Translesion DNA Synthesis Catalyzed by Human Pol η and Pol κ across 1,N^6-Ethenodeoxyadenosine*

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1,N^6-Ethenodeoxyadenosine, a DNA-adduct generated by exogenous and endogenous sources, severely blocks DNA synthesis and induces miscoding events in human cells. To probe the mechanism for in vivo translesion DNA synthesis across this adduct, in vitro primer extension studies were conducted using newly identified human DNA polymerases (pol) η and κ, which have been shown to catalyze translesion DNA synthesis past several DNA lesions. Analysis of translesion products have revealed that the synthesis is >100-fold more efficient with pol η than with pol κ and that both error-free and error-prone syntheses are observed with these enzymes. The miscoding events include both base substitution and frameshift mutations. These results suggest that both polymerases, particularly pol η, may contribute to the translesion DNA synthesis events observed for 1,N^6-ethenodeoxyadenosine in human cells.

In the last few years, several new human DNA polymerases (pol), which are likely to be involved in translesion DNA synthesis (TLS), were discovered. This list includes pol η (1), pol κ (2, 3), pol ι (4), and pol ζ (5). Pol η, pol ι, and pol κ are encoded by the hRAD30A, hRAD30B, and DNB1 genes, respectively. These new pols form a Rad30/UmuC/DinB/REV1 superfamily (2, 3, 6). Pol ζ consists of two gene products: hREV3 containing pol activity and hREV7 with an unknown function (5). In general, these pols catalyze TLS more efficiently than previously known pols. They synthesize DNA in a distributive manner and tend to show lower replication fidelity than other pols such as pol α, pol β, and pol δ when unmodified DNA is used as a template (7–10).

There are several pieces of evidence for the involvement of human pol η and pol ζ in TLS in vivo (5, 11–13). Although the involvement of human pol κ or pol ι has not yet been established in vivo, the Escherichia coli homologue of pol κ, pol IV, has been shown to play a role in TLS in vivo (14). Pol η, which is missing in xeroderma pigmentosum variant cells (13, 15), is shown to catalyze efficient TLS across the cis-syn cyclobutane thymine-thymine dimer by inserting two dAMPs opposite the lesion (16). Therefore, the cancer proneness of xeroderma pigmentosum variant patients is thought to be caused by the lack of accurate TLS across this and/or other UV photo products. Pol η also catalyzes TLS across other DNA lesions such as cisplatin G-G intrastrand cross-link (16), acetylaminofluorene-dG (16), and 8-oxodeoxyguanosine (17) with relatively high fidelity. On the other hand, TLS across (±)-trans-anti-benz[a]pyrene-N^2-dG is reported to be error-prone (18). Pol κ is also shown to conduct TLS across an abasic site (19, 20), acetylaminofluorene-dG (19, 20), (−)-trans-anti-benz[a]pyrene-N^2-dG (20), and 8-oxodeoxyguanosine (20).

Based on these findings, we are motivated to study the efficiency and fidelity of TLS catalyzed by these novel pols across 1,N^6-ethenodeoxyadenosine (edA) to probe the in vivo TLS mechanism. We have shown that edA is miscoding in simian and human cells by inducing edA→T, edA→G, and edA→C (21, 22). This adduct is produced in animals exposed to vinyl compounds such as the human carcinogen vinyl chloride. Surprisingly, this adduct is also found in unexposed animals and humans with lipid peroxidation products being the suspected source of this adduct (23). Our in vitro primer extension studies indicate that pol η catalyzes TLS more efficiently than pol κ and that both pols catalyze error-free and error-prone TLS.

MATERIALS AND METHODS

Materials—[γ-32P]ATP was purchased from Amersham Pharmacia Biotech. Human pol η and pol κ were purified as described (13, 19). Pol δ was purified to apparent homogeneity from calf thymus (24). Human proliferating cell nuclear antigen (PCNA) was a generous gift from Paul Fisher (State University of New York, Stony Brook, NY). T4 polynucleotide kinase and EcoRI were purchased from New England Biolabs. Ultrapure deoxyribonucleic acid triphosphates were purchased from Roche Molecular Biochemicals.

