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Evidence for the kinetic partitioning of polymerase activity on G-quadruplex DNA

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Abstract

We have investigated the action of the human DNA polymerase epsilon (hpol ϵ) and eta (hpol η) catalytic cores on G-quadruplex (G4) DNA substrates derived from the promoter of the c-MYC proto-oncogene. The translesion enzyme hpol η exhibits a 6.2-fold preference for binding to G4 DNA relative to non-G4 DNA, while hpol ϵ binds both G4 and non-G4 substrates with near equal affinity. Kinetic analysis of single-nucleotide insertion by hpol η reveals that it is able to maintain greater than 25% activity on G4 substrates compared to non-G4 DNA substrates, even when the primer template junction is positioned directly adjacent to G22 (the first tetrad-associated guanine in the c-MYC G4 motif). Surprisingly, hpol η fidelity increases ~15-fold when copying G22. By way of comparison, hpol ϵ retains ~4% activity and has a 33-fold decrease in fidelity when copying G22. The fidelity of hpol η is ~100-fold more accurate than hpol ϵ when comparing the mis-insertion frequencies of the two enzymes opposite a tetrad-associated guanine. The kinetic differences observed for the B- and Y-family pols on G4 DNA support a model where a simple kinetic switch between replicative and TLS pols could help govern fork progress during G4 DNA replication.

Many obstacles must be overcome to accurately replicate the genome. Exposure to reactive chemicals or radiation can produce a variety of damaged DNA structures that block replication and are etiological factors in many types of cancer.^(1, 2) The human genome encodes at least 17 different DNA polymerases (pols), including high-fidelity replicative pols and lower fidelity translesion synthesis (TLS) pols. Nucleotide selection by high-fidelity replicative pols, such as the B-family member pol epsilon (hpol ϵ), is restricted by structural and kinetic parameters that impair catalysis on damaged templates.⁽³⁾ TLS pols, on the other hand, are able to function in the replication of damaged templates because they possess structural attributes that allow them to accommodate bulky and/or distorted DNA

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ASSOCIATED CONTENT

Supporting Information. Supplemental Figures S1–S4 detailing some of the steady-state kinetic experiments with the two polymerases are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

lesions.⁽⁴⁾ Foremost among the TLS enzymes are the Y-family, which in humans includes pols η , ι , κ and Rev1. Mutations in the gene that encodes hpol η can cause the skin cancer-prone disease xeroderma pigmentosum variant.⁽⁵⁾ Accordingly, the TLS activity of hpol η is found to be involved in the bypass of UV-induced cyclobutane pyrimidine dimers (CPDs).^(6, 7) Additional studies have implicated hpol η action in resistance to anti-cancer treatments, especially platinum-based drugs, as well as in the replication of common fragile sites (CFSs).^(8, 9) The structural properties that facilitate hpol η -catalyzed bypass of DNA lesions are multi-faceted, but two features are central to the effective bypass of the aforementioned blocks to replication (i.e. CPDs, Pt-GG adducts and CFSs): (i) hpol η can accommodate CPD and Pt-GG adducts in its large active site and (ii) the hpol η DNA binding cleft acts as a “molecular splint” that is able to maintain damaged/distorted template DNA in an undistorted form.⁽⁶⁾ Additionally, it has been postulated that the back of the little finger domain serves as a “wedge” to mediate bypass of non-B-form DNA based on contacts between symmetry-related molecules in hpol η crystal structures.⁽⁶⁾

In addition to DNA adducts, blocks to replication arise from natural barriers, such as certain G-rich sequences that form G-quadruplex DNA (G4 DNA, also called QDNA or GQ).⁽¹⁰⁾ G4 DNA sequences adopt secondary structures with unusually slow unfolding rates, and in cells, they appear to form maximally during S-phase.^(11, 12) G4 motifs are positioned non-randomly in functional regions of prokaryotic and eukaryotic genomes.⁽¹³⁾ Several different G-quadruplexes have been studied in great structural and biophysical detail, including G4 structures derived from the *c-MYC* promoter.⁽¹⁴⁾ The relevance of these sequences to human disease is illustrated by the enrichment of G4 DNA sites in the promoters of many oncogenes, at telomeres, at replication origins and at chromosomal breakpoints in a variety of cancer types.^(14–20) Thus, defective maintenance of G4 DNA sequences contributes to genomic instability and is especially relevant to cancer.

Replication of G4 motifs requires proteins and enzymes with specific functions. It is well known that Y-family pols are important for TLS past DNA adducts^(21, 22), and it is becoming increasingly recognized that Y-family DNA pols are important for maintaining fork progress past non-B-form DNA structures, such as G4 DNA.^(23–28) As early as 2006, studies in *Caenorhabditis elegans* revealed that Y-family pols η and κ are responsible for preventing deletions in G4 motifs in strains deficient in the FANCD1 helicase.⁽²⁸⁾ Additional studies showed that human cells depleted of either pol η or κ exhibit increased double-strand break formation and cell death following exposure to G4 DNA-binding ligands when extra genomic copies of the *c-MYC* G4 DNA sequence are present.⁽²³⁾ More recently, two reports have illustrated that the Y-family member Rev1 is required for maintaining fork progression past G4 DNA sites in avian cells.^(26, 27) One study used complementation assays in *rev1* deficient DT40 cells to implicate both the catalytic activity and the polymerase interacting region of Rev1 in the successful replication of G4 DNA.⁽²⁷⁾ A second study from the same groups revealed that Rev1 functions in coordination with FANCD1 to maintain replication fork integrity during bypass of G4 DNA and that loss of Rev1 could be supplanted by either BLM or WRN RecQ helicases.⁽²⁶⁾ Thus, a model has been proposed recently for G4 DNA replication that involves the actions of either RecQ helicases or the TLS pol Rev1 functioning in coordination with FANCD1 helicase.⁽²⁶⁾ Still, the precise function of Y-family

pols (and proteins associated with their activity) during G4 DNA replication remains unclear, as the appropriate structure-function studies are lacking.

In order to elucidate the mechanistic basis for TLS pol action during replication of G4 DNA, we have investigated human pols from two different families: the catalytic cores of the B-family member hpol ϵ (a.a. 1-1189) and the Y-family member hpol η (a.a. 1-437). In an effort to systematically analyze the catalytic competence of each enzyme on G4 DNA, we performed DNA binding assays along with kinetic analysis of nucleotide insertion on both control non-G4 DNA substrates and DNA substrates possessing a G4-forming sequence derived from the *c-MYC* promoter. We find that, at the binding step, the TLS enzyme hpol η^{1-437} interacts preferentially

with G4 DNA substrates, while hpol ϵ^{1-1189} binds non-G4 and G4 DNA substrates with equal affinity. Comparing the polymerization activities, the catalytic core of the leading strand enzyme, hpol ϵ^{1-1189} , exhibits inhibition of activity near G4 structures, whereas hpol η^{1-437} maintains relatively robust activity when copying tetrad-associated guanines. Finally, we observe a curious increase in hpol η^{1-437} fidelity on G4 DNA substrates relative to non-G4 control substrates, whereas exonuclease-deficient (exo^-) hpol $\eta^{1-1189/\text{exo}^-}$ fidelity decreases sharply near G4 DNA. Our results show that a partitioning of pol activity can occur during replication of G-quadruplex structures based on DNA binding and kinetic parameters of nucleotidyl transfer alone (i.e. in the absence of accessory factors and/or protein-protein interactions). Taken together, our *in vitro* study is consistent with the idea that binding and catalytic properties of individual pols at sites of G4 DNA could help govern fork progress past these endogenous barriers to replication.

