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DNA Polymerase Fidelity: Comparing Direct Competition of Right and Wrong dNTP Substrates with Steady State and Pre-Steady State Kinetics[†]

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ABSTRACT: DNA polymerase fidelity is defined as the ratio of right (R) to wrong (W) nucleotide incorporations when dRTP and dWTP substrates compete at equal concentrations for primer extension at the same site in the polymerase−primer−template DNA complex. Typically, R incorporation is favored over W by 10^3−10^5-fold, even in the absence of 3′-exonuclease proofreading. Straightforward in principle, a direct competition fidelity measurement is difficult to perform in practice because detection of a small amount of W is masked by a large amount of R. As an alternative, enzyme kinetics measurements to evaluate k_{cat}/K_m for R and W in separate reactions are widely used to measure polymerase fidelity indirectly, based on a steady state derivation by Fersht. A systematic comparison between direct competition and kinetics has not been made until now. By separating R and W products using electrophoresis, we have successfully taken accurate fidelity measurements for directly competing R and W dNTP substrates for 9 of the 12 natural base mispairs. We compare our direct competition results with steady state and pre-steady state kinetic measurements of fidelity at the same template site, using the proofreading-deficient mutant of Klenow fragment (KF^-) DNA polymerase. All the data are in quantitative agreement.

The fidelity or accuracy of DNA synthesis in relation to mutagenesis has been studied for more than half a century. Recognizing that the biochemical basis of spontaneous mutation was first considered by Watson and Crick, one might even say that the fidelity of DNA synthesis predates the discovery of *Escherichia coli* DNA polymerase I (pol[1] I) (*1*). From the mid-1960s to early 1970s, studies using pol I, along with in vivo analyses with T4 mutator (*2*−*4*) and antimutator phage (*5*) and in vitro mutator and antimutator polymerases (*6*−*9*), showed that DNA polymerases influence mutation frequency in two ways: (1) by the base selection step on the primer−template motif in the DNA polymerase active site (*8*, *10*, *11*) and (2) by a proofreading step governed by associated 3′-exonuclease activity common to many replicative DNA polymerases (*6*, *12*−*15*). Since then, an arsenal of tools, including pre-steady state quench-flow and stopped-flow fluorescence kinetics, X-ray structural analysis, and theoretical computational analysis, have been used to dissect the enzymatic mechanisms governing fidelity at the nucleotide insertion and excision steps (*16*−*26*). These mechanistic tools were buttressed by reporter gene techniques for monitoring polymerase copying in vitro and detecting mutations in vivo, developed initially to measure reversions of single-site bacteriophage markers (*27*, *28*), and later expanded to measure mutational frequencies and spectra for base substitutions and frameshifts (*29*).

What can be made of measurements of pol fidelity per se? Early on Kornberg (*30*), Lehman (*31*), and Bessman (*6*), the discoverers of pol I (*1*), and later on we (*9*) performed fidelity measurements on purified pols, using a radioactively labeled "right" dNTP (e.g., [^{3}H]dRTP) competing directly with a differently labeled "wrong" dNTP (e.g., [^{32}P]dWTP) for incorporation at the same DNA template site. Here, by definition, DNA polymerase fidelity is equal to the ratio of the moles of R and W incorporated at equimolar dRTP and dWTP concentrations. This type of direct competition measurement, while simple in theory, is not simple to make in practice; a minute misincorporation of W in the presence of R is typically undetectable, even in the presence of heavily biased dNTP pools. It is akin to measuring a "needle in a haystack".

An alternative approach is to measure pol fidelity using steady state kinetics as proposed by Fersht (*32*). The analysis is straightforward. The incorporation of either a right (R) or a wrong (W) deoxynucleotide is measured as a function of dNTP concentration, and a rectangular hyperbola is fitted to the data to measure kinetic parameters and evaluate k_{cat}/K_m, for R or W incorporation. As proposed by Fersht, DNA polymerase fidelity can be calculated as $(k_{cat}/K_m)_R/(k_{cat}/K_m)_W$, where k_{cat} and K_m are the familiar Michaelis−Menten parameters in steady state enzyme kinetics. Pre-steady state kinetic techniques have also been introduced to express pol fidelity in terms of two parameters, k_{pol}, the maximum rate constant for polymerase incorporation of a right or wrong substrate, and K_d, the equilibrium dissociation constant for right or wrong dNTP substrate binding to the polymerase−primer−template DNA complex. In the pre-steady state experiments, the ratio $(k_{pol}/K_d)_R/(k_{pol}/K_d)_W$ is used as a substitute for $(k_{cat}/K_m)_R/(k_{cat}/K_m)_W$ to measure polymerase fidelity (*19*, *33*).

However, there is a caveat to consider, which is that the steady state kinetic method used to deduce pol fidelity is based on the "gedanken" experiment proposed by Fersht (*32*). The thought experiment envisions dWTP and dRTP competing simultaneously for the same template site in the polymerase−DNA complex. The

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[1]Abbreviations: pol, DNA polymerase; KF^-, Klenow fragment DNA polymerase lacking 3′-exonuclease proofreading activity.

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steady state velocities for incorporation of R and W are as follows: $v_R = (k_{cat}/K_m)_R$[pol−DNA][dRTP] and $v_W = (k_{cat}/K_m)_W$[pol−DNA][dWTP], respectively. The parameter k_{cat}/K_m is the apparent second-order rate constant for nucleotide incorporation when dNTP is bound in the pol−DNA complex. One obtains pol fidelity by taking the ratios of the two velocities when [pol−DNA] is the same and [dRTP] = [dWTP], in which case, $F = v_R/v_W = (k_{cat}/K_m)_R/(k_{cat}/K_m)_W$. Without question, the thought experiment makes a sound logical argument and predicts that the fidelity obtained by measuring R and W in direct competition can be deduced instead by measuring k_{cat}/K_m for R and W in separate steady state reactions. Whether it is self-evident, nevertheless, the deduction needs to be verified experimentally.

