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Protein Displacement by Herpes Helicase-Primase and the Key Role of UL42 During Helicase-Coupled DNA Synthesis by the Herpes Polymerase

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Abstract

The herpes helicase-primase (UL5-UL8-UL52) very inefficiently unwinds double stranded DNA. To better understand the mechanistic consequences of this inefficiency, we investigated protein displacement activity by UL5-UL8-UL52, as well as the impact of coupling DNA synthesis by the herpes polymerase with helicase activity. While the helicase can displace proteins bound to the lagging strand template, bound proteins significantly impede helicase activity. Remarkably, UL5-UL8-UL52, an extremely inefficient helicase, disrupts the exceptionally tight interaction between streptavidin and biotin on the lagging strand template. It also unwinds DNA containing streptavidin bound to the leading strand template, although it does not displace the streptavidin. These data suggest that the helicase may largely or completely wrap around the lagging strand template, with minimal interactions with the leading strand template. We utilized synthetic DNA minicircles to study helicase activity coupled with the herpes polymerase-processivity factor (UL30-UL42). Coupling greatly enhances unwinding of DNA, although bound proteins still inhibit helicase activity. Surprisingly, while UL30-UL42, as well as two non-cognate polymerases (Klenow Fragment and T4 DNA polymerase) all stimulate unwinding of DNA by the helicase, the isolated UL30 polymerase (i.e., no UL42 processivity factor) binds to the replication fork but in a manner incompetent for coupled helicase-polymerase activity. When present, UL42 increases the processivity of UL30 ~ 8-fold and makes a critical interaction with the DNA 24 nucleotides behind the primer 3'-terminus.

Graphical Abstract

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SUPPORTING INFORMATION AVAILABLE

These data include: (1) the binding curve for NEAT DBD (Figure S1); (2) effect of lac repressor on helicase DNA when the DNA lacks a lac repressor binding site (Figure S2); (3) bound streptavidin does not affect DNA unwinding by the helicase (Figure S3); (4) dissociation of streptavidin from duplex DNA in the presence of free biotin (Figure S4); (5) effect of UL30 on Klenow Fragment activity (Figure S5); (6) effect of streptavidin on DNA synthesis by UL30 and UL30-UL42 (Figure S6); (7) specificity of lac repressor for DNA substrates containing the lac binding site (Figure S7), and; (8) comparison of the processivity of UL30 and Klenow Fragment (Figure S8).

Herpes Simplex Virus-1 (HSV-1) contains a double-stranded DNA genome and is one of the most common viruses of the *Herpesviridae* family that infects humans.³ Viral DNA replication requires at least seven viral proteins, including the helicase-primase heterotrimer (UL5-UL8-UL52), the DNA polymerase-processivity factor complex (UL30-UL42), the single-stranded DNA binding protein (UL29), and the origin binding protein (UL9).⁴⁻⁶

The primase and helicase activities of the UL5-UL8-UL52 heterotrimer require both UL5 and UL52.⁷ Based on sequence homology, the helicase is a member of the SF1 superfamily of helicases.⁸⁻¹⁰ The complex tracks along the lagging strand template of the replication fork with 5'-3' polarity.¹¹ The heterotrimer does not require dimerization or multimerization for helicase activity,⁷ unlike some other replicative helicases. However, primase activity requires at least dimerization of the UL5-UL8-UL52.⁷

Compared to other helicases,¹²⁻¹⁴ Herpes helicase unwinds DNA very inefficiently. Even in the presence of excess enzyme, completely unwinding as few as 20 base-pairs of DNA can require many minutes.¹⁵ More efficient systems unwind DNA much faster, for example, Bacteriophage T4 Dda helicase unwinds 12 bp under single-turnover conditions in less than 0.05 seconds.¹² Bianco, *et al.* used optical trapping to show that RecBCD helicase unwinds DNA at 972 bp s⁻¹ and exhibits high processivity, unwinding 42,3000 bp before dissociating.¹³ Under precise conditions of ionic strength and the inclusion of a large excess of the single-stranded DNA binding protein UL29, the rate of unwinding by UL5-UL8-UL52 was around 60 bp s⁻¹.¹⁶

In addition to unwinding double-stranded DNA, replicative helicases presumably must displace proteins bound to the DNA to clear the path for the DNA polymerase. The ability of other helicases (T4 Dda, *E. coli* Rep protein, RecBCD, DnaB, etc.) to displace different proteins varies greatly.¹⁷⁻²⁰ Some helicases display minimal ability to displace proteins while others display robust protein displacement activity. Currently, no data exist regarding protein displacement by UL5-UL8-UL52.

HSV-1 polymerase (UL30) is a B-family polymerase that contains 3'-5' exonuclease activity and exhibits mild strand displacement activity.^{21,22} The UL42 processivity factor forms a heterodimer with the polymerase subunit, UL30. UL30-UL42 binds DNA more tightly than UL30 alone, likely due to UL42 tethering UL30 to the DNA.²³ Compared to other processivity factors, UL42 has several unique properties. Most processivity factors form a multimeric ring encircling the DNA and do not directly bind DNA. UL42 binds double-stranded DNA with high affinity ($K_d \sim 2$ nM), does not multimerize, and does not require a clamp loading complex.^{24,25} Surprisingly, the tertiary structure of UL42 bears a

striking similarity to that of proliferating cell nuclear antigen (PCNA), even though they have only 13% sequence homology and enhance processivity via very different mechanisms.²⁶

Presumably, efficient DNA replication requires that the helicase and polymerase activities function in a highly coordinated and synergistic manner. Several polymerase/helicase pairs exhibit enhanced activity as compared to the individual proteins, including T4 polymerase (gp43) with gp41 helicase and *E. coli* DNA Pol IV with Rep helicase.^{27,28} Several data suggest this will also be true for the herpes polymerase/helicase pair, but this has not been explicitly tested. First, the polymerase/helicase pair can synthesize long products using a minicircle replication system even though the helicase alone struggles to unwind more than 20 base-pairs of DNA. Second, UL30-UL42 binds to UL5-UL8-UL52 via interactions between UL30 and UL8.²⁹ The potential role(s) of UL42 in the coupled activity of the helicase and polymerase activities remains unclear.

