

Structural Insight into Processive Human Mitochondrial DNA Synthesis and Disease-Related Polymerase Mutations

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SUMMARY

Human mitochondrial DNA polymerase (Pol γ) is the sole replicase in mitochondria. Pol γ is vulnerable to nonselective antiretroviral drugs and is increasingly associated with mutations found in patients with mitochondrialopathies. We determined crystal structures of the human heterotrimeric Pol γ holoenzyme and, separately, a variant of its processivity factor, Pol γ B. The holoenzyme structure reveals an unexpected assembly of the mitochondrial DNA replicase where the catalytic subunit Pol γ A interacts with its processivity factor primarily via a domain that is absent in all other DNA polymerases. This domain provides a structural module for supporting both the intrinsic processivity of the catalytic subunit alone and the enhanced processivity of holoenzyme. The Pol γ structure also provides a context for interpreting the phenotypes of disease-related mutations in the polymerase and establishes a foundation for understanding the molecular basis of toxicity of anti-retroviral drugs targeting HIV reverse transcriptase.

INTRODUCTION

DNA Pol γ , in contrast to the many nuclear DNA polymerases (DNAPs) that have specialized functions, is solely responsible for DNA replication and repair in mitochondria. Human mitochondrial DNA (mtDNA) codes for a subset of proteins involving the oxidative phosphorylation electron transfer chain, plus two ribosomal rRNAs and 22 transfer RNAs (tRNAs) (Anderson et al., 1981). Accordingly, Pol γ is critically important for mtDNA maintenance, cellular energy supply, and viability. Reduced activities of Pol γ lead to mtDNA depletion and impairment of cellular metabolism. Mutations affecting the catalytic subunit Pol γ A cause a wide range of genetic syndromes with disease manifestations such as progressive external ophthalmoplegia (PEO, a disorder characterized by slow paralysis of external eye muscle and exercise intolerance), myopathy, epilepsy,

neonatal hypotonia, encephalopathy, and Alpers' syndrome (a fatal childhood disease leading to brain and liver failure). In animal studies, homozygous mice with a proofreading-defective mutant Pol γ A exhibit increased accumulation of point and deletion mutations in mtDNA, as well as premature aging and a reduced life span (Trifunovic et al., 2004). To date, Pol γ mutants have been implicated in more than 30 human diseases (Zeviani and Di Donato, 2004; Chinnery and Zeviani, 2008; Wallace, 2005). The clinical manifestations of many mutations are perplexing as they can be both autosomal dominant and recessive. Understanding the pathology of mitochondrial disorders can thus be a challenge.

Human Pol γ is known to be more susceptible than nuclear DNA polymerases to inhibition by certain nucleoside reverse transcriptase inhibitors (NRTIs) that target HIV; Pol γ is therefore probably responsible for most cellular toxicity of this class of antiviral drugs. The basis for the high susceptibility of Pol γ to inhibition by NRTIs has thus far been limited to modeling Pol γ with bacteriophage T7 DNAP. The active sites of Pol γ and HIV reverse transcriptase (RT) may exhibit features not found in nuclear DNAPs. However, drug efficacy against HIV is not well correlated with cellular toxicity: Some NRTIs (e.g., zalcitabine [ddC], didanosine [ddI], and stavudine [d4T]) are potent inhibitors of both HIV RT and Pol γ , causing both time-dependent and dose-dependent decreases in mtDNA content and secondary cellular toxicity, whereas others (e.g., tenofovir [PMPA] and abacavir [CBV]) are more selective for HIV RT (see review by Kohler and Lewis, 2007). These observations suggest that significant differences exist in the kinetics of NRTI incorporation into DNA by Pol γ and HIV RT and/or their active-site architectures. Such differences can be exploited in the rational design of more selective antiviral agents.

Pol γ , like other DNA replicases, has a catalytic subunit, Pol γ A, which possesses both polymerase and proofreading exonuclease activities, and an accessory subunit, Pol γ B, which increases enzyme processivity. Unlike other processivity factors that enhance processivity by increasing enzyme affinity for template DNA, Pol γ B enhances processivity by simultaneously accelerating polymerization rate and suppressing exonuclease activity, in addition to increasing affinity for DNA (Johnson and Johnson, 2001). Reduced exonuclease activity was suggested to help maintain the integrity of the replisome at mitochondrial

Table 1. Statistics of Data Analysis and Structural Refinement

	Pol γ Holoenzyme			Pol γ B- Δ I4
	Native	Se-Met Derivative	K ₂ OsO ₄ Soaked	Native
Data Collection				
Resolution (Å)	50.0–3.2	50.0–4.0	50.0–4.0	50.0–3.3
Wavelength (Å)	0.979	0.979	1.140	1.140
Space group	P3 ₂	P3 ₂	P3 ₂	P4 ₁
Cell dimensions				
a, b, c (Å)	138.4, 138.4, 226.4	138.94, 138.94, 227.35	139.25, 139.25, 227.70	64.42, 64.42, 260.64
α , β , γ (°C)	90.0, 90.0, 120.0	90.0, 90.0, 120.0	90.0, 90.0, 120.0	90.0, 90.0, 90.0
Unique reflections	76,667	41,728	41,530	11,917
Completeness ^a (%)	100 (100)	96.3 (82.4)	94.8 (87.9)	99.2 (94.7)
Redundancy	5.7 (5.7)	3.8 (3.7)	3.9 (3.7)	8.2 (3.8)
R _{Linear} ^b	0.09 (1.00)	0.072 (0.658)	0.070 (0.622)	0.167 (0.511)
I/ σ I	21.5 (2.3)	19.5 (3.0)	23.3 (1.8)	5.8 (2.0)
SAD FOM density modification on combined phases	0.72	0.35	0.40	
Refinement				
R _{work} ^c (%)	28.4			25.7
R _{free} ^d (%)	30.3			29.4
Number of amino acids	1,850			358
RMS deviations from ideal values				
Bond (Å)	0.0108			0.0090
Angle (°)	1.97			1.71

^a Values in parentheses are for the highest-resolution shell.

^b $R_{\text{Linear}} = \sum |I_i - \langle I \rangle| / \sum I_i$ where I_i is the i^{th} measurement and $\langle I \rangle$ is the weighted mean of all measurements of I .

^c $R_{\text{work}} = \sum_{hkl} |F_{\text{obs}}(hkl) - F_{\text{calc}}(hkl)| / \sum_{hkl} |F_{\text{obs}}(hkl)|$ for reflections in the working data set.

^d R_{free} is the same as R_{work} for 5% of data randomly omitted from refinement.

replication forks (Farge et al., 2007). Structurally, Pol γ B resembles class II aminoacyl tRNA synthetases and differs significantly from other processivity factors, including sliding clamps and thioredoxin. The structural basis for the enhanced DNA synthesis processivity by Pol γ B is unknown.

Two mechanisms of mtDNA synthesis have been proposed. A conventional synchronous mode where leading and lagging-strand synthesis occur simultaneously (Yang et al., 2002), and a displacement mode where synthesis initiating from the O_H origin displaces the parental H strand to form a D loop. Only when the newly synthesized H strand DNA crosses a second origin (O_L) does initiation of L strand synthesis occur. The nascent H and L strands are therefore extended asymmetrically (Clayton, 1982). This model was recently modified to allow initiation of L strand synthesis from a number of origins in addition to O_L (Brown et al., 2005).

Here, we report a crystal structure of human DNA Pol γ holoenzyme. The holoenzyme is a heterotrimer containing one Pol γ A subunit and a dimeric Pol γ B subunit. The Pol γ A active-site domain adopts a canonical polymerase configuration. Between the *pol* and *exo* domains lies a spacer domain containing a unique fold that interacts primarily with only one Pol γ B monomer. This study provides a structural basis for the processivity enhancement in the holoenzyme by the accessory subunit Pol γ B and lays a foundation for understanding the mechanisms

of antiviral drug toxicity and mutant Pol γ -related human diseases.

