JOURNAL OF VIROLOGY, Nov. 1997, p. 8886–8892 Vol. 71, No. 11
0022-538X/97/$04.00+0

Functional Complementation of UL3.5-Negative Pseudorabies Virus by the Bovine Herpesvirus 1 UL3.5 Homolog

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Received 4 June 1997/Accepted 28 July 1997

The UL3.5 gene is positionally conserved but highly variable in size and sequence in different members of the *Alphaherpesvirinae* and is absent from herpes simplex virus genomes. We have shown previously that the pseudorabies virus (PrV) UL3.5 gene encodes a nonstructural protein which is required for secondary envelopment of intracytoplasmic virus particles in the *trans*-Golgi region. In the absence of UL3.5 protein, naked nucleocapsids accumulate in the cytoplasm, release of infectious virions is drastically reduced, and plaque formation in cell culture is inhibited (W. Fuchs, B. G. Klupp, H. Granzow, H.-J. Rziha, and T. C. Mettenleiter, J. Virol. 70:3517–3527, 1996). To assay functional complementation by a heterologous herpesviral UL3.5 protein, the UL3.5 gene of bovine herpesvirus 1 (BHV-1) was inserted at two different sites within the genome of UL3.5-negative PrV. In cells infected with the PrV recombinants the BHV-1 UL3.5 gene product was identified as a 17-kDa protein which was identical in size to the UL3.5 protein detected in BHV-1-infected cells. Expression of BHV-1 UL3.5 compensated for the lack of PrV UL3.5, resulting in a ca. 1,000-fold increase in virus titer and restoration of plaque formation in cell culture. Also, the intracellular block in viral egress was resolved by the BHV-1 UL3.5 gene. We conclude that the UL3.5 proteins of PrV and BHV-1 are functionally related and are involved in a common step in the egress of alphaherpesviruses.

The *Alphaherpesvirinae* subfamily of the *Herpesviridae* encompasses a large number of mammalian and avian pathogens (27). Their DNA genomes exhibit great similarities in general organization. They consist of two unique regions, of which only the shorter one (group D) or both (group E) are flanked by inverted repeat sequences resulting in isomerization of the intervening genome parts (27). Within the respective genomic regions, gene arrangement is largely collinear. However, differences were also detected.

At one end of the invertible unique long region of the herpes simplex virus type 1 (HSV-1) and HSV-2 genomes the genes for glycoprotein L (gL) (UL1), uracil-DNA glycosylase (UL2), and a component of the viral helicase primase complex (UL5) were found to be separated by two genes (UL3 and UL4) encoding proteins of unknown function (20, 21, 28). Homologs of these genes were localized in a similar arrangement near the right ends of the unique long regions of the group D genomes of varicella-zoster virus (VZV), equine herpesvirus 1 (EHV-1), pseudorabies virus (PrV), bovine herpesvirus 1 (BHV-1), and infectious laryngotracheitis virus (ILTV) (3, 4, 5, 7, 15, 29, 31). However, in these virus genomes an additional open reading frame (ORF) located downstream of UL3 was detected. This ORF, which was designated gene 57 (VZV), gene 59 (EHV-1), or UL3.5 (PrV, BHV-1, and ILTV), is absent from HSV-1 and HSV-2. Thus, the UL3.5 homologs belong to the very few genes which are not conserved throughout the *Alphaherpesvirinae*. These genes are of particular interest since they point to specific differences between the different alphaherpesviruses and may, at least in part, explain their different biology. Sizes of deduced UL3.5 proteins range from 71 amino acids (aa) in VZV to 224 aa in PrV, and overall amino acid sequence homology is limited. However, all predicted UL3.5 gene products share a highly basic character and a short stretch of highly conserved amino acids close to their amino termini (7), which could imply a common conserved function.

