Key Role of Template Sequence for Primer Synthesis by the Herpes Simplex Virus 1 Helicase−Primase†

Kathryn A. Ramirez-Aguilar, Nisha A. Low-Nam, and Robert D. Kuchta*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309

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ABSTRACT: We investigated the effects of ssDNA template sequence on both primer synthesis and NTP hydrolysis by herpes simplex virus 1 helicase−primase. Primer synthesis was found to be profoundly dependent upon template sequence. Although not absolutely required, an important sequence feature for significant production of longer primers (beyond four nucleotides in length) is a deoxyguanylate−pyrimidine−pyrimidine (3′-G-pyr-pyr-5′) triplet in the template. The deoxyguanylate serves both to direct primase to initiate synthesis opposite the adjacent pyrimidine and to dramatically increase primer length. While primase synthesized significantly more long primers on those templates containing a G-pyr-pyr triplet, the enzyme still synthesized massive quantities of di- and trinucleotides on many templates containing this sequence. Varying the sequences around the G-pyr-pyr recognition sequence dramatically altered both the rate of primer synthesis and the fraction of primers longer than four nucleotides, indicating that primase must interact with both the G-pyr-pyr and flanking sequences in the template. In contrast to the large effects that varying the template sequence had on primase activity, ssDNA-dependent NTPase activity was essentially unaffected by changes in template sequence, including the presence or absence of the G-pyr-pyr trinucleotide. In addition to hydrolyzing NTPs the NTPase could also hydrolyze the 5′-terminal phosphate from newly synthesized primers.

Since all known DNA polymerases are unable to initiate DNA synthesis de novo, primer synthesis is almost always a vital component of DNA replication (*2*, *3*). DNA primases synthesize short RNA oligonucleotides that DNA polymerases further elongate via dNTP polymerization. During herpes DNA replication, the herpes DNA polymerase elongates the 3′-OH of a short RNA primer synthesized by a herpes-encoded DNA primase (*4*−*6*).

The herpes simplex virus 1 (HSV-1)[1] primase activity resides within a three-subunit helicase−primase complex and is essential for HSV-1 DNA replication (*6*−*8*). This heterotrimeric complex consists of three gene products, UL8, UL5, and UL52, and contains primase, ssDNA-dependent NTPase, and 5′−3′ helicase activities (*6*, *8*−*11*). Although many conserved helicase motifs have been identified in the UL5 subunit (*12*, *13*) and two conserved primase motifs in the UL52 subunit (*14*−*16*), individually the subunits lack either activity (*4*, *17*, *18*). Only complexes containing both UL5 and UL52 retain the activities of the heterotrimeric protein (*4*, *17*, *18*). While the UL8 subunit neither binds DNA nor possesses catalytic activity (*18*, *19*), it is essential for HSV-1 replication and can modify the catalytic properties of the UL5/UL52 subcomplex (*6*, *8*, *20*). Potential activities of UL8 include (1) stimulating primase and helicase activities (*21*, *22*), (2) overcoming the inhibition of NTPase and primase activities caused by coating ssDNA with the HSV-1 ssDNA binding protein, ICP8 (*22*, *23*), (3) recruiting HSV-1 helicase−primase to origins of replication via interaction with the origin binding protein, UL9 (*24*), and (4) recruiting the HSV-1 DNA polymerase (UL30/UL42 complex) to the HSV-1 helicase−primase via interactions with UL30 (*4*, *5*).

Direct primer synthesis by HSV-1 helicase−primase has been observed on three ssDNAs (M13, ϕX174 and pBS), producing primers up to ∼10−13 nucleotides in length (*1*, *4*, *11*, *12*, *14*, *16*, *21*). Interestingly, primer synthesis occurred only at discrete sites on these long ssDNAs, therefore indicating that HSV-1 helicase−primase has specific template requirements for primer synthesis. The principal sites of HSV-1 primer synthesis have been identified for both the pBS and ϕX174 templates (*1*, *25*). Furthermore, sequence variations of the ϕX174 site significantly affected HSV-1 primer synthesis (*1*). Template length, on the other hand, did not appear to impact primer synthesis as greatly. In fact, primer synthesis was observed for ssDNA templates as short as nine nucleotides. While primer synthesis has been observed on templates of defined sequence, the feature(s) that allow primer synthesis has (have) not been closely defined.

In this investigation, we have utilized synthetic ssDNA templates of defined sequence to further probe the sequence requirements of HSV-1 helicase−primase for primer synthesis and test the effects of template sequence on ssDNA-

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* To whom correspondence should be addressed. Telephone: (303) 492-7027. Fax: (303) 492-5894. E-mail: kuchta@spot.colorado.edu.

[1] Abbreviations: DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSV-1, herpes simplex virus 1; Ni-NTA, nickel nitrilotriacetic acid; pBS, pBluescript; PNK, polynucleotide kinase; G-pyr-pyr, deoxyguanylate−pyrimidine−pyrimidine; SAP, shrimp alkaline phosphatase; ssDNA, single-stranded DNA; SV40, simian virus 40.

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dependent NTPase activity. While length and quantity of primers produced varied significantly with template sequence, template sequence had little effect on the rate of NTPase activity. Furthermore, the sequence of the template that actually coded for the primer as well as regions flanking the primer synthesis site greatly affected primase activity. The implications of these results with respect to the mechanism of HSV-1 helicase−primase are discussed.

EXPERIMENTAL PROCEDURES

Materials

SF9 cells were obtained from the Tissue Culture Core Facility at the University of Colorado Cancer Center. Baculoviruses expressing the UL5 and UL52 subunits and monoclonal antibodies for the UL8, UL5, and UL52 subunits, a baculovirus for expressing UL5MI, and a baculovirus expression vector for a His_9-tagged UL8 subunit were generously provided by Dr. Robert Lehman (Stanford University), Dr. Sandra Weller (University of Connecticut Health Center), and Dr. Heidi Giordano (Tularik), respectively. 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 (*26*). Synthetic templates of defined sequence were obtained from Oligos Etc. Concentrations of ssDNA templates were determined spectrally and are given in terms of 5′-termini. [^{32}P]NTPs were purchased from New England Nuclear. Shrimp alkaline phosphatase (SAP) was purchased from Amersham Life Science, T4 polynucleotide kinase (PNK) was from New England Biolabs, and leupeptin and pepstatin A were from Sigma. All other reagents were of the highest purity available.

Methods

Expression and Purification of HSV-1 Helicase−Primase. We expressed and purified the enzyme using a modification of a previously published procedure (*27*, *28*). SF9 cells (2×10^8) were coinfected with three baculoviruses containing the genes for HisUL8 (moi = 5), UL52 (moi = 10), and either UL5 (moi = 10) or UL5MI (moi = 10), respectively. At 48 h postinfection, the cells were removed from the medium by centrifugation (10 min at 125*g*) and resuspended in 45 mL of ice-cold cell wash buffer (20 mM HEPES, pH 7.6, 150 mM NaCl, and 1 mM DTT). The suspension was centrifuged for 10 min at 125*g* and the cell pellet collected. The cells were then snap frozen in N_2(l) and stored at −80 °C. The cells were thawed just enough to be resuspended in 8 mL of ice-cold lysis buffer (0.1% Nonidet P-40, 400 mM NaCl, 20 mM Tris-HCl, pH 8.0, 5% glycerol, 8 mM β-mercaptoethanol, 1.5 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride, 5 μg/mL leupeptin, and 5 μg/mL pepstatin A), left on ice for 15 min, and then sonicated for 5 min in an ice bath using a Branson 1510 sonicator. All remaining steps were performed at 4 °C. After sonication, the cellular debris was removed by centifugation (2×18 min at 11750*g*). The supernatant fluids were then applied to 2 mL of Ni-NTA agarose beads (Qiagen) which had previously been equilibrated with buffer A (0.1% Nonidet P-40, 400 mM NaCl, 20 mM Tris-HCl, pH 8.0, 5% glycerol, 8 mM β-mercaptoethanol, 1.5 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride, and 15 mM imidazole). After 2 h of incubation while being slowly shaken, the mixture was added to a poly-prep chromatography column (Bio-Rad). The beads were washed with 20 mL of buffer A, followed by 20 mL of buffer B (buffer A with 30 mM imidazole), and the protein eluted from the beads with buffer C (buffer A with 200 mM imidazole). Positive fractions (500 μL), identified by Bradford reagent, were combined and dialyzed overnight against 20 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10% glycerol, 1 mM DTT, 1.5 mM $MgCl_2$, 2 μg/mL leupeptin, and 2 μg/mL pepstatin A. The enzyme solution was stored as 10 μL aliquots at −80 °C. The identity of the enzyme was verified by Western blotting with antibodies against the UL8, UL5, and UL52 subunits. The enzyme purity was determined to be >95% by SDS−polyacrylamide gel electrophoresis and Coomassie Blue staining.