MATERIALS AND METHODS

Materials

All chemicals were molecular biology grade or better. 2'-deoxy-nucleoside triphosphates (dNTPs) were obtained from Promega (Piscataway, NJ). All oligonucleotides used in this work were synthesized by Integrated DNA Technologies (Coralville, IA). All primers used in the polymerization assays were labeled with 5'-6-carboxyfluorescein (FAM). For DNA polymerization assays, seven primer-template DNA (p/t-DNA) substrates were prepared with one of two 42-mer template strands (i.e. seven non-G4 control p/t-DNA substrates and seven G4 p/t-DNA substrates) for a total of fourteen substrates (Table 1).

DNA substrate preparation

Oligonucleotide stock solutions were prepared as described previously ⁽²⁹⁾. Briefly, oligonucleotides were re-suspended in 50 mM HEPES (pH 7.5) buffer. The primer-template substrates were prepared in 50 mM HEPES (pH 7.5) buffer containing either KCl (100 mM) or NaCl (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.

Protein expression and purification

The core pol domain of hpol η (residues 1-437) was expressed in bacteria and purified as described previously.⁽³⁰⁾ The core pol domain of hpol ε (residues 1-1189) was expressed in bacteria and purified similar to what has been described previously.⁽³¹⁾ Both wild-type and exonuclease deficient (exo^-) versions of hpol ε^{1-1189} were prepared. Site directed mutagenesis was used to introduce Pol ε - exo^- mutations D275A and E277A. Pol ε -pGEX4T3 expression vectors were co-transformed into Rosetta cells (EMD Millipore) with the TEV-pRK603 vector to allow for intracellular cleavage of the N-terminal GST tag by TEV protease. An aliquot of an overnight culture of Rosetta cells containing the hpol ε -N140 expression vector was added to 6 L of LB media to a final $A_{600\text{nm}}$ of 0.1. Cells were grown at 37°C with shaking at 220 rpm to an OD_{600} of 0.6, then expression was induced by 4 μM IPTG for 4 hours at room temperature. Cells were harvested by centrifugation and resuspended in lysis buffer: 150 mM Tris (pH 7.8) buffer containing 50 mM NaCl, 10 mM K_2SO_4 , 0.25 mM EDTA, and protease inhibitor tablets (Roche) at 1.5 ml per 1 g cells. A french press was used to lyse cells, and clarified lysates were purified with a HisTrapFF column (GE Healthcare). The column equilibration/wash solution consisted of 150 mM Tris (pH 7.8) buffer containing 200 mM NaCl, 20 mM K_2SO_4 , and 2 mM dithiothreitol (DTT). Bound protein was washed with 25 column volumes (c.v.) of wash buffer, followed by 10 c.v. of wash buffer containing 75 mM imidazole, and then eluted in 150 mM imidazole. Protein was dialyzed overnight into size-exclusion chromatography (SEC) buffer, which consisted of 150 mM Tris (pH 7.8) buffer containing 200 mM NaCl, 20 mM K_2SO_4 , and 2mM DTT. The protein was then resolved by a Superdex 200 10/300 GL column (GE Healthcare). Purified samples were stored in SEC buffer supplemented with 10% glycerol (v/v) at -80°C.

Measurement of DNA binding affinity by fluorescence polarization

Two DNA substrates were prepared for binding assays (Table 1). The sequence of the unlabeled primer was 5'-ATCCTCCCCTA-3'. Substrates were prepared as described above. DNA binding affinity was measured by incubating DNA substrates (1 nM) with varying concentrations of enzyme (0 to 1 μM for hpol η^{1-437} and 0 to 0.4 μM for hpol ε^{1-1189}). Fluorescence polarization was measured in a Biotek Synergy4 plate reader using the appropriate filter sets ($\lambda_{\text{ex}} = 485 \pm 20$ nm and $\lambda_{\text{em}} = 525 \pm 20$ nm). All titrations were performed at 25 °C in 50 mM HEPES (pH 7.5) buffer containing 10 mM KOAc, 10 mM KCl, 5 mM MgCl_2 , 0.1 mM EDTA, 2 mM β -mercaptoethanol (β -ME) and 0.1 mg/mL bovine serum albumin (BSA). Polarization was determined using equation 1:

$$P = \frac{(F_{\parallel} - F_{\perp})}{(F_{\parallel} + F_{\perp})} \quad \text{Equation 1}$$

where $F_{\parallel}$ equals fluorescence intensity parallel to the excitation plane and $F_{\perp}$ equals the fluorescence intensity perpendicular to the excitation plane. Fluorescence polarization data were plotted as a function of enzyme concentration and then fit to a quadratic equation to estimate $K_{d,\text{DNA}}$ using the equation: $P = P_0 + (P_{\text{max}}) ((D_t + E_t + K_{d,\text{DNA}}) - ((D_t + E_t + K_{d,\text{DNA}})^2 - (4D_tE_t))^{1/2}) / (2D_t)$, where P is the measured change in polarization (millipolarization units, mP), P_0 is initial polarization value derived from the fit, P_{max} is

maximum change in polarization, D_t is DNA concentration, E_t is enzyme concentration, and $K_{d,DNA}$ is the equilibrium dissociation constant for enzyme binding to DNA.

Pol extension assays

A FAM-labeled primer (18 nt in length) was annealed to either a 42 nt non-G4 control template oligonucleotide or a 42 nt G4-forming sequence derived from the *c-MYC* promoter for use in pol extension assays (Table 1). DNA substrates (200 nM) were incubated at 37 °C with 5 nM hpol η^{1-437} or hpol ε^{1-1189} . Reactions were initiated by adding dNTP·MgCl₂ solution (0.25 mM of each dNTP and either 10 mM or 8 mM MgCl₂ for hpol η^{1-437} or hpol ε^{1-1189} , respectively) to the pre-incubated pol-DNA complex. All enzymatic reactions were carried out at 37 °C in 40 mM HEPES (pH 7.5) buffer containing 5 mM DTT, 100 µg/mL BSA, 5% (v/v) glycerol, and either 100 mM KCl or 100 mM NaCl. At time points of up to 6 hours, 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 were separated by electrophoresis on a 12% 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.⁽³²⁾

Steady-state kinetic analysis of dNTP insertion by pols

Single nucleotide insertion by both hpols η and ε was measured over a range of dNTP concentrations. Fourteen p/t-DNA substrates were used (Table 1; seven non-G4 p/t-DNA substrates and seven G4 p/t-DNA substrates) to assess the effect of varying the primer length on DNA pol activity. To obtain estimates for Michaelis-Menten kinetic parameters, non-G4 and G4 p/t-DNA substrates (200 nM) were pre-incubated with either hpol η^{1-437} (2 nM) or hpol $\varepsilon^{1-1189/exo-}$ (5 nM), and the reactions were initiated by addition of the appropriate dNTP (0 – 200 µM) and MgCl₂ (5 mM and 8 mM for hpol η^{1-437} and hpol $\varepsilon^{1-1189/exo-}$, respectively). All the enzymatic reactions were carried out at 37 °C in 40 mM HEPES (pH 7.5) buffer containing 100 mM KCl, 5 mM DTT, 0.1 mg/mL BSA and 5% glycerol (v/v). Experiments with hpol η^{1-437} proceeded for up to 30 min before 4 µL aliquots of the reaction were quenched with 36 µL of 95% formamide (v/v)/20 mM EDTA/0.1% bromophenol blue (w/v) solution (Figure S1). In experiments with hpol $\varepsilon^{1-1189/exo-}$, dNTP concentrations greater than 10 µM were quenched at 1.5 hours, while dNTP concentrations below 10 µM were allowed to proceed for 3 hours before quenching (Figure S2). 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.⁽³²⁾ Because hpol $\varepsilon^{1-1189/exo-}$ is highly processive, conditions for single nt insertion on the 23/42-mer substrate could not be obtained since the primer positions the enzyme across from three consecutive template guanines; therefore, the three product bands were quantified for reactions with G4 and non-G4 23/42-mer DNA to yield a value for total product as opposed to quantification of a single band for all other p/t-DNA substrates. The initial rate of product formation was plotted as a function of dNTP concentration and fit to the one-site hyperbolic equation in Prism (GraphPad, San Diego, CA). The turnover number (k_{cat}) and Michaelis constant ($K_{M,dNTP}$) are reported.

