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Herpes Simplex Virus-1 Helicase−Primase: Roles of Each Subunit in DNA Binding and Phosphodiester Bond Formation†

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ABSTRACT: The helicase−primase complex from herpes simplex virus-1 contains three subunits, UL5, UL52, and UL8. We generated each of the potential two-subunit complexes, UL5−UL52, UL5−UL8, and UL52−UL8, and used a series of kinetic and photo-cross-linking studies to provide further insights into the roles of each subunit in DNA binding and primer synthesis. UL8 increases the rate of primer synthesis by UL5−UL52 by increasing the rate of primer initiation (two NTPs → pppNpN), the rate-limiting step in primer synthesis. The UL5−UL8 complex lacked any detectable catalytic activity (DNA-dependent ATPase, primase, or RNA polymerase using a RNA primer−template and NTPs as substrates) but could still bind DNA, indicating that UL52 must provide some key amino acids needed for helicase function. The UL52−UL8 complex lacked detectable DNA-dependent ATPase activity and could not synthesize primers on single-stranded DNA. However, it exhibited robust RNA polymerase activity using a RNA primer−template and NTPs as substrates. Thus, UL52 must contain the entire primase active site needed for phosphodiester bond formation, while UL5 minimally contributes amino acids needed for the initiation of primer synthesis. Photo-cross-linking experiments using single-stranded templates containing 5-iodouracil either before, in, or after the canonical 3′-GPyPy (Py is T or C) initiation site for primer synthesis showed that only UL5 cross-linked to the DNA. This occurred for the UL5−UL52, UL5−UL52−UL8, and UL5−UL8 complexes and whether the reaction mixtures contained NTPs. Photo-cross-linking of a RNA primer−template, the product of primer synthesis, containing 5-iodouracil in the template generated the same apparent cross-linked species.

Herpes simplex virus-1 primase−helicase is one of the major virally encoded complexes essential for herpes DNA replication (*1*). The helicase−primase serves two essential functions during herpes replication. The helicase activity tracks along the lagging strand and unwinds the double-stranded DNA in front of the leading strand DNA polymerase, leaving in its wake single-stranded templates both for the leading strand polymerase and for primase-coupled polymerase activity on the lagging strand. The primase activity synthesizes short RNA primers for a DNA polymerase to further elongate, thereby providing a means of initiating new DNA strands.

The helicase−primase complex consists of three subunits (UL5, UL8, and UL52) and exhibits three activities: primase, helicase, and DNA-dependent ATPase activity (*2*, *3*). This last activity provides the energy necessary for unwinding double-stranded DNA. The UL5−UL52 complex is the minimal subcomplex that has been shown to either synthesize primers on single-stranded DNA or unwind double-stranded DNA (*4*, *5*). The UL5 protein contains seven motifs conserved among superfamily I helicases, including the Walker A and B motifs involved in nucleotide and metal ion binding (*6*). The UL52 subunit contains the conserved DhD (where h is a hydrophobic amino acid) motif found in most nucleotide polymerases and that likely helps chelate a pair of divalent metal ions (*7*), and a zinc-binding motif found in most primases (*8*, *9*).

Even though both UL5 and UL52 contain distinct helicase and primase motifs, respectively, neither subunit has demonstrated catalytic activity in the absence of the other (*4*, *10*, *11*). Moreover, the two activities demonstrate a complex relationship such that they are kinetically and mechanistically entangled. Weller and colleagues showed that eliminating helicase activity by mutating the conserved helicase motifs in UL5 can stimulate primase activity of the resulting complex (*12*). Additionally, some mutations of this Zn^{2+}-binding motif in UL52 primarily inhibit primase activity, whereas others abrogate both primase and helicase activity (*8*, *13*).

The UL8 subunit possesses no known catalytic function but has several proposed functions as an essential herpes DNA replication protein (*10*, *14*−*17*). On single-stranded DNA, the UL5−UL8−UL52 complex has greater activity than the UL5−UL52 complex. Furthermore, coating single-stranded DNA with the herpes single-stranded DNA binding protein ICP8 blocks primase activity by the UL5−UL52 complex, whereas the UL5−UL8−UL52 complex demonstrates robust activity. This effect of UL8 likely involves specific interactions with ICP8 since coating the DNA with *Escherichia coli* single-stranded DNA binding protein blocks primer synthesis by both the UL5−UL52 and UL5−UL8−UL52 complexes. In addition, we found that UL8 enhances primase-coupled polymerase activity (i.e., primase-catalyzed primer synthesis followed by

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polymerase-catalyzed dNTP polymerization) by enhancing primer utilization (*18*).

Herpes primase can initiate primer synthesis (i.e., synthesize the dinucleotide pppNpN from two NTPs) at any pair of template pyrimidines. However, the synthesis of primers more than three nucleotides long requires that primer initiation occur at a 3′-GPyPy-5′ sequence in which the G is cryptic (noncoding) and Py[1] is either pyrimidine (*19*). The presence of a single 3′-GPyPy-5′ in a longer sequence eliminates initiation at any other pair of pyrimidines, showing that the cryptic G directs where primase binds. After initiation of primer synthesis, the efficiency with which the enzyme further elongates the dinucleotide into longer products varies tremendously depending upon the sequences surrounding the initiation site. How the surrounding template sequence alters primer synthesis remains unclear, however.

We used a combination of photo-cross-linking and kinetic assays to explore DNA binding and primer synthesis by the three-subunit primase−helicase complex as well as each potential two-protein subcomplex. While the UL5−UL8 complex lacks detectable DNA-dependent NTPase activity, it cross-links to single-stranded DNA and RNA primer−DNA templates in the absence of UL52. The UL52−UL8 complex catalyzes formation of the phosphodiester bond using a RNA primer−DNA template but cannot initiate primer synthesis de novo. UL8 lacks detectable DNA binding activity but enhances primase activity by stimulating dinucleotide synthesis.