DNA Substrates—Oligonucleotides were purchased from Oligo Etc. (Wilsonville, OR) or synthesized in the laboratory of Francis Johnson (State University of New York, Stony Brook, NY). Oligomers were purified by electrophoresis on a 20% polyacrylamide gel containing 7M urea, detected by UV shadowing, excised from the gel, eluted from gel slices, and desalted using a SEP-PAK C18 cartridge (Waters). Purified oligonucleotide primers were labeled at the 5′ end with [γ-32P]ATP and T4 polynucleotide kinase. Primers were annealed to templates by mixing at a 1:1.2 molar ratio in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 mM NaCl by heating to 80 °C followed by slow cooling. For primer extension and standing primer kinetic studies (25) of nucleotide incorporation and extension, the [γ-32P]labeled primers (5′-GGTTCTAGGCT- TAGGT, 5′-GGTTCTAGGCTAGTTAGAT, and 5′-GGTTCTAGGCTAGTAGTATN (where N = A, C, G, or T)) were annealed to a 28-mer template (5′-CTGCTCCTCTXATACTACAGGCTAGAAAC (where X = dA or edA)), generating substrates 1, 2, and 3, respectively.

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‡ The abbreviations used are: pol, DNA polymerase; TLS, translesion DNA synthesis; edA, 1,N^6-ethenodeoxyadenosine; PCNA, proliferating cell nuclear antigen; DTT, dithiothreitol; BSA, bovine serum albumin.

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Fig. 1. Translesion DNA synthesis catalyzed by DNA polymerases. A, template-primer (substrate 1) used in B and C. B, calf thymus pol δ (0 (lanes 1 and 7), 0.09 (lanes 2 and 8), 0.19 (lanes 3 and 9), 0.38 (lanes 4 and 10), and 0.75 units (lanes 5, 6, 11, and 12)) was incubated with substrate 1 (where X = dA in lanes 1–6 and X = edA in lanes 7–12) containing 532P-labeled primer in a 10-µl reaction mixture at 30 °C for 30 min. Lanes 1–5 and 7–11 contained 70 ng of human PCNA, C, human pol η (0 (lanes 1 and 6), 0.29 (lanes 2 and 7), 1.5 (lanes 3 and 8), 7.3 (lanes 4 and 9), and 36.3 fmol (lanes 5 and 10)) or pol κ (0 (lanes 11 and 16), 0.25 (lanes 12 and 17), 1.2 (lanes 13 and 18), 6.2 (lanes 14 and 19), and 31 fmol (lanes 15 and 20)) was incubated with the substrate 1 (where X = dA in lanes 1–5 and 11–15 and X = edA in lanes 6–10 and 16–20) containing 532P-labeled primer in a 10-µl reaction mixture at 37 °C for 10 min. To quantify various TLS products, the 38-mer template (5’-CATGCT-GATGAACTCTCCATCTCCGTC (where X = dA or edA; EcoRI site shown in bold)) was annealed to the 32P-labeled primer (5’-AGAGGAAGTAG), yielding substrate 2 (Fig. 2).

