The UL8 subunit of the helicase/primase complex of herpes simplex virus promotes DNA annealing and has a high affinity for replication forks

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ABSTRACT

During lytic infection, herpes simplex virus (HSV) DNA is replicated by a mechanism involving DNA recombination. For instance, replication of the HSV-1 genome produces X- and Y-branched structures, reminiscent of recombination intermediates. HSV-1’s replication machinery includes a trimeric helicase/primase is composed of helicase (UL5) and primase (UL52) subunits and a third subunit, UL8. UL8 has been reported to stimulate the helicase and primase activities of the complex in the presence of ICP8, an HSV-1 protein that functions as an annealase, a protein that binds complementary single-stranded (ss)DNA and facilitates its annealing to duplex DNA. UL8 also influences the intracellular localization of the UL5/UL52 subunits, but UL8’s catalytic activities are not known. In this study, we used a combination of biochemical techniques and transmission electron microscopy. First, we report that UL8 alone forms protein filaments in solution. Moreover, we also found that, UL8 binds to ssDNAs >50 nt long and promotes the annealing of complementary ssDNA to generate highly branched duplex DNA structures. Finally, UL8 has a very high affinity for replication fork structures containing a gap in the lagging strand as short as 15 nt, suggesting that UL8 may aid in directing or loading the trimeric complex onto a replication fork. The properties of UL8 uncovered here suggest that UL8 may be involved in the generation of the X- and Y-branched structures that are the hallmarks of HSV replication.

Herpes simplex virus type 1 (HSV-1) has a 152-kb linear double-stranded (ds) DNA genome. During HSV-1 infection, the viral genome is released from the capsid into the nucleus and is replicated to form concatemeric DNA that is recognized by the packaging machinery to produce infectious virus. Seven virus-encoded proteins are required for viral DNA synthesis including an origin binding protein (UL9) and six core replication proteins: a two subunit DNA polymerase (UL30 and UL42), a three subunit helicase/primase complex (UL5, UL8 and UL52) and a multifunctional ssDNA binding protein (ICP8) (reviewed in ref.1). The six purified core replication proteins can function to produce linear concatemers in vitro if provided a primed template (2). On the other hand, in vivo, late replicating viral DNA has been reported to adopt a mixture of complex structures such as X- and Y-shaped branches and tangled masses, suggesting that recombination may play a role in the replication of HSV-1 DNA (3-7). Additional support for this concept was provided by Blümel et al. who showed that when SV40 genomes were replicated by the six HSV-encoded replication factors and SV40 large T antigen, concatemers composed of X-shaped DNA structures were observed (8). Since SV40 replication normally produces two circular daughter molecules, the complex DNA generated in the presence of HSV replication proteins suggests that the HSV replication machinery is inherently recombinogenic. A better understanding of the functions and properties of the essential viral replication proteins is expected...
to provide insight into the mechanism of HSV-1 DNA replication.

ICP8, the ssDNA binding protein (SSB), is a multifunctional protein essential for viral DNA replication including ssDNA binding, stimulation of HSV polymerase and helicase/primase activities and has a major role in the formation of replication compartments in which DNA replication takes place (9-11). ICP8 forms helical protein filaments in the absence of DNA (12), and filament formation appears to be required for pre-replicative site and replication compartment formation (13). ICP8 also forms superhelical structures on ssDNA, and these structures are believed to promote annealing of complementary ssDNAs (14,15). ICP8, in conjunction with the viral 5` to 3` exonuclease (UL12) has been shown to carry out a strand exchange reaction that is analogous to the activity of the lambda red alpha/beta proteins (16,17). Lambda red alpha/beta play a role in recombination-dependent DNA replication during lambda DNA replication and also promote in vivo recombination-mediated genetic engineering using short homologies—recombineering in bacteria (18). The similarities between ICP8 and lambda beta may reflect their roles in promoting recombination-dependent DNA replication in HSV-1 and lambda (19). Thus, ICP8 and UL12 have been implicated in single strand annealing, and in this paper we focus on previously unrecognized properties of the UL8 subunit of the helicase/primase complex.

The helicase/primase complex is comprised of a 1:1:1 association of three subunits, UL5, UL8 and UL52. A sub-complex of UL5 and UL52 exhibits DNA-dependent ATPase, primase, and helicase activities (20). The UL5 subunit is a member of the SF1 helicase superfamily (21,22). The UL52 subunit contains two conserved regions shared with other known primases: a signature DID motif required for catalytic activity and a putative zinc-binding domain (23,24). UL8 is essential for viral DNA replication in vivo (25), yet no enzymatic activities have been described and one study reported that it failed to bind to short (48 nt) ssDNAs (26). UL8 has been reported to influence the intracellular localization of the UL5/UL52 subunits (27). UL8 is not required for helicase and primase activities per se but stimulates primase and helicase activities in the presence of ICP8, suggesting a functional interaction between UL8 and ICP8 (10,28-32). No physical interaction between UL8 and ICP8 has been reported, and the mechanism by which helicase and primase activities are stimulated is not understood. It is unclear what features of the 3-subunit complex are responsible for directing it to a replication fork. UL5/UL52 does bind small fork-like structures and UL8 stimulates the binding of UL5/UL52 to the forked DNA substrate (33). Unlike the T4 system, which employs a specific helicase loader protein (34), no such factor is known for HSV. These issues are explored in this study.