Several techniques for direct competition measurements have been reported (*6*, *9*, *30*, *31*, *34*−*36*). Fidelity measured by direct competition is model-independent, whereas kinetic measurements are deduced from k_{cat}/K_m steady state or k_{pol}/K_d pre-steady state parameters and are, therefore, indirect and model-dependent, as discussed by Fersht (*32*). However, there has not been a systematic comparison of pol fidelities obtained using kinetics with direct measurements of fidelity using dRTP versus dWTP competition. Nor has a comparison been made for fidelities deduced by steady state and pre-steady state kinetics. In this paper, we determine DNA polymerase fidelity at the same template base using dNTP direct competition, steady state, and pre-steady state kinetics. We have performed the direct competition experiments in a manner that eliminates the needle-in-a-haystack difficulty for 9 of the 12 possible base mispairs. The measurements are performed with *E. coli* Klenow fragment exo$^-$ (D355A/E357A), i.e., KF$^-$, lacking 3′-proofreading activity as well as 5′-exonuclease activity (*37*, *38*). The data allow us to compare fidelity deduced by kinetics with a direct competition measurement of fidelity and to compare fidelity measured by the two different kinetic approaches, steady state and pre-steady state, which employ lower and higher polymerase concentrations, respectively, relative to that of the primer−template DNA.

MATERIALS AND METHODS

DNA Synthesis and Purification, Radiolabeling, and Annealing. All DNA primers and templates were synthesized on a solid-phase DNA synthesizer, purified by polyacrylamide gel electrophoresis, and desalted using an oligonucleotide purification cartridge purchased from Applied Biosystems. Primer DNA was 5′-end labeled, using T4 polynucleotide kinase (USB) and [γ-^{32}P]ATP (MP Biomedicals) with the supplied kinase protocol. The radiolabeled primer was mixed with 1.2 molar equiv of the appropriate template and annealed in kinase buffer by being heated to 95 °C and cooled slowly to room temperature. The primer and template DNA sequences used are as follows, with the target site on the template shown in bold: primers (^{32}P 5′ → 3′), TATTAGCGCGCTCGA (used in all standing-start experiments) (P1) and TATTAGCGCGCTCG (used only in running-start experiments) (P2); templates (3′ → 5′), ATAATCGCGCGAGCT**G**TTGGCCTTGTTGCG (T1), ATAATCGCGCGAGCT**G**AAGGCCTTGTTGCG (T2), ATAATCGCGCGAGCT**A**TTGGCCTTGTTGCG (T3), ATAATCGCGCGAGCT**A**GGGGCCTTGTTGCG (T4), ATAATCGCGCGAGCT**T**AAGGCCTTGTTGCG (T5), ATAATCGCGCGAGCT**T**GTGGCCTTGTTGCG (T6), ATAATCGCGCGAGCT**C**AAGGCCTTGTTGCG (T7), and ATAATCGCGCGAGCT**C**TTGGCCTTGTTGCG (T8).

Protein, Reaction Buffer, and dNTP Preparation. KF$^-$ was prepared as previously described (*37*, *38*). All experiments were conducted in triplicate in the same reaction buffer [50 mM NaCl, 20 mM Tris-HCl, and 8 mM $MgCl_2$ (pH 7.4)]. Stop solution contained 20 mM EDTA (pH 8.0) and formamide. Stock solutions of 100 mM dNTP were used as purchased (GE Healthcare).

Direct Competition Assay. For direct competition between dRTP and dWTP, we used dRTP concentrations of 50, 100, and 200 nM and a dWTP concentration of 1 mM to yield pool bias ratios of 1:20000, 1:10000, and 1:5000, respectively. A 2× solution of these dNTP mixtures contained activated calf thymus DNA (GE Healthcare), at a concentration (A_{260} = 0.005) sufficient to trap released polymerase. Trap DNA was used in direct competition assays to minimize the possibility of a second enzyme extending a primer that had been previously extended. Using primer P1 annealed to one of the templates (T1−T8), polymerase reactions at 37 °C were initiated by mixing 10 μL of the 2× dNTP mixture with 10 μL of 100 nM radiolabeled primer−template DNA and 40 nM KF$^-$. The reaction mixture containing 50 nM primer−template DNA, 20 nM KF$^-$, and 1× dNTP (50−200 nM dRTP and 1 mM dWTP) was incubated for 2 min at 37 °C and the reaction quenched with 80 μL of stop solution. Reaction products were separated by high-voltage electrophoresis using a 25% polyacrylamide denaturing gel (84 cm × 33 cm × 0.4 mm). Dehydrated gels were exposed to a phosphor screen, and products were analyzed with a phosphorimager (GE Healthcare Storm 860).

Steady State Standing-Start Kinetics Assay (17, 39). A 10 μL aliquot of 20 nM radiolabeled primer−template DNA (using P1) was incubated with 0.5 nM KF$^-$ in reaction buffer for 2 min and then mixed with a 10 μL aliquot of different concentrations of 2× dNTP (dRTP or dWTP). The mixture was allowed to react at 37 °C for 15 s for dRTP and 45 s to 45 min for dWTP. Reaction concentrations initially were 10 nM primer−template DNA, 0.25 nM KF$^-$, 5−400 nM correct dNTP, and 6−1600 μM incorrect dNTP. All reactions were quenched with 40 μL of stop solution after the appropriate reaction time. Reaction products were separated by high-voltage electrophoresis using a 20% polyacrylamide denaturing gel (39 cm × 33 cm × 0.4 mm). Dehydrated gels were exposed to a phosphor screen, and products were analyzed with a phosphorimager.

Pre-Steady State Kinetics Assay. Radiolabeled primer−template DNA (100 nM) was incubated with 400 nM KF$^-$ in reaction buffer for 3 min at 37 °C. Using a KinTek (model RQF-3) quench flow apparatus, equal volumes of the 2× protein/DNA mixture were rapidly combined with different concentrations of a 2× solution of dRTP or dWTP. Right incorporations used dRTP concentrations of 1−80 μM and reaction times from 0.005 to 3 s. Wrong incorporations used dWTP concentrations of 30−2000 μM and times from 0.25 s to 30 min. Reaction times of >15 s were mixed by hand. Reactions were quenched using 0.5 M EDTA. All reaction products were separated by high-voltage electrophoresis using a 20% polyacrylamide denaturing gel (39 cm × 33 cm × 0.4 mm). Dehydrated gels were exposed to a phosphor screen, and products were analyzed with a phosphorimager.

