DNA polymerase β nucleotide-stabilized template misalignment fidelity depends on local sequence context

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DNA polymerase β has two DNA-binding domains that interact with the opposite sides of short DNA gaps. These domains contribute two activities that modify the 5′ and 3′ margins of gapped DNA during base excision repair. DNA gaps greater than 1 nucleotide (nt) pose an architectural and logistical problem for the two domains to interact with their respective DNA termini. Here, crystallographic and kinetic analyses of 2-nt gap-filling DNA synthesis revealed that the fidelity of DNA synthesis depends on local sequence context. This was due to template dynamics that altered which of the two template nucleotides in the gap served as the coding nucleotide. We observed that, when a purine nucleotide was in the first coding position, DNA synthesis fidelity was similar to that observed with a 1-nt gap. However, when the initial templating nucleotide was a pyrimidine, fidelity was decreased. If the first templating nucleotide was a cytidine, there was a significantly higher probability that the downstream template nucleotide coded for the incoming nucleotide. This dNTP-stabilized misalignment reduced base substitution and frameshift deletion fidelities. A crystal structure of a binary DNA product complex revealed that the cytidine in the first templating site was in an extrahelical position, permitting the downstream template nucleotide to occupy the coding position. These results indicate that DNA polymerase β can induce a strain in the DNA that modulates the position of the coding nucleotide and thereby impacts the identity of the incoming nucleotide. Our findings demonstrate that “correct” DNA synthesis can result in errors when template dynamics induce coding ambiguity.

DNA repair intermediates include short gapped DNA substrates. These short gaps are generally utilized by DNA polymerases from the X family. These enzymes bind avidly to the 5′ margin of the gap, utilizing an 8-kDa DNA-binding domain (Fig. S1). This domain includes a helix–hairpin–helix motif that interacts with the DNA backbone via a monovalent cation in a sequence-independent manner. DNA polymerase (pol) β, an X family member, is a critical enzyme of the base excision repair pathway and well-suited for filling short DNA gaps (1–5 nt) (1). It has also been implicated recently to participate in an error-prone alternative nonhomologous end joining pathway (2). Pol β is the simplest mammalian pol, comprised of an 8-kDa lyase domain and a 31-kDa polymerase domain (Fig. S1A). Each domain contributes an enzymatic activity at the 5′ and 3′ DNA gap termini (deoxyribose phosphate lyase and DNA synthesis, respectively). The amino-terminal lyase domain targets the enzyme to the 5′ margin of a DNA gap (3). Additionally, the helix–hairpin–helix motifs in the lyase and polymerase domains stabilize the noncontiguous DNA strand downstream and upstream of the gap, respectively (Fig. S1B).

The polymerase domain is composed of functionally distinct subdomains: DNA binding (D), catalytic (C), and nascent base pair binding (N) (4). Several global conformational changes occur when pol β binds substrates. The most notable change occurs when the N subdomain of the binary enzyme–DNA complex closes around the nascent base pair upon binding dNTP. This subdomain repositioning is accompanied by subtle protein side-chain and DNA adjustments (5).

Pol β has moderate fidelity that is both DNA sequence– and DNA gap size–dependent (6). For a single-nucleotide gap, pol β misinserts 1 nt for 10^4–10^7 insertions (7). Previous studies

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have shown that X family pols (e.g., pol λ and pol μ) exhibit low frameshift fidelity (8–10). Pol β can bypass an abasic site by using the downstream nucleotide (11) and can reposition a templating pyrimidine, but not purine, from a coding template position (12). These attributes suggest that template ambiguity exists at the polymerase active site and that the incoming nucleotide may preferentially utilize a downstream template nucleotide rather than the templating nucleotide nearest the primer terminus. This has been referred to as dNTP-stabilized misalignment (13). Pol μ can utilize this strategy during nonhomologous end joining (14). If the downstream nucleotide codes for correct nucleotide insertion, then the mutagenic result would be either deletion of a single nucleotide or an apparent base substitution error when realignment of the primer terminus occurs, permitting insertion of an additional nucleotide (i.e., dislocation) (15). In this study, we characterize the sequence dependence of the fidelity of 2-nt gap filling and structurally capture a frameshift intermediate.

