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Mechanisms by Which Human DNA Primase Chooses To Polymerize a Nucleoside Triphosphate[†]

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ABSTRACT: Human DNA primase synthesizes short RNA primers that DNA polymerase α then elongates during the initiation of all new DNA strands. Even though primase misincorporates NTPs at a relatively high frequency, this likely does not impact the final DNA product since the RNA primer is replaced with DNA. We used an extensive series of purine and pyrimidine analogues to provide further insights into the mechanism by which primase chooses whether or not to polymerize a NTP. Primase readily polymerized a size-expanded cytosine analogue, 1,3-diaza-2-oxophenothiazine NTP, across from a templating G but not across from A. The enzyme did not efficiently polymerize NTPs incapable of forming two Watson−Crick hydrogen bonds with the templating base with the exception of UTP opposite purine deoxyribonucleoside. Likewise, primase did not generate base pairs between two nucleotides with altered Watson−Crick hydrogen-bonding patterns. Examining the mechanism of NTP polymerization revealed that human primase can misincorporate NTPs via both template misreading and a primer-template slippage mechanism. Together, these data demonstrate that human primase strongly depends on Watson−Crick hydrogen bonds for efficient nucleotide polymerization, much more so than the mechanistically related herpes primase, and provide insights into the potential roles of primer-template stability and base tautomerization during misincorporation.

DNA polymerases lack the capacity to initiate the synthesis of new DNA strands *de novo* on single-stranded DNA templates. Rather, they require the 3′ end of a preexisting primer bound to the template in order to replicate the template. In most replicative systems, DNA primases solve this problem by synthesizing short RNA primers that a replicative DNA polymerase then elongates (*1*, *2*). In eukaryotes, primase synthesizes a short RNA primer (8−12 nucleotides long) that DNA polymerase α (pol α) elongates by another ∼20 nucleotides to generate a DNA primer (*3*). Pol α dissociates, and either pol δ or pol ε finishes the bulk of DNA replication on the leading and lagging strands (*4*, *5*). After completion of Okazaki fragment synthesis on the lagging strand, the RNA primer is removed and replaced with DNA. Consequently, errors during primer synthesis do not become part of the genome, and therefore, primase need not have high fidelity. Indeed, human primase misincorporates NTPs at a frequency of around 1 mistake per 100 NTPs polymerized (*6*), while herpes primase has a misincorporation frequency near 1 in 30 (*7*).

Eukaryotic primase consists of two subunits, p49 and p58. The p49 subunit contains the catalytic core of primase; it binds to single-stranded DNA and catalyzes phosphodiester bond formation (*8*, *9*). In the presence of Mn^{2+}, p49 alone can both initiate primer synthesis and elongate the growing primer, whereas in the presence of just Mg^{2+}, p49 cannot initiate primer synthesis (*10*). p58 stabilizes p49 and plays important roles in primer initiation, the ability of primase to synthesize primers of defined length (i.e., count), and passing newly synthesized primers from primase to pol α (*11*).

Unlike primases from other sources, eukaryotic primase does not require a specific template sequence in order to synthesize a primer. Rather, purified eukaryotic primase only requires two consecutive template pyrimidines, although a pyrimidine-rich template generally enhances the overall rate of primer synthesis *in vitro* (*12*). In addition to synthesizing primers *de novo* on single-stranded DNA, primase can also polymerize NTPs onto RNA primer-templates (*10*).

We previously examined the interaction of human primase with a series of purine NTP analogues (*13*, *14*). Removal of N-1 from ATP resulted in a NTP that primase did not effectively incorporate, while removal of N^6 resulted in a NTP (purine NTP) that primase polymerized extremely inefficiently opposite T as compared to ATP. Addition of a halogen to C-2 of ATP or removal of N^2 from GTP had only small effects on NTP incorporation and suggested a model whereby primase required the formation of Watson−Crick hydrogen bonds between N-1 and N^6/O^6 of a purine NTP and the templating base.