Recently, we identified in PrV the first herpesviral UL3.5 protein as a 30-kDa nonstructural protein which is located in the cytoplasm of infected cells (8). In vitro studies performed with UL3.5-negative PrV mutants revealed a substantial growth defect, which could be compensated for in *trans* by propagation on cells expressing the PrV UL3.5 protein. Electron microscopic analyses of infected cells demonstrated that the observed defect was caused by an inhibition of envelopment of intracytoplasmic nucleocapsids in the *trans*-Golgi region (8). However, a PrV mutant lacking the 3′ terminus of the UL3.5 gene and expressing a truncated protein exhibited a wild-type (wt)-like phenotype in cell culture (8). This finding indicated that functional domains are located in the N-terminal part of the PrV UL3.5 protein, which is conserved within the deduced UL3.5 gene products of other alphaherpesviruses.

To analyze whether the function of the UL3.5 protein in virus maturation and egress is specific for PrV or represents a common property of the corresponding gene products of other alphaherpesviruses, we assessed the ability of the UL3.5 gene of BHV-1 to compensate for the lack of UL3.5 in PrV. The BHV-1 gene was chosen for these studies since phylogenetic studies indicate a close relationship between PrV and BHV-1 (22). In agreement with this notion, of all deduced UL3.5 proteins BHV-1 UL3.5 (15) exhibits the highest level of amino acid homology to the PrV protein, with 26% identical amino acids as determined by the Genetics Computer Group sequence analysis package program GAP and the BLOSUM62 scoring matrix (6, 12).

In the past, several BHV-1 proteins, such as gB, gE, and gI, were demonstrated to complement the respective PrV mutants. Nonessential gE and gI of BHV-1 inserted into PrV mutants lacking the homologous PrV genes were shown to restore in vivo virulence (17). The essential BHV-1 gB was also

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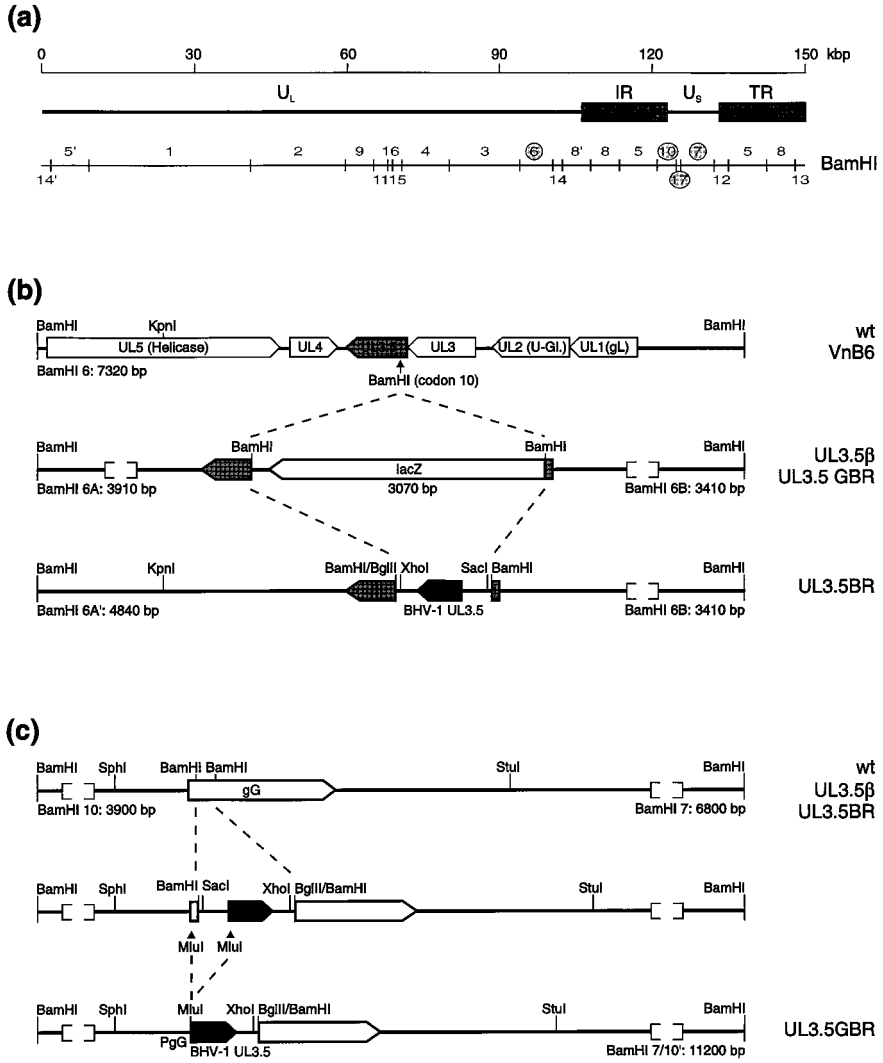