Steady State Running-Start Kinetics Assay (17, 39). Using primer P2, 20 nM radiolabeled primer−template DNA was incubated with 0.5 nM KF$^-$ in reaction buffer (10 μL) for 2 min at 37 °C and then mixed with 10 μL of different concentrations of dRTP or dWTP with the running-start nucleotide. The

running-start nucleotide (dATP) was present at a final concentration of 10 μM for right and wrong insertions except when measuring misincorporation of A opposite A, in which case the concentration of dATP was varied (see, e.g., Figure 3B, right panel). The final concentrations for correct dNTP ranged from 0.125 to 18 μM and for incorrect incorporation from 10 to 600 μM. Reactions were quenched with stop solution after 15 s. Reaction products were separated by high-voltage electrophoresis using a 20% polyacrylamide denaturing gel (39 cm × 33 cm × 0.4 mm). Dehydrated gels were exposed to a phosphor screen, and products were analyzed with a phosphorimager.

RESULTS

DNA polymerase KF$^-$ was used to extend a DNA template-bound primer with correct or incorrect dNTP substrates to evaluate error frequencies by three different experimental techniques: (a) direct competition, (b) steady state kinetics, and (c) pre-steady state kinetics. The objective is to determine error frequencies by direct competition of right (R) and wrong (W) dNTPs at the same template site, for comparison with evaluations based on the ratio of R and W insertion efficiencies measured separately at the same primer−template sites by kinetics assays. The template sequences used for assaying primer extensions on template DNA, at the site shown in bold (Materials and Methods), were designed to minimize multiple extensions in competition experiments. KF$^-$, an altered form of *E. coli* pol I lacking 5′- and 3′-exonuclease activities, has been widely used to study polymerase fidelity (*25*, *26*, *40*−*42*).

Direct Competition. DNA primer (P1) labeled at the 5′-end with ^{32}P and annealed to its complementary unlabeled DNA template sequence was incubated with KF$^-$ in conducting primer extension experiments in which a wrong dNTP competes with the right dNTP for insertion opposite the same template site. The reactions were stopped when both right and wrong extensions were observed, while the unextended primer still remained close to 50%. Extended primers resulting from incorporation of right and/or wrong nucleotides were in most cases (for 10 of the 12 possible base mispairs) successfully separated from each other, and from unextended primer, by high-voltage electrophoresis on a long (84 cm) 25% polyacrylamide denaturing gel. A polymerase trap (unlabeled calf thymus DNA) was included to bind the polymerase when it dissociated from the primer−template DNA following the insertion of a nucleotide.

As shown in Figure 1, and Figure 1S of the Supporting Information, extended primers are resolved as bands clearly separated from each other as well as from unextended primers (lowest bands). In Figure 1A, for example, the outer lanes marked dCTP and dTTP show the bands obtained using each of these substrates alone. The band labeled C → G shows extension by correct insertion of C opposite template base G, while T → G shows extension by incorrect insertion of T opposite the same G. The inner lanes marked with [dCTP]:[dTTP] ratios of 1:20000, 1:10000, and 1:5000 show the misinsertion of T opposite G compared to C opposite G at corresponding concentration ratios of right (dCTP) to wrong (dTTP) substrate. To obtain the observed bands, the dCTP concentration was varied in the submicromolar range (0.05, 0.10, and 0.20 μM) while the dTTP concentration was held constant at 1 mM. The corresponding [dCTP]:[dTTP] concentration ratios are shown (Figure 1A) as 1:20000, 1:10000, and 1:5000, respectively.

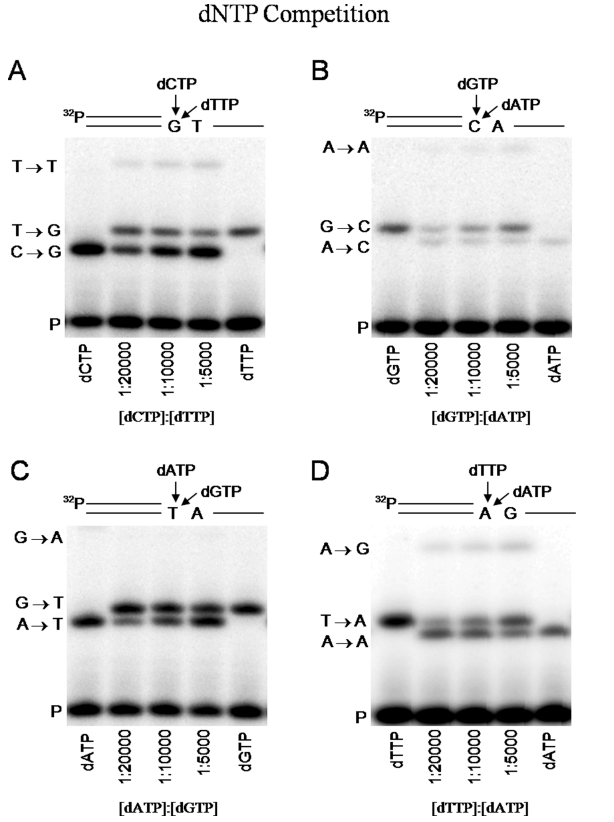

FIGURE 1: Direct competition assay for measuring DNA polymerase fidelity. Radiolabeled primer and extended primer bands are shown separated by denaturing gel electrophoresis, for a wrong dNTP competing with the right dNTP for insertion at the same target site on primer−-template DNA, as illustrated in the sketches above each gel. In each case (A−D), the concentration of the primer−template motif is 50 nM and that of KF$^-$ polymerase is 20 nM. The outer lanes show the bands obtained with right dNTP alone (lane 1) and wrong dNTP alone (lane 5). Inner lanes 2−4 show the bands obtained when right and wrong dNTPs compete at [dRTP]:[dWTP] ratios of 1:20000, 1:10000, and 1:5000. The band indicated by "P" is an unextended primer. (A) dCTP vs dTTP, competing for insertion opposite template G. The bands labeled C → G and T → G denote primer extension by right incorporation of C and wrong incorporation of T, respectively, opposite template G. The T → T band results from misincorporation of T opposite the next template base T, after a right (C → G) incorporation. (B) dGTP vs dATP, competing for insertion opposite template C. The G → C and A → C bands denote right and wrong incorporations, respectively. The A → A band results from a wrong incorporation opposite the next template base A, after a right (G → C) incorporation. (C) dATP vs dGTP, competing for insertion opposite template T. The A → T and G → T bands denote right and wrong incorporations, respectively. A potential additional misincorporation band (G → A) is not detected. (D) dTTP vs dATP, competing for insertion opposite template A. The T → A and A → A bands denote right and wrong incorporations, respectively. The A → G band results from a wrong incorporation opposite the next template base G, after a right (T → A) incorporation.

