Fidelity and Processivity of Reverse Transcription by the Human Mitochondrial DNA Polymerase*

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We have characterized, by transient-state kinetic methods, the polymerase and exonuclease activities of the human mitochondrial DNA polymerase (pol γ) during reverse transcription, employing a synthetic oligonucleotide consisting of a DNA primer and an RNA template. In comparison with the kinetic parameters observed with a DNA template, the rate of correct deoxynucleotide incorporation was reduced 25-fold (5.5 ± 0.2 s⁻¹), whereas the dissociation constant \( K_d \) for nucleotide binding was increased 4-fold (12 ± 1 μM). In addition, discrimination against mismatches was reduced ~20-fold to only 15,000 on average. The proofreading exonuclease favored the removal of an incorrect nucleotide (0.0021 ± 0.0002 s⁻¹ for correct versus 0.034 ± 0.004 s⁻¹ for incorrect), and the partitioning between incorporation beyond a mismatch (5.5 × 10⁻⁵ ± 0.4 × 10⁻⁵ s⁻¹), and exonuclease removal of that mismatch favors removal of the mismatch. These data suggest that the “reverse transcriptase activity” of mitochondrial polymerase could be physiologically relevant. However, the enzyme stalls and is unable to efficiently incorporate beyond a single nucleotide with an RNA template. Additionally, we present a refined method for calculating net discrimination, which more accurately describes the contributions of correct and incorrect incorporation. The biological and biotechnological significance of these results are discussed.

Reverse transcription is the process of DNA polymerization using an RNA template, and it is interesting to note that mitochondrial DNA polymerases (pol γ) were initially characterized by their ability to reverse-transcribe DNA from RNA (1). In fact, over the past 30 years, human polγ has been shown to be an effective reverse transcriptase in steady state single nucleotide incorporation assays using DNA/RNA heteroduplexes to probe for its presence and to assay its replication fidelity (1–4). RNase digestion of mammalian mitochondrial DNA has shown that there are at least 30 ribonucleotides incorporated in the mitochondrial genome (5), but the origin and significance of these ribonucleotides are unknown. They could be the products of incorporation by the DNA polymerase or merely remnants of RNA priming by the mitochondrial RNA polymerase after lagging strand synthesis and incomplete primer removal.

Steady state assays have been used to characterize reverse transcriptase activity and fidelity of pol γ largely because steady state rates are faster with an RNA template. However, steady state, single nucleotide turnover rates are limited by polymerase dissociation from product complexes and do not provide reliable measures of nucleotide discrimination. Therefore, presteady state kinetic approaches have been used to begin to characterize more accurately the reverse transcriptase activity of human pol γ (6). In our previous studies we were surprised to see how rapidly pol γ catalyzed a single DNA incorporation with an RNA template. Here we demonstrate a marked reduction in fidelity during reverse transcription and show that the polymerase stalls after a single incorporation event.

MATERIALS AND METHODS

Expression and Purification of Enzyme Subunits—Expression and purification of the catalytic subunit (pol γA) and the accessory subunit (pol γB) were accomplished as described previously (7, 8). An exonuclease-deficient mutant of the catalytic subunit (E200A), pol γA exo⁻, was purified as described (9); this mutation reduced the exonuclease rate greater than 10⁷-fold without affecting the kinetic parameters governing polymerization.

Experiments to assess the polymerization fidelity were performed as described (9) using the reconstituted holoenzyme, consisting of a catalytic subunit containing a C-terminal His₆ tag and a 56-amino acid N-terminal truncation combined with an accessory subunit with 29-amino acid N-terminal truncation. These N-terminal truncations were designed to mimic the native protein formed by cleavage of the N-terminal mitochondrial leader sequence.

Protein concentrations were determined by \( A_{280} \) measurements, and active enzyme concentrations were determined by active site titration against a known concentration of duplex DNA (7). A 1:5 ratio of catalytic subunit to accessory subunit was used for holoenzyme reconstitution based upon a measured \( K_d \) of 35 nM (7) and a catalytic subunit concentration of 50–100 nM. Contrary to observations based upon steady state rate measurements (10), we do not see inhibition caused by excess accessory subunit.

