Mitochondrial DNA polymerase γ (pol γ) is responsible for replication and repair of mtDNA and is mutated in individuals with genetic disorders such as chronic external ophthalmoplegia and Alpers syndrome. pol γ is also an adventitious target for toxic side effects of several antiviral compounds, and mutation of its proofreading exonuclease leads to accelerated aging in mouse models. We have used a variety of physical and functional approaches to study the interaction of the human pol γ catalytic subunit with both the wild-type accessory factor, pol yB, and a deletion derivative that is unable to dimerize and consequently is impaired in its ability to stimulate processive DNA synthesis. Our studies clearly showed that the functional human holoenzyme contains two subunits of the processivity factor and one catalytic subunit, thereby forming a heterotrimer. The structure of pol γ seems to be variable, ranging from a single catalytic subunit in yeast to a heterodimer in Drosophila and a heterotrimer in mammals.

Mitochondria contain a single DNA polymerase, polγ, responsible for replication and repair of mtDNA (reviewed in Ref. 1). Human pol γ is isolated from mitochondria as a complex containing two subunits, a catalytic subunit, pol γA, of 139 kDa and an accessory subunit, pol γB, of 53 kDa (2–5). The catalytic subunit is a family A DNA polymerase with separate polymerase and 3’–5’ exonuclease domains. Two recent developments have emphasized the importance of pol γ. Mutations in the catalytic subunit of human pol γ cause mitochondrial disorders (6, 7), and a proofreading ability accumulates errors in mtDNA and undergoes accelerated aging (8, 9).

The processivity and substrate binding properties of pol γA are enhanced by complex formation with the accessory subunit (2, 4, 5). Most interestingly, the presence of the accessory subunit has been shown to decrease the fidelity of DNA synthesis by the catalytic subunit because it increases the ability of the enzyme to extend a mismatched primer (10). Initial characterizations of pol γ suggested that the enzyme forms a heterodimer containing one copy of each subunit. However, when we solved the crystal structure of mouse pol γB, it became apparent that this accessory factor is itself a homodimer with remarkable structural similarity to prokaryotic tRNA synthetases (Protein Data Bank code 1G5H (11)). We considered it to be very unlikely that this dimerization is a crystal packing artifact, especially because we were able to show that wild-type pol γB sedimented more rapidly than a deletion derivative lacking a major portion of the dimerization interface (11).

The incorporation of pol yB in the pol γ holoenzyme appears to be a relatively recent event in evolutionary terms. The protein has not been reported in yeast, where pol γ was first cloned as a product of the mip1 gene (12), and efforts to find evidence for it have been unsuccessful (13). Drosophila pol γ differs from the enzyme in vertebrates as it is reported to contain only one subunit each of pol γA and pol yB (14, 15), because the amino acids required for dimerization of human pol γB are absent in the Drosophila ortholog. Thus, pol γ provides an interesting example of a eukaryotic DNA polymerase with variable quaternary structure. A number of publications have speculated on the possibility that pol γA or pol yB alone may have roles in mtDNA replication or repair independent of the other subunit (5, 10, 16, 17). Therefore, we have undertaken the studies described here to examine the stoichiometry and association kinetics for human pol γB by using a range of different methods. Our studies represent the first extensive investigation of the interaction between subunits of human pol γ and show that the native enzyme is a heterotrimer that contains one molecule of the catalytic subunit and two molecules of the processivity factor. The tight binding we observe between the two subunits suggests that the free subunits do not exist in vivo and that individual subunits do not play important roles in mitochondrial DNA replication and repair.

**EXPERIMENTAL PROCEDURES**

**Purification of Proteins**—His-tagged recombinant exo− pol γA was purified from SF9 cells infected with the pVL1393 baculovirus engineered to express exo− pol γA as described by Longley et al. (18). Cells were grown in suspension culture in JRH Excell 420 culture medium supplemented with 2.5% fetal calf serum and 100 IU penicillin plus 100 μg/ml streptomycin. 5-Liter batches of cells were harvested on the 4th day of incubation and centrifuged at 96,000g for 1 h at 4°C. The supernatant was treated with 0.05–1 mM imidazole (0.05–1 mM imidazole) and loaded onto Ni-IDA HisTrap resin (Amersham Biosciences) equilibrated in Buffer II (20 mM Hepes, pH 8.0, 5% glycerol, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.2 mM PMSF, 1 μg/ml pepstatin A, 5 μg/ml leupeptin) with 0.3 M KCl and 0.1% Triton X-100. The lysate was centrifuged at 96,000 × g for 1 h at 4°C. The supernatant was treated with 0.04% polyethyleneimine and centrifuged for 30 min at 96,000 × g to remove the DNA. The lysate was adjusted to contain 60 mM imidazole and 5 mM MgCl₂ and loaded onto Ni-IDA HisTrap resin (Amersham Biosciences) equilibrated in Buffer II (20 mM Hepes, pH 8.0, 5% glycerol, 0.2 mM PMSF, 1 μg/ml pepstatin A, 5 μg/ml leupeptin) with 0.2 M KCl and 50 mM imidazole. After washing with 10 column volumes of Buffer II, the protein was eluted with a linear gradient of imidazole (0.05–1 M) in Buffer II with 0.1 M KCl. Fractions of the eluate containing pol γA were diluted with Buffer I to adjust the conductivity to below 0.1 M KCl.
The enzyme was applied to a 1.0-ml HR 5/5 Mono S column equilibrated in Buffer I with 0.08 M KCl. After washing with 5 column volumes, the protein was eluted with a 15-column volume linear gradients of 0.08 to 0.7 M KCl in Buffer I. Fractions containing peak absorbance at 280 nm were loaded onto a Hiloald 16/60 Superdex 200 column (Amerham Biosciences) equilibrated in Buffer I containing 0.3 M KCl. The exo- pol γA eluted from this column at 62 ml. All chromatographic steps were performed at 4 °C. The enzyme was concentrated to 1 mg/ml using a 100,000 M, Vivaspin ultrafiltration device. The yield of the protein was about 0.7 mg/liter culture.

