Characterization of Novel Reverse Transcriptase and Other RNA-associated Catalytic Activities by Human DNA Polymerase γ

IMPORTANT IN MITOCHONDRIAL DNA REPLICATION

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During mitochondrial DNA (mtDNA) replication, DNA/RNA heteroduplex intermediates are formed. To understand how and why ribonucleotides are involved in mtDNA replication, we have studied the novel RNA-associated activities of human mitochondrial DNA polymerase (Pol γ), including reverse transcription, RNA-directed 3′ → 5′ DNA excision, RNA-primed DNA synthesis, and ribonucleotide incorporation. Remarkably, Pol γ catalyzes reverse transcription with a slightly higher efficiency than HIV-1 reverse transcriptase, suggesting that the activity may be physiologically significant, and furthermore, proofreading activity with an RNA template was also observed. RNA-primed DNA synthesis activity is required for initiation of mtDNA replication, and we have found that Pol γ holoenzyme is capable of performing this reaction at a physiologically relevant rate and that the accessory subunit plays an essential role in the initiation steps. Single ribonucleotides have been found scattered in the mtDNA genome, although their role and significance are not yet defined. Our finding that Pol γ also incorporates ribonucleotide triphosphates into a DNA primer offers a plausible enzymatic pathway for the origin of the RNA-containing mtDNA genome.

Mammalian mitochondrial DNA polymerase (Pol γ) is a key enzyme found in the mitochondria and is essential for mitochondrial DNA replication. It is a multifunctional enzyme that is involved in DNA synthesis, proofreading, and base excision repair by possessing DNA-directed DNA polymerization, 3′ → 5′ exonucleolytic excision, and apurinic/apyrimidinic (AP) lyase activities (1–3). In addition to these activities, Pol γ is known to perform a novel RNA-directed DNA synthesis (reverse transcription) activity in vitro (4, 5); however, the physiological role of this activity cannot be elucidated in the absence of relevant rate measurements. The reverse transcriptase activity has been also found in bacterial DNA polymerase I, and its steady state catalytic efficiency (V_max/K_m) was ~80–120-fold less than that for the DNA-directed DNA synthesis (6). Because of the absence of the pre-steady state rates, the relevance of this activity remains unclear.

The mammalian mitochondrial DNA (mtDNA) genome is ~16 kilobase pair of circular DNA consisting of a heavy strand (H-strand) and a light strand (L-strand) (7, 8). The first step of mtDNA replication involves transcription (i.e. RNA synthesis) of the L-strand DNA at the L-strand promoter located downstream of the origin of the H-strand DNA synthesis (9). The RNA is then processed by mitochondrial RNA processing RNome to form an RNA primer (10, 11). The H-strand DNA is synthesized using the RNA primer by Pol γ. After about two-thirds of the entire H-strand is synthesized, the displaced single-stranded H-strand forms a hairpin structure that is recognized as the origin of L-strand synthesis (12). A small RNA primer is created at the origin, and the L-strand DNA is synthesized. Because the mtDNA replication events appear to occur in an asynchronous fashion and an intermediate containing a large single-stranded DNA is formed, this proposed mechanism for mtDNA synthesis is termed a strand-asymmetric model.

Recently, another mechanism of mtDNA replication, a strand-coupled model, has been proposed (13, 14). In this model, both leading and lagging strands are synthesized simultaneously, and they are essentially in duplex throughout replication. Surprisingly, newly synthesized lagging L-strand is partly ribonucleotide-rich and forms a DNA/RNA heteroduplex with the parent H-strand. As a result, in the next step, the ribonucleotides in the lagging strand need to be replaced by corresponding deoxyribonucleotides. It is suggested that the RNA-directed DNA polymerase (reverse transcriptase) activity by Pol γ might be involved in this event.

Recent studies of RNase digestion of mtDNA showed that mammalian mtDNA contains at least 30 ribonucleotides with ~500-bp intervals (14). Single ribonucleotides were found at scattered positions in the entire mtDNA. Because Pol γ is highly processive, it is unlikely that it falls off during mtDNA synthesis, and RNA polymerase incorporates one ribonucleotide, thus suggesting that perhaps Pol γ may incorporate ribonucleotides during DNA synthesis. The role and significance for the presence of ribonucleotides in mtDNA has not been defined.