Preparation of Synthetic Oligonucleotide Substrate—Primer strands were 5’-32P-labeled using T4 polynucleotide kinase.
according to the manufacturer’s instructions (Invitrogen). Termination of the reaction was accomplished by heating to 95 °C for 5 min. Excess 32P-labeled nucleotide was removed using a Biospin 6 column (Bio-Rad). Primer-template annealing was accomplished by mixing equimolar concentrations of 25-mer primer and 45-mer template, heating the mixture to 95 °C, and allowing it to cool slowly to room temperature. The sequences of primer-template substrates used are listed in Table 1.

Assay Conditions—Single nucleotide incorporation assays were performed at 37 °C in a buffer containing 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 25 mM MgCl2. The holoenzyme-DNA complex was assembled in buffer lacking MgCl2, then mixed with a solution containing nucleotide and MgCl2. Mixing of the enzyme-DNA complex with the nucleotide was performed at a 1:1 ratio, and each solution was assembled at twice the reported final concentration. For reaction times less than 10 s, mixing was accomplished using a KinTek RQF-3 rapid quench flow instrument (Austin, TX). Longer reaction times were achieved by manual mixing.

Exonuclease assays were set up as described for the polymerization assays, except that the wild-type catalytic subunit was used, and deoxyribonucleotide triphosphates (dNTP) were omitted. Both the exonuclease-deficient mutant and the wild-type catalytic subunit were used in processive polymerization experiments, performed under conditions described for the single nucleotide incorporation assays, except that all four dNTPs were included at a final concentration of 100 μM each.

Product Analysis—Products were resolved on 15% denaturing polyacrylamide sequencing gels, imaged using a Storm 860 PhosphorImager, and quantified using the manufacturer’s ImageQuaNT software (GE Healthcare).

Kd and Maximum Rate of Polymerization for Incorrect Nucleotide Incorporation—Single nucleotide incorporation experiments were performed to measure the nucleotide concentration dependence of the rate of polymerization. These assays were carried out under single turnover conditions, with enzyme in slight excess over DNA, because the rates of incorporation for incorrect nucleotides were slower than the rate of dissociation of the enzyme from the DNA substrate. This protocol enables measurement of the rate of incorporation by eliminating the contributions because of turnovers of the enzyme with DNA in excess. The time course of product formation was fit to a single exponential ([product] = Ae−kt + C). Throughout the text, we will refer to the exponent derived by fitting to this equation as the observed “rate” of the reaction and reserve the term “rate constant” to refer to an intrinsic constant governing a single molecular step in a model.

Rates of polymerization determined from the single exponential fit to the primary date were then plotted against nucleotide concentration and fitted to a hyperbola (observed rate = kpol[dNTP] / (Kd + [dNTP])) to determine the apparent dissociation constant, Kd, and the maximum rate of polymerization, kpol, for each incorrect nucleotide.

RESULTS

Incorporation of Nucleotides into a 25d/45r Substrate—The kinetics of incorporation and exonuclease excision were examined in single turnover experiments and interpreted according to the model shown in Fig. 1. The concentration dependence of the presteady state burst of product formation during this series of experiments, and Table 2 summarizes the rate constants for incorporation of deoxyribonucleotides during reverse transcription. Representative data from this study are presented in Fig. 2. The concentration dependence of the observed rate provides an estimate of the maximum rate of 39 ± 2 s⁻¹ and an apparent dissociation constant of 18 ± 4 μM. The specificity constant for incorporation of dCTP during reverse transcription is 2.2 μM⁻¹ s⁻¹. Results of the single nucleotide incorporation assays for each of the four nucleotides are summarized in Table 2. The specificity constant varied from 0.5 to 2.2 μM⁻¹ s⁻¹ and had an average value ~40-fold lower than that measured with a DNA template (9, 12).

We next examined the misincorporation of dNTPs opposite a template rU to quantify discrimination by pol γ during reverse transcription. Fig. 3 illustrates representative data generated during this series of experiments, and Table 2 summarizes the kinetic parameters governing misincorporation. Discrimination ranged from 5,000 to 25,000 giving an average value 25-fold lower than that observed with a DNA template (9, 12).

