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Herpes Simplex Virus-1 DNA Primase: A Remarkably Inaccurate yet Selective Polymerase[†]

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ABSTRACT: Herpes simplex virus-1 primase misincorporates the natural NTPs at frequencies of around one error per 30 NTPs polymerized, making it one of the least accurate polymerases known. We used a series of nucleotide analogues to further test the hypothesis that primase requires Watson−Crick hydrogen bond formation to efficiently polymerize a NTP. Primase could not generate base pairs containing a complete set of hydrogen bonds in an altered arrangement (isoguanine·isocytosine) and did not efficiently polymerize dNTPs completely incapable of forming Watson−Crick hydrogen bonds opposite templating bases incapable of forming Watson−Crick hydrogen bonds. Similarly, primase did not incorporate most NTPs containing hydrophobic bases incapable of Watson−Crick hydrogen bonding opposite natural template bases. However, 2-pyridone NTP and 4-methyl-2-pyridone NTP provided striking exceptions to this rule. The effects of removing single Watson−Crick hydrogen bonding groups from either the NTP or templating bases varied from almost no effect to completely blocking polymerization depending both on the parental base pair (G·C vs A·T/U) and which base pair of the growing primer (second, third, or fourth) was examined. Thus, primase does not absolutely need to form Watson−Crick hydrogen bonds to efficiently polymerize a NTP. Additionally, we found that herpes primase can misincorporate nucleotides both by misreading the template and by a primer−template slippage mechanism. The mechanistic and biological implications of these results are discussed.

The herpes simplex virus-1 (HSV-1)[1] primase-helicase is an essential component of the herpes DNA replication machinery and consists of three subunits encoded by three genes, UL5, UL8, and UL52. This heterotrimeric complex possesses three main activities: primase, DNA-dependent NTPase, and 5′−3′ helicase. The helicase tracks along the lagging strand and unwinds the DNA in front of the replication fork, and the NTPase provides the energy needed for unwinding. Primase synthesizes short RNA primers (up to 10−13 nucleosides long) on single-stranded DNA that a replicative polymerase extends via dNTP polymerization (*1*−*3*). The helicase-primase can initiate primer synthesis at any consecutive template pyrimidine residues, but for longer primer synthesis (more than three nucleotides), the 3′-G-Pyr-Pyr trimer is strictly required. Even on templates containing 3′-G-Pyr-Pyr, however, primase still synthesizes very large quantities of dinucleotides and trinucleotides (*1*).

A subassembly containing UL5 and UL52 subunits retains all three activities, while the UL8 subunit modulates these activities. UL8 also binds to other herpes replication proteins and may be involved in the organization of the herpes replisome (*1*, *2*, *4*−*9*). UL52 contains the complete primase active site in terms of phosphodiester bond formation but cannot initiate primer synthesis in the absence of UL5 (*7*, *10*). Thus, UL5 plays a critical but as yet undefined role during initiation of primer synthesis.

The question of how polymerases choose whether to polymerize a (d)NTP remains unresolved, although enzymes from different evolutionary families clearly use different mechanisms. A and B family DNA polymerases do not require the formation of Watson−Crick hydrogen bonds to polymerize a dNTP (*11*, *12*). B family enzymes use specific functional groups on the base of the incoming dNTP to encourage correct dNTP incorporation and prevent misincorporation (*13*−*17*). Data for the A family are less clear-cut, with some strongly supporting shape as a primary determinant and others suggesting shape does not play a critical role (*16*, *18*−*27*). Human primase, an enzyme that shares a small amount of sequence similarity with herpes primase, may require the formation of Watson−Crick hydrogen bonds to polymerize a NTP (*28*, *29*). Likewise, it has been suggested that the Y family DNA polymerases also require the formation of Watson−Crick hydrogen bonds to polymerize a dNTP (*30*, *31*).

HSV-1 DNA primase misincorporates the natural NTPs extremely efficiently, on average only 30-fold less efficiently than it correctly incorporates a NTP, and readily generates all types of mismatches: purine·purine, purine·pyrimidine, and pyrimidine·pyrimidine (*32*). Misincorporation largely, but not necessarily exclusively, occurs via misreading of the templating base as opposed to occurring via template or primer slippage (*32*). Herpes primase clearly is a low-fidelity polymerase, although this infidelity is not a problem biologically since the RNA primer is removed and replaced with DNA.

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[1]Abbreviations: HSV-1, herpes simplex virus-1; Pyr, pyrimidine.

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Previous work examined polymerization of NTP analogues containing modified bases as the first, second, or third nucleotide of the primer and suggested that efficient NTP incorporation requires formation of Watson−Crick hydrogen bonds (*33*). Loss of either N-1 or N^6 from ATP reduced the level of incorporation much more severely than converting ATP to CTP, GTP, or UTP. The natural NTPs can always form Watson−Crick hydrogen bonds with any natural base by using either the major or a minor tautomer (*28*, *33*, *34*). Adding a halogen at C-2 of ATP, a modification that only weakens the ability of the resulting base to form Watson−Crick hydrogen bonds (*35*, *36*), likewise had relatively weak effects on incorporation.

We examined how HSV-1 primase interacts with a series of NTP analogues to better define the role of Watson−Crick hydrogen bonds and minor groove hydrogen bond acceptors during NTP polymerization. Surprisingly, primase exhibited very unpredictable requirements for polymerizing a NTP. The requirements varied depending upon the base pair being generated, whether the incoming NTP will become the second, third, or fourth nucleotide of the primer, and if the NTP was a purine or pyrimidine. The enzyme also did not require formation of any Watson−Crick hydrogen bonds between the incoming NTP and templating base with a few select NTPs. Besides showing that herpes primase can be a remarkably selective enzyme, these studies raise the question of whether other polymerases proposed to require Watson−Crick hydrogen bonds between the incoming and templating nucleotides actually do require them.

EXPERIMENTAL PROCEDURES

Materials. All reagents were of the highest quality commercially available. Unlabeled natural NTPs were from Sigma, and radiolabeled NTPs were from Perkin-Elmer. Protected phosphoramidites of 2′-deoxyribonucleosides containing the bases 5-methyl-2-1*H*-pyrimidinone, 3-deazaadenine, and 5-methylisocytosine were purchased from Glen Research. C-2′-Deoxyribonucleosides (pyridin-3-yl, 4-oxopyridin-3-yl, and 4-aminopyridin-3-yl) were synthesized as described previously (*37*). ITP and iso-GTP were purchased from Trilink. 2-Hydroxypyridine, 2-hydroxy-4-methylpyridine, 2,3-diaminopyridine, 2-amino-4-methyl-3-nitropyridine, benzimidazole, and 2,3-diaminotoluene were purchased from Sigma-Aldrich. Purine and protected 1-chlorodeoxyribose were purchased from Berry and Associates. Synthetic DNA oligonucleotides with a defined sequence were purchased from IDT or Biosearch Technologies. HSV-1 primase (UL5−LU8−UL52 complex) was purified and stored as previously described (*1*, *33*).

Methods. (*i*) *Primase Assays*. Assays (10 μL) were performed as previously described (*1*, *33*) and typically contained 600 nM HSV-1 primase, 2 μM DNA template, 50 mM tris(hydroxymethyl)aminomethane, HCl salt (pH 8.0), 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 10 mM $MgCl_2$, 1% glycerol, 800 μM GTP, and various concentrations of a NTP (analogue). Reaction mixtures were incubated at 37 °C for 60 min and quenched with 25 μL of a formamide/0.05% xylene cyanol mixture and bromophenol blue. Products were separated using 20 to 25% polyacrylamide, 8 M urea gels and analyzed using a Typhoon Phosphorimager (Molecular Dynamics). The relative V_{max}/K_M for polymerization of a NTP (analogue) versus GTP was determined using a partitioning analysis as described previously (*38*).

(*ii*) *Synthesis of Nucleotide Analogues*. (*a*) *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 from 0 to 10% MeOH in CH_2Cl_2. The product was crystallized from a mixture of MeOH and EtOAc. The yield of yellow crystals was 3.43 g (65%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (s, 1H, H-8), 8.35 (dd, 1H, J_1 = 4.7 Hz, J_2 = 2.4 Hz), 8.01 (bd, 1H, J = 7.9 Hz), 7.21 (dd, 1H, J_1 = 8.3 Hz, J_2 = 4.8 Hz, H-1), 3.41 (bs, 1H, NH).

(b) 6-Methyl-1-deazapurine. 2-Amino-4-methyl-3-nitropyridine (5 g, 32.6 mmol) was dissolved in a mixture of THF (50 mL), isopropyl alcohol (20 mL), and water (5 mL). Pd (1 g, 10% on charcoal, 0.9 mmol) was added, and the mixture was repeatedly evacuated and saturated with H_2 at room temperature (rt) and laboratory pressure. The mixture was vigorously stirred for 3 h; the Pd/C was filtered off, and organic solvents were removed under reduced pressure. The resulting 2,3-diamino-4-methylpyridine was dissolved (without any further purification) in formic acid (70 mL, 90% in water) to yield 6-methyl-1-deazapurine with the same procedure that was used for 1-deazapurine. The whole procedure yielded 2.2 g (51%) of 6-methyl-1-deazapurine: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.38 (s, 1H, H-8), 8.20 (d, 1H, J = 4.9 Hz), 7.04 (dd, 1H, J_1 = 4.9 Hz, J_2 = 0.7 Hz), 3.17 (bs, 1H, NH), 2.54 (s, 3H, Me).

(c) 7-Methylbenzimidazole. 2,3-Diaminotoluene (5 g, 41 mmol) was dissolved in formic acid (100 mL, 90% in water) and heated to yield 7-methylbenzimidazole with the same procedure that was used for 1-deazapurine. This yielded 4.1 g (76%) of 7-methylbenzimidazole: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (s, 1H, H-2), 8.16 (bs, 1H, NH), 7.41 (d, 1H, J = 6.8 Hz), 7.09 (dd, 1H, J_1 = 7.3 Hz, J_2 = 7.3 Hz), 6.98 (dt, 1H, J_1 = 7.1 Hz, J_2 = 0.9 Hz), 2.51 (s, 3H, Me).

(d) 9-β-D-(1-Deazapurin)-3′,5′-di-*O*-(4-toluoyl)-2′-deoxyribofuranose. 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 for 3 h at rt, and then 1-chloro-3,5-bis(*p*-toluoyl)-2-deoxy-β-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_4Cl 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%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.38 (dd, 1H, J_1 = 4.9 Hz, J_2 = 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 4H-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_{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_3-Tol), 2.40 (s, 3H, CH_3-Tol).

(e) 9-β-D-(1-Deazapurine)-2′-deoxyribofuranose. 9-β-D-(1-Deazapurin)-2′-deoxy-3′,5′-di-*O*-(4-toluoyl)-D-ribofuranose (0.5 g, 1.08 mmol) was deprotected over 30 min using 0.1 M MeONa in MeOH and purified by silica gel chromatography (20 mL) using a gradient from 0 to 20% MeOH in $CHCl_3$. This procedure gave 0.2 g (78%) of product: ^{1}H NMR (400 MHz, $CDCl_3$) δ 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_2O), 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_{gem} = 13.5 Hz, $J_{2'b,1'}$ = 6.1 Hz, $J_{2'b,3'}$ = 2.7 Hz, H-2′b).

