Role of the UL25 Protein in Herpes Simplex Virus DNA Encapsidation

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DNA packaging is a critical step in the lytic replicative cycle of herpes simplex virus type 1 (HSV-1). During the packaging reaction, replicated DNA is resolved from branched concatemers to single-unit-length genomes that are inserted into preformed capsids (10, 31). Three types of capsids are found in the nuclei of cells infected with HSV-1: A (empty), B (intermediate), and C (full) (reviewed in reference 14). The three are distinguishable morphologically in electron micrographs, and they can be separated from each other preparatively by sucrose density gradient ultracentrifugation. They differ in the material present inside the capsid cavity. C capsids contain the virus DNA, and A and B capsids lack DNA. The B capsid cavity is formed primarily with VP22a, the cleaved form of the scaffolding protein, while in A capsids, the cavity lacks both DNA and protein. While B capsids may form after spontaneous maturation of the procapsid, empty A capsids result from abortive DNA packaging (39). Seven HSV-1 genes have been identified that are required for DNA encapsidation (1, 2, 21–23, 27, 30, 34). Null mutants of the UL6, UL15, UL17, UL28, UL32, and UL33 genes produce only B capsids, and these mutations block cleavage of viral genomes from the replicated concatamer. In contrast, disruption of the UL25 gene results in the accumulation of the nucleus of both A and B capsids in addition to unpackaged, genome-length DNA (23, 33). Thus, UL25 is not required for cleavage or insertion of DNA into the capsid but rather for maintenance of the viral genome after the packaging event.

UL25 is a capsid-associated protein, and it is present on all capsid forms in increasing amounts from procapsid to B, A, and C capsids and virions (24, 32). UL25 attaches to the external vertices of herpes simplex virus type 1 capsids and is required for the stable packaging of viral DNA. To define regions of the protein important for viral replication and capsid attachment, the 580-amino-acid UL25 open reading frame was disrupted by transposon mutagenesis. The UL25 mutants were assayed for complementation of a UL25 deletion virus, and in vitro-synthesized protein was tested for binding to UL25-deficient capsids. Of the 11 mutants analyzed, 4 did not complement growth of the UL25 deletion mutant, and analysis of these and additional mutants in the capsid-binding assay demonstrated that UL25 amino acids 1 to 50 were sufficient for capsid binding. Several UL25 mutations were transferred into recombinant viruses to analyze the effect of the mutations on UL25 capsid binding and on DNA cleavage and packaging. Studies of these mutants demonstrated that amino acids 1 to 50 of UL25 are essential for its stable interaction with capsids and that the C terminus is essential for DNA packaging and the production of infectious virus through its interactions with other viral packaging or tegument proteins. Analysis of viral DNA cleavage demonstrated that in the absence of a functional UL25 protein, aberrant cleavage takes place at the unique short end of the viral genome, resulting in truncated viral genomes that are not retained in capsids. Based on these observations, we propose a model where UL25 is required for the formation of DNA-containing capsids by acting to stabilize capsids that contain full-length viral genomes.

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the UL25 protein appears to play an important role during the later stages of DNA packaging prior to release of capsids into the cytoplasm. Here, we show that this late defect is due to the premature cleavage of the viral genome as it is being packaged, resulting in truncated genomes that failed to be retained in capsids.

MATERIALS AND METHODS

Cells, viruses, and antibodies. African green monkey kidney cells (Vero; American Type Culture Collection, Rockville, MD) and UL25-transformed 8-1 cells were propagated as previously described (23). HSV wild-type KOS and UL25-null virus KUL25S have been previously described (23). The rabbit polyclonal antibody NC1 (7) was used in Western blots to detect the HSV-1 major capsid protein. UL19, Baculovirus-expressed UL25 protein (23) was purified and used to prepare a UL25 monoclonal antibody, 25E10, that was provided by W. Newcomb, University of Virginia (unpublished data).

Transposon mutagenesis. The transposition reaction was done following the GPS-LS protocol (New England Biolabs). The GPS-LS system is an in vitro method for random insertion of a 1.5-kbp transposon (Tn7-based transposi) that encodes a kanamycin resistance gene. The mutagenesis is accomplished by introduction of a transposant that contains the 8-bp PmeI site; the majority of the transposon is then removed by restriction digestion with PmeI. Religation results in a 15-bp insertion, which retains the unique PmeI site. Plasmid pAPV-UL25 contains the UL25 gene expressed from the HSV-1 ICP0 promoter and was used as the template for the transposition (23). Following transposition, the plasmid was reamplified from E. coli and plated on 40 ng/lamp antigen containing ampicillin and ampicillin. DNA extracted from individual colonies was analyzed using a restriction enzyme (PmeI) to map the site of the transposition. Plasmids with insertions that mapped within the UL25 open reading frame (ORF) were digested with PmeI to remove transposon sequences, religated, and transformed into bacteria. The mutants were sequenced (University of Pittsburgh DNA sequencing core laboratory) to confirm the positions and residues inserted as a result of the transposition.

Construction of UL25 mutants. UL25-expressing plasmids were made by PCR using pAPV-UL25 as a template followed by Topo cloning of the gel-purified PCR products into pcDNA3.1/V5-HisTOPO (Invitrogen) such that the UL25 gene was under the transcriptional control of the cytomegalovirus (CMV) promoter. PCR primers contained BamHI and EcoRI restriction sites that were used for further subcloning. UL25 plasmids pFH274, pFH284, pFH368, and pFH379 contained the coding sequences for UL25 amino acids 37 to 590, 1 to 580, 1 to 590, and 51 to 580, respectively. The following primers were used to amplify UL25: pFH274 (5'-GGATCCCTAAACCGCCGACATGTTTCGAT-3'), pFH284 (5'-GAACTGGATCATGGACCCGTACTGCCCAT-3'), pFH368 (5'-GGATCCCTAAACCGCCGACATGTTTCGAT-3'), and pFH379 (5'-GGATCCCTAAACCGCCGACATGTTTCGAT-3'). The UL25 Tope clones were digested with BamHI and UL25 sequences were then ligated into the BamHI site of plasmid pFH118. pFH118 expresses proteins with the UL25-null mutant virus, vKUL25S, into bacteria. The mutants were sequenced (University of Pittsburgh DNA sequencing core laboratory) to confirm the positions and residues inserted as a result of the transposition.

