Kinetic investigation of the polymerase and exonuclease activities of human DNA polymerase ε holoenzyme

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In eukaryotic DNA replication, DNA polymerase ε (Pole) is responsible for leading strand synthesis, whereas DNA polymerases α and δ synthesize the lagging strand. The human Pole (hPole) holoenzyme is comprised of the catalytic p261 subunit and the noncatalytic p59, p17, and p12 small subunits. So far, the contribution of the noncatalytic subunits to hPole function is not well understood. Using pre-steady-state kinetic methods, we established a minimal kinetic mechanism for DNA polymerization and editing catalyzed by the hPole holoenzyme. Compared with the 140-kDa N-terminal catalytic fragment of p261 (p261N), which we kinetically characterized in our earlier studies, the presence of the p261 C-terminal domain (p261C) and the three small subunits increased the DNA binding affinity and the base substitution fidelity. Although the small subunits enhanced correct nucleotide incorporation efficiency, there was a wide range of rate constants when incorporating a correct nucleotide over a single-base mismatch. Surprisingly, the 3′→5′ exonuclease activity of the hPole holoenzyme was significantly slower than that of p261N when editing both matched and mismatched DNA substrates. This suggests that the presence of p261C and the three small subunits regulates the 3′→5′ exonuclease activity of the hPole holoenzyme. Together, the 3′→5′ exonuclease activity and the variable mismatch extension activity modulate the overall fidelity of the hPole holoenzyme by up to 3 orders of magnitude. Thus, the presence of p261C and the three noncatalytic subunits optimizes the dual enzymatic activities of the catalytic p261 subunit and makes the hPole holoenzyme an efficient and faithful replicative DNA polymerase.

Three B-family DNA polymerases are responsible for replicating the majority of the eukaryotic nuclear genome: DNA polymerases α (Polα), δ (Polδ), and ε (Polε) (1). DNA replication is initiated on both the leading and lagging strands by the Polα-primase complex. Primase synthesizes short primers of 7-12 ribonucleotides (rNTPs) that are extended by Polα with an additional 20-25 deoxyribonucleotides (dNTPs) (2). Following primer synthesis, processive DNA synthesis is taken over by Polε and Polδ on the leading and lagging strands, respectively (3-6).

The eukaryotic replicative DNA polymerases are present as multisubunit complexes in vivo (1). Each of the replicative DNA polymerases shares a common core consisting of a single large catalytic subunit and a smaller B-subunit, e.g. Polε p180-p70, Polδ p125-p50, and Polε p261-p59 in humans. In addition to this core complex, each replicative DNA polymerase associates with a set of accessory subunits to form its heterotetrameric holoenzyme. For example, human Polδ (hPolδ) core complex associates with p66 (sometimes called p68) and p12 accessory subunits (7), whereas human Polε (hPolε) core complex interacts with p17 and p12 (distinct from hPolδ p12) accessory subunits (8). In the case of human Polα (hPolα), however, the p180-p70 core complex forms a heterotetrameric complex with the primase heterodimer, which is comprised of the p49 catalytic subunit and the p58 regulatory subunit (9).

Structures of Saccharomyces cerevisiae Polα (10), Polδ (11, 12), and Polε (13) as well as human Polδ (14) illustrate that all three replicative DNA polymerases are characterized by a globular catalytic core and an extended structure, with the latter element largely composed of the catalytic subunit C-terminal domain as well as the B- and accessory subunits. The structures further reveal that the extended regions play a role in modulating the coordination and dynamics of the replicative DNA polymerases. For example, the crystal structure of the hPolα-primase complex reveals that the catalytic subunits of Polα and primase are linked to each other through the primase p58 regulatory subunit to allow for efficient coordination of the polymerase and primase activities during primer synthesis (15). Additionally, in the recent high-resolution cryo-EM structure of human Polδ in complex with human proliferating cell nuclear antigen (PCNA), DNA, and dNTP, hPolδ is tethered to one of the three PCNA monomers through the extended C-terminal domain of the p125 catalytic subunit, whereas the p50, p66, and p12 small subunits extend laterally away from the DNA- and PCNA-binding sites (14). In this configuration, p12 likely stabilizes p125, while providing a scaffold for the p50 and p66 subunits to contact p125 and regulate its polymerization activity (14).

In the absence of the three small subunits, the catalytic subunit of each of the replicative DNA polymerases is capable of catalyzing template-dependent DNA synthesis in vitro. However, the B- and accessory subunits either regulate or enhance the DNA polymerization activities of the replicative DNA polymerase holoenzymes. For example, the hPolδ p70 B-subunit limits the processivity of RNA primer extension by the p180 catalytic subunit (16). Furthermore, the polymerase activity of the trimeric hPolδ complex (p125-p50-p66) is enhanced by 4.6-fold in the presence of the p12 accessory subunit, whereas its 3′→5′ exonuclease activity is reduced by as much as 5-fold on a DNA
substrate containing a single-base mismatch at the primer 3’ terminus (17). Additionally, the polymerization processivity of *S. cerevisiae* Polε is enhanced in the presence of all three of its small subunits (13, 18). Thus, to accurately characterize the activities of the eukaryotic replicative DNA polymerases *in vitro*, it is imperative that kinetic studies are carried out with the full, intact DNA polymerase complexes.

Previously, we kinetically characterized the N-terminal fragment of the hPolε p261 catalytic subunit (residues 1–1189, p261N) and established a minimal mechanism for DNA polymerization catalyzed by p261N (19). However, detailed analysis of the hPolε heterotetrameric complex has been hampered by the difficulty of preparing sufficient quantities of pure complex. Initial studies of hPolε heterotetramer overexpressed in insect cells revealed that the three small subunits do not enhance the activity of the catalytic subunit alone (20). However, these studies were performed with excess enzyme on a large M13 DNA plasmid template (20), which does not allow for a quantitative comparison of DNA binding and nucleotide binding and incorporation kinetics between p261N and the hPolε heterotetramer. Furthermore, these studies did not investigate the impact of the small subunits on the 3’→5’ exonuclease activity of hPolε. To determine whether the small subunits affect the DNA binding and polymerization kinetics of hPolε, we overexpressed and purified fully-assembled hPolε heterotetramer in insect cells and performed a pre-steady-state kinetic analysis of the heterotetramer. We determined that the p261 C-terminal domain (p261C) and the three small subunits enhance the DNA binding affinity of hPolε by reducing the DNA dissociation rate constant (19). Although p261C and the small subunits moderately affect base substitution fidelity, the contribution of 3’→5’ exonuclease activity to hPolε fidelity is surprisingly reduced compared with that of p261N, primarily due to a nearly 10-fold attenuation of the excision rate constant on a DNA substrate containing a single-base mismatch (19, 21). Together, these observations suggest that p261C and the small subunits drive hPolε toward processive DNA synthesis to ensure rapid synthesis of the leading strand *in vivo*.

### Results

Previously, we carried out the kinetic characterization of p261N, the N-terminal fragment (residues 1–1189) of the hPolε p261 catalytic subunit. Our initial characterization of p261N alone was motivated by the relative ease of its overexpression and purification from *Escherichia coli* in suitable quantities for pre-steady-state kinetic studies. Importantly, the p261N fragment contains all of the conserved polymerase and 3’→5’ exonuclease motifs (22). From our pre-steady-state kinetic analysis, we defined a minimal kinetic mechanism of nucleotide incorporation, a simplified version of which is shown in Scheme 1A (19). We also established a kinetic basis for the high fidelity of DNA polymerization catalyzed by p261N (21). In a study that followed, we examined WT hPolε holoenzyme prepared from baculovirus-infected insect cells and performed burst assays with both hPolε and p261N under identical conditions. We observed that both exhibited a fast burst phase and a slow linear phase of product formation (23). The multiphasic kinetics of correct nucleotide incorporation suggests that hPolε likely utilizes a similar minimal kinetic mechanism as p261N (Scheme 1A) for DNA polymerization. To provide more evidence for the kinetic mechanism and to further kinetically characterize the hPolε holoenzyme, we overexpressed and purified an exonuclease-deficient variant (D275A/E277A/D368A, hPolε exo−) and carried out the following kinetic analysis at 20 °C:

**Measurement of burst kinetics, the steady-state rate constant of correct nucleotide incorporation, and the dissociation rate constant of the E–DNA complex**

If the mechanism in Scheme 1A can be applied to hPolε, then it must display multiphasic burst kinetics during single-nucleotide incorporation under enzyme-limiting conditions.
To confirm that hPole exo− displays burst kinetics as previously observed for p261N (19, 23) and the WT hPole holoenzyme (23), we performed a burst assay in which a preincubated solution of hPole exo− (20 nM, UV concentration) and a 4-fold excess of D-1 DNA substrate (80 nM, Table 1) was rapidly mixed with dTTP (100 μM) for various time points. The time course of product formation showed a clear burst of nucleotide incorporation followed by a slow linear phase (Fig. 1A), indicating that hPole follows a similar mechanism of single-nucleotide incorporation as p261N. The data were best fit to Equation 1 in which the initial burst phase is described by two exponential rate constants, $k_{fast}$ and $k_{slow}$, and the second linear phase is described by $k_{linear}$. The rate constants for $k_{fast}$ and $k_{slow}$ were 139 ± 27 s⁻¹ and 1.3 ± 0.5 s⁻¹, respectively, whereas $k_{linear}$ was 0.0044 ± 0.0008 s⁻¹. Although Scheme 1A does not explain why two exponential phases are present in the initial burst of product formation, we address this observation in more detail in the analysis of our pre-steady-state nucleotide incorporation assays (see below).

Typically, the linear phase of the burst time course is equal to the steady-state rate constant of nucleotide incorporation. To verify this assumption, we directly measured the steady-state DNA polymerization rate constant ($k_{ss}$) by mixing hPole exo− (1 nM, active site concentration) and a large excess of D-1 DNA substrate (400 nM) with a solution of dTTP (100 μM) and Mg²⁺ (see “Experimental procedures”). The time course of product formation was fit to Equation 2 and the $k_{ss}$ was determined to be 0.0070 ± 0.0003 s⁻¹ (Fig. 1B), which is in good agreement with the linear phase rate constant measured in the burst assay (Fig. 1A).

Finally, for hPole to follow a similar mechanism as in Scheme 1A, the dissociation of hPole from the E•DNA complex must be the slowest step in a multiple turnover reaction and its rate must be equal to the steady-state rate constant of nucleotide incorporation (24–27). Subsequently, we directly measured the rate constant of dissociation of hPole exo− from DNA ($k_{-1}$, Scheme 1A). A preincubated solution of hPole exo− (50 nM, UV concentration) and 5'−radiolabeled D-1 DNA substrate (100 nM) was mixed with a large excess of unlabeled D-1 DNA trap (2.5 μM) for varying incubation times. During the incubation period, any hPole exo− that dissociated from the labeled D-1 would rebind to the unlabeled D-1, which was present in a 25-fold molar excess over the labeled D-1. Thus, when the reaction was initiated with the addition of dTTP (100 μM) and Mg²⁺, only hPole exo− that was still bound to labeled D-1 would catalyze observable product formation. Product concentration was plotted against DNA trap incubation time and the data were fit to Equation 3, resulting in a $k_{-1}$ of 0.0058 ± 0.0007 s⁻¹ (Fig. 1C). The $k_{-1}$ value is in good agreement with the measured $k_{ss}$ value and confirms that multiple enzyme turnovers are limited by the rate constant of DNA dissociation as observed for p261N and that hPole follows a similar mechanism as in Scheme 1A. Thus, the presence of the triple mutations and C-terminal residues 1190-2257 of p261 as well as the p59, p17, and p12 subunits did not change the kinetic mechanism and the rate-limiting step of multiple turnovers of DNA polymerization.

**Active site titration**

To determine whether the triple mutations (D275A/E277A/D368A), p261C, and the presence of the small subunits (p59, p17, and p12) affect the binding affinity of hPole to a DNA substrate, we measured the apparent equilibrium dissociation constant of hPole exo− binding to DNA to form the E•DNA binary complex ($K_{DNA}^{d(app)}$) using an active site titration assay. Because correct nucleotide incorporation during the first turnover was previously shown to be much faster than the equilibration of hPole binding to DNA (E + DNA → E•DNA) (23), a titration of the polymerase active site with DNA can be used to measure $K_{DNA}^{d(app)}$ by examining the DNA concentration dependence of the first-turnover amplitude (24–27). Specifically, a fixed concentration of hPole exo− (50 nM, UV concentration) was preincubated with varying concentrations of D-1 DNA substrate (10-125 nM) to allow the E•DNA complex to form prior to initiation of the reaction with the addition of correct dTTP (100 μM) and Mg²⁺. The concentration of the product formed during the first turnover was measured by quenching each reaction after 50 ms, which allowed adequate time for the dTTP incorporation to reach the maximum first-turnover amplitude with a negligible contribution of multiple turnovers (23). Importantly, the amplitude of product formation from the first turnover was a direct measurement of the amount of productive E•DNA complex that formed during the preincubation period. Therefore, the maximum amplitude corresponds to the active enzyme concentration. As observed previously for p261N (19) and WT hPole (23), the active hPole exo− concentration is low (14.7 nM, 29%), suggesting that the majority of hPole may not bind to DNA or binds to DNA in a nonproductive conformation. Notably, we have observed that hPole is prone to forming aggregates, even after purification by size-exclusion chromatography. Such hPole aggregates certainly contribute to the low active enzyme concentration. Therefore, the $K_{DNA}^{d(app)}$ determined by active site titration mostly reflects the hPole population that is bound to DNA in a productive, polymerization-ready state (see “Discussion”).

The concentration of E•DNA complex was plotted against the concentration of total D-1 DNA substrate and the data were fit to a quadratic equation (Equation 4) to give a $K_{DNA}^{d(app)}$ of 22 ± 4 nM (Fig. 2). Notably, the $K_{DNA}^{d(app)}$ measured here is comparable to the $K_{DNA}^{d(app)}$ of 33 nM that we measured previously for WT hPole (23), indicating that the triple mutations to inactivate the 3′−5′ exonuclease activity of hPole did not impact the ability of hPole to bind to a DNA substrate. Previously, a $K_{DNA}^{d(app)}$ of 79 nM was measured using the same assay to monitor the binding of p261N to the same D-1 DNA substrate (19). Thus, the C-terminal 1,068 residues of p261 and the three small
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Figure 1. Pre-steady-state and steady-state kinetics of correct dTTP incorporation by hPol exo−. A, the burst kinetics of dTTP incorporation were measured by mixing a preincubated solution of hPol exo− (20 nM, UV concentration) and 5'-radiolabeled D-1 DNA substrate (80 nM) with 100 μM dTTP and Mg²⁺ and then quenching at various time intervals with addition of EDTA. The data were fit to Equation 1 to yield a fast exponential rate constant of 139 ± 1 s⁻¹. B, the steady-state DNA polymerization rate constant was measured by mixing a preincubated solution of hPol exo− (1 nM, active site concentration) and 5'-radiolabeled D-1 DNA substrate (400 nM) with dTTP (100 μM) and Mg²⁺ for varying reaction times and then quenching the reaction with the addition of EDTA. Product concentration was plotted against time and the data were fit to Equation 2 to yield a k₉ of 0.0070 ± 0.0003 s⁻¹. C, to directly measure the DNA dissociation rate constant, kᵣ, a preincubated solution of hPol exo− (50 nM, UV concentration) and 5'-radiolabeled D-1 DNA substrate (100 nM) was mixed with unlabeled D-1 DNA substrate (2.5 μM) for varying incubation times before the reaction was initiated with the addition of dTTP (100 μM) and Mg²⁺. The reaction was allowed to proceed for 15 s and then quenched with the addition of EDTA. Product concentration was plotted against time and the data were fit to Equation 3 to yield a kₛ of 0.0058 ± 0.0007 s⁻¹.