Primer Extension Assays—Each reaction mixture (10 µl) contained 40 nM substrate 1 and 100 µM dNTPs. Reactions with pol η (1) or pol κ (19) contained 40 mM Tris-HCl (pH 8.0), 60 mM KCl, 5 mM MgCl₂, 10 mM dithiothreitol (DTT), 2.5% glycerol, and 250 µg/ml bovine serum albumin (BSA). Reactions with pol δ contained 7 ng/µl PCNA, 40 mM Bis-Tris (pH 6.8), 6 mM MgCl₂, 2 mM DTT, 4% glycerol, and 40 µg/ml BSA. Pol κ was diluted in 20 mM potassium phosphate (pH 7.5), 0.3 M KCl, 0.1 mM EDTA, 0.1 mg/ml BSA, 1 mM DTT, and 50% glycerol. Pol δ was diluted in 40 mM Bis-Tris (pH 6.8), 1 mM DTT, 0.2 mg/ml BSA, and 10% glycerol. Reactions were initiated by adding enzyme and were incubated at 37 ± 1 °C for 10 min (pol η or pol κ) or 30 ± 1 °C for 30 min (pol δ). Reactions were stopped by adding 10 µl of 95% formamide dye mixture (95% formamide, 10 mM EDTA, 0.001% xylene cyanol, and 0.001% bromophen blue), and then the mixture was heated to 95 °C for 5 min. Aliquots (1 µl) were subjected to electrophoresis in a denaturing 20% polyacrylamide gel. Kinetic Studies of Nucleotide Insertion and Extension—Standing-start reactions (25) (10 µl) contained 40 nM substrate 2 or 3 (substrate 2 for insertion analysis and substrate 3 for extension analysis), 0–2 mM dNTP(s), and a reaction buffer (see above). Initiation and termination of reactions were conducted as described above. Aliquots (1 µl) were subjected to electrophoresis in a denaturing 20% polyacrylamide gel.

Data Analysis—Integrated gel band intensities were measured using a PhosphorImager and ImageQuant software (Molecular Dynamics). Nucleotide incorporation parameters were determined (25). Less than 20% of the primers were extended in these steady-state kinetic analyses, ensuring single-hit kinetics (26). Values for the Michaelis-Menten constant (Kₘ) and Vₘₐₓ for incorporation opposite dA and edA were obtained by least squares nonlinear regression to a rectangular hyperbola. Kₘ was calculated by dividing Vₘₐₓ by the enzyme concentration. The frequency of insertion (F_ins) and extension (F_ext) were calculated using the equation F_ins or ext = (k_ins/K_m)substrate(k_ext/K_m)control (25). Standard errors derived from the curve-fitting are included.

Analysis of TLS Products—DNA synthesis reaction mixtures (10 µl) contained 50 nM substrate 4, 100 µM dNTPs, the appropriate buffer (see above), and enzyme (1.5 units of pol δ, 36 fmol of pol η, or 56 fmol pol κ) were incubated at 23 ± 1 °C for 15 min and then 37 ± 1 °C for 45 min (27). Reactions were stopped by adding 10 µl of a formamide dye mixture and heating to 95 °C for 5 min. Samples were subjected to electrophoresis in a denaturing 20% polyacrylamide gel (35 × 42 × 0.04 cm). Full-length products were extracted from the gel and annealed to a complementary 38-mer. The annealed products were digested with EcoRI (100 units) for 1 h at 30 °C and then 1 h at 15 °C. This digestion generates 32P-labeled 18-mers from the fully extended products. The products were separated in a two-phase polyacrylamide gel (15 × 72 × 0.04 cm) (27). This method allows the separation of four base substitution products and frameshift products.

The DNA template of substrate 4 is different from the template of substrates 1, 2, and 3 in the DNA sequence surrounding edA.
sequence context used in the template of substrates 1, 2, and 3 is identical to that used in miscoding studies in human cells (22). It was not possible to separate various TLS products by the method described above when this sequence context was employed. Therefore, we used the sequence (substrate 4) that has been shown to permit separation of various TLS products by gel electrophoresis (27).

RESULTS

DNA Polymerase Activity on Control and edA-Modified Templates—Pol η, pol κ, and pol δPCNA were assayed for polymerase activity on both unmodified and edA-modified templates. The primer (substrate 1; Fig. 1A) allowed the addition of two nucleotides before encountering the adduct. Although all three pols were capable of synthesizing across edA (Fig. 1, B and C), this lesion posed a much stronger block to pol δ than to pol η and pol κ when compared with the control templates. A very small amount of the full-length product was generated by pol δ only when PCNA was added to the reaction mixture (compare lanes 10 and 11 with lane 12 in Fig. 1B), revealing the enhancing role for PCNA in TLS. Pol η seems to catalyze TLS more efficiently than pol κ.