We examined the ability of the herpes helicase-primase to displace proteins from DNA, the synergistic activity of polymerase and helicase activities and the role of UL42. As with other helicases, UL5-UL8-UL52 could displace some proteins from DNA although bound proteins presented significant obstacles. While polymerase activity greatly enhanced helicase activity, bound proteins still significantly impeded the helicase. UL42 plays a remarkably essential role during coupled activity – in the absence of UL42, the polymerase still binds at the replication fork but in a manner incompetent for coupled helicase-polymerase activity.

EXPERIMENTAL PROCEDURES

Materials

dNTPs were purchased from Invitrogen and [α -³²P]dNTPs and [γ -³²P]ATP were from Perkin-Elmer. T4 polynucleotide kinase, T4 DNA polymerase and Klenow Fragment were from New England Biolabs. The plasmid for lac repressor expression, pLS1, and the cells in which they were expressed, BLIM cells, were purchased from Addgene. Streptavidin and biotin were from Sigma Aldrich. Purified NFAT DBD was kindly provided by Drs. Jennifer Kugel and Jim Goodrich (University of Colorado). Purified *E. coli* DnaB was kindly provided by Dr. Charles McHenry (University of Colorado). SF-900 SFM cell culture media was purchased from Gibco. HisUL8, UL52, and UL5 baculoviruses were produced and titered at the Tissue Culture Core Facility at the University of Colorado Cancer Center.

Oligonucleotides

All oligonucleotides were purchased from Integrated DNA Technologies, Inc. and purified by gel electrophoresis (Table 1). Minicircles were produced as described previously.² DNA_{Bridge1} was used to ligate DNA_{MC70}, DNA_{MC70-Nick}, DNA_{MC70-SA LAGG}, DNA_{MC70-SA LEAD}, DNA_{MC70-64bp}, and DNA_{MC150-Lac}. DNA_{Bridge2} was used to ligate DNA_{MC70-21bp} and DNA_{MC70-NFAT DBD}. DNA_{Bridge3} was used to ligate DNA_{MC70-2}.

Enzymes

Expression and purification of HSV-1 Helicase-Primase—Sf9 cells were coinfecting (moi = 3:3:3) in shaker flasks with three baculoviruses containing the genes for Hisg-UL8, UL52 and UL5. The heterotrimer was affinity purified using nickel-NTA chromatography, as described previously with minor modifications.³⁰ The cells were thawed on ice and resuspended in cold lysis buffer (0.1% Nonidet P-40, 400 mM NaCl, 20 mM Tris-HCl, pH 8.0, 5% glycerol, 1.5 mM MgCl₂, 8 mM β-mercaptoethanol, and 1 Complete mini-EDTA tablet (Roche)/10 mL lysis buffer). The remaining steps were followed as described.³⁰ The resin was washed with 40 mL Wash Buffer (15 mM Imidazole) and eluted with Wash Buffer + 200 mM imidazole). SDS-polyacrylamide gel electrophoresis and coomassie blue staining confirmed that the purity of the heterotrimer was >95%.

Purification of HSV-1 polymerase and polymerase-processivity factor—SF9 cells were infected with the appropriate baculoviruses (HIS-UL30 and HIS-UL42/UL30, moi = 5) and grown at the Tissue Culture Core Facility at the University of Colorado Cancer Center. Purification of UL30 and the heterodimer (UL30-UL42) were performed as described for UL5-UL8-UL52 above. Baculovirus harboring HIS-UL30 was kindly provided by Dr. Don Coen from Harvard University and amplified at the Protein Production, Monoclonal Antibody, Tissue Culture Shared Resource at the University of Colorado – Denver Medical School.

Expression and Purification of Lac repressor—Lac repressor was expressed in BLIM cells and purified as previously described.^{31,32}

Methods

Helicase displacement of streptavidin (SA)—All helicase reactions (15 μL) contained: 20 mM HEPES, pH 7.6, 1 mM MgCl₂, 1 mM DTT, 0.1 mg/mL bovine serum albumin, 10% glycerol and 5 mM ATP. Helicase reactions for ssDNA bound by streptavidin contained 50 nM HSV-1 UL5-UL8-UL52, 5 nM 5′-[³²P]-ssDNA template, 200 nM SA, and 1 μM free biotin. SA was added to the reactions with ssDNA and allowed to bind for 5 minutes at 37°C before free biotin was added. Reactions were initiated by adding ATP and incubated at 37°C. Helicase reactions were quenched by adding 2 volumes gel-loading buffer (15 mM EDTA, pH 7.8, 50% glycerol, bromophenol blue, and xylene cyanol). Products were separated by native polyacrylamide gel electrophoresis (6% acrylamide in 2.5 mM tris-19.2 mM glycine buffer). Gels were visualized by phosphorimager (Molecular Dynamics). The amount of free and bound DNA was quantified with ImageQuant software (Molecular Dynamics).

Helicase displacement of NFAT DBD and Lac repressor—The forked DNA substrate used for these studies was designed with the NFAT DBD 7-bp binding sequence and the lagging strand template was labeled at the 5′-end ([γ-³²P]ATP). All reactions contained 5 nM labeled dsDNA template, 10 nM unlabeled lagging strand template, and 200 nM NFAT DBD. NFAT DBD was added to the reactions and allowed to bind dsDNA for 10 minutes at 37°C. Reactions were initiated by adding ATP and incubated at 37°C for various periods of time. The reactions were quenched by adding 2 volumes gel-loading buffer (as

above except with added 1% Sodium dodecyl sulfate). Products were separated by native polyacrylamide gel electrophoresis and analyzed as for NFAT DBD displacement. Lac repressor displacement experiments were performed analogously using a DNA containing the lac repressor 40-bp binding sequence.³¹

Helicase Assays using minicircle DNA—DNA_{MC70-21bp} was labeled at the 5'-end ([γ -³²P]ATP) and annealed to the DNA_{MC70} minicircle for experiments containing 21 bp of duplex DNA. DNA_{MC70-Lac} was labeled at the 5'-end ([γ -³²P]ATP) and annealed to DNA_{MC70-Lac} Primer for experiments containing 64 bp of duplex DNA. Helicase activity was monitored as described above.

