In the ternary substrate complex of DNA polymerase (pol), the nascent base pair (templating and incoming nucleotides) is sandwiched between the duplex DNA terminus and polymerase. To probe molecular interactions in the dNTP-binding pocket, we analyzed the kinetic behavior of wild-type pol β on modified DNA substrates that alter the structure of the DNA terminus and represent mutagenic intermediates. The DNA substrates were modified to 1) alter the sequence of the duplex terminus near the nascent base pair, and 3) insert extra bases in the primer or template strands to mimic frameshift intermediates. The results indicate that the nucleotide insertion efficiency (k_{cat}/K_m, dGTP-dC) is highly dependent on the sequence identity of the matched (i.e. Watson-Crick base pair) DNA terminus (template/primer, G/C → A/T or T/A → C/G). Mismatches at the primer terminus strongly diminish correct nucleotide insertion efficiency but do not affect DNA binding affinity. Transition intermediates are generally extended more easily than transversions. Most mismatched primer termini decrease the rate of insertion and binding affinity of the incoming nucleotide. In contrast, the loss of catalytic efficiency with homopurine mismatches at the duplex DNA terminus is entirely due to the inability to insert the incoming nucleotide, since k_{cat}/K_m, dGTP is not affected. Abasic sites and extra nucleotides in and around the duplex terminus decrease catalytic efficiency and are more detrimental to the nascent base pair binding pocket when situated in the primer strand than the equivalent position in the template strand.

DNA polymerases select (bind and incorporate) a nucleoside triphosphate (dNTP) from a pool of structurally similar molecules to preserve Watson-Crick base pairing rules. DNA polymerase (pol)β is a model polymerase to study mechanisms utilized to assure efficient and faithful DNA synthesis. Its small size, lack of essential accessory proteins, and absence of a proofreading exonuclease have facilitated its biochemical, kinetic, and structural characterization. More importantly, it shares many general structural and mechanistic features exhibited by other DNA polymerases (1). DNA polymerase β contributes two important enzymatic activities during single-nucleotide base excision DNA repair. A deoxyribose phosphate lyase activity is associated with the amino-terminal 8-kDa lyase domain. This activity excises a deoxyribose phosphate intermediate during repair of abasic sites and generates a 5’-phosphate in a single-nucleotide gap. The nucleotidyl transferase activity of pol β is associated with the 31-kDa polymerase domain that fills the single-nucleotide gap. In addition, the polymerase activity of pol β is necessary for several alternate repair pathways that require longer gap-filling DNA synthesis (e.g. long patch base excision repair) (2, 3).

A general feature observed in the structures of all DNA polymerases that include a template overhang (i.e. single-stranded DNA) is that the trajectory of the template strand bends dramatically as it enters the polymerase active site (1). This serves at least two functions. First, it provides the polymerase the ability to assess whether geometrical constraints imposed by correct Watson-Crick hydrogen bonding occur, and second, it discourages the next templating base from prematurely entering the polymerase active site, which could result in the downstream template base coding for nucleotide insertion (deletion mutagenesis) (4). To accurately replicate DNA, polymerases need to stabilize the coding templating base as well as the correct, but not incorrect, incoming nucleotide (5). This is achieved through a series of protein- and substrate-induced conformational changes that result in a dNTP-binding pocket formed by the templating base, DNA duplex terminus, and enzyme. In the absence of an incoming nucleotide, the carboxyl-terminal N-subdomain (residues 262–335) of pol β is in an open conformation so that key polymerase side chains do not interact with the templating nucleotide. Upon binding the correct nucleotide, the N-subdomain closes on the nascent base pair (templating and incoming nucleotides), creating several key interactions with the enzyme (6). Accordingly, the constraints imposed by the dNTP-binding pocket are determined by DNA sequence (i.e. structure) as well as the conformational fluctuations that occur in response to enzyme and substrate binding.