Kinetic Studies of Nucleotide Incorporation and Extension—To determine the efficiency and fidelity of TLS catalyzed by pol η and pol κ, we first determined steady-state kinetic parameters ($K_m$ and $k_{cat}$) for nucleotide incorporation opposite dA and edA using substrate 2. The internal 13 nucleotides (5'-CTCTTCXCATACCT) of this template are identical to those used in the miscoding studies in human cells (22). The kinetic data ($F_{ins}$) indicate that pol η incorporates a nucleotide opposite edA more efficiently than pol κ. Pol η inserts the correct dTMP opposite edA twice as efficiently as dAMP and dGMP and 13 times more efficiently than dCMP. This dTMP insertion is ~68 times less efficient than that opposite dA. Similarly, pol κ also inserts dTMP most efficiently opposite edA, followed by dGMP and then dAMP, but its efficiency is ~1000 times less than the incorporation opposite dA. These results indicate that dTMP, the correct nucleotide, is preferentially inserted opposite edA by both pols.

We then determined steady-state kinetic parameters for nucleotide extension from four different 3' termini located opposite dA or edA using substrate 3. The kinetic data ($F_{ext}$) indicate that pol η extends from all the termini more efficiently than pol κ. Pol η extends the primer with the correct dTMP more efficiently than pol κ. Activity for extension from the dTMP terminus more efficiently than the other three termini when the modified template was used. This extension from the dTMP terminus is ~55 times less efficient than that from the dTMP terminus located opposite dA. In experiments using pol κ, the efficiency of extension from the 3' terminus followed the order of dAMP > dGMP > dTMP > dCMP, indicating that unlike pol η, the incorrect pairings are extended better than the correct dA/T pairing.

Based on these insertion and extension kinetic parameters,
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the relative efficiency of TLS was determined by multiplying $F_{\text{inc}}$ and $F_{\text{ext}}$. The results indicate that pol $\eta$ catalyzes TLS across dA more efficiently than pol $\kappa$. With pol $\eta$, TLS with dA:T is dominant, and its efficiency is 3.0, 4.4, and 45 times greater than with dA:A, dA:G, and dA:C, respectively. The same analysis for pol $\kappa$ shows that the efficiency of TLS with dA:T is 2.1 and 2.7 times greater than with dA:A and dA:G, respectively. These results indicate that accurate TLS is dominant but not exclusive with both pols.

Miscoding Specificity of $\eta$—Because steady-state kinetic analysis includes only one of four dNTPs in the reaction mixture, and frameshift mutations are not detected, we determined the miscoding specificity of $\eta$ in the presence of four dNTPs. We analyzed polymerization products using substrate 4 (Fig. 2). Although fully extended products were observed with the unmodified template for all three pols, only pol $\eta$ and pol $\kappa$ produced full-length products with the modified template (data not shown). One possible explanation is that the 10-mer primer is not long enough to accommodate both pol $\delta$ and PCNA (28), and pol $\delta$ alone cannot catalyze TLS as shown in Fig. 1B.

Fully extended products were digested with EcoRI and electrophoresed in a two-phase polyacrylamide gel. When the unmodified template was used, pol $\delta$-catalyzed products showed only one band (Fig. 2, lane 2) that co-migrated with the dT marker, indicating accurate DNA synthesis. Pol $\kappa$ also catalyzed faithful synthesis (Fig. 2, lane 5). Pol $\eta$ mainly catalyzed error-free DNA synthesis, but two additional bands were also observed, co-migrating with the dG and two-base deletion error products (Fig. 2, lanes 1 and 7). These products were quantified based on the amount of radioactivity in the bands (Table II). Consistent with the results of the steady-state kinetic analysis, pol $\eta$ dominantly catalyzed accurate TLS with dT on the modified template. However, substantial amounts of products containing dA, dG, dC, or one-base deletion markers (Fig. 2, lanes 1 and 7) were also observed on the other hand, pol $\kappa$ dominantly catalyzed TLS with one-base deletion, followed by dT, dA, and dC incorporation. The results shown in Table II indicate that error-prone TLS is dominant for both pols when frameshift mutations are included and that pol $\eta$ catalyzes accurate TLS more frequently than pol $\kappa$, but still more than 50% of the TLS is error-prone.

