A Domain of Herpes Simplex Virus pUL33 Required To Release Monomeric Viral Genomes from Cleaved Concatemeric DNA

Kui Yang, Xiaoqun Dang,* Joel D. Baines
Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana, USA

ABSTRACT  Monomeric herpesvirus DNA is cleaved from concatemers and inserted into preformed capsids through the actions of the viral terminase. The terminase of herpes simplex virus (HSV) is composed of three subunits encoded by UL15, UL28, and UL33. The UL33-encoded protein (pUL33) interacts with pUL28, but its precise role in the DNA cleavage and packaging reaction is unclear. To investigate the function of pUL33, we generated a panel of recombinant viruses with either deletions or substitutions in the most conserved regions of UL33 using a bacterial artificial chromosome system. Deletion of 11 amino acids (residues 50 to 60 or residues 110 to 120) precluded viral replication, whereas the truncation of the last 10 amino acids from the pUL33 C terminus did not affect viral replication or the interaction of pUL33 with pUL28. Mutations that replaced the lysine at codon 110 and the arginine at codon 111 with alanine codons failed to replicate, and the pUL33 mutant interacted with pUL28 less efficiently. Interestingly, genomic termini of the large (L) and small (S) components were detected readily in cells infected with these mutants, indicating that concatemeric DNA was cleaved efficiently. However, the release of monomeric genomes as assessed by pulsed-field gel electrophoresis was greatly diminished, and DNA-containing capsids were not observed. These results suggest that pUL33 is necessary for one of the two viral DNA cleavage events required to release individual genomes from concatemeric viral DNA.

IMPORTANCE  This paper shows a role for pUL33 in one of the two DNA cleavage events required to release monomeric genomes from concatemeric viral DNA. This is the first time that such a phenotype has been observed and is the first identification of a function of this protein relevant to DNA packaging other than its interaction with other terminase components.

KEYWORDS  herpes simplex virus, UL33, DNA cleavage/packaging
and C capsids, and an inner scaffold layer; A capsids lack the internal layer, and the C capsid contains DNA in place of the inner scaffold. A capsids are believed to be the result of aborted packaging events in which the inner shell was expelled or degraded and DNA was not inserted (10, 11).

All herpesviruses encode an enzyme called the terminase; in HSV, the terminase consists of three subunits encoded by the genes UL15, UL28, and UL33 (12–19). The UL28 protein (pUL28) has been shown to bind Pac1 DNA, which is important for the generation of short component termini (20), whereas UL15 has nonspecific cation-dependent nuclease activity in vitro and contains an essential Walker box motif, suggesting an ability to hydrolyze ATP (21). The UL89-encoded homolog of cytomegalovirus retains the ATPase motif and cation-dependent nuclease activity (22, 23). The structures of the C-terminal portions of the HSV-1 UL15 gene product (pUL15) and human cytomegalovirus (HCMV) pUL89 are virtually superimposable and reveal a conserved RNase H-like structure seen in a number of nucleases and bacteriophage terminase subunits responsible for endonucleolytic DNA cleavage (18, 21, 24).

Cleavage of viral DNA requires both intact capsids and all terminase components (18, 25–27). Mutations outside the ATP binding and nuclease active sites in pUL15 can prevent DNA packaging but do not preclude DNA cleavage or the release of intact genomes from concatemeric DNA, indicating that pUL15 has a packaging function separate from its nuclease activity (28). These observations are consistent with its roles as both a packaging motor and an endonuclease. pUL28, on the basis of its sequence-specific DNA binding activity, may act to restrict pUL15’s nuclease activity to correct sites at genomic termini.

The predominant model to explain HSV DNA packaging proposed that cleavage at the long terminus is followed by packaging and scanning of DNA until the short terminus is encountered in the concatamer, at which time it is cleaved (29, 30). The α sequences that signal cleavage contain subsequences designated DR1-Uc-DR4m-DR2n-Ub-DR1, where DR indicates a direct repeat and m and n indicate the number of repeats (31, 32). Ub and Uc are two unique sequences. Ub has been designated Pac1, and Uc has been designated Pac2. Although two cleavage events that are required to release genomes from the concatamer occur at different times and require different cis-acting sequences, they cleave DR1 identically, leaving single 3’ overhangs with 18 bp of DR1 at the L terminus and a single base pair of DR1 at the S terminus (32). The initial cleavage renders the S terminus on the concatamer nonfunctional because it removes much of DR1. Thus, it is believed that only the L terminus is packaged, whereas the S terminus generated from the first cleavage event is degraded. While most genomes in the concatemer are separated by a single α sequence, some genomic junctions and L component termini contain multiple α sequences that share an intervening DR1 region. During packaging, these tandem α sequences presumably pass by the docked terminase and remain uncleaved. Scanning continues until DR1 in the proper orientation is cleaved to generate the S component terminus of DNA to be packaged and to release the genome from the concatamer. The idea that the S component terminus is packaged last is supported by the observations that (i) the S terminus in packaged DNA is most susceptible to DNase digestion and (ii) the short component of packaged DNA never bears more than one α sequence, suggesting that it is generated when the terminase cleaves the first encountered packaging sequence in the proper orientation after scanning/packaging of the long and short components (7, 11, 33).

The main goal of the present study was to understand the role of pUL33 in the packaging reaction. pUL33 interacts with pUL28 and enhances the pUL28-pUL15 interaction (13). Extensive mutagenic analyses previously identified regions of UL33 important for DNA packaging and the generation of L component termini (34, 35). In extending these analyses, we have identified several novel mutations in a conserved region of pUL33 with a positive charge that do not prevent the cleavage of concatemeric DNA but preclude the release of monomeric genomes. These data suggest that the pUL33 component of the terminase is necessary for one of the two DNA cleavage events necessary to release unit-length genomes from concatemeric DNA.
RESULTS

In a previous mutagenesis study, the insertion of amino acids VRPQR at position 111 and AAAAA at position 116 of the 130-codon U₃3 open reading frame precluded the packaging of viral DNA (35). Neither of these insertions precluded interactions with the U₂₈ protein (pU₂₈), although the insertion at codon 116 diminished this interaction, as assessed by a coimmunoprecipitation assay. Alignment of HSV-1 U₃₃ with homologs in other herpesviruses showed that two regions, residues 46 to 77 and 101 to 130, were the most conserved regions of pU₃₃. Basic amino acids were highly conserved at positions corresponding to codons 110 to 113 (encoding KRER in HSV-1). These amino acids were followed by invariant phenylalanine and alanine residues at positions 114 and 115, respectively (Fig. 1).

