Structural basis for processivity and antiviral drug toxicity in human mitochondrial DNA replicate

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Abstract

The human DNA polymerase gamma (Pol γ) is responsible for DNA replication in mitochondria. Pol γ is particularly susceptible to inhibition by deoxynucleoside-based inhibitors designed to fight viral infection. Here, we report crystal structures of the replicating Pol γ–DNA complex bound to either substrate or zalcitabine, an inhibitor used for HIV reverse transcriptase. The structures reveal that zalcitabine binds to the Pol γ active site almost identically to the substrate dCTP, providing a structural basis for Pol γ-mediated drug toxicity. When compared to the apo form, Pol γ undergoes intra- and inter-subunit conformational changes upon formation of the ternary complex with primer/template DNA and substrate. We also find that the accessory subunit Pol γB, which lacks intrinsic enzymatic activity and does not contact the primer/template DNA directly, serves as an allosteric regulator of holoenzyme activities. The structures presented here suggest a mechanism for processivity of the holoenzyme and provide a model for understanding the deleterious effects of Pol γ mutations in human disease. Crystal structures of the mitochondrial DNA polymerase, Pol γ, in complex with substrate or antiviral inhibitor zalcitabine provide a basis for understanding Pol γ-mediated drug toxicity.

Keywords DNA replication; drug toxicity; human DNA polymerase gamma; mitochondrial toxicity; nucleoside reverse transcriptase inhibitors

Subject Categories DNA Replication, Repair & Recombination; Structural Biology

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Introduction

A human mitochondrion DNA (mtDNA) codes for a subset of components of the oxidative phosphorylation electron transfer chain for ATP synthesis. The integrity of mtDNA is critically important for energy supplies, the cell cycle, and programmed cell death. Mutations in both human mtDNA and mitochondrial DNA polymerase (Pol γ) have been associated with multisystem clinical disorders (Stumpf & Copeland, 2013).

Unlike nuclear DNA polymerases that have specialized functions in either replication or repair, Pol γ is responsible for both mtDNA replication and repair. Human Pol γ holoenzyme consists of a catalytic subunit Pol γA and a dimeric accessory subunit Pol γB. Pol γA possesses all active sites for holoenzyme activities, including polymerization (pol) for DNA synthesis, 3’–5’ exonuclease (exo) for proofreading, and 5’ deoxyribophosphatase (5’dRP) lyase for base excision repair. Pol γB has no intrinsic enzymatic activity, but regulates all Pol γA activities in the holoenzyme (Longley et al., 1998; Johnson et al., 2000; Gellon et al., 2008).

Structurally, Pol γ belongs to the DNA Pol I family. Polymerases in this family dissociate from DNA frequently and are therefore unsuitable for efficient highly processive DNA replication. Indeed, Pol γA alone displays low processivity (Graves et al., 1998; Lim et al., 1999). However, in contrast to most Pol I family members, Pol γA has a spacer domain that enables it to associate with Pol γB; the resulting holoenzyme exhibits drastically increased processivity (Graves et al., 1998; Lim et al., 1999). Pol γB enhances the holoenzyme’s processivity by both increasing the affinity of the holoenzyme for DNA and accelerating the DNA synthesis reaction rate (Johnson et al., 2000; Lee et al., 2010b). Pol γB also increases 5’dRP lyase and reduces exonucleolytic activities (Pinz & Bogenhagen, 2006). A crystal structure of apo Pol γ shows that the pol and exo active sites are 35 Å apart, and it is unclear how Pol γB can regulate multiple activities. Pol γA contacts dimeric Pol γB asymmetrically: It interacts extensively with the proximal Pol γB monomer, but only minimally with the distal monomer (Lee et al., 2009). Nevertheless, subunit interactions at the distal monomer region are important for proper functioning of the holoenzyme, as evidenced by the observation that Pol γA mutants (R232G or R232H) that reside at Pol γA–distal Pol γB subunit interface show significantly reduced DNA synthesis activity and elevated exonucleolytic activity. Patients carrying Pol γA mutations R232G/H display muscular and neurological disorders (Ferrari et al., 2005; Kollberg et al., 2006; Lee et al., 2010a; Rouzier et al., 2014). The apo structure provides little insight as to why these mutations give rise to the resulting phenotype.
The error frequency of Pol γ is $10^{-5}$ during DNA replication, comparable to other high-fidelity DNA polymerases (Longley et al., 2001; Lee & Johnson, 2006). However, Pol γ displays lower discrimination than its nuclear counterparts against nucleoside analog HIV reverse transcriptase inhibitors (NRTIs), particularly dideoxynucleoside triphosphates (Johnson et al., 2001; Lee et al., 2003). Thus, the NRTIs that inhibit Pol γ result in moderate to life-threatening mitochondrial toxicity (McKenzie et al., 1995; Koczor & Lewis, 2010).