RESULTS

UL8 monomers can assemble into rings and long protein filaments—As described above, HSV-1 UL8 is a subunit of the heterotrimeric helicase/primase complex; however, no catalytic activities have been identified. To further examine the physical properties of UL8, it was expressed in insect cells infected with recombinant baculovirus and purified using affinity chromatography as described in Experimental Procedures (Fig. 1). The results of size exclusion chromatography indicated that UL8 eluted from a Superose 6 column in buffer containing 150 mM NaCl showing one peak (Supplemental Fig. 1). The molecular mass of the UL8 peak was estimated to be 80 kDa, consistent with a previous report suggesting that UL8 is a monomer (26). UL8 was diluted in a buffer containing 150 mM NaCl, to a concentration of 0.8 micrograms/ml and applied to a glow discharge treated carbon EM support followed by negative staining with uranyl acetate (Experimental Procedures). Fields of particles predominantly ranging from 5 to 7 nm diameter (Fig. 2A) were observed. Seventy percent of particles have a diameter of 5 nm, 19% 6 nm and 11% 7 nm (n=200). Based on its molecular weight, a UL8 monomer would be predicted to have a diameter of ~5 nm, and we conclude that UL8 at this protein concentration is predominantly monomeric with some oligomeric forms. When UL8 was incubated in the same buffer at a 20-fold higher concentration, fixed with 0.6% glutaraldehyde, and then diluted prior to EM, the same distribution of small particles was observed arguing that UL8
exists as primarily a monomer at concentrations of up to at least 200 nM.

Magnesium ions often favor protein oligomerization, thus UL8 was incubated on ice for 30 min in the presence of 5 mM MgCl₂ and 50 mM NaCl followed by negative staining for EM. Examination of fields of molecules following incubation at increasing protein concentration revealed three distinct forms: irregular particles, regular oligomeric rings and linear protein filaments (Table 1). At concentrations less than 9 nM, UL8 appeared predominantly monomeric with some irregularly shaped oligomeric forms (5-15 nm) similar to the ones seen in the absence of Mg²⁺ and with 150 mM NaCl (Fig. 2A). At 10-50 nM, with 5 mM MgCl₂, at RT or on ice UL8 formed regular, donut shaped structures (oligomeric rings), 20 nm in diameter (Fig. 2B). The rings were highly consistent in size and likely contain between 4 and 6 monomers. When the UL8 concentration was increased to ≥50 nM with or without Mg²⁺ the protein self-assembled into long protein filaments ranging in length from 100 to >3000 nm (n=164) (Fig. 2C and Supplemental Fig. S2). The width of the filaments measured 15 nm. Filamentation required incubation on ice for 30 min and further incubation did not produce longer filaments. Although outside the scope of this publication, a 3D reconstruction of the oligomeric rings and filaments will be of considerable interest.

The general width and length of the UL8 filaments was similar to filaments formed by HSV-1 ICP8 and KSHV Orf6 proteins (12,35). However while the ICP8 and Orf6 filaments appear to be round and exhibit a clear helical axial repeat, no repeating structure was observed in the negative stained images. Furthermore, the UL8 filaments occasionally appeared as twisted flattened ribbons or a twisted flattened ribbon (Supplemental Fig. S3). In addition, the parameters of formation were different: UL8 requires 30 min incubation on ice, ICP8 requires 12-16 h incubation on ice and Orf6 requires overnight incubation at RT for long filaments. The UL8 filaments (15 nm in diameter) do not require Mg²⁺, while ICP8 filaments (18 nm in diameter) require 5 mM Mg²⁺. Orf6 filaments (14 nm in diameter) do not require Mg²⁺ but do require DTT for filament formation.

UL8 binds ssDNA—ICP8, which forms self-filaments in the absence of DNA, also binds tightly to ssDNA forming helical nucleoprotein filaments (12). While a previous study using a linear 48 nt oligonucleotide reported that UL8 failed to bind (26), longer substrates were not tested. Here we employed electrophoretic mobility shift assays (EMSA) using oligonucleotides of 42, 55, 69, 82 and 90 nt in length (Table 2). The UL8 concentration was held constant at 100 nM (160 ng) and the amount of each oligonucleotide was adjusted to provide 100 nM in oligonucleotide amounting to 50 ng for the 82 and 90-mers, 40 ng for the 70-mer, 30 ng for the 55-mer and 20 ng for the 42-mer. This provided close to one monomer of UL8 for each oligonucleotide molecule in the incubation mixture. Following incubation in a buffer containing 20 mM Hepes pH 7.6 (Mg²⁺ ions were omitted to avoid potential nuclease degradation) for 30 min at 30°C, the DNA-protein complexes were fixed with 0.6% glutaraldehyde for 5 min at RT and subjected to electrophoresis on 10% native polyacrylamide gels. In standard EMSA binding assays, when protein interacts with DNA, the protein-bound DNA complex migrates into the gel but at a slower rate and is said to be shifted. However, when UL8 was incubated with the oligonucleotides, slower moving bands were not observed, rather the protein-DNA complexes appeared trapped in the well of the gel (Fig. 3A). As the size of the oligomer increased, the amount of free oligonucleotide shifted into the well increased: with the 42-mer, 15% was shifted, with the 55-, 69- and 82-mers, ~60 was shifted, and with the 90-mer 96% was shifted into the well. Similar results were observed when the 90-mer was incubated with UL8 in the presence of magnesium (Fig. 3B). The examination of two other oligonucleotides (82 and 90 nt) with different sequences indicated that UL8 does not have any preferred DNA sequence (data not shown), confirming that the binding is length-dependent without any nucleotide bias. Since strong bands corresponding to a single oligonucleotide bound to a single monomer of UL8 were not observed, the best explanation for these results is that once a UL8 monomer binds an oligonucleotide, it has a high probability of interacting with other UL8-oligonucleotide complexes to generate higher order UL8-
UL8 was incubated with the 90-mer (1:1 molar ratio) as above in a buffer containing 5 mM Mg\(^{2+}\) and examined by negative staining. Regular spherical structures with varying diameters were observed (Fig. 3C). Of 100 complexes scored, 68% had diameters of 20 nm, 21% measured 30 nm and 11% measured 40 nm. EM analysis of complexes formed with shorter oligonucleotides (42-, 55- and 69-mers) also revealed regular spherical structures that ranged between 20 and 50 nm (data not shown). Similar structures were also formed in the absence of Mg\(^{2+}\) ions (Fig. 3D) but they were somewhat less regular in appearance. Formation of these structures thus explains the results of the gel shift analysis. In addition, these large structures may be similar to structures described in Tolun et al formed by ICP8 that are involved in DNA annealing (15).