RESULTS AND DISCUSSION

Structure of the Catalytic Subunit

Successful crystallization of the Pol γ holoenzyme required altered forms of both subunits. An exonuclease-deficient mutant of Pol γ A was crystallized with the deletion mutant Pol γ B- Δ I4, which lacks a four-helix bundle at the dimer interface (Carrodeguas et al., 2001; Yakubovskaya et al., 2006). DNA polymerase activity (see the Supplemental Experimental Procedures and Figure S1 available online) of the enzymes used for crystallization is comparable to the activity of the *exo*⁻ holoenzyme containing wild-type Pol γ B (Johnson et al., 2000; Yakubovskaya et al., 2006). The structure of Pol γ was determined to 3.2 Å resolution. Phases were calculated by combining those from single-wavelength anomalous diffraction using selenomethionine-substituted Pol γ A and osmium-derivatives of holoenzyme, and molecular replacement using human Pol γ B (Fan et al., 2006) as a search model. Density modification applied to the initial combined phases significantly improved the quality of electron density maps; the structure was refined to an R_{factor} of 28.4% and R_{free} 30.3%. Statistics for data collection and refinement are shown in Table 1.

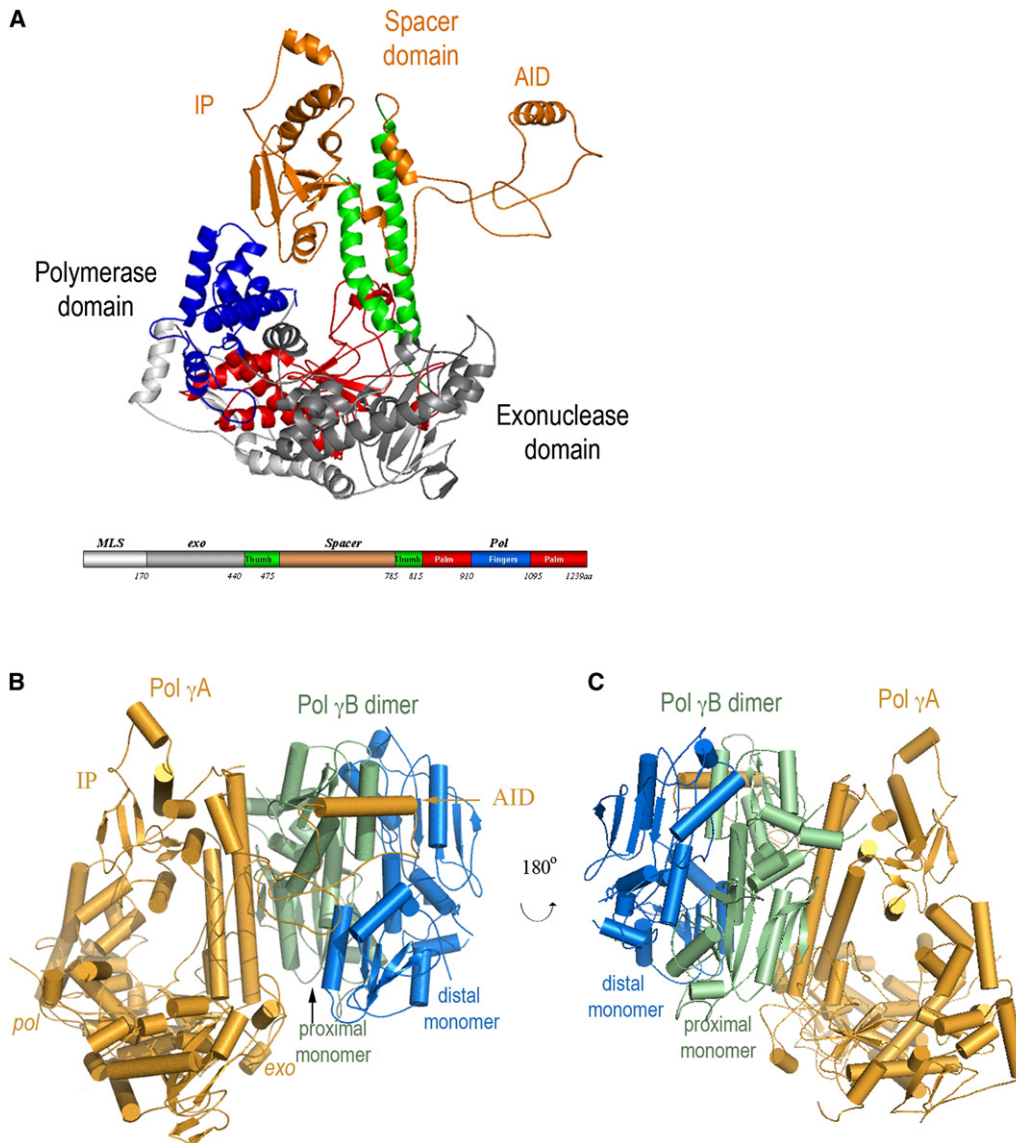


Figure 1. Structure of Pol γ

(A) Structure of Pol γ A. The *pol* domain shows a canonical “right-hand” configuration with thumb (green), palm (red), and fingers (blue) subdomains and the *exo* domain (gray). The spacer domain (orange) presents a unique structure and is divided into two subdomains. Domains are shown in a linear form where the N-terminal domain contains residues 1–170; *exo*, 171–440; spacer, 476–785; and *pol*, 441–475 and 786–1239. All figures are made with Pymol (DeLano, 2002). (B and C) Structure of the heterotrimeric Pol γ holoenzyme containing one catalytic subunit Pol γ A (orange) and the proximal (green) and distal (blue) monomers of Pol γ B. Pol γ A primarily interacts with the proximal monomer of the dimeric Pol γ B.

The catalytic subunit Pol γ A contains domains for exonuclease (*exo*) and polymerase (*pol*) activities separated by a linker or spacer. Pol γ A adopts the canonical polymerase “right-hand” configuration with subdomains of “fingers,” “palm,” and “thumb” that bind template DNA and substrate nucleotide triphosphate, as well as catalyze phosphodiester bond formation (Figure 1A). The conserved aspartic acids, D⁸⁹⁰ and D¹¹³⁵, are located in the palm at positions consistent with their known roles in catalysis.

Although the overall fold of Pol γ A confirms its classification as a member of the Pol I family, many features of Pol γ A are clearly

absent in the other enzymes. Most obviously, Pol γ A possesses a large spacer domain (~400 residues) between the *exo* and *pol* domains. In light of the atomic structure, it was necessary to modify the earlier sequence homology-based domain definition of Pol γ A because a portion of the originally assigned spacer is actually the thumb subdomain (Figure 1).

The spacer domain is spatially far from the *exo* and *pol* domains and connects to them only through the long helices of the thumb subdomain. The spacer has two obvious subdomains, a globular *intrinsic processivity* (IP) subdomain (residues 475–510 and 571–785) and an extended *accessory-interacting*

determinant (AID) subdomain (residues 511–570) that reaches more than 50 Å away from the main body of Pol γ A (Figure 1). We will show that the IP subdomain explains the intrinsic processivity of Pol γ A and the AID subdomain forms an important interface with Pol γ B that is essential for increased processivity of the holoenzyme. A homology search against structures in the protein data bank yields a Z score of ~ 0.2 , suggesting that the spacer domain has a novel fold (Holm and Sander, 1996).

Holoenzyme Formation and Subunit Interface

In agreement with solution studies (Figure S2), the crystal form of Pol γ is a heterotrimer containing one catalytic Pol γ A subunit (135 kDa) and a dimeric Pol γ B- Δ I4 (2×50 kDa) with a subunit contact area ~ 3500 Å². The deleted helical bundle in Pol γ B is distance from the subunit interface and not involved in the subunit interaction with Pol γ A (Figure S3).

The trimeric holoenzyme shows unequal subunit interactions: Pol γ A primarily interacts with only one monomer of the Pol γ B dimer (Figures 1B and 1C). An electron cryomicroscopic analysis of Pol γ at ~ 17 Å resolution came to a similar conclusion (Yakubovskaya et al., 2007), although the subunit interface, modeled onto the crystal structure of Pol γ B, appears to be different from that found in the crystal structure of the holoenzyme. The asymmetrical interactions of Pol γ A with the proximal monomer of the Pol γ B dimer suggests that a monomeric accessory subunit could be fully functional, which is indeed the case for *Drosophila* Pol γ (Wernette and Kaguni, 1986).

