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The Bovine Herpesvirus 1 Maturational Proteinase and Scaffold Proteins Can Substitute for the Homologous Herpes Simplex Virus Type 1 Proteins in the Formation of Hybrid Type B Capsids

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We determined the nucleotide sequence of a 3.5-kb region of the bovine herpesvirus 1 (BHV-1) genome which contained the complete BHV-1 homologs of the herpes simplex virus type 1 (HSV-1) UL26 and UL26.5 genes. In HSV-1, the UL26 and UL26.5 open reading frames encode scaffold proteins upon which viral capsids are assembled. The UL26-encoded protein is also a proteinase and specifically cleaves both itself and the UL26.5-encoded protein. The overall BHV-1-encoded amino acid sequence showed only 41% identity to the HSV-1 sequences and was most divergent in the regions defined to be involved in the scaffolding function. We substituted the proteins encoded by the BHV-1 homologs of the UL26 and UL26.5 open reading frames, expressed in baculovirus, for the corresponding HSV-1 proteins in an in vitro HSV-1 capsid assembly system. The proteins expressed from the BHV-1 UL26 and UL26.5 homologs facilitated the formation of hybrid type B capsids indistinguishable from those formed entirely with HSV-1-encoded proteins.

Seven viral proteins involved in herpesvirus capsid assembly have been identified, primarily through work done with herpes simplex virus type-1 (HSV-1) (32). VP5, VP19C, VP23, and VP26, encoded by genes UL19, UL38, UL18, and UL35, respectively, make up the capsid shell (4–6, 8–10, 20–26, 28, 29, 31). VP22a, VP21, and VP24, encoded by genes UL26.5 and UL26, are located as a core inside the outer shell of type B capsids (9, 12, 16, 23, 24, 38). VP22a, and to a lesser extent VP21, forms a scaffold upon which the outer capsid shell is built (23, 30, 35, 38). The HSV-1 UL26 gene encodes two coterminal proteins translated from separate transcripts (16, 17). The larger protein contains proteinase activity and cleaves itself as well as the smaller protein (encoded by the smaller UL26.5 transcript), resulting in three capsid assembly proteins,

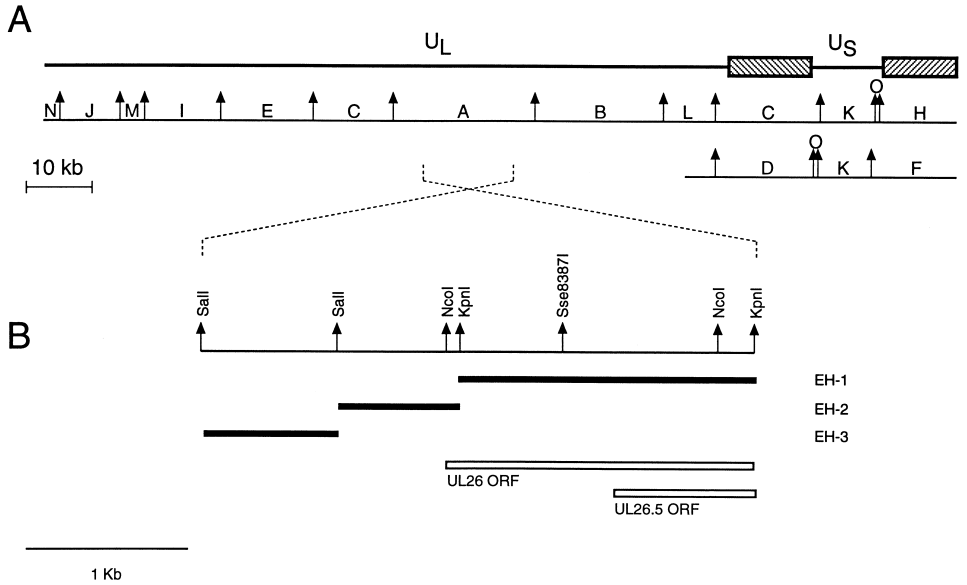

FIG. 1. (A) *Hin*dIII restriction map of the bovine herpesvirus 1 Cooper strain (19). (B) Enlarged map of the *Sal*I-to-*Kpn*I fragment sequenced. The bars marked EH-1, EH-2, and EH-3 are the three subclones used for sequencing and subsequent expression cloning analyses.