The His-tagged recombinant pol γB and pol γBΔ14 mutant proteins were expressed in *Escherichia coli* BL21 RIL codon+ cells (Novagen) transfected with plasmids pET22b+ constructs as described by Carrodugas et al. (11). When proteins were prepared without a His tag, the construct was modified by introducing a stop codon at the Nt1 restriction site of the pET22b+ vector. Pellets from 2 liters of bacterial culture expressing pol γB were suspended in 40 ml of Buffer I with 0.3 M KCl and 0.1% Triton X-100, and cells were broken in a French press. The lysate was centrifuged at 16,000 × g for 30 min at 4 °C. The supernatant was treated with 0.14% polyethyleneimine and centrifuged under the same conditions to remove the DNA. The lysate was adjusted to contain 50 mM imidazole and 5 mM MgCl₂, and the protein was purified by nickel affinity, cation exchange, and gel filtration chromatography as described for pol γA above. The enzyme was concentrated to 8 mg/ml using a 30,000 M, Centricon ultrafiltration device. The yields of pol γB and pol γBΔ14 proteins were about 2 and 8 mg/liter culture, respectively.

Reconstituted pol γ holoenzyme was prepared by mixing 1 volume of S9 lysate containing His-tagged exo- pol γA and 0.7 volume *E. coli* lysate containing recombinant pol γB without a His tag. The relative volumes used for this reconstitution varied somewhat from one preparation to another based on the specific protein concentrations of the lysates as assessed by SDS-PAGE. The lysates were mixed for 30 min at 4 °C on a rotator and adjusted to contain 60 mM imidazole and 5 mM MgCl₂. The holoenzyme was purified and concentrated using the same protocol as for pol γA. The extinction coefficients used to quantify protein were determined following Gill and von Hippel (19). The ε 280 for pol γA was 243,790 A 280/M and that used for both WT pol γB and pol γBΔ14 was 71,940 A 280/M, because the deletion did not remove any residues that contribute to A 280. The absorbance measurements were routinely made on native protein preparations and were blanked against the dialysate used in preparing the protein. Controls indicated that absorbances at 280 nm were identical within 3% when the proteins were denatured with guani-
dine hydrochloride.

**Analytical Ultracentrifugation**—Sedimentation equilibrium experiments were conducted using a Beckman XL-1 Optima analytical ultracentrifuge and a Ti-60a rotor. Six-channel, charcoal-filled Epon centerpieces with quartz windows were filled with 100 μl of sample (in 20 mM Hepes, pH 8.0, 300 mM KCl, 1 mM EDTA, 2 mM β-mercaptoethanol, 5% glycerol) at protein concentrations between 1.88 and 5.64 μM. Absorbance profiles were acquired at 280 nm. Sedimentation equilibrium studies were performed at 4 °C, using three different speeds (15,000, 18,000, and 22,000 rpm for wild-type pol γB and 20,000, 25,000, and 30,000 rpm for pol γBΔ14). The data were analyzed by two programs, Heteroanalysis (National Analytical Ultracentrifugation Facility at the University of Connecticut) and the XL-A/XL-I data analysis software (Beckman), and consistent results were obtained. Solvent density was calculated as 1.026 (Sednterp software (20)), and the partial specific volumes of protein were approximated from their amino acid compositions (0.7299 for wild-type pol γB and 0.7283 for pol γBΔ14) (Sednterp).