Table 2

| Parameter | p261N⁹ | hPol | p261N/hPol⁶ |
|-----------|--------|------|-------------|
| k₁       | 0.021 s⁻¹ | 0.0058 s⁻¹ | 3.6 |
| KᵣDNA    | 79 nM   | 22 nM | 3.6 |
| Kᵣmax    | 252 s⁻¹ | 411 s⁻¹ | 0.6 |
| Kᵣd     | 23 μM   | 11 μM | 2.1 |
| Kₛmax    | 11 μM s⁻¹ | 37 μM s⁻¹ | 0.3 |
| Kₛ, matched | 10⁻² s⁻¹ | 10⁻² - 10⁻³ s⁻¹ | 10⁻²-10⁻¹ |
| Kₛ, mismatched | 0.17 s⁻¹ | 0.015 s⁻¹ | 11 |
| Kₛ_mismatched | 2.6 s⁻¹ | 0.45 s⁻¹ | 5.8 |

*References 19 and 21.
*Calculated by dividing the kinetic parameter for p261N by the corresponding value measured for hPol.
*Average of measured Kᵣ values for the M-1 and M-8 substrates.

2.7 × 10⁵ M⁻¹ s⁻¹ measured for p261N (19). Consequently, the 3.6-fold binding affinity difference between hPol and p261N is almost completely contributed by the 3.6-fold difference in their k₁ values (Table 2).

Pre-steady-state kinetics of correct nucleotide incorporation

We then sought to determine whether the presence of p261C, the B-subunit, and the accessory subunits (p17 and p12) affects the kinetics of nucleotide binding and incorporation. To explore this possibility, we measured the equilibrium dissociation constant for correct nucleotide binding (KᵣDNA) and the maximum nucleotide incorporation rate constant (Kₛmax) by monitoring the dependence of the observed rate constant of nucleotide incorporation on nucleotide concentration under single-turnover kinetic conditions. Briefly, a preincubated solution of hPol exo− (100 nM, UV concentration) and D-1 DNA substrate (20 nM) was rapidly mixed with increasing concentrations of correct dTTP in the presence of Mg²⁺. After varying reaction times, the reaction was quenched with the

Figure 2. Active site titration assay to measure the KᵣDNA for hPol exo− binding to DNA. A preincubated solution of hPol exo− (50 nM, UV concentration) and increasing concentrations of 5'-radiolabeled D-1 DNA substrate (10-125 nM) was rapidly mixed with dTTP (100 μM) and Mg²⁺ for 50 ms and then quenched with the addition of EDTA. Measurements were performed in triplicate and the average concentration of E-DNA complex that formed during the preincubation period, given by product concentration, was plotted against total D-1 DNA concentration and the data were fit to Equation 4 to yield a KᵣDNA of 22 ± 4 nM and an active enzyme concentration (E₀) of 14.7 ± 0.8 nm. Error bars represent the S.D. from the calculated average product concentration.

Subunits (p59, p17, and p12) collectively increase the binding affinity of hPol by 3.6-fold (Table 2). Furthermore, the second-order rate constant of DNA and hPol association (k₁ = KᵣDNA/Kₛ_app, K₁ = 0.0058 s⁻¹, Kₛ_app = 22 nm) was calculated to be 2.6 × 10⁶ M⁻¹ s⁻¹, which is nearly identical to the k₁ value of...
addition of 0.37 mM EDTA. The time course of product formation at each nucleotide concentration was fit to Equation 5 to yield a fast \( k_{\text{fast}} \) and a slow \( k_{\text{slow}} \) observed rate constant for nucleotide incorporation (Fig. 3A), similar to the double-exponential kinetics in the burst assay. The fast observed rate constants were plotted against dTTP concentration and the data were fit to Equation 7 (Fig. 3B). The resulting \( k_{\text{max}} \) and \( K_{d}^{\text{dTTP}} \) values were 411 ± 26 s\(^{-1}\) and 11 ± 2 \( \mu \)M, respectively, and the substrate specificity \( k_{\text{max}}/K_{d}^{\text{dTTP}} \) was calculated to be 37 \( \mu \)M\(^{-1}\) s\(^{-1}\).

The dependence of \( k_{\text{fast}} \) on dTTP concentration indicates that \( k_{\text{fast}} \) reports on the rate constant for nucleotide incorporation onto DNA that is productively bound to the polymerase active site \( (E_p\bullet\text{DNA}) \), and the maximal rate \( (k_{\text{max}}) \) therefore reflects the rate-limiting pre-chemistry conformational change \( (k_3) \) preceding the chemistry step \( (k_{\text{chem}}) \) (Scheme 1A). In contrast, the observed slow rate constants of product formation, which ranged from 0.32 to 0.65 s\(^{-1}\), showed only a weakly negative correlation with nucleotide concentration (Fig. 3B), which is suggestive of an additional slow step prior to rapid nucleotide binding and incorporation that is exhibited by a significant population of hPol bound to DNA. A similar result was observed for single-turnover nucleotide incorporation assays performed with \( S.\ cerevisiae \) Pole heterotetramer and the slow phase was attributed either to switching of the DNA primer terminus from the 3′→5′ exonuclease active site to the polymerase active site or to slow binding of Pole to DNA (18). Although plausible, the former case is unlikely given that strand transfer from the 3′→5′ exonuclease active site to the polymerase active site was shown to be very fast (> 700 s\(^{-1}\)) for the replicative DNA polymerase from T7 bacteriophage (28). Additionally, we believe that the latter situation is unlikely because a 10-fold molar excess of \( S.\ cerevisiae \) Pole was preincubated with DNA before adding a correct dNTP to initiate nucleotide incorporation (18). Kinetically, hPole (Fig. 3A) likely bound to DNA in a productive \( (E_p\bullet\text{DNA}) \) and a nonproductive state \( (E_a\bullet\text{DNA}) \) at the polymerase active site, leading to the observed fast and slow nucleotide incorporation phases, respectively (29–34). To illustrate these two possible binding modes, we have proposed a revised Scheme 1B, which shows that hPole may bind directly to DNA in a productive conformation \( (E + \text{DNA} \rightarrow E_p\bullet\text{DNA}) \) or in a nonproductive conformation \( (E + \text{DNA} \rightarrow E_a\bullet\text{DNA}) \). hPole that is bound to DNA in a productive, polymerization-ready state will catalyze rapid nucleotide incorporation with a rate constant \( k_p \), which is equal to \( k_{\text{max}} \). On the other hand, hPole that is bound to DNA in a nonproductive state must first transition to the productive state at a rate constant \( k_{N\rightarrow P} \), which is equivalent to \( k_{\text{slow}} \) (Scheme 1B). This isomerization is intrinsic and its rate does not depend on nucleotide concentration (28). Thus, the slow phase in Fig. 3A was likely limited by isomerization and, thus, its rate constant was not significantly affected by dNTP concentration. Importantly, inclusion of an excess of an unlabeled DNA trap in the reaction mixture does not eliminate the slow phase or alter its rate constant (Fig. 3B), supporting our conclusion that the slow phase results from the transition of DNA-bound hPole from a nonproductive to a productive state. Finally, we note that the amplitude of the fast phase \( (A_{\text{fast}}) \) shows a weak positive correlation with nucleotide concentration, whereas the amplitude of the slow phase \( (A_{\text{slow}}) \) is relatively unaffected (Fig. 3C). The relationship between the amplitudes of the two phases and nucleotide concentration is unclear and is not further explored in the present study.