Primase Assays. Unless otherwise noted, reaction mixtures (10 μL) contained 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/mL bovine serum albumin, 5% glycerol, 6 μM ssDNA template, 500 μM [α- or γ-^{32}P]NTPs, and 100−500 nM HSV-1 helicase−primase. Reactions were initiated by adding enzyme, incubated at 37 °C for 1 h, and then quenched by adding 1.25 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. Primer standards produced by human primase (p58/p49) were loaded concurrently with primase assays on denaturing polyacrylamide gels. A ladder of adenylate primers (i.e., pppApA, pppApApA, pppApApApA) was produced by incubating a reaction (10 μL) containing 50 mM Tris-HCl, pH 8.0, 10 mM MgCl, 1 mM DTT, 0.1 mg/mL bovine serum albumin, 200 μM [α-^{32}P]ATP, 1 μM dT_{40}, and 200 nM human primase at 37 °C for 30 min. The reaction was then quenched with 2.5 volumes of gel-loading buffer (90% formamide). A ladder of $pppG(pG)_n$ primers was produced in a similar manner, except that reactions now contained 200 μM [α-^{32}P]GTP and 1 μM C_3R template instead of ATP and dT_{40} (*29*). pppApG and pppApGpA primer standards were produced similarly except that reactions now contained 200 μM [α-^{32}P]ATP and GTP and either 1 μM $d(TCA)_{20}$ [for pppApG only (*30*)] or 1 μM $d(TCTA)_{15}$ (for both pppApG and pppApGpA). A pApG primer was produced similarly except that reactions contained 2 mM AMP, 200 μM [α-^{32}P]GTP, and 1 μM $d(TCA)_{20}$ (*31*).

Dinucleotide Verification Experiment. Primase assays were performed on the templates $d(CT)_{30}$ and $d(TCTA)_{15}$ as described above, except that 20 μL reaction volumes were used. Likewise, primer standards, pppApG and pApG, were produced by human primase as described above, except that 20 μL reaction volumes were used instead. Rather than quench the reactions with gel-loading buffer, primase was heat inactivated at 65 °C for 10 min. For each reaction, 4 μL was then placed in 12.5 μL of gel-loading buffer, and 13 μL was incubated with 1 unit of SAP at 37 °C for 30 min. The SAP was then heat inactivated at 65 °C for 15 min, and 5 μL was mixed with 12.5 μL of gel-loading buffer. Six microliters of the remaining solution was supplemented

with 1.5 μL of a solution containing 16.6 mM $MgCl_2$, 167 mM Tris-HCl, pH 7.6, and 666 μM ATP prior to the addition of 10 units of T4 PNK. The resulting mixture was incubated at 37 °C for 30 min and then quenched with 12.5 μL of gel-loading buffer. The products from all steps of the experiment were then separated by denaturing polyacrylamide gel electrophoresis (20% polyacrylamide, 8 M urea) and visualized by phosphorimagery (Molecular Dynamics).

DNA-Dependent NTPase Assays. Reactions (10 μL) contained the same components as the primase assays, except where noted. In all cases, 100 nM HSV-1 helicase−primase was used, except for DNA concentration studies where only 10 nM enzyme was used. Reactions were initiated by adding enzyme. After 60 min at 37 °C, 0.75 mL of an acidic ammonium molybdate/malachite green solution (3:1 mixture of 0.045% malachite green hydrochloride and 4.2% ammonium molybdate in 4 M HCl) was added to detect the formation of free phosphate (*9*, *32*). After precisely 3 min, the absorbance at 650 nm was measured.

Experiment Verifying Removal of the 5′-Terminal Phosphate from a Primer. Human primase was used to produce oligo(G) ladders (pppGpG, pppGpGpG, pppGpGpGpG, etc.) labeled with either [α-^{32}P]GTP or [γ-^{32}P]GTP as described above, except that reaction volumes were 13 μL. Rather than quenching with gel-loading buffer, the human primase was heat inactivated at 65 °C for 10 min. After heat inactivation, one reaction (for each radiolabel) received 2 μL of distilled water while another reaction (for each radiolabel) received 2 μL of 3.4 μM HSV-1 helicase−primase. After 3, 15, and 60 min at 37 °C, 3.5 μL of each mixture was removed and quenched with 7 μL of gel-loading buffer. The products from all steps of the experiment were then separated by denaturing polyacrylamide gel electrophoresis (20% polyacrylamide, 8 M urea) and visualized by phosphorimagery.

RESULTS

As mentioned in the introduction, the discrete sites where HSV-1 helicase−primase preferentially initiates primer synthesis on the pBS ssDNA [$^{3'}$...GTCCTTCCG...$^{5'}$ (*25*)] and the ϕX174 ssDNA [$^{3'}$...GCCCTCCCATC...$^{5'}$ (*1*), Table 2] have been identified. Comparing the sequence of these two reported template initiation sites (underlined above) revealed three similarities: (1) in both cases, primer initiation began opposite a template pyrimidine; (2) the template sequence just after the initiation site is pyrimidine rich, and (3) a noncoding template deoxyguanylate is found just prior to the base that codes for the 5′-terminal nucleotide of the primer.

Primer Synthesis on the ϕX174 50mer Using Various Radiolabeled Nucleotides. Although the ϕX174 50mer template has previously been reported to have only one initiation site [opposite a template deoxycytidylate (*1*)], we found that more than one initiation site must exist. We initially examined primer synthesis on the ϕX174 50mer template ("unmodified" sequence in Table 2) using a variety of radiolabeled nucleotides: [α-^{32}P]ATP, [α-^{32}P]GTP, [α-^{32}P]-CTP, [α-^{32}P]UTP, [γ-^{32}P]ATP, and [γ-^{32}P]GTP. While the [α-^{32}P]NTPs will label all primers that contain this nucleotide, the [γ-^{32}P]NTPs will only label those primers that contain the radiolabeled nucleotide at the primer 5′ end since only this nucleotide retains its γ-phosphate. As shown in Figure 1, radioloabeled primers were observed when using either [γ-^{32}P]GTP or [γ-^{32}P]ATP to detect primer synthesis on the ϕX174 50mer template. Therefore, primer initiation on the ϕX174 50mer template must occur opposite both a template deoxycytidylate and a template thymidylate. Interestingly, initiation using ATP as the 5′-terminal nucleotide occurred about eight times more frequently than initiation using GTP.

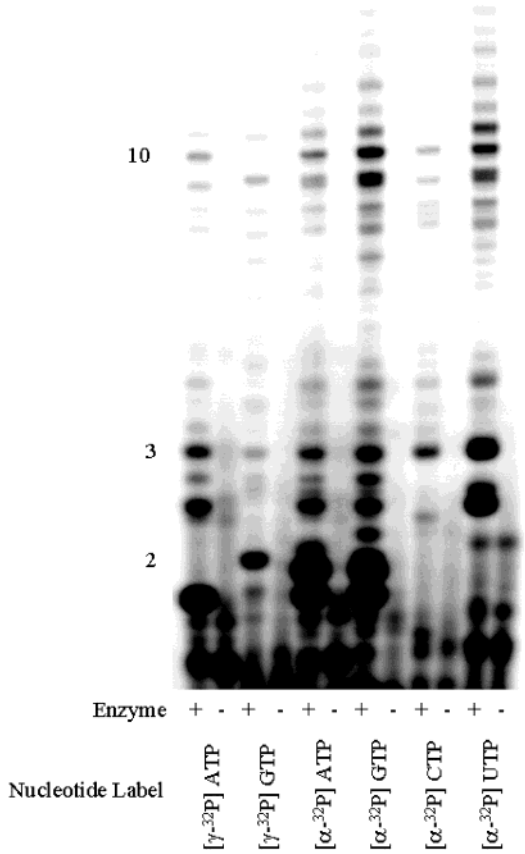

FIGURE 1: HSV-1 primer synthesis on the ϕX174 50mer. Assay conditions are described under Experimental Procedures and contained the noted [^{32}P]NTP. The presence or absence of 300 nM enzyme in each assay is noted. The electrophoretic mobility of oligoguanylates [pppG(pG)$_n$] is shown to the left of the image. It should be noted that the base composition of the products will affect their electrophoretic mobility.