Southern blots. A T-175 flask of 8-1 or Vero cells was infected with virus at a multiplicity of infection (MOI) of 5 PFU per cell. At 18 h postinfection, the medium was removed and the cells were washed in 1× PBS, scraped off the plate, and pelleted. The cells were lysed and viral DNA was prepared as previously described (23). The final DNA was digested with BamHI and PmeI and electrophoresed in 0.8% agarose gel, and the gel was transferred to nitrocellulose. The nitrocellulose was washed twice in Tris-buffered saline (TBS) and blocked overnight in TBS-T (20 mM Tris, 0.5 M NaCl [pH 7.5] plus 0.5% Tween 20) supplemented with 10% nonfat dry milk. The membranes were hybridized as previously described (15). Southern blots were scanned with Storm 840 PhosphoImager, and specific bands were quantified with ImageQuant software.

Immunoblotting. Protein extracts were separated on a 5 to 12% SDS-polyacrylamide gel, and proteins were transferred to nitrocellulose. The nitrocellulose was washed twice in Tris-buffered saline (TBS) and blocked overnight in TBS-T (20 mM Tris, 0.5 M NaCl [pH 7.5] plus 0.5% Tween 20) supplemented with 10% nonfat dry milk. The membranes were hybridized as previously described (15). Southern blots were scanned with Storm 840 PhosphoImager, and specific bands were quantified with ImageQuant software.

Complementation assay. Vero cells (1.0 × 10^7 cells/well in a 24-well plate) were transfected with 0.5 μg of plasmid DNA and 1 μl of Lipofectamine (Invitrogen) diluted in 100 μl of serum-free medium. The DNA-lipid complexes were allowed to form at room temperature for 20 min, added to the cells, and incubated overnight at 37°C. The next day, the cells were infected with UL25-null virus (vUL25) at an MOI of 5 PFU/cell. After 90 min at 37°C, the medium was removed and unabsorbed virus was inactivated by washing the cells three times with citrate buffer (pH 3). The cultures were incubated for an additional 18 h, after which the cells were scraped from the plate and rinsed with PBS. A 10% aliquot of total cells was frozen and used to determine virus titers on 8-1 cells and Vero cells. The remaining of the cell pellet was resuspended in 2× PAGE loading buffer (Invitrogen) and used for Western blot analysis.

Caspid purification. Vero cells (1.5 × 10^7) were infected overnight (18 h at 37°C) at an MOI of 5 PFU/cell. Infected cells were harvested, rinsed with PBS, and resuspended in 20 mM Tris (pH 7.5) plus protease inhibitors (Roche), adjusted to 1% Triton X-100, and incubated for 30 min on ice. The resulting nuclei were harvested by low-speed centrifugation, resuspended in 10 mM TNE (50 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5) and then sonicated to lyse the nuclei. The nuclear lysate was adjusted to 20 mM MgCl2 and incubated with DNase I (100 μg/ml) at room temperature for 30 min. The lysate was then cleared by low-speed centrifugation, and the resulting supernatant was layered on top of a 10-mL cushion of 35% sucrose (SW28 rotor; 23,000 rpm for 1 h). The
RESULTS
Transposon mutagenesis and functional assessment of UL25 mutants. In order to identify regions of UL25 protein that are important for viral replication and for attachment to capsids, the UL25 ORF was randomly disrupted by linker scanning mutagenesis. A plasmid vector (pAPV-UL25) containing the UL25 gene was subjected to Tn7 transposon-mediated insertion of a 15-bp fragment that contained a PmeI restriction site. Mutations are underlined. Uppercase letters represent the template sequence (pEP-Kan-S and pcGFP-in). Lowercase letters represent HSV UL25 gene sequences.

![TABLE 1. Primers used for generation of recombinant HSV-BACs](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>BAC</th>
<th>UL25 mutation</th>
<th>Primer sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>bFH439</td>
<td>Deletion replaced with rpsL-neo</td>
<td>5'-gacaagcagcagcttcctgtgtagttctggcttcacctgaagcttagctgtgcagatcccaccggagatcgtgagcggcggagATGGTGAGCAAGGGCGAGGA-3'</td>
</tr>
<tr>
<td>bFH407</td>
<td>143i</td>
<td>5'-gcggctggagaatttcgctgccagtaatgcaggtgcgcaacaggagcagcggccggcggagATGGTGAGCAAGGGCGAGGA-3'</td>
</tr>
<tr>
<td>bFH416</td>
<td>212s</td>
<td>5'-gcggcagctgcggagcagcagcagcagcttcctgtgtagttctggcttcacctgaagcttagctgtgcagatcccaccggagatcgtgagcggcggagATGGTGAGCAAGGGCGAGGA-3'</td>
</tr>
<tr>
<td>bFH418</td>
<td>560s</td>
<td>5'-gcggcagctgcggagcagcagcagcagcttcctgtgtagttctggcttcacctgaagcttagctgtgcagatcccaccggagatcgtgagcggcggagATGGTGAGCAAGGGCGAGGA-3'</td>
</tr>
<tr>
<td>bFH421</td>
<td>Δ1-50</td>
<td>5'-gcggcagctgcggagcagcagcagcagcttcctgtgtagttctggcttcacctgaagcttagctgtgcagatcccaccggagatcgtgagcggcggagATGGTGAGCAAGGGCGAGGA-3'</td>
</tr>
<tr>
<td>bFH422</td>
<td>GFP insertion</td>
<td>5'-gcggcagctgcggagcagcagcagcagcttcctgtgtagttctggcttcacctgaagcttagctgtgcagatcccaccggagatcgtgagcggcggagATGGTGAGCAAGGGCGAGGA-3'</td>
</tr>
<tr>
<td>bFH423</td>
<td>155i</td>
<td>5'-gcggcagctgcggagcagcagcagcagcttcctgtgtagttctggcttcacctgaagcttagctgtgcagatcccaccggagatcgtgagcggcggagATGGTGAGCAAGGGCGAGGA-3'</td>
</tr>
</tbody>
</table>

