

A Single Mutation in Human Mitochondrial DNA Polymerase Pol γ A Affects Both Polymerization and Proofreading Activities of Only the Holoenzyme*

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Common causes of human mitochondrial diseases are mutations affecting DNA polymerase (Pol) γ , the sole polymerase responsible for DNA synthesis in mitochondria. Although the polymerase and exonuclease active sites are located on the catalytic subunit Pol γ A, in holoenzyme both activities are regulated by the accessory subunit Pol γ B. Several patients with severe neurological and muscular disorders were reported to carry the Pol γ A substitutions R232G or R232H, which lie outside of either active site. We report that Arg²³² substitutions have no effect on independent Pol γ A activities but show major defects in the Pol γ A–Pol γ B holoenzyme, including decreased polymerase and increased exonuclease activities, the latter with decreased selectivity for mismatches. We show that Pol γ B facilitates distinguishing mismatched from base-paired primer termini and that Pol γ A Arg²³² is essential for mediating this regulatory function of the accessory subunit. This study provides a molecular basis for the disease symptoms exhibited by patients carrying those substitutions.

Ever since the discovery of the first mitochondrion-associated disease, mitochondrial dysfunctions have been implicated in a wide range of clinical disorders (reviewed by Wallace (1)). Major causes of the dysfunction are mutations on mitochondrial DNA (mtDNA)² and/or the DNA polymerase (Pol γ). Pol γ mutations clinically manifest different onset times and multisystem disorders (2, 3). Although there is a clear phenotype-genotype association, the molecular or structural basis for many mutations is unknown.

Mitochondrial DNA codes for a subset of components for ATP synthesis via the oxidative phosphorylation electron transfer chain and is maintained by Pol γ . Reduced Pol γ activity leads to depletion of mtDNA and impairment of cellular metabolism. Human Pol γ is a heterotrimeric holoenzyme of two subunits: a catalytic subunit, Pol γ A, which contains polymerase and exonuclease activities, and a dimeric accessory subunit, Pol

γ B. Although it has no known independent function, Pol γ B alters Pol γ A activity by increasing its processivity (4, 5). Pol γ B is known to suppress the exonuclease activity of the holoenzyme (6, 7). The genes for both Pol γ A (*POLG*) and Pol γ B (*POLG2*), and other components of the mitochondrial replisome are encoded in the nucleus, and the protein products are transported into mitochondria. Humans heterozygous for *POLG* may therefore have multiple forms of Pol γ holoenzyme in each mitochondrion.

Neurological disorders associated with mutations affecting Pol γ A often present different clinical symptoms (for an overview, see Ref. 8). Pol γ A is the most commonly affected gene in both dominant and recessive progressive external ophthalmoplegia (a disorder characterized by slow paralysis of external eye muscle and exercise intolerance), as well as Alpers syndrome. A patient who died at 6 months of age was found to carry an arginine to glycine substitution at position 232 (R232G) on one chromosomal copy of Pol γ A and the double substitution T251I/P587L on the other (9). The child was diagnosed with progressive generalized hypotonia and presented severe hypomyelinating peripheral neuropathy. Her mtDNA levels were only 3–5% of normal (9). In a separate case, a 5-month-old patient stopped growing and presented muscular hypotonia and later developed epilepsy. Pathological examinations revealed that he was heterozygous for Pol γ A: R232H in *trans* to W748S (and the SNP E1143G), and his mtDNA content was 12% of normal (10). In addition, a 6-month-old patient with Leigh syndrome harbored Pol γ A R232H in *trans* to G848S. The patient had low levels of mtDNA (11). Finally, three of four siblings exhibited various degrees of reduced dexterity, sensory redistribution, and muscle wasting. Two of the three (the third was not tested) were found to carry Pol γ A R232H in *trans* to G737R. The healthy fourth child was wild type for Pol γ A (12). These data strongly suggest that substitutions of Pol γ A Arg²³² are intimately associated with clinical disorders.

The Pol γ holoenzyme crystal structure (13) provides a clue to the phenotype of Arg²³² substitutions. Pol γ A makes an asymmetrical interaction with a Pol γ B homodimer; the Pol γ B monomer that forms the major interaction with Pol γ A is thus termed the proximal monomer, and the other monomer was termed distal. Among the limited interactions between Pol γ A and the distal Pol γ B monomer, Pol γ A Arg²³² and Pol γ B Glu³⁹⁴ make the only strong interaction. Arg²³² is spatially removed from both the polymerase and exonuclease active sites, so clinical disorders may be predicted to result from dis-

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² The abbreviations used are: mtDNA, mitochondrial DNA; Pol, polymerase; nt, nucleotide(s); wt, wild type; AID, accessory-interactive determinant.

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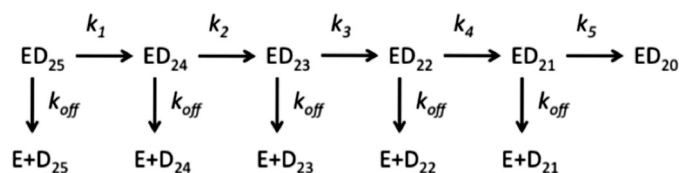
ruption of the subunit interaction between the catalytic subunit and the Pol γ B distal monomer (see Fig. 1*a*). However, abolishing a single interaction in the context of an extensive subunit interface would not be expected, *a priori*, to confer a major defect in enzyme activity. For example, various point mutations in the Pol γ A accessory-interacting determinant (AID) subdomain, which provides the major interacting surface with Pol γ B, confer at most a limited defect in holoenzyme activity (13). In addition, Pol γ A Arg²³² is conserved, even in species that do not possess a distal Pol γ B monomer, implying that Arg²³² may have an independent function.

To further our understanding of the function of Arg²³², we have analyzed substitutions both at that locus and at its interacting partner on Pol γ B. We report that Arg²³² serves as a sensor that monitors the accuracy of DNA synthesis by facilitating editing of misincorporated nucleotides. In addition, we show that the processivity factor Pol γ B confers on holoenzyme a selective exonucleolytic activity toward mismatch removal and that Pol γ A Arg²³² is critical for relaying the regulatory activity conferred by Pol γ B to the exonuclease active site. Substitutions of Arg²³² cause severe defects in DNA synthesis, and this study therefore provides a molecular basis for the human diseases.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Protein Purification—Wild type Pol γ B, cloned into pET22b(+) (14), was modified to incorporate the substitutions E394A, E394R, E449A, and E449R. Wild type Pol γ A and the *exo*⁻ mutant E200A (14), both cloned into pET22b(+), were modified to incorporate substitutions of Arg²³². Site-specific mutagenesis was performed using the QuikChange (Stratagene) kit. Pol γ A constructs were transferred into the baculovirus genome using the shuttle vector pBacPak9 (Clontech) and expressed in infected Sf9 insect cells ($1-2 \times 10^6$ cells/ml) grown at 27 °C in SF900 medium. The cells were harvested 72 h post-infection. Wild type Pol γ B and variants were expressed in *Escherichia coli* Rosetta (DE3) (Novagen), grown in LB at 37 °C. Isopropyl β -D-thiogalactopyranoside (0.4 mM) was added at $A_{600} = 0.6$, and the culture was subsequently incubated at 25 °C for 6 h before harvesting. The C-terminal His-tagged proteins were purified by sequential application to nickel-nitrilotriacetic acid, SOURCE S, and Superdex 200 columns as previously described (14).

Steady-state Polymerization Assay—Steady-state polymerization experiments with Pol γ A and Pol γ B wild type or variants were performed as described (14). Unless stated otherwise, the reactions contained 50 nM Pol γ A (all concentrations given are final), 100 nM Pol γ B, and 50 nM single-stranded M13mp18 DNA annealed to a 26-nt primer (5'-GGATTATTTACATTG-GCAGATTCACC-3') in buffer (10 mM HEPES, pH 7.5, 80 mM KCl, 12.5 mM NaCl, 50 μ g/ml bovine serum albumin, and 3 mM β -mercaptoethanol) were preincubated for 5 min at 37 °C. The reactions were initiated by adding 10 mM MgCl₂, dNTPs (50 μ M dGTP, dATP, dTTP, 5 μ M [α -³²P]dCTP, and 500 nM poly(dA-dT)·poly(dA-dT)) (Sigma P0883) as “trap” DNA. After 10 min at 37 °C, the reactions were stopped by the addition of 1% SDS, 20 mM EDTA, and 0.1 mg/ml Protease K and were incubated at 42 °C for 30 min. After removing free nucleotides on Micro



SCHEME 1. Minimal sequential exonuclease model. This model shows the sequential exonuclease reactions starting with DNA 25 nucleotides in length (ED₂₅). This model was used in global data fitting to derive estimates for each rate constant shown. At each step of the reaction, the DNA can dissociate from the enzyme at the rate defined by k_{off} . After dissociation, DNA was allowed to rebind in the computer simulation with a second-order rate constant of 10 $\mu\text{M}^{-1} \text{s}^{-1}$, according to previous estimates (26).