Human and herpes simplex virus-1 primase bear some sequence similarity, suggesting that they are evolutionarily related (*15*, *16*). Consistent with this sequence similarity, both enzymes interact with the sugar of a NTP very similarly (*17*, *18*) and also had very similar interactions with a set of purine NTP analogues (*13*, *19*). More recently, however, we examined the base specificity of herpes primase using an extended series of both purine and pyrimidine NTPs, as well as base analogues in both the template and incoming NTP (*20*). This extended series of

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analogues showed that herpes primase often shows a large asymmetry when polymerizing a NTP analogue (i.e., the enzyme polymerizes XTP opposite a templating Y but not YTP opposite a templating X) and that the enzyme does not absolutely require the formation of Watson−Crick hydrogen bonds in order to efficiently polymerize a NTP.

In light of these data with herpes primase and to better understand the mechanism by which human primase chooses to polymerize a NTP, we examined an extended series of both purine and pyrimidine analogues. The analogues were incorporated into both the template and incoming NTP. Human primase did not efficiently generate novel base pairs between bases incapable of Watson−Crick hydrogen bonding or with altered Watson−Crick hydrogen-bonding patterns and did not efficiently incorporate natural NTPs across from templating bases incapable of forming Watson−Crick hydrogen bonds. In those cases when primase misincorporated a NTP, this could occur via both misreading of the template and primer-template slippage. The mechanistic implications of these data are discussed.

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. 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. NTP analogues and the phosphoramidites of nucleoside analogues were synthesized as previously described (*20*). Synthetic DNA oligonucleotides of defined sequence containing only natural nucleotides were purchased from IDT or Biosearch Technologies. Synthetic DNA oligonucleotides containing nucleotide analogues were synthesized as previously described (*20*). The RNA primer was purchased from Dharmacon and 5′-^{32}P-labeled using [γ-^{32}P]ATP and polynucleotide kinase as previously described (*21*, *22*). Human primase (p49/p58 complex) was purified and stored as previously described (*23*).

Methods. *Primase Assays*. Assays (10 μL) were performed as previously described and typically contained 200 nM human primase, 2 μM 5′-[^{32}P]-primer-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, and various concentrations of a NTP (analogue) (*10*). Reactions were incubated at 37 °C for 60 min and quenched with 25 μL of gel loading buffer (formamide/0.05% xylene cyanol and bromophenol blue). For incorporation of a correct, natural NTP, the concentration of enzyme was lowered to 10−50 nM, and the reaction time was shortened to 5−10 min. Products were separated using 20−25% polyacrylamide and 8 M urea gels and analyzed using a Typhoon Phosphorimager (Molecular Dynamics).

RESULTS

Human primase incorporates incorrect, natural NTPs at a high frequency (around 1 every 100 correct NTPs), yet very strongly discriminates against purine NTP analogues that cannot form a complete set of Watson−Crick hydrogen bonds (*13*). To better understand how the enzyme chooses whether or not to polymerize a NTP, we examined an expanded series of both purine and pyrimidine analogues when present in both the template and incoming NTP (Figure 1).

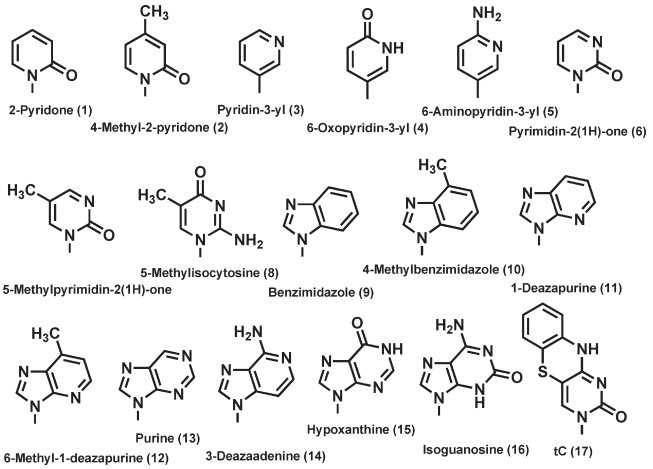

FIGURE 1: Base analogues used in these studies.

