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Human translesion polymerase kappa exhibits enhanced activity and reduced fidelity two nucleotides from G-quadruplex DNA

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Abstract

We have investigated the *in vitro* properties of human Y-family polymerase kappa (hpol κ) on G-quadruplex DNA (G4 DNA). Similar to hpol η , another Y-family member implicated in replication of G4 motifs, hpol κ bound G4 DNA with a 5.7-fold preference over control, non-G4 DNA. Results from pol extension assays are consistent with the notion that G-quadruplexes present a stronger barrier to DNA synthesis by hpol κ than they do to hpol η . However, kinetic analysis revealed that hpol κ activity increases considerably when the enzyme is 2-3 nucleotides away from the G4 motif, a trend that was reported previously for hpol η , though the increase was less pronounced. The increase in hpol κ activity on G4 DNA was readily observed in the presence of either potassium or sodium but much less so when lithium was used in the reaction buffer. The increased activity 2-3 nts from the G4 motif was accompanied by a decrease in fidelity of hpol κ when the counterion was either potassium or sodium but not in the presence of lithium. The activity of hpol κ decreased progressively as the primer was moved closer than two nts from the G4 motif when either potassium or sodium was used to stabilize the G-quadruplex. Interestingly, the decrease in catalytic activity at the site of the quadruplex observed in potassium-containing buffer was accompanied by an increase in fidelity on G4 substrates versus control non-G4 substrates. This trend for increased fidelity in copying a tetrad-associated guanine was observed previously for hpol η , but not for the B-family member hpol ϵ , which exhibited a large decrease in both efficiency and fidelity in the attempt to copy the first guanine in the G4 motif. In summary, hpol κ activity was enhanced relative to other Y-family members when the enzyme is 2-3 nucleotides from the G4 motif, but hpol κ appears to be less competent than hpol η at copying tetrad-associated guanines.

Barriers to DNA replication, including endogenous secondary structures and damage caused by exogenous radiation or chemical exposure, pose a problem for replicative polymerases.^(1,2) Unchallenged, these obstacles impede processive DNA synthesis by high-fidelity replicative polymerases (pols) and result in replication fork stalling followed by replication stress and activation of DNA damage response mechanisms, both of which are enabling characteristics of cancer.^(2,3) High-fidelity pols are characterized by restrictive active sites allowing accurate and efficient selection of the correct nucleotide during normal

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DNA replication at the expense of significantly diminished activity on damaged or structured DNA.⁽⁴⁾ Thus, specialized translesion synthesis (TLS) enzymes have been retained across species to overcome blocks to replication by copying past DNA damage and avoiding the deleterious effects of replication fork stalling.^(5,6)

TLS polymerases possess unique structural attributes that allow them to accommodate unusual DNA templates during replication.⁽⁷⁾ In humans, Y-family polymerases eta (hpol η), kappa (hpol κ), iota (hpol ι), and Rev1 (hRev1) are the primary enzymes involved in TLS.^(8,9) Translesion synthesis by pol κ has been well studied in the context of bulky minor-groove N^2 -dG DNA adducts.^(10,11) As an example, benzo[*a*]pyrene is metabolized to intermediates that react with template guanines to produce bulky lesions that are efficiently and accurately replicated by pol κ .^(12,13) The crystal structure in ternary complex with DNA and an incoming nucleotide reveals that, unique among the Y-family enzymes, pol κ possesses a “N-clasp” structure at its N-terminus.⁽¹⁴⁾ This region encircles DNA and constrains the active site of the enzyme, diminishing its activity on certain types of DNA damage and perhaps contributing to the higher fidelity of pol κ versus its Y-family pol counterparts.⁽¹⁴⁾ However, the N-clasp also acts as a “lock” to promote efficient DNA primer extension after a nucleotide is incorporated across from a lesion and has been demonstrated to be important in bypass of bulky N^2 -dG DNA adducts.^(14,15)

Natural blocks to replication can arise from DNA sequences possessing separate tracts of 2–4 guanine bases that interact to form highly stable G-quadruplex DNA (G4 DNA) structures.^(16,17) The existence of G-quadruplexes in human cells has been probed using small-molecule ligands and antibody-based methods of detection, and somewhat surprisingly, G4 structures were found to form maximally during S-phase of the cell cycle.^(18,19) Furthermore, bioinformatic analyses indicate that G4 motifs are enriched in specific regions of eukaryotic genomes, such as near telomeres, in promoters, and at breakpoint sites in many cancers.^(20–25) Thus, it has become apparent that defective maintenance of these structures can induce genomic instability and impair cellular homeostasis, hallmarks especially relevant to human disease and cancer.^(26–28)

Successful replication of highly stable G4 DNA requires the coordinated effort of specialized proteins.⁽²⁹⁾ Certain DNA helicases, including Pif1, FANCI, and RecQ helicases, have been shown to unwind G4 DNA structures.^(30–32) Long recognized as key components of TLS in bypass of common DNA adducts, recent studies have also implicated three of the four human Y-family pols in successful G4 DNA replication, namely, hpol η , hpol κ , and hRev1.^(7,8,26,30,33–35) Experiments in FANCI helicase-deficient *Caenorhabditis elegans* have demonstrated a requirement for pols η and κ in preventing deletions within G4-forming genomic sequences.⁽³⁴⁾ Knockdown of either pol η or κ in human cells treated with the G4 stabilizing ligand telomestatin (TMS) exhibited decreased viability and increased double-strand break formation upon stable transfection and genomic integration of *c-MYC* G4 DNA sequence copies.⁽³³⁾ Furthermore, two studies by Sarkies *et al.* conducted in avian DT40 cells have demonstrated hRev1 catalytic activity and its interaction with other Y-family pols are necessary for fork progression past G4 DNA, preventing single-stranded gaps of DNA, and maintaining epigenetic marks.^(26,30) A model was recently proposed in which hRev1 functions in parallel with specialized helicases to disrupt G4 DNA during replication. Our

group has demonstrated that a simple kinetic switch between the replicative B-family pol epsilon and Y-family pol η could facilitate replication across G4 sites.^(36,37)

It has remained unclear how hpol κ contributes to bypass of G4 structures. Despite being in the same family as hpol η , there are considerable structural and functional differences between the two enzymes. Therefore, we sought to investigate the biochemical properties of hpol κ on G4 DNA substrates. We performed DNA binding, primer extension, and single-nucleotide insertion assays with the hpol κ catalytic core (amino acids 19-526) using both G4 and non-G4 DNA substrates. The effect of varying the distance of the primer from the G4 motif was examined, as was the effect of using different monovalent cations in the reaction buffer. By comparing hpol κ activity on G4 DNA substrates in buffers containing K^+ , Na^+ , or Li^+ , we were able to examine TLS action against highly stable (K^+), moderately stable (Na^+), or destabilized (Li^+) G4 structures. The results reported here represent a step toward greater understanding of how fork progression is maintained during G4 DNA replication through the coordinated action of Y-family pols.

