Mechanism of Primer Synthesis by the Herpes Simplex Virus 1 Helicase−Primase†

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ABSTRACT: We utilized templates of defined sequence to investigate the mechanism of primer synthesis by herpes simplex virus 1 helicase−primase. Under steady-state conditions, the rate of primer synthesis and the size distribution of products remained constant with time, suggesting that the rate-limiting step(s) of primer synthesis occur(s) during primer initiation (at or before the formation of the pppNpN dinucleotide). Consistent with this idea, increasing the concentration of NTPs required for dinucleotide synthesis increased the rate of primer synthesis, whereas increasing the concentration of NTPs not involved in dinucleotide synthesis inhibited primer synthesis. Due to these effects on primer initiation, varying the NTP concentration could affect start site selection on templates containing multiple G-pyr-pyr initiation sites. Increasing the NTP concentration also increased the processivity of primase. However, even at very high concentrations of NTPs, elongation of the dinucleotide into longer products remained relatively inefficient. Primase did not readily elongate preexisting primers under conditions where free template was present in large excess of enzyme. However, if template concentrations were lowered such that primase synthesized primers on all or most of the template present in the reaction, then primase would elongate previously synthesized primers.

Herpes simplex virus 1 (HSV-1)[1] primase is an essential component of the HSV-1 DNA replication apparatus (*1*, *2*). All known DNA polymerases are unable to initiate synthesis of a new DNA strand de novo, and almost all require the activity of a DNA primase to prime the template (*3*, *4*). Herpes primase produces short oligoribonucleotide primers up to ca. 10−13 nucleotides long on ssDNA (*5*−*7*). These primers then allow herpes DNA polymerase to begin replicating DNA via dNTP polymerization (*8*−*10*).

Herpes primase is part of a heterotrimeric primosome which contains three gene products, UL5, UL8, and UL52, and exhibits primase, ssDNA-dependent NTPase, and helicase activities (*2*, *7*, *11*−*14*). The UL5 subunit has multiple conserved helicase motifs (*15*, *16*), and the UL52 subunit has two conserved primase motifs (*17*−*19*). However, the individual subunits lack either activity, and only complexes containing both UL5 and UL52 retain the activities of the heterotrimeric complex (*20*−*22*). The UL8 subunit is essential for HSV-1 DNA replication (*14*, *23*), although it exhibits neither catalytic activity nor DNA binding capacity (*22*, *24*). Possible roles of UL8 include (1) stimulating primase and helicase activities of the UL5/UL52 holoenzyme (*25*, *26*), (2) overcoming the inhibition of NTPase and primase activities caused by coating the ssDNA with the HSV-1 ssDNA binding protein, ICP8 (*26*, *27*), (3) recruiting HSV-1 helicase−primase to origins of replication via interaction with the origin binding protein, UL9 (*28*), and (4) recruiting the HSV-1 DNA polymerase (UL30/UL42 complex) to the HSV-1 helicase−primase via interactions with UL30 (*20*, *29*).

Unlike eukaryotic primases, which initiate primer synthesis at preferred but nonspecific template sequences, prokaryotic and viral primases usually have specific template sequence requirements for primer initiation (*30*). We previously demonstrated that herpes primase requires a 3′-deoxyguanylate−pyrimidine−pyrimidine (3′-G-pyr-pyr) template sequence for significant production of longer primer products (beyond four nucleotides in length) and that initiation occurs at the second nucleotide of the trinucleotide template sequence (*5*). Therefore, like all primases (*30*), herpes primase preferably initiates primer synthesis with a purine. The deoxyguanylate of the template 3′-G-pyr-pyr sequence is not a coding entity of the primer and therefore is a cryptic nucleotide (*5*). Single cryptic nucleotides are a common feature of required template sequences for prokaryotic and phage primases (*30*). However, dissimilar from other systems, the cryptic deoxyguanylate of the 3′-G-pyr-pyr start site is not absolutely essential for herpes primer initiation but instead serves to dramatically increase primer length (*5*). In the absence of the cryptic deoxyguanylate, herpes primase produces almost exclusively di- and trinucleotides. While the presence of the cryptic deoxyguanylate results in the production of products ca. 10 nucleotides long, the primary products typically still remain the di- and trinucleotides.

We have employed templates of define sequence to better define the mechanism of primer start site selection, initiation, and extension by herpes primase. Primer initiation (at or before synthesis of a pppNpN dinucleotide) limits the overall rate of primer synthesis, and increasing the concentration of either NTP involved in dinucleotide synthesis increases the

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[1] Abbreviations: HSV-1, herpes simplex virus 1; G-pyr-pyr, deoxyguanylate−pyrimidine−pyrimidine; ssDNA, single-stranded DNA.