To investigate whether these conserved regions are important for U₃₃ function, we generated a series of mutant viruses using a bacterial artificial chromosome (BAC) system and assessed their ability to replicate on CV₁ cells and complementing cells expressing U₃₃. Consistent with previous work, recombinant viruses with a deletion of residues 50 to 60 or 110 to 120 replicated only in pU₃₃-expressing cells (Table 1). In contrast, a mutant virus lacking codons 120 to 130 replicated in noncomplementing cells at levels similar to those of the wild-type virus (Table 1), suggesting that the last 10 amino acids are dispensable for U₃₃ function.

To further investigate the importance of charged residues for pU₃₃ functions, we generated a series of mutant viruses in which basic residues were replaced with alanine, using the recombinant bacterial artificial chromosome system. As shown in Table 1, single-alanine-residue substitutions at positions 52, 54, 60, 65, 85, 110, 113, and 120 reduced the replication of the corresponding mutant viruses by 2- to 14-fold, suggesting modest effects on pU₃₃ function. In contrast, mutants with multiple amino changes (110-KR-111 changed to 110-AA-111 and 110-KRER-113 changed to 110-AAEA-113, designated KR and KRER, respectively), could be propagated only on cells expressing pU₃₃ inasmuch as the ratio of yields from U₃₃-expressing cells infected with these viruses to those from CV₁ cells was over 10,000-fold (Table 1). These results were similar to those obtained from analyses of the U₃₃-null mutant performed at the same time (Table 1). We conclude that there was no detectable replication of the KR or KRER mutation in CV₁ cells.

To determine if these lethal substitution mutations interfered with the pU₂₈ interaction, cells were mock infected or infected with 5.0 PFU per cell of wild-type HSV-1(F) or U₃₃ mutant viruses. Cells were lysed at 18 h postinfection (p.i.), and clarified lysates were reacted separately with antibody against pU₂₈ or pU₃₃. Antigen-antibody complexes were purified, eluted in SDS-containing buffer, electrophoretically separated on a denaturing SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane, which was then probed with the same pU₂₈- or pU₃₃-specific antibodies. As shown in Fig. 2, the U₃₃ mutant proteins bearing the 110-AA-111 (KR) and 110-AAEA-113 (KRER) mutations were efficiently immunoprecipitated by the U₃₃ antibody. Unlike wild-type pU₃₃, the mutant proteins failed to coimmunoprecipitate pU₂₈. In the reciprocal interaction, pU₂₈-specific antibody efficiently immunoprecipitated pU₃₃ from cells infected with HSV-1(F), as shown previously (13, 15, 35). Less pU₂₈ was immunoprecipitated with the pU₂₈ antibody from lysates of cells infected with the U₃₃ mutants, consistent with previous results indicating that an optimal interaction of pU₂₈ confers stability to the pU₂₈ protein (13). Despite the lower levels of pU₂₈ in the lysates of cells infected with the U₃₃ mutant viruses, low levels of both mutant U₃₃ proteins were coimmunoprecipitated with the pU₂₈ antibody. We conclude that the U₃₃ mutations diminished the pU₂₈-pU₃₃ interaction but did not completely eliminate it.

Experiments were then conducted to assess effects of the mutations on the generation of genomic termini. As shown in Fig. 3A, terminase cleavage combined with BamHI cleavage should generate BamHI S fragments of around 2.9 kbp at the L component terminus and a P fragment of 3.45 kbp that reflects the terminus of the S
FIG 1  Multiple-sequence alignment of pUL33 from 19 alphaherpesviruses. Multiple sequences were aligned with Clustal Omega on-line software (http://www.ebi.ac.uk/Tools/msa/clustalo/). Conversed residues mutated in this research are highlighted in red boxes. HHV-3, human herpesvirus 3 (varicella-zoster virus); CHV, chimpanzee alphaherpesvirus 1; McHV-1, macacine alphaherpesvirus 1; PaHV-2, papine alphaherpesvirus 2; PRV, pseudorabies virus; BoHV-1, bovine alphaherpesvirus 1; SaHV-1, saimirine alphaherpesvirus 1; CeHV-2, cercopithecine alphaherpesvirus 2; FBHV-1, fruit bat alphaherpesvirus 1; LeHV-4, leporid alphaherpesvirus 4; EHV-1, equid alphaherpesvirus 1; FeHV-1, felid alphaherpesvirus 1; GaHV-1, gallid alphaherpesvirus 1; MeHV-1, meleagrid alphaherpesvirus 1.
component. Multiple BamHI S fragments were expected because one or more a sequences are present at the L termini of different genomes (Fig. 3A). Both the S and P fragments are covalently linked in the S-P fragment derived from the junction of the long and short components in monomeric DNA and the junctions of tandem genomes within concatemeric DNA. Therefore, to test for terminase cleavage activity, viral DNA was purified from cells infected with wild-type and mutant viruses, digested with BamHI, transferred to a positively charged nylon membrane, and probed with radiolabeled fragments specific for the termini of either the long or short components. The results are shown in Fig. 3B.

BamHI S fragments were readily detectable in cells infected with the wild-type virus (Fig. 3B, lane 1) but were hardly detectable in DNA from cells infected with the U133-null virus, indicating that pU133 was necessary for optimal terminase cleavage at the L component terminus. In contrast to these results, S fragments from cells infected with the KR or KREK mutant were readily detectable, indicating that the mutations did not block the cleavage that generates the L component terminus. Similarly, BamHI P fragments representing the S component terminus were readily detected in cells infected with both wild-type and U133 point mutant viruses, but P fragments were barely detected in U133-null-virus-infected cells.