We also note that competition between dCTP and dTTP for insertion opposite template base G yields an additional band, labeled T → T (Figure 1A), to indicate extension of primer by misinsertion of T opposite the base T that follows G on the template. We identify the T → T band as the misinsertion of T opposite T following the right insertion of C opposite G. Since the dTTP concentration is very much greater than the dCTP concentration, the second incorporation is almost certainly T rather than C, opposite template T.

The relative amounts of 5′-^{32}P-labeled primer extended by right and/or wrong incorporations are determined by integrating

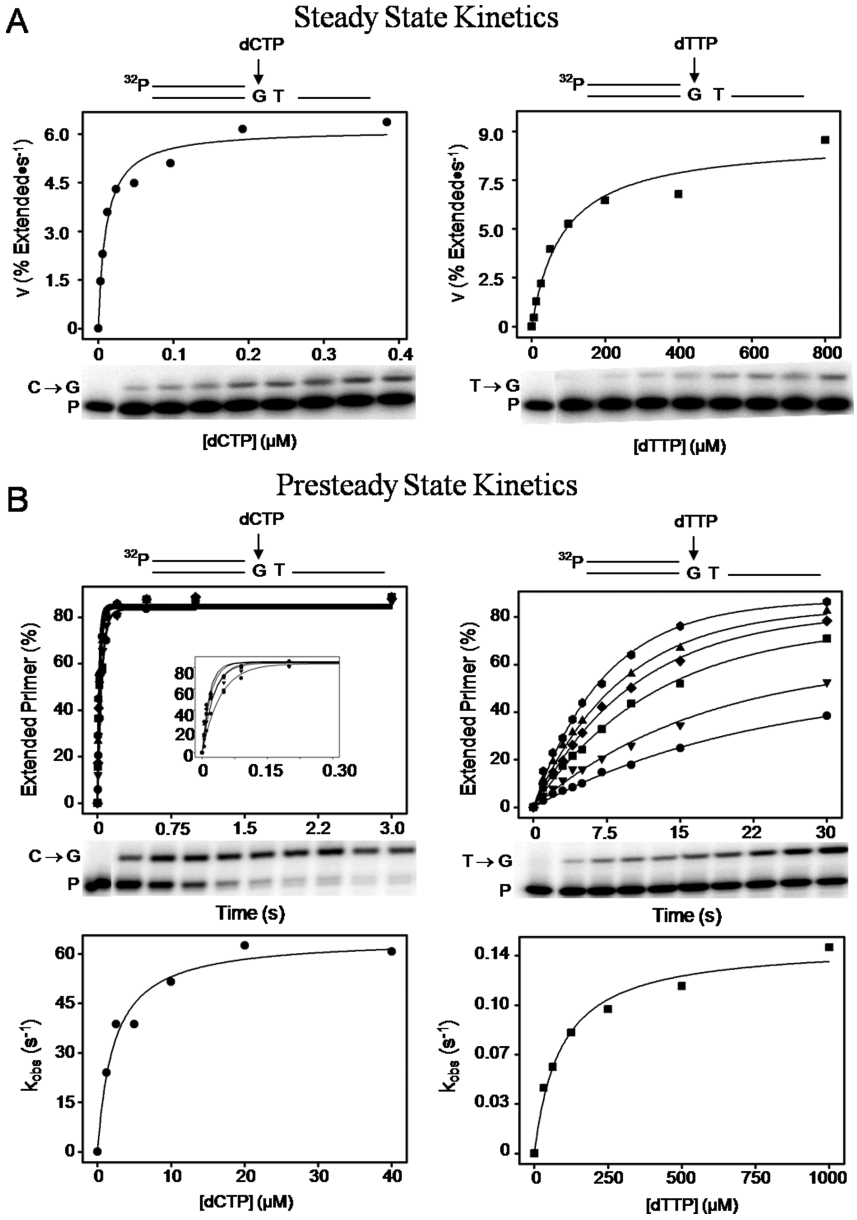

FIGURE 2: Separate kinetics assays for deducing DNA polymerase fidelity. (A) Steady state standing-start velocity for extension of primer (P), by either the incorporation of a right C (C → G, left panel) or wrong T (T → G, right panel), plotted against the corresponding dNTP concentration, [dCTP] or [dTTP]. A rectangular hyperbola fit to the data is used to determine V_{max}/K_m for right and wrong incorporations (see Results). The misinsertion frequency, deduced as $f_{ins} = (V_{max}/K_m)_W/(V_{max}/K_m)_R$, is shown for the steady state in Table 1. The concentrations of primer−template DNA and KF$^-$ are 10 and 0.25 nM, respectively. (B) Pre-steady state rate constant, k_{obs}, for extension of primer, at the same site as above, by right incorporation (C → G, top left panel) or wrong (T → G, top right panel), determined (top panels) by measuring the fractional amount (%) of primer extended as a function of time, at each dNTP concentration used (see Materials and Methods). In the inset, the top left panel shows an expanded view of the time scale for the rapid C → G incorporation. The percentage of primer extended is described by the exponential $a(1 - e^{-kt})$, where a is the maximum amount of primer extended and k is the observed first-order rate constant (k_{obs}), at each dNTP concentration. In the bottom panels, the k_{obs} values for right and wrong incorporation are plotted as a function of dNTP concentration to evaluate k_{pol}/k_d, by fitting a rectangular hyperbola (see Results). The resultant misinsertion frequency, deduced as $f_{ins} = (k_{pol}/K_d)_W/(k_{pol}/K_d)_R$, is shown for the pre-steady state in Table 1. The concentrations of primer−template DNA and KF$^-$ polymerase are 50 and 200 nM, respectively.

the intensity of each of the bands in a lane. Since the right incorporation (C opposite G) enables a second incorporation (T opposite T), the C → G and T → T band intensities are added together to obtain the "corrected" C → G band intensity for evaluating correct insertion of C opposite G. The misinsertion frequency for T opposite G is obtained by measuring the intensity ratio of the T → G band relative to the corrected C → G band and multiplying by the pool bias ratio, [dCTP]:[dTTP]. From the experimental data shown in Figure 1A, we find nearly identical misinsertion frequencies of 3.4×10^{-5}, 3.5×10^{-5}, and 3.4×10^{-5} for [dCTP]:[dTTP] ratios of 1:20000, 1:10000, and 1:5000, respectively. The experiments were performed in triplicate to yield the misinsertion frequency [$f_{ins} = (3.4 \pm 0.2) \times 10^{-5}$] reported in Table 1 for template/dNTP = G/T by competition.