We took advantage of primase's ability to polymerize NTPs onto a RNA primer-template to measure incorporation of NTP (analogues). During *de novo* primer synthesis on a single-stranded template, one cannot constrain primase to initiate primer synthesis at a specific site. Thus, one cannot define exactly when during primer synthesis that primase will interact with a templating nucleotide analogue or a NTP analogue. In contrast, measuring primer-template elongation allows us to precisely calculate the kinetic parameters for NTP incorporation during a defined polymerization event. Polymerization of both natural and analogue NTPs was measured across from both natural and analogue template bases. Importantly, the primer-templates only varied in the identity of the next templating nucleotide to minimize the possibility that sequence context affected the incorporation efficiencies (Table 1). Consistent with previous studies, primase incorporated each canonical NTP opposite its complementary natural template base (Table 2), with only small differences in the efficiency with which the enzyme generated each natural base pair.

We next examined a series of base analogues when present in either the template or the incoming NTP to better understand the role of Watson−Crick hydrogen bonds (Figure 2, Tables 3−5). Previous work showed that primase did not incorporate hydrophobic purine NTP analogues during *de novo* primer synthesis (*13*). Using a primer-template as substrate, primase likewise did not detectably polymerize purine NTP analogues where the base (benzimidazole, 4-methylbenzimidazole, 1-deazapurine, 6-methyl-1-deazapurine, and purine) lacked one or more Watson−Crick hydrogen-bonding groups (N^6/O^6 and/or N-1) opposite a natural templating base. Similarly, primase did not polymerize two pyrimidine NTP analogues, 2-pyridone NTP and 4-methyl-2-pyridone NTPs, opposite natural templating bases (Table 3). The lack of polymerization did not result from unsatisfied Watson−Crick hydrogen-bonding groups on the templating base since primase also did not polymerize these hydrophobic NTPs opposite hydrophobic templating bases (benzimidazole, 4-methylbenzimidazole, 1-deazapurine, 6-methyl-1-deazapurine, 2-pyridone, 4-methyl-2-pyridone). However, primase polymerized UTP across from purine only 4-fold less

Table 1: RNA Primer-Templates Used in These Studies

DNAX	5′-AAAAAAAAAACGGG **X** = Variable nucleotide 3′-TTTTTTTTTTTTTTTTTTTGCCC**X**ATTTTTTTTTTTTTT
DNA1	5′-AAAAAAAAAACGGG 3′-TTTTTTTTTTTTTTTTTTTGCCC**AG**TTTTTTTTTTTTT
DNA2	5′-AAAAAAAAAACGGG 3′-TTTTTTTTTTTTTTTTTTTGCCC**AT**TTTTTTTTTTTTT
DNA3	5′-AAAAAAAAAACGGG 3′-TTTTTTTTTTTTTTTTTTTGCCC**(isoC)A**TTTTTTTTTTTTT
DNA4	5′-AAAAAAAAAACGGG 3′-TTTTTTTTTTTTTTTTTTTGCCC**(isoC)G**TTTTTTTTTTTTT

Table 2: Incorporation of Natural Correct NTPs Opposite Natural Templates

NTP	template	k_{cat} (h^{-1})	K_M (μM)	k_{cat}/K_M (h^{-1} μM^{-1})
ATP	dT	110 ± 3	0.06 ± 0.008	1800
UTP	dA	250 ± 4	0.160 ± 0.01	1600
CTP	dG	150 ± 2	0.08 ± 0.004	2000
GTP	dC	140 ± 4	0.05 ± 0.008	3100

efficiently than opposite A (Figure 3, Tables 1 and 4), showing that formation of both Watson−Crick hydrogen bonds is not always essential for efficient NTP polymerization. This relatively efficient incorporation of UTP across from purine also contrasts with the lack of detectable incorporation of purine NTP across from T (Figure 3).

Two sets of experiments demonstrated that formation of Watson−Crick hydrogen bonds alone is not sufficient to drive NTP polymerization. We first asked if primase could generate a base pair containing a complete set of Watson−Crick hydrogen bonds, albeit in an altered arrangement (iso-C:iso-G). However, primase did not detectably generate this base pair, nor did it polymerize any analogue NTPs or natural NTPs, with the exception of UTP (see below, however), opposite iso-C (Tables 3 and 4). Then, we examined NTP polymerization across from 6-aminopyridin-3-yl and 6-hydroxypyridin-3-yl, two bases that can form Watson−Crick hydrogen bonds but lack the equivalent of a pyrimidine O^2 (Tables 3 and 4). With the exception of a low level of UTP incorporation, primase did not detectably polymerize NTPs across from either of these bases, even though they can form two Watson−Crick hydrogen bonds with G and A, respectively. Likewise, primase only polymerized small amounts of UTP across from pyridin-3-yl, a pyrimidine analogue only capable of forming a single Watson−Crick hydrogen bond. In contrast to the almost complete lack of NTP polymerization opposite these pyrimidine analogues lacking O^2, primase inefficiently polymerized ATP, GTP, and UTP across from pyrimidin-2(1*H*)-one, a pyrimidine analogue that lacks N^4/O^4 (Table 5). Thus, primase clearly requires more than Watson−Crick hydrogen bonds to polymerize a NTP.