In the pol β closed ternary substrate complex, the nascent base pair is sandwiched between the duplex DNA terminus and α-helix N (Fig. 1). Lys260 and Asp276 of α-helix N contribute van der Waals interactions with the templating and incoming nucleotide bases, respectively, whereas Asn279 and Arg283 contribute DNA minor groove interactions. Structure-based site-directed mutagenesis of these residues has identified interac-

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‡ The abbreviations used are: pol, polymerase; THF, tetrahydrofuran.

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DNA polymerase β stacking interaction with the nascent base pair. The nascent base pair (template and incoming nucleotides) is sandwiched between α-helix N of the N-subdomain of pol β and the growing DNA duplex terminus. In addition to the nascent base pair, two base pairs of duplex DNA are illustrated (stippled gray boxes) and the polarity of each strand (template and primer) indicated. Hydrophobic portions of the Asp276 and Lys280 side chains interact with the bases of the incoming and templating nucleotides, respectively. In the closed ternary complex structure, Asp276 and Lys280 also hydrogen-bond to Arg86 and the templating nucleotide 5′-phosphate, respectively (not illustrated).

EXPERIMENTAL PROCEDURES

Materials—Poly(dA), p(DT)$_{10}$, ultrapure deoxynucleoside triphosphates, [γ-$^32$P]ATP, and MicroSpin G-25 columns were from Amersham Biosciences. [α-$^32$P]dTTP was from PerkinElmer Life Sciences, and DE81 filters were from Whatman.

Protein Purification—Human DNA polymerase β was purified as described previously (14). Enzyme concentration was determined by Coomassie dye binding using purified pol β as a standard (15). The concentration of purified pol β was determined by total amino acid analysis.

DNA Preparation—The sequence, structure, and nomenclature of the DNA substrates used in this study are illustrated in Fig. 2. An oligonucleotide DNA substrate containing a single-nucleotide gap at position 16 was prepared by annealing three gel-purified oligonucleotides (Oligos Etc., Wilsonville, OR, or Integrated DNA Technologies) at position 16 was prepared by annealing three gel-purified oligonucleotides (Oligos Etc., Wilsonville, OR, or Integrated DNA Technologies, Coralville, IA). Each oligonucleotide was suspended in 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, and the concentration was determined from their UV absorbance at 260 nm. The annealing reactions were carried out by incubating a solution of 10 μM primer with 12 μM of downstream and template oligonucleotides at 90–100 °C for 3 min followed by 30 min at 65 °C and then slow cooling to room temperature.

Kinetic Assays—Steady-state kinetic parameters for single-nucleotide gap-filling reactions were determined by initial velocity measurements as described previously (13). Unless noted otherwise, enzyme activities were determined using a standard reaction mixture (50 μl) containing 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 5 mM MgCl$_2$, and 200 nM single-nucleotide gapped DNA. In some instances requiring high dNTP concentrations (e.g. misinsertion reactions), the MgCl$_2$ concentration was increased to assure that there was at least 5 mM free Mg$^{2+}$ in the reaction mixture. Enzyme concentrations and reaction time intervals were chosen so that substrate depletion or product inhibition did not influence initial velocity measurements. Reactions were stopped with 20 μl of 0.5 M EDTA and mixed with an equal volume of formamide dye, and the products were separated on 12% denaturing polyacrylamide gels. The dried gels were analyzed using a PhosphorImager.
THE MICHAELIS CONSTANT, THE $E$ CONCENTRATION, OF DECREASING CATALYTIC EFFICIENCIES RELATIVE TO A MATCHED TERMINUS. THE TEMPLATE BASE OF THE TERMINAL MISMATCH IS USED AS THE REFERENCE NUCLEOTIDE