**Surface Plasmon Resonance**—Surface plasmon resonance (SPR) experiments were performed on a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden) at 25 °C. pol γA was immobilized on a Sensor Chip C CM5 using the amine coupling kit (Biacore AB) in 10 mM phosphate buffer, pH 6.0, with immobilization levels of 1400 resonance units (RU). Analytes (WT pol γB and pol γBΔ14) were injected at concentrations ranging from 0 to 320 nM over the sensor chip with immobilized pol γA for 3 min followed by 8 min of dissociation and 10 s of regeneration using 3 mM NaOH, 1 mM KCl. Mass transfer and linked reaction experiments were performed. The kinetics of pol γB and pol γBΔ14 interaction with immobilized pol γA was evaluated using alternative predefined models in the BIAevaluation 4.1 software (Biacore AB). A model was judged appropriate if it fit to every experiment with χ² < 1.5. The conformational change model assumes a two-state reaction, where analyte B binds to ligand A followed by a conformational change of the complex AB to AB⁺. The overall dissociation constant K D was calculated from the rate constants as follows: K D = 1/(k off/(k on × (1 + k d/k s))). For stoichiometry determinations, a sensor Chip C CM5 with immobilization levels of pol γA at 600 RU was used. Analytes (pol γB and pol γBΔ14) were injected at increasing concentrations (from 50 to 500 nM) over immobilized pol γA for 5 min followed by 8 min of dissociation and 10 s of regeneration using 3 mM NaOH, 1 mM KCl. When the saturated concentration was achieved, R max values for pol γB (420 RU) pol γBΔ14 (217 RU) were measured, and the stoichiometry was calculated based on the equation: S n = (M r(pol γA)/M r(pol γΔ14)) × R max/RL, where RL is the resonance signal at a given ligand concentration.

The affinity of the enzymes for DNA was investigated using the streptavidin-coated sensor chip (SA chip; Biacore). A solution (2 nM) of a 5‘-biotinylated oligonucleotide (5‘-BioTET) in 26/45-mer (Qiagen) in HBS-EP BIACore buffer was used to yield an increase of 10 RU (1 min) on flow cell 1 and 150 RU (10 min) on flow cell 2. Two other flow cells without an oligonucleotide were used as reference cells. After immobilization, running buffer with low salt was used (30 mM KCl, 0.005% surfactant P20, 10 mM Hepes, pH 7.4). To compare the affinity of enzymes for DNA at different salt concentrations, 50 nM analytes (pol γA, pol γAB⁺, and pol γAΔ14) in buffers with different ionic strengths (30–200 mM for KCl and 1–40 mM for MgCl₂) were injected over immobilized oligonucleotide for 8 min followed by 30 s of regeneration using 1 μM KCl. In experiments with varied MgCl₂ concentration, 30 mM KCl was used.

**Isothermal Titration Calorimetry**—Isothermal titration calorimetry (ITC) experiments were performed with a VP-ITC calorimeter (Microcal) at 25 °C with a mixing speed of 302 rpm. The WT pol γB (160 μM) or pol γBΔ14 (130 μM) proteins were titrated as 5-μL injections (first injection 2 μL) into the sample cell containing 7.2 μM exo- pol γA. Samples were prepared by dialyzing all interacting components extensively against a buffer containing 20 mM Hepes, pH 8.0, 300 mM KCl, 5% glycerol, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.2 mM PMSF, 1 μg/ml pepstatin A, 5 μg/ml leupeptin. The heats of dilution of both proteins with buffer were determined and subtracted prior to analysis. Data were analyzed using the ORIGIN software supplied with the instrument according to the one- and two-binding sites models.

**Electrophoretic Mobility Shift Assays**—Native gel electrophoretic mobility shift assays (EMSA) were conducted as described (11) using the 26/45-mer primer-template labeled on the 5′ end of the 45-mer (16), except that the assays were conducted as challenge assays to assess the stability of the DNA-protein complexes. Binding reactions were assem-
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bled in 30 μl containing 4-nM labeled primer-template, 4 μM pol γA, and where indicated, 8 μM pol γB or pol γBΔ14 (both forms of pol γB calculated as a monomer). Binding reactions continued for 10 min. At this time 4 μl were removed as a binding control, and 3 μl of 200-nM cold 26:45-mer were added as a competitor to bind any protein that dissociated from the labeled complex. A second 4-μl sample was removed within 10 s after the competitor was mixed into the reaction and was considered a 0.2-min time point during the time series. The binding control and 0.2-min sample were loaded directly into the lanes of a running native gel. Additional 4-μl samples were removed at 1, 2, 4, 8, and 16, and in some cases 32 min after addition of the competitor. Following electrophoresis, gels were dried onto Whatman DE81 paper and exposed to a PhosphorImager to quantify the radioactivity of bound and free DNA.

**DNA Polymerase Assays**—DNA polymerase assays were performed as described (2) with 0.2 pmol of pol γA and varied amounts of pol γB or pol γBΔ14 in 30-μl reactions using either 120 μg/ml activated calf thymus DNA or 10 μg/ml poly(dA)-oligo(dT) as template. The standard polymerase reaction buffer was 50 mM KCl, 8 mM MgCl2, 10 mM Tris, pH 8, 2 mM dithiothreitol, 100 μg/ml bovine serum albumin. Where indicated, reactions contained 140 mM KCl. Primer-extension pro-cessivity experiments were performed as described by Carrodeguas et al. (2) and in the legend of Fig. 9.