MATERIALS AND METHODS

DNA substrate preparation

For DNA polymerization assays eight primer-template DNA (p/t-DNA) substrates were prepared with one of two 42-mer template strands (i.e. eight non-G4 control p/t-DNA substrates and eight G4 p/t-DNA substrates) for a total of sixteen substrates (Table 1), each prepared in 50 mM HEPES buffer (pH 7.5) containing either KCl (100 mM), NaCl (100 mM), or LiCl (100 mM). Oligonucleotide stock solutions were prepared as described previously.^(36,37) The primer-template substrates were prepared in 50 mM HEPES (pH 7.5) buffer containing either KCl (100 mM), NaCl (100 mM), or LiCl (100 mM) by adding the primer and template strands (1:2, primer:template molar ratio) and heating the sample to 95 °C for five minutes and then slow cooling to room temperature. Our model G4 DNA sequence, Myc2345 14/23, is derived from the *c-MYC* promoter and folds into a highly stable, parallel-stranded G-quadruplex with a T_m of 75–85 °C.^(38,39) Changing the cation in the DNA substrate solution allowed preparation of G-quadruplex substrates exhibiting a range of stabilities, as KCl promotes G4 folding and stabilization, NaCl reduces the melting temperature of G4 DNA to produce G-quadruplexes of intermediate stability, and LiCl does not promote G-quadruplex formation.^(29,38,39)

Protein expression and purification

The core pol domain of hpol κ (residues 19-536) was expressed in *Escherichia coli* BL21 Gold cells (Stratagene) and purified as described previously.⁽⁴⁰⁾ Purified hpol κ^{19-536} was then stored at –80 °C in the HEPES buffer (pH 7.5) containing 0.1 M NaCl, 5 mM β -ME, and 30% (v/v) glycerol.

Measurement of DNA binding affinity by fluorescence polarization

Two DNA substrates were prepared as described previously for binding assays (Table 1).⁽³⁶⁾ DNA binding affinity was measured by incubating DNA substrates (1 nM) with increasing concentrations of hpol κ^{19-536} (0 to 2 μ M), similar to our previous reports.⁽³⁶⁾

hpol κ^{19-536} extension assays

DNA substrates (200 nM) were incubated at 37 °C with 2 nM hpol κ^{19-536} . Reactions were initiated by adding dNTP•MgCl₂ solution (0.25 mM of each dNTP and 5 mM MgCl₂) to the pre-incubated pol•DNA complex. All extension assays were carried out at 37 °C in 50 mM HEPES (pH 7.5) buffer containing 5 mM DTT, 100 µg/mL BSA, 5% (v/v) glycerol, and either 100 mM KCl, 100 mM NaCl, or 100 mM LiCl. At time points up to 1 hour, 4 µl aliquots were quenched with 36 µl of a 95% formamide (v/v)/20 mM EDTA/0.1% bromophenol blue (w/v) solution and the extension products were separated from substrate by electrophoresis on a 16% polyacrylamide (w/v)/7 M urea gel. The products were visualized and quantified as described previously.⁽³⁷⁾

Steady-state kinetic analysis of dNTP insertion by hpol κ^{19-536}

The rate of single nucleotide insertion by hpol κ^{19-536} was measured in the presence of varying concentrations of dNTP. Sixteen p/t-DNA substrates prepared in KCl, NaCl, or LiCl were used (Table 1; eight non-G4 p/t-DNA substrates and eight G4 p/t-DNA substrates) to assess the effect of varying the primer length and cation identity on DNA pol activity. Experiments with substrates annealed in KCl were performed with a minimum of three replicates for correct dNTP insertion to demonstrate reproducibility of kinetic results. Additional steady-state kinetic measurements were the result of single experiments with a minimum of ten concentrations of dNTP used to define the Michaelis-Menten plot. Steady-state kinetic analysis was performed by pre-incubating non-G4 and G4 p/t-DNA substrates (200 nM) with 2 nM hpol κ^{19-536} , and reactions were started by adding the indicated dNTP (0 – 100 µM) and MgCl₂ (5 mM). All polymerase reactions were carried out at 37 °C in 50 mM HEPES (pH 7.5) buffer containing 100 mM salt (KCl, NaCl, or LiCl), 5 mM DTT, 0.1 mg/mL BSA and 5% (v/v) glycerol. The reactions were quenched by mixing 4 µL aliquots of the reaction with 36 µL of 95% formamide (v/v)/20 mM EDTA/0.1% bromophenol blue (w/v) solution. Reaction products were separated by electrophoresis on a 12% polyacrylamide (w/v)/7 M urea gel except for reactions with substrates possessing the 13-mer primer, which were separated on a 16% polyacrylamide (w/v)/7 M urea gel. The products were then visualized using a Typhoon imager (GE Healthcare Life Sciences) and quantified using ImageJ software.⁽⁴¹⁾

Analysis of hpol κ^{19-536} mis-insertion frequency

The mis-insertion frequency of hpol κ^{19-536} was measured using the 18/42-mer, 21/42mer, and 23/42-mer G4 and non-G4 DNA substrates annealed in 100 mM KCl, NaCl, and LiCl (Table 1). A pilot study was first conducted with each of the substrates to determine the identity of the most favored mis-insertion product. Kinetic analysis of dNTP mis-insertion was then performed with hpol κ^{19-536} (20 nM) for the six DNA substrates (200 nM) in reactions containing each of the three salts as described above. Reactions were initiated by adding dNTP (0 – 6 mM) and MgCl₂ (5 mM). The reactions were quenched as before. Mis-insertion of dCMP was measured on the 18/42mer and dGMP misinsertion was measured on both the 21/42mer and 23/42mer in all three salts; dAMP misinsertion was also measured in KCl and NaCl for the 21/42mer, as its preference by hpol κ^{19-536} rivaled that of dGMP. The reactions were stopped by adding 4 µl aliquots from the reactions to 36 µl of quench solution

at time points up to 10 hours. Reaction products were separated and visualized as described in the previous section.

RESULTS

hpol κ^{19-536} preferentially binds G4 DNA versus non-G4 DNA

By titrating in hpol κ^{19-536} and measuring changes in fluorescence polarization, we were able to determine parameters for hpol κ^{19-536} binding interactions with 11/28mer G4 and non-G4 DNA substrates (Table 1) annealed in KCl (100 mM). Fluorescence polarization data were fit to a quadratic equation and provided an estimate of the equilibrium dissociation constant for pol binding to G4 DNA ($K_{d,DNA}$) of 15 nM (± 2 nM), 5.7-fold lower than for the non-G4 control substrate ($K_{d,DNA} = 86$ nM ± 8 nM) (Figure 1). Thus, hpol κ^{19-536} exhibits a preference for interacting with G4 DNA over non-G4 DNA, binding it with a markedly higher affinity.

Running-start primer extension activity for hpol κ^{19-536} on G4 DNA structures of varying stability

Pol extension assays were conducted with non-G4 and G4 13/42mer DNA substrates prepared in KCl, NaCl, or LiCl as a qualitative assessment of differences in hpol κ^{19-536} activity toward G4 DNA of differing stability. The 13-mer primer used for these experiments positions the terminal 3'-OH group ten nts away from the first G4-associated guanine (G22) in the template strand, allowing the enzyme a "running start" before encountering the G-quadruplex-containing portion of the substrate (Figure 2a).

