Herpes Simplex Virus-1 Primase: A Polymerase with Extraordinarily Low Fidelity†

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ABSTRACT: We utilized templates of defined sequence to investigate the fidelity and mechanism of NTP misincorporation by DNA primase from herpes simplex virus-1. Herpes primase generated a wide range of mismatches during primer synthesis, including purine−purine, pyrimidine−pyrimidine, and purine−pyrimidine mismatches, and could even polymerize consecutive incorrect NTPs. Polymerization of noncognate NTPs resulted from primase misreading the template, as opposed to a primer slippage or dislocation mutagenesis mechanism. Primase did not efficiently misincorporate NTPs during the initiation reaction (i.e., dinucleotide synthesis). However, during primer elongation (after dinucleotide formation), herpes primase was extraordinarily inaccurate. It misincorporated NTPs at frequencies as high as 1 in 7, although frequencies of 1 in 25 to 1 in 60 were more common. In every case, however, misincorporation frequencies were no less than 1 in 100. For a specific mismatch, the DNA sequences flanking the site where misincorporation occurred could influence the frequency of misincorporation. This remarkably low level of fidelity is as low as that observed for the least accurate members of the Y class DNA polymerases involved in lesion bypass. Thus, herpes primase is one of the least accurate nucleotide polymerizing enzymes known.

Herpes simplex virus-1 (HSV-1)[1] primase is an essential component of the HSV-1 DNA replication apparatus and is required for the initiation of all new DNA strands (*1*, *2*). Herpes primase is part of a heterotrimeric primosome that contains three gene products, UL5, UL8, and UL52. This complex exhibits 3 activities: primase, ssDNA-dependent NTPase, and helicase activities (*2*−*7*). The helicase activity unwinds the DNA in front of the replication fork. On ssDNA, primase synthesizes short oligoribonucleotide primers up to ca. 10−13 nucleotides long (*3*, *8*, *9*). After transfer to the herpes polymerase complex, the polymerase elongates these primers via dNTP polymerization (*10*−*12*).

Herpes primase can initiate primer synthesis at any consecutive template pyrimidine residues (*8*). However, synthesis of primers longer than 4 nucleotides requires that initiation occur at a 3′-deoxyguanylate−pyrimidine−pyrimidine (3′-G−pyr−pyr) template sequence (*8*). Initiation occurs at the pyrimidine adjacent to the template deoxyguanylate, hence both the 5′-terminal nucleotide and second nucleotide of the primer will be purines. Formation of this initial pppNpN dinucleotide limits the overall rate of primer synthesis (*13*). Increasing the NTP concentration increases both the rate and the processivity of primase. However, even at very high concentrations of NTPs, elongation of the dinucleotide into longer products remains relatively inefficient (*13*). Thus, the dinucleotide remains the primary product at both high and low NTP concentrations.

A unique feature of both mammalian (calf and human) and bacterial (*E. coli*) primase is their relatively low fidelity. Calf primase can misincorporate NTPs at frequencies occasionally in excess of 1/30, although the enzyme typically exhibits somewhat higher fidelity (≥100-fold discrimination against noncognate NTPs) (*14*). Both calf and human primase can generate primers containing multiple mismatches, and remarkably, can even polymerize several consecutive incorrect NTPs (*14*, *15*). *E. coli* primase also misincorporates NTPs quite readily, although the actual error frequency remains unknown (*16*).

Primases from different herpes viruses show significant sequence homology (*17*). However, with the exception of two conserved motifs (*18*−*20*), herpes primase is not homologous to eukaryotic or bacterial primases (*18*, *19*) and appears to be unrelated to these primases. To determine if the herpes enzyme shares the property of low fidelity observed with other primases, we used templates of defined sequence. Herpes primase proved to be even more inaccurate than eukaryotic primase, previously thought to be the most error-prone primase known (*15*). Misincorporation frequencies varied from "as low as" 1 in 100 to as high as 1 in 7 for every misincorporation event examined. Importantly, misincorporation results from primase misreading the template, as opposed to slipping. The biological and mechanistic implications of these results are discussed.

EXPERIMENTAL PROCEDURES

Materials. HSV-1 helicase-primase (UL5/UL8/UL52) was expressed in baculovirus-infected SF9 cells and purified as previously described (*8*). SF9 cells were from the Tissue