**RESULTS**

**Influence of the Identity of the DNA Duplex Terminus on Catalytic Efficiency**—Steady-state kinetic analysis previously revealed a strong dependence of catalytic efficiency ($k_{cat}/K_m$) on the identity of the primer terminus for single-nucleotide gap filling by pol $\beta$ (19). That study examined the efficiency of dCTP insertion into a single-nucleotide gapped substrate with a templating guanine and systematically altered the primer terminus or downstream sequence to characterize the influence of DNA sequence on insertion efficiency. The downstream DNA sequence did not significantly alter the catalytic efficiency of nucleotide insertion. We have reexamined the influence of the identity of the primer terminus on insertion efficiency in a different DNA sequence context and with a different incoming nucleotide (i.e. dGTP rather than dCTP). The results are tabulated in Table I and indicate that the efficiency of dGTP insertion is strongly dependent on the identity of the matched (i.e. Watson-Crick base pair) primer terminus. As observed previously (19), primer termini with a pyrimidine are extended more efficiently than those with a purine situated at the 3′ terminus (DNA terminus: template/primer, G/C $\sim$ A/T $>$ C/G).

### **Table I**

| $X$/Y $\sim$ Z $\sim$ a | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $f_{ext}$ |
|--------------------------|----------|-------|---------------|----------|
| (A/T)/C                   | 16 (2)   | 0.5 (0.01) | 3200 (400)    | $10^{-4}$ |
| (A/C)/C                   | 2.33 (0.08) | 21 (2) | 11 (1) | 34 (5) |
| (A/A)/C                   | 0.035 (0.002) | 4.9 (1.5) | 0.7 (0.2) | 2.2 (0.7) |
| (A/G)/C                   | 0.041 (0.001) | 65 (16) | 0.065 (0.017) | 0.2 (0.059) |
| (C/G)/C                   | 5.5 (0.3) | 1.4 (0.2) | 400 (60) | $10^{-4}$ |
| (C/T)/C                   | 1.74 (0.06) | 260 (20) | 0.67 (0.06) | 17 (3) |
| (C/A)/C                   | 0.28 (0.07) | 160 (20) | 0.18 (0.05) | 4 (1) |
| (C/C)/C                   | 0.283 (0.009) | 400 (40) | 0.071 (0.007) | 1.8 (0.3) |
| (G/C)/C                   | 34 (3) | 0.34 (0.06) | 10000 (2000) | $10^{-4}$ |
| (G/T)/C                   | 10.1 (0.6) | 20 (3) | 50 (6) | 50 (10) |
| (G/A)/C                   | 0.22 (0.04) | 47 (4) | 0.47 (0.09) | 0.47 (1.1) |
| (G/G)/C                   | ND $^b$ | ND | ND | ND |
| (G/G)/T                   | 0.0123 (0.0009) | 8 (2) | 0.15 (0.04) | 0.19 (0.07) |
| (T/A)/C                   | 7 (1) | 1.2 (0.2) | 600 (100) | $10^{-4}$ |
| (T/G)/C                   | 7.7 (0.4) | 42 (16) | 18 (7) | 300 (100) |
| (T/C)/C                   | 7.7 (0.1) | 89 (3) | 8.6 (0.3) | 140 (20) |
| (T/T)/C                   | 0.31 (0.03) | 25 (2) | 1.3 (0.2) | 22 (5) |

$^a$ Identity of the template $(X)$ primer $(Y)$ terminus adjacent to the templating base $(Z)$ at position $n$.  
$^b$ As calculated from the ratio of catalytic efficiencies for correct insertion on a mismatched/matched primer terminus. Since $K_{d(mismatched)}$ $\sim$ $K_{d(mismatched)}$, $f_{ext}$ $\sim$ $f_{min}{^c}$.

$^c$ ND, not detected.

$^d$ Calculated from the corresponding efficiency for dATP insertion with the (G/C)/T gapped DNA substrate.

