Transcriptional fidelities of human mitochondrial POLRMT, yeast mitochondrial Rpo41, and phage T7 single-subunit RNA polymerases

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Edited by Ronald C. Wek

Single-subunit RNA polymerases (RNAPs) are present in phage T7 and in mitochondria of all eukaryotes. This RNAP class plays important roles in biotechnology and cellular energy production, but we know little about its fidelity and error rates. Herein, we report the error rates of three single-subunit RNAPs measured from the catalytic efficiencies of correct and all possible incorrect nucleotides. The average error rates of T7 RNAP (2 × 10⁻⁶), yeast mitochondrial Rpo41 (6 × 10⁻⁶), and human mitochondrial POLRMT (RNA polymerase mitochondrial) (2 × 10⁻⁵) indicate high accuracy/fidelity of RNA synthesis resembling those of replicative DNA polymerases. All three RNAPs exhibit a distinctly high propensity for GTP misincorporation opposite dT, predicting frequent A-G errors in RNA with rates of ~10⁻⁴. The A→C, G→A, A→U, C→U, G→U, U→C, and U→G errors mostly due to pyrimidine–purine mismatches were relatively frequent (10⁻⁵–10⁻⁶), whereas C→G, U→A, G→C, and C→A errors from purine–purine and pyrimidine–pyrimidine mismatches were rare (10⁻⁷–10⁻¹⁰). POLRMT also shows a high C→A error rate on 8-oxo-dG templates (~10⁻⁵). Strikingly, POLRMT shows a high mutagenic bypass rate, which is exacerbated by TEFM (transcription elongation factor mitochondrial). The lifetime of POLRMT on terminally mismatched elongation substrate is increased in the presence of TEFM, which allows POLRMT to efficiently bypass the error and continue with transcription. This investigation of nucleotide selectivity on normal and oxidatively damaged DNA by three single-subunit RNAPs provides the basic information to understand the error rates in mitochondrial and, in the case of T7 RNAP, to assess the quality of in vitro transcribed RNAs.

Transcription errors are made frequently during the enzymatic synthesis of RNA by DNA-dependent RNA polymerases, and such errors can have serious consequences to the cell (1–3).

This work was supported by NIGMS, National Institutes of Health Grant R35118086. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains supplemental Figs. S1–S5.

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For example, the intrinsic error rates of multisubunit RNAPs, such as Escherichia coli RNAP and nuclear Pol II, are high and estimated to be around 10⁻³–10⁻⁴ (4–6). Mutations in RNA can affect numerous post-transcriptional processes, including RNA processing and translation. Moreover, one mRNA molecule is translated multiple times; hence, aberrant RNAs can produce multiple copies of aberrant proteins. Mitochondria produce polycistronic RNAs that are extensively processed to generate tRNAs, rRNAs, and mRNAs, and transcription errors can alter these RNA-processing reactions to affect protein levels. Damaged DNA, including oxidized bases that are frequently found in the mitochondrial DNA, also affects transcription and error rates (7–10). Additionally, misincorporation can cause pausing or stalling of transcriptional complexes, which are major hurdles to active transcription and replication, resulting in genome instability (11, 12). Understanding the fidelity and mechanism of posterror processes of RNAPs is critically important.

Single-subunit RNAPs represent a distinct class of enzymes found in phage T7 and in the mitochondria of all eukaryotes. T7 RNAP is the simplest enzyme in this class that can processively transcribe the DNA without requiring any accessory factors (13–15). Mitochondrial RNAPs are related to T7 RNAP (16–18), but they depend on accessory factors for transcription initiation (19–24). For example, the human POLRMT requires TFAM and TFB2M (25, 26) for promoter opening, and the yeast Rpo41 requires Mtf1 (27, 28). T7 RNAP is widely used in in vitro transcription reactions for RNA synthesis, and mitochondrial RNAPs play a key role in cellular energy production. It is estimated that the mitochondrial transcripts comprise 10–30% of the total RNA in energy-demanding tissues, including heart, kidney, and brain (29). Consequently, errors in transcription can contribute to mitochondrial dysfunctions. Despite their importance, our understanding of the fidelity of RNA synthesis by T7 and mitochondrial RNAPs is largely incomplete.

Previous in vitro studies of transcription error measurements of T7 RNAP were carried out with promoter-initiated paused elongation complexes, which provided average error rates of 10⁻³–10⁻⁶ depending on the base misincorporated (30).

The abbreviations used are: RNAP, RNA polymerase; 8-oxo-dG, 8-oxodeoxyguanine; Pol, RNA polymerase; TF, transcription factor; ES, elongation bubble substrate; POLRMT, RNA polymerase mitochondrial; TEFM, transcription elongation factor mitochondrial.
Because of the many constraints in studying transcription elongation starting from the promoter sequence, we chose to use a promoter-free elongation substrate that allows one to bypass the nonprocessive stages of initiation and study the fidelity of RNA synthesis only in the elongation phase. Promoter-free elongation substrates with 9-bp RNA-DNA hybrid in a DNA bubble are excellent substrates of both single- and multisubunit RNAPs (18, 31, 32). Furthermore, promoter-free elongation substrates have been used to dissect the kinetic pathway of T7 RNAP and POLRMT during transcription elongation (33–35).