**RESULTS**

**High Level Expression of Both Subunits of Human pol γ and Reconstitution of the Holoenzyme**—We and others have been unable to express pol γA in bacteria in an intact form. To obtain quantities of recombinant enzyme sufficient for biophysical studies, we expressed the protein in insect cells from a baculovirus construct. Although it is possible to express the holoenzyme entirely in insect cells by co-expressing the A and B subunits, we chose to express the B subunit in bacteria using the T7 system. This permits us to test the activity of a number of pol γB variants without making an independent baculovirus construct for each protein of interest. In this study, we compared the binding of wild-type pol γB to a deletion derivative that we refer to as pol γBΔ14. This derivative, which contains a glycine residue in place of residues 147–178 of pol γB, was first reported by Carrodeguas et al. (11) to test the role of the four-helix bundle observed in the mouse pol γB crystal structure in dimerization of the accessory factor. This derivative has some ability to bind to primer-templates along with pol γA but has little ability to stimulate activity of the catalytic subunit. Thus, we set out to compare the association of wild-type pol γB and pol γBΔ14 to pol γA in this study.

Mixing a lysate of insect cells containing His-tagged pol γA with a lysate of bacterial cells expressing nontagged pol γB permits association of the subunits and purification of pol γ holoenzyme by using a three-step procedure. As described under "Experimental Procedures," this procedure consists of affinity chromatography on a Ni-IDA (His-trap®) resin followed by ion exchange chromatography and gel filtration. In a typical preparation beginning with 21 g of SF9 cells expressing pol γA and 15 g of BL21 cells expressing pol γB, we obtained a final yield of ~4.5 mg of pol γ holoenzyme. pol γA is prepared similarly without the addition of pol γB. These procedures have provided a sufficient quantity of enzyme for the biophysical studies of the association of the polymerase subunits reported here. The purity of typical proteins is shown in Fig. 1. It is apparent that the amount of the small subunit bound to pol γA is diminished by approximately half when the reconstitution is performed with pol γBΔ14 rather than with wild-type pol γB.

We previously compared the sedimentation properties of pol γB and pol γBΔ14 by using velocity sedimentation in glycerol gradients using a preparative ultracentrifuge (11). In this paper, we characterized these proteins in more detail by using equilibrium ultracentrifugation as...
shown in Fig. 2. For wild-type pol γB, sedimentation equilibrium distributions were fit to a single species. When the molar mass was treated as the unknown parameter in the global analysis, an average molecular mass of 105,747 Da was obtained (compared with the expected molecular mass of 106,916 Da for dimeric pol γB). Attempts to fit the data to a self-association model failed to yield better results. This is consistent with other data suggesting that the wild-type protein is a tightly bound homodimer with effectively no monomer present under these conditions. We estimated that the pol γB dimer association is characterized by a $K_{d}$ somewhat tighter than 0.1 nM. In contrast, analysis of data for the I4 mutant was complex. When the data were analyzed using a single-component model the apparent molecular mass calculated by the software was 70 kDa, a value intermediate between a monomer and a dimer. Moreover, the residuals indicated that the single-component model was
a poor fit to the data (data not shown). A better fit was obtained using a self-association model for a monomer-dimer equilibrium. The fitted monomer molecular mass was 48,370 Da, and the dissociation constant \( (K_D) \) was 5.8 \( \mu \)M. The fact that pol \( \gamma B\Delta I4 \) retains a weak ability to dimerize is consistent with the inference drawn from the crystal structure of mouse pol \( \gamma B \). In this model, we estimated that domain 2, which is deleted in pol \( \gamma B\Delta I4 \), contributes 2675 Å² to the total dimerization interface of 6850 Å². Thus, it is reasonable that pol \( \gamma B\Delta I4 \) is unable to form a dimer at these low protein concentrations, demonstrated as an exothermic process with \( \Delta H \) of \(-6.4\) kcal/mol and a \( K_D \) of \(-0.15\) \( \mu \)M. Association of pol \( \gamma A \) with the pol \( \gamma B\Delta I4 \) dimer is characterized as an exothermic process with \( \Delta H \) of \(-8522\) kcal/mol and \( \Delta S \) of \(-822\) kcal/mol.

Because the final step in our purification is a high resolution gel filtration step, we applied this as an analytical method to study the stoichiometry of the holoenzyme. FIG. 3 shows the results of a titration step, which known molar quantities of pol \( \gamma A \) and pol \( \gamma B \) were applied to a 60-cm Amersham Biosciences Superdex 200 gel filtration column. The elution profiles of free pol \( \gamma A \) and pol \( \gamma B \) are shown in the background of each panel. All added pol \( \gamma B \) was bound to pol \( \gamma A \) until saturation was reached at a 1.2 molar ratio of A to B. As the quantity of pol \( \gamma B \) was further increased, a peak of excess free pol \( \gamma B \) was apparent. This is consistent with the crystallographic finding that pol \( \gamma B \) is a dimer. A similar binding titration performed with pol \( \gamma A \) and pol \( \gamma B\Delta I4 \) showed saturation at a ratio of 1:1 (data not shown).