(f) 9-β-D-(6-Methyl-1-deazapurin)-3′,5′-di-*O*-(4-toluoyl)-2′-deoxyribofuranose. The nucleoside was prepared from 6-methyl-1-deazapurine (490 mg, 2.67 mmol) using the same procedure that was used for 1-deazapurine nucleoside. The whole procedure yielded 580 mg (44%) of colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, 1H, J_1 = 4.0 Hz, H-2), 8.18 (s, 1H, H-8), 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, 4H, 4H-Tol), 7.06 (dd, 1H, J_1 = 3.7 Hz, J_2 = 0.8 Hz, H-1), 6.67 (dd, 1H, J_1 = 8.4 Hz, J_2 = 5.7 Hz, H-1′), 5.82 (m, 1H, H-3′), 4.6−4.8 (m, 3H, H-5′a, H-5′b, H-4′), 3.15 (ddd, 1H, J_{gem} = 14.3 Hz, $J_{2'a,1'}$ = 8.7 Hz, $J_{2'a,3'}$ = 6.3 Hz, H-2′a), 2.82 (ddd, 1H, J_{gem} = 14.3 Hz, $J_{2'b,1'}$ = 5.7 Hz, $J_{2'b,3'}$ = 1.9 Hz, H-2′b), 2.65, 2.42, 2.38 (3s, 3H, $3CH_3$).

(g) 9-β-D-(6-Methyl-1-deazapurine)-2′-deoxyribofuranose. 9-β-D-(6-Methyl-1-deazapurin)-3′,5′-di-*O*-(4-toluoyl)-2′-deoxyribofuranose (530 mg, 1.1 mmol) was deprotected using the same procedure that was used for 1-deazapurine nucleoside to give 200 mg (76%) of crystals: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.55 (s, 1H, H-8), 8.41 (dd, 1H, J_1 = 4.7 Hz, J_2 = 1.4 Hz, H-2), 7.36 (d, 1H, J_1 = 4.1 Hz, H-1), 6.57 (dd, 1H, J_1 = 8.2 Hz, J_2 = 5.9 Hz, H-1′), 4.80 (bs, 3H, OH, H_2O), 4.60 (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_{gem} = 12.2 Hz, $J_{5'a,4'}$ = 3.1 Hz, H-5′a), 3.75 (dd, 1H, J_{gem} = 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_{gem} = 14.5 Hz, $J_{2'b,1'}$ = 5.8 Hz, $J_{2'b,3'}$ = 1.9 Hz, H-2′b).

(h) 1-β-D-(2-Oxopyridine)-3′,5′-di-*O*-(4-toluoyl)-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 rt 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 rt and worked up as described above. Silica gel chromatography (200 mL) in a 28% EtOAc/hexane mixture 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. ^{1}H NMR (400 MHz, $CDCl_3$) δ 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 = 1.9 Hz, 1H-Pyr), 7.31 (ddd, 1H, J_1 = J_2 = 6.5 Hz, J_3 = 2.0 Hz, 1H-Pyr), 7.28 (bd, 2H, 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_1 = 9.15 Hz, J_2 = 1.3 Hz, J_3 = 0.6 Hz, 1H-Pyr), 6.12 (dd, J_1 = 6.8 Hz, J_2 = 1.1 Hz, 1H-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_{gem} = 14.5 Hz, $J_{2'a,1'}$ = 5.7 Hz, $J_{2'a,3'}$ = 1.9 Hz, H-2′a), 2.43 (s, 3H, CH_3-Tol), 2.41 (s, 3H, CH_3-Tol), 2.16 (ddd, 1H, J_{gem} = 14.5 Hz, $J_{2'b,1'}$ = 7.9 Hz, $J_{2'b,3'}$ = 6.6 Hz, H-2′b). The NMR spectrum was identical to that reported in the literature (*39*).

(i) 1-β-D-(2-Oxopyridine)-2′-deoxyribofuranose. The 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 from 0 to 20% MeOH in $CHCl_3$. This procedure gave 224 mg (78%) of product: ^{1}H NMR (400 MHz, DMSO-d_6) 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′), 3.84 (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_{gem} = 13.2 Hz, $J_{2'b,1'}$ = 6.1 Hz, $J_{2'b,3'}$ = 6.1 Hz, H-2′b).

(j) 1-β-D-(4-Methyl-2-oxopyridine)-3′,5′-di-*O*-(4-toluoyl)-2′-deoxyribofuranose. This nucleoside was prepared from 4-methyl-2-hydroxypyridine (583 mg, 5.34 mmol) using the same procedure that was used for 2-oxopyridine nucleoside which yielded 790 mg (32%) of the desired 1-β-D-(4-methyl-2-oxopyridine)-3′,5′-di-*O*-(4-toluoyl)-2′-deoxyribofuranose (along with 40% of the mixture of desired product with the α anomer): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (bdd, 2H, J_1 = 6.7 Hz, J_2 = 1.6 Hz, Tol), 7.87 (bdd, 2H, J_1 = 6.7 Hz, J_2 = 1.6 Hz, Tol), 7.56 (d, 1H, J = 7.2 Hz, 1H-Pyr), 7.28 (dd, 2H, J_1 = 8.5 Hz, J_2 = 0.6 Hz, Tol), 7.23 (dd, 2H, J_1 = 8.5 Hz, J_2 = 0.6 Hz, Tol), 6.61 (dd, 1H, J_1 = 8.2 Hz, J_2 = 5.6 Hz, H-1′), 6.35 (1H, s, H-3-Pyr), 5.96 (dd, J_1 = 7.2 Hz, J_2 = 1.7 Hz, H-Pyr), 5.60 (dt, 1H, $J_{3',2'b}$ = 6.6 Hz, $J_{3,2'a}$ = 1.9 Hz, H-3′), 4.70 (m, 2H, H-5′a, H-5′b), 4.60 (m, 1H, H-4′), 2.96 (ddd, 1H, J_{gem} = 14.4 Hz, $J_{2'a,1'}$ = 5.6 Hz, $J_{2'a,3'}$ = 1.8 Hz, H-2′a), 2.44 (s, 3H, CH_3-Tol), 2.42 (s, 3H, CH_3-Tol), 2.24 (ddd, 1H, J_{gem} = 14.6 Hz, $J_{2'b,1'}$ = 7.2 Hz, $J_{2'b,3'}$ = 6.6 Hz, H-2′b). The NMR spectrum was identical to that reported in the literature (*39*).

(k) 1-β-D-(4-Methyl-2-oxopyridine)-2′-deoxyribofuranose. The 1-β-D-(2-oxopyridine)-3′,5′-di-*O*-(4-toluoyl)-2′-deoxyribofuranose (730 mg, 1.6 mmol) was deprotected using 0.1 M MeONa in MeOH and purified by silica gel chromatography (20 mL) using a gradient from 0 to 20% MeOH in $CHCl_3$. This procedure gave 330 mg (93%) of product: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.80 (d, 1H, J_1 = 7.1 Hz, 1H-Pyr), 6.34 (t, 1H, J_1 = 6.9 Hz, 1H-Pyr), 6.14 (m, 3H, 2H-Pyr, H-1′), 5.25 (d, 1H, J = 4.1 Hz, 3-OH), 5.03 (t, 1H, J = 5.2 Hz, 5-OH), 4.23 (m, 1H, H-3′), 3.84 (m, 1H, H-4′), 3.58 (m, 2H, H-5′a, H-5′b), 2.21 (ddd, 1H, J_{gem} = 13.2 Hz, $J_{2'a,1'}$ = 5.9 Hz, $J_{2'a,3'}$ = 3.3 Hz, H-2′a), 2.11 (s, 1H, Me), 1.92 (ddd, 1H, J_{gem} = 13.2 Hz, $J_{2'b,1'}$ = 7.1 Hz, $J_{2'b,3'}$ = 6.1 Hz, H-2′b).

(*iii*) *General Procedure for the Preparation of Purine Ribonucleoside Analogues*. Glycosylation was performed essentially as previously described (*28*, *40*). Benzimidazole (591 mg, 5 mmol) was dissolved in MeCN (50 mL), and *N*,*O*-bis(trimethylsilyl)acetamide (1.8 mL, 7.5 mmol) was added dropwise. The mixture was heated under reflux for 15 min. After the mixture had cooled to rt, 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (1.8 g, 5.6 mmol) in MeCN (20 mL) was added followed by 1.2 mL (6.25 mmol) of trimethylsilyl trifluoromethanesulfonate (1.3 mL, 7.2 mmol), and the mixture was heated under reflux for an additional 3 h. After cooling to rt, the mixture was poured into saturated $NaHCO_3$, and the product was extracted to EtOAc. The organic layer was dried with $MgSO_4$, and solvents were removed under vacuum. The product was purified by chromatography on silica gel (100 g) with a gradient from EtOAc to 5% of MeOH in EtOAc to give a colorless oil in yields of 25−60%. The nucleoside was then deprotected using MeONa in MeOH (0.1 M) and purified by chromatography on silica gel (25 g) with a gradient from CH_2Cl_2 to 30% MeOH in CH_2Cl_2.

(a) 1-β-D-(Benzimidazole)-2′,3′,5′-tri-*O*-acetyl-ribofuranose. The yield was 51%, and the ^{1}H NMR spectrum was identical to that reported previously (*40*).

(b) 1-β-D-(Benzimidazole)-ribofuranose. The yield was 85%, and the ^{1}H NMR spectrum was identical to that reported previously (*40*).

(c) 1-β-D-(4-Methylbenzimidazole)-2′,3′,5′-tri-*O*-acetyl-ribofuranose. The yield was 25% along with 20% of the other isomer [3-β-D-(4-Methylbenzimidazole)-2′,3′,5′-tri-*O*-acetyl-ribofuranose].

(d) 1-β-D-(4-Methylbenzimidazole)-ribofuranose. The yield was 90%, and the ^{1}H NMR spectrum was identical to that reported previously (*41*).

(e) 9-β-D-(1-Deazapurine)-2′,3′,5′-tri-*O*-acetyl-ribofuranose. Three products were obtained from the reaction, 18% of the desired 9-β-D-(1-Deazapurine)-2′,3′,5′-tri-*O*-acetyl-ribofuranose along with 7-β-D-(1-deazapurine)-2′,3′,5′-tri-*O*-acetyl-ribofuranose and 14% of the α-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (dd, 1H, $J_{2,1}$ = 4.9 Hz, $J_{2,6}$ = 1.5 Hz, H-2), 8.21 (s, 1H, H-8), 8.07 (dd, 1H, $J_{6,1}$ = 8.1 Hz, $J_{6,2}$ = 1.5 Hz, H-6), 7.27 (dd, 1H, $J_{1,6}$ = 8.1 Hz, $J_{1,2}$ = 4.8 Hz, H-1), 6.29 (d, 1H, $J_{1',2'}$ = 5.2 Hz, H-1′), 6.04 (t, 1H, $J_{2',1'} = J_{2',3'}$ = 5.3 Hz, H-2′), 5.72 (t, 1H, $J_{3',2'} = J_{3',4'}$ = 5.1 Hz, H-3′), 4.44 (m, 2H, H-4′ and H-5′a), 4.37 (dd, 1H, J_{gem} = 13.0 Hz, $J_{5b',4'}$ = 5.6 Hz, H-5′b), 2.13 (s, 3H), 2.10 (s, 3H), 2.06 (s, 3H, 3OAc). The structure of this compound was confirmed by COSY, 2D NOESY, HSQC, and HMBC spectra. 2D NOESY cross-peaks of H-1′ with H-4′ and H-8 with H-2′ confirmed that the compound is the β anomer, and HMBC cross-peaks of H-1′ with C-5, C-3, and C-8 and H-8 with C-5 and C-6 confirmed that the structure is 1-deazapurin-9-yl.