Table 1. Primers used for generation of recombinant HSV-BACs


produced proteins of the expected sizes (Fig. 2A). Eight of the transposon mutants expressed a UL25 protein that was similar in size to the wild-type UL25 protein. For these mutants, a size difference would not be apparent since only five amino acids were added to the mutant UL25 proteins. The two nonsense mutants 212s and 560s produced proteins of 23 and 60 kDa, respectively. No immunoreactive proteins were observed for

![FIG. 1. (Top) Physical map of the HSV-1 genome showing the location of the UL25 gene.](https://example.com/fig1.png)

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pellets were suspended in 3 ml TNE and adjusted to 1 mM dithiothreitol, and capsids were centrifuged by ultracentrifugation (SW41 rotor at 24,000 rpm for 1 h). The positions of A, B, or C capsids were observed as light-scattering bands, with A capsids being found highest (least dense) on the gradient and C capsids found lowest (dense fractions) on the gradient. The different capsid fractions can also be identified based on the presence or absence of the scaffold protein, VP22a, since only B capsids contain the scaffold protein. The fractions were collected using a Beckman fraction collector and stored at -80°C until analysis.

Three of these mutants contained in-frame stop codons after amino acids 12, 212, and 560.

The ability of the UL25 transposon mutants to support virus replication was tested using a genetic complementation assay. Vero cells were transfected with wild-type and mutant plasmids, and the next day the cells were infected with the UL25-null virus, vΔUL25. Following another day of incubation, the cell lysates were harvested, tested for UL25 protein expression by Western blot analysis, and assayed for virus progeny yield by plating the lysates on UL25-complementing cells (8-1 cells). With the exception of the 12s mutant, all of the plasmids produced proteins of the expected sizes (Fig. 2A). Eight of the transposon mutants expressed a UL25 protein that was similar in size to the wild-type UL25 protein. For these mutants, a size difference would not be apparent since only five amino acids were added to the mutant UL25 proteins. The two nonsense mutants 212s and 560s produced proteins of 23 and 60 kDa, respectively. No immunoreactive proteins were observed for
the 12s mutant, but this plasmid was predicted to make only a small peptide that would probably not be recognized by the UL25 antiserum. Using the HSV major capsid protein (UL19) as a loading control, all of the mutants except the 12s mutant were found to express similar amounts of UL25 (Fig. 2A). The 12s mutant contains a stop codon that would result in a truncated UL25 protein of only 12 amino acids that would not be detected on the gel. The ability of the UL25 mutants to complement growth of the UL25-null mutant was determined by titrating the cell lysates on 8-1 cells. Figure 2B shows that, with the exception of the three truncation mutants (12s, 212s, and 560s) and the 143i mutant, the remaining mutants were able to complement the UL25-null virus, although the 155i and 403i mutants complemented at less than 10% of the wild-type UL25 protein.

The N terminus of UL25 is required for capsid attachment in vitro. Previously we showed that [35S]methionine-labeled UL25 synthesized in a coupled in vitro transcription-translation system will attach to the surface of UL25-deficient capsids during in vitro incubation (24). To determine if mutations that eliminated complementation also affected UL25 capsid attachment, the 12s, 143i, 212s, and 560s mutants were tested for in vitro capsid binding. In vitro translation of plasmids containing the 143i, 212s, and 560s proteins produced proteins corresponding to their predicted molecular weights (Fig. 3A). Unexpectedly, in vitro translation of the 12s mutant produced a nearly wild-type-size UL25 protein (Fig. 3A). Capsid binding was determined by adding an aliquot of the [35S]methionine-labeled UL25 to pooled A and B capsids derived from cells infected with the UL25-null virus, v/H9004UL25 (Table 2). After incubation to allow UL25 attachment, the capsids were separated from other reaction components by centrifugation on a sucrose gradient, and gradient fractions were analyzed by SDS-PAGE followed by autoradiography. Capsid attachment was indicated by comigration of UL25 protein with capsids in the sucrose gradient (Fig. 3B and C). The 143i, 212s, and 560s proteins attached to capsids. Although the 212s protein was found to be primarily associated with the fractions containing A and B capsids, significant levels of the protein were found in fractions denser than capsids. This was probably the result of aggregation of the truncated UL25 protein in the gradient lacking M and B capsids (bottom of panel C). IVT, in vitro transcription-translation; M, molecular mass markers (from top, 194, 104, 60, 41, 27, 21, 16, and 7 kDa). Lane 1 is the bottom of the gradient and lane 10 (B) or 12 (C) is the top.
that there may be some low-level binding since the amount of radiolabeled 12s protein was reduced compared to the amounts of the other UL25 proteins. Examination of the UL25 coding sequence revealed that after the first AUG, the next in-frame AUG start codon was located ~120 bp downstream. This start site corresponded to UL25 amino acid M37. To determine if the 12s mutant was initiating at this downstream start site, the codons for amino acids 1 to 36 were deleted from the UL25 expression plasmid (Fig. 4A). The resulting in vitro-synthesized protein, containing UL25 amino acids 37 to 580, was found to be identical in size to the 12s mutant in vitro translation product (Fig. 4B). Similar to the result with the 12s protein, the UL25 N-terminal truncation (37 to 580) mutant did not bind capsids when tested in the in vitro capsid-binding assay (Fig. 4C). In addition, this mutant did not complement the UL25-null virus (data not shown). These results demonstrated that the sequences within the N terminus of the UL25 protein are required for capsid binding.

