Bypass of an Abasic Lesion Catalyzed by a Y-family DNA Polymerase

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DNA damage that eludes cellular repair pathways can arrest the replication machinery and stall the cell cycle. However, this damage can be bypassed by the Y-family DNA polymerases. Here, Dpo4, an archetypal Y-family member from the thermophilic Sulfolobus solfataricus, was used to extend our kinetic studies of the bypass of an abasic site, one of the most mutagenic and ubiquitous cellular lesions. A short oligonucleotide sequencing assay is developed to directly sequence DNA bypass products synthesized by Dpo4. Our results show that incorporation upstream of the abasic lesion is replicated error-free; yet dramatically, once Dpo4 encounters the lesion, synthesis became sloppy, with bypass products containing a myriad of mutagenic events. Incorporation of dAMP (29%) and dCMP (53%) opposite the abasic lesion at 37 °C correlates exceptionally well with our kinetic results and demonstrates two dominant bypass pathways via the A-rule and the lesion loop-out mechanism. Interestingly, the percentage of overall frameshift mutations increased from 71 (37 °C) to 87% (75 °C). Further analysis indicates that lesion bypass via the A-rule is strongly preferred over the lesion loop-out mechanism at higher temperatures and concomitantly reduces the occurrence of “−1 deletion” mutations observed opposite the lesion at lower temperatures. The bypass percentage via the latter pathway is confirmed by an enzymatic digestion assay, verifying the reliability of our sequencing assay. Our results demonstrate that an abasic lesion causes Dpo4 and possibly all Y-family members to switch from a normal to a very mutagenic mode of replication.

Cellular DNA is continually damaged by agents both endogenous and exogenous to an organism. This damage structurally modifies DNA, arresting replication by stalling the replicative DNA polymerase. The inability to copy the genome leads to cell cycle stalling and possibly cell death. Although a large portion of DNA lesions is restored by cellular repair pathways, a biologically significant amount of damage persists. Fortunately, organisms have evolved specialized enzymes known as the Y-family DNA polymerases that are capable of replicating damaged DNA in either an error-free or error-prone fashion (1, 2), thus rescuing a stalled replisome.

Although the Y-family DNA polymerases collectively synthesize one to a few nucleotides per binding event, they all lack proofreading exonuclease activity and generate up to 100,000-fold more errors on undamaged DNA when compared with the astonishingly accurate replicative DNA polymerases (1, 3, 4). This high error rate is due to the loose active sites in the structures of the eukaryotic polymerases: human polymerase ι (5), yeast polymerase η (6), human polymerase κ (pol κ) (7), and yeast Rev1 (8), as well as the thermophilic archaeal Sulfolobus solfataricus DNA polymerase IV (Dpo4)3 (9) and Sulfolobus acidocaldarius Dbh (10). These more permissive active sites impart unique functional attributes to the Y-family, including the ability to replicate through aberrant and bulky DNA lesions, which is conferred by the limited contacts between the Y-family polymerase active site residues and DNA.

Among DNA damage, apurinic/apyrimidinic (AP) sites are one of the most common lesions in mammalian cells (11) with a steady state frequency approaching 50,000 sites per genome (12). These noncoding lesions are generated enzymatically, via spontaneous hydrolysis, or by more complex mechanisms involving free radicals or alkylating agents (13, 14). Because S. solfataricus grows at 75–85 °C, it is reasonable to expect more genomic AP sites compared with the relative amount present in humans and other mesophilic systems. Although replicative polymerases like S. solfataricus pol B1 lack the ability to bypass AP sites (42), they can inefficiently incorporate dATP opposite the lesion in a phenomenon known as the “A-rule” (15). Thus, due to the large number of AP sites and the fact that Dpo4 is the only known Y-family polymerase in S. solfataricus, Dpo4 is an excellent model for mechanistic and structural studies of AP bypass. In our accompanying article (42), we show that during efficient bypass, Dpo4 paused when incorporating nucleotides directly opposite and one position downstream from an AP lesion due to a decrease in catalytic efficiency. In addition, incorporation opposite the lesion partitioned primarily between two distinct pathways, one involving the A-rule (Scheme 1A) and the second involving the novel lesion loop-out mechanism (Scheme 1B). This latter mechanism involved incorporation directed by the template base 5′ to the AP lesion via an extrahelical AP site that was extended in-frame without primer/template realignment generating a “−1 frameshift.” However,

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Schemes 4–7.