**Results**

**Fidelity of 2-nt gap-filling DNA synthesis**

The catalytic efficiency of single-nucleotide insertion catalyzed by pol β with 1- and 2-nt gapped DNA substrates in different sequence contexts was measured (Fig. 1 and Tables S1–S4). In general, the catalytic efficiency of inserting the cor-
rect nucleotide with the 2-nt gapped DNA substrate compared with 1-nt gapped DNA substrate is only modestly altered when a purine occupies the first templating position (AN and GN, where N represents an alternate downstream templating nucleotide). In contrast, the catalytic efficiency for correct insertion decreases 4- to 9-fold for CN gaps and 11- to 40-fold for TN sequences as compared with the 1-nt gapped DNA substrates.

To determine whether the fidelity of nucleotide insertion catalyzed by pol β is altered with the 2-nt gapped DNA substrates, we measured the catalytic efficiency for nucleotide misinsertion. This analysis revealed a striking trend for 2-nt gapped substrates containing a CN sequence where the catalytic efficiency for misinsertion increased significantly (100-fold) and depended on the identity of the downstream templating base. The misinsertion efficiency increased for the incoming nucleotide that was complementary to the downstream templating base but not for those that were not. For instance, the catalytic efficiency for dNTP-stabilized misalignment depended on the identity of the downstream templating base. Furthermore, the observation that the incoming dNTP can utilize the coding potential of the downstream templating base suggests that a dNTP-stabilized template misalignment intermediate is formed that alters fidelity.

**Influence of lyase domain interactions on fidelity**

In the crystal structure of pol β bound to 1-nt gap DNA, the lyase domain interacts with downstream duplex DNA (16). To ascertain whether these interactions are important for the fidelity of DNA synthesis, we altered these interactions and measured the fidelity within the CG sequence. If the lyase domain anchors pol β to the 5'-phosphate in the gap and influences the coding template register, then reducing or eliminating these interactions should increase fidelity (i.e., reduce dNTP-stabilized misalignment). To alter 5'-phosphate binding, we examined the fidelity of a triple-lysine mutant, where the lysines that coordinate the 5'-phosphate (Fig. S1C) were substituted with alanine (K35A/K68A/K72A (3KA)) (17). The catalytic efficiency for dGTP insertion with the 2-nt CG gapped substrate was reduced ~6-fold for the mutant compared with the WT enzyme, whereas dCTP misinsertion decreased ~3-fold (Figs. 1B and 2B and Tables S1 and S5). These results suggest that interactions with the lyase domain and the 5'-phosphate in the gap are not fully responsible for dNTP-stabilized misalignment synthesis.

Another approach to modulate downstream interactions with duplex DNA was to remove the downstream oligonucleotide to eliminate the gapped DNA structure. This substrate is referred to as CG-open, where the first two templating nucleotides are C and G, respectively (Fig. 2A). Removal of the downstream oligonucleotide modestly increases the fidelity of dCTP misinsertion from 250 (CG 2-nt gap) to 480 (CG-open). This is due to an 18-fold decrease in efficiency for the incorrect insertion (dCTP) coupled with a 9-fold decrease in correct (dGTP) insertion relative to a 2-nt gapped DNA substrate (Figs. 1B and 2B and Tables S2 and S5). These results indicate that 5'-phosphate and downstream duplex interactions have very little effect on dNTP-stabilized misalignment.

**RB69exo − fidelity of the CG-open substrate**

To determine whether properties inherent to the CG sequence give rise to the observed dNTP-stabilized misalignment synthesis, we examined the fidelity of the B family RB69 pol. This polymerase serves as a high-fidelity model replicative polymerase and does not make specific interactions with gapped DNA. We measured the catalytic efficiency for dNTP insertion using an exonuclease-deficient mutant with the CG-open sub-

![Image](324x378 to 552x733)