An asymmetric heterotrimer provokes the question whether the human holoenzyme could also be an A₂B₂ tetramer, which could position two polymerases at a replication fork—a necessary requirement for coupling leading- and lagging-strand DNA synthesis. We thus modeled a tetrameric enzyme, adding a second Pol γ A to the heterotrimer by following the symmetry operator constraining the Pol γ B dimer. The modeled tetramer reveals steric clashes between the AID subdomains of the two Pol γ As that preclude formation of an actual A₂B₂ tetramer. Although this result could be used as support for the displacement model of mtDNA replication (Clayton, 1982), it should be noted that a trimeric holoenzyme does not rule out other mechanisms for positioning two polymerases at a replication fork (Yang et al., 2002).

The only contact regions between Pol γ A and the distal Pol γ B monomer are a salt bridge (2.8 Å) between R²³² of Pol γ A and E³⁹⁴ of Pol γ B (Figure 2E) and a weak van der Waals contact (5.3 Å) between Pol γ A Q⁵⁴⁰ and Pol γ B R¹²² (Figure 2G). A R232G substitution, together with T251I and P587L, has been reported in a child with neonatal hypotonia and hepatic failure (Ferrari et al., 2005). Healthy siblings of this patient carried T251I and P587L, suggesting that the R232G substitution is associated with disease. This clinical case suggests that either R²³² is critical for Pol γ A activity or that the contact between Pol γ A and the distal Pol γ B monomer is important for human holoenzyme function.

In contrast to its limited interaction with the distal monomer, Pol γ A makes extensive interactions with the proximal Pol γ B monomer (Figure 2B). Examination of the subunit interface shows two major areas of hydrophilic interactions: between

Pol γ B R²⁶⁴, K³⁷³, and D⁴⁵⁹ and the Pol γ A thumb domain area (E⁴⁵⁴-D⁴⁶⁹, and R⁵⁷⁹) (Figure 2A) and between Pol γ B (D²⁵³ and D²⁷⁷) and Pol γ A (K¹¹⁹⁸, R¹²⁰⁸, and R¹²⁰⁹). In addition, hydrophobic interactions occur between a Pol γ B hydrophobic core (V³⁹⁸-L⁴⁰⁶, V⁴⁴¹-L⁴⁵⁵) in the C-terminal region and Pol γ A AID subdomain L helix (V⁵⁴³-L⁵⁵⁸) (Figure 2C). Pol γ A AID causes the steric clash in the modeled A₂B₂ tetramer; in the absence of stabilizing hydrophobic forces for a second Pol γ A monomer, the holoenzyme is therefore heterotrimeric. In turn, the modeling suggests that the hydrophobic interface is dominant in subunit interaction.

To test this idea, we made four L helix mutants: L549N and L552N, which reduce hydrophobicity with only minimal structural alteration, a complete deletion (Δ L), and K553A (Figures 2C and 2D). The latter change nullifies the electrostatic interaction between Pol γ A K⁵⁵³ and E⁴⁰⁴ of Pol γ B. In the absence of Pol γ B, all mutants exhibited activities comparable to wild-type Pol γ A, demonstrating that the alterations do not disrupt the active site (Figure 3 and the Supplemental Experimental Procedures). The L helix is therefore not directly involved in DNA binding, in agreement with our structural observation that the entire AID subdomain, which includes the L helix, is connected to the body of Pol γ A by flexible linkers, and would likely be disordered in the absence of Pol γ B.

However, the addition of Pol γ B reveals a very different outcome. In 90 mM salt, the Pol γ A Δ L deletion not only severely lowers the stimulation by Pol γ B, but it also reduces the length of product DNA (Figure 3). In contrast, the L helix missense mutants had little effect. At higher ionic strength (190 mM salt), wild-type Pol γ A is inactive but retains considerable activity when complexed to Pol γ B. However, holoenzymes containing Pol γ A L549N, L552N, or the Δ L mutant are completely inactive in high salt. This salt-dependent reduction of activity strongly suggests that the mutations disrupt the hydrophobic interactions between Pol γ A and Pol γ B. Interestingly, holoenzyme containing K553A appeared equally active as that of the wild-type; simply disrupting one electrostatic interaction between Pol γ A and Pol γ B therefore has only a minor effect when the hydrophobic interactions in this region are preserved. These data provide strong support to our conclusion that hydrophobic interactions between the Pol γ A L helix and the C-terminal domain of Pol γ B are the dominant attractive forces that stabilize the AID subdomain so that it can support processive DNA synthesis by the holoenzyme.

Although there is low overall sequence similarity in the Pol γ A L helix, residues involved in the hydrophobic interaction with Pol γ B are conserved and are predicted to be α -helical in the mouse, *Drosophila*, and *Xenopus* proteins (Figure 2D). The hydrophobic residues on the respective Pol γ B proteins are also conserved. Conservation of interacting residues in both subunits suggests that all these mitochondrial holoenzymes likely possess a common subunit interface that involves an AID subdomain.

Interestingly, the spacer containing AID is not only missing in the nonprocessive DNA polymerases, it is also largely absent in fungal Pol γ A (Figure S4). Perhaps not coincidentally, these enzymes also lack a Pol γ B-type processivity factor. It seems likely that the ancestor of human Pol γ A first acquired a spacer domain, which then allowed a Pol γ B-like protein to interact,

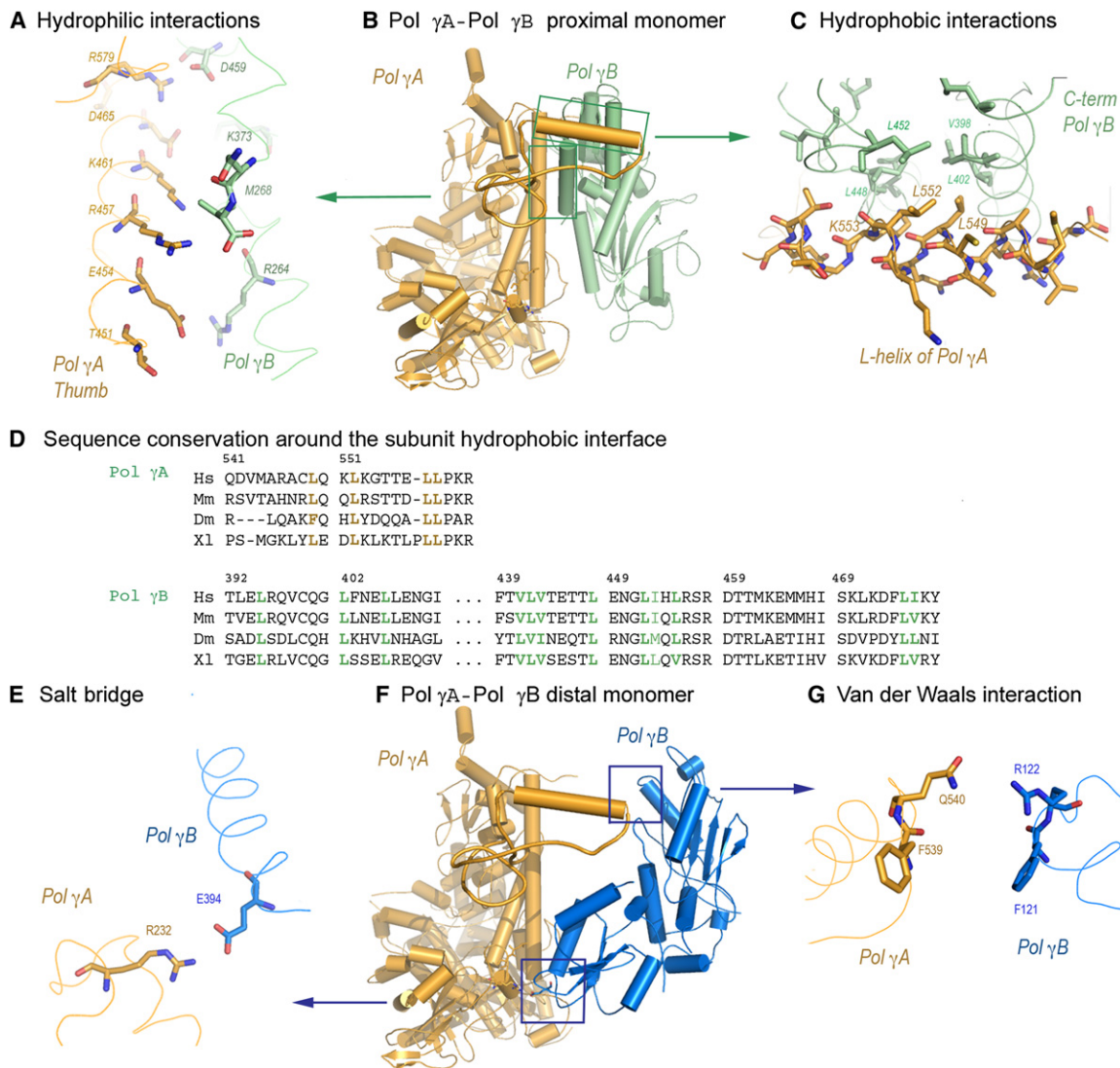


Figure 2. The Major Pol γ Subunit Interfaces

(A–C) Pol γ A-Pol γ B proximal monomer interactions. The distal monomer is omitted for clarity.