While 6-hydroxypyridin-3-yl and 6-aminopyridin-3-yl can form Watson−Crick hydrogen bonds, they lack the minor groove hydrogen bond acceptor (O^2 in a pyrimidine). Thus, the weak polymerization of ATP opposite 6-hydroxypyridin-3-yl or

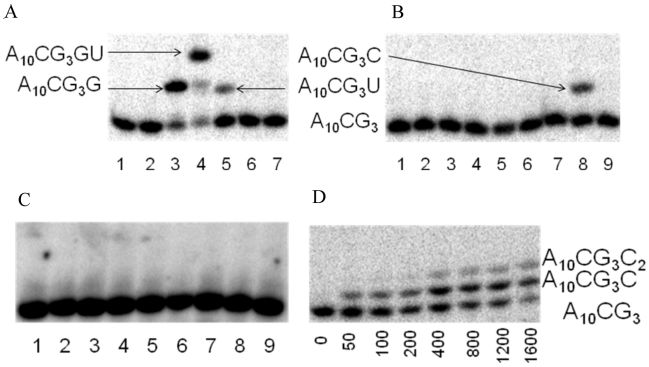

FIGURE 2: Incorporation of NTP (analogues). Experiments contain 400 nM human primase, 1 mM NTP, and 2 μM 5′-[^{32}P]-DNAX. Products are labeled. Panels A−C represent incorporation of various NTPs opposite a templating cytidine (panel A), hypoxanthine (panel B), and 2-pyridone (panel C). Panel A: In DNAX, X = C. Lanes: 1, no enzyme, no NTP; 2, no NTP; 3, GTP; 4, GTP and UTP; 5, UTP; 6, CTP; 7, ATP. Panel B: In DNAX, X = G. Lanes: 1, benzimidazole NTP; 2, 2(1*H*)-pyrimidinone; 3, isoGTP; 4, ITP; 5, purine NTP; 6, GTP; 7, ATP; 8, CTP; 9, UTP. Panel C: In DNAX, X = 2-pyridone. Lanes: 1, iso-GTP; 2, benzimidazole NTP; 3, 2-pyridone NTP; 4, 2(1*H*)-pyrimidinone NTP; 5, 1-deazapurine NTP; 6, GTP; 7, ATP; 8, UTP; 9, CTP. Panel D: DNAX, X = hypoxanthine. Assays contained the noted concentration of CTP (μM).

GTP opposite 6-aminopyridin-3-yl suggested that efficient NTP polymerization requires the minor groove hydrogen bond acceptor on the templating base. To further test this hypothesis, we measured NTP polymerization across from a templating 3-deazaadenine. Converting a template adenine to 3-deazaadenine significantly inhibited polymerization of UTP (Table 4), providing further evidence for primase specifically recognizing the minor groove hydrogen bond acceptor on the templating base.

As a final test of primase's ability to incorporate modified nucleotides, we examined incorporation of tCTP (1,3-diaza-2-oxophenothiazine NTP), a fluorescent CTP analogue that retains the hydrogen-bonding capacity of C but that also readily tautomerizes to resemble U (Figure 4 (*24*−*26*)). Figure 5 shows that primase incorporates the analogue opposite a templating G but, surprisingly, even at elevated concentrations did not incorporate it opposite a templating A. We also observed that even at moderate concentrations (400 μM), tCTP caused significant substrate inhibition, thus preventing us from directly measuring

Table 3: Incorporation of NTPs Opposite a Variety of Templates[a]