Bio-Spin 6 columns (Bio-Rad), DNAs were heat-denatured in gel loading buffer (70% formamide, 1× Tris borate-EDTA, 100 mM EDTA), electrophoresed on a 6% polyacrylamide, 7 M urea gel, and then visualized by phosphorimaging.

Pre-steady-state Polymerization Assay—The experiments were carried out using a RQF-3 Rapid Chemical Quench Flow instrument (KinTek Co.) as described (14). Briefly, the reactions were initiated by rapidly mixing a preincubated Pol γ -DNA complex (70 nM Pol γ A, 300 nM Pol γ B, and 200 nM 25/45-nt DNA with 50 μ M dATP, 10 mM MgCl₂, 100 mM NaCl, 20 mM HEPES, pH 7.5) and incubated at 37 °C. After various times (5 ms to 5 s), the reactions were stopped by the addition of 0.5 M EDTA. After resolving the 5' ³²P-labeled 25-nt primer from the 26-nt product by electrophoresis on a 15% polyacrylamide, 7 M urea gel and phosphorimaging, the amounts of primer and product were quantified using QuantityOne (Bio-Rad). The time-dependent product formation was fitted to a burst equation,

$$[\text{product } 26 - \text{nt}] = A(1 - e^{-k_{\text{pol}} \cdot t}) + k_{\text{ss}} \cdot t$$

where A represents the amplitude of the burst, k_{pol} is the burst rate for single nucleotide incorporation, and k_{ss} is the steady-state rate.

Pre-steady-state Exonuclease Assay—Kinetic experiments using wild type (*exo*⁺) Pol γ A and its R232G derivative also employed the RQF-3 instrument. The reactions were performed at 37 °C in buffer (20 mM HEPES, pH 7.5, 100 mM NaCl) and were initiated by the addition of 10 mM MgCl₂ into a preincubated Pol γ -DNA complex (100 nM Pol γ A, with or without 500 nM Pol γ B, and 75 nM substrate DNA). At various times (10 ms to 15 s), the reactions were quenched by rapid mixing with 0.5 M EDTA. The labeled DNA strands were resolved on a denaturing 15% polyacrylamide, 7 M urea gel, visualized by phosphorimaging, and quantified using QuantityOne (Bio-Rad). The kinetics of sequential exonuclease reactions were modeled using Scheme 1 based upon computer simulation using KinTek Explorer (KinTek Corporation) (24, 25). Rate constants for each excision were obtained as independent parameters during fitting, while the DNA dissociation rates were assumed to be the same for a given enzyme. In the model, once DNA dissociated from the enzyme, it was allowed to rebind with a second-order rate constant of 10 $\mu\text{M}^{-1} \text{s}^{-1}$ (26). Data were fit globally to the complete model by nonlinear regression.

RESULTS

Pol γ A Variants Construction and Mutation—To further our understanding of two Pol γ A Arg²³² mutants implicated in

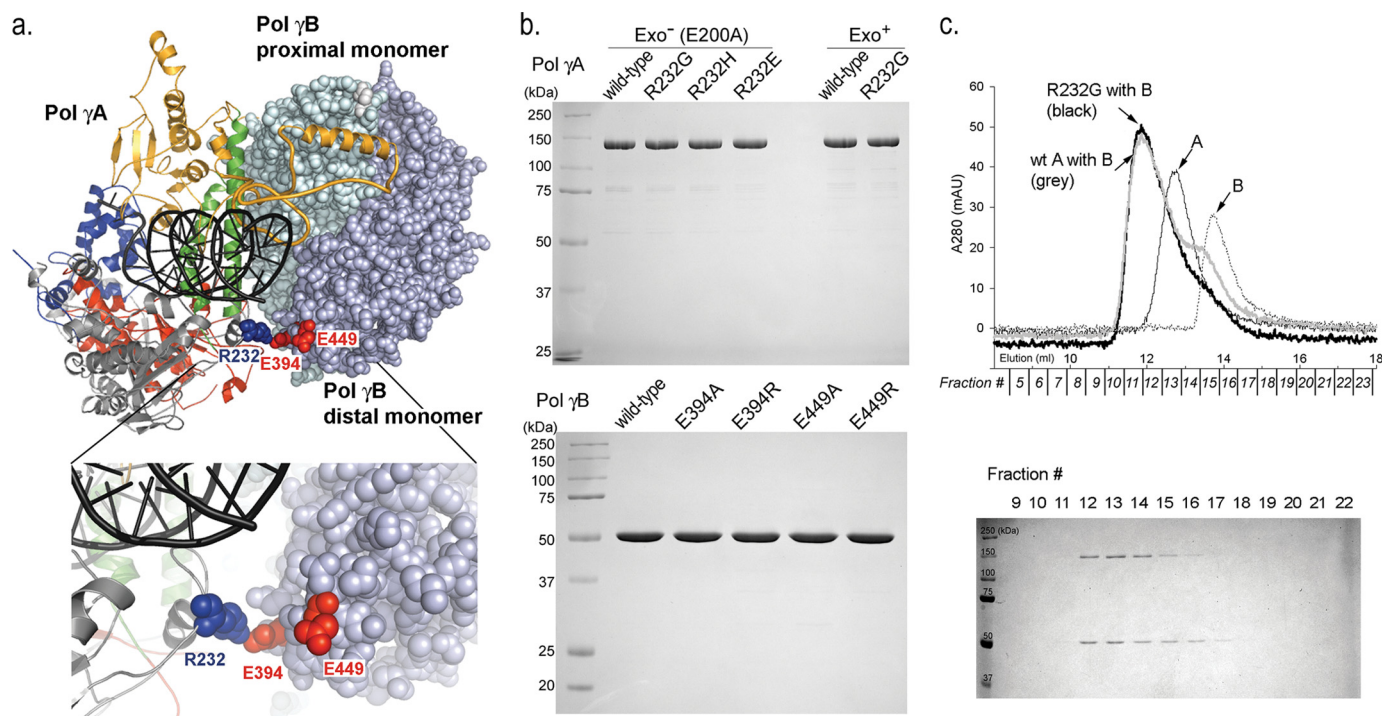


FIGURE 1. Structure of the heterotrimeric Pol γ holoenzyme with Pol γ A (ribbon representation) and dimeric Pol γ B (space filling). The proximal and distal monomers of Pol γ B are colored *light green* and *light gray*, respectively. *a*, the salt bridge between Pol γ A Arg²³² and Glu³⁹⁴ Pol γ B constitutes the major interaction of Pol γ A with the Pol γ B distal monomer in the apo-enzyme. Residues Pol γ A Arg²³² (blue sphere) and Pol γ B Glu³⁹⁴ and Glu⁴⁴⁹ (red spheres) are illustrated. *b*, purified Pol γ A wild type and variants (*top panels*) and Pol γ B variant proteins (*bottom panels*) (1 μ g/lane) analyzed on Coomassie-stained SDS-polyacrylamide gels. *c*, superimposed elution profiles of analytical gel filtration chromatography using 1 μ M Pol γ A R232G (1 μ M) or wild type (gray) in the presence of 2 μ M wild type Pol γ B. The profiles of uncomplexed Pol γ A (1 μ M) or Pol γ B (2 μ M) are shown in *thin solid* or *dashed lines*, respectively. Proteins in the peak fractions from the Pol γ A R232G-Pol γ B elution were visualized following SDS-PAGE gel electrophoresis.

human diseases, we made Gly and His substitutions, yielding R232G and R232H. In addition, because Arg²³² forms a charge-charge interaction with Glu³⁹⁴ of the distal Pol γ B monomer, we also constructed R232E. This mutant should display a repulsive interaction with Pol γ B Glu³⁹⁴. If the charge interaction between the subunits is important for the enzymatic activity of holoenzyme, R232E should be the most defective of the Arg²³² mutants. Further, to reveal any function of Arg²³² distinct from its making a salt bridge with the distal Pol γ B monomer, we constructed Pol γ B E394A and E394R. The former substitution abrogates the salt bridge, whereas E394R introduces a charge repulsion with Pol γ A Arg²³². If formation of a salt bridge with Pol γ B Glu³⁹⁴ is the only function of Pol γ A Arg²³², holoenzyme containing Pol γ B E394R should have properties similar to that containing Pol γ A R232E.

To assess DNA synthesis by Pol γ A variants without the complications of nucleolytic activity, Pol γ A R232G, R232H, and R232E were also constructed on an exonuclease-deficient enzyme where the catalytic residue Glu²⁰⁰ was changed to alanine, yielding Pol γ A R232G *exo*⁻, R232H *exo*⁻, and R232E *exo*⁻. All of the proteins were purified to an estimated $\geq 95\%$ homogeneity after SDS-PAGE (Fig. 1*b*).

Interaction between Pol γ A Arg²³² Variants and the Accessory Subunit—We first considered whether the reduced mtDNA synthesis observed in patients is caused by Arg²³² variants weakening subunit interactions in the holoenzyme. To assess the impact of disruption of the salt bridge between Pol γ A Arg²³² and Pol γ B Glu³⁹⁴, we analyzed the stability of holoen-

zyme formed by R232G or R232H variants with wild type Pol γ B using analytical gel filtration chromatography.