(A) Charge-charge interactions between the thumb domain of Pol γ A and the C-terminal domain of Pol γ B.

(B) L-shaped support between Pol γ A and the proximal monomer of Pol γ B.

(C) Hydrophobic interactions between the L helix of Pol γ A and a hydrophobic core of Pol γ B. Mutated residues L⁵⁴⁹, L⁵⁵², and K⁵⁵³ are shown.

(D) Sequence alignments of residues involved in hydrophobic interactions between Pol γ A and Pol γ B.

(E–G) Pol γ A-Pol γ B distal monomer interactions. The proximal monomer is omitted for clarity.

(E) The salt-bridge (2.8 Å) between Pol γ A R²³² and the distal Pol γ B E³⁹⁴.

(F) Pol γ A-Pol γ B distal monomer.

(G) The weak van der Waals interaction (5.3 Å) between Pol γ A and the distal Pol γ B monomer.

and the interacting domains subsequently co-evolved to increase the processivity of synthesis.

Processivity of the Holoenzyme

The ability to catalyze processive synthesis is essential for replisomal complexes. However, most replicases have little processivity by themselves, generally synthesizing 15 nt or less per primer-binding event. Pol γ A is somewhat exceptional in that it can synthesize ≥ 100 nt (Graves et al., 1998; Johnson et al.,

2000). However, when bound to their accessory proteins, all replicases exhibit high processivity, synthesizing thousands of nucleotides without dissociation (Hori et al., 1979; McHenry and Kornberg, 1977).

To begin to understand the mechanism of Pol γ processivity, we modeled a Pol γ -DNA complex by docking the primer-template DNA from the T7 DNAP-DNA complex (Briebe et al., 2004) onto the Pol γ holoenzyme after superimposing the active-site domains of the two polymerases. Despite strong

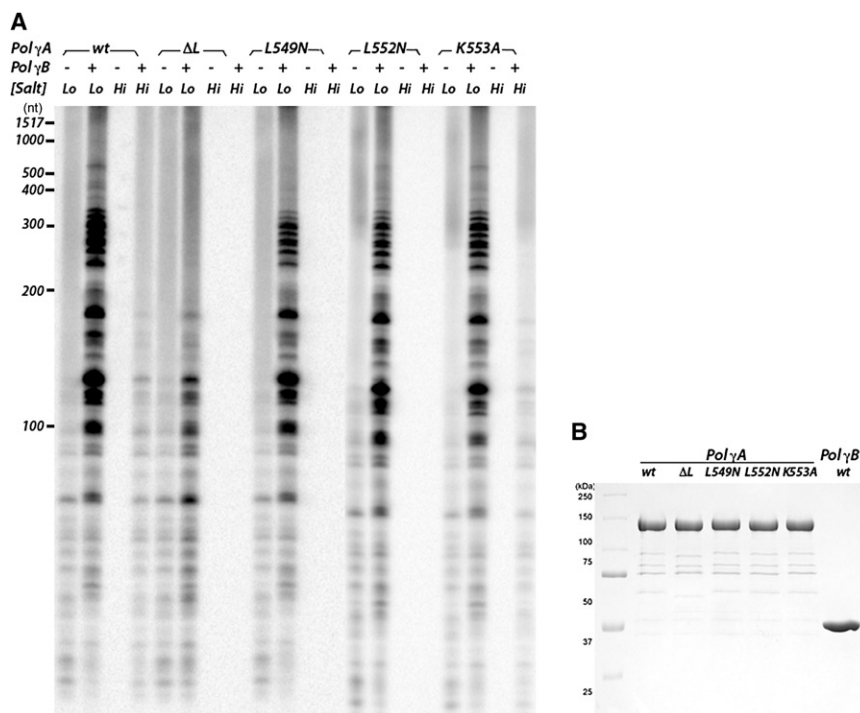


Figure 3. Activity of Pol γ A Mutants

(A) DNA synthesis activities of Pol γ A mutants Δ L, L549N, L552N, and K553A were assayed without or with Pol γ B at different ionic strengths. “Lo” and “Hi” denote 90 mM and 190 mM NaCl, respectively. Denatured products were separated by electrophoresis on an acrylamide gel. (B) The purity of Pol γ A wild-type and mutant proteins are shown after SDS-PAGE.

circumstantial evidence for a bacterial origin of mitochondria, the catalytic subunit Pol γ A is more closely related to bacteriophage T7 DNAP than to any bacterial replicase. The two active-site domains show high similarity and superimpose with an rmsd of 2.3 Å (Figure S5), which enables modeling an enzyme-DNA complex with confidence.

The docked DNA is cradled by a positively charged channel formed by the thumb, palm, and fingers of Pol γ A (Figures 4 and 5); this *pol* domain makes contact with \sim 10 bp of template DNA that includes the primer terminus. In Pol γ A holoenzyme, the hydrophobic interaction between Pol γ B and the L helix of the AID subdomain exposes a surface on Pol γ A containing a high density of positively charged residues (496 KQKKAKKV KK 505 , termed the K tract, Figures 4B and 5A). The K tract interacts with the negatively charged phosphodiester backbone of DNA upstream to that bound in the *pol* domain, thus increasing the contact of holoenzyme to DNA to \sim 25 bp (Figure 5A). The modeled complex reveals no direct contact between Pol γ B and primer-template DNA, suggesting that the increased DNA-binding affinity of holoenzyme by Pol γ B is mediated entirely through Pol γ A. In support of this conclusion, weakening the hydrophobic interaction between the AID subdomain and Pol γ B, which likely causes additional flexibility of the K tract, reduces activity and processivity of holoenzyme (Figure 3). This model now provides a structural basis for the known increased affinity of holoenzyme, relative to Pol γ A, to DNA.

Further evidence supporting the model includes limited proteolysis of the holoenzyme with and without primer-template DNA. Comparison of protease digestion patterns suggests several regions of Pol γ are protected by DNA (Figure 4A). Taking advantage of the fact that both Pol γ A and Pol γ B are His tagged at their C termini, western blot analyses with anti-His antibody