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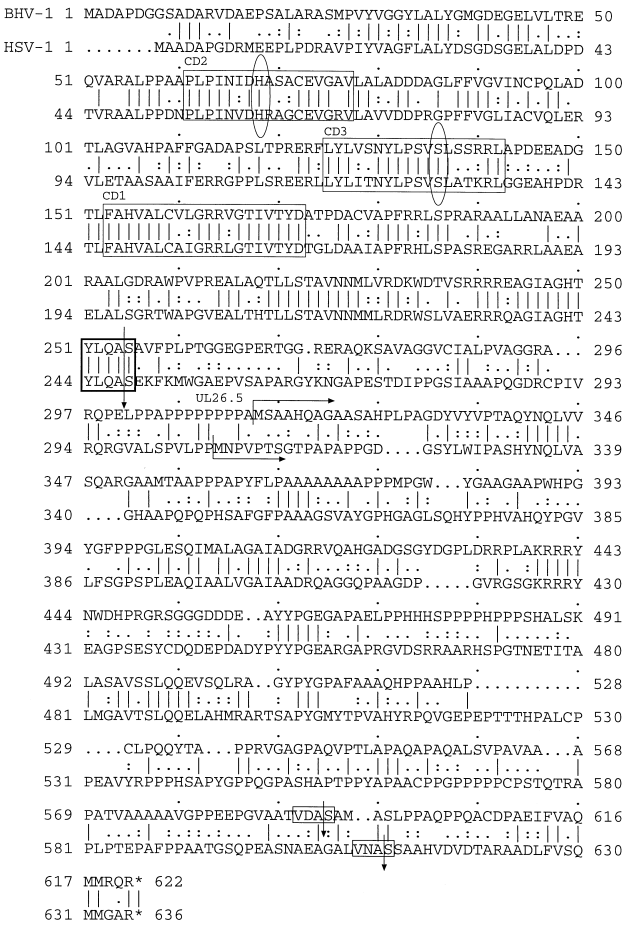

FIG. 2. Comparison of the deduced amino acid sequences of the BHV-1-encoded protein (top) and the HSV-1 UL26-encoded proteins (bottom). The previously defined conserved domains (39, 40) are boxed and labeled (CD1, CD2, and CD3), and the defined H and S residues of the catalytic site are circled. The R and M cleavage consensus sites are boxed, and the probable cleavage residues are separated by vertical arrows. The putative initiation residue for the BHV-1 UL26.5-encoded protein and the initiation residue for the HSV-1 UL26.5-encoded protein are marked with horizontal arrows.