When 1 μ M Pol γ A wild type (molecular mass, 135 kDa) is mixed with 2 μ M Pol γ B (monomer molecular mass, 50 kDa) and chromatographed, a single peak elutes that corresponds to an ~ 220 -kDa species (Fig. 1*c*), suggesting that at this concentration the two subunits complex completely. An identical profile is obtained with Pol γ A variants R232G (Fig. 1*c*), R232H, and R232E (data not shown). Peak fractions corresponding to the 220-kDa species were analyzed on SDS gels, showing that the complex contains both Pol γ A R232G and Pol γ B (Fig. 1*c*). The intensity ratio of the two bands is 160:150, consistent with formation of a heterotrimeric Pol γ AB₂. The peak heights of holoenzymes containing wild type Pol γ A or all Arg²³² variants are identical, suggesting that the variants form holoenzyme normally at this concentration.

DNA Synthesis Activity by Pol γ A Arg²³² Variants—Because Arg²³² variants showed no differences in holoenzyme formation, it was necessary to confirm that these mutations are indeed the cause of clinical disorders. We next analyzed the activities of Pol γ A Arg²³² *exo*⁻ variants (R232G, R232H, or R232E) on primed M13 DNA. All of the variants synthesized approximately equal amounts and similar lengths of DNA as wild type Pol γ A (Fig. 2*a*), suggesting that the polymerization domain of Pol γ A is unaffected by the substitutions.

However, holoenzymes containing Pol γ A variants showed altered DNA synthesis activities. Relative to wild type, holoenzyme containing Pol γ A R232H shows a reduced level of DNA

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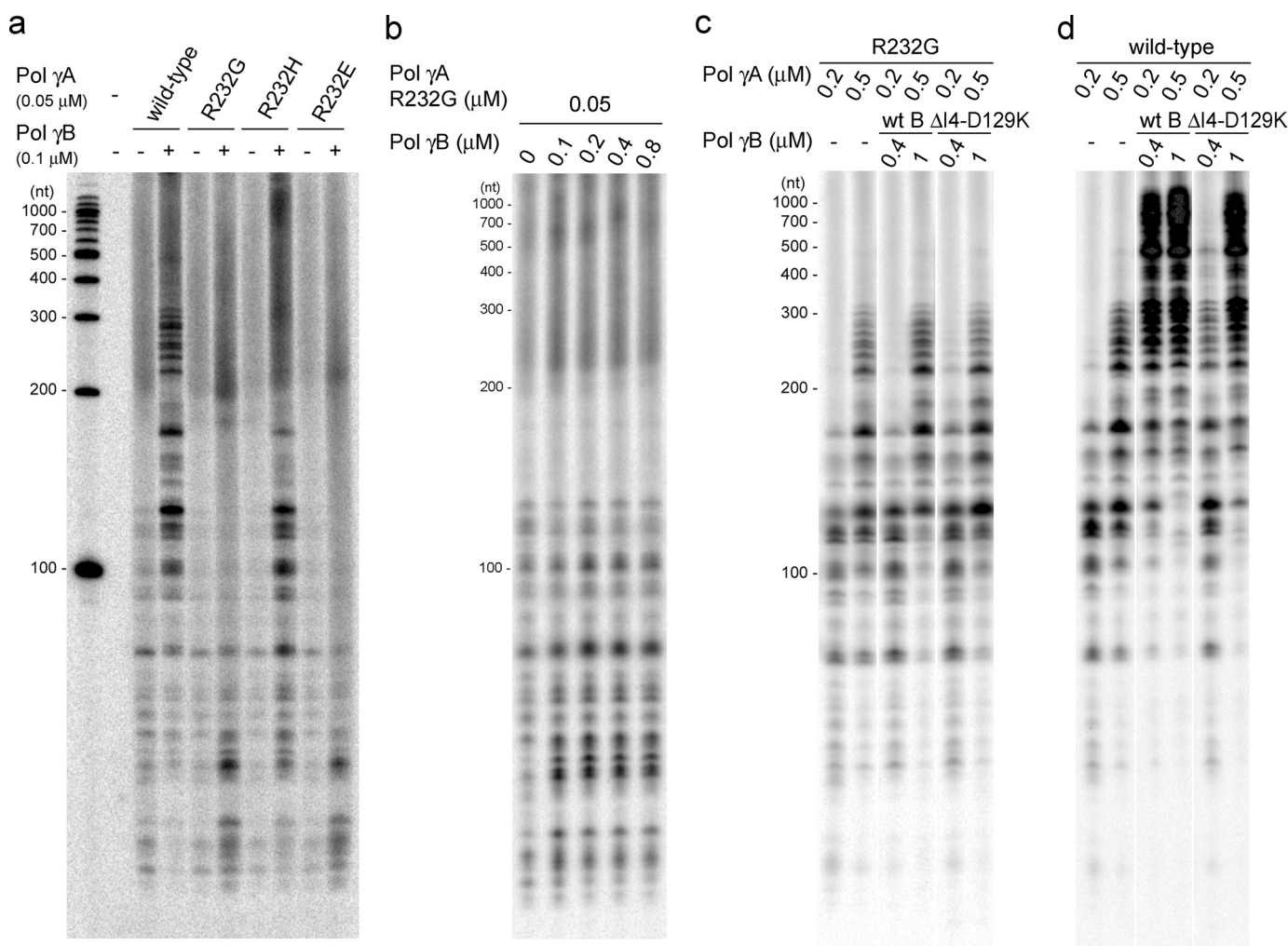


FIGURE 2. **Steady-state DNA synthesis activities of Pol γ A Arg²³² mutants.** The assays were performed on primed M13 DNA in the presence of ~10-fold molar excess of “trap” DNA. Products of Pol γ A variants, with or without Pol γ B, are visualized after separation on a denaturing polyacrylamide gel. *a*, 0.05 μ M Pol γ A variant, 0.1 μ M Pol γ B, 0.05 μ M M13 DNA. *b*, 0.05 μ M Pol γ A R232G and increasing concentrations of Pol γ B. *c* and *d*, Pol γ A R232G (*c*) or wild type (*d*) in the presence of dimeric wild type or monomeric Pol γ B Δ 14-D129K.

synthesis, albeit greater than that of the corresponding mutant Pol γ A alone, suggesting that processivity enhancement by Pol γ B is defective. More strikingly, holoenzymes containing Pol γ A R232G or R232E are completely nonresponsive to Pol γ B stimulation. The yields and lengths of DNA synthesized by the mutant Pol γ A in the presence of Pol γ B are identical to those synthesized by the catalytic subunit alone (Figs. 2*a* and 3*b*). This result was unexpected because both mutants form holoenzyme with Pol γ B. To test whether the lack of sensitivity to the accessory subunit is due to the lower concentration used in this assay than in gel filtration (Fig. 1*c*), we increased protein concentrations up to 1 μ M. No effect of Pol γ B was observed (Fig. 2, *b* and *c*), again showing that holoenzyme containing Pol γ A R232G or R232E cannot synthesize DNA processively.

We showed previously that processivity of human Pol γ B is conferred by both monomers in separate ways: the proximal Pol γ B monomer enhances DNA binding, whereas the distal monomer, which harbors the Pol γ A Arg²³² binding partner Glu³⁹⁴, accelerates the polymerization rate (14). The reduced activity of holoenzyme containing Pol γ A R232H can, in theory, be rationalized by minor local distortions because of the loss of the salt

bridge between Arg²³² and Glu³⁹⁴ of Pol γ A and Pol γ B. However, the synthesis defects of holoenzymes containing Pol γ A R232G or R232E are far more severe, even more so than that of wild type Pol γ A complexed to a monomeric Pol γ B, Δ 14-D129K (14), which shows a reduced but clear stimulation (Fig. 2*d*). Interestingly, Pol γ A R232G behaves differently than the wild type in their respective heterodimeric holoenzymes; Pol γ A R232G is much less active than the wild type enzyme (Fig. 2, *c* and *d*). Evidently, fulfilling the maximum potential of the normal heterotrimeric holoenzyme requires both Pol γ A Arg²³² and the distal Pol γ B monomer and requires more than merely the local interaction between Glu³⁹⁴ of that monomer and Pol γ A Arg²³².

