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Discrimination between Right and Wrong Purine dNTPs by DNA Polymerase I from *Bacillus stearothermophilus*†

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ABSTRACT: We used a series of dATP and dGTP analogues to determine how DNA polymerase I from *Bacillus stearothermophilus* (BF), a prototypical A family polymerase, uses N-1, N^2, N-3, and N^6 of purine dNTPs to differentiate between right and wrong nucleotide incorporation. Altering any of these nitrogens had two effects. First, it decreased the efficiency of correct incorporation of the resulting dNTP analogue, with the loss of N-1 and N-3 having the most severe effects. Second, it dramatically increased the rate of misincorporation of the resulting dNTP analogues, with alterations in either N-1 or N^6 having the most severe impacts. Adding N^2 to dNTPs containing the bases adenine and purine increased the degree of polymerization opposite T but also tremendously increased the degree of misincorporation opposite A, C, and G. Thus, BF uses N-1, N^2, N-3, and N^6 of purine dNTPs both as negative selectors to prevent misincorporation and as positive selectors to enhance correct incorporation. Comparing how BF discriminates between right and wrong dNTPs with both B family polymerases and low-fidelity polymerases indicates that BF has chosen a unique solution vis-à-vis these other enzymes and, therefore, that nature has evolved at least three mechanistically distinct solutions.

A key issue facing DNA polymerases is how to minimize incorporation of incorrect dNTPs to avoid the potentially deleterious effects of mutagenesis. For "high-fidelity" DNA polymerases, the measured error frequencies vary from around 10^{-3} to 10^{-5} errors per dNTP polymerized (*1*, *2*). However, despite substantial study by a variety of techniques, including protein mutagenesis, substrate mutagenesis, and high-resolution structural approaches (*3*−*11*), the key features that differentiate right and wrong dNTPs as well as the mechanisms that polymerases employ to accomplish this differentiation remain rather unclear.

Several different mechanisms that could allow a DNA (or RNA) polymerase to accurately copy a template have been proposed. Discrimination based on a requirement to form Watson−Crick hydrogen bonds was one of the first mechanisms proposed, and several polymerases may well employ this mechanism (*12*−*15*). For example, both human and herpes DNA primases, two very low-fidelity enzymes, appear to require formation of Watson−Crick hydrogen bonds to efficiently polymerize a NTP. The low-fidelity DNA pol η and DNA pol IV from *Sulfolobus solfataricus* also did not efficiently polymerize several dNTPs incapable of forming Watson−Crick hydrogen bonds. A second model posits that the shape of the base pair formed by the incoming dNTP and template base provides the key factor for discriminating between right and wrong (d)NTPs (*16*−*20*). Indeed, Kool and co-workers showed that both T7 DNA polymerase and Klenow fragment, two high-fidelity A family polymerases, will efficiently and accurately generate base pairs between adenine and 2,4-difluorotoluene nucleotides. Importantly, the shape of the latter base closely mimics the shape of thymine. Furthermore, they showed that varying the shape of the modified toluene significantly altered the efficiency of polymerization in a manner consistent with the "shape selectivity" model. However, several groups have shown that Klenow fragment also has the ability to efficiently generate base pairs between nucleotides whose bases cannot form a correctly shaped base pair (*7*, *9*, *21*−*23*). Finally, a combined use of both positive and negative selectivity has been proposed for two B family replicative polymerases, pol α and herpes DNA polymerase (*7*, *24*, *25*). In this model, the enzyme used specific chemical features of the incoming dNTP to both prevent misincorporation (N-1, N-3, and N^6 in the case of dATP) and enhance correct incorporation (formation of Watson−Crick hydrogen bonds).

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The large fragment of DNA polymerase I from *Bacillus stearothermophilus* (BF)[1] is an A family, high-fidelity polymerase (*26*). It exhibits substantial homology with other A family polymerases that have been characterized kinetically and structurally, including Klenow fragment of DNA polymerase I (*Escherichia coli*), T7 DNA polymerase, and KlenTaq from *Thermus thermophilus* (*3*, *27*−*30*). BF has been extensively characterized structurally, with high-resolution structures of the apoenzyme, BF−DNA binary complexes, and BF−DNA−dNTP ternary complexes available (*4*, *5*, *31*, *32*). Indeed, BF even retains catalytic activity in the crystal such that the products of multiple rounds of replication can be observed using X-ray crystallography.

We have examined the interactions of BF with a panel of purine dNTP analogues to improve our understanding of how this enzyme uses N-1, N^2, N-3, and N^6 to discriminate between right and wrong dNTPs. Remarkably, all four nitrogens played major roles in both preventing misincorporation and promoting correct incorporation by BF. Furthermore, addition of N^2 to dATP and related purines dNTPs enhanced both correct and incorrect polymerization of the resulting dNTPs.

MATERIALS AND METHODS

Materials. BF was prepared as previously described (*5*). All reagents were of the highest quality commercially available. Radiolabeled nucleotides were purchased from Perkin-Elmer, and unlabeled nucleotides were either purchased from Sigma and Trilink Biotechnologies or synthesized as previously described (*7*, *21*, *24*). Synthetic oligonucleotides were purchased from Biosearch Technologies, and their concentrations were determined spectrally. All materials from commercial sources were used without further purification.

5′ ^{32}P Labeling of Primers and Annealing of Primer-Templates. DNA primers were 5′-^{32}P-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP, gel purified, and annealed to the appropriate template as described previously (*33*, *34*). Stocks were stored at −20 °C.

Polymerization Assays with BF Polymerase. All reactions were carried out under steady-state conditions. Assays contained enzyme (2−10 nM, depending upon the analogue), 500 nM 5′-^{32}P-labeled primer-template, 50 mM tris(hydroxymethyl)aminomethane, HCl salt (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 2.5% glycerol, and various concentrations of dNTPs or dNTP analogues. Polymerization reactions were initiated by the addition of enzyme, and depending on the ability of BF to polymerize the analogue, the reaction mixtures were incubated for 5−20 min at 37 °C. Reactions were quenched with a 15 μL of gel loading buffer (90% formamide). Control experiments showed that the rates remained constant over the times used. Polymerization products were separated by denaturing gel electrophoresis (20% acrylamide, 7.5 M urea) and analyzed via phosphorimagery (ImageQuant). Kinetic parameters and errors were determined by fitting the data to the Michaelis−Menten equation as previously described (*24*).