Pol γ consists of two different subunits, a catalytic subunit and an accessory subunit. The catalytic subunit alone is able to catalyze DNA polymerization and excision. The accessory subunit does not have known enzymatic activities, but mutational studies have shown that the subunit is essential in vivo (15). When the accessory subunit is reconstituted with the catalytic
RNA-Associated Activities by Pol γ

subunit, the rate of DNA synthesis is dramatically enhanced (16, 17). The three-dimensional structure of the accessory subunit is known, and it is highly homologous to bacterial glucyl-tRNA synthetase (18). Based on the sequences of mtDNA, it has been suggested that a tRNA-like secondary structure is formed close to the origin of replication, and the accessory subunit alone binds to both single- and double-stranded DNA (19). Thus, it has been proposed that the subunit not only activates DNA polymerization but also is involved in primer recognition during initiation of mtDNA replication and serves as processivity clamp (20). However, direct measurement of the effect of the enzyme on dissociation rate demonstrates that the accessory protein does little to “clamp” the polymerase to the DNA; rather it acts by stimulating polymerization (17).

Although the reverse transcriptase activity by Pol γ has been known for a long time, the mechanism has not been studied in detail, and the physiological relevance of the activity remains unknown. As a first step in elucidating the physiological role, it is important to know how efficiently the enzyme catalyzes this reaction. In addition, the initiation step of mtDNA replication where the RNA primer is elongated with deoxyribonucleotides is known to be catalyzed by Pol γ; however, no kinetic characterization has been performed on this activity. Therefore, the present work addresses the following important questions on novel activities by human Pol γ related to mtDNA replication: 1) How efficiently does Pol γ perform reverse transcription, and how does this compare with HIV-1 reverse transcriptase? 2) Knowing that Pol γ catalyzes 3′ → 5′ DNA excision reaction, does Pol γ possess the same activity when the template is RNA? 3) Is Pol γ holoenzyme sufficient in incorporating deoxyribonucleotides into an RNA primer without additional proteins or cofactors? If so, how efficient is it? 4) Does the accessory subunit play a role in the elongation of the RNA primer during DNA synthesis? and 5) Does Pol γ incorporate ribonucleotides? Studying these novel activities of Pol γ is important not only to provide insight into mechanism and function, but also because these activities may be associated with actions of nucleoside analog drugs because it has been shown that mitochondrial toxicity of anti-HIV drugs highly correlates with the drug incorporation and removal by Pol γ (21, 22). We have used pre-steady state and steady state kinetic approaches to examine those novel activities by Pol γ and address these important questions related to mitochondrial DNA replication.

EXPERIMENTAL PROCEDURES

Materials—The dCTP and [γ-32P]dATP were obtained from Amer sham Biosciences. Ribopolyribonucleotides (R18 and R45) were synthesized by the Keck Oligonucleotide Synthesis Facility at Yale University and purified by 10% polyacrylamide gel electrophoresis.

Overexpression and Purification of Pol γ—Both the wild type and exonuclease-deficient mutant (exo−) catalytic subunits of Pol γ were overexpressed and purified from S.9 insect cells using a recombinant baculovirus expression system as described previously (23). The catalytic subunit of Pol γ was overexpressed and purified from Escherichia coli as described previously (17). Total protein concentration was determined using extinction coefficients at 280 nm of 334,420 and 71,894 M⁻¹ cm⁻¹ for the catalytic and accessory subunits, respectively. The holoenzyme was reconstituted by mixing the catalytic and accessory subunits at a 1:1 ratio for 10 min prior to mixing with substrates.

Labeling and Annealing of Oligonucleotides—Primers were labeled at the 5′ end by T4 polynucleotide kinase and [γ-32P]ATP as described previously (24). For annealing, the labeled primers and desired templates were mixed at a 1:1.4 ratio and underwent heat treatment at 90 °C for 5 min followed by slow cooling, typically 50 °C for 10 min and on ice for 10 min. The complete annealing was confirmed by a native gel electrophoresis containing 15% polyacrylamide. The sequences of the primers and templates used in this study are: D23 (5′-GCC TCG CAG CGC TCC AAG CAA CT-3′), D25 (5′-GCC TCG CAG CGC TCC AAG CAA CT-3′), R18 (5′-gac ccc guu gag ggg cca-3′), D45 (5′-CCG AGC GTC GCC AGG TTG GTG TCG TAG GAG CTA GTG TAC GCC AGG-3′), R45 (5′-ggg agg ggc agg ugg uag gag gaa ugg ggc agg-3′), and D36 (3′-CAG AAG TCC AGG GCC AAG CCC GCG GTG AGC ATC TCT-5′). Sets of the primer/template used in this study are D23/D45, D25/D45, D23/R45, and R18/D36. Except for R18/D36, the primer/templates were blunt-ended at the 5′ end of the primer.