The importance of the salt bridge between Pol γ A Arg²³² and Pol γ B Glu³⁹⁴ in the apo-enzyme structure was independently tested by altering the latter protein. In experiments comparable with that shown in Fig. 2, wild type Pol γ A was combined with Pol γ B mutants E394A, E394R, E449A, and E449R. Substitutions of Glu⁴⁴⁹ serve as a negative control; the residue lies close to Glu³⁹⁴ but from the atomic structure of Pol γ holoenzyme is not expected to interact with Pol γ A (Fig. 1*a*). In reactions on

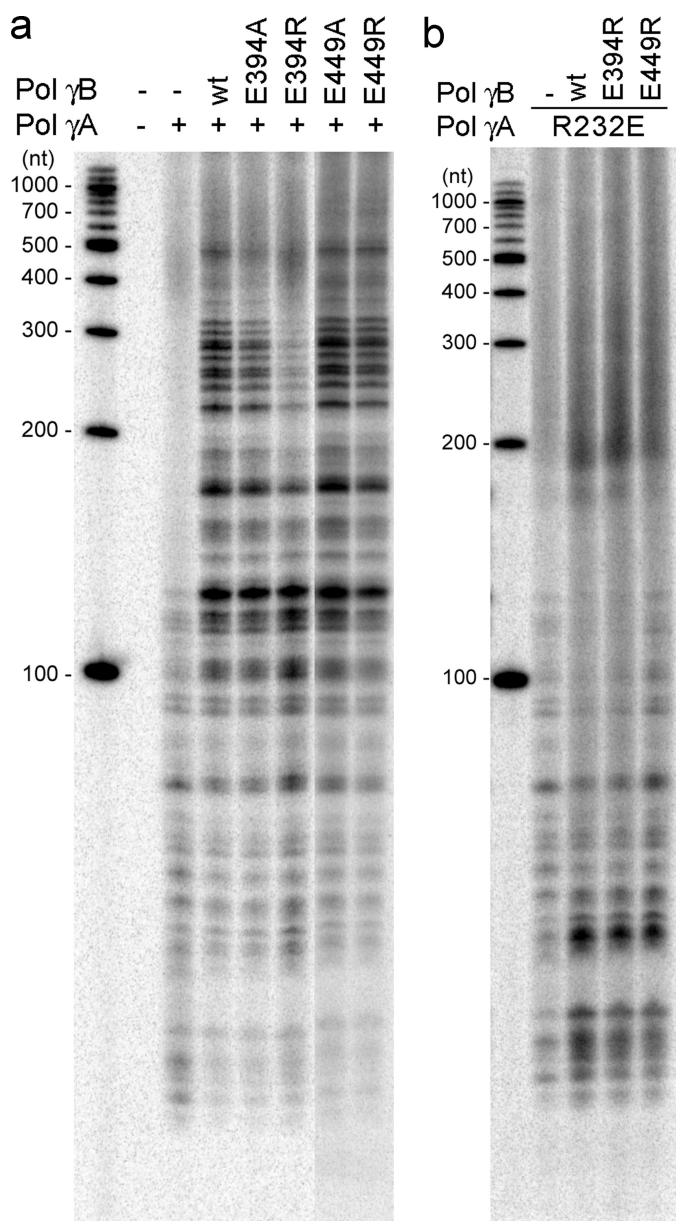


FIGURE 3. DNA polymerase activities of holoenzymes containing Pol γ B Glu³⁹⁴ variants. Products of wild type Pol γ A (a) or Pol γ A R232E (b) paired with a Pol γ B variant are visualized after separation on a denaturing polyacrylamide gel. The reactions contained 0.05 μ M wild type or R232E Pol γ A, with or without 0.1 μ M wild type or variant Pol γ B, 0.05 μ M M13 DNA, and 500 nM trap DNA.

primed M13 DNA, holoenzyme containing Pol γ B E394A, which abrogates the salt bridge with Pol γ A Arg²³², showed near wild type activity (Fig. 3a). This result clearly demonstrates that the salt bridge between Pol γ A and the distal Pol γ B monomer is not essential for processive DNA synthesis. The Pol γ B E394R substitution should generate a repulsive force with Pol γ A Arg²³², and although this form of holoenzyme is less active than the wild type, the mutant accessory protein still confers a substantial enhancement of activity to Pol γ A. Importantly, when a charge reversal combination of holoenzyme is made using Pol γ A R232E and Pol γ B E394R, there is only a small enhancement of activity relative to Pol γ A R232E alone (Fig. 3b) and at a level far below wild type holoenzyme. Pol γ B

E394R should collide with Pol γ A Arg²³² to a comparable degree as Pol γ A R232E to Pol γ B Glu³⁹⁴; the observation that Pol γ B E394R does not mirror the defect of Pol γ A R232E provides more support for the idea that in the presence of DNA, the interaction between subunits is more extensive than a salt bridge.

Effect of Pol γ A Arg²³² Mutation on Processivity—Pre-steady-state kinetic analyses were performed to provide a mechanistic explanation for the defects of the Pol γ A Arg²³² variants. Polymerization activities of Pol γ A *exo*⁻ forms of R232H and R232G were measured by monitoring the incorporation of a single nucleotide to a 25-nt primer annealed to a 45-nt template (Fig. 4a). The time-dependent primer extension to 26-nt product was plotted (Fig. 4b), and the data were fitted to the following burst equation,

$$[\text{product } 26 - \text{nt}] = A(1 - e^{-k_{\text{pol}} \cdot t}) + k_{\text{ss}} \cdot t \quad (\text{Eq. 1})$$

where A is the burst amplitude, k_{pol} is the burst rate of polymerization, and k_{ss} is the steady-state rate. Other important parameters, k_{off} and processivity, which correspond, respectively, to the time Pol γ remains associated with the template and the length of DNA synthesized per binding event, can be derived from the above parameters. The data are summarized in Table 1 for the wild type and Arg²³² variants, both with and without Pol γ B.

In the absence of Pol γ B, Pol γ A R232G and R232H have amplitudes ($A = 22.1$ and 25.6 nM) and burst rates ($k_{\text{pol}} = 10.5$ and 12.1 s⁻¹) similar to those of wild type ($A = 27.9$ nM, $k_{\text{pol}} = 11.4$ s⁻¹). These values are consistent with our steady-state measurements on M13 DNA and confirm that substitutions of Arg²³² do not substantively alter the intrinsic polymerase activity. In the presence of wild type Pol γ B, Pol γ A R232H and R232G both show increases in amplitudes to near the wild type level, indicating that the accessory protein increases DNA binding almost normally. However, the Pol γ A R232H-containing holoenzyme is defective, exhibiting only 60% of the wild type burst rate and an increased steady-state rate k_{ss} that reflects a higher rate of dissociation. Consequently, Pol γ A R232H-containing holoenzyme exhibits only 43% of wild type processivity.

Remarkably, the burst rate of the R232G-containing holoenzyme fails to increase from that of the catalytic subunit alone; it actually decreases to give a nearly 4-fold reduction from wild type holoenzyme. Further, the steady-state or dissociation rate of Pol γ A R232G-containing holoenzyme is almost twice that of wild type; the two defects result in an 8-fold drop in processivity. Thus, although Pol γ A R232G has only a minor defect in DNA binding and no significant defect in DNA synthesis, the presence of the wild type Pol γ B accessory factor in the mutant holoenzyme drastically reduces polymerization activity.

Note that the burst rate is for incorporation of a single nucleotide. A small reduction in burst rate is more apparent during synthesis of longer products because the amount of an N -nucleotide product is related to the burst rate by the N^{th} power. Thus, the lack of processivity enhancement in Pol γ A R232G-containing holoenzyme (Fig. 2) is the combination of increased DNA binding but a severely reduced reaction rate.

Similar experiments were performed using wild type Pol γ A and mutant Pol γ B (Fig. 4c); they also support observations

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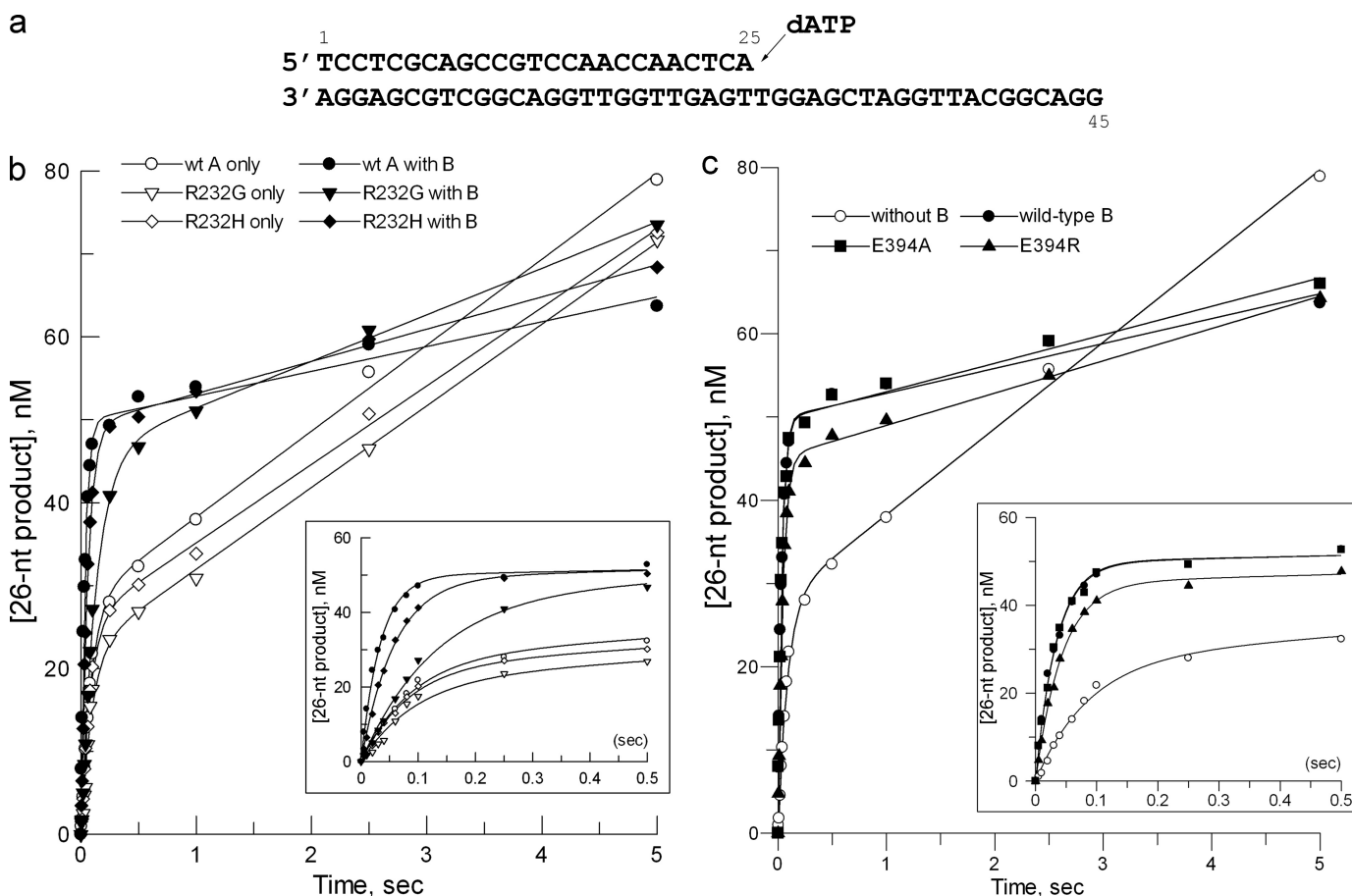