RESULTS

To understand how BF discriminates between right and wrong dNTPs, we examined polymerization of a panel of purine dNTP analogues containing modified bases (Figure 1). The modifications ranged from relatively minor (e.g., loss of N-1, N^2, N-3, or N^6 and/or conversion to another group) to severe (e.g., loss of N-1, N-3, and N^6 from adenine and addition of a CF_3 or NO_2 group). Polymerization of both the natural and analogue dNTPs was measured using primer-templates of defined sequence (Table 1). With one exception, the four primer-templates vary only in the identity of the template base being copied, thereby minimizing the possibility of DNA sequence affecting the results. In the case of DNAt, the second single-stranded template base was also changed to eliminate the possibility of consecutive dATP polymerizations. Consistent with the reported high fidelity of BF, the enzyme efficiently discriminated against polymerization of incorrect, natural dNTPs on these primer-templates (Table 2). In this, and in all subsequent tables, discrimination opposite a template T or C is defined by how much less efficiently BF polymerized the tested dNTP than it polymerized either dATP (15 μM^{-1} min^{-1}) or dGTP (3.4 μM^{-1} min^{-1}), respectively. Opposite a template A or G (i.e., correct TTP and dCTP incorporation), we defined discrimination by comparing (analogue) dNTP incorporation to the average k_{cat}/K_M for correct incorporation of dATP and dGTP opposite T and C, respectively (9.2 μM^{-1} min^{-1}).

Incorporation of a series of hydrophobic purine dNTPs was assessed opposite the four natural template bases under steady-state conditions (Table 3). In most cases, BF polymerized the analogues opposite a natural template base no better than or only slightly more efficiently than an incorrect, natural dNTP. In a few cases, however, BF incorporated the analogue dNTP significantly faster than a natural, incorrect dNTP (e.g., 6-trifluoromethylbenzimidazole dNTP opposite either A or G).

N-3 Affects both Correct and Incorrect dNTP Polymerization. We then examined the roles of four nitrogens in a purine, N-1, N^2, N-3, and N^6, by systematically varying their presence or absence in purine analogues. Adding N-3 to dZTP (4-methylbenzimidazole dNTP) to generate dQTP (1-deaza-6-methylpurine dNTP), a substitution that replaces an electropositive hydrogen with a free pair of electrons, had only modest effects; at most, the average efficiency of polymerization increased very

[1]Abbreviations: 2-aminoadenine dNTP or d2AATP, 2-aminoadenine-2′-deoxyriboside triphosphate; 2-amino-1-deazapurine dNTP or d2A1DPTP, 2-amino-1-deazapurine-2′-deoxyriboside triphosphate; 2-aminopurine dNTP or d2APTP, 2-aminopurine-2′-deoxyriboside triphosphate; benzimidazole dNTP or dBTP, benzimidazole-2′-deoxyriboside triphosphate; BF, large fragment of DNA polymerase I from *B. stearothermophilus*; 6-chloropurine dNTP or d6ClPTP, 6-chloropurine-2′-deoxyriboside triphosphate; 1-deazaadenine dNTP or d1DATP, 1-deazaadenine-2′-deoxyriboside triphosphate; 3-deazaadenine dNTP or d3DATP, 3-deazaadenine-2′-deoxyriboside triphosphate; 3-deazaguanine dNTP or d3DGTP, 3-deazaguanine-2′-deoxyriboside triphosphate; 1-deazapurine dNTP or d1DPTP, 1-deazapurine-2′-deoxyriboside triphosphate; 3-deazapurine dNTP or d3DPTP, 3-deazapurine-2′-deoxyriboside triphosphate; pol α, DNA polymerase α; pol η, DNA polymerase η; 4-methylbenzimidazole dNTP or dZTP, 4-methylbenzimidazole-2′-deoxyriboside triphosphate; 6-methyl-1-deazapurine dNTP or dQTP, 6-methyl-1-deazapurine-2′-deoxyriboside triphosphate; d6MePTP, 6-methylpurine-2′-deoxyriboside triphosphate; d5NO_2BTP, 5-nitrobenzimidazole-2′-deoxyriboside triphosphate; d6NO_2BTP, 6-nitrobenzimidazole-2′-deoxyriboside triphosphate; purine dNTP or dPTP, purine-2′-deoxyriboside triphosphate; d4CF_3BTP, 4-trifluoromethylbenzimidazole-2′-deoxyriboside triphosphate; 6-trifluoromethylbenzimidazole dNTP or d6CF_3BTP, 6-trifluoromethylbenzimidazole-2′-deoxyriboside triphosphate; d7CF_3BTP, 7-trifluoromethylbenzimidazole-2′-deoxyriboside triphosphate.