**Isothermal Titration Calorimetry Confirms the 1:2 Stoichiometry of A and B Subunits and Estimates the Affinity of the Interaction**—Isothermal titration calorimetry (ITC) was used to measure the enthalpy of holoenzyme formation, stoichiometry of the complex, and the dissociation constant. ITC was performed in parallel with both wild-type pol \( \gamma B \) and pol \( \gamma B\Delta I4 \) (FIG. 4). The results show that pol \( \gamma A \) is weaker by approximately a factor of 2. These measurements are comparable with the apparent \( K_D \) of 35 nM for the pol \( \gamma B-pol \gamma A \) interaction estimated from polymerase stimulation kinetics by Johnson et al. (5).

**TABLE 1**

| pol | \( K_D \) | \( \Delta G^{\text{obs}} \) | \( \Delta H^{\text{obs}} \) | \( -T\Delta S^{\text{obs}} \) |
|-----|----------|----------------|----------------|----------------|
| WT pol \( \gamma B \) | 1.97 (0.011) | \( 0.15 (0.014) \) | \(-9305\) | \(-6373 (52.5)\) | \(-2932\) |
| pol \( \gamma B\Delta I4 \) | 1.06 (0.062) | \( 0.14 (0.014) \) | \(-9344\) | \(-8522 (75.0)\) | \(-822\) |

\( ^* \) Indicates stoichiometry of binding.
\( ^\dagger \) \( \Delta G^{\text{obs}} \) estimated from titration curve using MicroCal software (experiments were performed at 25 °C).
\( ^\ddagger \) The errors shown in parentheses are the means ± S.D.

**Figure 5.** Kinetic analysis by SPR of pol \( \gamma B \) and pol \( \gamma B\Delta I4 \) interactions with pol \( \gamma A \). Different concentrations (0, 40, 80, 160, and 320 nM) of pol \( \gamma B \) or (0, 40, 80, and 160 nM) of pol \( \gamma B\Delta I4 \) (B) were injected in duplicate over 1400 RU of amine-coupled pol \( \gamma A \). The black points correspond to the experimental data and the solid lines to the fit using the two-state reaction model (conformational change). Residuals for the fit are shown. The kinetic and thermodynamic constants and systematic error related to the fits are listed in Table 2. The overall dissociation constant \( K_D \) is calculated from the rate constants: \( K_D = 1/(k_{-a}/k_{+a}) \times (1 + k_{a2}/k_{a2}) \).
The I4 Mutant Stabilizes the Interaction of pol γA with Primer-Template DNA Nearly as Well as Wild-type pol γB—We were surprised at the relatively small difference in affinity of pol γA for binding pol γBΔI4 as compared with the wild-type subunit. Therefore, we conducted experiments to examine the relative ability of these proteins to stabilize the interaction of pol γA with DNA primer-templates. One of the standard methods used to study DNA polymerases is to characterize their procedures” using pol γA alone (A), pol γA plus wild-type pol γB (B), or pol γA plus pol γBΔI4 (C). D shows the fraction of complex remaining intact in the presence of the cold binding competitor as a function of time.

FIGURE 6. The monomeric I4 deletion derivative of pol γB stabilizes pol γA on an oligonucleotide primer-template nearly as well as the wild-type dimeric pol γB. EMSA challenge assays were conducted as described under “Experimental Procedures” using pol γA alone (A), pol γA plus wild-type pol γB (B), or pol γA plus pol γBΔI4 (C). D shows the fraction of complex remaining intact in the presence of the cold binding competitor as a function of time.

The I4 mutant participates with pol γA in binding primer-template, but we have not studied previously the stability of this interaction. We therefore designed a binding challenge assay in which complexes containing different forms of pol γ bound to radiolabeled primer-template were assembled and then challenged with a large excess of cold primer-template to follow the kinetics of dissociation. Following addition of a 50-fold excess of cold primer-template, any polymerase released from the labeled complex would be much more likely to bind the unlabeled competitor than the original radiolabeled oligonucleotide. The kinetics of the dissociation reaction can be followed by withdrawing aliquots from the reaction at various times after addition of competitor and loading them directly on a running native gel. Similar use of EMSA to study dissociation kinetics has been employed since the initial development of this method (21).

shows that pol γA alone dissociates quickly from a 26-45 primer-template. The pol γAB2 holoenzyme complex migrates more slowly on the native gel, as expected, and shows considerable stabilization, with a half-life of almost 2 min (Fig. 6B). A parallel EMSA challenge assay performed with pol γ reconstituted with the I4 mutant protein revealed that this complex was almost as stable as that formed with wild-type pol γB (Fig. 6C).