Other examples of well-resolved bands observed in direct competition experiments are shown for the misincorporation of A opposite C (Figure 1B), G opposite T (Figure 1C), and A opposite A (Figure 1D), listed as C/A, T/G, and A/A, respectively, in Table 1. The data were acquired using the same procedures as described above. Misinsertion f_{ins} values obtained for these mismatches and others (presented in Figure 1S of the Supporting Information) are listed in Table 1. Our gel electrophoresis

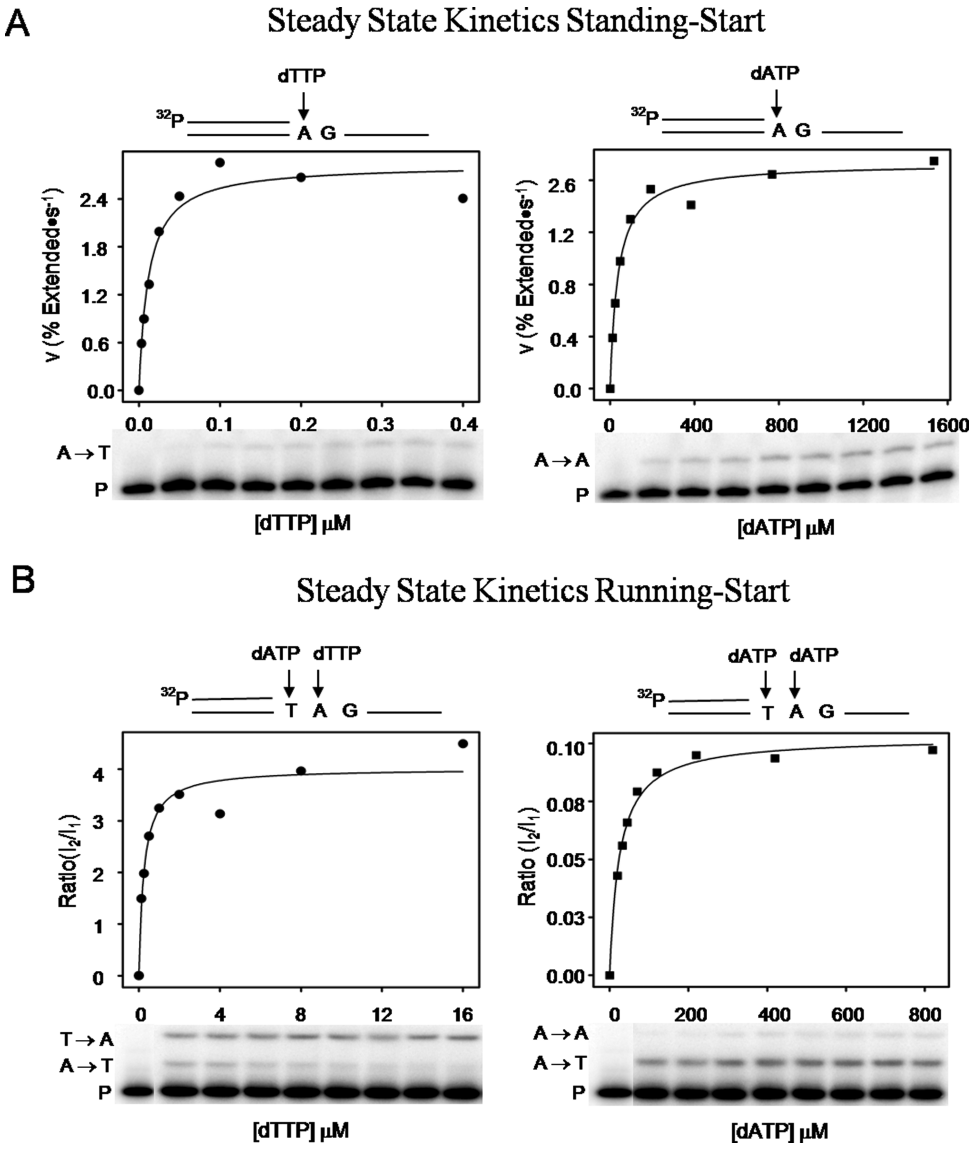

FIGURE 3: Steady state kinetics measurements for a standing start compared with a running start. Standing-start (A) and running-start (B) velocities for extension of primer (P) by incorporation of a right T (T → A, left panel) or wrong A (A → A, right panel) plotted as a function of the corresponding dNTP concentration. The ratio I_2/I_1 is the velocity of the extended primer, where I_2 is the band intensity at site 2 (T → A or A → A) and I_1 is the band intensity at site 1 (A → T). A rectangular hyperbola fit to the data is used to determine V_{max}/K_m for right and wrong incorporations (see Results). The standing- and running-start misinsertion frequencies [$f_{ins} = (V_{max}/K_m)_W/(V_{max}/K_m)_R$] are listed in Table 1 and the footnote of Table 1, respectively. The running-start dATP is kept at a constant concentration (10 μM) when right incorporation of T opposite template A (T → A, left panel) is measured. When wrong incorporation of A opposite template A (A → A, right panel) is measured, the running-start dATP concentration is varied from 10 to 600 μM. The concentrations of primer−template DNA and KF$^-$ are 10 and 0.25 nM, respectively.

technique has not been successful in resolving the product bands ending in T from those ending in G; therefore, we were unable to measure either C/T or A/G misinsertion frequencies, listed as not detectable in Table 1. Although we are able to readily resolve product bands ending in C from those ending in G (Figure 1AS of the Supporting Information), we did not observe the formation of C/C mispairs using KF$^-$ (Figure 1FS of the Supporting Information). Therefore, C/C is also listed in Table 1 as not detectable.