Exonuclease Removal of Nucleotides from 25d/45r Substrate—To calculate the contribution to net fidelity afforded by the exonuclease activity when pol γ is employed as a reverse transcriptase, we measured the rates of excision of the 3’-terminal base from primers containing either correct (dA-rU) or mismatched (dG-rU) base pairs. Removal of a mismatch from a 25d/45d substrate follows a double exponential, and the rate of the fast phase defines the rate of excision, and the rate of the slow phase appears to be limited by the rate of transfer of the primer strand from the polymerase to the exonuclease active site (13). In this

![Figure 1. Mechanism for incorporation of dNTPs onto a 25d/45r heteroduplex.](image)
study, we only measured a single phase, which was quite slow for both the correctly paired and mismatched primers. The results from these assays are summarized in Table 3. The terminal base was removed from a correct base pair at a rate of 0.0021 s⁻¹, whereas a mismatch was removed at a 16-fold greater rate, 0.034 s⁻¹. However, this rate is ~100-fold slower than the rate of removal of a mismatch from a DNA duplex (13).

Incorporation of the Next Correct Nucleotide after a Mismatch—To fully assess the contribution to fidelity afforded by the exonuclease domain of pol γ, when employed as a reverse transcriptase, it was necessary to determine the kinetic parameters governing the forward polymerization to bury a mismatch to assess the probability of extension versus excision (16).

The incorporation of a dCTP opposite a template G to bury a terminal dG-rU mispair was characterized at 100, 250, and 500 μM dCTP. The time dependence of product formation is presented in Fig. 4A, and the concentration dependence of the

| TABLE 2 | Kinetic parameters of reverse transcription by polymerase γ |
|---|---|---|---|---|---|
| Template base | dNTP | $k_{pol}$ | $K_d$ | Specificity | Discrimination |
| U | dATP | 5.5 ± 0.2 | 12 ± 1 | 0.5 ± 0.05 | 1 |
| | dCTP | 0.0016 ± 0.0002 | 60 ± 10 | 30 ± 6 × 10⁻⁶ | 16,700 |
| | dGTP | 0.0014 ± 0.0001 | 60 ± 30 | 2 ± 1 × 10⁻⁵ | 25,000 |
| G | dCTP | 39 ± 2 | 18 ± 4 | 2.2 ± 0.5 | 1 |
| C | dGTP | 8 ± 2 | 10 ± 10 | 1 ± 1 | 1 |
| A | dTTP | 1.79 ± 0.08 | 2.4 ± 0.9 | 0.8 ± 0.3 | 1 |

FIGURE 2. Correct incorporation of dCTP during reverse transcription. A, the kinetic parameters of dCTP incorporation during reverse transcription were assayed at dCTP concentrations of 10 (□), 25 (△), 50 (▲), 100 (■), and 250 μM (○) dCTP. B, the observed rates were plotted against [dCTP] and fit to a hyperbola. The hyperbolic fit generated a $k_{pol}$ of 39 ± 2 s⁻¹ and a $K_d$ of 18 ± 4 μM.

FIGURE 3. Misincorporation of dCTP onto U during reverse transcription. The kinetic parameters of dCTP misincorporation onto U during reverse transcription were assayed over a concentration range of 10 (□), 25 (△), 50 (▲), 100 (■), 250 (▲), 500 (▲) and 1000 μM (○) dCTP. The observed rates were plotted against [dCTP] and fit to a hyperbola to obtain $k_{pol}$ of 0.0016 ± 0.0002 s⁻¹ and a $K_d$ of 58 ± 10 μM.

| TABLE 3 | Exonuclease removal by polymerase γ |
|---|---|---|---|---|---|
| Template base | dNMP | Rate |
| U | rU | dA | 0.0021 ± 0.0002 |
| | dG | 0.034 ± 0.004 |

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observed rate is depicted in Fig. 4B. To generate a fit to the data, the end point of product formation was constrained in each of the three time courses to 90 nM, the concentration of oligonucleotide duplex present in the reaction. The rates and amplitudes of the product formation observed are very low, and therefore the errors of the rate determinations are large, but it is clear that the rate of burying a mismatch is extremely low. From these experiments, the $k_{\text{pol}}$ for burying a mismatch was calculated to be $5.5 \times 10^{-10}$ s$^{-1}$ and a $K_d$ of 150 ± 30 μM.

This rate implies a high selectivity toward mismatch removal even though the rate of excision is low. The exonuclease would contribute a factor of 1,700 toward the overall fidelity. However, the slow extension makes this conclusion irrelevant physiologically and raises the question of the rate of extension beyond a correct base pair during reverse transcription.

**Processive Reverse Transcription**—In order for pol γ to function as a competent reverse transcriptase, it must incorporate multiple dNTPs sequentially. To test the processivity of pol γ when acting as a reverse transcriptase, a series of experiments was designed to assay for multiple incorporation reactions. The wild-type enzyme-DNA complex was rapidly mixed with a solution containing 100 μM of each of the four dNTPs. Samples were removed at various times, and the products of reaction were resolved on denaturing polyacrylamide gels.