Coupled Helicase-Polymerase Assays—Reactions typically contained 20 nM primer-annealed minicircle DNA, 100 nM UL30 or UL30-UL42 and 100 nM UL5-UL8-UL52. Reactions were initiated by adding 10 μ M dNTPs ([α -³²P]TTP) and 5 mM ATP and incubated at 37°C. The reactions were quenched by adding 2 volumes gel-loading buffer (60 mM NaOH, 2 mM EDTA, 20% glycerol, and bromocresol green). Products were separated using 1.5% alkaline agarose gel electrophoresis and analyzed as described previously.²

Processivity Assays for UL30 and UL30-UL42—Polymerase assays were performed as previously described.³³ Assays contained the standard reaction mixtures as used above for the helicase assays, with the addition of 1 μ M primer-template (DNA_{12/90}), 1 nM UL30, UL30-UL42 or Klenow Fragment, 1 μ M dNTPs, and 1 μ Ci [α -³²P]dTTP. Reactions were incubated at 37°C and quenched with 2 volumes gel loading buffer (0.05% xylene cyanol and bromophenol blue in formamide). Products were separated by denaturing gel electrophoresis (15% polyacrylamide, 8 M urea) and analyzed using ImageQuant software (Molecular Dynamics). To calculate the processivity of the polymerase when elongating a substrate of length n (i.e., how frequently the polymerase adds the next dNTP versus dissociating from that substrate), we measured how frequently a product of length n was converted into products of length $n+1$ via dNTP polymerization versus the frequency with which the polymerase dissociated from the length n species. Thus, processivity is given by the amount of products $>n$ nucleotides long divided by the amount of total product n nucleotides long. For example, to calculate the processivity during elongation of the 23mer, we divided the amount of 23mer by all products ≤ 23 nucleotides long. The overall increase in processivity was calculated from the average processivity of the 21–23mers divided by the average processivity for the ≤ 24 mers.

RESULTS

Similar to previous reports, we found that UL5-UL8-UL52 very inefficiently unwinds a forked DNA (Figure 1A. Table 1 lists the oligonucleotides used in this work). Under conditions of excess helicase over DNA, the helicase unwound only 27% of the 37 base-pair duplex DNA after 30 minutes. To determine if the herpes helicase-primase can unwind DNA when they contain bound proteins, we first studied the effect of three DNA-binding proteins on helicase activity – lac repressor, the DNA binding domain of NFAT (NFAT DBD) and streptavidin.

Bound proteins impede HSV-1 helicase-primase

We initially examined unwinding of DNA in the presence of NFAT DBD bound to its 7 bp DNA binding site (apparent $K_d \sim 35$ nM, Supplemental Figure 1). The helicase unwound DNA containing bound NFAT DBD (dsDNA_{NFAT DBD}), although the bound protein reduced the rate of dsDNA unwinding by 2-fold compared to the identical DNA lacking bound NFAT DBD (Figure 1A). Omitting ATP or helicase from the assays eliminated any DNA unwinding.

We next examined *E. coli* lac repressor, a DNA binding protein with much greater affinity for its binding site ($K_d \sim 5 \times 10^{-12}$ M).³² Figure 1B shows that even under conditions of a 10-fold excess of helicase-primase over DNA (dsDNA_{Lac}), bound lac repressor severely impedes dsDNA unwinding by the helicase such that we observed <2% detectable unwinding of the DNA. An alternative explanation for the ability of bound lac repressor to inhibit helicase would be the presence of a helicase inhibitor in the lac repressor. However, adding lac repressor to a helicase assay containing a DNA that lacks a lac repressor binding site does not prevent unwinding (Supplemental Figure 2). Thus, herpes helicase does not effectively displace lac repressor from DNA.

The ability of the helicase to unwind DNA containing streptavidin bound to one strand and/or disrupt the streptavidin-biotin interaction was likewise examined. Streptavidin binds biotin extremely tightly ($K_d \sim 10^{-14}$ M),³⁴ much more tightly than either lac repressor or NFAT DBD bind DNA. Assays contained a forked dsDNA substrate, dsDNA_{Biotin} (Table 1), bearing a biotinylated nucleotide on either strand. In the presence of excess streptavidin (200 nM), the helicase unwound the DNA in both cases. Furthermore, the presence of bound streptavidin on either strand did not substantially affect the rate of unwinding, showing that a large mass on either strand does not prevent unwinding (Supplemental Figure 3).

While the helicase could clearly bypass the streptavidin, it remained unclear if the streptavidin remained bound to the biotinylated DNA or if the helicase displaced it. We first tested if the helicase could displace streptavidin from biotinylated single-stranded DNA. Assays contained ssDNA_{Biotin}, streptavidin, and excess free biotin to bind any streptavidin not bound to the biotinylated DNA. Under these conditions, if the helicase displaces the streptavidin while it translocates along the DNA, the streptavidin-ssDNA_{Biotin} complex will be converted to free ssDNA_{Biotin}. Figure 1C shows that the helicase indeed converts the streptavidin-ssDNA_{Biotin} complex into free ssDNA_{Biotin} in an ATP-dependent manner. In the absence of either ATP or helicase, no free ssDNA_{Biotin} was produced, indicating that the streptavidin remains bound to ssDNA during the time course of the reaction.

