

# Role of the 2-Amino Group of Purines during dNTP Polymerization by Human DNA Polymerase $\alpha^{\dagger}$

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**ABSTRACT:** We used a series of dNTP analogues in conjunction with templates containing modified bases to elucidate the role that N<sup>2</sup> of a purine plays during dNTP polymerization by human DNA polymerase  $\alpha$ . Removing N<sup>2</sup> from dGTP had small effects during correct incorporation opposite C but specifically increased misincorporation opposite A. Adding N<sup>2</sup> to dATP and related analogues had small and variable effects on the efficiency of polymerization opposite T. However, the presence of N<sup>2</sup> greatly enhanced polymerization of these dATP analogues opposite a template C. The ability of N<sup>2</sup> to enhance polymerization opposite C likely results from formation of a hydrogen bond between the purine N<sup>2</sup> and pyrimidine O<sup>2</sup>. Even in those cases where formation of a wobble base pair, tautomerization, and/or protonation of the base pair between the incoming dNTP and template base cannot occur (e.g., 2-pyridone•purine (or purine analogue) base pairs), N<sup>2</sup> enhanced formation of the base pair. Importantly, N<sup>2</sup> had similar effects on dNTP polymerization both when added to the incoming purine dNTP and when added to the template base being replicated. The mechanistic implications of these results regarding how pol  $\alpha$  discriminates between right and wrong dNTPs are discussed.

Accurate DNA replication is crucial for cell survival. Fortunately, most replicative DNA polymerases rarely misincorporate dNTPs, exhibiting typical error rates of  $10^{-3}$ – $10^{-6}$  errors per nucleotide replicated (3). When misincorporation does occur, the rate of elongation decreases substantially, thereby allowing for exonucleolytic proofreading by a 3'–5' exonuclease (4).

Presently, the specific mechanisms different polymerases employ to distinguish between right and wrong dNTP substrates during the polymerization reaction remain unclear. Structural and biophysical analysis of various polymerases has suggested that polymerases differentiate between substrates by the opening and closing of the polymerase•DNA complex (5–8). When the complex is in an open conformation, the active site of the enzyme becomes solvent accessible and allows the polymerase to test its dNTP substrates. After initial binding of a dNTP, the mechanism employed for dNTP selection, however, remains a topic of debate. One model posits that Watson–Crick hydrogen bonding between the incoming dNTP and the template base provides the driving force for the incorporation of correct dNTPs. Consistent with this model, the low-fidelity enzymes human primase and herpes primase only efficiently incorporate NTPs that can form Watson–Crick hydrogen bonds with the template base (9, 10). Alternatively, the shape selectivity model posits that the polymerase discriminates between correct and incorrect base pairs based on shape (11–13). To further complicate this issue, recent work with a variety of

polymerases has suggested that different polymerases may use distinct mechanisms (14, 15).

DNA polymerase  $\alpha$  (pol  $\alpha$ )<sup>1</sup> is one of three DNA polymerases essential for replication of nuclear DNA in eukaryotes (16). Biologically, this enzyme elongates primase-synthesized primers to initiate the synthesis of all new DNA strands. This B-family polymerase exhibits relatively low processivity and moderate fidelity (ca.  $10^{-3}$ – $10^{-5}$  errors per nucleotide polymerized (17, 18). Pol  $\alpha$  also lacks 3'–5' exonuclease activity; hence it cannot remove just added incorrect nucleotides.

Recent studies with nucleotide analogues have provided some insight into the chemical properties of the dNTP substrate that pol  $\alpha$  uses to distinguish between right and wrong dNTPs. Similar to some other polymerases, efficient dNTP polymerization by pol  $\alpha$  does not require formation of either a correctly shaped base pair or Watson–Crick hydrogen bonds between the incoming dNTP and the template base (15, 19–22). Instead, during polymerization of dATP and related analogues, N-3 and N-1 prevent misincorporation of a dNTP (analogue) while N-1 and N<sup>6</sup> enhance the correct incorporation of the dNTP, in this case

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<sup>1</sup> Abbreviations: 2-amino-6-chloropurine dNTP or ACldPTP, 2-amino-6-chloropurine-2'-deoxyribose triphosphate; 2-amino-1-deazapurine dNTP or 2A1DdPTP, 2-amino-1-deazapurine-2'-deoxyribose triphosphate; 2-amino-dATP or d2AATP, 2-amino-2'-deoxyadenosine triphosphate; 2-aminopurine dNTP or d2ATP, 2-aminopurine-2'-deoxyribose triphosphate; 6-chloropurine dNTP or ClldPTP, 6-chloropurine-2'-deoxyribose triphosphate; 1-deazapurine dNTP or d1DPTP, 1-deazapurine-2'-deoxyribose triphosphate; 3-deazapurine dNTP or d3DPTP, 3-deazapurine-2'-deoxyribose triphosphate; pol  $\alpha$ , DNA polymerase  $\alpha$ ; purine dNTP or dPTP, purine-2'-deoxyribose triphosphate; pyridone dNTP or d2PyrTP, 2-pyridone-2'-deoxyribose triphosphate; TEAB, triethylamine bicarbonate; Tris-HCl, tris(hydroxymethyl)aminomethane.

opposite a template T. Thus, pol  $\alpha$  uses a combination of positive and negative selectivity to accurately replicate DNA.

While pol  $\alpha$  will frequently polymerize dNTPs bearing highly modified and unusually shaped bases opposite the four natural bases in the template, the converse does not appear to be true: pol  $\alpha$  incorporates natural dNTPs opposite modified template bases much less efficiently (15, 19). Thus, there exists a tremendous asymmetry in how pol  $\alpha$  recognizes base pairs between a natural base and a highly modified base analogue. This result suggests that pol  $\alpha$  recognizes different base pairs between the natural four bases via distinct mechanisms.

To better understand the chemical features of purine bases that impact substrate discrimination by pol  $\alpha$ , we examined the effects of the exocyclic amine at C-2 of purines. The polymerase did not require N<sup>2</sup> of guanine for generating a correct base pair. On the other hand, adding a N<sup>2</sup> to purine bases (e.g., 2-aminopurine) that cannot form any other Watson–Crick hydrogen bonds with cytosine greatly enhanced polymerization of the resulting 2-aminopurine dNTPs opposite cytosine. The implications of these results with respect to the mechanism of substrate selection by pol  $\alpha$  are discussed.

## EXPERIMENTAL PROCEDURES

**Materials.** All reagents were of the highest quality commercially available. Unlabeled natural dNTPs were from Sigma, and radiolabeled dNTPs were from Perkin-Elmer. dITP, 2-amino-dATP, 2-aminopurine dNTP, and 2-amino-6-chloropurine dNTP were from Trilink. Protected phosphoramidites of nucleosides containing the bases 2-aminoadenine, 2-aminopurine, and hypoxanthine were from Glen Research. 2,3,6-Triaminopyridine was from Anichem LLC. Synthetic DNA oligonucleotides were purchased from IDT or Biosearch. The two subunit p180–p70 polymerase complex was expressed in baculovirus-infected SF9 cells at the Tissue Culture Core Facility of the University of Colorado Health Sciences Center and purified as previously described with the following modifications (46). The enzyme was stored in 50% glycerol, 1 mM ethylenediaminetetraacetic acid, 50 mM Tris-HCl, pH 8.8, and 1 mM dithiothreitol.

**5'-End Labeling of Primers and Annealing of Primer–Templates.** DNA primers were 5'-<sup>32</sup>P-labeled using polynucleotide kinase (New England Biolabs) and [ $\gamma$ -<sup>32</sup>P]ATP (Perkin-Elmer). The labeled primer was gel purified and annealed to the template as previously described (24, 25).

**Incorporation Assays with Pol  $\alpha$ .** All kinetic data were determined under steady-state conditions. Reactions (5  $\mu$ L) typically contained 5 nM pol  $\alpha$ , 1  $\mu$ M 5'-<sup>32</sup>P-primer–template, 50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 10 mM MgCl<sub>2</sub>, 5% glycerol, and various concentrations of a dNTP (analogue). Reactions were incubated at 37 °C for 5–30 min and quenched with 5  $\mu$ L of formamide/0.05% xylene cyanol and bromophenol blue. Products were separated using 25% polyacrylamide and 8 M urea gels and imaged using a Typhoon phosphorimager (Molecular Dynamics). Kinetic parameters were determined by fitting data to a Michaelis–Menten curve using Origin 6.1 graphing software.

