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Assembly of the Herpes Simplex Virus Capsid: Requirement for the Carboxyl-Terminal Twenty-Five Amino Acids of the Proteins Encoded by the UL26 and UL26.5 Genes

DARRELL R. THOMSEN,[1] WILLIAM W. NEWCOMB,[2] JAY C. BROWN,[2] AND FRED L. HOMA[1]*

Molecular Biology Research, The Upjohn Company, Kalamazoo, Michigan 49001,[1] and Department of Microbiology and Cancer Center, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908[2]

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Herpes simplex virus type 1 (HSV-1) intermediate capsids are composed of seven proteins, VP5, VP19C, VP21, VP22a, VP23, VP24, and VP26, and the genes that encode these proteins, UL19, UL38, UL26, UL26.5, UL18, UL26, and UL35, respectively. The UL26 gene encodes a protease that cleaves itself and the product of the UL26.5 gene at a site (M site) 25 amino acids from the C terminus of these two proteins. In addition, the protease cleaves itself at a second site (R site) between amino acids 247 and 248. Cleavage of the UL26 protein gives rise to the capsid proteins VP21 and VP24, and cleavage of the UL26.5 protein gives rise to the capsid protein VP22a. Previously we described the production of HSV-1 capsids in insect cells by infecting the cells with recombinant baculoviruses expressing the six capsid genes (D. R. Thomsen, L. L. Roof, and F. L. Homa, J. Virol. 68:2442–2457, 1994). Using this system, we demonstrated that the products of the UL26 and/or UL26.5 genes are required as scaffolds for assembly of HSV-1 capsids. To better understand the functions of the UL26 and UL26.5 proteins in capsid assembly, we constructed baculoviruses that expressed altered UL26 and UL26.5 proteins. The ability of the altered UL26 and UL26.5 proteins to support HSV-1 capsid assembly was then tested in insect cells. Among the specific mutations tested were (i) deletion of the C-terminal 25 amino acids from the proteins coded for by the UL26 and UL26.5 genes; (ii) mutation of His-61 of the UL26 protein, an amino acid required for protease activity; and (iii) mutation of the R cleavage site of the UL26 protein. Analysis of the capsids formed with wild-type and mutant proteins supports the following conclusions: (i) the C-terminal 25 amino acids of the UL26 and UL26.5 proteins are required for capsid assembly; (ii) the protease activity associated with the UL26 protein is not required for assembly of morphologically normal capsids; and (iii) the uncleaved forms of the UL26 and UL26.5 proteins are employed in assembly of 125-nm-diameter capsids; cleavage of these proteins occurs during or subsequent to capsid assembly. Finally, we carried out in vitro experiments in which the major capsid protein VP5 was mixed with wild-type or truncated UL26.5 protein and then precipitated with a VP5-specific monoclonal antibody. Analysis of the precipitate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the two proteins coprecipitated only when the UL26.5 gene product contained the C-terminal 25 amino acids. The overall results of these experiments support a model for capsid assembly in which the uncleaved forms of the UL26 and UL26.5 proteins are used to assemble the HSV-1 B capsid. The C-terminal 25 amino acids of the UL26 and UL26.5 proteins are suggested to be directly involved in interaction of the scaffold with VP5 in the capsid shell.

The capsid of herpes simplex virus type 1 (HSV-1) is approximately 15 nm thick and 125 nm in diameter; its major structural features are 162 capsomers that lie on a T-16 icosahedral lattice (8, 39, 49). The existence of an icosahedral capsid composed of 162 capsomers is characteristic of all members of the family *Herpesviridae*. Morphogenesis of HSV-1 includes a step in which a precursor capsid called the B capsid is formed in the infected cell nucleus (10, 27–29, 40, 41). The B capsid is similar to the capsid found in infectious HSV-1 particles, except that it lacks DNA. The cavity of the B capsid is occupied instead by a proteinaceous core that is removed when DNA enters.

The HSV-1 B capsid is composed of seven proteins (36). The outer icosahedral shell is made up of four of the seven capsid proteins. The 162 capsomers that make up the outer shell are composed of 150 hexavalent capsomers (hexons) and 12 pentavalent capsomers (pentons) (21, 22, 25, 42). Hexons and pentons differ in their order of rotational symmetry but are otherwise morphologically similar. The major capsid protein VP5, which is the product of the HSV-1 UL19 gene, is the predominant structural subunit of both the hexons and pentons, with hexons containing six copies of VP5 and pentons containing five copies (25, 47). The three other proteins that make up the outer shell are VP19C, VP23, and VP26, which are the products of the UL38, UL18, and UL35 genes, respectively (3, 5, 20, 31, 38). VP26 is found at the distal tips of the hexons, while VP19C and VP23 together form trigonal nodules called triplexes, which lie at the capsid floor and connect capsomers in groups of three (1, 25, 50).

The core of the B capsid is composed of three proteins, VP21 and VP24 (products of the UL26 gene) and VP22a (product of the UL26.5 gene) (3, 15, 30). The capsids of most large double-stranded DNA viruses are built initially as double-shelled capsids that have a core of scaffolding protein contained within the icosahedral shell (35). The scaffold of the HSV-1 B capsid is composed of mainly one protein, VP22a (44, 46). VP22a is the most abundant protein found in the B capsid, with approximately 1,100 copies of this protein being used to form the inner core (21, 22). The scaffold serves to

* Corresponding author. Mailing address: The Upjohn Company, 7242-267-5, 301 Henrietta St., Kalamazoo, MI 49001. Phone: (616) 384-9724. Fax: (616) 385-4599. Electronic mail address: flhoma@upj.com.

promote the assembly of the outer shell into properly dimensioned capsids. In the absence of the scaffolding function, the proteins that form the outer shell assemble into aberrant structures (6, 44, 46).

The UL26 and UL26.5 genes are expressed as 3′-coterminal transcripts, with the promoter for the UL26.5 gene located within the coding region of the UL26 gene (4, 6, 9, 15–18). The UL26.5 open reading frame (329 amino acids) overlaps and is in frame with the carboxyl-terminal end of the HSV-1 UL26 open reading frame (635 amino acids). The UL26 gene encodes a protease that cleaves itself between amino acids 247 and 248 and between amino acids 610 and 611 to generate the capsid proteins VP21 and VP24 (7, 17). VP24 comprises the first 247 amino acids of the UL26 protein and is the region of the protein that contains proteolytic activity, while VP21 comprises amino acids 248 through 610 of the UL26 protein. The UL26 proteinase also cleaves the product of the UL26.5 gene at a site 25 amino acids from the C terminus of this protein (15). Because the UL26.5 open reading frame starts with codon 307 of the UL26 protein, the C-terminal cleavage site is the same as the 610/611 cleavage site of the UL26 protein. Both VP21 and VP22a are removed from B capsids during DNA encapsidation, while VP24 is retained (10, 36). Cleavage of the UL26 and UL26.5 proteins is not required for capsid assembly, but the cleavage event is essential for DNA encapsidation (9, 33). It is most probable, therefore, that B capsids are assembled with the uncleaved forms of the UL26 and UL26.5 proteins; cleavage occurs later and is required to release the core proteins from the capsid shell as DNA enters.

We have recently described the production of HSV-1 intermediate capsids in insect cells by infecting the cells with recombinant baculoviruses expressing the six capsid genes (46). Using this system, we demonstrated that the products of the UL26 and/or UL26.5 genes are required as scaffolds for assembly of HSV-1 capsids. The studies reported here had two main objectives. The first was to determine what role the C-terminal 25 amino acids of the UL26 and UL26.5 proteins serve in capsid assembly. The second was to determine the role of proteolytic cleavage in capsid assembly. Our approach was to introduce specific changes in the UL26 and UL26.5 genes by site-directed mutagenesis and then determine the phenotypic effect of the change by testing the mutant constructs in the insect cell assembly system.

MATERIALS AND METHODS

Cells, viruses, and antibodies. Previously described procedures were employed for growth and maintenance of African green monkey kidney cells (Vero; ATCC CCL 81 [12]), and the HSV-1 wild-type strain KOS (12). Baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) was grown in *Spodoptera frugiperda* (Sf9) cells (ATCC CRL 1711) as previously described (43). Monoclonal antibody MCA406 (Serotec, Inc.) was used to analyze the UL26 and UL26.5 proteins (4). Monoclonal antibody 6F to the UL19 gene product has been described previously (24, 25). The UL19, UL38, and UL18 proteins were analyzed with rabbit polyclonal antisera NC1 (UL19), NC2 (UL38), and NC5 (UL18), which were provided by Roselyn Eisenberg and Gary Cohen of the University of Pennsylvania, Philadelphia (2).

Construction of recombinant baculoviruses expressing mutant forms of the UL26 and UL26.5 genes. Cloning and expression of the six HSV-1 capsid genes from recombinant baculoviruses were performed as previously described (46). The UL26 and UL26.5 open reading frames in plasmids pAC-UL26 and pAC-UL26.5 were mutagenized to give rise to the constructs pAC-UL26/61, pAC-UL26/114, pAC-UL26/247, pAC-UL26/610, and pAC-UL26.5/304 by PCR-mediated mutagenesis (Fig. 1). The oligonucleotides used for PCR were purchased from Genosys Biotechnologies, Inc. (The Woodlands, Tex.). For pAC-UL26/61, PCR primers 1 (5′-CCGTGCCCATTTACGTGGC-3′) and 2 (5′-TCGCAGC CAGCGCGTTGGTCC-3′) were used to amplify sequences between the two *Bst*XI sites (nucleotides 50874 to 50995) of the UL26 gene. The fragment generated by PCR was digested with *Bst*XI and used to replace the same *Bst*XI fragment of pAC-UL26. Primer 2 contained sequences that changed the His-61 codon of the UL26 gene to glutamine. For pAC-UL26/114, PCR primers 3 (5′-GTCTATCAATATATAGTTG-3′) and 4 (5′-GTTGGTGATCAGGTAC AACAGGCGGCCGGCCCGGGAGAGCGG-3′) were used to amplify sequences between the *Bcl*I site (nucleotide 51167) of the UL26 gene and the *Eco*RV site of the pAc373 vector, located 100 bp 5′ of the UL26 ATG codon. The fragment generated by PCR was digested with *Bcl*I and *Eco*RV and used to replace the same *Bcl*I-*Eco*RV fragment of pAC-UL26. Primer 4 contained sequences that changed the Glu-114 and Glu-115 codons of the UL26 gene to Ala-114 and Gly-115. For pAC-UL26/247, PCR primers 5 (5′-GCGACTGGC CGCCGAGGC-3′) and 6 (5′-CATTTTGAATTTTTCAGGCCTCTGGAGG TAGG-3′) were used to amplify sequences between the *Sst*I and *Apo*I sites (nucleotides 51392 and 51556) of the UL26 gene. The fragment generated by PCR was digested with *Sst*I and *Apo*I and used to replace the same *Sst*I-ApoI fragment of pAC-UL26. Primer 6 contained sequences that changed the Ala-247 and Ser-248 codons of the UL26 gene to Arg-247 and Pro-248. For pAC-UL26/610 and pAC-UL26.5/304, PCR primers 7 (5′-GCTGATGGGGGCGGTGA CGTC-3′) and 8 (5′-AGAGGTACCTCAGGCGTTGACAAGGGCCCCG-3′) were used to amplify sequences between the *Pst*I and *Kpn*I sites (nucleotides 52274 and 52736) of the UL26 gene. The fragment generated by PCR was digested with *Pst*I and *Kpn*I and used to replace the same *Pst*I-*Kpn*I fragment of pAC-UL26 and pAC-UL26.5. Primer 8 was designed so that the coding region for the C-terminal 25 amino acids of the UL26 and UL26.5 genes was deleted and replaced with a TGA stop codon. The mutations were confirmed by DNA sequence analysis of the entire region that was generated by PCR. Recombinant baculoviruses expressing the mutant UL26 and UL26.5 proteins were generated as previously described (14) with the baculoGold transfection kit supplied by Pharmingen.

