Fidelity of the Human Mitochondrial DNA Polymerase*

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We have quantified the fidelity of polymerization of DNA by human mitochondrial DNA polymerase using synthetic DNA oligonucleotides and recombinant holoenzyme and examining each of the possible 16-base pair combinations. Although the kinetics of incorporation for all correct nucleotides are similar, with an average $K_d$ of 0.8 $\mu M$ and an average $k_{\text{cat}}$ of 37 s$^{-1}$, the kinetics of misincorporation vary widely. The ground state binding $K_d$ of incorrect bases ranges from a low of 25 $\mu M$ for a dATP:A mispair to a high of 360 $\mu M$ for a dCTP:T mispair. Similarly, the rates of incorporation of incorrect bases vary from 0.0031 s$^{-1}$ for a dCTP:C mispair to 1.16 s$^{-1}$ for a dGTP:T mispair. Due to the variability in the kinetic parameters for misincorporation, the estimates of fidelity range from 1 error in 3563 nucleotides for dGTP:T to 1 error in 2.3 $\times$ 10$^6$ nucleotides for dCTP:C. Interestingly, the discrimination against a dGTP:T mismatch is 16.5 times lower than that of a dTTP:G mismatch due to a tighter $K_d$ for ground state binding and a faster rate of incorporation of the dGTP:T mismatch relative to the dTTP:G mismatch. We calculate an average fidelity of 1 error in 440,000 nucleotides.

The mitochondrial genome encodes 13 proteins involved in oxidative phosphorylation, 2 rRNAs, and 22 tRNAs. The observed mutation rates in human mitochondrial DNA are 20–100-fold higher than that of nuclear DNA (1), and mutations within, and depletion of the mitochondrial genome are known to cause several chronic degenerative diseases affecting all systems of the body. Point mutations within the mitochondrial DNA (mtDNA) are associated with diseases including but not limited to autism, hearing loss, cardiomyopathy, and adult onset leukemia (2–7). Large scale depletions are known to be associated with diseases including Kearns-Sayre syndrome and Leber hereditary optic neuropathy (8, 9).

Administration of nucleoside analogs as part of the highly active antiretroviral therapy to combat human immunodeficiency virus infections (10) causes duration-dependent mitochondrial dysfunction. Inhibition of mtDNA replication and the resulting mitochondrial DNA depletion leads to oxidative stress, increased mtDNA mutation, and decreased mitochondrial function (11). It has been proposed that energy decline resulting from mtDNA depletion increases oxidative stress, which can ultimately lead to mutations in the mitochondrial genome (12). Side effects experienced by patients treated with nucleoside analogs include peripheral neuropathy, cardiac and skeletal muscle myopathy, pancreatitis, and bone marrow suppression (12, 13), attributable to decreased mtDNA content.

Previous analysis of the discrimination against nucleoside analog reverse transcriptase inhibitors (NRTIs) by human mitochondrial DNA polymerase (Pol $\gamma$)$^2$ has defined a toxicity index based upon the increased time required to replicate the mitochondrial genome when NRTIs are present (14, 15). The toxicity index reflects the toxicity observed when these nucleoside analog reverse transcriptase inhibitors are assayed in vivo and used clinically. Future drug design efforts, with the aim of decreasing toxicity, should be facilitated by a detailed understanding of the function and fidelity of the human mitochondrial DNA polymerase.

Pol $\gamma$ is a nucleus-encoded enzyme responsible for the replication of the 16.5-kil-base mitochondrial genome. Mutation rates have been estimated using gain or loss of gene function assays or nucleotide reversion frequency assays to be $\sim$1 in 500,000 (16–19). Recently, a link between mitochondrial DNA mutation and aging has been established using homozygous knock-in mice (20). Mice expressing an exonuclease deficient form of Pol $\gamma$ exhibit a 3–5-fold increase in the level of point mutations. This increase in mitochondrial DNA mutation is associated with the onset of age-related phenotypes including hair loss, subcutaneous fat loss, weight loss, curvature of the spine, reduced fertility, enlargement of the heart, osteoporosis, and anemia (20).