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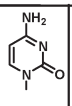
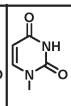
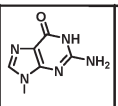
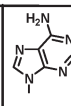
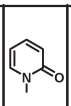
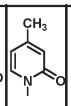
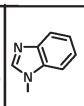
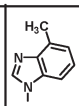
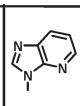
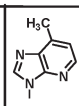
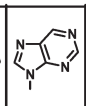
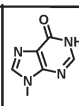
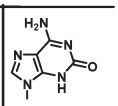
	 C	 U	 G	 A	 1	 2	 6	 9	 10	 11	 12	 13	 15	 16
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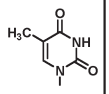
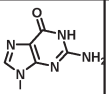
	+	+	ND	++	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	++	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
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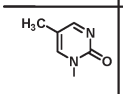
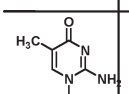
	ND	+	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
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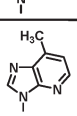
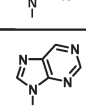
	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	ND	++	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
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[a]ND = no detectable product (k_{cat}/K_M < 10 h^{-1} μM^{-1}); (+) is k_{cat}/K_M < 100 h^{-1} μM^{-1}; (++) is k_{cat}/K_M > 100 h^{-1} μM^{-1}. Where incorporation occurred, rates were quantified in Tables 1, 3, and 4.

the efficiency of polymerization onto a primer-template by simply varying the tCTP concentration. To overcome this problem, we resorted to competition experiments using assays containing [^{32}P]-primer-template and varying ratios of tCTP and CTP

Table 4: Polymerization of UTP Opposite a Variety of Templates

Template Base	k_{cat} (h^{-1})	K_M (μM)	k_{cat}/K_M ($h^{-1}\mu M^{-1}$)
	3.7 ± 0.02	0.20 ± 0.03	19
	0.6 ± 0.015	0.017 ± 0.005	35
	2.8 ±0.04	0.036 ± 0.003	78
	3.0 ± 0.08	0.060 ± 0.007	50
	2.2 ± 0.06	0.034 ± 0.006	65
	1.7 ± 0.08	0.13 ± 0.02	13
	24 ±1.2	0.053 ± 0.01	450
	2.3 ± 0.05	0.091 ± 0.007	25
	3.3 ± 0.1	0.12 ± 0.02	28

(*20*, *27*). At each polymerization cycle the enzyme has a choice of incorporating either tCTP or CTP, and the frequency with which primase will incorporate each NTP is dependent upon the k_{cat}/K_M for each NTP. Thus, by measuring the relative amounts of product due to incorporation of CTP and tCTP as a function of the tCTP/CTP ratio, one can directly obtain the ratio of k_{cat}/K_M (tCTP) versus k_{cat}/K_M (CTP) (*20*, *27*). Interestingly, these studies showed that primase preferred to polymerize tCTP by a factor of 2.5, indicating that the larger size and/or hydrophobicity of tC increases polymerization efficiency opposite G.

Primase Can Misincorporate NTPs via a Primer-Template Slippage Mechanism. A curious feature of the above studies was the polymerization of UTP opposite a surprising number of unnatural templating bases. Potentially, this could indicate a special relationship between primase and UTP or that primase can misincorporate NTPs via a primer-template slippage mechanism (Scheme 1). For all of these studies we used a common sequence where only the first templating base varied to eliminate the possibility that different rates of NTP (analogue) polymerization resulted from variations in the primer-template sequence. This, however, resulted in an A always being the second templating base. Thus, the frequent UTP polymerization might have resulted from primase allowing the first template base to loop out and then copying the second template base, A.

We tested the hypothesis that primase can misincorporate a NTP via a primer-template slippage mechanism using the set of templates in Table 6. If misincorporation resulted from primer-template slippage, then changing the identity of the second template base should change the identity of the misincorporated NTP. As a first test of this hypothesis, we compared misincorporation of ATP and CTP opposite A on DNA1 (3′-...**A**GTTT..., Table 1) and DNA2 (3′-...**A**TTTT...). Primase did not detectably misincorporate ATP opposite the templating A on either template but misincorporated CTP opposite A with identical efficiency on both templates, 1.6 h^{-1} μM^{-1} (NTP). Thus, misincorporation of CTP did not result from a primer-template slippage mechanism in this case. As a second probe of primer-template slippage, we examined misincorporation of UTP and CTP opposite 5-methylisocytosine in DNA3 (5′-...**isoC**ATT...) and DNA4 (5′-...**isoC**GTT...). Primase polymerized UTP on DNA3 with an efficiency of 13 h^{-1} μM^{-1} but did not detectably incorporate CTP. In contrast, the enzyme polymerized CTP on DNA4 with an efficiency of 70 h^{-1} μM^{-1} but did not detectably incorporate UTP. The ability of the nucleotide following the iso-C to direct which base the enzyme incorporated opposite the iso-C indicates that, in this case, misincorporation likely occurs via a primer-template slippage mechanism. Together, these data indicate that primase can incorporate NTPs via primer-template slippage, but the extent to which slippage occurs depends upon the templating base.