FIGURE 4. **Pre-steady-state single nucleotide incorporation.** *a*, the 25/45-nt duplex DNA substrate. *b*, the 26-mer product was quantified from reactions using Pol γ A wild type or Arg²³² variants in the presence or absence of Pol γ B. *c*, same as *b*, but with Pol γ B wild type or Glu³⁹⁴ variants in the presence of wild type Pol γ A. Short time points are shown as an *insets*. The reactions contain 200 nM 25/45-nt DNA and 70 nM Pol γ A, with or without 300 nM Pol γ B.

TABLE 1
Polymerization activities of Pol γ A mutants

Pol γ A (exo-)	Pol γ B	Amplitude (A) ^a	Burst rate (k_{pol})	Steady state rate (k_{ss}) ^a	Processivity ^b
		<i>nM</i>	<i>s</i> ⁻¹	<i>nM s</i> ⁻¹	<i>nt</i>
wt		27.9 (± 0.8)	11.4 (± 0.8)	10.3 (± 0.2)	31
R232H		25.6 (± 0.7)	12.1 (± 0.8)	9.5 (± 0.3)	32
R232G		22.1 (± 1.1)	10.5 (± 1.4)	9.9 (± 0.4)	23
wt	wt	50.0 (± 0.8)	29.7 (± 1.5)	3.0 (± 0.8)	495
R232H	wt	49.3 (± 0.6)	17.2 (± 0.5)	3.9 (± 0.2)	217
R232G	wt	45.8 (± 1.0)	7.7 (± 0.4)	5.6 (± 0.4)	63
wt	E394A	49.6 (± 0.7)	29.4 (± 0.5)	3.4 (± 0.3)	428
wt	E394R	45.1 (± 0.5)	23.2 (± 0.7)	3.9 (± 0.4)	268

^a The numbers in parentheses are the root mean square standard deviations for data fitting.

^b Processivity is calculated by $k_{\text{pol}}A/k_{\text{ss}}$.

made in steady-state reactions with primed M13 DNA (Fig. 3a). The Pol γ B E394A mutant holoenzyme gives an amplitude and burst rate indistinguishable from wild type (Table 1). However, holoenzyme containing Pol γ B E394R, which may alter the position of Pol γ A Arg²³² by charge repulsion, results in a slightly reduced amplitude and a burst rate that is 80% of wild type. Together, the data support the idea that the salt bridge between Pol γ A Arg²³² and Pol γ B Glu³⁹⁴ simply maintains the correct spatial conformation of Pol γ A Arg²³² in the holoenzyme. Importantly, the changes in kinetic parameters of holoenzyme containing Pol γ B E394R do not match those that contain Pol γ A R232E, confirming the idea that substitutions of Pol γ A Arg²³² affect enzyme activity beyond that expected for a simple salt bridge interaction between subunits.

Effects of the Arg²³² Mutation on Exonuclease Activity—Spatially, Arg²³² is located on the periphery of the 3' \rightarrow 5' exonuclease domain (consisting of residues 171–440), 45 Å away from the polymerase domain. How do substitutions of Arg²³² abolish the stimulation normally conferred by the accessory protein Pol γ B and, in the case of Pol γ A R232G, make the holoenzyme a less efficient enzyme than the catalytic subunit alone? Examination of the DNA synthesized by holoenzyme containing Pol γ A R232G reveals that shorter products predominate (Fig. 2). This could be due to lower processivity, but it could also reflect increased degradation of newly synthesized DNA. This last idea led us to examine the exonuclease activity of Pol γ A variants.

Exonuclease activity was tested using a partial duplex containing mismatched nucleotides (Fig. 5a). This substrate allows

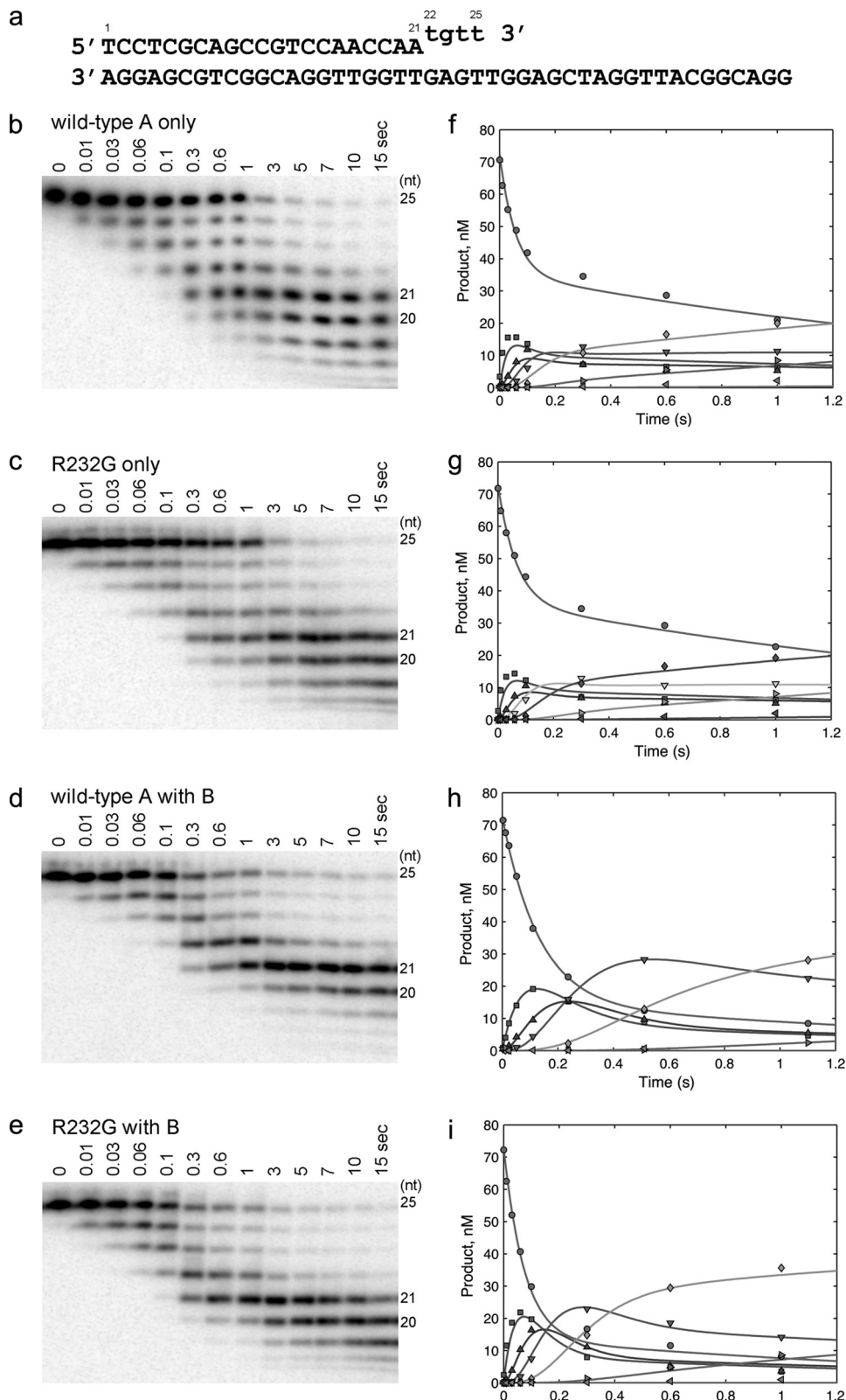


FIGURE 5. Exonuclease activities of Pol γ A Arg²³² variants. *a*, the partial duplex substrate. Products of each excision event with the catalytic subunit (*open symbols*) or holoenzyme (*filled symbols*) were visualized (*b–e*) and quantified (*f–i*). All of the reactions contained 75 nM DNA substrate, 100 nM wild type Pol γ A (*circles*), or R232G (*triangles*), with or without 500 nM Pol γ B.

us to examine the rates of excision of primers containing from four to one mismatched nucleotides and determine the rate of excision for each. Furthermore, in the same reaction the rate of

fully duplexed. The concentration of products resulting from hydrolysis of the original 25-nt primer can then be expressed as the sum of both products,

excision of correctly base-paired primers can also be determined. This substrate therefore enables examination of consecutive primer hydrolytic reactions at single nucleotide resolution (Fig. 5).