To understand antiviral drug toxicity and Pol γ mutations in human diseases, we determined crystal structures of Pol γ holoenzyme replication complexes, captured with either a substrate or an antiviral agent, zalcitabine. These structures are the first intact multi-subunit DNA replicase captured during DNA synthesis. The structures illustrate how the two subunits coordinate functions in the holoenzyme to achieve high processivity and to regulate structures. The antiviral agent, zalcitabine. These structures are the first intact multi-subunit DNA replicase captured during DNA synthesis. The structures illustrate how the two subunits coordinate functions in the holoenzyme to achieve high processivity and to regulate pol/exo activities. The structures show that Pol γ binds zalcitabine and a substrate almost identically, providing a molecular basis for Pol γ’s susceptibility to inhibition by dideoxynucleotides. The structures provide new insight in understanding diseases associated with Pol γ mutations that were inexplicable by the apo structure.

**Results and Discussion**

**Overall structure of replicating holoenzyme**

Crystals of human Pol γ replicating complexes (~250 kDa) were formed in the presence of the catalytic subunit Pol γA, the accessory subunit Pol γB, a 24/28-mer primer/template duplex DNA, CaCl₂, and a substrate (dCTP), or a NRTI (the triphosphate form of zalcitabine, ddCTP) (Fig 1). Pol γB adopts a pyramidal configuration resembling class II aminoacyl tRNA synthetases with domains corresponding to the base, middle, and apex of the pyramid. Pol γA has three domains: a polymerase (pol) domain that has the canonical “right-hand” DNA polymerase fold with subdomains called the thumb, fingers, and palm; an exonuclease (exo) domain; and a spacer domain that lies between pol and exo. The Pol γA spacer domain contains two subdomains: a globular intrinsic processivity (IP) subdomain and an extended accessory-interacting determinant (AID) subdomain that forms a major binding site for Pol γB at the proximal monomer (Supplementary Fig S1). The primer/template DNA is located in a deep positively charged cleft on Pol γA. Relative to the apo holoenzyme, where Pol γA has almost no interaction with the distal Pol γB monomer, the replication complex has significantly enhanced interaction between the distal Pol γB monomer and Pol γA at the R²³² region, resulting from DNA-binding-induced conformational changes. The newly formed subunit interface plays an important role in enabling Pol γB to allosterically regulate polymerase and exonuclease activities in the holoenzyme. The structures illustrate the division of functions in a multi-subunit DNA polymerase.

**Substrate, inhibitor, and DNA-binding sites**

In the replication complex, the substrate dCTP is coordinated by the catalytic residues D⁹⁰⁰ and D¹¹₃⁵ and two metal ions (M²⁺) in the pol active site bounded by the palm and fingers subdomains (Fig 2; Supplementary Fig S2). Although as many as four metal ions are seen in various DNA polymerases, a minimum of two metal ions are critical for the phosphotransfer reaction. One divalent metal ion participates in nucleophilic reaction by reducing the pKa of the attacking group, 3’OH of the primer. The other metal ion stabilizes the leaving group PPI after catalysis (Steitz & Steitz, 1993). As the primer terminus is a nonreactive dideoxynucleotide, the structure represents a pre-chemistry substrate complex (Steitz, 1999). The triphosphate moiety of dCTP is stabilized by R⁹⁴³ and K⁵⁴⁷ of the O-helix (residues 943–955) in the fingers subdomain (Fig 3). The template DNA bends 90° at the single-/double-strand junction; the bent conformation is enforced by Y⁹⁵⁵ and a short connecting loop (357AG358) (Fig 2). Compared to apo Pol γ, the O-helix pivots around its center so that R⁹⁴³ and K⁵⁴⁷ close the active site concurrent with Y⁹⁵⁵ rotation to facilitate productive dNTP binding (Fig 4B). This extreme template bending and Y⁹⁵⁵ rotation are important to maintain template register, and to reduce slippage and backtracking during replication (Graziewicz et al., 2004; Estep & Johnson, 2011). The open–closed conformational change occurs at each nucleotide addition, as shown in many high-fidelity DNA polymerases (Doublié & Ellenberger, 1998; Steitz & Yin, 2004).