Transmission electron microscopy. Monolayers of Vero cells were infected with KOS at a multiplicity of infection (MOI) of 10, and at 12 h postinfection, the cells were harvested. Monolayers of Sf9 cells were infected with mixtures of recombinant baculoviruses expressing either wild-type or mutant HSV capsid genes at a MOI of 5 (each virus), and at 64 h postinfection, the cells were harvested. Thin sections were prepared for electron microscopy as described by Tengelsen et al. (45).

Capsid purification. Suspension cultures (100 to 500 ml) of Sf9 cells were infected with baculovirus recombinants at a MOI of 5 (each virus), and at 64 h postinfection, the cells were harvested. Monolayers of Vero cells were infected with KOS at a MOI of 10, and at 12 h postinfection, the cells were harvested. The cell pellets from Vero or Sf9 cells were resuspended in 2× capsid lysis buffer (1 M NaCl, 40 mM Tris-HCL [pH 7.5], 2 mM EDTA, 2% Triton X-100), freeze-thawed three times, sonicated, and centrifuged for 5 min at 5,000 rpm in a Sorvall SA600 rotor, and the cleared extract was layered on a 20 to 65% sucrose gradient and centrifuged at 24,000 rpm in an SW41 rotor for 60 min. Capsids were observed as visible light-scattering bands and were harvested and concentrated by dilution in phosphate-buffered saline (PBS) and centrifugation for 1 h at 20,000 rpm in an SW41 rotor. The pellet was resuspended in 0.1 to 0.2 ml of PBS, and the capsids were then rebanded on a second 20 to 65% sucrose gradient as described above.

Immunoprecipitation and Western immunoblots. Sf9 cells were infected at a MOI of 5 with recombinant baculovirus, and at 24 or 36 h postinfection, the cells were labelled with 50 μCi of [^{35}S]methionine per ml in Grace's methionine-free insect cell medium (Gibco) containing 10% fetal bovine serum and 10% of the normal concentration of methionine. The cells were harvested at various times postinfection, and proteins were immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (11). Western immunoblots were performed as described previously (45). Detection of the immune complexes on nitrocellulose filters was done with an alkaline phosphatase color reaction. The antibodies were used at a 1:1,000 dilution.

Coimmunoprecipitation. Sf9 cells (100-ml spinner cultures) were infected at a MOI of 5 with recombinant baculovirus expressing either the UL19, UL26.5, or the UL26.5/304 genes, and 64 h postinfection, the cells were harvested and stored at −70°C. Cell extracts were prepared by resuspending the cells in 4 volumes (wet weight of the cells) of PBS, and the samples were freeze-thawed four times and then cleared by centrifugation (16,000 × *g* for 1 min). The resulting supernatants were removed to clean tubes and adjusted to 50 mM EDTA–5 mM dithiothreitol. Five microliters of the cleared extract was mixed with 45 μl of dissociation buffer (5 mM Tris-HCL [pH 7.5], 5% 2-mercaptoethanol, 2% SDS, 5% glycerol, 0.1 mg of bromophenol blue per ml), and 20 μl of each sample was examined by SDS-PAGE (see Fig. 9, lanes 2 to 4). Coimmunoprecipitation was done by mixing 15 μl of the UL26.5 or UL26.5/304 extracts with 15 μl of the UL19 extract (Fig. 9, lanes 8 and 9, respectively), or as a control, 15 μl of each extract was combined with 15 μl of PBS (Fig. 9, lanes 4 to 7). After a 1-h incubation at 27°C, 6 μl of monoclonal antibody 6F (specific for VP5) was added to each sample, incubated for an additional 45 min at 27°C, and then centrifuged (16,000 × *g* for 0.5 min). The resulting pellets were washed once by resuspension in 50 μl of PBS and recentrifuged (16,000 × *g* for 2 min). The pellet was then resuspended in 70 μl of dissociation buffer, and 20 μl of each sample was examined by SDS-PAGE.

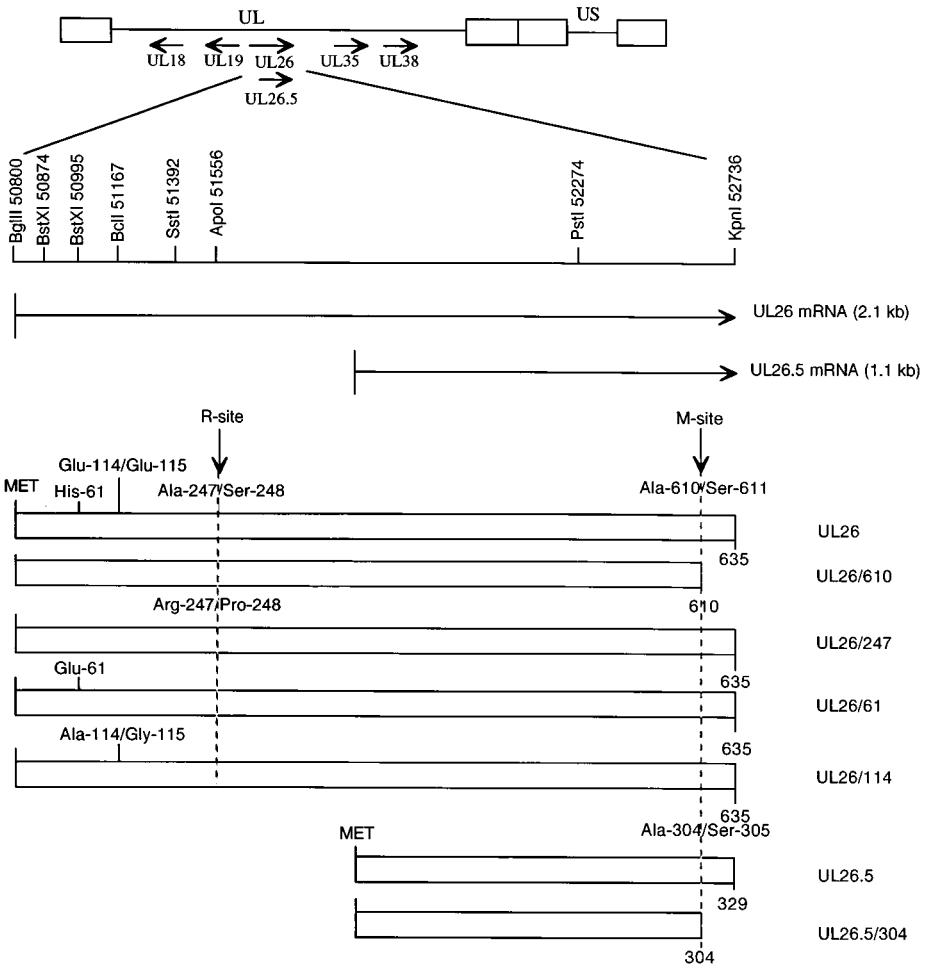

FIG. 1. Schematic representation of the UL26 and UL26.5 genes and their products. The HSV-1 genome is shown at the top, with UL and US referring to the long and short unique region sequences, respectively. The approximate locations and direction of transcription of the six HSV-1 capsid genes are indicated. An expanded region of the genome that contains the UL26 and UL26.5 genes with relevant restriction sites and the locations of the UL26 and UL26.5 mRNA coding sequences are shown below the HSV-1 genome. The numbering of the restriction sites follows that of McGeoch et al. (19). At the bottom are shown the 635-amino-acid protein encoded by the UL26 gene and the locations of the R and the M cleavage sites. The four UL26 mutants used in this study are shown with the amino acids deleted or changed relative to the wild-type protein indicated. At the very bottom are shown the 329-amino-acid protein encoded by the UL26.5 gene and the truncated mutant protein used in this study.

RESULTS

Expression of mutant UL26 and UL26.5 genes in Sf9 cells. In order to examine the role of proteolytic cleavage of the UL26 and UL26.5 proteins in HSV-1 capsid assembly, we constructed a number of baculoviruses that expressed altered UL26 and UL26.5 proteins (Fig. 1). DiIanni et al. (7) have shown that the UL26 gene encodes a protease that cleaves itself and the UL26.5 protein at a position 25 amino acids from the C-terminal end (between amino acids Ala-610 and Ser-611 of the UL26 protein [M site]) of both proteins. In addition, the UL26 protease cleaves itself at a second site (between amino acids Ala-247 and Ser-248 [R site]) to yield two peptides of 45 kDa (VP21) and 29 kDa (VP24). Liu and Roizman (16) have also shown that mutations which alter the histidine at amino acid 61 or the two glutamines at amino acids 114 and 115 of the UL26 protein abolish protease activity. Five mutants were constructed with a PCR-based mutagenesis procedure (Materials and Methods [Fig. 1]). In the UL26/610 and the UL26.5/304 mutants, DNA sequences coding for the last 25 amino acids of the protease and scaffold protein were deleted and replaced with a stop codon. In the UL26/247 mutant, amino acids 247 and 248 of the protease were changed from Ala-Ser to Arg-Pro. In the UL26/61 mutant, amino acid 61 of the protease was changed from His to Glu, and in the UL26/114 mutant, amino acids 114 and 115 of the protease were changed from Glu-Glu to Ala-Gly.