To determine if the UL25 N terminus was sufficient for capsid binding, three plasmids were generated that expressed portions of UL25 protein fused at its C terminus to a TAP tag (Fig. 4A). The TAP tag consists of the IgG-binding peptide protein A, the cleavage site for the tobacco etch virus protease, TABLE 2. Virus growth assay results

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>UL25 protein expressed (amino acid positions)</th>
<th>Virus growth (PFU/ml) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vero cells</td>
</tr>
<tr>
<td>KOS</td>
<td>Wild type (1–580)</td>
<td>2 × 10⁸</td>
</tr>
<tr>
<td>KUL25NS</td>
<td>Null (1–107)</td>
<td>3 × 10⁴</td>
</tr>
<tr>
<td>vΔUL25</td>
<td>Null</td>
<td>&lt;1 × 10²</td>
</tr>
<tr>
<td>v143i</td>
<td>143i (1–580)</td>
<td>&lt;1 × 10²</td>
</tr>
<tr>
<td>v212s</td>
<td>212s (1–212)</td>
<td>&lt;1 × 10²</td>
</tr>
<tr>
<td>v560s</td>
<td>560s (1–560)</td>
<td>&lt;1 × 10²</td>
</tr>
<tr>
<td>vΔ1-50</td>
<td>Δ1-50 (51–580)</td>
<td>&lt;1 × 10²</td>
</tr>
<tr>
<td>vUL25-GFP</td>
<td>UL25-GFP (1-50-GFP-51-580)</td>
<td>4 × 10⁷</td>
</tr>
<tr>
<td>v155i</td>
<td>155i (1–580)</td>
<td>1 × 10⁷</td>
</tr>
</tbody>
</table>

FIG. 4. Capsid binding by UL25 N-terminal mutants. (A) UL25 N-terminal truncation and TAP fusion constructs are shown. The numbers listed on the left indicate the UL25 amino acids included in each construct. The TAP constructs contained the TAP tag fused to the C terminus of UL25. The predicted molecular mass (MM) of each protein is listed in parentheses. wt, wild type. (B) Autoradiograph of in vitro translation (IVT) products after SDS-PAGE. Molecular mass markers in kDa are shown at left. Each protein showed the appropriate molecular mass due to either addition of the TAP tag or deletion of the UL25 N-terminal amino acids. (C) In vitro capsid-binding assay. In vitro-translated protein was incubated with pooled A and B capsids isolated from Vero cells infected with the UL25-null virus, vΔUL25, and then purified by sucrose gradient centrifugation. A representative Coomassie-stained gel of the gradient fractions is shown (top panel). Autoradiographs of dried gels demonstrating the binding of 1-580-TAP or 1-50-TAP but not 51-580-TAP or the 37-580 to the two capsid types (bottom panel). (D) In vitro-translated protein was incubated with pooled wild-type KOS A, B, and C capsids and then purified by sucrose gradient centrifugation. A representative stained gel of the gradient fractions is shown (top panel). Autoradiographs of dried gels demonstrating the binding of 1-50-TAP but not 51-580-TAP to all three capsid types (bottom panel). The fractions containing A, B, and C capsids are indicated. The capsid proteins visible in the stained gel are listed to the right. IVT, in vitro transcription-translation; M, molecular mass markers (from top, 194, 109, 59, 30, 22, 13, and 6 kDa). Lane 1 is the bottom of the gradient, and lane 10 or 11 is the top.
and the calmodulin binding domain; the tag adds 178 amino acids (~20 kDa) to the C terminus of the UL25 fusion protein (29). The three constructs 1-580-TAP, 51-580-TAP, and 1-50-TAP contained the full-length UL25 protein, UL25 amino acids 51 to 580, and UL25 amino acids 1 to 50 fused to the TAP tag, respectively (Fig. 4A). In vitro translation produced proteins corresponding to their predicted size, and each protein was tested for binding to UL25-deficient A and B capsids (Fig. 4A to C). Like the 12s and 37-580 mutants, the UL25 fusion protein 51-580-TAP did not attach to capsids. In contrast, the full-length 1-580-TAP and truncated 1-50-TAP fusion proteins were able to bind capsids. When incubated with A, B, and C capsids purified from KOS-infected cells, the 1-50-TAP but not the 51-580-TAP was found to bind all three capsid types by binding to unoccupied sites on the capsids and/or competitively displacing wild-type protein from capsids (Fig. 4D). These results demonstrated that UL25 amino acids 1 to 50 mediate capsid attachment in the in vitro capsid-binding assay.

**Introduction of UL25 mutations into the virus genome.** Several of the UL25 mutations were introduced into the viral genome through the genetic manipulation of an HSV-1 (KOS) genome maintained in a recombinant bacterial artificial chromosome (BAC). The genotypes of the viruses used in these studies are listed in Table 2. Virus vAUL25 contains the rpsL-neo antibiotic resistance genes, which replace the entire UL25 ORF. Recombinant viruses v143i, v155i, v212s, and v560s contain the indicated UL25 linker insertion mutations (Fig. 5A). Recombinant virus vΔ1-50 contains a deletion that removed the coding sequences for UL25 amino acids 1 to 50, which were replaced with an ATG start codon. Recombinant virus vUL25-GFP contains DNA that encodes the GFP gene inserted in frame between codons 50 and 51 of the UL25 gene (Fig. 5A). Since there are no PmeI sites in the HSV-1 genome, the presence of the PmeI linker in the UL25 gene and the resulting BACs and viruses are indicated on top. UL25 fragments of v143i BAC, 155i BAC, 212s BAC, and 560s BAC) are listed on top. The BACs used to generate each virus were confirmed by digesting them with BamHI and PmeI. The UL25 ORF is contained within the HSV-1 2,300-bp BamHI U fragment (Fig. 5A). Since there are no PmeI sites in the HSV-1 genome, the presence of the PmeI linker in the UL25 gene of the HSV BACs 143i, 155i, 212s, and 560s resulted in the loss of the 2,300-bp BamHI fragment, which was shifted to a smaller fragment that comigrates with other HSV-1 BamHI fragments (Fig. 5B). The HSV BACs Δ1-50 and UL25-GFP contained the deletion of 150 bp and the insertion of 726 bp in UL25, respectively, which were detected as shifts in the BamHI U fragments (Fig. 5B).