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Table 1: Synthetic ssDNA Templates[a] Used

d(T_{20}GTCCT_{19})	3′TTTTTTTTTTTTTTTTTTTTGTCCTTTTTTTTTTTTTTTTTTT5′
d(T_{20}GTCCT_{36})	3′TTTTTTTTTTTTTTTTTTTTGTCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT5′
d(C_{20}GTCCA_{19})	3′CCCCCCCCCCCCCCCCCCCCGTCCAAAAAAAAAAAAAAAAAAA5′
d(T_{20}GTCT_{20})	3′TTTTTTTTTTTTTTTTTTTTGTCTTTTTTTTTTTTTTTTTTTT5′
d(C_{20}GCCA_{20})	3′CCCCCCCCCCCCCCCCCCCCGCCAAAAAAAAAAAAAAAAAAAA5′
d(C_{20}GTCA_{20})	3′CCCCCCCCCCCCCCCCCCCCGTCAAAAAAAAAAAAAAAAAAAA5′
d(T_{20}GTCG_{20})	3′TTTTTTTTTTTTTTTTTTTTGTCGGGGGGGGGGGGGGGGGGGG5′
d(C_{20}GCTA_{20})	3′CCCCCCCCCCCCCCCCCCCCGCTAAAAAAAAAAAAAAAAAAAA5′
d(C_{20}GCCTA_{19})	3′CCCCCCCCCCCCCCCCCCCCGCCTAAAAAAAAAAAAAAAAAAA5′
d(C_{20}GCCCTA_{18})	3′CCCCCCCCCCCCCCCCCCCCGCCCTAAAAAAAAAAAAAAAAAA5′
d(C_{20}GCCCCTA_{17})	3′CCCCCCCCCCCCCCCCCCCCGCCCCTAAAAAAAAAAAAAAAAA5′
d(C_{20}GCCTCCA_{17})	3′CCCCCCCCCCCCCCCCCCCCGCCTCCAAAAAAAAAAAAAAAAA5′
d(C_{20}GCTA_{12}GTCA_{20})	3′CCCCCCCCCCCCCCCCCCCCGCTAAAAAAAAAAAAGTCAAAAAAAAAAAAAAAAAAAA5′
d(C_{20}GCCA_{12}GTTA_{20})	3′CCCCCCCCCCCCCCCCCCCCGCCAAAAAAAAAAAAGTTAAAAAAAAAAAAAAAAAAAA5′
d(C_{20}GTTA_{12}GCCA_{20})	3′CCCCCCCCCCCCCCCCCCCCGTTAAAAAAAAAAAAGCCAAAAAAAAAAAAAAAAAAAA5′

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

rate of primer synthesis. In contrast, increasing the concentration of NTPs involved in latter polymerization events increased the processivity of these polymerization events, but did not increase the rate of primer synthesis. The implications of these results with respect to the mechanism of primer synthesis by HSV-1 helicase−primase are discussed.

EXPERIMENTAL PROCEDURES

Materials. HSV-1 helicase−primase (UL8/UL5/UL52) was expressed in baculovirus-infected SF9 cells and purified as previously described (*5*). SF9 cells were from the Tissue Culture Core Facility at the University of Colorado Cancer Center. Baculoviruses expressing UL5 and UL52 subunits were generously provided by Dr. Robert Lehman (Stanford University), and a baculovirus expression vector for a His_9-tagged UL8 subunit was generously provided by Dr. Heidi Giordano (Tularik). 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 (*5*). Synthetic templates of defined sequence (Table 1) were obtained from Biosearch Technologies, Inc., and Oligos Etc. 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(hydroxymethyl)aminomethane (HCl salt), pH 8.0, 10−16 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 5% glycerol, 20 μM ssDNA template, 0.8 mM [α- or γ-^{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. Unless noted otherwise, the quantity of primers at each and all primer lengths were calculated and summed together as a measurement of total primer synthesis.

RESULTS

To determine if the concentration of NTPs affects start site selection by herpes primase, we selectively varied individual NTP concentrations in the presence of templates containing two 3′-G-pyr-pyr initiation sites. d(C_{20}GCTA_{12}-GTCA_{20}) contains two potential start sites (Table 1). While each start site codes for a single adenylate and a single guanylate during dinucleotide synthesis, the two sites code for primers with different nucleotides at the 5′-terminus (guanylate with the GCT site and adenylate with the GTC site). Since only the 5′-terminal nucleotide of the primer

Table 2: Steady-State Kinetic Parameters of NTPs for Primer Synthesis on Various Templates[a]

template	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
d(C_{20}GTCCA$_{19}$)	1.5 ± 0.5	0.06 ± 0.01	0.040 ± 0.015
d(T_{20}GTCT$_{20}$)	1.6 ± 0.5	0.32 ± 0.05	0.20 ± 0.07
d(C_{20}GCCA$_{20}$)	1.5 ± 0.4	0.048 ± 0.007	0.032 ± 0.010
d(C_{20}GCCCCTA$_{17}$)	1.2 ± 0.5	0.042 ± 0.007	0.035 ± 0.016

[a] Primase reactions contained all four NTP concentrations which were concurrently and incrementally ranged from 300 μM to 3 mM while the enzyme concentration was held constant at 100 nM and the template concentration at 20 μM. This concentration of DNA is >100-fold higher than the K_M for DNA (*5*); hence the measured kinetic parameters do not reflect DNA binding.

retains its γ-phosphate, the frequency with which primase uses each start site can be determined by performing assays containing either [γ-^{32}P]GTP to measure initiation at the GCT site or [γ-^{32}P]ATP to measure initiation at the GTC site. While increasing either ATP or GTP (from 0.8 to 3.2 mM) increased the frequency with which primase initiated primer synthesis at each site (by ca. 75%), the ratio of initiation events at the GCT start site verses the GTC start site remained constant. Thus, neither the first nor the second NTP of the primer dominates start site selection. Furthermore, while increasing the concentration of either UTP or CTP (from 0.8 to 3.2 mM) decreased the rate of primer synthesis (by ca. 40%), the ratio of initiation events between the two sites again remained unaffected. Thus, NTPs not involved in dinucleotide synthesis do not affect start site selection.

