Herpes Simplex Virus 1 Primase Employs Watson−Crick Hydrogen Bonding To Identify Cognate Nucleoside Triphosphates†

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ABSTRACT: We utilized NTP analogues containing modified bases to probe the mechanism of NTP selection by the primase activity of the herpes simplex virus 1 helicase−primase complex. Primase readily bound NTP analogues of varying base shape, hydrophobicity, and hydrogen-bonding capacity. Remarkably, primase strongly discriminated against incorporating virtually all of the analogues, even though this enzyme misincorporates natural NTPs at frequencies as high as 1 in 7. This included analogues with bases much more hydrophobic than a natural base (e.g., 4- and 7-trifluoromethylbenzimidazole), a base of similar hydrophobicity as a natural base but with the Watson−Crick hydrogen-bonding groups in unusual positions (7-*β*-D-guanine), bases shaped almost identically to the natural bases (4-aminobenzimidazole and 4,6-difluorobenzimidazole), bases shaped very differently than a natural base (e.g., 5- and 6-trifluoromethylbenzimidazole), and bases capable of forming just one Watson−Crick hydrogen bond with the template base (purine and 4-aminobenzimidazole). The only analogues that primase readily polymerized into primers (ITP and 3-deaza-ATP) were those capable of forming Watson−Crick hydrogen bonds with the template base. Thus, herpes primase appears to require the formation of Watson−Crick hydrogen bonds in order to efficiently polymerize a NTP. In contrast to primase's narrow specificity for NTP analogues, the DNA-dependent NTPase activity associated with the herpes primase−helicase complex exhibited very little specificity with respect to NTPs containing unnatural bases. The implications of these results with respect to the mechanism of the helicase−primase and current fidelity models are discussed.

Herpes simplex virus 1 (HSV-1)[1] primase, an essential component of the herpes DNA replication apparatus, provides short RNA primers that the herpes DNA polymerase then elongates (*1*−*5*). Primase initiates primer synthesis on ssDNA opposite two template pyrimidines by binding and converting two purine NTPs into a dinucleotide (*6*). It produces an abundance of short primers (<3 nucleotides) but can also produce longer products (from 4 to ∼10 nucleotides) if initiation occurs at a preferred 3′-deoxyguanylate−pyrimidine−pyrimidine (3′-G-pyr-pyr) template start site sequence (*6*). Dinucleotide synthesis is the rate-limiting step for primase (*7*).

Herpes primase is part of a heterotrimeric enzyme complex containing three gene products: UL5, UL8, and UL52. In addition to primase activity, the complex also exhibits helicase and ssDNA-dependent NTPase activities (*2*, *8*−*12*). The UL5 subunit contains conserved helicase motifs (*13*, *14*), and the UL52 contains conserved primase motifs (*15*−*17*). Importantly, an interdependence exists between the subunits such that neither subunit by itself exhibits helicase or primase activities (*18*−*20*). The UL8 subunit has no known catalytic activity (*18*, *19*, *21*) but can stimulate both primase and helicase activities (*22*, *23*) and overcome the inhibitory effect of coating ssDNA with the HSV-1 ssDNA binding protein on NTPase and primase activities (*23*, *24*).

The mechanism(s) utilized by polymerases to discriminate between correct and incorrect nucleotides remain(s) a topic of heated discussion. Since the ΔG difference between correct and incorrect base pairs in solution cannot account for the high fidelity obtained by many polymerases (often an error frequency of 10^{-4} to 10^{-6}), multiple models have been proposed (*25*). One model postulates that enzymes can distinguish between the shape of correct base pairs and the shape of incorrect base pairs (*26*). Alternatively, it has been proposed that hydrophobic interactions may be critical for polymerases to differentiate between right and wrong base pairs (*27*). Finally, it is unclear to what extent polymerases obtain fidelity by discriminating for the correct base pair as opposed to discriminating against wrong base pairs (*28*, *29*).

While no universal mechanism has yet been elucidated to explain how polymerases distinguish between correct and incorrect (d)NTPs, it is clear that polymerases have a wide range of fidelities with error rates ranging from 10^{-1} to 10^{-6} errors per nucleotide polymerized (*25*, *30*). Thus, it seems plausible that different polymerases will employ different mechanisms to identify correct nucleotides. Recently, we found that a low-fidelity polymerase, human primase, utilizes Watson−Crick hydrogen bonding to discriminate between correct and incorrect base pairing (*31*). This enzyme only polymerized NTP analogues that could form Watson−Crick

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[1] Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; G-pyr-pyr, deoxyguanylate−pyrimidine−pyrimidine; HSV-1, herpes simplex virus 1; NTP, nucleoside triphosphate; ssDNA, single-stranded DNA; Tris, tris(hydroxymethyl)aminomethane (HCl salt).

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Table 1: Synthetic ssDNA Templates Used[a]

d(GCCC)$_{15}$	3′**GCC**C**GCC**C**GCC**C**GCC**C**GCC**C**GCC**C**GCC**C**GCC**C**GCC**C**GCC**C**GCC**C**GCC**C**GCC**C**GCC**C5′
d(GTTT)$_{15}$	3′**GTT**T**GTT**T**GTT**T**GTT**T**GTT**T**GTT**T**GTT**T**GTT**T**GTT**T**GTT**T**GTT**T**GTT**T**GTT**T5′
d(TCTG)$_{15}$	3′TCT**GTC**T**GTC**T**GTC**T**GTC**T**GTC**T**GTC**T**GTC**T**GTC**T**GTC**T**GTC**T**GTC**T**GTC**T**GTC**T**GTC**T**GTC**T**G**5′
d(C$_{20}$GCCTA$_{19}$)	3′CCCCCCCCCCCCCCCCCCCC**GCC**TAAAAAAAAAAAAAAAAAA5′
d(C$_{20}$GCTA$_{20}$)	3′CCCCCCCCCCCCCCCCCCCC**GCT**AAAAAAAAAAAAAAAAAAAA5′
d(T$_{20}$GTCCT$_{19}$)	3′TTTTTTTTTTTTTTTTTTTT**GTC**CTTTTTTTTTTTTTTTTTT5′

[a] The 3′-G-pyr-pyr herpes primase start sites are shown in bold, and the initiation sites and direction of primer synthesis are indicated by the arrows.

hydrogen bonds between the base of the incoming NTP and the template base being replicated. Interestingly, human primase demonstrates error rates (around 10^{-2} per NTP polymerized) consistent with that expected for a selection mechanism based solely on hydrogen bonding (*32*, *33*).