(f) 9-β-D-(1-Deazapurine)-ribofuranose. This compound was obtained by deprotection of 9-β-D-(1-deazapurine)-2′,3′,5′-tri-*O*-acetyl-ribofuranose in the usual procedure to give an 87% yield: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.71 (s, 1H, H-8), 8.38 (dd, 1H, $J_{2,1}$ = 4.8 Hz, $J_{2,6}$ = 1.3 Hz, H-2), 8.14 (dd, 1H, $J_{6,1}$ = 8.0 Hz, $J_{6,2}$ = 1.5 Hz, H-6), 7.34 (dd, 1H, $J_{1,6}$ = 8.0 Hz, $J_{1,2}$ = 4.8 Hz, H-1), 6.07 (d, 1H, $J_{1',2'}$ = 6.1 Hz, H-1′), 5.50 (d, 1H, J = 6.2 Hz, OH-3′), 5.27 (t, 1H, J = 5.0 Hz, OH-5′), 5.23 (d, 1H, J = 4.8 Hz, OH-2′), 4.68 (m, 1H, H-4′), 4.19 (m, 1H, H-3′), 3.99 (m, 1H, H-2′), 3.69 (ddd, 1H, J_{gem} = 12.0 Hz, $J_{5a',4'}$ = 4.5 Hz, $J_{5a',OH'}$ = 4.5 Hz, H-5a′), 3.59 (ddd, 1H, J_{gem} = 12.0 Hz, $J_{5b',OH'}$ = 6.6 Hz, $J_{5b',4'}$ = 3.9 Hz, H-5b′).

(g) 9-β-D-(6-Methyl-1-deazapurine)-2′,3′,5′-tri-*O*-acetyl-ribofuranose. Two products were obtained, 33% of the desired 9-β-D-(6-methyl-1-deazapurine)-2′,3′,5′-tri-*O*-acetyl-ribofuranose along with 12% of 7-β-D-(6-methyl-1-deazapurine)-2′,3′,5′-tri-O-acetyl-ribofuranose. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (d, 1H, $J_{2,1}$ = 4.9 Hz, H-2), 8.16 (s, 1H, H-8), 7.07 (dd, 1H, $J_{1,2}$ = 4.9 Hz, J_2 = 0.7 Hz, H-1), 6.27 (d, 1H, $J_{1',2'}$ = 5.3 Hz, H-1′), 6.02 (t, 1H, $J_{2',1'} = J_{2',3'}$ = 5.4 Hz, H-2′), 5.71 (t, 1H, $J_{3',2'} = J_{3',4'}$ = 5.1 Hz, H-3′), 4.43 (m, 2H, H-4′ and H-5′a), 4.37 (dd, 1H, J_{gem} = 12.8 Hz, $J_{5b',4'}$ = 5.5 Hz, H-5′b), 2.65 (s, 3H, Ar-Me), 2.13 (s, 3H), 2.10 (s, 3H), 2.05 (s, 3H, 3xOAc).

(h) 9-β-D-(6-Methyl-1-deazapurine)-ribofuranose. This compound was obtained by the deprotection of 9-β-D-(6-methyl-1-deazapurine)-2′,3′,5′-tri-*O*-acetyl-ribofuranose with the usual procedure in an 84% yield: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.64 (s, 1H, H-8), 8.22 (d, 1H, $J_{2,1}$ = 4.9 Hz, H-2), 7.17 (d, 1H, $J_{1,2}$ = 4.8 Hz, H-1), 6.03 (d, 1H, $J_{1',2'}$ = 6.0 Hz, H-1′), 5.47 (d, 1H, J = 6.1 Hz, OH-2′), 5.37 (dd, 1H, J_1 = 6.8 Hz, J_2 = 4.8 Hz, OH-5′), 5.22 (d, 1H, J = 4.7 Hz, OH-3′), 4.67 (m, 1H, H-2′), 4.18 (m, 1H, H-3′), 3.99 (m, 1H, H-4′), 3.69 (m, 1H, H-5′a), 3.59 (m, 1H, H-5′b), 2.59 (s, 1H, Me).

(i) 9-β-D-(Purine)-2′,3′,5′-tri-*O*-acetyl-ribofuranose. Purine (330 mg, 2.75 mmol) was dissolved in MeCN (20 mL), and *N*,*O*-bis(trimethylsilyl)acetamide (0.6 mL, 2.95 mmol) was added dropwise. The mixture was heated under reflux for 1 h at rt. After the mixture had cooled, 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (1 g, 3.14 mmol) in MeCN (10 mL) was added. Trimethylsilyl trifluoromethanesulfonate (0.65 mL, 3.59 mmol) was added, and the mixture was heated under reflux for 4 h. Upon cooling to rt, the mixture was extracted to EtOAc twice with saturated $NaHCO_3$ and once with saturated NaCl. The organic layer was dried over Mg_2SO_4. Solvents were removed under vacuum. The product was purified by chromatography on silica gel (200 mL) eluted with a 3:1 EtOAc/hexane mixture and a 0 to 5% MeOH gradient. Purification yielded two distinct products; the desired product was a colorless oil (390 mg, 1.18 mmol) with a 43% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.13 (s, 1H, Ar), 8.97 (s, 1H, Ar), 8.25 (s, 1H, Ar), 6.23 (d, 1H, $J_{1',2'}$ = 5.2 Hz, H-1′), 5.95 (t, 1H, $J_{2',1'} = J_{2',3'}$ = 5.4 Hz, H-2′), 5.66 (t, 1H, $J_{3',2'} = J_{3',4'}$ = 5.2 Hz, H-3′), 4.30−4.70 (m, 3H, H-4′, H-5′a, and H-5′b), 2.12 (s, 3H, Ac), 2.07 (s, 3H, Ac), 2.04 (s, 3H, Ac).

(j) 9-β-D-(Purine)-ribofuranose. 9-β-D (Purine)-2′,3′,5′-tri-*O*-acetyl-ribofuranose (390 mg, 1.18 mmol) was deprotected by being stirred for 30 min in 0.1 M NaOMe in MeOH. The mixture was purified with silica gel chromatography (20 mL) using a gradient from 0 to 20% MeOH in CH_2Cl_2. Deprotection and purification yielded 71 mg of product (24% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 9.13 (s, 1H, Ar), 8.95 (s, 1H, Ar), 8.81 (s, 1H, Ar), 6.18 (d, 1H, $J_{1',2'}$ = 5.6 Hz, H-1′), 4.76 (dd, 1H, $J_{2',1'} = J_{2',3'}$ = 5.3 Hz, H-2′), 4.39 (dd, 1H, $J_{3',2'}$ = 5.1 Hz, $J_{3',4'}$ = 3.7 Hz, H-3′), 4.18 (ddd, 1H, $J_{4',3'} = J_{4',5'a} = J_{4',5'b}$ = 3.3 Hz, H-4′), 3.90 (dd, 1H, J_{gem} = 12.3 Hz, $J_{5a',4'}$ = 3.0 Hz, H-5′a), 3.79 (dd, 1H, J_{gem} = 12.3 Hz, $J_{5b',4'}$ = 3.3 Hz, H-5′b).

(k) 1-β-D-(Oxopyridine)-ribofuranose. 2-Hydroxypyridine (0.48 g, 5.5 mmol) and 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (1.6 g, 5.0 mmol) were dissolved in 25 mL of MeCN, and $SnCl_4$ (1.25 mL) was added dropwise. The mixture was stirred for 8 h at rt. Then the solvents were evaporated, and the product was purified by chromatography on silica gel (100 mL) in EtOAc. An oily product was immediately deprotected using 0.1 M MeONa in MeOH and purified by chromatography on silica gel (30 mL) with a gradient from CH_2Cl_2 to 20% MeOH in CH_2Cl_2. The product was crystallized from a MeOH/EtOAc mixture to give 280 mg (25%) of the desired compound: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.99 (dd, 1H, J_1 = 7.1 Hz, J_2 = 1.6 Hz, H-Pyr), 7.39 (ddd, 1H, J_1 = 8.8 Hz, J_2 = 6.5 Hz, J_3 = 2.0 Hz, H-Pyr), 6.34 (ddd, 1H, J_1 = 9.2 Hz, J_2 = 0.6 Hz, J_3 = 0.6 Hz, H-Pyr), 6.03 (d, 1H, J_1 = 6.8 Hz, J_2 = 6.8 Hz, J_3 = 1.3 Hz, H-Pyr), 6.01 (d, 1H, $J_{1',2'}$ = 3.5 Hz, H-1′), 5.41 (d, 1H, J = 4.3 Hz, OH-3′), 5.11 (t, 1H, J = 5.0 Hz, OH-5′), 5.04 (bd, 1H, J = 3.4 Hz, OH-2′), 3.93 (m, 2H, H-4′ and H-3′), 3.88 (m, 1H, H-2′), 3.70 (m, 1H, H-5′a), 3.58 (m, 1H, H-5′b).

(l) 1-β-D-(4-Methyl-oxopyridine)-ribofuranose. This compound was prepared from 4-methyl-2-hydroxypyridine (600 mg, 5.5 mmol) using the same procedure that was used for 2-oxopyridin-1-yl nucleoside with an overall yield of 620 mg (44%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.83 (d, 1H, J_1 = 7.2 Hz, H-6), 6.15 (bs, 1H, H-3), 6.08 (dd, 1H, J_1 = 7.1 Hz, J_2 = 1.7 Hz, H-5), 6.01 (d, 1H, $J_{1',2'}$ = 3.7 Hz, H-1′), 5.36 (bs, 1H, OH-3′), 5.09 (bs, 1H, OH-5′), 5.00 (bs, 1H, OH-2′), 3.93 (m, 2H, H-4′ and H-3′), 3.87 (m, 1H, H-2′), 3.67 (bd, 1H, J_{gem} = 11.9 Hz, H-5′a), 3.58 (bd, 1H, J_{gem} = 12.2 Hz, H-5′b), 2.09 (s, 3H, Me-Ar).

(*iv*) *General Procedure for the Synthesis of DMTr-Protected Nucleosides*. Free nucleoside (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 $NaHCO_3$ and the product was extracted into EtOAc. Organic layers were washed with several portions of 1% $NaHCO_3$ and dried over $MgSO_4$, and solvents were evaporated under reduced pressure. The product was purified by silica gel chromatography (0−10% MeOH in EtOAc). The reaction yielded 60−90% of 5′-(4,4-dimethoxytrityl)-protected nucleoside.

(a) 3-β-D-(Pyridine)-5′-(4,4-dimethoxytrityl)-2′-deoxyribofuranose: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (d, 1H, $J_{2,4}$ = 2.1 Hz, H-2), 8.51 (dd, 1H, $J_{6,5}$ = 4.8 Hz, $J_{6,4}$ = 1.7 Hz, H-6), 7.71 (ddd, $J_{4,5}$ = 7.9 Hz, $J_{4,2}$ = 2.1 Hz, $J_{4,6}$ = 1.6 Hz, H-4), 7.44 (dm, 2H, J = 7.1 Hz, 2H-DMTr), 7.18−7.36 (m, 8H, 7H-DMTr, H-5), 6.81 (dm, 4H, J = 6.9 Hz, 4H-DMTr), 5.20 (dd, 1H, $J_{1',2'b}$ = 10.3 Hz, $J_{1',2'a}$ = 5.6 Hz, H-1′), 4.45 (m, 1H, H-3′), 4.10 (m, 1H, H-4′), 3.78 (s, 6H, CH_3-DMTr), 3.35 (dd, 1H, J_{gem} = 9.8 Hz, $J_{5'a,4'}$ = 4.5 Hz, H-5′a), 3.26 (dd, 1H, J_{gem} = 9.8 Hz, $J_{5'b,4'}$ = 5.3 Hz, H-5′b), 2.29 (ddd, 1H, J_{gem} = 13.0 Hz, $J_{2'a,1'}$ = 5.6 Hz, $J_{2'a,3'}$ = 1.9 Hz, H-2′a), 2.02 (ddd, 1H, J_{gem} = 13.1 Hz, $J_{2'b,1'}$ = 10.3 Hz, $J_{2'a,3'}$ = 5.9 Hz, H-2′a).