Expression of the mutant UL26 and UL26.5 proteins from recombinant baculoviruses was examined by immunoprecipitation. Baculovirus-infected cells were labeled from 24 to 48 h postinfection with [^{35}S]methionine, and the UL26 and UL26.5 proteins were immunoprecipitated with monoclonal antibody MCA406. The results (Fig. 2) of these experiments showed the following. (i) As expected, the UL26.5/304 mutant expressed a truncated form of the scaffold protein which was identical in size to the cleaved (missing C-terminal 25 amino acids) UL26.5 protein (Fig. 2, compare lanes 1, 3, and 4). (ii) The UL26/610 mutant expressed a protein (45 kDa) identical in size to the protein expressed from the wild-type UL26 gene (Fig. 2, lanes 2 and 5). Pulse-chase experiments demonstrated that the full-length form of the UL26/610 protein was 75 kDa in size (data not shown), indicating that the UL26/610 mutant was missing the C-terminal 25 amino acids. Removal of the C-terminal 25

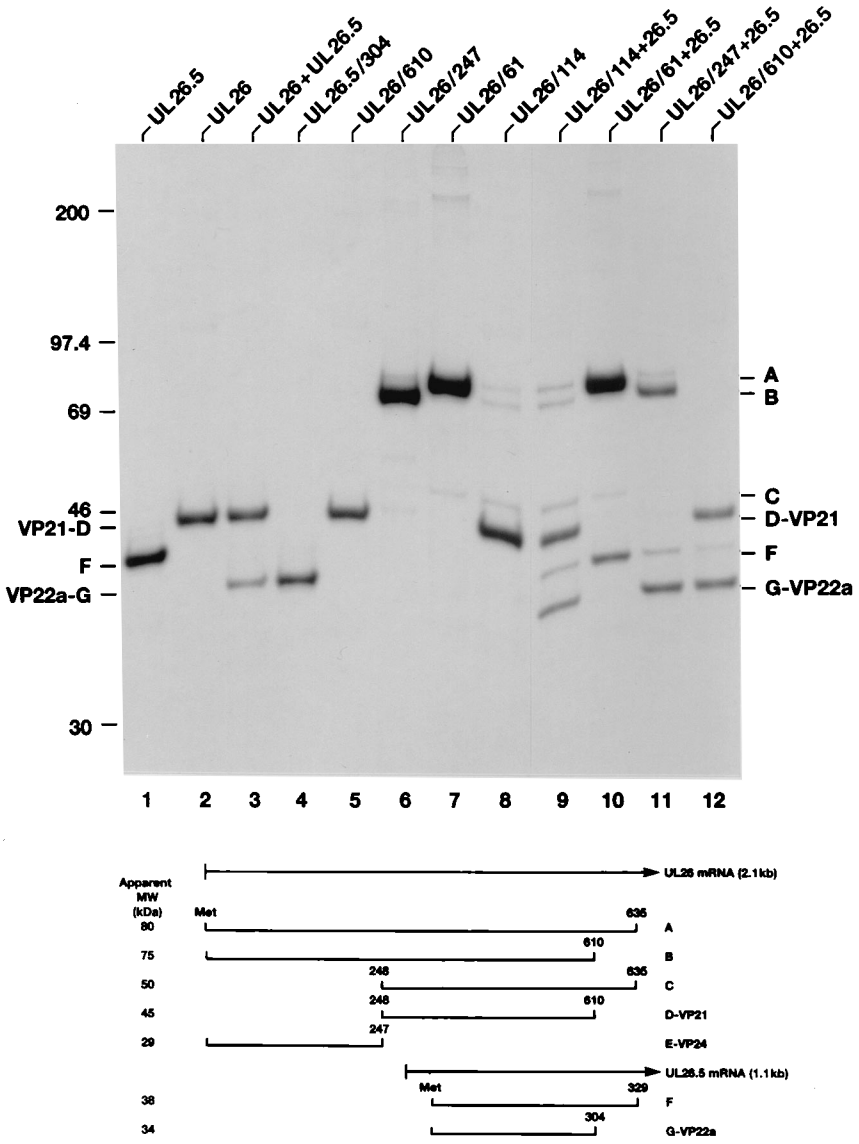

FIG. 2. Polypeptides synthesized by recombinant baculoviruses. (Top) Proteins synthesized in Sf9 cells infected with the indicated viruses were labelled with [^{35}S]methionine from 24 to 48 h postinfection, and proteins were immunoprecipitated with a mouse monoclonal antibody, MCA406. The immunoprecipitated material was separated on an SDS–12.5% polyacrylamide gel. (Bottom) Diagram of the HSV-1 UL26 and UL26.5 genes and their products. The different cleaved forms of the UL26 and UL26.5 proteins are shown, and the size of each product is indicated. The different proteins are listed as A to G along the right margin, and the corresponding protein is indicated in the polyacrylamide gel above. The proteins that correspond to HSV capsid proteins VP21, VP22a, and VP24 are indicated. MW, molecular mass.

amino acids did not affect the ability of the UL26/610 protein to cleave the UL26.5 protein (Fig. 2, lane 12). (iii) Cells infected with the UL26/247 mutant yielded a 75-kDa protein which corresponded in size to a UL26 protein that was cleaved at the M cleavage site but not at the R cleavage site (Fig. 2, compare lanes 2 and 6), although a trace amount of fully cleaved (45-kDa) UL26 protein was also detected. The UL26/247-expressed protein also cleaved the UL26.5 protein, but with slightly reduced efficiency compared with the wild-type protease (Fig. 2, compare lanes 3 and 11). (iv) The UL26/61 mutant expressed a protein that lacked protease activity, because only the uncleaved (80-kDa) form of this protein was detected in UL26/61-infected cells (Fig. 2, lane 7). In addition, the UL26/61-expressed protein failed to cleave the UL26.5 protein in cells infected with both the UL26/61 and UL26.5 genes (Fig. 2, lane 10). (v) The predominant form of the UL26 protein found in cells infected with the UL26/114 mutant was the fully processed 45-kDa peptide, although trace amounts of uncleaved (80-kDa) and singly cleaved (75- and 50-kDa) forms of this protein were also present (Fig. 2, lane 8). Thus, the UL26/114 mutant retained protease activity, but the efficiency of cleavage was reduced at both the M and R cleavage sites compared with that of the wild-type protease. The UL26/114-expressed protein also cleaved the UL26.5 protein, but again with reduced efficiency compared with the wild-type protease (Fig. 2, lane 9).

Assembly of capsids in Sf9 cells infected with recombinant baculoviruses expressing HSV-1 capsid proteins. To examine what affect the mutant UL26 and UL26.5 proteins would have on capsid assembly, Sf9 cells were infected with a mixture of recombinant baculoviruses expressing the HSV-1 UL18, UL19, UL35, and UL38 genes along with either wild-type or mutant

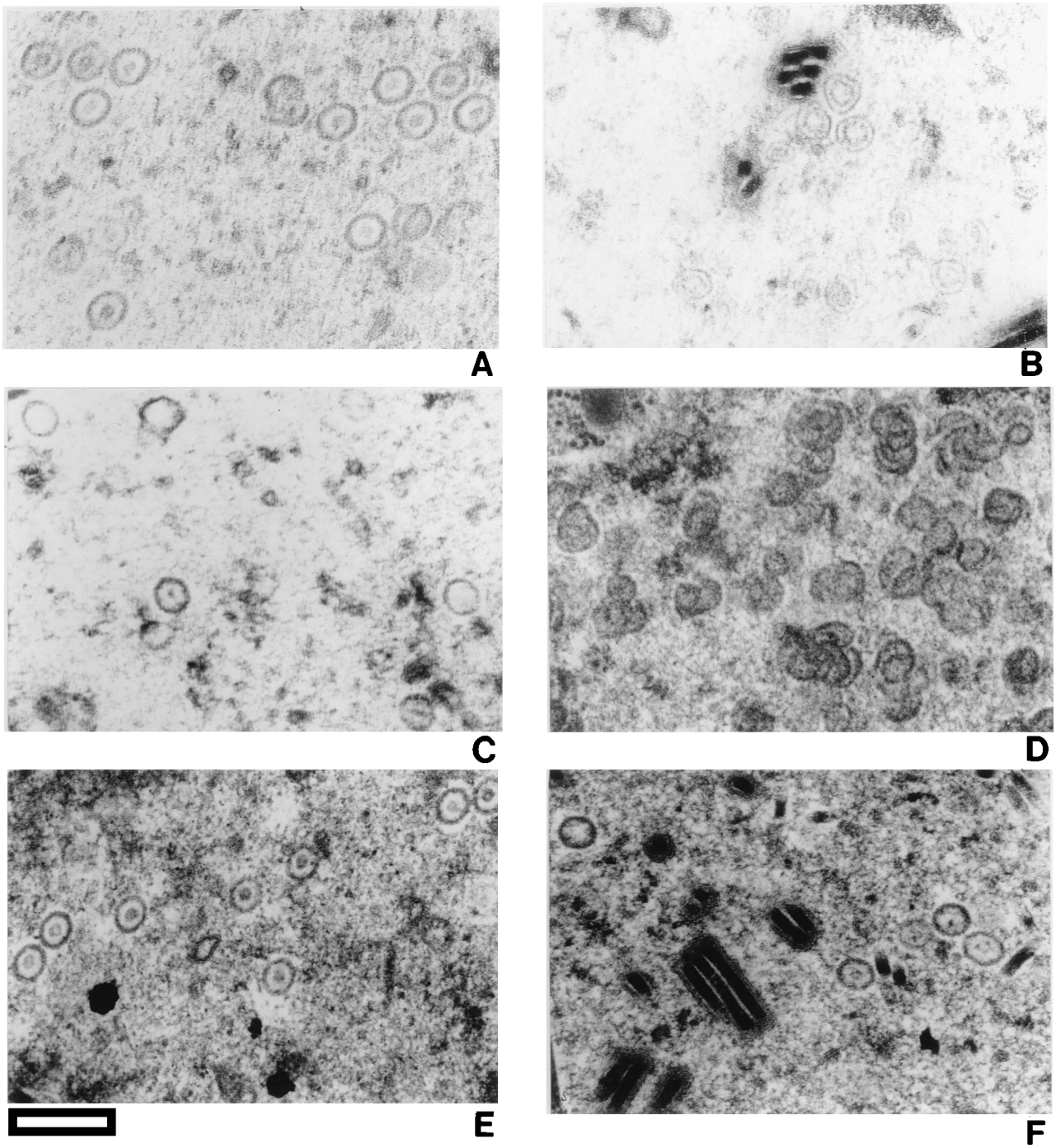

FIG. 3. Electron micrographs of thin sections of Sf9 cells infected with recombinant baculoviruses. A portion of the cells harvested (64 h postinfection) for isolation of virus particles (see Fig. 4 and 5) was pelleted and fixed, and thin sections were prepared for electron microscopy. Cells were infected with recombinant baculoviruses expressing UL18, UL19, UL35, or UL38 plus UL26 and UL26.5 (A), UL26.5 (B), UL26 (C), UL26/610 and UL26.5/304 (D), UL26/610 and UL26.5 (E), UL26 and UL26.5/304 (F), UL26/61 (G), UL26/61 and UL26.5 (H), UL26/247 (I), UL26/247 and UL26.5 (J), UL26/114 (K), or UL26/114 and UL26.5 (L). Bar, 200 nm.