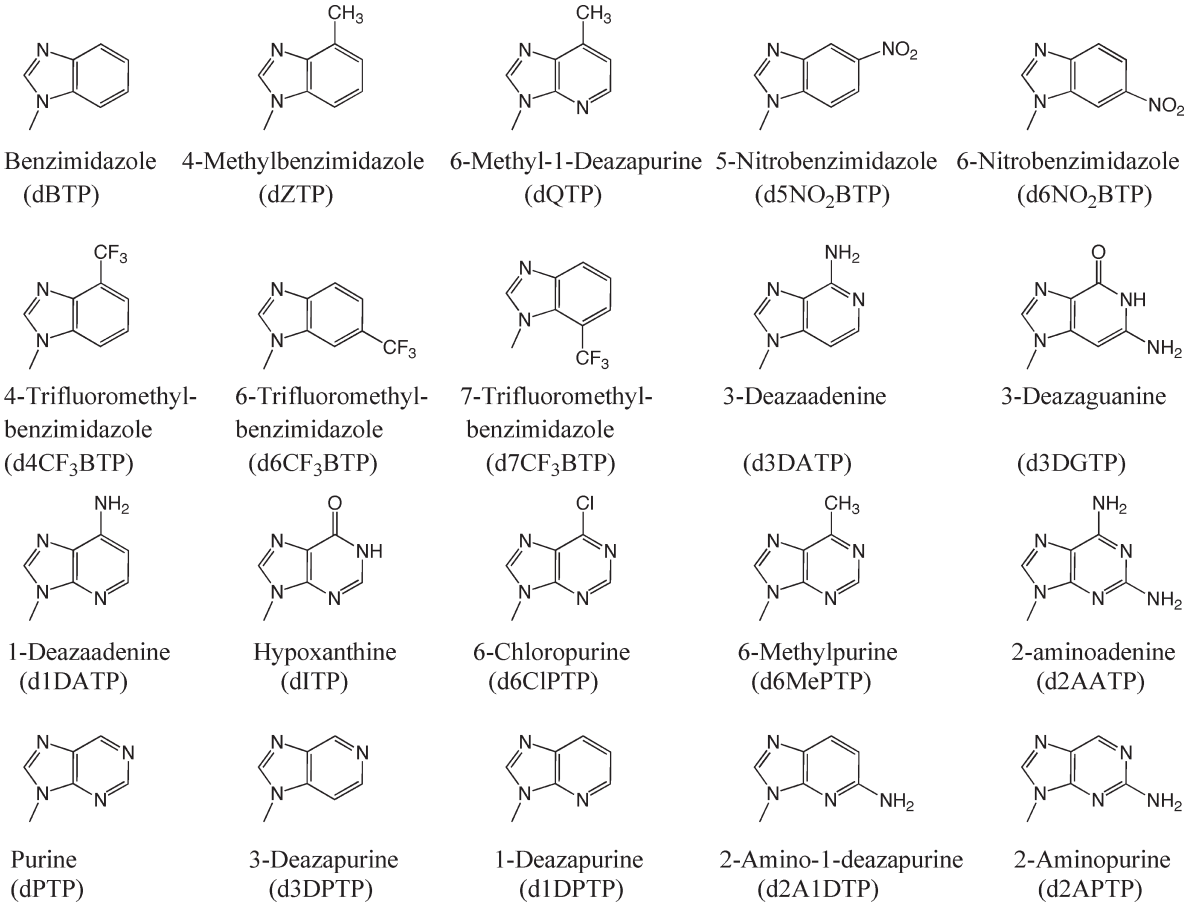


FIGURE 1: Structures of the bases on the dNTPs. The structures of the various bases examined are shown along with the abbreviations for the resulting dNTPs used in Tables 3 and 4.

Table 1: Sequences of Primer-Templates[a]

DNAa	5′- TCCATATCACAT 3′- AGGTATAGTGTA**A**TTCTTATCATCT
DNAc	5′- TCCATATCACAT 3′- AGGTATAGTGTA**C**TTCTTATCATCT
DNAg	5′- TCCATATCACAT 3′- AGGTATAGTGTA**G**TTCTTATCATCT
DNAt	5′- TCCATATCACAT 3′- AGGTATAGTGTA**T**TATCTTATCATCT

[a] The letter after DNA designates the template base being replicated.

slightly (Table 3).[2] In contrast, adding N-3 (purine numbering system) to benzimidazole dNTP and thereby generating 1-deazapurine dNTP dramatically increased the efficiency of incorporation, even though a base pair between any natural base and 1-deazapurine will still lack any Watson−Crick hydrogen bonds and the shape will be distinct from a canonical base pair (Tables 3 and 4). Similarly, adding N-3 to 3-deazapurine dNTP also increased the degree of polymerization of the resulting purine dNTP opposite A, T, and C by factors of 2.5, 21, and 8, respectively, while marginally decreasing the degree of polymerization opposite G [by 10% (Table 4)].

We further probed the role of N-3 by removing it from two canonical, high-fidelity nucleotides, dATP and dGTP (Table 4). The resulting dNTPs, 3-deaza-dATP and 3-deaza-dGTP, differed from dATP and dGTP in two significant ways. First, they were polymerized much less efficiently opposite their complementary template nucleotides, T and C, respectively. Second, the efficiency of misincorporation of 3-deaza-dATP and 3-deaza-dGTP often increased substantially compared to that of their parent compounds. For example, BF misincorporated 3-deaza-dGTP opposite either A or G ≥20-fold more efficiently than it misincorporated dGTP. Curiously, in two cases, polymerization of 3-deaza-dGTP opposite T and 3-deaza-dATP opposite G, removing N-3 decreased the level of misincorporation. Thus, N-3 appears to have two distinct roles, enhancing incorporation and, at least within the context of adenine and guanine, managing misincorporation frequencies.

Effects of N^2 on dGTP and dATP Polymerization. The role of N^2 was examined within the context of both guanine- and adenine-related bases (Table 4). Removing N^2 from dGTP, thereby generating dITP, both decreased the efficiency of polymerization opposite C and increased the level of misincorporation, particularly opposite A and T. The 10-fold decrease in polymerization efficiency opposite C corresponds to a ΔG of 1.4 kcal mol^{-1}, in the range of what one would expect for a hydrogen

[2] In these studies, we have only compared the efficiency of polymerization of each analogue (V_{max}/K_M). BF is a moderately processive enzyme; hence, DNA dissociation limits the steady-state turnover rate after incorporation of a correct dNTP. Since we do not know the rate-limiting step during polymerization of the analogues, the V_{max} values for polymerization of different dNTPs cannot be directly compared. Likewise, K_M and K_D cannot safely be compared due to the potential for different rate-limiting steps.

Table 2: Polymerization of dATP and dGTP Opposite Each Natural Template Base

dNTP	template	V_{max} (min^{-1})	K_M (μM)	V_{max}/K_M ($min^{-1}\,\mu M^{-1}$)	discrimination[a]
dGTP	DNAa	0.28 ± 0.05	720 ± 260	0.00039	24000
	DNAt	0.57 ± 0.18	1140 ± 760	0.00050	30000
	DNAc	4.1 ± 3	1.2 ± 0.3	3.4	1
	DNAg	0.15 ± 0.02	380 ± 130	0.00039	24000
dATP	DNAa	0.73 ± 0.07	590 ± 130	0.0012	7700
	DNAt	15 ± 1	1.0 ± 0.3	15	1
	DNAc	0.11 ± 0.01	130 ± 60	0.00085	4000
	DNAg	0.14 ± 0.01	110 ± 46	0.0013	7100

[a] Discrimination values are defined as V_{max}/K_M for the correct dNTP opposite that template base (i.e., dATP:T, dGTP:C, etc.) divided by V_{max}/K_M for the noted dNTP (or dNTP analogue) opposite that template base.

bond. Adding N^2 to dATP, thereby forming 2-amino-dATP, had unexpectedly large effects during polymerization opposite all four natural template bases. BF polymerized 2-amino-dATP opposite a template T slightly more efficiently than it polymerized dATP. More surprisingly, the presence of N^2 significantly weakened the ability of BF to identify 2-amino-dATP as wrong opposite A, C, and G. Depending upon the mismatch examined, BF misincorporated 2-amino-dATP 37−85-fold more efficiently.