Analysis of pol mis-insertion frequency

The mis-insertion frequency of both hpol η^{1-437} and hpol $\epsilon^{1-1189/exo-}$ was measured over a range of dNTP concentrations for 21/42-mer, 22/42-mer and 23/42-mer G4 and non-G4 DNA substrates. A pilot study was first conducted with both hpol η^{1-437} and hpol $\epsilon^{1-1189/exo-}$ to determine the identity of the most favoured mis-insertion product (supplemental Figures S3 and S4). Kinetic analysis of dNTP mis-insertion was then performed with hpol η^{1-437} (2 nM) and hpol $\epsilon^{1-1189/exo-}$ (5 nM) for the six DNA substrates (200 nM). Reactions were initiated by adding dNTP (0 – 1 mM for hpol η^{1-437} and 0 – 2.5 mM for hpol $\epsilon^{1-1189/exo-}$) and $MgCl_2$ (5 mM for hpol η^{1-437} and 8 mM $MgCl_2$ for hpol $\epsilon^{1-1189/exo-}$). The reactions were quenched as described above. Mis-insertion of dTMP was measured for hpol η^{1-437} on the 21/42-mer, 22/42-mer and 23/42-mer G4 and non-G4 DNA substrates. For hpol $\epsilon^{1-1189/exo-}$, mis-insertion of dGMP opposite template dG was measured for 21/42-mer and 23/42-mer DNA substrates with both G4 and non-G4 sequences. Mis-insertion of dCMP opposite template dT was measured for hpol $\epsilon^{1-1189/exo-}$ with both G4 and non-G4 22/42-mer DNA substrates. Incorporation of dCMP opposite template dT by hpol $\epsilon^{1-1189/exo-}$ resulted in multiple mis-insertion product bands on 22/42-mer non-G4 DNA, as did mis-insertion of dGMP on 23/42-mer DNA substrates. Therefore, total product was quantified for hpol $\epsilon^{1-1189/exo-}$ mis-insertion reactions on 22/42-mer and 23/42-mer DNA substrates. For experiments with hpol η^{1-437} , 4 μ l aliquots were added to 36 μ l of quench solution at time points up to 3 hours; 4 μ l aliquots of hpol $\epsilon^{1-1189/exo-}$ reactions were quenched at 8 hours. Reaction products were separated, visualized, and analyzed as described in the previous section.

RESULTS

Comparison of hpol ϵ^{1-1189} and hpol η^{1-437} binding to c-MYC G4 DNA substrates

We first analyzed the binding properties of recombinant hpol η^{1-437} and hpol ϵ^{1-1189} by titrating enzyme into a solution of FAM-labeled p/t-DNA and measuring changes in fluorescence polarization for either non-G4 DNA or G4 DNA substrates (Figure 1). Two 11/28-mer DNA substrates (Table 1) were prepared in a solution containing K^+ (100 mM), and salt-dependent G-quadruplex formation for G4 substrates was assessed using circular dichroism (CD) and dimethyl sulfate (DMS) foot-printing (data not shown). Fitting the fluorescence polarization data to a quadratic equation resulted in an estimate of the equilibrium dissociation constant for pol binding to DNA ($K_{d,DNA}$). The Y-family member hpol η^{1-437} binds G4 DNA-containing substrates with approximately 6.2-fold higher affinity than non-G4 substrates (Figure 1A). The measured $K_{d,DNA}$ values for hpol η^{1-437} binding were 87 nM ($\pm$ 11 nM) for the non-G4 control substrate and 14 nM ($\pm$ 2 nM) for the G4 DNA substrate. We then measured the affinity of the catalytic core of the eukaryotic leading strand pol, hpol ϵ^{1-1189} . The measured $K_{d,DNA}$ values for hpol ϵ^{1-1189} binding were 280 nM ($\pm$ 39 nM) for the non-G4 control substrate and 223 nM ($\pm$ 42 nM) for the G4 DNA substrate. These values are slightly higher than but comparable to the $K_{d,DNA}$ (79 nM) reported for the hpol ϵ^{1-1189} catalytic core recently using chemical quench assays.⁽³³⁾ However, the authors of the previous study performed the pol reactions at 20 °C and they did not add any salt to the reaction buffers used to measure pol activity, which will undoubtedly alter the DNA binding properties of hpol ϵ . We observe a 1.3-fold difference in

measured $K_{d,DNA}$ values for the two substrates used here (Figure 1B), which suggests that hpol ϵ^{1-1189} does not discriminate between G4 and non-G4 DNA at the binding step. Although these results were obtained with the hpol ϵ catalytic core, it is likely the same would hold true for the holoenzyme since a previous study reported that the small subunits of yeast pol ϵ had no impact on ssDNA binding capacity.⁽³⁴⁾ Comparing the two enzymes, it would seem that hpol η has a stronger preference for binding G4 DNA substrates than the replicative enzyme.

Running-start primer extension activities for hpol ϵ^{1-1189} and η^{1-437} on G4 DNA substrates

Running start pol extension assays were conducted as an initial, qualitative assessment of differences in enzymatic activity toward G4 DNA substrates. Total extended product formed in each of the four reactions was quantified to calculate the percent activity of each pol on G4 DNA by dividing the amount of product in the G4 reaction by the amount of product in the control reaction. Contrasting the percent activity values allowed us to make a relative comparison of pols activity toward G4 DNA. The 18-mer primer used for these experiments positions the terminal 3'-OH group five nts away from the first tetrad-guanine (G22) in the template strand (Figure 2A). Initially, we compared both G4 and non-G4 DNA substrates in reaction solutions containing 100 mM potassium. As expected, both enzymes pause in the presence of G4 DNA (Figure 2B). However, there are two important differences that are noticeable when comparing the gel results for the two enzymes. First, hpol ϵ^{1-1189} exhibits a strong pause two nts away from G22, whereas hpol η^{1-437} can synthesize DNA up to the first tetrad-guanine without much pausing. The second feature of note in the running start-experiment is the amount primer converted to product by each enzyme. In the reaction with hpol ϵ^{1-1189} , there is clearly less primer extension occurring in the experiment with G4 DNA compared to the non-G4 experiment, where essentially all of the primer is extended (Figure 2B; note the different intensities of the 18-mer substrate band on the gel for the two hpol ϵ^{1-1189} experiments). This is in contrast to the amount of primer extended by the Y-family member, hpol η^{1-437} , for which the difference in unutilized primer between the control and G4 DNA substrates is less dramatic (note the intensity of the 18-mer substrate band in the experiments with hpol η). These results indicate that the Y-family pol is able to perform catalysis on G4 substrates more effectively than the B-family member, hpol ϵ^{1-1189} . Comparing the quantified results, hpol ϵ^{1-1189} exhibits 79.5% ($\pm 1.8\%$) activity on G4 substrates, while hpol η^{1-437} is 92.2% ($\pm 1.9\%$) active when comparing G4 substrates to non-G4 DNA (Figure 2C; $P = 0.0084$). We conclude that primer extension by the TLS enzyme hpol η^{1-437} is more efficient and proceeds closer to the G4 structure than the replicative enzyme, hpol ϵ^{1-1189} .