(b) 3-β-D-(6-Isobutyrylaminopyridine)-5′-(4,4-dimethoxytrityl)-2′-deoxyribofuranose. The free nucleoside (100 mg, 0.48 mmol) was dissolved in pyridine (5 mL); trimethylsilyl chloride (300 μL, 3 equiv) was added, and the mixture was stirred at rt for 5 h. Then isobutyryl chloride (0.2 mL) was added, and the mixture was stirred at rt overnight. The product was poured into saturated $NaHCO_3$ and extracted to EtOAc. Organic layers were extracted three times with water and then dried over $MgSO_4$. Organic solvents were evaporated to give the crude oil of silylated nucleoside, which was deprotected overnight using TBAF in THF (2 mL, 0.1 M). THF was evaporated, and the product was chromatographed on silica gel (15 mL) with a gradient from CH_2Cl_2 to 25% MeOH in CH_2Cl_2: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81 (bs, 1H, NH), 8.20 (d, 1H, J = 6.1 Hz, H-2), 8.20 (bs, 1H, H-5), 7.70 (dd, 1H, $J_{4,5}$ = 8.8 Hz, $J_{4,2}$ = 2.2 Hz, H-4), 5.14 (dd, 1H, $J_{1',2'b}$ = 9.9 Hz, $J_{1',2'a}$ = 5.8 Hz, H-1′), 4.45 (dd, 1H, $J_{3',4'}$ = 8.8 Hz, $J_{3',2'a}$ = 3.2 Hz, H-3′), 4.06 (m, 1H, H-4′), 3.62−3.80 (m, 2H, 2H-5′), 2.50−2.64 (m, 1H, CH-iBu), 2.29 (ddd, 1H, J_{gem} = 13.1 Hz, $J_{2'a,1'}$ = 5.8 Hz, $J_{2'a,3'}$ = 2.4 Hz, H-2′a), 2.02 (ddd, 1H, J_{gem} = 13.1 Hz, $J_{2'b,1'}$ = 9.9 Hz, $J_{2'a,3'}$ = 3.4 Hz, H-2′a), 1.23 (d, 1H, J = 7.1 Hz, Me-iBu), 1.23 (d, 1H, J = 7.1 Hz, Me-iBu).

This intermediate was tritylated according to the general procedure to give an overall yield of 60 mg (22%) of protected nucleoside: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, 1H, $J_{2,4}$ = 2.2 Hz, H-2), 8.99 (d, 1H, $J_{5,4}$ = 8.6 Hz, H-5), 8.15 (bs, 1H, NH), 7.69 (dd, $J_{4,5}$ = 8.5 Hz, $J_{4,2}$ = 2.2 Hz, H-4), 7.44 (dm, 2H, J = 7.2 Hz, 2H-DMTr), 7.18−7.36 (m, 7H, 7H-DMTr), 6.81 (dm, 4H, J = 6.9 Hz, 4H-DMTr), 5.15 (dd, 1H, $J_{1',2'b}$ = 10.2 Hz, $J_{1',2'a}$ = 5.4 Hz, H-1′), 4.43 (m, 1H, H-3′), 4.07 (m, 1H, H-4′), 3.79 (s, 6H, CH_3-DMTr), 3.34 (dd, 1H, J_{gem} = 9.8 Hz, $J_{5'a,4'}$ = 4.7 Hz, H-5′a), 3.25 (dd, 1H, J_{gem} = 9.8 Hz, $J_{5'b,4'}$ = 5.3 Hz, H-5′b), 2.54 (septet, 1H, J = 6.8 Hz, CH-iBu), 2.24 (ddd, 1H, J_{gem} = 13.1 Hz, $J_{2'a,1'}$ = 5.7 Hz, $J_{2'a,3'}$ = 1.9 Hz, H-2′a), 2.02 (ddd, 1H, J_{gem} = 13.1 Hz, $J_{2'b,1'}$ = 10.3 Hz, $J_{2'a,3'}$ = 5.9 Hz, H-2′a), 1.24 (d, 3H, J = 7.2 Hz, Me-iBu), 1.24 (d, 3H, J = 7.2 Hz, Me-iBu).

(c) 3-β-D-(6-Oxopyridine)-5′-(4,4-dimethoxytrityl)-2′-deoxyribofuranose: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (dd, $J_{4,5}$ = 9.4 Hz, $J_{4,2}$ = 2.5 Hz, H-4), 7.44 (dm, 2H, J = 7.2 Hz, 2H-DMTr), 7.18−7.36 (m, 7H, 7H-DMTr), 7.16−7.23 (m, 1H, H-2), 6.82 (dm, 4H, J = 6.9 Hz, 4H-DMTr), 6.52 (d, 1H, $J_{5,4}$ = 9.4 Hz, H-5), 4.93 (dd, 1H, $J_{1',2'b}$ = 10.3 Hz, $J_{1',2'a}$ = 5.4 Hz, H-1′), 4.41 (m, 1H, H-3′), 4.01 (m, 1H, H-4′), 3.79 (s, 6H, CH_3-DMTr), 3.31 (dd, 1H, J_{gem} = 9.8 Hz, $J_{5'a,4'}$ = 4.5 Hz, H-5′a), 3.20 (dd, 1H, J_{gem} = 9.8 Hz, $J_{5'b,4'}$ = 5.4 Hz, H-5′b), 2.24 (ddd, 1H, J_{gem} = 13.1 Hz, $J_{2'a,1'}$ = 5.5 Hz, $J_{2'a,3'}$ = 1.8 Hz, H-2′a), 2.02 (ddd, 1H, J_{gem} = 13.1 Hz, $J_{2'b,1'}$ = 10.3 Hz, $J_{2'a,3'}$ = 6.0 Hz, H-2′a).

(d) 9-β-D-(1-Deazapurine)-5′-(4,4-dimethoxytrityl)-2′-deoxyribofuranose: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 (dd, 1H, J_1 = 4.7 Hz, J_2 = 1.4 Hz, H-2), 8.24 (s, 1H, H-8), 8.07 (dd, 1H, J_1 = 8.1 Hz, J_2 = 1.5 Hz, H-6), 7.44 (dm, 2H, J = 7.2 Hz, 2H-DMTr), 7.18−7.36 (m, 8H, 7H-DMTr, H-1), 6.82 (dm, 4H, J = 6.9 Hz, 4H-DMTr), 6.64 (t, 1H, J = 6.6 Hz, H-1′), 4.72 (m, 1H, H-3′), 4.23 (m, 1H, H-4′), 3.78 (s, 6H, CH_3-DMTr), 3.41 (m, 2H, 2H-5′), 2.86 (dt, 1H, J_{gem} = 13.4 Hz, $J_{2'a,1'}$ = $J_{2'a,3'}$ = 5.8 Hz, H-2′a), 2.84 (ddd, 1H, J_{gem} = 13.4 Hz, $J_{2'b,1'}$ = 6.3 Hz, $J_{2'b,3'}$ = 3.9 Hz, H-2′b).

(e) 9-β-D-(1-Deaza-6-methylpurine)-5′-(4,4-dimethoxytrityl)-2′-deoxyribofuranose: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (d, 1H, $J_{2,3}$ = 4.9 Hz, H-2), 8.16 (s, 1H, H-8), 7.44 (dm, 2H, J = 7.2 Hz, 2H-DMTr), 7.18−7.36 (m, 7H, 7H-DMTr), 7.03 (d, 1H, $J_{3,2}$ = 4.9 Hz, H-1), 6.82 (dm, 4H, J = 6.9 Hz, 4H-DMTr), 6.58 (t, 1H, J = 6.7 Hz, H-1′), 4.67 (m, 1H, H-3′), 4.18 (dd, 1H, $J_{4',5'}$ = 5.7 Hz, $J_{4',3'}$ = 4.9 Hz, H-4′), 3.75 (s, 6H, CH_3-DMTr), 3.67 (m, 2H, 2H-5′), 2.80 (ddd, 1H, J_{gem} = 13.5 Hz, $J_{2'a,1'}$ = $J_{2'a,3'}$ = 6.4 Hz, H-2′a), 2.65 (s, 3H, Ar-Me), 2.53 (ddd, 1H, J_{gem} = 13.5 Hz, $J_{2'b,1'}$ = 6.2 Hz, $J_{2'b,3'}$ = 2.3 Hz, H-2′b).

(f) 1-β-D-(2-Oxopyridine)-5′-(4,4-dimethoxytrityl)-2′-deoxyribofuranose: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.94 (dd, 1H, J_1 = 6.0 Hz, J_2 = 1.8 Hz, H-6), 7.42 (dm, 2H, J = 7.2 Hz, 2H-DMTr), 7.14−7.37 (m, 8H, 7H-DMTr, 1H-Pyr), 6.82 (dm, 4H, J = 6.9 Hz, 4H-DMTr), 6.54 (t, 1H, J = 6.1 Hz, H-1′), 6.49 (d, 1H, J = 8.8 Hz, H-Pyr), 6.03 (td, 1H, J_1 = 6.9 Hz, J_2 = 1.1 Hz, H-5), 4.51 (m, 1H, H-3′), 4.17 (ddd, 1H, $J_{4',3'}$ = 7.2 Hz, $J_{4',5'a}$ = $J_{4',5'b}$ = 3.7 Hz, H-4′), 3.78 (s, 6H, CH_3-DMTr), 3.48 (dd, 1H, J_{gem} = 10.6 Hz, $J_{5'a,4'}$ = 3.3 Hz, H-5′a), 3.40 (dd, 1H, J_{gem} = 10.6 Hz, $J_{5'a,4'}$ = 3.8 Hz, H-5′b), 2.74 (ddd, 1H, J_{gem} = 13.7 Hz, $J_{2'a,1'}$ = 6.0 Hz, $J_{2'a,3'}$ = 4.3 Hz, H-2′a), 2.19 (dt, 1H, J_{gem} = 13.0 Hz, $J_{2'b,1'}$ = $J_{2'b,3'}$ = 6.3 Hz, H-2′b).

(g) 1-β-D-(4-Methyl-2-oxopyridine)-5′-(4,4-dimethoxytrityl)-2′-deoxyribofuranose: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.79 (d, 1H, $J_{6,5}$ = 7.1 Hz, H-6), 7.42 (dm, 2H, J = 7.2 Hz, 2H-DMTr), 7.14−7.37 (m, 7H, 7H-DMTr), 6.82 (dm, 4H, J = 6.9 Hz, 4H-DMTr), 6.57 (t, 1H, $J_{1',2'a}$ = $J_{1',2'b}$ = 6.3 Hz, H-1′), 6.29 (s, 1H, H-3), 5.89 (dd, 1H, $J_{5,6}$ = 7.1 Hz, $J_{5,?}$ = 1.7 Hz, H-5), 4.50 (dt, 1H, $J_{3',4'}$ = 6.1 Hz, $J_{3',2'a}$ = $J_{3',2'b}$ = 3.6 Hz, H-3′), 4.17 (ddd, 1H, $J_{4',3'}$ = 7.1 Hz, $J_{4',5'a}$ = $J_{4',5'b}$ = 3.5 Hz, H-4′), 3.78 (s, 6H, CH_3-DMTr), 3.45 (dd, 1H, J_{gem} = 10.5 Hz, $J_{5'a,4'}$ = 3.2 Hz, H-5′a), 3.37 (dd, 1H, J_{gem} = 10.5 Hz, $J_{5'a,4'}$ = 4.0 Hz, H-5′b), 2.73 (ddd, 1H, J_{gem} = 13.7 Hz, $J_{2'a,1'}$ = 6.0 Hz, $J_{2'a,3'}$ = 3.7 Hz, H-2′a), 2.19 (m, 1H, H-2′b), 2.13 (s, 3H, Ar-Me).

(*v*) *General Procedure for the Synthesis of DMTr-Protected Phosphoramidites of Nucleosides*. Tritylated nucleoside **5** (0.22 mmol) was dissolved in CH_2Cl_2 (8 mL), and DMAP (catalytic amount) and ethyl-diisopropylamine (0.3 mL) were added. The mixture was cooled to 0 °C, and then 2-cyanoethyl diisopropylaminochlorophosphoramidite (0.3 mL) was added. The mixture was stirred for 30 min at 0 °C and then for 30 min while warming to 25 °C. The solvents were evaporated, and the crude oily product was dissolved in a mixture of ethyl acetate, hexane, and triethylamine (49:49:2) and chromatographed over silica gel (10 mL) in the same mixture to give an oil of two

diastereiosomers of phosphoramidites. The oil was dried by codistillation with MeCN (three times) which yielded 50−90% phosphoramidite.