**Elemental effect on correct nucleotide incorporation**

To further determine whether p261C, the B-subunit, and the accessory subunits affect the rate-limiting step of correct nucleotide incorporation by hPole \( \text{exo}^- \), we compared the incorporation rate constants of dTTP and \( \alpha \)-thiophosphate-substituted dTTP \( (S_\alpha\text{-dTTP}) \). A preincubated solution of hPole \( \text{exo}^- \) (100 nM, UV concentration) and D-1 DNA substrate (20 nM) was rapidly mixed with dTTP (or \( S_\alpha\text{-dTTP} \)) (5 \( \mu \)M) and Mg\(^{2+}\) for varying incubation times. The reaction was quenched with the addition of 0.37 mM EDTA. The time courses of product formation were fit to Equation 5 to yield fast observed incorporation rate constants of 127 ± 15 s\(^{-1}\) and 118 ± 21 s\(^{-1}\) for dTTP and \( S_\alpha\text{-dTTP} \), respectively (Fig. 4A). The elemental
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by the intrinsic isomerization rate expected, the slow phase of nucleotide incorporation, limited to dCTP - 1.4 dATP 0.26 6

Figure 4. Elemental effect on correct and incorrect nucleotide incorporation. A, a preincubated solution of hPol exo- (100 nM, UV concentration) and 5'-radiolabeled D-1 DNA substrate (20 nM) was rapidly mixed with 5 μM dTTP (●) or Sα-dATPαS (○) and Mg2+ for varying incubation times before quenching with the addition of EDTA. Product concentration was plotted against time and the data were fit to Equation 5 to yield fast rate constants of 127 ± 15 s−1 and 118 ± 21 s−1 for dTTP and Sα-dATPαS, respectively, resulting in an elemental effect of 1.1. B, the elemental effect on incorrect dATP incorporation was tested under identical conditions, except the reaction was initiated with the addition of 100 μM dATP (●) or Sα-dATPαS (○) and Mg2+. Product concentration was plotted against time and the data for dATP incorporation were fit to Equation 6, whereas the data for Sα-dATPαS were fit to a line. The incorporation rate constants were 0.073 ± 0.007 s−1 for dATP and 0.0016 s−1 for Sα-dATPαS, giving an elemental effect of 46.

Table 3
Kinetic parameters for correct and incorrect nucleotide incorporation onto the D-1 DNA substrate catalyzed by hPol ε exo- at 20 °C

| dNTP     | kmax [s−1] | Kd[dNTP] [μM] | kmax/Kd[dNTP] [μM−1 s−1] | Fpol a | Fpol, p261N/Fpol b |
|----------|------------|---------------|--------------------------|--------|-------------------|
| dTTP     | 411 ± 26   | 11 ± 2        | 37                       | -      | -                 |
| dATP     | 0.26 ± 0.01| (4.6 ± 0.5) × 102 | 5.7 × 10−4               | 1.5 × 10−5 | 8                 |
| dCTP     | -          | (1.8 ± 0.1) × 103 | 1.4 × 10−3               | 3.8 × 10−5 | 8.4               |
| dGTP     | (1.50 ± 0.06) × 10−2 | 8.3 × 10−6                       | 2.2 × 10−7               | -      | -                 |

a Calculated as kmax/Kd[dNTP]incorrect/kmax/Kd[dNTP]correct + kmax/Kd[dNTP]incorrect.
b Values for Fpol, p261N reported in Table 2 of Ref. 21.

effect (Kd[dTTP] / Kd[dATPαS]) was calculated to be 1.1 for hPol exo-. Previously, an elemental effect of 4–11 was used as suggestive evidence for a rate-limiting chemistry step during nucleotide incorporation (19, 24–26, 35). Thus, the protein conformational change (Ep′•DNAα•dTTP → Ep′•DNAβ•dATP), rather than phosphodiester bond formation (Ep′•DNAα•dTTP → Ep′•DNAβ•dATP), is likely rate-limiting for each round of correct nucleotide incorporation catalyzed by hPol exo- (Scheme 1B). Notably, a similarly small elemental effect (0.9) was observed for correct nucleotide incorporation catalyzed by p261N (19). Thus, both hPol and p261N share the same rate-limiting step during correct nucleotide incorporation (Scheme 1). Finally, the slow phases for incorporation of dTTP and Sα-dATPαS yielded an elemental effect of 1.2, indicating that, as expected, the slow phase of nucleotide incorporation, limited by the intrinsic isomerization rate kNε−p (Scheme 1B), was not affected by the α-thiophosphate substitution in Sα-dATPαS.

Pre-steady-state kinetics of incorrect nucleotide incorporation

The p261N fragment of hPol exhibits high base substitution fidelity (10−4–10−7) resulting from decreases in both kmax and the ground-state binding affinity (1/Kd[dNTP]) for incorrect relative to correct nucleotides (21). To probe whether p261C, the B-subunit, and the accessory subunits affect the base substitution fidelity of hPol, we measured the pre-steady-state kinetic parameters for the incorporation of all three incorrect nucleotides with D-1 as described above. The kmax and Kd[dNTP] values for all four nucleotides are listed in Table 3. As for correct dTTP incorporation, we calculated the substrate specificities for each of the incorrect nucleotides and subsequently determined the base substitution fidelity (Fpol) of hPol exo-, which was 10−5–10−7 (Table 3). We also measured the elemental effect for incorrect nucleotide incorporation onto D-1 by comparing the incorporation rate constant of dATP and Sα-dATPαS (500 μM). The observed incorporation rate constants were 0.073 ± 0.007 s−1 for dATP and 0.0016 s−1 for Sα-dATPαS (Fig. 4B), yielding an elemental effect of 46, which indicates that phosphodiester bond formation is at least partially rate-limiting for incorrect nucleotide incorporation (see above). This is similar to the large elemental effect (167) on incorrect nucleotide incorporation observed with p261N (19).

Interestingly, only a single phase of product formation was observed for incorporation of dATP and Sα-dATPαS (Fig. 4B). Likewise, misincorporation of dCTP lacked a second phase (data not shown). Unlike correct nucleotide incorporation, the rate of intrinsic conversion from the nonproductive to productive bound state for DNA binding to hPol exo- is faster than or comparable to the rate of misincorporation, and the overall observed rate constant of nucleotide incorporation was
either limited by slow incorrect nucleotide incorporation or was a function of both $k_{N \leftrightarrow p}$ (Scheme 1B) and slow nucleotide incorporation. In the case of dGTP misincorporation, a fast phase of incorporation was observed for a very small population of the total DNA concentration (< 5%), and the reaction time course was dominated by very slow nucleotide incorporation (data not shown). The significance of the small population of more rapidly misincorporated dGTP is unclear and requires further studies.

**Pre-steady-state kinetics of mismatch extension**

Previously, we determined that p261N exo− very poorly extends single-base mismatches due to large decreases in both $k_{\text{max}}$ and $1/K_{\text{dNTP}}^2$ for the next correct nucleotide (21). To determine whether the presence of p261C, the B-subunit, and the accessory subunits affect the ability of hPol exo− to extend a single-base mismatch, we measured the kinetic parameters for incorporation of the next correct nucleotide, dCTP, onto two different mismatched substrates containing either a C:A mismatch (M-1, Table 1) or a C:C mismatch (M-8, Table 1) at the primer 3′ terminus. For extension of both the C:A and the C:C mismatched DNA substrates, the $k_{\text{max}}$ of correct nucleotide incorporation was reduced by 100- and 63,000-fold (Table 4), respectively, relative to extension of a correctly-matched A:T base pair in D-1 (Tables 1 and 3). Furthermore, the binding affinity for the correct nucleotide was reduced by 22-fold when extending from the C:A base pair, and 64-fold when extending from the C:C base pair. Overall, the substrate specificity of the correct nucleotide was reduced by $2.2 \times 10^5$- and $4.0 \times 10^6$-fold with the C:A and C:C mismatched DNA substrates (Tables 3 and 4), respectively, indicating that hPol exo− very poorly extended mismatched DNA substrates compared to matched substrates as observed with p261N exo− (21). However, both hPol exo− and p261N exo− demonstrate a marked variability in their abilities to extend different mismatched base pairs. Based on the current study, hPol exhibits an 1,800-fold preference for extension of a C:A mismatch versus a C:C mismatch (Table 4), whereas p261N only displayed a 28-fold preference for C:A versus C:C (21). The reason for this difference in mismatch type discrimination remains to be determined. We further tested the ability of hPol exo− to extend the C:A mismatch with an incorrect nucleotide, dGTP. From the measured kinetic parameters, we calculated a substrate specificity of $1.6 \times 10^{-7} \, \mu M^{-1} \, s^{-1}$ for dGTP (Table 4), which is similar to the substrate specificity values determined for extension of a mismatched base pair with an incorrect nucleotide by p261N exo− (21). Thus, both hPol exo− and p261N exo− are similarly unlikely to bury a mismatched base pair with an additional mismatch. Based on both a 20,000-fold decrease in $k_{\text{max}}$ and a 5.4-fold decrease in binding affinity relative to extension of the same mismatch with correct dCTP, we determined the base substitution fidelity during mismatch extension ($F_{\text{exo}}$) to be $9.4 \times 10^{-6}$ (Table 4), demonstrating that hPol exo− remains highly selective for the correct nucleotide even when extending from a mismatch.