To date there has been no exhaustive study of the fidelity of Pol $\gamma$, examining the incorporation kinetics of all 16 possible base pairs. Steady-state experiments have suggested that the majority of errors made by Pol $\gamma$ are substitution errors and those caused by the incorporation of dNTPs opposite bases that are not their Watson-Crick base pairing partners (21). A study using another Pol A family enzyme, the Klenuv fragment of DNA Pol I, showed that of all of the 12 possible incorrect pairings, a dGTP incorporated onto a template dTMP was the most common mutation (22). Additionally, single nucleotide incorporation assays showed that the fidelity of Klenuv fragment manifests from a $\sim$25-fold increase in the $K_d$ for ground state binding and an $\sim$3000-fold decrease in the rate of incorporation on the average. For dGTP onto a dTMP, the $K_d$ increase is 25-fold.

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2 The abbreviations used are: Pol $\gamma$, human mitochondrial DNA polymerase holoenzyme; BF, Bacillus DNA polymerase I fragment; Pur, purine; Pyr, pyrimidine.
assembled in a MgCl₂-free buffer, then mixed with the appropriate nucleotide, previously diluted into a MgCl₂ containing buffer. The catalytic holoenzyme-DNA complex was formed as described previously (23) using the reconstituted enzyme (23) and has been shown to be identical to the wild type enzyme. Experiments to assess the polymerization fidelity were performed as described previously (23) using the reconstituted holoenzyme, consisting of a catalytic subunit containing a 29-amino acid truncation and an accessory subunit containing a His₆ tag and a 56-amino acid terminal truncation. Protein concentrations were determined by active site titration against a known concentration of duplex DNA (25). A 1:5 ratio of catalytic subunit to accessory subunit was used for holoenzyme reconstitution based upon a measured Kₐ of 35 nM and a typical enzyme concentration of 50–100 nM.

Preparation of DNA-DNA Substrate—Primer strands were 5'-32P-labeled with T4 polynucleotide kinase as per manufacturer’s instructions (Invitrogen). Termination of the reaction was accomplished by heating the reaction 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 ratios 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—Assays were performed at 37 °C in a buffer containing 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 2.5 mM MgCl₂. The catalytic holoenzyme-DNA complex was assembled in a MgCl₂-free buffer, then mixed with the appropriate nucleotide, previously diluted into a MgCl₂ containing buffer, at a 1:1 ratio to initiate the reaction. Because mixing of the enzyme-DNA with the nucleotide is at a 1:1 ratio, each solution is assembled at twice the reported final concentration.

~5-fold, and the rate decrease is but ~900-fold. The authors suggest that the differences in kinetic data between dGTP: dTMP and dTTP:dGMP misincorporations may be due to the geometric asymmetry of the purine-pyrimidine mispairs relative to normal Watson-Crick base pairs.

This report expands upon previous work from this laboratory (23) detailing the kinetic parameters governing the fidelity of human Pol γ polymerization under single turnover conditions utilizing a defined primer-template system. Pol γ is thought to be a heterotrimer (24) consisting of a single catalytic subunit (140 kDa) and two accessory subunits (54 kDa) with no known catalytic activity. Because both subunits are nucleoside- encoded and transported to the mitochondria, the genes of the recombinant proteins were truncated to mimic the removal of the mitochondrial import leader sequences (25, 26). The catalytic and accessory subunits were combined in a 1:5 molar ratio needed to saturate the binding to reconstitute the holoenzyme. To probe the fidelity of human Pol γ, we challenged an exonuclease deficient mutant (E200A) with incorrect dNTPs and measured the kinetic parameters of mismatch polymerization. In the accompanying paper we examine the kinetics of incorporation of 8-oxo-7,8-dihydroguanosine triphosphate, a common oxidative metabolite of dGTP (27).