In this study, we have measured the transcriptional fidelity of the human mitochondrial POLRMT, yeast (Saccharomyces cerevisiae) mitochondrial Rpo41, and phage T7 RNAP during transcription elongation (33–35).

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In this study, we have measured the transcriptional fidelity of the human mitochondrial POLRMT, yeast (Saccharomyces cerevisiae) mitochondrial Rpo41, and phage T7 RNAP by measuring the single-nucleotide incorporation rate constant ($k_{pol}$), the nucleotide dissociation constant (nucleotide triphosphate (NTP) $K_d$), and the catalytic efficiency ($k_{pol}/K_d$) of correct and all 12 incorrect nucleotides. The nucleotide selectivity defined as the $k_{pol}/K_d$ of incorrect nucleotide incorporation divided by the $k_{pol}/K_d$ of correct nucleotide incorporation estimates the transcription error rates. The error rates predict the types of expected base changes in the transcribed RNA, and their measurements provide basic information to compare the error rates of single-subunit RNAPs, multisubunit RNAPs, and replicative DNA polymerases. Additionally, a detailed study of POLRMT was carried out to investigate posterror processes, such as mutagenic bypass, translesion bypass on oxidatively damaged 8-oxo-dG template, and the propensity of POLRMT to form paused transcription complexes on oxidized and misincorporated templates.

**Results**

**Equilibrium dissociation constant $K_d$ and off-rate of POLRMT from the elongation substrate**

Elongation substrates were prepared by annealing a 12-mer RNA (5’-end fluorescein) to a complementary DNA template to generate 9-bp RNA-DNA hybrid and a 3-nucleotide overhang at the 5’-end of the RNA. This RNA-DNA hybrid was annealed to a partially complementary non-template DNA strand to make the elongation bubble substrate (ES) (Fig. 1A).
Four such ES substrates were prepared with different +1 templating bases, \( X \) (dA, dG, dC, or dT), which enabled us to measure the rates of correct and incorrect nucleotide incorporations. The elongation substrates are abbreviated as dX-ES where +1 \( X \) represents the templating base. To measure the fidelity of transcription, we measured the incorporation rates of correct and incorrect nucleotides. It was important to use the same set of ES substrates to compare the fidelities of the three single-subunit RNAPs because the individual misincorporation efficiencies can depend on the local sequence around the templating base. Correct nucleotide addition kinetics of T7 RNAP have been studied previously (33, 34, 36); hence, the following sections focus on measuring the kinetics of correct nucleotide by the mitochondrial RNAPs, in particular the POLRMT.

First, we measured the affinity of POLRMT for the ES using equilibrium DNA binding and kinetic off-rate experiments. To determine the equilibrium dissociation constant (\( K_d \)) of the ES-POLRMT complex, 10 nM fluorescein-labeled dT-ES was titrated with increasing concentrations of POLRMT (Fig. 1B). We observed stoichiometric binding of POLRMT to ES (Fig. 1B). The data were fit to the quadratic equation (37) to assess the 10 pm \( K_d \) of the ES-POLRMT complex. Because of the stoichiometric nature of the binding curve, this value is an upper limit of the true \( K_d \) value, and it indicates that POLRMT forms an extremely high-affinity complex with the elongation substrate.

To measure the off-rate of POLRMT from the elongation complex, a preformed fluorescent ES-POLRMT complex was chased with an excess of unlabeled ES. Dissociation of the fluorescent complex was measured through the time-dependent decrease in fluorescence anisotropy (Fig. 1C). Consistent with its high affinity, the ES-POLRMT complex dissociated with a slow rate constant of \( 5 \times 10^{-5} \, \text{s}^{-1} \), which indicates a life-time (1/off-rate) of 5.5 h. This off-rate is about 30 times slower than the reported off-rate of POLRMT (60 \( \mu \text{M} \)). Having measured the kinetic constants of POLRMT, we can now compare the elongation kinetics of POLRMT with that of T7 RNAP. The NTP \( K_d \) of POLRMT (60 \( \mu \text{M} \)) measured here resembles the reported NTP \( K_d \) of T7 RNAP (80 \( \mu \text{M} \)); hence the two RNAPs have similar binding affinities for the correct nucleotide. However, the \( K_d \) of T7 RNAP is 18 times faster than that of POLRMT (2 \( \mu \text{M} \) and 0.5 \( \mu \text{M} \)), and \( K_d \) of T7 RNAP is about 220 \( \text{s}^{-1} \) (33, 34). We can also compare the elongation efficiency of POLRMT \( (2 \times 10^6 \, \text{M}^{-1} \, \text{s}^{-1}) \), T7 RNAP \( (-2 \times 10^6 \, \text{M}^{-1} \, \text{s}^{-1}) \), and Rpo41 \( (1 \times 10^5 \, \text{M}^{-1} \, \text{s}^{-1}) \). The \( k_{pol}/K_d \) of Rpo41 was estimated from the correct nucleotide incorporation rate constant and NTP concentration from Fig. 2F (50 \( \text{s}^{-1}/50 \, \mu \text{M} \)). This comparison shows that the elongation efficiency of T7 RNAP is 10 times higher and of Rpo41 is about 5 times higher than that of POLRMT.