UL26 and UL26.5 genes, and at 64 h postinfection, the cells were harvested. The harvested cells were either fixed and then thin sectioned for electron microscopy analysis, or cell lysates were prepared and layered onto 20 to 65% sucrose gradients. After sedimentation, the B capsid band was harvested, and the sample was rebanded on a second 20 to 65% sucrose gradient. The protein composition of the double-banded sample was then determined by SDS-PAGE and immunoblot analysis. The results of gel and electron microscopy analyses are shown in Fig. 3 to 5, and a summary of the data is presented in Fig. 6.

UL26/610 and UL26.5/304. When baculoviruses expressing the UL26/610 and UL26.5/304 mutants were used in place of viruses expressing the wild-type UL26 and UL26.5 genes, structures resembling incomplete or aberrant capsids were observed (Fig. 3D). Analysis of the protein composition of the capsid structures showed that they were composed of the four

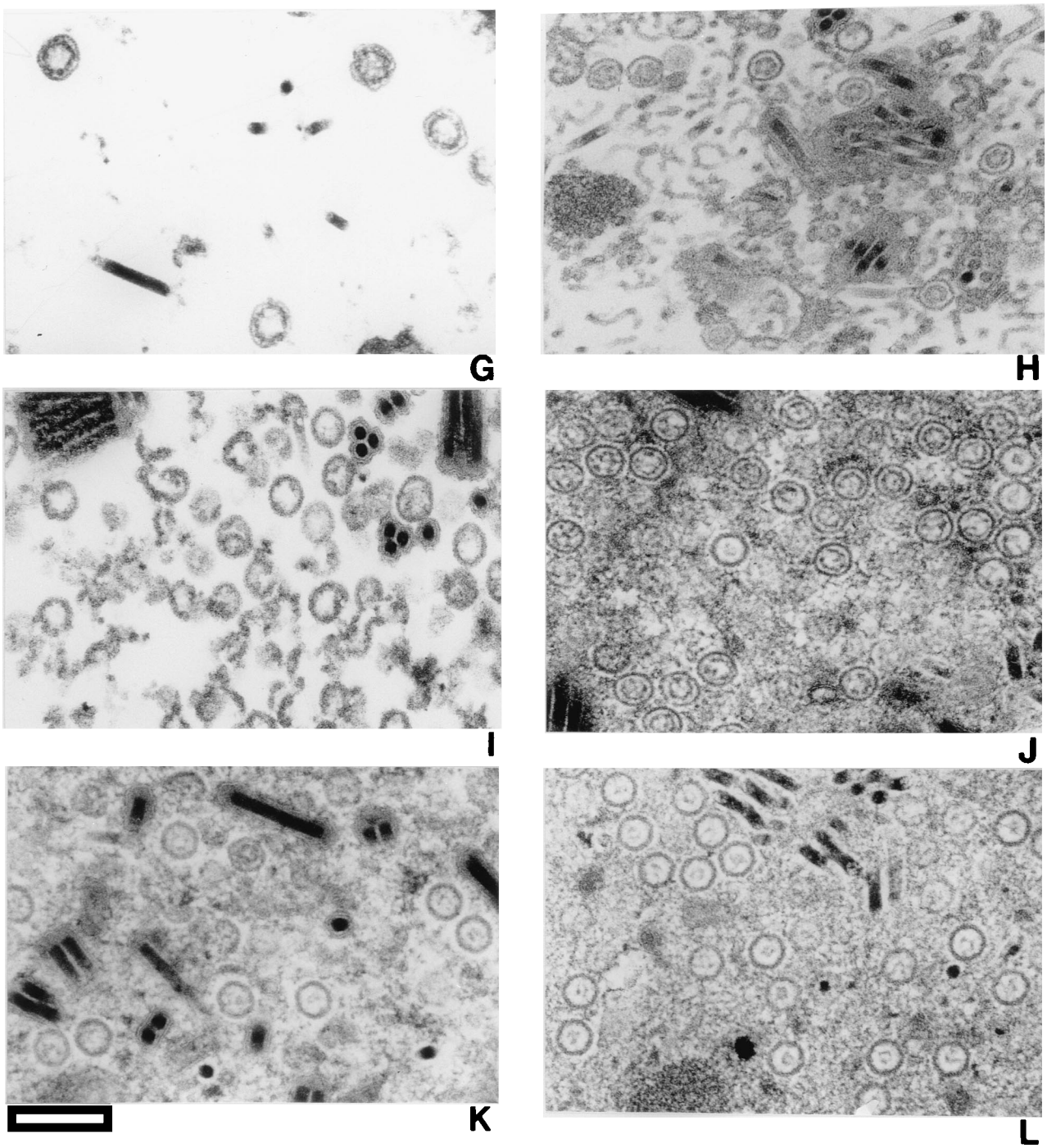

FIG. 3—*Continued.*

proteins (VP5, VP19C, VP23, and VP26) that make up the outer shell of the HSV capsid (Fig. 4A to C, lane 4, and Fig. 6). These structures are identical in composition to the aberrant capsids made in Sf9 cells infected with recombinant baculoviruses expressing just the UL18, UL19, UL35, and UL38 genes (Fig. 4A to C, lane 3). Thin-section preparations of Sf9 cells infected with the wild-type UL26.5 gene along with the mutant UL26/610 gene revealed that the nuclei of these cells contained capsids (Fig. 3E) which were very similar in appearance to capsids found in cells infected with the wild-type UL26 and UL26.5 genes (Fig. 3A); in addition, the protein composition of these capsids was similar to that of capsids isolated from cells infected with both wild-type genes (Fig. 4A to C, compare lanes 1 and 5). In contrast, the capsids made in cells infected with the wild-type UL26 gene along with the mutant UL26.5/304 gene were missing the UL26.5 protein (Fig. 4C, lane 6) and the capsids appeared to lack a visible internal core (Fig. 3F). The predominant capsid forms found in cells expressing the UL26 and UL26.5/304 genes were aberrant capsids (see Fig. 6). The results of these experiments indicated that the C-

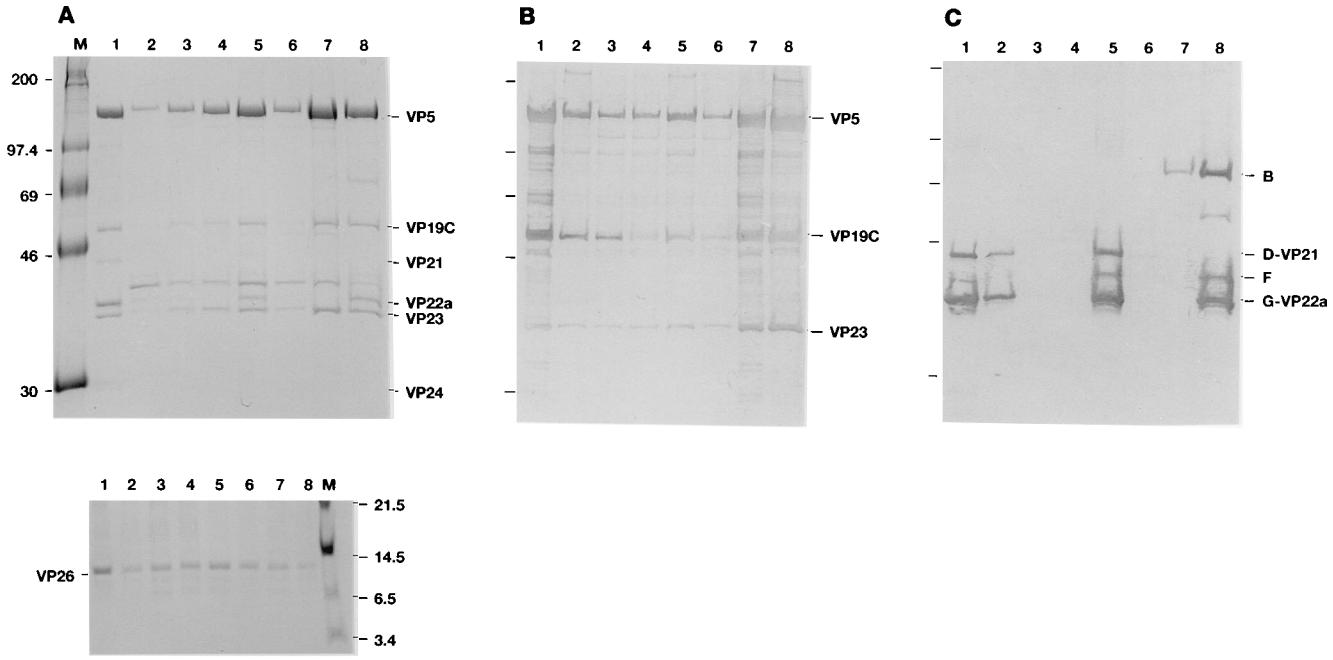

FIG. 4. Protein composition of particles harvested from sucrose gradients. (A) Vero cells were infected with HSV-1 (lane 1) or Sf9 cells were infected with recombinant baculoviruses expressing UL18, UL19, UL35, and UL38 plus UL26 and UL26.5 (lane 2), no addition (lane 3), UL26/610 and UL26.5/304 (lane 4), UL26/610 and UL26.5 (lane 5), UL26 and UL26.5/304 (lane 6), UL26/247 (lane 7), or UL26/247 and UL26.5 (lane 8). Cells were harvested at 12 h (Vero cells) or 64 h (Sf9 cells) postinfection, and particles were purified by double banding on 20 to 65% sucrose gradients as described in Materials and Methods. Capsid proteins were run on 12.5% (top gel) or 10 to 20% (bottom gel, which shows only proteins with a molecular mass below 21 kDa) polyacrylamide–SDS gels, and proteins were visualized by staining with Coomassie blue. Lane M, marker proteins with molecular masses listed in kilodaltons. (B and C) Western blots of the gel shown in the top part of panel A, with NC1, NC2, and NC5 (B) or MCA406 (C) as the primary antibodies. The mobilities of the capsid proteins are marked, and the letters on the right side of the Western blot in panel C refer to the different forms of the UL26 and UL26.5 proteins shown at the bottom of Fig. 2.

terminal 25 amino acids of the UL26 and UL26.5 proteins are essential for interaction of the scaffold proteins with the outer capsid shell during capsid assembly. In addition, the results demonstrated that the wild-type UL26.5 protein can assist the truncated UL26 protein (VP21) into the capsid, while the wild-type UL26 protein does not do the same for the truncated UL26.5 protein.