FIG. 1. Construction of PrV UL3.5 mutants. (a) The organization of the PrV genome and the *Bam*HI restriction map are shown. Relevant *Bam*HI fragments 6, 10, 17, and 7 are highlighted. (b) *Bam*HI fragment 6 of the wt PrV genome contains six ORFs (UL1 to UL5 and UL3.5). This fragment was used to establish the PrV UL3.5-expressing cell line VnB6 (8). PrV-UL3.5β carries the *E. coli lacZ* gene inserted into a newly created *Bam*HI site at codon 10 of the UL3.5 gene. This insertion is maintained in PrV-UL3.5GBR. In PrV-UL3.5BR *lacZ* was replaced with the UL3.5 gene of BHV-1 contained in a 920-bp *Sac*I-*Xho*I fragment, which was recloned as a *Bam*HI-*Bgl*II fragment. (c) The nonessential gG gene located within *Bam*HI fragments 10, 17, and 7 of the wt PrV genome is unaltered in PrV-UL3.5β and PrV-UL3.5BR. In PrV-UL3.5GBR, the UL3.5 gene of BHV-1 was fused to the gG gene promoter (PgG) at two *Mlu*I sites, which had been created behind the initiation codons of both the gG and UL3.5 ORFs. The expected sizes of the *Bam*HI fragments of wt and mutant PrV DNA are noted below the bars, and brackets indicate that the fragments were not drawn to scale.

found to complement replication-deficient gB$^-$ PrV in *trans* and in *cis* (18, 26).

Our previously described UL3.5-negative PrV mutants (8) were generated by heterologous *cis* complementation of PrV from which the gB gene was deleted (25) with the gB gene of BHV-1. Since in these PrV recombinants the foreign gB gene disrupting the PrV UL3.5 ORF is essential for virus replication, rescue, as well as further manipulations of the UL3.5 gene, is difficult. Therefore, we constructed a novel virus mutant by insertion of the *Escherichia coli lacZ* gene into the 224-codon UL3.5 ORF of wt PrV strain Ka (14). For this approach, plasmid pWF-41B (8) was used; this plasmid contains a 6-kbp *Kpn*I-*Bam*HI subfragment of PrV *Bam*HI fragment 6 (Fig. 1a) with a unique *Bam*HI site at codon 10 of the UL3.5 ORF introduced by oligonucleotide-directed mutagenesis (Fig. 1b). At this site the *lacZ* ORF was inserted as a 3,072-bp *Bam*HI fragment derived from plasmid pMC1871 (Pharmacia, Freiburg, Germany) and fused in frame to the 5′ end of the PrV UL3.5 ORF (Fig. 1b). This insertion should prevent expression of the 3′-terminal 214 codons of the UL3.5 ORF, since no possible initiation codon is present downstream from the stop codon of *lacZ*. After cotransfection of PrV UL3.5-expressing cell line VnB6 (8) with resulting plasmid pWF-41Z and wt PrV DNA, blue virus plaques were detected by Bluo-Gal staining (23) and purified to homogeneity, and a single plaque isolate, designated PrV-UL3.5β (Fig. 1b), was further analyzed (see below).