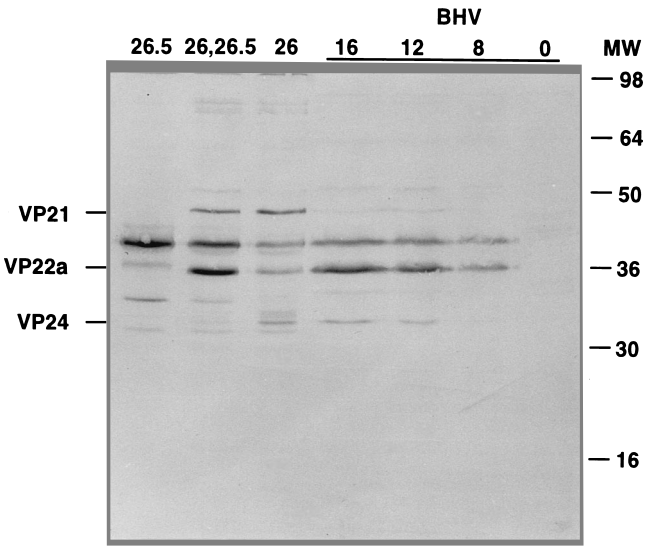

FIG. 3. Western blot showing expression of the BHV-1 UL26- and UL26.5-encoded proteins in baculovirus. The blot was processed with rabbit antiserum raised against *E. coli*-expressed BHV-1 UL26 fusion protein. The left side of the blot shows the migration distances of the BHV-1 homologs of the three capsid assembly proteins encoded by the UL26 complex, and the right side of the blot shows molecular weight standards (in thousands). Lane 26.5, Sf9 cells infected with a recombinant baculovirus expressing the BHV-1 UL26.5-encoded protein; lane 26,26.5, Sf9 cells coinfected with recombinant baculoviruses expressing the BHV-1 UL26- and UL26.5-encoded proteins; lane 26, Sf9 cells infected with recombinant baculoviruses expressing the BHV-1 UL26-encoded protein; lanes 16, 12, 8, and 0, BT cells infected with BHV-1 (Cooper strain) for 16, 12, 8, and 0 h, respectively.

VP24 (the proteinase) and VP21 and VP22a (the scaffolding proteins). Recently it has been shown that coinfection of insect cells with recombinant baculoviruses expressing the seven herpesvirus capsid assembly proteins resulted in the formation of type B herpesvirus capsids indistinguishable from the type B capsids formed by HSV-1-infected cells (35, 38).

The HSV-1 UL26 and UL26.5 gene products are well conserved among the different herpesvirus pathogens, particularly in the N-terminal proteinase (VP24) domain (40). The C-terminal portions of the UL26 (VP21) and UL26.5 (VP22a) homologs are not as well conserved, although all probably share a similar (scaffolding) function (1, 3, 7, 13, 20, 34, 36, 40). In this study, we set out to test whether the proteinase and scaffold proteins expressed by a heterologous herpesvirus can substitute in the assembly of HSV-1 capsids.

We first determined the sequence of the bovine herpesvirus 1 (BHV-1) UL26 and UL26.5 genes (GenBank accession no. U31809). Nucleotide sequencing was performed by Lark Sequencing Technologies, Inc. (Houston, Tex.). The source of the DNA was plasmid pBH92, obtained from Leonard Bello, University of Pennsylvania (15). A restriction map of the region is shown in Fig. 1. Standard molecular biology protocols were followed as previously described (2, 33). Figure 2 shows a comparison of the deduced proteins encoded by the BHV-1 and HSV-1 UL26 and UL26.5 genes. We predicted the probable N terminus of the BHV-1 UL26.5 open reading frame (nucleotide 954, amino acid 314) by homology with the corresponding HSV-1 protein. Although sharing only 41% overall similarity, the predicted BHV-1-encoded proteins showed conservation of several functionally important features. The active site histidine and serine residues of the HSV-1 proteinase were conserved in the BHV-1 homolog (11, 39). The three conserved domains (CD1, CD2, and CD3) of the proteinase, identified by Welch et al. (39, 40), were conserved in the corresponding BHV-1 protein. Two putative proteolytic cleavage sites were also conserved in the BHV-1 amino acid sequence. The R (release) site releases the proteinase (VP21) from the rest of the molecule. The putative BHV-1 R site, Y-L-Q-A$^\wedge$S, fits the consensus sequence for herpesvirus R sites, Y-V/L-K/Q-A$^\wedge$S (39), where $^\wedge$ is the cleavage site. The M (maturation) site is located 25 amino acids (in HSV-1) from the C terminus of the UL26 and UL26.5 proteins. The consensus sequence for this cleavage site is V/L-X-A$^\wedge$S, compared with the most likely BHV-1 site, V-D-A$^\wedge$S. The C-terminal portions of the BHV-1 UL26 and UL26.5 proteins, the portions involved in scaffold formation, were not appreciably similar to the corresponding HSV-1 sequences.

