HERPES SIMPLEX VIRUS TYPE 1 HELICASE-PRIMASE: DNA BINDING AND
CONSEQUENT PROTEIN OLIGOMERIZATION AND PRIMASE ACTIVATION

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ABSTRACT

The heterotrimeric helicase/primase complex of herpes simplex virus type I (HSV-1), consisting of UL5, UL8, and UL52, possesses 5' to 3' helicase, ssDNA-dependent ATPase, primase and DNA binding activities. In this study, we confirm that the UL5/8/52 complex has higher affinity for forked DNA than for ssDNA and fails to bind to fully annealed dsDNA substrates. In addition, we show that a single-stranded overhang of greater than 6nt is required for efficient enzyme loading and unwinding. Electrophoretic mobility shift assays and surface plasmon resonance analysis provide additional quantitative information about how the UL5/8/52 complex associates with the replication fork. Although it has previously been reported that in the absence of DNA and NTPs, the UL5/8/52 complex exists as a monomer in solution, we now present evidence that in the presence of forked DNA and AMP-PNP, higher order complexes can form. Electrophoretic mobility shift assays reveal two discrete complexes with different mobilities only when helicase/primase is bound to DNA containing a single-stranded region, and surface plasmon resonance analysis confirms higher amounts of the complex bound to forked substrates compared to single overhang substrates. Furthermore, we show that primase activity exhibits a cooperative dependence on protein concentration, while ATPase and helicase activities do not. Taken together, these data suggest that the primase activity of the helicase/primase requires formation of a dimer or higher order structure, while ATPase activity does not. Importantly, this provides a simple mechanism for generating a two-polymerase replisome at the replication fork.
INTRODUCTION

Replication of DNA genomes is a highly coordinated process that guarantees accurate and efficient inheritance of genetic information. Viruses provide important models for studying the molecular mechanisms involved in eukaryotic DNA replication and its regulation. In fact, much of what we know about cellular DNA replication has come from studying viral systems. Furthermore, viral enzymes involved in replication provide clinically useful targets for antiviral therapy against many viral pathogens. Herpes simplex viruses encode seven viral proteins required for viral DNA replication - an origin binding protein (UL9), a single strand binding protein (ICP8), a two-subunit polymerase (UL30/UL42) and a three-subunit helicase/primase (H/P) complex (UL5, UL8 and UL52). The viral polymerase and H/P complex proteins have both been exploited as targets for antiviral therapy (reviewed in (17)).

During HSV-1 replication, the origin binding protein UL9, in conjunction with the viral ssDNA binding protein, ICP8, is believed to interact with an HSV origin causing an initial distortion (6, 8). By analogy with other well-characterized replication systems, the heterotrimeric HSV-1 H/P complex, is believed to be recruited to the replication fork, where it subsequently unwinds the duplex DNA (helicase activity) and synthesizes short RNA primers to initiate DNA replication (primase activity) (7, 15, 39). Several lines of evidence suggest that, in addition to enzymatic functions, HSV-1 H/P acts as a scaffold for recruitment of viral proteins to pre-replicative sites leading to the formation of replication compartments (9, 13, 37, 50). In addition to interactions among the subunits of the H/P itself, UL5, UL8 and UL52 have also been reported to interact with other replication proteins such as UL9, ICP8 and UL30/42 (7, 10, 15, 23, 27, 35, 39, 40, 42, 47). Thus, the H/P complex is thought to play a critical role in assembly of
the replication machinery at the replication fork as well as in the replication process itself.

Despite the recognition more than two decades ago that UL5, UL52 and UL8 comprise the HSV H/P (19, 20), many questions remain regarding the mechanism of action of this complex at the replication fork. For instance, an unambiguous assignment of functions to the individual subunits has been complicated by the fact that UL5 and UL52 are functionally interdependent. It is known that UL5 contains seven motifs that are conserved in other helicase superfamily I proteins and that mutations in these motifs abolish ATPase and helicase activity of the H/P complex (25, 52). UL52 contains an internal DXD motif that is highly conserved in different primases. Mutations in this motif abolish the primase activity but not the helicase activity of the H/P (22, 31). On the other hand, mutations in the UL52 zinc finger motif affect DNA binding of the entire complex, and mutations in UL5 affect primase activity (4, 16). Furthermore, mutations causing resistance to H/P inhibitors have been mapped to both UL5 and UL52 subunits, suggesting that interactions of these two subunits create a composite substrate binding surface (2, 5, 30, 36). Recent studies with subcomplexes containing various subsets of the H/P subunits suggest that UL52/UL8 complex can polymerize NTPs onto a RNA primer-template, indicating that UL52 contains the active site for phosphodiester bond formation (14). However initiation of primer synthesis on ssDNA, requires a UL5/UL52 subcomplex suggesting that UL5 contributes residues essential for the initiation of primer synthesis. Taken together these observations indicate that UL5 and UL52 likely encode the helicase and primase subunits, respectively, as originally suggested (21); however, the functional interdependence between the UL5 and UL52 subunits (3, 14, 16, 25) presents interesting challenges in terms of mapping functional domains such as DNA binding sites.