**Extension Assays with Pol  $\alpha$ .** The kinetic parameters for addition of the next correct dNTP onto a just incorporated

nucleotide analogue were determined using the Goodman “running start” methodology (26, 27). Extension past an incorporated dNTP (analogue) was determined by adding 25  $\mu$ M analogue dNTP and varying amounts of the next correct dNTP. Assays were performed and analyzed as described above. In those cases where we wanted to measure elongation where the base pair at the primer 3'-terminus would consist of a natural nucleotide in the primer and an analogue in the template, extension was measured directly rather than using the running start methodology. Chemically synthesized primers and templates were annealed together (DNA<sub>X</sub> in Figure 2, where X is any nucleotide analogue), and assays were performed as described above for incorporation assays.

**Synthesis of Nucleotide Analogues. 1-Deazapurine.** 1,2-Diaminopyridine (4.36 g, 40 mmol) was dissolved in formic acid (90%) and refluxed for 48 h. Formic acid was evaporated under reduced pressure, and the crude product was chromatographed on silica gel (250 mL) using a gradient of 0–10% MeOH in CH<sub>2</sub>Cl<sub>2</sub>. The product was crystallized from a mixture of MeOH/EtOAc. The yield of yellow crystals was 3.43 g (65%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 8.45 (s, 1H, H-8), 8.35 (dd, 1H, *J*<sub>1</sub> = 4.7 Hz, *J*<sub>2</sub> = 2.4 Hz), 8.01 (bd, 1H, *J* = 7.9 Hz), 7.21 (dd, 1H, *J*<sub>1</sub> = 8.3 Hz, *J*<sub>2</sub> = 4.8 Hz, H-1), 3.41 (bs, 1H, NH). MS (MALDI<sup>+</sup>) 121 (M + 2), calcd 121.

**2-Amino-1-deazapurine.** 2,3,6-Triaminopyridine (1 g, 8 mmol) was dissolved in formic acid (25 mL) and refluxed for 24 h. The formic acid was evaporated under reduced pressure, and the black residual oil dissolved in DMF (50 mL). The mixture was heated to 150 °C for 48 h. After being cooled to room temperature, the mixture was poured into 200 mL of hexane/ethyl acetate (1:1) and purified by silica gel chromatography (300 mL). After the mixture was loaded onto the column, it was washed with a mixture of hexane/ethyl acetate (1:1, 500 mL) to remove DMF, and the product was eluted with a gradient of 0–20% MeOH in EtOAc. This yielded a mixture of the desired product and the N<sup>2</sup> formamide. To remove the formamide group, the product mixture was dissolved in ammonia (25% in water, 100 mL) and heated for 4 days at 50 °C. Then ammonia and water were evaporated, and pure 2-amino-1-deazapurine was recrystallized from a mixture of MeOH, EtOAc, and hexane, originally 1:1:1, but the MeOH and EtOAc were slowly removed from the mixture by distillation, while hexane was replaced with new portions. This gave 1.16 g (89%) of 2-amino-1-deazapurine. <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD): 8.31 (s, 1H, NH), 8.03 (s, 1H, H-8), 7.73 (d, 1H, *J* = 8.7 Hz, H-6), 7.57 (d, 1H, *J* = 8.7 Hz, H-1), 4.94 (bs, NH<sub>2</sub>, MeOH). MS (MALDI<sup>+</sup>) 136 (M + 2), calcd 136.

**9- $\beta$ -D-(1-Deazapurine)-2'-deoxy-3',5'-di-O-(4-toluoyl)-D-ribofuranose.** 1-Deazapurine (500 mg, 3.78 mmol) was dissolved in MeCN (11 mL), and NaH (5.67 mmol, 60% in oil, 1.5 equiv) was added. The mixture was stirred 3 h at room temperature, and then 1-chloro-3,5-bis(*p*-toluoyl)-2-deoxy- $\beta$ -D-ribofuranose (1.5 g, 3.78 mmol) was added. The resulting slurry was stirred overnight, resulting in most of the slurry dissolving. The mixture was then poured into saturated aqueous NH<sub>4</sub>Cl and extracted with EtOAc, and the product was purified by chromatography on silica gel (100 mL) using a gradient from 50% to 100% EtOAc in hexanes. The yield of colorless oil was 750 mg (43%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 8.38 (dd, 1H, *J*<sub>1</sub> = 4.9 Hz, *J*<sub>2</sub> = 1.5 Hz, H-2),

8.23 (s, 1H, H-8), 8.07 (dd, 1H,  $J_1 = 4.9$  Hz,  $J_2 = 1.5$  Hz, H-6), 7.98 (bdd, 2H,  $J_1 = 6.7$  Hz,  $J_2 = 1.7$  Hz, Tol), 7.93 (bdd, 2H,  $J_1 = 6.7$  Hz,  $J_2 = 1.7$  Hz, Tol), 7.20–7.30 (m, 5H, H-1 and 4 × H-Tol), 6.70 (dd, 1H,  $J_1 = 8.5$  Hz,  $J_2 = 5.8$  Hz, H-1'), 5.82 (m, 1H, H-3'), 4.6–4.8 (m, 3H, H-5'a, H-5'b, H-4'), 3.18 (m, 1H, H-2'a), 2.84 (ddd, 1H,  $J_{\text{gem}} = 12.1$  Hz,  $J_{2'b,1'} = 5.8$  Hz,  $J_{2'b,3'} = 2.0$  Hz, H-2'b), 2.44 (s, 3H, CH<sub>3</sub>-Tol), 2.40 (s, 3H, CH<sub>3</sub>-Tol). MS (MALDI<sup>+</sup>) 473 (M + 2), calcd 473.

**9-β-D-(1-Deazapurine)-2'-deoxyribofuranose.** 9-β-D-(1-Deazapurine)-2'-deoxy-3',5'-di-*O*-(4-toluoyl)-D-ribofuranose (0.5 g, 1.08 mmol) was deprotected in 30 min using 0.1 M MeONa in MeOH and purified by silica gel chromatography (20 mL) using a gradient of 0–20% MeOH in CHCl<sub>3</sub>. This procedure gave 0.2 g (78%) of product. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 8.61 (s, 1H, H-8), 8.37 (dd, 1H,  $J_1 = 4.9$  Hz,  $J_2 = 1.5$  Hz, H-2), 8.10 (dd, 1H,  $J_1 = 8.1$  Hz,  $J_2 = 1.5$  Hz, H-6), 7.37 (dd, 1H,  $J_1 = 8.1$  Hz,  $J_2 = 4.9$  Hz, H-1), 6.57 (dd, 1H,  $J_1 = 8.0$  Hz,  $J_2 = 6.0$  Hz, H-1'), 4.83 (bs, 3H, OH, H<sub>2</sub>O), 4.61 (m, 1H, H-3'), 4.09 (dd, 1H,  $J_1 = 6.0$  Hz,  $J_2 = 3.2$  Hz, H-4'), 3.85 (dd, 1H,  $J_{5'a,5'b} = 12.2$  Hz,  $J_{5'a,4'} = 3.1$  Hz, H-5'a), 3.75 (dd, 1H,  $J_{5'b,5'a} = 12.3$  Hz,  $J_{5'a,3'} = 3.4$  Hz, H-5'a), 2.88 (m, 1H, H-2'a), 2.43 (ddd, 1H,  $J_{\text{gem}} = 13.5$  Hz,  $J_{2'b,1'} = 6.1$  Hz,  $J_{2'b,3'} = 2.7$  Hz, H-2'b). MS (MALDI<sup>+</sup>) 237 (M + 2), calcd 237.