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[1] Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; *E. coli*, *Escherichia coli*; G−pyr−pyr, deoxyguanylate−pyrimidine−pyrimidine; HSV-1, herpes simplex virus 1; ssDNA, single-stranded DNA.; SAP, shrimp alkaline phosphatase; 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**C**GCC**C5′
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**GTC**TG5′
d(TCTCTG)$_{10}$	3′TCTCT**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**TG5′
d(T$_{20}$GCCCCAAT$_{16}$)	3′TTTTTTTTTTTTTTTTTTTT**GCC**CCAATTTTTTTTTTTTTTTT5′
d(T$_{20}$GCCCCAT$_{17}$)	3′TTTTTTTTTTTTTTTTTTTT**GCC**CCATTTTTTTTTTTTTTTTT5′
d(C$_{20}$GCCCCTTA$_{16}$)	3′CCCCCCCCCCCCCCCCCCCC**GCC**CCTTAAAAAAAAAAAAAAAA5′
d(C$_{20}$GCCCCTA$_{17}$)	3′CCCCCCCCCCCCCCCCCCCC**GCC**CCTAAAAAAAAAAAAAAAAA5′
d(C$_{20}$GCCCTA$_{18}$)	3′CCCCCCCCCCCCCCCCCCCC**GCC**CTAAAAAAAAAAAAAAAAAA5′
d(C$_{20}$GCCCCGGA$_{16}$)	3′CCCCCCCCCCCCCCCCCCCC**GCC**CCGGAAAAAAAAAAAAAAAA5′
d(C$_{20}$GCCCCGA$_{17}$)	3′CCCCCCCCCCCCCCCCCCCC**GCC**CCGAAAAAAAAAAAAAAAAA5′
d(C$_{20}$GCCCTCA$_{17}$)	3′CCCCCCCCCCCCCCCCCCCC**GCC**CTCAAAAAAAAAAAAAAAAA5′
d(C$_{20}$GCCTCCA$_{17}$)	3′CCCCCCCCCCCCCCCCCCCC**GCC**TCCAAAAAAAAAAAAAAAAA5′

[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.

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. Human primase (p58/p49) was expressed and purified as previously described (*21*). Synthetic 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. All other reagents were of the highest purity available.

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, 5% glycerol, 20 μM ssDNA template, 0.8 mM [α -^{32}P]NTPs, and 100 nM HSV-1 helicase-primase. 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 denaturing polyacrylamide gel electrophoresis (20% polyacrylamide, 8 M 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.

Synthesis of Primer Standards by Human Primase. ppApG, pppApGpA, pppG(pG)$_n$, and pppA(pA)$_n$ primer standards were produced by human primase (p58/p49), as described previously (*8*). ApA, GpG, and ApG standards were produced by first making pppApA, pppGpG, and pppApG standards with human primase on the templates d(GTT)$_{20}$, d(CT)$_{30}$, and d(TCA)$_{20}$, respectively, followed by exposure to SAP (see below). Human primase reactions (10 μL) were incubated at 37 °C for 30 min and contained 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/mL BSA, 1 μM ssDNA template, 200 μM [α -^{32}P]NTPs ([α -^{32}P] ATP for pppApA, [α -^{32}P] GTP for pppGpG, and ATP and [α -^{32}P]GTP for pppApG), and 200 nM human primase.

Removal of the 5′-Terminal Phosphates from Primers. Primase reactions were performed as described above for both herpes and human primase, except reactions contained 13 μL. Rather than quench the reactions with gel-loading buffer, primase was heat inactivated at 65 °C for 10 min. After heat inactivation, the 5′-terminal phosphates of the primers were removed by incubating the reaction with 1 unit of SAP at 37 °C for 20 min (*8*). The SAP was heat inactivated at 65 °C for 10 min, and 2.5 volumes of gel-loading buffer (90% formamide) was added. The NpN products were separated by polyacrylamide gel electrophoresis (40% polyacrylamide, no urea) and visualized by phosphorimagery (Molecular Dynamics). ImageQuant Software (Molecular Dynamics) was utilized for quantitative analysis of primer products.

RESULTS

Herpes Primase Misincorporates NTPs in the Absence of the Cognate NTP. To initially determine whether herpes primase could misincorporate nucleotides, we compared primer synthesis with or without CTP on templates consisting of the following repeating sequences (written 3′ to 5′): GCCC, TCTG, and TCTCTG (Table 1). In all cases, omitting CTP drastically decreased the amount of long primers (10−13 nucleotides in length) and increased the amount of short primers (Figure 1). Despite this shift toward shorter primer products, primase produced longer-than-expected primers on all three templates when lacking CTP. For example, if primase did not readily misincorporate NTPs, primase should