Pre-steady State Kinetic Assays—Radiochemical quench experiments were performed using a BioTek Corporation (Austin, TX) model RQF-3 rapid quench flow apparatus as described previously (24, 25). All concentrations of reactants were reported in the final concentrations. Deoxyribonucleotide incorporation experiments were performed under burst conditions where slightly excess primer/template is mixed with the enzyme. For burst experiment analyses, time-dependent product formation was fit to an equation: (Product) = A.ksam exp(-ktₐₙ) + A₀km.ₐₙ, where A is the amplitude, kmₐₙ is the observed burst rate, t is time, and A₀kmₐₙ is the observed steady state rate. The program Kaleidograph (Synergy Software, Reading, PA) was used for the data analyses.

RNA-directed DNA Synthesis—Reverse transcriptase activity by Pol γ was studied under burst conditions. The reaction was initiated by mixing a reactant containing varied concentrations of dCTP, 2.5 mM MgCl₂ in 50 mM Tris-HCl, 100 mM NaCl buffer, pH 7.8, and another reactant containing 300 nM D23/R45, 80 nM Pol γ holoenzyme. The reaction was quenched in 0.3 M EDTA. The products were separated on a 20% acrylamide gel containing 8% urea and analyzed on a Bio-Rad GS-525 Molecular Image System.

Kₐ for DNA-DNA—The dissociation constant (Kₐ) for D23/R45 was determined as described for DNA/DNA previously (17). A reactant containing 250 μM dCTP and 2.5 mM MgCl₂ was mixed with 80 nM holoenzyme with varied concentrations of D23/R45 using chemical quench apparatus. The reaction was quenched 1.0 s after mixing, and the formation of the product DNA was measured. The data were fit to the quadratic equation: [E-DNA/RNA] = 0.5(K₀ + [E]ₐₙ + [DNA/RNA])² – (E₀-DNA/RNA)², where [E-DNA/RNA] is the enzyme DNA/RNA complex, and E₀-DNA/RNA is active enzyme.

Exonuclease Activity Assays—Exonuclease activity was studied under steady state conditions in the absence of dCTP using 40 nM wild type Pol γ and varied concentrations of the primer/template from 0.2 to 2 μM. The initial rate of cleavage was determined by following the products. Maximum rate of excision, kₐₙ for primer/template were determined by fitting the data to the following Michaelis-Menten equation, kₐₙ = kₐₙ₀/Pₐₙ[(Kₐₐₙ + [Pₐₙ])], where kₐₙ₀ is the observed steady state rate, and P is primer/template.

DNA-directed RNA-primed DNA Synthesis—Incorporation of one or two ribonucleotides into an RNA primer was studied using rapid chemical quench as described above. In case of single nucleotide incorporation, a solution containing 0–200 μM dCTP and 2.5 mM MgCl₂ was rapidly mixed with 80 nM exo− enzyme preincubated with 300 nM R18/D36. For two-nucleotide incorporation, 200 μM dCTP and dTTP were used. In both cases, the reaction was quenched by mixing with 0.3 M EDTA. The incorporation experiments was performed in the absence and in the presence of the accessory subunit.

For steady state kinetic experiments of elongation of the RNA primer, the reaction was initiated by injecting 5 mM MgCl₂ into a mixture containing 200 μM of all four dNTPs, 4 μM R18/D36, and 200 nM wild type Pol γ in the presence and the absence of the accessory subunit. The reaction was then quenched at different times by adding 0.3 M EDTA.

Ribonucleotide Incorporation—Ribonucleotide incorporation by Pol γ was studied under burst conditions. The reaction was initiated by mixing a reactant containing varied concentrations of dCTP, 2.5 mM MgCl₂ in 50 mM Tris-HCl, 100 mM NaCl buffer, pH 7.8, and another reactant containing 300 nM D23/D45, 100 nM exo−Pol γ holoenzyme. The same experiments were done for ATP incorporation using D25/D45. Because the ribonucleotide incorporation reaction was slow, the reaction was quenched by manually mixing the reaction mixture with 0.3 M EDTA.