The DNA duplex is bound exclusively to Pol γA in a deep cleft formed by the spacer and pol domains, adopting the A-form in the active site, and transforming to B-form around base pair –4. All subdomains of Pol γA pol domain participate in interacting with the primer/template DNA (Fig 3). The thumb binds the minor groove of the duplex at the –5 to –7 positions, and a cluster of positively charged residues, R⁸⁰², K⁸⁰⁶, and R⁸⁰⁷, forms electrostatic interactions with the DNA backbone (Figs 3 and 4A; Supplementary Fig S3). These interactions made us predict that the thumb forms firm grip on the DNA duplex such that movements of the thumb are coupled to movements of the primer terminus.

Additional DNA interactions are formed with the spacer domain: The IP subdomain binds the primer strand at positions –3 to –5. Together with pol, the IP subdomain encircles the primer/template, reducing enzyme dissociation from the DNA. The flexible AID subdomain contains a lysine-rich K-tract that interacts with the negatively charged phosphodiester backbone (Fig 4A). Binding to Pol γB stabilizes the flexible AID subdomain and increases the holoenzyme DNA-binding area to 20 bp from that of 10 bp with Pol γA alone. The increased interaction provides a structural basis for Pol γB increasing DNA affinity in the holoenzyme.

The replication complex containing ddCTP shows that the inhibitor is bound nearly identically as dCTP (Fig 2; Supplementary Fig S2), providing a structural basis for the high toxicity for ddN-based antiviral inhibitors. In both structures, Y⁹⁵¹ stacks with the incoming nucleotide, with its –OH group that is 3.5 Å from O³’ and C²’ of the deoxyribose moiety. The hydroxyl group of Y⁹⁵¹ is the main source of drug toxicity, because the Pol γA Y951F mutant exhibits significantly reduced sensitivity to ddN (Lim et al., 2003; Graziewicz et al., 2004). E895 is situated in the pol site, and its ε-oxygen is 3.4 and 4.3 Å from the C²’ and O³’ of the ribose moiety of dCTP, confirming its contributions to nNTP and minor ddNTP discrimination (Supplementary Fig S4). In Pol I family members, this residue, conserved as either a Phe or Tyr, is important for substrate sugar moiety specificity (Tabor & Richardson, 1995; Astatke et al., 1998; Feng et al., 2004). Although both Phe and Tyr are able to discriminate against the ribonucleotides that are structurally larger than dNTP, only Phe exhibits selectivity against smaller ddNTP (Tabor & Richardson, 1995; Astatke et al., 1998; Wang et al., 2012). Pol γA Y951F substitution is likely to
cause a slight misalignment of the O-helix, resulting in exclusion of ddNTP in a fashion similar to that shown in bacillus DNAP ternary complexes (Wang et al., 2012).

**DNA induced intra- and inter-subunit structural changes**

Upon binding to DNA, the holoenzyme undergoes conformational changes that reveal the mechanism for holoenzyme processivity and pol and exo activity regulation. In the apo enzyme, the subunit interface is formed between proximal Pol γB monomer and Pol γA L-helix in the AID and the thumb subdomains; only a single salt bridge exists between R232 and E394 of the distal Pol γB monomer (Fig 5A and B). Formation of a productive replication complex results in conformational changes at the subunit interface: Pol γB rotates by 22° toward pol of Pol γA. However, the rotation does not alter the interface of Pol γA–Pol γB proximal monomer because the thumb and the

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**Figure 1. Structure of Pol γ replication complex.**

A Pol γ holoenzyme ternary complex contains Pol γA, a dimeric Pol γB with the proximal (green) and the distal monomers (blue), a primer/template (pink/gray) DNA duplex, and an incoming nucleotide.