DISCUSSION

Pol $\eta$ has been reported to catalyze TLS across several DNA lesions in a relatively error-free manner (1, 16, 17). Our steady-state kinetic analyses and the analysis of TLS products have revealed that TLS catalyzed by pol $\eta$ across dA, like the benzo[α]pyrene dG adduct (18), is significantly erroneous. Although accurate TLS with dTMP insertion opposite dA is predominant, pol $\eta$ also frequently catalyzes erroneous TLS, causing base substitutions and frameshift mutations (Tables I and II and Fig. 2). Pol $\kappa$ catalyzes TLS across several DNA lesions in relatively error-free and error-prone manners (19, 20). Our steady-state kinetic analysis shows that pol $\kappa$ preferentially incorporates dTMP, followed by dAMP, opposite dA (Table I). The product analysis experiment (Table II and Fig. 2), however, has revealed that one-base deletion events were dominant followed by dTMP insertion products, indicating that pol $\kappa$-catalyzed TLS is also erroneous. Neither pol $\eta$ nor pol $\kappa$ can be characterized as simply error-free or error-prone polymerases.

The overall efficiency of TLS, determined by $F_{\text{inc}} \times F_{\text{ext}}$, for A:dA and G:dA is similar for both pols: $9.0 \times 10^{-5}$ versus $6.1 \times 10^{-5}$ for pol $\eta$ and $1.5 \times 10^{-5}$ versus $1.2 \times 10^{-5}$ for pol $\kappa$ (Table I). However, analysis of TLS products shows that dA incorporation is preferred to dG by both pols: 19.8% dA versus 9.1% dG for pol $\eta$ and 18.8% dA versus 2.9% dG for pol $\kappa$ (Table II). It is likely that some dGMP incorporated opposite dA misaligned (Fig. 3, step 3) to generate a one-base deletion, whereas dAMP incorporation does not cause this misalignment. Accordingly, dAMP incorporation opposite dA leads to a base substitution, whereas dGMP incorporation results in both a base substitution and a one-base deletion. Another mechanism envisioned is “dNTP-stabilized misalignment,” which was observed for pol $\beta$ in TLS across abasic sites (29). According to this mechanism, the slippage event occurs first, causing dA to be extrahelical (step 2), and the incoming dGTP stabilizes this misalignment (step 4). Continuous extension from this terminus results in a one-base deletion (step 5), whereas realignment (step 7) and extension (step 8) result in a base substitution. The hallmark of the dNTP-stabilized misalignment mechanism is the relatively low $K_m$ for dNMP insertion at the terminus opposite a DNA lesion, which suggests that the incorporation is actually opposite the base 5′ to the lesion (29). In the insertion kinetic studies with pol $\eta$, the $K_m$ value for dGMP insertion opposite dA (42.7 $\mu$M) is not much different from that for dTTP insertion opposite dA (21.4 $\mu$M) (Table I), which suggests that dGMP is inserted opposite dC, 5′ to dA, and extension from this terminus results in a one-base deletion (steps 2 to 4 to 5). On the other hand, with pol $\kappa$ the $K_m$ value for dGMP insertion opposite dA (126 $\mu$M) is very different from that for dTTP insertion opposite dA (9.4 $\mu$M) (Table I). This suggests that dGMP is incorporated opposite dC, followed by misalignment and subsequent extension of the primer. This is the likely mechanism for the induction of one-base deletions (the dominant event) by pol $\kappa$.

Our mutagenesis experiments (22) have shown that miscoding events account for 10–20% of TLS in human cells. Although it is not possible to speculate as to what extent these pols contribute to TLS in vivo, our results suggest that these pols are involved in TLS in vivo, then it is likely to be error-prone. In vivo experiments using human cells lacking these pols are necessary to clarify this point.

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