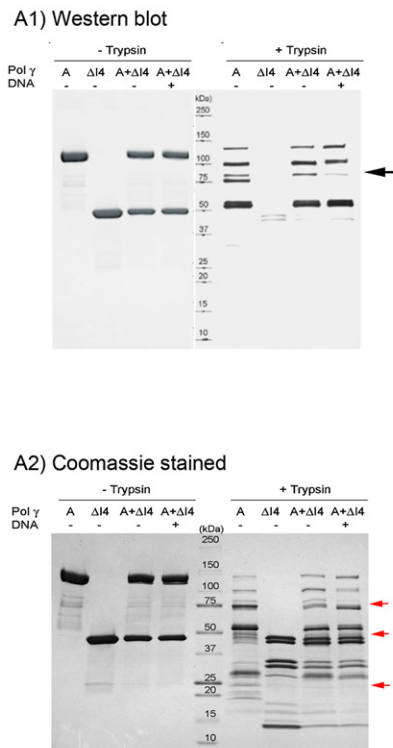
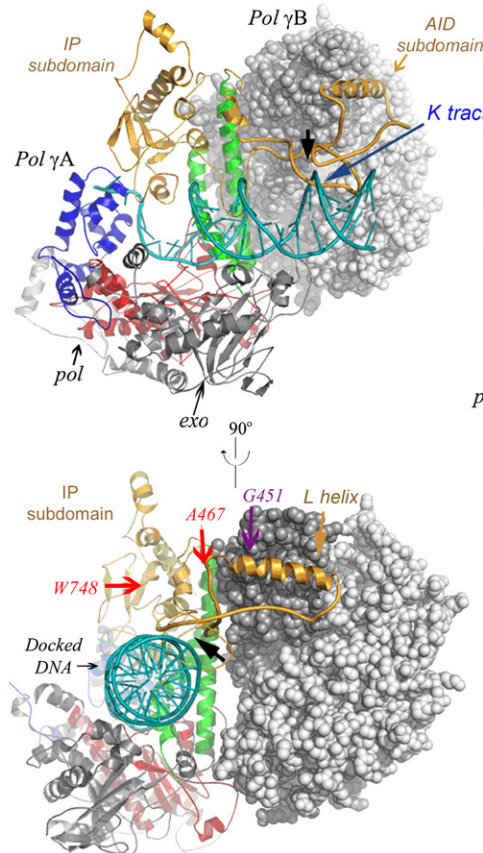
aided identification of proteolytic fragments. Digestion of the catalytic subunit Pol γ A generated three C-terminal major fragments with apparent molecular masses of 105 kDa, 77 kDa, and 56 kDa, and a minor fragment of 84 kDa. The 77 kDa C-terminal fragment is absent when Pol γ B is present, suggesting that the region around residue 560, corresponding to AID domain L helix of Pol γ A, is involved in subunit interactions. When holoenzyme is bound to DNA, the intensity of the 84 kDa fragment is significantly reduced, suggesting that it contains a DNA-binding site. The DNA-protected

region lies near residue 500, corresponding to the K tract, which is in good agreement with the modeled Pol γ -DNA complex. Other differentially protease-sensitive bands are apparent in Figure 4A, but the protected regions cannot be unequivocally identified.

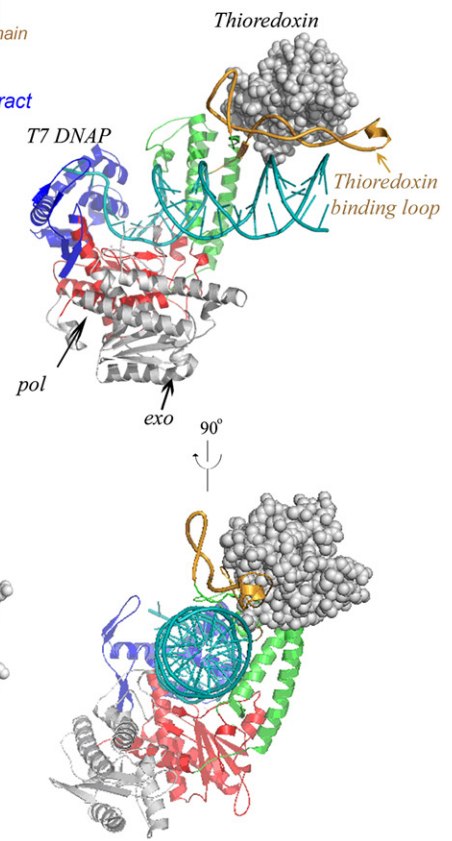
Although Pol γ A and T7 DNAP's catalytic subunit, gene 5 protein (gp5), have similar active-site domains (Figures 4 and S5), simply referring to Pol γ A as a T7-like DNAP is only partially correct. Pol γ A is more processive than T7 gp5 in the absence of an accessory protein (Graves et al., 1998; Tabor et al., 1987), and the corresponding processivity factors are different in structure and function. *E. coli* thioredoxin, the processivity factor for gp5, is a 104 aa monomeric protein, whereas the dimeric Pol γ B contains 970 aa. Functionally, the two accessory proteins use different mechanisms for processivity enhancement: thioredoxin increases T7 DNAP affinity for primer/template DNA by decreasing k_{off} without affecting the rate of nucleotide incorporation, therefore effectively prolonging the time of each binding event (Huber et al., 1987). In contrast, Pol γ B accelerates the polymerization rate, thereby increasing the number of nucleotides incorporated per binding event (Johnson et al., 2000).

The model suggests that the relatively high processivity of Pol γ A in the absence of Pol γ B can be attributed to the IP subdomain of the spacer, which provides a binding site for the upstream primer-template DNA duplex (Figure 4B). An IP subdomain is not found in T7 gp5 (Figure 4C), precluding significant DNA synthesis in the absence of thioredoxin. The modeled DNA-holoenzyme complex can also fully explain the remarkable ability of Pol γ B to increase polymerase and decrease exonuclease activity simultaneously. Binding of Pol γ B to Pol γ A causes the primer terminus to be preferentially bound in the

A Proteolysis digestion

B Human Pol γ holoenzyme

C T7 DNAP-DNA complex

**Figure 4. Comparison of a Modeled Pol γ -DNA Complex with that of the T7 DNAP-DNA Complex**

(A) Limited proteolysis of Pol γ visualized after SDS-PAGE by western blot or by Coomassie blue staining.

(B) Modeled Pol γ -DNA complex containing Pol γ A (shown in ribbons), Pol γ B (gray CPK), and a docked DNA (blue ribbons) shows that IP and AID subdomains enhance DNA binding. Mutations and the region protected by DNA from proteolytic digestion (black arrow) are indicated.

(C) Crystal structure of T7 DNAP-DNA complex containing gp5 (ribbons), thioredoxin (gray CPK), and a primer-template DNA (blue ribbons).

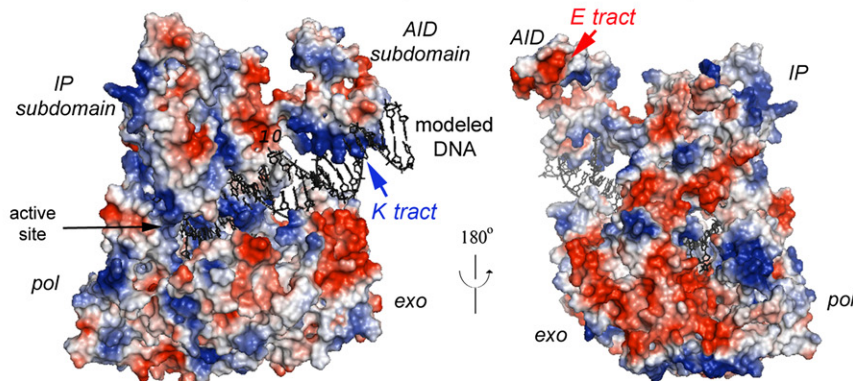
pol rather than the *exo* site, probably because less DNA bending is required.

Furthermore, Pol γ B may function beyond processivity enhancement and play a role in replisome assembly. Despite it not contacting DNA directly in the modeled Pol γ -DNA complex, Pol γ B is able to bind DNA. This activity, however, appears to be important only for DNA synthesis on duplex templates. Changing the positively charged residues ³⁶³RKK³⁶⁵ and, separately, ³²⁸RK³²⁹ to alanines abolishes DNA-binding activity. ³⁶³RKK³⁶⁵ is part of the I7 loop (residues 356–369) that contains several positively charged residues; the corresponding region in threonyl-tRNA synthetase (structurally homologous to Pol γ B) is involved in tRNA binding. Deletion of I7 abolishes Pol γ B DNA binding (Carrodeguas et al., 2001). Nonetheless, mutant Pol γ B-containing holoenzyme retains normal activity in copying single-stranded DNA (Farge et al., 2007), but is defective in replicating duplex DNA in the presence of SSB and helicase. These data are consistent with the substitutions lying distant from the primer-template channel (Figure S3). Other Pol γ B mutants are analyzed in the Supplemental Data.