The UL52 subunit contains a C-terminal zinc finger motif that is conserved in prokaryotic, eukaryotic and viral primases (28, 43). By analogy with the T7 primase, the zinc finger motif may function in sequence-specific DNA recognition (29, 33, 34). As mentioned above, mutations
in this region abolish not only primase activity but also DNA binding and helicase activities suggesting that at least one DNA binding site in the H/P complex resides within UL52 (3, 16). It is likely that at least one additional DNA binding site exists within the UL5 helicase subunit itself. The functional interdependence between UL5 and UL52 may reflect the presence of shared DNA binding sites between these two subunits.

A major unanswered question relates to how the helicase and primase activities are coordinated at the replication fork. For instance, the UL5 helicase tracks along the lagging strand with 5' to 3' polarity (19, 20), but the primase would be expected to synthesize primers in the opposite direction. Interestingly, primase activity can be detected in assays that use a single strand oligonucleotide as a substrate; however, primase activity is inefficient in assays using forked substrates (Graves-Woodward and Weller, unpublished data; Ramirez-Aguilar and Kuchta, unpublished data). In this paper, we demonstrate that primase activity exhibits cooperative dependence on protein concentration, while ATPase and helicase activities do not. These results suggest that primase activity requires multimerization of the H/P complex, while helicase activity does not. Models for H/P function at the replication fork will be discussed.
MATERIALS AND METHODS

Materials and reagents - Wild type UL5/8/52 complex was expressed in insect cells co-infected with recombinant baculoviruses encoding UL5, his-tagged UL8, and UL52 (45). The heterotrimeric complex was purified on a HIS-Select nickel affinity column (purchased from Sigma) as previously described (16). DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). 32P-labeled NTPs were purchased from Perkin-Elmer Life Science (Boston, MA).

Substrate Preparation – Structures and sequences of the artificial substrates for each experiment are described in the individual figures. Single stranded oligonucleotides were 5’ end-labeled using [γ-32P] ATP (6000 (222 TBq) Ci/mmol) and polynucleotide kinase (New England Biolabs). To generate duplex, triplex or cruciform structures, labeled oligos were annealed to one or more unlabeled DNA oligo(s) at a ratio of 1:3 in 50mM Tris-HCl (pH 8.0), 80mM KCl, and 1mM dithiothreitol (DTT). Components were mixed together, boiled for 10 min, and slow-cooled to room temperature. All substrates used in this study were PAGE purified, resuspended in 10mM Tris-HCl, 1mM EDTA (pH 8.0), and quantified by GeneQuant 2.0.

Electrophoretic mobility shift assay (EMSA) – DNA binding reactions were performed as previously described (16). Reactions contained 20mM Tris-HCl (pH 8.0), 4% glycerol, 0.1 mg/ml BSA, 0.5mM DTT, 5mM MgCl2, 10nM 32P-labeled substrate, as well as 50 or 200nM of UL5/8/52, unless otherwise indicated. Reactions were incubated at room temperature for 30 min, followed by addition of 1 μl of 10x gel loading buffer containing 250mM Tris-HCl, pH 8.0, 40% glycerol, and 0.1% bromophenol blue. Products were resolved by 4% nondenaturing PAGE, visualized and analyzed using a Storm Phosphorimager (Amersham Biosciences) and ImageQuant software (version 2.1). Binding efficiency was defined as: [free DNA] / ([free DNA] + [DNA+protein]) x 100%. Each measurement was repeated at least three times.
Helicase Assay – DNA unwinding reactions were performed as previously described (16). Reactions contained 20mM Hepes (pH 7.6), 10% glycerol, 0.1 mg/ml BSA, 1mM DTT, 5mM MgCl₂, 10mM ATP, 10nM ³²P-labeled substrate and increasing amounts of UL5/8/52 protein complex. In order to prevent re-annealing of the labeled strand after unwinding, reactions also contained excess (50nM) unlabeled oligonucleotide corresponding to the labeled strand, as a molecular trap. Reactions were incubated at 37°C for 30 min and terminated with 5x stop buffer containing 250mM EDTA (pH 8.0), 40% glycerol, and 0.1% bromophenol blue. Products were resolved by 10% nondenaturing PAGE, visualized and analyzed using a Storm Phosphorimager (Amersham Biosciences) and ImageQuant software (version 2.1). Unwinding efficiency was defined as: [ssDNA] / ([ssDNA] + [substrate]) x 100%. Each measurement was repeated at least three times.