Steady State Kinetics. Fidelity is measured using a steady state gel kinetics assay that we developed previously (*39*, *43*, *44*). As in the direct competition experiments, we use the same DNA polymerase, primer (P1), and templates, with the primer labeled at the 5′-end with ^{32}P and the template unlabeled. However, the DNA polymerase (KF$^-$) concentration used in steady state kinetics experiments is much lower than that of the primer−template DNA, to ensure that initial primer and dNTP substrate concentrations are not significantly altered in the course of primer extension. While the concentrations of DNA polymerase and primer−template DNA are constant, the velocity (v) of primer extension by one nucleotide is measured as a function of dNTP concentration, for each dNTP separately, be it right (R) or wrong (W).

The extended primer band is separated by electrophoresis from the unextended primer band on a standard polyacrylamide denaturing gel and scanned using a phosphorimager to determine the percent of primer extended. The velocity at different concentrations of dNTP is determined by evaluating the percent of primer extended per unit of reaction time. By plotting v versus dNTP concentration and fitting the Michaelis−Menten rectangular hyperbola [$v = V_{max}$[dNTP]/(K_m + [dNTP])] by a least-squares method as previously described (*39*), we obtain the kinetic parameters V_{max} and K_m. The misinsertion frequency

Table 1: Misinsertion Frequencies Determined by Direct dNTP Competition Compared to Steady State and Pre-Steady State Kinetics[a]

template/dNTP	competition	steady state[b]	pre-steady state
G/G	$(4.6 \pm 0.4) \times 10^{-5}$	$(5.7 \pm 0.2) \times 10^{-5}$	$(6.7 \pm 0.9) \times 10^{-5}$
G/T	$(3.4 \pm 0.2) \times 10^{-5}$	$(2.2 \pm 0.8) \times 10^{-5}$	$(4.0 \pm 0.5) \times 10^{-5}$
G/A	$(1.3 \pm 0.2) \times 10^{-5}$	$(6.0 \pm 0.1) \times 10^{-6}$	$(1.1 \pm 0.1) \times 10^{-5}$
C/C	nd[c,d]	nd[c]	$(1.1 \pm 0.2) \times 10^{-7}$
C/T	nd[c]	$(4.9 \pm 0.2) \times 10^{-5}$	nd[c]
C/A	$(2.2 \pm 0.4) \times 10^{-5}$	$(2.2 \pm 0.3) \times 10^{-5}$	$(9.2 \pm 0.7) \times 10^{-6}$
T/T	$(5.1 \pm 0.9) \times 10^{-6}$	$(4.7 \pm 0.9) \times 10^{-6}$	$(1.0 \pm 0.2) \times 10^{-5}$
T/G	$(1.3 \pm 0.1) \times 10^{-4}$	$(2.4 \pm 0.4) \times 10^{-4}$	$(1.3 \pm 0.1) \times 10^{-4}$
T/C	$(9.8 \pm 0.3) \times 10^{-6}$	$(2.3 \pm 0.2) \times 10^{-6}$	$(3.1 \pm 0.2) \times 10^{-6}$
A/A	$(1.2 \pm 0.1) \times 10^{-4}$	$(1.7 \pm 0.4) \times 10^{-4}$	$(1.9 \pm 0.3) \times 10^{-4}$
A/G	nd[c]	$(6.5 \pm 0.6) \times 10^{-6}$	$(1.1 \pm 0.5) \times 10^{-5}$
A/C	$(6.8 \pm 0.2) \times 10^{-5}$	$(5.2 \pm 0.8) \times 10^{-5}$	$(8.4 \pm 0.3) \times 10^{-5}$

[a]Values are reported as means ± the standard error of three replicates. [b]Results shown are for standing-start conditions; under running-start conditions, the misinsertion frequency for an A/A mismatch is $(2.5 \pm 0.2) \times 10^{-4}$ and for a G/T mismatch is $(8.5 \pm 0.8) \times 10^{-5}$. [c]Not detectable. [d]See Figure 1S of the Supporting Information.

for a wrong dNTP is then calculated as $f_{ins} = (V_{max}/K_m)_W/(V_{max}/K_m)_R = (k_{cat}/K_m)_W/(k_{cat}/K_m)_R$ (*17*). $V_{max} = k_{cat}$[total polymerase−primer−template complex]; the total polymerase DNA concentration is held constant by using constant concentrations of polymerase and primer−template DNA.

Figure 2A (left side) shows the plot of primer extension velocity, v, for the correct incorporation of C opposite G as a function of dCTP concentration. The curve represents the least-squares fit of the Michaelis−Menten rectangular hyperbola used to evaluate V_{max} and K_m. Below the plot is the gel band labeled C → G, at increasing dCTP concentrations of ≤0.4 μM, along with the band labeled P representing unextended primer. Corresponding results are shown in Figure 2A (right side) for misinsertion of T opposite G as a function of much higher dTTP concentrations (≤800 μM). We see that right (dCTP) and wrong (dTTP) substrates obey rectangular hyperbolas with very different initial slopes (V_{max}/K_m), indicating very different insertion efficiencies.

The determination of kinetic parameters, V_{max} and K_m, by a least-squares fit to the rectangular hyperbola in each case provides a reliable V_{max}/K_m measure of insertion efficiency for both right dCTP and wrong dTTP at the same template G site. The misinsertion frequency for T opposite G is then calculated as the ratio of V_{max}/K_m (for dTTP) to V_{max}/K_m (for dCTP). The resultant misinsertion frequency of $(2.2 \pm 0.8) \times 10^{-5}$ for the G/T mispair is shown in Table 1, for template/dNTP = G/T by steady state, along with corresponding results obtained for other mispairs by similar steady state experiments.