To examine the binding of UL8 to a 7249-nt long natural ssDNA, linearized M13mp18 ssDNA (Experimental Procedures) at 1 nM (2.5 µg/ml) was incubated with increasing UL8 from 5 nM to 100 nM (0.4 to 8 µg/ml) in the absence of Mg\(^{2+}\). Fixed DNA-protein complexes were subjected to electrophoresis on 0.8% agarose gels (Fig. 4A). As the concentration of UL8 increased, the M13 DNA band disappeared, and material appeared in the well of the gel, similar to the UL8-oligonucleotide complexes shown in Figure 3. The decrease of unbound DNA occurred at and above 5 nM UL8, and all of the M13 ssDNA was shifted to the well by the addition of 100 nM UL8. Although a small amount of complex could be detected as a smear above unbound ssM13 at UL8 concentrations of 5, 10 and 25 nM, the majority of bound material was detected in the well. If, following incubation, the samples were treated with proteinase K and SDS prior to electrophoresis, no material was present in the well of the gel showing that this material consists of M13 ssDNA bound by UL8 (Fig. 4B). In the gel shift experiments with the oligonucleotides, we observed that a stoichiometry of one UL8 monomer per each oligonucleotide was sufficient to generate a higher order complex that would not enter the gel, even with the 90 nt oligos. Using M13 ssDNA all the ssDNA was shifted into a higher order structure at an input ratio of 1 UL8 monomer for every 72 nt of M13 ssDNA. This is unlikely to reflect the site size for binding which would be considerably smaller, but rather the amount of UL8 needed to condense the large M13 ssDNA into a compact nucleoprotein structure that would not enter the gel.

To examine the architecture of the UL8-ssM13 DNA complexes, the fixed complexes (after removing the unbound material) were adsorbed to carbon grids, shadow cast with tungsten and examined by EM (Experimental Procedures). By this preparative method M13 ssDNA alone appears as bush-like structures due to base pairing within ssDNA (Fig. 4C). With 1 nM M13 ssDNA and 5 nM UL8, a few protein clusters were present on the ssDNA (Fig. 4C). With 10 and 100 nM UL8, the UL8-ssDNA complexes were condensed into highly collapsed structures (Figs. 4E and F). Addition of up to a 10-fold higher concentration of UL8 (1 µM) failed to generate any regular extended nucleoprotein filaments of the kinds seen with ICP8 and Orf6 at high protein concentrations (14,36). The implications of the difference in DNA binding properties between UL8 and ICP8 will be explored in the Discussion.

The incubation of complementary ssDNA strands with UL8 results in the formation of high molecular weight DNA products—The class of proteins known as annealases bind complementary ssDNA and facilitates their annealing to duplex forms. Given the well-documented activity of ICP8 as an annealase (14,15,37), we tested UL8 for its ability to anneal complementary ssDNA. Linearized, blunt-ended 3987 bp pSAK dsDNA (38) (50 ng, 1 nM dsDNA, 1014 nt) was heat-denatured to produce ssDNA, rapidly quenched on ice and then incubated in the presence or absence of UL8. At the end of each reaction, the samples were deproteinized and electrophoresed on 0.8% agarose gels (Fig. 5). The band in lane 1 of the panels (A-D) corresponds to dsDNA. In the absence of UL8 (Figs. 5A-D, lane 2), a faint band corresponding to dsDNA (Fig. 5). The band in lane 1 of the panels (A-D) corresponds to ssDNA. In the absence of UL8 (Figs. 5A-D, lane 2), a faint band corresponding to dsDNA was present due to spontaneous annealing of the ssDNA over a 30-min time period. Addition of UL8 at concentrations between 4 nM and 25 nM (0.32 to 2 ng/µl of protein) (Fig. 5A, lanes 6-10), resulted in a slightly higher amount of dsDNA; however, at 50 nM UL8 (lane 11), the band corresponding to ssDNA started to diminish significantly, and a faint band appeared in the well of the gel. At 100