Expression of the BHV-1 UL26 and UL26.5 proteins. Recombinant baculoviruses were grown and titers were determined by standard methods (27). Baculovirus transfer vectors containing both the UL26- and UL26.5-homologous genes from BHV-1 were cotransfected into Sf9 cells with BaculoGold DNA (Pharmingen) (38). Recombinant viruses were screened for protein expression by Western blotting (immunoblotting) with rabbit antiserum raised against a portion of the BHV-1

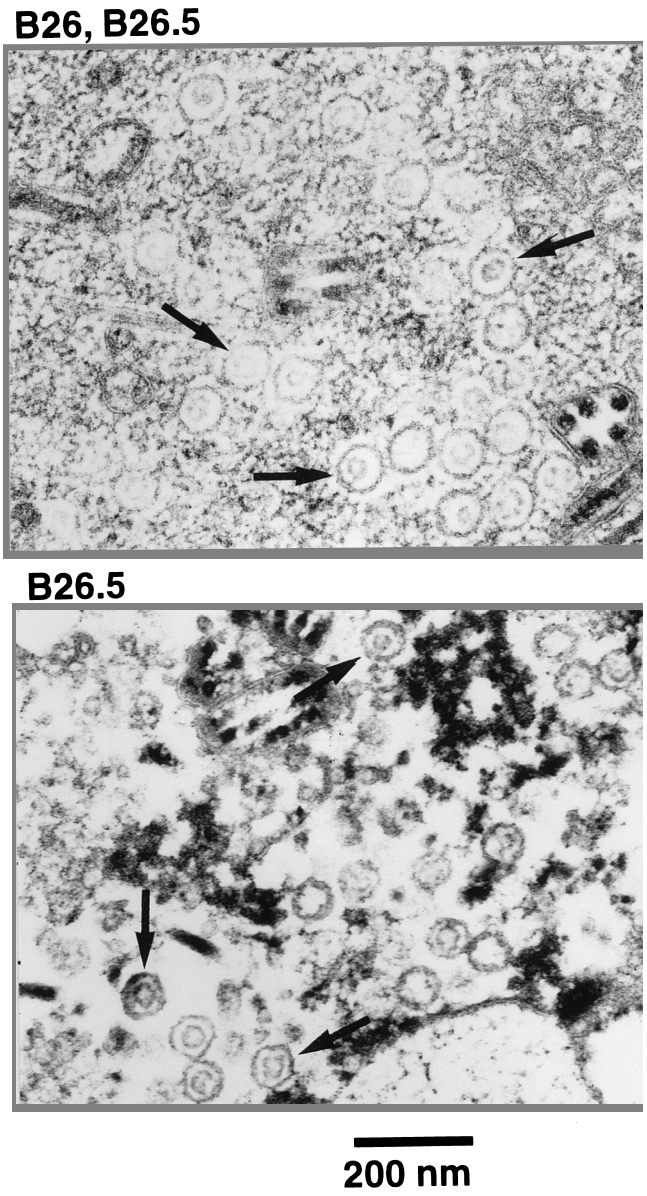

FIG. 4. Thin-section electron micrographs showing the assembly of type B HSV-1 capsids. Each panel shows Sf9 cells coinfected with recombinant baculoviruses expressing the HSV-1 VP5, VP19C, VP23, and VP26 proteins plus recombinant baculoviruses expressing proteins from the BHV-1 UL26 and UL26.5 genes (top panel) or from just the UL26.5 gene (bottom panel). Typical type B capsids are denoted by arrows in each panel.