B Pol γ (shown in electrostatic surface) interaction with primer/template DNA (green).

C Primer/template DNA sequence. The structurally visible region of the primer/template is bolded in the sequences. For convenience, bases are numbered such that the incoming nucleotide is +1, and the upstream residues are −1, −2, . . . , n, and the downstream residues +2, +4.
L-helix of Pol γA rotate together with Pol γB as a single rigid body, despite being on separate polypeptide chains. These conformational changes bring the Pol γB distal monomer 16 Å closer to Pol γA in the R232 region (Fig 5C). Consequently, a more substantial subunit interaction is formed between Pol γA and distal Pol γB monomer; the contact surface area increases from 226 Å² in the apo enzyme to 1,610 Å² in the ternary replication complex. Using the conversion factor of 25 cal per Å² contact surface area (Richards, 1977), the conformational changes enhance the interaction of Pol γA with the distal Pol γB monomer by 34 kcal. Importantly, Pol γA R232 is situated in a pocket of the distal Pol γB, forming H-bonds with H467 and S469, and Pol γA W235 forms hydrophobic interactions with Pol γB V338 (Fig 5C). The replication complex adopts a more compact structure than apo Pol γ in good agreement with solution measurement performed by small-angle X-ray scattering (He et al, 2013). While Pol γA displays several intra-subunit-independent structural changes in the fingers, thumb and L-helix, and the IP subdomains, Pol γB shows no internal structural changes and moves as a rigid body. The locations of Pol γB and the Pol γA thumb in the replication complex orient the primer terminus in pol and away from exo, tilting the pol/exo activity balance toward replication. The structure provides an explanation for the deleterious effects on mtDNA synthesis in patients carrying Pol γA R232 substitutions and highlights the importance of an intact Pol γA–distal Pol γB monomer interface for mitochondrial DNA replication.

The two subdomains in the spacer undergo different modes of structural changes upon binding to DNA (Figs 4A and 5A). The IP globular subdomain clamps down on the DNA duplex toward the 3'-end of the primer, enlarging the Pol γA DNA-binding site, whereas the AID subdomain undergoes more complex changes.
lysine-rich K-tract binds DNA duplex in the minor groove at base pairs -20 to -22 (Figs 3 and 4A), and additional interactions are seen between AID and the distal Pol γB monomer (Fig 4A). These structural changes are consistent with solution studies where binding to DNA strengthens the affinity of Pol γA for Pol γB (Lee et al., 2010b). A pre-steady state kinetic study reported that Pol γ exhibits reduced
synthesis rate on an 18-nt primer, which is sevenfold slower than that on a 24-nt primer (3.2 nt s\(^{-1}\) versus 22 nt s\(^{-1}\)) (Murakami et al., 2003), in good agreement with our structural results.

To confirm our structural results, we substituted each lysine in the K-tract to cysteine and analyzed the pre-steady state kinetics of multiple nucleotide addition by Pol γ variants using primer/template substrate in the presence of all dNTPs. The rationale for the substitutions was to examine the effect of positive-charge changes on DNA synthesis activity. 25-nt primer was used as Pol γ synthesis rate on primers 25 nt or longer provided maximum reaction rate, as analyses using longer primers (up to 37 nt) showed that Pol γ replication proceeds at comparable rate as on the 25-nt primer. Thus, 25-nt primer is the minimal length primer that enables the maximum reaction rate for Pol γ. Pol γA elongates the primer at an average rate of 14 nt s\(^{-1}\), whereas Pol γ holoenzyme is more efficient at an average rate of 43 nt s\(^{-1}\). This processive elongation rate of the holoenzyme is very similar to the rate of single-nucleotide addition reported in the literature (Estep & Johnson, 2011). The K-tract substitutions (K498C, K499C, and K501C) have a significant effect on the activity of both Pol γA and holoenzyme (Fig 6). In the absence of Pol γB, the mutants add the first nucleotide rapidly, but subsequent nucleotides are added at two- to fivefold slower rates relative to wild-type. Therefore, in these mutants, there is visible accumulation of the one-nucleotide elongation product even in the presence of all dNTPs.