Herpes primase is also an extremely error-prone polymerase, with typical misincorporation frequencies between $10^{-1}$and 10^{-2} (*34*). To elucidate how herpes primase chooses to polymerize a NTP, we examined a series of NTPs containing base analogues that varied in terms of shape, hydrophobicity, and Watson−Crick hydrogen-bonding capacity. While herpes primase bound all of the base analogues tested, it only readily polymerized the analogue if it could form Watson−Crick hydrogen bonds with the template base. The implication of these results with respect to the mechanism of herpes primase and how different polymerases discriminate between right and wrong (d)NTPs is discussed.

EXPERIMENTAL PROCEDURES

Materials

HSV-1 wild-type helicase−primase (UL5/UL8/UL52) and HSV-1 mutant helicase−primase lacking NTPase activity (UL5MI/UL8/UL52) were expressed in SF9 cells via baculovirus infection and purified by Ni-NTA chromatography via a His$_9$-tagged UL8 subunit, as previously described (*6*). SF9 cells were from the Tissue Culture Core Facility at the University of Colorado Cancer Center. Baculoviruses were amplified and titered by the Tissue Culture Core Facility at the University of Colorado Cancer Center. Primer standards of defined length and base composition were produced by human primase (p58/p49 complex) as previously described (*6*). Synthetic ssDNA templates of defined sequence (Table 1) were obtained from Biosearch Technologies, Inc., and Oligos Etc. and are always written in the 3′ to 5′ direction. Concentrations of ssDNA templates were determined spectrally and are reported in terms of 5′-termini. NTPs were from Sigma, and concentrations were determined spectrally. [^{32}P]NTPs were purchased from Perkin-Elmer. 2-Chloro-ATP was purchased from Sigma. 2-Fluoroadenosine was a generous gift from Dr. Jack A. Secrist (Southern Research Institute) and was converted to 2-fluoro-ATP as previously described (*35*). 3-Deaza-ATP was a generous gift from Merck Research. Synthesis and characterization of all other nucleotide analogues are described previously (*31*, *35*). All other reagents were of the highest purity available.

Methods

Primase Assays. Unless otherwise noted, reaction mixtures (10 μL) contained 50 mM Tris, pH 8.0, 10−16 mM $MgCl_2$, 1 mM DTT, 0.1 mg/mL BSA, 2−6% glycerol, 20 μM ssDNA template, 0.8 mM [α-^{32}P]NTPs, and 100 nM HSV-1 wild-type helicase−primase (UL5/UL8/UL52). Reactions were initiated by adding enzyme, incubated at 37 °C for 30 min, and then quenched by adding 2−4 volumes of gel-loading buffer (90% formamide). Control reactions that lacked enzyme were conducted under identical conditions. Products were separated by polyacrylamide gel electrophoresis (20% polyacrylamide and 7.5 M urea or 40% acrylamide and no urea) and visualized by phosphorimagery (Molecular Dynamics). ImageQuant Software (Molecular Dynamics) was utilized for quantitative analysis of primer products. Primer standards of defined length and sequence were loaded concurrently with the products from primase assays to determine the product lengths and identity (*6*).

DNA-Dependent NTPase Assay. Reactions (10−20 μL) contained the same components as the primase assays and were initiated by adding wild-type helicase−primase (UL5/UL8/UL52). After 60 min at 37 °C, 0.75 mL of an acidic ammonium molybdate/malachite green solution (3:1 mixture of 0.045% malachite green HCl and 4.2% ammonium molybdate in 4 M HCl) was added to detect the formation of free phosphate (*11*, *36*). After precisely 3 min, the absorbance at 650 nm was measured. Control reactions that lacked either NTP or enzyme were conducted under identical conditions.

RESULTS

We used a series of NTP analogues to elucidate how herpes primase chooses whether to polymerize a NTP. The analogues contained the sugar ribose conjugated to bases that varied substantially in terms of shape, electron distribution, hydrogen-bonding capacity, and hydrophilicity (Chart 1).

Primase Tightly Binds a Variety of Nucleotide Analogues. Initially, we examined the ability of these compounds to inhibit primase activity. Inhibition of ATP polymerization was measured in assays containing the template d(GTTT)$_{15}$ (sequence written in the 3′ to 5′ direction) and only [α-^{32}P]ATP, while inhibition of GTP polymerization was measured in assays containing the template d(GCCC)$_{15}$ and only [α-^{32}P]GTP. As shown in Table 2, all of the analogues tested inhibited both ATP and GTP polymerization with similar potency. Interestingly, these analogues inhibited