In contrast to the large effects upon addition of N^2 to dATP, adding N^2 to purine dNTP and 1-deazapurine dNTP had much milder effects (Table 4). BF polymerized 2-aminopurine dNTP opposite all four template bases 3−11-fold more efficiently than purine dNTP. Comparing polymerization of 1-deazapurine dNTP and 2-amino-1-deazapurine dNTP showed that the presence of N^2 affected polymerization opposite A, T, and G by <4-fold and only increased the degree of polymerization opposite C by a factor of 8. Thus, the effects of N^2 appear to be very dependent upon the rest of the base.

Removing N^6 from dATP Inhibits Polymerization Opposite T and Stimulates Misincorporation. N^6 of adenine normally forms a hydrogen bond with O^4 of thymine. Converting dATP into purine dNTP resulted in a 23-fold decreased efficiency of polymerization opposite T (Table 4). Additionally, BF misincorporated purine dNTP much more efficiently opposite the other three template bases. The loss of N^6 most severely impacted misincorporation opposite C, where the level of misincorporation increased by a factor of 22.

In a different context, removing N^6 from 1-deaza-dATP to generate 1-deazapurine dNTP, the loss of N^6 had quite different effects than with dATP. Compared to 1-deaza-dATP, BF incorporated 1-deazapurine dNTP with similar efficiency opposite A, only 2-fold less efficiently opposite T, but 13- and 6-fold less efficiently opposite C and G, respectively (Table 4). Comparison of these two sets of data indicates that the effects of N^6 vary substantially depending upon the rest of the base.

The role of exocyclic substituents at C-6 was further probed by replacing N^6 with either an electron-withdrawing Cl or a slightly electron-donating CH_3 (Table 4). As occurred upon replacement of N^6 with H, both of these latter two replacements inhibited polymerization opposite T and increased the level of misincorporation opposite A, C, and G. Comparing the effects of replacing N^6 shows that Cl and CH_3 replacements gave less misincorporation opposite A than replacement with H, while the CH_3 group gave more misincorporation opposite G and less misincorporation opposite C than replacement with H and Cl.

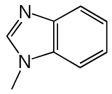
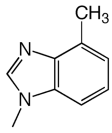
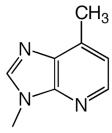
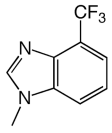
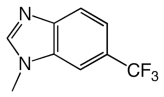
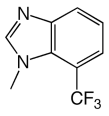
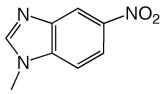
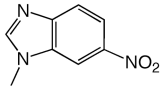
N-1 Is Important for both Correct Incorporation and Preventing Misincorporation. N-1 normally forms a Watson−Crick hydrogen bond with the NH-3 group of thymine. Not surprisingly, removing N-1 from either purine dNTP or dATP significantly impairs polymerization opposite T (Table 4). Additionally, BF misincorporates 1-deaza-dATP much more efficiently than dATP, indicating that N-1 plays a role in preventing misincorporation. In contrast, converting purine dNTP into 1-deazapurine dNTP had variable effects on fidelity. It decreased the level of misincorporation opposite C by 9-fold, had little effect on misincorporation opposite G (25% increase), and increased the level of misincorporation opposite A by 4-fold. Thus, just like the effects of N^2 and N^6, the impact of N-1 depends upon the rest of the base.

DISCUSSION

We examined the roles of N-1, N^2, N-3, and N^6 for both adenine- and guanine-based dNTPs during polymerization by BF. Removing any one of these nitrogens had two effects: decreased level of polymerization of the resulting dNTP within the context of a correct base pair and enhanced misincorporation. Importantly, the effects of each nitrogen depended greatly on the structure of the rest of the base.

The effects of specific functional groups exhibited a tremendous dependence upon the rest of the base. For example, with most bases examined, the presence of N-3 greatly increased the degree of polymerization, but not in the case of converting 4-methylbenzimidazole dNTP into 1-deaza-6-methylpurine dNTP. Analogously, adding N^2 to both dATP and purine dNTP increased the level of incorporation, whereas adding N^2 to 1-deazapurine dNTP had minimal effects. In other words, removing N-1 from dATP increased the level of misincorporation opposite A, C, and G, but removing N-1 from either purine dNTP or 1-deazapurine dNTP inhibited polymerization. Using another A family polymerase, Klenow fragment, Romesberg et al. have also shown that the effects of modifying a specific functional group can vary depending upon the rest of the base (*35*). Modifying furo[2,3-*c*]pyridin-7(6*H*)-one dNTP into furo[2,3-*c*]pyridine-7-thiol dNTP versus modifying furo[3,2-*c*]pyridin-4(5*H*)-one dNTP into furo[3,2-*c*]pyridine-4-thiol dNTP (Figure 2), each of which involves converting an oxygen into sulfur, had very different effects on polymerization of the thiol-containing dNTPs, up to >3000-fold in the case of polymerization opposite a template C. This base dependence, in combination with each functional group playing a role in both enhancing correct incorporation and preventing it, suggests that BF uses a holistic, integrative approach in its interactions with the base of the incoming dNTP. Rather than using specific chemical features of the base for specific functions, BF "sees" and "interprets" the entire base when deciding whether to polymerize an incoming

Table 3: Polymerization of Hydrophobic dNTP Analogues by BF

dNTP	Base Structure	Template	V_{MAX} (min^{-1})	K_M (μM)	V_{max}/K_M ($min^{-1}\,\mu M^{-1}$)	Discrimination [a]
dBTP		DNAa	0.12 ±0.01	250 ±60	0.00048	19000
		DNAt	0.1 ±0.01	260 ±20	0.00047	32000
		DNAc	N.D. [b]	N.D.		>100000
		DNAg	0.57 ±0.03	200 ±30	0.0029	3200
dZTP		DNAa	0.060 ±0.006	170 ±54	0.00034	27000
		DNAt	0.040 ±0.001	11 ±1.6	0.0036	4200
		DNAc	0.032±0.002	170 ±28	0.00019	18000
		DNAg	0.020 ±0.002	68 ±33	0.00029	32000
dQTP		DNAa	0.37 ±0.07	360 ±100	0.0010	9200
		DNAt	0.13 ±0.02	140 ±46	0.00092	16000
		DNAc	0.98 ±0.03	1200 ±440	0.00082	4100
		DNAg	0.11 ±0.01	230 ±70	0.00047	20000
d4CF_3BTP		DNAa	0.65 ±0.57	290 ±200	0.0022	4200
		DNAt	0.23 ±0.16	1700 ±1100	0.00014	>100000
		DNAc	0.14 ±0.01	19 ±7	0.0074	460
		DNAg	0.010 ±0.003	230 ±100	0.000043	>100000
d6CF_3BTP		DNAa	5.6 ±0.02	62 ±14	0.09	100
		DNAt	0.19 ±0.0004	50 ±13	0.0038	3900
		DNAc	0.07 ±0.0001	13 ±4	0.0054	630
		DNAg	0.84 ±0.0007	30 ±4	0.028	330
d7CF_3BTP		DNAa	0.19 ±0.00009	120 ±50	0.0016	5800
		DNAt	0.013 ±0.00005	96 ±60	0.00014	>100000
		DNAc	N.D.	N.D.		>100000
		DNAg	0.0078 ±0.00002	64 ±30	0.00012	77000
d5NO_2BTP		DNAa	0.48 ±0.03	68 ±12	0.0071	1300
		DNAt	0.13 ±0.02	120 ±30	0.0011	14000
		DNAc	0.05 ±0.01	130 ±40	0.00038	8900
		DNAg	1.0 ±0.1	100 ±20	0.01	920
d6NO_2BTP		DNAa	0.89 ±0.03	47 ±5	0.019	480
		DNAt	0.11 ±0.10	77 ±14	0.0014	11000
		DNAc	0.10 ±0.01	140 ±20	0.0071	480
		DNAg	0.25 ±0.10	52 ±6	0.0048	1900