(a) [1β-(Pyridin-3-yl)-5′-(dimethoxytrityl)-2′-deoxy-D-ribofuranos-3′-yl]-2-cyanoethyl-*N*,*N*-bis(isopropylamino)-phosphoramidite. This compound was obtained as a colorless oil in 82% yield: MS (ESI, MeCN, LiCl added, negative) 733 (M + 35 [Cl]) calcd 733; MS (ESI, MeCN, LiCl added, positive) 705 (M + 7 [Li]) calcd 705; ^{31}P NMR (400 MHz, MeCN-d_3) δ 148.37 (s, first diastereoisomer), 148.33 (s, second diastereoisomer).

(b) [1β-(6-Isobutyrylamino-pyridin-3-yl)-5′-(dimethoxytrityl)-2′-deoxy-D-ribofuranos-3′-yl]-2-cyanoethyl-*N*,*N*-bis-(isopropylamino)phosphoramidite. This compound was obtained as a colorless oil in 79% yield: MS (ESI, MeCN, LiCl added, negative) 818 (M + 35 [Cl]) calcd 818; MS (ESI, MeCN, LiCl added, positive) 790 (M + 7 [Li]) calcd 790; ^{31}P NMR (400 MHz, MeCN-d_3) δ 148.52 (s, first diastereoisomer), 148.49 (s, second diastereoisomer).

(c) [1β-(6-Oxopyridin-3-yl)-5′-(dimethoxytrityl)-2′-deoxy-D-ribofuranos-3′-yl]-2-cyanoethyl-*N*,*N*-bis(isopropylamino)-phosphoramidite. This compound was obtained as a colorless oil in 20% yield: MS (ESI, MeCN, LiCl added, negative) 749 (M + 35 [Cl]) calcd 749; MS (ESI, MeCN, LiCl added, positive) 721 (M + 7 [Li]) calcd 721; ^{31}P NMR (400 MHz, MeCN-d_3) δ 148.30 (s, first diastereoisomer), 148.27 (s, second diastereoisomer).

The yield was small, because 20% of a byproduct, bis-phosphoramidite (3′-*O*-phosphoramidite along with and 6-*O*-phosphoramidite), existed which was proven by the spectral data: MS (ESI, MeCN, LiCl added, negative) 948 (M + 35 [Cl]) calcd 948; MS (ESI, MeCN, LiCl added, positive) 920 (M + 7 [Li]) calcd 920; ^{31}P NMR (400 MHz, MeCN-d_3) δ 148.31 (s, 1P), 148.28 (s, 1P), 144.59 (s, 1P), 144.56 (s, 1P).

(d) [1β-(1-Deazapurin-9-yl)-5′-(dimethoxytrityl)-2′-deoxy-D-ribofuranos-3′-yl]-2-cyanoethyl-*N*,*N*-bis(isopropylamino)-phosphoramidite. This compound was obtained as a colorless oil in 57% yield: MS (ESI, MeCN, LiCl added, negative) 772 (M + 35 [Cl]) calcd 772; MS (ESI, MeCN, LiCl added, positive) 744 (M + 7 [Li]) calcd 744; ^{31}P NMR (400 MHz, MeCN-d_3) δ 148.97 (s, first diastereoisomer), 148.87 (s, second diastereoisomer).

(e) [1β-(6-Methyl-1-deazapurin-9-yl)-5′-(dimethoxytrityl)-2′-deoxy-D-ribofuranos-3′-yl]-2-cyanoethyl-*N*,*N*-bis(isopropyl-amino)phosphoramidite. This compound was obtained as a colorless oil in 84% yield: MS (ESI, MeCN, LiCl added, negative) 797 (M + 35 [Cl]) calcd 797; MS (ESI, MeCN, LiCl added, positive) 759 (M + 7 [Li]) calcd 759; ^{31}P NMR (400 MHz, MeCN-d_3) δ 148.95 (s, first diastereoisomer), 148.84 (s, second diastereoisomer).

(f) [1β-(2-Oxopyridin-1-yl)-5′-(dimethoxytrityl)-2′-deoxy-D-ribofuranos-3′-yl]-2-cyanoethyl-*N*,*N*-bis(isopropylamino)-phosphoramidite. This compound was obtained as a colorless oil in 89% yield: MS (ESI, MeCN, LiCl added, negative) 748 (M + 35 [Cl]) calcd 748; MS (ESI, MeCN, LiCl added, positive) 720 (M + 7 [Li]) calcd 720; ^{31}P NMR (400 MHz, MeCN-d_3) δ 149.07 (s, first diastereoisomer), 149.01 (s, second diastereoisomer).

(g) [1β-(4-Methyl-2-oxopyridin-1-yl)-5′-(dimethoxytrityl)-2′-deoxy-D-ribofuranos-3′-yl]-2-cyanoethyl-*N*,*N*-bis(isopropyl-amino)phosphoramidite. This compound was obtained as a colorless oil in 71% yield: MS (ESI, MeCN, LiCl added, negative) 762 (M + 35 [Cl]) calcd 762; MS (ESI, MeCN, LiCl added, positive) 734 (M + 7 [Li]) calcd 734; ^{31}P NMR (400 MHz, MeCN-d_3) δ 149.03 (s, first diastereoisomer), 149.02 (s, second diastereoisomer).

(*vi*) *General Procedure for the Synthesis of Triphosphates of Nucleosides*. Nucleoside (0.27 mmol) was dissolved in trimethyl phosphate (0.6 mL), and the mixture was cooled to 0 °C. Then $POCl_3$ (27 μL, 1.1 equiv) in 0.2 mL of trimethylphosphate was added dropwise. The mixture was stirred for 3 h, and then tetrabutylammonium pyrophosphate (0.8 g, 5 equiv) in DMF (1 mL) was added. The mixture was stirred for 4 h, while the temperature reached rt. The crude product was poured into triethylammonium bicarbonate (0.1 M in water); water and TEAB were removed under vacuum, and the crude product was diluted with water (100 mL) and added directly to a TEAB-equilibrated ion-exchange column (Sephadex-DEAE A-25, Aldrich) and eluted using a 0 to 1 M TEAB gradient. Fractions were individually spotted on a MALDI plate with THAP as the matrix, and triphosphate fractions were identified by their MALDI$^-$ peak (negative ion mode). Fractions were collected, lyophilized, and purified by HPLC using a gradient from 0 to 60% MeCN in 20 mM TEAA. The spectrophotometric yields were 2−50%.

(a) 1-β-D-(2-Oxopyridine)-3′,5′-di-*O*-(4-toluoyl)-2′-deoxy-ribofuranose triphosphate: MS (MALDI, negative) 466 (M − 1) calcd 466; ^{31}P NMR (400 MHz, D_2O) δ −10.00 (d, 1P, J = 40.8 Hz, γ-P), −10.50 (d, 1P, J = 40.0 Hz, α-P), −22.34 (m, 1P, β-P).

(b) 1-β-D-(6-Methyl-2-oxopyridine)-3′,5′-di-*O*-(4-toluoyl)-2′-deoxyribofuranose triphosphate: MS (MALDI, negative) 481 (M − 1) calcd 481; ^{31}P NMR (400 MHz, D_2O) δ −9.89 (bs, 1P, γ-P), −10.46 (d, 1P, J = 46.4 Hz, α-P), −22.31 (m, 1P, β-P).

(c) 1-β-D-(Benzimidazol-1-yl)-β-D-ribofuranose triphosphate: MS (MALDI, negative) 489 (M − 1) calcd 489; ^{31}P NMR (400 MHz, D_2O) δ −9.67 (d, 1P, J = 46.4 Hz, γ-P), −10.40 (d, 1P, J = 49.2 Hz, α-P), −22.20 (t, 1P, J = 40.8 Hz, β-P).

(d) 1-β-D-(4-Methylbenzimidazol-1-yl)-β-D-ribofuranose triphosphate: MS (MALDI, negative) 503 (M − 1) calcd 503; ^{31}P NMR (400 MHz, D_2O) δ −9.44 (m, 1P, γ-P), −10.38 (d, 1P, J = 46.0 Hz, α-P), −22.20 (m, 1P, β-P).

(e) 1-β-D-(1-Deazapurin-9-yl)-β-D-ribofuranose triphosphate: MS (MALDI, negative) 490 (M − 1) calcd 490; ^{31}P NMR (400 MHz, D_2O) δ −9.83 (bs, 1P, γ-P), −10.33 (d, 1P, J = 43.2 Hz, α-P), −22.18 (m, 1P, β-P).

(f) 1-β-D-(6-Methyl-1-deazapurin-9-yl)-β-D-ribofuranose triphosphate: MS (MALDI, negative) 504 (M − 1) calcd 504; ^{31}P NMR (400 MHz, D_2O) δ −9.88 (bs, 1P, γ-P), −10.35 (d, 1P, J = 49.2 Hz, α-P), −22.24 (m, 1P, β-P).

(*g*) *Zebularine triphosphate:* MS (MALDI, negative) 467 (M − 1) calcd 467; ^{31}P NMR (400 MHz, D_2O) δ −9.88 (bs, 1P, γ-P), −10.50 (d, 1P, J = 45.2 Hz, α-P), −22.36 (m, 1P, β-P).

(h) 1-β-D-(Purin-9-yl)-β-D-ribofuranose triphosphate: MS (MALDI, negative) 491 (M − 1) calcd 490; ^{31}P NMR (400 MHz, D_2O) δ −9.87 (bs, 1P, γ-P), −10.46 (d, 1P, J = 43.2 Hz, α-P), −22.45 (m, 1P, β-P).

(*vii*) *5′-Dimethoxytrityl-3′-phosphoramidites and Oligonucleotides*. The 5′-hydroxy groups of the nucleosides were protected with the 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 (*42*, *43*).

RESULTS

HSV primase misincorporates the natural NTPs at an extremely high frequency (1 every 30 correct NTPs) yet very strongly

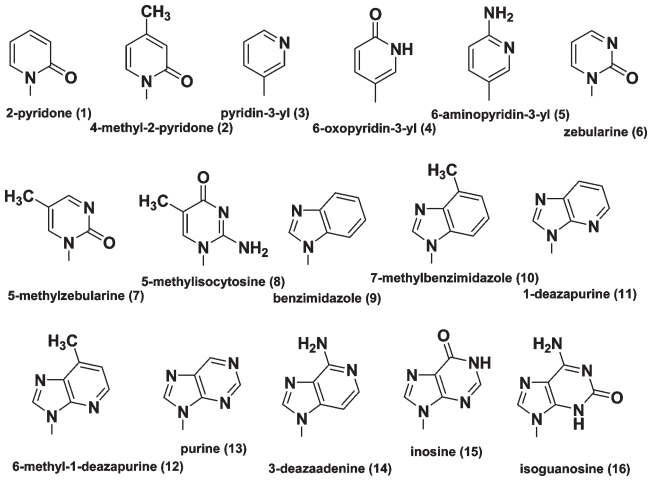


FIGURE 1: Base analogues used in this study.

discriminated against purine NTP analogues that cannot form Watson−Crick hydrogen bonds involving N-3 and N^4/O^4 of the templating pyrimidine during synthesis of the second and third nucleotides of the primer (*33*). To improve our understanding of how the enzyme discriminates so strongly against NTPs that cannot form a complete set of hydrogen bonds with the template base, we examined a series of templates and NTPs containing modified bases (Figure 1).