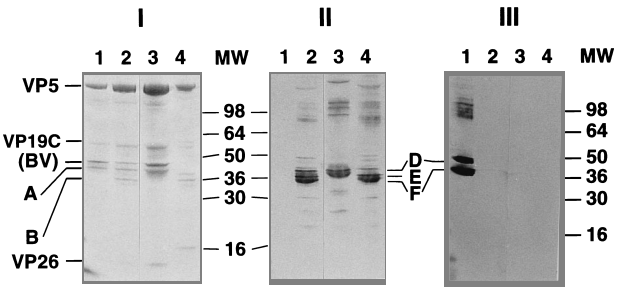

FIG. 5. (I) Coomassie blue-stained SDS-PAGE gel of proteins contained in HSV-1 or hybrid type B capsids purified from sucrose gradients. The migration distances of molecular weight standards (in thousands) are shown. Each lane shows the visible proteins in capsids formed from Sf9 cells infected with recombinant baculoviruses (BV) expressing gene products from the HSV-1 UL18, UL19, UL35, and UL38 genes, plus the BHV-1 or HSV-1 UL26 and/or UL26.5 genes as follows. Lane 1, HSV-1 UL26- and UL26.5-encoded proteins; lane 2, BHV-1 UL26- and UL26.5-encoded proteins; lane 3, BHV-1 UL26.5-encoded protein; lane 4, type B capsids purified from BHV-1-infected BT cells. HSV-1 proteins VP5, VP19C, and VP26 are marked. (II) Western blot of a gel identical to that shown in panel I reacted with the rabbit antiserum raised against the BHV-1 UL26 *E. coli* fusion protein. (III) Western blot of a gel identical to that shown in panel I reacted with a monoclonal antibody, MCA406, directed to an epitope in the HSV-1 VP22a protein. The protein bands migrating with A, B, D, E, and F are as follows. In panel I, the HSV VP22a and VP23 bands comigrate as a single band, which is labeled A. Band B is the BHV-1 VP22a homolog. In panels II and III, bands D are the VP21 homologs from both viruses, bands E are the uncleaved forms of the BHV-1 UL26.5 protein, and bands F are the two VP22a homologs.

UL26-encoded protein (missing 33 N-terminal amino acids) expressed as a hexahistidine fusion in *Escherichia coli*. Figure 3 shows a Western blot of Sf9 cells infected with the recombinant baculoviruses expressing the indicated BHV-1 proteins. The proteolytic cleavage pattern of the UL26 protein was similar to that previously reported for the HSV-1 homolog, as shown in lane 26. Two faint protein bands migrating at approximately 71 and 68 kDa probably represent the full-length and M-cleaved UL26 protein fragments, respectively, while the proteins migrating at approximately 42 and 32 kDa result from both M and R cleavage (homologs of the HSV-1 VP21 and VP24 proteins, respectively). Protein bands of comparable sizes were seen in BHV-1 infected-cell proteins (Fig. 3, BHV lanes 8, 12, and 16).

The apparent uncleaved molecular mass of the baculovirus-expressed BHV-1 UL26.5, shown in lane 26.5, was approximately 38 kDa. Lane 26,26.5 (coinfection with both the UL26 and UL26.5 baculovirus clones) contained an abundant amount of an ~36-kDa protein band (the putative homolog of the HSV-1 VP22a), suggesting that the UL26 proteinase cleaved the UL26.5 protein. The cleavage patterns of the native and baculovirus-expressed UL26.5 proteins were identical (compare the 38- and 36-kDa protein bands in lane 26,26.5 and the BHV lanes), suggesting that we correctly predicted the initiation codon of the BHV-1 UL26.5 protein.

Protein bands comigrating with the putative digested and undigested forms of the BHV-1 UL26.5 protein were consistently observed with the baculovirus clone containing the complete UL26 open reading frame (lane 26). These protein bands were variable in quantity from infection to infection relative to the VP21 and VP24 protein bands. Northern (RNA) analysis of total RNA verified that the only transcript hybridizing to a UL26 DNA probe in the recombinant baculovirus expressing UL26 was the full-size transcript we expected. Therefore, either a small amount of the UL26.5 protein was being produced as a result of internal initiation from the full-size UL26 transcript in baculovirus or else the comigrating immunoreactive protein bands were specific proteolytic breakdown products of the full-size UL26 protein. In addition, we determined that the BHV-1 proteinase could cleave both the R and M sites in baculovirus-expressed HSV-1 UL26-encoded proteins and that the HSV-1-encoded UL26 proteinase could cleave the M site of the BHV-1-encoded UL26.5 protein (data not shown).

The BHV-1 UL26 and UL26.5 proteins complement in vitro HSV-1 capsid assembly. In order to examine the functionality of the BHV-1 UL26 and UL26.5 homologs, we substituted baculoviruses expressing the BHV-1 proteins for their HSV-1 counterparts in an in vitro capsid assembly system (38). In vitro capsid assembly was evaluated by thin-section transmission electron microscopy and by density gradient centrifugation of

capsids on sucrose gradients followed by Western immunoblotting (38). As shown in the thin-section electron micrographs in Fig. 4, substitution of the BHV-1 UL26 and UL26.5 homologs for the HSV-1 counterparts in this system resulted in formation of type B capsids (arrow in lower panel) that were indistinguishable from those formed with the HSV-1 UL26 and UL26.5 proteins (38). Inclusion of only the BHV-1 UL26.5 protein with no UL26 protein (the bottom panel in Fig. 4) resulted in the assembly of 125-nm B capsids that contained visible cores, identical to the result obtained when only the HSV-1 UL26.5 protein is included (38). Without either the BHV-1 UL26 or the UL26.5 protein, capsid assembly was not observed (data not shown).