**Figure 2. Effect of modifying pol β interactions with downstream DNA on DNA synthesis discrimination.** A, steady-state catalytic efficiencies ($k_{cat}/K_{m,dNTP}$) were determined for 1-nt (templating C), 2-nt (CG in the gap), and open (no downstream oligonucleotide; i.e., no DNA gap) substrates; the relevant DNA sequence is given in the cartoon, and the full sequence is provided under “Experimental procedures.” In addition, 3KA, which perturbs 5'-phosphate gapped binding, was utilized. RB69exo− (B family replicative pol) was used as a control. B, discrimination plot for the steady-state catalytic efficiencies tabulated in Table S5. The identities of the incoming nucleotides are dCTP (green), dGTP (black), and dTTP (blue).
Mutagenically correct nucleotide insertion

Figure 3. Single-turnover analysis of mutagenic 2-nt gap-filling DNA synthesis. Single-turnover primer extension was assayed and quantified as described under “Experimental procedures.” A, illustration of the 5′-FAM–labeled 2-nt gapped DNA substrate. The sequence in the gap is 3′-CG-5′, and dCTP was added to initiate DNA synthesis. B, gel image illustrating the time course for 2-nt gap-filling (shown above). The substrate primer band (S) is extended by two dCMP insertions (+2 band). An intermediate band (+1) accumulates at short time intervals (<10 s). C, plot of the time course for the product bands (+1 and +2 as well as their sum, Total P). The progress curves were fit to either a single-exponential (+2 time course: $A = -0.93$, $k = 0.077$ s$^{-1}$, $C = 0.89$; Total P time course: $A = -0.88$, $k = 0.30$ s$^{-1}$, $C = 0.91$) or double-exponential equation (+1 time course: $A_1 = -0.78$, $k_1 = 0.49$ s$^{-1}$, $A_2 = 0.75$, $k_2 = 0.09$ s$^{-1}$, $C = 0.05$). D, illustration of the 5′-FAM–labeled 2-nt gapped DNA substrate. The sequence in the gap is 3′-GC-5′, and dGTP was added to initiate DNA synthesis. E, gel image illustrating the time course for 2-nt gap-filling (shown above). The substrate primer band (S) is extended by at least two dGMP insertions (P). An intermediate band can be observed that accumulates at later intervals. F, plot of the time course for all product bands (Total P). The progress curve was fitted to a single-exponential equation ($A = -0.90$, $k = 0.0007$ s$^{-1}$, $C = 0.90$).

To further explore the mechanistic basis of the enhanced catalytic efficiency with the CN substrates, dCTP insertion with a CG 2-nt gapped DNA substrate was measured under single-turnover conditions (i.e. pol/DNA = 10; Fig. 3). These reactions were performed with saturating concentrations of enzyme and dCTP so that substrate binding was not rate-limiting. The observed rate constant ($k_{obs}$) for dCTP insertion with a 2-nt gapped DNA was significantly higher than the corresponding rate constant for misinsertion into a 1-nt gapped DNA with a template C (0.3 s$^{-1}$ and 0.009 s$^{-1}$, respectively; Fig. S2A, left plot). In contrast, $k_{obs}$ for primer extension of a GC 2-nt gapped DNA substrate with dGTP was comparable with that observed with a 1-nt gapped DNA substrate (template G; Fig. S2A, right plot). These data are consistent with the sequence-dependent effects observed in the steady-state reactions and indicate that the incoming nucleotide can base-pair with the downstream templating nucleotide in a CG 2-nt gap nucleotide as opposed to direct misincorporation of dCTP opposite C.

Crystallographic structures of 2-nt gapped DNA intermediates

To provide molecular insight into the interactions of pol β with 2-nt gapped DNA substrates, crystals of binary enzyme–DNA complexes were grown with DNA substrates that included different sequence contexts in a 2-nt gap. To create the 2-nt gap, the primer strand was 1 nt shorter (9-mer) than that used to create a 1-nt gap (10-mer). The nucleotide complementary to the second template base in the gap was soaked into the crystal to form a ternary substrate complex and initiate DNA synthesis. Not surprisingly, the structures revealed that the incoming nucleotide formed a Watson–Crick base pair with the downstream templating nucleotide. The structures were nearly identical to that observed with a 1-nt gapped ternary substrate complex, except that the primer strand was 1 nt shorter. Thus, there was no indication that the 2-nt gapped DNA substrate was altered to hasten nucleotide insertion (i.e. the shortened primer strand was too far from the incoming nucleotide). For example, soaking dCTP into a crystal of a DNA binary complex with two G residues in the gap resulted in a closed ternary substrate complex (Table S6 and Fig. S3, A and B). The
incoming dCTP is base-paired with the downstream G in the gap, but because the primer strand was 1 nt shorter, O3’ of the primer terminus was too far (~7 Å) from P0 of the incoming nucleotide, precluding DNA synthesis. Because pol β can efficiently fill 2-nt gaps, the structure represents a trapped precatalytic intermediate because of crystallographic constraints.