oligonucleotide aggregates. EM was employed to further examine this.
nM UL8 (lane 12), all of the ssDNA was converted to a product that remained at the top of the gel. The reaction products generated at 100 nM UL8 were further examined as a function of time. At 1 min, DNA species that cannot enter the well started to form. By 30 min, the input ssDNA disappeared completely and appeared in the well of the gel. The presence of magnesium did not have a significant effect on the appearance of the well-trapped DNA (Fig. 5C). In these experiments, the highest amount of UL8 added corresponds to input ratios of 1 UL8 monomer per 75 nt and addition of 5 to 10 fold higher amounts corresponding to 1 monomer per ~7 nt did not alter the results obtained. In the case of ICP8, input ratios on the order of 1 ICP8 monomer per 20 nt are optimal for ssDNA annealing (37) due to the requirement for the formation of large nucleoprotein filaments. Fig. 5D shows that ICP8 anneals in a concentration-dependent manner the denatured pSAK ssDNA to produce dsDNA forms that migrated in the gel. However, unlike UL8, no well-trapped DNA was formed in the presence of ICP8. To demonstrate that the well-trapped DNA requires complementarity, two non-homologous linearized ssDNAs, M13mp18 and φX174, were incubated together in the presence and absence of UL8. No well-trapped DNA was observed either in the presence or absence of UL8 (Fig. 5E).

EM analysis of the annealing products reveals highly branched duplex DNA structures—The gel electrophoresis experiments demonstrate that incubation of complementary ssDNA with UL8 results in the formation of DNA trapped in the well of the gel following deproteinization. To characterize this DNA further, 100 nM UL8 was incubated with 1 nM denatured pSAK DNA in the presence of 5 mM MgCl₂ for 30 min at 30°C. Following deproteinization one half was electrophoresed on an agarose gel to verify that the reactions paralleled the results described above and the other half prepared for EM by spreading on the surface of a denatured film of cytochrome C protein (Experimental Procedures). In the absence of UL8, the DNA appeared as bush-like ssDNA clusters except for a few monomer length dsDNAs (Fig. 6A). Incubation with 100 nM UL8 for 0.5 min resulted in a slightly higher amount of dsDNAs (Fig. 6B), consistent with the results presented in Figure 5B. After 15 min the predominant forms observed by EM were X-shaped DNA molecules (Figs. 6C and D). In addition, some dsDNAs containing Y junctions measuring two pSAK plasmid lengths were present (Fig. 6E). To examine the junctions of the branched structures at higher resolution, the samples were incubated with E. coli SSB and prepared for EM by mounting onto thin carbon supports followed by tungsten shadow casting (Experimental Procedures). EM examination revealed frequent SSB-stained ssDNA segments at the junctions of the molecules (Fig. 6F). At 30 min, only highly branched DNA networks were observed (Fig. 6G) and their complexity made it impossible determine the number of molecules involved in each. Incubation of linear pSAK dsDNA with UL8 under the annealing conditions failed to produce any X- and Y-shaped molecules or DNA networks (data not shown) showing that they are a product of ssDNA annealing by UL8.

In summary, the networks seen by EM are largely duplex in nature and represent the material trapped in the wells. Their formation requires complementary ssDNA templates, they are present following deproteinization, and are stable to 55 degrees. This points to a highly base paired nature but also for the presence of multiple complex DNA junctions resulting in highly branched DNA products. As discussed below catalysis of annealing by UL8 differs in a number of respects from the reactions catalyzed by ICP8 both in the final products and also the amount of protein required. The presence of highly branched products suggested that UL8 might have a specific affinity for branched DNA.

UL8 has a high affinity for DNA replication forks—During HSV-1 DNA replication in vivo the UL5/UL8/UL52 heterotrimer acts specifically at replication forks to catalyze DNA unwinding and synthesize new RNA primers. What features of this complex target it to the replication fork and provide a strong enough affinity to allow it to remain bound is not fully understood. The experiments above suggested that UL8 may be involved in annealing DNAs at complex junctions raising the possibility that UL8 itself might have an affinity for fork-like structures. To examine this, we employed a model replication fork template we constructed and used in previous studies (39). This template consists of a 3 kb duplex DNA circle containing replication fork at a single site with a 400 bp dsDNA arm. A
gap is present between the fork and the 5' terminus of what would be the first Okazaki fragment on the displaced lagging strand (Fig. 7A). For these experiments templates with gaps of 1, 5, 15, and 25 nt were generated. This DNA (100 ng; 2 nM) was incubated with UL8 (50 ng; 31 nM) in a buffer of 20 mM Hepes pH 7.6, 50 mM NaCl for 30 min at 30°C. The protein was fixed in place with 0.6% glutaraldehyde for 5 min at RT followed by gel filtration through agarose beads to remove free protein and fixatives followed by adsorption to thin carbon substrates, dehydration, and shadow casting with tungsten (Experimental Procedures). At the EM, fields of sequentially encountered molecules were scored (200 for each sample). This revealed replication fork templates, and with the larger gaps, many with a single UL8 particle bound at the fork junction. No binding was seen with the templates containing a 1 nt or 5 nt gap, while 45% of the DNA with a 15 nt gap had UL8 present at the fork and 60% of the DNA with a 25 nt gap had UL8 bound at the fork (Fig. 7B). The size of the UL8 particle was consistent with its being a single protein monomer. At this protein to DNA ratio, no UL8 binding was observed anywhere else on the DNAs, either along the circle, along the displaced arm or at the terminus of the arm. Based on the concentration of UL8 in the incubation and the roughly 50% template binding at 7.5 nM, we can estimate an affinity constant of 7.5 nM suggesting a very high affinity with great specificity for the gap at the fork. It is of note that while strong binding was observed with a 15 nt ss gap in a fork structure, with linear ss oligos, no binding was observed in the gel shift experiments until the length approached 90 nt. This raises the possibility that UL8 might also bind to small simple internal gaps present in the viral genome.