UL26/61. Thin-section preparations of cells infected with the UL26/61 and UL26.5 genes demonstrated that large numbers of capsids were made in these cells (Fig. 3H). However, the

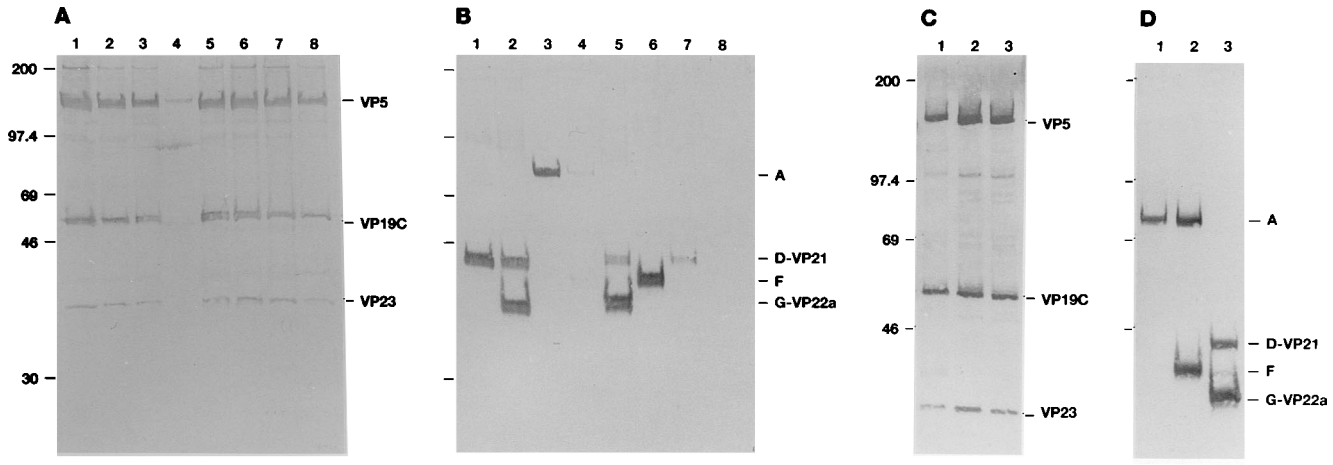

FIG. 5. Protein composition of particles harvested from sucrose gradients. (A and B) Sf9 cells were infected with recombinant baculoviruses expressing UL18, UL19, UL35, and UL38 plus UL26/114 (lane 1), UL26/114 and UL26.5 (lane 2), UL26/61 (lane 3), UL26/61 and UL26.5 (lane 4), UL26 and UL26.5 (lane 5), UL26.5 (lane 6), UL26 (lane 7), or no addition (lane 8). Cells were harvested at 64 h postinfection, and particles were purified as described in the legend to Fig. 4. Capsid proteins were run on 12.5% polyacrylamide–SDS gels. (C and D) Because the amount of protein loaded in lanes 4 of panels A and B was too low to detect in the Western blots, a second gel was run in which more protein for that sample was loaded. Lanes: 1, UL26/61; 2, UL26/61 and UL26.5; 3, UL26 and UL26.5. (A and C) Western blot analysis with NC1, NC2, and NC5 as the primary antibodies. (B and D) Western blot analysis with MCA406 as the primary antibody. The mobilities of the capsid proteins are marked, and the letters on the right side of the Western blots in panels B and D refer to the different forms of the UL26 and UL26.5 proteins shown at the bottom of Fig. 2.

RECOMBINANT BACULOVIRUS INFECTION	PROTEIN COMPOSITION BY SDS-PAGE	VIRUS STRUCTURE ELECTRON MICROSCOPY	
		MAJOR	MINOR
UL26 + UL26.5	VP21, VP22a		
UL26	VP21		
UL26.5	UL26.5F		
--	--		--
UL26/610 + UL26.5/304	--		--
UL26/610 + UL26.5	VP21, UL26.5F, VP22a		
UL26 + UL26.5/304	VP21		
UL26/61	UL26A		
UL26/61 + UL26.5	UL26A, UL26.5F		
UL 26/247	UL26B		
UL26/247 + UL26.5	UL26B, UL26.5F, VP22a		
UL26/114	VP21, VP24		
UL26/114 + UL26.5	VP21, VP22a, VP24		

-Large-Cored B capsid - Small-Cored B capsid - Capsid without core - Aberrant capsid

FIG. 6. Summary of mixed infections with recombinant baculoviruses expressing HSV-1 capsid genes. Briefly, Sf9 cells were infected with recombinant baculoviruses expressing the HSV-1 UL18, UL19, UL35, and UL38 genes plus the indicated forms of the UL26 and UL26.5 genes. To determine protein composition, samples isolated from sucrose gradients were run on SDS-polyacrylamide gels; HSV-1 capsid proteins that were present in the sample are indicated. All samples were composed of VP5, VP19C, VP23, VP26, and the indicated forms of the UL26 and UL26.5 gene products. UL26A, UL26B, and UL26.5F corresponded to the unprocessed forms of the UL26 and UL26.5 gene products shown in the bottom part of Fig. 2. VP24 is listed only for capsids in which this protein was observed on stained gels. For a description of the different capsid structures observed in electron microscopy thin sections see text.

appearance of these capsids differed from that of those made in cells expressing the UL26 and UL26.5 genes by virtue of the larger diameter of the internal core structures they contained (compare Fig. 3A and H). The cores found in these capsids were nearly identical to the cores found in cells infected with the UL26.5 gene alone (Fig. 3B). An even larger internal core structure was observed in the capsids made in cells infected with just the UL26/61 gene (Fig. 3G). The presence of a visible internal core structure in the capsids made in UL26/61-infected cells was surprising, because we had previously shown (46) that an observable core structure was found only when a baculovirus expressing the UL26.5 protein was included in the mixed infection. Fig. 3C shows empty capsids made when the UL26.5 gene was omitted. Analysis of the capsids made in UL26/61-infected cells showed that they contained the unprocessed form (80 kDa) of the UL26 protein (Fig. 5B, lane 3, and Fig. 5D, lane 1), while the capsids made in UL26/61- plus UL26.5-infected cells contained the unprocessed forms of both the UL26 and UL26.5 proteins (Fig. 5D, lane 2). These results demonstrated that proteolytic processing of the UL26 and UL26.5 proteins by the UL26-encoded protease was not required for capsid assembly. In addition, these results demonstrated that cleavage of the UL26 and UL26.5 proteins results in a change in the morphology of the B capsid inner core. When the scaffold and protease remained uncleaved, the capsids contained large cores (Fig. 3G and H), while cleavage resulted in the absence of a visible core in cells infected with the UL26 gene alone (Fig. 3C) and in capsids with small cores in cells infected with both UL26 and UL26.5.

UL26/247. Thin-section preparations of cells infected with the UL26/247 and UL26.5 genes (Fig. 3J) showed that the nuclei of these cells contained both large- and small-cored capsids. The large-cored capsids were identical in appearance to the capsids observed in either UL26/61- plus UL26.5-infected cells (Fig. 3H) or in cells infected with just the UL26.5 gene alone (Fig. 3B), while the small-cored capsids were identical in appearance to the capsids observed in cells infected with wild-type UL26 and UL26.5 genes (Fig. 3A). In contrast, thin-section preparations of cells infected with the UL26/247 gene alone showed mainly aberrant capsids along with a few complete capsids that lacked a visible internal core structure (Fig. 3I). Analysis of the protein composition of these capsid structures showed that the capsids made in cells infected with the UL26/247 gene contained a UL26 protein (75 kDa) that was cleaved at the M but not the R cleavage site (Fig. 4A to C, lane 7). The capsids isolated from cells infected with the UL26/247 and UL26.5 genes also contained the 75-kDa form of the UL26 protein (Fig. 4A to C, lane 8), along with both the cleaved and uncleaved forms of the UL26.5 protein. The presence of both forms of the UL26.5 protein in these capsids would account for why both large- and small-cored B capsids are found in these cells.

UL26/114. The UL26/114 mutant expresses a protease that has reduced activity relative to the wild-type protein (Fig. 2). The reduced protease activity of the UL26/114 protein did not affect capsid assembly, because large numbers of capsids were present in the nuclei of cells infected with either the UL26/114 gene alone (Fig. 3K) or in cells infected with both the UL26/114 and UL26.5 genes (Fig. 3L). The capsids made in cells expressing the UL26/114 and UL26.5 proteins were all of the small-cored type, while equal numbers of capsids with small cores and without cores were found in cells expressing just the UL26/114 gene. Cells infected with the UL26/114 gene alone showed few if any aberrant capsids (Fig. 3K), which was surprising because cells infected with the wild-type UL26 gene contain mainly aberrant capsids (46). Western blot analysis of the purified capsids showed that the protein composition of the capsids was similar to that of the capsids made when the wild-type UL26 gene was used, with the exception that the capsids made with the mutant appeared to contain more VP21 (Fig. 5A and B, compare lanes 1 and 2 with lanes 5 and 7, respectively).