In reactions with KCl, the most strongly G4 DNA stabilizing ion, we observed a strong pause in hpol κ^{19-536} activity upon encountering the first tetrad-associated guanine (G22), and product formation past G22 was negligible (Figure 2b). There was also a pause in extension at the 14mer position, but it occurred in reactions with both the non-G4 and G4 DNA substrates. Finally, the amount of substrate that was not converted to extended product by hpol κ was more apparent in the G4 reactions, especially for the early time points, than that for the control reactions (Figure 2b, note the different intensities of the 13mer substrate bands on the gel for the KCl experiments). These results indicate that hpol κ^{19-536} extension activity is impaired on highly stable G-quadruplex substrates compared to non-structured control substrates especially when hpol κ attempts to copy the tetrad-associated guanines in a K^+ -stabilized G-quadruplex structure.

We next examined the extension activity of hpol κ^{19-536} on less stable versions of our model G4 substrate prepared in NaCl and LiCl. As expected, the ability of hpol κ^{19-536} to catalyze extension of the substrates was less impaired on the less stable Na^+ -bound G-quadruplex. While the enzyme paused at G22, hpol κ -catalyzed incorporation of nucleotides along the G4 motif was significantly improved in Na^+ compared to reactions performed in the presence of K^+ (Figure 2c, note the number of product bands above the pause at G22). However, hpol κ is more inhibited by Na^+ -stabilized G4 structures than hpol η , as hpol η exhibits almost no reduction in activity on Na^+ -stabilized G4 DNA.⁽³⁷⁾

For reactions in LiCl, there was less difference in extended product formation between reactions with control substrates and those with G4 substrates (Figure 2d, note the similar intensities of the 13mer substrate bands on the gel for the LiCl experiments). The extension results with hpol κ in the reaction buffer containing Li^+ are not surprising, since Li^+ does not effectively stabilize G4 structures. Thus, while hpol κ^{19-536} extension activity is hindered by the presence of a stable G-quadruplex (i.e. in the presence of K^+), the enzyme can copy a destabilized G4 motif.

Steady-state kinetic analysis of hpol κ^{19-536} activity on G4 DNA substrates

To more rigorously measure the effect of G4 DNA on hpol κ^{19-536} activity, steady-state kinetic experiments were performed with a series of p/t-DNA substrates. Single nucleotide insertion kinetic parameters were measured for the correct (i.e. Watson-Crick) nascent base pair on DNA substrates with varying primer lengths that position the terminal 3'-OH from ten (13-mer primer) to zero (23-mer primer) nts away from G22, the first G4-associated guanine in the *c-MYC* G4 motif. The p/t-DNA substrates were prepared in buffer containing KCl, NaCl, or LiCl. This resulted in sixteen DNA substrates being prepared in three different reaction buffers for a total of 48 DNA substrates (Table 1; eight non-G4 control substrates and eight G4 DNA substrates for each salt). Because K^+ is generally considered to be the most biologically relevant cation for stabilizing G-quadruplexes *in vivo*⁽²⁴⁾ and to ensure reproducibility of kinetic results, assays with KCl were performed in triplicate. The relative activity of hpol κ^{19-536} on G4 DNA was calculated by measuring the specificity constant ($k_{\text{cat}}/K_{\text{m,dNTP}}$) on G4 DNA and dividing that value by the specificity constant of the enzyme on non-G4 DNA substrates for individual sets of experiments.

Surprisingly, hpol κ^{19-536} activity decreased to 10% on G4 DNA when the primer terminus was positioned ten nts from the G4 motif (Table 2, see data for 13/42mer DNA substrates). The activity of hpol κ was also low on G4 substrates relative to non-G4 DNA when the primer was positioned 9, 5, and 4 nts from the G4 motif (Table 2, see results for 14/42, 18/42, and 19/42mer substrates). The decrease in hpol κ^{19-536} catalytic efficiency is primarily driven by an increase in the Michaelis constant ($K_{\text{m,dNTP}}$) for the incoming dNTP and a decrease in the turnover number describing nucleotidyl transfer by hpol κ^{19-536} (k_{cat}). We observed a remarkable increase in hpol κ^{19-536} activity when the primer is positioned 2-3 nts from the G4 motif (Table 2, see results for 20/42 and 21/42mer substrates). The increased activity is due to a decrease in the $K_{\text{m,dNTP}}$ relative to the control. Compared to the robust activity of hpol κ noted 2-3 nts in advance of the G-quadruplex, the activity of hpol κ on G4 DNA decreases sharply to ~15% when the primer is positioned one nucleotide from G22. The activity of hpol κ^{19-536} is further diminished to less than 1% for insertion opposite G22, as the k_{cat} decreases and the $K_{\text{m,dNTP}}$ increases compared to the non-structured substrate (Table 2).

Compared to K^+ -bound G4 structures, hpol κ^{19-536} catalytic efficiency on substrates annealed in NaCl is generally less perturbed. At a distance of 10 nts from G22, hpol κ^{19-536} was equally active on the Na^+ -stabilized G-quadruplex substrate and the control non-G4 DNA substrate (Table 3, see results for 13/42mer substrates). Similar to the results observed in K^+ , hpol κ activity remained relatively low when the primer terminus is positioned 9, 5,

and 4 nts from the Na⁺-stabilized G4 structure in comparison to the activity observed on additional substrates positioning the primer terminus closer to the G4 motif (Table 3). The relative activity of hpol κ^{19-536} began trending upward on the 20/42-mer G4 substrate before increasing dramatically 2 nts from G22 to 143% as a result of an increase in both the k_{cat} and $K_{\text{m,dNTP}}$ parameters (Table 3). Relative hpol κ^{19-536} activity on NaCl-annealed G4 DNA decreased to 11% on the 23/42-mer substrate when attempting dNTP insertion across from a G-quadruplex-associated nucleotide, primarily because of a decrease in the k_{cat} (Table 3). Overall, the results in Na⁺ were similar to those obtained in K⁺ but with less pronounced inhibition of hpol κ action.

In contrast to experiments with K⁺- and Na⁺-stabilized G-quadruplexes, hpol κ^{19-536} activity was least disrupted by G4 DNA substrates annealed in LiCl. The catalytic activity of hpol κ^{19-536} was nearly equal on Li⁺-bound G4 DNA and control non-G4 DNA 10 nts from G22 on the 13/42-mer (Table 4). Relative activity of hpol κ^{19-536} toward G4 DNA was maintained at ~60–90% at all other primer positions tested, including insertion across from G22, reaching a maximum of 132% when the primer was positioned 3 nts from the G-quadruplex on the 20/42-mer substrate (Table 4). Together with the single-nucleotide kinetics for hpol κ^{19-536} and the other salts, our results demonstrate hpol κ^{19-536} to exhibit a pronounced increase in activity ~2 nts from G4 structures (either K⁺ or Na⁺ stabilized), but the enzyme was found to be less tolerant of G4 structures than hpol η when the primer was positioned adjacent to tetrad-associated guanines (Figure 4a).⁽³⁷⁾