The autoradiogram in Fig. 5 shows that the polymerase stalls after a single nucleotide incorporation event and that the rate of the exonuclease reaction is comparable with the rate of subsequent polymerization steps. Therefore, products of both reactions accumulate during the time course of the experiment. Because these experiments generated many products from a single assay, we quantified net product formation as follows. At each time point, all products longer than the 25-mer primer were summed and treated as the total product of forward polymerization. All products shorter than the 25-mer substrate were summed and treated as the total product of exonuclease removal. To a first approximation, these measurements define the kinetics of the first step in each direction (polymerase or exonuclease).

Fig. 6 shows the time dependence of the accumulation of products of the polymerase and exonuclease reactions. Both
plots fit best to a double exponential equation, and the values obtained from fitting the data are presented in Table 4. Formation of polymerase products essentially stalls after the incorporation of the first nucleotide during reverse transcription, and only minor amounts of longer products can be seen. Although the fast phase is not resolved in the experiment, the data show that it occurs at a rate of at least 6 s$^{-1}$. In contrast, the exonuclease products accumulate at a rate of 0.004 s$^{-1}$. The gradual decrease in exonuclease products at long reaction times may be due to errors in summing over all products. As a first approximation, these data define the kinetic partitioning between polymerization and excision reactions governing depletion of the starting material. Note also that the polymerization reaction failed to go to completion in the fast phase, which we believe is because of weak binding of the polymerase to the DNA/RNA heteroduplex.

The exonuclease reaction interfered with a more complete analysis of processive polymerization because it appeared that this activity may prevent the accumulation of longer products after the first incorporation event, because the rates of the second extension and excision reactions were comparable. Therefore, the processive polymerization experiments were repeated with an exonuclease-deficient enzyme. An autoradiogram for this experiment is shown in Fig. 7A, and the time dependence of product formation is plotted in Fig. 7B. All products, at each time point longer than the substrate 25-mer, were summed and fit to a single exponential. This fit generated a rate of 13 ± 1 s$^{-1}$ and an amplitude of 56 ± 2 nm for the reaction.

To test the possibility that sequence context effects were preventing the polymerase from continuing past a single incorporation event, we examined whether the heteroduplex primer-template could be extended by a true reverse transcriptase, namely HIV reverse transcriptase. To get the results shown in Fig. 8, wild-type pol γholoenzyme (100 nM) was mixed in excess over the heteroduplex (90 nM) and allowed to react with 100 μM of each nucleotide for 10 or 20 s. After 20 s of reaction, HIV RT was added to the mixture. Additional time points were collected after 30, 60, and 90 s of reaction with RT. These results show that HIV RT can continue the processive synthesis after pol γ has stalled following the first incorporation.

**DISCUSSION**

**Single Nucleotide Incorporation Assays**—Single nucleotide incorporation assays were performed to determine the specificity constant for incorporation of dNTPs by pol γ onto a 25d/45r oligonucleotide DNA/RNA heteroduplex. In line with previous
models for DNA polymerization, the concentration dependence of the rate single nucleotide incorporation (Fig. 2B) defined the apparent nucleotide dissociation constant, $K_{d}$, and the maximum rate of polymerization, $k_{pol}$ (9, 17–19). The rate of the slow phase (Fig. 2A) is most probably a function of the release of the oligonucleotide duplex from the polymerase and subsequent rebinding of the enzyme to the substrate from solution. This weakened binding of the enzyme to the oligonucleotide duplex explains the low amplitudes of single nucleotide incorporation observed in these reactions.

The specificity constants for correct nucleotide incorporation ranged from a high of $2.2 \mu M^{-1} s^{-1}$ to a low of $0.5 \mu M^{-1} s^{-1}$ as detailed in Table 2. These values for RNA-dependent DNA polymerization are roughly 25–100-fold lower than the specificity constant observed for DNA-dependent DNA replication under single turnover conditions (12). This loss of efficiency is most likely because of differences in specific contacts between the oligonucleotide duplex and the enzyme. DNA/RNA heteroduplexes have been previously shown to differ in structure from either DNA/DNA or RNA/RNA structures (20). The RNA strand of the heteroduplex tends toward an A-form conformation, whereas the DNA strand tends toward a B-form conformation. Overall, the duplex tends to be more A-form than B-form, which leads to a fatter duplex and a narrower and deeper major groove. This difference in structure may account for the lower rates and lower fidelity seen when pol copies an RNA template.