Since the Myc2345 14/23 G4 structure used in our assays is a highly stable quadruplex with a T_m of 75-85 °C (depending on the K^+ concentration)^(35, 36), we wanted to ascertain whether the two pols under investigation could copy a less stable version of the parallel-stranded *c-MYC* G4 structure. To test this idea we prepared both the G4 and non-G4 DNA substrates in Na^+ (100 mM) and performed running-start extension assays. While Na^+ can stabilize G-quadruplex structures, it is known to decrease the melting temperature of G4 DNA.⁽¹²⁾ Indeed, experiments with hpol η^{1-437} reveal the less stable nature of a Na^+ -bound G-quadruplex, as the Y-family member synthesizes across the G4 motif with a noticeably

smaller reduction in the amount of fully extended product compared to hpol ϵ (Figure 3A). The replicative enzyme, hpol $\epsilon^{1-1189/exo-}$, on the other hand, still exhibits a strong pause when attempting to copy the G4 motif, although the pause two nts away from G22 is diminished for Na^+ -stabilized G4 DNA (Figure 3B). It becomes apparent from these results that the Na^+ -stabilized G4 structure is not an impediment to hpol η activity, while hpol ϵ still has some difficulty in dealing with the less stable G-quadruplex.

Steady-state kinetic analysis of the TLS Y-family member hpol η^{1-437} activity on G4 DNA substrates

To more closely examine the effect of G4 DNA on polymerization, we prepared a series of p/t-DNA substrates with varying primer lengths that positioned the 3'-OH from ten (13-mer primer) to zero (23-mer primer) nts away from G22, the first tetrad-associated guanine in the *c-MYC* G4 motif. A total of fourteen p/t-DNA substrates were prepared (Table 1; seven non-G4 control substrates and seven G4 DNA substrates). We performed steady-state kinetic analysis of single nucleotide insertion for the correct dNTP by hpol η^{1-437} on each of the fourteen DNA substrates. The relative activity of hpol η^{1-437} 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. In this way, we were able to systematically evaluate how the catalytic efficiency of hpol η^{1-437} changes as the enzyme approaches the G4 structure.

When positioned ten nts from G22, hpol η^{1-437} activity is essentially unperturbed (Table 2). Somewhat surprisingly, there is a drop in hpol η^{1-437} relative activity to ~40% when the primer terminus is positioned five nts from the G4 structure. However, hpol η^{1-437} is able to maintain fairly robust activity on G4 substrates as the primer terminus is moved closer to the G-quadruplex structure. Interestingly, hpol η^{1-437} activity on G4 substrates increases back to levels comparable to the control substrate when the primer is positioned two to three nts away from the G-quadruplex (Table 2, see results for the 20/42 and 21/42mer substrates). These kinetic results correspond well to the qualitative running-start primer extension assays (Figure 2B), in which there is very little pausing by hpol η^{1-437} as the primer is extended to 20 and 21 nts, indicative of efficient pol activity. The catalytic efficiency of hpol η^{1-437} decreases to 31% when the primer is positioned one nt away from G22 (Table 2, see results for 22/42mer DNA substrate). The relative activity of hpol η^{1-437} is ~25% when the enzyme inserts dCMP opposite the tetrad guanine G22 (Table 2, see results for 23/42-mer substrates). The general trend that underlies the decrease in hpol η^{1-437} catalytic efficiency on G4 substrates is an increase in the Michaelis constant ($K_{\text{m,dNTP}}$), although there is a 43% reduction in the k_{cat} for dCMP insertion opposite G22 (Table 2, see results for 23/42mer substrates). For most of the substrates, the turnover number (k_{cat}) describing hpol η^{1-437} -catalyzed nucleotidyl transfer is essentially unchanged by the presence of G4 DNA compared to changes in the measured $K_{\text{m,dNTP}}$ values. Overall, the single-nucleotide results for hpol η^{1-437} are indicative of an enzyme that is reasonably tolerant of a very stable intra-molecular G-quadruplex in the template strand, even when the G4 DNA structure is in close proximity to the pol active site.

Measurement of hpol η^{1-437} mis-insertion frequency on G4 DNA substrates

We next wanted to examine the effect of G4 DNA on the mis-insertion frequency (f_{ins}) of hpol η^{1-437} . We focused our efforts on the three substrates that position the primer terminus two, one or zero nts from G22 (i.e. 21/42-mer, 22/42-mer and 23/42-mer G4 and non-G4DNA, for a total of six DNA substrates). We determined the nucleotide that was most likely to be mis-inserted by performing a time-course experiment measuring hpol η^{1-437} -catalyzed insertion of each of the incorrect dNTPs on the six DNA substrates used in the mis-insertion experiments. In all cases, we found that hpol η^{1-437} preferred to mis-insert dTMP. Steady-state kinetic analysis of dTMP mis-insertion by hpol η^{1-437} was performed, and the mis-insertion frequency on each substrate was estimated (Table 3). On the three non-G4 DNA substrates, hpol η^{1-437} makes a mistake roughly once every ~13 to 300 catalytic events. The range of hpol η^{1-437} accuracy was found to be one mistake every 30 to 4,800 catalytic events for the three G4 DNA substrates. Directly comparing the mis-insertion frequency for each substrate pair, we find that the fidelity of DNA synthesis by hpol η^{1-437} is increased slightly (~8-fold) when the primer is positioned two nts from the G4 motif (Table 3, compare f_{ins} for the 21/42-mer DNA substrates). There is an approximate 5-fold drop in fidelity when hpol η^{1-437} is placed one nt from the G4 motif (Table 3, compare f_{ins} for the 22/42-mer DNA substrates). Perhaps the most interesting change in fidelity was observed with the 23/42-mer substrate, for which hpol η^{1-437} fidelity is increased ~15-fold when it attempts to copy the tetrad guanine G22 (Table 3, compare f_{ins} for the 23/42-mer substrates). We conclude from these experiments that the fidelity of hpol η is impacted by the presence of G4 DNA, with mis-insertion of dTMP across from the G22 tetrad-guanine being so strongly inhibited by the G-quadruplex that the fidelity of hpol η is increased 15-fold.

Steady-state kinetic analysis of the replicative B-family member hpol $\epsilon^{1-1189/\text{exo-}}$ activity on G4 DNA substrates

So that we might gain some perspective on how the changes in hpol η^{1-437} catalytic efficiency and fidelity compared to other pols, we then went on to investigate how the replicative enzyme, hpol ϵ , was affected by G-quadruplexes. Exonuclease deficient hpol $\epsilon^{1-1189/\text{exo-}}$ was used to measure single-nucleotide insertion kinetics. The same set of fourteen p/t-DNA substrates was used to determine the relative activity of hpol $\epsilon^{1-1189/\text{exo-}}$ on G4 DNA. The absolute values of the steady-state kinetic parameters obtained with hpol $\epsilon^{1-1189/\text{exo-}}$ are different from those reported in a previous study⁽³¹⁾, resulting in a lower specificity constant for the DNA substrates tested here (Table 4). This is likely due to the higher salt concentrations required to investigate G-quadruplexes, as a previous report showed that 100 mM KCl or NaCl strongly inhibits the pol ϵ holoenzyme.⁽³⁷⁾ Recent pre-steady-state experiments have reported k_{pol} values of ~250–300 s⁻¹ with yeast and human pol epsilon, in line with the values reported for other B-family pols.^(33, 38) However, the reaction buffer used in the pre-steady-state experiments with pol epsilon did not have any salt in them. Thus, the absolute values of the pre-steady-state kinetic constants reported might not represent what occurs at more physiological salt concentrations, such as the concentrations we have employed in our steady-state assays. The k_{cat} values reported here are ~5- to 10-fold lower than those calculated for hpol $\epsilon^{1-1189/\text{exo-}}$ previously under the no

salt reaction conditions.^(31, 33) However, the mis-insertion frequencies measured by us (Table 5) are very similar to steady-state values reported previously under conditions of no salt⁽³¹⁾, which is a good indicator that the mechanism of nucleotide selection by hpol $\epsilon^{1-1189/exo-}$ remains intact.