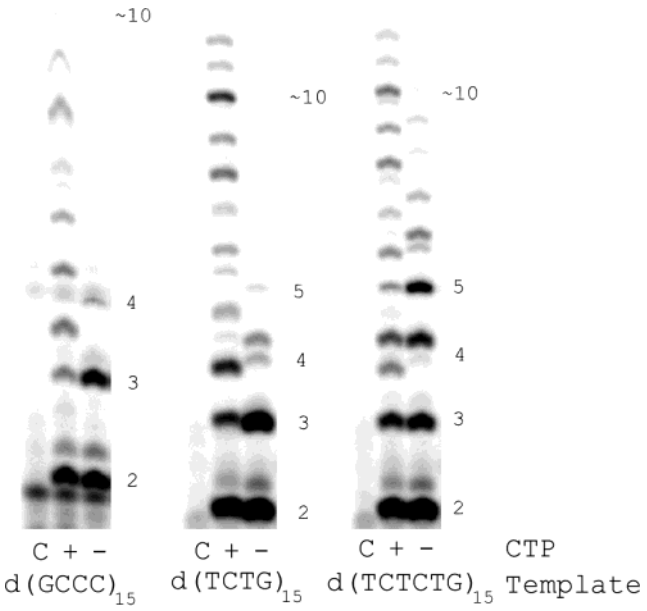


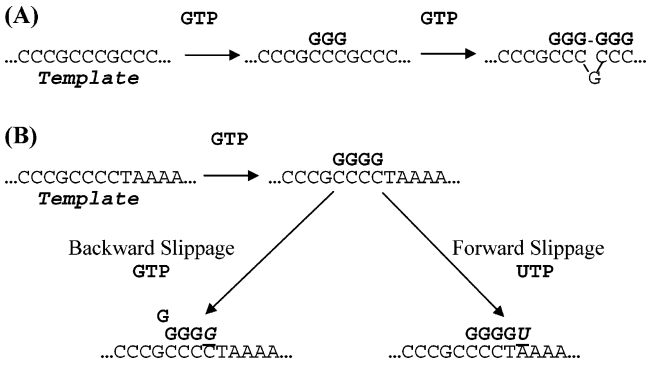
FIGURE 1: Herpes primase misincorporates NTPs. Primase reactions were performed as described under Experimental Procedures and contained 100 nM primase and 20 μM template ($d(GCCC)_{15}$, $d(TCTG)_{15}$, and $d(TCTCTG)_{10}$), except for the control reactions, which lacked enzyme (C). Reactions with $d(GCCC)_{15}$ contained 800 μM [α-^{32}P]GTP and, when indicated, 800 μM CTP. Reactions with $d(TCTG)_{15}$ and $d(TCTCTG)_{10}$ contained 800 μM [α-^{32}P]ATP, 800 μM GTP, and when indicated, 800 μM CTP. Primer lengths (in nucleotides) are noted to the right of the images.

have only synthesized primers up to 3 nucleotides long on $d(GCCC)_{15}$ when only GTP was present. However, we observed primers up to 7 nucleotides long (only primers of 4 nucleotides are clearly visible in Figure 1). Furthermore, these primers are of altered electrophoretic mobility when compared to those produced when CTP was present. Therefore, primase misincorporated GTP opposite the template deoxyguanylate and then continued primer synthesis. Similar results were observed on the templates $d(TCTG)_{15}$ and $d(TCTCTG)_{10}$ in the absence of a "required" NTP (Figure 1).

Nucleotide Misincorporation Occurs Via Template Base Misreading. Nucleotide misincorporation could occur via three possible mechanisms: dislocation mutagenesis, primer slippage, or template base misreading. As shown in Scheme 1A, dislocation mutagenesis involves displacing a template base and continuing strand extension by inserting the cognate NTP for the template base succeeding the displaced base (*22*). Primer slippage (Scheme 1B) slides one strand along the other and then incorporates the next cognate NTP based upon the newly misaligned primer−template (*23*). Finally, template base misreading directly produces base-pair mismatches by incorporating a noncognate NTP opposite a template base.

Two different approaches were used to discriminate among these possibilities. First, we examined the effects on misincorporation when primase faced either one or two consecutive template nucleotides for which it lacked the cognate NTP (Ex., templates $d(T_{20}GCCCCAT_{17})$ and $d(T_{20}GCCCCAAT_{16})$ in the absence of UTP). If misincorporation occurred via dislocation mutagenesis and/or the primer sliding forward over a template base, then the presence of consecutive template bases would be expected to inhibit misincorporation.

Scheme 1: Misincorporation by Dislocation Mutagenesis (A) and Primer Slippage (B)



However, Table 2 shows that for three different template pairs ($d(T_{20}GCCCCAT_{17})$ and $d(T_{20}GCCCCAAT_{16})$, $d(C_{20}GCCCCTA_{17})$ and $d(C_{20}GCCCCTTA_{16})$, $d(C_{20}GCCCCGA_{17})$ and $d(C_{20}GCCCCGGA_{16})$), the presence of consecutive bases for which primase lacked the cognate NTP minimally affected misincorporation of the NTP coded for by the following template base (ATP with $d(T_{20}GCCCCAT_{17})$, and UTP with $d(C_{20}GCCCCTA_{17})$ and $d(C_{20}GCCCCGA_{17})$). For example, on template $d(T_{20}GCCCCAT_{17})$ in the absence of UTP, the fraction of tetraguanylate primers elongated by ATP misincorporation (0.09) is essentially the same as the fraction elongated on template $d(T_{20}GCCCCAAT_{16})$ in the absence of UTP (0.072).

We next investigated whether herpes primase could misincorporate nucleotides by misreading the template sequence and directly forming mismatched base pairs. To test this mechanism, we measured misincorporation of NTPs not coded for by any template bases (Ex., UTP misincorporation on template $d(GCCC)_{15}$). If herpes primase primarily misincorporates NTPs by primer slippage and/or dislocation, NTPs not coded for by the template should not be misincorporated since these mechanisms utilize correct base pairing on misaligned primer−templates. However, if misincorporation occurs via misreading of a template base, an NTP not specified by the template should be readily incorporated. In the presence of the template $d(GCCC)_{15}$, we compared primer synthesis with GTP alone to primer synthesis with (1) GTP and UTP, (2) GTP and ATP, and (3) GTP and CTP. Since neither UTP nor ATP are coded for by the template sequence, any new primer bands observed when either one of these NTPs is added to the reaction can only mean that they were incorporated into the primer by misreading of the template. As shown in Figure 2, when comparing primer synthesis with GTP alone to primer synthesis with GTP and UTP or to primer synthesis with GTP and ATP, new products were observed. These new bands are of altered electrophoretic mobility when compared to primer synthesis with GTP and CTP and are visible when assays contained unlabeled GTP and either [α-^{32}P UTP] or [α-^{32}P ATP], respectively. Thus, they can only be a result of UTP or ATP misincorporation. In a similar experiment, we observed ATP misincorporation in reactions containing the template $d(C_{20}GCCCCGA_{17})$ (data not shown). Together, these data demonstrate that herpes primase misincorporates nucleotides by misreading the template sequence. While we cannot exclude the possibility that herpes primase *can* misincorporate nucleotides via dislocation mutagenesis and/