Incorrect nucleotide incorporation by POLRMT

The error frequency is best estimated from the nucleotide selectivity, which is the ratio of the \( k_{pol}/K_d \) of incorrect and correct nucleotides. As expected, each incorrect nucleotide is added by the POLRMT with a different rate (Fig. 4, A and B). Therefore, the misincorporation rates were used as a guide to identify a time of reaction to carry out the [NTP] dependence of the misincorporation reaction to obtain the \( k_{pol} \) and \( K_d \) values. For example, because of the fast rates of GTP misincorporation across dT, this reaction was monitored for 20 s, whereas CTP addition across dT was slow and monitored for 15 min. In these measurements, we made sure that, under no conditions, more than 20–30% of the RNA was extended to products, assuring initial rate conditions. The misincorporation rates versus [NTP] plots were fit to a hyperbola (Equation 2) to obtain the misincorporation \( k_{pol} \) and incorrect NTP \( K_d \) values. If the misincorporation rates did not become saturated at the highest [NTP] used, then the initial slope estimated the catalytic efficiency \( k_{pol}/K_d \) of misincorporation.

The first templating nucleotide in the dT-ES is dT, and +2 is dG (Fig. 1A). In the presence of CTP alone, POLRMT misincorporates CTP across +1 dT and then adds another CTP
across the +2 dG, elongating the 12-mer RNA to 14-mer (Fig. 4C). No intermediate 13-mer was observed, which indicates a fast mutagenic bypass rate (studied in more detail in later sections below). The hyperbolic fit of the misincorporation rates versus [CTP] provided a dT:CTP misincorporation $k_{pol}$ of 1.1 $\times$ 10$^{-3}$ s$^{-1}$ and CTP $K_d$ of 1110 $\mu$M (Fig. 4F). This indicates that, relative to correct nucleotide, the incorrect CTP binds across dT with a 20-fold weaker affinity but incorporated at an ~10,000 times slower rate. Thus, the nucleotide selectivity of POLRMT for CTP versus ATP across dT is 5 $\times$ 10$^{-6}$, which means that this misincorporation will occur once in 2 $\times$ 10$^5$ correct addition reactions. The dT:CTP misincorporation results in the A→C base change in the RNA; hence the A→C error rate is 5 $\times$ 10$^{-6}$.

Similar experiments and analyses were carried out to assess the dT:UTP and dT:GTP misincorporation rates on the dT-ES. POLRMT misincorporates UTP across dT with a $k_{pol}$ of 1.7 $\times$ 10$^{-2}$ s$^{-1}$ and UTP $K_d$ of 4820 $\mu$M (Fig. 4E). Misincorporation of GTP across dT was very efficient with a $k_{pol}$ of 1.4 $\times$ 10$^{-2}$ s$^{-1}$ and GTP $K_d$ of 580 $\mu$M (Fig. 4F). These kinetic parameters indicate that the A→G error rate is 1 $\times$ 10$^{-4}$ and the A→U error rate is 2 $\times$ 10$^{-5}$ (Table 1).

Next, we used the dA-ES to measure the dA:ATP, dA:GTP, and dA:CTP misincorporation rates. POLRMT misincorporated ATP across dA with a very slow rate, which did not become saturated even at 5 mM ATP (Fig. 5A). Thus, we could assess only the $k_{pol}/K_d$ of dA:ATP misincorporation as 0.006 M$^{-1}$ s$^{-1}$ (Fig. 5, A and E), indicating that the U→A error rate is very
Transcription error rates of POLRMT, Rpo41, and T7 RNAP

Figure 3. The $k_{\text{pol}}$ and $K_d$ values of correct nucleotide incorporation by POLRMT. A, single-turnover kinetics of UTP incorporation into 100 nM dA-ES by 200 nM POLRMT. The single-turnover kinetics were measured at increasing concentrations of UTP at 25 °C in a rapid chemical quench-flow instrument, and the data were fit to a single exponential equation to obtain the rate constants. $FAM$, 6-carboxyfluorescein. B, the rate constants from A are plotted against [UTP], and the dependence was fit to a hyperbola to obtain the indicated $k_{\text{pol}}$ and $K_d$ of correct UTP incorporation by POLRMT. The errors are standard errors of fitting. The experiment was carried out twice and representative data are shown. Error bars represent S.E.

Summary of the transcription errors of the yeast Rpo41

A complete misincorporation study was carried out with the yeast Rpo41 (supplemental Figs. S1–S4). Interestingly, the yeast Rpo41 shows a similar general trend of transcription errors as the POLRMT (Table 2 and Fig. 8). Rpo41 also shows a high propensity of GTP addition across dT with an A→G error rate of $10^{-4}$. Errors from purine–pyrimidine mismatches ($4 \times 10^{-5}$) are about 7 times higher than the error rates of purine–purine and pyrimidine–pyrimidine mismatches ($6 \times 10^{-6}$). Specifically, the G→A, U→G, A→U, U→C, A→C, G→U, and C→U errors are more frequent ($10^{-5}$–$10^{-6}$), and C→G, U→A, C→A, and G→C errors are rare ($10^{-7}$ and $10^{-8}$). In sum, the average transcription error rate of POLRMT is $2 \times 10^{-5}$.