Surface Plasmon Resonance (SPR) – SPR measurements were performed using a Biacore T100 (Biacore). Biotinylated DNA substrates were immobilized on a streptavidin-coated sensor chip (Biacore type SA). The DNA sequence and structure of substrates used for SPR were as shown in Fig 5 A. For 3’ overhang DNA the amount of immobilized ligand corresponded to 700 resonance units (RUs), or 29 fmoles of DNA, resulting in a concentration of 240 µM in the surface matrix. For 5’ overhang DNA the amount of immobilized ligand corresponded to 560 RUs, or 23 fmoles of DNA, resulting in a surface concentration of 190 µM. For forked DNA the amount of immobilized ligand corresponded to 690 RUs, or 23 fmoles of DNA, resulting in a surface concentration of 190 µM. Different concentrations (2.5 to 40 nM) of protein analyte (UL5/8/52) in HBS buffer (10mM Hepes, pH 7.4, containing 3mM EDTA, 0.15M NaCl, 5mM MgCl₂ and 0.05% Surfactant P20), with or without 10mM AMP-PNP, were injected over the sensor surface at a flow rate of 30 µl/min for 180 sec. Post-injection dissociation was monitored in HBS buffer for 180 sec at the same flow rate. BSA was used as a control for nonspecific binding. The surface was regenerated between injections using 2.5M NaCl at a flow rate of 1000 µl/min.
100µl/min for 20 sec. Sensorgrams were fitted to a 1:1 Langmuir binding model using Biacore T100 Evaluation software.

**Primase assay** – Primase activity was measured as previously described (44). Assays (10 µL) contained 1 µM ssDNA template, [α-32P]NTPs, 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, and 10 mM MgCl₂. Reactions were incubated at 37°C for 60 min and quenched with 25 µL formamide/0.05% xylene cyanol and bromophenol blue. Products were separated by 20% denaturing PAGE and analyzed using a Typhoon Phosphorimager (Molecular Dynamics) and ImageQuant software (version 2.1). Data were fitted to a monomer-dimer equilibrium model where only the dimer has activity, as described in(26).

**DNA-dependent ATPase assay** – Assays (5 µL) typically contained (200 nM-1 µM) ssDNA, 100 µM-5 mM [α-32P]ATPs, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 5% glycerol, 0.1 mg/mL Bovine Serum Albumin, and 1 mM DTT. Reactions were incubated at 37°C for 30 minutes and quenched with 2.5 µL of 1M EDTA. Products were separated on PEI cellulose TLC plates by (J.T. Baker) in 0.34 M potassium phosphate buffer, pH 7.5. To enhance resolution, the plates were prerun in H₂O and dried prior to sample spotting and chromatography. The products were analyzed using a Typhoon Phosphorimager and ImageQuant software.

**RESULTS**

We have shown previously that HSV-1 H/P subcomplex containing only two subunits (UL5 and UL52) displays a preference for binding to forked substrates over ssDNA and exhibits minimal binding to fully annealed dsDNA (4), suggesting that a single stranded region is required for efficient enzyme binding. These studies lacked UL8, an essential component of the H/P, and examined a limited set of substrates. Therefore, we extended this analysis to the heterotrimeric UL5/8/52 complex and additional DNA structures to provide insights into the function of UL8 and further characterize the DNA binding preferences of the complex. The
UL5/8/52 complex was purified from insect cells infected with recombinant baculoviruses as described under Materials and Methods. Figure 1 shows a Coomassie stained gel indicating that all three subunits are present in roughly equimolar amounts and the level of purity is high.

A single stranded DNA region is required for efficient HSV-1 H/P loading and unwinding – In order to test the hypothesis that a ssDNA region is required for efficient enzyme loading, we examined the DNA binding activity of the H/P UL5/8/52 by electrophoretic mobility shift assay (EMSA) (Fig. 2). Substrates tested included: ssDNA (Fig. 2B), fully annealed double stranded DNA (dsDNA, Fig. 2C), forked DNA (Fig. 2D), a three-way junction substrate (Fig. 2E), a cruciform substrate (Fig. 2F) and a T-shape substrate without any ssDNA regions (T-shape, Fig. 2G). The heterotrimeric H/P complex (UL5/UL8/UL52) was able to efficiently bind substrates containing ssDNA regions - ssDNA, forked DNA and DNA with three way junctions. H/P showed a preference for binding to forked DNA over ssDNA. This result is consistent with our previous finding that the UL5/UL52 subcomplex also showed a strong preference for forked substrates (4) and indicates that UL8 does not alter this preference. Substrates that contain no ssDNA, including cruciform and T-shaped substrates, did not bind detectably to the enzyme.

To further probe the interactions of different DNAs with H/P, we examined the ability of the helicase to unwind various DNA substrates (Figure 3). Consistent with the results observed in the DNA binding assays, H/P efficiently unwound the forked substrate (Fig. 3B) and the three-way junction substrate (Fig. 3C), resulting in release of ssDNA products that migrate at the bottom of the gel. Partial unwinding of the three-way junction substrate produced some forked intermediate, shown at the middle of the gel. A clear preference for substrates containing ssDNA is evident since no significant unwinding was observed with dsDNA (Fig. 3A), cruciform (Fig. 3D) and T-shape (Fig. 3E) substrates. These results indicate that a single stranded region is required for efficient enzyme binding and unwinding.

Since recombination is thought to play a major role in HSV DNA replication (39, 51), we
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investigated whether the HSV-1 H/P might play a role in Holliday Junction branch migration. It was shown that the bacteriophage T4 UvsW helicase can bind and unwind cruciform substrates (12), indicating a possible role in branch migration during recombination. Fig. 3D shows that the HSV-1 H/P was unable to bind and unwind the cruciform substrate, suggesting that H/P is not likely to participate directly in HSV-1 Holliday Junction branch migration.