The addition of Pol γB partially rescues this defect of the Pol γA mutants and there is relatively less stalling after the addition of one nucleotide, but the mutant proteins remain less processive. Consequently, they synthesize DNA at three- to fivefold slower rates relative to wild-type. The rest of the K-tract mutants show similar activity as the wild-type (Fig 6). Overall, the results indicate that the K-tract is not directly involved in the nucleotide addition catalytic activity, but the inability to interact with upstream DNA decreases the processivity of DNA synthesis. Interestingly, this defect is observed in lysines that are close to mutation Q497H substitution, which is associated with Alpers syndrome (Winterthun et al., 2005).

**Communication between pol and exo sites**

An incorporated NRTI is technically an incorrect nucleotide and should therefore provoke proofreading by Pol γ exonuclease. However, the most toxic nucleotide analog inhibitors, including ddC, are hydrolyzed at significantly slower rates than drugs with lower toxicity, indicating that exo contributes to Pol γ-mediated drug toxicity (Feng et al., 2001; Lim & Copeland, 2001).

The exo domain (residues 170–440) of Pol γ is both spatially separated and structurally distinct from pol; the two active sites are 35 Å apart and exo consists of an array of antiparallel β-sheets sandwiched between α-helices (Fig 7A). This configuration of exo and its distance from pol are generally conserved in other members of the DNA Pol I family. When pol incorporates an incorrect nucleotide, the 3'-end has to move to exo for excision. There must therefore be a mechanism to link recognition of a misincorporation event to shutting of the 3'-end to exo.

Analysis of the Pol γ-DNA structure suggests that an antiparallel hairpin, consisting of residues 835–858, could perform this function. The hairpin is located between pol and exo, and the hairpin tip contains two adjacent arginines in a conserved hexapeptide R\(^{852}\)TRRAVE\(^{856}\) (Fig 7; Supplementary Fig S6). In the Pol γ replicating complex, R\(^{853}\) reaches into pol and forms an H-bond with base pair −1 in the minor groove, as well as with the incoming nucleotide at the +1 position (Fig 7B and C; Supplementary Fig S6). These H-bonds can serve as checkpoints for correct Watson–Crick (W-C) base complementarity. The other conserved arginine, R\(^{852}\), points away from the DNA duplex and is embedded in a deep protein core, forming bipartite H-bonds with residues K\(^{1107}\) and S\(^{1103}\) in a helix of the fingers subdomain (Fig 7). These interactions may be important in positioning R\(^{853}\). The base of the connecting hairpin is close to the thumb and thus could be able to sense both the thumb rotation and the fingers open–close conformational changes that occur during nucleotide incorporation. These structural features lead us to hypothesize that the antiparallel hairpin may physically and functionally link the base complementarity check with, in the case of misincorporation, shutting the primer end from pol to exo. In support of this idea, Pol γA substitutions at R\(^{852}\) and R\(^{853}\) were found to dramatically reduce polymerase activity and, not surprisingly, are associated with mitochondrial disorders: Patients carrying Pol γA R852C/H display increased mtDNA mutations, and Parkinsonism patients carrying R853W/Q show multiple mtDNA deletions (Davidzon et al., 2006; Gonzalez-Vioque et al., 2006; Kasiviswanathan et al., 2009; Vasta et al., 2012). In silico, the substitutions R852C/H abrogate the H-bonds that facilitate positioning R853, whereas substitution of R853 with the bulkier tryptophan residue abolishes
Switch between the pol and exo sites

Interactions with the nacent bp (+1)

Interactions with the upstream bp (-1)

Figure 7. Regulator for pol and exo activity.

A. The β-hairpin is situated between pol and exo active sites and is connected to the thumb subdomain. R853 interacts with residues on the fingers subdomain.

B. R853 forms H-bonds with the nascent bp of incoming nucleotide and the template residue (T+1).

C. Upstream, R853 interacts with bp of the T-1 template and P-1 primer residues.

the important H-bond with the -1 base pair. The loss of this checkpoint for base complementarity in pol could increase the mutation rate, and/or cause slippage during replication.