Summary of the transcription errors of the T7 RNAP

A complete misincorporation study of the T7 RNAP was carried out with a slightly different construct of ES but with the same RNA-DNA hybrid sequence (supplemental Fig. S5). T7
RNAP also shows a similar trend of misincorporation as the mitochondrial RNAPs (Table 3 and Fig. 8). However, T7 RNAP shows a much higher discrimination at the chemical step relative to the mitochondrial RNAPs. On average, the binding affinity of incorrect NTPs is 30-fold weaker, and the incorporation rate is 15,000-fold slower than correct NTPs. Errors from purine–pyrimidine mismatches are about 3 times higher than the errors from purine–purine and pyrimidine–pyrimidine mismatches. T7 RNAP also shows a high rate of dT:GTP misincorporation, predicting a high A→G error rate of 10^{-5} followed by A→U, A→C, C→U, G→A, G→U, and U→G error rates of 10^{-6} and G→C, C→A, C→G, and U→A error rates of 10^{-7}. The average transcription error rate of T7 RNAP is 2 \times 10^{-6}, which is 10-fold lower than that of POLRMT.

**Pausing, bypass, and dissociation kinetics of POLRMT after the misincorporation event**

Unlike multisubunit RNAPs and replicative DNA polymerases, the single-subunit RNAPs do not have error proofreading activity. To investigate the fate of the elongation complex after
Table 1
Nucleotide selectivity of POLRMT

| DNA-NTP | POLRMT $k_{pol}$ | $K_d$ | $k_{pol}/K_d$ | Transcription error rate |
|---------|------------------|-------|---------------|---------------------------|
| dA:UTP | 12 ± 1.1 s$^{-1}$ | 63 ± 17 μM | 1.9 × 10$^5$ | 1.8 × 10$^4$ | 1 |
| dA:ATP | ND | ND | 0.006 ± 0.001 | 3.2 × 10$^{-8}$ | 1.4 × 10$^{-5}$ |
| dA:CTP | ND | ND | 2.7 ± 0.6 | 3.7 ± 1 | 2.1 × 10$^{-5}$ |
| dA:GTP | 4.6 ± 0.4 × 10$^{-2}$ | 1230 ± 350 | 3.5 ± 0.6 | 3.5 ± 0.6 | 1.8 × 10$^{-5}$ |
| dG:ATP | ND | ND | 24 ± 8 | 1 ± 0.3 | 1.3 × 10$^{-4}$ | 5.2 × 10$^{-6}$ |
| dC:ATP | 3 ± 0.3 × 10$^{-2}$ | 600 ± 240 | 1 × 10$^{-3}$ | 1 × 10$^{-3}$ | 5 × 10$^{-5}$ |
| dC:GTP | 4 ± 0.2 × 10$^{-3}$ | 630 ± 120 | 0.6 ± 0.1 | 0.54 ± 0.3 | 2.8 × 10$^{-6}$ | 5.2 × 10$^{-9}$ |
| dC:TTP | 3 ± 0.4 × 10$^{-3}$ | 560 ± 310 | 0.54 ± 0.3 | 0.001 ± 1 × 10$^{-5}$ | 2.6 × 10$^{-5}$ | 3.2 × 10$^{-6}$ |
| dG:UTP | ND | ND | 0.03 ± 0.01 | 0.03 ± 0.01 | 4.3 × 10$^{-4}$ | 1.6 × 10$^{-7}$ |
| dC:UTP | 1.5 ± 0.08 × 10$^{-2}$ | 180 ± 40 | 83 ± 19 | 83 ± 19 | 4.3 × 10$^{-4}$ | 4.3 × 10$^{-4}$ |
| 8-Oxo-dG:ATP | 9 ± 0.3 × 10$^{-3}$ | 692 ± 98 | 1.3 | 1.3 | 6.8 × 10$^{-6}$ | 6.8 × 10$^{-6}$ |

Figure 5. The POLRMT misincorporation rates across the dA templating base. A, the gel image shows the dA:ATP misincorporation with increasing [ATP]. The reaction conditions are the same as in Fig. 4C. The dA:ATP misincorporation rates plotted against [ATP] were fit to a line, and the slope provided the $k_{pol}/K_d$ listed in E. FAM, 6-carboxyfluorescein. B shows dA:G misincorporation by POLRMT at increasing [GTP]. The data were fit to a hyperbola to determine the $k_{pol}$ and $K_d$ kinetic parameters. C, the time course of CTP incorporation on the dA-ES by POLRMT at various [CTP] (25, 50, 100, 250, 500, and 1000 μM). D, the slopes from C were plotted against [CTP] to obtain the $k_{pol}/K_d$ of dA:C misincorporation. E, the table lists the $k_{pol}$, $K_d$, and $k_{pol}/K_d$ of all possible misincorporations across dA by POLRMT. ND, not determined.