We first developed an assay to quantify how efficiently primase incorporates NTPs containing both natural and synthetic bases opposite a natural or synthetic template base. The DNA template 3′-T_{20}**GCC**CXAT_{14}, where X represents either a natural or synthetic nucleotide, supports high levels of primase activity. Primase exclusively initiates synthesis opposite the two underlined C residues in the canonical GCC initiation site and efficiently incorporates an additional GTP before coming to the variable nucleotide. When assays contain only 800 μM GTP and X is either a natural nucleotide or an analogue, primase synthesizes large amounts of the correct ppp$(G)_3$ trinucleotide but also readily misincorporates GTP to generate the ppp$(G)_4$ tetranucleotide (Figure 2A,B). Adding a different NTP, either correct or incorrect, decreased the amount of 5′-ppp$(G)_4$ misincorporation product and generated a new product of altered electrophoretic mobility due to incorporation of this additional NTP (Figure 2B−D).[2]

We quantified the ability of primase to incorporate a correct versus an incorrect NTP across from X as shown in Scheme 1. Since initiation [i.e., ppp$(G)_2$ dinucleoide synthesis] is the rate-limiting step in primer synthesis, the rate of polymerization of the NTP onto the trinucleotide cannot be directly measured. Instead, we measured the partitioning of the primase−ppp$(G)_3$ complex to determine the relative efficiency of polymerizing a NTP (*38*). After generating the trinucleotide ppp$(G)_3$, primase has three choices. (i) It can dissociate, thus resulting in the formation of the ppp$(G)_3$ product. (ii) It can incorporate a GTP to form ppp$(G)_4$. (iii) It can incorporate another nucleotide (N) to form the ppp$(G)_3$N product. Thus, by comparing the relative amounts of ppp$(G)_4$ and ppp$(G)_3$N products as a function of NTP concentration at a fixed GTP concentration, we can quantify the relative ability of primase to incorporate GTP versus NTP (*38*). It should be emphasized that these data do not provide

[2]Many of the short products appear as doublets due to the helicase removing the γ-phosphate from ppp$(G)_n$ to generate pp$(G)_n$ (*1*).

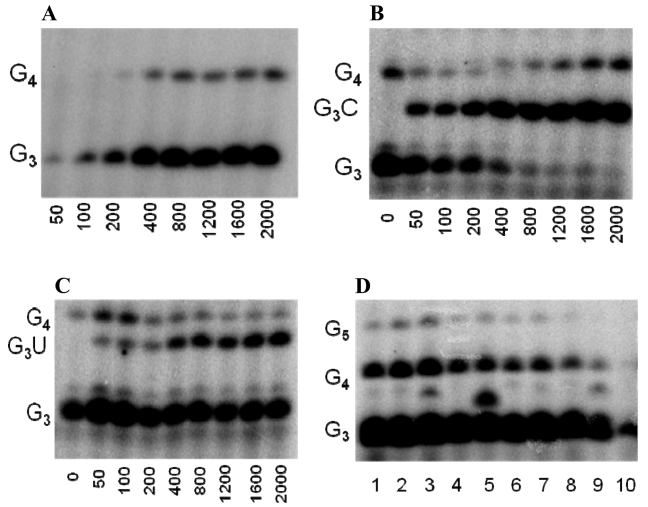
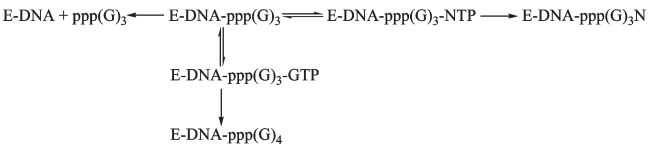


FIGURE 2: Experiments contain 400 nM HSV-1 primase, 2 μM template, 0.8 mM [α-^{32}P]GTP, and 0−2 mM investigated NTPs. (A) Noted concentration of GTP and the template $T_{20}GC_3$[2-pyridone]AT_{14}. (B) Noted concentration of CTP and the template $T_{20}GC_3GAT_{14}$. (C) Noted concentration of UTP and the template $T_{20}GC_3GAT_{14}$. (D) Incorporation of various NTPs opposite purine in the template $T_{20}GC_3$[purine]AT_{14}. Reaction mixtures contained 1 mM NTP analogue: lane 1, no analogue; lane 2, benzimidazole; lane 3, 4-methyl-2-pyridone; lane 4, 7-methylbenzimidazole; lane 5, 2-pyridone; lane 6, 6-methyl-1-deazapurine; lane 7, 1-deazapurine; lane 8, isoG; lane 9, purine; and lane 10, 2-1*H*-pyrimidinone. The presence of products with altered electrophoretic mobilities indicates the analogue's incorporation.

Scheme 1: Partitioning of the Trinucleotide among Dissociation, Polymerization of GTP opposite X, and Polymerization of NTP opposite X[a]

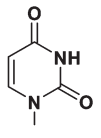
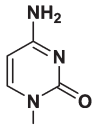
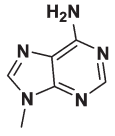
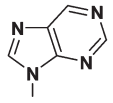
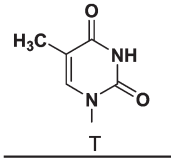
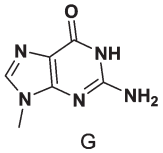
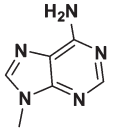
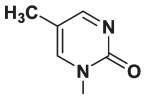
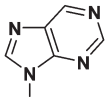
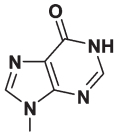


[a]The polymerization of NTPs is effectively irreversible because of the low pyrophosphate concentration in the assays, and previous work showed that primase does not elongate short oligonucleotides once they dissociate from the enzyme, thereby making the dissociation step effectively irreversible (*1*, *56*).

the actual efficiency with which primase incorporates a NTP (analogue). However, by using a constant GTP concentration with the different analogues, the relative efficiency with which primase polymerizes the different NTPs can be determined.

Table 1 shows that as expected, producing equal amounts of products due to polymerization of GTP and of the tested NTP required less of the tested NTP when it was correct than when it was incorrect. For example, opposite a template T, primase produced equal amounts of products due to ATP polymerization and GTP polymerization when assays contained only the correct ATP at 8 μM. In contrast, producing equal amounts of product due to CTP polymerization and GTP polymerization required the incorrect CTP at 1900 μM. Consistent with the low fidelity of primase, however, the difference in the amounts of the correct NTP versus the amounts of an incorrect NTP needed to give equal effects was relatively low, varying from 17 in the case of polymerization of UTP opposite G as compared to CTP opposite G to 420 during polymerization of ATP opposite G as compared to CTP opposite G.

Table 1: Incorporation of Natural NTPs and Purine NTPs opposite either Natural Bases or Analogue Bases Lacking One Watson−Crick Hydrogen Bonding Group[a]

Template \ Triphosphate	 U	 C	 A	 13
 T	240 ± 20	1900 ± 50	25 ± 10	40 ± 5
 G	260 ± 20	15 ± 1	6300 ± 200	ND
 A	8 ± 1	920 ± 40	2500 ± 100	3500 ± 100
 7	1200 ± 50	ND	14000 ± 1000	ND
 13	90 ± 10	ND	5000 ± 100	ND
 15	30 ± 20	23 ± 3	6300 ± 300	ND

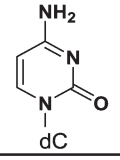
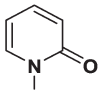
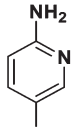
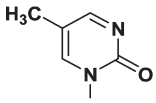
[a]Listed is the concentration (micromolar) of the NTP that gives equal elongation of the ppp$(G)_3$ trinucleotide with that NTP and with GTP. ND means no detectable incorporation.

Effect of Removing N^4 from CTP. Previously, we showed that removing one of the Watson−Crick hydrogen bonding groups from ATP strongly inhibited polymerization when the template required ATP as either the second or third nucleotide of the primer (*33*). To test if removal of one of the Watson−Crick hydrogen bonding groups also affected pyrimidines, we examined polymerization of 2-1*H*-pyrimidinone NTP (zebularine), a C analogue that lacks N^4. We found similar results, no detectable polymerization of 2-1*H*-pyrimidinone NTP opposite a template G at concentrations up to 2 mM.

Effect of Removing a Watson−Crick Hydrogen Bonding Group from the Templating Base. The effect of removing a single Watson−Crick hydrogen bonding group from the template base was explored using templates in which X is 2-1*H*-pyrimidinone (no N^4), X is purine (no N^6), and X is hypoxanthine (no N^2). Primase very efficiently incorporated CTP opposite hypoxanthine (Table 1), indicating that primase does not require the hydrogen bond normally found between N^2 of G and O^2 of C. Whereas the loss of N^2 did not increase the level of misincorporation of ATP, it significantly increased the level of misincorporation of UTP. Surprisingly, primase incorporated UTP across from purine only 11-fold less efficiently than across from A, but it discriminated against incorporation of ATP and CTP more effectively than if the template contained A (Table 1).

Opposite a templating 5-methyl-2-1*H*-pyrimidinone, primase did not efficiently polymerize ATP, CTP, or UTP (Table 1). To measure the efficiency of GTP polymerization, we measured the fraction of the trinucleotide elongated to the tetranucleotide as a function of GTP concentration. Incorporation of GTP demanded this alternative analysis since the reaction mixtures contained only GTP. Table 2 shows that primase incorporated GTP opposite 5-methyl-2-1*H*-pyrimidinone much less efficiently than opposite C, and only slightly more efficiently than if the template contained a base completely incapable of

Table 2: Incorporation of GTP opposite the Noted Template Base in the Template $T_{20}GCCCXAT_{14}$[a]

	Template	V_{max}	K_M (μM)	V_{max}/K_M
GTP	 dC	0.9 ± 0.025	57.1 ± 9	0.016
	 1	0.12 ± 0.002	188 ± 14	0.00064
	 3	0.19 ± 0.016	528 ± 121	0.00036
	 5	0.32 ± 0.009	146 ± 17	0.0022
	 7	0.26 ± 0.006	217 ± 29	0.0012

[a]The F_{max} is the fraction of $ppp(G)_3$ elongated into $ppp(G)_4$ with saturating GTP, and the K_M is the concentration of GTP that gives half-maximal elongation. It should be emphasized that F_{max} is based on the partitioning of the E−$ppp(G)_3$ complex between addition of another GTP and dissociation of $ppp(G)_3$ and is not V_{max}.

Watson−Crick hydrogen bonding (2-pyridone). Thus, primase does not efficiently polymerize GTP across from a templating 5-methyl-2-1*H*-pyrimidinone, and the loss of Watson−Crick hydrogen bonds clearly interferes with polymerization. As we will describe in greater detail below, the low level of GTP misicorporation opposite 2-pyridone and 2-1*H*-pyrimidinone likely largely results from template−primer slippage.

Then, to determine if primase will efficiently incorporate a natural NTP opposite a template base incapable of forming any Watson−Crick hydrogen bonds, we generated a series of $T_{20}GCCCXGT_{14}$ templates, where X was 2-pyridone, 4-methyl-2-pyridone, 1-deazapurine, or 6-methyl-1-deazapurine, and measured the level of incorporation of the natural NTPs (Table 3). When X was a natural nucleotide, primase misincorporated small amounts of GTP. However, it did not efficiently incorporate any other natural NTPs opposite these hydrophobic template bases. In each case examined, incorporation of a natural NTP was no more efficient than polymerization of a natural NTP opposite an incorrect, natural template base (compare Tables 1 and 3).