EXPERIMENTAL PROCEDURES

Reagents. Unlabeled NTPs and dNTPs were purchased from Sigma and radiolabeled NTPs and dNTPs from Perkin-Elmer. Synthetic DNA oligonucleotides of defined sequence were obtained from Oligos, etc. or BioSearch Technologies, Inc.; RNA oligonucleotides were obtained from Dharmacon. Oligonucleotide concentrations were determined spectrally and are reported in terms of 5′-termini. All other reagents were of the highest purity available.

Protein Purification. HSV-1 helicase−primase subcomplexes (UL5−UL8 and UL52−UL8), HSV-1 holoenzyme (UL5−UL52−UL8), and UL8 were all expressed in baculovirus-infected *SF9* cells by infecting cells with the appropriate baculoviruses and grown at the Tissue Culture Core Facility at the University of Colorado Health Sciences Center. In each case, the UL8 subunit was His_8-tagged such that the complexes could be purified using Ni-NTA affinity chromatography as previously described (*19*). The UL5−UL52 subcomplex was purified as previously described (*8*). All purified enzymes were identified and analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis, followed by Western blot using monoclonal antibodies for each subunit. The antibodies were generously provided by I. Robert Lehman (Stanford University, Stanford, CA).

Primase Assay. Primase assays (10 uL) were performed as previously described and typically contained 10−15 μM single-stranded DNA template, 0.4−1 mM [α-^{32}P]NTPs, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5% glycerol, 1 mM DTT, and 0.1 mg/mL bovine serum albumin (*20*). Reactions were initiated via addition of 50−100 nM helicase−primase and quenched after 30 min at 37 °C.

[1]Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Py, pyrimidine; Tris-HCl, tris(hydroxymethyl)aminomethane, hydrochloride salt.

Table 1: Effects of UL8 on Total Primer Synthesis Using the Noted Single-Stranded Template in Assays Containing 400 μM NTPs

DNA	*x*-fold stimulation by UL8
$T_{20}GTCCT_{19}$	2.4
$(TCTG)_{15}$	1.9
$(TCTA)_{15}$	4.0
$(TC)_{30}$	4.5
$A_{20}GTCA_{20}$	1.2

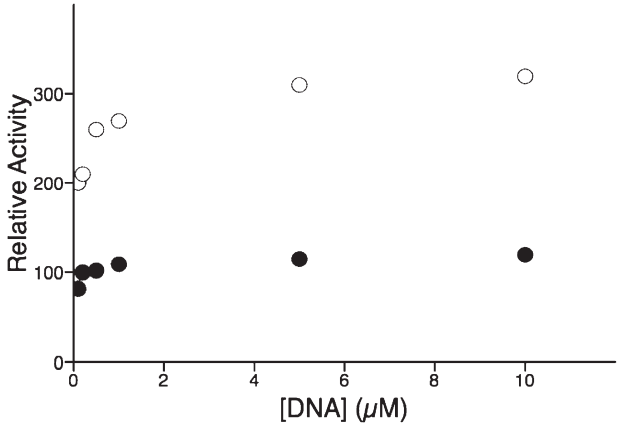

FIGURE 1: Effect of increasing DNA concentrations on the rate of primer synthesis by the UL5−UL52 (●) and UL5−UL52−UL8 (○) complexes.

Primer−Template Elongation. Assays for assessing NTP polymerization onto a RNA primer−template contained a 5′-^{32}P-labeled primer−template and NTPs and were performed as previously described (*21*). Products were separated by denaturing gel electrophoresis (20% acrylamide and 7.5 M urea) and analyzed by phosphorimagery (Molecular Dynamics, Inc.).

DNA-Dependent ATPase. Assays for assessing the DNA-dependent ATPase contained $T_{20}GTCCT_{19}$ and were performed as previously described (*20*).

Cross-Linking Reactions. DNA templates containing 5-iodouracil were 5′-^{32}P-radiolabeled using [α-^{32}P]ATP and T4 polynucleotide kinase as previously described (*22*, *23*). Samples (usually 20−50 μL) typically contained 0.1−2 μM protein, 0.04−2 μM ^{32}P-labeled DNA, 50 mM Tris-HCl (pH 8.0), and 1 mM DTT. Samples were lased at 325 nm for 10−30 min using a Coherent laser as previously described (*24*). Products were separated by denaturing gel electrophoresis (7 or 8% polyacrylamide) and analyzed by phosphorimagery. Cross-linking to a RNA−[^{32}P]template was performed analogously.

Western Blotting of the Cross-Linked Proteins. To isolate cross-linked protein and remove un-cross-linked protein from the photolysis reactions, the 5′-^{32}P-labeled templates used for photo-cross-linking now contained a biotinylated uracil at the 3′-end. Reaction mixtures typically contained 1 μM primase and 1 μM [^{32}P]DNA in a volume of 200 μL. After photolysis, the buffer was brought to 50 mM Tris-HCl (pH ~7.5), 100 mM DTT, 2% SDS, and 10% glycerol. The samples were added to streptavidin−agarose beads (400 μL) pre-equilibrated with 500 μL of 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, and 10% glycerol. These mixtures were incubated for 30 min at room temperature followed by an overnight incubation at 4 °C with gentle mixing on a nutator. The beads were then washed with 7−10×1 mL of the buffer described above. After removal of the wash, the beads were placed in the wash buffer to elute the

Table 2: DNA Sequences Used[a]