Table 2: Single and Consecutive Bases for Which Primase Lacks the Cognate NTP Have Similar Misincorporation Frequencies

template[a]	NTPs present[b]	fraction of pppGpGpGpG elongated with[c]: GTP	fraction of pppGpGpGpG elongated with[c]: other NTP present
$d(T_{20}GCCCCAT_{17})$	GTP & ATP	0.21 ± 0.01	0.09 ± 0.01
$d(T_{20}GCCCCAAT_{16})$	GTP & ATP	0.26 ± 0.01	0.072 ± 0.001
$d(C_{20}GCCCCTA_{17})$	GTP & UTP	0.082 ± 0.002	0.095 ± 0.004
$d(C_{20}GCCCCTTA_{16})$	GTP & UTP	0.094 ± 0.002	0.077 ± 0.007
$d(C_{20}GCCCCGA_{17})$	GTP & UTP	0.094 ± 0.001	0.24 ± 0.02
$d(C_{20}GCCCCGGA_{16})$	GTP & UTP	0.101 ± 0.003	0.23 ± 0.04

[a] The italic base in the template corresponds with the primer position at which NTP misincorporation was measured. [b] Each NTP present in the assay was at 800 μM. [c] Values shown are the average of 3 experiments.

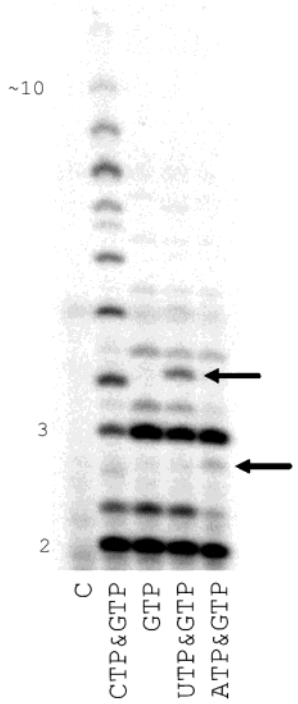


FIGURE 2: Nucleotide misincorporation occurs via template base misreading. The upper arrow pinpoints a primer band resulting from UTP misincorporation (verified by [α-^{32}P]UTP incorporation) and the lower arrow pinpoints a primer band resulting from ATP misincorporation (verified by [α-^{32}P]ATP incorporation). Assays were performed as described under Experimental Procedures and contained 100 nM primase, 20 μM $d(GCCC)_{15}$, and [α-^{32}P]GTP, except for the control reaction, which lacked enzyme (C). Each indicated NTP was also present at a concentration of 800 μM, except ATP, which was increased to 3.2 mM to provide easier viewing of ATP misincorporation. Primer lengths (in nucleotides) are noted to the left of the image.

or primer slippage, these do not appear to be primary mechanisms of misincorporation.

Primer Initiation Remains Rate-Limiting in the Absence of a Cognate NTP. In the presence of all required NTPs, primer initiation (i.e., dinucleotide synthesis) is the rate-limiting step (*13*). Potentially, the absence of a cognate NTP could alter the rate limiting step. For example, primase might pause before either dissociating from the growing primer or misincorporating a noncognate NTP. Alternatively, and as occurs with calf thymus primase, the absence of a cognate NTP could greatly enhance the rate of primer synthesis (*14*). Three lines of evidence indicate that misincorporation does not change the rate-limiting step from primer initiation. **(i)** The effects of varying the concentration of NTPs on primer synthesis was measured in assays that contained either all cognate NTPs or where one cognate NTP, not involved in primer initiation, was replaced with a noncognate NTP (Table 3). Similar effects were observed under both sets of conditions. For example, on template $d(GCCC)_{15}$, increasing the concentration of GTP (by 4-fold), the NTP involved in primer initiation, stimulated primer synthesis to a similar degree (3−4-fold) regardless of whether the reaction also contained the cognate CTP or noncognate ATP or UTP. Similarly, increasing the concentration of the NTP not involved in primer initiation for $d(GCCC)_{15}$ (CTP, ATP, or UTP, by 4-fold) decreased primer synthesis to a similar degree (by a factor of 0.5−0.75) regardless of whether that NTP was cognate or noncognate for a polymerization reaction. If a change in the rate-limiting step had occurred upon removing a cognate NTP from the reaction, we would not have expected such similar effects upon varying the NTP concentrations. **(ii)** Both in the presence of all required NTPs and absence of a cognate NTP, increasing the concentration of the NTP involved in initiation, GTP, increased primer synthesis (Table 3). In contrast, increasing the concentration of a NTP not involved in initiation (ATP, UTP, or CTP for templates $d(GCCC)_{15}$ and $dC_{20}GCCCCTA_{17}$) inhibited primer synthesis (Table 3). Thus, the rate-limiting step of the reaction involves GTP. If a step other than primer initiation had been rate limiting, then increasing the concentration of the NTP involved in this other step should have increased primer synthesis. **(iii)** The size distribution of products remains constant with time, both in the presence and absence of a required NTP. Figure 3 shows the time course of primer synthesis on $d(TCTG)_{15}$ in the presence of all required NTPs (GTP, ATP, and CTP) and when CTP has been replaced with UTP. While the distribution of products is quite different between the two reactions, in each case the size distribution of products (i.e., the fraction of total products that are dinucleotide, the fraction that are trinucleotide, etc.) remained constant with time. Quantitation of the data in Figure 3B showed that during the course of reaction, the product distribution remained at $69 \pm 2\%$ dinucleotide, $25 \pm 1\%$ trinucleotide, $4.6 \pm 0.7\%$ tetranucleotide (lower band), and $1.6 \pm 0.3\%$ tetranucleotide (upper band). If nucleotide misincorporation had become rate limiting in the absence of the cognate CTP, we should have observed accumulation of the 5′-AGA trinucleotide prior to formation of the 5′-AGAX misincorporation products. Since this did not occur (Figure 3B), misincorporation must be fast relative to initiation. We obtained similar results on the templates $d(GCCC)_{15}$ and $d(C_{20}GCCCCTA_{17})$ (data not shown).