**9-β-D-(2-Amino-1-deazapurine)-2'-deoxyribofuranose.** This compound was prepared from 2-amino-1-deazapurine (1 g, 7.5 mmol) as described above except that the crude 9-β-D-(2-acetamido-1-deazapurine)-2'-deoxy-3',5'-di-*O*-(4-toluoyl)-ribofuranose was immediately deprotected using MeONa in refluxing MeOH overnight followed by the addition of NH<sub>4</sub>Cl and refluxing for a further 6 h (without this additional NH<sub>4</sub>Cl step, N<sup>2</sup> remained protected). The overall yield was 0.48 g (26%). <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD): 8.10 (s, 1H, H-8), 7.70 (d, 1H,  $J = 8.7$  Hz, H-1), 6.53 (d, 1H,  $J = 8.7$  Hz, H-6), 6.38 (dd, 1H,  $J_1 = 8.4$  Hz,  $J_2 = 5.9$  Hz, H-1'), 4.9 (bs, 4H, OH, NH<sub>2</sub>, H<sub>2</sub>O), 4.59 (m, 1H,  $\Sigma J = 10.0$  Hz, H-3'), 4.08 (dd, 1H,  $J_1 = 5.2$  Hz,  $J_2 = 2.9$  Hz, H-4'), 3.87 (dd, 1H,  $J_{5'a,5'b} = 12.3$  Hz,  $J_{5'a,4'} = 2.9$  Hz, H-5'a), 3.75 (dd, 1H,  $J_{5'b,5'a} = 12.3$  Hz,  $J_{5'a,3'} = 3.1$  Hz, H-5'a), 2.86 (m, 1H, H-2'a), 2.33 (ddd, 1H,  $J_{\text{gem}} = 13.5$  Hz,  $J_{2'b,1'} = 6.0$  Hz,  $J_{2'b,3'} = 2.3$  Hz, H-2'b). MS (MALDI<sup>+</sup>) 252 (M + 2), calcd 252.

**1-β-D-(2-Oxopyridine)-2'-deoxyribofuranose.** 2-Hydroxypyridine (510 mg, 5.34 mmol) was dissolved in MeCN (11 mL) and bis(trimethylsilyl)acetamide (1.32 mL, 5.34 mmol) was added dropwise. The mixture was stirred for 70 min at room temperature and cooled on ice, and a mixture of MeCN (11 mL) and 1-chloro-3,5-bis(*p*-toluoyl)-2-deoxy-β-D-ribofuranose (1.7 g, 4.35 mmol) was then added. The mixture was stirred overnight while being warmed to room temperature and worked up as described above. Silica gel chromatography (200 mL) in 28% EtOAc/hexane yielded 1.8 g (37%) of the desired 1-β-D-(2-oxopyridine)-3',5'-di-*O*-(4-toluoyl)-2'-deoxyribofuranose, along with 0.5 g (10%) of a mixture of α and β anomers and 1.4 g (29%) of the α anomer of the product. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 7.97 (bdd, 2H,  $J_1 = 6.4$  Hz,  $J_2 = 1.7$  Hz, Tol), 7.87 (bdd, 2H,  $J_1 = 6.4$  Hz,  $J_2 = 1.7$  Hz, Tol), 7.71 (dd, 1H,  $J_1 = 7.1$  Hz,  $J_2 = 1.7$  Hz, 1H-Pyr), 7.31 (ddd, 1H,  $J_1 = J_2 = 6.5$  Hz,  $J_3 = 2.0$  Hz, 1H-Pyr), 7.28 (bd,  $J = 6.5$  Hz, Tol), 7.22 (bd, 2H,  $J = 6.5$  Hz, Tol), 6.61 (dd, 1H,  $J_1 = 8.1$  Hz,  $J_2 = 5.6$  Hz, H-1'), 6.54 (bd, 1H,  $J = 9.15$  Hz, 1H-Pyr), 6.12 (dd,  $J_1 = 6.8$  Hz,

$J_2 = 1.1$  Hz, H-Pyr), 5.60 (dt, 1H,  $J_1 = 6.6$  Hz,  $J_2 = 2.0$  Hz, H-3'), 4.71 (m, 2H, H-5'a, H-5'b), 4.62 (m, 1H, H-4'), 3.00 (ddd, 1H,  $J_{\text{gem}} = 14.5$  Hz,  $J_2 = 5.7$  Hz,  $J_3 = 1.9$  Hz, H-2'a), 2.43 (s, 3H, CH<sub>3</sub>-Tol), 2.41 (s, 3H, CH<sub>3</sub>-Tol), 2.16 (ddd, 1H,  $J_{\text{gem}} = 14.5$  Hz,  $J_2 = 6.6$  Hz,  $J_3 = 6.6$  Hz, H-2'b). The NMR spectrum was identical to that reported in the literature (28). MS (MALDI<sup>+</sup>) 449 (M + 2), calcd 449.

**1-β-D-(2-Oxopyridine)-3',5'-di-*O*-(4-toluoyl)-2'-deoxyribofuranose** (525 mg, 1.2 mmol) was deprotected using 0.1 M MeONa in MeOH and purified by silica gel chromatography (20 mL) using a gradient of 0–20% MeOH in CHCl<sub>3</sub>. This procedure gave 224 mg (78%) of product. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): 7.93 (dd, 1H,  $J_1 = 7.0$  Hz,  $J_2 = 1.8$  Hz, 1H-Pyr), 7.42 (ddd, 1H,  $J_1 = J_2 = 6.5$  Hz,  $J_3 = 2.0$  Hz, 1H-Pyr), 6.35 (m, 2H, 2H-Pyr), 6.28 (td,  $J_1 = 6.9$  Hz,  $J_2 = 1.2$  Hz, H-1'), 5.26 (bd, 1H,  $J = 4.3$  Hz, 3-OH), 5.04 (t, 1H,  $J = 5.2$  Hz, 5-OH), 4.22 (m, 1H, H-3'), 4.84 (m, 1H, H-4'), 3.60 (m, 2H, H-5'a, H-5'b), 2.24 (m, 1H, H-2'a), 1.94 (ddd, 1H,  $J_{\text{gem}} = 13.2$  Hz,  $J_2 = 6.1$  Hz,  $J_3 = 6.1$  Hz, H-2'b). MS (MALDI<sup>+</sup>) 213 (M + 2), calcd 213.

**9-β-D-(2-Isobutyrylamido-6-chloropurine)-2'-deoxyribofuranose.** 9-β-D-(2-Amino-6-chloropurine)-2'-deoxyribofuranose (0.5 g, 1.75 mmol) was dissolved in pyridine (30 mL), and Me<sub>3</sub>SiCl (1.32 mL, 10.5 mmol) was added. The mixture was stirred for 2 h at room temperature, and then isobutyryl chloride (0.38 mL, 3.5 mmol) was added. The mixture was stirred overnight and then poured into cold water. Crude product was extracted into EtOAc (3×). The collected organic fractions were washed with water and dried over MgSO<sub>4</sub>, and the solvents were evaporated under reduced pressure to yield 0.61 g (69%) of a brown oil. This oil was dissolved in THF (50 mL), and excess TBAF (2 mL) was added. The mixture was stirred overnight, and the solvents were removed under reduced pressure. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and chromatographed on silica gel (100 g) with 0–16% MeOH in CH<sub>2</sub>Cl<sub>2</sub>. Collected fractions gave pure 9-β-D-(2-isobutyrylamido-6-chloropurine)-2'-deoxyribofuranose (0.32 g, 74%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): 10.84 (s, 1H, NH), 8.70 (s, 1H, H-8), 6.35 (t, 1H,  $J_{1',2'} = 6.7$  Hz, H-1'), 5.40 (d, 1H,  $J = 4.12$  Hz, OH-3'), 4.95 (t, 1H,  $J = 5.5$  Hz, OH-5'), 4.44 (m, 1H,  $\Sigma J = 15.2$  Hz, H-3'), 3.86 (dd, 1H,  $J_1 = 7.7$  Hz,  $J_2 = 4.7$  Hz, H-4'), 3.48–3.64 (m, 2H, 2 × H-5'), 2.75 (septet, 1H,  $J_{\text{CH},\text{CH}_3} = 6.7$  Hz, CH-*i*Bu), 2.75 (m, 1H, H-2'a), 2.31 (ddd, 1H,  $J_{\text{gem}} = 13.4$  Hz,  $J_{2'b,1'} = 6.3$  Hz,  $J_{2'b,3'} = 3.6$  Hz, H-2'b), 1.08 (d, 6H,  $J_{\text{CH}_3,\text{CH}} = 6.9$  Hz, 2 × CH<sub>3</sub>-*i*Bu). MS (MALDI<sup>+</sup>) 357 (M + 2), calcd 357.