_H/P translocates in a 5’ to 3’ direction_ - We next examined the minimal length of the ssDNA region needed for H/P to bind and confirmed the previously reported 5’ to 3’ polarity of this enzyme (19). We performed a series of helicase assays on partially double-stranded substrates that contained either 5’ or 3’ ssDNA overhangs of various lengths (Fig. 4A). Reactions were performed for 30 minutes, and unwinding efficiency was determined. Forked DNA and dsDNA were used as positive and negative controls, respectively (Fig. 4B). At the 30 min time point, forked DNA showed an unwinding efficiency of 26%, while dsDNA exhibited very little unwinding.

When comparing unwinding of substrates with 5’ overhangs (Fig. 4C) to those with 3’ overhangs (Fig. 4D), we observed that substrates with 5’ overhangs are preferred over substrates with 3’ overhangs. These results confirm that H/P translocates along ssDNA primarily in a 5’ to 3’ direction with minimal ability to translocate in the 3’ to 5’ direction (19). We also observed that, within each group, substrates with longer ssDNA regions displayed more robust helicase activity than those with shorter ssDNA regions. A significant increase in unwinding efficiency was observed when the length of the ssDNA region was changed from 6nt to 9nt, suggesting that >6 nt of single stranded DNA are required for efficient enzyme unwinding.

Previous reports suggested that binding of UL5/52 to DNA requires at least 12nt of ssDNA if the entire DNA is single-stranded (18). The difference between the previous study and this one could reflect the fact that we are using a heterotrimeric complex of UL5/UL8/UL52, and UL8 reduces the amount of DNA needed for efficient protein binding. Additionally, the length of the
ssDNA region required to bind a forked DNA may be smaller than that needed to bind purely ssDNA due to interactions of the H/P with the dsDNA at the fork (3).

HSV-1 H/P binds to substrates with 3’ and 5’ overhangs with comparable affinities - As shown above, UL5/8/52 prefers to unwind substrates with a 5’ overhang compared to those with a 3’ overhang. Since DNA binding precedes unwinding, we next asked whether the preference for unwinding substrates with a 5’ overhang reflects preferential binding of UL5/8/52 to substrates with 5’ overhangs. Binding of UL5/8/52 to forked, 5’ overhang and 3’ overhang DNA substrates was measured by EMSA. Figure 5 shows that all three DNA substrates were shifted in the presence of H/P at concentrations of 50 or 200 nM (Fig. 5). The binding efficiencies at 200 nM protein were 82% for the forked substrate, 60% for the substrate containing a 30 nt 5’ overhang (5’-30) and 75% for the substrate containing a 30 nt 3’ overhang (3’-30), indicating that H/P can bind all three substrates efficiently. The forked substrate contains two 30nt single strand regions. In each case, two shifted bands were observed, and at higher protein concentrations the slower migrating band increased in prominence in the reactions with the fork and the 5’ overhang substrate. As described below, formation of this super-shifted, higher order complexes may be critical for primase activity.

Binding kinetics of the H/P to the forked and overhang substrates were analyzed by surface plasmon resonance analysis (SPR). Various DNA ligands containing 5’ or 3’ overhangs were conjugated with biotin and immobilized on a streptavidin-coated chip. SPR measurements were performed using multiple concentrations of H/P (from 2.5 to 40 nM) as analyte. Sensorgrams, shown in Figure 6, were fitted to a 1:1 Langmuir binding model to obtain on- and off-rates and \( K_D \) values, which are plotted on a rapID plot to facilitate comparison of different substrates under different conditions (Figure 7). \( K_D \) values for 3’ overhang, 5’ overhang and forked substrates are comparable, 8.1 X 10^-8 M, 6.4 X 10^-8 M and 4.3 X 10^-8 M, respectively (Fig 6, top), indicating that H/P binds all three substrates with comparable affinities, which is consistent
with the EMSA results. These data also indicate that on- and off-rates are slightly faster for 3’
overhangs than for 5’ overhangs. Interestingly, on- and off-rates are almost 10 fold faster for
forked substrates (Figs. 6 and 7). The increased on-rate for binding of H/P to forked substrate
compared to single overhang substrates suggests a difference in the way DNA is contacted by
H/P possibly because in the forked substrate, both 5’ and 3’ overhangs are available for binding.
The number of RUs for H/P binding to a forked substrate is greater than for H/P binding to
single overhang substrates, indicating higher binding stoichiometry for binding to forked
substrates. This is consistent with the hypothesis that H/P can bind to both arms of the fork
simultaneously. The increased on- and off-rates for binding and dissociation of H/P from forked
substrate compared to single overhang substrates also suggest that the mechanism for H/P
binding to the fork is different than for H/P binding to single overhang substrates.