The BACs were transfected onto UL25-complementing 8-1 cells, and the recovered viruses were plaque purified on Vero or 8-1 cells. Each mutant was titrated on Vero and 8-1 cells to determine the effects of UL25 mutations on viral replication (Table 2). The vUL25-GFP mutant was able to replicate on Vero cells, while Vero cells were nonpermissive for the replication of vAUL25, v143i, v212s, v560s, and vΔ1-50 (Table 2). The virus v155i displayed an intermediate phenotype with a fivefold reduction of growth on Vero cells as compared to the UL25-complementing cell line. To verify the presence of the mutations in the progeny virus, viral DNA was digested with BamHI and PmeI and subjected to Southern blot analysis with the 2,300-bp BamHI fragment (32P labeled) UL25 probe (Fig. 5C). As seen in the restriction enzyme digestion analyses of the individual BAC preparations, a prominent 2,300-bp fragment was detected in the KOS lane, which was reduced to two

**FIG. 5.** Characterization of BAC DNA and HSV-infected cell DNA. (A) Schematic representation of the BamHI region located between nucleotides 48635 and 50929 of the HSV-1 genome that contains the UL25 ORF. The sites where unique PmeI restriction sites are found in the UL25 gene and the resulting BACs and viruses (143i BAC, 155i BAC, 212s BAC, and 560s BAC) are listed on top. (B) DNA was digested with BamHI and PmeI and run on a 1.2% agarose gel. The wild-type 2.3-kbp BamHI fragment is present in the KOS BAC lane (arrow). This fragment was cleaved to smaller fragments by PmeI in 143i BAC, 155i BAC, 212s BAC, and 560s BAC. The UL25 fragments of Δ1-50 BAC and UL25-GFP BAC are shifted to 2.1 kbp (which comigrates with another viral band) and 3.2 kbp (arrow), respectively. M, DNA markers in kbp. (C) Southern blot of viral DNA. Viral DNA was purified from cells infected with the indicated viruses. Viral DNA (10 μg) was digested with BamHI and PmeI and run on a 1.2% agarose gel. DNA was blotted to a nylon membrane, and the blot was hybridized with the 32P-labeled UL25-containing BamHI U fragment. The wild-type BamHI fragment containing UL25 is visible at 2.3 kbp in the KOS lane and was cleaved to smaller fragments by PmeI in v143i, v155i, v212s, and v560s. The UL25 fragments of vΔ1-50 and vUL25-GFP are shifted to 2.1 and 3.2 kbp, respectively.
smaller fragments in v143i, v155i, v212s, and v560s and one
smaller fragment in vΔ1-50. The 400-bp fragment for v560s was
observed on longer exposure (not shown). The 2,300-bp frag-
ment containing the UL25 ORF was not detected in the UL25
deletion virus vΔUL25. The insertion of the 726-bp GFP gene
in the vUL25-GFP virus resulted in a larger BamHI fragment.
These results demonstrated that the UL25 mutants had
the desired insertions or deletions.

Association of UL25 with purified capsids. Intracellular cap-
sids were purified from Vero cells infected with wild-type KOS
or UL25 mutant viruses. The nuclear lysates were subjected to
sucrease gradient centrifugation, and capsids were viewed as
visible light-scattering bands (Fig. 6A). In cells infected with
wild-type KOS or the two UL25 mutants vUL25-GFP and
v155i, which replicate on Vero cells, all three capsid forms
were observed. The KOS gradients contained nearly equal
amounts of both B and C capsids, with only minor amounts of
A capsids (Fig. 6A). In contrast, the gradients for the vUL25-
GFP and v155i mutants contained less C capsids and more A
and B capsids, and the C capsid band was barely detectable in
the v155i gradient. The lysates of the UL25 mutants that did
not replicate on Vero cells (vΔUL25, v143i, v212s, v560s, vΔ1-
50, and KUL25NS) contained approximately equal numbers of
A and B capsids but no DNA-containing C capsids (Fig. 6).
The high proportion of A capsids found along with the absence
of C capsids in all of these UL25 mutants is identical to what
was previously described with the UL25-null mutant
KUL25NS (23). To determine whether the UL25 protein was
present in these capsids, the sucrose gradient fractions were
analyzed by Western blot analysis to detect the UL25 protein
(Fig. 6). The KOS gradient showed that the UL25 protein was
found in the fractions that contained A, B, and C capsids. This
result was replicated in capsids purified from cells infected with
the vUL25-GFP virus. The 155i mutant was found to associate
with A and B capsids, but since there were so few C capsids
present on the gradient, there was little if any of this protein
detected with these DNA-containing capsids. The UL25 linker
insertion mutant 143i and the nonsense mutants 212s and 560s
were found associated with A and B capsids isolated from cells
infected with viruses expressing those proteins. The vΔ1-50
mutant expressed a truncated UL25 protein that was missing
residues 1 to 50, and although this protein could be detected in
the cell extract, it failed to bind either the A or B capsids. This
result demonstrates the importance of the N terminus of the
UL25 protein for capsid association of UL25. These data dem-
strate that similar to the result of the in vitro capsid-binding
assay (Fig. 3), the UL25 capsid-binding domain resides within
the first 50 amino acids of the UL25 protein. Furthermore, the
studies with the UL25 mutant viruses demonstrate that the
UL25 capsid-binding domain is essential but not sufficient for
production of infectious virus, as both virus progeny and C
capsids were significantly reduced in noncomplementing cells
infected with the 155i mutant (Table 2).