We also attempted to analogously measure streptavidin displacement from double-stranded DNA (dsDNA_{Biotin}) in assays containing excess free biotin. However, the streptavidin rapidly dissociated from the double-stranded DNA in all reactions, including during control reactions that lacked either ATP or helicase. After just one minute, >80% of the streptavidin dissociated (Supplemental Figure 4), a result similar to that of Yardimci, H. and coworkers¹ (Footnote ^a). Therefore, all remaining experiments in this study containing dsDNA bound by streptavidin excluded free biotin.

Coupled helicase-polymerase activity and the role of UL42

As noted earlier, the herpes helicase and polymerase appear to function cooperatively since they can synthesize long DNA products on a DNA minicircle⁷ despite the apparent inefficiency of the helicase and the weak strand displacement activity of the polymerase. Using the minicircle system, one can indirectly measure helicase activity via the polymerization of dNTPs under conditions that require strand displacement.^{2,35} One concern was that the minicircles (70 nucleotides) are shorter than the persistence length of DNA (150 bp).^{36,37} Therefore, it was possible that the small size of the minicircle and resultant helical stress enhanced helicase activity. Under conditions of excess enzyme and a substrate containing a 21 nucleotide long double-stranded region, 50% of the dsDNA was unwound in ~3 minutes (Figure 2). However, using a substrate with 64 nucleotides of double-stranded DNA (i.e., >90% of the minicircle is double stranded), no detectable unwinding was observed. Thus, the persistence length of DNA was not a concern for the MC70 and the helicase also inefficiently unwinds DNA on a minicircle substrate.

Similar to previous studies,^{2,16} including both the helicase and UL30-UL42 resulted in long leading strand products (>4 kb long after 5 minutes, Figure 3). However, the UL30 polymerase subunit alone, gave short products ~200 nucleotides long, indicating that UL42 plays a critical role for the synthesis of long products. Likewise, omitting the helicase also results in short products, with the products synthesized by UL30-UL42 being marginally longer than those from UL30.

We tested two non-cognate polymerases for their ability to function in the minicircle system, Klenow Fragment and T4 DNA polymerase. Both polymerases could replace UL30-UL42, with Klenow Fragment generating products ~1 kb long. T4 polymerase generated products >4 kb long, only slightly shorter than those formed using UL30-UL42 (Figure 4). Surprisingly, however, the two non-cognate polymerases generated products much longer than those formed with the cognate UL30 (Figure 3B). In assays containing a constant helicase concentration and low polymerase concentration, the length of products synthesized by the cognate UL30-UL42 were now much longer than with the non-cognate polymerases (Figure 4). Thus, while non-cognate polymerases can replace UL30-UL42, they function much less efficiently than UL30-UL42.

Since previous work indicates UL30 can bind to the helicase via interaction with UL8²⁹, the large decrease in product length when using UL30 alone raised the possibility that UL30 was present at the replication fork but in a state that cannot perform coupled helicase-polymerase activity. We tested this idea by asking if adding UL30 to assays containing helicase and Klenow Fragment inhibited the ability of Klenow Fragment to generate long products. As shown in Figure 5, adding increasing amounts of UL30 to assays containing helicase and Klenow Fragment inhibited the production of the longer products synthesized by Klenow Fragment. Analogously, adding increasing concentrations of Klenow Fragment

^aWe designed two forked dsDNA substrates in which 5-biotinylated thymidine was placed midway through the duplex region on the lagging strand template (Supplemental Figure 4A). Upon the addition of free biotin (BioFree), greater than 90% of bound streptavidin dissociated from duplex DNA (Supplemental Figure 4B). Similar results have been observed and reported by Yardimci, H, *et al.*¹ It remains unclear why this dissociation occurs only for dsDNA and not for ssDNA.

to reactions containing a fixed concentration of UL30 results in the formation of the longer, Klenow Fragment-synthesized products at the higher Klenow Fragment concentrations. We excluded the possibility that the effect of UL30 resulted from a Klenow Fragment specific inhibitor in the UL30 in two ways. Neither heat denatured UL30 nor active UL30 inhibited Klenow Fragment during elongation of a synthetic primer template (Supplemental Figure 5C). Thus, in the absence of UL42, UL30 likely still binds at the replication fork but in a manner incompetent for coupled polymerase-helicase activity.

Just as the herpes helicase can function with a non-cognate polymerase, herpes polymerase (UL30-UL42) can function with a non-cognate helicase. Figure 6 shows that in assays containing *E. coli* DnaB helicase, UL30-UL42 synthesizes much longer products than in the absence of any helicase. However, the products are marginally shorter than those synthesized in assays containing the herpes helicase. Thus, specific interactions between the helicase and herpes polymerase are not required to allow the herpes polymerase to replicate double stranded DNA.

Protein displacement by the combined action of UL30-UL42 and UL5-UL8-UL52

Herpes polymerase clearly increases the efficiency with which the helicase unwinds DNA, however, it remains unknown if it combined polymerase-helicase activity can more efficiently bypass bound proteins. We initially examined displacement of streptavidin from the lagging strand template using a biotinylated lagging strand template annealed to a minicircle (DNA_{MC70}, Figure 7A). Compared to the products synthesized in the absence of streptavidin, adding streptavidin slightly decreased the length and amount of polymerase products. Thus, while the bound streptavidin slows the replication machinery, it does not block replication.

The impact of streptavidin bound to the leading strand template was likewise measured. Control experiments showed that UL30-UL42 alone cannot synthesize DNA past a streptavidin bound to a linear template (Supplemental Figure 6). Similarly, using the minicircle template shown in Figures 7B and 8, streptavidin bound to the leading strand template prevented the synthesis of long products by UL30-UL42 when present in either the single- or double-stranded region of the minicircle. Since the polymerase cannot synthesize DNA past a streptavidin bound to the template, these data indicate that the combined action of the helicase and polymerase cannot significantly dissociate the streptavidin from the leading strand template. In combination with the earlier results showing that the helicase efficiently unwinds DNA containing streptavidin bound to the leading strand template, these data also indicate that during unwinding, the helicase does not displace streptavidin from the leading strand template.