[a] Discrimination values are defined as V_{max}/K_M for the correct dNTP opposite that template base (i.e., dATP:T, dGTP:C, etc.) divided by V_{max}/K_M for the noted dNTP analogue opposite that template base. [b] ND, none detected.

Table 4: Polymerization of Purine dNTPs Modified at N-1, N^2, N-3, and N^6

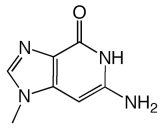
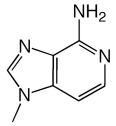
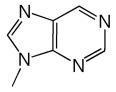
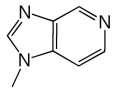
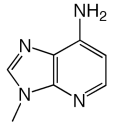
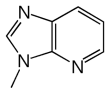
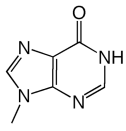
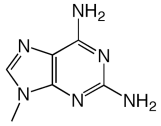
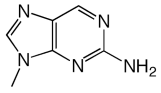
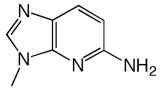
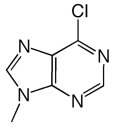
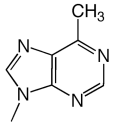
dNTP	Base Structure	Template	V_{MAX} (min^{-1})	K_M (μM)	V_{max}/K_M ($min^{-1}\,\mu M^{-1}$)	Discrimination [a]
d3DGTP		DNAa	1.4 ±0.1	130 ±16	0.011	840
		DNAt	0.041 ±0.008	800 ±260	0.000051	>100000
		DNAc	1.7 ±0.3	210 ±60	0.0081	420
		DNAg	3.2 ±1	410 ±250	0.0078	1200
d3DATP		DNAa	1.0 ±0.2	490 ±170	0.002	4600
		DNAt	51 ±9	200 ±55	0.25	60
		DNAc	0.38 ±0.07	50 ±20	0.0076	450
		DNAg	0.83 ±0.18	1400 ±970	0.00059	16000
dPTP		DNAa	3.9 ±0.2	530 ±50	0.0074	1200
		DNAt	4.4 ±0.2	6.7 ±1.0	0.66	23
		DNAc	1.4 ±0.2	73 ±29	0.019	180
		DNAg	1.2 ±0.1	420 ±100	0.0029	3200
d3DPTP		DNAa	0.19 ±0.02	62 ±15	0.0031	3000
		DNAt	0.47 ±0.02	15 ±3	0.031	480
		DNAc	0.044 ±0.004	20 ±7	0.0022	1500
		DNAg	0.31 ±0.03	97 ±8	0.0032	2900
d1DATP		DNAa	12 ±6	470 ±300	0.026	350
		DNAt	22 ±12	1000 ±600	0.022	680
		DNAc	2.6 ±0.5	100 ±70	0.026	130
		DNAg	3.8 ±0.8	160 ±90	0.024	380
d1DPTP		DNAa	1.1 ±0.1	39 ±3	0.028	330
		DNAt	1.2 ±0.2	100 ±20	0.012	1300
		DNAc	3.8 ±0.5	1900 ±440	0.002	1700
		DNAg	0.51 ±0.12	130 ±30	0.0039	2400
dITP		DNAa	0.22 ±0.01	31 ±7	0.0071	1300
		DNAt	0.32 ±0.03	43 ±16	0.0074	2000
		DNAc	7.1 ±0.4	21 ±3	0.34	10
		DNAg	0.096 ±0.09	210 ±42	0.00046	20000

Table 4. Continued.

dNTP	Base Structure	Template	V_{MAX} (min^{-1})	K_M (μM)	V_{max}/K_M ($min^{-1}\,\mu M^{-1}$)	Discrimination [a]
d2AATP		DNAa	9.3 ±0.5	210 ±47	0.044	210
		DNAt	12 ±0.4	0.38 ±0.04	32	0.47
		DNAc	8.7 ±0.4	120 ±16	0.073	47
		DNAg	15 ±1	150 ±30	0.1	92
d2APTP		DNAa	3.4 ±1	93 ±51	0.037	250
		DNAt	16 ±4	4 ±1	4.0	3.8
		DNAc	4.8 ±0.4	68 ±12	0.049	69
		DNAg	3.5 ±0.7	110 ±41	0.032	290
d2A1DPTP		DNAa	4.2 ±0.4	500 ±90	0.0084	1100
		DNAt	2.5 ±0.2	350 ±60	0.0071	2100
		DNAc	5.2 ±0.2	320 ±40	0.016	210
		DNAg	3.2 ±0.3	480 ±80	0.0067	1400
d6ClPTP		DNAa	1.6 ±0.3	74 ±7	0.022	420
		DNAt	11 ±1	10 ±2	1.1	14
		DNAc	4.4 ±0.2	160 ±30	0.028	121
		DNAg	0.57 ±0.05	220 ±50	0.0026	3500
d6MePTP		DNAa	3.6 ±0.2	190 ±30	0.019	484
		DNAt	20 ±2	14 ±3	1.4	11
		DNAc	2.6 ±0.5	430 ±100	0.0060	570
		DNAg	0.7 ±0.1	55 ±15	0.013	710

[a] Discrimination values are defined as V_{max}/K_M for the correct dNTP opposite that template base (i.e., dATP:T, dGTP:C, etc.) divided by V_{max}/K_M for the noted dNTP analogue opposite that template base.

dNTP. This approach differs from the B family enzymes pol α and HSV pol where specific functional groups serve precise purposes independent of the rest of the base (*7*, *24*, *25*).