RESULTS

Active Site Determination—As is often the case, the enzymes are not always 100% active, and the total enzyme concentration is not necessarily the active enzyme concentration. For a careful kinetic characterization of Pol γ, the active site concentration of the enzyme was determined. Active site concentration of the reconstituted holoenzyme was determined by measuring the burst amplitudes as described previously (24). The burst


Fig. 1. RNA-directed DNA synthesis by Pol γ. A, pre-steady state burst curve at dCTP concentration of 20 μM. The observed burst rate was 10.5 ± 1.3 s⁻¹, and the amplitude was 28.4 ± 1.0 nM. B, binding affinity for DNA/RNA. Duplicate experiments were performed, and all of the data were fit into a quadratic equation (see “Experimental Procedures”). The dissociation constant (Kₐ) for D23/R45 was 16.2 ± 2.7 nM, and the total active site concentration was determined to be 47.1 ± 1.5 nM. C, dependence of observed rate of dCTP incorporation into D23/R45 at varied concentrations of dCTP. The hyperbolic fit gave a Kₐ value for dCTP of 25.7 ± 4.1 μM and a kₐ value of 21.9 ± 1.1 s⁻¹.

Experiments were performed by combining 97.5 nM holoenzyme (based on extinction coefficient at 280 nm) preincubated with 1 μM D23/D45 (sequence shown under “Experimental Procedures”) and a mixture containing 2.5 mM MgCl₂ and 10 and 100 μM dCTP. In both cases (10 and 100 μM dCTP), the burst amplitude was 80 nM, indicating that the purified and reconstituted enzymes were 82% active (data not shown). When D23/R45 was used, a burst phase was observed, and the amplitude was consistently 30 nM (Fig. 1A), indicating that 30% of the total protein was observed to be active. The lower amplitude could be due to low binding affinity of DNA/RNA or due to nonproductive binding of the DNA/RNA heteroduplex (26).

Binding of DNA/RNA—A mixture containing 250 μM dCTP and 2.5 mM MgCl₂ was rapidly mixed with 80 nM holoenzyme preincubated with various concentrations of DNA/RNA, and the reaction was quenched 1.0 s after mixing with 0.3 M EDTA. The amount of D24-mer formed was plotted versus DNA/RNA concentration, and the data were fit to a quadratic equation (Fig. 1B). The best fit gave the Kₐ of 16.2 nM, which is similar to the value previously determined for DNA/DNA of 9.9 nM (17). The results indicate that binding of D23/R45 is not limiting under our experimental conditions, and there is a high affinity for this primer/template substrate.

A Pre-steady State Burst in Incorporation of dCTP into DNA/RNA—The tight binding of a DNA/RNA substrate implies that reverse transcription by Pol γ may be the result of a mechanism paralleling DNA-directed DNA synthesis. The RNA-directed activity was further studied to measure the nucleotide concentration dependence of the pre-steady state burst of polymerization. The rate of single nucleotide incorporation into a DNA/RNA primer/template was measured by mixing the enzyme preincubated with D23/R45 and a mixture of dCTP and magnesium. Fig. 1A shows a kinetic trace at 20 μM dCTP that was fit to a burst equation. The curve clearly showed two phases. The fast burst phase represents rapid conversion of the pre-bound substrate to the product, and the slow steady state phase indicates rate-limiting product release. The observed burst rate at different concentrations of dCTP was plotted and fitted into hyperbolic equation to determine the maximum rate of polymerization (k₄pol) of 21.9 ± 1.1 s⁻¹ and equilibrium dissociation constant (Kₐ) for dCTP of 25.7 ± 4.1 μM (Fig. 1C). Efficiency (or specificity constant) for incorporation (k₄pol/Kₐ) was calculated to be 0.85 μM⁻¹ s⁻¹. These values were compared with previously determined numbers with HIV-1 reverse transcriptase (RT) using both D23/D45 and D23/R45 and with Pol γ using D23/D45 under the same experimental conditions (Table I (21, 27). The efficiency of incorporation by Pol γ was ~50-fold less with D23/R45 than D23/D45. However, comparison between Pol γ and HIV-1 RT showed that the k₄pol, Kₐ, and efficiency values were very similar (Table I). Surprisingly, the efficiency of incorporation was slightly (~10%) better with Pol γ. These results suggest that the reverse transcription activity by Pol γ may be physiologically significant.