As an alternative approach, a new 2-nt gapped DNA substrate was created with a longer template (17-mer rather than the 16-mer used to create the 1-nt gapped DNA substrate). This approach conserves the upstream and downstream duplex nature of the DNA substrate used for the 1-nt gap substrate. In addition, the structure was co-crystallized with all necessary components. The sequence in the gap was CG, and dCTP was included. The resulting structure was a “nicked” product complex after a single dCMP insertion (Fig. 4). The first templating C was in an extrahelical position to permit the downstream G to occupy the coding template position. The global pol conformation was open compared with the open conformation of the binary DNA complex with a 1-nt DNA gap (18) (Fig. 4A; root mean square deviation = 0.54 Å). The active-site Mg2+ ions and PPi had dissociated, and the nascent GC base pair was severely buckled, weakening the hydrogen bonds between these complementary bases (Fig. 4B). The poor density for the extrahelical C indicates that this nucleoside is highly dynamic and lacks stabilizing enzyme interactions (Fig. S4).

dNTP-stabilized misalignment leading to a base substitution error

For the 2-nt gapped DNA substrate with the CT sequence context, dATP rapidly filled the gap (i.e. two dAMP insertions) (Fig. 5). In this case, the n+1 product accumulated only to a minor extent, suggesting that the downstream T served as the coding template base less often than that observed in the CG sequence context (Fig. 3C). The modestly elevated rate of insertion, compared with direct misinsertion into a 1-nt gap (Fig. S2B), is consistent with only a proportion of the product being generated through dATP-stabilized misalignment. Subsequent to dATP-stabilized misalignment, the primer terminus must rapidly realign to create a mismatched primer terminus (primer
terminus A opposite C) that is extended with a second dAMP insertion opposite T. This creates nicked DNA with a matched 3' terminus. If this DNA product is ligated, it will generate a base substitution error. In contrast to the product structure determined for the CG gapped DNA substrate, crystals were not formed with the CT gapped DNA, probably because of the dynamic nature of events with this sequence context and the constraints necessary to form crystals.

**Discussion**

DNA polymerases must select the correct incoming nucleotide that is complementary to the templating base to maintain high fidelity. An essential aspect of this selection is that the proper template base must direct nucleotide insertion. In reiterated DNA sequences (e.g., mono-, di-, and trinucleotide repeats), polymerases can be error-prone because of DNA slippage events that realign nucleotides in the primer stem. Because this occurs in runs of a reiterated sequence, correct base pairing is maintained, albeit with fewer base pairs, and generally leads to frameshift insertion or deletion errors. In contrast to upstream slippage events that require DNA melting and annealing, downstream DNA template dynamics can alter the proper register of the single-stranded template strand that can confound proper identification of the coding template nucleotide. It has been noted previously that if pol β can utilize the downstream template nucleotide to direct nucleotide insertion, and this has been referred to as dNTP-stabilized misalignment (13, 15). This event has been clearly demonstrated for pol β when it encounters an abasic site centrally positioned in a 5-nt DNA gap (11). Rather than insert dATP opposite an abasic site, as observed for most pols, pol β utilizes the downstream templating base to direct DNA synthesis. If DNA synthesis continues, then this results in a 1 deletion. If on the other hand, the template strand realigns, then further DNA synthesis would generate a base substitution error (Fig. 6).