**5'-(4,4-Dimethoxytrityl)-9-β-D-(2-isobutyrylamido-6-chloropurine)-2'-deoxyribofuranose.** 9-β-D-(2-Isobutyrylamido-6-chloropurine)-2'-deoxyribofuranose (0.32 g, 0.9 mmol) was dissolved in pyridine (10 mL), and dimethoxytrityl chloride (880 mg, 2.6 mmol) was added. After the reaction was completed (3 h, analyzed by silica TLC in EtOAc), the mixture was poured into saturated NaHCO<sub>3</sub>, and the product was extracted into EtOAc. Organic layers were washed with several portions of 1% NaHCO<sub>3</sub> and dried over MgSO<sub>4</sub> and solvents were evaporated under reduced pressure. The product was purified by silica gel chromatography (0–10% MeOH in EtOAc). The reaction yielded 0.44 g (74%) of 5'-(4,4-dimethoxytrityl)-9-β-D-(2-isobutyrylamido-6-chloropurine)-2'-deoxyribofuranose. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 8.37 (s, 1H, NH), 8.27 (s, 1H, H-8), 7.11–7.44 (m, 9H, 9 × H-DMTr), 6.85 (t, 1H,  $J_{1',2'} = 7.3$  Hz, H-1'), 6.6 (m, 4H, 4



× H-DMTr), 4.79 (d, 1H,  $J_{3',4'} = 2.0$  Hz, H-3'), 4.51 (bs, 1H, OH-3'), 4.33 (d, 1H,  $J_{4',3'} = 2.0$  Hz, H-4'), 3.75 (s, 3H, CH<sub>3</sub>-DMTr-a), 3.75 (s, 3H, CH<sub>3</sub>-DMTr-b), 3.41 (m, 2H, 2 × H-5'), 2.55–2.80 (m, 3H, CH-iBu, 2 × H-2'), 1.16 (d, 6H,  $J = 6.9$  Hz, 2 × CH<sub>3</sub>-iBu). MS (MALDI<sup>+</sup>) 659 (M + 2), calcd 659.

**Synthesis of Nucleoside 5'-Triphosphates.** Nucleosides were converted to nucleoside 5'-triphosphates as previously described (15). Crude nucleoside triphosphates were purified by loading them onto an anion-exchange column (Sephadex-DEAE A-25, Aldrich, TEAB equilibrated) followed by elution with a 0–1 M TEAB gradient. Fractions were identified by MALDI mass spectrometry (negative M – 1 ion) with a THAP matrix. Collected fractions were evaporated and purified by reverse-phase (C18) HPLC using a gradient of 0–50% MeCN in 20 mM triethylammonium acetate. The structure and purity were confirmed by a single peak in HPLC, MALDI MS, and <sup>31</sup>P NMR. Analytical data for the novel triphosphates synthesized are given in Supporting Information.

**5'-Dimethoxytrityl-3'-phosphoramidites and Oligonucleotides.** The 5'-OH of the nucleosides was protected with a dimethoxytrityl group as described above, and established procedures were used for generation of the phosphoramidites and synthesis of the oligonucleotides on an Applied Biosystems 394 automatic DNA synthesizer (29, 30). Analytical data for the phosphoramidites are given in Supporting Information. HPLC analysis of the purified oligonucleotides gave a single product and ESI-mass spectrometry showed the appropriate mass for the modified oligonucleotides.

## RESULTS

Previous work showed that pol α readily polymerizes synthetic dNTPs whose bases do not closely resemble the shape of the canonical bases and cannot form Watson–Crick hydrogen bonds, whereas the enzyme rarely misincorporates the canonical bases (15, 19, 20). In the case of dATP, the low rate of misincorporation and rapid rate of incorporation opposite a template deoxythymidylate results from specific effects of N-1, N-3, and N<sup>6</sup>. Here, we examined the role of the exocyclic N<sup>2</sup> in a purine using a series of base analogues that only differed by the presence or absence of N<sup>2</sup> (Figure 1).

Incorporation of both the natural and analogue dNTPs was measured on synthetic primer–templates of defined sequence (Figure 2). Similar to previous results, pol α rarely misincorporated incorrect dNTPs and incorporated dGTP opposite a template dC slightly more efficiently than it incorporated dATP opposite a template T (Table 1 (19)).

**Role of N<sup>2</sup> during Generation of G•C Base Pairs.** We initially examined the role of the N<sup>2</sup> by comparing incorporation of dGTP and dTTP opposite all four natural bases (Table 1). Removing N<sup>2</sup> from dGTP had two effects. First, it slightly decreased (~6-fold) the efficiency of polymerization opposite C. More significantly, losing N<sup>2</sup> dramatically increased misincorporation opposite A (≥180-fold), with minimal effects on misincorporation opposite T or G. Similar effects occurred upon removal of N<sup>2</sup> from a template G. Pol α still efficiently polymerized dCTP opposite I (only 3-fold slower), but the rate of dATP misincorporation increased dramatically (~70-fold) (Table 2).

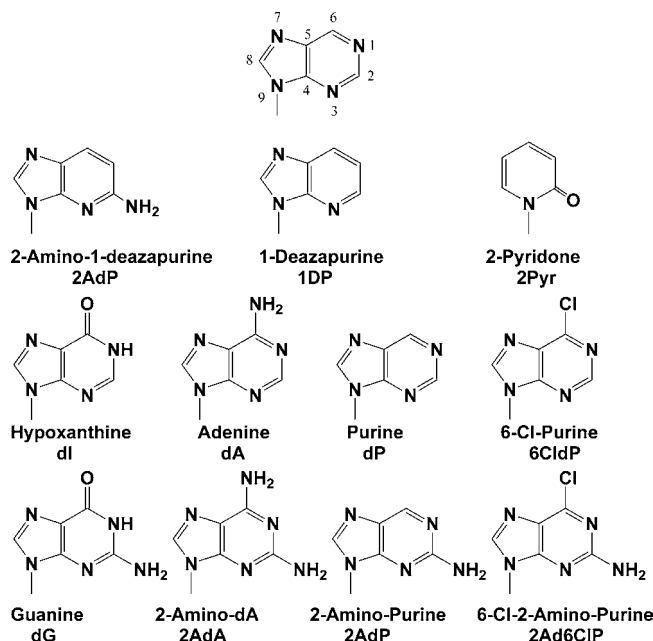


FIGURE 1: Analogues and natural purine bases used. The numbering system for a purine is shown.












DNA <sub>A</sub>	5'- TCC ATA TCA CAT
	3'- AGG TAT AGT GTA <b>A</b> TT CTT ATC ATC T
DNA <sub>C</sub>	5'- TCC ATA TCA CAT
	3'- AGG TAT AGT GTA <b>C</b> TT CTT ATC ATC T
DNA <sub>G</sub>	5'- TCC ATA TCA CAT
	3'- AGG TAT AGT GTA <b>G</b> AT CTT ATC ATC T
DNA <sub>T</sub>	5'- TCC ATA TCA CAT
	3'- AGG TAT AGT GTA <b>T</b> AT CTT ATC ATC T
DNA <sub>X</sub>	5'- TCC ATA TCA CAT
	3'- AGG TAT AGT GTA <b>X</b> CT CTT ATC ATC T

FIGURE 2: The DNA primer–template sequences used. In DNA<sub>X</sub>, X represents any nucleotide analogue.