To determine whether the first and second nucleotides together could influence start site selection, we tested the effects of ATP and GTP concentrations on primer synthesis in reactions containing either d(C_{20}GTTA$_{12}$GCCA$_{20}$) or d(C_{20}GCCA$_{12}$GTTA$_{20}$). These templates also have two start sites; however, the two start sites, GTT and GCC, require different NTPs for dinucleotide synthesis. For each template, increasing the ATP concentration by 4-fold (from 0.8 to 3.2 mM) greatly enhanced primer synthesis at the GTT site (11- and 5.6-fold, respectively), whereas increasing the GTP concentration by 4-fold (from 0.8 to 3.2 mM) enhanced primer synthesis at the GCC site (4.4- and 3.0-fold, respectively). Concomitant with the increase at the primer synthesis site complementary to the increased NTP, primer synthesis at the other site decreased (by 28−39%). Thus, individually varying the ATP or GTP concentration alters the ratio of primer synthesis between the two sites by decreasing primer synthesis at one site while stimulating synthesis at the other. However, simultaneously increasing the concentration of ATP and GTP (by 4-fold each) had little effect on start site selection (the ratio remained unchanged), since primer synthesis was enhanced similarly at both sites under these conditions (data not shown).

Influence of NTP Concentration on the Rate of Primer Synthesis. To further investigate the effects of NTP concentration on the rate of primer synthesis, we measured changes in the rate of total primer synthesis on templates containing a single 3′-G-pyr-pyr start site while varying NTP concentrations. The four templates tested, d(C_{20}GTCCA$_{19}$), d(T_{20}GTCT$_{20}$), d(C_{20}GCCA$_{20}$), and d(C_{20}GCCCCTA$_{17}$) (Table 1), all support the synthesis of longer primers (greater than four nucleotides in length). The V_{max} for primer synthesis varied significantly among the templates (Table 2), while the K_Ms for NTPs were identical within experimental error (1.2−1.6 mM).

Increasing the concentration of individual NTPs increased the rate of primer synthesis but only if that NTP was required for dinucleotide synthesis. The three templates tested, d(T_{20}GTCG$_{20}$), d(C_{20}GTCA$_{20}$), and d(C_{20}GCTA$_{20}$), each code for a single adenylate and a single guanylate in either the first or second primer position. Table 3 shows that increasing the concentration of either ATP or GTP from 0.8 to 3.2 mM increased total primer synthesis. In contrast, increasing the concentration of NTPs not involved in dinucleotide synthesis did not stimulate primer synthesis but rather inhibited it. This occurred even if these NTPs were required for polymerization events after dinucleotide synthesis.

Further investigation determined that both purines and pyrimidines are capable of inhibiting primer synthesis. We measured primer synthesis on the templates d(C_{20}GCCTA$_{19}$), d(C_{20}GCCCTA$_{18}$), and d(C_{20}GCCCCTA$_{17}$), each of which only requires GTP for dinucleotide formation. Table 4 shows that increasing the ATP concentration to 3.2 mM while holding the concentration of the other NTPs constant at 0.8 mM resulted in 30−40% inhibition on each template. Interestingly, the inhibition with ATP was similar to inhibition observed with UTP and CTP, suggesting that purines and pyrimidines have similar interactions with the primase active site.

The fact that those NTPs required for dinucleotide formation stimulate the rate of primer synthesis suggests that primer initiation (the steps leading to the synthesis of the pppNpN dinucleotide) limits the overall rate of primer synthesis. If a polymerization event following dinucleotide synthesis was rate limiting, then increasing the concentration of the NTP involved in this latter polymerization event should have stimulated primer synthesis, and increasing the concentration of those NTPs involved in dinucleotide synthesis should have had no effect. However, this was not the case. Rather, increasing the concentration of an NTP other than one required for dinucleotide formation always decreased the rate of primer synthesis.

The effects of increasing the concentrations of the two NTPs involved in dinucleotide synthesis appear largely

Table 3: Effect of Increasing Individual NTP Concentrations on Primer Synthesis

	ratio of total primer synthesis[a,b] (4-fold increase in indicated [NTP(s)])/(all [NTPs] equivalent)				
template	ATP	GTP	ATP + GTP	UTP	CTP
d(T_{20}GTCG$_{20}$)	1.86 ± 0.04	1.81 ± 0.12	3.16 ± 0.13	0.76 ± 0.06	0.65 ± 0.04
d(C_{20}GTCA$_{20}$)	1.71 ± 0.22	1.67 ± 0.12	2.83 ± 0.49	0.64 ± 0.03	0.74 ± 0.08
d(C_{20}GCTA$_{20}$)	2.71 ± 0.03	1.17 ± 0.07	3.65 ± 0.43	0.61 ± 0.04	0.68 ± 0.10

[a] Average of ratios calculated from three separate experiments. [b] All four NTPs were present in each assay. "All [NTPs] equivalent" means each NTP concentration was at 800 μM. "4-fold increase in indicated [NTP(s)]" means that the designated NTP concentration(s) was (were) increased to 3.2 mM while the other NTP concentrations remained at 800 μM.