Primase Misincorporates NTPs During Initiation. Each of the misincorporation examples described thus far took place during primer extension. To examine whether herpes

Table 3: Dinucleotide Synthesis Remains Rate Limiting When Primase Misincorporates an NTP

		ratio of total primer synthesis[a]		
		(4-fold increase in indicated [NTP])/([NTP] equiv)		
template	NTPs present	GTP	2nd NTP listed	3rd NTP listed
$d(GCCC)_{15}$	GTP, CTP	3.3 ± 0.2	0.62 ± 0.04	N/A
$d(GCCC)_{15}$	GTP, UTP	3.8 ± 0.3	0.75 ± 0.07	N/A
$d(GCCC)_{15}$	GTP, ATP	3.0 ± 0.3	0.56 ± 0.03	N/A
$d(C_{20}GCCCCTA_{17})$	GTP, ATP, UTP	2.6 ± 0.2	0.61 ± 0.06	0.57 ± 0.03
$d(C_{20}GCCCCTA_{17})$	GTP, CTP, UTP	3.6 ± 0.1	0.61 ± 0.05	0.63 ± 0.03

[a] 4-fold increase in indicated [NTP] means that the designated NTP concentration was increased to 3.2 mM, while the other NTP concentrations remained at 800 μM. [NTP] equiv means all NTP concentrations were at 800 μM. The values shown are the ratio of the rates of primer synthesis under these two sets of conditions and thus are unitless. 2nd and 3rd NTP listed refers to the NTPs noted under NTPs present.

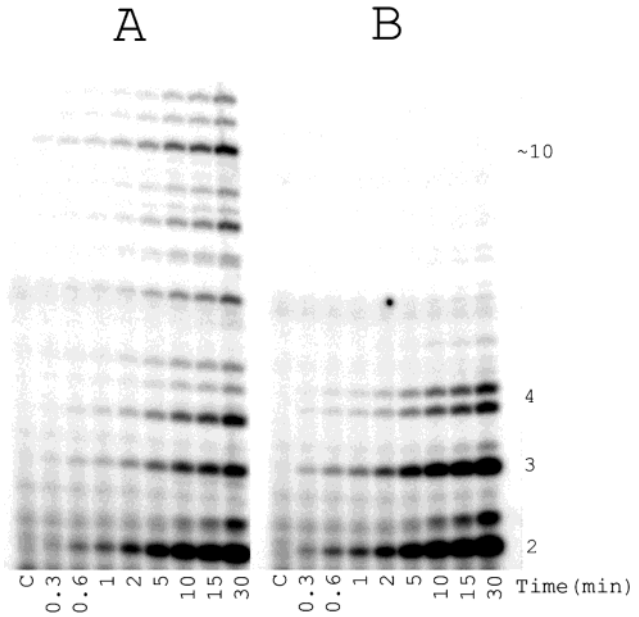


FIGURE 3: The product distribution does not change with time. The time course of primer synthesis on $d(TCTG)_{15}$ was measured in the presence of either (A) [α-^{32}P]GTP, ATP, and CTP or (B) [α-^{32}P]GTP, ATP, and UTP. Assays contained 100 nM primase, 20 μM $d(TCTG)_{15}$, and 800 μM of the indicated NTPs, except the control reaction (C), which lacked enzyme. Primer lengths are indicated to the right of the images.