$T_{20}GTCCT_{19}$	3′-TTTTTTTTTTTTTTTTTTTTGTCCTTTTTTTTTTTTTTTTTT
$T_{20}GCCCGAT_{14}$	3′-TTTTTTTTTTTTTTTTTTTTGCCCGATTTTTTTTTTTTTT
$(TC)_{30}$	3′-TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
$(TCTG)_{13}$	3′-TCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTG
$(TCTA)_{13}$	3′-TCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTA
$C_{20}GTCCA_{19}$	3′-CCCCCCCCCCCCCCCCCCCCGTCCAAAAAAAAAAAAAAAAAAA
DNA_1	3′-TTTTTTTTTTTTTTTTXTTTTGTCCTTTTTTTTTTTTTTTTTT
DNA_2	3′-TTTTTTTTTTTTTTTTTTTTGXCCTTTTTTTTTTTTTTTTTT
DNA_3	3′-TTTTTTTTTTTTTTTTTTTTGTCCXTTTTTTTTTTTTTTTTTT
DNA_{1A}	3′-TTTTTTTTTTTTTTTTXTTTTATCCTTTTTTTTTTTTTTTTTT
DNA_{2A}	3′-TTTTTTTTTTTTTTTTTTTTAXCCTTTTTTTTTTTTTTTTTT
DNA_{3A}	3′-TTTTTTTTTTTTTTTTXTTTTATCCXTTTTTTTTTTTTTTTTTT
DNA_{1B}	3′-TBTTTTTTTTTTTTTTXTTTTGTCCTTTTTTTTTTTTTTTTTT
DNA_{2B}	3′-TBTTTTTTTTTTTTTTTTTTTGXCCTTTTTTTTTTTTTTTTTT
DNA_{3B}	3′-TBTTTTTTTTTTTTTTTTTTTGTCCXTTTTTTTTTTTTTTTTTT
P/T_1	5′-AAAAAACAGGA 3′-TTTTTTTTTTTTTTTTXTTTTGTCCTTTTTTTTTTTTTTTTTT
P/T_2	5′-AAAAAACAGGA 3′-TTTTTTTTTTTTTTTTTTTTGXCCTTTTTTTTTTTTTTTTTT
P/T_3	5′-AAAAAACAGGA 3′-TTTTTTTTTTTTTTTTXTTTTGTCCXTTTTTTTTTTTTTTTTTT
P/T_4	5′-AAAAAACGGG 3′-TTTTTTTTTTTTTTTTTTTTGCCCGATTTTTTTTTTTTTT
P/T_5	5′-AAAAAACGGG 3′-TTTTTTTTTTTTTTTTTTTTGCCCAGTTTTTTTTTTTTTT
DNA_G	5′- GGGGUAAA 3′-GCCCCATTTGTAATGCATGC-5′

[a] **X** is 5-iodouracil, and **B** is biotinylated deoxyuridine. All primer strands are RNA, and all template strands are DNA.

biotinylated DNA and subjected to denaturing polyacrylamide gel electrophoresis as described above. Western blotting was performed as described previously (*23*, *25*).

RESULTS

To clarify the roles of each subunit in the heterotrimeric helicase−primase complex, we purified each possible dimeric complex and compared its properties to those of the ternary holoenzyme. As described below, this was facilitated by using baculovirus expression systems to generate each complex followed by either classical purification (UL5−UL52 complex) or affinity chromatography (UL5−UL8 and UL52−UL8 complexes).

UL5−UL52 Complex and Stimulation of Primase Activity by UL8. We initially examined how UL8 stimulates the primase activity of the UL5−UL52 complex. Comparing primase activity on several different templates containing a single primer initiation site showed that the extent of stimulation varied from almost no stimulation to 4-fold (Table 1). The greatest stimulation occurred on a template that supports only short primer synthesis due to the lack of a 3′-GPyPy-5′ start site, while UL8 had weaker effects on templates that contained a canonical start site. Stimulation did not change as the DNA concentration varied (Figure 1), consistent with the low K_M for DNA (*19*) and demonstrating that UL8 does not dramatically alter binding of UL5−UL52 to DNA. Since previous studies have shown that initiation (dinucleotide synthesis) limits the overall rate of primer synthesis and the greatest stimulation occurred on templates that support almost exclusively dinucleotide synthesis, UL8 stimulates primer synthesis via enhanced initiation.

In addition to synthesizing primers de novo on single-stranded templates, primase also can directly elongate a RNA primer−template via NTP polymerization (*21*). This latter reaction avoids the initiation step and only requires phosphodiester bond formation. UL5−UL52 and UL5−UL52−UL8 polymerized CTP onto DNA_G (Table 2) with catalytic efficiencies (k_{cat}/K_M) of 0.302 ± 0.002 and 0.43 ± 0.01 h^{-1} mM CTP^{-1}, respectively.

These similar efficiencies indicate that the loss of UL8 does not significantly affect polymerization of a single NTP onto a primer−template.

DNA Binding by the UL5−UL52−UL8 Complex. To improve our understanding of how helicase−primase interacts with DNA and any potential role of UL8, we measured photo-cross-linking of three DNAs containing a photoactivatable cross-linking agent that structurally resembles thymine, 5-iodouracil. The 5-iodouracil was located before, within, or just after the 3′-GPyPy-5′ trinucleotide initiation site (Table 2). Primase assays showed that the 5-iodouracil minimally impacted primer synthesis compared to the identical templates containing thymine at each position both in terms of rate and in terms of the size distribution of products (Figure 2 and data not shown).