Figure 6. The POLRMT misincorporation rates across the dC templating base. A, the dC-ES is shown. FAM, 6-carboxyfluorescein. B, C, and D show the gel images of dC:C, dC:A, and dC:U misincorporations, respectively, across the dC templating base by POLRMT at increasing concentrations of the NTP. In B, very little product is observed. In D, formation of the 14-mer due to dG:U mismatch is observed at higher UTP concentrations. The data were fit to a line to obtain the $k_{pol}$, $K_d$, and $k_{pol}/K_d$ parameters shown in E. ND, not determined.
misincorporation events, we investigated several of the possible processes with the following questions. After misincorporation, does POLRMT bypass the error, stall after misincorporation to generate paused transcription complexes, or abort the RNA? To explore these possibilities, we prepared several terminally mismatched ES, such as dT:U, dT:G, and dA:G. We measured the mutagenic bypass rates of POLRMT. Surprisingly, POLRMT efficiently bypasses both dT:U and dT:G mismatches by adding the next correct NTP with rates almost similar to those for correct nucleotide (Fig. 9, A and B). We had to use rapid kinetic methods to measure the mutagenic bypass rates, which were 7–8 s⁻¹ at 50 μM NTP. A similarly fast rate of correct nucleotide over incorrect base pair is noted above during measurements of CTP misincorporation on the dT-ES (Fig. 4C). The fast mutagenic bypass rates of POLRMT contrast with DNA polymerases that have very slow rates of correct addition over mismatches (39). Given that dT:G mismatch is most frequently introduced and dT:U is introduced with moderate efficiency, the fast mutagenic bypass rates would indicate that A→G and A→U errors once made will be sealed into the RNA. In contrast to the fast mutagenic bypass rates past dT:U and dT:G mismatches, the mutagenic bypass rate past the purine–purine dA:G mismatch was ~1000 times slower (Fig. 9, C and D), which indicates that the mutagenic bypass rate of POLRMT is dependent on the type of mismatch.

To explore the possibility that POLRMT may abort after making an error, we measured the off-rates of POLRMT from matched and various mismatched terminated ESs (Fig. 10A). A preformed complex of POLRMT with fluorescein-labeled ES was chased with an excess of unlabeled ES (Fig. 10B). The time-dependent decrease in fluorescence provided the off-rates and lifetimes of POLRMT complexes. The lifetime of POLRMT on a matched primer-end ES is ~5.5 h (Fig. 1C). In contrast, the

Figure 7. The POLRMT misincorporation rates across the dG templating base. A, the dG-ES is shown. FAM, 6-carboxyfluorescein. B, C, and D show the gel images of dG:U, dG:A, and dG:G misincorporations, respectively, across the dG templating base by POLRMT at increasing concentrations of the NTP. The data were fit to a line to obtain the $k_{pol}$, $K_a$, and $k_{pol}/K_a$ parameters shown in E. "ND," not determined.

Figure 8. Summary of the transcription error rates of POLRMT, Rpo41, and T7 RNAP for all possible base pair combinations. The plots summarize the transcription errors rates (from Tables 1–3), organizing the rates from the highest to the lowest for the three RNAPs. The A→G base change occurs with the highest probability (~10⁻³) in all three RNAPs. Similarly, the U→A, C→G, and C→A, and G→C base changes occur with the lowest probabilities (~10⁻⁷–10⁻¹⁰) in all three RNAPs. The G→A, U→G, A→U, U→C, A→C, G→U, and C→U base changes occur with intermediate probabilities (~10⁻³–10⁻⁵). The errors bars show the errors associated with $k_{pol}/K_a$ values of correct and misincorporations from Tables 1–3 calculated using the error propagation method. The errors bars for Rpo41 are missing because the $K_a$ values of correct nucleotide addition by Rpo41 was estimated and assumed to be the same as POLRMT.
The correct CTP across 8-oxo-dG with a $K_d$ of 4.7 × 10$^{-3}$ s$^{-1}$ (Fig. 10, C and D). This indicates that the terminal mismatch affects the stability of the elongation complex. The mutagenic bypass rate past dA:G (2 × 10$^{-3}$ s$^{-1}$) is comparable with the off-rate (3 × 10$^{-3}$ s$^{-1}$); hence there is a high probability that POLRMT will abort after the dA:G mismatch incorporation event, decreasing the frequency of T→G errors in productive RNAs. In contrast, POLRMT will seal in the A→U and A→G mistakes in the RNA because the bypass rates of POLRMT past dT:U and dT:G mismatches are much faster (6–8 s$^{-1}$) than the off-rates (1 × 10$^{-3}$ s$^{-1}$).

### Effect of an oxidized 8-oxo-dG templating base on elongation by POLRMT

8-Oxo-dG is a common oxidative damage in the DNA. We created an ES with 8-oxo-dG as the +1 templating base (Fig. 11A) to measure the incorporation of correct CTP and incorrect ATP across 8-oxo-dG. Although elongation studies of POLRMT and T7 RNAP have been conducted with 8-oxo-dG (10, 40), the rates of pausing, mutagenic bypass, and error-free bypass have not been measured. We show that POLRMT adds the correct CTP across 8-oxo-dG with a $k_{pol}$ of 9 × 10$^{-4}$ s$^{-1}$ (Fig. 11, B and D), which is ~13,000 times slower than the normal elongation rate. The $K_d$ of CTP across 8-oxo-dG is 700 μM, which is 12 times weaker than normal base pairing. Thus, the catalytic efficiency $k_{pol}/K_d$ of CTP addition across 8-oxo-dG is 150,000 times lower than the efficiency of normal elongation. Once CTP is added across 8-oxo-dG, the next correct nucleotide is added at a fast rate (Fig. 11B).