One limitation of the EMSA to study nucleic acid-protein interactions is that this method provides little ability to vary solution conditions because ions in the reaction buffer are quickly separated from macromolecules as complexes enter the gel. We used SPR to study the primer-template binding of pol γA alone and complexes of pol γA with either wild-type pol γB or pol γBΔI4 at varied concentrations of monovalent and divalent ions (Fig. 7). Each curve in Fig. 7 represents the results of 10 titration experiments conducted at varied KCl or MgCl2 concentrations to determine the maximal amount of protein bound to the biotinylated 26-45 mer oligonucleotide immobilized on a streptavidin chip. The data are normalized to the maximum resonance signal obtained at low ionic strength. As expected, high concentrations of KCl or MgCl2 destabilized the binding of pol γA to the oligonucleotide, whereas the presence of wild-type pol γB in the pol γ holoenzyme significantly stabilized the binding of the polymerase to DNA. pol γBΔI4 provided an intermediate level of stabilization.

### Table 2: Kinetic parameters for pol γB and or pol γBΔI4 binding to the exo− pol γA in SPR experiments

| Protein Complex | $k_{a1} \times 10^5$ | $k_{d1} \times 10^{-5}$ | $k_{a2} \times 10^5$ | $k_{d2} \times 10^{-5}$ | $K_{D}$ |
|-----------------|---------------------|---------------------|---------------------|---------------------|--------|
| WT pol γB       | 2.81 × 10^4 (166)   | 6.21 × 10^{-3} (1.26 × 10^{-3}) | 4.93 × 10^{-5} (1.14 × 10^{-5}) | 7.09 × 10^{-4} (5.44 × 10^{-5}) | 2.78 × 10^{-7} (3.15 × 10^{-11}) |
| pol γBΔI4       | 3.61 × 10^4 (5268)  | 7.79 × 10^{-3} (3.25 × 10^{-3}) | 7.13 × 10^{-5} (1.75 × 10^{-5}) | 1.46 × 10^{-3} (3.42 × 10^{-5}) | 4.6 × 10^{-10} (2.5 × 10^{-10}) |

* For $k_{a1}$, $k_{d1}$, $k_{a2}$, and $k_{d2}$, the rate constants were obtained from nonlinear least squares global fitting of the respective sensorgrams using SF Revolution software version 3.0 and a model permitting conformational change.

b $K_{D}(a)$ is the equilibrium dissociation constant derived from the rate constants by the equation $K_{D} = 1/(k_{a2}/k_{d1})(1 + k_{a2}/k_{d2})$.

The errors shown in parentheses are the means ± S.D.
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The foregoing results showing that pol γΔI4 binds pol γA with high affinity and stabilizes the polymerase on primer-template oligonucleotides were somewhat surprising in light of our previous work showing that this monomeric derivative had very limited ability to stimulate DNA synthesis by pol γA (11). Previous polymerase reactions with this mutant used only low quantities of pol γBΔI4, <400 fmol, and used only poly(dA)-oligo(dT) as the primer-template. Therefore, we reinvestigated the ability of this derivative relative to wild-type pol γB to stimulate pol γA activity at higher protein concentrations and on heteropolymeric DNA as well as on the synthetic homopolymer. As can be seen in Fig. 8A, in reactions using 0.2 pmol of pol γA (6.7 nM) and a poly(dA)-oligo(dT) primer-template, DNA synthesis is maximally stimulated by 1 pmol of pol γB (0.5 pmol of B2 dimer; 16 nM). Significantly higher concentrations of pol γBΔI4 were required to stimulate pol γA, and the maximal activity obtained was ∼50% compared with wild-type pol γB. This reduced ability to stimulate pol γA was observed when activated DNA was used as substrate as well, but a dependence on pol γB was only seen at elevated KCl concentrations, as reported previously (2, 4) (Fig. 8B). Although the monomeric pol γBΔI4 did retain some ability to stimulate pol γA, the overall reaction rate was always diminished.

We performed single-round primer-extension reactions (Fig. 9) to study polymerase processivity in greater detail. pol γ was preincubated with a primer-template consisting of 5′-32P-oligo(dT)16-poly(dA). Extension of primers bound by pol γ was initiated by addition of TTP mixed with a nonradioactive primer-template to serve as a binding competitor to trap polymerase molecules that dissociated from the radioactive primers. This 26:45-mer oligonucleotide would require dATP as the first incoming nucleotide and is incapable of supporting polymerization with only TTP. Lanes 5 and 6 of Fig. 9 show that wild-type pol γ extended primers to products as long as 200–300 nucleotides (median extension about 60–80 nucleotides) before dissociation within the first 1.5 min. The size range of products did not continue to increase when the reaction time was doubled. These results and the controls in lanes 7 and 8 of Fig. 9 indicate that the oligonucleotide competitor provided an effective trap to prevent re-utilization of labeled primers. Thus, we conclude that the distribution of product lengths shown in lanes 5 and 6 of Fig. 9 represent the products synthesized following a single polymerase binding event. When pol γΔI4 was used in parallel reactions, the primers were typically extended by fewer than 40 nucleotides, indicating that the monomeric pol γBΔI4 was greatly impaired as a processivity factor. Virtually no extension of the primer was seen using pol γA alone.