Photolysis of the helicase−primase complex and 5′-^{32}P-labeled DNA_1, DNA_2, or DNA_3 at 325 nm gave a series of cross-linked species. A typical gel of the cross-linked species is shown in Figure 3A. All three DNA templates gave similar products, although the relative intensities of the major bands changed depending on the position of the 5-iodouracil in the template sequence. The maximum yield of cross-linking varied by 2−3-fold among the three DNA templates. Reaction mixtures containing 1.4 μM UL5−UL52−UL8 and 200 nM 5′-[^{32}P]DNA_1, -DNA_2, and -DNA_3 gave 25, 9, and 11% cross-linking of the DNA after photolysis for 30 min, respectively. Control experiments showed that omitting photolysis or omitting protein eliminated cross-linking.

DNA−protein cross-linking did not require the cryptic G in the 3′-GPyPy-5′ initiation site (Figure 3B). Cross-linking of templates identical to DNA_1, DNA_2, and DNA_3, except that the cryptic G was replaced with A [i.e., DNA_{1A}, DNA_{2A}, and DNA_{3A}, respectively (Table 2)], gave similar major cross-linked

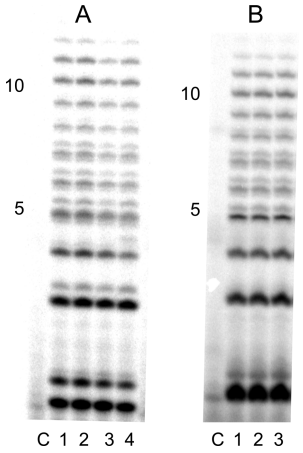

FIGURE 2: Primase activity on both normal and modified templates. Assays were performed as described in Experimental Procedures: (A) lane 1, $T_{20}GTCCT_{19}$; lane 2, DNA_1; lane 3, DNA_2; lane 4, DNA_3; lane C, no enzyme; and (B) lane 1, $T_{20}GTCCT_{19}$; lane 2, DNA_1; lane 3, DNA_{1B}; lane C, no enzyme. Primer length is noted on the side of each gel.

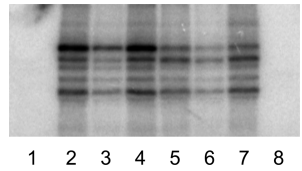

FIGURE 4: Photo-cross-linking of UL5−UL52−UL8 to primer−templates. In lanes 1 and 8, the photolysis reaction mixtures contained DNA_2 or P/T_2 and no enzyme. Lanes 2−7 contained UL5−UL52−UL8 (2 μM) and either DNA_2 (lane 2), P/T_2 (lanes 3 and 4), DNA_3 (lane 5), and P/T_3 (lanes 6 and 7). The concentration of DNA was 2 μM. Reaction mixtures in lanes 3 and 6 were photolyzed for 10 min, while all other reaction mixtures were photolyzed for 20 min.

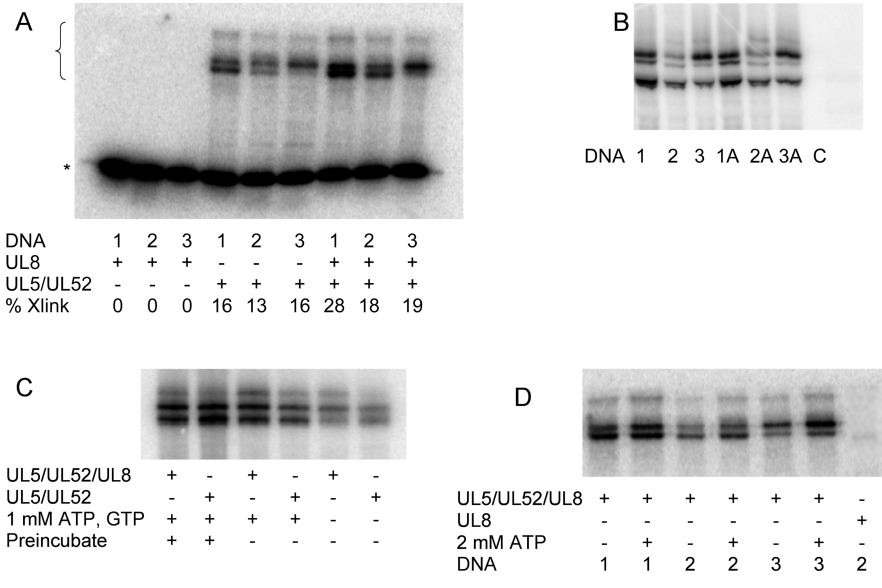

FIGURE 3: (A) Photo-cross-linking of DNA_1, DNA_2, or DNA_3 to UL8, UL5−UL52, or UL5−UL52−UL8. Photo-cross-linking was performed as described in Experimental Procedures in reaction mixtures containing the noted protein (280 nM) and the noted 5′-^{32}P-labeled DNA (40 nM). Cross-linked products are noted with the bracket, and the unreacted DNA is noted with an asterisk. The % Xlink data are the percentages of the DNA cross-linked. (B) Effect of eliminating the canonical 3′-GPyPy-5′ initiation site for primer synthesis. The gel shows the products of UL5−UL52−UL8 (840 nM) cross-linking to templates (120 nM) containing or lacking the 3′-GPyPy-5′ start site. Each reaction mixture contained the noted DNA. Lane C contained no protein. (C) Effect of NTPs on cross-linking. Reaction mixtures contained 100 nM DNA_2 and the noted enzyme and NTPs (350 nM each). In the first two lanes, the enzyme, NTPs, and DNA were preincubated for 20 min to allow primer synthesis prior to photolysis. (D) Effect of one NTP (ATP) on cross-linking. Reaction mixtures contained either 280 nM UL5−UL52−UL8 or 2.8 μM UL8 and 40 nM indicated DNA (5′-^{32}P-labeled) in the presence or absence of 2 mM ATP.