Table 4: ATP, UTP, and CTP Each Inhibit Primer Synthesis Similarly on Templates Requiring Only GTP for Dinucleotide Formation

	ratio of total primer synthesis[a,b] (4-fold increase in indicated [NTP(s)])/(all [NTPs] equivalent)			
template	ATP	UTP	CTP	GTP
$d(C_{20}GCCTA_{19})$	0.67 ± 0.06	0.64 ± 0.06	0.57 ± 0.07	4.2 ± 0.3
$d(C_{20}GCCCTA_{18})$	0.72 ± 0.04	0.60 ± 0.05	0.55 ± 0.02	3.1 ± 0.5
$d(C_{20}GCCCCTA_{17})$	0.65 ± 0.08	0.63 ± 0.04	0.55 ± 0.01	3.3 ± 0.2

[a] Average of ratios calculated from three separate experiments. [b] All four NTPs were present in each assay. All [NTPs] equivalent means each NTP concentration was at 800 μM. 4-fold increase in indicated [NTP(s)] means that the designated NTP concentration was increased to 3.2 mM while the other NTP concentrations remained at 800 μM.

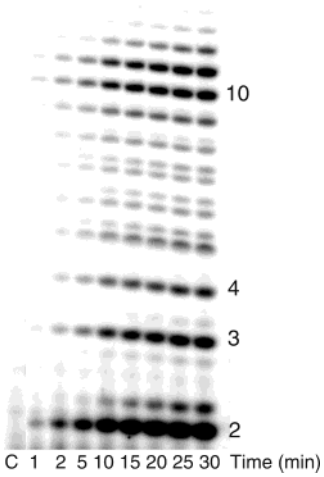

FIGURE 1: The product distribution does not change with time. As described under Experimental Procedures, the assay contained 100 nM primase, 20 μM template $d(T_{20}GTCCT_{19})$, 800 μM ATP, 800 μM GTP, and [α-^{32}P]GTP, except for the control reaction (C) which lacked enzyme. Primer lengths (in nucleotides) are indicated to the right of the image.

additive (Table 3). For example, multiplying the effect of increasing just the concentration of ATP by that of the effect of increasing just GTP predicts that simultaneously increasing the concentration of both NTPs should increase the rate of primer synthesis by 3.4, 2.9, and 3.2 on the templates $d(T_{20}GTCG_{20})$, $d(C_{20}GTCA_{20})$, $d(C_{20}GCTA_{20})$, respectively. These values are remarkably close to the observed effects, 3.2, 2.8, and 3.7, respectively.

The Rate-Limiting Step(s) of Primer Synthesis Occur(s) during Primer Initiation. To further verify that steps after dinucleotide formation are not rate limiting under steady-state conditions, we examined the time course of primer synthesis. Figure 1 shows the primer products synthesized on template $d(T_{20}GTCCT_{19})$ as function of time. Both the rate of primer synthesis and 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. If the rate-limiting step had occurred after dinucleotide formation, then shorter primers should have accumulated followed by a conversion of these shorter products to longer products with time. However, this was not observed, indicating that all polymerization events after dinucleotide must be fast compared to dinucleotide synthesis. We repeated this experiment for templates $d(C_{20}GTCCA_{19})$ and $d(C_{20}GCCA_{20})$ and again found that the rate of primer synthesis and the product distribution remained constant with time despite the fact that these templates gave rates of primer synthesis 2- and 4-fold slower than $d(T_{20}GTCCT_{19})$, respectively (data not shown). Furthermore, even during the first turnover event on the template $d(C_{20}GTCCA_{19})$, where the total amount of primers synthesized was less than the amount of enzyme present in the assay, there was no detectable accumulation of short products prior to the formation of longer primers (data not shown). Thus, all polymerization events after dinucleotide formation must be comparatively fast, and the rate-limiting step(s) in primer synthesis occur(s) either before or during dinucleotide formation (i.e., primer initiation).

Influence of NTP Concentration on Primase Processivity. A surprising feature of primase is the relative inefficiency with which it elongates short primers into longer primers. Even on a template where primase demonstrates comparatively good processivity and fast rates of synthesis ($d(T_{20}GTCCT_{19})$), only ca. 10% of the primers are at least eight nucleotides long (with 800 μM NTPs). To better understand the reason behind this low processivity, we examined the effect of NTP concentrations on processivity.