DISCUSSION

pol γ Holoenzyme Subunit Stoichiometry—We report the first extensive investigation of the assembly of the pol γ holoenzyme. Our sedimentation equilibrium data show that pol γB forms a dimer in solution, while analytical gel filtration and isothermal titration calorimetry establish that the pol γB dimer binds tightly to the pol γA monomer to form a heterotrimer with the structure AB2. This is the first detailed study of the association of these subunits in the absence of DNA. Interestingly, the $K_D$ of 27 nM for the subunit interaction, which we determined by using SPR, agrees well with the binding affinity measured in the presence of DNA in the enzyme kinetic studies of Johnson et al. (5). The recombinant pol γAβ2 complex would have a calculated mass of 245
FIGURE 9. Processivity of pol γAB₂ and pol γABΔI4 analyzed by single-round primer extension. 500 pmol of either pol γA, pol γAB₂ or pol γABΔI4 were mixed with 5'-[32P]-labeled oligo(dT)₆-poly(dA) primer-template in standard polymerase reactions (50 mM KCl, 8 mM MgCl₂) in a final volume of 15 µl and preincubated on ice for 5 min and then at 30 °C for 1 min. For the reaction shown in lanes 1 and 2, the polymerase reaction was started by addition of TTP to a final concentration of 25 µM along with 4 pmol of 26:45 unlabeled oligonucleotide competitor. 4-µl samples were removed at 1.5 (lane 1) and 3 min (lane 2) and added to 6 µl of formamide loading solution. The reaction shown in lanes 3 and 4 was conducted in the same manner, except that the nonradioactive 26:45 competitor was added to the preincubation mixture before polymerase was added. Lanes 5–8 and 9–12 show the products of reactions similar to those in lanes 1–4, except that pol γAB₂ and pol γABΔI4 were used, respectively. A PhosphorImager analysis of a polyacrylamide-urea gel to analyze the products of primer extension is shown. Lane M shows the starting labeled oligonucleotide in the primer-template. Lane 0 shows the starting labeled oligonucleotide in the primer-template. Lane M shows mobility markers of end-labeled MspI fragments of pUC 18 DNA labeled according to size in nucleotides.

kDa. This appears to be somewhat larger than estimates of the molecular mass of the polymerase isolated from mitochondria (3, 4). We cannot rigorously rule out the possibility that differences in post-translational modification of the recombinant polypeptides or their inclusion of His tags may influence the higher order structure of the polymerase. However, considering the high biological activity of the recombinant enzyme, we think it is more likely that the smaller apparent size of the enzyme purified from mitochondria may be due to the fact that the hydrodynamic measurements in these studies used much lower protein concentrations. It is possible that the holoenzyme may be in equilibrium with A and B₂ subunits during overnight glycerol gradient sedimentation of dilute protein samples, leading to an underestimate of the sedimentation coefficient.

Unfortunately, there is currently no way to estimate the concentrations of polymerase subunits in mitochondria. The interaction between the pol γA and B₂ subunits is probably sufficiently tight to prevent frequent dissociation of the holoenzyme to the free subunits in vivo. Our previous experiments revealed no excess of free pol γA or pol γB subunits when the holoenzyme was purified from crude mitochondrial lysates (2, 16). Therefore, we think it unlikely that models for the role of pol γ in mtDNA replication and repair should consider any independent roles for the pol γ subunits under most circumstances. An exception to this generalization derives from the fact that these two subunits are synthesized and imported into mitochondria separately. Very little is known about the kinetic process by which single subunits are assembled into a holoenzyme in the organelle. Although we cannot rule out the possibility that single subunits may be free to engage in nucleic acid binding, no pool of free subunits has been documented.

We also compared the behavior of wild-type pol γB with that of a mutant form designated I4 that lacks a two-helix bundle domain important for dimerization. Analytical ultracentrifugation experiments showed that dimerization of this mutant protein is markedly impaired, with an apparent K₉₅ of 5–14 µM for the monomer-dimer equilibrium. Nevertheless, pol γBΔI4 retains the ability to bind to pol γA in a 1:1 complex with an affinity comparable with that of the wild-type pol γB dimer. Despite the difference in stoichiometry, both complexes are formed with nearly the same ΔG° (see Table 1). This implies that most of the interactions that pol γA forms with pol γB are preserved when it binds pol γBΔI4. As has been suggested for the Drosophila pol γ subunits, it is likely that human pol γA and pol γB share an extensive interaction interface (22). One simple model for the structure of the holoenzyme might propose that a dimeric pol γB may provide two identical interfaces for the interaction with pol γA. Such a model would be consistent with the assembly of an A₂B₂ tetramer. This structure, however, is not compatible with our results, which clearly indicate the formation of a heterotrimeric complex. We suggest an asymmetrical model for the interaction between pol γA and pol γB. According to this model, pol γA mainly interacts with one pol γB subunit in the pol γB dimer, but this interaction sterically inhibits interaction of a second catalytic subunit with the other pol γB subunit. When pol γA binds to the pol γBΔI4 mutant it is possible that pol γA forms additional contacts with pol γB surfaces that are exposed in the monomeric pol γBΔI4 permuting binding of this complex to primer-template but inhibiting polymerization. Both models are consistent with our data, and it will be extremely interesting to obtain a crystal structure of the pol γ holoenzyme to understand how this unusual processivity factor functions to stimulate the catalytic subunit.