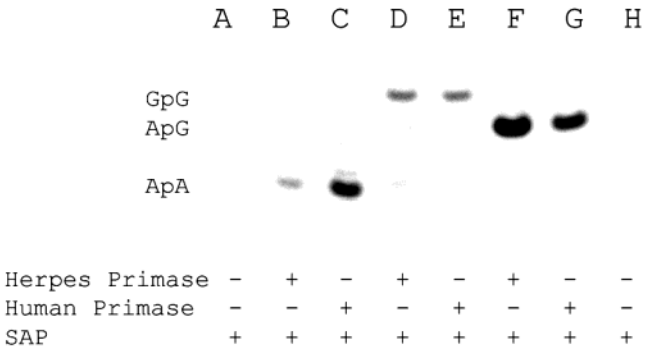


FIGURE 4: Nucleotide misincorporation during primer initiation. NpN dinucleotide primers were produced as described under Experimental Procedures on $d(TCTG)_{15}$ by herpes primase followed by SAP exposure in the presence of (B) [α-^{32}P]ATP only, (D) [α-^{32}P]GTP only, and (F) ATP and [α-^{32}P]GTP. To visualize misincorporation products, the specific activity of (B) and (D) was increased 20-fold in comparison to (F). (C) ApA, (E) GpG, and (G) ApG standards were produced using human primase followed by SAP treatment. Control reactions, lacking primase, were conducted in the presence of (A) [α-^{32}P]ATP and (H) [α-^{32}P]GTP with the same specific activity as (B) and (D), respectively.

primase could misincorporate nucleotides during initiation (i.e., pppNpN formation), we used a template that codes for both ATP and GTP during initiation, $d(TCTG)_{15}$. When we provided the enzyme with only GTP, we observed a product that comigrated with a pppGpG standard, and when we provided the enzyme with only ATP, we observed a product that comigrated with a pppApA standard (data not shown). To ensure that these products were dinucleotides, we treated them with SAP to remove any phosphates on the 5′-terminus. This results in primers with a lower electrophoretic mobility. As shown in Figure 4, the dinucleotide primers comigrated with their respective NpN standards. Control experiments showed that no dinucleotide was generated in the absence of template (data not shown), indicating that this misincorporation event was template directed.

For misincorporation to occur during dinucleotide synthesis, at least one of the NTPs must be complementary to the template. In assays containing the template $d(C_{20}GCCA_{20})$, which requires only GTP for initiation, we were unable to detect any dinucleotide in the presence of only ATP (data not shown). Similarly, no dinucleotide was generated on the template $d(GTT)_{20}$ in the presence of only GTP (data not shown).

While herpes primase can misincorporate NTPs during initiation, the efficiency of misincorporation is rather low. Quantitative analysis of the data in Figure 4 showed that the rate of initiation on $d(TCTG)_{15}$ when both ATP and GTP are present is 220-fold and 270-fold greater than the rate when only GTP or ATP, respectively, are present (all concentrations at 800 μM). Considering that the frequency of ATP or GTP misincorporation during primer initiation is likely to significantly decrease in the presence of both ATP and GTP, these results suggest that misincorporation during primer initiation is a rare event.

Primase Has Astoundingly Low Fidelity During Elongation. We measured the frequency of misincorporation at specific template positions under conditions of equimolar cognate and noncognate NTP. Under these conditions, the frequency of misincorporation is a direct measure of the $\{V_{MAX}/K_M\}_{correct}/\{V_{MAX}/K_M\}_{incorrect}$. This method is feasible because the electrophoretic mobility of the products varies depending upon whether the correct or incorrect NTP was incorporated. Several controls were performed to ensure that we correctly identified the misincorporation product. **(i)** For those reactions where incorporation of either the cognate or noncognate NTP resulted in a homopolymer (e.g., pppG-$(pG)_n$), standards of the identical sequence were independently synthesized and shown to comigrate with the primer

Table 4: Frequency of Misincorporation during Primer Elongation[a]

mismatch	template	misincorporation frequency
T−G	d(C_{20}GCCCC*T*TA_{16})	0.11
dC−A	d(C_{20}GCCCC*C*TTA_{16})	0.027
dG−G	d(*G*CCC)$_{15}$	0.016
dG−U	d(*G*CCC)$_{15}$	0.039
dC−C	d(GCCC)$_{15}$	0.040
dG−G	d(TCT*G*)$_{15}$	0.039
dG−A	d(TCT*G*)$_{15}$	0.010
dG−U	d(TCT*G*)$_{15}$	0.14
T−C	d(TC*T*G)$_{15}$	0.014
T−U	d(TC*T*G)$_{15}$	0.017
dA−G	d(T_{20}GCCCC*A*AT$_{16}$)	0.037
dC−U	d(T_{20}GCCCC*A*AT$_{16}$)	0.060
dC−U	d(T_{20}GCCC*C*AAT$_{16}$)	0.019

[a] The cognate NTP for the italic template base and the noncognate NTP of interest were present at 800 μM. Reactions also contained other NTPs at 800 μM as needed for primer synthesis. The italic base corresponds with the primer position at which the mismatch frequency was measured. Mismatches are written as template base−NTP base.