Because genomic DNA within input virions is linear and would be incorporated into preparations of infected cell DNA, we wanted to ensure that the BamHI S and P fragments that we observed were not due to contaminating input virion DNA. Thus, cells were infected with wild-type and mutant viruses in the presence and absence of phosphonoacetic acid (PAA), a potent viral DNA synthesis inhibitor, and viral DNAs were purified, digested with BamHI, and analyzed on Southern blots probed with the radiolabeled HSV-1(F) P fragment, as detailed above. As shown in Fig. 4, P fragments were detected in input viral DNA in all samples from cells treated with PAA. Moreover, the P fragment was present with the S-P junction fragment at a consistent ratio in all PAA-treated samples. This was expected and reflected the presence of exclusively linear DNA in input virions. Also as expected, the amount of viral DNA in samples from cells infected in the presence of PAA was reduced. Most importantly, the ratios of P to S-P fragments were similar in all samples except those from cells infected with the U133-null virus in the absence of PAA. In this sample, the P/S-P ratio was reduced by approximately 55%. These data suggest that while pU133 is necessary for the optimal generation of genomic termini, both genomic termini were generated in cells infected with two U133 substitution mutants.

| Virus     | Mutation(s) in pU133 | Titer in CV33 cells^a | Titer in CV1 cells | Interaction with pU33^b |
|-----------|----------------------|-----------------------|-------------------|------------------------|
| 33 null   | Deletion of pUL33    | 6 × 10^6              | <10^3             | −                      |
| Del50–60  | Deletion of residues 50–60 | 7 × 10^6              | <10^3             | ?                      |
| Del110–120| Deletion of residues 110–120 | 1.5 × 10^6            | <10^3             | ?                      |
| Del120–130| Deletion of residues 120–130 | 5.5 × 10^6            | 2 × 10^6          | +                      |
| P52A      | Proline to alanine   | 4 × 10^6              | 8 × 10^7          | +                      |
| E54A      | Glutamic acid to alanine | 8 × 10^4              | 1 × 10^7          | +                      |
| P60A      | Proline to alanine   | 7 × 10^4              | 5 × 10^6          | +                      |
| K65A      | Lysine to alanine    | 2 × 10^7              | 1.2 × 10^7        | +                      |
| K86A      | Lysine to alanine    | 2.6 × 10^6            | 8 × 10^7          | +                      |
| K110A     | Lysine to alanine    | 4 × 10^6              | 1.2 × 10^7        | +                      |
| R113A     | Arginine to alanine  | 2.2 × 10^6            | 4 × 10^7          | +                      |
| R120A     | Arginine to alanine  | 9.5 × 10^7            | 5 × 10^7          | +                      |
| KR        | Lysine and arginine at positions 110 and 111 to alanine | 5 × 10^6 | <10^3 | +/−                |
| KRER      | Lysine and arginine at positions 110, 111, and 113 to alanine | 2 × 10^6 | <10^3 | +/−                |

^aStock viruses were propagated in CV33 cells, while virus titers were determined separately in CV33 or CV1 cells. Virus titers are expressed as PFU per ml.

^bThe interaction between pU28 and pU33 was determined by coimmunoprecipitation followed by immunoblotting. −, no interaction; ?, unknown; +, interaction; +/−, diminished interaction.
To determine whether the terminase cleavage events in UL33 mutant viral DNA were sufficient to release genomes from concatemeric viral DNA, we analyzed viral DNA using pulsed-field gel electrophoresis. Cells were infected with HSV-1(F) and UL33 mutants in the presence and absence of PAA, and agarose plugs containing the infected cell DNA was subjected to electrophoresis on an agarose gel in a pulsed-field

FIG 2 Assessment of pUL28 and pUL33 interactions in cells infected with wild-type and mutant viruses. CV1 cells were mock infected or infected at an MOI of 5 PFU/cell with HSV-1(F) or UL33 mutants, as indicated. The cells were harvested at 18 h postinfection and lysed in RIPA buffer. Precleared lysates were reacted with anti-pUL33 (A) or anti-pUL28 (B) antibodies. Antigen-antibody complexes were eluted in 2× SDS sample buffer and separated on denaturing 12% polyacrylamide gels. Separated proteins were transferred onto a nitrocellulose membrane and probed with anti-pUL28 or anti-pUL33 antibodies. Arrows indicate the positions of the indicated proteins and the heavy chain of rabbit immunoglobulin. IP, immunoprecipitation.
The separated DNA was then transferred to a nitrocellulose membrane and probed with radiolabeled viral DNA. As shown in Fig. 5, both concatemeric DNA (well DNA) and monomeric DNAs were detected in untreated HSV-1(F)-infected cells, whereas no concatemeric DNA and small amounts of monomeric DNA were detected after infection in the presence of PAA. We attribute the signal in the PAA-treated sample to input virion DNA. Unlike the results with the wild-type virus, the UL33 mutant viruses produced substantially less monomeric DNA than did the wild-type virus inasmuch as virtually all replicated DNA remained in the well, reflecting its presence within DNA concatemers. Small amounts of monomeric DNA were detected in cells infected with UL33 mutant viruses in the presence of PAA. Although most of this monomeric DNA signal was attributable to input virion DNA, we could not rule out some generation of monomeric DNA by the UL33 mutant viruses because a slightly higher monomeric signal was obtained from cells infected in the absence of PAA than in PAA-treated cells. In the absence of PAA, the monomeric mutant DNA bands were less distinct and exhibited broad bands extending below the migration position of monomeric DNA. This observation suggested that monomeric DNA was cleaved non-specifically or was partially degraded in these samples.

To assess DNA packaging of the mutant viruses, two sets of experiments were conducted. In the first set of these experiments, thin sections of cells infected with wild-type and mutant viruses were examined by electron microscopy. As shown in Fig. 6, HSV-1(F) produced electron-dense capsids containing DNA (type C), capsids with an inner electron-lucent core (type B), and empty capsids (type A). In contrast, while type B capsids were readily detected in cells infected with the UL33 mutants, neither type A...
nor type C capsids were detected in these cells. These results suggested that the UL33 mutants exhibited profound defects in DNA packaging.