Exonuclease Activity—Pol γ possesses a 3’ → 5’ DNA exonuclease proofreading activity, and because Pol γ is capable of utilizing RNA as a template in DNA synthesis as shown above, it is possible that the enzyme also catalyzes 3’ → 5’ DNA excision with an RNA template. To test this hypothesis, a steady state kinetic method is employed using D23/R45 as a substrate, as well as D23/D45 as a side-by-side comparison. The rate of excision was measured by adding magnesium into a mixture containing 40 nM wild type Pol γ and 1.5 μM primer/template and quantitating the total amount of DNA cleaved by Pol γ. The kinetic traces were plotted and fit to lines (Fig. 2). The observed rates for DNA/RNA and DNA/DNA were 0.038 ± 0.001 and 0.0120 ± 0.0002 s⁻¹, respectively.

Elongation of RNA Primer with dNTPs—During initiation of mitochondrial DNA replication, an RNA primer is formed at the origin of the heavy strand synthesis. Although evidence that Pol γ can initiate DNA synthesis using an RNA primer has been shown previously (28), no detailed kinetic characterization is available. Therefore, we have examined whether Pol γ is capable of adding dNTPs to an RNA primer at a physiologically relevant rate. A model primer/template (R18/D36) as an initiation substrate was used, and incorporation of the next correct deoxyribonucleotide, dCMP, was studied by pre-steady state burst experiments. A burst phase with the rate of 2.11 s⁻¹ was observed at dCTP concentration of 100 μM (Fig. 3A). The observed burst rates at varying concentrations of dCTP were plotted, and the Kₐ value for dCTP of 37 μM and the k₄pol value of 3.2 s⁻¹ were determined based on a hyperbolic fit (Fig. 3B).

Role of the Accessory Subunit—The accessory subunit is known to significantly enhance the efficiency of dNTP incorporation into a primer during DNA synthesis. Thus, in the ab-
sence of the accessory subunit, there may be a change in the rate-limiting step because the incorporation rate with an RNA-primed substrate was relatively slow compared with a DNA-primed substrate in the presence of the accessory subunit. Therefore, effects of the accessory subunit in incorporation of dNTP into an RNA primer were examined. Pre-steady state burst experiments monitoring two nucleotides (dCMP and dTMP) incorporation were performed in the presence (Fig. 4A) and in the absence of the accessory subunit (Fig. 4B). The rates for single and two nucleotides incorporation were confirmed to be the same, and the two-nucleotide incorporation gave more distinct separation on the acrylamide gel. Elongation of the RNA primer was observed both in the presence and the absence of the accessory subunit. However, in the absence of the accessory subunit, no burst was observed, indicating that the rate-limiting step is prior to the release of elongated product and is most likely to be a conformational change or chemistry step. Whereas in the presence of the accessory subunit, a burst phase with the rate of 2.58 ± 0.58 s⁻¹ and an amplitude of 18.7 ± 1.2 nM were observed. Assuming the active enzyme concentration in these experiments is 18.7 nM based on the burst amplitude in the presence of the accessory subunit, the rate for the reaction in the absence of the accessory subunit was determined to be 0.027 s⁻¹. These results indicate that the accessory subunit enhanced the rate of incorporation for 100-fold. This compares with the 6-fold enhancement seen for the DNA-dependent DNA polymerization reaction (17).

Previous studies showed that in the exonucleolytic cleavage by Pol γ is 2-fold faster in the absence of the accessory subunit than in the presence (2). Therefore, based on our excision rate described above (0.012 s⁻¹), in the absence of acces-

Table I
Comparison of kinetic parameters between Pol γ and HIV-1 RT

|       | Pol γ | HIV-1 RT |
|-------|-------|----------|
| kpol  | s⁻¹   | s⁻¹      |
| Kd    | μM    | μM       |
| Efficiency | μM⁻¹ s⁻¹ | μM⁻¹ s⁻¹  |
|-------|-------|----------|
| D23/D45 | 44 ± 2a | 1.1 ± 0.1b | 0.080b |
|       | 1.9 ± 0.1b | 24 ± 5b   |        |
|       | 21.9 ± 1.1 | 25.7 ± 4.1 | 0.85   |
|       | 22.9 ± 0.7b | 30 ± 4b   | 0.76b  |

a Ref. 21.
b Ref. 27.
CTP concentration. The dependence of observed initial rates on the Michaelis-Menten analysis was performed by varying the concentration of Fig. 6. Because the reaction is very slow, no initial velocity of the reaction was measured (Fig. 6, inset), and the initial velocity was analyzed by fitting the earlier points to a line, and the rate was determined to be $0.0052 \pm 0.00004$ s$^{-1}$.