The effect of G4 DNA stability on hpol κ^{19-536} mis-insertion frequency

After determining the kinetic parameters for correct nucleotide insertion, we next examined the effect of G4 DNA stability on the mis-insertion frequency (f_{ins}) of hpol κ^{19-536} . We chose the substrates that positioned the primer terminus five, two, or zero nts from G22 (*i.e.* 18/42-mer, 21/42-mer, and 23/42-mer G4 and non-G4DNA, for a total of six DNA substrates), each prepared in KCl, NaCl, or LiCl (Table 1). By performing a pilot time-course experiment measuring hpol κ^{19-536} -catalyzed insertion of each of incorrect dNTP, we determined the identity of the nucleotide most likely to be mis-inserted at each position on the different substrates. In each of the three salts, we found that on both the control and G4 substrates, hpol κ^{19-536} preferred to mis-insert dCMP for the 18/42mer; and dGMP for the 23/42-mer (data not shown). The 21/42-mer was unique in that hpol κ^{19-536} mis-inserted dGMP on both control and G4 substrates in all three salts but was also likely to mis-insert dAMP on substrates annealed in KCl and NaCl (data not shown). Steady-state kinetic analysis of nucleotide mis-insertion by hpol κ^{19-536} was performed and the mis-insertion frequency on each substrate in the presence of the three cations was estimated (Tables 5–7).

The fidelity of hpol κ^{19-536} was not substantially changed for G4 substrates relative to non-G4 controls when the primer terminus was positioned –5 nts from the G4 motif (Figure 3b and Tables 5–7, compare f_{ins} for the 18/42-mer DNA substrates), regardless of the monovalent cation used to prepare the substrate. These results suggest that G4 structures do not impact hpol κ^{19-536} fidelity when the primer is positioned more than 5 nts from the G4 motif. A similar conclusion was drawn from experiments with hpol η performed with the

18/42-mer substrates.⁽³⁷⁾ We next went on to test for changes in fidelity when the primer was positioned closer than 5 nts to the G4 motif.

The most interesting changes in hpol κ^{19-536} fidelity were observed when the primer was positioned 2 nts from the G4 motif. As noted above, we observed two major mis-insertion events with the 21/42-mer substrates annealed in either K^+ or Na^+ . Both dGMP and dAMP are mis-inserted against template dG equally well. However, comparing the f_{ins} for dAMP mis-insertion on the 21/42-mer (–2 nts from the G4 motif) reveals a ~9-fold decrease in fidelity on G4 substrates stabilized with K^+ (Table 5, compare dAMP f_{ins} for the 21/42-mer DNA substrates) and a ~6-fold decrease in fidelity on G4 substrates stabilized with Na^+ (Table 6, compare dAMP f_{ins} for the 21/42-mer DNA substrates) versus mis-insertion on 21/42mer non-G4 control substrates; dAMP was not mis-inserted at the –2 position in reactions with LiCl. Surprisingly, no change in fidelity on G4 substrates compared to non-G4 controls was observed at the 21/42-mer position for dGMP insertion with LiCl, KCl, or NaCl (Table 5–7, compare dGMP f_{ins} for the 21/42-mer DNA substrates) (Figure 3b).

The trend towards decreased hpol κ^{19-536} fidelity was reversed at the 23/42-mer position. In reactions containing KCl, there was a ~9-fold increase in hpol κ^{19-536} accuracy on 23/42-mer G4 substrates (Table 5, compare f_{ins} for the 23/42-mer DNA substrates) versus non-G4 control 23/42-mer substrates. The fidelity of hpol κ^{19-536} on G4 substrates was only slightly better than the non-G4 control in reactions with NaCl (Table 6, compare f_{ins} for the 23/42-mer DNA substrates) (Figure 3b). Finally, there was no change in hpol κ^{19-536} fidelity on G4 substrates relative to non-G4 control substrates in reactions including LiCl (Table 7, compare f_{ins} for the 23/42-mer DNA substrates) (Figure 3b). From these results, we conclude that the dramatic increase in hpol κ activity on G4 DNA compared to non-G4 controls observed 2 nts from the G4 motif is accompanied by a decrease in accuracy. By way of comparison, the accuracy of nucleotide selection increases when hpol κ attempts to insert across from a tetrad-associated guanine versus insertion at the same position on a control, non-G4 substrate, in spite of a markedly reduced catalytic efficiency at this site. These results indicate that the presence of a G-quadruplex promotes an increase in fidelity of hpol κ^{19-536} specific to catalysis of nucleotide insertion at G4 sites.

DISCUSSION

Once believed to be an *in vitro* anomaly, G4 DNA has become widely recognized as an important feature of the human genome.^(38,39,42–45) G4-forming motifs have been found proximal to various promoters, telomeres, and ribosomal DNA.^(18,20,22,25,26,46–48) Thus, G4 structures clearly serve to regulate expression of certain genes and protect telomeres, but they can also act as barriers to replication to produce double-strand break (DSBs) formation following fork collapse, a phenomenon that contributes to genomic instability in cancer.^(23,46,49,50) Further evidence for the importance of G4 motifs as endogenous barriers to replication comes from recent studies in human cells demonstrating maximum formation of G-quadruplex structures during S phase when the DNA double helix exists in a single-stranded state.⁽¹⁹⁾

In addition to phenomenological studies, it is apparent from biochemical and biological experiments that certain proteins and enzymes have been retained during evolution to promote successful maintenance and replication of G4 DNA. Prior studies have shown replicative helicases, including FANCD1 and certain RecQ family helicases, as well as certain single-stranded binding proteins to be competent at unfolding G4 DNA during replication *in vitro*.^(30,51–53) Based on *in vitro* folding kinetics and the enrichment of G-quadruplexes in S-phase, it is likely that pols will encounter G4 structures that have refolded in the wake of helicase action during replication.⁽⁵⁴⁾ Despite experiments in cells indicating the necessity of TLS pol action on G4 DNA, few studies have investigated the precise role of the Y-family class of translesion polymerases in G-quadruplex replication.^(30,33,34) We have previously examined the activity of human Y-family hRev1 and hpol η on G4 DNA structures.^(36,37) The current study focused on the biochemical properties of the third Y-family pol implicated in G4 maintenance, hpol κ , in an effort to better understand how Y-family translesion pols contribute to successful bypass of G4 DNA.

Our initial fluorescence anisotropy-based pol-DNA binding assays demonstrated that hpol κ^{19-536} binds a G4 DNA substrate with a 5.7-fold preference over a control, non-structured substrate. This data is in line with our previous binding analyses for the catalytic cores of hpol η^{1-437} and hRev1³³⁰⁻⁸³³, which bound G4 DNA with 6.2- and 15-fold greater affinities than non-G4 DNA, respectively (Figure 1).^(36,37) It is possible the increased affinity of the Y-family pols for G4 substrates exists to aid in targeting these enzymes to replication forks at G-quadruplex sites.