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3 The abbreviations used are: Dpo4, Sulfolobus solfataricus DNA polymerase IV; AP, abasic; SOSA, short oligonucleotide sequencing assay.
these kinetic predictions need to be verified by actual DNA sequence data. In addition, kinetic analysis cannot capture infrequent mutation events and thus cannot provide a complete picture of the mutagenic spectrum, especially downstream from the abasic lesion. Here we determined the precise sequences of short DNA products synthesized by Dpo4 during AP lesion bypass in vitro, using a novel short oligonucleotide sequencing assay. These sequences will reveal the mutagenic impact of AP lesion bypass by the Y-family.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human AP endonuclease and Taq DNA polymerase were purchased from Trevigen and Invitrogen, respectively. Dpo4 was expressed and purified as described previously (16). The oligodeoxynucleotides listed in Table 1 were purchased from Integrated DNA Technologies and were purified, labeled, and annealed as described previously (17).

**Reaction Buffer R**—The optimized buffer contained 50 mM HEPES (pH 7.5 at 37 °C), 5 mM MgCl₂, 50 mM NaCl, 5 mM dithiothreitol, 10% glycerol, 0.1 mM EDTA, and 0.1 mg/ml bovine serum albumin (16).

resolved within this sequence window; however, there remains a small possibility that some bypass products contained additional mutations outside this window. The amplified AP bypass product was then ligated into a pCRII-TOPO vector using a TOPO TA cloning kit (Invitrogen) followed by transformation into TOP10 Escherichia coli strain (Invitrogen). Plasmid was isolated from 55 independent colonies using a miniprep kit (Promega) and were then sequenced using an automated 3730 DNA analyzer (Applied Biosystems, Inc.). The method was summarized in Scheme 2. Controls for human AP endonuclease show its ability to completely digest DNA containing either intrahelical or extrahelical AP sites, and showing no degradation of an undamaged DNA control (data not shown).

**BmgBI Digestion Assay**—A preincubated solution of Dpo4 (120 nM) and 5'-radiolabeled 17-mer/41AP (30 nM; Table 1) was mixed with four dNTPs (200 μM each) to generate a full-length AP bypass product at 37 °C. The full-length AP bypass product was purified from Dpo4 and the template 41AP via phenol/chloroform extraction, human AP endonuclease treatment, denaturing PAGE, and C18 column chromatography as described above. The pool of purified AP bypass product was
annulled to 40Te (Table 1) in molar excess containing half of the BmgBI restriction site, in 1 × NEB Buffer, and then overdi-gested with BmgBI (20 units; New England Biolabs) for 3 h at 37 °C. Digestion reactions were then heated to 65 °C for 30 min to thermally denature BmgBI and subsequently run on a 20% native polyacrylamide gel for 2 h to resolve digested product from full-length duplex DNA. The percent digestion was quan-titated using a PhosphorImager. Reactions at 75 °C were per-formed in Buffer R with potassium phosphate instead of HEPES.