In contrast to CTP, the catalytic efficiency of ATP addition across 8-oxo-dG is ~75-fold higher than the higher $k_{pol}$ and the lower $K_d$ (Fig. 11, C and D). The affinity of ATP across 8-oxo-dG is 4 times greater than CTP across 8-oxo-dG. This also indicates that the 8-oxo-dG templating base assumes a syn conformation to form a stable Hoogsteen base pair with the incoming ATP. If 8-oxo-dG assumed the anti conformation in the active site of POLRMT, it would bind preferably to CTP (41). In summary, our results indicate that POLRMT will undergo mutagenic translesion bypass at 8-oxo-dG, introducing C→A base changes with rates of 4 × 10$^{-4}$. Note that the C→A error rates on a normal dG template are very low (5 × 10$^{-9}$). Furthermore, we predict that POLRMT will generate paused transcription complexes on 8-oxo-dG oxidized templates. This is because the mutagenic and error-free translesion bypass rates, respectively, are ~800–15,000 times slower than normal elongation rates.

### Effect of TEFM on the transcriptional fidelity of POLRMT

The mitochondrial transcription elongation factor TEFM was recently identified as a transcription elongation factor (42). TEFM promotes POLRMT processivity and thus helps in the synthesis of longer transcripts. In addition, it prevents pausing...
of POLRMT at various sites on the DNA, thereby aiding in continuation of transcription (43, 44). However, the roles of TEFM in transcriptional fidelity of POLRMT are not known. We tested the effect of TEFM on the misincorporation rate of GTP across dT as this is the most efficient mismatch. However, TEFM had no effect on the rate of dT:G mismatch formation (Fig. 12A). Interestingly, TEFM increased the efficiency of the mutagenic bypass over the dA:G mismatch by 8-fold (Fig. 12B). This suggests that TEFM allows POLRMT to continue with transcription after a misincorporation event. This possibly could be due to stabilization of POLRMT by TEFM on the dA:G template. Therefore, we measured the off-rate of POLRMT from a mismatched dA:G elongation complex in the presence and absence of TEFM and observed that TEFM substantially increases the stability of the mismatched elongation complex. The off-rate of POLRMT from the dA:G elongation complex in the presence of TEFM (2 × 10^{-5} s^{-1}) is 60-fold slower than that in the absence of TEFM (Fig. 12C). In fact, the lifetime of the mismatched elongation complex with TEFM was similar to that of POLRMT on matched template. Thus, TEFM aids in the continuation of transcription by preventing the pausing of POLRMT at a mismatch site.

Discussion

We have carried out a comprehensive nucleotide selectivity study of three single-subunit RNAPs, including the human mitochondrial POLRMT, yeast mitochondrial Rpo41, and phage T7 RNAP, by determining the catalytic efficiencies of correct and all 12 incorrect nucleotide incorporations on a promoter-free elongation substrate. From the nucleotide selectivity values, we can predict that the average transcription error rate of T7 RNAP is 2 × 10^{-6}, that of yeast mitochondrial Rpo41

Figure 9. Mutagenic bypass rates of the POLRMT. A, the dT-U mismatched ES (200 nM) was incubated with GTP (50 μM) in the presence of POLRMT (400 nM), and the gel image shows the elongation of 12-mer to 13-mer. The kinetics of mutagenic bypass were measured in a rapid chemical quench-flow instrument and fit to a single exponential equation. FAM, 6-carboxyfluorescein. B, the same reaction as in A was carried out with the dT-G mismatched ES in the presence of 400 nM POLRMT and 1600 nM ES. C, the same reaction as in A was carried out with the dA:G mismatched ES (1600 nM) by POLRMT (400 nM) were measured at increasing [CTP] to obtain the $k_{pol}$ and $K_d$ values shown. The errors are standard errors of fitting.
is $6 \times 10^{-6}$, and that of human mitochondrial POLRMT is $2 \times 10^{-5}$. Thus, T7 RNAP is about 10 times more accurate than POLRMT, and the yeast Rpo41 is about 3 times more accurate than POLRMT. The intrinsic error rates of single-subunit RNAPs are close to or lower than the error rates of replicative DNA polymerases (45, 46). The transcription error rate of POLRMT is close to the replication error rate of the proofreading-deficient human Poly ($2 \times 10^{-5}$) (46), and the T7 RNAP error rate ($\sim 2 \times 10^{-6}$) is actually 10 times lower.

The intrinsic error rates of single-subunit RNAPs are also lower than the intrinsic error rates of multisubunit RNAPs. For example, the intrinsic error rates of *E. coli* RNAP and nuclear Pol II are estimated to be around $10^{-3}$ to $10^{-4}$ (4–6). However, multisubunit RNAPs either contain an intrinsic proofreading activity (47) or use accessory factors such as GreA/B and TFIIS to proofread errors, which increases their accuracy/fidelity of RNA synthesis (5, 48–51). Such proofreading activities are absent in single-subunit RNAPs, but the higher fidelity likely compensates for their lack of proofreading capabilities.