To test for complementation of PrV UL3.5 function by the BHV-1 homolog, we inserted the 126-codon UL3.5 ORF of BHV-1 into the genome of PrV-UL3.5β at two different sites. To this end, the UL3.5 gene of BHV-1, including its promoter and polyadenylation signal, was isolated as a 922-bp *Sac*I-*Xho*I subfragment from genomic *Hin*dIII fragment K of the wt BHV-1 strain Schönböken (obtained from G. Keil, Insel

Riems, Germany). The isolated subfragment was inserted into plasmid pSP73 (Promega, Madison, Wis.) which had been doubly digested with *Bgl*II and *Sac*I, after Klenow fill-in of the noncompatible *Bgl*II and *Xho*I ends, which leads to restoration of both restriction sites in the resulting plasmid, pSP-BHKSX.

For the generation of a PrV recombinant which carries the BHV-1 UL3.5 gene under the control of its own promoter instead of *lacZ*, the insert of pSP-BHKSX was recloned as a *Bgl*II-*Bam*HI fragment into the *Bam*HI site of pWF-41B so that the BHV-1 UL3.5 gene was in parallel transcriptional orientation to the disrupted PrV UL3.5 ORF (Fig. 1b, UL3.5BR). The resulting plasmid was cotransfected with PrV-UL3.5β DNA into normal Vero cells. Since parental PrV-UL3.5β is unable to form plaques on Vero cells (see below), the appearance of infectious progeny indicated the insertion of the BHV-1 UL3.5 gene into the PrV genome and already pointed to the propensity of the BHV-1 gene to compensate for the loss of PrV UL3.5 function. Furthermore, under a Bluo-Gal overlay progeny virus plaques remained colorless as a consequence of the *lacZ* gene substitution. One single plaque isolate, PrV-UL3.5BR, was further analyzed.

To obtain a second, independent recombinant which, however, still expresses β-galactosidase, the *lacZ* insertion within the UL3.5 gene of PrV-UL3.5β was maintained and the BHV-1 UL3.5 gene was inserted into the cloned gG gene derived from the unique short region of the PrV genome (Fig. 1c). Deletion of the gG gene has no detectable effects on PrV replication either in vitro or in vivo (1, 24) and thus represents a convenient insertion site. In addition, the PrV gG gene promoter proved to be suitable for high-level expression of foreign genes (23). Therefore, the BHV-1 UL3.5 ORF was fused to the PrV gG promoter. A 4,012-bp *Sph*I-*Stu*I fragment derived from the unique short region of wt PrV was cloned into phagemid vector pBS$^-$ (Stratagene, Heidelberg, Germany) that had been doubly digested with *Sph*I and *Sma*I. A 196-bp *Bam*HI fragment located within the 5′-terminal part of the PrV gG gene ORF was replaced with the *Bgl*II-*Bam*HI insert of pBS-BHKSX, which encompasses the BHV-1 UL3.5 gene (Fig. 1c). Two *Mlu*I sites were introduced by oligonucleotide-directed mutagenesis (19) at the third codons of the PrV gG and BHV-1 UL3.5 genes. In addition, the second codon of the PrV gG ORF was altered to encode alanine, which is the second amino acid of the BHV-1 UL3.5 protein. After selection of recombinant plasmids the authentic BHV-1 UL3.5 ORF was fused to the PrV gG gene promoter by deletion of a 400-bp *Mlu*I fragment (Fig. 1c, UL3.5GBR). The plasmid obtained, pBS-SMX, was cotransfected with PrV-UL3.5β DNA, and virus progeny was screened by blue plaque assay on Vero cells. Since the desired virus recombinants formed blue plaques, a single blue plaque isolate, designated PrV-UL3.5GBR, was further analyzed. Correct insertion of the foreign gene and the genetic homogeneity of the PrV mutants were verified by Southern blot hybridization of *Bam*HI-digested virion DNA (data not shown). The sizes of all observed restriction fragments were as expected (Fig. 1).