RESULTS AND DISCUSSION

Our previous kinetic results (42) reveal numerous branched pathways used by Dpo4 to bypass an AP lesion. Although elucidating the kinetics of each branched pathway would be extremely tedious, various aspects of these kinetic assays require careful consideration. While these pre-steady state kinetic methods are mechanistically informative and provide a relative measure of polymerization fidelity, they cannot generate DNA sequence information or the mutation profile. The most common kinetic assay for fidelity determina-tion involves measuring and comparing the incorporation preference of each individual deoxynucleoside triphosphate (dNTP) into a DNA substrate via single nucleotide incorpo-ration assays under either steady state or pre-steady state reaction conditions. Since the selection of dNTPs at the pol-ynomerase active site for incorporation opposite a specific canonical template base forms a correct or an incorrect Watson-Crick base pair, polymerase fidelity can be calcu-lated under the assumption that there will be exactly one nucleotide incorporated opposite each template base. Although almost exclusively true for a replicative polymer-ase that replicates undamaged DNA with high fidelity, bypass of a DNA lesion by Dpo4, a low fidelity enzyme, has been shown by us here and others previously (18–22) to generate a variety of deletion and addition mutations in the vicinity of the lesion, indicating an equilibrium between several conformationally distinct DNA species exploited for bypass. This equilibrium is particularly relevant at structurally flexible AP sites (23, 24) and thus invalidates the assumption of “one dNTP for each template base” for lesion bypass. As such, the definition of fidelity, although suitable for studies with undamaged DNA, cannot be strictly applied to these incorporation events. Furthermore, these single nucleotide incorporation assays are subject to pyrophospho-lysis, the reverse of forward synthesis, if the incoming dNTP does not form a correct base pair with the downstream template base after the first incorporation event. Dpo4 has been shown to have somewhat robust pyrophosphorolysis activity that is notably attenuated when an incorrect incor-poration event can be extended by correct nucleotide incor-poration (25). Thus the kinetic results of lesion bypass from single nucleotide incorporation assays may not accurately reflect all actual bypass events, especially those through non-canonical pathways (see below), and requires cautious inter-pre-tation. To extend our previous kinetic studies (42) and provide a complete mutagenic spectrum, we designed an assay for sequencing short DNA products synthesized by Dpo4 during AP lesion bypass to elucidate the entire muta-tion profile.

Development of a Novel Assay for Sequencing Short DNA Products—Traditional strategies for sequencing short DNA products include the Maxam-Gilbert method and the Sanger method. However, both of these methods are limited by the inability to resolve sequence information from a DNA mixture of variable lengths and sequences (20). Because our previous kinetic results (42) indicated Dpo4 generated a population of bypass products, these techniques could not be used. Although the liquid chromatography/tandem mass spectrometry tech-nique used by Zang et al. (20) is fundamentally better than the aforementioned methods, it still only provides a semi-quantita-tive analysis of a population of DNA substrates and disregards the sequence contexts of minor species. In addition, the modified reversion assay used by Kokoska et al. (18), although eleg-ant, only detects roughly 60% of single base errors made during in vitro lesion bypass. This is because of the lack of a completely ligated plasmid prior to transformation into E. coli. In addition, the assay itself is less likely to observe minor species because it lacks DNA product amplification before plasmid assembly. Because of the shortcomings of these assays, we developed a short oligonucleotide sequencing assay (SOSA) integrating PCR amplification and an efficient cloning tech-nique to unambiguously determine the sequence of a popu-lation of short DNA bypass products synthesized by Dpo4 in the exact sequence context used in our previous kinetic analysis (42).

Analysis of DNA Products Using SOSA at 37 °C—We exploited this novel assay to determine the mutation profile for the bypass of an AP site by Dpo4 using a 17-mer/41AP (Table 1) that contained a template guanine 5′ to the lesion. Ultimately, the method (Scheme 2) was designed to isolate and ligate the AP bypass products synthesized by Dpo4 into a vector for auto-mated sequence analysis. Analysis of the DNA sequences from
55 independent colonies is summarized in Fig. 1A. The results of the assay performed at 37 °C revealed that upstream from the AP site all incorporation events catalyzed by Dpo4 were sequence-dependent, and all incorporation events (220 nucleotides) were replicated error-free and in-frame. Strikingly, once Dpo4 encountered the lesion, a spectrum of mutagenic bypass events was generated. Interestingly, 13% of the time (7/55 colonies) Dpo4 synthesized a full-length (41-mer) AP bypass product lacking any downstream mutations with dAMP located opposite the AP site (Fig. 1A). Other AP bypass products contained only single/double base substitutions (9%, 5/55 colonies), only single/multiple base deletions (56%, 31/55 colonies), only base additions (2%, 1/55 colonies), or complex transactions involving a combination of deletion(s), addition(s), and substitution(s) (13%, 7/55 colonies). The remaining 7% (4/55 colonies) of AP bypass products were full-length and in-frame but had dCMP, dGMP, or dTMP inserted opposite the AP site (Fig. 1A). Although our results demonstrated the ability to incorporate all nucleotides directly opposite the lesion, 82% of the time (45/55 colonies) Dpo4 incorporated either dAMP (via A-rule) or dCMP (mostly via the lesion loop-out mechanism) coinciding with our previous kinetic results (42) showing that 90% of AP lesion bypass events occurred via these two pathways. Yet this initial partitioning opposite the lesion did not represent the full extent of mutagenic bypass of the AP bypass. Our results showed 21 unique sequence contexts revealing that unregulated bypass of the lesion by Dpo4 was extremely sloppy.