To first assess how G-quadruplex stability affected the ability of hpol κ^{19-536} to extend substrates, we prepared our model G4 DNA substrate derived from the G-quadruplex-forming region of the *c-MYC* promoter in the presence of three different cations: KCl, NaCl, and LiCl. G4 structures stabilized by K^+ exhibit melting temperatures that are ~5–15 °C higher than Na^+ -stabilized structures.^(29,38,39) Thus, K^+ -bound G4 substrates would be expected to present a stronger barrier to replication than Na^+ structures. In the presence of Li^+ , G4 structures are not stable. Therefore, pol activity should be inhibited to varying degrees depending on the identity of the monovalent cation used to prepare the G4 substrate. In running-start extension assays, hpol κ^{19-536} activity past G22 (the first G-quadruplex-associated guanine) was completely inhibited on the KCl-stabilized G4 substrate with no production of product past this point, unlike reactions with the control substrate which resulted in nearly complete conversion of substrate to fully-extended product. Surprisingly, the enzyme was also found to pause upon encountering the G-quadruplex at G22 on less stable substrates annealed in NaCl (Figure 2b and c). Similar running-start primer extension experiments with hpol η^{1-437} revealed that Na^+ -stabilized G-quadruplexes did not represent a barrier to hpol η^{1-437} , which was able to fully extend the less stable G-quadruplex substrates with almost no reduction in activity compared to reactions with control substrates.⁽³⁷⁾

We also performed a systematic, steady-state kinetic analysis on a range of G4 substrates to determine the effect of primer position relative to the G-quadruplex, as well as G-quadruplex stability, on hpol κ^{19-536} catalytic activity. For reactions containing LiCl, the relative activity of nt insertion by hpol κ^{19-536} on G4 substrates was ~60% or greater at all positions, and

there was effectively no change in fidelity, supporting the notion that hpol κ^{19-536} activity on substrates with G4 motifs that are not stably folded as G-quadruplex structures is similar to that observed with control substrates (Figure 3a and b, Tables 4 and 7). Conversely, hpol κ^{19-536} activity on K^+ -stabilized G4 substrates was dramatically reduced even when the primer was positioned ten nts away from the G4 motif (Figure 3a, Table 2). Nucleotide insertion by hpol κ^{19-536} exhibited an unusually large increase in activity when the primer was positioned 2-3 nts from the G4 motif. This trend mirrors that observed for hpol η^{1-437} activity on G4 substrates, which also peaks 2 nts in advance of the G4 motif (Figure 4a).⁽³⁷⁾ However, this peak in activity decreases rapidly to less than 1% of the control when the primer is positioned next to the G4 structure (Figure 4a). By way of comparison, hpol η maintains >25% activity when inserting nucleotides directly adjacent to or opposite tetrad-associated guanines.⁽³⁷⁾ The activity observed for hpol κ^{19-536} for nt insertion on the G-quadruplex is similar to that established for hRev1³³⁰⁻⁸³³, which had a relative activity of ~200% when the primer is positioned ten nts from the G4 structure, and ~2% when inserting opposite G22 (Figure 4a).⁽³⁶⁾ Based on these results, it seems that hRev1 and hpol κ are more inhibited than hpol η when the primer is in close proximity to the G4 structure. Instead, hRev1 and hpol κ display higher catalytic proficiency when positioned ten or two nts from the G4 motif, respectively.

G-quadruplex substrates annealed in NaCl exerted less of an effect on hpol κ^{19-536} catalysis, as pol activity on the G4 substrate remained ~30% or greater at all positions tested except for insertion across from G22 (Figure 3a, Table 3). Advancing the primer terminus closer to G22 revealed an increase in hpol κ^{19-536} activity similar to what was observed with K^+ -stabilized G4 DNA relative to control substrates beginning 4 nts away, with a peak in maximum activity 2 nts from G22 (Figure 3a, Table 2). The activity of hpol κ^{19-536} decreased to ~11% for nucleotide insertion opposite a Na^+ -stabilized tetrad-associated guanine (Figure 4a). The trends for hpol κ^{19-536} activity against Na^+ -stabilized G4 structures are similar to those obtained for G4 substrates prepared in K^+ , but the degree of inhibition is less, as would be expected for a less stable G-quadruplex.

The fidelity of hpol κ^{19-536} decreased ~6-9-fold on K^+ - and Na^+ -stabilized G4 substrates when the primer was placed 2 nts away from the G4 motif (Figure 4b). This is in stark contrast to the fidelity of hpol η^{1-437} 2 nts from a G4 structure, which increases ~9-fold at this position.⁽³⁷⁾ Interestingly, both hpol κ^{19-536} and hpol η^{1-437} increase in fidelity ~9- and ~15-fold, respectively, when replicating G22, primarily due to large decreases in the k_{cat} for mis-insertion on the 23/42-mer G4 substrate (Figure 4b, Table 5).⁽³⁷⁾ The increased accuracy of Y-family hpol η and κ support the idea that the presence of G4 structures at biologically relevant sites within the genome provides protection from deleterious mutations in these important regions.⁽³⁷⁾ This idea is not without precedent, as hpol κ has been shown to copy microsatellite regions of the genome, notable for their high mutation rates and subsequent contribution to genomic instability, in an accurate manner.⁽⁵⁵⁾

Together, the kinetic parameters describing pol catalytic activity toward G4 DNA fits a model for G-quadruplex replication involving multiple Y-family pols. Experiments in avian DT40 cells deficient in hRev1 and subsequently complemented with various hRev1 constructs demonstrated a requirement for hRev1 catalytic activity in bypass of G-

quadruplex structures.⁽²⁶⁾ Cells complemented with a mutant hRev1 construct lacking the C-terminal portion of the protein responsible for binding hpols η and κ were defective in G4 replication.⁽²⁶⁾ We have previously shown that hRev1 is able to disrupt G4 structures in the absence of catalysis by the enzyme.⁽³⁶⁾ Thus, it is possible that hRev1 can interact favorably with G4 sites and assist in the unfolding of these structures, while simultaneously serving as a scaffold for recruitment of hpol κ and hpol η . Unfolding of G4 structures through the combined properties of hRev1 and G4 helicases, such as FANCI and RecQ enzymes, could accompany nucleotide insertion by either hpol κ or hpol η just upstream from the G4 structure. The involvement of hpols η and κ in G4 replication is supported by the formation of deletions in G4-forming regions of the genomes of *C. elegans* mutants lacking the FANCI helicase and functional pols η or κ .⁽³⁴⁾ Extension through the G4 motif may be catalyzed by hpol η , which possesses an active site large enough to house two template bases in multiple conformations.^(56,57) This is in line with our observations that, of the pols tested, hpol η maintains the highest intrinsic activity for nt insertion on G4 substrates.