We extended these studies to examine the effects of bound NFAT DBD and lac repressor. Using a minicircle with a NFAT DBD binding site (DNA_{MC70-NFAT DBD}, Figure 9A), the presence of bound NFAT DBD resulted in shorter products and a 2.5-fold decrease in amount (Figure 9B and C). Likewise, when replicating a minicircle containing a lac repressor binding site, the presence of lac repressor greatly decreased the length of the products, as well as the amount of products (6-fold, Figure 10). Control experiments showed that inhibition by lac repressor requires that the DNA contain a lac repressor binding site

(Supplemental Figure 7). Thus, the combined action of the polymerase and helicase can unwind DNA past both lac repressor and NFAT DBD, although they significantly slow the rate of DNA synthesis. The distinct banding pattern observed during replication of DNA containing bound lac repressor likely results from the lac repressor rebinding to the newly synthesized DNA. After the replication machinery displaces the lac repressor and copies the DNA, it has regenerated the lac repressor binding site to which lac repressor can rebind. Since the bound lac repressor inhibits the coupled activity of the helicase and polymerase, pause sites in newly synthesized products should occur every 150 nucleotides (Footnote ^b). Indeed, we observed 6 distinct pause sites during synthesis of a 1 kB product (Figure 10B).

We also considered the possibility that allowing simultaneous lagging strand synthesis might enhance replication past these bound proteins. However, including all 4 NTPs so as to allow lagging strand synthesis did not detectably enhance replication as measured by the rate and length of products (Data not shown).

In addition to proteins bound to DNA, a nicked template provides an additional obstacle the herpes replication machinery might face. Since UL5-UL8-UL52 binds both the lagging strand template and UL30-UL42, we considered the possibility that UL5-UL8-UL52 could allow the polymerase to synthesize DNA past a nick in the leading strand template by enhancing binding of the polymerase to the replication fork. However, the presence of a nick on the leading strand template in the double-stranded region of the minicircle completely blocked DNA synthesis (DNA_{MC70-Nick}, Figure 11).

UL42 enhances the processivity of UL30 by 8-fold

In light of UL42s ability to both enhance the processivity of UL30 and UL42s critical role for ensuring a catalytically competent UL30 at the replication fork, we undertook a quantitative analysis of how UL42 enhances the processivity of UL30. Elongation of a 12-nucleotide primer bound to a 90 nucleotide template, by either UL30 or UL30-UL42 was measured (Figure 12). Processivity reflects a competition between dissociation of the product formed after polymerization of a dNTP versus addition of the next dNTP. Thus, under conditions of excess starting DNA (DNA_{12/90}) whereby the polymerase will not rebind a product once it has dissociated, processivity can be quantified by measuring how frequently the polymerase dissociates versus adds the next correct dNTP. Starting with a 12-nucleotide long primer, the banding pattern of the products generated and the fraction elongated by both UL30 and UL30-UL42 remain similar for polymerization of the first 11 nucleotides. However, upon addition of the 12th dNTP to generate a 24 nucleotide long double-stranded region on the primer-template, the banding pattern abruptly changes and the processivity of UL30-UL42 increases 8-fold compared to UL30 (Figures 12B and C). Furthermore, the processivity of UL30-UL42 compared to UL30 does not appear to further increase as the double-stranded region increases in length. Thus, the key interaction(s) of UL42 that result in increased processivity occurs 24 nucleotides upstream of the primer 3'-terminus.

^bDue to the large size of the lac repressor binding site (40 nucleotides), the requirement that the minicircle primer-template contain ~20 nucleotides of single-stranded template for efficient loading of the enzymes² and that UL30-UL42 binds ~30 nucleotides of double-stranded DNA, we used a 150 nucleotide minicircle (MC150).

DISCUSSION

Herpes helicase-primase very inefficiently unwinds DNA and bound proteins significantly impede unwinding. The UL30-UL42 polymerase-processivity factor complex dramatically improves the efficiency of unwinding via a mechanism that requires the processivity factor. Curiously, in the absence of UL42, UL30 binds at the replication fork but in a manner incompetent for efficient coupled helicase-polymerase activity.

As with other helicases, UL5-UL8-UL52 displayed varied ability to displace different bound proteins. UL5-UL8-UL52 alone gets by the NFAT DBD and displaces streptavidin from single-stranded DNA. In contrast, it did not detectably displace lac repressor, a double-stranded DNA binding protein that binds ~7,000-fold more tightly than the NFAT DBD. Analogously, *E. coli* DnaB displaces Epstein-Barr virus nuclear antigen 1 protein, but not lac repressor, while Bacteriophage T4 Dda displaces lac repressor and streptavidin but not GAL4.^{18,20,38,39}

While UL5-UL8-UL52 can unwind DNA having a bound NFAT DBD, it may not be actively displacing it. Rather, the helicase-primase may wait for the protein to dissociate and then continue unwinding DNA. In contrast, the very tight binding of streptavidin to biotin on single stranded DNA requires that the helicase actively dissociates the streptavidin. Under the experimental conditions employed, streptavidin did not detectably dissociate from single stranded DNA even after one hour. It should be noted that the streptavidin displacement assay only determined if the helicase could displace streptavidin during translocation and it remains unclear if the streptavidin affected the translocation rate.

During translocation along the lagging strand template, the helicase may largely or completely wrap around the DNA. Displacement of streptavidin from 5-biotinylated thymidine on the lagging strand template indicates that the helicase interacts with the “back-side” of this strand and this interaction generates significant force. In contrast, the helicase either does not interact or only weakly interacts with the “back-side” of the leading strand template. The helicase unwound double-stranded DNA with a streptavidin bound to 5-biotinylated thymidine on the leading strand template, indicating that the streptavidin did not block the helicase while it translocated along the lagging strand template. Importantly, the helicase likely did not displace the streptavidin from the leading strand template during unwinding. In the minicircle replication studies, the presence of streptavidin on the leading strand template blocked formation of long products, presumably due to the ability of template-bound streptavidin to block polymerase activity (vide infra). If the helicase had displaced the streptavidin, the polymerase could have replicated the leading strand template and long products should have been synthesized, but this was not observed. Collectively, these data indicate that the helicase-primase complex may interact exclusively with the lagging strand template. Furthermore, these data also suggest that even though the HSV-1 helicase-primase functions as a monomer, it may largely or completely wrap around the lagging strand template much like multimeric helicases that form rings around the DNA.