In this study, we characterized the fidelity of 2-nt gap-filling DNA synthesis by pol β to ascertain which nucleotides in the template strand may be prone to repositioning and thereby impact fidelity. Previous studies with pol β indicated that an adenine mispair at the primer terminus can displace a templating cytosine (19), but not adenine (20), in 1-nt gapped DNA. In the former case, the templating cytosine is moved to an extrahelical position. Similarly, spectroscopic analysis of pol β binding to 1-nt DNA gaps indicated that addition of divalent metals can form a productive enzyme when pyrimidines serve as the templating nucleotides, but not purines (12). To accomplish this, the templating pyrimidine must be displaced to permit the primer terminus to move into the nascent base pair binding pocket. This behavior correlates with a robust reverse reaction, pyrophosphorolysis, observed with a template pyrimidine in the gap. Because the reverse reaction requires the primer terminus to occupy the nascent base pair binding pocket, the templating pyrimidine in the gap must be removed from the coding template position.

The results presented here indicate that the fidelity of 2-nt gap filling is highly dependent on the sequence in the gap. The distance between the respective insertion efficiencies plotted in Fig. 1 is a representation of the fidelities for the apparent misinsertions and provides insight into the origin of the altered discrimination. In general, when the first templating nucleotide in the gap is a purine (A or G), then the catalytic efficiency for correct nucleotide insertion is hardly affected when going from a 1- to a 2-nt gap (Fig. 1, A and C). Likewise, the catalytic efficiencies for misinsertion are not significantly affected by gap size; accordingly, fidelity is not significantly altered.

In contrast, when the first templating base in a 2-nt gap is a pyrimidine (C or T), then there is a significant decrease in fidelity. This is the result of two changes: a general decrease in the efficiency of correct insertion when a 2-nt gap is utilized and an increase in catalytic efficiency of the incoming incorrect nucleotide when it is complementary to the downstream templating base (Fig. 1, B and D), suggesting that the downstream templating nucleotide provided the coding base (i.e., dNTP-stabilized misalignment; Fig. 6, path ii) (13). This behavior was most pronounced in the CG gap. The fidelity of insertion of dCTP opposite C in a 1-nt gap is 290,000 ((kcat/Km,dGTP)/(kcat/Km,dCTP)), but in the 2-nt gap it is 250 (1160-fold lower). This is primarily due to a 290-fold increase in the insertion efficiency of dCTP in the CG gap (Table S2). These results are consistent with a dCTP-stabilized misalignment where the downstream G in the CG gap is utilized as the coding template base. Additionally, the stability of this nascent base pair is consistent with the rapid accumulation of primer extended by 1 nt (Fig. 3, B and C), indicating that a step after initial insertion is slow.

The crystallographic structure of the binary product complex after insertion of a single dCMP (Fig. 4) is also consistent with a stable misaligned intermediate resulting in a slow primer realignment step. The structure reveals that the first templating C is extrahelical and dynamic (Fig. S4). If this intermediate is ligated, then the result would be a 1 deletion mutation (Fig. 6, path ii, ligation). In contrast to the extrahelical displacement of C observed here, pol μ preferentially utilizes the downstream templating nucleotide in a 2-nt gap even when the first templating nucleotide is a purine (14). This structure is similar to that of pol β with 2 G residues in the gap (Fig. S3B). In the pol μ structure, however, the primer terminus is well-positioned for catalysis through compression of the templating nucleotides near the active site. This strain in the template strand is relieved after nucleotide insertion.

Over the past 20 years, high-resolution crystal structures of pol β with gapped DNA substrates have been determined with 16-mer templates. This presumably optimizes crystal packing because attempts to crystalize pol β with longer substrates have been unsuccessful. The 2-nt CG-gapped 17-mer substrate utilized here produced a low-resolution structure by flipping the cytosine in the original gap outside of the DNA helix, generating a semistable 16-mer template mimic. The low resolution of the complex probably reflects the stress introduced into the active site with this premutagenic DNA intermediate. In contrast, a stable 16-mer intermediate mimic could not be formed with the CT substrate. This interpretation is consistent with the
kinetics of gap-filling DNA synthesis with these two substrates and failure to solve a structure with the CT 2-nt gap substrate. In contrast to the dCTP-stabilized misalignment in the CG context described above, the dATP-stabilized misalignment in the CT context does not result in a stable product complex (Fig. 5). Because the rate of dAMP insertion into a 1-nt gap with a templating C is similar to that observed for misinsertion into the CT 2-nt gap (Fig. S2B), the poor accumulation of the n/H11001 product is consistent with slow misinsertion followed by rapid extension. A closer examination of the results illustrated in Fig. 5 suggests that both misinsertion and dATP-stabilized misalignment are occurring during CT-gap filling. The time course for the n+1 band indicates that a population of enzymes can rapidly insert dAMP ($k_{obs} = 2 \text{ s}^{-1}$) into the 2-nt CT gap (Fig. 5C). In this instance, however, the amplitude (i.e. the population of enzymes utilizing the downstream T residue, $\sim 10\%$) is smaller than that observed in Fig. 3 for the CG gap (n+1 band amplitude $\sim 80\%$). Additionally, the decay of this intermediate suggests that rapid primer realignment and correct insertion occur to fill the 2-nt gap.