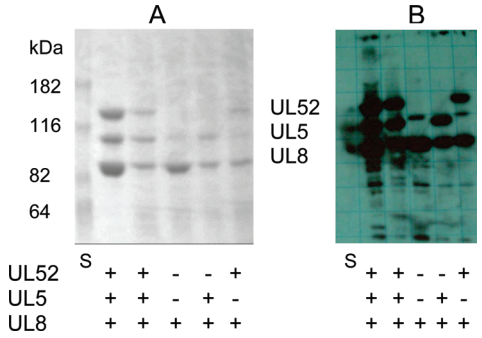

FIGURE 5: Purification of UL5−UL8 and UL52−UL8. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gels (7% acrylamide) of proteins purified from SF9 insect cells infected with baculoviruses for the noted proteins and probed with either Coomassie Blue staining (A) or Western blotting using a polyclonal antibody raised against UL5−UL52−UL8 (B). Molecular weights for the Benchmark Prestained Protein Ladder from Invitrogen (lane S) and the UL5, UL52, and UL8 subunits are shown at the sides of the gels.

products as did the templates containing the cryptic G. Thus, cross-linking of the helicase−primase to DNA does not require an initiation site, although the cryptic G may subtly affect how the protein binds to the DNA.

Since the helicase uses NTPs to power DNA unwinding and primase uses NTPs as substrates, we considered the effects of NTPs on cross-linking. Panels C and D of Figure 3 show that adding a single NTP to allow an active helicase but not allow primer synthesis or adding two NTPs to allow both primer synthesis and an active helicase did not alter the primary cross-linked species, although the band intensities varied slightly. Likewise, incubating the enzyme with ATP and GTP to allow primer synthesis prior to photolysis also did not alter the cross-linked species.

We synthesized a series of RNA primer−templates, the products of primase activity, based on DNA_{1-3} [i.e., P/T_{1-3} (Table 2)] and examined their ability to cross-link to UL5−UL52−UL8. Figure 4 shows that these constructs efficiently react with the protein complex but again gave similar patterns of bands as did the single-stranded templates alone.

UL8 Does Not Affect Cross-Linking of DNA to UL5−UL52. Previous studies using gel shift assays suggested that UL8 did not bind DNA (*14*, *26*). Consistent with these results, photolyzing 280 nM UL8 with any of the DNAs containing iodouracil at 40 nM resulted in no detectable cross-linking products (Figure 3A). At an extremely high UL8 concentration (2.8 μM), an extremely faint band results from photolysis with DNA_2 or DNA_3 (Figure 3D). By comparison, cross-linking with only 280 nM UL5−UL52−UL8 gives >40-fold more cross-linked products (Figure 3D). As a second probe of UL8 cross-linking to DNA, we compared the UL5−UL52 and UL5−UL52−UL8 complexes. Panels A and C of Figure 3 shows that both complexes gave a similar cross-linking pattern under several different conditions, and Western blotting of the products with an anti-UL8 antibody gave no detectable signal at the position of the cross-linked species (data not shown). Additionally, the extent of cross-linking was similar for both UL5−UL52 and UL5−UL52−UL8 (Figure 3). Thus, UL8 does not greatly alter how the DNA binds to UL5−UL52 and by itself reacts at best extremely weakly with iodouracil in DNA.

UL5−UL8 Complex. To determine if UL5 and UL8 form a stable and isolatable complex, we co-infected insect cells with

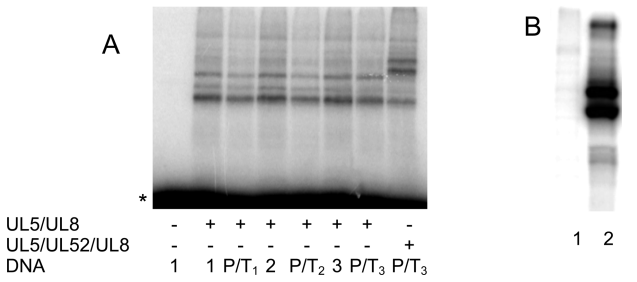

FIGURE 6: Cross-linking of DNA to UL5−UL8 or UL52−UL8. (A) Photolysis reactions were performed as described in Experimental Procedures, and the mixtures contained either 500 nM UL5−UL8 or 500 nM UL5−UL52−UL8 and the 5′-^{32}P-labeled DNA (500 nM) as noted. The primer−templates were labeled on the primer strand. (B) Photolysis reaction mixtures contained either 1 μM UL52−UL8 (lane 1) or UL5−UL52−UL8 (lane 2) and 1 μM DNA1.

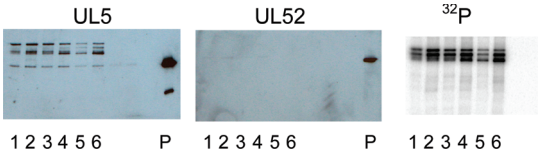

FIGURE 7: Western blots of cross-linked proteins. Photolysis reactions and separation of cross-linked protein from un-cross-linked protein was performed as described in Experimental Procedures. Reaction mixtures contained 350 nM protein and 100 nM 5′-[^{32}P]DNA_{1B}. The images show the cross-linked products generated from photolysis of [^{32}P]DNA_{1B} with UL5−UL52−UL8 (right side) and the results of a Western blot using a mAb against UL5 (left) and a mAb against UL52 (center): lane 1, UL5−UL52−UL8; lane 2, UL5−UL52; lane 3, UL5−UL52−UL8 and 1 mM ATP and GTP; lane 4, UL5−UL52 and 1 mM ATP and GTP; lane 5, UL5−UL52−UL8 and 1 mM ATP and GTP with a 20 min preincubation prior to photolysis; and lane 6, UL5−UL52 and 1 mM ATP and GTP with a 20 min preincubation prior to photolysis. The lane labeled P contained UL5−UL52.