Radiolabeled primers were generated using both [α-^{32}P]-CTP and UTP, indicating that primase incorporates both nucleotides into primers (Figure 1). However, little, if any, radiolabeled products two nucleotides in length were detected using either [α-^{32}P]CTP or [α-^{32}P]UTP, suggesting that primase does not readily polymerize these NTPs during formation of the pppNpN dinucleotide. When assays contained either [α-^{32}P]ATP or [α-^{32}P]GTP, labeled products as short as two nucleotides were readily detected, consistent with the results using [γ-^{32}P]ATP and [γ-^{32}P]GTP, showing that primase indeed polymerizes these nucleotides during dinucleotide synthesis. Surprisingly, however, assays containing [α-^{32}P]NTPs exhibited products of altered electrophoretic mobility as compared to assays containing [γ-^{32}P]-NTPs. As will be discussed later, this results from the NTPase activity of the primase−helicase complex hydrolytically removing the γ-phosphate from short primers.

Table 1: Synthetic ssDNA Templates Used

$d(CT)_{30}$	$^{3'}$CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT$^{5'}$
$d(TCTA)_{15}$	$^{3'}$TCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTA$^{5'}$
$d(TCTG)_{15}$	$^{3'}$TCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTG$^{5'}$
$d(TCTCTA)_{10}$	$^{3'}$TCTCTATCTCTATCTCTATCTCTATCTCTATCTCTATCTCTATCTCTATCTCTA$^{5'}$
$d(TCTCTG)_{10}$	$^{3'}$TCTCTGTCTCTGTCTCTGTCTCTGTCTCTGTCTCTGTCTCTGTCTCTGTCTCTG$^{5'}$
$d(TCTCTCTCTCA)_5$	$^{3'}$TCTCTCTCTCATCTCTCTCTCATCTCTCTCTCATCTCTCTCTCATCTCTCTCTCATCTCT$^{5'}$
$d(TCTCTCTCTCG)_5$	$^{3'}$TCTCTCTCTCGTCTCTCTCTCGTCTCTCTCTCGTCTCTCTCTCGTCTCTCTCTCGTCTCT$^{5'}$
$d(GTC)_{20}$	$^{3'}$GTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTCGTC$^{5'}$
$d(GCC)_{20}$	$^{3'}$GCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCC$^{5'}$
$d(GCT)_{20}$	$^{3'}$GCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT$^{5'}$
$d(GTT)_{20}$	$^{3'}$GTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTT$^{5'}$
$d(GCCC)_{15}$	$^{3'}$GCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCC$^{5'}$
$d(GTTT)_{15}$	$^{3'}$GTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTT$^{5'}$
dC_{40}	$^{3'}$CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC$^{5'}$
$d(C_2GTC_{41})$	$^{3'}$CCGTCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC$^{5'}$
$d(C_{20}GTC_{21})$	$^{3'}$CCCCCCCCCCCCCCCCCCCCCGTCCCCCCCCCCCCCCCCCCCCCC$^{5'}$
$d(C_{20}ATC_{21})$	$^{3'}$CCCCCCCCCCCCCCCCCCCCCATCCCCCCCCCCCCCCCCCCCCCC$^{5'}$
$d(C_{20}GAC_{21})$	$^{3'}$CCCCCCCCCCCCCCCCCCCCCGACCCCCCCCCCCCCCCCCCCCCC$^{5'}$
$d(C_{20}GTAC_{20})$	$^{3'}$CCCCCCCCCCCCCCCCCCCCGTACCCCCCCCCCCCCCCCCCCCC$^{5'}$
$d(C_{20}GTTC_{20})$	$^{3'}$CCCCCCCCCCCCCCCCCCCCGTTCCCCCCCCCCCCCCCCCCCCC$^{5'}$
$d(C_{20}GCTC_{20})$	$^{3'}$CCCCCCCCCCCCCCCCCCCCGCTCCCCCCCCCCCCCCCCCCCCC$^{5'}$
$d(C_{20}GC_{22})$	$^{3'}$CCCCCCCCCCCCCCCCCCCCGCCCCCCCCCCCCCCCCCCCCCC$^{5'}$
$d(A_{20}GTC_{21})$	$^{3'}$AAAAAAAAAAAAAAAAAAAAGTCCCCCCCCCCCCCCCCCCCCC$^{5'}$
$d(C_{20}GTCA_{20})$	$^{3'}$CCCCCCCCCCCCCCCCCCCCGTCAAAAAAAAAAAAAAAAAAAA$^{5'}$
$d(A_{20}GTCA_{20})$	$^{3'}$AAAAAAAAAAAAAAAAAAAAGTCAAAAAAAAAAAAAAAAAAAA$^{5'}$
$d(C_{20}GTCCA_{19})$	$^{3'}$CCCCCCCCCCCCCCCCCCCCGTCCAAAAAAAAAAAAAAAAAAA$^{5'}$
$d(T_{20}GTCT_{20})$	$^{3'}$TTTTTTTTTTTTTTTTTTTTGTCTTTTTTTTTTTTTTTTTTTT$^{5'}$
$d(T_{20}GTCCT_{19})$	$^{3'}$TTTTTTTTTTTTTTTTTTTTGTCCTTTTTTTTTTTTTTTTTTT$^{5'}$
$d(T_{20}GTCG_{20})$	$^{3'}$TTTTTTTTTTTTTTTTTTTTGTCGGGGGGGGGGGGGGGGGGGG$^{5'}$
dT_{40}	$^{3'}$TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT$^{5'}$
C_3R	$^{3'}$GTAGCATCCCGTAGCATCCCGTAGCATCCCGTAGCATCCC$^{5'}$
$d(TCA)_{20}$	$^{3'}$TCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCA$^{5'}$
$d(CCA)_{20}$	$^{3'}$CCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCA$^{5'}$

Importance of Template Deoxyguanylate for Primer Synthesis. A noncoding template deoxyguanylate is found just prior to the previously reported primer initiation sites for both the ϕX174 and pBS viral ssDNAs (see above and refs *1* and *25*). To assess the importance of a template deoxyguanylate for HSV-1 primase activity, primer synthesis was compared on a series of 60mer ssDNA templates with the following repeating sequences (written 3′ to 5′): CT, TCTA, TCTG, TCTCTA, TCTCTG, TCTCTCTCTCA, and TCTCTCTCTCG (Table 1). As shown in Figure 2A, large quantities of dinucleotides and some 3mer and 4mers were produced on all of the templates in assays containing 500 μM NTPs and 6 μM DNA. However, only those template sequences containing deoxyguanylate supported the synthesis of significant quantities of primers with lengths greater than four nucleotides.

To determine if a lack of longer primer synthesis for the non-deoxyguanylate templates was due to insufficient quantities of NTPs or template, primer synthesis was again compared at (1) 500 μM NTPs and 30 μM DNA and (2) 2 mM NTPs and 6 μM DNA. Increasing the template concentration from 6 to 30 μM DNA (while NTPs remained at 500 μM), increased neither the quantity nor average length of primers (data not shown), indicating that 6 μM DNA saturates the enzyme. In fact, the rate of primer synthesis with only 300 nM $d(TCTG)_{15}$ was similar to that at 6 μM DNA, indicating that the enzyme has a very low K_m for DNA. Increasing the NTP concentration from 500 μM to 2 mM (while DNA concentration remained at 6 μM) increased the rate of primer synthesis by ca. 3-fold and resulted in detectable quantities of longer primers for the non-deoxyguanylate templates (Figure 2B). Even under these conditions, however, the templates containing deoxyguanylate continued to produce far greater quantities of longer primers than the templates lacking deoxyguanylate. Therefore, template deoxyguanylates are important for elongation of short primers into longer products.

To gain further information about the initiation sites of HSV-1 primer synthesis on the $d(CT)_{30}$, $d(TCTA)_{15}$, and $d(TCTG)_{15}$ templates, primer synthesis was again compared using various radiolabeled nucleotides (Figure 2C). With all three templates, primer synthesis began almost exclusively opposite a template thymidylate, as evidenced by the large amounts of labeled products generated using [γ-^{32}P]ATP and lack of products detected using [γ-^{32}P]GTP. The large quantities of dinucleotide primers observed with [γ-^{32}P]ATP, for all three templates, comigrated with a 5′-pppApG dinucleotide standard, again consistent with initiation occurring opposite a template thymidylate. Furthermore, primase synthesizes small to undetectable quantities of dinucleotide primers when labeling with [α-^{32}P]CTP for the template $d(TCTG)_{15}$ and [α-^{32}P]UTP for the template $d(TCTA)_{15}$, suggesting that primase does not readily polymerize these NTPs during dinucleotide formation. Together, these data indicate that, on $d(TCTA)_{15}$ and $d(TCTG)_{15}$, primase greatly prefers to initiate primer synthesis opposite the template thymidylate on the 5′ side of a template purine.