The amount of each hydrolytic product was monitored over time and resolved by electrophoresis on a denaturing polyacrylamide gel (Fig. 5, *b–e*). Each band corresponding to a defined primer length was quantified and is presented graphically in Fig. 5, *f–i*.

For all enzymes, the rate for excision of primers containing more mismatched nucleotides is greater than for those with less; the primer with one mismatch is excised most slowly. This is in agreement with earlier pre-steady-state analyses (6) and fluorescence resonance energy transfer analyses (15). However, the rate of hydrolysis of a perfectly base-paired primer (yielding products of <21 nt) is strikingly slower than any mismatched primer (Fig. 5, *f–i*). Thus, when the products of mismatch removal are analyzed over longer times, the reaction is clearly biphasic with a sharp initial rise phase that plateaus at a maximum amplitude. The plateau is followed by a gradual decline (Fig. 6*a*), because it represents two combined reactions of a fast hydrolysis of mismatched primer (21–24 nt) and a slower degradation of correctly base-paired primer (<21 nt) generated after mismatch removal. It would not have the second phase if the exonuclease activity of Pol γ were directed exclusively toward a mismatched 3'-end.

Because our primary interest here is in the comparison of wild type and mutant enzyme, we took advantage of the drastic rate difference for excision of mismatch and correctly paired primers and simplified the analysis by pooling data for primers containing one or more mismatches and, separately, for primers that are

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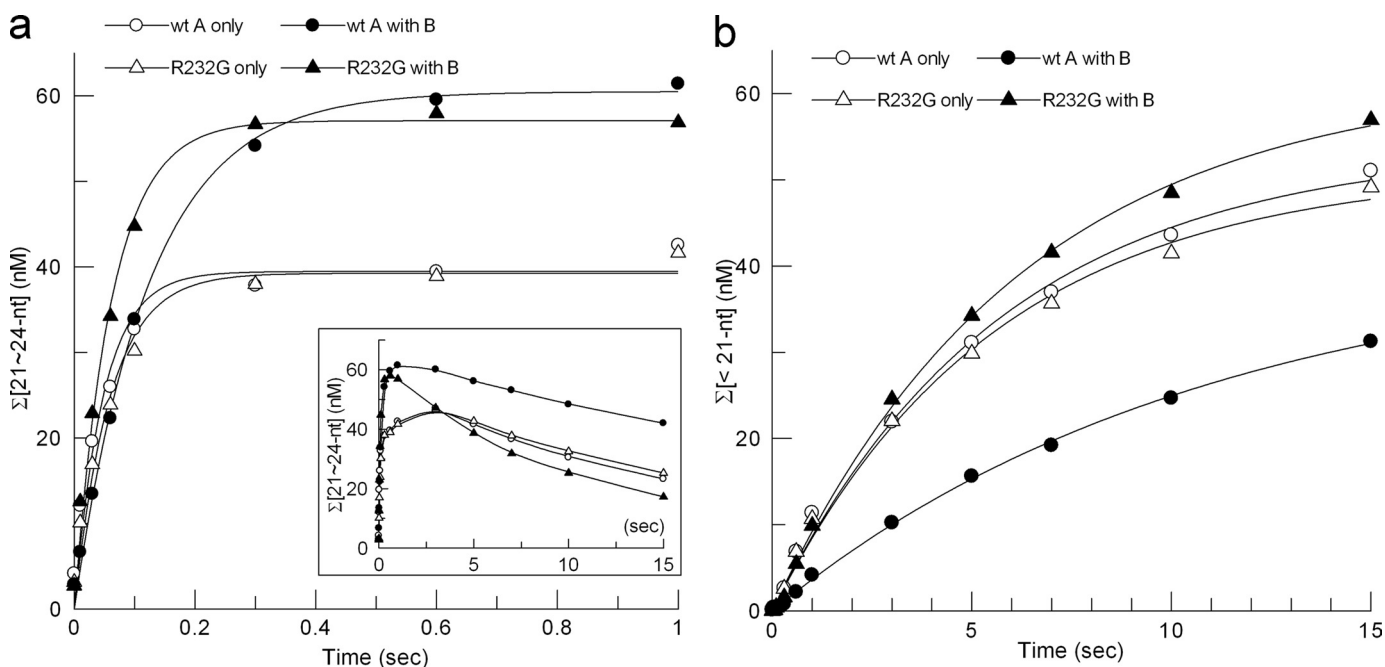


FIGURE 6. **Exonuclease analyses.** Products of all mismatches (21–24-mer; *a*) and duplex DNA (<21-mer; *b*) fitted into their respective burst equations. *wt*, wild type.

TABLE 2
Exonuclease activity of Pol γ A variants

Enzyme	Mismatch excision		Duplex excision	
	Amplitude (A_m) ^a	Average exo rate ^a	Amplitude (A_c) ^a	Average exo rate ^a
	<i>nm</i>	<i>s</i> ⁻¹	<i>nm</i>	<i>s</i> ⁻¹
Pol γ A wt	40.0 (± 2.4)	20.7 (± 3.4)	53.8 (± 2.2)	0.2 (± 0.01)
Pol γ A R232G	39.2 (± 1.9)	16.8 (± 2.2)	51.2 (± 2.1)	0.2 (± 0.01)
Pol γ A wt and Pol γ B wt	60.4 (± 1.7)	8.0 (± 0.5)	41.2 (± 1.6)	0.1 (± 0.00)
Pol γ A R232G and Pol γ B wt	57.1 (± 2.8)	16.2 (± 1.2)	61.8 (± 1.5)	0.2 (± 0.01)

^a The numbers in parentheses are the root mean square standard deviations for data fitting.

$$[25nt]_0 - [25nt]_t = \sum(21-24nt)_t + \sum(<21nt)_t \quad (\text{Eq. 2})$$

where $[25nt]_0$ and $[25nt]_t$ are concentrations of the 25-nt primer at times 0 and t (Fig. 6*a*, inset). $\sum(21-24nt)_t$ and $\sum(<21nt)_t$ are the respective sums of nucleolytic products of primers originally containing a mismatched or a base-paired 3'-end.

The two processes are sequential, which allows us to simplify the kinetic analysis by fitting the formation of all mismatched primers $\sum(21-24nt)_t$ at short times (≤ 1 s; Fig. 6*a*) to the following equation.

$$\sum(21-24nt)_t = A_m[1 - \exp(-k_{\text{exo}}^m \cdot t)] \quad (\text{Eq. 3})$$

Data of hydrolysis of duplex DNAs shorter than 21 nt (Fig. 6*b*) were then fitted to the following equation,

$$\sum(<21nt)_t = A_c[1 - \exp(-k_{\text{exo}}^c \cdot t)] \quad (\text{Eq. 4})$$

where A_m and A_c are the respective amplitudes for mismatched and correctly paired primer-templates; they represent the amount of primer-template bound to polymerase subject to excision during the first cycle of reaction, and k_{exo}^m and k_{exo}^c represent excision rates of mismatched and correctly paired primers.

To provide details on intermediate steps of exonuclease reaction, we fit data to a minimal model shown in Scheme 1 based

upon computer simulation. The observed kinetics of the disappearance of the starting material and the transient rise and fall of each intermediate are a function of the rates of excision *versus* dissociation of the DNA from the enzyme. For example, the loss of the starting material in the absence of Pol B (Fig. 5, *f* and *g*) is biphasic due to the partitioning between excision and dissociation, whereas in the presence of the Pol B, the reaction goes largely to completion. Dissociation of the DNA from the enzyme during the exonuclease reaction also accounts for the observed accumulation of intermediate products; otherwise, each intermediate would disappear as the reaction to form the next product proceeded. To fit the data, we assumed a single dissociation rate for each intermediate and achieved a good fit. Allowing different dissociation rates for intermediates of different lengths slightly improved the fit, but the improvement was insufficient to justify the increased number of parameters.