The most logical mechanisms for each of these 21 DNA sequences were diagrammed in supplemental Schemes 4–7 and were based on previous structural studies of Dpo4. Roughly 52% (30/55 colonies) of these AP bypass products were characterized by either dAMP incorporation opposite the AP site (A-rule) lacking downstream mutations or a −1 deletion mechanism due to incorporation via the lesion loop-out mechanism (see above). The proposed mechanism for the former, as illustrated in supplemental Scheme 4B, was supported by two crystal structures from Ling et al. (19) of Dpo4 with a DNA substrate containing an AP site. The first crystal structure (structure Ab-4A) provides partial evidence for dAMP incorporation by Dpo4 opposite the lesion in an intrahelical fashion, whereas crystal structure Ab-2A captures the ability of Dpo4 to extend from this incorporation event retaining the extrahelical AP lesion (19). The remaining 19 AP bypass products were each observed only 2–4% of the time and contain primarily deletion

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**Figure 1.** Mutation spectrum of AP site bypass catalyzed by Dpo4. A, 37 °C; B, 75 °C. Results from the SOSA are shown separately based on specific nucleotide incorporated opposite the AP site. Only the full-length "AP bypass product" (FABP) is shown in its entirety. Sequences corresponding to primers used for amplification are shown in lowercase and sequenced nucleotides are shown in uppercase. Individual base substitutions (blue) are shown above the expected FABP. Base deletions (red) and base additions (green) are shown below the FABP, and complex mutations are color-coded based on specific mutation in order of occurrence and shown either above or below the expected FABP. Boldface letters correspond to incorporation opposite the AP lesion. Relative ratios are shown at right in parentheses.
and substitution mutations. For example, the bypass event in supplemental Scheme 4A occurred via incorporation of dAMP opposite the lesion followed by a substitution mutation that proceeded either by a simple mismatch incorporation of dTMP opposite the downstream 5′ template guanine or via a mechanism reminiscent of the type II Dpo4 structure reported with undamaged DNA, where the incoming dTTP instead base pairs with the downstream template adenine (9), similar to that observed in the Ab-2B structure (19). Other proposed mechanisms involved more complicated DNA structural rearrangements as in supplemental Scheme 4F where, after dAMP incorporation opposite the AP site, the proposed pathway splits into two subpathways involving the following: (i) either a type II-like intermediate followed by a subsequent two-nucleotide loop-out in the template strand to generate the double deletion, or (ii) an intermediate involving an extrahelical base in the primer similar to what was observed in Ab-4B (19) followed by extension and rearrangement of the primer-template to generate the “−2 deletion.” Importantly, although the assay temperature is not physiologically relevant for Dpo4, it is relevant for other mesophilic Y-family DNA polymerases, especially those from the DinB subfamily (i.e. human pol κ and E. coli DNA polymerase IV) which, like Dpo4, have all been shown to generate −1 deletions even on undamaged DNA (26–28).