Chart 1: Structures of NTP Analogues Used in These Studies

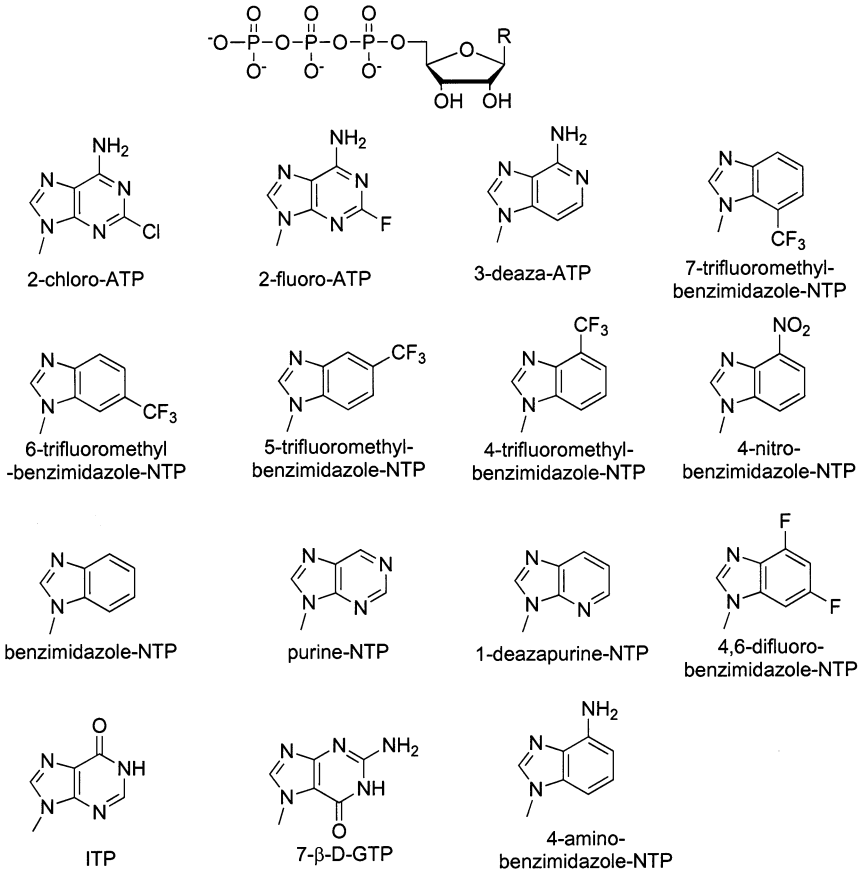

Table 2: IC_{50} Values (μM) of NTP Analogues

NTP analogue	IC_{50} vs ATP[a]	IC_{50} vs GTP[a]
2-chloro-ATP	1070 ± 130	ND[b]
2-fluoro-ATP	490 ± 140	470 ± 160
3-deaza-ATP	810 ± 170	370 ± 140
7-trifluoromethylbenzimidazole-NTP	NM[c]	59 ± 3
6-trifluoromethylbenzimidazole-NTP	34 ± 3	60 ± 10
5-trifluoromethylbenzimidazole-NTP	100 ± 10	150 ± 20
4-trifluoromethylbenzimidazole-NTP	87 ± 3	80 ± 10
4-nitrobenzimidazole-NTP	NM[c]	200 ± 20
benzimidazole-NTP	310 ± 10	500 ± 70
purine-NTP	110 ± 20	160 ± 20
1-deazapurine-NTP	170 ± 10	210 ± 30
4,6-difluorobenzimidazole-NTP	540 ± 120	610 ± 120
7-β-D-GTP	40 ± 10	70 ± 10

[a] ATP polymerization was measured on template $(GTTT)_{15}$ in the presence of 800 μM ATP, and GTP polymerization was measured on template $d(GCCC)_{15}$ in the presence of 800 μM GTP. In both cases, no other natural NTPs were present. [b] ND, not determined. An IC_{50} could not be determined because of the stimulating effect of low concentrations of 2-chloro-ATP (~100−1000 μM) on GTP polymerization. At high concentrations of 2-chloro-ATP, however, GTP polymerization was inhibited 50% at a concentration of ~3500 μM. [c] NM, not measured.

primer synthesis as well as or better than a natural NTP. GTP and UTP inhibited ATP polymerization on $d(GTTT)_{15}$ with IC_{50}s of 1230 ± 50 and 2700 ± 170 μM, respectively, and UTP inhibited GTP polymerization on $d(GCCC)_{15}$ with an IC_{50} of 6100 ± 500 μM. To determine the type of inhibition (competitive, noncompetitive, etc.), we conducted a complete kinetic analysis for the inhibition of GTP polymerization by 5-trifluoromethylbenzimidazole-NTP and 2-fluoro-ATP. In both cases, we observed competitive inhibition between the analogue and GTP (data not shown).

Two compounds, ATP and 2-chloro-ATP, had rather surprising effects on primase activity. Rather than simply inhibiting GTP polymerization on the template $d(GCCC)_{15}$, low concentrations (~100−1000 μM) of these compounds initially stimulated GTP polymerization (by 28 ± 3% and 35 ± 9%, respectively) while higher concentrations gave inhibition (Figure 1). Interestingly, low concentrations of 2-chloro-ATP as well as any of the other NTPs or NTP analogues did not stimulate ATP polymerization on the template $d(GTTT)_{15}$.

Incorporation of NTP Analogues opposite T. Despite the extraordinarily low fidelity of primase and its ability to bind

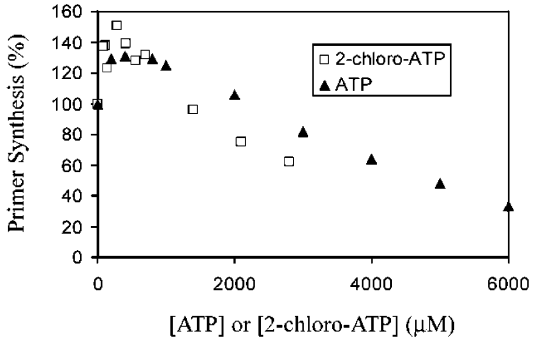

FIGURE 1: ATP and 2-chloro-ATP stimulate GTP polymerization by primase on $d(GCCC)_{15}$ at low concentrations and inhibit at higher concentrations. Reactions were performed as described under Experimental Procedures and contained 100 nM wild-type herpes helicase−primase, 20 μM $d(GCCC)_{15}$, 800 μM [α-^{32}P]GTP, and various concentrations of either ATP or 2-chloro-ATP. The rate of primer synthesis in the absence of ATP and 2-chloro-ATP was normalized to 100%.