While hpol κ is not efficient at copying tetrad-associated guanines, it does exhibit the highest activity of the pols tested when the primer is positioned 2-3 nts from the G4 motif. This presents an alternative explanation for the requirement of hpol κ in maintenance of G4 DNA that may involve its role in priming synthesis upstream of the G4 motif and promoting ATR/Chk1 activation. ATR-mediated phosphorylation of Chk1 upon replication stalling induced by DNA damage or endogenous barriers can lead to activation of the replication checkpoint and cell cycle arrest, providing an additional window of time for resolution of barriers to replication fork progression.⁽⁵⁸⁾ A recent study by Bétous *et al.* found that depletion of pol κ in unstressed MRC-5 cells significantly increased γ -H2AX and replication protein A (RPA) foci formation, indicating replication fork stalling, accumulation of regions of ss-DNA, and ds-DNA break formation.⁽⁵⁹⁾ The observed increase in replication stress markers was not accompanied by a corresponding increase in levels of Chk1 phosphorylation, as would be expected. In that same study, *Xenopus laevis* egg extracts were immunodepleted for pol κ and UV-irradiated to induce DNA lesions and replication stress to implicate pol κ in promotion of Chk1 phosphorylation upon replication fork stalling, as phosphorylated Chk1 levels were strongly reduced in the absence of pol κ .⁽⁵⁹⁾ A second study investigating the effects of pol κ depletion on replication signaling activation demonstrated that prolonged fork pausing at endogenous minisatellite repeats, which represent natural barriers to replication, results in increased mutagenesis and genomic instability.⁽⁶⁰⁾ It is possible that in the absence of pol κ , cells are unable to promote replication checkpoint signaling and downstream Chk1 activation at G4 sites within the genome, which would lead to increased replication fork stalling and collapse near G4 sites.

Successful bypass of G-quadruplex structures *in vivo* is undoubtedly a complex process, requiring the coordination of a host of replicative enzymes including DNA binding proteins, helicases, and polymerases. The current study has evaluated the biochemical properties of hpol κ on G4 DNA in a systematic fashion. Of the Y-family pols, hpol κ activity is particularly high on G4 substrates 2-3 nts from the stabilized structure, but this increase in activity is accompanied by a large decrease in the fidelity of nucleotide selection. Based on these findings, we have proposed a model for Y-family pol action near G4 sites that incorporates biochemical properties with observations made by others in cell culture. Ours

are the first studies to provide detailed biochemical insight into the mechanism by which Y-family translesion pols might serve to promote successful maintenance of G-quadruplex structures in cells. Future experiments will investigate Y-family pol activity during G4 replication in the presence of additional proteins/enzymes and within the context of native cellular environments.

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1 Abbreviations

G4	G-quadruplex
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
pol	(DNA) polymerase
FAM	6-carboxyfluorescein
nt(s)	nucleotide(s)
TLS	translesion DNA synthesis

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Figure 1. hpol κ^{19-526} preferentially binds G4 DNA-containing substrates

Hpol κ^{19-526} was titrated into a solution containing either G4 DNA ($\square$) or non-G4 DNA ($\bullet$) FAM-labeled 11/28-mer primer-template oligonucleotides (1 nM). Changes in fluorescence polarization were measured and the resulting data fit to a quadratic equation to yield the following equilibrium dissociation constants: G4 DNA, $K_{d,DNA} = 15 \pm 2$ nM; non-G4 DNA, $K_{d,DNA} = 86 \pm 8$ nM. The reported values represent the mean $\pm$ s.e.m. (n = 3).

Figure 3. Kinetic analysis of relative hpol κ^{19-526} on G4 structures of different stability
a. The relative activity of hpol κ^{19-526} (2 nM) on G4 DNA (200 nM) is plotted as a function of primer terminus distance from the first tetrad guanine (G22) in reaction mixtures containing 100 mM KCl, NaCl, or LiCl. The results for experiments performed in KCl represent the mean $\pm$ s.d. (n = 3-4). **b.** hpol κ^{19-526} (5 or 20 nM) change in fidelity on G4 DNA substrates relative to control non-G4 is shown for p/t-DNA substrates (200 nM) annealed in 100 mM KCl, NaCl, or LiCl and positioning the primer terminus ten, five, two, and zero nts away from the first tetrad guanine (G22).

Figure 4. Comparison of human pols relative kinetic properties on G4 DNA yields insight into accurate and efficient G-quadruplex replication

a. The relative activity of Y-family translesion pols hpol κ^{19-526} , hpol η^{1-437} , and hRev1³³⁰⁻⁸³³ on G4 DNA is plotted as a function of primer terminus distance from the first tetrad guanine (G22). The results for experiments performed with hpol κ^{19-526} in KCl represent the mean $\pm$ s.d. ($n = 3-4$). **b.** The change in Y-family translesion pols hpol κ^{19-526} , and hpol η^{1-437} fidelity on G4 DNA substrates relative to control non-G4 is shown for p/t-DNA substrates positioning the primer terminus ten, five, two, one and zero nts away from the first tetrad guanine (G22). The data for hpol η^{1-437} , and hRev1³³⁰⁻⁸³³ were re-plotted from our previous studies to illustrate differences in pol activity on G4 DNA. Please see reference number 36 for original source of hRev1 data and reference number 37 for original source of hpol η data.

Table 1

Sequences of DNA substrates used in the study

Substrates used in DNA binding assays:**1. 11/28-mer non-G4 DNA**

5'-ATCCTCCCCTA-3'

3'-TAGGAGGGGATGGGTCGTATCAGTGTAT-/FAM/-5'

2. 11/28-mer G4 DNA ^a

5'-ATCCTCCCCTA-3'

3'-TAGGAGGGGATGGGTGGGATGGGTGGGT-/FAM/-5'

Substrates used in DNA pol activity assays:**1. 13/42-mer non-G4 DNA**

5'-/FAM/-TTTGCCTCGAGCCAGC-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

2. 14/42-mer non-G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCC-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

3. 18/42-mer non-G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGAG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

4. 19/42-mer non-G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGAGCA-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

5. 20/42-mer non-G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGAGAC-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

6. 21/42-mer non-G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGAGACG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

7. 22/42-mer non-G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGAGACGC-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

8. 23/42-mer non-G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGAGACGCA-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

9. 13/42-mer G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGC-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTGGGATGGGTGGGAGT-5'

10. 14/42-mer G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCC-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTGGGATGGGTGGGAGT-5'

11. 18/42-mer G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGAG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGT**GGGTGGG**ATGGGTGGGAGT-5'

12. 19/42-mer G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGCAGA-3'

3'-CGGAGCTCGGTCGGCGTCTGCGT**GGGTGGG**ATGGGTGGGAGT-5'

13. 20/42-mer G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGCAGAC-3'

3'-CGGAGCTCGGTCGGCGTCTGCGT**GGGTGGG**ATGGGTGGGAGT-5'

14. 21/42-mer G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGCAGACG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGT**GGGTGGG**ATGGGTGGGAGT-5'

15. 22/42-mer G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGCAGACGC-3'

3'-CGGAGCTCGGTCGGCGTCTGCGT**GGGTGGG**ATGGGTGGGAGT-5'

16. 23/42-mer G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGCAGACGCA-3'

3'-CGGAGCTCGGTCGGCGTCTGCGT**GGGTGGG**ATGGGTGGGAGT-5'

^aTetrad-associated guanines are shown in bold with the G22 guanine underlined.