Analysis of AP Bypass Products at 75 °C—The same assay was performed at 75 °C, the in vivo temperature for S. solfataricus, to determine the effect of temperature on the mutation profile for AP lesion bypass catalyzed by Dpo4. This assay was buffered with 50 mM potassium phosphate instead of 50 mM HEPES because of the pH stability of this buffer over a range of temperatures, although all other components in Buffer R (see “Experimental Procedures”) remained unchanged. Notably, the change in buffer conditions did not affect the burst kinetics reported previously (data not shown (29)). To prevent the melting of the 17-mer/41AP duplex at 75 °C prior to polymerization, the DNA substrate was preincubated with Dpo4 at room temperature for 10 min prior to incubation at 75 °C such that Dpo4 binding would stabilize the DNA duplex. For the reaction at 75 °C, we were able to extract sequencing information from 45 individual colonies using SOSA (Fig. 1B). Again, as observed at 37 °C, the results showed all four incorporations upstream of the AP site were replicated faithfully and lacked a single mutation (180 total incorporation events). However, once Dpo4 encountered the AP lesion, it generated a myriad of frameshift and substitution mutations resulting in 16 distinct AP bypass products. The corresponding pathways were not illustrated as were those at 37 °C because of their modest redundancy. Yet the precise mutational spectrum differed from that observed at 37 °C. While at 37 °C, 13% of the AP bypass products were in-frame with dAMP located opposite the AP site (Fig. 1A), and we similarly found 11% (5/45 colonies) had this sequence context at 75 °C (Fig. 1B). Likewise, at both 37 and 75 °C there were very few AP bypass products that contained only base additions (2% at 37 °C and none at 75 °C). However, at 75 °C, 80% (36/45 colonies) contained only single/multiple base deletions (versus 56% at 37 °C); 7% (3/45 colonies) were complex transactions (versus 13% at 37 °C), and only 2% (1/45 colonies) had base substitutions (versus 9% at 37 °C). Thus, at higher temperature, Dpo4 generated more deletion mutations at the expense of a similar magnitude decrease in complex mutations, base substitutions, and in-frame incorporations with dCMP, dGMP, or dTMP opposite the AP site (Table 2). In addition, regardless of specific mutations, the percentage of overall frameshift mutations (both addition and deletion mutations) increased from 71% (39/55 colonies) at 37 °C to 87% (39/45 colonies) at 75 °C. It was not clear what caused the shift in the frameshift frequency. It is possible that the energy barriers for nucleotide incorporation into noncanonical ternary complexes could be overcome by more conformationally active Dpo4 at higher temperatures. We have observed an increase in the rate constant for an incorrect dGTP by 27-fold with a 30 °C increase in the reaction temperature (data not shown).

Yet even more fascinating was the dramatic increase in bypass events characterized by dAMP incorporation opposite

### Table 2: Mutational spectrum of short oligonucleotide sequencing assay at 37 and 75 °C

| Mutation type       | Reaction at 37 °C | Percentage<sup>a</sup> | Reaction at 75 °C | Percentage<sup>a</sup> |
|---------------------|-------------------|-------------------------|-------------------|-------------------------|
|                     | No. of AP bypass products<sup>b</sup> |                        | No. of AP bypass products<sup>b</sup> |                        |
| 1-Base substitution | 5                 | 9.1                     | 1                 | 2.2                     |
| 2-Base addition     | 1                 | 1.8                     | 0                 | 0.0                     |
| 1-Base deletion     | 27                | 49.1                    | 11                | 24.4                    |
| 2-Base deletion     | 1                 | 1.8                     | 10                | 22.2                    |
| 3-Base deletion     | 2                 | 3.6                     | 0                 | 0.0                     |
| 4-Base deletion     | 1                 | 1.8                     | 9                 | 20.0                    |
| 5-Base deletion     | 0                 | 0.0                     | 6                 | 13.3                    |
| Complex transaction | 7                 | 12.7                    | 3                 | 6.7                     |
| Full-length         | 11                | 20.0                    | 5                 | 11.1                    |
| Total               | 55                | 100                     | 45                | 100                     |

<sup>a</sup> Data designate the number of colonies containing AP bypass products with a particular mutation.

<sup>b</sup> Percentage refers to percentage of a particular mutation calculated as ((number of AP bypass products)/total) × 100.

<sup>c</sup> Data designate the colonies possessing full-length DNA substrates containing no additional downstream mutations.