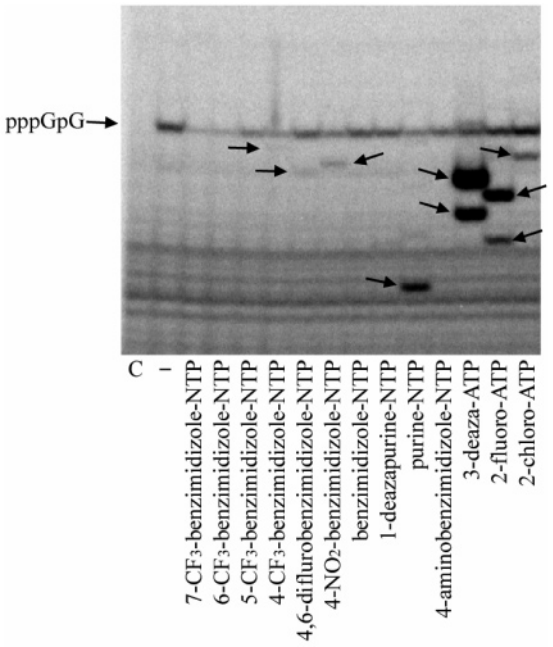

FIGURE 2: Herpes primase incorporates some analogues opposite a template T in the absence of cognate ATP. Reactions contained 100 nM wild-type herpes helicase−primase, 20 μM d(TCTG)$_{15}$, 800 μM [α-^{32}P]GTP, and 200 μM specified analogue, except for the control reactions which lacked enzyme (C) or lacked analogue (−). New products of altered electrophoretic mobility due to analogue incorporation are identified by arrows on the image of the 40% acrylamide gel. The electrophoretic mobility of pppGpG dinucleotide primers is noted to the left of the image.

a wide variety of NTP analogues, primase did not appear to readily incorporate these NTP analogues. Including various analogues in assays containing the template d(GTTT)$_{15}$ and 800 μM [α-^{32}P]ATP significantly inhibited primase activity (Table 2) but did not result in new products of altered electrophoretic mobility. We have previously observed that incorporating a nucleotide analogue into a primer usually results in a shift in electrophoretic mobility (*31*, *32*), thus suggesting that primase did not readily incorporate these NTPs. (We could have easily detected new products that corresponded to <3% of the amount of product generated in the absence of an analogue.)

Since the lack of analogue incorporation opposite T could have resulted from an inability of the analogues to effectively compete with the ATP in the assay, incorporation of each analogue in Chart 1 was measured in assays that lacked the cognate ATP. When reactions contained the template d(TCTG)$_{15}$ and only [α-^{32}P]GTP, primase still synthesized the pppGpG dinucleotide due to misincorporation of GTP opposite the template T that codes for the 5′-terminal nucleotide of the primer (Figure 2). While it is conceivable that primase initiated synthesis at the second nucleotide after the G (i.e., the template C) and misincorporation actually occurred at the following T, this seems extremely unlikely since we have never observed primase to initiate primer synthesis at positions other than the first nucleotide after the template G.

In several cases, including an analogue NTP in these assays resulted in new products of altered electrophoretic mobility, indicating that primase incorporated the analogue. To better understand the chemical features that allow primase to polymerize a NTP, we examined incorporation of ana-

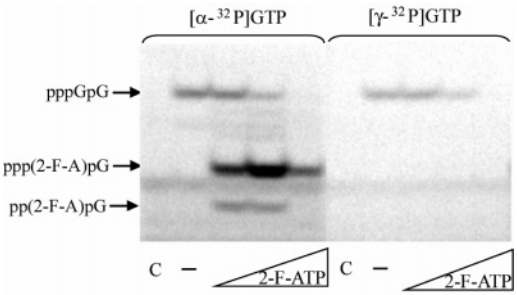

FIGURE 3: Herpes primase incorporates 2-fluoro-ATP as the 5′-terminal nucleotide opposite a template T of d(TCTG)$_{15}$. Reactions contained 100 nM wild-type herpes helicase−primase, 20 μM d(TCTG)$_{15}$, 800 μM [^{32}P]GTP, and 100, 300, or 850 μM analogue, except for the control reactions which lacked either enzyme (C) or 2-fluoro-ATP (−). The electrophoretic mobility of pppGpG, as well as the deduced mobility of ppp(2-fluoro-A)pG and pp(2-fluoro-A)pG dinucleotide primers (based on the experimental results shown in Figure 4), is noted to the left of the image of the 40% acrylamide gel.

logues into the primer at the first (5′-terminal), second, and third nucleotide positions.

5′-Terminal Nucleotide Position. To determine if primase could incorporate an analogue as the NTP that becomes the 5′-terminal nucleotide of the primer, assays contained 20 μM d(TCTG)$_{15}$, 800 μM [α-^{32}P]GTP, and 200 μM analogue. As noted above, primase synthesizes the pppGpG dinucleotide due to misincorporation of a GTP. Addition of many of the analogues (7-trifluoromethylbenzimidazole-NTP, 6-trifluoromethylbenzimidazole-NTP, 5-trifluoromethylbenzimidazole-NTP, benzimidazole-NTP, 1-deazapurine-NTP, 4,6-difluorobenzimidazole-NTP, 7-β-D-GTP, and 4-aminobenzimidazole-NTP) resulted in decreased levels of the pppGpG dinucleotide without the appearance of any new products (Figure 2 and data not shown). Since changing the nucleotide composition of a dinucleotide generally results in large changes in electrophoretic mobility (refs *31* and *32* and see below), these results indicate that primase will not readily incorporate these analogues as the 5′-terminal nucleotide. Primase did, however, incorporate detectable amounts of seven NTP analogues: 3-deaza-ATP, 2-fluoro-ATP, 2-chloro-ATP, purine-NTP, 4-trifluoromethylbenzimidazole-NTP, ITP, and 4-nitrobenzimidazole-NTP (Figure 2 and data not shown). Furthermore, we verified that incorporation of the analogues 3-deaza-ATP, 2-fluoro-ATP, purine-NTP, and ITP occurred at the 5′-terminal position. Whereas both the pppGpG and the product due to analogue incorporation were detected when assays contained [α-^{32}P]GTP, only the pppGpG product was detected when assays contained [γ-^{32}P]GTP (an example is shown for 2-F-ATP in Figure 3). If analogue incorporation had occurred at the second nucleotide in the primer, the product, pppGpX, where X = analogue, would still have been radiolabeled since it retains the γ-phosphate of GTP. (The other analogues could not be similarly tested because the amount of product due to analogue incorporation was too low for conclusive results.)