**Pre-steady-state kinetics of matched and mismatched base pair excision**

Previous pre-steady-state kinetic analysis of mismatch extension and excision by p261N revealed that the 3′−5′ exonuclease activity of p261N increased its fidelity of DNA synthesis by $3.5 \times 10^{-7}$- to $1.2 \times 10^{-6}$-fold due to a strong preference for excision of a mismatched DNA substrate coupled with poor efficiency of extension (21). Here, we used a similar approach to measure the excision rate constants for a matched A:T base pair in D-1 and a mismatched C:A base pair in M-1 (Table 1) by WT hPol heterotetramer. A preincubated solution of WT hPol (100 nM, UV concentration) and a 5′-radiolabeled DNA substrate (20 nM) was rapidly mixed with Mg$^{2+}$ in the absence of nucleotides. The concentration of remaining DNA substrate was plotted against time (Fig. 5). The data for excision of the matched D-1 DNA substrate were fit to Equation 9 to yield an excision rate constant of 0.015 ± 0.003 s$^{-1}$. In contrast to the matched D-1 DNA substrate, the mismatched M-1 DNA substrate displayed two clear phases of excision (Fig. 5). Accordingly, the data were fit Equation 10 to yield a fast excision rate constant of $0.4 \pm 0.1 \, s^{-1}$ and a slow excision rate constant of 0.0099 ± 0.0006 s$^{-1}$. Thus, in the presence of a single mismatch, the excision rate constant increased by 27-fold. This is comparable with the 15-fold 3′−5′ exonuclease activity enhancement measured previously for p261N exo+ in the presence of a single-base mismatch (Table 2). Similarly, for the M-8 substrate, which contains a C:C mismatch, we measured a fast excision rate constant of $0.5 \pm 0.1 \, s^{-1}$ and a slow mismatch excision rate constant of 0.0041 ± 0.0002 s$^{-1}$ (data not shown), indicating that the identity of the mismatched base pair does

### Table 4

Kinetic parameters for mismatch extension and excision catalyzed by hPol exo− and hPol exo+ at 20°C

| dNTP    | $k_{\text{max}}$ | $K_{\text{dNTP}}^2$ | $k_{\text{max}}/K_{\text{dNTP}}^2$ | $F_{\text{ext}}$ | $k_{\text{dCTP D-1}}^b$ | $k_X^c$ | $F_{\text{exo}}^c$ |
|---------|------------------|----------------------|-------------------------------------|------------------|--------------------------|---------|------------------|
| C:A mismatch (M-1) |       |                     |                                    |                  |                          |         |                  |
| dCTP    | $4.1 \pm 0.3$   | $(2.4 \pm 0.5) \times 10^2$ | $1.7 \times 10^{-2}$ | -                | $1.2$                     | -       | -                |
| dGTP    | $(2.1 \pm 0.2) \times 10^{-4}$ | $(1.3 \pm 0.2) \times 10^3$ | $1.6 \times 10^{-7}$ | $9.4 \times 10^{-6}$ | $1.5 \times 10^{-5}$ | -       | -                |
| C:C mismatch (M-8) |       |                     |                                    |                  |                          |         |                  |
| dCTP    | $(6.5 \pm 0.5) \times 10^{-2}$ | $(7 \pm 1) \times 10^2$ | $9.3 \times 10^{-6}$ | $8.1 \times 10^{-4}$ | -                        | $0.4 \pm 0.1$ | $617$            |

$^a$ Calculated as $(k_{\text{max}}/K_{\text{dNTP}}^2)^{\text{incorrect}}/(k_{\text{max}}/K_{\text{dNTP}}^2)^{\text{correct}} + (k_{\text{max}}/K_{\text{dNTP}}^2)^{\text{incorrect}}$.

$^b$ Calculated as $k_{\text{dNTP D-1}}^b/(k_d^b + [\text{dNTP}])$ during extension from a mismatched primer terminus at an intracellular dCTP concentration of 100 μM.

$^c$ Calculated as $k_X^c/(K_{\text{dCTP}}^c)$.
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Discussion

Kinetic mechanism for DNA polymerization catalyzed by the hPol ε holoenzyme

Using pre-steady-state kinetic methods, we previously determined that p261N, the N-terminal 140-kDa fragment of the p261 catalytic subunit, catalyzes correct nucleotide incorporation via an induced-fit mechanism (19) that is generally followed by all DNA polymerases (Scheme 1) and is characterized by a rate-limiting pre-chemistry conformational change following nucleotide binding (37, 38). In the present study, as well as in our previous study (23), we demonstrated that the hPol ε holoenzyme, like p261N, incorporated correct nucleotides by following multiphasic kinetics (Fig. 1A). We then determined that the multiple-turnover (or steady-state) rate (0.007 s⁻¹) of nucleotide incorporation was governed by the hPol εDNA complex dissociation (0.0058 s⁻¹) (Fig. 1). Through an active site titration assay, the apparent binding affinity of D-1 DNA substrate to hPol εDNA (1/KᴰΝΑₓₓ, KᴰΝΑₓₓ = 22 nM) was measured (Fig. 2), indicating that the first turnover of nucleotide incorporation was much faster than the equilibration of hPol and DNA binding (E + DNA ↔ EₓDNA) (23).

Based on the results of our pre-steady-state kinetic assays (discussed below), hPol ε likely binds to DNA in three different modes during preincubation with a duplex DNA substrate: a productive polymerization-ready state (EₓDNA), a nonproductive state (EₓDNA), and with the DNA bound at the 3’→5’ exonuclease active site (EₓDNA). Thus, the kᴰΝΑ_app measured by the active site titration assay (Fig. 2) is a function of KᴰΝΑₓₓ, KᴰΝΑₓₓ, and KᴰΝΑₓₓ (Scheme 1B). However, KᴰΝΑ_app is expected to be a good approximation of KᴰΝΑ_app for the following reasons. First, the reaction is quenched after 50 ms, which is too short to observe any additional product formation resulting from the slow conversion of EₓDNA to EₓDNA (Eᴺ→P; 0.32-0.65 s⁻¹; t₁/₂ = 1.1-2.2 s). Second, excision of a matched DNA substrate is dominated by the rate of strand transfer from the polymerase active site to the 3’→5’ exonuclease active site (Eₓ→Eₓ). The lack of a fast phase of excision indicates that no appreciable population of DNA is bound to the 3’→5’ exonuclease active site of hPol εDNA. Thus, KᴰΝΑ_app is expected to represent a good approximation of the equilibrium dissociation constant for productive binding of DNA to the polymerase active site of hPol εDNA (EₓDNA).

Through the single-turnover kinetic assay, we determined the maximum correct dTTP incorporation rate (k_max = 411 s⁻¹) and ground-state binding affinity (1/K_d, K_d = 11 μM) of correct dTTP with hPol (Fig. 3). Furthermore, we measured the elemental effect (1.1) of dTTP incorporation onto D-1 by hPol (Fig. 4A), suggesting that phosphodiester bond formation did not limit the single-turnover rate of correct nucleotide incorporation (19, 24–26, 35). Together, these kinetic assays demonstrate that hPol ε and p261N possess similar kinetic behaviors and likely follow the similar minimal kinetic mechanism for productive nucleotide incorporation (compare Scheme 1, A and B) involving a rate-limiting conformational change (k₃ in Scheme 1A or k₃ in Scheme 1B) that is triggered by correct
nucleotide binding \( (K_d^{dNTP} = k_{-2}/[k_2[dNTP]]) \) and precedes phosphodiester bond formation \( (k_{chem}) \) (Scheme 1).