### Table 3: Comparison of nucleotide incorporation opposite an AP site at 37 and 75 °C

| dNTP   | Probability at 37 °C | Probability at 75 °C<sup>a</sup> |
|--------|----------------------|-----------------------------------|
|        | %                    | %                                 |
| dATP   | 29                   | 69                                |
| dCTP   | 53                   | 13                                |
| dGTP   | 4                    | 2                                 |
| dTTP   | 14                   | 2                                 |

<sup>a</sup> The remaining 14% (6/45 colonies) contained no incorporation opposite the AP site.
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FIGURE 2. BmgBI digestion assay. 32P-Labeled AP bypass product was purified (see “Experimental Procedures”) and digested with BmgBI for 3 h, and the resulting digestion fragments were resolved by native PAGE. The digestion reaction and controls were loaded as follows (all lanes were digested with BmgBI unless mentioned otherwise): lane 1, 40Pr/40Te duplex with intact BmgBI restriction site in the absence of BmgBI; lane 2, 41Pr/40Te duplex with single unpaired base within BmgBI restriction site; lane 3, 40Pr/40Te duplex with intact BmgBI restriction site; lane 4, 41Pr/41Te duplex with single base pair addition in BmgBI restriction site; lane 5, Dpo4 bypass product catalyzed at 37°C. Assay performed in triplicate with reaction indicating 32% digestion is shown in lane 5.

TABLE 4
Comparison of methods for the preference of nucleotide incorporation opposite an AP site at 37 °C

| dNTP | Kinetic probability | SOSA probability |
|------|---------------------|------------------|
| dATP | 33                  | 29               |
| dCTP | 57                  | 53               |
| dGTP | 1                   | 4                |
| dTTP | 9                   | 14               |

* The kinetic probability = \( \frac{kp}{(kp + ka + acp + actp + attp)} \) and the SOSA probability = \( \frac{kp}{(kp + ka + acp + actp + attp)} \); the data were taken from our accompanying article (42).

In the following sequence, the AP site is designated as X:

21-mer/41AP

5′-CCGACCGGCAACCCACCTCA-3′

3′-GCGACCGGCAACCTGTTGACTXACGCTAGGTTACGCGAGG-5′

sequenced AP bypass product was either incorporated directly opposite the lesion or via some other mechanism involving the local sequence context, e.g. lesion loop-out mechanism (see above). This was suggested by the error-free replication observed at all four upstream nucleotides (see “Experimental Procedures”), corresponding to the 18th to 21st nucleotides, and the subsequent chaotic incorporation events downstream from the lesion, specifically the 22nd to 26th nucleotides. We chose to compare this particular incorporation event opposite the lesion instead of events further downstream because, as mentioned above, Dpo4 generated 21 unique “bypass products” each via a distinctive pathway. Thus, this first incorporation event opposite the lesion possessed the largest sample size and thus the greatest reliability over subsequent events for analysis. In addition, the anomalous nature of downstream events precluded accurate comparison (supplemental Schemes 4–7). Interestingly, the results from our novel SOSA matched the results obtained from our kinetic studies. Specifically, the sequencing assay showed dATP (16/55 colonies, 29%) and dCTP (29/55 colonies, 53%) were the major incorporation events. These percentages coincided with those calculated from our kinetic studies (dATP, 33%; dCTP, 57%) (Table 4). Furthermore, the probabilities for incorporation of dGTP (2/55 colonies, 4%) and dTTP (8/55 colonies, 15%) were much less frequent in this sequence context yet were very similar to those probabilities gleaned from our kinetic assays (1 and 9% for dGTP and dTTP, respectively; see Table 4). Thus, this novel assay correlated well with our kinetic studies of incorporation opposite the AP lesion and demonstrated strong competition between the A-rule and the lesion loop-out mechanism for AP lesion bypass.