DISCUSSION

UL8, although essential for viral DNA replication in vivo (25), is the only component of the HSV-1 replisome that had not previously been associated with enzymatic activity. This study examines the in vitro properties of the UL8 subunit of the UL5/UL8/UL52 heterotrimeric complex when it is expressed in the absence of the UL5 and UL52 subunits. Here, we report novel biochemical properties of UL8 including the propensity to form protein filaments in solution, and the ability to bind ssDNA. Moreover, our study reveals that UL8 can promote annealing of complementary ssDNA strands; however, the renaturation reaction promoted by UL8 is distinct from that promoted by other ssDNA annealing proteins. Annealing by UL8 results in the formation of complex branched structures, rather than typical duplex annealing products. Finally, UL8 was found to have a very high specificity for binding to a replication fork containing a short gap at the 5' terminus of the Okazaki fragment, suggesting a role for UL8 in targeting the heterotrimer to its site of action.

Our findings place UL8 in the family of proteins that include ICP8 and Orf6. First, we showed that UL8 self-assembles into protein filaments in solution. ICP8 is also known to form filaments in the absence of DNA (12). Furthermore, ICP8 mutants that disrupt its ability to form filaments are defective for viral growth and the formation of prereplicative sites and replication compartments (13). UL8 and ICP8 form filaments with similar diameters (15 and 18 nm respectively) however they differ considerably in other ways. ICP8 assembles into cylindrical double helical filaments with an asymmetric unit containing a dimer of ICP8 (12) and a clear axial repeat as seen in the EM. Negative staining of the UL8 filaments failed to reveal any such substructure and some images suggested that the UL8 filaments are not cylindrical but rather flat. UL8 at concentrations as low as 4 µg/ml can readily form filaments within 30 min, while ICP8 requires much higher concentrations (50 µg/ml) and prolonged incubation (12-16 h) (12). Filament formation by UL8 does not strictly require divalent cations as identical filaments can be formed without Mg²⁺, whereas ICP8 filaments cannot form in the absence of Mg²⁺. The differences in the appearance of the protein filaments and the conditions required for their formation suggest that the filamentous structures formed by these two proteins may be involved in different functions. It will be of interest to generate filament-deficient UL8 mutants to probe the biological function of the filaments.

Another finding of our study is that UL8 binds ssDNA in a length-dependent fashion. The chain length required for the most efficient binding was found to be 90 nt. The gel shift studies were done at stoichiometries of 1 UL8
monomer per oligonucleotide and at this ratio, all of the 90-mer was shifted into the well of the gel, arguing against the requirement for an oligomer forming on each and every oligonucleotide. This points to a strong interaction between UL8-oligomer complexes once the individual complexes have formed. When UL8 binds to longer ssDNA substrates such as ssM13 (7.4 kb), UL8 compacts the ssDNA. At a ratio of one UL8 monomer per ~70 nt all of the M13 ssDNA was shifted into the well of the gel. Since the site size for UL8 binding to ssDNA should be much less (ICP8 binds ~ 20 nt at saturation), one explanation is that the M13 ssDNA contains significant secondary structure, which UL8 is unable to bind to and also unable to open up. In contrast, ICP8 will remove secondary structure in ssDNA, holding it in an extended configuration to generate highly regular helical filaments on ssDNA (14) as contrasted to the condensed structures seen here with UL8. The helical structure of the ICP8 nucleoprotein is thought to be important for the annealing activity of ICP8 such that the ssDNA-ICP8 filaments facilitate its interaction with its complement in the other ICP8-ssDNA filament (14,15). Two nucleoprotein filaments then twine around each other to form a paired coiled-coil structure within which annealing occurs throughout their length. However, the condensed ssDNA-UL8 binary complex lacks such superhelical structures. The compacted character of the binary complex may account for the extensive pairing/branching between homologous ssDNA strands promoted by UL8 described in this study. UL8 pairs homologous regions of the complementary ssDNAs on the multiple DNA strands yielding highly branched DNA molecules. The branched networks could be formed by extensive pairing of two or more homologous ssDNA strands on multiple DNA molecules.

It was of note that while UL8 fails to form stable complexes on ssDNA oligomers until the length approaches 90 nt, if the ss segment is embedded in a true replication fork structure, then a ss segment as short as 15 nt provides strong stable UL8 binding. The EM images showed particles of a size consistent with single UL8 monomers at the fork, arguing against binding requiring an oligomer of UL8. This finding suggests that an additional function for UL8 may be to either load the heterotrimer onto replication forks, or to hold it in place once bound. In this respect it might parallel the gene 59 protein in the T4 system which acts as a helicase loader and then remains bound at moving forks as seen in the EM using nanoscale tags (41). It has previously been shown that UL5/UL8/UL52 binds preferentially to the oligo-based fork-like structures (42). Whether this binding is dictated by UL8 is to be seen.