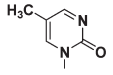
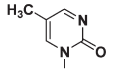
DISCUSSION

We examined the ability of human primase to polymerize NTPs containing both natural and modified bases opposite both natural and modified templating bases. Consistent with previous work (*13*), the enzyme strongly discriminated against generating base pairs where the base on either the incoming NTP or template could not form Watson−Crick hydrogen bonds. Thus, the formation of Watson−Crick hydrogen bonds appears to be a key driving force for NTP polymerization by human primase.

We tested the hypothesis that the requirement for Watson−Crick hydrogen bonds actually reflected an inability to generate base pairs that would require the presence of unsatisfied Watson−Crick hydrogen bond acceptors and donors in its active site. However, primase did not polymerize NTPs whose bases lack Watson−Crick hydrogen-bonding groups opposite template bases that also lacked Watson−Crick hydrogen-bonding groups. Thus, the simple lack of unsatisfied Watson−Crick hydrogen-bonding groups does not result in efficient NTP polymerization. Furthermore, in at least one case, UTP across from a templating purine, primase relatively efficiently generated this base pair even though O^4 of UTP will not be paired with a hydrogen bond donor on the template.

Why might primase require the formation of Watson−Crick hydrogen bonds? Potentially, binding of the base on the incoming NTP within the primase active site could be enhanced by hydrophobic stacking of the NTP base onto the end of the primer and/or by hydrogen bonding between the NTP and templating base. If primase contained a relatively hydrophobic active site where the terminal base of the primer and the base of the NTP bind, stacking interactions would provide little or no energetic advantage for binding the NTP. Rather, formation of Watson−Crick hydrogen bonds between the incoming NTP and templating base would now be needed to provide significant stabilization. Since hydrophobic NTPs cannot interact with the Watson−Crick hydrogen-bonding groups of the template base (and *vice versa*), they would not bind in a catalytically competent orientation.

Table 5: Misincorporation of Natural NTPs Opposite Natural and Modified Template Bases

NTP	Template	k_{cat} (h^{-1})	K_M (μM)	k_{cat} (h^{-1})/K_M (μM)
CTP	T	1.2 ± 0.02	0.038 ± 0.003	32
CTP	 dI	100 ± 4	0.078 ± 0.002	1600
CTP	 3-Deaza-dA	1.3 ± 0.05	0.24 ± 0.04	18
ATP	 5-Methylpyrimidin-2(1H)-one	0.3 ± 0.01	0.01 ± 0.009	30
GTP	 5-Methylpyrimidin-2(1H)-one	0.54 ± 0.01	0.044 ± 0.005	11

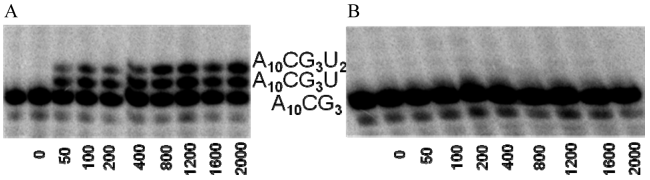


FIGURE 3: Efficient incorporation of UTP opposite purine but not purine NTP opposite T. Experiments contain 400 nM human primase, NTP, and 2 μM 5′-[^{32}P]-DNAX. Products are labeled. Panel A: In DNAX, X = purine. The first lane contained no enzyme. Other lanes contained primase and the noted concentration of UTP (μM). Panel B: In DNAX, X = T. Assays contained the noted concentration of purine NTP (0−2000 μM).