The bottom panel of Figure 6 shows sensorgrams for binding in the presence of the non-
hydrolyzable ATP analogue, AMP-PNP. The sensorgrams were again fitted with a 1:1 Langmuir
binding model. Especially during the dissociation phase, the fit is not as good in the presence of
AMP-PNP as in the absence, suggesting that this ligand alters the binding mechanism. Based
on fitting to the 1:1 Langmuir binding model, AMP-PNP increases both $k_{on}$ and $k_{off}$
proportionately for all substrates, such that overall $K_D$ values are comparable in the presence
and absence of AMP-PNP. More significantly, AMP-PNP increases the number of RUs for H/P
binding to different DNA ligands approximately two fold compared to sensorgrams in the
absence of AMP-PNP. Thus, AMP-PNP appears to increase the stoichiometry of binding at
these low protein concentrations.

Multimeric H/P binds to DNA substrate at high protein concentration - It is not known how HSV-1
H/P balances its two activities, helicase and primase, as these two activities function in opposite
directions on the lagging strand. To address this question, we measured the protein
concentration dependence of the various activities exhibited by H/P. In Figure 8, DNA binding
activity to a forked substrate was measured over a concentration range of 25 to 200 nM. The percent substrate bound increased with protein concentration. At protein concentrations from 25 to 100 nM a single shifted protein/DNA species was detected; however beginning at 125 nM, a slower migrating form was also observed (also see Figure 5). The formation of this super-shifted species suggests that at higher protein concentration, multiple heterotrimeric complexes of H/P bind to a single DNA molecule.

Primase but not helicase and DNA-dependent ATPase activities are cooperative - The appearance of both shifted and suppershifted primase-helicase-DNA species in the gel shift assays along with the increased stoichiometry of binding in the presence of AMP-PNP in the SPR experiments suggest that the helicase-primase complex can assemble into higher-order complexes that associate with DNA. Since the formation of higher order helicase-primase complexes might significantly affect enzyme activity, we measured the effects of varying enzyme concentrations on both primase and DNA-dependent NTPase activity.

Primase activity was measured on 3’-G<sub>20</sub>G<sub>20</sub>C<sub>20</sub>-5’, a template that has a single canonical initiation site for primer synthesis (the underlined 3’-GCC). Figure 9A shows that in assays containing 500 µM [α<sup>32</sup>P]GTP, the rate of primer synthesis increases non-linearly with increasing protein concentration. In contrast, the rate of DNA-dependent ATP hydrolysis increases linearly with respect to increasing enzyme concentrations (Figure 9B). The non-linear increase in primase activity with increasing enzyme concentration indicates that optimal primase activity minimally requires association between at least two UL5/L8/UL52 heterotrimers. To ensure that the apparent cooperativity of primase activity is not just a function of the particular experimental conditions used in Figure 9, we measured both primase and DNA-dependent ATPase activity with three different templates (T<sub>20</sub>G<sub>20</sub>C<sub>20</sub>C<sub>20</sub>C<sub>17</sub>, T<sub>20</sub>G<sub>2</sub>T<sub>20</sub>C<sub>19</sub> and T<sub>20</sub>G<sub>2</sub>C<sub>2</sub>TAT<sub>14</sub>), with varying NTP concentrations (0.5-5 mM), and with varying DNA concentrations (0.2-1 µM). In each case, NTPase activity increased linearly with protein concentration while primase
activity showed distinct cooperativity (Fig 9A and 9C, also see Supplemental Data) Thus, whereas DNA-dependent ATPase activity behaves non-cooperatively, primase activity exhibits cooperativity – i.e., active primase requires at least a \((UL5/UL8/UL52)_2\) complex.

These observations suggest a model where a single UL5/UL8/UL52 heterotrimeric complex lacks primase activity, but when UL5/UL8/UL52 forms higher order complexes \(((UL5/UL8/UL52)_n\)\), these complexes exhibit primase activity. The simplest model to explain the data is shown in Figure 10. According to this model UL5/UL8/UL52 exists in two forms, a “monomer” that lacks primase activity and a “dimer” \([(UL5/UL8/UL52)_2]\) that has primase activity (Fig. 10B). (Note: For simplicity, we will call the single UL5/UL8/UL52 complex a monomer and the \((UL5/UL8/UL52)_2\) complex a dimer.) In this model, formation of the dimer does not affect the DNA-dependent ATPase activity of each monomer. When H/P binds at the replication fork at low concentration, the helicase activity can unwind the DNA duplex, but shows limited primase activity (Fig. 10A). At higher H/P concentrations, the primase activity is activated to synthesize RNA primers, which can be extended by the DNA polymerase UL30/42. This model is consistent with the data shown in Figure 9.