Structurally, the I7 region in both monomers is disordered in the apo Pol γ B structure, but in the proximal monomer becomes ordered in the holoenzyme structure. To assess whether the distal monomer remains disordered because of asymmetrical interaction between dimer Pol γ B with Pol γ A or whether it is due to the loss of the four-helical bundle in the Pol γ B- Δ I4 mutant, we crystallized and determined its structure to 3.3 Å resolution. Deletion of the helical bundle changed the crystal packing from that of the wild-type, but the structure still remained a perfect dimer (Figure S6). Aside from the deleted helical bundle, the structure of Pol γ B- Δ I4 is essentially identical to that of the wild-type protein. As in the wild-type protein, the two I7 regions are disordered in Pol γ B- Δ I4, indicating that the differential folding of the Pol γ B loops in the holoenzyme is not a function of the deletion. Most likely, the ordering of I7 in holoenzyme is a direct consequence of the Pol γ B proximal monomer interacting with Pol γ A.

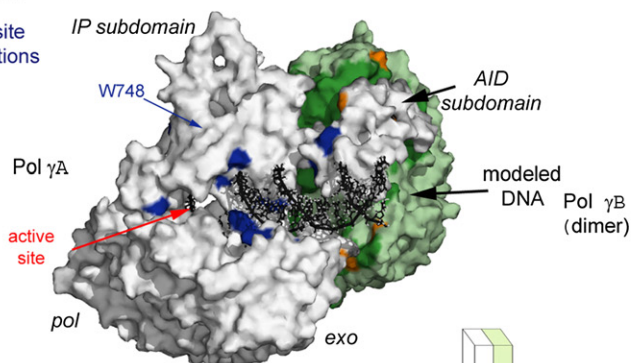
Although we do not have sufficient data to model the replisome, the electrostatic surface potential of Pol γ A is informative. As expected, the putative DNA-binding channel is lined

A Electrostatic surface potential of Pol γ A with modeled primer-template DNA

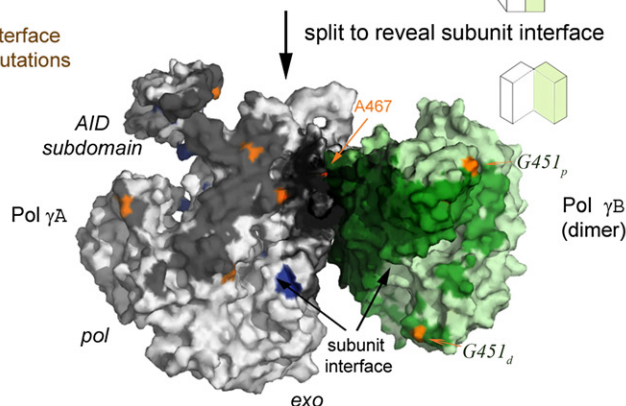


B Pol γ mutations

i) DNA binding site (class II) mutations



ii) subunit interface (class III) mutations



with positively charged residues, but the opposite surface of the protein presents a large negatively charged region near the exo domain, and the tip of the AID subdomain also contains four sequential glutamates (⁵³⁵EEEE⁵³⁸, E tract; Figure 5A). The human mitochondrial helicase, Twinkle, has a highly positively charged C-terminal region that could contact one of these regions. If the interaction is through the negatively charged E tract in the replisome, Twinkle would be positioned in a location close to that of the ³⁶³RKK³⁶⁵ and ³²⁸RK³²⁹, residues important in Twinkle-dependent replication of duplex DNA. In support of this idea, the corresponding region in T7 DNAP, the thioredoxin-binding domain, contacts the C terminus of T7 helicase via charge-charge inter-

Figure 5. Pol γ A Charge Distribution and Mutational Analysis

(A) The electrostatic surface potential of Pol γ A is shown in two views, with positively charged regions highlighted in blue and negatively charged regions in red. A primer-template DNA duplex is modeled with black sticks.

(B) Locations of Pol γ mutants. (i) Pol γ A mutants in the DNA-binding channel (class II) are highlighted in blue. (ii) A split open view of the subunit interface showing class III mutants (orange) affecting Pol γ A and Pol γ B (subscripts denote the proximal and distal monomer) interaction.

actions, and this interaction is essential for replisome assembly (Hamdan et al., 2005).

Distinct Mode of Substrate Binding

Polymerases are classic examples of enzymes using the induced-fit mechanism to achieve substrate specificity. The apo form of most DNA Pol I members adopt an “open” conformation, where catalytically important residues on the fingers domain lie some distance away from the palm active-site residues. After DNA binding, the fingers domain undergoes structural changes to the “closed” conformation, enabling the enzyme to align the important residues for catalysis and substrate selection. The desolvation effect generated by the conformational changes further enhances substrate specificity (Fersht, 1985; Petruska et al., 1986). This mechanism is utilized by all high-fidelity polymerases and is apparently absent in error-prone polymerases. Interestingly, the fingers domain of apo Pol γ A directly abut where the primer terminus will be positioned, and apo Pol γ thus presents a partially “closed” conformation. Nevertheless, the catalytically important residues on

the fingers domain are still too far for catalysis, a rotational conformational change is still necessary after DNA binding to position the catalytic residues correctly. The configuration of the Pol γ A active site suggests that the conformational change in the fingers domain is coaxial with the duplex DNA, whereas it is perpendicular in other DNAPs.

Apo Pol γ A further differs from other DNAPs in the active site by containing a small subdomain (residues 1050–1095) that partially blocks the DNA-binding channel. This type of subdomain has not been described before in other DNAPs, but the apo form of phage N4 virion miniRNAP contains a similar subdomain that rotates out of the channel after DNA binding (Gleghorn et al., 2008). If Pol γ indeed undergoes different conformational

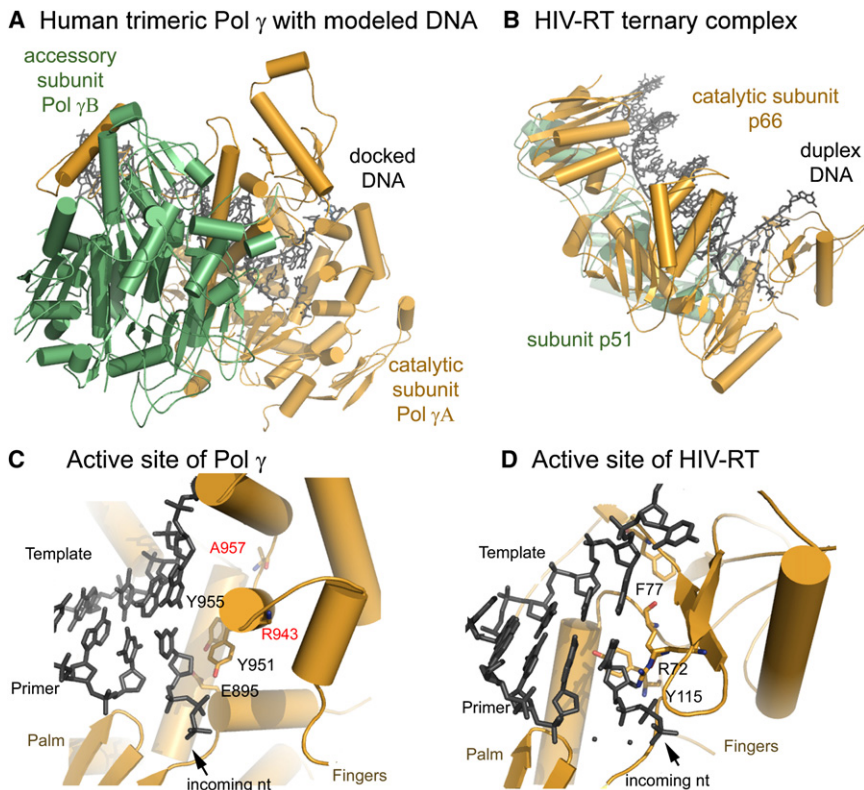


Figure 6. Structural Differences between Human Pol γ and HIV Reverse Transcriptase

Overall structures of the two enzymes illustrate differences in the interaction between the catalytic and accessory subunits, and the modes of DNA binding (A and B). The active site of Pol γ A, comprised of an α -helical fingers domain (C), differs significantly from that of HIV RT (D), where the incoming dNTP binding site is comprised of β sheet fingers.

ered, not all substitutions shown in Table S1 have been shown biochemically to adversely affect enzyme activity.