### MATERIALS AND METHODS

**Enzyme Subunits**—Expression and purification of Pol γA and Pol γB were accomplished as previously described (25, 26). The expression, purification, and characterization of an exonuclease-deficient mutant of Pol γA has been previously reported (23) and has been shown to be identical to the wild type enzyme. Experiments to assess the polymerization fidelity were performed as described previously (23) using the reconstituted holoenzyme, consisting of a catalytic subunit containing a 29-amino acid truncation and an accessory subunit containing a His₆ tag and a 56-amino acid terminal truncation. Protein concentrations were determined by active site titration against a known concentration of duplex DNA (25). A 1:5 ratio of catalytic subunit to accessory subunit was used for holoenzyme reconstitution based upon a measured Kₐ of 35 nM and a typical enzyme concentration of 50–100 nM.

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**Results and Discussion**

Exonuclease-deficient Pol γ was used for these misincorporation experiments because neither creation of a mismatch nor extension beyond a mismatch have been observed in the presence of exonuclease function (23, 28, 29). Moreover, because the rate of polymerization is as slow or slower than the rate of dissociation of the enzyme from the DNA substrate and to eliminate the complications associated with multiple turnovers of the enzyme, these assays were carried out under single turnover conditions, with enzyme in slight excess over DNA. Reactions were generally carried out from 0 to 30 min, and the time course of product formation was fit to a single exponential ([product] = A × e⁻kt + C). Rates of polymerization determined from the single exponential were plotted against nucleotide concentration and fit to a hyperbola (observed rate = kₕpol × [dNTP] / (Kₐ + [dNTP])) to determine the dissociation constant, Kₐ, and the maximum rate of polymerization, kₕpol, for each incorrect nucleotide.

**A Note on Terminology**—Throughout the paper we will use the shorthand notation for a base pair as dGTP:dC, for example, where dGTP is the incoming nucleoside triphosphate, and dC is the template base.

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Fig. 1 illustrates representative data defining the kinetics of formation of an A:A mismatch. The rates were determined by fitting to a single exponential function defining the formation of product versus time. The rates were then plotted against the nucleotide concentration and fit to a hyperbola to yield a Kₐ of 25 ± 7 and a kₕpol of 0.0036 ± 0.0003. Kinetic parameters for the

### TABLE 1

| DNA primer/template sequences used to examine incorporation |
|------------------------------------------------------------|
| 5' - TCCTCGCCAGCCTCCAGCACTACA |
| 3' - AGAGGCTCGAGGAAGGTTGGTTGAAGAGCTAGGTTACCCGGAGG |
| 5' - TCCCGACGCGCTCCAAACCAAATA |
| 3' - AGAGGCTCGAGGAAGGTTGGTTGAAGAGCTAGGTTACCCGGAGG |
| 5' - TCCCGACGCGCTCCAAACCAAATA |
| 3' - AGAGGCTCGAGGAAGGTTGGTTGAAGAGCTAGGTTACCCGGAGG |

**Product Analysis**—Products were separated on 15% denaturing polyacrylamide sequencing gels, imaged on a Storm 860, and quantified using ImageQuant software (GE Healthcare).

Kₐ and Maximum Rate of Polymerization for Incorrect Nucleotide Incorporation—To examine the effects of nucleotide concentration upon the rate of polymerization, single nucleotide incorporation experiments were performed. Because it was expected that the rates of incorporation of incorrect nucleotides would be slower than the rate of dissociation of the enzyme from the DNA substrate and to eliminate the complications associated with multiple turnovers of the enzyme, these assays were carried out under single turnover conditions, with enzyme in slight excess over DNA. Reactions were generally carried out from 0 to 30 min, and the time course of product formation was fit to a single exponential ([product] = A × e⁻kt + C). Rates of polymerization determined from the single exponential were plotted against nucleotide concentration and fit to a hyperbola (observed rate = kₕpol × [dNTP] / (Kₐ + [dNTP])) to determine the dissociation constant, Kₐ, and the maximum rate of polymerization, kₕpol, for each incorrect nucleotide.