In the second set of experiments, capsids were purified from cells infected with wild-type or mutant viruses and were separated by rate-zonal centrifugation on continuous sucrose gradients. As shown in Fig. 7, only the wild-type virus produced light-refracting bands at positions consistent with all three capsid types, whereas the UL33 mutants produced only 1 band consistent with type B capsids that lack DNA. These data further indicated that the UL33 mutants were defective in DNA packaging.

**DISCUSSION**

To define the function of pUL33, we extended mutagenesis studies reported previously, and we report here the generation of new UL33 mutants using a bacterial artificial chromosome system. The deletion of the conserved regions between either amino acids 50 and 60 or amino acids 110 and 120 of pUL33 impaired its function, and these mutants were able to replicate only in UL33-expressing cell lines. Our results are consistent with data from previous work in which pUL33 mutations with insertions in these regions were unable to complement the growth of a UL33-null virus in transient-packaging assays (35). The last 10 amino acids of pUL33 are highly conserved in all alphaherpesviruses but were found to be dispensable for HSV replication.

One goal of this work was to define the region of the pUL33 interaction with pUL28. Our previous work showed that pUL33 bearing a point mutation at position 61 failed to interact with pUL28 at a nonpermissive temperature (34). Consistent with this work, the deletion of codons 50 to 60 precluded the interaction of pUL33 with pUL28 (data not shown), and this mutant virus was unable to replicate in noncomplementing cells.

**FIG 4** Ratio of terminal to junction BamHI fragments in replicated and input HSV DNA from infected cells. (A) CV1 cells were infected with the indicated viruses at an MOI of 5 PFU per cell in the presence or absence of phosphonoacetic acid (PAA). At 18 h postinfection, viral DNAs were extracted, digested with BamHI, separated on an agarose gel by electrophoresis, denatured, and transferred onto a nylon membrane. Bound DNAs were probed with the denatured radiolabeled P fragment. (B) The levels of the P or S-P fragments in all samples were quantified by using ImageJ software, and the ratios of P fragments to S-P fragments are plotted on a graph.
Table 1), suggesting that this region may mediate protein-protein interaction. Surprisingly, mutants with alanine replacements in the conserved residues (52P, 54E, 60P, or 65K) in this region replicated in noncomplementing cells nearly as well as the wild-type virus (Table 1), suggesting that these point mutations did not dramatically affect pUL33 function or interactions with pUL28. However, we have not tested whether these mutations affect pUL33 function at higher temperatures (e.g., 39°C). Whether this conserved region is sufficient to mediate the interaction of pUL33 with pUL28 needs further study. A virus lacking UL33 codons 110 to 120 was also unable to replicate in noncomplementing cells (Table 1). Attempts to assess pUL28 interactions with a pUL33 mutant lacking codons 110 to 120 were unsuccessful because the deletion of this region precluded detection by the pUL33-specific antibody (data not shown). Therefore, it remains unclear whether the deletion of codons 110 to 120 in pUL33 affects the interaction with pUL28.

Previously characterized terminase component mutants either do not cleave DNA or allow both cleavage events but preclude DNA packaging (11, 18, 26, 28, 36–40). The UL33 mutants KR and KRER (Fig. 3 and 4) are unusual because both long and short termini were generated, yet little to no monomeric DNA was released from the concatemer. Because type A capsids that result from aborted packaging events were not detected, it is likely that the UL33 mutants abort the packaging reaction after DNA cleavage but before the initiation of DNA packaging. Although there are other possibilities, the simplest explanation for the lack of released monomers is a failure of the second DNA cleavage event. Thus, in noncomplementing cells, the short and long termini of these mutants are generated from a single successful DNA cleavage event, but this is not followed by a second coordinated cleavage event that releases genomes from the concatemer. If true, this would suggest that the first DNA cleavage event always occurs at DR1 in the α sequence most distal to UL, leaving an S terminus on the concatemer with an α sequence bearing a single base pair of DR1.

Indirect evidence also favors the possibility that the KR and KRER mutants are defective in the second DNA cleavage event. Specifically, in coimmunoprecipitation experiments, these UL33 mutants were coimmunoprecipitated with pUL28 by anti-pUL28 antibodies, but pUL28 was not pulled down with the pUL33 mutant by anti-pUL33 antibodies. These observations suggest that these mutations inhibit the pUL33-
pUL28 interaction but do not completely eliminate it. This imperfect interaction may inhibit proper scanning, which may be required for the coordination of the two cleavage events to release monomeric genomes. The lack of an optimal pUL33-pUL28 interaction may also preclude the second DNA cleavage event, which is likely augmented by pUL28’s recognition of Pac1 DNA (20, 41).
It is notable that low levels of L termini are detectable in concatemeric DNA (well DNA) from cells infected with the wild-type virus (42, 43). Concatemeric DNA has been designated well DNA experimentally because it is large enough to be retained in loading wells even after lengthy pulsed-field gel electrophoresis (44). Remarkably, S termini have not been detected in well DNA (44, 45), suggesting that they are absent from concatemeric ends. This observation is surprising because each DNA cleavage event should generate both an S terminus and an L terminus. It is therefore likely that S termini are degraded (when produced from the first terminase DNA cleavage event) or, when produced from the second DNA cleavage event, removed from the concatemer through DNA packaging. Unlike the wild-type virus, the UL33 KR and KRER mutants described here produce concatemeric DNA almost exclusively, yet both S and L termini were abundant and readily detected. Taken together, these results suggest that pUL33 may also play a direct or indirect role in the degradation of S termini on the concatemer. Another possibility is that both terminase DNA cleavage events occur in the KR and KRER UL33 mutants, but the monomers are somehow retained with the concatemer in well DNA. We do not favor this possibility because monomers are released when well DNA is digested with SpeI, a restriction enzyme that cuts each genome within the concatemer only once (44). Thus, tandem cleavages a genome apart should be sufficient to release monomeric genomes. It follows that the cleavages that we detected in the KR and KRER mutants are much farther apart than genome length, suggesting a discoordination of the two cleavage events relevant to DNA packaging.

In summary, these data reveal previously unknown functions of pUL33 and support its role as an important terminase component.