To assess the possible physiological significance of the reverse transcriptase activity of pol, we quantified the discrimination in single turnover assays designed to determine the specificity of incorporation opposite a template rU. From these values and the specificity constants calculated for the correct incorporation of dATP opposite rU, the discrimination against misincorporation was calculated as shown in Table 2. Although the discrimination against a dG-rU (25,000) is higher than the corresponding discrimination against a dG-dT (3600), the average discrimination against misincorporation during reverse transcription is only 15,600, compared with 440,000 for DNA polymerization (12). With a DNA template, the least accurate replication was seen with the dG-dT wobble base (9). In contrast, when RNA was the template, the lowest fidelity was seen with the dT-rU mispair. pol is 50-fold more likely to make a dT-rU pair as a reverse transcriptase than it is to make a dT-dT mispair when replicating DNA, and greater than 30-fold more likely to make a dC-rU mispair than a dC-dT pair. Although we have not yet performed a comprehensive screen of all possible mismatches, our existing data suggest that pol can be expected to exhibit a vastly different mutation profile when operating as a reverse transcriptase compared with reactions with a DNA template (12). Moreover, the overall fidelity is reduced 25-fold.

Reverse transcriptase assays have often been employed for steady state kinetic measurements of discrimination by pol, in part because the rates of steady state single nucleotide incorporation assays are faster than with DNA (21). The steady state rates are limited by the rate of release of DNA from the enzyme and RNA templates provide a faster steady state rate, because they are poorer substrates. The limitations of steady state meth-
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ods in determining specificity and discrimination constants have been discussed previously and will not be repeated here (12, 22). Our current data further call into question the validity of steady state reverse transcriptase assays to assess polymerase fidelity or discrimination against nucleoside analogs (21, 24).

Net Discrimination—Average discrimination is a parameter typically used for comparing polymerases and is one that we have used here to compare the reverse transcriptase activity of pol γ to the DNA-dependent DNA polymerase activity of pol α. Discrimination is defined as the number of correct nucleotides incorporated per misincorporation event and is approximately equal to the reciprocal of the misincorporation frequency, more precisely the frequency, $f = 1/(D + 1)$, where $D$ is the discrimination. Traditionally the “average discrimination” has been calculated by averaging the individual discrimination values, which is perfectly appropriate as defined, but perhaps a better method should be used to estimate net discrimination during DNA replication, representing the sum of all misincorporation probabilities at each position.

For each templating base, there are three possible mismatched bases and only one correct base. Therefore, the frequency of making a mismatch at a given position is the sum of the individual misincorporation frequencies. Accordingly, the net discrimination, $D_{net}$ is defined by Equation 1,

$$D_{net} = \frac{1}{\sum 1/(D + 1)}$$

(Eq. 1)

The data in Table 2 show discrimination values ranging from 5,000 to 25,000 giving an average value of 15,600. However, the net discrimination, calculated from the sum of the frequencies of forming each mismatch, is only 3,300. This value more accurately reflects the frequency of forming the sum of all possible mismatches opposite rU, but it is obviously dominated by high frequency of forming a dTTP:rU mismatch. The average discrimination for incorporation opposite dT during DNA-dependent DNA synthesis was 275,000, but the net discrimination, as defined here, was only 3,500 because of the fast misincorporation and the subsequent kinetic partitioning governing extension versus excision of the mismatch.

### Exonuclease Removal of Nucleotides from a 25d/45r—To test the activity and specificity of the exonuclease domain of pol γ, single turnover assays were performed to determine the rate of removal of a correctly paired and an incorrectly paired nucleotide. The results of these experiments are detailed in Table 3. The rate of removal of a correctly paired nucleotide was determined to be 16-fold less than the removal of a mismatch, showing that the exonuclease domain is both active against a 25d-45r oligonucleotide duplex and specific for mismatched nucleotides.