Unsatisfied Watson−Crick Hydrogen Bonding Groups. We then tested the hypothesis that primase's inability to efficiently generate base pairs between a natural base and a base incapable of forming Watson−Crick hydrogen bonds resulted from the presence of unsatisfied Watson−Crick hydrogen bonding groups on one of the bases. Polymerization of 2-pyridone, 4-methyl-2-pyridone, benzimidazole, 4-methylbenzimidazole, 1-deazapurine, and 6-methyl-1-deazapurine NTPs was measured in assays containing GTP and the template $T_{20}GCCCXGT_{14}$, where X was 2-pyridone, 4-methyl-2-pyridone, 1-deazapurine, or 6-methyl-1-deazapurine. However, even at elevated concentrations of these hydrophobic NTP analogues (2 mM), primase only detectably incorporated 2-pyridone NTP and 4-methyl-2-pyridone NTP (Figures 3 and 4) (the Supporting Information contains a summary of the hydrophobic dNTP analogues that primase did not incorporate opposite hydrophobic template nucleotides). Polymerization of 4-methyl-2-pyridone and 2-pyridone NTP opposite several hydrophobic bases was further examined and quantified as described above (Table 4). Primase incorporated these NTP analogues with an efficiency similar to that observed for polymerization of a natural NTP opposite an incorrect, natural template base. Together, these data indicate that the simple lack of unsatisfied H-bonds between the bases of the incoming NTP and the template base does not result in rapid NTP polymerization.

We next tested how efficiently primase incorporated pyridinone and 4-methyl-2-pyridinone NTPs opposite the natural bases. Generally, primase incorporated the two pyrimidine analogues much more efficiently than analogous purine analogues, although incorporation opposite A and T was no better than misincorporation (Table 4). Surprisingly, primase incorporated the pyridone NTPs very efficiently across from G, much better than an incorrect natural NTP, but not quite as efficiently as CTP (compare Tables 1 and 4). The relatively efficient

Table 3: Incorporation of Natural NTPs opposite Template Bases Lacking Any Watson−Crick Hydrogen Bonding Groups[a]

Template	Triphosphate: U	Triphosphate: C	Triphosphate: A
1	490 ± 30	2100 ± 20	2800 ± 300
2	170 ± 30	1900 ± 80	4200 ± 250
11	460 ± 40	2200 ± 100	5400 ± 400
12	1800 ± 30	1700 ± 30	7700 ± 700

[a]Listed is the concentration (micromolar) of the NTP that gives equal elongation of the ppp(G)$_3$ trinucleotide with that NTP and with GTP.

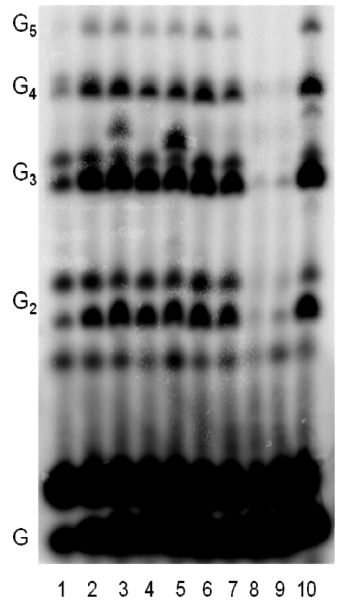


FIGURE 3: Incorporation of a variety of NTPs opposite 1-deazapurine in template T$_{20}$GCCC[1-deazapurine]AT$_{14}$. Experiments contained 400 nM HSV-1 primase, 2 μM template, 0.8 mM [α-^{32}P]GTP, and the following (analogue) NTPs at 2 mM: lane 1, no further addition; lane 2, benzimidazole; lane 3, 4-methyl-2-pyridone; lane 4, 7-methylbenzimidazole; lane 5, 2-pyridone; lane 6, 6-methyl-1-deazapurine; lane 7, 1-deazapurine; lane 8, isoG; lane 9, 2-1*H*-pyrimidinone; and lane 10, ATP. The presence of products with altered electrophoretic mobilities in lanes 3 and 5 indicates incorporation of 4-methyl-2-pyridone and 2-pyridone, respectively. In the case of isoGTP and 2-1*H*-pyrimidinone (lanes 8 and 9, respectively), a large amount of these NTPs caused inhibition of the enzyme.

incorporation of the pyridones opposite guanine might have resulted from formation of a hydrogen bond between O^2 of the pyridones and N^2 of guanine. However, primase also efficiently incorporated the pyridone NTPs opposite hypoxanthine, a guanine analogue that lacks N^2, indicating that the efficient incorporation of the pyridones did not result from a hydrogen bond involving N^2 (Table 4).

We further examined the role of unsatisfied hydrogen bonding groups by measuring the level of polymerization of purine NTP opposite a templating T. In contrast to previous studies showing that primase strongly discriminated against purine polymerization when T encoded the second or third nucleotide of the primer (*33*), primase exhibited only modest discrimination (< 2-fold) against polymerization of purine NTP when incorporated at the fourth position (Table 1). This result is also consistent with the efficient polymerization of UTP opposite purine described earlier.

Alternative Hydrogen Bonding Patterns. We tested the possibility that primase would incorporate NTPs with a complete set of Watson−Crick hydrogen bonds, albeit in an unusual arrangement. However, primase did not detectably incorporate isoGTP opposite 5-methyl-isoC, even when assays contained 2 mM isoGTP. Likewise, primase polymerized ATP and CTP opposite isoC no better than it misincorporated these natural NTPs opposite a natural template base (Table 5), and polymerized UTP only slightly better.

Minor Groove Hydrogen Bonding Groups. The role of the minor groove hydrogen bond acceptor in the template base was probed using purines that lacked N-3 and pyrimidine analogues that lacked O^2. Primase incorporated UTP opposite 3-deazaadenine (Table 5) and adenine (Table 1) with similar efficiencies, while the efficiency of misincorporation changed slightly. In contrast to these minimal effects upon removal of N-3 from a purine, removing the equivalent minor groove substituent, O^2, from pyrimidines had a somewhat greater effect, even when the template base retained the capacity to form two correct Watson−Crick hydrogen bonds [ATP polymerization opposite 6-hydroxypyridin-3-yl and GTP polymerization opposite 6-aminopyridin-3-yl (Tables 2 and 6)].

Primase Can Misincorporate NTPs via Primer−Template Slippage. Primase generated the ppp(G)$_4$ tetranucleotide with all of the T$_{20}$GCCC**X**AT$_{14}$ templates. Potentially, this could have occurred via primase actually incorporating GTP opposite X or via the primer or template slipping after generating the ppp(G)$_3$ trinucleotide. In the slippage mechanism, the formation of ppp(G)$_4$ results from re-replication of the third template C. Two approaches were used to examine this question. First, we examined the efficiency with which primase incorporated GTP opposite X as a function of GTP concentration (Table 2). Using a series of template bases, primase incorporated GTP much less efficiently opposite the analogues than opposite C. Then, we measured the level of misincorporation with the template T$_{20}$GCCT**X**AT$_{14}$. If the polymerization of GTP opposite X on the template T$_{20}$GCCC**X**AT$_{14}$ resulted from a primer−template slippage mechanism, removing the C just before X should reduce or eliminate polymerization of GTP and may give enhanced ATP polymerization opposite X. In contrast, if incorporation of GTP opposite X resulted from primase misreading X and actually polymerizing GTP opposite X, then replacing the GTP prior to X should have little or no effect.

To test for slippage, we examined incorporation of GTP and ATP opposite 2-pyridone in the template T$_{20}$GCCTXAT$_{14}$, where X is 2-pyridone (Figure 4). In the presence of ATP and GTP, primase synthesized large amounts of a trinucleotide that

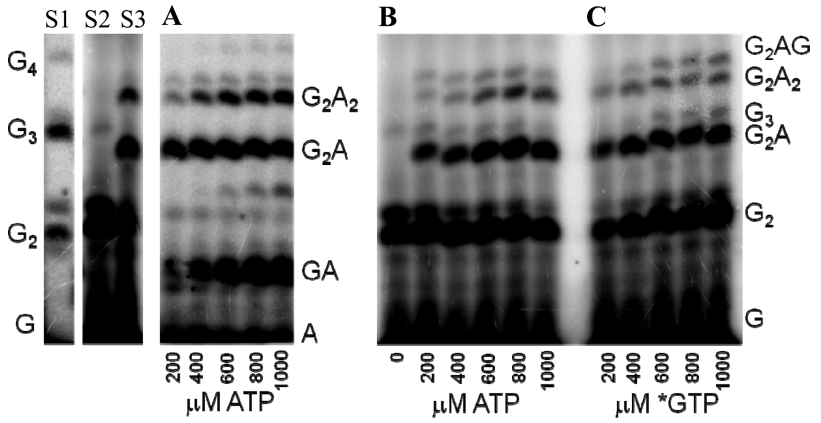


FIGURE 4: Detecting primer−template slippage during misincorporation. Reactions were performed as described in Experimental Procedures, and mixtures contained 400 nM HSV-1 primase and 2 μM template $T_{20}GC_2TXAT_{14}$, where X is 2-pyridone, unless noted otherwise. Primer standards of known composition were generated in assays containing $T_{20}GCCCTAAT_{14}$ and 0.6 mM [α-^{32}P]GTP (lane S1), $T_{20}GCCTTAAT_{14}$, 0.6 mM [α-^{32}P]GTP, and 0.6 mM ATP (lane S2), or $T_{20}GC_2T_2A_2T_{14}$ and 0.6 mM [α-^{32}P]GTP (lane S3). The identities of the products are listed beside the gels. (A) Reaction mixtures contained 0.6 mM GTP and 0.2−1 mM [α-^{32}P]ATP. (B) Reaction mixtures contained 0.6 mM [α-^{32}P]GTP and 0−1 mM ATP. (C) Reaction mixtures contained 0.6 mM ATP and 0.2−1 mM [α-^{32}P]GTP.

contains both A and G, presumably pppGGA, the product encoded by the first three nucleotides of the template at the primer initiation site, and two four-nucleotide products. Each product contained A and G since including either [α-^{32}P]ATP or [α-^{32}P]GTP in the assays resulted in labeled products. The primary four-nucleotide product comigrated with pppGGAA, while the minor four-nucleotide long product migrated slightly slower. Increasing the concentration of ATP significantly increased the amount of pppGGAA product relative to the slower migrating product and pppGGA. Quantitation of the data showed that 50% conversion of the pppGGA to pppGGAA required only 2.8 ± 0.2 mM ATP. In contrast, when the template nucleotide just before the 2-pyridone was C, 50% conversion of the $ppp(G)_3$ trinucleotide to $ppp(G)_3A$ required 41 ± 2 mM ATP. This ability of the template nucleotide just prior to the 2-pyridone to direct polymerization opposite 2-pyridone suggests that this polymerization can occur via a primer−template slippage mechanism. We also measured the effects of increasing GTP concentrations on the synthesis of the two four-nucleotide products. Figure 4 shows that the amount of the slower migrating four-nucleotide product increased relative to the amount of pppGGAA. Thus, this slower migrating product likely has the sequence pppGGAG and results from incorporation of GTP opposite 2-pyridone. Quantitation of the data showed that 50% elongation of pppGGA to the apparent pppGGAG required 18 ± 1 mM GTP, while only 6.1 ± 0.4 mM was required for 50% elongation of pppGGG to pppGGGG on the template $T_{20}\underline{GCC}CXAT_{14}$.

DISCUSSION

We used a series of base analogues to further probe how herpes primase chooses whether to polymerize a NTP. Surprisingly, the extent to which the formation of Watson−Crick hydrogen bonds enhances NTP polymerization varied with both the identity of the base pair and the location of the base pair in the primer (second, third, etc.). In some cases, the effects of modifying the base had similar effects when either the template or incoming NTP contained the modified base, whereas in other cases, the effects were very asymmetrical.

Primase polymerized purine NTP opposite T only 2-fold worse than it polymerized ATP opposite T as the fourth nucleotide of the primer. Previously, we found that primase strongly discriminated against purine NTP incorporation as the second or third nucleotide of the primer, presumably because of the missing hydrogen bond between N^6 of the purine and O^4 of T. The decreasing level of discrimination against purine NTP polymerization with increasing primer length suggests that for polymerization across from T, increased primer−template stability weakens the requirement for Watson−Crick hydrogen bonds. Furthermore, these data also demonstrate that primase does not absolutely require the formation of a complete set of Watson−Crick hydrogen bonds to efficiently polymerize a NTP.