Several lines of evidence suggest that during lytic infection, HSV DNA is replicated by a process that requires DNA recombination (19,43-45). For instance, replication of the HSV-1 genome produces X and Y branched structures that can be visualized by EM and 2D gel electrophoresis (5,7). These structures are reminiscent of recombination intermediates. The novel properties of UL8 revealed in this study suggest the possibility that UL8 may be involved in the generation of X and Y branched structures. To understand the full implications of the in vitro branching activity of UL8, genetic and cell based studies will be required.

**EXPERIMENTAL PROCEDURES**

**Proteins**—The recombinant baculovirus encoding His<sub>6</sub>-tagged UL8 was generated in the Bac-to-Bac system (Invitrogen) as described previously (46). Sf21 insect cells were infected with the recombinant His-UL8 baculovirus and harvested at 72 h postinfection. The protein was purified on a Ni<sup>2+</sup>-NTA affinity chromatography (Qiagen) as described previously (47). Purified proteins were resolved by 10% SDS-PAGE and were visualized by Coomassie blue staining. Fractions containing the highest purity were dialyzed against storage buffer (20 mM Tris-HCl pH 7.6, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA and 50% glycerol) and were stored at -20°C. ICP8 was purified using the chromatography protocol described previously (48).

**Size exclusion chromatography and light scattering**—Five micrograms of purified UL8 was injected into a Superose 6 HR (24 ml, GE life Sci) size exclusion column and eluted with 150 mM NaCl at a flow rate of 500 µl/min. The column effluent was passed through an in-line enhanced optimal system laser photometer (Helios II, Wyatt Technologies) coupled to a refractometer (optilab tREX, Wyatt Technologies) to measure the molecular weight and sample concentrations.
respectively. The data were analyzed using the Astra software (Wyatt Technologies).

**DNA substrates**— Oligonucleotides were purchased from Eurofins Scientific. The oligonucleotides (42, 55, 69, 82 and 90 nt) described in Table 1 were used in the binding assays. M13mp18 (7249 nt) and ΦX174 (5386 nt) circular ssDNAs were from New England Biolabs. Their linearized forms were prepared by generating a restriction enzyme recognition site formed by annealing the following oligonucleotides: The oligonucleotide (5’-ACTCTAGAGGATCCCCGGGTAC-3’) annealed to ssM13 was complementary to the region (6243-6264) encompassing the BamHI restriction site. The oligonucleotide (5’AGCACGAGAGCGGTCAGTAGCA-3’) used to generate linear ΦX174 was complementary to the region (521-542) containing the BsrBI restriction site. Following hybridization, the partial duplex DNAs were digested with the appropriate restriction enzymes. The linear ssDNA fragments were purified from agarose gels with the QIAquick Gel Extraction kit (Qiagen). pSAK dsDNA (3987 bp) (38) used in the annealing reactions was linearized at the unique restriction SmaI site to generate a linear, blunt-ended, duplex DNA. The product was gel purified as above. Replication fork templates containing 1, 5, 15 and 25 nt gaps were prepared as described previously (39).

**Filament formation**—UL8 filaments were formed by incubating purified protein in a buffer containing 20 mM Hepes pH 7.6, 50 mM NaCl in the presence or absence of 5 mM MgCl₂ for 30 min on ice. Samples were then directly analyzed without fixation by negative staining EM (see below).

**Electrophoresis mobility shift assays**—The ssDNA binding ability of UL8 was assayed by gel mobility shift assays using oligonucleotides and linearized M13mp18 ssDNA. Oligonucleotide-binding reactions (20 µl) contained 20 mM Hepes pH 7.6, 100 nM UL8 and 100 nM oligonucleotide at different lengths (Table 1). MgCl₂ (5 mM) was added in the reaction mixture as indicated in the figure legends. Reactions were incubated for 30 min at 30°C. Oligonucleotide-protein complexes were cross-linked by the addition of glutaraldehyde (0.6% final concentration) for 5 min at RT. The cross-linking reaction was terminated by the addition of 1 µl of 1 M Tris-HCl pH 8.0. Products were resolved on 10% native polyacrylamide gels in TBE buffer. Binding assays using M13mp18 ssDNA as DNA substrate were carried out as described above except that 1 nM substrate was used and samples were electrophoresed on 0.8% agarose gels in TAE buffer. The protein bound DNAs were detected by SYBR Gold (Life Technologies) staining. The extent of the oligonucleotide-UL8 binding was measured by quantification of the remaining unbound ssDNA using ImageJ software.

**Strand annealing assays**—Double-stranded pSAK DNA was denatured in boiling water for 1 min and immediately placed on ice. Unless otherwise indicated, the reaction (20 µl) was performed for 30 min at 30°C in a buffer containing 5 mM MgCl₂, 1 nM heat-denatured pSAK and 100 nM of UL8. For kinetic experiments, 150 µl reactions were initiated and 20 µl aliquots were removed at 0.5, 1, 2, 5, 10, 15 and 30 min. Reactions were terminated by addition of EDTA to a final concentration of 50 mM. Protein was removed by treating with 1% SDS and 100 µg/ml Proteinase K (final concentrations) for 1h at RT. Samples were electrophoresed on 0.8% agarose gels and stained with SYBR Gold.