To determine the contribution to fidelity afforded by the exonuclease domain, the rate of incorporation to bury a mismatch was measured. In Fig. 9 we summarize the kinetics of misincorporation and the subsequent kinetic partitioning governing extension versus excision of the mismatch. The maximum rate of incorporation to bury a mismatch was calculated to be $5.5 \pm 0.4 \times 10^{-5} \text{ s}^{-1}$ and the apparent nucleotide dissociation constant was estimated to be $150 \pm 30 \mu\text{M}$. In contrast, the mismatch was excised at a rate of 0.034 s$^{-1}$. Accordingly, the contribution to fidelity by the proofreading exonuclease is defined by the rate of mismatch excision relative to the rate of polymerization to bury the mismatch (13) calculated as $k_{exo}/(k_{exo} + k_{bury})$. These data show that greater than 99.9% of mismatches will be removed by the exonuclease proofreading activity before they are buried by forward polymerization. The proofreading activity of pol γ contributes a factor of 1700-fold to the fidelity of the polymerase. In comparison, the exonuclease only contributes a 200-fold enhancement to the fidelity of the polymerase when replicating a DNA/DNA oligonucleotide substrate (13). However, the apparently higher exonuclease contribution to fidelity during reverse transcription is due solely to the significant reduction in the rate of forward polymerization. Although polym + can incorporate a dNTP over a mismatch during DNA replication at a rate as fast as $12 \text{ s}^{-1}$, it incorporates a dNTP to bury a mismatch in a 25d/45r 200,000-fold slower because rates of the second incorporation are inherently slow. Although the selective removal of mismatches and stalling of the polymerase after a mismatch would improve fidelity, the extension reaction is so slow that it would be a rare event inside the mitochondria with either a correct or mismatched primer.

The cost of proofreading can also be computed by comparing the rates of excision versus extension on top of a correct base pair. Even though the rate of excision of a correct base pair is low, the rate of extension is also low, so the cost is quite high. Although we have not accurately measured the rate of extension after the first incorporation with an RNA template, it appears that most of correctly incorporated nucleotides would be removed faster than they are extended.
Reverse Transcription by pol γ—Our results show that pol γ binds weakly to a DNA/RNA duplex, incorporates a single dNTP, and then stalls. Active site titrations attempting to determine the binding affinity of the polymerase and the oligonucleotide duplex proved to be difficult because of the low amplitude of the burst phase of product formation during presteady state RNA-dependent DNA polymerization assays; so an accurate measure of the binding affinity of pol γ to a DNA/RNA heteroduplex was not possible. However, this weak binding, as determined by pulse-chase experiments, explains the low burst amplitude observed under single turnover, single nucleotide incorporation assays and the apparent double exponential nature of these reactions. Even though the enzyme is in molar excess over the 25d/45r heteroduplex, only a small fraction of the primer-template is bound by the enzyme at the beginning of the reaction, leading to low burst amplitude and a slow second phase of the reaction because of multiple enzyme turnovers.

Reverse transcriptases have been used extensively to construct cDNA libraries from cellular mRNA, and in this capacity they are extremely powerful tools for the molecular biologist. Currently available reverse transcriptases could be improved considerably, however, if these enzymes were more processive and showed proofreading activity. However, we believe that pol γ cannot be employed as a viable reverse transcriptase for molecular biological applications, because of its lack of processivity. Mutagenesis to increase the binding of the polymerase to the DNA/RNA duplex may allow for pol γ to function as a processive reverse transcriptase, but this may not be biochemically feasible or economically viable.

The overall discrimination seems to be great enough that pol γ could be employed as a passable reverse transcriptase, if the exonuclease proofreading activity selectively removed mismatches. However, our measurements show that the exonuclease rates are ~100-fold slower than observed with DNA templates. Moreover, it was not possible to accurately assess the selectivity of the exonuclease because extension of the next correct base was so slow even with a correctly paired primer terminus. Following the incorporation of a single base opposite an RNA template, the polymerase stalls and idles because of subsequent excision and reinsertion of nucleotides at a single site. Over time, the 16-fold selectivity of the exonuclease toward removal of mismatches would improve the overall fidelity of insertion at this position.

The possible origins and eventual fate of the DNA/RNA heteroduplex in the mitochondria is not known. However, it is well established that during RNA polymerase-dependent initiation of DNA synthesis, a mitochondrial RNA processing RNase cleaves the RNA strands to produce primers for initiation of DNA synthesis (23, 27).

Because of the inability of the enzyme to polymerize processively, we believe that the reverse transcriptase activity is not likely to contribute significantly to the biology of mitochondrial DNA replication. However, the remarkably efficient incorporation of a single base opposite an RNA template is intriguing and will require further study to establish its possible physiological significance.

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