baculoviruses encoding UL5 and His_8-UL8 and purified the resulting proteins via Ni^{2+}-NTA affinity chromatography. Figure 5 shows that this resulted in purification of the UL5−UL8 complex, and the yield of complex was comparable to that of either His_8-UL8 alone or the UL5−UL52−UL8 ternary complex (0.51 mg of UL5−UL8, 0.27 mg of UL8, and 0.42 mg of UL5−UL52−UL8 from 2×10^7 cells). Thus, UL5 and UL8 stably interact, and the resulting complex is readily purified.

Previous studies reported that the UL5 subunit lacks catalytic activity in the absence of UL52 (*4*, *5*). Likewise, we found that the UL5−UL8 complex also lacked detectable catalytic activity in three different assays: DNA-dependent ATPase, primer synthesis on single-stranded DNA, and polymerization of NTPs onto a RNA primer−template (<0.01% of the activity of UL5−UL52−UL8).

An inability to bind DNA and/or altered DNA binding by the UL5−UL8 complex could explain the lack of DNA-dependent ATPase activity by the UL5−UL8 complex. Thus, we probed DNA binding using the DNA templates containing 5-iodouracil as described above. UL5−UL8 clearly binds DNA, as evidenced by the cross-linking between DNA_{1-3} and UL5−UL8 (Figure 6). However, the pattern of cross-linked products varied substantially between the UL5−UL8 and UL5−UL52−UL8 complexes, indicating that UL52 affects how the helicase−primase complex binds DNA (Figure 6). Cross-linking of a RNA primer−template, P/T_1, P/T_2, or P/T_3, gave cross-linked products identical to those using just single-stranded DNA (Figure 6), and

Table 3: Comparison of RNA Primer−Template Elongation by UL5−UL52−UL8 and UL52−UL8

protein	primer−template	k_{cat} (h^{-1})	K_M(NTP) (μM)	k_{cat}/K_M ($h^{-1}\,\mu M^{-1}$)
UL5−UL52−UL8	P/T_4	8.1 ± 0.1	64 ± 4	0.13
UL52−UL8	P/T_4	2.3 ± 0.3	450 ± 180	0.0051
UL5−UL52−UL8	P/T_5	7.3 ± 0.3	190 ± 30	0.038
UL52−UL8	P/T_5	0.58 ± 0.04	280 ± 50	0.0021

the absence of UL52 also altered the cross-linking pattern of the protein with primer−templates.

UL52−UL8 Complex. To determine if the UL52−UL8 complex could be stably expressed, we co-infected insect cells with baculoviruses encoding UL52 and His_8-UL8 and purified the resulting proteins via Ni^{2+}-NTA affinity chromatography. Figure 5 shows that the purified complex contains both UL52 and UL8, and the UL52−UL8 yield is comparable to the yield of either UL8 alone or the ternary UL5−UL52−UL8 complex (0.43 mg of UL52−UL8, 0.27 mg of UL8, and 0.42 mg of UL5−UL52−UL8 from 2×10^7 cells). Thus, UL52 and UL8 form a stable complex.

The catalytic properties of the UL52−UL8 complex were analyzed using three different assays: DNA-dependent ATPase, primer synthesis on a single-stranded template, and elongation of a RNA primer−template. Consistent with previous work indicating that UL5 contains the ATPase activity and primase activity requires UL52 and UL5 (*4*−*6*), the UL52−UL8 complex lacked detectable ATPase activity and could not synthesize primers on single-stranded templates (< 0.01% of UL5−UL52−UL8). However, UL52−UL8 did elongate RNA primer−templates via polymerization of the next required NTP. UL52−UL8 elongated primer−templates ~20−25-fold less efficiently than UL5−UL52−UL8 (k_{cat}/K_M), due both to an increased $K_{M(NTP)}$ and a decreased k_{cat} (Table 3). Importantly, these results indicate that UL52 contains all of the amino acids required for phosphodiester bond formation.

UL52−UL8 must bind a RNA primer−template to catalyze phosphodiester bond formation. However, the inability of this complex to synthesize a primer on single-stranded DNA might have resulted from an inability to bind single-stranded DNA. To exclude this possibility and test the extent to which DNA sequence affects the affinity of UL52−UL8 for DNA, we measured the effect of adding single-stranded DNA to assays that measured elongation of a primer−template. If UL52−UL8 could not bind single-stranded DNA, then adding single-stranded DNA to these assays should have no effect. However, the single-stranded DNA did inhibit primer−template elongation, indicating that UL52−UL8 does bind single-stranded DNA. Single-stranded DNA inhibits elongation of primer−templates by both UL5−UL52−UL8 and UL52−UL8 with similar potency (Table 4), suggesting that the loss of UL5 does not greatly affect binding of single-stranded DNA to UL52. Additionally, the presence or absence of a canonical GPyPy initiation sequence did not affect binding of the single-stranded DNAs, indicating that this feature is not a major determinant of DNA binding by UL52.

We next asked if we could detect cross-linking of DNA to UL52 within the context of the UL52−UL8 complex. However, even with high concentrations of DNA and UL52−UL8, at most trace amounts of DNA cross-linked to the protein (in Figure 6B, compare cross-linking to UL52−UL8 and UL5−UL52−UL8).