Somewhat surprisingly, including either ATP or an analogue in the assays occasionally gave rise to two new products that migrated in the region expected for a dinucleotide (e.g., 3-deaza-ATP and 2-fluoro-ATP, Figure 2, as well

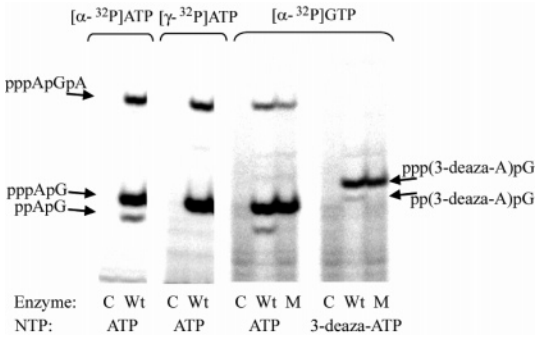

FIGURE 4: NTPase activity removes the 5′-terminal phosphate from a pppNpN dinucleotide to form a ppNpN dinucleotide. Reactions contained 20 μM d(TCTG)$_{15}$, 800 μM GTP, 200 μM ATP or 3-deaza-ATP, the indicated radiolabel, and either wild-type helicase−primase (Wt) or mutant helicase−primase (M) that lacks NTPase activity, except for the control reactions which lacked either enzyme (C). The amount of Wt ([α-^{32}P]GTP) sample applied to the gel was reduced by a factor of 2 to ensure that similar quantities of pppNpN dinucleotide were present for both enzymes. As a result, the Wt ([α-^{32}P]GTP) samples have a lower background in comparison to the C and M samples. The electrophoretic mobility of pppApG and pppApGpA and the deduced mobility of ppApG, ppp(3-deaza-A)pG, and pp(3-deaza-A)pG dinucleotide primers (based on the results of this experiment) are noted on the side of the image of the 40% acrylamide gel.

as ATP, Figure 4).[2] Since we had previously shown that the NTPase activity of the primase−helicase complex could remove the 5′-terminal phosphate from a pppNpN dinucleotide to generate the ppNpN dinucleotide (*6*), we suspected that this was the cause of the extra products. To test this hypothesis, we first determined if the faster migrating product observed in assays containing ATP, GTP, and d(TCTG)$_{15}$ retained a triphosphate at its 5′-terminus. When assays contained [α-^{32}P]ATP and GTP, both authentic pppApG and a slightly faster migrating product were observed. In contrast, when assays contained [γ-^{32}P]ATP and GTP, only the product that comigrated with authentic pppApG was observed (Figure 4). Thus, the faster migrating product lacks a γ-phosphate at its 5′-end. Additionally, we examined the products generated in assays containing ATP or 3-deaza-ATP, [α-^{32}P]GTP, and d(TCTG)$_{15}$ and either wild-type helicase−primase or a mutant helicase−primase that lacks NTPase activity. As shown in Figure 4, eliminating the NTPase activity eliminates the "extra" new product, indicating that it results from the NTPase activity removing the 5′-terminal phosphate of the dinucleotide.[3] Since the NTPase always has a choice of hydrolyzing either one of the NTPs in the assay or a dinucleotide, detection of ppNpN products in Figure 2 is likely limited to only those dinucleotides that accumulate to relatively high levels.

For those analogues that primase incorporated, we determined their efficiency of incorporation. Assays contained the template d(TCTG)$_{15}$, [α-^{32}P]GTP, and increasing concentrations of an analogue. In the absence of any analogue, primase synthesizes the pppGpG dinucleotide. Thus, we can compare the ability of primase to polymerize either ATP (the natural, correct NTP) or an analogue by determining how much of each of these compounds must be present in assays to cause primase to synthesize equimolar amounts of the pppGpG and (pppAnalogue)pG dinucleotide (*31*). In assays containing 800 μM GTP, including only 1.6 ± 0.1 μM ATP resulted in synthesis of equimolar amounts of pppApG and pppGpG. While primase incorporated 3-deaza-ATP almost as efficiently as ATP, it incorporated the other analogues substantially less efficiently (Table 3).

Table 3: Incorporation of NTP Analogues opposite T in the Absence of ATP

NTP analogue	analogue concn (μM), where analogue incorporation = GTP misincorporation,[a] at primer position: 5′-terminal	second	third
ATP	1.6 ± 0.1	17 ± 2	30 ± 2
3-deaza-ATP	2.9 ± 0.4	87 ± 5	103 ± 8
2-fluoro-ATP	31 ± 3	>500[b]	320 ± 50
2-chloro-ATP	220 ± 20	ND[c]	6600 ± 400
4-trifluoromethylbenzimidazole-NTP	>300[b]	ND[c]	>400[b]
4-nitrobenzimidazole-NTP	180 ± 20	ND[c]	ND[c]
purine-NTP	48 ± 3	ND[c]	580 ± 45
ITP	360 ± 50	ND[c]	ND[c,d]

[a] Assays contained 800 μM GTP. Templates d(T̲CTG)$_{15}$, d(C$_{20}$GCT̲A$_{20}$), and d(C$_{20}$GCCT̲A$_{19}$) were used to measure analogue incorporation at the 5′-terminal, second, and third primer positions, respectively. The underlined base in the template sequence indicates the site where analogue incorporation and GTP misincorporation were measured. [b] High concentrations of these analogues inhibited primer synthesis to such an extent that it was not possible to determine the concentration at which analogue incorporation equaled GTP misincorporation. Thus, only a lower limit is provided. [c] ND, incorporation was not detectable above background. [d] On d(C$_{20}$GCCTA$_{19}$), detectable incorporation of ITP only occurred opposite C.