The most prominent misincorporation event that was observed in all three single-subunit RNAPs was GTP across dT,

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**Figure 10. Kinetic stability of the 3′-end mismatched elongation complexes of POLRMT.**

*Panel A.* The 3′-end mismatched ES is shown. FAM, 6-carboxyfluorescein. *Panel B.* The experimental design to measure the off-rate of ES from POLRMT. The reaction conditions are the same as in Fig. 1. *Panel C.* Time courses of ES dissociation from POLRMT as monitored by the kinetics of fluorescence anisotropy decay. The experiments were repeated twice, and representative traces are shown. *Panel D.* The kinetics in C were fit to a single exponential equation to obtain the off-rates and lifetimes of the POLRMT-ES complexes. The experiments were carried out at least twice. The errors are standard errors of the fit.
which introduces A→G errors in the RNA. Our studies predict that this error will occur with a rate of $\sim 10^{-4}$. The single-subunit RNAPs are structurally related to the Pol I family of DNA polymerases, including the Klenow fragment of *E. coli* Pol I and human Pol. Interestingly, the Pol I family DNA polymerases also show a high rate of dT:G misincorporation (46, 52), indicating a structural basis for efficient misincorporation of GTP across dT. One reason is that the dT:G forms a wobble base pair, which is accommodated well within the active site of these polymerases (53). However, base-stacking interactions are also important because the corresponding dG:U misincorporation occurs with a 100-fold lower rate in all three RNAPs and Pol (46). This indicates that, in addition to wobble base pairing, base-stacking interactions of the incoming purine GTP make a significant contribution to the high rate of dT:G misincorporation.

It is possible that the individual errors rates are influenced by the neighboring sequences, but in general misincorporations from purine–pyrimidine base pairs are more frequent in all three RNAPs. The nucleotide selectivity predicts that A→C, G→A, A→U, C→U, G→U, U→C, and U→G errors occur with rates of $10^{-5}$–$10^{-6}$. Conversely, C→G, U→A, G→C, and C→A resulting from purine–purine and pyrimidine–pyrimidine mismatches are rare with rates of $10^{-7}$–$10^{-10}$. In general, discrimination against the incorporation of incorrect NTPs is both due to a weak NTP binding (14–20-fold) and a slower chemical step (2000–15,000-fold) relative to correct NTP.

We wished to determine how the most prominent base changes in RNA predicted from *in vitro* measurements compare with those observed *in vivo*. The most prominent errors observed in *in vivo* *E. coli* RNAs are G→A, C→U, and C→A (6). The G→A base change is a highly probable error resulting from dC:A misincorporation, which is a wobble base pair that is most likely accommodated well in the active sites of polymerases. This mismatch is also frequently found in the mitochondrial DNA polymerase Poly reactions (46). The C→U and...
A errors prominently found in vivo show low occurrences in our in vitro transcription reactions. A possible explanation is that the C→U base change observed prominently in vivo results from deamination of cytosines in single-stranded RNA (54). Similarly, the C→A base change most prominently found in the in vivo RNAs may arise from oxidized guanines in the template DNA. This is consistent with our observations that the C→A error rate is high and of the order of ~10^{-4} on 8-oxo-dG template. Thus, in addition to misincorporation, damaged bases in the template DNA and deamination of cytosines are major sources of transcription errors in vivo. Mitochondrial DNA is prone to oxidative damage (55); thus we expect a high frequency of C→A base change in the mitochondrial RNAs.

We also measured the rates of correct nucleotide addition over mismatches to investigate posterror consequences. First, we saw no evidence for any proofreading activity of POLRMT. Second, we found that POLRMT efficiently extends the pyrimidine–pyrimidine and purine–pyrimidine mismatches, including dT:U and dT:G, with rates as fast as extending a matched primer end. This is surprising because DNA polymerases slow down considerably after all misincorporation events, which allows the proofreading activity to excise the mismatches (56). The rates of correct nucleotide addition over dT:U and dT:G mismatched primer ends are faster than the POLRMT off-rates, which indicates that POLRMT will not stall or abort after these misincorporation events, and A→U and A→G errors will be efficiently sealed into the transcribed RNA. Other mismatches, such as the dG:A, behaved differently. The correct nucleotide addition past the dA:G mismatched primer end was slower and comparable with the POLRMT off-rate, which indicates that POLRMT will frequently stall after making this mistake and abort the RNA. Thus, depending on the type of mismatch, POLRMT may pause, efficiently bypass, or abort the RNA.

It has been reported that paused transcription complexes pose a barrier to transcriptional and moving replisome, contributing to genome instability (11, 12). Our studies indicate that except for certain misincorporations, such as dT:U and dT:G, POLRMT is expected to form paused transcription complexes after misincorporation events and upon encou-
tering oxidized bases, such as 8-oxo-dG, in the template. In multisubunit RNAPs, pausing is greatly reduced by proof-reading factors such as GreA (49, 50). In human mitochondria, TEFM was shown to increase the bypass rate at 8-oxo-dG (44). Herein, we show that TEFM increases the mutagenic bypass rate of POLRMT by stabilizing the elongation complex. Thus, TEFM prevents stalling of POLRMT at mismatched sites.

In summary, we show that T7 RNAP is about 10 times more efficient at adding the correct nucleotide during transcription elongation than POLRMT, and Rpo41 is about 5 times more efficient than POLRMT. The misincorporation studies indicate that all three RNAPs are highly accurate with transcription error rates lower than those of multisubunit RNAPs and resembling those of replicative DNA polymerases. The average error rate of T7 RNAP is 10-fold lower than that of POLRMT, and that of Rpo41 is 3-fold lower. It is interesting that T7 RNAP, which does not require any transcription factors, has the highest fidelity and that POLRMT and Rpo41, which depend on transcription factors, have lower fidelities. Although POLRMT is efficient at catalyzing elongation on its own, TEFM is known to stimulate transcription elongation (43, 44). Although TEFM does not affect the misincorporation rate, it increases the mutagenic bypass rate, thereby allowing continuation of transcription. Like other Pol I family polymerases, we found that all three RNAPs misincorporate GTP across dT with a high rate, predicting frequent A→G errors in the transcribed RNAs. Frequent C→U errors are also predicted from deamination, and C→A errors are predicted in RNA from high efficiency of incorrect ATP addition across 8-oxo-dG in the template. Additionally, we show that misincorporation events and oxidized templates promote paused transcription complexes, which can be overcome by the presence of TEFM.