The high rate of dAMP misinsertion opposite C or into the CT 2-nt gap is also noteworthy (Fig. S2B). A crystallographic ternary substrate complex structure with a templating C and incoming dATP analog indicates that the template C residue is removed from the active site and positioned upstream upon nucleotide binding, effectively creating an abasic site (21). This is consistent with the relatively robust misinsertion rate relative to other misinsertions and similar to the rate observed for dAMP insertion opposite an authentic abasic site (18).

Taken together, the results suggest that the ability of the first templating base to stack with upstream DNA and/or form van der Waals contact with pol $\beta$ influences template dynamics, which, in turn, will dictate which path will prevail (Fig. 6). In the

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Figure 6. Mutagenically correct nucleotide insertion. Shown is a diagram illustrating how correct nucleotide insertion can result in mutagenic DNA synthesis because of a dNTP-stabilized misalignment. In this general example, a 2-nt gapped DNA substrate is shown, with template nucleotides A (green) and B (red) in the gap. For pol $\beta$, when a purine occupies the first templating position (left, error-free path i), gap-filling DNA synthesis is faithful, inserting the complementary nucleotide (lowercase and in the same color; i.e. daTP opposite A and dbTP opposite B). In this situation, the downstream noncoding nucleotide (i.e. B) must relocate outside of the active site. Structures of pol $\lambda$ with such an intermediate indicate that it occupies a position near $\alpha$-helix N and has been termed scrunching (22). Translocation creates a 1-nt gap that is efficiently filled with high fidelity. In contrast to this error-free path, when a pyrimidine occupies the first templating position in a 2-nt gap (mutagenic path ii), template instability (looping out or dislocation) can lead to dNTP-stabilized misalignment (dotted box). In this case, the pyrimidine is moved out of the active site into an extrahelical position, permitting the downstream templating base to occupy the coding template position; in this case, B serves as the coding template base for the correct incoming nucleotide, dbTP. After insertion, the primer terminus could realign, generating a mispaired primer terminus and 1-nt gap. Correct insertion from this mispaired terminus completes gap filling but results in a base substitution error. Alternatively, if the first nascent base pair is stable and does not realign, then this pseudonicked structure provides a substrate for DNA ligation. Ligation of this pseudonicked substrate would generate a $\sim 1$ deletion.
Mutagenically correct nucleotide insertion

In the case of a dNTP-stabilized misalignment (Fig. 6, path ii), the stability of the nascent base pair will influence the competition between subsequent steps (ligation versus realignment). Although the influence of such intermediates on DNA ligase efficiency has not been examined in detail, these intermediates can be ligated after pol β-dependent synthesis on short gaps (9).

Although DNA synthesis fidelity is compromised during 2-nt gap filling relative to that observed in a 1-nt gap when the first templating nucleotide is a pyrimidine, fidelity is not significantly altered when a purine occupies the first templating position. As suggested above, this is probably due to stabilizing interactions: stacking with the primer terminus base pair and hydrogen bonding with the incoming nucleotide. In this situation, the downstream templating nucleotide must be accommodated either in an extrahelical position or in an alternative binding pocket. The first possibility seems unlikely because the template strand is bent 90° at the coding templating nucleotide, binding pocket. The second possibility, however, is likely because the downstream oligonucleotide is in a stabilized conformation (24).

X family pols interact with duplex DNA on opposite sides of short DNA gaps. For pol β, the amino-terminal lyase domain avidly binds to the downstream 5′-phosphate–binding site because there are positively charged residues in this region that could provide a good binding pocket for negatively charged backbone of the displaced downstream strand (Fig. S5). This general scenario is consistent with the minimal effect of altering interactions with the downstream duplex on catalytic efficiency and fidelity (Fig. 2).