Influence of pol γB on pol γA DNA Binding and Polymerase Activity—We have taken advantage of the contrast between wild-type pol γB and pol γBΔI4 to explore the contribution of the accessory subunit to the interaction of the polymerase with DNA primer-templates. By using both electrophoretic mobility shift assays and SPR experiments, we showed that the heterodimeric enzyme formed by association of pol γBΔI4 to pol γA binds tightly to primer-template and dissociates slowly in the presence of competitor primer-template. The apparent K₉₅ value for the binding of wild-type holoenzyme to a 26:45-mer oligonucleotide at 30 mM KCl is 0.06 mM, although pol γA and the pol γA-pol γBΔI4 complex bind the same substrate with K₉₅ values of 0.6 and 0.4 mM, respectively. These binding affinities are considerably tighter than those reported by Johnson et al. (5) based on polymerase kinetic measurements made at a higher salt concentration, 100 mM NaCl. Several labs have shown that activity of pol γA is reduced at high salt but is stimulated by pol γB (2, 4). Our SPR experiments show both pol γB and pol γBΔI4 dramatically stabilize binding of pol γA to primer-template at higher salt concentrations (Fig. 7). Like other DNA-binding proteins, pol γA presumably displaces cations upon binding to DNA (23). pol γB and, to a lesser extent, pol γBΔI4 appear to modify the interaction of pol γA with DNA to enable it to resist competition by free cations.

Although pol γB clearly increases the processivity of pol γA, it affects other activities of the catalytic subunit as well. Johnson et al. (5) have shown that the accessory subunit decreases the K₉₅ value for binding nucleotides and increases the polymerization rate, although Longley et al. (10) have demonstrated that these effects result in decreased polym-
erase fidelity. Our physical studies comparing the interaction of pol γB and pol γBΔ14 with pol γA have not directly addressed the influence of these proteins on kinetic parameters of polymerase function. However, we note that the tight binding of pol γBΔ14 to pol γA and the consequent stabilization of the enzyme on primer-templates does not lead to stimulation of polymerase activity (Figs. 8 and 9). Indeed, the monomeric pol γBΔ14 may have a weak ability to act as a dominant negative form of pol γB, because it competes well for binding to pol γA but is very ineffective in stimulating polymerization. Experiments to test this proposal are under way.

Comparison of pol γB to Other Processivity Factors—The available data suggest that the interaction between pol γB and pol γA is structurally distinct compared with that of most other pairs of processivity factors with their cognate polymerases. pol γB is structurally unlike any of the sliding clamp processivity factors such as proliferating cell nuclear antigen, E. coli pol III B, and T4 phage glycoprotein 45 that stabilize polymerase binding to DNA by encircling the DNA duplex (24). In these examples, as well as in the binding of thioredoxin to T7 phage DNA polymerase, the interaction of the accessory subunit with the polymerase is mainly mediated by a small interface that can be delimited to a peptide domain (25, 26). This is also true for cytomegalovirus UL44 (27, 28) and herpes simplex virus UL42 (29) proteins that do not form complete toroidal rings. Breyer and Mathews (30) have suggested that processivity can be conferred in either of two ways, by topological linkage, as illustrated by sliding clamps, or through the involvement of a large interaction surface. We suggest that pol γ may exemplify this latter category.

As noted in the Introduction, pol γ provides a unique example of an essential DNA polymerase that has a variable structure in different organisms. The obvious relationship between mammalian pol γB and homodimeric prokaryotic tRNA synthetases (11) has been cited as an illustration of horizontal gene transfer during evolution (31). An exceptional contrast is provided by Drosophila pol γ, which is a heterodimer containing one copy each of the A and B subunits (1, 15). Although Drosophila pol γB shares only 15% sequence identity with human pol γB, the only significant internal deletion in the alignment of the two proteins suggests that the Drosophila protein contains a discrete deletion of domain 2 of the mammalian proteins containing the two-helix structure, a deletion similar to that used to generate human pol γBΔ14. We suggest that the Drosophila protein may have experienced a deletion of this domain during evolution from a common ancestor with the mammalian lineage. Our observation that pol γBΔ14 is able to stabilize pol γ on primer-templates suggests that this deleted protein could retain substantial function. Additional mutations may have occurred to enable monomeric Drosophila pol γB to stimulate its catalytic partner. These mutations may have occurred either before or after deletion of this domain. It will be of great interest to compare the structures of Drosophila and mammalian pol γ holoenzymes.

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