Since polymerization events after dinucleotide formation are fast compared to dinucleotide formation, we cannot directly measure the rates of these latter polymerization events. Therefore, to study the influence of NTPs on these latter polymerization events, we examined the effect of NTP concentrations on the ability of primase to extend primers of specific lengths. We first measured the effect of varying the NTP concentration on the ability of primase to elongate short primers synthesized on the templates $d(C_{20}GTCCA_{19})$, $d(T_{20}GTCCT_{19})$, $d(T_{20}GTCT_{20})$, $d(C_{20}GCCCCTA_{17})$, and $d(C_{20}GCCA_{20})$. As expected, simultaneously increasing the concentration of all four NTPs increased the overall rate of primer synthesis. However, the processivity of primase increased only slightly (Figure 2). Even at 3 mM NTPs, primase only elongated about 50% of the dinucleotide to a trinucleotide for all five templates assayed. Interestingly, even though the rate of primer synthesis on the five templates varied by 7-fold, the percentage of dinucleotide elongated was remarkably similar at each NTP concentration. This was not the case for other length primers, as the templates $d(C_{20}GTCCA_{19})$, $d(T_{20}GTCCT_{19})$, $d(T_{20}GTCT_{20})$, and $d(C_{20}GCCCCTA_{17})$ supported more efficient elongation of the 3-mer, 4-mer, and 5-mer than did the template $d(C_{20}GCCA_{20})$. Consequently, the fraction of primers greater than five nucleotides long on the templates $d(C_{20}GTCCA_{19})$, $d(T_{20}GTCCT_{19})$, $d(T_{20}GTCT_{20})$, and $d(C_{20}GCCCCTA_{17})$ was ca. 7-, 9-, 6-, and 8-fold greater, respectively, than on the template $d(C_{20}GCCA_{20})$.

To further probe the effects of NTP concentration on processivity, we used the templates ($d(C_{20}GCCTA_{19})$, $d(C_{20}GCCCTA_{18})$, and $d(C_{20}GCCCCTA_{17})$ (Table 1). These templates allowed us to specifically examine polymerization of ATP onto a di-, tri-, and tetraguanylate within an almost identical sequence context. Consistent with the results

Table 5: Effect of NTP Concentration on Single Polymerization Events during Primer Elongation

template[a]	polymerization event analyzed[b]	ratio of fraction of primers elongated[c,d] (10-fold increase in indicated [NTP(s)])/(all [NTPs] equivalent)				
		all NTPs	ATP	GTP	UTP	CTP
$d(C_{20}GCCTA_{19})$	ATP	1.7 ± 0.2	2.5 ± 0.1	0.60 ± 0.07	1.1 ± 0.1	1.0 ± 0.1
$d(C_{20}GCC\underline{C}TA_{18})$	ATP	1.28 ± 0.08	1.60 ± 0.03	0.51 ± 0.08	0.93 ± 0.03	1.05 ± 0.07
$d(C_{20}GCCC\underline{C}TA_{17})$	ATP	1.9 ± 0.4	2.2 ± 0.7	0.63 ± 0.02	1.0 ± 0.3	1.0 ± 0.3
$d(C_{20}GCC\underline{T}C\underline{C}A_{17})$	ATP	1.6 ± 0.2	2.3 ± 0.5	0.55 ± 0.05	1.2 ± 0.2	0.9 ± 0.3

[a] The underlined base in the template corresponds with the primer position at which NTP incorporation was measured. [b] NTP polymerized onto the primer as coded for by the underlined base in the template. [c] Average of ratios calculated from three separate experiments. [d] Numbers listed are a ratio of (1) the fraction of primers elongated when the indicated NTP(s) concentration(s) is (are) increased 10-fold to 3 mM (while the others remain at 300 μM each) divided by (2) the fraction of primer elongated when all four NTP concentrations are at 300 μM each.

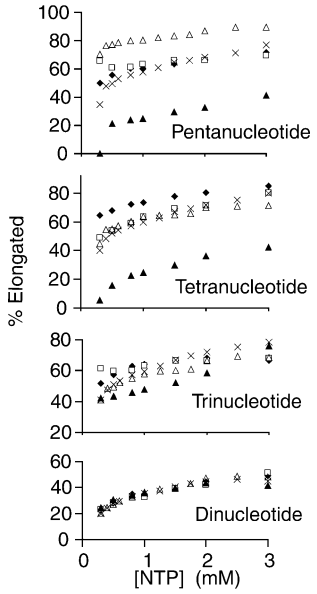

FIGURE 2: Increasing the NTP concentration increases primase processivity. The percent elongation of individual primer lengths was calculated at various NTP concentrations on five templates [$d(T_{20}GTCCT_{19})$ (◆), $d(C_{20}GTCCA_{19})$ (□), $d(C_{20}GCCA_{20})$ (▲), $d(T_{20}GTCT_{20})$ (×), and $d(C_{20}GCCCCTA_{17})$ (△)]. Primase assays were performed as described in the Experimental Procedures and contained 100 nM herpes primase, 20 μM template, [α-^{32}P]GTP, and all four NTPs each at the indicated NTP concentration.

presented earlier, increasing all four NTPs simultaneously from 300 μM to 3 mM increased polymerization of ATP at the targeted position for each template (Table 5). Even larger increases occurred when ATP alone was increased to 3 mM while the other NTPs were maintained at 300 μM, suggesting that GTP, CTP, and/or UTP were inhibiting polymerization of ATP. Indeed, increasing GTP alone was found to inhibit ATP polymerization, while increasing UTP or CTP alone had little effect. To ensure that these results were not caused by the template sequence after the site coding for ATP polymerization, we also examined the template $d(C_{20}GCCTCCA_{17})$. Table 5 shows that varying NTP concentrations with both $d(C_{20}GCCTA_{19})$ and $d(C_{20}GCCTCCA_{17})$ had similar effects on ATP polymerization.