**Effect of Adding N<sup>2</sup> to Adenine Analogues.** We further examined the role of N<sup>2</sup> by adding it to three purines whose N-1 can form a Watson–Crick hydrogen bond with thymine, adenine, purine, and 6-chloropurine, and examined the effects on polymerization of the resulting 2-amino-dNTPs. Adding N<sup>2</sup> to purine dNTP, 6-chloropurine dNTP, and dATP had relatively modest effects during polymerization opposite T, either slightly increasing or inhibiting polymerization. Thus, the potential formation of an additional Watson–Crick hydrogen bond is not necessarily advantageous for correct incorporation (Table 1). While the presence of N<sup>2</sup> did not impact misincorporation opposite G or A, N<sup>2</sup> increased misincorporation opposite C.










The effects of adding N<sup>2</sup> to adenine, purine, or 6-chloropurine when present in the template were likewise examined (Table 2). Adding N<sup>2</sup> to these bases had variable effects on

Table 1: Kinetic Parameters of Incorporation of dNTPs Containing Modified Bases Opposite Natural Nucleotides

dNTP	Base Structure	Template Base	$k_{cat}/K_M$ ( $\mu\text{M}^{-1} \text{min}^{-1}$ )	Discrimination <sup>a</sup>
dCTP		G	$6.5 \pm 0.8$	1
TTP		A	$2.4 \pm 0.5$	1
dGTP		A	$< 1.0 \times 10^{-1b}$	$> 20000$
		C	$9.0 \pm 1.4$	1
		G	$1.0 \pm 0.1 \times 10^{-4}$	90000
		T	$3.9 \pm 0.5 \times 10^{-3}$	1800
dTTP		A	$1.8 \pm 0.4 \times 10^{-2}$	120
		C	$1.6 \pm 0.9$	6
		G	$< 1.0 \times 10^{-4}$	$> 20000$
dATP		T	$< 1.0 \times 10^{-4}$	$> 20000$
		A	$1.1 \pm 0.5 \times 10^{-3}$	2100
		C	$1.8 \pm 0.9 \times 10^{-2}$	500
		G	$4.7 \pm 2.8 \times 10^{-4}$	13800
2AdATP		T	$7.0 \pm 1.6$	1
		A	$5.6 \pm 0.9 \times 10^{-4}$	4100
		C	$7.4 \pm 1.9 \times 10^{-2}$	120
		G	$4.3 \pm 2.0 \times 10^{-4}$	15000
dPTP		T	$1.9 \pm 0.1$	4
		A	$< 1.0 \times 10^{-4}$	$> 20000$
		C	$3.8 \pm 0.9 \times 10^{-3}$	2400
		G	$< 1.0 \times 10^{-4}$	$> 20000$
2AdPTP		T	$2.9 \pm 1.0$	2.4
		A	$8.2 \pm 3.9 \times 10^{-4}$	2800
		C	$1.4 \pm 0.4 \times 10^{-1}$	64
		G	$2.9 \pm 0.1 \times 10^{-3}$	2200
6ClJPTP		T	$5.3 \pm 0.2 \times 10^{-1}$	13
		A	$< 1.0 \times 10^{-4}$	$> 20000$
		C	$4.1 \pm 2.8 \times 10^{-2}$	220
		G	$< 1.0 \times 10^{-4}$	$> 20000$
2Ad6ClJPTP		T	$4.1 \pm 2.0 \times 10^{-1}$	17
		A	$9.1 \pm 4.1 \times 10^{-4}$	2600
		C	$2.3 \pm 0.8 \times 10^{-1}$	40
		G	$5.6 \pm 1.4 \times 10^{-4}$	12000
dIDPTP		T	$7.4 \pm 2.5 \times 10^{-1}$	9
		A	$< 1 \times 10^{-4}$	$> 20000$
		C	$< 1 \times 10^{-4}$	$> 20000$
		G	$< 1 \times 10^{-4}$	$> 20000$
2AdIDPTP		T	$3.1 \pm 0.2 \times 10^{-3}$	2300
		A	$6.1 \pm 1.8 \times 10^{-4}$	3800
		C	$1.2 \pm 0.08 \times 10^{-2}$	750
		G	$2.4 \pm 0.2 \times 10^{-4}$	30000
d2PYRTP		T	$1.4 \pm 0.4 \times 10^{-2}$	500
		A	$6.9 \pm 2.8 \times 10^{-5}$	33000
		2AA	$1.4 \pm 0.5 \times 10^{-2}$	170
		P	$< 1 \times 10^{-4}$	$> 20000$
		2AP	$5.8 \pm 1.8 \times 10^{-2}$	40

<sup>a</sup> All values are the average of three to five independent experiments. Discrimination values are defined as  $k_{cat}/K_M$  for the correct dNTP opposite that template base (e.g., dATP·T, dCTP·G) divided by  $k_{cat}/K_M$  for the noted dNTP or dNTP analogue opposite that template base. Thus, for example, the discrimination for 2AdATP opposite T would be  $[k_{cat}/K_M(\text{dATP} \cdot \text{T})]/[k_{cat}/K_M(\text{2AdATP} \cdot \text{T})]$ .  
<sup>b</sup> Values less than  $1 \times 10^{-4}$  could not be specifically determined due to low levels of incorporation ( $< 1\%$ ) even at high ( $> 2$  mM) substrate concentration.

Table 2: Kinetic Parameters for Incorporation of Natural dNTPs Opposite Modified Bases in the Template

dNTP	Template Base	Base Structure	$k_{cat}/K_M$ ( $\mu\text{M}^{-1} \text{min}^{-1}$ )	Discrimination <sup>a</sup>
dATP	A		$1.1 \pm 0.5 \times 10^{-3}$	2100
dCTP			$6.2 \pm 2.7 \times 10^{-4}$	3700
dGTP			$< 1.0 \times 10^{-1}$	$> 20000$
TTP			$2.4 \pm 0.5$	1
dATP	2AA		$1.4 \pm 1.0 \times 10^{-3}$	1650
dCTP			$1.1 \pm 0.3 \times 10^{-2}$	600
dGTP			$< 1.0 \times 10^{-4}$	$> 20000$
TTP			$1.7 \pm 0.7$	1.4
dATP	P		$5.8 \pm 1.7 \times 10^{-4}$	4000
dCTP			$< 1.0 \times 10^{-4}$	$> 20000$
dGTP			$< 1.0 \times 10^{-4}$	$> 20000$
TTP			$8.3 \pm 3.2 \times 10^{-2}$	28
dATP	2AP		$7.6 \pm 3.8 \times 10^{-4}$	3000
dCTP			$3.0 \pm 0.5 \times 10^{-2}$	77
dGTP			$< 1.0 \times 10^{-4}$	$> 20000$
TTP			$5.6 \pm 1.7 \times 10^{-1}$	4
dATP	6ClP		$1.2 \pm 0.4 \times 10^{-2}$	200
dCTP			$8.8 \pm 1.4 \times 10^{-4}$	2600
dGTP			$1.5 \pm 0.2 \times 10^{-4}$	15300
TTP			$9.5 \pm 1.1 \times 10^{-1}$	4
dATP	2A6ClP		$< 1.0 \times 10^{-4}$	$> 20000$
dCTP			$7.8 \pm 2.4 \times 10^{-2}$	30
dGTP			$< 1.0 \times 10^{-4}$	$> 20000$
TTP			$1.2 \pm 0.4 \times 10^{-1}$	20
dATP	G		$2.8 \pm 0.5 \times 10^{-4}$	23000
dCTP			$6.5 \pm 0.8$	1
dGTP			$6.3 \pm 4.4 \times 10^{-4}$	10000
TTP			$2.7 \pm 0.1 \times 10^{-3}$	2400
dATP	I		$3.2 \pm 0.4 \times 10^{-2}$	200
dCTP			$2.1 \pm 0.3$	3
dGTP			$6.2 \pm 0.9 \times 10^{-4}$	10500
TTP			$5.6 \pm 0.2 \times 10^{-3}$	1200
dATP	2PYR		$1.9 \pm 0.4 \times 10^{-3}$	4000
2AdATP			$1.4 \pm 0.6 \times 10^{-2}$	500
dPTP			$7.7 \pm 1.5 \times 10^{-3}$	910
2AdPTP			$1.3 \pm 0.5 \times 10^{-4}$	54
1DdPTP			$2.5 \pm 0.1 \times 10^{-2}$	280
2A1DdPTP			$3.1 \pm 0.4 \times 10^{-1}$	23