The ability of N-1 (dATP), N^2 (dGTP), and N^6 (dATP) to drive correct polymerization is consistent with a key role for Watson−Crick hydrogen bonds. Removing N^6 from dATP reduced the level of correct incorporation by 23-fold, while the loss of N^2 from dGTP reduced the level of correct incorporation by 10-fold. These decreases correspond to losses of 1.9 and 1.4 kcal mol^{-1} in transition-state stabilization, respectively, consistent with the loss of a hydrogen bond. Replacing N-1 of dATP with a CH group decreased the level of polymerization opposite T by 680-fold, or 4.0 kcal mol^{-1}. While the much greater decrease in the degree of polymerization due to removing N-1 might indicate that the hydrogen bond involving N-1 is stronger than those involving either N^6 of adenine or N^2 of guanine, it may also result from the replacement of a favorable interaction with an unfavorable interaction. Whereas N-1 of adenine is electron rich and ideally suited to interact with the electron deficient NH-3 group of thymine, the hydrogen of the CH-1 group of 1-deazaadenine will also be electron deficient such that its interactions with the NH-3 group of thymine may become energetically repulsive.

While the effects of deleting N^2 from dGTP were analogous to the effects of losing either N-1 or N^6 from dATP, the consequences of adding N^2 to dATP were completely unexpected. The slightly enhanced polymerization of 2-amino-dATP opposite T presumably results from the extra hydrogen bond one can form between N^2 of the purine dNTP and O^2 of T. Consistent with this hypothesis, adding N^2 to purine dNTP also resulted in more efficient polymerization opposite T. More remarkably,

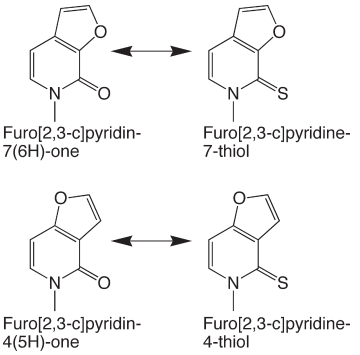


FIGURE 2: Structures of furopyridine dNTP analogues tested with Klenow fragment in ref *35*.

adding N^2 to dATP significantly reduced the capacity of BF to identify the resulting 2-amino-dATP as wrong opposite any template base. Indeed, adding N^2 to dATP largely overcomes the negative consequences of incorrect hydrogen bonding groups at N-1 and N^6 and/or consequences of altered base pair shape. While the formation of a hydrogen bond between N^2 of 2-amino-dATP and O^2 of C could account for this misincorporation event, how N^2 increases the level of misincorporation opposite A and G is less clear. Since preventing misincorporation ultimately requires a polymerase to not recognize an incoming dNTP as correct, i.e., the dNTP does not bind in a polymerizable conformation, the presence of N^2 on dATP allows the 2-aminoadenine to bind in the active site in a manner that BF does not effectively recognize as being wrong.

Why N-3 significantly alters both correct and incorrect dNTP polymerization remains unclear. Similar to BF, Klenow fragment also polymerized 3-deaza-dGTP less efficiently than dGTP (10-fold); hence, it seems likely that these effects will be general to A family polymerases (*36*). Curiously, in structures of E−DNA−dNTP closed complexes with various A family polymerases (KlenTaq, T7 DNA polymerase, and BF), N-3 is stacked against a highly conserved Phe (*3*, *29*, *32*). Potentially, interactions between the electron rich N-3 and electron deficient edge of this Phe could mediate the kinetic effects. Alternatively, the effects of N-3 might occur via an interaction with the protein that occurs prior to formation of the closed complex, such as when the template base resides within the preinsertion site (*37*).

BF could incorporate a variety of dNTPs containing hydrophobic bases opposite all four natural bases. However, it generally discriminated against polymerizing these dNTPs quite strongly, indicating that hydrophobicity alone is not sufficient to drive rapid polymerization of unnatural dNTPs. Using a series of trifluorobenzimidazole dNTPs, all of whose bases will have similar hydrophobicity and size, BF clearly found the 6-trifluorobenzimidazole dNTP to be the most attractive substrate. Indeed, the enzyme polymerized this dNTP opposite a natural template base 4−80-fold more efficiently than an incorrect, natural dNTP. This clear preference for the 6-trifluoromethylbenzimidazole dNTP suggests that shape can contribute to base selectivity, although not necessarily in the way that one would predict. However, it should be noted that these bases will have different dipoles, which may also contribute to the enzymes preferences.

An outstanding and often hotly debated question is the relative importance of base pair shape versus Watson−Crick hydrogen bonds to DNA polymerase fidelity. Among the A family polymerases, different studies have led to very different conclusions (*17*, *38*, *39*). Part of this discrepancy may result from different A family polymerases using different mechanisms, yet studies have clearly shown that Klenow fragment, the paradigm for polymerases using shape, can efficiently polymerize dNTP analogues whose base cannot form a correctly shaped base pair with the template base being replicated (*7*, *21*−*23*). These apparently conflicting observations can be reconciled if the extent to which the polymerase requires Watson−Crick hydrogen bonds and/or correct shape depends upon the overall chemistry and structure of the base. As noted earlier, the effects of Watson−Crick hydrogen bonding groups vary depending upon the base. Additionally, Waksman and colleagues showed that the A family polymerase KlenTaq replicates the four natural template bases with different dynamics and that the structures of the four correct E−DNA−dNTP ternary complexes vary depending upon the template base (*29*, *30*). Both of these results again suggest that the identity of the template base affects kinetic and/or mechanistic details of the reaction.

Different families of polymerases use at least three different mechanisms for discriminating between right and wrong dNTPs. Two very low fidelity RNA polymerases, human and herpes DNA primase, appear to incorporate efficiently only those NTPs that can form Watson−Crick hydrogen bonds with the template base (*12*, *13*). Likewise, two low fidelity DNA polymerases, pol η and DNA pol IV from *S. solfataricus*, also may require formation of Watson−Crick hydrogen bonds to efficiently incorporate a dNTP (*14*, *15*). Thus, direct employment of Watson−Crick hydrogen bonds may be a general strategy of low fidelity polymerases. Two B family polymerases, pol α and herpes DNA polymerase, use a combination of positive and negative selectivity to identify incoming dNTPs as being right or wrong (*7*, *24*, *25*). Unlike the low fidelity enzymes, they often efficiently incorporate dNTPs whose bases lack Watson−Crick hydrogen bonding groups. These polymerases do not require N-3 of purine dNTPs for correct incorporation but do use N-3 to prevent misincorporation. N-1 both helps prevent misincorporation and enhances correct incorporation, while N^6 plays a lesser role. N^2 plays a minor role during correct polymerization of dGTP but is critical for minimizing polymerization opposite A. Since B family polymerases have well-conserved active sites (*10*, *40*, *41*), these general observations will probably apply to most B family enzymes.