Table 2

Steady-state kinetic parameters for hpol κ^{19-526} catalysis on G4 DNA and non-G4 DNA substrates prepared in KCl

	k_{cat} (min^{-1})	$K_{\text{M,dNTP}}$ (μM)	$k_{\text{cat}}/K_{\text{M,dNTP}}$ ($\text{min}^{-1} \mu\text{M}^{-1}$)	Relative activity ^a
<u>13/42-mer: dCMP insertion opposite template dG –10 nt from G22</u>				
non-G4 DNA	7.5 ± 0.9	13 ± 3	0.58	-
G4 DNA	1.7 ± 0.2	30 ± 9	0.06	$10.4 \pm 1.4\%$
<u>14/42-mer: dGMP insertion opposite template dC –9 nt from G22</u>				
non-G4 DNA	3.7 ± 1.0	26 ± 3	0.15	-
G4 DNA	1.2 ± 0.8	169 ± 37	0.007	$5.0 \pm 1.0\%$
<u>18/42-mer: dAMP insertion opposite template dT –5 nt from G22</u>				
non-G4 DNA	8.9 ± 4.1	13 ± 2	0.65	-
G4 DNA	3.8 ± 0.8	85 ± 29	0.05	$7.5 \pm 2.1\%$
<u>19/42-mer: dCMP insertion opposite template dG –4 nt from G22</u>				
non-G4 DNA	9.0 ± 3.5	20 ± 1	0.45	-
G4 DNA	2.3 ± 0.3	25 ± 8	0.10	$22.8 \pm 2.4\%$
<u>20/42-mer: dGMP insertion opposite template dC –3 nt from G22</u>				
non-G4 DNA	5.1 ± 0.3	4.9 ± 1.5	1.10	-
G4 DNA	4.4 ± 1.0	4.1 ± 0.7	1.13	$103 \pm 18\%$
<u>21/42-mer: dCMP insertion opposite template dG –2 nt from G22</u>				
non-G4 DNA	8.6 ± 1.8	28 ± 1	0.31	-
G4 DNA	7.9 ± 1.9	14 ± 2	0.57	$188 \pm 25\%$
<u>22/42-mer: dAMP insertion opposite template dT –1 nt from G22</u>				
non-G4 DNA	7.3 ± 1.5	4.4 ± 0.9	1.67	-
G4 DNA	2.6 ± 0.4	11 ± 1	0.24	$14.6 \pm 3.1\%$
<u>23/42-mer: dCMP insertion opposite G22</u>				
non-G4 DNA	6.4 ± 0.3	6.1 ± 0.4	1.06	-
G4 DNA	0.21 ± 0.03	26 ± 3	0.0081	$0.76 \pm 0.11\%$

^aRelative activity was calculated as $[(k_{\text{cat}}/K_{\text{M,dNTP}})_{\text{G4 DNA}} / (k_{\text{cat}}/K_{\text{M,dNTP}})_{\text{non-G4 DNA}}] \times 100$ for three to four individual sets of experiments. For determination of k_{cat} and $K_{\text{M,dNTP}}$, graphs of the initial rate of product formation versus dNTP concentration were plotted using non-linear regression analysis (one-site hyperbolic fit) in the GraphPad Prism program. The mean and standard deviation of three to four individual sets of experiments is reported for each k_{cat} and $K_{\text{M,dNTP}}$ value.

Table 3

Steady-state kinetic parameters for hpol κ^{19-256} catalysis on G4 DNA and non-G4 DNA substrates prepared in NaCl

	k_{cat} (min^{-1})	$K_{\text{M,dNTP}}$ (μM)	$k_{\text{cat}}/K_{\text{M,dNTP}}$ ($\text{min}^{-1} \mu\text{M}^{-1}$)	Relative activity ^a
<u>13/42-mer: dCMP insertion opposite template dG –10 nt from G22</u>				
non-G4 DNA	2.7 ± 0.2	4.1 ± 1	0.66	-
G4 DNA	2.5 ± 0.2	3.8 ± 1	0.66	100%
<u>14/42-mer: dGMP insertion opposite template dC –9 nt from G22</u>				
non-G4 DNA	2.0 ± 0.2	51 ± 10	0.039	-
G4 DNA	2.3 ± 0.2	140 ± 20	0.016	41%
<u>18/42-mer: dAMP insertion opposite template dT –5 nt from G22</u>				
non-G4 DNA	10 ± 0.9	13 ± 4	0.77	-
G4 DNA	6.6 ± 0.3	19 ± 3	0.35	45%
<u>19/42-mer: dCMP insertion opposite template dG –4 nt from G22</u>				
non-G4 DNA	7.3 ± 0.2	15 ± 1	0.49	-
G4 DNA	3.0 ± 0.08	21 ± 2	0.14	29%
<u>20/42-mer: dGMP insertion opposite template dC –3 nt from G22</u>				
non-G4 DNA	4.1 ± 0.1	2.2 ± 0.3	1.86	-
G4 DNA	4.4 ± 0.2	1.9 ± 0.4	2.31	121%
<u>21/42-mer: dCMP insertion opposite template dG –2 nt from G22</u>				
non-G4 DNA	5.0 ± 0.2	24 ± 3	0.21	-
G4 DNA	6.0 ± 0.3	20 ± 2	0.30	143%
<u>22/42-mer: dAMP insertion opposite template dT –1 nt from G22</u>				
non-G4 DNA	4.7 ± 0.3	2.7 ± 0.7	1.74	-
G4 DNA	3.1 ± 0.1	4.5 ± 0.8	0.69	41%
<u>23/42-mer: dCMP insertion opposite G22</u>				
non-G4 DNA	6.4 ± 0.2	5.0 ± 0.5	1.28	-
G4 DNA	1.1 ± 0.05	7.7 ± 1	0.14	11%

^aRelative activity was calculated as $[(k_{\text{cat}}/K_{\text{M,dNTP}})_{\text{G4 DNA}} / (k_{\text{cat}}/K_{\text{M,dNTP}})_{\text{non-G4 DNA}}] \times 100$. For determination of kinetic values, graphs of the initial rate of product formation versus dNTP concentration were plotted using non-linear regression analysis (one-site hyperbolic fit) in the GraphPad Prism program. The standard error of the fit is reported for each value.

Table 4

Steady-state kinetic parameters for hpol κ^{19-526} catalysis on G4 DNA and non-G4 DNA substrates prepared in LiCl

	k_{cat} (min^{-1})	$K_{\text{M,dNTP}}$ (μM)	$k_{\text{cat}}/K_{\text{M,dNTP}}$ ($\text{min}^{-1} \mu\text{M}^{-1}$)	Relative activity ^a
<u>13/42-mer: dCMP insertion opposite template dG –10 nt from G22</u>				
non-G4 DNA	8.8 ± 0.4	18 ± 3	0.49	-
G4 DNA	9.0 ± 0.8	17 ± 4	0.53	108%
<u>14/42-mer: dGMP insertion opposite template dC –9 nt from G22</u>				
non-G4 DNA	1.6 ± 0.1	42 ± 7	0.039	-
G4 DNA	1.6 ± 0.2	47 ± 20	0.034	87%
<u>18/42-mer: dAMP insertion opposite template dT –5 nt from G22</u>				
non-G4 DNA	6.8 ± 0.3	4.9 ± 0.8	1.39	-
G4 DNA	6.3 ± 0.3	7.5 ± 1	0.84	60%
<u>19/42-mer: dCMP insertion opposite template dG –4 nt from G22</u>				
non-G4 DNA	9.3 ± 0.6	26 ± 5	0.36	-
G4 DNA	7.6 ± 0.7	25 ± 6	0.30	83%
<u>20/42-mer: dGMP insertion opposite template dC –3 nt from G22</u>				
non-G4 DNA	1.4 ± 0.04	3.7 ± 0.4	0.38	-
G4 DNA	2.4 ± 0.07	4.8 ± 0.6	0.50	132%
<u>21/42-mer: dCMP insertion opposite template dG –2 nt from G22</u>				
non-G4 DNA	3.8 ± 0.2	31 ± 4	0.12	-
G4 DNA	2.6 ± 0.2	23 ± 6	0.11	92%
<u>22/42-mer: dAMP insertion opposite template dT –1 nt from G22</u>				
non-G4 DNA	1.2 ± 0.03	3.4 ± 0.4	0.35	-
G4 DNA	1.1 ± 0.03	3.9 ± 0.5	0.28	80%
<u>23/42-mer: dCMP insertion opposite G22</u>				
non-G4 DNA	1.2 ± 0.05	19 ± 2	0.063	-
G4 DNA	1.0 ± 0.04	28 ± 3	0.036	57%