Table 4: Inhibition of Primer−Template Elongation by Single-Stranded DNAs

single-stranded DNA	enzyme	IC_{50} (μM)
$T_{20}GC_3AGT_{14}$	UL5−UL52−UL8	2.9 ± 0.6
$T_{20}GC_3AGT_{14}$	UL52−UL8	2.3 ± 0.5
$T_{20}GC_3GAT_{14}$	UL5−UL52−UL8	3.5 ± 0.5
$T_{20}GC_3GAT_{14}$	UL52−UL8	1.6 ± 0.9
$(TTC)_{20}$	UL52−UL8	1.6 ± 0.2
$(CCT)_{20}$	UL52−UL8	1.3 ± 0.5

DNA Cross-Links to UL5. We determined the subunit(s) of the UL5−UL52−UL8 complex to which DNA cross-linked by Western blotting. Since UL5, UL8, and UL52 all have similar molecular masses (99, 80, and 114 kDa, respectively) and covalently bound DNA could affect the electrophoretic mobility of the cross-linked products, we first separated the cross-linked protein from the native protein. To accomplish this, the DNA templates for these studies contained both a 5-iodouracil and a biotin near its 3′-terminus [DNA_{1B}−DNA_{3B} (Table 2)]. Control experiments showed that the biotin did not affect primase activity (Figure 2). To eliminate un-cross-linked protein prior to Western blotting, the photolyzed primase−helicase complex was first denatured, the biotinylated DNA and any covalently bound proteins were captured onto streptavidin−agarose beads, and the beads washed to remove un-cross-linked proteins. Finally, the products bound to the beads were analyzed by denaturing polyacrylamide gel electrophoresis and phosphorimagery. This purification procedure efficiently captured any protein that cross-linked to the DNA as evidenced by the following. (i) Analysis of the material that did not bind to the column showed that it lacked any detectable cross-linked species, only trace amounts of ^{32}P that comigrated with the input [^{32}P]DNA. (ii) The relative amounts of each cross-linked species captured by the beads were the same as the relative amounts prior to capture.

Western blot analysis of the cross-linked products using antibodies to UL5 and UL52 showed that all of the major cross-linked species contained UL5, with no detectable UL52 (Figure 7, cross-linking with DNA_{1B}). As noted previously, we were unable to detect cross-linking of DNA to UL8. The Western blot also shows only trace amounts of un-cross-linked protein, demonstrating the effectiveness of the purification. Similarly, we generated biotinylated versions of DNA_{2B} and DNA_{3B} and found that all of the cross-linked species contained only UL5 as detected by Western blotting (not shown).

The relative abilities of the anti-UL5 and anti-UL52 antibodies to detect UL5 and UL52, respectively, were compared to provide a limit of detection. Western blots with different amounts of UL5 and UL52 loaded onto the gel showed that the anti-UL52 antibody could detect ≤0.75 fmol of protein under the conditions used while the anti-UL5 antibody could detect ≤0.075 fmol of protein (data not shown). The amount of sample loaded onto the gel for Western blotting contained approximately 37 fmol of cross-linked protein for the samples containing UL5−UL52−UL8 and 20 fmol of cross-linked protein for the samples containing UL5−UL52. The lack of a detectable signal from the Western blot with the UL52 antibody in combination with the data showing that all of the cross-linked species contain UL5 indicates that only UL5 reacts significantly with the DNA, even though the primase active site resides within UL52.

DISCUSSION

Herpes primase−helicase consists of three subunits whose precise roles in primase and helicase activity have not been well-defined. Here, we used a combination of kinetic and DNA cross-linking approaches to better define the roles and catalytic properties of each subunit.

All three potential two-protein subcomplexes of the ternary UL5−UL52−UL8 helicase−primase complex can be readily expressed and purified using a baculovirus expression system. The yields of UL5−UL8 and UL52−UL8 were comparable to the yields of UL8 alone or the UL5−UL52−UL8 complex, indicating that the absence of one subunit does not significantly impede expression and folding of either UL5 or UL52 in the presence of UL8. Purification of UL5−UL52 cannot be directly compared to that of these other complexes since it employs a very different purification protocol (*8*).

The ability to purify the UL5−UL8, UL52−UL8, and UL5−UL52 complexes indicates that each subunit of the primase-helicase complex binds to the other two subunits with sufficiently strong interactions to allow purification of each complex. Thus, UL8 binds to a remarkable number of viral proteins, including UL5, UL52, ICP8, UL9, and UL30 (*15*, *16*, *27*, *28*). Stow and colleagues used UL8 deletion mutants to identify regions important for the interaction with UL5 and UL52 and found that residues 340−470 of UL8 likely play critical roles (*28*). It is unknown, however, if all of the protein−protein interactions can occur simultaneously and if different interactors share a common binding domain on UL8.

Previous studies have suggested that the primase and helicase/DNA-dependent ATPase activities require both UL5 and UL52, although these studies did not explicitly examine purified UL5 or UL52 (*3*, *4*). Consistent with helicase/ATPase requiring both UL5 and UL52, the UL5−UL8 complex showed no detectable DNA-dependent ATPase activity. The UL5−UL8 and UL5−UL52−UL8 complexes gave distinctly different cross-linking patterns to DNA, indicating that UL52 alters the binding of DNA to UL5−UL8 as compared to UL5−UL52−UL8. How binding changes, however, cannot be derived from these data. Potentially, this altered DNA binding could account for the lack of ATPase activity. Consistent with this idea, mutations in the Zn^{2+} binding domain of UL52, a domain that likely binds DNA, also affect helicase activity (*8*). It should be noted that the changes in cross-linking pattern do not rule out the possibility that UL52 also contributes catalytically essential residues for helicase/DNA-dependent ATPase activity.