Despite structural and mechanistic differences, the HSV-1 replication machinery can utilize non-cognate enzymes at high concentrations, albeit much less efficiently than with the

cognate enzyme pair. Falkenberg, M., *et al.* used T7 DNA polymerase to indirectly measure the rate at which the helicase-primase complex unwinds DNA.⁴⁰ In the presence of ICP8, the rate of DNA synthesis did not vary significantly between T7 DNA polymerase and HSV-1 UL30-UL42. Even without ICP8, we found that non-cognate polymerases such as Klenow Fragment and T4 DNA polymerase allow the herpes helicase to more efficiently unwind DNA and allow the synthesis of long products. The ability of non-cognate polymerases to enhance helicase activity suggests that a major cause of the inefficiency of the herpes helicase alone is rapid reannealing of the two strands. Replication of the leading strand template by a polymerase would minimize the potential for the two strands to reanneal.

While non-cognate polymerases enhanced helicase activity, the cognate UL30-UL42 polymerase more efficiently enhanced helicase activity, especially at low concentrations of polymerase. The higher efficiency with the cognate polymerase likely results from UL5-UL8-UL52 binding to UL30-UL42. Since UL5-UL8-UL52 binds at the replication fork, polymerase-helicase interactions will enhance binding of the herpes polymerase to the replication fork – i.e., the helicase-primase in effect acts as an additional “processivity factor” for the polymerase. Conversely, this also predicts that the herpes polymerase should help tether the helicase at the replication fork.

Even though UL30-UL42 significantly enhances helicase activity, bound proteins still significantly impede unwinding. Lac repressor severely inhibits the isolated helicase activity, and reduces the rate of coupled polymerase-helicase activity by 6-fold. The banding pattern observed in Figure 10B may be a result of the replication fork pausing at the bound lac repressor, either waiting for the protein to dissociate or only slowly actively displacing it. The $t_{1/2}$ of lac repressor dissociation can be as short as 5 minutes;⁴¹ however, the banding pattern appears at time points as early as 2.5 minutes. These results suggest that the replication machinery actively displaces the lac repressor rather than just waiting for lac repressor to dissociate. Likewise, NFAT DBD and streptavidin bound to the lagging strand template also inhibited the coupled polymerase-helicase activity by ~3 and 2.5-fold, respectively. Including all 4 NTPs in the reactions to allow primer synthesis and Okazaki fragment synthesis on the lagging strand template did not increase the ability of coupled helicase-polymerase to synthesize leading strand products (data not shown). Thus, while coupled helicase-polymerase activity likely enhances displacement of one protein (lac repressor), bound proteins still greatly impede activity. This raises the question of how the herpes replication apparatus deals with proteins bound to DNA. Most likely, other herpes-encoded or cellular proteins are needed to further enhance replication past bound proteins.

Surprisingly, even though UL30 binds at the replication fork and retains polymerase activity on a primer-template, it is not competent for coupled helicase-polymerase activity in the absence of UL42. This contrasts with both Klenow Fragment and T4 DNA polymerase, two non-cognate polymerases that result in significant helicase-polymerase activity. Indeed, high T4 polymerase concentration resulted in products only slightly shorter than those synthesized using the cognate UL30-UL42 polymerase. The absence of any products longer than ~100 nucleotides in assays containing UL30 is not due to either the lower processivity of UL30 relative to UL30-UL42 since UL30 and Klenow Fragment have similar processivity

(Supplemental Figure 8) and Klenow Fragment generates long products. UL30 also can clearly bind at the replication fork since in the presence of UL5-UL8-UL52, it synthesized products ~100 nucleotides long and inhibits the synthesis of longer products by Klenow Fragment. Rather, these data suggest that UL42 plays a critical and as yet undefined role in organizing the UL30-UL42/UL5-UL8-UL52 complex that permits coupled activity.

UL42 enhanced the processivity of UL30 by a factor of 8 in a surprising all-or-nothing manner. This 8-fold increase in processivity correlates closely with the 10-fold increased affinity of UL30-UL42 for a primer-template as compared to UL30,⁴² suggesting that the increase in processivity results from an increased affinity for the primer-template. When the double-stranded region of the primer-template was 23 nucleotides long, UL30 and UL30-UL42 had similar processivities. However, adding just one more nucleotide greatly enhanced the processivity (~8-fold) of the UL30-UL42 complex compared to UL30 and polymerization of additional nucleotides did not further increase the processivity. Thus, UL42 makes a key contact with the base-pair 24 nucleotides behind the primer terminus to give increased processivity. Unfortunately, while the structure of the C-terminus of UL30 bound to UL42 is known, no structural data of the UL30-UL42 complex bound to DNA exist to allow us to identify the contact points.²⁶ Footprinting studies showed that the UL30-UL42 complex binds ~19 – 28 nucleotides of double-stranded DNA, with UL30 alone binding ~14 base-pairs.⁴² Thus, assuming B-form DNA and that UL42 does not grossly alter where UL30 binds DNA, the key interaction for enhanced processivity occurs approximately one helical turn after the DNA emerges from UL30.