<sup>a</sup> Discrimination is defined as described in Table 1. For all of the template bases except for G and I, correct incorporation is defined as polymerization of TTP opposite A. For G and I, correct incorporation is defined as polymerization of dCTP opposite G.

dTTP polymerization, ranging from a ca. 5-fold increase in polymerization efficiency (purine  $\rightarrow$  2-aminopurine) to around a 6-fold decrease in polymerization efficiency (6-chloropurine  $\rightarrow$  2-amino-6-chloropurine). With the exception of decreasing the efficiency of dATP misincorporation opposite 2-amino-6-chloropurine relative to 6-chloropurine, adding N<sup>2</sup> did not significantly alter misincorporation of either dATP or dGTP. However, analogous to what was observed upon adding N<sup>2</sup> to dNTPs, adding N<sup>2</sup> to the

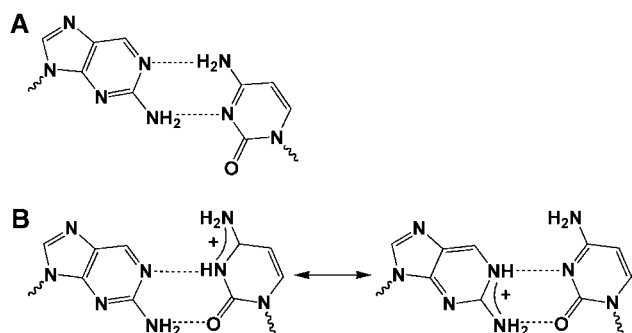


FIGURE 3: Potential structures of a 2-aminopurine•C base pair in a wobble geometry (A) and after protonation of the base pair (B) (31–33, 44, 45).

template bases greatly increased the rate of dCTP polymerization opposite the aminated bases. Thus, the effects of adding N<sup>2</sup> to purine nucleotides is extremely symmetrical in terms of the effect on a purine dNTP versus a purine nucleotide in the template.

Potentially, the enhanced formation of C•2-aminopurine, C•2-amino-6-chloropurine, and C•2-amino-adenine base pairs could have resulted from the additional hydrogen bond between N<sup>2</sup> of the purine and O<sup>2</sup> of the pyrimidine, protonation of the base pair, or formation of a wobble base pair between the 2-aminopurines and cytosine (Figure 3). Previous NMR studies suggested that, in solution, a cytosine•2-aminopurine base pair exists in a protonated and/or wobble structure and that these structures may account for the ability of a DNA polymerase to relatively rapidly incorporate 2-aminopurine dNTP opposite a template C (31–33). To differentiate between the rapid incorporation resulting from a hydrogen bond between N<sup>2</sup> of a purine and O<sup>2</sup> of C versus a wobble structure/unusual protonation of the 2-aminopurine•C base pair, we examined pol  $\alpha$  catalyzed formation of 2-pyridone•purine ( $\pm$ N<sup>2</sup>) base pairs. 2-Pyridone can form a hydrogen bond between O<sup>2</sup> and the purine N<sup>2</sup> but cannot form a wobble base pair. Additionally, protonation of a 2-aminopurine•2-pyridone base pair would not result in any obvious advantages for stabilizing this base pair. Adding N<sup>2</sup> to a purine dNTP greatly increased the efficiency of polymerization opposite a template 2-pyridone (Table 2), and adding N<sup>2</sup> to a purine nucleotide in the template likewise enhanced the incorporation of 2-pyridone dNTP (Table 1).

As a further test of this idea, we synthesized 1-deaza-2-aminopurine dNTP and compared its polymerization to that of 1-deazapurine dNTP (Table 1). Pol  $\alpha$  strongly discriminated against polymerization of 1-deazapurine dNTP opposite all of the natural template bases. Upon addition of N<sup>2</sup>, however, pol  $\alpha$  incorporated the resulting 1-deaza-2-aminopurine dNTP more rapidly across from all four natural bases, but especially C. Clearly, adding a hydrogen bond donor to a purine that can interact with O<sup>2</sup> of C can dramatically enhance polymerization. Consistent with the ability of N<sup>2</sup> of 1-deaza-2-aminopurine dNTP to form a hydrogen bond with O<sup>2</sup> of a natural pyrimidine in the

Table 3: Kinetic Parameters for Extension of Primer–Templates Containing either Canonical Bases or Base Analogues

base pair at 3'-end of primer		$k_{cat}/K_M$ ( $\mu\text{M}^{-1} \text{min}^{-1}$ )	discrimination <sup>a</sup>
primer base	template base		
A	C	N/D <sup>b</sup>	
	T	$3.4 \pm 0.5$	1
C	A	$<1.6 \times 10^{-3}$	$>2130$
	C	$(3.1 \pm 0.2) \times 10^{-4}$	11000
	G	$0.67 \pm 0.12$	1
	T	$(3.3 \pm 2.5) \times 10^{-3}$	1030
T	A	$1.3 \pm 0.7$	1
	C	$<4.3 \times 10^{-3}$	$>790$
	T	$(1.0 \pm 0.3) \times 10^{-2}$	340
2AA	C	N/D	
2AA	T	$2.6 \pm 0.2$	1.4
C	2AA	$(1.2 \pm 0.5) \times 10^{-3}$	1100
T	2AA	$0.9 \pm 0.3$	1.4
P	C	$<6.9 \times 10^{-3}$	$>500$
P	T	$(1.9 \pm 0.5) \times 10^{-1}$	18
C	P	$(3.7 \pm 1.7) \times 10^{-5}$	3400
T	P	$(5.1 \pm 2.5) \times 10^{-3}$	250
2AP	C	$<1.6 \times 10^{-2}$	$>210$
2AP	T	$(2.2 \pm 0.01) \times 10^{-1}$	15
C	2AP	$(8.2 \pm 3.6) \times 10^{-4}$	1600
T	2AP	$(3.0 \pm 0.7) \times 10^{-2}$	43
6CIP	C	N/D	
6CIP	T	$(6.2 \pm 0.1) \times 10^{-2}$	55
C	6CIP	$<1.0 \times 10^{-4}$	$>20000$
T	6CIP	$<1.0 \times 10^{-4}$	$>13000$
2A6CIP	C	$(3.7 \pm 0.5) \times 10^{-2}$	92
2A6CIP	T	$(2.4 \pm 0.5) \times 10^{-1}$	14
C	2A6CIP	$(3.2 \pm 1.4) \times 10^{-3}$	720
T	2A6CIP	$(3.0 \pm 0.8) \times 10^{-2}$	80
2PYR	A	$<1 \times 10^{-4}$	$>20000$
2PYR	2AA	$(2.6 \pm 0.6) \times 10^{-2}$	130
2PYR	2AP	$(3.0 \pm 0.2) \times 10^{-2}$	120
A	2PYR	$(2.7 \pm 1.3) \times 10^{-2}$	130
2AA	2PYR	$(4.4 \pm 0.2) \times 10^{-2}$	77
P	2PYR	$(1.3 \pm 0.2) \times 10^{-1}$	26
2AP	2PYR	$(2.1 \pm 0.8) \times 10^{-1}$	16
I	A	N/D	
I	C	$2.2 \pm 0.2$	1.5
A	I	$(8.1 \pm 0.6) \times 10^{-4}$	790
C	I	$(1.6 \pm 0.6) \times 10^{-1}$	4.2

<sup>a</sup> Discrimination is defined as described in Table 1. <sup>b</sup> N/D, not determined. The next correct dNTP inhibited incorporation of the modified dNTP; hence the rate of polymerization of the next correct dNTP onto the modified dNTP could not be measured.

template, pol  $\alpha$  incorporated 2-amino-1-deazapurine dNTP opposite 2-pyridone 10-fold more efficiently than it incorporated 1-deazapurine dNTP.<sup>2</sup>

**Effects of N<sup>2</sup> on Polymerase Extension.** Stable misincorporation of an incorrect nucleotide requires two different polymerase related reactions: incorporation of the incorrect dNTP and continued polymerization of additional dNTPs. Thus, to better understand the role of N<sup>2</sup>, we examined the effect of adding N<sup>2</sup> to the base at the primer 3'-terminus on polymerization of the next correct dNTP. Extension past an incorporated analogue was measured using running start conditions (26). Consistent with previous studies, pol  $\alpha$  only slowly polymerized the next correct dNTP onto incorrect, natural base pairs (Table 3) (15, 20). Furthermore, the assay conditions resulted in no detectable product due to consecutive incorporation of the next correct dNTPs (i.e., products where the primer was extended by two nucleotides). Thus, primers extended by two nucleotides in the presence of an analogue dNTP and the next correct dNTP result from consecutive incorporation of the analogue dNTP followed by the next correct dNTP.