Steady-state kinetic analysis of hpol $\epsilon^{1-1189/exo-}$ -catalyzed correct nucleotide insertion was consistent with the running-start extension assays in that the relative efficiency of catalysis by hpol $\epsilon^{1-1189/exo-}$ drops substantially when the primer is positioned two nts away from G22 (Table 4). Unlike hpol η^{1-437} , the drop in efficiency for hpol $\epsilon^{1-1189/exo-}$ is driven by both an increase in the $K_{m,dNTP}$ and a decrease in the k_{cat} . The changes in both the k_{cat} and $K_{m,dNTP}$ values are also observed with the 22/42-mer and 23/42-mer substrates, with hpol $\epsilon^{1-1189/exo-}$ activity dropping to less than 4% when the enzyme attempts to copy G22 in an accurate fashion. In short, hpol $\epsilon^{1-1189/exo-}$ maintains good activity until the primer is positioned two to three nts from G22, at which point the relative efficiency of the reaction decreases substantially.

Measurement of hpol $\epsilon^{1-1189/exo-}$ mis-insertion frequency on G4 DNA substrates

Finally, we measured the effect of G4 DNA on the fidelity of hpol $\epsilon^{1-1189/exo-}$ by measuring the steady-state mis-insertion frequencies on the 21/42-mer, 22/42-mer and 23/42-mer DNA substrates. We assessed the preference for mis-insertion by hpol $\epsilon^{1-1189/exo-}$ and found that hpol $\epsilon^{1-1189/exo-}$ preferred to mis-insert dGMP on the 21/42-mer and 23/42-mer substrates, while favoring dCMP mis-insertion on the 22/42-mer DNA. Comparing the mis-insertion frequencies for non-G4 and G4 DNA substrates reveals that the fidelity of nucleotide selection by hpol $\epsilon^{1-1189/exo-}$ is decreased when a G4 structure is present in the template strand (Table 5). The decrease in hpol $\epsilon^{1-1189/exo-}$ fidelity occurs for all three G4 substrate tested, but it is most pronounced when the enzyme attempts to copy G22 (Table 5, see results for 23/42-mer DNA). The accuracy of nucleotidyl transfer by hpol $\epsilon^{1-1189/exo-}$ is decreased over 30-fold on the 23/42-mer G4 DNA substrate. In fact, nucleotidyl transfer by hpol $\epsilon^{1-1189/exo-}$ is around two orders of magnitude more error-prone than hpol η^{1-437} on the 23/42-mer G4 DNA substrate (Tables 3 and 5; compare the value of f_{ins} for each enzyme with the 23/42-mer G4 DNA substrate). Based on the change in fidelity for the two enzymes tested here, it would seem that hpol η can copy G4 motifs in a more accurate and efficient manner than the catalytic core of the replicative enzyme, hpol ϵ .

DISCUSSION

Although originally thought to be an *in vitro* curiosity⁽³⁹⁾, landmark studies have elucidated the structural diversity of G-quadruplexes *in vitro*^(18, 20, 35, 36, 40–43), and experimental strategies have shown that G4 DNA forms *in vivo* with a wide range of biological ramifications.^(13, 17, 27, 44–49) Recent studies have visualized G4 DNA in human cells and found that they are formed maximally during S-phase when the double-helix is converted to single-stranded intermediates.^(11, 50) Additionally, analysis of over 2,700 cancer samples revealed that G4-motifs are enriched near chromosomal breakpoints and act as “mutagenic factors” in a variety of tumors.⁽¹⁷⁾ Yet, the proteins and enzymes required for successful G4 DNA replication are only now being elucidated, and many catalytic features relevant to

DNA synthesis at G4 motifs remain unclear. The visualization of non-telomeric G4 structures during S-phase and the ability of some intramolecular G-quadruplexes to fold on the millisecond time scale suggest the possibility that DNA pols might encounter fully or partially folded G4 DNA during replication.^(11, 51) Certain helicases and single-stranded DNA binding proteins are critical for maintenance of G4 motifs and a number of these proteins and enzymes are capable of unwinding G-quadruplexes *in vitro*.^(26, 48, 52–55) What is less clear is why certain DNA pols appear to be required for replication of G4 DNA. We set out to determine if the biochemical properties of recombinant B- and Y-family DNA pols could help explain results obtained in genetic and cell culture based experiments implicating a role for translesion enzymes in the maintenance of G-quadruplexes.^(23, 28) The binding and catalytic properties of the Y-family enzyme hpol η^{1-437} were compared to the B-family enzyme, hpol ϵ^{1-1189} , in an effort to better understand mechanisms of pol action during G4 DNA replication. We also compared the results reported here to those we reported previously for hRev1 using identical DNA substrates.⁽²⁹⁾

Our biochemical results reveal important differences for B- and Y-family pols. First, we find that hpol η^{1-437} binds to G4 substrates with a 6.2-fold preference over non-G4 DNA. We do not observe a similar change in binding affinity for the replicative enzyme, hpol ϵ^{1-1189} . The increased binding affinity observed for hpol η^{1-437} with G4 DNA is not without precedent. We have shown previously that the catalytic core of another Y-family pol, hRev1³³⁰⁻⁸³³, binds G4 DNA substrates with 15-fold greater affinity than non-G4 DNA.⁽²⁹⁾ The increased binding affinity for TLS pols could certainly aid in the localization of these specialized enzymes to G4 structures during replication fork stalling. However, preferred binding does not necessarily translate into robust catalytic properties on G4 DNA. For example, while hRev1 showed a considerable preference for binding to G4 substrates, the relative activity of nucleotide insertion by hRev1 opposite G22 was quite low (~2%) when compared to that measured for hpol η in the current study (25%). The relative activity of hRev1 was considerably better (~70%) when the enzyme is positioned four nts away from G22 (i.e. catalyzing dCMP insertion opposite template dG on the 19/42-mer substrate). We were initially surprised by the lack of robust catalysis from hRev1 when copying the tetrad guanine G22 given its purported role in avian G4 replication. However, these results may not be so perplexing when one considers the fact that Rev1 is thought to function as a central scaffold for recruiting TLS pols to sites of replication stress through interactions in its C-terminus. Previous experiments from the Sale group implied that if Rev1 cannot recruit the other TLS pols then DNA synthesis across the G4 motif is impaired.⁽²⁷⁾ The low activity for hRev1 in catalyzing nucleotidyl transfer across from tetrad guanines could mean that DNA synthesis across the G4 motif is mainly performed by hpol η (or hpol κ), which was suggested previously by others and is consistent with both the kinetics results reported here, as well as the defects in G4 replication reported in DT40 cells expressing a Rev1 C-terminal truncation mutant that cannot bind pol η or κ .⁽²⁷⁾ A model emphasizing a role for pol η (and/or pol κ) in performing DNA synthesis past G4 DNA is also in line with the deletion of G4 motifs observed in experiments with *C. elegans* mutants lacking FANCI and either pol η or pol κ .⁽²⁸⁾ However, it is likely that Rev1 does copy some portion of the G4 motif in the template strand with the assistance of a helicase, such as FANCI, since DT40 mutant cell lines expressing a catalytically inactive version of Rev1 exhibit a defect in G4 maintenance

that is roughly half as strong as Rev1-null or Rev1 C-terminal deletion mutant cell lines.^(26, 27)