Structural studies of UL42 indicate the presence of a relatively large positively charged surface,²⁶ and mutation of a number of positively charged amino acids to a neutral amino acid at multiple locations in UL42 reduces both the affinity of UL42 for DNA and the processivity of the UL30-UL42 complex.^{43,44} A priori, therefore, one might have expected a gradual increase in processivity as the amount of DNA with which UL42 can interact increases. However, each mutation reduced UL42 binding much more than they reduced the processivity of the UL30-UL42 complex. The effects of each individual mutation on DNA binding by UL42 significantly exceeded the 10-fold increase in primer-template binding afforded by adding UL42 to UL30. In combination with processivity increasing so abruptly at a specific length DNA, these data suggest that the positively charged surface in UL42 is involved in more than just localizing the UL30-UL42 complex to the primer terminus and increasing processivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABREVIATIONS

BSA	bovine serum albumin
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSV-1	herpes simplex virus type 1
MC70	minicircle 70 bases long
dNTP	2'-deoxyribonucleoside 5'-triphosphate
UL5-UL8-UL52	HSV-1 helicase-primase complex
UL30-UL42	HSV-1 DNA polymerase and processivity factor
SA	Streptavidin
NFAT DBD	Nuclear Factor of Activated T-cells DNA Binding Domain
nts	nucleotides
bp	base-pairs

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Figure 1. Protein displacement by UL5-UL8-UL52

(A) EMSA comparing dsDNA unwound in the absence and presence of bound NFAT DBD (dsDNA_{NFAT DBD}). Control reactions lacking ATP or enzyme were quenched after 30 minutes. (B) EMSA comparing dsDNA unwound in the absence and presence of lac repressor (dsDNA_{LAC}). Control reactions were quenched after 30 minutes. (C) EMSA for displacement of streptavidin (SA) from biotinylated ssDNA (ssDNA_{Biotin}) during translocation. Control reactions were quenched after 15 minutes. Reactions without ATP or helicase are noted as (-) ATP and (-) Hel, respectively. DNA labeled at the 5'-end with [α - 32 P]ATP are noted with (*). Percent strand displacement was measured using ImageQuant.

Figure 2. Helicase activity on minicircles with varying lengths of duplex DNA
(A) DNA substrates utilized. (B) In the absence of the polymerase, minicircles with a duplex region of 21 bp (DNA_{MC70-21bp}) or 64 bp (DNA_{MC70-64bp}) were used to measure helicase activity over time (0, 1, 2.5, 5, 10, 15 and 30 minutes). DNA products were separated using 15% native polyacrylamide gel electrophoresis and quantified using ImageQuant.

Figure 3. UL42 plays a key role during minicircle replication

Assays contained DNA_{MC150-Lac} (A) and UL5-UL8-UL52 as noted. (B) UL30-UL42 activity and (C) UL30 activity. DNA products were separated using 1.5% alkaline agarose gel electrophoresis. DNA length markers (M).

Figure 4. Non-cognate polymerases can replace UL30-UL42 during minicircle replication

Either Klenow Fragment or T4 DNA Polymerase were titrated into assays containing DNA_{MC70} (**A**) and 100 nM UL5-UL8-UL52. (**B**) DNA products were separated with 1.5% alkaline agarose gel electrophoresis.

Figure 6. DNA replication by HSV-1 UL30-UL42 and *E. coli* DnaB helicase
(A) DNA_{MC70}. **(B)** Comparison of DNA products synthesized by 100 nM UL30-UL42 in the presence of 100 nM UL5-UL8-UL52 or 100 nM DnaB helicase. 20 nM DNA (DNA_{MC70}) were used. DNA products were separated by 1.5% alkaline agarose gel electrophoresis.

Figure 7. SA displacement from within duplex regions of a MC70

The biotin tag was located on: **(A)** the lagging strand template ($\text{DNA}_{\text{MC70-SA LAGG}}$) or **(B)** the leading strand template ($\text{DNA}_{\text{MC70-SA LEAD}}$). 1.5% alkaline agarose gel electrophoresis was used to separate DNA products.

Figure 8. Effect of streptavidin on DNA replication by UL30-UL42

(A) DNA_{MC70-Biotin}. (B) Assays contained 100 nM UL30-UL42, 100 nM UL5-UL8-UL52 and 20 nM DNA_{MC70-Biotin}. DNA products were separated by 1.5% alkaline agarose gel electrophoresis. (C) Amount of products synthesized (filter binding assay).²

Figure 9. Effect of bound NFAT DBD during DNA replication by UL30-UL42 and UL5-UL8-UL52

(A) DNA_{MC70}-NFAT DBD (B) were used to study NFAT DBD displacement by UL30-UL42 and UL5-UL8-UL52. DNA products were separated by 1.5% Alkaline agarose gel electrophoresis. (C) Amount of products synthesized (filter binding assay).²

Figure 10. Effect of bound lac repressor during DNA replication by UL30-UL42 and UL5-UL8-UL52

(A) DNA_{MC150-LAC}. (B) Assays contained UL30-UL42, UL5-UL8-UL52 and DNA_{MC150-LAC}. DNA products were separated by 1.5% alkaline gel electrophoresis. (C) Amount of products synthesized (filter binding assay).²

Figure 11. Effect of nicked DNA on HSV-1 replication

(**A**) DNA synthesis on ligated MC70 annealed and unligated MC70 annealed to the 20mer (DNA_{MC70-Nick}). (**B**) Products from the reaction containing nicked DNA analyzed using 15% polyacrylamide/8 M urea gel electrophoresis. Nucleotides (nts).

Figure 12. Interaction between UL42 and DNA enhances processivity of UL30 by 8-fold (A) DNA_{12/90}. (B) Assays contained either UL30 or UL30-UL42 and products were analyzed using a 15% polyacrylamide/8 M urea gel electrophoresis. (C) Bar graph represents the fraction of UL30-UL42 to UL30 dissociating during processive elongation of the noted length products (Normalized to peak signal).