In order to directly compare the protein compositions of the capsids made in cells infected with either the mutant or wild-type UL26 genes, purified capsids were run on an SDS–12.5% polyacrylamide gel, and the proteins were visualized by staining the gel with Coomassie blue (Fig. 7). The capsids isolated from cells infected with either the UL26/114 gene alone or with both the UL26/114 and UL26.5 genes contained a prominent VP21 band (Fig. 7, lanes 4 and 5). Compared with B capsids isolated from HSV-infected Vero cells (Fig. 7, lane 6), the capsids made with the UL26/114 protein appeared to contain slightly more VP21. Even more striking was the difference in the amount of VP21 found in capsids made in insect cells when the UL26/114 protein was used in place of the wild-type UL26 protein (Fig. 7, compare lanes 1 and 2 with lanes 4 and 5, and

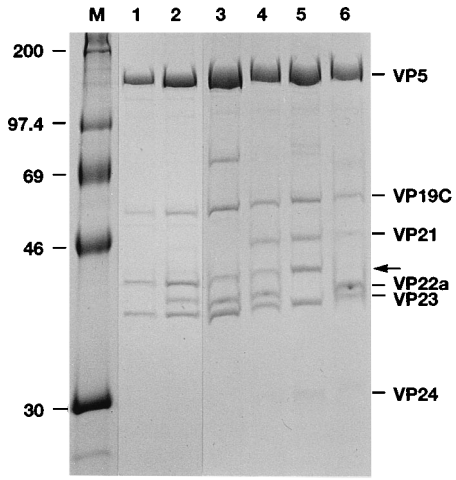

FIG. 7. Protein composition of particles harvested from sucrose gradients. Vero cells were infected with HSV-1 (lane 6), or Sf9 cells were infected with recombinant baculoviruses expressing UL18, UL19, UL35, and UL38 plus UL26 (lane 1), UL26 and UL26.5 (lane 2), UL26/247 and UL26.5 (lane 3), UL26/114 and UL26.5 (lane 4), or UL26/114 (lane 5). Cells were harvested at 12 h (Vero cells) or 64 h (Sf9 cells) postinfection, and particles were purified as described in the legend to Fig. 4. Proteins were run on 12.5% polyacrylamide–SDS gels, and proteins were visualized by staining with Coomassie blue. The mobilities of the capsid proteins are marked. A contaminating Sf9 cell or baculovirus protein is indicated by an arrow. Lane M, marker proteins with molecular masses in kilodaltons.

Fig. 5A and B, compare lanes 1 and 2 with lanes 5 and 7). A visible VP21 band was present in the capsids made with the UL26/114 protein alone (Fig. 7, lane 5), but little if any VP21 was detected in the capsids made with the UL26 protein (Fig. 7, lane 1). Increased amounts of VP21 were also observed in cells infected with the UL26/114 and UL26.5 genes (Fig. 7, lane 4) compared with when the wild-type UL26 gene was used (Fig. 7, lane 2). The amount of VP24 also appeared to increase when the UL26/114 mutant was used (Fig. 7, lane 5), but because this protein is difficult to see on a stained gel, we were unable to make a direct comparison with capsids made with the wild-type UL26 protein. An increase in the UL26 gene product (prominent 75-kDa band) was also seen in capsids isolated from cells infected with the UL26/247 and UL26.5 genes (Fig. 7, lane 3). Little if any change in the amount of VP22a was seen when either the UL26/114 or UL26/247 mutant was used in place of the wild-type UL26 gene (Fig. 7, compare lane 2 with lanes 3 and 4).

Pulse-chase analysis of the UL26, UL26/114, and UL26/247 proteases. The kinetics and cleavage-site specificity of the wild-type and mutant UL26 proteins were examined by pulse-chase radiolabelling and immunoprecipitation of proteins expressed from baculovirus-infected insect cells (Fig. 8). Sf9 cells were infected with recombinant baculoviruses expressing either the six (Fig. 8, ALL) HSV-1 capsid genes or with viruses expressing just the UL26, UL26/114, or UL26/247 gene plus the UL26.5 gene, and 36 h postinfection, the cells were pulse-labelled with [^{35}S]methionine for 5 min and then chased with nonradioactive methionine. Samples were taken at 0, 5, 15, 30, and 60 min after the addition of nonradioactive methionine-containing media and analyzed by immunoprecipitation with the MCA406 antibody followed by SDS-PAGE. The protease expressed from the wild-type UL26 gene rapidly cleaved itself (80-kDa full-length protein [peptide A of Fig. 8]) at the two internal cleavage sites to yield three proteolytic products with sizes of 75, 50, and 45 kDa (Fig. 8, lanes 6 to 10; peptides B, C, and D, respectively). The 80-kDa band was most intense in the 5-min-chase sample and then steadily decreased in intensity in the 15-, 30-, and 60-min-postchase samples, consistent with it being

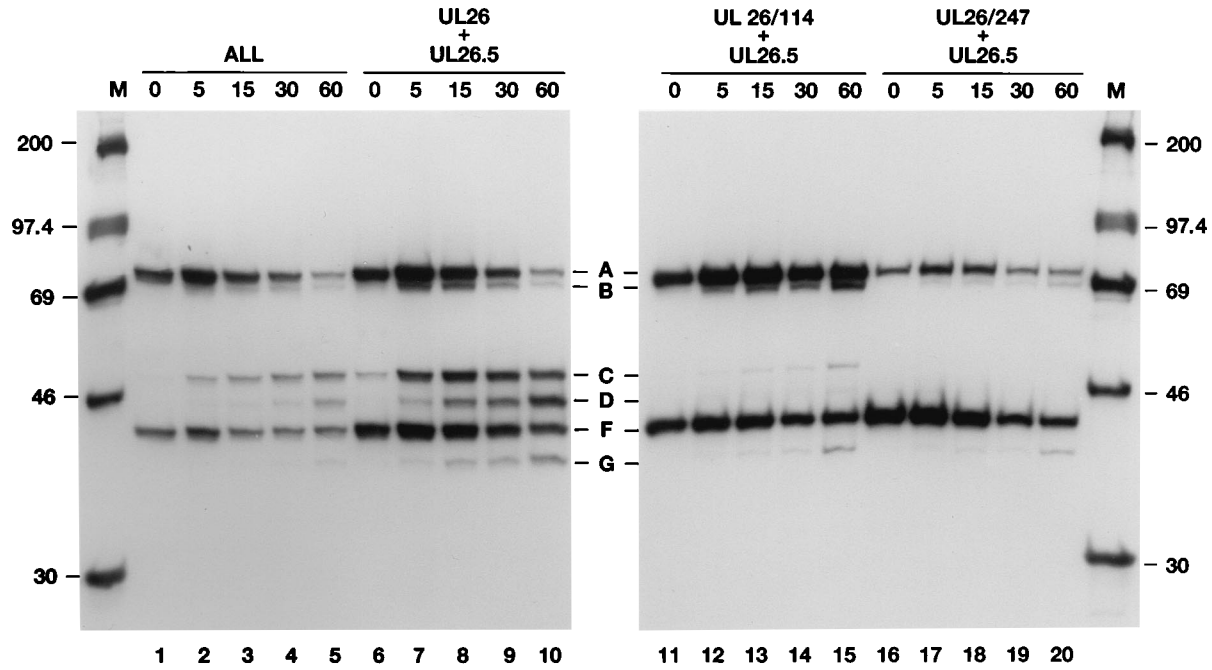

FIG. 8. Pulse-chase analysis of the protease activity associated with the proteins expressed by the UL26, UL26/114, and UL26/247 gene products. Sf9 cells were infected with the indicated viruses. (ALL indicates cells infected with baculoviruses expressing the six HSV-1 capsid genes UL18, UL19, UL26, UL26.5, UL35, and UL38.) At 36 h postinfection, cells were pulsed with [^{35}S]methionine for 5 min and chased with unlabelled methionine-containing media for the indicated times (in minutes). Labeled proteins were precipitated from the lysates of infected cells with monoclonal antibody MCA406, and the material was separated on a 12.5% polyacrylamide–SDS gel. The letters between the panels identify the different forms of the UL26 and UL26.5 proteins as described in the legend to Fig. 2.

processed to lower-molecular-mass bands. The 75-kDa (M site cleavage) and 50-kDa (R site cleavage) bands were first detected in the 5-min-pulse period, and both bands increased in intensity through the 15-min-chase period. The intensity of the 75-kDa band decreased in the 30-min-chase period and then decreased further in the 60-min-chase period, while the intensity of the 50-kDa band decreased only slightly in the 30-min-chase period and remained at the same intensity in the 60-min period. The 45-kDa band was barely detectable in the 5-min-pulse sample and increased steadily through the 60-min-chase period. Thus, the full-length (80-kDa) UL26 protein was first cleaved at either the M or R site with the efficiency of cleavage at each site being equivalent. The 75-kDa protein was then rapidly converted to the 45-kDa form by cleavage at the R site. In contrast, the 50-kDa protein was slowly converted to the 45-kDa form by cleavage at the M site. These results indicate that the M site was cleaved rapidly when present in the full-length protease but more slowly when the M site was not directly attached to the N-terminal 247 amino acids of the UL26 protein. This conclusion is supported by the fact that cleavage of the M site present in the UL26.5 protein (38 kDa, peptide F of Fig. 8) followed the same pattern as that seen with cleavage of this site in the 50-kDa protein. The UL26.5 protein (38 kDa) was first detected in the 5-min-pulse sample, increased in intensity through the 15-min-chase period, and then decreased in intensity through the next two chase periods, while the cleaved form of the UL26.5 protein (34 kDa, peptide G of Fig. 8) was first detected during the 5-min-chase period and slowly increased at later times postchase. The order of cleavage for the HSV protease is similar to what Welch et al. (48) and Jones et al. (13) have shown for the cytomegalovirus protease, although they found that the M site is cleaved slightly faster than the R site in the full-length cytomegalovirus protease.

Because assembly of a B capsid requires the products of four additional HSV-1 genes, pulse-chase analysis of the UL26 protease was examined with insect cells infected with the six (Fig. 8, ALL) HSV-1 capsid genes (Fig. 8, lanes 1 to 5). Although the intensity of the UL26 and UL26.5 proteins isolated from cells infected with the six capsid proteins was reduced, the pattern of cleavage of these two proteins by the UL26-encoded protease was identical to that observed in cells infected with just the UL26 and UL26.5 genes. Cleavage of the full-length protease at both the M and R sites was observed in the 5-min-chase sample; the 75-kDa protein (M site) was rapidly chased to the 45-kDa form of the protease, while the amount of the 50-kDa protein (R site) steadily increased at later times postchase. In addition, the 38-kDa form of the UL26.5 protein was slowly chased out to the 34-kDa form, similar to what was observed in cells infected with just the UL26 and UL26.5 genes. These results demonstrated that the kinetics and cleavage-site specificity of the UL26 protease were not altered by the presence of the four proteins (VP5, VP19C, VP23, and VP26) that make up the outer icosahedral shell of the HSV-1 capsid.