**MATERIALS AND METHODS**

**Cells, viruses, and plasmids.** CV1 cells and CV1-derived complementing cells expressing UL33 (designated CV33) were described previously (13). The wild-type F strain of HSV-1 [HSV-1(F)] and the UL33 deletion virus were described previously (46, 47). The HSV-1(F) bacterial artificial chromosome (HSV-BAC) was obtained from Y. Kawaguchi, University of Tokyo (48), and all the recombinant mutants were derived from this HSV-BAC. The pEPKan-S plasmid containing aphA1 (encoding kanamycin resistance) was a gift from Klaus Osterrieder, University of Berlin. Plasmid pCAGGS-nlsCre expressing Cre recombinase was a
by using a plaque assay. All the recombinant viruses were generated with the BAC system as described previously by Tischer et al (49). The primers used to construct recombinant BACs are listed in Table 2. Detailed procedures to generate recombinant BACs were described above, and the expected mutations were confirmed by DNA sequencing. The corresponding recombinant viruses were reconstituted by cotransfecting BAC DNA with the Cre expression plasmid into CV33 cells. Stock viruses of these mutants were prepared in CV33 cells, and the virus titers in CV33 and CV1 cells were determined by using a plaque assay.

Coimmunoprecipitation and Western blotting. CV1 cells were infected with the indicated viruses at a multiplicity of infection (MOI) of 5.0 PFU per cell. Eighteen hours after infection, cells were washed with phosphate-buffered saline (PBS) and lysed in cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, and a protease inhibitor cocktail). The lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4°C, and the precleared lysates were reacted with anti-pUL28 or anti-pUL33 antibodies, followed by the addition of GammaBind Sepharose beads for immunoprecipitation. Immune complexes bound on the beads were washed extensively with RIPA buffer, eluted in 2× Laemmli sample buffer (Bio-Rad), separated on an SDS–12% polyacrylamide gel, and transferred onto a nitrocellulose membrane for immunoblotting. Both anti-pUL28 and anti-pUL33 antibodies were diluted at 1:100 for immunoprecipitation and at 1:1,000 for immunoblotting. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G and enhanced chemiluminescence were used for immunodetection.

Southern blotting. CV1 cells were infected with wild-type HSV-1 or U333-null mutant viruses at an MOI of 5 PFU per cell in the presence or absence of 300 μg/ml PAA. At 18 h postinfection, viral DNA was extracted as described previously (28). Briefly, cells were lysed in 1% NP-40 and digested with proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. Viral DNAs were digested with BamHI and electrophoretically separated on a 0.8% agarose gel. The separated DNAs were denatured with a denaturation solution (1.5 M NaCl, 0.5 M NaOH), neutralized with neutralization buffer (1 M Tris-HCl [pH 8.0], 1 mM NaCl), and transferred onto a positively charged nylon membrane. Bound DNAs were cross-linked with a UV cross-linker (FB-UVXL-1000; Fisher Scientific) and hybridized with denatured 32P-labeled BamHI P or S fragments of HSV-1 DNA. The membrane was washed extensively with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the positions of the bound probe were determined by fluorography using X-ray film exposed at −80°C in the presence of an intensifying screen. The film was scanned and processed with Adobe Photoshop software, and the signal intensity was quantified with ImageJ software.

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis experiments were carried out as described previously (28). Briefly, about 3.2 × 108 CV1 cells in a 60-mm-diameter dish were infected with the indicated viruses at an MOI of 5 PFU per cell in the presence or absence of 300 μg/ml PAA for 18 h. Cells were collected in cold PBS and pelleted by spinning them at 4,000 rpm for 5 min in a refrigerated tabletop centrifuge. The pellets were resuspended in 150 μl of PBS and mixed with 300 μl of 1.2% agarose, and the mixture was poured onto the bottom of a 10-mm-diameter dish to form an agarose block. The agarose block wasdigested with 100 μg/ml proteinase K in digestion buffer (10 mM Tris [pH 8.0], 100 mM EDTA, 1% [w/v] N-lauroylsarcosine sodium salt [Sarkosyl]) for 20 h at 37°C and washed with storage buffer (10 mM Tris [pH 8.0], 10 mM EDTA). Roughly equally sized agarose plugs were sliced and loaded into the wells of a 0.8% agarose gel, and the wells were sealed with 0.8% low-melting-point agarose. The gel was run in 0.5× TBE buffer (1× TBE buffer contains 89 mM Tris, 89 mM boric acid, and 2 mM EDTA [pH 8.0]) at 6 V/cm for 16 h at 14°C, with an angle of 120° and a pulse time of 45 to 70 s, with a Bio-Rad CHEF-DR II pulsed-field electrophoresis system. After electrophoresis, the gel was soaked in 0.25 M HCl for 45 min to depurinate the DNAs, and the DNAs were further denatured, neutralized, and transferred onto a positively charged nylon membrane as described above. DNAs were UV cross-linked to the membrane and hybridized with the denatured 32P-labeled BamHI P fragment of the HSV-1 genome as described above. The bound probe was revealed by exposure of the membrane to X-ray film at −80°C in the presence of intensifying screens.