The antiparallel hairpin is spatially conserved in other Pol I family members. The Pol γA R451 that performs the minor groove checking at base pair -1 is conserved as R450 and R451 in T7 DNA polymerase and E. coli Pol I, respectively (Doublié et al., 1998). The amino acid composition of this hairpin is one of the most conserved sequences in known bacterial Pol I family enzymes (TGRLSSXXPNLQNIP, the conserved R is bolded), and the sequence of the antiparallel hairpin in Pol γ is highly conserved in mitochondrial polymerases from invertebrates to mammals (Supplementary Fig S5), suggesting a common and important function.

This β-hairpin is seemingly structurally conserved even in polymerases with proofreading activity from other families, such as the Pol B family members bacteriophage RB69 and T4 DNA polymerases (Hogg et al., 2007; Subuddhi et al., 2008). Studies of the synthesis and editing complexes of RB69 identified a β-hairpin that appears to perform the same function. The β-hairpin of RB69 is located on the surface of the molecule and, although different from Pol γ, may serve as a communicator between the pol and exo sites (Hogg et al., 2007; Subuddhi et al., 2008). Additional residues, typically conserved Gln and Asn in Pol I family polymerases, also participate in minor groove readout: Q1102 and N1098 in Pol γ, Q615 and N611 in T7 DNA, and Q745 and N750 in Taq DNA. The linearly aligned Arg, Gln, and Asn extend the entire distance of the minor groove of the nascent base pair and are predicted to be critically important to maintain DNA synthesis fidelity.

Allosteric regulatory switch for pol/exo activities

The conformational changes in the replication complex suggest a structural mechanism for pol/exo activity regulation by Pol γA–distal Pol γB interface. Mutations on both subunits in this region display severe loss of function in patients. In addition to the Pol γA R232G/H mutant holoenzymes as discussed above, the Pol γB G451E substitution, located in the same interface in the replication complex, shows equivalently decreased synthesis and increased nucleolytic activity defects as the Pol γA R232-containing holoenzyme (Longley et al., 2006; Lee et al., 2010a; Young et al., 2011). Pol γB G451E has a severely reduced association with Pol γA, which decreases the stimulation of catalytic activity and processivity (Longley et al., 2006; Young et al., 2011). Both G451 residues in the Pol γB dimer interact with Pol γA in the replication complex, and therefore, the substitution weakens the holoenzyme: The distal G451E causes a steric clash with E230 of Pol γA, whereas the proximal G451E weakens the interaction with the hydrophobic Pol γA L-helix (Fig 8). These studies strongly suggest that the interaction between Pol γA and the distal Pol γB monomer is critical for holoenzyme pol activity and that disruption of the subunit interface favors binding of the primer in exo. Lack of such interaction in the holoenzyme containing Pol γB G451E substitution makes it structurally resemble the apo enzyme.

To investigate this idea, we modeled the transition of Pol γ holoenzyme from the apo to the ternary complex, assuming that the DNA binding to the thumb was unchanged. The resulting complex positions the 3’-end of the primer away from pol and 16 Å closer to exo (Supplementary Fig S7). This exercise suggests that interactions between Pol γA and distal Pol γB monomer contribute to pol/exo
activity regulation. The model thus provides a structural explanation for how Pol γB can simultaneously regulate both pol and exo activities by serving as an allosteric regulator through dynamic interactions at the Pol γA–distal Pol γB interface. The ternary structures of Pol γ presented here are the first intact human multi-subunit replicating complexes. The structures reveal that the holoenzyme possesses a strong inter-subunit core that maintains the constant contacts between the catalytic subunit Pol γA and the proximal monomer of Pol γB, which are necessary for highly processive DNA synthesis, but leaves the Pol γA–distal Pol γB monomer interface dynamic, such that the interface can regulate both DNA synthesis and proofreading. Thus, the Pol γA–distal Pol γB monomer interface functions as an allosteric regulator for pol and exo activities. We show that the nucleoside inhibitor designed against HIV reverse transcriptase binds to Pol γ almost identically as the substrate, providing a structural basis for Pol γ's susceptibility to inhibition by dideoxynucleotides. These structures provide a literal and conceptual framework for assessing the impact of Pol γ mutations implicated in mitochondrial diseases.