Data were fitted to Equation 1 for competitive inhibition by nonlinear regression methods.

$$k_{obs} = \frac{(k_{cat} \times S)}{S + K_m \left(1 + \frac{C}{K_C}\right)} \quad \text{(Eq. 1)}$$

The Michaelis constant, $K_m$, and $k_{cat}$ for the homopolymeric DNA substrate (S) were determined in the absence of heteropolymeric competitor DNA (C). When competitor DNA binds tightly ($K_C <$ polymerase concentration, $E$), an alternate form of Equation 1 (Equation 2) is necessary to account for the depletion of free inhibitor and enzyme as the EC complex is formed (18).

$$v_{obs} = E - C - K_{app} + \sqrt{E - C - K_{app} + 4EK_{app}} \quad \text{(Eq. 2)}$$

For a competitor inhibitor, the apparent inhibitor constant is given by Equation 3.

$$K_{app} = K_m \left(1 + \frac{S}{K_C}\right) \quad \text{(Eq. 3)}$$

This expression is the relative rate of adding a correct nucleotide (dNTP) onto a wrong (w, mismatched) and right (r, matched) primer terminus at equal concentrations under steady-state conditions. The parameters $K_m$, $K_{app}$, and $P$ refer to the equilibrium DNA binding affinity, dNTP Michaelis constant, and processivity for correct insertion on a matched or mismatched primer terminus. Processivity is defined as the ratio of rate constants describing nucleotide insertion and DNA dissociation (i.e. $k_{pol}/K_{app}$). Thus, the relative efficiency for extending a mismatch is dependent on the correct nucleotide concentration with maximum discrimination occurring at infinitely low dNTP concentrations. This intrinsic mismatch extension efficiency is referred to as $f_{min}$ (Equation 5).

$$f_{min} = \frac{\left(k_{cat}/K_m\right)(P + P_d[nDNTP])}{\left(k_{cat}/K_{app}\right)(P + P_d[nDNTP])} \quad \text{(Eq. 4)}$$

If the polymerase binds DNA with matched and mismatched primer termini with equal affinities, then $f_{min}$ is simply the
ratio of catalytic efficiencies for insertion on a mismatched relative to that on a matched terminus.

**DNA Binding Affinity**—We have previously utilized a simple competition assay to assess the equilibrium DNA binding affinity of gapped DNA substrates with pol β (16). The assay follows DNA synthesis on a homopolymeric template/primer system and determines the competitive inhibition constant, \( K_i \), for a heteropolymeric DNA substrate that does not support DNA synthesis (i.e., the templating bases on the two competing DNA substrates are different). Fig. 3 illustrates the results from an assay to determine the binding affinity of single-nucleotide gapped DNA that has a G/C or C/C (template/primer) terminus with a templating G or C, respectively. These DNA substrates have similar binding affinities (Table III). The binding affinity of the matched terminus with a templating G is the same as that determined by following the DNA concentration dependence of the pre-steady-state burst amplitude (\( K_d = 20 \) nM) (11). The binding affinities for a series of single-nucleotide gapped DNA substrates with matched or mismatched primer termini were determined and are tabulated in Table III. Although the binding affinities for the one-nucleotide gapped DNA substrates are modestly sensitive to DNA sequence, the mean binding affinities for matched and mismatched primer termini are very similar. Likewise, introduction of an abasic site opposite the primer terminus (i.e., \( T_{-1} \)) or an extra base directly behind the primer terminus (i.e., \( P_{-2} \)) does not significantly affect DNA binding affinity.

To determine crystallographic structures of DNA polymerases with bound substrates, an abortive ternary substrate complex is produced (23). This is typically achieved with a polymerase-DNA complex, where the first two templating nucleotides are complementary to the included ddNTP. The polymerase inserts the first ddNTP, resulting in a terminated primer and binds another ddNTP, resulting in an abortive ternary complex. Utilizing a 3'-deoxycytosine at the primer terminus, the addition of the complementary dNTP results in a significant increase in the apparent binding affinity for the gapped DNA substrate (Table III), suggesting that important conformational changes have occurred.

**Pol β-Dependent Mismatch Extension**—Since the DNA bind-
mismatched primer nucleotide is the incorrect partner. It should be noted, however, that mismatches can arise through correct nucleotide insertion on a misaligned template strand followed by realignment (i.e., dislocation) (24). In this scenario, the incorrect nucleotide in the primer strand does not represent a misincorporation event.