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Table 1

DNA Substrates Used

ssDNA _{Biotin} ^a	5' GGGTTGACTGGGAAACCCCTGGCGTTACCCCAACTTAATCATCTCATGCGGGAAGACTTAAACACGATAGGAAGCAATAG
dsDNA _{Lead Biotin} ^a	5' GGGTTGACTGGGAAACCCCTGGCGTTACCCCAACTTAATCATCTCATGCGGGAAGACTTAAACACGATAGGAAGCAATAG
	3' CCCAACTGACCCCTTTTGGGACCCGCAATGGGTTGAATTAGT ₍₃₅₎
dsDNA _{Lagg Biotin} ^a	5' GGGTTGACTGGGAAACCCCTGGCGTTACCCCAACTTAATCATCTCATGCGGGAAGACTTAAACACGATAGGAAGCAATAG
	3' CCCAACTGACCCCTTTTGGGACCCGCAATGGGTTGAATTAGT ₍₃₅₎
dsDNA _{Lac} ^b	5' GTATATACCACTCCACCATAAACCCGTCTGTGTGAAATTGTTATCCGCTCACAAATTCACACAAACATTAGCATATA
	3' CATATATGGTGAAGGTGGTATTTGGCAGGACACACTTTTAAACAATAGGCGGAGTGTGTTAAGGTGTGTGT ₍₁₇₎
dsDNA _{NEAT DBD} ^c	5' GGGATGACTGCCCTGGCGTGGAAAAACCTTACCACAATTCACTCATGAAGACCCGAGAAGCAGGAATAG
	3' CCTACTGACGGGACCGCACCTTTTGGAAATGGGTTGAAAGT ₍₂₈₎
DNA _{12/90}	
5' CGGAACACACAGAA	
3' GCCTTGTGTCTTCACCTTCGTCCTGTCTTCGCCCTTCGTCCTTCGCTTCGCCGGTCCCTCTCTCTGTCTCTCGTCCTTCTC	
DNA _{30/90}	
5' CGGAACACACAGAAGCGGAAGCAGGGACAGAA	
3' GCCTTGTGTCTTCGCCCTTCGTCCTGTCTTCACCTTCGTCCTCTCTTCGCCCTTCGTCCTCCGGTCCCTCTCTCTGTCTCTCGTCCTTCTC	
DNA _{Linear MC70-Biotin} ^a	
5' GAGTGGTATAGTGGAGTGAAGTG	
3' CTCACCATATATCACCTCACTTCACCTCACCTCCAACTTACCACTTATAACCCCTCCACCTCCAATACCAC/5Phos/	
DNA _{MC70} ^a	
5' /Phos/CACCATATAACCTCCACCCCTCCCCAATATTCACCATCAACCCCTTCACCTCACCTCACCTATATACCACTC	
3' GGAGGTGGGAGGGGTATATAAGTGGTAGTTGGGAAGTGGAGTGAAGTTTTTTTTTTTTT	
DNA _{MC70-Niek}	

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5' /Phos/CACCATAACCTCCACCCCTCCCCAATATTCACCATCAACCTTCACCTCACTTCACTCCACTATACCACTC

3' ATATGGTGAGGTGGTATTGG

DNA_{MC70} + Lac

5' /5Phos/CACCATAACCGTCTGTGTGAATTGTTATCCGCTCACAAATTCACACAACTTAGCATATATACCACTC

3' ATATATGGTGAGGTGGTATGGCAGGACACACTTACAATAGCGAGTGTTAAGGTGTTGTTT

DNA_{MC70-SA LAGG}^a

5' /Phos/CACCATAACCTCCACCCTCCCCAATATTCACCATCAACCCCTCACCTCACTCCACTATACCACTC

3'GGAGGTGGGAGGGGTTATAAGTGGTAGTTGGGAAGTGGAGTGAAGTTT'TTTT'TTTT

DNA_{MC70-SA LEAD}^a

5' /Phos/CACCATAACCTCCACCCTGCCCAATATTCACTTCACTCCTCACTTCACCTATACCACTC

3'GAGGTGATATGCTGAGGTGCTATTGGAGGTGGAGGGGTTATAAGT₍₁₅₎

DNA_{MC70-21bp}

5'/Phos/CGGAACACAGAGCGGAGCAGGACAGAGCGGAGCCGAGCCAGCACCGGA

3' TGTCCTCGCCCTTCGTCCCTGTTTCTTTT

DNA_{MC70-64bp}

5' /Phos/CACCATAAACCGTCCGTGTGTAATTGTTATCCGCTCACAAATCCACACAACATTAGCATATATACCACTC

3' ATATATGGTGAGGTGGTATTGGCAGGACACACTTTAACAATAGCGAGTGTTAAGGTGTTGTTTGTGTTT

DNA_{MC70-NEAT DBD}^c

5'/Phos/CGGAACACAGAACCGGTGAAAAAAGCAGGGACAGAGCAGGCAGAAAGCGACCTCAGTCGCACCACCGA-3'

3' GCCTTGTC^TTTCGCCACCTTTTTCGTCCCTGTCTCGTCCCTTGGCCTTGGTCCGGCTCTCTCTGTCTCTCGTCCCCTTC

b
DNA_{MCI50-Lac}

5' / Phos / CACCATTACCAAGCGGAACAGAAGCGGATGTGGTGTGGAAATTTGTAGCGGGATAACAATTTTCACACAGGAGAGGCGTCCGCGAGACTTAGCGCCCATAAACGGAATTACAGCGGGCTATTACCACCT

3' CTGCTTCGCCCTTCGTCCTTGCTTCGGCAACAAACACACCTTAACACTCGCCTATTGTTAAAGTGTGCTCTTTT

DNA_{MC70-2}

5' /Phos/AGGAAATGTGGTGGTTTGCTACACTTCCATATAGGATACGAGATACAGAATAGCCGATACCCAAGAT

3'CGATGTGAAGGTATATCCTATGCTCTATGCTTATCGGCTATGGGTTCTATCTGTCTCTGTCCCTTCTC

DNA_{Bridgel}

5'GGTATGGTGGAGTGGTATA

DNA_{Bridge2}

5'CTGTGTTCCGTGGTGGTGC

DNA_{Bridge3}

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CACATTTCCTATCTTGGGTA

The underlined thymidine (T) represents the 5-biotinylated thymidine
The lac operator sequence is underlined
The underlined region indicates where the NFAT DBD binds the DNA