The protease expressed by the UL26/114 mutant cleaved both its own M site and that of the UL26.5 protein with approximately the same efficiency as the wild-type UL26 protein but was less efficient than the wild-type protease in cleaving its R site (Fig. 8, lanes 11 to 15). Only small amounts of the 50- and 45-kDa forms of the UL26 protein were detected in the 60-min-chase sample, indicating limited cleavage of the UL26 protein at the R site. The majority of the radiolabel accumulated in either the 80-kDa (full-length) or 75-kDa (M site) form of the UL26 protein at the latest postchase time point. The overnight labeling experiment shown in Fig. 2 demonstrates that the R site is eventually cleaved in the UL26/114 protein but at a much reduced rate compared with the wild-type UL26 protein. These results indicate that the two glutamines located at amino acids 114 and 115 of the UL26 protein are critical for efficient cleavage of the R site.

Mutation of the R site in the UL26/247 mutant resulted in the accumulation of the 80-kDa protein and the absence of the 50- and 45-kDa proteins (Fig. 8, lanes 16 to 20). However, the 80-kDa precursor was still cleaved at the M site to yield the 75-kDa protein, and the UL26/247-encoded protease cleaved the M site of the UL26.5 protein. The efficiency of cleavage at the M site was reduced compared with that of the wild-type protease, which is consistent with what was observed in long-term radiolabeling experiments (Fig. 2, lane 11).

Coimmunoprecipitation of VP5 and uncleaved UL26.5. In order to examine the importance of the C-terminal 25 amino acids of the UL26.5 protein in formation of a stable complex with VP5, the VP5-specific monoclonal antibody 6F was tested for its ability to precipitate complexes containing VP5 and VP22a. Sf9 cells were separately infected with recombinant baculoviruses expressing either VP5 (UL19 gene), uncleaved VP22a (UL26.5 gene), or cleaved VP22a (UL26.5/304 gene), and at 64 h postinfection, the cells were harvested and the cell pellet was resuspended in a small volume of PBS (see Materials and Methods). An aliquot of each sample was then subjected to PAGE, either before or after immunoprecipitation with the 6F antibody, and the proteins were detected by staining the gel with Coomassie blue (Fig. 9). These experiments were performed by adding antibody to the cell extracts, incubating the sample for a short time, and then pelleting any precipitate that formed. The pelleted sample was then washed once with PBS, and an aliquot of the washed pellet was loaded on the gel. There were no secondary antibodies or protein A beads added to facilitate pelleting of the samples. The cell extracts isolated from UL26.5/304- and UL19-infected cells each contained a major protein-staining band which corresponded to the HSV-1 capsid proteins VP22a and VP5, respectively (Fig. 9, lanes 3 and 4). The cell extract isolated from UL26.5-infected cells contained a major protein-staining band which was 4 to 5 kDa larger than VP22a and corresponded to the uncleaved form of the UL26.5 gene product (Fig. 9, lane 2). When the cell extracts were mixed with the 6F antibody, small amounts of VP5 and VP22a were precipitated (Fig. 9, lanes 6 and 7), but no uncleaved VP22a was precipitated from the UL26.5 extract (Fig. 9, lane 5). It should be noted that if protein A beads are added, the 6F antibody efficiently precipitates VP5 but not VP22a (cleaved or uncleaved) (23). When the extract isolated from UL19-infected cells was mixed with the extract isolated from UL26.5-infected cells, the 6F antibody precipitated large amounts of both VP5 and uncleaved VP22a (Fig. 9, lane 8). In contrast, the 6F antibody precipitated very little VP5 or VP22a when extracts from UL19-infected cells were mixed with extracts isolated from UL26.5/304-infected cells (Fig. 9, lane 9). Precipitation of VP5 and uncleaved VP22a was dependent on addition of the 6F monoclonal antibody, because neither protein sedimented in the absence of the antibody (23). These results demonstrated that interaction of VP5 and VP22a requires the C-terminal 25 amino acids of the UL26.5 protein.

DISCUSSION

The primary findings of this report are that (i) the C-terminal 25 amino acids of the UL26 and UL26.5 proteins are required for assembly of 125-nm HSV-1 capsids; (ii) coimmunoprecipitation of VP5 and UL26.5 with a VP5-specific mono-

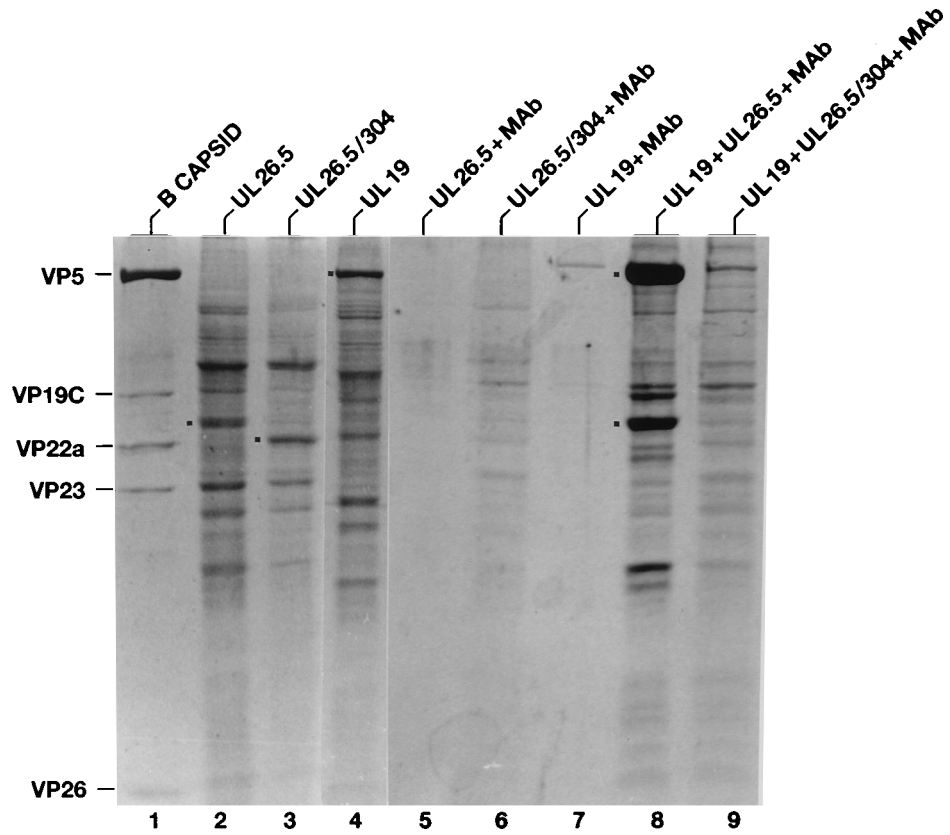

FIG. 9. Coimmunoprecipitation of VP5 and VP22a (uncleaved). Lane 1 contains purified HSV-1 B capsids, and the mobilities of the capsid proteins are marked. Lanes 2 to 4 show extracts from Sf9 cells infected with recombinant baculoviruses expressing the indicated gene, and the HSV-1 capsid proteins expressed by each virus are indicated by dots. Lanes 5 to 6 show proteins immunoprecipitated from these extracts with the VP5-specific monoclonal antibody (MAb) 6F. Lanes 8 and 9 show proteins immunoprecipitated when extracts from UL19- and UL26.5-infected cells were mixed (lane 8) or when extracts from UL19- and UL26.5/304-infected cells were mixed (lane 9). In lane 8, the protein bands that correspond to VP5 and VP22a (uncleaved) are indicated by dots. Proteins were separated by electrophoresis through an SDS–12.5% polyacrylamide gel, and proteins were detected by staining with Coomassie blue.

clonal antibody requires the C-terminal 25 amino acids of the UL26.5 protein; (iii) the uncleaved forms of the UL26 and UL26.5 proteins are used in assembly of the HSV B capsid, and cleavage of these proteins occurs during or subsequent to assembly; and (iv) the efficiency of cleavage at the R cleavage site determines the level of VP21 incorporated into B capsids assembled in insect cells.

The C-terminal 25 amino acids of the UL26 and UL26.5 proteins are critical for capsid assembly. The HSV-1 UL26 gene encodes a maturational proteinase that cleaves itself and the product of the HSV-1 UL26.5 gene at a site 25 amino acids from the C terminus of these two proteins. Without this cleavage, infectious virus is not produced (9, 33, 34, 37). In order to test the importance of the C-terminal 25 amino acids in capsid assembly, we constructed recombinant baculoviruses that expressed truncated UL26 and UL26.5 proteins. When these two mutant constructs were tested in the insect cell assembly assay, it was found that only aberrant capsid structures were made. The lack of complete capsids in cells infected with viruses expressing the truncated UL26 and UL26.5 proteins clearly demonstrated that the C terminus of these two proteins is essential for assembly of morphologically normal capsids.

The importance of the C-terminal 25 amino acids suggested that this region is required either for oligomerization of the scaffolding protein to form the inner core of the B capsid or for interaction of the scaffold protein with the capsid shell. The former is probably not the case, because Newcomb and Brown (22) have demonstrated that VP22a (cleaved form of UL26.5) isolated from B capsids will self-assemble into scaffoldlike particles in the absence of the other capsid proteins. In addition, Preston et al. (32) have shown that thin-section preparations of insect cells infected with baculoviruses expressing both the UL26 and UL26.5 genes contain large numbers of scaffoldlike particles. These structures were not found in cells infected with the UL26 gene alone, and only small numbers of them were observed in cells infected with the UL26.5 gene alone. Therefore, the cleaved form of the UL26.5 protein appears to form better scaffoldlike structures than the full-length protein.

Experiments performed with extracts from infected insect cells indicated that the C-terminal 25 acids of the UL26.5 protein are important for interaction of the scaffold with the capsid shell. Formation of a stable complex between the major capsid protein, VP5, and the uncleaved form of the UL26.5 protein was demonstrated by coprecipitating the two proteins with the VP5-specific monoclonal antibody 6F (Fig. 9, lane 8). The 6F antibody failed to precipitate a VP5/UL26.5 complex when the C-terminal 25 amino acids were missing from the UL26.5 protein (Fig. 9, lane 9). In addition, the 6F antibody precipitated only small amounts of the major capsid protein from extracts of UL19-infected cells (Fig. 9, lane 7), indicating that VP5 must form a large complex in order to be efficiently precipitated. The complex would consist of VP5 bound to preformed scaffold structures (composed of the uncleaved UL26.5 protein), and addition of the 6F antibody results in the

precipitation of this large complex. Therefore, the overall results suggest that the scaffold interacts with capsid shell (VP5) through its C terminus, with the last 25 amino acids of the UL26.5 protein strongly implicated as containing the actual site of interaction. We have recently shown that HSV-1 capsids will assemble spontaneously in a cell-free system consisting of extracts prepared from insect cells infected with baculoviruses expressing the capsid genes (24). The C-terminal 25 amino acids of the UL26.5 protein are also required for capsid assembly in the cell-free system (23).