The presence of two distinct phases of correct nucleotide incorporation by hPole provides evidence for hPole binding to DNA in at least two modes: a productive, polymerization-ready state \( (E_p\bullet DNA) \) and a nonproductive state \( (E_a\bullet DNA) \). Although our pre-steady-state exonuclease assays demonstrate that DNA may also bind directly to the 3′→5′ exonuclease active site of hPole \( (E_a\bullet DNA) \), we argue that the slow phase of nucleotide incorporation is not rate-limited by strand transfer from the 3′→5′ exonuclease active site to the polymerase active site \( (k_{x→p}) \) as the rate constant for this strand transfer was previously measured to be very fast \( (>700 \text{ s}^{-1}) \) (28). Moreover, the absence of a fast excision phase in our exonuclease assays with D-1 (Fig. 5) shows that almost no matched DNA substrate is bound to the 3′→5′ exonuclease active site after the pre-incubation phase. Therefore, the slow phase of polymerization most likely results from DNA-bound enzyme isomerizing from the nonproductive to the productive state \( (k_{N→P}) \). In contrast, the strong dependence of the fast phase of nucleotide incorporation on nucleotide concentration indicates that the maximal rate of the fast phase \( (k_{max}) \) represents the rate-limiting pre-chemistry conformational change \( (k_p) \). Accordingly, Scheme 1B illustrates the interconversion between nonproductive and productive polymerization states, the latter of which binds nucleotide to catalyze rapid correct nucleotide incorporation.

**Effect of the C-terminal domain of p261 and the three small subunits on nucleotide incorporation kinetics with hPole**

Previously, we determined that p261N rapidly incorporates correct nucleotides with an average \( k_{max} \) of 252 s\(^{-1} \) at 20 °C and an average \( K_d^{dNTP} \) of 23 μM (Table 2), and exhibits large decreases in both \( k_{max} \) and ground-state nucleotide binding affinity \( (1/K_d^{dNTP}) \) for incorrect nucleotides thereby achieving the high base substitution fidelity \( (10^{-4}−10^{-7}) \) (21). In this study, we determined that hPole catalyzes correct dCTP incorporation onto D-1 at 20 °C with a slightly higher \( k_{max} \) (411 s\(^{-1} \), 1.6-fold), a slightly lower \( K_d^{dNTP} \) (11 μM, 2.1-fold), and a moderately higher \( k_{max}/K_d^{dNTP} \) correct \( (37 \mu M^{-1} s^{-1}, 3.4\text{-fold}) \) (Tables 2 and 3). Furthermore, we measured the kinetic parameters for all three misincorporations onto D-1 and calculated the base substitution fidelity of hPole to be \( 10^{-5}−10^{-7} \), which is 8- to 20-fold higher than that of p261N (Table 3). The high base substitution fidelity of hPole is a hallmark of replicative DNA polymerases (39). Taken together, the presence of p261C and the three small subunits favorably affect the nucleotide incorporation kinetics and fidelity of hPole. In comparison, the pre-steady-state kinetic parameters determined for correct nucleotide incorporation by S. cerevisiae PolE are nearly identical to those measured for the C-terminal truncated Pol2 catalytic subunit, indicating that the small subunits do not significantly affect nucleotide incorporation kinetics with S. cerevisiae PolE (18). This conclusion is supported by the positioning of the small subunits away from the globular Pol2 catalytic domain in the low-resolution cryo-EM structure of S. cerevisiae PolE (13). Other than the solution structure of an N-terminal fragment of the p59 subunit (40), there are currently no structures of the hPole heterotetramer. Although *in vitro* transcription/translation and immunoprecipitation studies have assigned the binding sites for the three small subunits within the p261C domain (20), the structure of the polymerase active site within p261N is likely affected by the association of the small subunits in the hPole holoenzyme.

Interestingly, hPole catalyzes correct dCTP incorporation during extension from a single-base mismatch with a \( k_{max} \) of 4.1 s\(^{-1} \) for a C:A mismatch and 0.0065 s\(^{-1} \) for a C:C mismatch (Table 4). This wide \( k_{max} \) range \( (10^{-3}−10^{-1} \text{ s}^{-1}) \) contrasts significantly from the values determined for p261N, which generally catalyzes single-base mismatch extension with a \( k_{max} \) on the order of \( 10^{-2} \text{ s}^{-1} \) regardless of mismatch identity (Table 2). The wide \( k_{max} \) range also affects the contribution of the 3′→5′ exonuclease to the overall fidelity of hPole, which was calculated to be 0.3- or 617-fold under a typical intracellular nucleotide concentration of 100 μM (Table 4). Consequently, the wide-ranging contribution to fidelity by the 3′→5′ exonuclease activity of hPole is likely one of the factors to cause sequence-dependent DNA replication errors in *vivo* (41, 42).

**Enhanced DNA binding affinity of hPole by the C-terminal domain of p261 and three small subunits**

Earlier structural and biochemical studies of *S. cerevisiae* PolE implicated a direct role for the three small subunits in modulating the polymerase processivity of PolE. A low-resolution cryo-EM structure of *S. cerevisiae* PolE heterotetramer revealed an extended tail-like structure that is hypothesized to encircle dsDNA that trails PolE as it synthesizes the leading strand, thereby tethering PolE to DNA and enhancing processivity (13). This hypothesis is supported by the observation that *S. cerevisiae* PolE heterotetramer is able to synthesize longer products than the Pol2 catalytic subunit alone under single-hit conditions but only when the length of dsDNA is at least 40 nucleotides long (43). Consistently, the Dpb3/Dpb4 (corresponding to p12/p17 in humans) heterodimer forms a stable complex with dsDNA in the absence of Pol2/Dpb2 (corresponding to p261/p59 in humans) (44) and significantly enhances the polymerase and 3′→5′ exonuclease processivities of the Pol2/Dpb2 heterodimer (43), suggesting that the increased processivity results from additional interactions between *S. cerevisiae* PolE and DNA afforded by the small subunits. In the presence of p261C and the three small subunits, the hPole heterotetramer binds to D-1 DNA substrate with a \( K_{DNA_{app}} \) of 22 nm (Fig. 2), which is 3.6-fold lower than the \( K_d^{DNA} \) previously measured for p261N binding to the same D-1 DNA (Tables 1 and 2). Additionally, the D-1 dissociation rate constant of 0.0058 s\(^{-1} \) was determined using unlabeled DNA trap (Fig. 1C). Notably, this DNA dissociation rate constant is 3.6-fold slower than that measured for p261N (Table 2) and essentially accounts for the 3.6-fold difference in DNA binding affinity, indicating that the C-terminal residues 1190-2257 of p261 and the p59, p17, and p12 subunits collectively enable hPole to form a tighter, more stable complex with its DNA substrate. Surprisingly, our previous data show that the processivities of p261N and hPole heterotetramer are indistinguishable when the polymerases are
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in excess over a DNA substrate (23). In addition, p261N and hPole are comparably active on a primed M13 DNA template and their activities are similarly enhanced in the presence of PCNA, replication factor C, and replication protein A (20). Thus, the increased DNA binding affinity does not appear to affect the polymerization processivity of hPole, contrasting sharply with the effect of the small subunits on the polymerase processivity of *S. cerevisiae* Pole. The difference will be answered by future high-resolution structures of hPole and *S. cerevisiae* Pole in complex with DNA and an incoming dNTP. Surprisingly, the recent high-resolution cryo-EM structure of the complex of hPolδ, PCNA, DNA, and dNTP shows that the C-terminal domain of hPolδ and the three subunits do not directly interact with DNA but may indirectly facilitate DNA binding through stabilizing the complex by interactions between the subunits and with one of the three PCNA monomers (14). Thus, the contributions of p261C and the three small subunits to DNA binding by hPole may be different in the presence and absence of PCNA.

**Modulation of hPole proofreading activity by the C-terminal domain of p261 and the three small subunits**

WT hPole excised a matched A:T in D-1 with a single-turnover excision rate constant of 0.015 s⁻¹. In contrast, the C:A mismatch in M-1 and the C:C mismatch in M-8 were excised with fast rate constants of 0.4 s⁻¹ and 0.5 s⁻¹ and slow rate constants of 0.0099 s⁻¹ and 0.0041 s⁻¹, respectively (Tables 1, 2, and 4). Thus, the excision rate constant is enhanced roughly 27- to 33-fold in the presence of a single-base mismatch compared with a matched base pair.