Materials and Methods

Cloning and expression

Wild-type and exonuclease-deficient (exo<sup>-</sup>), D198A/E200A Pol γA, lacking residues 1–29 (mitochondrial localization sequence) and 10 of the 13 sequential glutamines (residues 43–52), were placed in pET22b(+) (Novagen). K-tract (K<sup>496</sup>KQKKAKKKVKK<sup>505</sup>) mutants were produced by substituting each lysine to cysteine in exo<sup>-</sup> Pol γA using the QuikChange kit (Stratagene), resulting in exo<sup>-</sup> Pol γA K496C, K498C, K499C, K501C, K502C, K504C, and K506C. Clones were transferred into the baculovirus genome using the shuttle vector pBacPak9 (Clontech). The C-terminal His-tagged proteins were synthesized in Sf9 insect cells (Invitrogen), grown at 27° C in SF900-II SFM growth medium, and infected at a density of 1–2 × 10<sup>6</sup> cells/ml. Cells were harvested 72 h post-infection. Selenomethionine-substituted exo<sup>-</sup> Pol γA was purified by a modification of our earlier protocol (Lee et al., 2009). In brief, SF900 II SFM growth medium (Invitrogen) was exchanged 12 h after infection with unsupplemented Grace's insect medium (Invitrogen), and 50 mg/l L-(+)-Se-Met (Acros) was added. Cells were harvested 48 h after medium exchange. Pol γB-D14 was expressed and purified as described (Lee et al., 2009). Selenomethionine-substituted Pol γB-D14 was expressed in E. coli B834(DE3) RIL (Novagen) in M9 minimal media supplemented with 100 mg/l L-(+)-Se-Met (Acros).

Protein purification

Pol γA wild-type and variants Pol γA exo<sup>-</sup>, K496C, K498C, K499C, K501C, K502C, K504C and K506C, and Pol γB wild-type and variant Δ14 were purified by sequential application to TALON (Clontech) for Pol γA exo<sup>-</sup> and Ni-NTA agarose (Qiagen) for Pol γB-Δ14, SOURCE S, and Superdex 200 columns (Lee et al., 2009). Selenomethionine-substituted exo<sup>-</sup> Pol γA and Pol γB-Δ14 were purified similarly. Holoenzyme used for crystallization is formed by combining exo<sup>-</sup> Pol γA...
Table 1. Data collection and refinement statistics.

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<td>0.79</td>
</tr>
<tr>
<td>Ramachandran favored (%)</td>
<td>95</td>
<td>99.3</td>
<td>99.0</td>
</tr>
<tr>
<td>Ramachandran outliers (%)</td>
<td>4.9</td>
<td>0.69</td>
<td>0.98</td>
</tr>
<tr>
<td>Clashscore</td>
<td>18.54</td>
<td>11.75</td>
<td>10.8</td>
</tr>
<tr>
<td>Average B-factor</td>
<td>58.30</td>
<td>53.66</td>
<td>61.57</td>
</tr>
<tr>
<td>Macromolecules</td>
<td>49.40</td>
<td>46.30</td>
<td>57.60</td>
</tr>
<tr>
<td>Nucleic acids</td>
<td>86.90</td>
<td>82.20</td>
<td>99.30</td>
</tr>
</tbody>
</table>

Statistics for the highest resolution shell are shown in parentheses.

and Pol γB–Δ4 at a 1:2 molar ratio and isolation on Superdex 200. The peak fractions corresponding to 240-kDa complex were pooled and verified by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were judged to be ~98% pure. Concentrations of Pol γA and Pol γB were determined spectrophotometrically using extinction coefficients: ε280 = 2.4379 × 10^4 M⁻¹ cm⁻¹ and 1.4388 × 10^4 M⁻¹ cm⁻¹, respectively.