**Electron microscopy**—To image the UL8 filaments and the UL8-bound oligonucleotides by negative staining, samples were adsorbed to glow-discharge treated thin carbon films supported by 400 mesh copper grids and stained with 2% uranyl acetate. Samples were examined in a Tecnai 12 transmission electron microscope (TEM) (FEI, Hillsboro, OR) at 80 kV. To visualize ssM13-UL8 complexes, samples were treated with 0.6% glutaraldehyde for 5 min at RT and chromatographed through 2-ml columns of 6% agarose beads (Agarose Bead Technologies), equilibrated in 10 mM Tris-HCl pH 7.6 and 0.1 mM EDTA. The samples were then adsorbed to thin carbon supports in the presence of 2 mM spermidine, washed, air-dried, and rotary shadow cast with tungsten (48). The grids were examined in the EM at 40 kV. To visualize deproteinized annealing reaction products, samples were treated with proteinase K (100 µg/ml) and SDS (1%) for 1h at RT and then passed over agarose bead columns as above. Following deproteinization, two methods were used. The DNA was either complexed with *E. coli* SSB and prepared for EM
as above or were directly prepared for EM by spreading on an air-water interface with cytochrome c protein (49). The grids were examined in EM at 40 kV. All images were captured on a Gatan Orius charge-coupled device (CCD) camera programmed with Digital Micrograph software (Gatan, Warrendale, PA).

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**Conflict of interest**—The authors declare that they have no conflict of interest with the contents of this article.

**Author contributions**—OB conducted all of the experiments, analyzed the data, and wrote the paper. SKW and JDG wrote the paper and analyzed the data with OB.

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FIGURES LEGENDS

FIGURE 1. Characterization of purified UL8. His-tagged UL8 was purified from Sf21 insect cells as described in Experimental Procedures. Products were resolved by 10% SDS-PAGE and subsequently stained with Coomassie blue. The protein marker is shown in the left lane, and purified UL8 is shown in the right lane.

FIGURE 2. Visualization of UL8 by negative staining. (A) An aliquot of the peak fraction from the gel filtration column shown in Supplemental Fig. S1 in elution buffer containing 150 mM NaCl was directly imaged by EM. The fields show small protein particles of relatively uniform size. Panels (B) and (C) show images of UL8 incubated in 20 mM Hepes pH 7.6 and 5 mM MgCl₂ for 30 min on ice. (B) At 10 nM, UL8 adopts ring-shaped structures. (C) The majority of the protein at 100 nM forms linear filaments. The samples were adsorbed directly onto glow-discharged thin carbon foils without fixation and stained with 2% (w/v) uranyl acetate. Scale bars equal 50 nm.

FIGURE 3. Effects of DNA length and Mg²⁺ ions on UL8 binding to oligonucleotide. (A) Gel electrophoretic analysis of oligonucleotide length dependence. The protein was incubated with five oligonucleotides at lengths indicated (Table 2) in the absence of Mg²⁺. The extent of binding as measured by the decrease of the oligonucleotide signal following addition of UL8 shows that there was 15% binding to the 42-mer oligonucleotide. UL8 binding to 55-, 69- and 82-mer oligonucleotides was ~60%. The most effective binding (96%) was observed with the 90-mer oligonucleotide. (B) Binding of UL8 to the 90-nt oligonucleotide in the absence or presence of 5 mM Mg²⁺. The samples were analyzed on 10% native polyacrylamide gels. Panels (C) and (D) show the electron micrographs of the well DNA corresponding to the UL8-Oligo90 complex shown in Panel (B) in the presence and absence of Mg²⁺ respectively. (C) The protein adopts very regular spherical structures with 20, 30 and 40 nm diameters in the presence of magnesium. (D) In the absence of magnesium, UL8 forms aggregates. The complexes were directly adsorbed onto glow-discharged thin carbon foils and stained with 2% (w/v) uranyl acetate. All experiments were performed by incubating 100 nM UL8 with 100 nM oligonucleotide for 30 min at 30°C. The protein-nucleic acid complexes were fixed with 0.6% glutaraldehyde to prevent the dissociation of the protein during electrophoresis. Scale bars equal 20 nm.

FIGURE 4. Agarose gel electrophoretic and EM analysis of UL8 binding to ssM13 DNA. (A) UL8 at the concentrations indicated was incubated with 1 nM linearized M13mp18 ssDNA (Experimental Procedures) in 20 mM Hepes pH 7.6 for 30 min at 30°C and the protein-DNA complexes fixed with 0.6% glutaraldehyde, prior to electrophoresis. The binding of UL8 was detected by the disappearance of ssDNA and the appearance of the material in the well in a concentration-dependent manner. (B) Biochemical analysis of the material in the well of the gel. Following the incubation of ssDNA with 100 nM UL8, the samples were treated or not treated with SDS and Proteinase K. The protein-DNA samples were found in the well of the gel and the well DNA disappeared after the removal of the protein. Panels (C-F) show representative images of ssM13 incubated with (C) 0, (D) 5, (E) 10 and (F) 100 nM UL8. The samples were chromatographed through 2 ml DNA agarose bead columns and mounted onto thin carbon films (Experimental Procedures). Scale bars equal 50 nm.