Elongation of Preexisting Primers. All experiments described thus far were conducted with DNA concentrations in large excess over enzyme concentrations. Thus, an ample supply of naked ssDNA template was always available for primase to use. To study the impact of significantly reducing the ssDNA concentration on primer synthesis, we decreased the template to enzyme ratio from 200:1 (Figure 1) to 6:1 and examined the time course of primer synthesis. Now, the rate of synthesis and size distribution of primers did not remain constant with time (Figure 3). At early times on the template $d(T_{20}GTCCT_{19})$, the primary products were 2−3 and 10−11 nucleotides long (Figure 3A), and the rate of total primer synthesis coincided with the linear rate of primer synthesis observed when template was in 200-fold excess over enzyme (Figure 3B). However, at longer reaction times, primase began to synthesize products up to approximately 18 nucleotides long (Figure 3A). Concomitantly, the accumulation rate for primers 10−11 nucleotides long decreased dramatically (by >90%) while the accumulation of very short products continued but at a rate about half as fast as occurred at early times in the reaction. These data suggest that, at later times, primase was converting preexisting primers of length 10−11 nucleotides into longer products rather than solely producing new primer strands. The rate of primer synthesis also decreased substantially at the later times (Figure 3B), in contrast to the linear rate of primer synthesis observed when the amount of DNA template was much greater than the amount of enzyme. These effects likely resulted from depletion of naked, single-stranded template. After 5 min, the amount of primers at least eight nucleotides long was equal to the amount of template present in the reaction while the amount of total primers present exceeded the amount of template by 6.5-fold. After 15 min, primers at least eight nucleotides long exceeded template by 2.3-fold, and the total moles of primer exceeded template by 13-fold.

To further explore the mechanism by which preexisting primers were further elongated and to exclude the possibility that the distance between the primer initiation site and the 5′-end of the template affected elongation, we analyzed primer synthesis on the templates $d(T_{20}GTCCT_{36})$ and $d(C_{20}GTCCA_{19})$ at a low template to primase ratio (6:1). While the rate of primer synthesis and product distribution again changed with time, the size distribution of longer products varied substantially from that observed on $d(T_{20}GTCCT_{19})$. On $d(T_{20}GTCCT_{36})$, a template where the primer initiation site is 17 nucleotides further away from the 5′-end than on the template $d(T_{20}GTCCT_{19})$, primers increased in length from ca. 10 nucleotides to 18 nucleotides more gradually and incrementally (Figure 4A) than on the template $d(T_{20}GTCCT_{19})$. In contrast, when primase further elongated primers synthesized on the template $d(C_{20}GTCCA_{19})$, they were elongated by only an additional one or two nucleotides (Figure 4B).

Decreasing the NTP concentration enhanced the conversion of 10−11 nucleotide long primers into products up to 18 nucleotides on template $d(T_{20}GTCCT_{19})$. In reactions

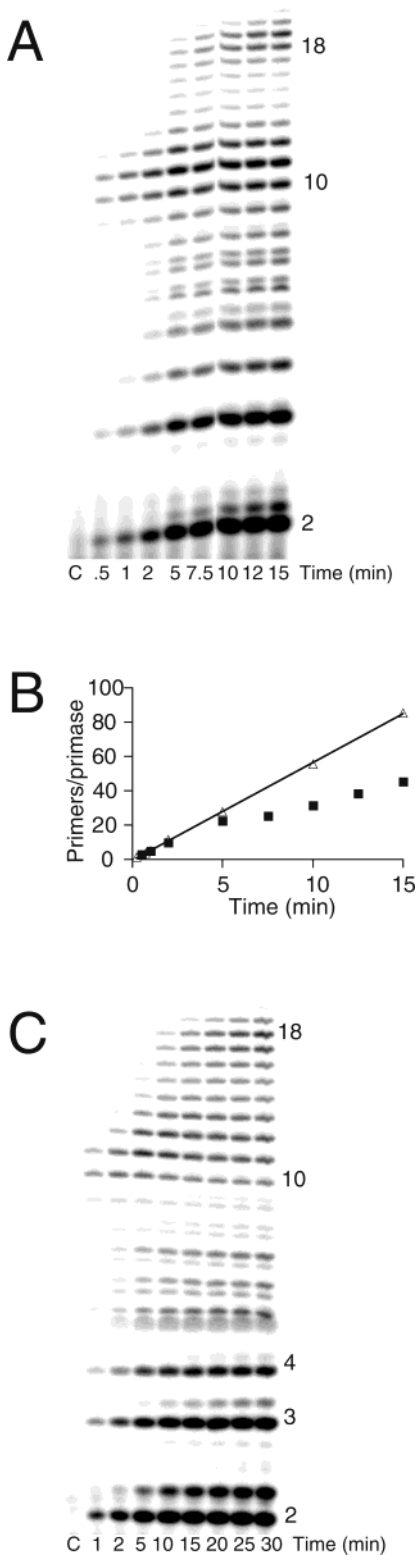

FIGURE 3: Elongation of preexisting primers in assays containing a low template:primase ratio (6:1). (Panel A) Assays contained 1 μM primase, 6 μM template d(T_{20}GTCCT$_{19}$), 4 mM ATP, 4 mM GTP, and [α-^{32}P]GTP. The control reaction lacked enzyme (C). Primer length is noted to the right of the image. (Panel B) The amount of primers synthesized in the reactions shown in Figure 2A was quantified (■). The solid line shows the linear rate of primer synthesis obtained at a template:primase ratio of 200:1 (△). (Panel C) Assays contained 1 μM primase, 6 μM template d(T_{20}GTCCT$_{19}$), 1 mM ATP, 1 mM GTP, and [α-^{32}P]GTP, except the control reaction which lacked enzyme (C). Primer lengths are noted to the right.