Pre-Steady State Kinetics. In contrast to steady state and direct competition experiments, much higher concentrations of DNA polymerase relative to that of primer−template DNA are used in pre-steady state experiments to ensure that each primer−template DNA has polymerase bound to it, ready to extend primer upon addition of dNTP substrate. The objective is to make primer extension with time obey first-order kinetics of the form $y = a(1 - e^{-kt})$, where a is the maximum amount of primer extended and k is the observed first-order rate constant (k_{obs}), at each dNTP concentration. The observed first-order rate constant k at each dNTP concentration used for primer extension is plotted against dNTP concentration to yield (by least-squares fit) the rectangular hyperbola [$k = k_{max}$[dNTP]/(K_d + [dNTP])] used to evaluate the pre-steady state parameters, K_d and k_{pol} ($= k_{max}$), thereby yielding the resultant extension efficiency, k_{pol}/K_d, for each dNTP (*19*).

The pre-steady state kinetic parameters, k_{pol} and K_d, were determined for each possible base pairing using the same DNA polymerase, primer (P1), and template sequences used in the other assays. The 5′-^{32}P-labeled primer was annealed to the unlabeled template as before, except that now a much greater amount of KF^- was added to saturate the primer−template DNA with the enzyme. Reaction products were separated from unreacted primer with a 39 cm 20% denaturing polyacrylamide gel as previously described (*45*).

The left panel of Figure 2B shows the plot of the percent primer extended versus time, for correct incorporation of C opposite template G, as illustrated by the accompanying diagram on top. The gel image below the plot shows, for a single dCTP concentration, how extended (C → G) and unextended (P) primer bands change with time from 0.005 to 3 s. The percentage of primer extended was determined by integration of the bands and plotted versus time (top graph) for each concentration of dCTP used.

The exponential rise in extended primer (Figure 2B, left panel), as a function of time, yielded a first-order rate constant (k) for each concentration; the k value was then plotted versus dCTP concentration (bottom graph). A rectangular hyperbola fitted to the plot of k versus dCTP concentration gave k_{pol}, the maximum rate constant for polymerase activity, and K_d, the dNTP substrate concentration needed to yield $k = 0.5k_{pol}$. The right panel of Figure 2B shows representative data for the incorrect incorporation of dTTP opposite G. The kinetic parameters were calculated using the method described above for the correct incorporation. As for the steady state assay, the misincorporation efficiency was obtained by dividing the incorporation efficiency (k_{pol}/K_d) for the mispair by that for the correct base pair. This experiment yields a misincorporation efficiency of $(4.0 \pm 0.5) \times 10^{-5}$, listed in Table 1 for template/dNTP = G/T by the pre-steady state, along with the other mismatches obtained under pre-steady state conditions.

Standing Start versus Running Start. We measured misinsertion frequencies for A/A and G/T in a running-start mode instead of the standing-start mode used in all three assays described above. Unlike standing-start experiments in which fidelity is measured at the first template site after the 3′-end of the annealed primer (Figures 1−3A), the running-start experiments examine fidelity at the second template site (Figure 3B). A "high" concentration of running-start dNTP [dATP (Figure 3B)] is present to ensure rapid insertion in the first template site [T (Figure 3B)]. The misinsertion frequency is measured at the second template site, by varying the concentrations of right and wrong dNTPs [dTTP and dATP, respectively (Figure 3B)] (*17*, *44*).

Kinetic parameters using a running-start assay were measured under single-completed hit conditions as described previously (*17*, *44*). The kinetic values, V_{max} and K_m, were obtained by measuring the amount of primer extended from site 1 (A → T) to site 2 [T → A (Figure 3B, left panel) or A → A (Figure 3B, right panel)] at varying concentrations of dTTP or dATP in a 15 s reaction. The velocities for right and wrong incorporation were determined by integrating the bands on the gel and taking the ratio of band intensity at template site 2 to band intensity at template site 1. The velocities were plotted as a function of dNTP concentration, and a least-squares fit to a rectangular hyperbola was used to determine V_{max} and K_m. The running-start misinsertion frequency found for the A/A mismatch is $(2.5 \pm 0.2) \times 10^{-4}$ (Figure 3B); the corresponding standing-start $f_{ins} = (1.7 \pm 0.4) \times 10^{-4}$ (Figure 3A and Table 1, A/A steady state). Similarly, for the

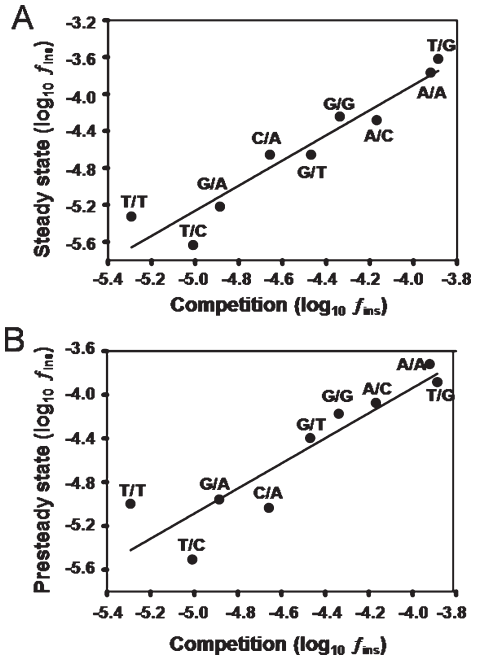

FIGURE 4: Comparison of misinsertion frequencies, f_{ins}, deduced from kinetics with those measured by direct competition of dRTP vs dWTP. The values are shown plotted as $\log(f_{ins})$. We are able to resolve 9 of the 12 possible base substitution mispairs by direct competition, as shown in Table 1. The plots of $\log(f_{ins})$ for (A) steady state and (B) pre-steady state, vs direct dNTP competition, show linear correlations with correlation coefficients of 0.95 and 0.91, respectively.

G/T mismatch measured under running-start conditions, we obtain an f_{ins} of $(8.5 \pm 0.8) \times 10^{-5}$, compared to $(2.2 \pm 0.8) \times 10^{-5}$, under standing-start conditions (Figure 2A and Table 1, G/T steady state). The running-start misinsertion frequencies are included in a footnote to Table 1.

The excellent agreement between dNTP competition and kinetics is illustrated by plotting $\log(f_{ins})$ determined by dNTP competition against $\log(f_{ins})$ determined by steady state kinetics (Figure 4A) and by pre-steady state kinetics (Figure 4B). The linear correlation coefficients comparing kinetics with direct competition are 0.95 for steady state kinetics and 0.91 for pre-steady state kinetics.