The data in Figure 2C also reveal that the dinucleotide is the primary product of primer synthesis, even when the template contained a deoxyguanylate. Since only the 5′-terminal nucleotide of the primer retains its γ-phosphate, labeling products with [γ-^{32}P]NTPs results in incorporation of one ^{32}P per primer, regardless of length. Hence, comparing the intensities of the bands for each length product gives the relative molar amount. In each case, the dinucleotide is by far the most intense band.

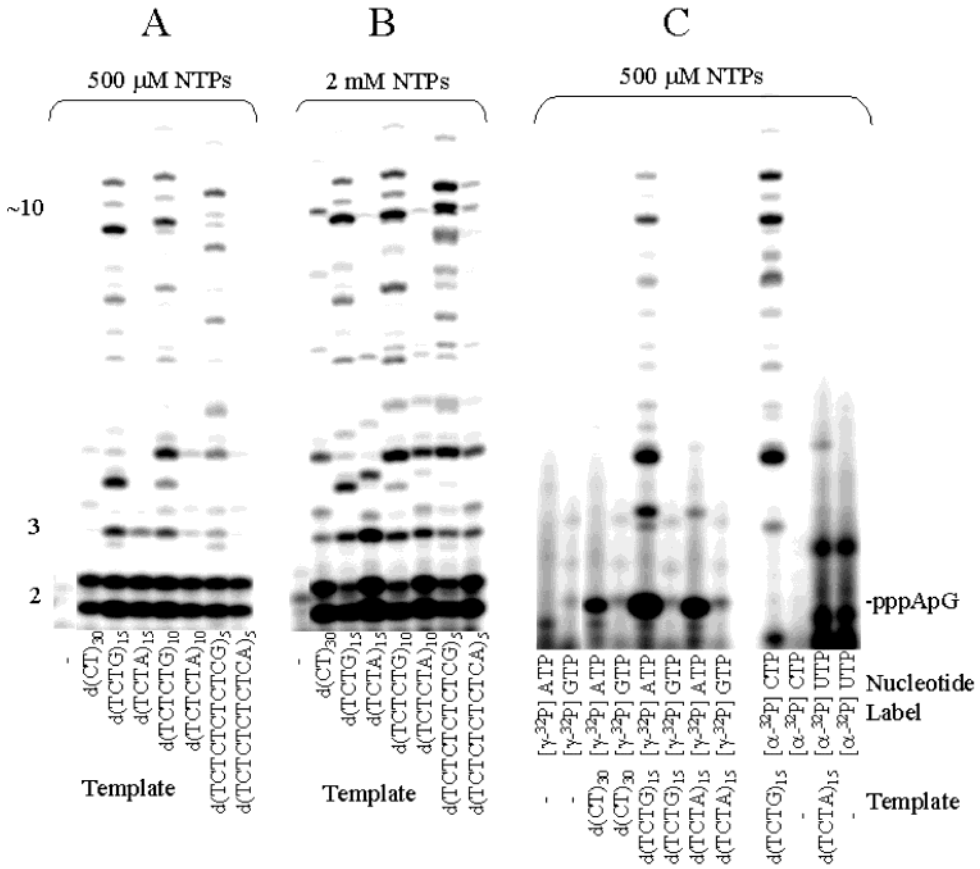

FIGURE 2: Template deoxyguanylate is important for long primer synthesis by HSV-1 primase. Primase assays, as described under Experimental Procedures, contained [α-^{32}P]GTP, 100 nM enzyme, 6 μM DNA, and either (A) 500 μM NTPs or (B) 2 mM NTPs. Control reactions, labeled as (−), were conducted identically but lacked enzyme. (C) Primase assays contained the noted radiolabeled NTP, 6 μM DNA, and 200 nM enzyme [except those lanes labeled (−) which lacked enzyme]. Primer length (in nucleotides) and the migratory position of pppApG, as determined by standards, are noted to the left and right of the images, respectively.

Table 2: Modifications to the ϕX174 50mer Template Sequence[a]

unmodified ϕX174	3′GTTTCCTATTTGTAGTATCCGTCAGCCCTCCCATCAGCCTTGGCTTCTTC5′
ϕX174 A	3′TTTTCCTATTTTTATTATCCGT̲CAGC̲CCTCCCATCATCCTTTTCTTCTTC5′
ϕX174 B	3′TTTTCCTATTTTTATTATCCGT̲CA**A**C̲CCTCCCATCATCCTTTTCTTCTTC5′
ϕX174 C	3′TTTTCCTATTTTTATTATCC**A**T̲CAGC̲CCTCCCATCATCCTTTTCTTCTTC5′
ϕX174 D	3′TTTTCCTATTTTTATTATCC**A**T̲CA**A**C̲CCTCCCATCATCCTTTTCTTCTTC5′
ϕX174 E	3′TTTTCCTATTTTTATTATCCG**C̲**CAG**T̲**CCTCCCATCATCCTTTTCTTCTTC5′

[a] Changes from the ϕX174 A sequence are shown in bold, and initiation sites are underlined.

Verification of Dinucleotide Primer Products. To ensure that the products identified as dinucleotides on the basis of their identical electrophoretic mobility with standards were, in fact, dinucleotides, several additional control experiments were performed (Figure 3). For simplicity, two templates were chosen that primarily resulted in the formation of dinucleotides: d(CT)$_{30}$, and d(TCTA)$_{15}$. The presumed pppApG dinucleotide primers were treated with SAP to remove any phosphate(s) on the 5′-terminus of the primer. As expected for a dinucleotide (*31*), the resulting product exhibited much lower electrophoretic mobility and comigrated with an ApG standard (Figure 3). This product was then treated with polynucleotide kinase and ATP, conditions that should result in the addition of a single phosphate to the 5′-terminus. The products now exhibited increased electrophoretic mobility and comigrated with a pApG standard. Together, these experiments show that the products initially identified as dinucleotides are true dinucleotides.

Identification of a Second Primer Initiation Site on the ϕX174 50mer. A surprising feature of our initial experiments on the ϕX174 50mer was the appearance of primers labeled by both [γ-^{32}P]ATP and [γ-^{32}P]GTP. Since under these conditions only the 5′-terminal nucleotide of the primer becomes labeled, these results indicated that primase initiated primer synthesis at, minimally, two different locations on the ϕX174 50mer. Examining the sequence of the ϕX174 50mer (see unmodified sequence in Table 2) revealed the presence of a 3′-GTC-5′, identical to the sequence where primase initiates on pBS and d(TCTG)$_{15}$. (As will be described in greater detail below, a trinucleotide is a key initiation sequence for the enzyme.) To probe primer initiation opposite both the template thymidylate in the 3′-GTC sequence and the previously reported deoxycytidylate in the sequence 3′-GCC (*1*), we studied the impact of selective ϕX174 50mer sequence modifications. For each modification (shown in Table 2), we compared primer initiation with ATP to initiation with GTP by labeling with [γ-^{32}P]ATP and [γ-^{32}P]GTP, respectively.

The first modification (template ϕX174 A), replacement of all template deoxyguanylates with thymidylate except

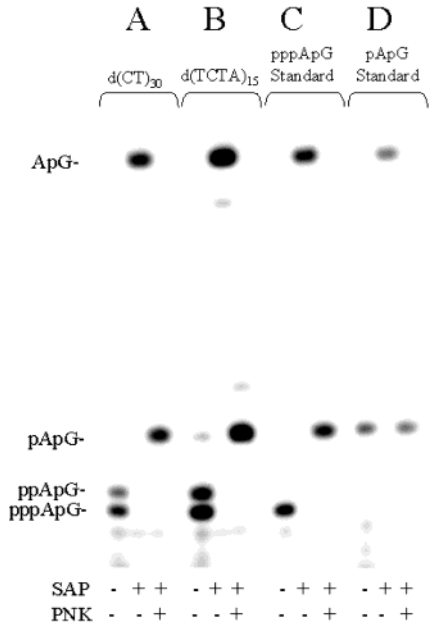

FIGURE 3: Verifying the identity of dinucleotide primers. Experiments were performed as described under Experimental Procedures. Dinucleotide primers labeled with [α-^{32}P]GTP were produced with 100 nM HSV-1 helicase−primase on (A) d(CT)$_{30}$ and (B) d(TCTA)$_{15}$. The resulting pppApG dinucleotides appear as doublets due to hydrolysis of the primer 5′-end phosphate (see Figure 6 and associated text). (C) pppApG and (D) pApG standards labeled with [α-^{32}P]GTP were produced using human primase as described under Experimental Procedures. All four products were exposed to SAP (which removed all 5′-end phosphates to produce ApG) followed by PNK (which adds a single phosphate to the 5′-terminus to produce pApG).