Second Nucleotide of the Primer. Incorporation of the NTP analogues as the second nucleotide of the primer was measured on the template d(C$_{20}$GCT̲A$_{20}$). Primer synthesis begins opposite the C immediately following the G, and analogue incorporation opposite the underlined T was measured. In the presence of only [α-^{32}P]GTP, primase synthesizes the pppGpG dinucleotide. In assays containing 800 μM GTP, 17 ± 2 μM ATP resulted in synthesis of equimolar amounts of pppGpA and pppGpG (Table 3). Primase discriminated much more strongly against analogues at this second position of the primer than it did at the 5′-terminal position. Indeed, adding most of the analogues resulted in no detectable incorporation, and primase readily incorporated only 3′-deaza-ATP (Table 3). To ensure that 3-deaza-ATP was incorporated at the second nucleotide position (i.e., pppGpX), we verified that incorporation was detectable when reactions contained either [α-^{32}P]GTP or [γ-^{32}P]GTP (data not shown).

Third Nucleotide of the Primer. Incorporation of the analogues as the third nucleotide of the primer was measured on the template d(C$_{20}$GCCT̲A$_{19}$). In the presence of only 800 μM [α-^{32}P]GTP, primase synthesizes both a correctly base-paired pppGpG dinucleotide and a pppGpGpG trinucleotide due to misincorporation of GTP opposite the underlined template T. In assays containing 800 μM GTP,

[2] Products were analyzed in 40% acrylamide gels, rather than the more typical 20% acrylamide, because of the much greater resolution of 40% gels. In gels containing only 20% acrylamide, products consisting of natural NTPs (e.g., pppGpG) and products containing an analogue NTP (e.g., pppXpG or pppGpX, where X is the analogue) often were not completely separated from each other.

[3] Whereas the ppNpN dinucleotides migrate more rapidly than the pppNpN dinucleotides in gels containing 40% acrylamide, the ppNpN dinucleotides migrate more slowly than the pppNpN dinucleotides in gels containing 20% acrylamide and 7.5 M urea.

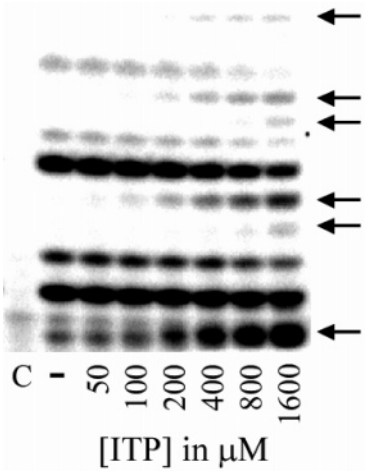

FIGURE 5: Herpes primase readily incorporates ITP as a GTP analogue. Reactions contained 100 nM wild-type herpes helicase−primase, 20 μM d(GCCC)$_{15}$, 800 μM [α-^{32}P]GTP, and the indicated concentration of ITP, except for the control reactions which lacked either enzyme (C) or ITP (−). New products of altered electrophoretic mobility are labeled with arrows on the right of the image of the 20% acrylamide, 7.5 M urea gel.

30 ± 2 μM ATP resulted in synthesis of equimolar amounts of pppGpGpA and pppGpGpG. Again, with the exception of 3-deaza-ATP, primase incorporated the other nucleotide analogues much less efficiently (Table 3).

Incorporation of NTP Analogues opposite C. With one exception, ITP, herpes primase did not readily incorporate NTP analogues opposite C. Including various NTP analogues in assays containing the template d(GCCC)$_{15}$ and 800 μM [α-^{32}]GTP resulted in inhibition of primase activity (Table 2) but no new products of altered electrophoretic mobility. The one exception was ITP, which primase readily polymerized as a GTP analogue (Figure 5). Including even low concentrations (100 μM) of ITP in assays on d(GCCC)$_{15}$ resulted in products of altered electrophoretic mobility.

We further tested for analogue incorporation opposite a template C in the absence of the cognate GTP. Adding a 200 μM amount of each analogue in Chart 1 to reactions containing 20 μM d(TCTG)$_{15}$ and 800 μM [α-^{32}]ATP resulted in detectable incorporation of only two NTP analogues (Figure 6): significant incorporation of ITP and only trace amounts of 3-deaza-ATP. Thus, primase strongly discriminates against polymerization of almost all of the analogues tested.

The DNA-Dependent NTPase Hydrolyzes Many NTP Analogues. We tested the ability of the DNA-dependent NTPase activity of the primase−helicase complex to hydrolyze NTP analogues (Table 4). Hydrolysis was measured in assays containing 20 μM d(T$_{20}$GTCCT$_{19}$) and 800 μM ATP or a NTP analogue. Table 4 shows that the helicase−primase hydrolyzed all of the NTP analogues at rates similar to or somewhat less than the rate at which it hydrolyzed ATP.

DISCUSSION

We measured the ability of herpes primase to bind and polymerize a series of NTP analogues containing bases that varied significantly in terms of shape, hydrophobicity, and hydrogen-bonding potential. While the enzyme bound all of these analogues with similar affinity, it only efficiently polymerized those analogues that could form Watson−Crick hydrogen bonds with the template base. In contrast, the DNA-dependent NTPase was very promiscuous in terms of substrate specificity.