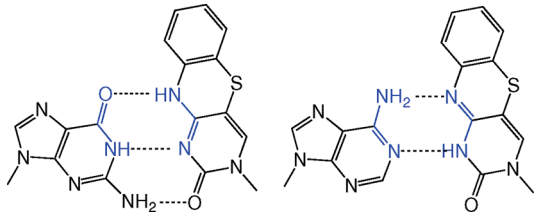


FIGURE 4: Tautomerization of tC.

Curiously, primase had substantially lower misincorporation rates on the primer-template than when initiating primer synthesis *de novo*. Since we cannot direct primer initiation to a specific nucleotide, previous studies to examine misincorporation have only looked at the early polymerization events when the primer is only around two to five nucleotides long (*6*, *13*). Under these conditions, the primer-template is relatively unstable due to its short length. Here, we examined a longer primer-template where the primer is stably bound. These observations raise the possibility that the reported high error frequency of primase results from primer-template instability, as opposed to an inherent inaccuracy. This latter model also suggests a biochemical rationale for the existence of primase. If a DNA polymerase initiated DNA synthesis and the instability of the primer-template resulted in greatly increased misincorporation rates, the accuracy of replication might be fatally compromised (*2*, *28*). First generating a stable RNA primer-template using primase would overcome this problem. Experiments to test the role of primer-template stability in primase fidelity are currently in progress.

A particularly surprising NTP in this regard is tCTP. Previous studies showed that high-fidelity DNA polymerases (pol α, herpes pol, BF, Klenow Fragment, Vent, and Taq) incorporated dtCTP and the closely related dtC^{O}TP as efficiently as dCTP opposite G and only around 10-fold less efficiently than TTP across from A (*24*, *26*, *29*). The extremely efficient polymerization opposite A correlates with the ease with which these bases tautomerize. In contrast, human primase very strongly discriminated against polymerizing tCTP across from A. These data provide further evidence of the distinct mechanisms by which primases and A family (Klenow Fragment, BF, and Taq) and B family (pol α, herpes pol, and Vent) choose whether or not to polymerize a (d)NTP. They also suggest that misincorporation by primase might not involve tautomerization of the base and would be consistent with a key role for primer-template stability.

While formation of Watson−Crick hydrogen bonds clearly enhances NTP polymerization, formation of Watson−Crick hydrogen bonds alone is not sufficient to drive efficient NTP

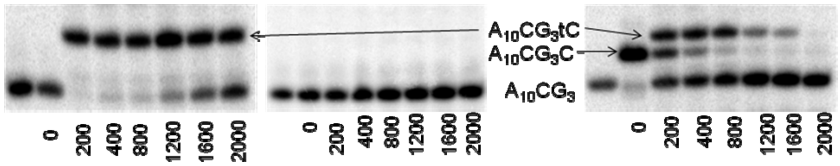
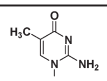
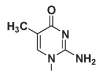
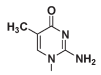
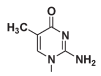
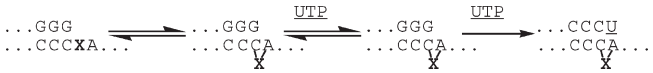


FIGURE 5: Incorporation of tCTP. Left panel: Assays contained 2 μM [^{32}P]DNAX, X = G. The first lane contained no enzyme. Other lanes contained 400 nM primase and the noted concentration of tCTP (0−2000 μM). Middle panel: Assays contained 2 μM [^{32}P]DNAX, X = A. The first lane contained no enzyme, while the latter lanes contained 400 nM primase and the noted concentration of tCTP (0−2000 μM). Right panel: Assays contained 2 μM [^{32}P]DNAX, X = G. The first lane contained no enzyme, while the other lanes contained primase, 800 μM CTP, and the noted concentration of tCTP (0−2000 μM).