BF polymerase, a prototypical A family polymerase, appears to be distinct from these two groups of enzymes. Similar to the B family enzymes, BF uses N-1, N^2, and N^6 to both increase the level of correct incorporation and prevent misincorporation; i.e., enzymes from both families use a combination of positive and negative selectivity. The similarity, however, ends there. While BF occasionally incorporates dNTPs containing hydrophobic bases much more rapidly than an incorrect natural dNTP, it more commonly incorporates hydrophobic dNTP analogues no better than a natural, incorrect dNTP. Klenow fragment, another A family polymerase, also usually, but not always, polymerizes hydrophobic dNTP analogues less efficiently than the B family enzymes, with the key exception being 2,4-dihalotoluenes opposite A (*7*, *21*). For both Klenow fragment and BF, N-3 is critical for rapid correct polymerization of dGTP and dATP (*36* and vide infra), whereas B family polymerases primarily use N-3 to prevent misincorporation. Unlike the results with B family polymerases, the effects of N-1, N^2, N-3, and N^6 depend heavily on the rest of the base. Additionally, these four nitrogens prevent or enhance different sets of mismatches for the different enzymes. Most notably, some B family polymerases specifically misincorporate

2-aminopurine dNTPs opposite C, likely due to formation of a hydrogen bond between the purine N^2 and O^2 atoms of C (*42*).

A key unanswered question is what chemical features of the active sites from polymerases allow different families of enzymes to use different mechanisms to accomplish the common task of discriminating between right and wrong dNTPs. Additionally, how do different polymerases from the same family tune their active sites for different levels of fidelity? Besides providing fundamental insights into polymerase function, answering this question could lead to the development of novel polymerase inhibitors, a medically extremely important class of compounds.