**DISCUSSION**

The HSV-1 H/P is believed to unwind DNA and synthesize RNA primers on viral DNA; however, little information is available concerning how the heterotrimeric complex is recruited to viral DNA. Although several lines of evidence suggest that HSV-1 H/P binds DNA (3, 4), it has not been possible to dissect the DNA binding domains on UL5 and UL52, in part because UL5 and UL52 have to be expressed together for de novo primase and helicase activity (10, 13, 20). Crosslinking studies have shown that on forked substrates, both subunits contact DNA and that UL52 binds at the ssDNA tail, while UL5 binds to the junction region (3). In this paper we have used a combination of gel shift, surface plasmon resonance and biochemical assays to study protein-DNA interactions of the H/P complex with various DNA substrates. Similar to UL5/52, the
UL5/8/52 complex has higher affinity for forked DNA than for ssDNA and fails to bind to fully
annealed dsDNA substrates. Thus, a single stranded region appears to be required for efficient
complex binding. During initiation of HSV DNA synthesis, it is thought that UL9 binds at the
origin and along with ICP8 causes an initial distortion/destabilization of viral DNA (1, 31, 38). It is
possible that this initial distortion event creates short ssDNA regions that allow H/P recruitment.
Consistent with this model, we demonstrate that efficient enzyme loading and unwinding of
dsDNA requires between 6-9 nt of ssDNA. Furthermore we confirm the results of Lehman and
colleagues (18) demonstrating that HSV-1 H/P exhibits a preference for unwinding substrates
with a 5’ overhang.

**DNA binding ability of the HSV-1 H/P complex** – The experiments presented in this paper
indicate that H/P binds to and dissociates from forked substrates significantly faster than from
single overhang substrates. Faster on-rates with forked substrates may reflect additive multi-site
binding because of the presence of 5’ and 3’ overhangs, both of which can bind to H/P. Since in
vivo, H/P is more likely to encounter forked substrates than single overhang substrates, the
kinetic properties for forked substrates are more physiologically relevant to the function of H/P.
Rapid association/dissociation of H/P with DNA at the fork may facilitate the various functions of
this protein better than slower binding and unbinding. The presence of AMP-PNP increases the
apparent binding stoichiometry approximately 2-fold as well as the apparent on and off rates for
binding of H/P to DNA substrates approximately 10 fold. The SPR data was fitted to a 1:1
binding model, which is the simplest possible model. However, it is possible that a more
complicated binding model involving dimers or higher order oligomeric structures would be more
appropriate. Experiments are in progress to more accurately define the parameters for binding
and dissociation of H/P to DNA to further constrain the binding model.

The increased binding stoichiometry in the presence of AMP-PNP implies that AMP-PNP
and by extension ATP facilitates oligomerization of H/P. Since cells generally contain high levels
of ATP, ATP-dependent oligomerization of H/P is likely to be physiologically relevant. In this
regard, Lehman and coworkers showed that in the absence of DNA and NTPs, H/P exists as a
monomer in solution (17). Our results indicate that in the presence of AMP-PNP H/P binds to
DNA as an oligomeric complex, suggesting that binding of these ligands (NTP and DNA) causes
a conformational change that allows H/P to dimerize or form higher order complexes.

We also report in this paper that the UL5/8/52 complex can bind, load and unwind DNA
substrates that contain between 6 and 9 nt of ss DNA. This is in contrast to previous reports
that binding of the UL5/52 complex to DNA requires at least 12 nt of ssDNA (21). We suggest
that the presence of UL8 in the heterotrimeric H/P reduces the length of ssDNA required for
efficient loading. Thus although UL8 does not exhibit DNA binding activity on its own, it appears
to modulate DNA binding by UL5/52.

H/P forms higher order complexes – Perhaps the most important finding is that dimers or higher
order oligomeric complexes of the UL5/8/52 heterotrimer form at the replication fork, as
demonstrated by three different experimental approaches. 1) Electrophoretic mobility shift
assays resolve two discrete complexes, with the slower migrating species becoming more
prominent at higher protein concentrations. 2) Surface plasmon resonance analyses indicate
that higher amounts of complex bind to DNA in the presence of AMP-PNP. 3) Primase activity
assays reveal cooperative dependence on protein concentration, while ATPase and helicase
activities do not. Together, these three different experimental approaches provide strong
evidence for formation of oligomeric complexes of UL5/8/52 at the replication fork. Besides
indicating that the ATPases of each UL5/UL8/UL52 “monomer” act independently, this result
allows us to rule out trivial explanations for the nonlinear kinetics of primase activity such as
protein denaturation at low concentrations or dissociation of the UL5/8/52 complex, since both
primase and ATPase activity require the same subunits. The cooperative behavior appears to be
independent of DNA sequence and NTP concentration since it occurred on multiple templates.
and over a range of NTP concentrations (Fig 9 and supplemental data).

The simplest model consistent with the data is that primase can exist as either a monomer or a dimer (Figure 10). Both the monomer and dimer have DNA-dependent ATPase activity, while only the dimer has primase activity. Fitting the various data to this model indicates a dimerization $K_D$ around 50 nM. Interestingly, previous studies by Lehman and coworkers using sucrose density gradient centrifugation showed that 70 nM UL5/UL8/UL52 in the absence of DNA and NTPs exists as a monomer (17). Thus, H/P dimerization either strictly requires these ligands or DNA and NTPs facilitate dimerization.