In addition to performing DNA binding studies, we systematically investigated the effect of G4 DNA on the single-nucleotide insertion kinetics of hpol η^{1-437} and hpol $\epsilon^{1-1189/exo-}$ by varying the length of the primer on templates containing G4 DNA. Neither pol can completely bypass G4 DNA stabilized by K^+ (i.e both enzymes pause on G4 substrates), but we do observe clear differences in the catalytic efficiencies of the two enzymes when the primer position is varied (Figure 4A). In agreement with the idea that replicative pols efficiently copy non-structured DNA, hpol $\epsilon^{1-1189/exo-}$ activity is higher than hpol η^{1-437} when the primer terminus is positioned four to five nucleotides from the G4 structure. However, hpol η^{1-437} activity increases dramatically when the 3'-OH group is placed two to three nts from the first tetrad guanine (G22) and remains relatively robust even when copying G22, consistent with a role for this enzyme in performing DNA synthesis near G4 DNA structures. The most striking change in pol activity was obtained when we studied the kinetics of mis-insertion by the two enzymes. Pol ϵ has the highest fidelity among yeast and human pols and importantly, copies homonucleotide repeats, such as those found in G4 DNA substrates, more accurately than pol δ .^(31, 56) However, we find that the fidelity of hpol $\epsilon^{1-1189/exo-}$ drops considerably on G4 substrates, especially when copying G22 (Table 5 and Figure 4B). Most surprisingly, hpol η^{1-437} fidelity increases 15-fold when the enzyme attempts to copy G22 (Table 3 and Figure 4B). These results are consistent with the idea that changes in binding and catalysis for pols η and ϵ can partition the action of each enzyme on G4 DNA substrates (Figure 4C). While the catalytic activity of hpol η^{1-437} on G4 substrates is more efficient than either hpol ϵ^{1-1189} or hRev1³³⁰⁻⁸³³ when the enzymes are positioned within three nts of the G4 structure, it is by no means unaffected by the presence of G4 DNA. Rather, the degree to which catalysis by hpol η^{1-437} is perturbed implies that the enzyme could help maintain forward progress at sites where intramolecular G-quadruplexes have refolded in the path of the replisome but that it probably cannot perform this function alone.

The TLS enzyme hpol η is probably best known for its role in copying CPD lesions.^(7, 57, 58) Binding of pol η to CPD-containing substrates is enhanced when a single 3'-adenosine is paired opposite the 3'-T in the dimer but destabilized once two adenosines are paired opposite the lesion⁽⁵⁹⁾, which supports the increase in processivity that has been observed for pol η synthesis near CPD lesions.⁽⁷⁾ The steady-state specificity constants describing hpol η -catalyzed insertion opposite each damaged thymine in the T-T dimer are essentially unchanged, consistent with the idea that hpol η copies UV-induced T-T dimers very efficiently.⁽⁶⁰⁾ Moreover, the fidelity of hpol η is unaffected by the presence of the CPD lesion⁽⁶⁰⁾, which is important for replication of CPDs. Another adduct that hpol η is thought to help bypass *in vivo* is the main intrastrand cross-linked product of cisplatin reactions with adjacent guanines (Pt-GG).^(8, 9, 61) Kinetic analysis has revealed that hpol η copies the 3'-dG and 5'-dG sites within a Pt-GG adduct with a catalytic efficiency of 82% and 35% relative to unmodified DNA.⁽⁶²⁾ However, the accuracy of nucleotide selection at the second insertion step (opposite the 5'-dG of the Pt-GG adduct) appears to be decreased based on robust mis-insertion of dAMP⁽⁶²⁾. By way of comparison, G4 DNA is a greater impediment to efficient

DNA synthesis by hpol η than either CPDs or Pt-GG adducts, but unlike bypass of the aforementioned adducts, the fidelity of nucleotide selection by hpol η is increased when inserting nucleotides opposite G22. The increased accuracy of hpol η on the 23/42-mer G4 substrate is primarily due to a large (~140-fold) decrease in the k_{cat} for mis-insertion of dTMP opposite G22 (Table 3). If we extrapolate the kinetic results with hpol η into a cellular context, the G-quadruplex structure might be thought of as protecting the genome from increased levels of error-prone DNA synthesis by inhibiting TLS pol-catalyzed mis-insertion. Loss of TLS pol action at these sites would be predicted to induce mutagenic replication, based on our results with hpol ϵ .

DNA pol strand-displacement activity has been reported on many occasions for short B-form dsDNA duplexes and is even the basis of a fluorescence-based screen for pol inhibitors.^(63, 64) However, it is unclear if DNA pols can promote unfolding of G4 DNA in the template strand by exerting a force on the nucleic acid structure as DNA synthesis is occurring. We have observed hRev1-dependent disruption of *cMYC* G4 DNA in the absence of DNA synthesis that appears to be related to the ssDNA binding properties of that Y-family member.⁽²⁵⁾ For most pols, it seems unlikely that disruption of K^+ -stabilized G4 structures with high melting temperatures, such as the *cMYC* motif used here, would accompany DNA synthesis, but as one might expect, the degree of pol inhibition appears to change in manner that is dependent on the stability of the structure. The biophysical properties of G-quadruplexes are complex when one considers the sheer number of different topological arrangements, loop lengths and folding dynamics associated with these structures.⁽¹²⁾ One trend that seems to be shared by most (if not all) quadruplexes studied to date is the fact that K^+ -bound G4 structures are more difficult to unfold than Na^+ -bound G4 DNA. Substituting Na^+ for K^+ can decrease the T_m of telomeric G4 DNA by ~5–15 °C and result in ~1–4 kcal/mol decreases in $G_{(310)}$ values.⁽¹²⁾ Unfolding of Na^+ -stabilized telomeric G4 DNA, which is not as stable as the *cMYC* G4 structure used in our assays, occurs when forces of less than 10 pN are applied.⁽⁶⁵⁾ The T7 DNA pol has been shown using single-molecule approaches to exert a force of ~30 pN during translocation.⁽⁶⁶⁾ Therefore it may be possible for DNA pols to promote the unfolding of low stability K^+ -stabilized G4 structures or Na^+ -stabilized G4 DNA, and it is reasonable to propose that both hpol ϵ and hpol η can act to varying degrees on Na^+ -stabilized G4 structures in the template strand. Based on our results, it would appear that hpol η does indeed cause quadruplexes to unfold completely since we observe full-length primer extension on Na^+ -stabilized G4 substrates (Figure 3A). Conversely, the highfidelity enzyme hpol $\epsilon^{1-1189/\text{exo-}}$ still exhibits a noticeable pause when it encounters the Na^+ -stabilized G4 structure (Figure 3B), perhaps due to unfavorable interactions between the G4 structure and the domains comprising the epsilon active site.

A recent study with yeast and human pol δ reported that some pausing within telomeric TTAGGG repeat sequences occurred even under conditions where G-quadruplex formation is suppressed.⁽⁶⁷⁾ Extension assays with yeast and human pol δ showed that pausing is stronger when G-quadruplex formation is promoted⁽⁶⁷⁾, although there does appear to be a non-G4-related factor that contributes to pol δ stuttering past homonucleotide repeats. Interestingly, both yeast and human pol δ show fairly strong pauses two nts away from the

first run of telomeric tetrad-guanines⁽⁶⁷⁾, which is similar to what is observed with hpol ϵ (Figure 2). Direct comparisons to our results with hpol ϵ ¹⁻¹¹⁸⁹ are hampered by differing template sequences, as well as the fact that an equimolar ratio of pol δ to DNA was used to test for pol activity on telomeric G4 DNA. However, the fact that DNA synthesis by both hpol ϵ and hpol δ appears to be perturbed at a similar location on two different G4 template sequences is intriguing.