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incorporation of all 16 correct and incorrect nucleotide base pairs are summarized in Table 2. In Fig. 2 comparisons between various kinetic parameters are illustrated bar graphs.

The studies presented here expand upon previous work examining the fidelity of Pol γ. These data provide estimates of the specificity constant for each base pair combination as the ratio $k_{pol}/K_d$. Nucleotide discrimination is then defined by the ratio $D = (k_{pol}/K_d)_{correct}/(k_{pol}/K_d)_{incorrect}$. The probability of a misincorporation can then be computed by $1/(D + 1)$ when competing nucleotides are at the same concentration.

Based upon evidence favoring a rapid equilibrium of nucleotide binding, $K_d$ and $k_{pol}$ measured in single turnover experiments define $K_m$ and $k_{cat}$, respectively, and thus, define the specificity constant, $k_{cat}/K_m$. In this context we consider the theoretical values for $k_{cat}$ and $K_m$ assuming these parameters could be measured accurately during steady-state processive synthesis and ignoring practical problems precluding accurate measurements in the steady state as discussed below. The equality $K_d = K_m$ has been shown to be approximately correct because nucleotide ground state binding appears to be in rapid equilibrium (30, 31). Evidence supporting rapid equilibrium of the ground state for human Pol γ has been reported previously based largely upon lack of evidence to the contrary (23).

The maximum rate of polymerization at saturating nucleotide, $k_{pol}$, defines a parameter equating to $k_{cat}$ for processive synthesis. For the past decade it has been debated whether the chemistry step or a conformational change limits $k_{pol}$ and further studies on Pol γ will be required to establish whether a nucleotide-induced conformational change limits the rate of nucleotide incorporation. However, recent evidence on T7 DNA polymerase has indicated that it does not matter which step is rate-limiting. Rather, the reverse of the conformational change step determines specificity in that a good substrate is committed to continued forward reaction by the slow release of tightly bound nucleotide (31). In contrast, a bad substrate dissociates from the enzyme faster than it is incorporated. Although we do not yet know whether a conformational change step contributes to selectivity by Pol γ, the more detailed analysis afforded in studies using T7 DNA polymerase justify the use of $k_{pol}/K_d$ to measure $k_{cat}/K_m$ most accurately. Pol γ and T7 DNA polymerase are both Pol A family enzymes and share significant sequence conservation near the active sites.

The specificity constant ($k_{cat}/K_m$) represents the limiting slope of the concentration dependence of the rate of incorporation at low substrate concentration. As such, it has been argued that one could measure $k_{cat}/K_m$ under steady-state conditions even though the rate of polymerization is limited by DNA release (32). That is, the concentration dependence of the rate of steady-state single nucleotide incorporation should, theoretically, give a measure of specificity even though this rate is limited by the rate of DNA dissociation (21). However, given that the average $k_{cat}/K_m$ for correct incorporation by Pol γ is 46 s$^{-1}$ (31), and the rate of DNA dissociation is 0.02 s$^{-1}$, then the expected apparent $K_m$ is 0.4 nM for steady-state single nucleotide incorporation. Concentrations of nucleotides required to accurately measure $k_{cat}/K_m$ using steady-state methods are too low to generate a signal in the assay, and this could explain the aberrant results reported using steady-state methods (33).