Mismatch Removal by Pol γ A and Holoenzyme—Wild type Pol γ A exhibits significant nucleolytic activity for mismatched DNA; with an amplitude of 40 nM, substrate is excised at a rate of 20.7 s^{-1} (Fig. 6*a* and Table 2). Pol γ A R232G alone displays mismatch removal activity comparable with wild type, suggesting that substitution has little impact on the integrity and function of the exonuclease active site. However, in the presence of Pol γ B, significant differences become apparent. The amplitude

TABLE 3
Exonuclease kinetic constant for Pol γ A variants^a

Enzyme		Rate Constant (s ⁻¹)					
		k_1	k_2	k_3	k_4	k_5	k_{off}
Pol γ A wt	–	9 (\pm 2)	25 (\pm 9)	26 (\pm 9)	12 (\pm 4)	2.3 (\pm 1.5)	7.7 (\pm 2.2)
Pol γ A R232G	–	8 (\pm 1)	24 (\pm 4)	25 (\pm 6)	11 (\pm 6)	2 (\pm 0.9)	6.6 (\pm 1.3)
Pol γ A wt	Pol γ B wt	6 (\pm 0.2)	11 (\pm 0.4)	9 (\pm 0.5)	2 (\pm 0.1)	0.2 (\pm 0.1)	1.3 (\pm 0.1)
Pol γ A R232G	Pol γ B wt	11 (\pm 0.9)	17 (\pm 2)	14 (\pm 3)	5 (\pm 0.7)	0.6 (\pm 0.2)	2.2 (\pm 0.5)

^a Rate constants were derived by global data fitting of the full time course of sequential exonuclease reactions for each enzyme (Fig. 5), based upon the model shown in Scheme 1. Standard error estimates were derived by nonlinear regression based upon the global fit to the model as described in the text. In fitting the data, the DNA dissociation rate was assumed to be the same for each intermediate for a given enzyme, and the DNA rebinding rate was also held fixed at 10 μ M⁻¹ s⁻¹ for all enzymes.

of wild type holoenzyme increases, indicating an increased affinity for a mismatched DNA substrate, and the burst rate is reduced.

The rate of the first excision reaction (k_1) is slower than the two subsequent excisions (k_2 and k_3). This may reflect some Mg⁺-dependent isomerization upon initiation of the reaction. The rate constants (k_2 , k_3 , and k_4) by holoenzymes containing either Pol γ A wt or R232G were slower than Pol γ A alone (Table 3). Thus, one effect of Pol γ B appears to be in stabilizing the duplex DNA at the *pol* site, thereby slowing the rate at which the DNA migrates to the *exo* site.

A primer containing a single mismatch is the most likely product of erroneous synthesis. Of primers containing mismatches, that with a single unpaired nucleotide is excised by wild-type at 2 s⁻¹ (k_4) (Table 3), in good agreement with earlier pre-steady state analyses (6) and FRET analyses (18). However, the rate of hydrolysis of a single mismatch by the R232G mutant holoenzyme is 2.5-fold faster than wild-type, suggesting that the mutant is less sensitive to the Pol γ B-induced rate reduction. This result supports the idea that R232 mediates communication between Pol γ A and γ B subunits.

Hydrolysis of a Correct Base Pair—In contrast to the increased amplitude for mismatched DNA excision, on perfect duplex DNA both the amplitude and burst rate by wild type holoenzyme are lower than by Pol γ A alone (Fig. 6*b* and Table 2). The rate of hydrolysis of the correctly paired base (k_5) of a perfectly base-paired primer is much slower than any mismatched primer. Both Pol γ A wild-type and the R232G mutant have rate constants (k_5) for removal of a base-paired terminus about one-fifth those for containing a single mismatch (k_4). The differential for base-paired *versus* a mismatch is about 10-fold for the holoenzymes. The accessory protein Pol γ B therefore not only increases the processivity and rate of polymerization but also simultaneously stimulates excision of incorrectly incorporated nucleotides and reduces unnecessary excision of correctly incorporated nucleotides. Performing both functions confers a significant Pol γ B contribution to DNA synthesis fidelity.

In stark contrast, the rate constant for the Pol γ A R232G holoenzyme was three times faster than wild-type. This is even apparent by visual inspection of Fig. 5, *d* and *e*. The amounts of 20 and 19 nucleotide-long primer strands, representing continued hydrolysis after all mismatches have been removed, appear earlier and attain significantly higher levels. These observations suggest that the mutant holoenzyme is less capable of distinguishing the conformations of mismatched and correctly paired primer-templates, and therefore, correctly incorporated nucleotides are excessively and unnecessarily hydrolyzed.

Taken together, holoenzyme containing Pol γ A R232G has elevated exonucleolytic activities for both correct and incorrect DNA. Nonetheless, because of increased removal of the correct DNA, the selective nucleolytic activity is reduced. Consequently, wild type holoenzyme is 60-fold more active on mismatches than correctly paired DNA, whereas the R232G holoenzyme is only 23-fold.

DISCUSSION

Among disease-associated Pol γ A mutations, those located in the polymerase (*pol*) or exonuclease (*exo*) active sites have been easiest to rationalize. The recognizable motifs and the crystal structure of the homologous T7 DNAP bound to DNA aided the interpretation of biochemical defects because of active site mutations. Such analyses have provided explanations for some severe disorders, *e.g.* active site mutations in *pol* have been linked to autosomal recessive progressive external ophthalmoplegia, and mutations in *exo* have been linked to pathological premature aging (16, 17).

However, correlating a clinical phenotype with mutations outside the active sites was more difficult until determination of crystal structure of human Pol γ (13). Several patients displaying severe clinical neurological and muscular disorders have been found to carry the Pol γ A Arg²³² substitutions R232G and R232H. Patients presented various degrees of mtDNA copy number reduction or deletions of mtDNA, both of which are indicative of defective mtDNA maintenance. Using an atomic structure of Pol γ holoenzyme to guide our biochemical investigation, we have described critical properties of Pol γ A Arg²³² that provide a molecular explanation for the role of Arg²³² substitutions in human disease.

Like most DNA replicases, the catalytic subunit of Pol γ A has low processivity. Not until Pol γ A associates with its accessory subunit Pol γ B does the resulting holoenzyme exhibit high processivity. Pol γ A Arg²³² forms the only strong interaction with Glu³⁹⁴ of the distal Pol γ B monomer in the holoenzyme. Although our biochemical investigation confirms that the interaction contributes to Pol γ activity, the salt bridge between the residues seen in the apo-enzyme is not absolutely required. However, Pol γ A Arg²³² is essential for mediating Pol γ B functions, explaining conservation of the residue across diverse Pol γ A proteins, even in species where the holoenzyme contains only monomeric Pol γ B.

Substitutions of Pol γ A Arg²³² Alter Both Polymerase and Exonuclease Activities of Holoenzyme—There are no detectable defects of Pol γ A Arg²³² substitutions on the activities of the catalytic subunit, suggesting that Arg²³² does not directly participate in either *pol* or *exo* activities. Surprisingly, Pol γ A

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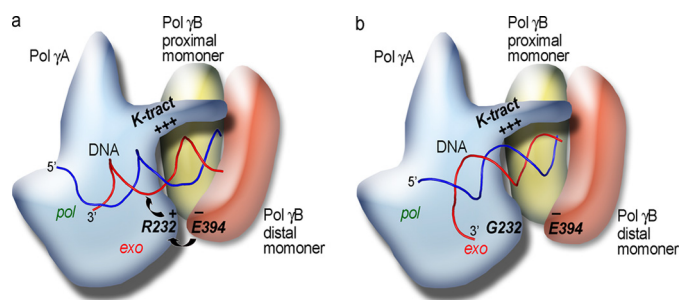


FIGURE 7. The signaling pathway in the modeled Pol γ holoenzyme-DNA complex. The 5'-end of the template and the 3'-end of the primer are indicated. *a*, Pol γ A undergoes conformational changes upon binding to DNA at the K-tract that results in concerted changes in Pol γ B. The Arg²³²-Glu³⁹⁴ region moves closer to the DNA-binding channel (in the direction indicated by the arrow). In this conformation, Pol γ A Arg²³² forms a bipartite charge interaction with both the DNA backbone and Pol γ B Glu³⁹⁴ that facilitates positioning the primer terminus in *pol*. *b*, the mutant Pol γ A G²³² cannot make either interaction and is thus unable to transduce the signal from Pol γ B. A more detailed discussion is given in the text.

mutants R232G or R232E show major defects only in holoenzyme, where, despite an apparently normal association of subunits, the effects of Pol γ B are completely abrogated. The R232H mutant behaves similarly, albeit to a lesser degree. We conclude that Pol γ A Arg²³² constitutes an essential part of the communication pathway with Pol γ B. Signal transduction is completely disrupted by the R232G or R232E substitutions; the mutant holoenzymes display markedly reduced processivity. Concurrently, they display altered exonuclease activity. The combined changes of *pol* and *exo* activities result in Pol γ A Arg²³² mutant-containing holoenzymes having a major defect in DNA synthesis. Pol γ A R232H-containing holoenzyme presents 43% of wild type activity, and that containing R232G presents only 13% of wild type activity.