The presence of two phases of excision for a mismatched DNA substrate suggests that some population of mismatched DNA substrate may bind directly to the 3′→5′ exonuclease active site of hPole. Although the fast phase excision rate constant (0.4-0.5 s⁻¹) is much faster than the single rate constant for excision of a matched DNA substrate (0.015 s⁻¹), the slow phase excision rate constants for the two mismatched DNA substrates tested here (0.0041-0.0099 s⁻¹) are more comparable with the rate constant for matched DNA excision. Therefore, these rate constants are likely representative of the rate constant for strand transfer from the polymerase active site to the 3′→5′ exonuclease active site (kₚ→₅ₓ), whereas the fast phase excision rate constants represent cleavage of DNA pre-bound to the 3′→5′ exonuclease active site of hPole. Accordingly, Scheme 1B shows a third DNA binding mode in which DNA directly binds to the 3′→5′ exonuclease active site of hPole or transfers from the polymerase active site to the 3′→5′ exonuclease active site. Once bound to the 3′→5′ exonuclease active site, the DNA substrate is cleaved with a rate constant k₅ₓ.

Surprisingly, the excision rate constants measured with WT hPole are significantly lower than those measured for p261N exo+ and are reduced by 11-fold for excision of a matched DNA substrate and 5.8-fold for excision of a mismatched DNA substrate (Table 2). Furthermore, excision of mismatched substrates by p261N exo+ was dominated by a single fast phase, whereas hPole displayed a low-population fast phase of excision in the presence of a mismatch. Thus, the presence of p261C and the three small subunits appears to modulate the 3′→5′ exonuclease activity of hPole by limiting both strand transfer from the polymerase active site to the 3′→5′ exonuclease active site (kₚ→₅ₓ) as well as direct binding of the DNA to the 3′→5′ exonuclease active site. Interestingly, this result contrasts sharply with the pre-steady-state kinetic analysis of *S. cerevisiae* Pole, which demonstrated that the excision rate constants for matched, mismatched, and ssDNA substrates were largely unaffected by the presence or absence of the Pol2 C-terminal domain and the three small subunits (18).

Previously, pre-steady-state kinetic methods have been used to examine the 3′→5′ exonuclease activities of hPoleδ, the major lagging strand replicative DNA polymerase, and hPoly, the mitochondrial replicative DNA polymerase, as well as their oligomeric assemblies. Notably, kinetic comparison of a heterotrimeric hPoleδ assembly (p125-p50-p66), and the fully assembled heterotetramer (p125+p50-p66-p12), revealed that addition of p12 to the hPoleδ heterotrimer results in a 4.6-fold increase in polymerase activity, but 8.3- and 4.8-fold decreases in the excision rates of matched and single-base mismatched DNA substrates, respectively (17). The high-resolution cryo-EM structure of the complex of hPolδ, PCNA, DNA, and dNTP shows that p12 bridges the exonuclease and C-terminal domains of p125 as well as the oligonucleotide-binding domain of p50 and stabilizes hPoleδ (14). The stabilization likely enhances hPoleδ polymerase activity but restricts the transfer of a mismatched primer from the polymerase to the 3′→5′ exonuclease active site for editing. Consequently, processive polymerization by hPoleδ heterotetramer is less likely to be interrupted by the slow switching of the DNA primer from the polymerase to the 3′→5′ exonuclease active site. Consistent with the enhanced polymerase activity and attenuated exonuclease activity, hPoleδ heterotetramer exhibits increased tolerance for translesion synthesis and mismatch incorporation and extension in the presence of p12 (45). Interestingly, it has been shown that treatment of human cells with UV, methyl methanesulfonate, and other DNA damaging agents results in degradation of p12 (46). Taken together, damage-dependent p12 degradation resulting in a more proofreading active form of hPoleδ is a potential mechanism that human cells have evolved for minimizing mutagenic DNA synthesis while maintaining the high rate of DNA synthesis required for timely genome replication under normal cellular conditions. Similarly, hPoly catalyzes nucleotide incorporation 5-fold faster and is 7.8-fold more processive in the presence of its p55 accessory subunit (47), but exhibits a 2-fold decrease in exonuclease activity and greater selectivity against excision of correctly matched dsDNA (36). However, unlike hPoleδ, a similar mechanism of proofreading activity control has not been identified for hPolγ in vivo. Finally, whereas the polymerization rate we have measured for hPole heterotetramer increases slightly from that of p261N (411 s⁻¹ versus 252 s⁻¹, 1.6-fold; Table 2), the 3→5′ decrease in exonuclease-activity is consistent with the regulatory effects exerted upon hPoleδ and hPolγ by their small subunits. As with hPolγ, the significance of such control is unknown. Overall, the proofreading activities of these three replicative DNA polymerases are comparably modulated in the presence of their respective small subunits,
although the physiological role for proofreading control may not be common between these DNA polymerases.

**Experimental procedures**

**Materials**

Reagents used for the experiments described were purchased from the following sources: [γ-32P]ATP from PerkinElmer Life Sciences (Boston, MA); OptiKinase from U.S. Biochemicals (Cleveland, OH); and dNTPs from Bioline (Taunton, MA).

**Preparation of the human DNA polymerase ε heterotetramer**

The 11A/hPole1exo− vector, which encodes an exonuclease-deficient (D275A/E277A/D368A) variant (19) of the full-length p261 catalytic subunit, and the 11A/hPole234 vector, which encodes the p59 B-subunit as well as the p17 and p12 accessory subunits, were kindly provided by Drs. Yoshihiro Matsumoto and Alan Tomkinson at the University of New Mexico (Albuquerque, NM). The exo− p261 sequence contains C-terminal PKA, FLAG, and His6 tags. The p59 sequence contains an N-terminal StreptII tag, a thrombin cleavage site, a PKA tag, and a hemagglutinin tag. The p17 and p12 sequences are WT and contain no tags. Recombinant baculoviruses were generated using the Bac-to-Bac expression system (Life Technologies) and amplified to a high titer according to the manufacturer’s instructions. The hPole exo− heterotetramer was overexpressed by simultaneously infecting 1.8 × 10^9 Sf9 cells in a 1-liter shaking culture with p261 exo− virus and virus encoding all three small subunits. The infected insect cells were incubated at 25°C for 60 h, and then harvested and lysed by Dounce homogenization. The lysate was clarified by ultracentrifugation at 20,000 × g and hPole exo− heterotetramer was purified from the cleared lysate essentially as described (23). The WT hPole heterotetramer was also prepared as described (23).

**DNA substrates**

The DNA substrates listed in Table 1 were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and purified by denaturing PAGE as described (48). All primers were radiolabeled by incubation with [γ-32P]ATP and OptiKinase at 37°C for 3 h. Following the labeling reaction, the kinase was inactivated by heating the reaction mixture at 95°C for 5 min, and unreacted [γ-32P]ATP was removed by passing the reaction mixture through a Bio-Spin 6 column (Bio-Rad). The 5′-radiolabeled primers were annealed to the template oligonucleotides in Table 1 by incubating the primer with a 1.15-fold molar excess of template at 95°C for 5 min and then slowly cooling the mixture to room temperature over several hours.

**Pre-steady-state kinetic assays**

All assays were performed at 20°C in reaction buffer containing 50 mM Tris-OAc (pH 7.4), 8 mM Mg(OAc)2, 1 mM DTT, 10% glycerol, 0.1 mg/ml BSA, and 0.1 mM EDTA. Fast reactions were performed using a rapid chemical quench-flow apparatus (KinTek). All reported concentrations are final after mixing. All reactions were quenched with the addition of EDTA. Use of 1 M HCl as a quenching solution decreases the observed rate constants by ∼2-fold (Fig. S3). However, there are still distinct fast and slow phases with acid quench as with EDTA quench (Fig. S3), indicating that the slow phase did not result from slow incorporation by the ε-DNA·dNTP complex that cannot be stripped of Mg2+ by the EDTA quench. Thus, the 2-fold rate constant decrease observed with acid quench relative to EDTA quench does not change our proposed kinetic mechanism (Scheme 1B). Accordingly, we chose to use an EDTA quench to avoid strong acid damage to our rapid chemical quench-flow apparatus. Unless otherwise specified, most experimental data were collected from only single trials due to the insufficient quantities of purified hPole holoenzyme to repeat all assays in triplicate.