product. **(ii)** Whenever possible, we ensured that a misincorporation product (and those products elongated from that misincorporation) contained the incorrect NTP by performing the reactions in the presence of the incorrect [α-^{32}P]NTP. In addition, whenever possible, we also ensured that the putative misincorporation product did not contain the cognate NTP by repeating the reactions using cognate [α-^{32}P]NTP. **(iii)** In cases where the misincorporated nucleotide was also a cognate NTP earlier in the primer (e.g., misincorporation of either GTP or ATP opposite the template dG on d(TCT*G*)$_{15}$), we distinguished between products by comparing the signal with different [α-^{32}P]NTP radiolabels. For example, primase generates two misincorporation products on d(TCTG)$_{15}$ in assays containing only ATP and GTP − 5′-AGAA and 5′-AGAG. Changing the radiolabel from [α-^{32}P]ATP to [α-^{32}P]-GTP changed the amount of signal from each product in the predicted manner. **(iv)** Finally, we identified misincorporation products by examining the effect of slowly increasing the ratio of incorrect to correct NTP. If the product resulted from misincorporation, this increased the amount of the product due to misincorporation at the expense of the product generated via incorporation of the correct NTP. Table 4 summarizes the frequency of a variety of misincorporation events on multiple templates. Amazingly, primase misincorporated NTPs at a frequency as high as 1 in 7. While misincorporation frequencies of 1 in 25 to 1 in 60 were more common, in no case did primase discriminate against a noncognate NTP by more than a factor of 100. The ability of primase to discriminate against formation of a specific mismatch (e.g., dG−U) varied among different templates. Thus, the template sequences surrounding the site of misincorporation play a role in determining the frequency of a particular mismatch.

Primase Elongates Mismatches Less Efficiently than Correct Base-Pairs. To directly measure the ability of primase to elongate a mismatch, we calculated the fraction of primers extended after misincorporation and compared it to the fraction of primers extended after a correct incorporation at the same position. To easily identify misincorporation events and the products that resulted from further NTP incorporation, we only used templates where misincorporation and continued NTP polymerization after misincorporation involved the polymerization of just one NTP. For example, in reactions containing d(GCCC)$_{15}$ and only GTP, primase must misincorporate GTP to generate the tetranucleotide pppGpGpGpG. Elongation from the mismatch is determined by the amount of products greater than 4 nucleotides long. As shown in Table 5, the fraction of primers elongated immediately after misincorporation is 3−8-fold lower than the fraction of primers elongated after cognate nucleotide incorporation at that position. Thus, misincorporation significantly inhibits further primer elongation.

Table 5: Effect of Nucleotide Misincorporation on Primer Elongation

	fraction of primers extended with GTP after:	
template[a]	misincorporation[b]	cognate nucleotide incorporation[c]
d(*G*CCC)$_{15}$	0.19 ± 0.03	0.55 ± 0.07
d(C_{20}GCCC*T*CA$_{17}$)	0.11 ± 0.02	0.89 ± 0.02
d(C_{20}GCC*T*CCA$_{17}$)	0.19 ± 0.10	0.75 ± 0.02

[a] The italic base in the template is where misincorporation or cognate nucleotide incorporation occurred. [b] To measure elongation after misincorporation, assays only contained 800 μM GTP. Each value is the average of three experiments. [c] To measure elongation after correct incorporation, assays contained 800 μM GTP and either 800 μM CTP (d(GCCC)$_{15}$) or 800 μM ATP (d(C_{20}GCCCTCA$_{17}$) and d(C_{20}GCCTCCCA$_{17}$)). Each value is the average of three experiments.

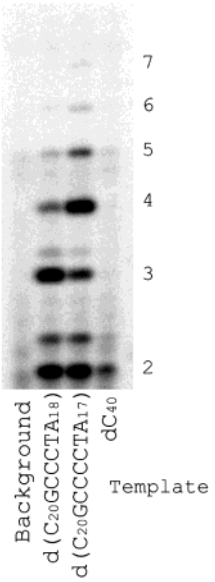


FIGURE 5: Herpes primase can misincorporate consecutive nucleotides. Assays contained 100 nM primase, 20 μM of the indicated template, and 800 μM [α-^{32}P]GTP, except the control reaction (Background), which lacked enzyme. The electrophoretic mobility of primer standards [pppG(pG)$_n$] is noted to the right of the image.

Primase Can Misincorporate Consecutive Noncognate NTPs. In addition to being able to elongate mismatches via polymerization of the next cognate NTP, primase can also elongate mismatches by polymerization of another incorrect NTP. For example, in assays containing either d(C_{20}GCCCTA$_{18}$) or d(C_{20}GCCCCTA$_{17}$) and only GTP, primase synthesized products greater than 4 and 5 nucleotides long, respectively (Figure 5). Since assays contained only GTP, primase must have misincorporated consecutive GTPs. While we previously showed that herpes primase initiates primer synthesis following the template deoxyguanylate (*8*), we none-the-less tested the possibility that the longer-than-expected primers on these templates resulted from primer

synthesis on the string of deoxycytidylates prior to the template deoxyguanylate. As shown in Figure 5, primer synthesis on dC_{40} only resulted in a small amount of dinucleotide primers. We also observed misincorporation of consecutive NTPs on the templates $d(T_{20}GCCCCAT_{17})$, $d(T_{20}GCCCCAAT_{16})$, and $d(C_{20}GCCCCGA_{17})$. In the presence of only GTP, primers greater than 5 nucleotides were produced (data not shown).

DISCUSSION

Herpes DNA primase exhibits an astoundingly low level of discrimination against incorrect NTPs during primer elongation. In every case examined, herpes primase discriminated against incorrect NTPs by less than or equal to 100-fold, and in some cases by less than or equal to 10-fold, and therefore, is one of the lowest fidelity nucleotide polymerases known. Misincorporation was found to readily occur via misreading of the template, as opposed to a template slippage and/or dislocation mutagenesis mechanism. Both the identity of the mismatch and the sequences flanking the template base could alter the rates of misincorporation. After misincorporating a NTP, primase polymerized the next correct NTP with less efficiency than after polymerizing a correct NTP.