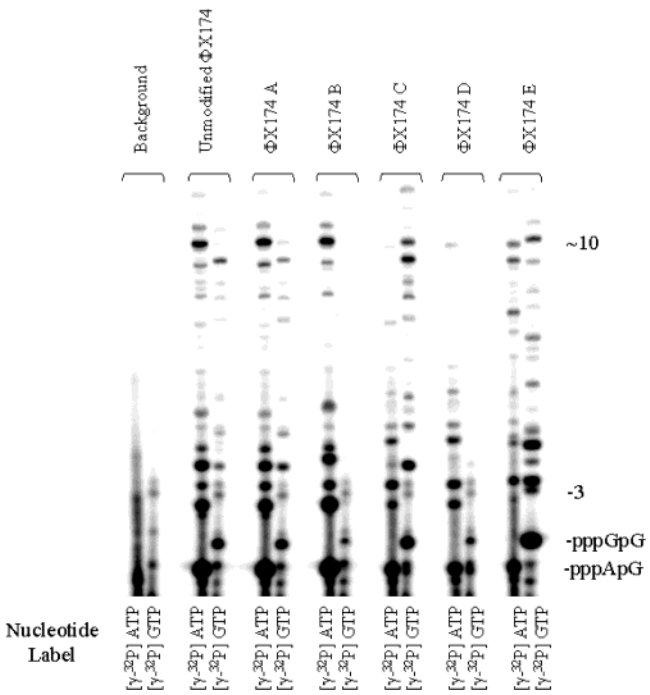

FIGURE 4: Effects of modifying the ϕX174 50mer on the location of primer initiation. Primase assays were performed as described under Experimental Procedures and contained 500 nM enzyme except those lanes labeled background, which lacked enzyme. Primer lengths in nucleotides (as determined by standards) are indicated on the right-hand side of the gel and templates at the top of the gel.

those at the two anticipated initiation sites, had virtually no measurable effect on primer initiation or product distribution in reactions containing either [γ-^{32}P]ATP or [γ-^{32}P]GTP (Figure 4). Then, we systematically eliminated either or both the 3′-GTC and 3′-GCC initiation sites (ϕX174 B, ϕX174 C, and ϕX174 D) by replacing the deoxyguanylates with deoxyadenylates. Replacing both deoxyguanylates virtually eliminated the synthesis of all long products and reduced the amount of short products synthesized in assays containing either [γ-^{32}P]ATP or [γ-^{32}P]GTP (Figure 4, ϕX174 D). Conversion of only the GCC site to ACC (template ϕX174 B) had no measurable effect on primer synthesis with a 5′-terminal adenylate; however, primer synthesis with a 5′-terminal guanylate was almost completely obliterated. Nearly the opposite occurred when only the GTC was converted to ATC (template ϕX174 C). Now, primer synthesis with a 5′-terminal adenylate decreased dramatically while primer synthesis with a 5′-terminal guanylate increased slightly (1.4-fold, average from four experiments). Together, these data indicate that two primary sites of HSV-1 primer initiation exist on the ϕX174 50mer, opposite the previously reported deoxycytidylate in the GCC sequence (*1*) as well as opposite the thymidylate in the neighboring GTC sequence.[2] The effects of eliminating the deoxyguanylate in each sequence also provide further evidence of the key role this residue plays during primer synthesis.

To address whether the preference for one primer initiation site versus another on the ϕX174 50mer template was a result of template sequence at the initiation site or position in the template, the positions of the two initiation sites were switched (compare ϕX174 A and ϕX174 E). Similar to the original ϕX174 50mer template, primase initiated synthesis using ATP about nine times more frequently than GTP on ϕX174 A (Figure 4). Interestingly, upon switching the 3′-GCC and 3′-GTC initiation sites (ϕX174 E), the identity of the 5′-terminal nucleotide of newly synthesized primers also switched. Now, primase initiated primer synthesis using GTP about four times more frequently than ATP (Figure 4). Thus, altering the sequences surrounding an initiation site significantly affects the ability of primase to use that site.

Primer Synthesis on Templates with Repeating Deoxyguanylate−(Pyrimidine)$_{2or3}$ Sequences. Rapid primer synthesis on the template d(TCTG)$_{15}$ along with the observation that on the ϕX174-derived templates primer synthesis began at the 3′-GCC and 3′-GTC sequences suggested that a template deoxyguanylate followed by two or three pyrimidines was important for primer synthesis. Therefore, we measured primer synthesis on a series of 60mer templates

[2] Two likely explanations exist for why Tenney and co-workers did not detect this second initiation site (*1*). First, since they mapped initiation sites using the entire ϕX174 DNA, sequences and/or secondary structure outside of the 50mer sequence may have altered initiation specificity. Alternatively, these results could have resulted from mapping initiation sites by elongating the RNA primers using T7 DNA polymerase and then comparing the length of restriction fragments to DNA markers of known length. Considering that the two initiation sites (GTC and GCC) are only separated by five bases on the ϕX174 template and the presence of RNA in a polynucleotide can alter its electrophoretic mobility, RNA/DNA strands resulting from either initiation site would appear nearly identical in length. Furthermore, initiation at the GTC initiation site explains why short primer products ($\ll$10 nucleotides) were observed when Tenney et al. utilized the [α-^{32}P]UTP label with the ϕX174 50mer template (Figure 2B of ref *1*). While initiation at the GCC site does not code for UTP insertion into the primer until the eighth base, initiation at the GTC site codes for UTP insertion at only the third base.