The finding that the H/P forms at least a dimer provides an explanation for several puzzling observations in herpes replication. First, the helicase moves toward the replication fork while the primase moves away from the fork. By analogy with the bifunctional T7 H/P (50), it is possible that during unwinding, the primase scans along the template until it recognizes a primer synthesis site, whereupon a conformational change occurs that slows the rate of unwinding leading to activation of primase activity. Alternatively, if the H/P forms a dimer where two H/P monomers bind in opposite orientations, this would solve the DNA polarity issue and would allow simultaneous helicase and primase activity.

Second, the observation of primase cooperativity provides a rationale for development of a reconstituted herpes replication system using purified proteins and a minicircle template that generates equal amounts of leading and lagging strand products. A valuable tool for studying coordinated leading and lagging strand synthesis has been to use a single strand circular DNA molecule with an annealed replication fork. Using purified bacteriophage T7, T4 and E. coli replication proteins, it has been possible to reconstitute both leading and lagging strand synthesis and to demonstrate that synthesis is coordinated and interconnected (47). Herpes replication has been examined using similar assays containing HSV polymerase, single strand DNA binding protein and the H/P (23, 24, 30). While this system performs highly efficient strand
displacement synthesis mimicking leading strand DNA production, it produces many fewer
lagging strand products. However, these reactions contained low concentrations of H/P (14 nM,
(23), much less than needed for optimal primer synthesis (vide infra), which may account for the
very inefficient lagging strand synthesis. We have recently generated a herpes replication
system on circular templates that generates equal amounts of leading and lagging strand
products (Unpublished data, G. Stengel and R. Kuchta). One of the key requirements is a high
concentration of H/P (100-200 nM). Both biophysical and kinetic assays indicate that the
functional form of the H/P complex likely contains at least two copies of H/P (i.e.,
(UL5/UL8/UL52)₂).

Finally, formation of a H/P dimer would also answer a major question regarding the herpes
replisome – how does one assemble two DNA polymerases for coordinated leading and lagging
strand synthesis? The UL8 subunit of UL5/UL8/UL52 reportedly binds to UL30 of the
UL30/UL42 polymerase complex (41). Thus, the presence of two interacting UL5/UL8/UL52
complexes at the replication fork would provide a simple mechanism for recruiting two
polymerases to the replication fork (Figure 10B). The assembly of an HSV replisome at the
replication fork is likely to be a complex process requiring all six replication fork proteins, H/P
(UL5/8/52), the DNA polymerase (UL30/42). ICP8 the single stranded binding protein of HSV
has been reported to stimulate the primase and DNA-dependent ATPase activities of the H/P,
but only if all three subunits are present (26, 48, 49). ICP8 bound to DNA may promote the
binding of UL5/8/52 to DNA through an interaction with UL8 (26). Thus, the UL8 subunit of the
H/P may play a pivotal role in the assembly of the replisome at the replication fork through its
interactions with ICP8 and UL30. According to this model, one UL5/UL8/UL52 would bind the
polymerase that replicates the leading strand while a second UL5/UL8/UL52 would bind the
polymerase(s) that generates Okazaki fragments on the lagging strand. As noted earlier, the
presence of two UL5/UL8/UL52 complexes at the replication fork would also solve the potential
problem of the helicase tracking along the lagging strand 5’-3’ while primase synthesizes primers 3’-5’ with respect to the lagging strand template.

It should be noted that while a DNA-dependent dimerization model accurately describes the observed cooperativity for primase activity, it is possible that the H/P complex forms higher order structures (trimer, hexamer, etc.). Additionally, it is currently unclear if complex formation absolutely requires DNA and NTPs, or if these ligands just lower the $K_D$ for complex formation. Experiments to completely define the mechanism of complex formation are in progress.

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FIGURE LEGENDS

Fig. 1. Purified UL5/UL8/UL52 complex The UL5/UL8/UL52 complex was purified from sf9 insect cells that had been coinfectected with recombinant baculoviruses encoding UL5, His-UL8 and UL52. The protein was purified on a HIS-Select nickel affinity column as described in Materials and Methods. Products were resolved on 10% SDS-PAGE subsequently stained with Coomassie blue. Molecular weight markers are shown in the left lane and purified H/P is shown in the right lane. The protein ladder.

Fig. 2. A single stranded region is required for efficient enzyme loading. A, The sequences of the oligonucleotides used to prepare artificial substrates are shown. B-G, DNA binding ability was examined by electrophoretic mobility shift assay (EMSA) between H/P and ssDNA (B), fully annealed double stranded DNA (dsDNA, C), forked DNA (D), a three-way junction substrate (E), a cruciform substrate (F), and a T-shape substrate without any ssDNA region (T-shape, G), respectively. A schematic of the substrate is indicated above each panel. The star (*) indicates the position of the $^{32}$P label. Binding efficiency is shown below the lanes. Substrates were prepared as described in Materials and Methods, by annealing different oligonucleotides shown in A. Reactions contained 50 or 200 nM enzyme. The lane marked ‘-’ corresponds to a control reaction without enzyme.