Enzymes

WT and mutant pol β were prepared as described previously (24). The concentration was determined by absorbance at 280 nm using an extinction coefficient of 23,380 M⁻¹ cm⁻¹. RB69exo² was kindly provided by William Konigsberg (Yale).

Steady-state kinetic assays

For correct nucleotide insertion, steady-state kinetic parameters for gap-filling reactions were determined by initial velocity measurements as described previously (4). Unless noted otherwise, enzyme activities were determined using a standard reaction mixture containing 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA, 200 nm gapped DNA, and 10% glycerol at 37°C. Enzyme concentrations and reaction time intervals were chosen so that substrate depletion or product inhibition did not influence initial velocity measurements. Reactions were initiated by addition of enzyme and quenched with 2× quencher buffer (200 mM EDTA, 80% formamide, ~0.1% bromphenol blue, and 0.1% xylene cyanol). Reaction mixtures were then heated at 95°C for 5 min, placed on ice, and loaded onto prerun 22% denaturing polyacrylamide gels. The gels were scanned using a Typhoon scanner and quantified using ImageQuant TL (GE Healthcare Life Sciences). Steady-state kinetics parameters and standard errors represent the results of least squares curve fitting of duplicated measurements to either the Michaelis–Menten equation (k_cat and K_m) or a modified form to extract catalytic efficiencies (k_cat/K_m). The catalytic efficiencies for most misinsertion reactions were determined by using sub-saturating concentrations (25–200 μM) of nucleotides. When [dNTP] ≪ K_m, then k_cat/K_m ≈ (k_cat/K_m)[dNTP].
Model RQF-3 chemical quench-flow apparatus (KinTek Corp., State College, PA). Samples were quenched, resolved, and quantified as described above. Progress curves were fit to either a single-exponential ($k_{obs} = A \times \exp(-kt) + C$) or double-exponential ($k_{obs} = A_1 \times \exp(-k_1t) + A_2 \times \exp(-k_2t) + C$) equation, as indicated, where $A$ is the amplitude, $k$ the rate constant, and $C$ the end point.

**Crystallography**

Ternary complex crystals with 2-nt gapped DNA were obtained by soaking the DNA binary complex crystals with dCTP as described earlier (25, 26). The sequences of the template strand (16-mer), primer strand (9-mer), and phosphorylated downstream primer strand (5-mer) were 5′-CCG ACG GCG CAT CAG C-3′, 5′-GCT GAT GCG C-3′, and 5′-pGTC GG-3′, respectively. The nucleotides in the gap are underlined. The oligonucleotides were annealed at a ratio of 1:1:1 by heating at 90 °C for 10 min and cooling to 4 °C (1 °C/min) using a PCR thermocycler, resulting in a 1 m mole mixture of 2 nt gapped DNA. This annealed mixture was crystallized by sitting drop vapor diffusion at 18 °C by mixing 2 μl of the complex with 2 μl of crystallization buffer. The crystallization buffer consisted of 14%–16% PEG3350, 350 mM sodium acetate, and 50 mM imidazole (pH 7.5).

Binary product DNA complex crystals were obtained by cocystalization of annealed oligonucleotides, creating 2-nt gapped DNA, pol β, and dCTP. The sequences of the template strand (17-mer), primer strand (10-mer), and phosphorylated downstream primer strand (5-mer) were 5′-CCG ACG GCG CAT CAG C-3′, 5′-GCT GAT GCG C-3′, and 5′-pGTC GG-3′, respectively. The nucleotides in the gap are underlined. Data were collected at 100 K on a charge coupled device detector system mounted on a MiraMax®-007HF (Rigaku Corp.) rotating anode generator. Data were integrated and reduced with HKL2000 software (27). All crystals belong to the space group $P2_1_1_2_1$. The structures were solved by molecular replacement using PDB codes 4KLM (binary) and 2FMS (ternary) as reference models. The structures were refined using PHENIX (28), and manual model building was done using Coot. The crystallographic statistics are reported in Table 56.

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