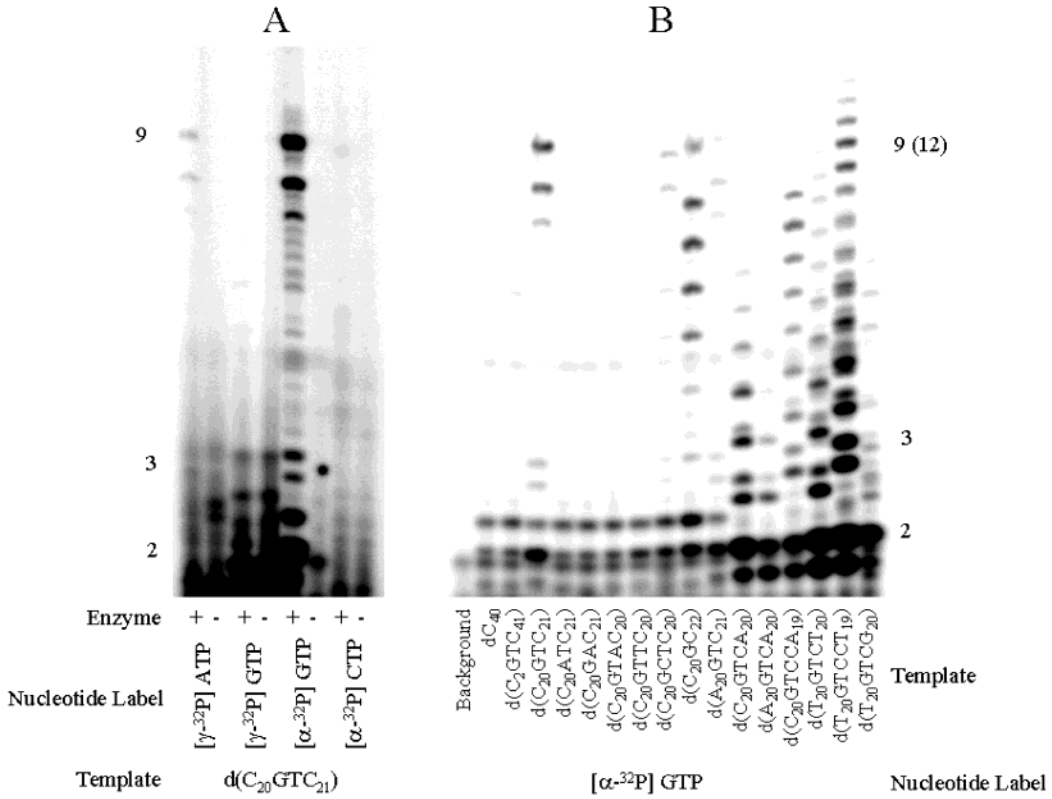

FIGURE 5: (A) Primer synthesis preferentially initiates opposite the template thymidylate of d($C_{20}GTC_{21}$). Assays were performed as described under Experimental Procedures. For each radiolabeled NTP tested, reactions with and without 300 nM enzyme are shown side by side. (B) Effects of varying the sequence of the template d($C_{20}GTC_{21}$) on primer synthesis. Assays were performed as described under Experimental Procedures and contained 300 nM enzyme except the lane labeled background, which lacked enzyme. Quantitation of the rate of primer synthesis for each template is provided in Table 3. The length of primer standards [pppG(pG)$_n$] is noted to the right of the image. Since pppA(pA)$_n$ and pppG(pG)$_n$ of identical lengths migrate differently, the migration of pppA(pA)$_{11}$ is also noted in parentheses.

consisting of repeating 3′-GCCC, GTTT, GTC, GCC, GCT, or GTT sequences. While all of these templates could support primer synthesis, primer lengths and rates of synthesis varied tremendously and in ways not easily rationalized. For example, while d$(GCC)_{20}$ is a relatively poor template for primer synthesis ($v = 4.1$ h^{-1}), d$(GCCC)_{15}$ is an excellent template ($v = 20$ h^{-1}). In contrast, d$(GTT)_{20}$ supports a very high rate of primer synthesis ($v = 62$ h^{-1}), while the rate supported by d$(GTTT)_{15}$ is significantly less ($v = 18$ h^{-1}). The different rates of primer synthesis were likely not due to the experimental conditions since varying the template concentration from 6 to 30 μM had little to no effect on primer synthesis and increasing the NTP concentration from 500 μM to 2 mM only slightly increased the rate of primer synthesis (<2-fold).

Systematic Study of the Effects of Template Sequence on HSV-1 Primer Synthesis. Since ssDNA templates with repeating sequences are likely to contain multiple HSV-1 primer initiation sites, we synthesized a template with a single preferred initiation site to study the effects of template sequence. This template, d($C_{20}GTC_{21}$), contains a single deoxyguanylate previously shown to be critical for primer synthesis. Figure 5A shows that primer synthesis preferentially begins opposite the thymidylate on this template since (1) greater quantities of primers are detected with [γ-^{32}P]-ATP than either [γ-^{32}P]GTP or [α-^{32}P]CTP (direct comparison of band intensities observed for these three labels in Figure 5A is possible since each primer can contain at most one ^{32}P from each [^{32}P]NTP) and (2) the primer products observed with [γ-^{32}P]ATP comigrate with the longer products observed with [α-^{32}P]GTP. The short primers observed with [α-^{32}P]GTP are likely not detected with [γ-^{32}P]ATP since, as described below (Figure 6), the NTPase activity of HSV-1 helicase−primase removes the 5′-γ-phosphate of short primers. Interestingly, the location of the initiation site in the d($C_{20}GTC_{21}$) template is clearly important, since moving the GT sequence to near the 3′ end of the template resulted in negligible levels of primer synthesis [d(C_2GTC_{41}), Figure 5B, Table 3].

We systematically varied the sequence of d($C_{20}GTC_{21}$) and examined the effects on primer synthesis. Those effects are discussed below and are qualitatively shown in Figure 5B. Since the amount of [α-^{32}P]GTP label incorporated into primers (shown in Figure 5B) varies with the coding template sequence, quantitative analysis of the rates of primer synthesis requires one to correct for the number of radiolabeled nucleotides present in the primer (Table 3).

To confirm the critical role of the template deoxyguanylate followed by the two pyrimidines, we replaced the template deoxyguanylate, thymidylate, and following deoxycytidylate of d($C_{20}GTC_{21}$) individually with deoxyadenylate to generate d($C_{20}ATC_{21}$), d($C_{20}GAC_{21}$), and d($C_{20}GTAC_{20}$). In all three cases, primer synthesis was essentially eliminated (Figure 5, Table 3). Primer synthesis levels diminished when changing the TC to TT and CT [d($C_{20}GTTC_{20}$) and d(C_{20}-$GCTC_{20}$)] but remained about the same when changing to CC [d($C_{20}GC_{22}$), Figure 5, Table 3]. Interestingly, of these three templates [d($C_{20}GTTC_{20}$), d($C_{20}GCTC_{20}$), and d(C_{20}-

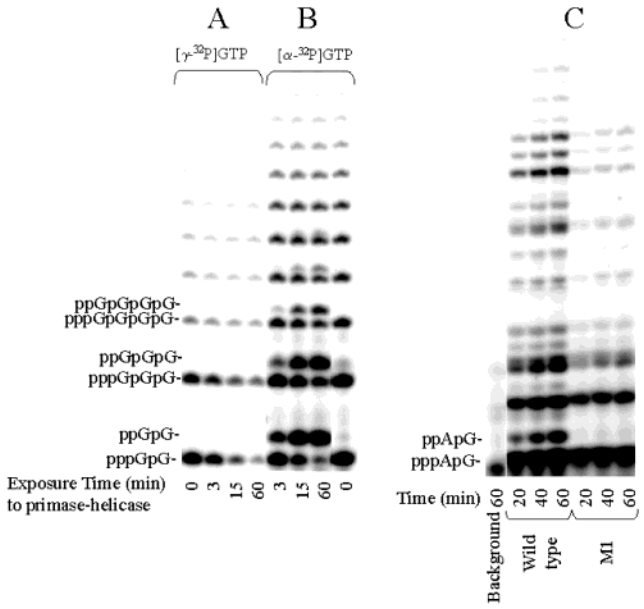

FIGURE 6: The NTPase activity of HSV-1 helicase−primase removes the 5′-terminal phosphate of short primers. A ladder of primers of the sequence $pppG(pG)_n$ were produced on C_3R by human primase as described under Experimental Procedures and then exposed to herpes primase−helicase. In (A), the primers were labeled with [γ-^{32}P]GTP such that only the 5′-terminal nucleotide of $pppG(pG)_n$ was radiolabeled, while in (B) the primers were labeled with [α-^{32}P]GTP such that all nucleotides of $pppG(pG)_n$ were radiolabeled. The products prior to treatment with herpes primase−helicase are also shown (zero exposure time). Control experiments showed that herpes primase−helicase does not synthesize detectable amounts of primers on C_3R (data not shown). In (C), primer synthesis on $d(TCTG)_{15}$ by 100 nM wild-type primase−helicase was compared to 500 nM M1−primase−helicase, a form of the enzyme that lacks NTPase activity. A control reaction lacking enzyme is also shown (background). Primer identities are noted to the left of the images.

Table 3: Rate of Primer Synthesis for Each Template in Figure 5B[a]

template	all primers (h^{-1})	primers >6 nucleotides (h^{-1})
dC_{40}	0.8	0.02
$d(C_2GTC_{41})$	1.0	0.03
$d(C_{20}GTC_{21})$	3.0	0.24
$d(C_{20}ATC_{21})$	0.9	0.02
$d(C_{20}GAC_{21})$	1.1	0.02
$d(C_{20}GTAC_{20})$	1.0	0.03
$d(C_{20}GTTC_{20})$	1.0	0.02
$d(C_{20}GCTC_{20})$	2.1	0.07
$d(C_{20}GC_{22})$	2.8	0.26
$d(A_{20}GTC_{21})$	2.9	0.07
$d(C_{20}GTCA_{20})$	19	0.33
$d(A_{20}GTCA_{20})$	7.6	<0.01
$d(C_{20}GTCCA_{19})$	9.2	0.73
$d(T_{20}GTCT_{20})$	49	0.87
$d(T_{20}GTCCT_{19})$	53	1.9
$d(T_{20}GTCG_{20})$	12	0.25

[a] Rates were obtained from the data in Figure 5B and are given as moles of primer per mole of enzyme per hour. The molar amount of each product was determined using the specific activity of the radiolabel and the number of radiolabeled nucleotides in each length primer.