Electron microscopy. Electron microscopic examination of infected cells was performed at the Shared Instrument Facility at Louisiana State University. Confluent CV1 cells in T25 flasks were infected with wild-type HSV-1 or U333 mutants for 18 h. Medium was removed, and the cells were incubated with a fixative solution (2% formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer [pH 7.0]) for 10 min and collected by scraping into a centrifuge tube, followed by shaking for 2 h. Cells were pelleted, and the supernatant was discarded. The pellets were mixed with equal volumes of 3% agarose and transferred onto a glass slide before solidification. The agarose cubes were rinsed 5 times (15 min each) with 0.1 M phosphate buffer (pH 7.4) containing 0.08 M glycine, followed by fixing the cells for 1 h in the dark with 2% osmium tetroxide prepared in 0.1 M phosphate buffer (pH 7.4). After 3 washes with distilled water, samples were dehydrated with a graduated series of ethanol concentrations (50%, 70%, 80%, 90%, and 100%, for 15 min under each concentration). This was followed by infiltration with a 1:1 mixture of ethanol (EOH) and LR White for 2 h and with 100% LR White for 2 h. Samples were dispersed into the bottom of a Beem capsule, and the resin was polymerized at 65°C for 24 h. Ultrathin sections were cut on a Leica EM UC7 microtome, and thin sections (90 nm thick) were collected on 300-mesh nickel grids. Thin sections were counterstained with 2% uranyl acetate for 20 min and then with lead citrate for 7 min. Stained grids were viewed with a JEOL JEM-1400 transmission electron microscope. Images were captured digitally and processed with Adobe Photoshop software.
| Primer                  | Sequence                                                                 |
|------------------------|--------------------------------------------------------------------------|
| Delete 50–60 aa Forward| TCGCGAGAGGCGCGATGACGCGGCCGTCTGAGGTATCTACGGAGCGCAAGCTGAACTAGGGATAACAGGGTAATCGATTT |
| Delete 50–60 aa Reverse| CCGCTGCGTCGCGTATCGGGAAGCAAACCTCCAGCCTCGGGGGTTCTTCCCTTGGACCTGCACCAGATTTAGGGATAACAGGGTAATCGATTT |
| P52A Forward           | GACCGGCGCATCGGCGGCGTGTCTGGAGGTATGACCACCCTGGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| P52A Reverse           | CAGCTTGGCGTGATCCGGAAGCAAACCTCCAGCCTCGGGGGTTCTTCCCTTGGACCTGCACCAGATTTAGGGATAACAGGGTAATCGATTT |
| E54A Forward           | GCTGCTGAGGTACGCGGCGTGTCTGGAGGTATGACCACCCTGGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| E54A Reverse           | GTAGTTCAGGCGCGTGTCTGGAGGTACGCGGCGTGTCTGGAGGTATGACCACCCTGGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| P60A Forward           | TCGCTGAGGATATGACCACCCTGGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| P60A Reverse           | CGGCGGCGGCGTGTCTGGAGGTACGCGGCGTGTCTGGAGGTATGACCACCCTGGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| K65A Forward           | ACCCCGGCGACCGTCGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| K65A Reverse           | CAGGAGGCGACCGTCGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| Delete 120–130aa Forward| CTGCTGCGCGCAAGGGAAAGATTCGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| Delete 120–130aa Reverse| CAAAGGGTTACGCGGCTGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| Delete 110–120aa Forward| ACCCGCGCGCGTGTCTGGAGGTACGCGGCGTGTCTGGAGGTATGACCACCCTGGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| Delete 110–120aa Reverse| GCACGCGCGCGTGTCTGGAGGTACGCGGCGTGTCTGGAGGTATGACCACCCTGGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| K86A Forward           | ACGGGCGCGCGTGTCTGGAGGTACGCGGCGTGTCTGGAGGTATGACCACCCTGGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| K86A Reverse           | GCTGCTGCGCGCGTGTCTGGAGGTACGCGGCGTGTCTGGAGGTATGACCACCCTGGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| K110A Forward          | ACCCGCGCGAGCTGGCGACGGTGAGGGGAAGATTCGCGGCGGTCATTAACCCAGGCAGCTCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| K110A Reverse          | CAGGAGGCGACCGTCGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| R113A Forward          | ACCGCGGCGCGTGTCTGGAGGTACGCGGCGTGTCTGGAGGTATGACCACCCTGGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| R113A Reverse          | AAGGTTGCTGCGGCTGACCGCTCGGCGTGTCTGGAGGTATGACCACCCTGGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| R120A Forward          | CTGCTGCTGCGCGCGGAAAGATTCGCGGCGGTCATTAACCCAGGCAGCTCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| R120A Reverse          | GACCGGCGCGGTCGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| 110KRAA111 Forward      | ACCCGCGCGAGCTGGCGACGGTGAGGGGAAGATTCGCGGCGGTCATTAACCCAGGCAGCTCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |
| 110KRAA111 Reverse      | AAGGTTGCTGCGGCTGACCGCTCGGCGTGTCTGGAGGTATGACCACCCTGGCGGCTGAGGCTGTACTTCCCTCCTGGTTCAGGCGGAAAGTGTTACAACCAATTAACC |

*Letters in boldface type represent introduced mutations, and underlined sequences prime to the 5' or 3' termini of the kanamycin gene of pEPKan-S.*
Capsid purification. Capsids were purified from virus-infected cells as described previously (50). About 8 × 10⁶ CV1 cells (four 150-mm dishes) were infected with wild-type HSV-1 or U133 mutant viruses at an MOI of 5.0 PFU per cell. At 18 h postinfection, cells were collected, lysed in lysis buffer (20 mM Tris-HCl [pH 7.6], 500 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, and a protease inhibitor), and precentrifuged by centrifugation at 10,000 rpm for 20 min. The precleared lysates were loaded onto a 5-Ml 35% (wt/vol) sucrose cushion and pelleted by spinning in a Beckman SW28 rotor at 24,000 rpm for 1 h. Capsids were resuspended in 600 μL TNE buffer (20 mM Tris-HCl [pH 7.6], 500 mM NaCl, 1 mM EDTA) and loaded onto a continuous 20% to 50% sucrose gradient, followed by centrifugation at 24,500 rpm for 1 h. After centrifugation, the light-reflecting capsids were photographed.

ACKNOWLEDGMENTS

We thank Caitlin Anglin for technical assistance and Ying Xiao in the Shared Instrument Facility at Louisiana State University for help in electron microscopy. This work was supported by NIH grant R21 AI107382-03, a pilot grant from the LSU-Tulane COBRE-Center for Experimental Infectious Disease Research, and an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number 5P30GM110760-03 to K.Y.