Preparation and crystallization of the Pol γAB–DNA–nucleotide complex

Synthetic DNA oligonucleotides were obtained from Midland Certified (Midland, TX) and IDT (Coralville, Iowa). Nucleic acid scaffolds (Fig 1C) were mixed at equimolar concentrations of template and primer to a final concentration of 1 mM of primer/template DNA and were then annealed by heating to 95°C and slowly cooling to room temperature. ddCTP and dCTP were purchased from TriLink BioTechnologies (San Diego, CA). For crystallization, the exo⁻ Pol γA and Pol γB–Δ4 complex (40–65 μM) was formed with 1 mM CaCl₂, 1 mM ddCTP or dCTP, and 24/28-mer primer/template DNA (60–95 μM) and incubated on ice for ~15 min. Crystals were obtained using the sitting-drop method by mixing the complex at 1:1 volume ratio with a solution: 3% PEG 8000, 150 mM NaCl, 10 mM CaCl₂, 100 mM MES pH 6, 2 mM 2-mercaptoethanol, 0.8 M NDSB-201, 1–6% sucrose, and 2% Jeffamine M-600. Crystallization experiments were performed against a
well solution containing 3% PEG 8000, 150 mM NaCl, 10 mM CaCl$_2$, 100 mM MES pH 6, and 2 mM 2-mercaptoethanol at 20°C. Crystals grew to a maximum size of 0.4 × 0.2 × 0.2 mm within 2–4 days. Selenomethionine-substituted exo$^-\text{Poly(}A\text{)AA}-\text{DNA-ddCTP}$ was grown similarly except that 2-mercaptoethanol was 50 mM. Before freezing in liquid nitrogen, crystals were cryo-protected by adding a mixture of glycerol and MPD (<20% final) and 1 mM ddCTP/dCTP into the crystallization drop.

**Data collection, refinement, and structural determination**

X-ray diffraction data were collected at 100 K using synchrotron sources at Advanced Photon Source and Advanced Light Source. Single wavelength anomalous dispersion (SAD) diffraction data were collected at peak of the Se anomalous wavelength (0.979 Å) using the inverse beam method, and the native data were collected at 1.0 Å wavelength. Diffraction data sets were processed with HKL (Minor et al., 2006), and data collection statistics are shown in Table 1. The structures were determined using combined phases from molecular replacement and SAD. Forty-two (of 46 total) Se atoms in the holoenzyme were identified, and the combined phases gave a figure of merit 0.704. Phases were further improved by density modification. The final structure was refined using Phenix (Adams et al., 2010). The experimental map clearly illustrates density for the double-helical DNA structure (Supplementary Fig S8), and enabled rebuilding of the regions that had undergone structural changes induced by DNA binding. Disordered regions of the holoenzyme are mainly located in the N- or C-terminal and fingers regions. These regions include residues 30–76, 248–261, 317–342, 663–740, 996–1,051, and 1,230–1,239. The disordered regions in Pol $\gamma$B involve 116 aa in the dimer and again are the same in both structures. They include residues 26–67, 222–228, and 356–361 in chain B and 26–66, 220–226, and 356–367 in chain C.

**Kinetic assays**

Pre-steady state polymerization assays were carried out using RQF-3 Rapid Chemical Quench Flow instrument (KinTek Co.). Briefly, reactions were initiated by rapid mixing of a pre-incubated Pol $\gamma$BA-DNA complex (200 nM Pol $\gamma$A, 800 nM Pol $\gamma$B) and 50 nM primer/template DNA with $5^{32}$P-labeled primer with 1 mM dNTP, 10 mM MgCl$_2$ in buffer containing 50 mM Tris acetate pH 7.5, 50 mM potassium glutamate, 0.05% Tween-20, 5% glycerol, and 2 mM DTT. Reactions were incubated at 37°C before being quenched with 0.5 M EDTA. The resulting polymerization patterns were resolved using 24% denaturing PAGE and were visualized by autoradiography. The reactions were carried out with a 65-mer template (CATATTAATTGATATAAGCCCATATAATATTATATCATTGGCAA TCTTTCCACTAACCAGC) annealed to a 25-mer primer (GCCCTGTGTTAATGGAAGAGATTCTA). Each of the DNA bands up to 17 nt additions was quantified and summed to estimate the number of nucleotides added in each reaction time, and the slopes after one-nucleotide addition are provided as estimates of the DNA synthesis rates.

**Accession numbers**

The coordinates of the structures reported here have been deposited in the Protein Data Bank (accession numbers 4ZTU and 4ZT2).

**Supplementary information** for this article is available online:
http://emboj.embo.org

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**Author contributions**

MRS, VBK, CS, QM, Y-SL, and YWW designed and performed the experiments and analyzed the experimental results. SP and GP performed and analyzed the kinetic data. MRS, SP, and YWY wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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