Inspection of Fig. 2 reveals several observations. Although the results may reflect sequence context effects for the particular oligonucleotides chosen for this study, some of the trends appear to be more generally applicable. As a general rule, purine-pyrimidine base pairs are favored over purine-purine and pyrimidine-pyrimidine base pairs. This observation supports the notion that the overall size of the base pair is an

TABLE 2

| dNTP:template | $K_d$ | $K_m$ | Specificity | Discrimination |
|---------------|------|------|-------------|----------------|
| dATP:dT     | 0.8 ± 0.06 | 45 ± 1 | 57 | 1 |
| dCTP:dT     | 360 ± 80 | 0.038 ± 0.003 | 0.0001 | 570,000 |
| dGTP:dT     | 70 ± 10 | 1.16 ± 0.06 | 0.016 | 3,600 |
| dCTP:dG     | 0.9 ± 0.2 | 43 ± 2 | 47 | 1 |
| dATP:dG     | 250 ± 40 | 0.042 ± 0.003 | 0.00017 | 280,000 |
| dCTP:dG     | 200 ± 50 | 0.16 ± 0.02 | 0.00080 | 59,000 |
| dGTP:dG     | 150 ± 40 | 0.066 ± 0.007 | 0.00044 | 110,000 |
| dATP:da     | 0.6 ± 0.16 | 25 ± 2 | 39 | 1 |
| dCTP:da     | 540 ± 100 | 0.1 ± 0.01 | 0.00019 | 210,000 |
| dGTP:da     | <1000 <0.1 | | |
| dATP:da     | 25 ± 7 | 0.0036 ± 0.0003 | 0.00014 | 280,000 |
| dGTP:dc     | 0.8 ± 0.13 | 37 ± 2 | 45 | 1 |
| dATP:dc     | 160 ± 40 | 0.10 ± 0.01 | 0.00063 | 71,000 |
| dCTP:dc     | 140 ± 30 | 0.003 ± 0.0003 | 0.00002 | 2,300,000 |
| dTTP:dc     | 180 ± 30 | 0.012 ± 0.0008 | 0.00007 | 640,000 |

*Previously reported in Johnson and Johnson (23). Specificity is defined as $k_{pol}/K_d$. Discrimination is defined as $specificity_{correct}/specificity_{incorrect}$. 
important determinant (34). With the possible exception of dGTP:dA, incorporations onto dT and dG appear to be favored over dC and dA, within a base pair subgroup (Pur-Pyr, Pur-Pur, etc). Rates of incorporation favor identical base pairings over all others, whereas ground state binding favors nonidentical purine-purine/pyrimidine-pyrimidine base pairs. However, the differences in specificity constants and discrimination observed when comparing mismatches to each other are small compared with the much greater differences observed comparing correct incorporations and mismatches to each other as groups. The largest difference in specificities observed is 800-fold when comparing mismatch to mismatch (dGTP:dT compared with dCTP:dG). Ignoring dGTP:dT, the difference is 40-fold. The smallest difference in specificities, comparing mismatch to correct, is 2400-fold (dGTP:dT compared with dCTP:dC). Ignoring dGTP:dT, the difference is 40-fold. The smallest difference in specificities, comparing mismatch to each other are small compared with the much greater differences observed comparing correct incorporations and mismatches to each other as groups. The largest difference in specificities observed is 800-fold when comparing mismatch to mismatch (dGTP:dT compared with dCTP:dG). Ignoring dGTP:dT, the difference is 40-fold. The smallest difference in specificities, comparing mismatch to correct, is 2400-fold (dGTP:dT and dTTP:dA).

Discrimination against misincorporation results from weaker ground state binding and slower rates catalyzed by T7 DNA polymerase, human immunodeficiency virus-1 reverse transcriptase, and rat polymerase β (35–37). According to $K_d$ and $k_{pol}$ values reported here and previously, exo− human Pol γ also exhibits weaker ground state binding and decreased rates of polymerization for mismatched nucleotides. A C:C mispair was formed at the slowest rate, whereas the weak binding of dGTP provided selection against the formation of a G:A mispair. G:T mispairs have been observed to be relatively easily formed by other polymerases (37, 38), and human Pol γ is no exception. Although T:T and G:T mispairings both have similarly tight dissociation constants (57 and 71 μM, respectively), disrupting catalytic site assembly (40). The study also showed that although this enzyme adopts a distorted open conformation when bound to a G:T terminally mispaired DNA substrate, the G:T pair adopts a conformation similar to the cognate base pairing and that the 3′ hydroxyl placement for catalysis remains nearly intact.