FIGURE 5. Agarose gel electrophoretic analysis of DNA annealing promoted by UL8. (A) Incubation of the heat-denatured linear pSAK ssDNA for 30 min with UL8 at 0.5, 1, 2, 4, 10, 15, 20, 25, 50 and 100 nM. (B) Time course of the reaction promoted by 100 nM UL8. (C) Magnesium titration of the annealing reaction promoted by 100 nM UL8 at a 30-min time point. The well DNA formation as measured by gel densitometry showed that there was a considerable amount of well DNA formation in the absence of magnesium, but the addition of 5 mM magnesium yielded the maximum conversion of the input ssDNA to the well DNA. (D) DNA annealing promoted by ICP8. The heat-denatured ssDNA was incubated with
ICP8 at 5, 10, 25, 50, 100 and 200 nM or 200 nM UL8 for 30 min. (E) Homology requirement of the reaction was assessed by incubating two linearized non-homologous substrates, M13mp18 and φ174X ssDNAs (Experimental Procedures), in the absence and presence of 100 nM UL8 for 30 min. At the end of each reaction, the protein was removed with Proteinase K/SDS treatment. All the reaction buffers included 5 mM MgCl₂ except (C). ds denotes SmaI digested, blunt-ended, duplex pSAK DNA; ss indicates heat-denatured pSAK ssDNA incubated in the reaction buffer for 30 min at 30°C, as control of spontaneous annealing of ssDNA in the absence of protein.

FIGURE 6. Visualization of the DNA renaturation products generated by UL8. (A) Heat-denatured pSAK ssDNA in the absence of UL8 incubated for 30 min at 30°C. DNA was mostly single-stranded in the absence of UL8 except a few dsDNAs formed due to spontaneous annealing of complementary strands. (B) Duplex DNA formation promoted by UL8 at 0.5 min. (C-F) Reaction intermediates at 15 min. (G) Incubation of ssDNA with UL8 for 30 min generated the conversion of all the input ssDNA into highly branched DNA structures. All the reactions included 5 mM Mg²⁺. The proteins were removed by treatment with SDS and Proteinase K for 1 h at RT. The deproteinized samples were cleaned through 2 ml agarose bead columns. Following chromatography, the DNAs shown in panels (A-E and G) were prepared by surface spreading with cytochrome c (Experimental Procedures). The samples in Panel (F) were mounted onto thin carbon supports followed by shadow casing with tungsten and the ssDNA regions were thickened by E.coli SSB. The scale bars: 200 nm (A-E,G), 100 nm (F).

FIGURE 7. Visualization of UL8 binding to DNA replication forks. (A) The plasmid pGLGAP (39) contains a single nicking site followed by a 400 bp G-less cassette which makes it possible to use a DNA polymerase to initiate at the nick and displace a 400 nt ssDNA arm when dGTP is omitted. The ss arm can be converted to a duplex with a variable sized gap on the displaced arm by extending an annealed oligonucleotide. (B) DNAs with 1 or 5 nt gaps at the fork fail to bind UL8. (C) DNAs with gaps of 15 or 25 nt show specific UL8 binding at the fork (arrow). UL8-DNA complexes were directly adsorbed to carbon supports, dehydrated, air dried and shadow cast with tungsten. Bar equals 100 nm.
Table 1. Physical state of UL8 under varying experimental conditions.

| Structure         | Irregular rounded particles | Regular oligomeric rings | Regular linear filaments |
|-------------------|-----------------------------|--------------------------|--------------------------|
| Concentration     | 1-9 nM                      | 10-49 nM                 | ≥50 nM                   |
| 5 mM MgCl₂        | (-) / (+)                   | (+)                      | (-) / (+)                |
| Width             | 5-15 nm                     | 20 nm                    | 15 nm                    |

The independence of the indicated parameter from the physical state of UL8 is designated as (-) / (+).
| Oligo  | Sequence                                      |
|--------|-----------------------------------------------|
| 42     | 5’-TCTTCCCTGCTCTGTCTCACTCTAGAGGATCCCGGGTAC-3’ |
| 55     | 5’-CCTGCTCTGTCTCTTTCCCTGCTCTGTCTCTACTCTAGAGGATCCCGGGTAC-3’ |
| 69     | 5’-CGGAACACAGAAGCGAAGCGAAGCAGGACAGGAAGCGAAGCGAAGCCCGGAGCCAAGCACCACCCG-3’ |
| 82     | 5’-TGAACATAAAAGCAATGACGGCAGCAATAAACTCAACAGGACAGGAAGAAAGCGAGGGTATCCCACAAAGTCCAGCGTACCAT-3’ |
| 90     | 5’-CTCTTTCCCTGCTCTGTCTCTCTCTGCGCCTCTCCCTGCTTTCTGTCTCCCTGCTTCCGTCCGGTCCGTTGTTCCG-3’ |
Figure 3
Figure 4

A

ssM13+UL8

ssM13

UL8 (nM) 0 1 2.5 5 10 25 50 100

B

ssM13+UL8

ssM13

UL8 - + +
Prot. K - - +

C

D

E

F

ssM13

UL8 0 5 nM 10 nM 100 nM

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Figure 5
The UL8 subunit of the helicase/primase complex of herpes simplex virus promotes DNA annealing and has a high affinity for replication forks
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