Steady-state kinetic parameters are tabulated in Table I in order of decreasing catalytic efficiencies for the respective mismatches relative to the corresponding matched terminus. Since insertion of dCTP on a G/G mismatch could not be readily measured (see below), the templating base was changed to thymidine. In general, pol β extends transition intermediates more efficiently than transversions. In most instances, this is due to a decrease in $k_{\text{cat}}$ and an increase in $K_m$. However, there are situations where changes in one kinetic parameter dominate over the other. For example, $k_{\text{cat}}$ for extension of T/G and T/C is hardly affected relative to T/A, but $K_m$ is increased 35- and 74-fold, respectively. The interpretation of these steady-state kinetic parameters is not straightforward due to the observation that product dissociation (i.e. $k_{\text{off}}$ for nicked DNA) and nucleotide insertion (i.e. $k_{\text{pol}}$) are partially rate-limiting for correct insertion on a matched terminus (11). Thus, $k_{\text{cat}}$ can be a reflection of both rate constants. 3 The observation that it is not significantly altered suggests that the increase in $K_m$ represents a diminished dNTP binding affinity rather than a change in processivity ($k_{\text{pol}}/k_{\text{cat}}$). It is not surprising that a mismatched primer terminus results in diminished dNTP binding and insertion, since the primer terminus forms part of the dNTP-binding pocket. However, the kinetic analysis of extension of an A/A or G/G mismatch indicates that the loss of catalytic efficiency is entirely due to the loss of insertion ($k_{\text{pol}}$) without altering the binding affinity for the incoming nucleotide. Because $k_{\text{cat}}$ for correct insertion is diminished 460- and 2800-fold respectively, $k_{\text{cat}}$ is a direct measure of $k_{\text{pol}}$. Since the DNA binding affinity is not significantly altered (Table III), $k_{\text{pol}}$ is now completely rate-limiting. In such a situation, $K_m$ is equivalent to $K_f$ for the incoming nucleotide (25). The $K_f$ for the correct incoming nucleotide on a matched terminus is $\sim 10 \mu M$ (11, 13). Thus, the increase in $K_m$ for these mismatches reflects the diminished $k_{\text{pol}}$ and not a lower binding affinity for the incoming nucleotide.

To verify that the correct nucleotide has a high affinity for the pol β active site when an A/A mismatch forms a portion of the binding pocket, the DNA binding affinity was assessed in the presence of the complementary nucleotide. With a matched primer terminus that cannot be extended (i.e., 3'-deoxynucleotide), binding of the correct nucleotide results in an abortive complex that binds DNA more tightly than the binary DNA complex (Table III). As with the matched primer terminus, the binding affinity for single-nucleotide gapped DNA substrate with an A/A terminal mismatch is significantly increased in the presence of dGTP, indicating that a ternary complex is formed. Although dGTP binds well with the A/A duplex terminus, its insertion rate is diminished 8- to 10-fold (Table I). In contrast, inclusion of dGTP with a C/C mismatched primer terminus, which does not support strong binding of a correct nucleotide, has only a small influence on DNA binding affinity (Table III).

Misalignment of the Free Primer Terminus—As noted above, dGTP insertion opposite a templating deoxycytidine was too low on a G/G mismatch for kinetic characterization. Accordingly, we surveyed other templating nucleotides (Fig. 4). Measurable correct nucleotide insertion could be observed with the other templating bases on this mismatch. With a templating deoxyguanosine, the insertion of two nucleotides occurs. Taken
Frameshift Intermediate

Fig. 5. Gap-filling DNA synthesis on frameshift intermediates. Correct dGTP insertion was characterized on DNA substrates where an extra nucleotide, typically C, was strategically included in an oligonucleotide so that proper annealing resulted in a frameshift intermediate. Steady-state kinetic parameters were determined as described under "Experimental Procedures." The nomenclature for the various substrates with an extra nucleotide (Bulge) is illustrated in Fig. 2B. Catalytic efficiencies (kcat/Km) are expressed relative to that determined for a DNA substrate that is properly annealed (None). No DNA synthesis was observed when the extra base was in the primer strand immediately upstream of the 3’-primer (i.e. P−2). Substituting T or THF for the C at P−2 did not restore DNA synthesis. P+4 represents a control frameshift intermediate substrate where the extra nucleotide is in the downstream oligonucleotide 14 nucleotides downstream of the templating cytosine residue. DNA polymerase β is not believed to significantly interact with this portion of the DNA duplex.