<sup>2</sup> Attempts to synthesize a template containing 2-amino-1-deazapurine were unsuccessful. While the protected nucleoside phosphoramidite was readily incorporated into an oligonucleotide, deprotection of N<sup>2</sup> was completely unsuccessful under conditions that did not simultaneously degrade the oligonucleotide. Protecting groups tested included isobutryl, acetyl, and benzyl.

When the base pair at the primer 3'-terminus consisted of an analogue and a natural nucleotide, pol  $\alpha$  polymerized additional nucleotides less efficiently than a correct base pair (Table 3). Curiously, pol  $\alpha$  elongated these analogue•natural base pairs very asymmetrically. In some cases, pol  $\alpha$  elongated the base pair with the analogue in the primer strand more efficiently than the base pair with the analogue in the template strand, in other cases the converse occurred, and in a couple cases it did not matter which strand contained the analogue. Perhaps the most dramatic examples of asymmetry were the 6-chloropurine•T and 2-pyridone•A base pairs. In one orientation, pol  $\alpha$  added the next correct dNTP reasonably well, but in the other orientation polymerization of the next correct dNTP was undetectable. With the exception of an A•T base pair as the primer 3'-terminal base pair, adding N<sup>2</sup> to the purine to the analogue significantly increased the efficiency with which pol  $\alpha$  elongated primer–templates containing either C or T as the pyrimidine. Although the effects were more modest, adding N<sup>2</sup> to the primer 3'-terminal base pair also stimulated polymerization onto substrates where this base pair involved 2-pyridone nucleotide, consistent with the formation of an additional hydrogen bond. Curiously, even in the absence of N<sup>2</sup>, pol  $\alpha$  elongated primer termini where the template contained 2-pyridone nucleotide much more efficiently than a natural mismatch.

The effects of N<sup>2</sup> on base pairs containing either I or G in the template were also measured (Table 3). Pol  $\alpha$  elongates a C•I base pair modestly slower than a C•G base pair, indicating that losing the hydrogen bond involving N<sup>2</sup> does not critically impair further DNA synthesis. Significantly, pol  $\alpha$  elongates an A•I base pair much less efficiently than either a C•G or C•I base pair, indicating that the polymerase recognizes the A•I base pair as defective. Incorporation of dITP opposite A in the presence of next correct nucleotide was too slow to measure, while the extension past an I•C base pair was almost as efficient as elongation past a canonical base pair (only 1.5-fold less efficient (Table 3)).

## DISCUSSION

The effect of purine N<sup>2</sup> on pol  $\alpha$  activity was investigated to better understand how this enzyme distinguishes between correct and incorrect dNTPs. N<sup>2</sup> does not play an essential role during generation of G•C base pairs. However, pol  $\alpha$  uses N<sup>2</sup> of G to prevent the formation of A•G mispairs. Adding N<sup>2</sup> to adenine analogues dramatically increases formation of A•C mispairs, likely due to the formation of an additional hydrogen bond between N<sup>2</sup> of the purine and O<sup>2</sup> of a pyrimidine.

Losing the hydrogen bond between N<sup>2</sup> of guanine and O<sup>2</sup> of cytosine reduced polymerization of dGTP opposite C by 6-fold and polymerization of dCTP opposite G by 3-fold. This difference corresponds to a  $\Delta G$  of 0.7–1.1 kcal mol<sup>-1</sup>, slightly less than the ca. 1.4 kcal mol<sup>-1</sup> loss in duplex stability for replacing a G•C base pair with an I•C base pair in DNA (34). These relatively small effects of losing N<sup>2</sup> are similar to the effects observed with other polymerases and indicate that the remaining two Watson–Crick hydrogen bonds are sufficient for efficient polymerization (35).

Surprisingly, losing N<sup>2</sup> from guanine nucleotides dramatically increased the efficiency (>100-fold) with which pol  $\alpha$

polymerized dATP opposite I and dITP opposite A vis-à-vis guanine nucleotides. These effects were remarkably base pair specific, since generation of either T•I/I•T or G•I/I•G mispairs did not increase. The higher rate of A•I base pair generation compared to I•T and I•G corresponds to the greater stability of the A•I base pair (36). Thus, N<sup>2</sup> serves as a critical barrier for preventing formation of incorrect A•G base pairs.<sup>3</sup>

What remains less obvious, however, is how N<sup>2</sup> in guanine would prevent generation of an A•G base pair. Both A•G and A•I base pairs will vary greatly from a correct base pair in terms of structure, especially if one wants to maintain any sort of hydrogen bonding between them, and it is difficult to imagine a scenario where N<sup>2</sup> would sterically block placement of an A across from G. The enhanced misincorporation opposite a template I may result from pol  $\alpha$  specifically interacting with N<sup>2</sup>. This interaction results in the active site of the E•primer•template having a structure that prevents dATP polymerization. In the absence of N<sup>2</sup>, however, the active site will not adopt this structure, thereby allowing misincorporation of dATP. Conversely, when pol  $\alpha$  replicates a template adenine, the active site of this enzyme•primer•template complex adopts a conformation where N<sup>2</sup> on an incoming dGTP indicates that it is wrong. Currently, no structures of ternary B-family DNA polymerase•primer•template•dNTP exist where the dNTP is incorrect, thus precluding a structure-based analysis of these data.

While no high-resolution structure of pol  $\alpha$  exists, the structure of the closely related polymerase RB69 has been solved as the closed ternary E•DNA•dTTP complex (37). In this complex, the enzyme is replicating a template A. In light of the tremendous conservation of the amino acids that surround the bases of the incoming dNTP and the template nucleotide being replicated, it seems very likely that the design of the pol  $\alpha$  and RB69 active sites will be very similar. N<sup>2</sup> of guanine will be on the minor groove side of the helix, near the electropositive edge of a Tyr that is very highly conserved among B family polymerases. Furthermore, mutation of this Tyr results in decreased fidelity of RB69 DNA polymerase (38, 39). Since this Tyr may “feel” the electronic effects of a nearby electropositive amino group, the decreased fidelity upon removing N<sup>2</sup> from guanine nucleotides may be mediated by this Tyr.

Another explanation for the formation of I•A base pairs is the template base flipping into the *syn* conformation to form a Hoogsteen base pair in order to maintain a more stable duplex (Figure 4) (40). This has been shown to occur with a common DNA lesion, 8-oxoguanine, when forming base pairs with A in double-stranded DNA (41, 42). However, this base-flipping mechanism would require a remarkable degree of flexibility of the pol  $\alpha$  active site. Generation of a dATP•I mispair would require flipping of adenine on the dNTP, whereas formation of a dITP•A mispair would require flipping of adenine in the template strand. Hence this mechanism would seem to require that the enzyme could

<sup>3</sup> Yasui et al. recently reported that removal of N<sup>2</sup> from a template G increases misincorporation of TTP, with minimal effects on dATP misincorporation by pol  $\alpha$  (I). We do not know the reason for this discrepancy between their and our results. Consistent with our data, transfection of NIH3T3 cells with a plasmid containing an I•T mismatch results in incorporation of primarily dCTP opposite I but also a small amount of dATP incorporation (2).



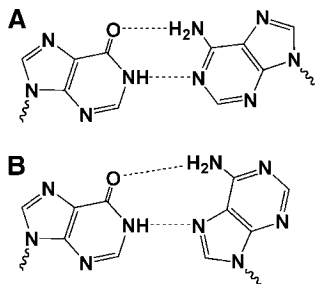


FIGURE 4: Potential structures of an I•A base pair. The structure of an I•A base pair with both nucleotides in the *anti* conformation (A) and with A in the *syn* conformation and I in the *anti* conformation (B).

accommodate extra mass on either side of the active site. We are presently testing how the absence of N<sup>2</sup> allows for facile generation of A•I base pairs.