GC_{22})], only $d(C_{20}GTTC_{20})$ retained the thymidylate adjacent to the deoxyguanylate, and yet, only this template led to nearly undetectable primer synthesis. For each template that resulted in greater than negligible levels of primer synthesis, the site of primer initiation was examined by comparing primer synthesis when labeling with [γ-^{32}P]ATP versus [γ-^{32}P]GTP. The results from those experiments indicate that, despite changes in template sequence, the first pyrimidine following the template deoxyguanylate preferentially codes for the 5′-terminal nucleotide of the primer (data not shown).

Just as varying the sequence of the 3′-deoxyguanylate−pyrimidine−pyrimidine (G-pyr-pyr) initiation sequence significantly impacted primer synthesis, varying the regions flanking this sequence strongly impacted primer synthesis (Figure 5, Table 3). Replacing the first 20 template deoxycytidylates with deoxyadenylates [$d(A_{20}GTC_{21})$] decreased long primer synthesis, whereas replacing the last 20 template deoxycytidylates with deoxyadenylates [$d(C_{20}GTCA_{20})$] both significantly increased total primer synthesis and decreased primer length. A combination of effects was observed when both flanking regions were replaced with deoxyadenylates; not only were the primers shorter but the quantities were increased [in comparison to $d(C_{20}GTC_{21})$]. Since replacing both flanking regions with thymidylate also resulted in a dramatic increase of short primers [$d(T_{20}GTCT_{20})$], we hypothesized that the larger number of relatively weak A·T base pairs generated during primer synthesis on $d(C_{20}GTCA_{20})$ and $d(T_{20}GTCT_{20})$ destabilized interactions between the growing primer and the template. Consistent with this hypothesis, altering the sequences slightly to permit an additional G·C base pair [$d(C_{20}GTCCA_{19})$ and $d(T_{20}GTCCT_{19})$] significantly increased long primer synthesis. Curiously, increasing potential G·C base pairs by replacing the last 20 thymidylates of $d(T_{20}GTCT_{20})$ with deoxyguanylate [to produce $d(T_{20}GTCG_{20})$] decreased primer synthesis considerably. Therefore, although greater G·C base pairing can enhance longer primer synthesis, a large number of deoxyguanylates in the primer synthesis site may be problematic. To control for the possibility that changing the flanking sequences changed where primase initiated primer synthesis, we again compared primer synthesis using either [γ-^{32}P]ATP or [γ-^{32}P]GTP. Primase only generated labeled primers when assays contained [γ-^{32}P]ATP, consistent with primase initiating synthesis opposite the first pyrimidine (thymidylate) after the template deoxyguanylate.

The NTPase Removes the 5′-Phosphate of Short Primers. A curious feature of primer synthesis was that the shorter primer products appeared as "doublets" when assays contained [α-^{32}P]NTPs (e.g., Figure 3), but only the faster migrating species were observed if assays contained [γ-^{32}P]-NTPs (e.g., Figure 5A). To test the hypothesis that this resulted from hydrolysis of the primer 5′-end phosphate by the NTPase activity of the helicase−primase, we synthesized oligonucleotides of the sequence 5′-$pppG(pG)_n$ where either only the 5′-terminal phosphate was radiolabeled or every α-phosphate was radiolabeled (i.e., those phosphates present at internucleotide linkages as well as the α-phosphate of the 5′-terminal nucleotide). When oligonucleotides containing only a 5′-terminal radiolabel were treated with the primase−helicase complex, shorter primers disappeared with time (Figure 6A). In contrast, adding the primase−helicase to $pppG(pG)_n$ where every α-phosphate was radiolabeled resulted in the appearance of the slower moving species (Figure 6B). Importantly, the disappearance of products containing a 5′-terminal radiolabeled phosphate corresponded to the appearance of new products when the α-phosphate was radiolabeled. For example, 91% of the dinucleotide, 68% of the 3mer, 46% of the 4mer, and 37% of the 5mer

Table 4: Rate of ssDNA-Dependent NTPase Activity on Various Templates

template	rate (h^{-1})[a]	template	rate (h^{-1})[a]
dC_{40}	3200	$d(TCTG)_{15}$	1700
dT_{40}	3100	$d(TCTA)_{15}$	2000
$d(C_2GTC_{41})$	2600	$d(GTC)_{20}$	1800
$d(C_{20}GTC_{21})$	2700	$d(GCC)_{20}$	2200
$d(A_{20}GTCA_{20})$	2400	$d(GCT)_{20}$	1400
$d(T_{20}GTCT_{20})$	2200	$d(GTT)_{20}$	830
$d(T_{20}GTCG_{20})$	2600	$d(TCA)_{20}$	2700
$d(CT)_{30}$	2500	$d(CCA)_{20}$	2400

[a] Assays contained 500 μM NTPs and 6 μM DNA.

disappeared after 60 min incubation of the 5′-terminally radiolabeled oligonucleotides with the primase−helicase. Likewise, 92% of the dinucleotide, 69% of the 3mer, 56% of the 4mer, and 34% of the 5mer were converted to slower migrating species after 60 min incubation of the α-radiolabeled oligonucleotides with the primase−helicase. Analysis of the 3 and 15 min time points showed a similar correspondence between the disappearance of the 5′-terminally radiolabeled oligonucleotides and appearance of slower migrating products when the oligonucleotides were α-radiolabeled (data not shown). Importantly, when we repeated these experiments using a mutant form of the primase−helicase complex that lacks detectable NTPase activity (*13*), the $pppG(pG)_n$ primers were not altered (data not shown). Furthermore, the NTPase-deficient primase−helicase did not generate the slower migrating species in primase assays (Figure 6C). Together, these results demonstrate that the NTPase activity of helicase−primase removes phosphate from the 5′ end of short primers. The NTPase activity likely only hydrolyzed the 5′-γ-phosphate since the hydrolyzed dinucleotide migrated slower than pppNpN and faster than pNpN.

ssDNA-Dependent NTPase Activity Is Independent of Template Sequence and Primase Activity. To provide further insights into DNA binding as well as how DNA sequence affects NTPase activity, we examined the ability of DNA templates of defined sequence to support NTPase activity. Similar to previously reported results (*1*), changing the template sequence minimally affected NTPase activity. We measured V_{max}/K_M as a function of NTP concentration on four different templates and found that it remained constant regardless of the ability of the template to support primer synthesis [10 μM^{-1} h^{-1} for dC_{40}, 11 μM^{-1} h^{-1} for $d(CT)_{30}$, 15 μM^{-1} h^{-1} for $d(TCTA)_{15}$, and 15 μM^{-1} h^{-1} for $d(TCTG)_{15}$]. Since these measurements were performed under conditions where primer synthesis could occur (i.e., all four NTPs present), these results show that conditions that allow primer synthesis do not block NTP hydrolysis. The rate of NTPase activity remained constant as the DNA concentration varied from 30 nM to 1 μM, suggesting that the enzyme binds DNA very tightly (data not shown). We also measured NTPase activity on a series of additional templates under a single set of experimental conditions (500 μM NTPs and 6 μM DNA) and found that the rate varied by at most by a factor of 4 (average = 2300 h^{-1}, Table 4). Importantly, even those templates that were poor substrates for primer synthesis were excellent substrates for NTPase activity, thereby indicating that a lack of primer synthesis was not due to a lack of DNA binding to the primase−helicase complex.

DISCUSSION

We used ssDNA templates of defined sequence to examine the effect of template sequence on primase activity and NTPase activity by HSV-1 helicase−primase. While NTPase activity was unaffected by changes in template sequence, primase activity was profoundly dependent upon template sequence. The key feature for a high level of primase activity was at least one 3′-G-pyr-pyr triplet in the template, although flanking sequences play a large role in determining the rate of primer synthesis. Intriguingly, a major role of the template deoxyguanylate appears to be to increase primer length.

Previous studies have indicated that the ssDNA-dependent NTPase activity is nonspecific with respect to ssDNA sequence (*1*). Consistent with these results, we found that, regardless of their ability to support primase activity, all of the tested templates bound to the primase−helicase complex, as evidenced by their ability to stimulate the ssDNA-dependent NTPase activity. Thus, the inability of the helicase−primase complex to produce more than negligible quantities of primers on some templates [e.g., $(dC)_{40}$] could not have resulted from the DNAs not binding the complex. While these templates can bind the complex, it remains unclear where they are bound. For example, $(dC)_{40}$ may bind very tightly to the helicase active site but only very weakly to the primase active site. Hence, a lack of primer synthesis on a specific template could result from where it binds on the complex.