A cluster of substitutions found in PEO patients (Lamantea et al., 2002; Van Goe-them et al., 2001), R943H/C, Y955C, and A957P/S, fall into class I. A modeled Pol γ -DNA complex with an incoming dNTP (Figure 6C) suggests that R⁹⁴³ may form a charged interaction with the triphosphate moiety. Substitution of the positively charged arginine should drastically reduce affinity for incoming nucleotides. Y⁹⁵⁵ abuts the templating base, in a position that is consistent with its known multifunctional roles in other Pol I family enzymes, including primer-template

changes than other DNAPs when binding DNA, it may then use different mechanisms to ensure replication fidelity. These differences could reflect the high susceptibility of Pol γ to NRTIs.

Pol γ Mutations and Human Diseases

The critical functions of Pol γ in mtDNA synthesis may, in part, rationalize the diversity and progressive effects of Pol γ mutations in degenerative human disorders. Many severe human diseases have been correlated with mutations affecting Pol γ (<http://tools.niehs.nih.gov/polg/>), and several mutant proteins have been characterized. Using the Pol γ structure, we can now begin to rationalize the effects of some of these substitutions. Previous mutational analyses using the T7 DNAP structure as a model have successfully explained mutations predicted to affect the active site (Graziewicz et al., 2004), and as we have shown here the active sites of the two proteins are homologous. However, mutations located in the spacer region—as originally defined by sequence alignment with *E. coli* Pol I—show diverse biochemical behaviors. The structure of Pol γ now enables us to use a structure-based domain definition and distinct subdomain functions to reanalyze the effects of these mutations.

We divided all reported disease-associated Pol γ mutations into three classes (Table S1). Class I contains active-site mutations that all result in reduced enzyme catalysis; class II includes substitutions located in the putative DNA-binding channel, thus reducing DNA-binding affinity directly. Class III contains subunit interface substitutions that disrupt the subunit interaction between Pol γ A and Pol γ B and thus naturally have a reduced processivity. It should be noted that, although clinically discov-

ered, not all substitutions shown in Table S1 have been shown biochemically to adversely affect enzyme activity. A cluster of substitutions found in PEO patients (Lamantea et al., 2002; Van Goe-them et al., 2001), R943H/C, Y955C, and A957P/S, fall into class I. A modeled Pol γ -DNA complex with an incoming dNTP (Figure 6C) suggests that R⁹⁴³ may form a charged interaction with the triphosphate moiety. Substitution of the positively charged arginine should drastically reduce affinity for incoming nucleotides. Y⁹⁵⁵ abuts the templating base, in a position that is consistent with its known multifunctional roles in other Pol I family enzymes, including primer-template alignment and substrate selection (Joyce and Benkovic, 2004). A⁹⁵⁷ is adjacent to a critical glycine (G⁹⁵⁸); the equivalent residue in T7 RNAP (another Pol I family member) serves as a fulcrum during enzyme translocation and coordinates substrate binding (Yin and Steitz, 2004). Substitutions of A⁹⁵⁷ with bulkier residues likely interfere with both enzyme translocation and binding to an incoming dNTP. In general, the predicted consequences of all these mutations are a decreased affinity for dNTPs, increased error rates and/or reduced catalysis, in good agreement with solution studies (Graziewicz et al., 2004). Mutations giving rise to these defective proteins should tend to confer a dominant phenotype, likely because the mutant enzymes compete effectively with the wild-type enzyme for binding to the template DNA and cause error-prone DNA synthesis.

Because of the multiple functions of the spacer, the phenotypes of what have traditionally been called spacer mutants vary with the spatial location of the substitution. Our structure suggests that the IP and AID subdomains of the spacer use distinct means to increase processivity: the IP subdomain functions independently of Pol γ B, whereas AID acts only through its interaction with Pol γ B. Accordingly, spacer mutations segregate into classes II and III, but in general both are likely to have reduced affinity for DNA.

A large number of class II mutations are arginine substitutions that are distributed along the modeled primer-template DNA binding channel. Substitution of positively charged arginine with neutral residues will decrease DNA binding and polymerase activity. Thus, class II mutants tend to be recessive, as the mutant Pol γ A is ineffective in competing with the wild-type enzyme for template DNA.

The class II mutation W748S is commonly associated with autosomal-recessive ataxia and Alpers' syndrome (Hakonen et al., 2005). W⁷⁴⁸ is located in the IP subdomain (Figures 4B and 5B), away from the subunit interface, and is likely important in maintaining the local structure of the IP domain that contacts the downstream single-stranded DNA. W⁷⁴⁸ forms stacking-interaction with F⁷⁵⁰ and H⁷³³ in the local structure. Destabilization of this stack by the W748S substitution will undermine the enzyme's interaction with template DNA, leading to lower polymerase activity. This interpretation is consistent with the biochemical observations of low DNA polymerase activity and processivity and a severe DNA-binding defect, but normal holoenzyme formation (Chan et al., 2006).

The most common substitution among all Pol γ mutations, A467T, is a representative of class III mutants and is associated with a wide range of mitochondrial disorders (Nguyen et al., 2005). Biochemical studies using the A467T mutant, which was thought to affect the spacer domain, unexpectedly was found to have reduced template binding, and the mutant-containing holoenzyme has lower processivity (Chan et al., 2005; Luoma et al., 2005), suggesting that the A467T mutant Pol γ A has both reduced polymerase activity and subunit interaction. This observation can now be explained because the substitution actually lies in the thumb domain of Pol γ A (Figure 4A and 5B), which is well known to interact with template DNA. Although Pol γ B interacts with the thumb containing A⁴⁶⁷, this residue faces away from the interaction surface; A467T may interrupt the local hydrophobic environment formed by L⁴⁶⁶ and L⁶⁰², causing a slight spatial shift of the thumb domain that interferes with the interaction between the subunits.

The only Pol γ B substitution that has been reported to be associated with disease is G451E, which was found in a single PEO patient with multiple mtDNA deletions (Longley et al., 2006). G451E is a class III mutant, as G⁴⁵¹ is located near the interface formed between the C-terminal domain of the proximal Pol γ B monomer and the AID subdomain of Pol γ A (Figure 5B). The G451E substitution may cause a steric clash with T⁵⁵⁶ on the AID subdomain; perhaps more importantly, it may disrupt the hydrophobic interaction that is essential for subunit interaction. This structural analysis agrees with the biochemical characterization of G451E-substituted Pol γ B, which revealed a compromised subunit interaction and incomplete stimulation of catalytic subunit activity (Longley et al., 2006).

From their structural and biochemical properties, mutations in class III are expected to be autosomal recessive. A defective subunit interface leads to reduced polymerase processivity and DNA binding, defects that can be at least partially compensated in heterozygotes by the presence of wild-type enzyme. In addition, all class III mutations identified to date are located in the subunit hydrophilic interface that plays a secondary role in subunit interaction.

Structural Dissimilarities between Human Pol γ and HIV Reverse Transcriptase Provides Exploitable Space for Drug Design

Antiviral NRTIs present a unique opportunity for drug design, because both the target HIV RT and the adverse target human

Pol γ are known. Although it has long been suspected that the two enzymes are dissimilar, we can now make detailed structural comparisons of the human and viral polymerases and exploit differences in a rational design of antiviral drugs with higher selectivity.

There are several structural differences between human Pol γ and HIV RT that may be utilized in designing selective inhibitors. The distinct subunit interactions of the two enzymes result in substrate DNA being bound in the active site of Pol γ at an angle of 45° to that in HIV RT (Figures 6A and 6B). More importantly, although the catalytic aspartates of the HIV RT p66 subunit and Pol γ A have a similar spatial arrangement, the incoming nucleotide-binding sites formed between the palm and fingers subdomains are structurally distinct. This portion of the fingers subdomain is α -helical in human Pol γ but β sheet in HIV RT (Figures 6C and 6D).