The structural basis for preferential interactions between Y-family pols and G4 DNA has yet to be defined. Crystal structures of hpol η provide some insight into how Y-family pols might accommodate G4 DNA in a manner that is distinct from other types of pols. The structure of hpol η reveals that two template bases are housed in the large active-site for both undamaged and damaged DNA templates.^(6, 62) Additionally, template guanines in the hpol η active-site have been observed in multiple conformations.⁽⁶²⁾ The accommodation of diverse template conformations is a hallmark of Y-family pol structure/function, and this property could promote productive interactions between hpol η and G4 DNA by allowing the enzyme to accept partially folded G4 structures. Furthermore, hpol η has been proposed to act as a “molecular splint” that maintains DNA in a B-form conformation even when adducts, such as CPD lesions, are present.⁽⁶⁾ It is conceivable that contacts with multiple tetrad guanines near the hpol η active site could help prevent refolding of an intramolecular G-quadruplex. An additional point of contact between hpol η and G-quadruplex structures may involve a DNA interacting surface that has been observed in the symmetry related molecules of hpol η and was proposed to serve as a “wedge to separate non-B-form” DNA.⁽⁶⁾ This secondary DNA binding interface has both electrostatic (Arg⁸¹ and Arg⁸⁴) and base-stacking (Trp³³⁹) interactions between hpol η residues on the backside of the little finger and the symmetry-related DNA that are apparent in the crystal structures. These contacts may contribute to the preferential binding to G4 DNA by hpol η observed in our study, but experiments to test such a notion have yet to be reported. It is unclear how B-family pols might accommodate a fully or partially folded G4 structure. The crystal structure of yeast pol ϵ reveals that the enzyme does not make direct contact with any ssDNA template residues beyond the +1 position (i.e. the nucleotide in the template strand that is just 5' of the nascent base pair).⁽⁶⁸⁾ The template residue in the +1 position must undergo a dramatic rotation as it moves into the yeast pol ϵ active site, a mechanistic feature that is conserved between B-family pols.^(69, 70) The inaccessibility of G4-associated guanines likely interferes with this base-flipping event and is consistent with our results showing a strong pause for hpol ϵ two nucleotides short of the first tetrad.

While DNA pols obviously function as part of the multi-subunit replisome, our study is the first to provide evidence for a model in which kinetic partitioning of pol activity could govern fork progress during G4 DNA replication, providing further insight into why hpol η -deficient cells have defects in the maintenance of sequences predicted to form G-quadruplexes.^(23, 28) Experiments to decipher the exact role for different Y-family pols at G4 sites in cells, as well as individual steps within the hpol η and hpol ϵ catalytic cycle that are modulated by G4 DNA, are ongoing. With regard to our results with hpol ϵ , it is possible that the hpol ϵ holoenzyme could display increased fidelity and processivity on G4 substrates since deletion of the two smallest subunits contributes to increased spontaneous mutagenesis in yeast.⁽⁷¹⁾ It is important to note that our experiments were conducted with

the catalytic core of human pol epsilon. G4 DNA replication *in vivo* would involve the pol epsilon holoenzyme, which contains a 3'-5' exonuclease domain that might affect fidelity of the enzyme. Thus, it will be interesting to assess the affect of G4 DNA on the proofreading action of hpol ε. Finally, it is interesting that G4 structures appear to be oxidized more readily than non-G4 sequences since the oxidation potential of G4 guanines is expected to be lower than that of guanines in dsDNA.^(72, 73) The potential role for Y-family pol activity in the replication of damaged G4 DNA is an active area of investigation that could provide important insights into the mechanism(s) of TLS across from these non-B-form structures. In summary, the results reported here provide direct evidence for a simple kinetic switch between pols during G4 DNA replication, a mechanism that could prevent fork stalling at these sites in cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

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

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Figure 1. The Y-family member hpol η^{1-437} preferentially binds G4 DNA-containing substrates
a. hpol η^{1-437} 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, $K_{d,DNA} = 14 \pm 2$ nM; non-G4 DNA, $K_{d,DNA} = 87 \pm 11$ nM. The reported K_d values represent the mean $\pm$ s.e.m. (n = 3).
b. hpol ϵ^{1-1189} 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: primer-template G4 DNA, $K_{d,DNA} = 223 \pm 42$ nM; primer-template non-G4 DNA, $K_{d,DNA} = 280 \pm 39$ nM. The reported K_d values represent the mean $\pm$ s.e.m. (n = 2).

Figure 2. hpol η^{1-437} and hpol ϵ^{1-1189} extension activity on G4 DNA substrates

a. Running-start pol extension assays were performed with non-G4 DNA (CTL) and G4 DNA (G4) 18/42-mer p/t-DNA substrates. The tetrad-associated guanines in the G4 substrate are shown in bold, with the G22 position underlined. **b.** Each enzyme (5 nM) was incubated with 18/42-mer p/t-DNA (200 nM) and pol extension was allowed to proceed in the presence of a mixture of all four dNTPs (250 μ M) for 3 hrs at 37 °C. The pol extension products were separated by electrophoresis on a 12% polyacrylamide (w/v)/7 M urea gel. The identities of the template bases just 3' to the first tetrad guanine (G22) are noted to the side of the gel. **c.** The total product formed in each of the pol extension assays was quantified. Dividing the total product formed for G4 DNA by the product formed in the CTL reaction and multiplying by 100 resulted in a % activity on G4 DNA value for both enzymes. The TLS pol, hpol η^{1-437} , retained 92.2 % ($\pm$ 1.9 %) activity on G4 substrates (n=3). The replicative enzyme, hpol ϵ^{1-1189} , retained 79.5 % ($\pm$ 1.8 %) on G4 substrates

(n=3). The reported values represent the mean $\pm$ s.e.m. A two-tailed unpaired t-test comparing the values for the two enzymes resulted in $P = 0.0084$.

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Figure 3. hpol η^{1-437} can efficiently copy G4 DNA stabilized by Na^+ but hpol ϵ^{1-1189} remains partially inhibited

a. Running-start pol extension time course experiments were performed with hpol η^{1-437} (5 nM) in the presence of either KCl or NaCl (100 mM) for both CTL and G4 18/42-mer p/t-DNA substrates (200 nM). DNA synthesis by hpol η^{1-437} proceeds essentially unperturbed when copying Na^+ -stabilized G4 DNA. **b.** Running-start pol extension time course experiments were performed with hpol $\epsilon^{1-1189/exo-}$ (5 nM) in the presence of either KCl or NaCl (100 mM) for both CTL and G4 18/42-mer p/t-DNA substrates (200 nM). DNA synthesis by hpol $\epsilon^{1-1189/exo-}$ still shows a considerable amount of pausing on Na^+ -stabilized G4 DNA substrates.

Figure 4. A simple kinetic switch between pols may promote more accurate and efficient replication of G4 DNA
a. The relative activity of hpol η^{1-437} and hpol $\epsilon^{1-1189/exo-}$ on G4 DNA is plotted as a function of primer terminus distance from the first tetrad guanine (G22). **b.** The change in fidelity on G4 DNA substrates relative to control non-G4 is shown for p/t-DNA substrates positioning the primer terminus two, one and zero nts away from the first tetrad guanine (G22). **c.** A cartoon illustration is shown to depict the idea that DNA binding and kinetic properties of nucleotidyl transfer could partition hpol η and hpol ϵ activity during encounters

with G4 DNA and that this division of labor may occur between two and three nts away from G-quadruplex structures.