Cleavage of the UL26 and UL26.5 proteins by the UL26 protease occurs during or subsequent to capsid assembly. Cleavage of the UL26.5 gene product by the UL26-encoded protease is not required for formation of the inner core structure of the B capsid (44, 46), although proteolytic processing of the scaffold results in a change in the morphology of the core (36). The role of cleavage in altering the morphology of the core was first described with the HSV-1 temperature-sensitive mutant *ts*1201 (33). This mutant has a lesion that maps within the UL26 gene and results in an inactive protease. At the nonpermissive temperature, the capsids made in *ts*1201-infected cells contain large cores which are converted to smaller cores when shifted to the permissive temperature. The results of studies with the protease-deficient UL26/61 mutant confirm that the large-cored phenotype results when the scaffold proteins remain uncleaved. Insect cells infected with baculoviruses expressing the UL26/61 and UL26.5 proteins contained only large-cored capsids. Isolation of these capsids demonstrated that the full-length forms of the UL26 and UL26.5 proteins were present (Fig. 5D, lane 2). In addition, we found that capsids made in cells infected with the UL26/61 gene alone also contained a large inner core. This result was surprising, because we had previously shown that the capsids made in the absence of the UL26.5 protein lacked a visible internal core structure (46). Therefore, the UL26 protein will form a visible core structure, and formation of the core requires the C-terminal 25 amino acids of the UL26 protein. This is based on the fact that a visible core is present in capsids made in UL26/61-infected cells (capsids contain the full-length UL26 protein) but not in capsids made in UL26/247-infected cells (the 75-kDa UL26 protein that these capsids contain is missing the C-terminal 25 amino acids).

A role for R site cleavage in capsid assembly. Previously Liu and Roizman (16) showed that substitution of glutamines 114 and 115 of the UL26 protein to Gly-Ala eliminated the ability of the protease to cleave the UL26.5 protein. In the present study, we found that a similar UL26 mutant (amino acids 114 and 115 changed to Ala and Gly, respectively) expressed from a recombinant baculovirus cleaved the UL26.5 protein but at much slower rate than the wild-type UL26 protein (Fig. 2). Pulse-chase analysis demonstrated that the UL26/114 mutant was extremely slow at cleaving the R cleavage site, suggesting a role for these two amino acids in determining R site specificity (Fig. 8). The difference in protease activity for the two UL26 mutants is not clear but might be explained by the fact that the mutant protease described in this study was expressed at a much higher level in baculovirus-infected cells than with the cotransfection method used by Liu and Roizman (16) to assay the mutant protease. Alternatively, changing the two glutamines to Gly-Ala instead of Ala-Gly may result in an inactive enzyme.

When we tested the UL26/114 mutant in the insect cell assembly system and compared capsids made with this mutant with capsids made with the wild-type UL26 protein, the UL26/114 capsids appeared to contain more VP21 (Fig. 7). The increased amounts of the UL26 gene product were also seen in capsids isolated from cells infected with the UL26/247 gene (Fig. 7, lane 2). These results suggest that R site cleavage plays an important role in determining the amounts of the UL26 gene products that are retained in the assembled B capsid.

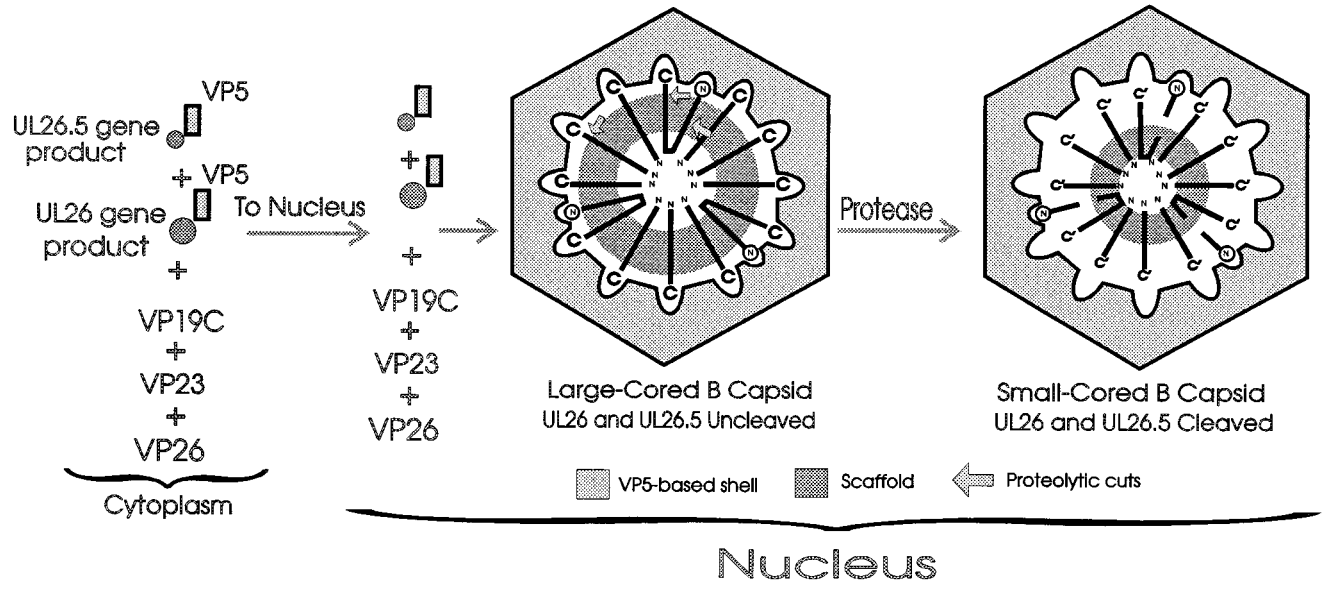

FIG. 10. Assembly pathway of HSV-1 capsids as suggested by the results presented here and in other relevant studies. The proposed pathway suggests that VP5 associates in the cytoplasm with the uncleaved forms of the UL26.5 and UL26 proteins. This association is required for efficient transport of VP5 to the nucleus, and it prevents cleavage of the UL26 and UL26.5 proteins prior to capsid assembly. In the nucleus, the capsid proteins assemble into large-cored B capsids composed of the uncleaved forms of the UL26 and UL26.5 proteins. As depicted in this model, the interaction of the UL26 and UL26.5 proteins with the VP5-based capsid shell would be via the common C-terminal 25 amino acids of the two proteins and, in the case of the UL26 protein, through its unique N-terminal sequence. Proteolytic cleavage at the M site in both the UL26 and UL26.5 proteins to generate a new C terminus and at the R site in the UL26 protein results in a small-cored B capsid that contains VP21, VP22a, and VP24.

Model for the assembly pathway of the HSV B capsid. The evidence presented in this report and previously shown with the HSV *ts*1201 mutant suggests that large-cored B capsids containing unprocessed protease and VP22a represent an early step in capsid assembly. A possible model for HSV-1 capsid assembly based on this evidence is shown in Fig. 10. The model proposes that processing of the UL26 and UL26.5 proteins occurs during or subsequent to capsid assembly. Because protease processing of the UL26 and UL26.5 proteins can occur in the absence of capsid structures (Fig. 2 and 8), cleavage of these proteins must be inhibited prior to assembly.

Capsid assembly is considered to begin when capsid proteins are transported to the nucleus. Recently it has been demonstrated that efficient localization of VP5 to the cell nucleus depends on its interaction with the UL26.5 gene product (18, 26). In these studies, the intracellular distribution of the HSV capsid proteins was examined by expressing individual capsid proteins in cells by using either recombinant vaccinia viruses or plasmid vectors. When expressed in the absence of the other capsid proteins, the UL26.5 protein was found exclusively in the nucleus, while VP5 was distributed throughout the cell. Coexpression experiments showed that VP5 was efficiently transported to the nucleus in the presence of the UL26.5 protein. These studies were done with the full-length form of the UL26.5 protein, so it is not known if the C-terminal 25 amino acids are essential for nuclear localization of VP5. As shown on the left-hand side of Fig. 10, the existence of a VP5/UL26.5 complex in the cytoplasm may serve to transport VP5 to the nucleus and may also serve to prevent cleavage of the UL26.5 protein prior to capsid assembly. Because all of the amino acid sequences in the UL26.5 protein are present in the UL26 protein, a similar VP5/UL26 complex may also form in the cytoplasm and as a result prevent the UL26 protease from cleaving itself.

After transport to the nucleus, VP5/UL26.5 complexes (along with VP5/UL26 complexes) come together by virtue of the ability of the UL26.5 protein to interact with itself, forming a structure to which the other capsid proteins attach to form large-cored B capsids. The interaction of the UL26 and UL26.5 proteins with the VP5-based capsid shell would be through their common C-terminal 25 amino acids and, in the case of the UL26 protein, through its unique N-terminal sequence. The interaction of the N terminus of the protease with the shell is suggested by the presence of VP24 in A, B, and C capsids (10, 36). Proteolytic cleavage at the M site alters the morphology of the scaffold and generates a new C terminus in both the UL26 and UL26.5 proteins. Cleavage at the R site releases the C terminus of the UL26 protein, with VP24 remaining attached to the capsid shell. The M and R site cleavages result in small-cored B capsids that contain VP21, VP24, and VP22a.

Finally, aberrant capsids are prominent structures in insect cells expressing the HSV-1 capsid genes (44, 46). The abundance of these structures is probably the result of overexpression of the capsid proteins from the recombinant baculoviruses. In contrast, HSV-infected cells contain few if any aberrant capsid structures (6, 40). Desai et al. (6) have recently reported the isolation of a mutant virus with a null mutation in the UL26 and UL26.5 genes. When this virus infects nonpermissive cells, only aberrant capsid structures are assembled. The assembly of these structures in cells infected with the UL26/UL26.5 null virus provides further evidence that in wild-type-infected cells, VP5 is in complexes with the products of the UL26.5 and/or UL26 genes and is not free to interact with the other proteins (VP19C, VP23, and VP26) that make up the capsid shell.

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