Previously, we showed that pol  $\alpha$  uses a combination of positive and negative selectivity to accurately replicate dNTPs. For example, N-3 of purine dNTPs prevents misincorporation while N-1 of adenine and adenine analogues both prevents misincorporation and increases correct polymerization opposite T. Pol  $\alpha$  employs N<sup>2</sup> of dGTP in an identical fashion: as a positive selector that slightly enhances correct incorporation opposite C and as a negative selector for specifically preventing misincorporation opposite A.

Adding N<sup>2</sup> to dATP and two related compounds, purine dNTP and 6-chloropurine dNTP, greatly increased the efficiency of polymerization of the resulting dNTPs opposite C. Symmetrically, pol  $\alpha$  incorporates dCTP opposite 2-aminoadenine, 2-aminopurine, and 6-chloro-2-aminopurine in the template much more efficiently than it polymerizes dCTP opposite adenine, purine, and 6-chloropurine, respectively. Thus, adding N<sup>2</sup> to adenine and related analogues has clear mutagenic effects, analogous to results from previous studies using 2-aminopurine nucleotides with T4 DNA polymerase, another closely related B family polymerase (43).

Potentially, the ability of N<sup>2</sup> to facilitate generation of incorrect base pairs with C could have resulted either from the formation of a new hydrogen bond between N<sup>2</sup> of the purine and O<sup>2</sup> of C or from protonation/tautomerization/wobble of the base pair (Figure 3). Solution structural studies have shown that, in aqueous solvent, formation of a base pair between 2-aminopurine and cytosine involves protonation and/or wobble of the 2-aminopurine•cytosine base pair (31–33). However, since an enzyme active site and aqueous solution will presumably have quite different energetic constraints, it is unclear whether the pol  $\alpha$  active site will allow formation of the same structures as will aqueous solution. Adding N<sup>2</sup> to the adenine, purine, and 6-chloropurine nucleotides increased the efficiency of polymerization of C from 3-fold in the case of adenine vs 2-aminoadenine in the template to 40-fold in the case of purine vs 2-aminopurine in the template. Energetically, this corresponds to N<sup>2</sup> stabilizing the transition state by 0.7–>1.2 kcal mol<sup>−1</sup>, values consistent with either mechanism.

To help differentiate between these two models, we employed nucleotides containing the bases 2-pyridone, 1-deazapurine, and 2-amino-1-deazapurine. Since 2-pyridone lacks N-3, adding N<sup>2</sup> to a purine analogue could only enhance polymerization via formation of a hydrogen bond between N<sup>2</sup> of the purine and O<sup>2</sup> of the pyridine (7–17-fold).

Importantly, in every case examined, adding N<sup>2</sup> to the purine increased polymerization opposite 2-pyridone, indicating that formation of a new hydrogen bond can facilitate polymerization. We also found that adding N<sup>2</sup> to 1-deazapurine to generate 2-amino-1-deazapurine significantly boosted polymerization opposite both C and 2-pyridone (Tables 1 and 2). Since neither 2-amino-1-deazapurine nor 2-pyridone can tautomerize/protonate/wobble to form additional hydrogen bonds in a 2-amino-1-deazapurine•2-pyridone base pair, these data also support the idea that the formation of a new hydrogen bond between N<sup>2</sup> of a purine and O<sup>2</sup> of C significantly enhances formation of a C•2-aminopurine (analogue) base pair by pol  $\alpha$ . With this latter set of analogues, N<sup>2</sup> enhanced polymerization from 8- to >200-fold, very similar to the increase observed with 2-aminopurine (analogues) opposite C.

So, how does N<sup>2</sup> of, for example, 2-aminopurine increase misincorporation opposite C: formation of an additional hydrogen bond or a tautomerization/protonation/wobble mechanism? The one explanation consistent with all of the data, and therefore our favored model, is formation of an additional hydrogen bond between N<sup>2</sup> of the purine and O<sup>2</sup> of C without any additional alterations to the structure of the C•2-aminopurine (analogue) base pair. Our data do not, of course, rule out the more complicated possibility that the enzyme uses different mechanisms with different bases.

Importantly, these data also demonstrate the power of Watson–Crick hydrogen bonds to drive dNTP polymerization by pol  $\alpha$ . For example, pol  $\alpha$  readily polymerizes benzimidazole dNTP opposite all four natural bases, indicating that pol  $\alpha$  cannot identify as wrong the “base pair” formed between a natural base and benzimidazole (15). Converting benzimidazole dNTP into 1-deazapurine dNTP by inserting N-3 changes the situation drastically, since pol  $\alpha$  readily identifies 1-deazapurine dNTP as incorrect for polymerization opposite all four natural dNTPs. Further addition of N<sup>2</sup>, however, overcomes the negative effects of N-3 and allows incorporation of the resulting dNTP much more rapidly opposite both T and C. Thus, in the absence of N<sup>2</sup>, 1-deazapurine dNTP cannot bind in a fashion that allows for efficient polymerization due to the presence of N-3. Adding N<sup>2</sup> to 1-deazapurine dNTP allows formation of a hydrogen bond between N<sup>2</sup> and O<sup>2</sup> of the pyrimidine that, presumably, changes the location of the 1-deazapurine dNTP and/or the template pyrimidine within the active site. The resulting base pair now looks less incorrect to pol  $\alpha$ , and as a consequence, the polymerization efficiency increases.

The effect of N<sup>2</sup> in one of the nucleotides at the primer 3′-terminus varied significantly depending upon the identity of the base pair. For the canonical A•T and G•C base pairs, adding or removing N<sup>2</sup>, respectively, only slightly inhibited addition of the next correct dNTP. If, however, the base pair at the primer 3′-terminus contained at least one nucleotide analogue, adding N<sup>2</sup> generally increased the efficiency of polymerization of the next correct dNTP. The smallest effect of adding N<sup>2</sup> occurred during polymerization onto a purine•T base pair (<2-fold, Table 3), but in some cases the effect exceeded 100-fold (e.g., adding N<sup>2</sup> to T•6-chloropurine or C•6-chloropurine base pairs). The effects of N<sup>2</sup> also demonstrated a tremendous asymmetry in some cases. Whereas adding N<sup>2</sup> to a 6-chloropurine•T base pair had a 4-fold effect,



adding N<sup>2</sup> to a T•6-chloropurine base pair increased addition of the next correct dNTP by >160-fold.

The effects of N<sup>2</sup> on polymerization are consistent with the shape of the base pair at the primer 3'-terminus playing a dominant role in determining the polymerization efficiency of the next correct dNTP. In most cases examined, adding N<sup>2</sup> to the purine in the base pair at the primer 3'-terminus significantly increased the rate of polymerization of the next correct dNTP. Presumably, the formation of an additional hydrogen bond between a purine N<sup>2</sup> and a pyrimidine O<sup>2</sup> will help to align the two bases into a geometry that more closely resembles a canonical base pair. However, the data also indicate that efficient elongation involves more than just geometry. First, the large asymmetries observed during elongation of some base pairs suggest that electronic features also affect polymerization (e.g., 6-chloropurine•T versus T•6-chloropurine, purine•T versus T•purine, and A•2-pyridone versus 2-pyridone•A base pairs). Second, the extremely strong discrimination against elongating primers where the 3'-terminal base pair contains 6-chloropurine as the template base suggests that the chlorine generates a particularly bad interaction with the enzyme.

While these and previous data have provided insights into the functional groups important for dNTP polymerization and the key role of the shape of the base pair at the primer 3'-terminus, a critical question is how the enzyme "reads" this information and then transmits it to the catalytic center (15, 19, 20). Solving this other half of the puzzle would provide fundamental insights into molecular recognition and could help lead to the development of novel base pairs that B family polymerases replicate with high fidelity as well as novel polymerase-directed chemotherapeutics.

## SUPPORTING INFORMATION AVAILABLE

NMR and mass spectra of selected compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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