Replication and cleavage of HSV-1 DNA. Previously, it has
been shown that the UL25-null virus KUL25NS was able to
cleave replicated viral DNA into unit-length genomes but
failed to stably package the cleaved genomes (23, 33). To
determine whether viruses bearing linker insertions or non-
sense mutations were impaired in DNA cleavage and packag-
ing, total DNA was isolated from Vero cells infected with
wild-type and mutant viruses and subjected to Southern blot
analysis. Viral DNA replication generates concatemers that
are cleaved into unit-length molecules and packaged into viri-
on. The cleaved viral DNA contains free chromosomal ter-

FIG. 6. Analysis of capsid-bound UL25. Vero cells were infected at an
MOI of 5 with KOS or the indicated UL25 mutants. Nuclear lysates were
subjected to sucrose gradient sedimentation. (A) Capsids from Vero cells
infected (MOI of 5) with KOS or the indicated UL25 mutant virus were
harvested at 18 h postinfection, layered onto 20 to 50% sucrose gradients,
and centrifuged at 24,000 rpm (SW41 rotor) for 1 h. The positions of A,
B, and C capsid bands are indicated. (B) The protein composition for
each gradient fraction was determined by SDS-PAGE (stained gel). Lane
1 is the bottom of the gradient, and lane 11 or 12 is the top. Gradient
fractions that contained A, B, and C capsids are indicated. (B) The protein composition for
each gradient fraction was determined by SDS-PAGE (stained gel). Lane
1 is the bottom of the gradient, and lane 11 or 12 is the top. Gradient
fractions that contained A, B, and C capsids are indicated. The positions
of the capsid proteins (major capsid protein, VP5; triplex proteins, VP19C
and VP23; scaffold protein, VP22a; smallest capsid protein, VP26) are
indicated. (Bottom panels) Gradient fractions were analyzed by SDS-
PAGE followed by immunoblotting for UL25 using the 25E10 antibody.
The stained gel in the bottom right panel contains capsids isolated from
UL25-null virus vΔUL25, which is representative of the mutants (vΔ1-50,
v143i, v212s, and v560s) that fail to replicate on Vero cells. The UL25
Western blots (WB) for these mutants are shown below the stained gel
with asterisks indicating the fractions that are equivalent to the capsid-
containing fractions (lanes 5 to 7) of the vΔUL25-stained gel. Lane a, cell
lysate from KOS-infected cells; lane b, cell lysate from UL25 mutant-
infected cells. Molecular mass standards are visible in lanes M (from top,
194, 109, 59, 30, 22, 13, and 6 kDa).
mini, and the presence of chromosomal ends can be monitored by Southern blot analysis of total infected cell DNA digested with BamHI and probed with the HSV-1 BamHI K fragment. The S fragment represents the unique long (U₄) terminus, and the Q fragment represents the unique short (U₃) terminus (Fig. 7A). The different sizes of the BamHI S and K fragments are due to the presence of one to multiple copies of the a sequence, while the Q fragment contains only a single copy of the a sequence. An example of a mutant that fails to cleave replicated viral DNA is the null mutant GCB, which does not express the product of the HSV UL28 gene (34). As shown in Fig. 7B, the terminal Q and S fragments were absent from DNA isolated from Vero cells infected with GCB. All of the UL25 mutants replicated viral DNA when grown on Vero cells, as assessed by the presence of the joint K fragments, but DNA isolated from Vero cells infected with replication-defective UL25 mutants lacked the terminal Q fragments (Fig. 7B). Since the Q and S fragments are similar in size, the blot was probed with a ³²P-labeled fragment that hybridizes specifically to the U₅ terminus. The probe detected the Q fragment in the digests for the three viruses that replicate on Vero cells but not in any of the viruses that do not; the junction fragment K was present in all samples (Fig. 7C). The amounts of radioactivity in the bands corresponding to the joint fragment, the long terminus, and the short terminus (BamHI fragments K, S, and Q, respectively) were measured, and the ratio of joint to each terminal fragment was calculated for each lane in Fig. 7B. In the case of the BamHI S fragments from the UL terminus, the measurement included both the S1 and S2 fragments. For wild-type HSV-1, the ratios of joint to end fragments were similar. However, for the UL25-null virus, vUL25-GFP, and the other UL25 mutants that do not replicate in Vero cells, the UL terminus was present at levels similar to those in the wild-type virus, but the US terminus was absent or significantly underrepresented compared to the joint fragment (Fig. 7B). The ratios of the end fragments for DNA isolated from cells infected with vUL25-GFP were similar to that of KOS, while there was a twofold reduction in the US terminus for the 155i linker insertion mutant, v155i. This is consistent with the fact that replication of v155i is reduced (Table 2) and demonstrates a direct correlation of the defect with the cleavage of viral DNA, the production of stable C capsids, and the production of infectious virus.

The absence of US terminal fragments from the UL25 mutants was surprising, because previous studies from our laboratory demonstrated that genome-size (152 kbp) HSV DNA is present in cells infected with the UL25-null virus, KUL25NS. To further examine the US terminus generated by the UL25 mutants, total infected cell DNA was digested with HindIII and subsequently analyzed by Southern blot analysis using the HSV-1 BamHI J fragment as a probe (Fig. 7A). The probe spanned the HindIII restriction site that links the genome fragments containing the 13-kbp fragment from the US terminus (HindIII G fragment) and an internal 5-kbp fragment from the US region (HindIII N fragment). The HindIII G fragment was readily detected in DNA isolated from Vero cells infected with KOS and the two UL25 mutants that replicate in Vero cells (vUL25-GFP and v155i), but this fragment was absent from DNA isolated from Vero cells infected with the UL28 mutant, GCB (Fig. 7D). Although