^aRelative activity was calculated as $[(k_{\text{cat}}/K_{\text{M,dNTP}})_{\text{G4 DNA}} / (k_{\text{cat}}/K_{\text{M,dNTP}})_{\text{non-G4 DNA}}] \times 100$. For determination of kinetic values, graphs of the initial rate of product formation versus dNTP concentration were plotted using non-linear regression analysis (one-site hyperbolic fit) in the GraphPad Prism program. The standard error of the fit is reported for each value.

Table 5

Steady-state kinetic parameters for hpol κ^{19-526} mis-insertion on G4 DNA and non-G4 DNA substrates prepared in KCl

	k_{cat} (min^{-1})	$K_{\text{M,dNTP}}$ (μM)	$k_{\text{cat}}/K_{\text{M,dNTP}}$ ($\text{min}^{-1} \mu\text{M}^{-1}$)	f_{ins}^a
<u>18/42-mer: dCMP insertion opposite template dT (-5 nt from G22)</u>				
non-G4 DNA	1.3 ± 0.3	1500 ± 570	0.00087	0.00087
G4 DNA	0.14 ± 0.05	1600 ± 860	0.000088	0.0018
<u>21/42-mer: dGMP insertion opposite template dG (-2 nt from G22)</u>				
non-G4 DNA	0.21 ± 0.01	350 ± 60	0.00060	0.0018
G4 DNA	0.42 ± 0.02	460 ± 70	0.00091	0.0014
<u>21/42-mer: dAMP insertion opposite template dG (-2 nt from G22)</u>				
non-G4 DNA	0.069 ± 0.01	1400 ± 500	0.000049	0.00014
G4 DNA	0.28 ± 0.01	350 ± 50	0.00080	0.0013
<u>23/42-mer: dGMP insertion opposite template dG (0 nt from G22)</u>				
non-G4 DNA	2.2 ± 0.3	1100 ± 300	0.0020	0.0056
G4 DNA	0.0053 ± 0.0007	420 ± 200	0.000013	0.00062

^aThe mis-insertion frequency (f_{ins}) was calculated as $(k_{\text{cat}}/K_{\text{M,incorrect dNTP}})/(k_{\text{cat}}/K_{\text{M,correct dNTP}})$. For determination of kinetic values, graphs of the initial rate of product formation versus dNTP concentration were plotted using non-linear regression analysis (one-site hyperbolic fit) in the GraphPad Prism program. The standard error of the fit is reported for each value.

Table 6

Steady-state kinetic parameters for hpol κ^{19-526} mis-insertion on G4 DNA and non-G4 DNA substrates prepared in NaCl

	k_{cat} (min^{-1})	$K_{\text{M,dNTP}}$ (μM)	$k_{\text{cat}}/K_{\text{M,dNTP}}$ ($\text{min}^{-1} \mu\text{M}^{-1}$)	f_{ins}^a
<u>18/42-mer: dCMP insertion opposite template dT (−5 nt from G22)</u>				
non-G4 DNA	1.6 ± 0.4	1400 ± 500	0.0011	0.0014
G4 DNA	1.1 ± 0.3	2600 ± 800	0.00042	0.0012
<u>21/42-mer: dGMP insertion opposite template dG (−2 nt from G22)</u>				
non-G4 DNA	0.12 ± 0.004	230 ± 30	0.00052	0.0025
G4 DNA	0.16 ± 0.007	290 ± 40	0.00056	0.0019
<u>21/42-mer: dAMP insertion opposite template dG (−2 nt from G22)</u>				
non-G4 DNA	0.073 ± 0.007	1200 ± 300	0.000061	0.00029
G4 DNA	0.24 ± 0.02	430 ± 90	0.00056	0.0019
<u>23/42-mer: dGMP insertion opposite template dG (0 nt from G22)</u>				
non-G4 DNA	2.0 ± 0.1	770 ± 100	0.0026	0.0020
G4 DNA	0.62 ± 0.09	2600 ± 600	0.00024	0.0017

^aThe mis-insertion frequency (f_{ins}) was calculated as $(k_{\text{cat}}/K_{\text{M,incorrect dNTP}})/(k_{\text{cat}}/K_{\text{M,correct dNTP}})$. For determination of kinetic values, graphs of the initial rate of product formation versus dNTP concentration were plotted using non-linear regression analysis (one-site hyperbolic fit) in the GraphPad Prism program. The standard error of the fit is reported for each value.

Table 7

Steady-state kinetic parameters for hpol κ^{19-526} mis-insertion on G4 DNA and non-G4 DNA substrates prepared in LiCl

	k_{cat} (min^{-1})	$K_{\text{M,dNTP}}$ (μM)	$k_{\text{cat}}/K_{\text{M,dNTP}}$ ($\text{min}^{-1} \mu\text{M}^{-1}$)	f_{ins}^a
<u>18/42-mer: dCMP insertion opposite template dT (-5 nt from G22)</u>				
non-G4 DNA	0.13 ± 0.01	590 ± 100	0.00022	0.00016
G4 DNA	0.14 ± 0.01	580 ± 200	0.00024	0.00029
<u>21/42-mer: dGMP insertion opposite template dG (-2 nt from G22)</u>				
non-G4 DNA	0.082 ± 0.005	190 ± 50	0.00043	0.0036
G4 DNA	0.10 ± 0.007	190 ± 50	0.00053	0.0048
<u>23/42-mer: dGMP insertion opposite template dG (0 nt from G22)</u>				
non-G4 DNA	0.34 ± 0.02	570 ± 100	0.00060	0.0095
G4 DNA	0.31 ± 0.03	1300 ± 300	0.00024	0.0067

^aThe mis-insertion frequency (f_{ins}) was calculated as $(k_{\text{cat}}/K_{\text{M,incorrect dNTP}})/(k_{\text{cat}}/K_{\text{M,correct dNTP}})$. For determination of kinetic values, graphs of the initial rate of product formation versus dNTP concentration were plotted using non-linear regression analysis (one-site hyperbolic fit) in the GraphPad Prism program. The standard error of the fit is reported for each value.