Primer synthesis on single-stranded DNA, a reaction that requires rate-limiting dinucleotide synthesis followed by polymerization of additional NTPs, also requires both UL5 and UL52. However, if one considers only the phosphodiester bond forming reaction (i.e., polymerization of a NTP onto a primer−template), then UL52 alone suffices. UL52 must, therefore, contain the entire primase active site for NTP polymerization but lacks some critical residues for initiation. Potential roles of UL5 during initiation include proper positioning of the DNA, perhaps including an interaction with the cryptic template G, or binding the NTP that becomes the 5′-terminal nucleotide of the primer. The active sites of DNA polymerases and primases typically contain three critical aspartates that chelate two essential divalent metal ions (*29*−*33*), suggesting that the herpes primase active site will also contain three essential aspartates. Two of the critical aspartates, D628 and D630, have been identified, while the identity of the third remains unknown (*7*). The ability of UL52 to catalyze phosphodiester bond formation indicates that the third aspartate also resides within UL52. Even though the complex analyzed contains UL8, UL8 cannot contain the third aspartate since primase activity does not require UL8.

This phenomenon of the catalytic subunit of primase catalyzing phosphodiester bond formation but not dinucleotide synthesis provides another commonality between herpes and human primase. Human primase also consists of two subunits, p58 and p49 (*9*). p49 contains the active site and can elongate primer−templates by itself but cannot initiate primer synthesis without p58 (*34*). p49 and UL52 also exhibit mild sequence similarity but, more significantly, appear to discriminate between right and wrong NTPs and between different sugars using virtually identical mechanisms (*21*, *35*−*37*).

In all of the cross-linking studies, we detected cross-linking to only UL5, even in the UL52−UL8 complex lacking UL5. This occurs even though UL52 can bind primer−templates and single-stranded DNA within the context of both the UL5−UL52−UL8 and UL52−UL8 complexes, as evidenced by our kinetic studies. Thus, the absence of cross-linking to UL52 within the context of the UL5−UL52 (with or without UL8) complex likely did not result from the DNA primarily binding to UL5. Rather, these data indicate that the DNA binding domain(s) in UL52 lacks amino acids to which 5-iodouracil can cross-link when probed using single-stranded DNA and primer−templates. Upon photoactivation, 5-iodouracil has only been observed to cross-link with Phe, Tyr, His, and Met (*38*). Thus, the DNA binding domain of UL52 likely lacks these amino acids in near proximity and/or in the appropriate orientation that would allow cross-linking to 5-iodouracil.

Regardless of the location of the 5-iodouracil in the template, the same primary cross-linked species resulted upon photolysis of the UL5−UL52 and UL5−UL52−UL8 complexes. The multiple species generated indicate that several different amino acids in UL5 can react with the photoactivated 5-iodouracil, and the apparently identical species observed with each template indicate that the same set of amino acids always reacts. This occurred whether (1) the reaction mixtures contained NTPs, (2) the 5-iodouracil was contained within a single-stranded template with or without the cryptic G needed for efficient synthesis of long primers, and (3) the 5-iodouracil was in a primer−template. The relative amount of each cross-linked species did, however, vary somewhat as the aforementioned parameters were varied. These results suggest that when bound in the UL5 DNA binding site(s), the different conditions do not change the amino acids with which the 5-iodouracil can react, although the accessibility of these amino acids may vary. Perhaps the simplest mechanism to account for this result is that the DNA can slide within the DNA binding domain. Additionally, the DNA binding domain appears to bind both single-stranded DNA and a RNA−DNA duplex since both nucleic acids give the same cross-linked species.

Weller and co-workers reported that within the context of the primase−helicase complex, UL52 can cross-link to a forked DNA substrate containing 5-iodouracil (*12*). This raises the possibility that when engaged at a forked junction, the DNA binding domain in UL52 undergoes a structural rearrangement that exposes reactive amino acids or that a forked DNA binds in a different portion of the UL52 DNA binding domain than either single-stranded DNA or a primer−template.

UL8 is a key component of the herpes replication apparatus, interacting with multiple proteins and stimulating the catalytically

active UL5−UL52 subcomplex. Comparing primer synthesis by the UL5−UL52 and UL5−UL52−UL8 complexes showed that stimulation resulted from increased rates of primer initiation. Consistent with UL8 primarily affecting initiation, UL8 had little effect on primer−template elongation by UL5−UL52. UL8 did not greatly affect binding of UL5−UL52 to DNA, as evidenced by similar extents of stimulation at different DNA concentrations and the similar patterns of cross-linking to DNA. Previous electromobility shift assays suggested that the UL5−UL52−UL8 complex binds DNA only slightly tighter than does the UL5−UL52 complex (*12*), consistent with the cross-linking studies (Figure 3). UL8 also did not alter the length of products synthesized on single-stranded DNA, indicating that it does not affect the ability of primase to count. Thus, the effects of UL8 on primer synthesis appear directed solely to increasing either a conformational change or phosphodiester bond formation associated with dinucleotide synthesis. Consistent with this conclusion, UL8 did not affect the ability of the UL5−UL52 subcomplex to polymerize a single NTP onto a RNA primer−template.

While both UL5 and UL52 contain DNA binding domains, their location and how they interact remain unclear. Weller and colleagues have shown that the primase and helicase activities exhibit a remarkably complex interdependence (*12*). How UL5 and UL52 communicate mechanistically remains unclear, but interacting DNA binding domains in the two proteins would help explain this interdependence. Indeed, a contiguous DNA binding domain would allow for direct information transfer between UL5 and UL52.

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