![FIG. 7. Processing of virus DNA. (A) Schematic diagram of the HSV genome showing the locations of the HindIII G, I, and N fragments and the BamHI J, K, Q, and S fragments. The different sizes of the BamHI S fragment are due to the presence of one to multiple copies of the a sequence at the U₄ terminus. (B to D) Vero cells were infected with the indicated virus at an MOI of 5 PFU per cell. At 18 h postinfection, total infected cell DNA was isolated, digested with BamHI (B and C) or HindIII (D), and subjected to Southern blot analysis. The blots were probed with ³²P-labeled BamHI K (B), BamHI Q (C), or BamHI J (D) fragments of the HSV-1 genome. The ratio of the BamHI joint K fragment to U₄-end Q or U₄-end S fragments and the ratio of the internal HindIII N fragment to the US-end G fragment were determined by quantification of the hybridizing bands with phosphorimaging software.](http://jvi.asm.org/articles/54/1/49/17240/F7fig07.jpg)
the HindIII G fragment was present in the blots of DNA isolated from the five UL25 mutants (vΔUL25, v143i, v212s, v560s, and vΔ1-50) that fail to grow on Vero cells, the hybridizing band was diffuse, suggesting that the probe detected multiple U₅ terminal fragments (Fig. 7D). The presence of multiple U₅ terminal fragments would indicate that either the cleavage reaction is taking place at several sites to generate HindIII G fragments of different sizes or that the full-length genomic DNA is not stable and is being degraded. Because the BamHI Q fragment is absent from all of the UL25-defective mutants, the U₅ terminus for these mutants appears to be located at least 3,000 bp 5' to the wild-type U₅ terminus. The ratio of HindIII G fragment to the internal HindIII N fragment was calculated for each virus and showed that the cleavage at the U₅ end is reduced approximately twofold for the UL25 mutants. The two large hybridizing fragments that are present in all of the samples are viral DNA fragments that result from cleavage of the replicated DNA concatamers and consist of the joint-spanning fragments HindIII G plus HindIII B or, when the UL is inverted, HindIII G plus HindIII I (Fig. 7A).

**DISCUSSION**

The HSV-1 UL25 protein is one of seven viral proteins that are required for DNA cleavage and packaging. UL25 is found at or near each of the vertices of DNA-containing C capsids on the capsid surface. The capsid-bound UL25 appears to be a multifunctional protein required: (i) to stabilize capsids after DNA is packaged; (ii) to trigger nuclear egress of the capsid once a stable DNA-containing capsid is formed; (iii) to anchor VP1/2, the major tegument protein to capsids; and (iv) to uncoat the incoming viral genome early during infection (8, 23, 24, 28, 33, 38). Random mutagenesis of the HSV-1 UL25 gene was used to identify regions of UL25 protein important for virus replication and for UL25 capsid attachment. Although the five amino acid insertions disrupted core portions of the folded protein, most UL25 transposon mutants were able to complement the UL25 deletion virus. Four mutants were defective in the complementation. Insertion of stop codons after amino acids 212 and 560 resulted in the expression of nonfunctional truncated proteins that attached to capsids in vitro and when expressed from a recombinant virus. Similarly the 143i insertion mutant expressed a nonfunctional protein that failed to complement the UL25-null virus but retained the ability to bind capsids. The 12s mutant failed to complement and was predicted to encode a small UL25 peptide, but instead, in vitro translation of 12s produced a nearly wild-type-size protein that did not bind capsids. Examination of the UL25 DNA sequence indicated that the resulting protein was probably the result of translation initiation at an internal start codon (M37) in the UL25 gene. This result was confirmed by constructing a UL25 mutant in which the codons for amino acids 1 to 37 were deleted and replaced with an ATG codon. In vitro translation of this construct produced a protein the same size as the 12s mutant that also failed to bind capsids. Deletion of the UL25 N terminus to amino acid 50 eliminated in vitro capsid binding; conversely, the UL25-TAP fusion protein containing UL25 amino acids 1 to 50 was able to bind capsids in vitro. A recombinant virus expressing the UL25 51–580 protein was replication defective and produced capsids lacking the virus-expressed mutant protein. The fact that insertion of GFP between UL25 amino acids 50 and 51 did not affect virus replication suggests that the capsid binding domain resides solely within amino acids 1 to 50. This conclusion is supported by the capsid-binding studies where 1-50-TAP was shown to bind capsids (Fig. 4C). This result indicated that separation of amino acids 1 to 50 from the rest of the UL25 N terminus did not preclude capsid attachment or virus replication. Taken together, these data indicate that amino acids 1 to 50 are necessary and sufficient for UL25 capsid binding and therefore comprise the UL25 capsid-binding domain. However, binding of UL25 to the capsid through the N-terminal capsid-binding domain is not sufficient for virus production. The two truncation mutants v212s and v560s both express proteins that associate with capsids, but these mutants do not produce infectious virus, indicating that the final 19 amino acids of UL25 are also essential for function.

**Mutational analysis of UL25 structure.** The crystal structure of UL25 (amino acids 134 to 580) has been determined (5). The UL25 protein consists of numerous flexible loops extending from a rigid core composed of several tightly packed alpha helices. We expected that mutations disrupting any of the alpha helices located in the core would alter the overall structure of the protein and hence inhibit its function. However, most of the mutants isolated in this study contained insertions in the UL25 core that did not reduce function of the plasmid-expressed mutant protein. For example, the 354i and 538i mutations were both located in internal alpha helices, but neither of these mutations caused significant reduction in complementation of the UL25-null virus. The 143i, 151i, and 155i insertion mutations occur within a region where the predicted amino acid sequence is strongly conserved within homologues of the UL25 gene of other herpesviruses. In the folded protein, this region is part of a loop that bridges the unstructured N terminus (residues 1 to 45) with the highly structured C terminus (residues 133 to 580); there are two small antiparallel beta sheets located between amino acids 133 and 155 (5). The 143i insertion maps to the turn between the two beta sheets, and the 151i insertion occurs shortly after the end of the second beta sheet. The 155i insertion is located within the first alpha helix found in the core structure. Interestingly, the 143i and 155i insertions altered virus replication, but the 151i insertion did not. Our results suggest that this region does not contribute to capsid binding but is important for UL25 function, possibly as a bridge from the flexible capsid-binding region (residues 1 to 50) of UL25 to the more structured C terminus. Other structural features of UL25 protein include electropositive and electronegative faces and clusters of conserved surface residues (5). The 151i and 155i mutations are located in the electropositive face of UL25, while the other mutations occur along the periphery or in the interior of the folded protein. The 151i and 155i mutations also fall within one of the conserved surface clusters identified by Bowman et al. (5).