A key template feature for efficient synthesis of long primers is the sequence 3′-G-pyr-pyr. The template G is necessary neither for binding of primase to the DNA nor for initiation of primer synthesis; rather, it is essential for the production of large amounts of long primers. Indeed, primase will initiate primer synthesis on pyrimidine-rich templates in the absence of this G; however, virtually all of the products are only two to four nucleotides long. Thus, a key role of this G is to enhance the elongation of short primers into longer primers. This G also serves to define the specific nucleotide at which primase prefers to initiate primer synthesis, namely, the first pyrimidine following this G, regardless of whether it is a T or C. When templates lack this G [e.g., $d(CT)_{30}$ or $d(TCTA)_{15}$, Figure 2C], primase almost exclusively initiates primer synthesis using ATP as the 5′-terminal nucleotide. This result indicates that, in the absence of a template G, primase either greatly prefers ATP as the 5′-terminal nucleotide or GTP as the nucleotide that becomes the second nucleotide of the primer.

Even though 3′-G-pyr-pyr is sufficient for primase activity, sequences surrounding this trinucleotide can dramatically affect the rate of primer synthesis. To a certain degree, increasing the number of G•C base pairs in the growing primer appears to increase the amount of long primers. More importantly, changes in these flanking regions can dramatically alter the rate of initiation. A previously mapped primer synthesis site was reported to be enriched in deoxycytidylates (*1*). While we found that deoxycytidylate-rich template sequences support primer synthesis, we also observed even higher rates of primer synthesis on some templates containing minimal amounts of deoxycytidylate. For example, replacing the flanking deoxycytidylate regions of $d(C_{20}GTC_{21})$ with thymidylates to generate $d(T_{20}GTCT_{20})$ increased primer synthesis 16-fold. Furthermore, primer initiation also was

not limited to templates solely rich in pyrimidines. For example, replacing the 5′ flanking region of d(C_{20}GTC_{21}) with deoxyadenylate [d(C_{20}GTCA_{20})], thereby leading to a pyrimidine-rich primer rather than a purine-rich primer, resulted in a 6-fold increase in total primer synthesis. Even what appear to be modest changes in the sequence of the flanking regions can greatly affect the rate of primer synthesis. For example, d$(GCCC)_{15}$ supports high levels of primer synthesis, but d$(GCC)_{20}$ does not. Likewise, the parental ϕX174 50mer contains two potential primer synthesis sites that are separated by only five nucleotides. Switching the location of the two initiation sites dramatically alters which initiation site primase prefers to utilize. Finally, previous work showed that, on phage DNA, primase synthesized a primer only once every 3000 nucleotides (*21*), yet the sequence G-pyr-pyr should occur once every 16 nucleotides. Why changes in flanking sequence affect primer synthesis remains obscure, however.

At least some of these effects of flanking sequences must involve specific sequence interactions. For example, the templates d$(GTT)_{20}$ and d$(GTTT)_{15}$ should not have defined secondary structures at 37 °C, yet these two templates with identical GTT trinucleotide recognition sequences vary dramatically in their ability to support primer synthesis. Likewise, neither d(T_{20}GTCCT_{19}) nor d(C_{20}GTC_{21}) should form stable secondary structures, yet they support very different levels of primase activity even though they both contain the same GTC initiation sequence. It would not, however, be surprising if secondary structure also greatly affected primer synthesis.

The mechanism by which herpes primase recognizes a primer recognition site has characteristics of both eukaryotic primase and bacterial and phage primases. Primers synthesized by primases from other sources almost always contain a 5′-terminal purine (*33*), similar to primers synthesized by the herpes enzyme. Eukaryotic primase initiates primer synthesis only at some locations on natural templates such as SV40 and M13 viral DNAs (*34*−*39*), thereby suggesting some sequence/structural requirements near the primer synthesis site and similar to the herpes enzyme (*21*). Significantly, eukaryotic primase has no specific sequence requirement analogous to herpes primase's requirement for 3′-G-pyr-pyr (*2*, *33*). This specific sequence requirement is, however, very similar to the prokaryotic and phage primases (*33*). For example, primases from *Escherichia coli*, T7, and T4 recognize the template sequences 3′-GTC, 3′-CTG, and 3′-TTG (or TCG), respectively (*40*−*42*). In each case, primer synthesis begins at the second nucleotide of the trinucleotide (*40*−*42*), again similar to the herpes enzyme. Compared to the prokaryotic and phage enzymes, herpes primase exhibits a somewhat relaxed specificity with respect to the trinucleotide sequence 3′-G-pyr-pyr. Either deoxycytidylate or thymidylate will suffice as pyrimidines, and the enzyme will recognize deoxyadenylate, albeit much more poorly than deoxyguanylate. The role of the first nucleotide in this trinucleotide sequence for primer synthesis by herpes primase appears to be distinct from the role of this nucleotide with other primases. The deoxyguanylate greatly enhances the production of long primers by herpes primase but is not essential for initiation. In contrast, T7 primase will not even initiate primer synthesis in the absence of the recognition trinucleotide (*43*).

While the primases from different herpes viruses share significant sequence homology (*44*), comparison of the amino acid sequence of herpes primase with those from eukaryotic, phage, and prokaryotic sources reveal essentially no sequence homology. Indeed, the only two motifs that herpes primase shares with other primases are a putative zinc finger motif (*16*) and a DhD (h = hydrophobic amino acid) motif (*14*, *15*). Mutation of these aspartates in the latter motif obliterates the activity of herpes primase (*15*), suggesting that these amino acids help to bind critical divalent metals in the active site, similar to what has been observed with other nucleotide polymerizing enzymes (*45*−*47*). Thus, at the level of both template recognition for primer synthesis and amino acid sequence, herpes primase does not closely resemble any other primases.

Herpes primase synthesizes products from 2 to ca. 13 nucleotides long; however, for many templates, the majority of the products are only 2−3 nucleotides long. Why herpes primase should synthesize such large amounts of short products is unclear. Other RNA polymerases, including the eukaryotic primase and RNA polymerase II, also synthesize large amounts of very short oligonucleotides (*48*, *49*), suggesting that this result is not an artifact of the assay. Biologically, the role of primase is to synthesize primers that a DNA polymerase can then elongate, thereby initiating the synthesis of a new strand of DNA. If the herpes DNA polymerase cannot elongate these short primers, they may simply be waste products of the primase reaction. Indeed, eukaryotic primase synthesizes copious quantities of di- and trinucleotides that pol α cannot elongate (*48*). Alternatively, these short primers may be substrates for the herpes polymerase. For example, phage DNA polymerases will often elongate extremely short primase-synthesized primers (*33*).

The NTPase activity of the primase−helicase complex could bind and hydrolyze the 5′-terminal phosphate from newly synthesized primers. Previous studies have shown that the NTPase prefers to hydrolyze ATP and GTP, but it will also use CTP and UTP, indicating that it is not highly specific (*9*). The studies described herein indicate that this lack of specificity extends beyond the base since the enzyme can use oligonucleotides at least two nucleotides long as substrates.

The ability of the NTPase to hydrolyze primers may also relate to the role of the UL5 subunit in primase activity. Previous studies have demonstrated that the helicase, NTPase, and primase activities have a complex interrelationship. UL5 contains a series of conserved helicase and NTPase motifs (*12*, *13*), and UL52 contains two conserved (d)NTP polymerase motifs (*14*−*16*). However, while the UL5/UL52 complex has primase, NTPase, and helicase activity (*6*, *8*, *10*), the isolated subunits are inactive (*4*, *17*, *18*). Interestingly, mutations in the conserved helicase domains of UL5 that inactivate helicase activity stimulate primase activity by 2- to >30-fold (*13*). Additionally, two recently discovered compounds simultaneously inhibit both helicase and primase activity by apparently forming an enzyme−DNA−inhibitor complex (*50*, *51*). While no high-resolution structures of the primase−helicase exist, the observation that locking DNA onto the complex (via the inhibitors) inhibits both activities is consistent with the idea that a single DNA binding site extends through both the helicase and primase active sites of the UL5/UL52 complex (*16*). Likewise, the ability of the

NTPase active site to interact with the 5′-terminus of a primer raises the possibility that the UL5 subunit is an intimate participant in primase activity. For example, UL5 might interact with the NTP that becomes the 5′-terminus of the incipient primer, thereby helping to define the template specificity of primer synthesis. Experiments to test this hypothesis are in progress.

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