REFERENCES

1. Drake, J. W. (1991) A Constant Rate of Spontaneous Mutation in DNA-based Microbes. *Proc. Natl. Acad. Sci. U.S.A. 88*, 7160–7164.
2. Kunkel, T. A., and Bebenek, K. (1988) Recent Studies on the Fidelity of DNA Synthesis. *Biochim. Biophys. Acta 951*, 1–15.
3. Doublie, S., Tabor, S., Long, A. M., Richardson, C. C., and Ellenberger, T.(1998) Crystal Structure of a Bacteriophage T7 DNA Replication Complex at 2.2 Å Resolution. *Nature 391*, 251–258.
4. Hsu, G. W., Kiefer, J. R., Burnouf, D., Becherel, O. J., Fuchs, R. P., and Beese, L. S. (2004) Observing Translesion Synthesis of an Aromatic Amine DNA Adduct by a High-Fidelity DNA Polymerase. *J. Biol. Chem. 279*, 50280–50285.
5. Kiefer, J. R., Mao, C., Hansen, C. J., Basehore, S. L., Hogrefe, H. H., Braman, J. C., and Beese, L. S. (1997) Crystal Structure of a Thermostable *Bacillus* DNA Polymerase I Large Fragment at 2.1 Å Resolution. *Structure 5*, 95–108.
6. Beckman, R. A., Mildvan, A. S., and Loeb, L. A. (1985) On the Fidelity of DNA Replication: Manganese Mutagenesis *in vitro*. *Biochemistry 24*, 5810–5817.
7. Kincaid, K., Beckman, J., Zivkovic, A., Halcomb, R. L., Engels, J., and Kuchta, R. D. (2005) Exploration of Factors Driving Incorporation of Unnatural dNTPs into DNA by Klenow Fragment (DNA Polymerase I) and DNA Polymerase α. *Nucleic Acids Res. 33*, 2620–2628.
8. Berger, M., Luzzi, S. D., Henry, A. A., and Romesberg, F. E. (2002) Stability and Selectivity of Unnatural DNA with Five-Membered-Ring Nucleobase Analogues. *J. Am. Chem. Soc. 124*, 1222–1226.
9. Henry, A. A., Olsen, A. G., Matsuda, S., Yu, C., Geierstanger, B. H., and Romesberg, F. E. (2004) Efforts to Expand the Genetic Alphabet: Identification of a Replicable Unnatural DNA Self-Pair. *J. Am. Chem. Soc. 126*, 6923–6931.
10. Yang, G., Wang, J., and Konigsberg, W. (2005) Base Selectivity Is Impaired by Mutants that Perturb Hydrogen Bonding Networks in the RB69 DNA Polymerase Active Site. *Biochemistry 44*, 3338–3346.
11. Minnick, D. T., Bebenek, K., Osheroff, W. P., Turner, R. M., Astatke, M., Liu, L., Kunkel, T. A., and Joyce, C. M. (1999) Side Chains That Influence Fidelity at the Polymerase Active Site of *Escherichia coli* DNA Polymerase I (Klenow Fragment). *J. Biol. Chem. 274*, 20967–20975.
12. Ramirez-Aguilar, K. A., Moore, C. L., and Kuchta, R. D. (2005) Herpes Simplex Virus I Primase Employs Watson-Crick Hydrogen Bonding to Identify Cognate NTPs. *Biochemistry 44*, 15585–15593.
13. Moore, C. L., Chiaramonte, M., Higgins, T., and Kuchta, R. D. (2002) Synthesis of Nucleotide Analogs That Potently and Selectively Inhibit Human DNA Primase. *Biochemistry 41*, 14066–14075.
14. Washington, M. T., Helquist, S. A., Kool, E. T., Prakash, L., and Prakash, S. (2003) Requirement of Watson-Crick Hydrogen Bonding for DNA Synthesis by Yeast DNA Polymerase η. *Mol. Cell. Biol. 23*, 5107–5112.
15. DeCarlo, L., Gowda, A. S., Suo, Z., and Spratt, T. E. (2008) Formation of Purine-Purine Mispairs by *Sulfolobus solfataricus* DNA Polymerase IV. *Biochemistry 47*, 8157–8164.
16. Sloane, D. L., Goodman, M. F., and Echols, H. (1988) The Fidelity of Base Selection by the Polymerase Subunit of DNA Polymerase III Holoenzyme. *Nucleic Acids Res. 16*, 6465–6475.
17. Morales, J. C., and Kool, E. T. (1998) Efficient Replication between non-Hydrogen Bonded Nucleoside Shape Analogs. *Nat. Struct. Biol. 5*, 950–954.
18. Morales, J. C., and Kool, E. T. (2000) Varied Molecular Interactions at the Active Sites of Several DNA Polymerases: Nonpolar Nucleoside Isosteres as Probes. *J. Am. Chem. Soc. 122*, 1001–1007.
19. Kim, T. W., Brieba, L. G., Ellenberger, T., and Kool, E. T. (2006) Functional Evidence for a Small and Rigid Active Stie in a High Fidelity DNA Polymerase: Probing T7 DNA Polymerase with Variably Sized Base Pairs. *J. Biol. Chem. 281*, 2289–2295.
20. Kim, T. W., Delaney, J. C., Essigmann, J. M., and Kool, E. T. (2005) Probing the Active Stie Tightness of DNA Polymerase in Subangstrom Increments. *Proc. Natl. Acad. Sci. U.S.A. 102*, 15803–15808.
21. Chiaramonte, M., Moore, C. L., Kincaid, K., and Kuchta, R. D. (2003) Facile Polymerization of dNTPs Bearing Unnatural Base Analogues by DNA Polymerase α and Klenow Fragment (DNA Polymerase I). *Biochemistry 42*, 10472–10481.
22. Leconte, A. M., Hwang, G. T., Matsuda, S., Capek, P., Hari, Y., and Romesberg, F. E. (2008) Discovery, Characterization, and Optimization of an Unnatural Base Pair for Expansion of the Genetic Alphabet. *J. Am. Chem. Soc. 130*, 2336–2343.
23. Matsuda, S., Henry, A. A., and Romesberg, F. E. (2006) Optimization of Unnatural Base Pair Packing for Polymerase Recognition. *J. Am. Chem. Soc. 128*, 6369–6375.
24. Beckman, J., Kincaid, K., Hocek, M., Spratt, T., Engels, J., Cosstick, R., and Kuchta, R. D. (2007) Human DNA Polymerase α Uses a Combination of Positive and Negative Selectivity to Polymerize Purine dNTPs with High Fidelity. *Biochemistry 46*, 448–460.
25. Cavanaugh, N. A., Urban, M., Beckman, J., Spratt, T., and Kuchta, R. D. (2009) Identifying the Features of Purine dNTPs that Allow Accurate and Efficient DNA Replication by Herpes Simplex Virus 1 DNA Polymerase. *Biochemistry 48*, 3554–3564.
26. Phang, S. M., Teo, C. Y., Lo, E., and Wong, V. W. (1995) Cloning and Complete Sequence of the DNA Polymerase-Encoding Gene (BstPolI) and Characterization of the Klenow-like Fragment from *Bacillus stearothermophilus*. *Gene 163*, 65–68.
27. Beese, L. S., Derbyshire, V., and Steitz, T. A. (1993) Structure of DNA Polymerase I Klenow Fragment Bound to Duplex DNA. *Science 260*, 352–355.
28. Beese, L. S., Friedman, J. F., and Steitz, T. A. (1993) Crystal Structure of the Klenow Fragment of DNA Polymerase I Complexed with dNTP and Pyrophosphate. *Biochemistry 32*, 14095–14101.
29. Li, Y., and Waksman, G. (2001) Crystal Structures of a ddATP-, ddTTP-, ddCTP-, and ddGTP-trapped Ternary Complex of Klentaq1: Insights into Nucleotide Incorporation and Selectivity. *Protein Sci. 10*, 1225–1233.
30. Rothwell, P. J., and Waksman, G. (2007) A Pre-equilibrium before Nucleotide Binding Limits Fingers Subdomain Closure by KlenTaqI. *J. Biol. Chem. 282*, 28884–28892.
31. Johnson, S. J., and Beese, L. S. (2004) Structures of Mismatch Replication Errors Observed in a DNA Polymerase. *Cell 116*, 803–816.
32. Keifer, J. R., Mao, C., Braman, J. C., and Beese, L. S. (1998) Visualizing DNA Replication in a Catalytically Active *Bacillus* DNA Polymerase Crystal. *Nature 391*, 304–307.
33. Kuchta, R. D., Benkovic, P., and Benkovic, S. J. (1988) Kinetic Mechanism Whereby DNA Polymerase I (Klenow) Replicates DNA with High Fidelity. *Biochemistry 27*, 6716–6725.
34. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Plainview, NY.
35. Henry, A. A., Yu, C., and Romesberg, F. E. (2003) Determinants of Unnatural Nucleobase Stability and Polymerase Recognition. *J. Am. Chem. Soc. 125*, 9638–9646.
36. McCain, M. D., Meyer, A. S., Schultz, S. S., Glekas, A., and Spratt, T. E. (2005) Fidelity of Mispair Formation and Mispair Extension Is Dependent on the Interaction between the Minor Groove of the Primer Terminus and Arg668 of DNA Polymerase I of *Escherichia coli*. *Biochemistry 44*, 5647–5659.
37. Johnson, S. J., Taylor, J. S., and Beese, L. S. (2003) Processive DNA Synthesis Observed in a Polymerase Crystal Suggests a Mechanism for the Prevention of Frameshift Mutations. *Proc. Natl. Acad. Sci. U.S.A. 100*, 3895–3900.
38. Lee, J. R., Helquist, S. A., Kool, E. T., and Johnson, K. A. (2008) Importance of Hydrogen Bonding for Efficiency and Specificity of the Human Mitochondrial DNA Polymerase. *J. Biol. Chem. 283*, 14402–14410.
39. Kool, E. T. (2002) Active Site Tightness and Substrate Fit in DNA Replication. *Annu. Rev. Biochem. 71*, 191–219.
40. Yang, G., Franklin, M., Li, J., Lin, T. C., and Konigsberg, W. (2002) A Conserved Tyr Residue Is Required for Sugar Selectivity in a Pol α DNA Polymerase. *Biochemistry 41*, 10256–10261.
41. Wang, J., Sattar, A. K., Wang, C. C., Karam, J. D., Konigsberg, W. H., and Steitz, T. A. (1997) Crystal Structure of a Pol α Family Replication DNA Polymerase from Bacteriophage RB69. *Cell 89*, 1087–1099.
42. Patro, J. N., Urban, M., and Kuchta, R. D. (2009) Role of the 2-Amino Group of Purines during dNTP Polymerization by Human DNA Polymerase α. *Biochemistry 48*, 180–189.