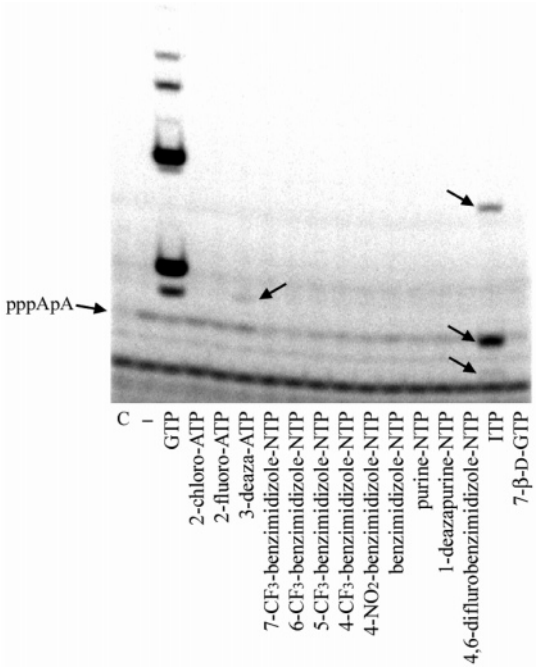

FIGURE 6: Herpes primase does not incorporate most analogues opposite a template C in the absence of the cognate GTP. Reactions contained 100 nM wild-type herpes helicase−primase, 20 μM d(TCTG)$_{15}$, 800 μM [α-^{32}P]ATP, and 200 μM specified analogue, except for the control reactions which lacked either enzyme (C) or an analogue (−). The products of altered electrophoretic mobility due to incorporation of either ITP or 3-deaza-ATP are identified by arrows. The electrophoretic mobility of the pppApA dinucleotide is noted to the left of the image of the 40% acrylamide gel.

Table 4: Rate of NTP Hydrolysis by the DNA-Dependent NTPase[a]

NTP	rate (h^{-1})[b]
ATP	5800 ± 1100
2-chloro-ATP	2100 ± 500
2-fluoro-ATP	1000 ± 200
3-deaza-ATP	5000 ± 1900
7-trifluoromethylbenzimidazole-NTP	900 ± 200
6-trifluoromethylbenzimidazole-NTP	3700 ± 1400
5-trifluoromethylbenzimidazole-NTP	2900 ± 600
4-trifluoromethylbenzimidazole-NTP	900 ± 300
4-nitrobenzimidazole-NTP	1100 ± 600
benzimidazole-NTP	3000 ± 300
purine-NTP	1000 ± 100
1-deazapurine-NTP	5800 ± 2500
7-β-D-GTP	2300 ± 400
ITP	2200 ± 700
4,6-difluorobenzimidazole-NTP	2900 ± 600

[a] Assays contained 20 μM d(T$_{20}$GTCCT$_{19}$) and 800 μM ATP or the indicated analogue. [b] Hydrolysis rates are an average of two to three separate experiments for each compound.

The DNA-dependent NTPase uses a remarkable broad range of substrates. The enzyme hydrolyzed every analogue tested and many at rates comparable to the natural substrate, ATP. Previous studies have shown that the helicase will hydrolyze all four NTPs (*11*) and will even remove the 5′-terminal phosphate from short oligonucleotides (pppNpN, pppNpNpN, etc.) (*6*). The ability of the NTPase to also hydrolyze NTPs whose bases do not closely resemble the canonical bases suggests that hydrolysis does not require specific enzyme−base interactions.

The interaction of NTPs with the helicase active site may account for the surprising ability of ATP and 2-chloro-ATP to stimulate primase activity. Previously, Weller and colleagues showed that the primase and helicase activities exhibit a complex interdependence (*13*). Mutations that impaired the NTPase activity that provides the ΔG for DNA unwinding often stimulated primase activity. Thus, it would not be surprising if binding and/or hydrolysis of ATP and 2-chloro-ATP in the NTPase site also affected primase activity. Why the effects were so specific to these compounds remains unclear. Further experiments to understand this process are underway.

Herpes primase promiscuously binds NTPs, as evidenced by the ability of all of the analogues to inhibit, and in some cases be incorporated by, primase. The studies showing that 5-trifluoromethylbenzimidazole-NTP and 2-fluoro-ATP inhibit primase competitively with respect to the NTP, the similarity in analogue structure, and polymerization of some of the analogues indicate that the analogues bind into the primase active site. Remarkably, in almost all cases the analogues inhibited primer synthesis more potently than a natural, noncognate NTP (Table 2). This included bases much more hydrophobic than a natural base (e.g., 4- and 7-trifluoromethylbenzimidazole), a base of similar hydrophobicity as a natural base but with the Watson−Crick hydrogen-bonding groups in unusual positions (7-β-D-guanine), a base shaped almost identically to a natural base (4,6-difluorobenzimidazole), bases shaped very differently than a natural base (e.g., 5- and 6-trifluoromethylbenzimidazole), and a base capable of forming just one Watson−Crick hydrogen bond with the template base (purine). The identity of the template base being replicated also has little effect on the ability of the NTP analogues to inhibit primase, as evidenced by similar IC_{50} values for inhibition of both ATP and GTP polymerization. This relatively nonspecific binding of a wide variety of NTP analogues suggests that primase does not make highly specific contacts with the base of an NTP during the initial binding event.

Whereas herpes primase very promiscuously binds NTP analogues, it strongly discriminates against polymerizing most nucleotide analogues with altered bases. The only NTPs that primase incorporated at rates similar to those of a natural NTP contained the bases 3-deazaadenine and hypoxanthine. This contrasts with the remarkably low fidelity observed with the natural bases. Previously, we found that primase generally misincorporates NTPs at frequencies around 1/30, although on occasion it misincorporates NTPs at frequencies as high as 1/7 (*34*).

The strong discrimination against polymerization of most analogues allows us to rule out several properties of the bases as dominating the selection process. Primase strongly discriminated against NTPs containing unnatural bases much more hydrophobic than the normal bases (e.g., benzimidazole, trifluoromethylbenzimidazole) and of similar hydrophobicity as a natural base (7-β-D-GTP), indicating that hydrophobicity is not a dominant factor. Shape also cannot be a dominant factor since primase strongly discriminated against NTPs whose bases are slightly smaller (benzimidazole), slightly larger (trifluoromethylbenzimidazole), and similarly sized and shaped (4,6-difluorobenzimidazole, 4-aminobenzimidazole) as the natural bases. Further evidence for the lack of importance of size and shape of the base pair comes from the ability of primase to efficiently synthesize both pyrimidine−pyrimidine and purine−purine mismatches (*34*).