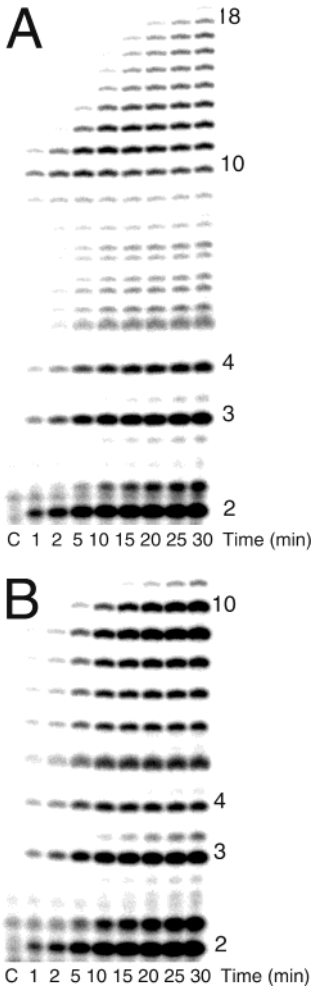

FIGURE 4: Elongation of preexisting primers. (Panel A) Assays contained 1 μM primase, 6 μM template d(T_{20}GTCCT$_{36}$), 4 mM ATP, 4 mM GTP, and [α-^{32}P]GTP, except the control reaction which lacked enzyme. Primer lengths are noted to the right. (Panel B) Assays contained 1 μM primase, 6 μM template d(C_{20}GTCCA$_{19}$), 4 mM ATP, 4 mM GTP, 4 mM UTP, and [α-^{32}P]GTP, except the control reaction which lacked enzyme. Primer lengths are noted to the right.

containing only 1 mM NTPs, 75% less than the reactions in Figure 3A, the amount of primers 10−11 nucleotides long actually decreased at later time points as the amount of primers ca. 18 nucleotides long increased (Figure 3C). The accumulation of dinucleotide products was again slowed, in this case by ca. 85%. The enhanced elongation of preexisting primers along with decreased accumulation of very short primers suggests that decreasing the NTP concentration impacts initiation of new primers to a greater extent than it impacts elongation of preexisting primers.

DISCUSSION

Templates of defined sequence were used to further elucidate the mechanism of primer synthesis by herpes primase. The template regions flanking a potential initiation site largely determine how efficiently primase will use that potential start site (*5*). However, when confronted with a choice of potential initiation sites, the NTP concentration can, to a lesser extent, influence start site selection both by increasing the rate of synthesis at one site and by inhibiting synthesis at another. Under steady-state conditions, the

overall rate of primer synthesis is limited by primer initiation (at or before formation of the pppNpN dinucleotide). While more rapid than dinucleotide synthesis, elongation of the dinucleotide is relatively inefficient even at high NTP concentrations, thus resulting in the accumulation of large amounts of dinucleotide. On templates where primase demonstrates comparably good processivity, primers reach a length of ca. 10 nucleotides, at which point primase terminates synthesis. In the presence of excess single-stranded template, primase will then synthesize another primer on these single-stranded templates. However, in the absence of excess single-stranded template, primase can elongate a preexisting primer or synthesize another primer.

Two lines of evidence indicate that primer initiation limits the rate of primer synthesis. First, short primers did not accumulate prior to the appearance of longer products, even during the first turnover of primase. If a step after dinucleotide formation had limited the rate of primer synthesis, shorter primer accumulation (dinucleotide, trinucleotide, etc., depending upon the position of the rate-limiting step) would have occurred prior to the formation of longer products. Second, increasing the concentration of those NTPs involved in dinucleotide synthesis increased the rate of primer synthesis, whereas increasing the concentration of NTPs not involved in dinucleotide synthesis inhibited primer synthesis. If a polymerization reaction other than dinucleotide synthesis was rate limiting, one would not have expected that (i) increasing the concentration of NTPs involved in dinucleotide synthesis would increase the rate of primer synthesis and (ii) increasing the concentration of a NTP required for later polymerization events would inhibit primer synthesis. Similar results were obtained on several different templates, suggesting that dinucleotide synthesis will be rate limiting on most, if not all, templates.

Rate-limiting initiation appears to be a common feature among primases from different sources. Steady-state studies with *Escherichia coli* primase and pre-steady-state studies with eukaryotic primase indicate that the rate-limiting step occurs either before or during dinucleotide synthesis (*31*, *32*), analogous to herpes primase. However, under steady-state conditions, the rate-limiting step for eukaryotic primase occurs after primer synthesis (*32*, *33*). This second rate-limiting step is alleviated if reactions also contain DNA polymerase α and dNTPs such that the primase-synthesized primers are immediately elongated (*33*). In contrast, the herpes reaction mechanism does not contain a rate-limiting step after primer synthesis, as evidenced by the linear kinetics of primer synthesis.