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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/-TTGCCTCGAGCCAGC-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

2. 18/42-mer non-G4 DNA

5'-/FAM/-TTGCCTCGAGCCAGCCGAG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

3. 19/42-mer non-G4 DNA

5'-/FAM/-TTGCCTCGAGCCAGCCGAG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

4. 20/42-mer non-G4 DNA

5'-/FAM/-TTGCCTCGAGCCAGCCGAG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

5. 21/42-mer non-G4 DNA

5'-/FAM/-TTGCCTCGAGCCAGCCGAG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

6. 22/42-mer non-G4 DNA

5'-/FAM/-TTGCCTCGAGCCAGCCGAG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

7. 23/42-mer non-G4 DNA

5'-/FAM/-TTGCCTCGAGCCAGCCGAG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTACCAGTTGTAGAGTG-5'

8. 13/42-mer G4 DNA

5'-/FAM/-TTGCCTCGAGCCAGC-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTGGGATGGGTGGGAGT-5'

9. 18/42-mer G4 DNA

5'-/FAM/-TTGCCTCGAGCCAGCCGAG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTGGGATGGGTGGGAGT-5'

10. 19/42-mer G4 DNA

5'-/FAM/-TTGCCTCGAGCCAGCCGAG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGTGGGTGGGATGGGTGGGAGT-5'

11. 20/42-mer G4 DNA

5'-/FAM/-TTGCCTCGAGCCAGCCGAG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGT**G****GGT****G****GG**AT**G****GG****T****GG**GAGT-5'

12. 21/42-mer G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGACGACG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGT**G****GGT****G****GG**AT**G****GG****T****GG**GAGT-5'

13. 22/42-mer G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGACGACG-3'

3'-CGGAGCTCGGTCGGCGTCTGCGT**G****GGT****G****GG**AT**G****GG****T****GG**GAGT-5'

14. 23/42-mer G4 DNA

5'-/FAM/-TTTGCCTCGAGCCAGCCGACGACGCA-3'

3'-CGGAGCTCGGTCGGCGTCTGCGT**G****GGT****G****GG**AT**G****GG****T****GG**GAGT-5'

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

Table 2Steady-state kinetic parameters for hpol η^{1-437} catalysis on G4 DNA and non-G4 DNA substrates

	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	10 $\pm$ 1	33 $\pm$ 3	0.30	-
G4 DNA	9.6 $\pm$ 0.2	44 $\pm$ 3	0.22	73%
<u>18/42-mer: dAMP insertion opposite template dT -5 nt from G22</u>				
non-G4 DNA	9.2 $\pm$ 0.7	14 $\pm$ 3	0.66	-
G4 DNA	12 $\pm$ 1	44 $\pm$ 6	0.27	41%
<u>19/42-mer: dCMP insertion opposite template dG -4 nt from G22</u>				
non-G4 DNA	26 $\pm$ 1	11 $\pm$ 2	2.4	-
G4 DNA	23 $\pm$ 2	25 $\pm$ 4	0.92	38%
<u>20/42-mer: dGMP insertion opposite template dC -3 nt from G22</u>				
non-G4 DNA	11 $\pm$ 1	20 $\pm$ 2	0.55	-
G4 DNA	9.2 $\pm$ 0.2	23 $\pm$ 1	0.40	73%
<u>21/42-mer: dCMP insertion opposite template dG -2 nt from G22</u>				
non-G4 DNA	18 $\pm$ 1	27 $\pm$ 5	0.67	-
G4 DNA	15 $\pm$ 1	21 $\pm$ 3	0.71	107%
<u>22/42-mer: dAMP insertion opposite template dT -1 nt from G22</u>				
non-G4 DNA	7.7 $\pm$ 0.3	26 $\pm$ 3	0.30	-
G4 DNA	5.1 $\pm$ 0.2	55 $\pm$ 3	0.09	31%
<u>23/42-mer: dCMP insertion opposite G22</u>				
non-G4 DNA	20 $\pm$ 1	5.4 $\pm$ 0.9	3.7	-
G4 DNA	11 $\pm$ 1	12 $\pm$ 2	0.92	25%

^a Relative 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 all kinetic values, initial estimates for the rate of product formation were followed by titration experiments (10–12 concentrations of dNTP, $n = 1$). 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 kinetic parameter.

Table 3Steady-state kinetic parameters for hpol η^{1-437} mis-insertion on G4 DNA and non-G4 DNA substrates

	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>21/42-mer: dTMP insertion opposite template dG (-2 nt from G22)</u>				
non-G4 DNA	2.6 ± 0.3	53 ± 22	0.049	0.073
G4 DNA	1.3 ± 0.2	190 ± 80	0.0068	0.0096
<u>22/42-mer: dTMP insertion opposite template dT (-1 nt from G22)</u>				
non-G4 DNA	1.4 ± 0.1	730 ± 140	0.0019	0.0063
G4 DNA	1.4 ± 0.1	510 ± 110	0.0027	0.030
<u>23/42-mer: dTMP insertion opposite template dG (0 nt from G22)</u>				
non-G4 DNA	5.6 ± 0.8	450 ± 140	0.012	0.0032
G4 DNA	0.039 ± 0.003	200 ± 50	0.00019	0.00021

^aThe mis-insertion frequency (f_{ins}) was calculated as $(k_{\text{cat}}/K_{\text{M,incorrect dNTP}})/(k_{\text{cat}}/K_{\text{M,correct dNTP}})$.

Table 4Steady-state kinetic parameters for hpol $\epsilon^{1-1189/exo-}$ catalysis on G4 DNA and non-G4 DNA substrates

	k_{cat} (min^{-1})	$K_{M,dNTP}$ (μM)	$k_{cat}/K_{M,dNTP}$ ($\text{min}^{-1} \mu\text{M}^{-1}$)	^a Relative activity
<u>13/42-mer: dCMP insertion opposite template dG -10 nt from G22</u>				
non-G4 DNA	0.31 ± 0.01	8.9 ± 2.1	0.035	-
G4 DNA	0.22 ± 0.01	8.6 ± 1.3	0.025	71%
<u>18/42-mer: dAMP insertion opposite template dT -5 nt from G22</u>				
non-G4 DNA	0.42 ± 0.01	3.3 ± 0.6	0.13	-
G4 DNA	0.23 ± 0.01	2.5 ± 0.6	0.09	72%
<u>19/42-mer: dCMP insertion opposite template dG -4 nt from G22</u>				
non-G4 DNA	0.27 ± 0.01	2.3 ± 0.6	0.12	-
G4 DNA	0.21 ± 0.01	2.0 ± 0.4	0.11	89%
<u>20/42-mer: dGMP insertion opposite template dC -3 nt from G22</u>				
non-G4 DNA	0.42 ± 0.02	2.0 ± 0.5	0.21	-
G4 DNA	0.16 ± 0.01	1.5 ± 0.5	0.11	51%
<u>21/42-mer: dCMP insertion opposite template dG -2 nt from G22</u>				
non-G4 DNA	0.33 ± 0.06	8.0 ± 1.5	0.041	-
G4 DNA	0.098 ± 0.004	19 ± 4	0.005	12%
<u>22/42-mer: dAMP insertion opposite template dT -1 nt from G22</u>				
non-G4 DNA	0.25 ± 0.01	4.4 ± 0.1	0.057	-
G4 DNA	0.12 ± 0.01	12 ± 2	0.010	18%
<u>23/42-mer: dCMP insertion opposite G22</u>				
non-G4 DNA	0.44 ± 0.03	3.1 ± 1.5	0.14	-
G4 DNA	0.29 ± 0.08	52 ± 33	0.0056	4%

^a Relative activity was calculated as described in Table 2.

Table 5Steady-state kinetic parameters for hpol $\epsilon^{1-1189/exo-}$ mis-insertion on G4 DNA and non-G4 DNA substrates

	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>21/42-mer: dGMP insertion opposite template dG (-2 nt from G22)</u>				
non-G4 DNA	0.065 ± 0.005	280 ± 40	0.00023	0.0056
G4 DNA	0.019 ± 0.001	380 ± 90	0.00005	0.010
<u>22/42-mer: dCMP insertion opposite template dT (-1 nt from G22)</u>				
non-G4 DNA	0.046 ± 0.005	390 ± 150	0.00012	0.0021
G4 DNA	0.017 ± 0.001	220 ± 70	0.00008	0.0080
<u>23/42-mer: dGMP insertion opposite template dG (0 nt from G22)</u>				
non-G4 DNA	0.052 ± 0.004	600 ± 130	0.00009	0.00063
G4 DNA	0.015 ± 0.005	120 ± 35	0.00012	0.021

^aThe mis-insertion frequency (f_{ins}) was calculated as described in Table 3.