Table 6: Misincorporation of the Noted NTPs Measured Using DNA1−4 (Table 1) in Assays Containing the Noted NTP

Template Base	Second Template Base	NTP	k_{cat} (h^{-1})	K_M (μM)	k_{cat}/K_M (h^{-1} μM^{-1})
A	T	ATP	-	-	0
A	T	CTP	0.25 ± 0.007	0.16 ± 0.02	1.6
A	G	ATP	-	-	0
A	G	CTP	0.5 ± 0.01	0.32 ± 0.03	1.6
	G	UTP	-	-	0
	A	UTP	3.5 ± 0.4	0.051 ± 0.02	70
	G	CTP	0.42 ± 0.07	0.032 ± 0.004	13
	A	CTP	-	-	0

Scheme 1: Misincorporation due to Primer-Template Slippage



incorporation. Primase did not efficiently polymerize ATP, GTP, or UTP opposite 6-hydroxypyridin-3-yl, 6-aminopyridin-3-yl, or 3-deazaadenine templating bases, respectively. Since all three of these template bases lack the minor groove hydrogen bond acceptor, these data suggest that primase interacts with O^2 of a template pyrimidine and N-3 of a template purine. While the structure of human primase is unknown, these data predict that it will contain a hydrogen bond donor on the minor groove side of the templating base, similar to what has been observed with some DNA polymerases (*30*, *31*). In contrast to its interactions with the templating base, human primase does not require the minor groove hydrogen bond acceptor on the incoming NTP as evidenced by the reasonably efficient polymerization of 3-deazaATP.

We previously showed that primase can misincorporate NTPs by misreading the template base, as evidenced by the incorporation of NTPs not coded for by any template base (*6*). However, primase in some cases can also misincorporate NTPs by a primer-template slippage mechanism, similar to how some DNA polymerases can also misincorporate dNTPs (*32*−*34*). When confronted with iso-C in the template, primase misincorporates a NTP by reading the template base following the iso-C, presumably by looping out the iso-C. Indeed, we suspect that the unusually high frequency of UTP incorporation opposite some of the unnatural bases largely or exclusively resulted from primer-template slippage since UTP was generally the only natural NTP misincorporated opposite the analogues. Curiously, many of the templates containing nucleotide analogues did not allow for primer-template slippage, as shown by the lack of UTP incorporation even though the templating base following the analogue was always A. Why some, but not other, templating bases allow for primer-template slippage remains unclear.

Human and herpes primase share both significant similarities and differences in how they choose to polymerize a NTP. Formation of Watson−Crick hydrogen bonds generally enhances NTP polymerization by both enzymes, and the presence of a hydrogen bond acceptor in the minor groove of the templating base greatly enhances incorporation (*20*). Both enzymes neither efficiently incorporated hydrophobic purine NTP analogues (e.g., 1-deazapurine NTP) nor polymerized NTP (analogues) opposite these hydrophobic bases analogues. Neither enzyme could generate base pairs with an altered arrangement of the Watson−Crick hydrogen-bonding groups (iso-C:iso-G).

Unlike human primase, however, herpes primase very efficiently incorporated two hydrophobic pyrimidine NTP analogues, 2-pyridone NTP and 4-methyl-2-pyridone NTP opposite both natural and modified templating bases (*20*). Remarkably, in some cases herpes primase incorporated these NTP analogues almost as efficiently as a natural NTP opposite its cognate template base even though these pyrimidine analogues cannot form any Watson−Crick hydrogen bonds. This last result raises the possibility of generating inhibitors that target herpes primase but not human primase.

The generally strong reliance on Watson−Crick hydrogen bonds distinguishes human and herpes primase from most other nucleotide polymerases. Both A and B family DNA polymerases will very efficiently polymerize a variety of dNTP analogues incapable of forming Watson−Crick hydrogen bonds, and various mechanisms have been posited to explain their ability to choose correct dNTPs (shape discrimination, positive and negative selectivity, etc. (*35*−*43*)). The mechanism by which the X-family polymerases discriminate between right and wrong dNTPs remains unclear. Similar to the situation with human and herpes primase, some of the low-fidelity, lesion bypass DNA polymerases have been suggested to require Watson−Crick hydrogen bond formation, albeit on the basis of a limited small set of analogues (*44*, *45*).

Sequence analysis of herpes and human primase indicates that the catalytic cores of these enzymes are distantly related (*16*). Human primase also bears significant sequence homology to the X family polymerases in the large, noncatalytic subunit, while no such homology exists in herpes primase (*23*). Consistent with the different strategies employed by these primases and the various polymerase families just described, these families appear evolutionarily distinct. It will be particularly interesting to ask how the chemistries employed by the different active sites vary to allow such different strategies of nucleotide discrimination.

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