together, these results are consistent with a realignment of the primer terminus to satisfy the hydrogen bonding capacity of the terminal primer base. Consequently, when the templating base is cytosine, the terminal deoxyguanosine pairs with the templating base, effectively producing nicked DNA and inhibiting further DNA synthesis. When the templating base is guanine, dCTP insertion results in a primer terminus that can pair with the templating deoxyguanosine or the preceding deoxyguanosine of the G/G mismatch. In this latter case, the realignment produces a one-nucleotide gap with a templating guanine that can support the insertion of a second dCTP (i.e. n + 2). The realignment of the primer terminus was not observed with correct insertion opposite thymidine (dATP) or deoxyadenosine (dTTP).

Influence of Frameshift Intermediates on Catalytic Efficiency—The ability of a polymerase to produce addition or deletion errors is partially dependent on the ability to extend these intermediates. An addition error (+1 frameshift) is characterized by intermediates with an “extra” unpaired base in the primer strand, whereas a deletion error (−1 frameshift) is characterized by intermediates with an extra base in the template strand (4, 24). In vitro, pol β is observed to produce significantly more deletion errors than addition errors (26).

Introduction of frameshift intermediates in the template/primer stem strongly diminishes the catalytic efficiency for correct nucleotide insertion (Fig. 5). Accordingly, such mutagenic intermediates influence the molecular organization of the polymerase active site. In general, an extra nucleotide in the primer strand was more detrimental than in the template strand in the same position. For example, nucleotide insertion was completely abrogated with an extra cytosine residue behind the primer terminus (i.e. P−2). In contrast, an extra cytosine residue at T−2 resulted in a 2000-fold loss of catalytic efficiency. Modifying the identity of the extra base (e.g. T) or using a synthetic abasic site (i.e. tetrahydrofuran (THF)) at this position in the primer strand did not restore measurable insertion. However, moving the extra base upstream of the polymerase active site at least 5 nucleotides or 14 base pairs downstream restored catalytic efficiency to nearly that observed with an unmodified gapped DNA substrate.

Effect of an Abasic Site in the Template/Primer Stem on Catalytic Efficiency—Base stacking represents an important energetic source for the stability of duplex DNA. Perturbing these stacking interactions by introducing an abasic site analogue, THF, at strategic positions in the primer or template strands dramatically diminished the catalytic efficiency for insertion of the correct nucleotide (Table IV). Positioning the THF residue opposite the cytosine at the primer terminus (i.e. position T−1) dramatically reduced single-nucleotide gap-filling DNA synthesis. Substituting adenine for cytosine at the primer terminus resulted in a 6-fold increase in catalytic efficiency. Moving the abasic site one nucleotide upstream of the growing DNA terminus resulted in a less detrimental effect on catalytic efficiency. Again, an abasic site in the primer strand was more detrimental to catalytic efficiency than when it was in the opposite template strand.

DISCUSSION

As observed previously for pol β in a different DNA sequence context (19), correctly matched base pairs at the growing DNA terminus influence the catalytic efficiency for the insertion of the correct nucleotide. Significantly, this result indicates that matched base pairs at the template/primer terminus for pol β are not symmetrical. In contrast, Watson-Crick base pairs in duplex DNA are geometrically symmetrical and have a sequence independent disposition of hydrogen bonding groups in the DNA minor groove (27). Crystallographic structures of several DNA polymerases indicate that duplex DNA deviates from the standard B-form near the polymerase active site (23, 28–31), suggesting that the polymerase may influence the structure of the primer terminus upon formation of a ternary substrate complex.