Herpes primase did not efficiently misincorporate NTPs during dinucleotide synthesis, in contrast to the facile misincorporation during latter polymerization events. Eukaryotic primase (calf thymus) has also been found to act similarly (*14*). Potentially, this may reflect the differential stabilities of the "primer" being elongated during dinucleotide synthesis versus latter polymerization events. After initiation, the primer−template duplex will be stabilized via at least 2 base-pairs and any direct interactions with primase. During initiation, however, the only stabilization will be a single base-pair and the interactions of each NTP with the enzyme.

Even though herpes primase appears unrelated to other primases based on sequence analysis (*18*, *19*), all primases examined to date share the property of extremely low fidelity (*14*−*16*, *24*). Calf thymus primase discriminates quite poorly against noncognate NTPs (*14*). In one case, a T−G mismatch, the calf enzyme discriminated against GTP misincorporation by <30-fold. More typically, however, calf primase discriminates against noncognate NTPs by greater than or equal to 100-fold. *E. coli* primase also appears to readily misincorporate NTPs, although the actual extent by which *E. coli* primase discriminates between correct and incorrect NTPs has not been quantified (*16*). In contrast, other RNA polymerases and DNA polymerases generally discriminate against incorrect (d)NTPs orders of magnitude more effectively than herpes primase (*25*−*30*). One exception is the Y class DNA polymerases. Some members of this class of DNA polymerases have misincorporation frequencies between $\sim 10^{-1}$ and 10^{-3} (*31*), although it is not completely understood why the Y-class polymerases are so inaccurate. In the case of primase, nature presumably tolerates this remarkably low fidelity because of the transient nature of the primase-synthesized primer and the fact that errors in the primer do not directly alter genomic integrity.

The extraordinarily low fidelity of herpes primase, however, raises the question of the effects of mismatches on the polymerase. Misincorporation of an incorrect nucleotide will result in a mismatched primer 3′-terminus with, presumably, significantly altered geometry as compared to a correctly base-paired 3′-terminus. While herpes primase, as well as calf thymus primase (*14*), is able to elongate from a misincorporation with only modest reductions in polymerization activity (3−8- fold after T−G or dG−G mismatches for herpes primase), most DNA polymerases, including that from herpes, do not readily elongate primers containing mismatches at or near the 3′-terminus (*25*). Parris and coworkers showed that the poor elongation of an exogenously produced primer containing a mismatch at the 3′-terminus by herpes polymerase results from a greatly increased K_M for dNTPs (*32*). Since a substantial fraction of herpes primase-synthesized primers will likely contain a mismatch at or near the 3′-terminus, the rate of herpes DNA synthesis could be significantly impacted by the low-fidelity of primase.

Potentially, herpes polymerase could overcome this issue by interacting with primase-generated primer−templates very differently than an exogenously added primer−template such that mismatches at or near the 3′-terminus have little effect on elongation. Indeed, this is what occurs with eukaryotic pol α-primase (*14*). Whereas pol α does not efficiently elongate exogenously added primer−templates that contain a mismatch at or near the 3′-terminus, it efficiently elongates primase-generated primer−templates that contain multiple mismatches. Alternatively, the 3′-5′ exonuclease of herpes polymerase may solve the problem of mismatches at the 3′-terminus of the primer by simply excising them.

The remarkably high rate of template misreading raises the question of what is it about how primase chooses NTPs that results in such low fidelity? Multiple theories exist as to how nucleotide polymerases discriminate between correct and incorrect (d)NTPs. Initially, differences in Watson−Crick hydrogen bonding between correct and incorrect base-pairs were posited to allow a polymerase to distinguish right from wrong. The difference in shape between correct and incorrect base-pairs has also been proposed to be a key feature that polymerases use for fidelity (*33*, *34*). Alternatively, it has been suggested that polymerases can obtain fidelity by strongly discriminating against incorrect base-pairs, as opposed to selecting for the correct base-pair (*35*).

Among nucleotide polymerases, the Y class enzymes are the only ones whose misincorporation rates even come close to those of herpes and other primases. The Y class polymerases are unique in that they are thought to replicate past lesions in the template strand and may not be designed for replicating "normal" DNA (*31*). As such, the active site constraints needed for replication past lesions may compromise constraints needed for accurate replication of normal bases. Primases, however, are not known to be involved in lesion bypass and are thought to copy normal DNA. Indeed, a fairly common lesion found in DNA, an abasic site, very strongly blocks the activity of human primase (*36*).

While our data does not allow us to discern how primase obtains fidelity, the fact that herpes primase only discriminates against incorrect NTPs by ca. 7−100- fold raises the possibility that it will use a very simple (and not very effective!) mechanism to differentiate between right and wrong. Interestingly, this difference is similar to the amount of discrimination predicted from a fidelity mechanism based on the thermodynamic stabilities of correct and incorrect

base-pairs (*37*). In light of the tremendous differences between the fidelity exhibited by herpes primase and other, higher fidelity polymerases, it will be interesting to determine how the herpes enzyme obtains fidelity.

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