REFERENCES

1. Roizman B. 1979. The structure and isomerization of herpes simplex virus genomes. Cell 16:481–494. https://doi.org/10.1016/0022-2836(79)90023-0.
2. Hayward GS, Jacob RJ, Wadsworth SC, Roizman B. 1975. Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. Proc Natl Acad Sci U S A 72:4243–4247. https://doi.org/10.1073/pnas.72.11.4243.
3. Deiss LP, Chou J, Frenkel N. 1986. Functional domains within the α sequence involved in the cleavage-packaging of herpes simplex virus DNA. J Virol 59:605–618.
4. Nasseri M, Mocarski ES. 1988. The cleavage recognition signal is contained within sequences surrounding an a-a junction in herpes simplex virus DNA. Virology 167:25–30. https://doi.org/10.1016/0042-6822(88)90050-5.
5. Newcomb WW, Juhas RM, Thomsen DR, Homa FL, Burch AD, Weller SK, Brown JC. 2001. The UL6 gene product forms the portal for entry of viral DNA into the herpes simplex virus capsid. J Virol 75:10923–10932. https://doi.org/10.1128/JVI.75.22.10923-10932.2001.
6. Varmuza SL, Smiley JR. 1985. Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. Cell 41:793–802. https://doi.org/10.1016/0092-8674(85)80060-X.
7. Stow ND. 2001. Packaging of genomic and amplicon DNA by the herpes simplex virus terminase subunits pUL28 and pUL33. J Virol 75:10755–10765. https://doi.org/10.1128/JVI.75.22.10755-10765.2001.
8. Tong L, Stow ND. 2010. Analysis of herpes simplex virus type 1 DNA packaging signal mutations in the context of the viral genome. J Virol 84:321–329. https://doi.org/10.1128/JVI.01489-09.
9. Trus BL, Booy FP, Newcomb WW, Brown JC, Homa FL, Thomsen DR, Steven AC. 1996. The herpes simplex virus procapsid: structure, conformational changes upon maturation, and roles of the triplex proteins VP19c and VP23 in assembly. J Mol Biol 263:447–462. https://doi.org/10.1006/jmbi.1996.0018.
10. Gibson W, Roizman B. 1972. Proteins specified by herpes simplex virus. 8. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J Virol 10:1044–1052.
11. McNab AR, Desai P, Person S, Roof LL, Thomsen DR, Newcomb WW, Brown JC, Homa FL. 1998. The product of the herpes simplex virus type 1 UL28 gene is required for encapsidation but not for cleavage of replicated viral DNA. J Virol 72:1060–1070.
12. Beard PM, Taus NS, Baines JD. 2002. DNA cleavage and packaging proteins encoded by genes U1L28, U1L15, and U1L33 of herpes simplex virus type 1 form a complex in infected cells. J Virol 76:4785–4791. https://doi.org/10.1128/JVI.76.12.4785-4791.2002.
13. Yang K, Baines JD. 2006. The putative terminase subunit of herpes simplex virus 1 encoded by UL28 is necessary and sufficient to mediate interaction between pUL15 and pUL33. J Virol 80:5733–5739. https://doi.org/10.1128/JVI.00125-06.
14. Yang K, Homa F, Baines JD. 2007. Putative terminase subunits of herpes simplex virus 1 form a complex in the cytoplasm and interact with portal protein in the nucleus. J Virol 81:6419–6433. https://doi.org/10.1128/JVI.00047-07.
15. Jacobson JG, Yang K, Baines JD, Homa FL. 2006. Linker insertion mutations in the herpes simplex virus type 1 UL28 gene: effects on UL28 interaction with UL15 and UL33 and identification of a second-site mutation in the UL15 gene that suppresses a lethal UL28 mutation. J Virol 80:12312–12323. https://doi.org/10.1128/JVI.01766-06.
16. Hemen JD, Huffman JB, Jones LM, Homa FL. 2014. Isolation and characterization of the herpes simplex virus 1 terminase complex. J Virol 88:225–236. https://doi.org/10.1128/JVI.02632-13.
17. Abbotts AP, Preston VG, Hughes M, Patel AH, Stow ND. 2000. Interaction of the herpes simplex virus type 1 packaging protein UL15 with full-length and deleted forms of the UL28 protein. J Gen Virol 81:2999–3009. https://doi.org/10.1099/0022-1317-81-12-2999.
18. Tengelsen LA, Pederson NE, Shaver PR, Watthen MW, Homa FL. 1993. Herpes simplex virus type 1 DNA cleavage and encapsidation require the product of the UL28 gene: isolation and characterization of two UL28 deletion mutants. J Virol 67:3470–3480.
19. Koslowski KM, Shaver PR, Casey JT, II, Wilson T, Yamanaka G, Sheaffer AK, Tenney DJ, Pederson NE. 1999. Physical and functional interactions between the herpes simplex virus UL15 and UL28 DNA cleavage and packaging proteins. J Virol 73:1704–1707.
20. Adelman K, Salmon B, Baines JD. 2001. Herpes simplex virus DNA packaging sequences adopt novel structures that are specifically recognized by a component of the cleavage and packaging machinery. Proc Natl Acad Sci U S A 98:3086–3091. https://doi.org/10.1073/pnas.011555698.
21. Selvarajan Sigamani S, Zhao H, Kamau YN, Baines JD, Tang L. 2013. The structure of the herpes simplex virus DNA-packaging terminase pUL15 nuclease domain suggests an evolutionary lineage among eukaryotic and prokaryotic virions. J Virol 87:7140–7148. https://doi.org/10.1128/JVI.00311-13.
22. Nadal M, Mas PJ, Blanco AG, Arnau C, Sola M, Hart DJ, Coll M. 2010. Structure and inhibition of herpesvirus DNA packaging terminase nuclease domain. Proc Natl Acad Sci U S A 107:16078–16083. https://doi.org/10.1073/pnas.1007144107.
23. Scheffczik H, Savva CG, Holzenburg A, Kolesnikova L, Bogner E. 2002. The terminase subunits pUL56 and pUL89 of human cytomegalovirus are DNA-metabolizing proteins with toroidal structure. Nucleic Acids Res 30:1695–1703. https://doi.org/10.1093/nar/30.7.1695.
24. Bogner E, Radsak K, Stinski MF. 1998. The gene product of human cytomegalovirus open reading frame UL56 binds the pac motif and has specific nuclease activity. J Virol 72:2259–2264.