As observed with other DNA polymerases (20, 21, 32), DNA substrates with matched or mismatched primer termini bind to pol β with similar affinities (Table III) (33). The similar affinities suggest that there are few polymerase interactions with the bases of the template/primer terminus in the polymerase-DNA binary complex. This suggestion is supported by the crystallographic structure of the open binary pol β-DNA complex (34). In contrast, the structure of the ternary substrate complex where the N-subdomain has closed upon the nascent base pair indicates that Arg283 and Tyr271 have been repositioned to form
and matched termini (Eq. 5), the magnitude of this parameter will be highly dependent on the fidelity of the polymerase. As noted previously (39), DNA polymerase fidelity is strongly coupled to the efficiency for correct, but not incorrect, nucleotide insertion. In other words, two DNA polymerases that exhibit identical insertion efficiencies on a mismatch but differ 100-fold in their efficiency to insert a correct nucleotide on a matched terminus would exhibit relative mispair extension efficiencies that differ by 100-fold. For example, pol β inserts a nucleotide on a G/T mismatch with a catalytic efficiency of $0.005 \text{M}^{-1} \text{s}^{-1}$ (Table I), whereas DNA polymerase ζ inserts a correct nucleotide with a catalytic efficiency that is 4-fold lower (35). Since the catalytic efficiencies for inserting a correct nucleotide on a matched G/C template/primer terminus are 1 and 0.037 $\mu\text{M}^{-1} \cdot \text{s}^{-1}$ for pol β and ζ, respectively, $f_{\text{pol}}^0$, would be 0.005 (pol β) and 0.033 (pol ζ). Thus, the elevated $f_{\text{pol}}^0$ observed for pol ζ is a reflection of its lower catalytic efficiency on matched termini rather than an intrinsic propensity to extend mispairs as has been suggested (35). This example highlights the care that must be used in attempting to extrapolate the intrinsic relative mispair extension efficiencies among different DNA polymerases.

Since the efficiency of correct nucleotide insertion is dependent on the identity of the terminal mismatched base pair, the polymerase is expected to have a significant influence on the structure(s) of the specific mispair. Molecular dynamics simulations of terminal mismatches in the confines of the pol β active site have suggested that the geometry of transversion mispairs (G/G and C/C) are more distorted than the A/C transition mispair (40), offering a structural origin for the kinetic consequence of these mispairs. DNA sequence is also expected to have a profound influence on the structure of the mismatch and therefore on polymerase-DNA interactions. As discussed above, such an instance was observed with extension of the G/G mismatch when the identity of the templating base was C (Fig. 4).

The structures of DNA mismatches at the template/primer terminus in the confines of an A-family DNA polymerase have recently been reported (41). In many instances, the structure of the mismatch is similar to those reported previously in duplex DNA in the absence of protein. However, several mismatches exhibited a frayed conformation (e.g. A/A) or were disordered (e.g. A/C). As noted by the authors, these later observations may indicate that there are several conformers in equilibrium underscoring the dynamic nature of the polymerase, DNA, and their interactions.

The binding affinities for the incoming correct nucleotide utilizing a mismatched primer terminus (Table I) are generally higher than the binding affinity for the incorrect nucleotide producing that specific mispair (Table II). Surprisingly, the binding affinity for the incoming nucleotide with several mismatched primer termini suggests that the binding pocket for the incoming nucleotide is not significantly perturbed, although the rate of nucleotide insertion is dramatically decreased. For example, the A/A or G/G primer termini do not perturb the $K_{\text{d(GNTP)}}$, but strongly inhibit nucleotide insertion. Consistent with this proposal is the observation that the addition of the next correct nucleotide with a gapped DNA substrate with an A/A mismatched terminus results in an apparent stronger DNA binding (Table III). Thus, dGTP binds but is not inserted. The observation that purine-purine mispairs do not occlude the dNTP-binding pocket suggests that one of the glycosidic torsion angles may be in syn-conformation to accommodate the large purine rings. The G/G mismatch situated at the primer terminus in the confines of an A-family DNA polymerase indicates that the primer G base has flipped into a syn-conformation (41). The ability of pol β to bind an incoming
nucleotide that is inserted very slowly such as on a mismatched primer terminus (e.g. G/G; Table I) could be biologically significant. In a cellular environment where all the dNTPs are present, correct dNTP binding after a base substitution error would result in a dead end complex. The apparent tighter DNA binding affinity would limit access to the mismatch for an extrinsic proofreading exonuclease. Fortunately, transcription intermediates are generally the most difficult base substitution errors for pol β to make (Table II).