**HSV-1 DNA packaging.** In its basic features, the pathway for assembly of the HSV-1 capsid resembles that observed in dsDNA bacteriophages (3, 11, 19). The process of DNA encapsidation in bacteriophages begins when the terminase makes a double-strand cut in the replicated concatamer DNA and binds to the end of the DNA. The terminase-DNA com-
plex then docks onto the procapsid at the portal vertex. DNA is then driven into the capsid by the terminase complex until it encounters a second cut site in the concatemer DNA. The second cut may occur at a second packaging signal or after a headful of DNA has been injected. As DNA is entering, the procapsid is transformed into its mature, icosahedral morphology. After the last DNA end has entered the capsid, the portal is closed, and the capsid is stabilized by addition of head completion proteins.

HSV DNA translocation into the capsid is mediated by the terminase complex comprised of the HSV UL15, UL28, and UL33 proteins (18, 40, 41). The cis-acting HSV-1 packaging sequences, designated pac1 and pac2, are found near the genomic ends of viral DNA. At the U<sub>1</sub> end of the genome, the pac2 site defines packaging directionality by mediating initiation, and pac1 serves to terminate packaging at the U<sub>S</sub> end after a unit-length genome has entered the capsid (6). Terminase cleaves viral DNA at the pac2 site and then docks the free end of DNA at the capsid portal vertex. Packaging proceeds from the U<sub>1</sub> end until a full-length genome has entered the capsid; DNA cleavage occurs at a pac1 site at the U<sub>S</sub> end. The packaged genome is then sealed within the capsid. Similar to the dsDNA bacteriophages, DNA cleavage is suppressed until a full genome (headful mechanism) has entered the capsid because otherwise shorter packaged genomes would be formed from cleavage at the packaging sequences within the HSV genome’s internal repeat sequences. The addition of UL25 to the capsid at or near the end of the packaging process suggests that it may function similarly to lambda phage head completion proteins, gpD, or the Soc and Hoc proteins of phage T4; these proteins bind to the phage capsid surface after DNA has entered and are thought to reinforce the capsid structure (12, 16, 17, 42). Although most of our UL25 mutant proteins were able to attach to capsids, the failure of recombiant viruses to replicate on Vero cells was coincident with premature cleavage of the U<sub>S</sub> end of the viral genome, as previously described for the UL25-null mutant KUL25NS (28, 33). In CMV, the halogenated benzimidazoles BDCRB (2-bromo-5,6-dichloro-1-β-D-riborfuranosyl benzimidazole riboside) and TCRB (2,5,6-trichloro-1-β-D-riborfuranosyl benzimidazole riboside) induce premature cleavage events during DNA packaging such that the site of cleavage is located at a similar distance from the end of the genome as was observed with the UL25 mutants (25). These compounds are not active against HSV-1, but resistance mutations to BDCRB and TCRB map to the human CMV terminase genes UL89 and UL56, which are the homologues of HSV-1 UL15 and UL28, respectively (20, 39). While the drugs’ mechanism of action is not known, they may relax the cleavage site sequence specificity of the terminase without relieving the headful requirement. By extension, the absence of functional UL25 on the capsid surface may relax the cleavage site specificity and allow aberrant cleavage by the terminase.

Role of UL25 in DNA packaging. In conclusion, we propose to integrate our current studies of UL25 into the HSV DNA encapsidation model (Fig. 8). DNA packaging is an energy-dependent process that requires conformational changes in the capsid proteins and in the viral DNA itself (4, 9, 37). Presumably, terminase supplies the force required to compact DNA within the capsid. UL25 association with the capsid may stabilize this transition, such that in the absence of functional UL25, the equilibrium between the internal pressure of packaged DNA and the force exerted by the terminase is breached. When the terminase can no longer propel DNA into the nascent capsid, it stalls on the DNA and cleaves to abort packaging, forming the truncated genomes observed in replication-deficient UL25 mutants. The UL25 capsid-binding domain is responsible for the binding of UL25 to the capsid surface, but other portions of the protein are essential for completion of DNA packaging. These essential functions could include a direct role in the cleavage reaction, either by UL25 interaction with the terminase and portal complexes or by UL25 binding to the pac1 site.

FIG. 8. Model for the role of UL25 in DNA packaging. Proposed model for the role of UL25 in HSV DNA encapsidation. (Step 1) DNA packaging initiates on concatemers when the terminase complex consisting of UL15, UL28, and UL33 binds (step 2) to packaging sequences and cleaves (step 3) the concatemer to generate a U<sub>S</sub> end with the terminase still bound. The terminase-DNA complex then docks at the portal vertex on the procapsid (step 4), and the terminase initiates DNA packaging (step 5). As the procapsid is filled (step 6A) with DNA, it angularizes and in the process, UL25 binding sites are exposed at the capsid vertexes. DNA cleavage is suppressed by a headful mechanism (step 7A) until a full-length genome has entered. Cleavage occurs at a packaging site located at the U<sub>S</sub> end of the genome (step 8A). More UL25 binds to the capsid, and the newly packaged genome is sealed within the C capsid (step 9A). In the absence of a functional UL25 protein, the packaging reaction proceeds (step 6B) with the capsid filling and expanding until close to a genome length has entered (step 7B). Without UL25 to stabilize the capsid against the pressure that the packaged genome generates, premature cleavage (step 8B) occurs just prior to the entry of the U<sub>S</sub>-end repeat, resulting in genomes with truncated U<sub>S</sub> termini. In the absence of UL25, the capsid is not stable. The DNA is released (step 9B), which generates an empty A capsid and a free viral genome that is truncated at the U<sub>S</sub> end.
REFERENCES