For both herpes and eukaryotic primases, varying the NTP concentration can alter where primase initiates primer synthesis. For herpes primase, this only occurred when the two sites used completely different NTPs. The most dramatic effects with eukaryotic primase occur upon varying the concentration of the NTP that becomes the second nucleotide of the primer (*32*). This NTP binds to primase first; hence increasing the concentration of a specific NTP greatly enhances the rate of primer synthesis at those template sequences that specify this NTP as the second nucleotide of the primer. In contrast, when herpes primase is confronted with two potential initiation sites that require the same two nucleotides but in opposite order (i.e., GTC versus GCT), varying the concentration of just one NTP did not affect the frequency with which the enzyme used the two sites. Thus, both NTPs appear to play equally important roles in determining start site selection.

Accumulation of very large amounts of short products is a common feature of primases and RNA polymerases(*30*, *34*, *35*), and herpes primase is certainly no exception. Even at high concentrations of NTPs (3 mM), only half of all dinucleotides were elongated on the templates d(C_{20}GTCCA$_{19}$), d(T_{20}GTCCT$_{19}$), d(T_{20}GTCT$_{20}$), d(C_{20}GCCCCTA$_{19}$), and d(C_{20}GCCA$_{20}$). These templates contain two of the four possible G-pyr-pyr start sites, GTC and GCC, and previous studies showed that primase also synthesizes large amounts of dinucleotide on templates containing GCT and GTT initiation sites (*5*). Interestingly, the fraction of dinucleotide elongated appears relatively independent of dinucleotide sequence, even though G·C base pairs are much more stable than A·T base pairs. Thus, the frequent release of dinucleotide appears to be largely independent of the stability of the dinucleotide−template duplex. This contrasts with eukaryotic primase, where the enzyme produces relatively small amounts of dinucleotide on templates that specify formation of a GG dinucleotide [poly(dC)], but generates massive amounts of dinucleotide on templates that result in AA dinucleotide formation [poly(dT)] (*36*). Additional factors that may affect dinucleotide elongation for herpes primase include the identity of the next NTP being polymerized, the sequences of the flanking regions, and, in vivo, the presence of other replication proteins bound to the UL5−UL8−UL52 primase−helicase complex.

When primase is provided with insufficient quantities of template to maintain steady-state conditions, preexisting primers are further elongated, and as a result, the product distribution changes with time. Since this only occurs after a large fraction of the potential initiation sites have been "consumed" via primer synthesis, these data indicate that primase would generally prefer to initiate synthesis of a new primer rather than elongate a preexisting primer. Interestingly, however, low NTP concentrations enhance the frequency with which primase further elongates a preexisting primer versus initiating synthesis of a new primer. Thus, the K_M for NTPs during initiation of a new primer is likely higher than the K_M for elongation of a preexisting primer, perhaps due to the greater stability of a primase−primer/template−NTP complex as compared to a primase−template−NTP−NTP complex.

During elongation of a preexisting primer, the size distribution of products generated varied significantly with different templates. On the template d(T_{20}GTCCT$_{19}$), elongation of the ca. 10-mers to ca. 18-mers occurred with minimal accumulation of intermediate length products. In contrast, this process resulted in many more intermediate length products on the template d(T_{20}GTCCT$_{36}$), while on d(C_{20}-GTCCA$_{19}$), the 9-mers were only elongated by one to two nucleotides. These results suggest that the different template sequences and/or the identity of the NTP being polymerized greatly affect(s) this reaction.

Primase appears to readily bind noncognate NTPs. Increasing the concentration of NTPs not involved in dinucleotide synthesis by only 4-fold significantly inhibited the rate of dinucleotide synthesis (by 20−50%). If primase strongly discriminated against binding of noncognate NTPs during

initiation, one would not have expected these concentrations of a noncognate NTP to significantly inhibit polymerization of the cognate NTP. An alternative possibility is that NTPs not involved in dinucleotide synthesis "trapped" primase at other positions on the template. For example, UTP could trap the enzyme opposite a template deoxyadenylate, thereby inhibiting the ability of primase to locate the G-pyr-pyr initiation sequence. However, NTPs for which the complementary deoxynucleotide was not present in the template also inhibited initiation, therefore suggesting that this is not a major mechanism of inhibition. As shown in Table 3, UTP inhibited initiation on the template d(T_{20}GTCG_{20}), even though this template lacks deoxyadenylate.

Facile binding of noncognate NTPs suggests that herpes primase should be relatively inaccurate, similar to primase from other sources (*31*, *33*, *37*). Indeed, preliminary studies indicate that herpes primase is a relatively inaccurate polymerase (K. Ramirez-Aguilar and R. Kuchta, unpublished data). Since herpes primase binds noncognate natural nucleotides so readily, it may also bind and potentially misincorporate synthetic nucleotide analogues, analogues that could be designed to specifically inhibit this essential herpes replication enzyme. Studies to investigate the fidelity of herpes primase and its ability to utilize nucleotide analogues during primer synthesis are in progress.

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