**Discussion**

**RNA-dependent DNA Synthesis**—Reverse transcriptase activity by human mitochondrial DNA polymerase was studied by rapid chemical quench methods that allowed us to investigate the individual steps of the reaction. As shown for HIV-1 RT previously, a burst phase was seen in dCTP incorporation into D23/R45 with Pol γ (Fig. 1A), indicating that the molecular mechanism for reverse transcription is similar to that for HIV-1 RT. Therefore, the values of dissociation constant ($K_d$), the maximum rate of polymerization ($k_{pol}$), and the efficiency of incorporation for dCTP into DNA/RNA were determined and compared with DNA/DNA and with those for HIV-1 RT (Table 1). Interestingly, those parameters for DNA/RNA were almost identical between the two enzymes. Our results suggest that the RT activity by Pol γ may be significant because it is slightly more efficient than that of HIV RT.

**RNA-directed DNA Excision**—The novel 3′ → 5′ exonuclease activity has never been reported with a DNA/RNA primer/template. As shown in Fig. 2, where the 3′ end has a correct base pairing, no burst phases with the amplitude of the enzyme concentration of 40 nM were observed using both D23/D45 and D23/R45, indicating that the rate-limiting step for the exonuclease reaction is prior to product release such as primer/template binding, chemistry, or conformational change. The rate for DNA/DNA (0.012 s$^{-1}$) was similar to the previously determined number with the same enzyme and primer/template concentrations but a different sequence of the primer (0.022 s$^{-1}$) (21). Interestingly, $k_{exo}$ for D23/R45 was 3-fold higher than for D23/D45 with this primer/template.

**Initiation of Mitochondrial DNA Replication**—During the initiation of mitochondrial DNA replication, RNA/DNA heteroduplex is formed, and the RNA fragment serves as a primer for DNA synthesis (8). We have investigated whether Pol γ is able to add dNTPs to the RNA primer and initiate DNA synthesis. For single nucleotide incorporation into an RNA primer, we obtained the rate constant of 3.2 s$^{-1}$. This rate must be faster than the overall mtDNA replication rate to be physiologically relevant. The mtDNA replication rate has been reported to be very slow because it takes 2 h to replicate the entire genome (7). This suggests that the rate of deoxyribonucleotide incorporation into the RNA primer (3.2 s$^{-1}$) at the initiation stage is
sufficiently fast. Furthermore, the elongation rate has to be significantly faster than the rate of excision by the same enzyme. Our results show that in the presence of the accessory subunit, the incorporation rate was more than 200-fold faster than the excision rate. Therefore, we conclude that Pol γ is able to initiate mtDNA replication at a physiologically relevant rate. However, in the absence of the accessory subunit, the rate of deoxyribonucleotide incorporation was about 100-fold slower than in the presence, and the burst phase was no longer observed. This indicates that there was a change in the rate-limiting step from product release to either chemistry or conformational change. In addition, our steady state elongation experiment clearly showed that the accessory subunit is required for efficient DNA elongation from an RNA primer (Fig. 5).

**Ribonucleotide Incorporation**—It is highly interesting that ribonucleotides are found in the mtDNA at scattered positions (14). To understand how those ribonucleotides are incorporated, single ribonucleotide incorporation into a DNA primer was studied under burst conditions. As shown in Fig. 6, CMP was incorporated by Pol γ at relatively slow rate (0.0096 s⁻¹), and no burst phase was observed. The rate of AMP incorporation was 0.0055 s⁻¹, about 2-fold slower that that of CMP. Because a burst phase was not observed, the rate-limiting step of overall ribonucleotide incorporation reaction is a step prior to the elongated primer/template product release. It is possible that the 2'-hydroxyl group of the incoming ribonucleotide interacts with the residue in the active site, which could interfere the conformational change or the chemistry. The rate for deoxyribonucleotide (dCMP) incorporation into D23/D45 was reported to be 44 s⁻¹, and the binding is very tight (Table I). Although it is difficult to correlate the frequency ribonucleotide incorporation found in mtDNA (~1 in every 500 base pairs) with our kinetic results, we have shown that Pol γ possesses the ability to incorporate a ribonucleotide into a DNA primer, and accordingly this activity could play a physiological role in incorporating ribonucleotide into mtDNA.