Rather, the data indicate that herpes primase likely requires the formation of Watson−Crick hydrogen bonds, and most importantly between N-1 of a purine with N-3 of a pyrimidine, to efficiently polymerize a NTP. Other than the natural NTPs, the only two NTPs that primase readily polymerized contained the bases 3-deazaadenine and hypoxanthine, and both of these bases can form the aforementioned Watson−Crick hydrogen bond to thymine and cytosine, respectively. While primase occasionally polymerized NTPs containing bases incapable of forming hydrogen bonds (4-trifluoromethylbenzimidazole-NTP and 4-nitrobenzimidazole-NTP), polymerization of these NTPs was very inefficient.

The moderate discrimination against purine-NTP and 2-fluoro-ATP and stronger discrimination against 2-chloro-ATP are also consistent with NTP incorporation based on Watson−Crick hydrogen bonding. Similarly to adenine, N-1 of purine can form a hydrogen bond with the proton on N-3 of thymine. Fluorine and chlorine decrease the pK_a of N-1 due to their electron-withdrawing properties (*37*, *38*), chlorine more so than fluorine. Importantly, this decreased proton affinity should inhibit the ability of N-1 to form hydrogen bonds. Thus, the lower efficiency with which primase polymerizes 2-fluoro-ATP as compared to ATP and even lower efficiency for 2-chloro-ATP are consistent with the enzyme requiring formation of a hydrogen bond at N-1 in order to efficiently polymerize a NTP. An alternative explanation for these data with 2-fluoro-ATP and 2-chloro-ATP is that the extra mass at C-2 inhibits polymerization. This seems unlikely, however, because primase readily polymerizes GTP and guanine contains extra mass at C-2 vis-à-vis ATP. The importance of hydrogen bonding at the N-1 is further supported by the fact that primase did not readily incorporate 4-aminobenzimidazole-NTP, which can only hydrogen bond to thymine and/or cytosine by the C-6 exocyclic amine.

The extremely rapid misincorporation of natural NTPs by herpes primase can also be explained via a Watson−Crick hydrogen-bonding model. The natural bases can exist in either a major or minor tautomeric form (*31*, *39*). Importantly, one can form Watson−Crick hydrogen bonds between any two natural bases by employing both tautomeric forms. While in aqueous solution the major tautomer is much more stable than the minor tautomer (*39*), it is clear that enzymes can dramatically alter the relative stabilities of the major and minor tautomer. Johnson and Beese examined the structure of a primer-template containing a G•T mismatch bound to the thermophillic *Bacillus* DNA polymerase I (*40*). Importantly, the structures of the G•T mismatch suggested that the tautomerization state of the bases varied depending upon where in the primer-template it was located. Thus, a nucleotide selection model that emphasizes the formation of Watson−Crick hydrogen bonds could account both for the ability of primase to discriminate against NTP analogues whose bases lack Watson−Crick hydrogen-bonding groups and for the high rate of natural NTP misincorporation.

A key issue in terms of fidelity is how many mechanisms exist to discriminate between right and wrong (d)NTPs. Both herpes and human primase appear to use similar mechanisms

in choosing whether to polymerize a NTP. Like herpes primase, human primase efficiently polymerizes a NTP if it can form hydrogen bonds involving both N-1 and the exocyclic group at C-6 of a purine (*31*). However, unlike herpes primase, human primase does not polymerize a NTP when hydrogen bonding is limited to just N-1 of purine. In contrast, rapid polymerization of dNTPs by Klenow fragment, an A family polymerase, does not require the incipient base pair between the incoming dNTP and template base to either contain Watson−Crick hydrogen bonds or resemble the shape of a correct base pair (*27*−*29*, *41*−*45*). Similarly, efficient dNTP polymerization by pol α, a B family polymerase, likewise does not require the incipient base pair to either contain Watson−Crick hydrogen bonds or resemble a correct base pair (*28*, *29*). Indeed, recent studies indicate that this enzyme obtains fidelity by using the hydrogen-bonding groups on the base to specifically discriminate against wrong dNTPs (*46*). Thus, nature appears to have evolved at least two different mechanisms to distinguish between right and wrong (d)NTPs.

Why should human and herpes primase share similar mechanisms to discriminate between right and wrong NTPs? One possibility is that this mechanism is common to all primases. Mechanistically, the enzymes share a number of similarities including identical biological roles and extremely low fidelity. Presumably, the low fidelity is tolerable since the primer will not become a permanent part of the genome. However, at the sequence level, these primases do not appear to be related to each other (*15*, *16*, *47*). Alternatively, the use of Watson−Crick hydrogen bonds to select the correct (d)NTP may be a general hallmark of low-fidelity polymerases. Previous work has suggested that the low-fidelity pol *η* discriminates between right and wrong dNTPs on the basis of Watson−Crick hydrogen bonds (*48*). It has also been previously pointed out that the differences in stability of correct and incorrect base pairs in solution are only sufficient to permit a very low level of discrimination between right and wrong (d)NTPs (*33*).

Certainly, further investigation is needed to determine if other primases and low-fidelity polymerases employ hydrogen bonding as a selection tool for nucleotide polymerization and to obtain a mechanistic understanding of how a polymerase uses a Watson−Crick hydrogen bond to determine whether to polymerize a (d)NTP. For example, satisfied hydrogen bonds between NTP base and template base may allow proper positioning of the NTP in the active site for polymerization to occur. A mechanistic understanding of nucleotide selection by herpes primase could also aid in the development of new drugs against herpes that specifically target herpes DNA replication.

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