With the exception of 2-pyridone NTP and 4-methyl-2-pyridone NTP, primase very strongly discriminated against polymerizing NTPs whose base cannot form any Watson−Crick hydrogen bonds opposite both natural and analogue bases. This occurred whether or not these analogues contained a minor groove hydrogen bond acceptor (N-3 of a purine). The inability of primase to efficiently generate base pairs between two bases that both lacked Watson−Crick hydrogen bonding groups indicates that the simple absence of unsatisfied Watson−Crick hydrogen bonding groups in the primase active site does not lead to rapid NTP polymerization. Indeed, primase polymerized natural NTPs across from the purine analogues 1-deazapurine and 6-methyl-1-deazapurine more efficiently than it polymerized 1-deazapurine NTP and 6-methyl-1-deazapurine NTP opposite any base (Tables 1 and 3 and Supporting Information).

Among the tested NTP analogues incapable of Watson−Crick hydrogen bonding, primase incorporated only the two pyrimidine analogues, 2-pyridone and 4-methyl-2-pyridone NTP. Primase polymerized them surprisingly well opposite both natural bases and unnatural bases. In most cases, the incorporation efficiency was similar to that of an incorrect natural NTP across from a natural base. However, polymerization opposite hypoxanthine and G was almost as efficient as generation of a canonical base pair, only 2- and 7-fold less efficient than polymerization of CTP across from hypoxanthine and G, respectively.

The efficient incorporation of 2-pyridone NTP and 4-methyl-2-pyridone NTP clearly indicates that herpes primase does not require the formation of Watson−Crick hydrogen bonds to efficiently polymerize a NTP, yet primase very potently discriminated against analogous NTPs in which the base mimics a purine, 1-deazapurine NTP, and 6-methyl-1-deazapurine NTP.

Table 4: Polymerization of Pyridone NTP and 4-Methylpyridone NTP opposite Various Bases[a]

Template	Triphosphate: 1	Triphosphate: 2	Template	Triphosphate: 1	Triphosphate: 2
T	520 ± 20	2300 ± 30	5	3500 ± 100	ND
G	70 ± 15	100 ± 30	7	ND	ND
A	1300 ± 40	2700 ± 100	11	700 ± 60	1100 ± 100
1	660 ± 50	470 ± 40	12	610 ± 40	910 ± 80
2	580 ± 50	810 ± 60	14	130 ± 40	1800 ± 200
3	520 ± 30	1100 ± 40	15	36 ± 30	70 ± 30
4	660 ± 20	800 ± 100	16	ND	ND

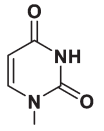
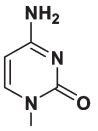
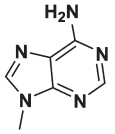
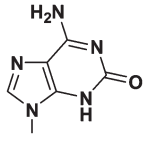
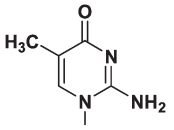
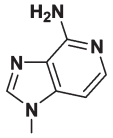
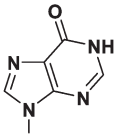
[a]Listed is the concentration (micromolar) of the NTP that gives equal elongation of the ppp(G)$_3$ trinucleotide with that NTP and with GTP.

Likewise, our previous studies on HSV-1 primase using a larger series of purine NTP analogues indicated a requirement for Watson−Crick hydrogen bonds (*33*). These contrasting results suggest that the requirements for incoming purine NTPs and incoming pyrimidine NTPs are not identical. Additionally, it raises the intriguing question of how one designs an active site to interact so very differently with these different analogues.

Surprisingly, converting 2-pyridone NTP into 2-1*H*-pyrimidinone NTP via re-introduction of N^3 greatly impaired incorporation opposite G or any other nucleotide. This occurred even though 2-1*H*-pyrimidinone NTP can now form a Watson−Crick hydrogen bond between N^3 of 2-1*H*-pyrimidinone and H-N^3 of G. Likewise, primase also did not efficiently polymerize any natural NTPs opposite a template 5-methyl-2-1*H*-pyrimidinone. Indeed, even 2-pyridone NTP and 4-methyl-2-pyridone NTP were not efficiently incorporated across from this base.

Remarkably, converting 2-pyridone into 2-1*H*-pyrimidinone had essentially the exact opposite effect of converting 1-deazapurine into purine. The former conversion severely inhibited formation of base pairs, whereas the latter conversion greatly stimulated generation of base pairs with U/T. Chemically, however, both changes are remarkably similar in that an electron deficient C-H group is converted into an electron rich N that can form a Watson−Crick hydrogen bond in the core of a duplex nucleic acid. Since 2-1*H*-pyrimidinone can be considered a C analogue while purine is an A analogue, these differences suggest that herpes primase differentially recognizes A·U/T and G·C base pairs.

Table 5: Polymerization of Natural NTPs and IsoGTP opposite IsoC, 3-Deazaadenine, and Hypoxanthine[a]

Template \ Triphosphate	 U	 C	 A	 16
 8	100 ± 30	3000 ± 100	5900 ± 200	ND
 14	9 ± 3	11 ± 1	5000 ± 100	ND
 15	30 ± 20	23 ± 3	6300 ± 300	ND

[a]Listed is the concentration (micromolar) of the NTP that gives equal elongation of the ppp(G)$_3$ trinucleotide with that NTP and with GTP. ND means no detectable incorporation.

Primase frequently demonstrated great asymmetry during generation of base pairs. For example, while it polymerized 2-pyridone NTP opposite G quite efficiently, it did not efficiently incorporate GTP opposite 2-pyridone. Likewise, primase polymerized 2-pyridone NTP across from 1-deazapurine and 6-methyl-1-deazapurine at least 10-fold more efficiently than it polymerized 1-deazapurine NTP and 6-methyl-1-deazapurine NTP opposite 2-pyridone [primase did not detectably polymerize these NTPs opposite 2-pyridone (Supporting Information)]. Thus, just like with some DNA polymerases (*20*, *44*−*48*), predicting how altering a template or NTP base will affect activity is problematic at best. Mechanistically, this may result from the interactions of the enzyme with the templating base subtly altering the structure of the active site and, therefore, how the enzyme interacts with the base of the incoming (d)NTP.

This asymmetry in base pair recognition extends to the minor groove hydrogen bond acceptor found on the canonical bases. Primase does not require a minor groove hydrogen bond acceptor in either an incoming ATP or a templating A, as evidenced by the minimal effects on generation of A·T/U base pairs upon removal of N-3 from A. In contrast, removing O^2 from a templating T or C more significantly impaired polymerization of ATP or GTP, respectively.

Primase likely misincorporates NTPs by misreading the templating base as well as by a template (or primer) slippage mechanism. Evidence of misreading comes from the ability of primase to polymerize NTPs not encoded by any template base (*32*). Primase also appears to misincorporate NTPs by re-replicating a template base. Regardless of the identity of X in the template ...GCCCX..., primase always generated significant amounts of ppp(G)$_4$, even if it did not efficiently polymerize ATP, CTP, or UTP opposite the base (i.e., 6-methyl-1-deazapurine and isoC). To explicitly test for primer slippage, we examined NTP polymerization opposite X with a template containing the sequence ...GCCTX... This resulted in a slightly decreased level of polymerization of GTP along with a 20-fold increased level of polymerization of ATP. Thus, the template nucleotide immediately before the analogue can direct what NTP primase incorporates opposite X. Potentially, this could occur by primase allowing the primer to slide backward or the template strand to slip forward, thereby resulting in re-replication of a template base.

Misincorporation of dNTPs by DNA polymerases, including high-fidelity polymerases, can also occur by a primer−template slippage mechanism (*49*−*52*). With DNA polymerases, this problem appears to be most acute in highly repetitive sequences. Indeed, triplet expansion diseases may result from primer−template slippage during DNA synthesis (*49*, *52*). Thus, this mechanism of misincorporation is clearly not limited to herpes primase and presents a general potential problem for nucleotide polymerizing enzymes. In the case of primase, however, slippage can occur even in the absence of highly repetitive sequences.

Incorporation of NTPs by herpes primase cannot be described by any one simple model. Primase clearly does not require the formation of Watson−Crick hydrogen bonds, as evidenced by the efficient incorporation of 2-pyridone and 4-methyl-2-pyridone NTPs opposite both natural bases and bases incapable of Watson−Crick hydrogen bonding. The simple absence of unsatisfied Watson−Crick hydrogen bonding groups is also not sufficient to allow rapid NTP polymerization, both in the presence and in the absence of minor groove hydrogen bond acceptors. For example, primase did not efficiently polymerize benzimidazole NTP or 1-deazapurine NTP opposite a templating 2-pyridone. Likewise, a correctly shaped base pair is not critical as shown by the efficient misincorporation of wrong natural NTPs as well as the efficient incorporation of 2-pyridone and 4-methyl-2-pyridone NTPs opposite a variety of differently shaped bases. Hydrophobicity by itself cannot drive efficient

Table 6: Polymerization of Natural NTPs opposite Pyrimidine Analogues[a]

Template	Triphosphate: U	Triphosphate: C	Triphosphate: A
3	1600 ± 100	1000 ± 100	4200 ± 400
4	120 ± 5	870 ± 40	120 ± 20
5	180 ± 20	ND	ND
7	1200 ± 50	ND	14000 ± 1000

[a]Listed is the concentration (micromolar) of the NTP that gives equal elongation of the ppp(G)$_3$ trinucleotide with that NTP and with GTP. ND means no detectable incorporation.

incorporation of NTPs since converting ATP or GTP into benzimidazole NTP or 4-methylbenzimidazole NTP greatly interferes with incorporation, whereas converting UTP or CTP into 2-pyridone NTP or 4-methyl-2-pyridone NTP greatly stimulates some polymerization events. Finally, the presence or absence of specific functional groups on either the templating base or the NTP base did not predictably enhance or inhibit NTP polymerization, indicating that herpes primase does not use a simple positive/negative selectivity model as proposed for B family DNA polymerases (*14*, *17*).

Rather, primase may have an extremely plastic active site that can relatively efficiently generate many unnatural base pairs. Multiple chemical solutions exist for generating a base pair that primase efficiently synthesizes. For example, UTP, CTP, and 2-pyridone NTP all satisfy the requirements for efficient polymerization opposite a templating hypoxanthine, even though the bases on the NTPs are very different chemically. Similarly, primase polymerizes UTP opposite isoC, purine, and 6-hydroxypyridin-3-yl with virtually identical efficiencies and only slightly worse than a correct, natural base pair, even though the structures and chemistry of any resulting base pairs must vary substantially. Elucidating the chemical features of the incipient base pair that enhance or inhibit polymerization will likely require elucidation of the structure of primase with multiple different base pairs in the active site.

In a general sense, these results with primase also demonstrate the difficulties inherent in drawing conclusions about the mechanism of any nucleotide polymerase based on a limited set of substrate analogues. It will be interesting to determine for other polymerases that have been posited to require Watson−Crick hydrogen bonds to polymerize a dNTP whether this is generally true or a consequence of the specific base(s) tested.

Herpes primase is a particularly tempting target for the development of novel anti-herpes therapeutics given its essential role in herpes replication. Indeed, various groups have recently described several potent non-nucleotide inhibitors of this enzyme (*53*−*55*). Unfortunately, the unique and unpredictable features of herpes primase with respect to how it interacts with nucleotide analogues suggest that it will be difficult to rationally design novel nucleotide-based primase inhibitors containing modified bases. Of the bases tested, the most promising are clearly the 2-pyridones in light of their relatively efficient polymerization.

SUPPORTING INFORMATION AVAILABLE

A summary of analogue NTPs that primase did not polymerize opposite analogue template bases. This material is available free of charge via the Internet at http://pubs.acs.org.

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