Type B capsids formed by substituting the BHV-1 UL26 and/or UL26.5 proteins for the HSV-1 counterparts were purified on sucrose gradients, and the protein compositions of the purified capsids were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (Fig. 5). Figure 5I shows the Coomassie blue-stained gel. Figure 5II and III show Western blots of duplicate gels reacted with either the BHV-1 UL26 antiserum or monoclonal antibody MCA406, which recognizes an epitope in HSV-1 VP22A. Figure 5I (lane 1) shows the protein bands present in type B capsids formed with the seven HSV-1 proteins. The prominent proteins visible by SDS-PAGE are VP5, VP19C, a doublet consisting of VP23 and (HSV-1) VP22a (the band migrating with A in Fig. 5I, lane 1), and VP26. A nonherpesvirus protein migrating at approximately 42 kDa consistently copurified with type B capsids assembled in the baculovirus system (band BV). VP21 and VP22a were visible in the Western blot with the MCA406 monoclonal antibody (bands D and F in Fig. 5III, lane 1). Fig. 5I (lane 2) shows the proteins present in the purified B capsids in which the BHV-1 UL26 and UL26.5 proteins were substituted for the respective HSV-1 proteins. The expected HSV-1 protein bands were still present, and, as mentioned earlier, the BHV-1 homolog of VP22a (band B in Fig. 5I, lane 2, and band F in Fig. 5II, lane 2) migrated considerably more rapidly than the homologous HSV-1 VP22a protein (band A in Fig. 5I). This protein was also a prominent protein band in the B capsids purified from BHV-1-infected BT cells (Fig. 5I and II, lanes 4). The uncleaved version of the BHV-1 UL26.5 protein in the absence of either UL26 protein also facilitated the assembly of B capsids and was present in the resultant capsid bands on sucrose gradients (Fig. 5I, lane 3, band A, and Fig. 5II, lane 3, band E).

Previous studies have shown that the C-terminal 25 residues of the HSV-1 UL26.5 protein, which are cleaved off during or subsequent to capsid assembly, are absolutely required for the assembly of 125-nm HSV-1 type B capsids (14, 18, 37). In addition, the C-terminal 25 amino acids appear to be directly involved in the interaction of the scaffold with VP5 in the capsid shell (37). This region is not particularly conserved between HSV-1 and BHV-1, and the BHV-1 C-terminal cleaved region is, in fact, four residues longer (Fig. 2). Nonetheless, the final 12 residues of the BHV-1 and HSV-1 proteins, as well as those from other herpesviruses, including HSV-2 (34), equine herpesvirus 1 (36), and varicella-zoster virus (7), contain a semiconserved motif, consisting of A-X-X-F-V/A-X-Q-M-M-X-X-R. The F-V/A and Q-M-M residues are also present in infectious laryngotracheitis virus (13). These conserved residues therefore may be involved in the interaction of the scaffold with the capsid shell. The human cytomegalovirus assemblin protein and the homologs in simian cytomegalovirus, herpesvirus saimiri, and Epstein-Barr virus do not contain this semiconserved C-terminal domain (1, 3, 40).

We conclude that the BHV-1 homologs of the HSV-1 VP22a, VP24, and VP21 proteins can substitute for the HSV-1 proteins in an in vitro capsid assembly system and that they are present in the resultant type B capsid particles. To our knowledge, this is the first description of the successful assembly of hybrid type B herpesvirus capsids. These results confirm a common functionality of the proteins encoded by the UL26 and UL26.5 genes for these two herpesviruses. The common functionality of the HSV-1 and BHV-1 scaffold proteins, despite their primary sequence divergence, should allow for the rational identification and mutational analysis of conserved residues to examine their possible contribution to scaffold formation and capsid assembly.

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