25. Baines JD, Cunningham C, Nalwanga D, Davison A. 1997. The UL15 gene of herpes simplex virus type 1 contains within its second exon a novel open reading frame that is translated in frame with the UL15 gene product. J Virol 71:2666–2673.
26. Patel AH, Rixon FJ, Cunningham C, Davison AJ. 1996. Isolation and characterization of herpes simplex virus type 1 mutants defective in the UL6 gene. Virology 217:111–123. https://doi.org/10.1006/viro.1996.0098.
27. Desai P, DeLuca NA, Glorioso JC, Person S. 1993. Mutations in herpes simplex virus type 1 genes encoding V5 and VP23 abrogate capsid formation and cleavage of replicated DNA. J Virol 67:1357–1364.
28. Yang K, Wills EG, Baines JD. 2011. A mutation in UL15 of herpes simplex virus type 1 that reduces packaging of cleaved genomes. J Virol 85:11972–11980. https://doi.org/10.1128/JVI.00857-11.
29. Conway JF, Homa FL. 2011. Nucleocapsid structure, assembly and DNA packaging of herpes simplex virus, p 175–193. In Weller SK (ed), Alpha-herpesviruses. Caister Academic Press, Norfolk, United Kingdom.
30. Baines JD, Weller SK. 2005. Cleavage and packaging of herpes simplex virus, p 135–150. In Catalano CE (ed), Viral genome packaging machines. Landes Bioscience, Georgetown, TX.
31. Mocarski ES, Roizman B. 1981. Site-specific inversion sequence of the herpes simplex virus genome: domain and structural features. Proc Natl Acad Sci U S A 78:7047–7051. https://doi.org/10.1073/pnas.78.11.7047.
32. Mocarski ES, Roizman B. 1982. Structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. Cell 31:89–97. https://doi.org/10.1016/0092-8674(82)90408-1.
33. Cockrell SK, Sanchez ME, Erazo A, Homa FL. 2009. Role of the UL25 protein in herpes simplex virus DNA encapsidation. J Virol 83:47–57. https://doi.org/10.1128/JVI.01889-08.
34. Yang K, Poon AP, Rixon B, Baines JD. 2008. Temperature-sensitive mutations in the putative herpes simplex virus type 1 terminase subunits pUL15 and pUL33 preclude viral DNA cleavage/packaging and interaction with pUL28 at the nonpermissive temperature. J Virol 82:487–494. https://doi.org/10.1128/JVI.01875-07.
35. Belislein F, Higgs MR, Stow ND. 2009. Mutation-specific analysis of the herpes simplex virus type 1 DNA packaging protein UL33. J Virol 83:8938–8945. https://doi.org/10.1128/JVI.01048-09.
36. Addison C, Rixon FJ, Preston VG. 1990. Herpes simplex virus type 1 UL28 gene product is important for the formation of mature capsids. J Gen Virol 71(Part 10):2377–2384.
37. Baines JD, Poon AP, Rovnak J, Roizman B. 1994. The herpes simplex virus 1 UL15 gene encodes two proteins and is required for cleavage of genomic viral DNA. J Virol 68:8118–8124.
38. Lamberti C, Weller SK. 1998. The herpes simplex virus type 1 cleavage/packaging protein, UL32, is involved in efficient localization of capsids to replication compartments. J Virol 72:2463–2473.
39. Lamberti C, Weller SK. 1996. The herpes simplex virus type 1 UL6 protein is essential for cleavage and packaging but not for genomic inversion. Virology 226:403–407. https://doi.org/10.1006/viro.1996.0668.
40. Salmon B, Cunningham C, Davison AJ, Harris WJ, Baines JD. 1998. The herpes simplex virus type 1 UL17 gene encodes virion tegument proteins that are required for cleavage and packaging of viral DNA. J Virol 72:3779–3788.
41. Hodge PD, Stow ND. 2001. Effects of mutations within the herpes simplex virus type 1 DNA encapsidation signal on packaging efficiency. J Virol 75:8977–8986. https://doi.org/10.1128/JVI.75.19.8977-8986.2001.
42. Severini A, Morgan AR, Tovell DR, Tyrell DL. 1994. Study of the structure of replicative intermediates of HSV-1 DNA by pulsed-field gel electrophoresis. Virology 200:428–433. https://doi.org/10.1006/viro.1994.1206.
43. Zhang X, Efstatious S, Simmons A. 1994. Identification of novel herpes simplex virus replicative intermediates by field inversion gel electrophoresis: implications for viral DNA amplification strategies. Virology 202:530–539. https://doi.org/10.1006/viro.1994.1375.
44. Martinez R, Sarrisky RT, Weber PC, Weller SK. 1996. Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates. J Virol 70:2075–2085.
45. Deshmange SL, Raengsakulrach B, Berson JF, Fraser NW. 1995. The replicating intermediates of herpes simplex virus type 1 DNA are relatively short. J Neurovirol 1:165–176. https://doi.org/10.3109/13550289509113963.
46. Ejercito PM, Kieff ED, Roizman B. 1968. Characterization of herpes simplex virus strains differing in their effects on social behaviour of infected cells. J Gen Virol 2:357–364. https://doi.org/10.1099/0022-1317-2-3-357.
47. Cunningham C, Davison AJ. 1993. A cosmid-based system for constructing mutants of herpes simplex virus type 1. Virology 197:116–124. https://doi.org/10.1006/viro.1993.1572.
48. Tanaka M, Kagawa H, Yamanashi Y, Sata T, Kawaguchi Y. 2003. Construction of an excisable bacterial artificial chromosome containing a full-length infectious clone of herpes simplex virus type 1: viruses reconstructed from the clone exhibit wild-type properties in vitro and in vivo. J Virol 77:1382–1391. https://doi.org/10.1128/JVI.77.2.1382-1391.2003.
49. Tischer BK, von Einem J, Kaufer B, Osterrieder N. 2006. Two-step re-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. Biotechniques 40:191–197. https://doi.org/10.2144/000112096.
50. Yang K, Baines JD. 2011. Selection of HSV capsids for envelopment involves interaction between capsid surface components pUL31, pUL17, and pUL25. Proc Natl Acad Sci U S A 108:14276–14281. https://doi.org/10.1073/pnas.1108564108.