Both human Pol γ and HIV RT utilize electrostatic interactions of positively charged residues on the fingers domain to bind the negatively charged triphosphate of an incoming dNTP. However, their interaction with the nucleoside moiety is different. In Pol γ A, the nucleoside binding site is likely bounded by E⁸⁹⁵, Y⁹⁵¹, Y⁹⁵⁵, and Q¹¹⁰²; in HIV RT it is bounded by R⁷², F⁷⁷, Y¹¹⁵, and Q¹⁵¹ (Figures 6C and 6D) (Huang et al., 1998). Not only is a charge of a residue reversed (E⁸⁹⁵ in Pol γ A versus R⁷² in RT) but the positions of Y⁹⁵¹ and Y¹¹⁵ are also altered. A highly conserved bulky residue (a Y or F) in members of the DNA Pol I family is known to play a major role in discriminating against incorporation of ddNMP. Pol γ A Y⁹⁵¹ is located on the O helix of the fingers domain and has been shown to be responsible for the lack of discrimination between dNTPs and ddNTPs (Lim et al., 2003). Y¹¹⁵ of the p66 subunit of HIV RT has been shown to be important in the discrimination of 3'-OH residues (Klarmann et al., 2007) and may be the equivalent residue to Pol γ A Y⁹⁵¹. However, Y¹¹⁵ in HIV RT p66 lies on a loop behind the ribose moiety of the incoming dNTP (Figures 6C and 6D). The different angles at which Y⁹⁵¹ and Y¹¹⁵ approach the sugar moiety of an incoming dNTP suggest that they differentially shape the active site and, further, that HIV RT and Pol γ may interact differently with nucleoside analogs. The HIV RT residue in the equivalent spatial position as Pol γ A Y⁹⁵¹ is R⁷², which actually functions in pyrophosphorolysis rather than discrimination against the 2'-OH of ribose (Sarafiannos et al., 1995). The differences between the two enzymes suggest that it may therefore be possible to design small molecules that exploit these structural and functional dissimilarities.

The modeled complex of Pol γ and DNA also illustrate how the fingers domain may undergo conformational changes in order to accommodate the primer/template DNA duplex, changes that can be contrasted with those in the thumb domain of HIV RT (Ding et al., 1998; Huang et al., 1998). The differences may influence how the two enzymes maintain their different degrees of substrate specificity and their different responses to nucleoside inhibitors. However, a clearer picture of how correct and incorrect or analog nucleotides are differentially accommodated within the active sites of HIV RT and Pol γ A must await cocrystal structures of the latter enzyme with DNA and dNTPs.

EXPERIMENTAL PROCEDURES

Preparation of Pol γ A and Pol γ B

The His-tagged, exonuclease-deficient (exo⁻) catalytic subunit Pol γ A (which also lacks the mitochondrial localization sequence [residues 1–29] and ten of 13 sequential glutamines [residues 43–52]), was prepared by substitution of catalytic residues D¹⁹⁸ and E²⁰⁰ to alanines. Other mutants were made using this construct as starting material. Details of these constructions and of the deletion mutant Pol γ B- Δ 14 are described in the [Supplemental Data](#). Pol γ A was expressed in insect Sf9 cells, and Pol γ B was expressed in *E. coli*.

Selenomethionine-substituted Pol γ A was produced by a variation of the procedure of [Bellizzi et al. \(1999\)](#). In brief, Sf9 growth medium (5% FBS in SF-900 SFM; Invitrogen) was exchanged 12 hr after infection for a methionine-deficient medium containing 5% dialyzed FBS; 7 hr later, the medium was made 50 mg/L L-(+)-Se-Met (Acros). After 48 hr, cells were harvested and lysed, and proteins were purified by sequential application to Ni-NTA, SOURCE S and Superdex 200 columns ([Yakubovskaya et al., 2006](#)). Holoenzyme was formed by combination of Pol γ A and Pol γ B monomer at a 1:2 molar ratio; the complex was then isolated by gel filtration through Superdex 200.

Polymerization Assay

The substrate was single-stranded M13mp18 DNA annealed to a 26 nt primer. Reaction mixtures contained 50 nM Pol γ A (wild-type, L549N, L552N, K553A, or Δ L), 100 nM Pol γ B (wild-type), and 50 nM primer/template DNA in 20 μ l reaction buffer (10 mM HEPES [pH 7.5], 50 μ g/ml BSA, and 3 mM β -mercaptoethanol). Reactions were initiated by the addition of MgCl₂ (10 mM) and dNTPs (50 μ M dGTP, dATP, dTTP, 5 μ M dCTP, and 0.1 μ M [α -³²P]dCTP) and NaCl (90 or 190 mM) and incubated at 37°C for 10 min. Reactions were stopped by addition of 1% SDS, 20 mM EDTA, and 0.1 mg/ml Protease K and incubation at 42°C for 30 min. Mixtures were then applied to Micro Bio-Spin 6 columns (Bio-Rad) to remove free nucleotides, heat-denatured at 95°C for 5 min in gel loading buffer (70% formamide, 1 \times TBE, 100 mM EDTA), and analyzed on a 6% polyacrylamide/7 M urea gel. Reaction products were visualized by autoradiography.

Crystallography

Crystals of Pol γ holoenzyme were grown with the hanging-drop method at 20°C at 2–3 mg/mL of the protein complex against a well solution containing 5.5%–7% PEG 8000, 100 mM NaH₂PO₄, and 100 mM ACES (pH 7.0). Osmium derivatives were prepared by soaking of crystals in mother liquor containing 3 mM K₂O₈OsO₄ for 7 hr. Prior to freezing in liquid nitrogen, crystals were transferred into solutions with stepwise increasing concentrations of glycerol up to 20%. Single-wavelength anomalous diffraction data (SAD) for Se and Os derivatives were collected at Advanced Photon Source 19-ID. All data sets were processed with the program *HKL* ([Otwinowski and Minor, 1997](#)).

Se atoms (30 out of 34 total) and Os atoms (eight) were located by the anomalous difference Fourier method using phases obtained from molecular replacement with apo Pol γ B as a search model. Initial phases (with a figure of merit of 0.56) were calculated using combined phases from Se-SAD, Os-SAD, and molecular replacement with the program *CNS* ([Brunger et al., 1998](#)). Density modification using *Solomon* in the CCP4 suite ([Collaborative Computational Project N, 1994](#)) was applied to the initial phases; this procedure and B factor sharpening drastically improved the quality of electron-density maps ([Figure S7](#)). The diffraction data of the holoenzyme were initially indexed to a hexagonal space group P3₂21 containing one complex per asymmetric unit (asu). Although the electron-density map was readily interpretable, the resulting atomic structure could only be refined to a high R_{free} (~49%). After careful examination, we reassigned the diffraction data to space group P3₂ with two copies per asu. In the new space group, diffraction intensity analysis indicated that the diffraction data were partially twinned with a twinning operator (h₁-h₂-k₁-) and a twinning factor of 0.46. Refinement was subsequently carried out in space group P3₂, with the detwinning procedure in the program *CNS*.

Crystals of Pol γ B- Δ 14 were grown by the hanging-drop method at 4°C with 10–15 mg/mL protein and a well solution containing 100 mM Tris-HCl (pH 7–7.5), 100 mM KCl, 6%–8% PEG8000, and 30% glycerol. Crystals were directly flash frozen in liquid nitrogen. Diffraction data were collected at

APS 19-ID. The structure was determined by the molecular replacement method using wild-type human Pol γ B as a search model with the program AMORE ([Navaza, 2001](#)) and was refined with *Refmac* ([Winn et al., 2003](#)).

Limited Proteolysis

Experiments were conducted with 20 μ g of purified Pol γ A, Pol γ B- Δ 14, and holoenzyme in 20 μ l of reaction buffer (20 mM HEPES [pH 7.5], 140 mM KCl, 1 mM EDTA, 5 mM β -mercaptoethanol) with or without equal molar of 25/45-mer DNA, to which trypsin (0.1 μ g) was added. Samples were incubated on ice for 3 min and treated with an equal volume of 2 \times SDS sample buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 100 mM DTT) to stop the digestion reaction. Reaction products were separated by SDS-PAGE; western blots were performed with anti-His antibody according to the manufacturer Abcam's protocol.

ACCESSION NUMBERS

The coordinates of the structures reported here have been deposited in the Protein Data Bank (accession numbers 3IKL and 3IKM).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at [http://www.cell.com/supplemental/S0092-8674\(09\)01044-7](http://www.cell.com/supplemental/S0092-8674(09)01044-7).

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