We showed previously that each monomer of Pol γ B dimer has a distinct role in processivity; the proximal monomer increases the affinity for DNA, whereas the distal monomer increases the polymerization rate (14). Holoenzymes containing R232H or R232G show no—or even a negative—reaction rate acceleration over that of Pol γ A alone, indicating that Pol γ A Arg²³² is mainly responsible for transmitting a signal from the distal Pol γ B monomer. Nonetheless, the mutant holoenzymes exhibit normal DNA binding affinity, suggesting that the function of the Pol γ B proximal monomer is unaffected by substitutions of Arg²³². The Pol γ A K-tract, a lysine-rich region (⁴⁹⁶KQKKAKK⁵⁰⁵) of the AID subdomain (13) (Fig. 7), most likely enhances DNA binding in holoenzyme. The distinct functions of the two Pol γ B monomers, strengthening DNA binding and enhancing the polymerization rate, may thus be independently mediated by the K-tract and Arg²³² of Pol γ A.

Pol γ A Arg²³² Senses the Conformation of Primer-Template for Selective Exo Activity—Pol γ B was previously reported to reduce the exonuclease activity of holoenzyme, which potentially could lower its proofreading ability, relative to Pol γ A alone (6, 7). This seemingly detrimental activity of a processivity factor is, however, a combination of excision of both a mismatched and a properly base-paired primer. By modifying the methods described in Ref. 6, we were able to analyze mismatch and correct nucleotide excision in the same reaction.

Although Pol γ A has an intrinsic ability to distinguish mismatched from correctly matched primer-template, Pol γ B further enhances this selectivity. By increasing the affinity and reaction rate of a mismatched primer in the *exo* site and a correctly paired primer in the *pol* site, Pol γ B facilitates the DNA geometry-dependent activity of the catalytic subunit, thereby making a positive contribution to the fidelity of DNA synthesis. As a result, the nucleolytic rate of Pol γ holoenzyme on a correctly paired primer is reduced to ~1% of a mismatch (Table 3).

The exonuclease activity of holoenzyme containing Pol γ A R232G is elevated for both correct and incorrect paired primers. However, the nucleolytic activity for the correct primer increases more than for mismatched primers, leading to a reduced ability to discriminate. Consequently, the mutant holoenzyme more frequently positions a correctly base-paired primer in the *exo* site, resulting in excessive degradation. This suggests that Arg²³² comprises part of the sensing system that directs a primer terminus to *pol* or *exo* as appropriate.

The *pol* and *exo* sites are 45 Å apart, similar to those in other DNA polymerases. How is a primer strand transferred between the two sites? Crystal structures of two DNAP editing complexes with the primer terminus in *exo* show that both maintain their respective apo-enzyme conformations (18–20); primer bound in the *exo* site may therefore be the low energy state. The low energy state for DNA bound to Pol γ A may also lie in *exo*, but for holoenzyme it is biased toward the *pol* site. Binding of dNTPs should also promote switching to *pol*, but when polymerization stalls, the primer strand falls back into the *exo* site for editing.

A mismatch has the highest probability to be edited immediately after its incorporation (6). The important comparison is therefore between hydrolysis of a single mismatch and perfectly duplexed DNA. Interestingly, mismatch removal occurs at a steadily decreasing rate as the number of mismatches reduces from four to one nucleotide. The rate reduction may reflect a requirement for an increasing number of base pairs to be unwound prior to excision (21, 22). The rate of hydrolysis of a single mismatch is slowest among mismatches, suggesting that the lowest energy barrier for a primer to partition between the *exo* and *pol* sites is for a single mismatch, in agreement with the fluorescence studies of Millar and co-workers (15). This suggests that the two sites are delicately balanced and can be interconverted by a subtle structural change in polymerase conformation. A low energy barrier would allow rapid switching between synthesis and editing modes so that the polymerase can achieve rapid replication with high fidelity.

Arg²³² and Primer Strand Transfer—We suggest the following model for why substitutions of Pol γ A Arg²³² have such a detrimental effect on holoenzyme activity when they confer only minor changes to the activities of the catalytic subunit alone. Substitution of Pol γ A Arg²³² changes both *pol* and *exo* activities, suggesting that the arginine residue directly interacts with DNA. In the current absence of atomic structures of Pol γ -DNA complexes, we constructed a Pol γ -DNA complex using the structure of the apo-holoenzyme with a docked primer-template DNA (Fig. 7).

Two Pol γ A DNA-binding regions, the AID subdomain and Arg²³², function only in the holoenzyme. Both regions are

essential for positioning the primer terminus in *pol* or *exo* site, and their functions are mutually dependent. The AID subdomain harbors the positively charged lysine-rich K-tract that could contact DNA (13). The opposite face of the K-tract forms the major subunit interaction with the Pol γ B proximal monomer, but deleting the interacting residues in Pol γ A has no effect on activity. In the absence of Pol γ B, the AID subdomain is simply too flexible to allow the K-tract to function. Furthermore, in the absence of a K-tract interaction to clamp the template-primer, Arg²³² cannot form a stable interaction with DNA. Thus, in the absence of Pol γ B, neither AID nor Arg²³² alone can contribute to Pol γ A function, which explains why Arg²³² substitutions only affect holoenzyme activity.

When a template-primer is docked onto the apo-enzyme structure, positioning the 3'-end of the primer in the *pol* site, neither Arg²³² nor the AID subdomain directly contacts DNA. We suggest that upon binding to DNA, the Pol γ A AID subdomain undergoes a rotational movement that aligns the K-tract with the phosphodiester backbone. Because of the strong interaction of the AID subdomain with Pol γ B, rotation of the AID subdomain should induce a concerted rotation of the dimeric Pol γ B in the direction that brings the distal monomer close to the Arg²³² loop region. Consequently, the local subunit contact surface area between Pol γ A and the distal Pol γ B monomer is extended beyond the single salt bridge of Arg²³²–Glu³⁹⁴ in the apo-enzyme. In addition, the Arg²³² loop is brought closer to the DNA (Fig. 7). Pol γ A Arg²³² may then form a bipartite charge-charge interaction with both the negatively charged backbone of the primer-template and Pol γ B Glu³⁹⁴ and may thus coordinate with the latter in positioning the primer-template in the *pol* site.

In the holoenzyme, whereas the K-tract firmly binds the duplex DNA upstream of the primer terminus, Pol γ A Arg²³² could act as a pivot between the *pol* and *exo* active sites by biasing a duplex primer toward *pol*. This conformational change model explains why Arg²³² only functions in the holoenzyme. Eliminating the positive charge in Pol γ A R232G disrupts both the salt bridge with Pol γ B and its interaction with DNA. This explains why Pol γ A R232G is completely unresponsive to Pol γ B-mediated stimulation of polymerase and suppression of exonucleolytic reaction rates. Similarly, Pol γ A R232E introduces charge repulsion to both DNA and Pol γ B Glu³⁹⁴, supporting the proposed role for Arg²³². Presumably, the histidine substitution in R232H retains enough positive charge character in its local environment to allow a small response to the presence of Pol γ B. Loss of Arg²³² function abolishes the balancing force to switch the primer strand back to the *pol* site, resulting in excessive nucleolytic activity. The Pol γ B E394R substitution, which leads to a partially defective holoenzyme (Fig. 3 and Table 1), should widen its separation from Pol γ A Arg²³², indicating that for optimal function the proper local geometry of Arg²³² and Glu³⁹⁴ is important. This geometry must be maintained with Pol γ B E394A, because holoenzyme containing this mutant is fully functional, but the defects of the charge-reversal combination of Pol γ A R232E/Pol γ B E394R holoenzyme indicate that a simple interaction is insufficient, and Pol γ A Arg²³² is of critical importance.

Pol γ A Arg²³² provides the balancing force to switch the primer strand from its likely low energy state in *exo* back to *pol*. Loss of Pol γ A Arg²³² therefore prolongs the residence time of the primer in the *exo* site, resulting in excessive nucleolytic activity.

Pol γ A Arg²³² Substitution and Human Diseases—Pol γ A Arg²³² has an essential role in both processive DNA synthesis and proofreading, providing a molecular explanation for the association of Pol γ A Arg²³² substitutions with human diseases. Estimating the reduction in synthetic capacity (2.5-fold for R232H and 7.7-fold for R232G) and an increase in exonucleolytic activity (3-fold), we predict that the overall mtDNA content for patients carrying Pol γ A R232H or R232G should be ~13 or 4%, respectively, of normal. These are in excellent agreement with clinical findings, where the patients who carried Pol γ A R232H and R232G had 12 and 3–5%, respectively, of the normal mtDNA content.

However, patients carrying Pol γ A Arg²³² substitutions are heterozygous for *POLG*, where the effects of the substitution are complicated by the presence of the other copy. Although recessive to wild type, an Arg²³² mutant holoenzyme may compete effectively with Pol γ A variants for template DNA, reducing their capacity for synthesis. The disease manifestations suggest that the substitutions T251I/P587L, G737R, and G848S that accompany R232G or R232H should also cause Pol γ A to be defective. Even if the intrinsic effects of these substitutions on Pol γ activity are mild, they will be enhanced in the presence of an Arg²³² substitution. Inducing expression of a dominant negative mutant Pol γ A in human cells quickly depleted mtDNA (23), an observation likely caused by the same mechanism.

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