Perhaps more interesting than the comparison of T:T and G:T mispairs is the comparison of a G:T to a T:G mispairing. Our data suggest that base pair hydrogen bonding of the T:G mispair does not stabilize it to the same degree as for the G:T mispair (35–37). According to $K_d$ and $k_{pol}$ values reported here and previously, exo− human Pol γ also exhibits weaker ground state binding and decreased rates of polymerization for mismatched nucleotides. A C:C mispair was formed at the slowest rate, whereas the weak binding of dGTP provided selection against the formation of a G:A mispair. G:T mispairs have been observed to be relatively easily formed by other polymerases (37, 38), and human Pol γ is no exception. Although T:T and G:T mispairings both have similarly tight dissociation constants (57 and 71 μM, respectively), disrupting catalytic site assembly (40). The study also showed that although this enzyme adopts a distorted open conformation when bound to a G:T terminally mispaired DNA substrate, the G:T pair adopts a conformation similar to the cognate base pairing and that the 3′ hydroxyl placement for catalysis remains nearly intact.

Nucleotide discrimination can be calculated by dividing the specificity constant for correct nucleotide binding by the constant for incorrect binding (Fig. 2D). For example, the specificity constant for T:A incorporation is 41.7 s$^{-1}$ μM$^{-1}$, and the specificity constant for A:A misincorporation is 7.6 × $10^{-5}$ s$^{-1}$ μM$^{-1}$ resulting in a discrimination value of 550,000. We have previously calculated and reported an average fidelity of Pol γ
polymerization to be 1 error in 280,000 base pairs catalyzed. The studies presented in this paper allow us to calculate a more complete average fidelity of polymerization of 1 error in 440,000 bases incorporated. Pre-steady-state kinetic studies of exonuclease activity have defined the exonuclease proofreading contribution to fidelity of Pol γ as a 4–200-fold increase (28). Combining the fidelity contributions from polymerization and exonuclease proofreading allows us to refine the previously reported overall fidelity of 1 error in 1.1–20 × 10⁹ to a new overall fidelity of 1 error in 1.8 × 10⁹–3.6 × 10⁹ bases incorporated.

Fidelity of nucleotide incorporation by a DNA polymerase is a complex phenomenon created by a host of interactions including, but not limited to Watson-Crick interactions, base stacking, nearest neighbor interactions, and enzyme-substrate contacts within the active site of the polymerase. Experiments in aqueous solution have shown a trend in base stacking interactions, where deoxyguanosine stacks most strongly followed by deoxyadenosine, deoxycytosine, and deoxythymidine in order (41). Additionally, it has been shown that due to the exclusion of water in the active site of DNA polymerases, base stacking interactions are stronger than would otherwise be expected (41, 42). In the experiments that are presented in this paper, only a single DNA primer was employed to investigate the fidelity of nucleotide incorporation. Because only deoxyadenosine was tested as the base directly 5′ of the position of incorporation, the results presented here may be biased. However, because only one primer sequence was employed, the data are more easily compared directly, as primer sequence is removed as a possible source of variability. It has been previously shown with T4 DNA polymerase that replication fidelity is highest when either deoxyguanosine or deoxyadenosine are present in the primer directly 5′ of the site of incorporation (43). Although experiments to determine the effects of nearest neighbors on discrimination have not been carried out with Pol γ, data from T4 DNA polymerase suggest that the overall discrimination that we have calculated for Pol γ may be a high estimate. Further experimentation employing primers and templates differing in sequence from those used previously should allow for further refinement of the estimate of nucleotide discrimination by human mitochondrial DNA Pol γ without ambiguity or bias.

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