**Roles of RNA-associated Activities**—In this study, four novel RNA-associated activities of Pol γ, reverse transcription, RNA-directed 3' → 5' DNA excision, RNA-primed DNA synthesis, and ribonucleotide incorporation, were investigated. Our proposed roles for these activities during mtDNA replication are summarized in Fig. 7. Because Pol γ catalyzes RNA-associated reactions at physiologically relevant rates, we support the strand-coupled mtDNA replication model in which an intermediate with newly synthesized ribonucleotide-containing lagging strand is formed. To complete the replication, those ribonucleotides have to be replaced by corresponding deoxyribonucleotides. It has been suggested that the RT activity may be involved in this conversion (14). In this case, the resulting transcript would be the complementary strand; thus, further DNA synthesis from the transcript to its complementary strand is required. The other possible mechanism of the conversion is that the ribonucleotides are digested by RNase H activity, and DNA is synthesized. The mitochondrial RNA processing RNase could be considered as RNase H. It is puzzling as to why the ribonucleotide-containing intermediate is formed during mitochondrial RNA processing RNase DNA replication because it requires additional steps to complete the replication. It is also possible there may be more complex processes where the RT activity is utilized during the replication and perhaps also is involved in mitochondrial DNA repair. For instance, it is known that DNA/RNA heteroduplex is formed during the replication, and if there is any DNA damage in the heteroduplex, the reverse transcriptase activity may be required to repair the damage. Nucleotide substitution mutations are estimated to occur in mtDNA 100-fold more frequently as in nuclear DNA (29). A major cause of those mutations is oxidative stress. During mitochondrial oxidative phosphorylation, reactive oxygen species are formed, and there are high chances for oxidative DNA damage in mitochondria. The repair of those mutations involves AP endonuclease incision at an AP site followed by removal of the AP site by deoxyribose phosphate lyase; then DNA polymerase activity is required to fill the gap. Finally, the two fragments are ligated by DNA ligase (see Ref. 30 for review). If there is DNA damage in a DNA/RNA heteroduplex, RT activity would be required for the gap filling reaction during the base excision repair. This RNA-templated DNA repair activity by Pol γ is currently under investigation.

The observation that Pol γ catalyzes RNA-directed DNA excision activity also suggests that the RNA-associated activities are important. The role of the RNA-directed DNA excision activity is likely to be proofreading during reverse transcription. The rate of excision with an RNA template was 3-fold higher than with DNA. One reason for this might be that the fidelity of reverse transcription is lower than DNA-directed DNA synthesis, and it requires more efficient proofreading activity. This might be associated with the fact that the fidelity of HIV-1 RT is much lower than other polymerases (1).

Our proposed role of RNA-primed DNA synthesis activity is elongation of an RNA primer that is formed during initiation of mtDNA replication as discussed above. It is also possible that this activity is involved in conversion of the ribonucleotide-containing strand into the DNA strand. The mitochondrial
RNA processing RNase could process the RNA-rich region of the strand, and Pol γ uses the RNA fragment as a primer.

In the present studies, novel RNA-associated activities by Pol γ have been kinetically investigated, and several significant findings are presented. First, Pol γ catalyzes reverse transcription slightly better than HIV RT. This indicates that the RT activity by Pol γ may be physiologically significant and provides strong evidence for the strand-coupled model of mtDNA replication. Second, RNA-directed DNA excision activity was shown for the first time, although this can be considered as a logical consequence of reverse transcriptase activity by a polymerase containing a proofreading exonuclease. Because the rate with an RNA template was 3-fold faster than with a DNA-template, this novel RNA-associated excision activity may be important. This activity is proposed to be a proofreader, but we have not yet shown selectivity in the excision of nucleotides from a DNA/RNA primer/template heteroduplex. Third, RNA-primed DNA synthesis activity, which is required for initiation of mtDNA replication, has been studied. We have shown that Pol γ holoenzyme is sufficient in catalyzing this reaction at a physiologically relevant rate without any other proteins or cofactors and that the accessory subunit has an essential role in the initiation step. Finally, we have found that Pol γ is capable of incorporating a ribonucleotide into a DNA primer, and this activity could take part in incorporating ribonucleotides into the mtDNA.

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