Base stacking is a major stabilizing force in DNA. Structural and thermodynamic analysis of abasic sites in duplex DNA indicates that they disturb local stacking interactions with the adjacent base pairs, but not the global DNA conformation (42, 43). Structures of a Y-family DNA polymerase, Sulfolobus solfataricus DNA polymerase IV, complexed with DNA containing abasic sites indicate that the conformation of the abasic site was dependent on the surrounding DNA sequence (44). In some instances, the abasic site was observed to be in equilibrium between two conformations, resulting in misaligned template and primer strands. These structural observations coincide with the low frameshift fidelity of this polymerase (45). For pol β, the introduction of an abasic site opposite the primer terminus resulted in a profound loss of catalytic efficiency (T1, Table IV). The catalytic efficiency was greater than 6-fold higher when the primer terminus was adenine rather than cytosine, consistent with potentially better base stacking attributes provided by adenine. When the abasic sites were positioned one base pair upstream of the primer terminus (i.e. position −2), the abasic site in the primer strand was more detrimental to active site function than when it was positioned in the template strand at the equivalent position.

As observed with the introduction of abasic sites near the primer terminus, introducing extra nucleotides in the primer strand appears to have a greater effect than when inserted in the equivalent position in the template strand (Fig. 5). When the extra base was positioned more than 5 nucleotides from the primer terminus, the catalytic efficiency was affected less than 10-fold. The structure of pol β with bound DNA indicates that there are protein-DNA interactions 4–5 base pairs upstream of the active site (34). The small influence of the extra base on catalytic efficiency beyond the −5-position may suggest that the extra cytosine produces a local structural change that is transmitted to the polymerase active site.

The observation that the effects on catalytic efficiency are larger when unpaired bases are introduced into the primer strand indicates that the primer terminus is extremely sensitive to primer strand modifications. Molecular dynamics simulations of terminal mispairs in the pol β active site have noted that the geometry of the primer terminus appears less stable than the noncomplementary template nucleotide (40). In the structure of the closed ternary substrate complex, Tyr271 and Arg283 hydrogen-bond to the minor groove edge of the primer base and the sugar of the template nucleotide, respectively (34). In the structure of the open binary complex, these interactions are absent, since the N-subdomain (residues 262–335) is positioned away from the nascent base pair binding pocket.

Alanine substitution for Arg283 results in a dramatic decrease in catalytic efficiency for insertion of the correct nucleotide on a matched primer terminus (7, 39), precluding characterization with a mismatched terminus. In contrast, alanine substitution for Tyr271 has little or no effect on insertion efficiency with matched (9) or mismatched termini.5 Additionally, the Y271A mutant exhibited an identical catalytic efficiency when an abasic site was situated opposite the primer terminus at T−1.5

 Unexpectedly, these results indicate that Tyr271 does not play a critically important role during correct nucleotide insertion independent of whether the primer terminus is properly base-paired. Alternatively, the hydrogen bond provided by Tyr271 may be very important to catalytic cycling, but the polymerase has compensated for the loss of this hydrogen bond by altering hydrogen bonding networks, as suggested by molecular dynamics simulations (46).

**Conclusion**—The results presented here demonstrate that DNA sequence and structure have a profound influence on the nascent base pair binding pocket of pol β that translates into altered active site function. In addition, it is not unexpected that the polymerase in turn will also induce local DNA conformational changes to optimize hydrogen bonding and stacking interactions. The conformational flexibility of the enzyme, substrates, and complexes makes it difficult to unambiguously predict the dynamic behavior of a polymerase during catalytic cycling. However, through a multifaceted approach employing structural, kinetic, and modeling techniques, general and distinct strategies utilized by DNA polymerases may be identified.

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