**Experimental procedures**

**Nucleic acids, proteins, and other reagents**

Oligodeoxynucleotides were custom-synthesized and HPLC-purified by Integrated DNA Technologies (Coralville, IA). DNA concentration was determined from its absorbance at 260 nm and the calculated molar extinction coefficients. RNAs were purchased PAGE-purified and 2′-deprotected/desalted from GE Dharamcen. RNAs were purchased with 5′-end fluorescein. High purity NTPs (100 mM solution) were purchased from Affymetrix Thermo Fisher Scientific.

The N-terminal His-tagged POLRMT, N-terminal His-tagged Rpo41, untagged TEFM, and untagged T7 RNAP were purified as described previously (25, 28, 36, 44, 57, 58). Enzyme concentration was calculated from its absorbance at 280 nm and the calculated molar extinction coefficient.

**Assembly of the promoter-free elongation substrate**

Template DNA, non-template DNA, and 5′-labeled RNA were mixed in a 1.25:1:5.1 ratio in the transcription buffer (50 mM Tris acetate, pH 7.5, 10 mM magnesium acetate, 100 mM sodium glutamate, 5 mM DTT, 0.025% Tween 20) at a final concentration of 20 μM, heated at 95 °C for 20 min, and then stepwise cooled from 75, 55, and 45 °C for 20 min each, 20 °C for another 25 min, and finally to 4 °C for an hour.

**Fluorescence anisotropy studies to measure the equilibrium K_d and off-rates of POLRMT complexes**

Fluorescence anisotropy measurements were carried out in a Fluoromax-4 (Horiba Jobin Yvon) at 25 °C. Fluorescein-labeled ES (10 nM) was titrated with increasing concentrations of POLRMT in the transcription buffer. Fluorescence anisotropy was recorded after excitation at 494 nm and emission at 516 nm and plotted against [POLRMT]. The data were fit to the quadratic equation to obtain the equilibrium dissociation constant (K_d) as described (37, 59).

The off-rates were determined by chasing a complex of fluorescein-labeled ES (10 nM) and POLRMT (40 nM) with unlabeled ES (400 nM) with or without TEFM (120 nM) and monitoring the decrease in fluorescence anisotropy due to the dissociation of fluorescein-labeled ES from the POLRMT. The kinetics were fit to a single exponential equation to obtain the off-rates.

**Correct nucleotide incorporation by using rapid chemical quench-flow kinetics**

Presteady-state kinetic experiments were conducted at 25 °C using a Model ROF-3 chemical quench-flow apparatus (KinTek Corp., Austin, TX). A mixture of RNAP and elongation substrate in the transcription buffer was loaded in one syringe of the quenched-flow instrument, and NTP was added from a second syringe of the instrument. The reactions were rapidly mixed and quenched with EDTA (0.2 mM final concentration) after predefined time intervals.

**Incorrect nucleotide incorporation**

The kinetics of incorrect nucleotide misincorporation were measured using 1600 nM elongation substrate and 400 nM RNAP at 25 °C. The reactions were quenched with 0.2 mM EDTA at various times ranging from 5 s to 60 min depending on the initially established reaction conditions. The misincorporation experiments were carried out multiple times (initially with Cy5- and then with fluorescein-labeled ES), and representative gels and figures are shown.

The EDTA-quenched correct and incorrect nucleotide reaction mixtures were loaded on a 24% acrylamide/bis (19:1), 4 M urea sequencing gel. The fluorescein-labeled RNAs were directly detected by scanning the gel on a Typhoon 9410 or Typhoon FLA 7000 Instrument (GE Healthcare) and quantified using ImageQuant software.

The correct nucleotide incorporation kinetics were fit to Equation 1 (single exponential equation) using SigmaPlot software (Jandel Scientific),

\[
Y = y_0 + (1 - A \exp(-k_{obs}t)) \tag{1}
\]

where Y is the fraction or molar amount of elongated primer products, y_0 is the y-intercept or background, A is the amplitude or the total amount of products at the completion of the reaction, and k_{obs} is the observed rate constant of product formation to completion.

The observed rate, k_{obs}, of correct and incorrect nucleotide incorporation was plotted as a function of [NTP] and fit to Equation 2,
Transcription error rates of POLRMT, Rpo41, and T7 RNAP

\[
k_{\text{obs}} = \frac{k_{\text{pol}[\text{NTP}]} - k_{d} + [\text{NTP}]}{18.7}
\]

where \(k_{d}\) is the equilibrium dissociation constant of the NTP from the polymerase complex and \(k_{\text{pol}}\) is the maximum rate constant of NMP incorporation into the RNA primer.

**Author contributions**—S. S. P. and S. S. designed the study. S. S. and M. S. generated and analyzed the data. S. S. P. wrote the manuscript. A. R. provided assistance in the T7 and TEFM study and in the critical review of the manuscript. All authors approved the final version of the manuscript.

**Acknowledgments**—We thank members of the Patel laboratory for valuable advice and critical insights throughout this study.

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