**Active site titration assay**

A preincubated solution of hPole exo− (50 nM, UV concentration) and 5′-radiolabeled D-1 DNA substrate (10-125 nM) was rapidly mixed with a solution containing dTTP (100 μM). The reaction was allowed to proceed for 50 ms and then quenched with the addition of 0.37 M EDTA. Each data point for the active site titration assay was measured in triplicate.

**Measurement of the ε-DNA complex dissociation rate constant**

A preincubated solution of hPole exo− (50 nM, UV concentration) and 5′-radiolabeled D-1 DNA substrate (100 nM) was mixed with an excess of unlabeled D-1 DNA substrate (2.5 μM). After varying incubation times, the reaction mixture was supplemented with dTTP (100 μM). The nucleotide incorporation reaction was allowed to proceed for an additional 15 s and then was quenched with addition of 0.37 M EDTA.

**Measurement of the steady-state rate constant of correct nucleotide incorporation**

A preincubated solution of hPole exo− (1 nM, active site concentration) and 5′-radiolabeled D-1 DNA (400 nM) was mixed with a solution containing dTTP (100 μM). After varying incubation times, an aliquot of the reaction mixture was quenched in 0.37 M EDTA.

**Measurement of the elemental effect on nucleotide incorporation**

A preincubated solution of hPole exo− (100 nM, UV concentration) and 5′-radiolabeled D-1 DNA substrate (20 nM) was rapidly mixed with a solution containing either dTTP or S5′-dTTPoS (5 μM) for varying reaction times and quenched with the addition of 0.37 M EDTA. The elemental effect on incorrect nucleotide incorporation was measured under identical assay conditions, but with the addition of incorrect dATP or S5′-dATPαS (500 μM).

**Single-turnover exonuclease assays**

A preincubated solution of WT hPole (100 nM, UV concentration) and 5′-radiolabeled DNA substrate (20 nM) was rapidly mixed with a solution containing 8 mM Mg(OAc)2 to initiate
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the 3’→5’ exonuclease reaction. After varying reaction times, the reaction was quenched with the addition of 0.37 M EDTA.

Product analysis

Reaction substrates and products were separated by denaturing PAGE (17% acrylamide, 8 M urea, and 1× TBE running buffer) and quantified using a Typhoon TRIO (GE Healthcare) and ImageQuant (Molecular Dynamics).

Data analysis

Data were fit by nonlinear regression using Kaleidagraph (Synergy Software). Data from the burst assay were fit to Equation 1,

\[
[\text{Product}] = A_{\text{fast}}[1 - \exp(-k_{\text{fast}}t)] + A_{\text{slow}}[1 - \exp(-k_{\text{slow}}t)] + (A_{\text{fast}} + A_{\text{slow}})k_{\text{linear}}t \quad \text{(Eq. 1)}
\]

where \(A_{\text{fast}}\) and \(A_{\text{slow}}\) are the amplitudes of product formation of the fast and slow phases, \(k_{\text{fast}}\) and \(k_{\text{slow}}\) are the observed rate constants of the fast and slow phases, and \(k_{\text{linear}}\) is the observed steady-state rate constant.

Data from the steady-state nucleotide incorporation assay were fit to Equation 2,

\[
[\text{Product}] = k_{\text{ss}}E_0t + E_0 \quad \text{(Eq. 2)}
\]

where \(k_{\text{ss}}\) is the steady-state rate constant of nucleotide incorporation at the initial enzyme concentration of \(E_0\).

Data from the DNA dissociation assay were fit to Equation 3,

\[
[\text{Product}] = A[\exp(-k_{\text{-1}}t)] \quad \text{(Eq. 3)}
\]

where \(A\) is the product concentration in the absence of the DNA trap and \(k_{\text{-1}}\) is the DNA dissociation rate constant.

Data from the active site titration assay were fit to Equation 4,

\[
[E \cdot \text{DNA}] = 0.5\left(K_{d}^{\text{DNA}_{d(app)}} + E_0 + D_0\right)
- 0.5\left(K_{d}^{\text{DNA}_{d(app)}} + E_0 + D_0\right)^2 - 4E_0D_0 \right)^{1/2} \quad \text{(Eq. 4)}
\]

where \(K_{d}^{\text{DNA}_{d(app)}}\) is the apparent equilibrium dissociation constant for hPole binding to DNA to form the \(E\bullet\text{DNA}\) binary complex, \(E_0\) is the enzyme concentration, and \(D_0\) is the DNA concentration.

Data from single-turnover polymerization assays were fit to Equation 5,

\[
[\text{Product}] = A_{\text{fast}}[1 - \exp(-k_{\text{fast}}t)] + A_{\text{slow}}[1 - \exp(-k_{\text{slow}}t)] \quad \text{(Eq. 5)}
\]

where \(A_{\text{fast}}\) and \(A_{\text{slow}}\) are the amplitudes of product formation of the fast and slow phases and \(k_{\text{fast}}\) and \(k_{\text{slow}}\) are the observed rate constants of the fast and slow phases. For some misincorporations, only a single exponential phase of product formation was observed, or the first fast phase was populated by less than 5% of the total DNA substrate. Accordingly, these data were fit to Equation 6,

\[
[\text{Product}] = A[1 - \exp(-k_{\text{obs}}t)] \quad \text{(Eq. 6)}
\]

where \(A\) is the amplitude of product formation and \(k_{\text{obs}}\) is the rate constant of nucleotide incorporation.

The \(k_{\text{fast}}\) (or \(k_{\text{obs}}\)) values were plotted against nucleotide concentration and the data were fit to Equation 7,

\[
k_{\text{fast}} = k_{\text{max}}[\text{dNTP}]/(K_{d}^{\text{dNTP}} + [\text{dNTP}]) \quad \text{(Eq. 7)}
\]

where \(k_{\text{max}}\) is the maximum rate constant of nucleotide incorporation and \(K_{d}^{\text{dNTP}}\) is the equilibrium dissociation constant for dNTP binding. When \(K_{d}^{\text{dNTP}}\) was very large, the data were fit to Equation 8,

\[
k_{\text{fast}} = \left(k_{\text{max}}/K_{d}^{\text{dNTP}}\right)[\text{dNTP}] \quad \text{(Eq. 8)}
\]

to yield the substrate specificity constant, \(k_{\text{max}}/K_{d}^{\text{dNTP}}\).

Data from single-turnover exonuclease assays with a matched DNA duplex were fit to Equation 9,

\[
[\text{Remaining DNA substrate}] = A_{\text{exo}}[\exp(-k_{\text{exo}}t)] + C \quad \text{(Eq. 9)}
\]

where \(A_{\text{exo}}\) is the amplitude of DNA substrate excision and \(k_{\text{exo}}\) is the observed excision rate constant.

Data from single-turnover exonuclease assays with a mismatched DNA duplex were fit to Equation 10,

\[
[\text{Remaining DNA substrate}] = A_{\text{exo}}[\exp(-k_{\text{exo}}t)] + A_{\text{exo2}}[\exp(-k_{\text{exo2}}t)] + C \quad \text{(Eq. 10)}
\]

where \(A_{\text{exo}}\) and \(A_{\text{exo2}}\) are the amplitudes of DNA substrate excision of the fast and slow phases and \(k_{\text{exo}}\) and \(k_{\text{exo2}}\) are the observed excision rate constants of the fast and slow phases.

All reported errors were generated by fitting the data to the above equations in Kaleidagraph.

Data availability

All data are in the manuscript and supporting information, or are available from the authors Walter J. Zahurancik (zahurancik.2@osu.edu) and Zucai Suo (zucai.suo@med.fsu.edu) upon request.

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Abbreviations—The abbreviations used are: hPol, human DNA polymerase α heterodimer; hPolε, human DNA polymerase ε heterotetramer; p261N, the N-terminal fragment (residues 1–1189) of p261; p261C, the C-terminal domain (residues 1190–2257) of p261; hPolδ, human DNA polymerase δ heterotetramer; cryo-EM, cryo-electron microscopy; PCNA, proliferating cell nuclear antigen; PKA, protein kinase A; dATP, diadenosine penta-phosphate; p-diastereoisomer of deoxythymidine α-thiotriphosphate; TBE, Tris-boric acid-EDTA; hPoly, human DNA polymerase γ.

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