DISCUSSION

In this paper, the fidelity of DNA polymerase KF^- was measured directly by dNTP substrate competition as well as indirectly by two kinetic approaches. Fidelity was measured directly as the incorporation of R relative to W when dRTP and dWTP compete for insertion opposite the same template base in the same reaction, or indirectly using either steady state or pre-steady state kinetics to determine k_{cat}/K_m for incorporation of dRTP or dWTP substrates opposite the same template base in separate reactions. The dNTP competition approach is a direct measure of polymerase fidelity, while the kinetic approaches are indirect although reasonably based in theory (*32*). Our objective was to perform a systematic quantitative test of Fersht's model that competition and steady state kinetics are alternative, equivalent methods for determining pol fidelity (*32*) and comparing steady state and pre-steady state fidelity measurements.

Polymerase Fidelity Determined by Direct dRTP versus dWTP Competition opposite the Same Template Base. We have described polymerase fidelity measurements by direct competition as model-independent because the competition assay actually measures the amount of R and W incorporation when dRTP and dWTP substrates compete for insertion at the same site in the same enzyme (polymerase−primer−template) complex. The resultant misinsertion frequencies, evaluated as the average for the three values of the [dRTP]:[dWTP] ratio, are shown for 9 of the 12 possible base mispairs (Table 1). For each of these 9 cases, product bands are clearly resolved by electrophoresis, as illustrated for 4 cases (Figure 1). There are 3 cases for which no competition results are presented (Table 1). For 2 of these, the product bands generated by the addition of T cannot be adequately resolved from those containing a terminal G by our gel electrophoresis method, precluding the measurement of C/T or A/G misinsertion frequencies. For the third case, although primers ending in C and G are well resolved (Figure 1AS of the Supporting Information), we did not detect the formation of C/C mispairs by KF^- (Figure 1FS of the Supporting Information).

The direct competition measurement could, in principle, be subject to possible error, since the concentrations of directly competing right and wrong dNTPs do not remain constant over the course of the reaction. Three pool bias ratios are used with the dRTP concentration varied over a 4-fold range (50, 100, and 200 nM) and the dWTP concentration kept constant at 1 mM (Figure 1). The concentrations of R incorporated ranged from 5 to 20 nM, for the three dRTP concentrations, while the level of incorporation of W was less than 20 nM. Therefore, the initial dWTP concentration remained essentially constant, and the dRTP concentration was reduced by no more than 10%. In fact, the robust nature of the competition assay is shown by the finding of almost no difference in misinsertion ratio (W/R) at the three pool bias ratios (Table 1 and Figure 1).

Comparison with Steady State Kinetic Measurements. Steady state kinetic measurements to ascertain k_{cat}/K_m for right and wrong dNTP substrates can be used as an alternative method to determine misinsertion frequency, namely, as $(k_{cat}/K_m)_W/(k_{cat}/K_m)_R$, according to the steady state analysis introduced by Fersht (*32*). The standing-start kinetic scheme (Figures 2 and 3A) conforms precisely to Fersht's description, in which an experiment that measures the incorporation of dRTP and dWTP competing simultaneously for insertion on the same enzyme (polymerase−primer−template) complex at the same site is replaced by a steady state kinetic measurement, in which the velocities of incorporation of dRTP and dWTP are measured in two separate reactions. The steady state kinetic measurement assumes that the polymerase−primer−template complex behaves as a Michaelis−Menten enzyme, that the data are collected using steady state conditions with respect to dNTP substrates and primer−template DNA molecules, and that when dRTP and dWTP compete, the incorporation of R competitively inhibits the incorporation of W and vice versa.

In the standing-start assay, the gel bands are generated by a polymerase that cycles between primer−template DNA (*39*). We have also investigated a running-start kinetic scheme in which the gel bands are generated by a single encounter between the polymerase and primer−template DNA (Figure 3B and Table 1, footnote) (*44*, *46*). Standing- and running-start measurements have been used extensively to measure the fidelity of a variety of polymerases, including nearest-neighbor base stacking perturbations on misinsertion efficiency (*43*), and 3′-exonuclease proofreading on misincorporation efficiency (*44*, *46*, *47*).

Comparison with Pre-Steady State Kinetic Measurements. Pre-steady state kinetic studies employ single-turnover analysis to determine k_{pol}, the nucleotide incorporation rate

constant, and K_d, the equilibrium dissociation constant for binding of dNTP to the polymerase−primer−template complex (*19*). A large excess of polymerase is used to ensure that almost all of the primer−template DNA has bound polymerase at ttime zero, so that nucleotide incorporation for R and W can be measured on millisecond and subsecond time scales, respectively. Although the pre-steady state measurements do not, strictly speaking, conform to the Fersht "thought model" for steady state kinetics, it has been suggested that for all intents and purposes, one can use $(k_{pol}/K_d)_W/(k_{pol}/K_d)_R$ to approximate $(k_{cat}/K_m)_W/(k_{cat}/K_m)_R$ as the misinsertion frequency (*19*, *33*).

The chief point of our study is that indirect methods using steady state and pre-steady state kinetics provide valid measurements of DNA polymerase fidelity. The kinetic data using steady state values, k_{cat}/K_m, or pre-steady state values, k_{pol}/K_d, to deduce fidelity give the same quantitative values as those obtained by measuring fidelity by direct competition (Table 1). The accuracy of both kinetic methods is exemplified by the excellent correlations obtained (Figure 4) when nucleotide misinsertion frequencies (f_{ins}) evaluated from kinetics measurements were plotted against the corresponding values determined directly by dRTP versus dWTP substrate competition, for the 9 mispairs detected in our competition assay (Table 1). From these plots, a linear correlation coefficient of 0.95 is obtained for steady state measurements (Figure 4A) and 0.91 for pre-steady state measurements (Figure 4B). A larger deviation is observed in the pre-steady state comparisons for the "harder to make" T/T, T/C, and C/A mispairs. These somewhat larger discrepancies for the pre-steady state measurements are extraneous regarding the central conclusion: determinations of polymerase fidelity by kinetics and competition are in good agreement.

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SUPPORTING INFORMATION AVAILABLE

Additional examples of well-resolved bands observed in direct competition experiments and a table containing the steady state and pre-steady state kinetic parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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