Fig. 3. A single stranded region is required for efficient unwinding. The unwinding activity of H/P was assayed with different substrates: fully annealed double stranded DNA (dsDNA, B), forked DNA (B), a three-way junction substrate (C), a cruciform substrate (D) or a T-shape substrate without any ssDNA region (T-shape, E). A schematic of the substrate is indicated above each panel. The asterisk (*) indicates the position of the $^{32}$P label. Concentrations of the enzyme were 25, 50, 100, 200, 400 nM. The lane marked ‘-’ corresponds to a control reaction without enzyme.

Fig. 4. H/P translocates along ssDNA primarily in a 5’ to 3’ direction. A, sequences of the substrates used. The labeled oligonucleotides are in italic. For fork-15, the complementary region is underlined. B. Helicase assay using fork-15 and dsDNA. C. Helicase assay using 5’ overhang substrates
containing 3nt, 6nt, 9nt, 12nt or 15nt extension, respectively. D. Helicase assay using 3’ overhang substrates containing 3nt, 6nt, 9nt, 12nt or 15nt extension, respectively. Unwinding efficiency is shown below the lane. A schematic of the substrate is shown above the panel. The asterisk (*) represents the location of the \(^{32}\)P label. The lane marked ‘-‘ corresponds to a control reaction without enzyme. Reactions contained 200nM of protein.

**Fig. 5.** H/P binding to forked and 5’ and 3’ overhang substrates. Electrophoretic mobility shift assays were performed with forked or overhang substrates. The forked substrate contains a 30nt duplex and two 30 nt single stranded regions. The 5’ and 3’ overhang substrates contain a 30 nt duplex plus a 30nt extension. The lane marked ‘0’ represents control reactions in the absence of protein. Reactions contained either 50nM or 200nM of protein.

**Fig. 6.** HSV-1 H/P binds to forked and 3’ and 5’ overhang substrates with comparable \(K_D\)s as assessed by Surface Plasmon Resonance (SPR). The DNA sequence and structure of substrates used for SPR were as shown in Fig. 5A. Sensorgrams for different concentrations of enzyme binding to various substrates are shown. Reactions were performed in the absence (top row) or presence (bottom row) of non-hydrolyzable ATP (AMP-PNP). Red lines show experimental sensorgram data. Black lines show fitted curves obtained by global fitting using a 1:1 Langmuir binding model. A summary of the DNA binding parameters determined from the fitted curves is shown beneath each set of sensorgrams. \(k_a\) indicates the association rate. \(k_d\) indicates the dissociation rate. \(K_D\) is calculated as \(k_d\) divided by \(k_a\).

**Fig. 7.** raPID Plot of DNA binding parameters determined in Figure 6. Association rates (\(k_a\)) are plotted on the X axis and dissociation rates (\(k_d\)) are plotted on the Y-axis. Dissociation constants (\(K_D\)) are indicated on the diagonal axis. Open symbols indicate parameters measured in the absence of AMP-PNP. Closed symbols indicate parameters measured in the presence of AMP-PNP. 5’, 3’ and forked substrates are shown in red, green and blue, respectively.

**Fig. 8.** EMSA binding assay showing H/P bound to DNA at increasing protein concentrations. DNA
binding of the H/P to the forked substrate described in the legend to Figure 5 was examined by electrophoretic mobility shift assay (EMSA) at increasing protein concentrations from 0 to 200 nM.

Fig. 9. Effects of varying the UL5/UL8/UL52 concentration on (Panels A and C) primase and (Panels B and D) DNA-dependent ATPase activity. Assays contained enzyme, 0.2 µM C20GCC(C)20, and 500 µM [α-32P]GTP (Panel A, primase activity), 1 mM [α-32P]GTP (Panel C, primase activity), 500 µM [α-32P]ATP (Panel B, DNA dependent ATPase), or 1 mM [α-32P]ATP (Panel D, DNA dependent ATPase) and were performed as described under Experimental Procedures. The line shows the fit with $K_D = 50$ nM.

Fig. 10. Model. A. Scheme showing activities monomer or higher order complexes of the UL5/UL8/UL52 heterotrimeric complex. Our model proposes that a single UL5/UL8/UL52 heterotrimeric complex lacks primase activity, but when UL5/UL8/UL52 forms higher order complexes $((UL5/UL8/UL52)_n)$, these complexes exhibit primase activity. B. Replication Fork Model. The HSV polymerase UL30 and its accessory protein UL42 are drawn as a spotted oval and a crescent, respectively. The UL5/UL8/UL52 H/P ternary complex is depicted as a shaded oval for simplicity. The saw-tooth line depicts the RNA primer, and the solid line depicts single-stranded DNA. In the top panel, low concentrations of the H/P helicase activity result in unwinding of the duplex DNA with little or no primase activity observed. At higher concentrations of H/P, a dimer or higher order multimer of H/P forms at the fork, and under these conditions, RNA primers are synthesized which can then be extended by HSV DNA pol/UL42. Low [HP] is shown in top panel, while high [HP] is shown in bottom panel.

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$k_d (s^{-1})$

$K_D (M)$

- 5' overhang [-ATP]
- 3' overhang [-ATP]
- fork [-ATP]
- 5' overhang [+AMP-PNP]
- 3' overhang [+AMP-PNP]
- fork [+AMP-PNP]