Quantitative Verification of the Bypass Percentage via the Lesion Loop-out Mechanism by an Enzymatic Digestion Assay—To provide an independent method for characterizing AP bypass catalyzed by Dpo4, an enzymatic digestion assay was also developed to quantitatively determine the percentage of AP bypass product that resulted from the lesion loop-out mechanism (Scheme 1B). Quantitative discrimination was based on the ability of a restriction enzyme, BmgBI, to digest a DNA substrate containing AP bypass product annealed to a complementary 40-mer template (40Te, Table 1) possessing an intact BmgBI restriction site located precisely in the region of the AP bypass product that corresponded to incorporation at the AP site. This complementary DNA substrate 40Te was
Broad Impact of Our Studies—Studies have shown that AP sites are lethal when introduced into biologically active DNA (30, 31). The primary reason for this lethality is because of the inefficient incorporation and extension past these lesions by high fidelity replicative DNA polymerases. Evidence of this inability for replicative polymerases to bypass AP sites are abundant (32–35), e.g. S. solfataricus pol B1 (42). However, it is clear from our analysis and the series of published Dpo4 structures bound to DNA with an AP site that there are multiple mechanisms that Dpo4 uses to bypass and extend from this lesion (19). Similarly, results from analysis of Dpo4 replication of a 1,N²-ethenoguanine lesion also indicate the existence of multiple bypass mechanisms that generate a subset of products even for a single sequence context (20). Additional crystal structures of Dpo4 with the bulky, covalently modified cis-syn thymine-thymine dimer (36) show that Dpo4 can accommodate this entire lesion into its active site, whereas structural analysis of Dpo4 bound to an adducted DNA containing benzo[a]pyrene suggests a mechanism by which Dpo4 can alternatively shift extremely bulky bases into the major groove to perform translesion synthesis (22). Dpo4 has also been shown to catalyze the error-free bypass of the bulky 8-oxodG through Watson-Crick base pairing with an incoming dCTP (37, 38). Taken together, these results suggest structural plasticity and flexibility in the Dpo4 active site as was recently described by Mizukami et al. (39). This flexibility translates into a more promiscuous mode of replication that is structurally justified by the limited contacts between Y-family members and the nascent base pair as seen in crystal structures of eukaryotic polymerases η, τ, κ, and REV 1 as well as archaeal Dpo4 and Dbh. This plasticity bestows the Y-family with the exceptionally unique ability to perform DNA transactions forbidden by the replicative polymerases to facilitate lesion bypass.

So what does the observed chaotic bypass mean in the context of an in vivo system? Our previous kinetic studies suggest two competing mechanisms for the bypass of an AP lesion by the Y-family polymerase Dpo4. However, unlike the conclusion reached by Ling et al. (19) suggesting that Dpo4 does not use the AP site to instruct nucleotide insertion but rather uses the 5’ template base, our previous kinetic studies (42) coupled with the two independent assays shown here involving direct and indirect sequencing methods, categorically demonstrate that there exist two primary mechanisms for AP site bypass. We believe that the basis for the preference of bypass involves the relative stability of the AP site (intrahelical or extrahelical) in the Dpo4 active site and is influenced by reaction temperature. Our assay showed that incorporation events downstream from the AP site were far more anomalous than a previous report (18) that did not specifically describe these events and were similar in this respect to results described by Zang et al. (20) for bypass of a 1,N²-ethenoguanine adduct by Dpo4. Yet the relevance of these complex downstream incorporation events is subject to debate in the context of an in vivo system. Besides the inherently low fidelity of Dpo4 on undamaged DNA, the observed spectrum of mutations downstream from the AP site indicates that it is absolutely critical that replication by Dpo4 be limited to the lesion to guarantee genome stability. Thus the role of polymerase switching in bypass synthesis, which has been suggested to be mediated by the ubiquitination of proliferating cell nuclear antigen (40, 41), will likely prove to be an important mechanism in regulating continued synthesis by Y-family members.

Overall, we have developed a novel short oligonucleotide sequencing assay that produced results that demonstrate two dominant mechanisms used by Dpo4 to bypass an AP lesion and provide a powerful new tool to directly sequence mixtures of small oligonucleotides synthesized by any DNA polymerase or reverse transcriptase. We are currently using this assay to characterize AP bypass by the eukaryotic Y-family polymerases. This technically feasible method can be applied to the bypass of any DNA lesion and can be used in multiple enzyme/protein systems.

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![Diagram](image-url)
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