For detection of the BHV-1 UL3.5 protein a monospecific antiserum was prepared after expression of the respective gene in *E. coli*. A 514-bp *Mlu*I-*Xho*I fragment was isolated from plasmid pBS-SMX (see above) after fill-in of the single-strand overhang at the *Mlu*I site by Klenow polymerase and was inserted into expression vector pGEX-4T-2 (Pharmacia) that had been doubly digested with *Sma*I and *Xho*I. The resulting plasmid, pGEX-UL3.5B, contains codons 3 to 126 of the BHV-1 UL3.5 gene 3′-terminally fused to the glutathione *S*-transferase gene. After transformation of *E. coli* DH5α, expression was induced as described previously (8) and bacterial

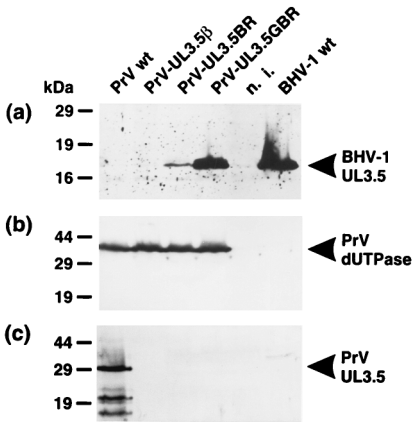

FIG. 2. Detection of the BHV-1 UL3.5 protein. Lysates of MDBK cells harvested 14 h after infection with wt PrV, PrV-UL3.5β, PrV-UL3.5BR, PrV-UL3.5GBR, or wt BHV-1, as well as lysates of noninfected cells (n. i.) were separated in SDS–14% polyacrylamide gels and transferred to nitrocellulose membranes. Western blots were reacted with rabbit antisera directed against a glutathione *S*-transferase–BHV-1 UL3.5 fusion protein (a), the PrV UL50 gene product, dUTPase (b), or the PrV UL3.5 protein (c). The locations of molecular mass markers are indicated on the left.

proteins were separated on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. The gels were stained in 0.2% Serva blue R (Serva, Heidelberg, Germany)–20% methanol–0.5% acetic acid for 20 min and destained in 30% methanol. The 40-kDa fusion protein was excised and electroeluted into centricon concentrators (Centrilutor; Amicon, Witten, Germany) in 25 mM Tris–200 mM glycine–0.1% SDS–10% methanol for 6 h at 200 V. The eluate was concentrated, washed twice with an equal volume of phosphate-buffered saline, and concentrated again. A rabbit was immunized four times at 2-week intervals by intramuscular injection of 100 μg of the purified fusion protein emulsified in mineral oil. Sera collected before and after immunization were used for Western blot analyses of infected bovine kidney (MDBK) cell lysates. These cells were chosen because they permit efficient replication of PrV and BHV-1. Cells were infected with either wt BHV-1, PrV-UL3.5β, PrV-UL3.5BR, PrV-UL3.5GBR, or wt PrV at a multiplicity of infection (MOI) of 5. After 16 h at 37°C the cells were scraped into the medium, collected by centrifugation, and lysed. Protein of ca. 10^5 cells per lane was separated by SDS-polyacrylamide gel electrophoresis, electrotransferred onto nitrocellulose filters, and reacted with rabbit antisera. The binding of peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany) was visualized by luminescence (ECL Western blot detection system; Amersham, Braunschweig, Germany) and recorded on X-ray films.

In BHV-1-infected cells, the BHV-1 UL3.5 protein-specific antiserum recognized a protein with an apparent molecular mass of 17 kDa (Fig. 2a), which was not detected by the respective preimmune serum (data not shown) and which was absent from noninfected cells (Fig. 2a, lane n. i.). Although the predicted molecular mass of the BHV-1 UL3.5 protein is only 13.4 kDa, a similar aberrant electrophoretic mobility of this protein was also found by others (16). The difference between the calculated and observed molecular masses might be a consequence of posttranslational modifications. On the other hand, the unusual amino acid composition of the BHV-1 UL3.5 protein, with an isoelectric point of 12.3, might cause aberrant electrophoretic mobility. It is noteworthy that the PrV UL3.5 protein, with a similar basic amino acid composition,

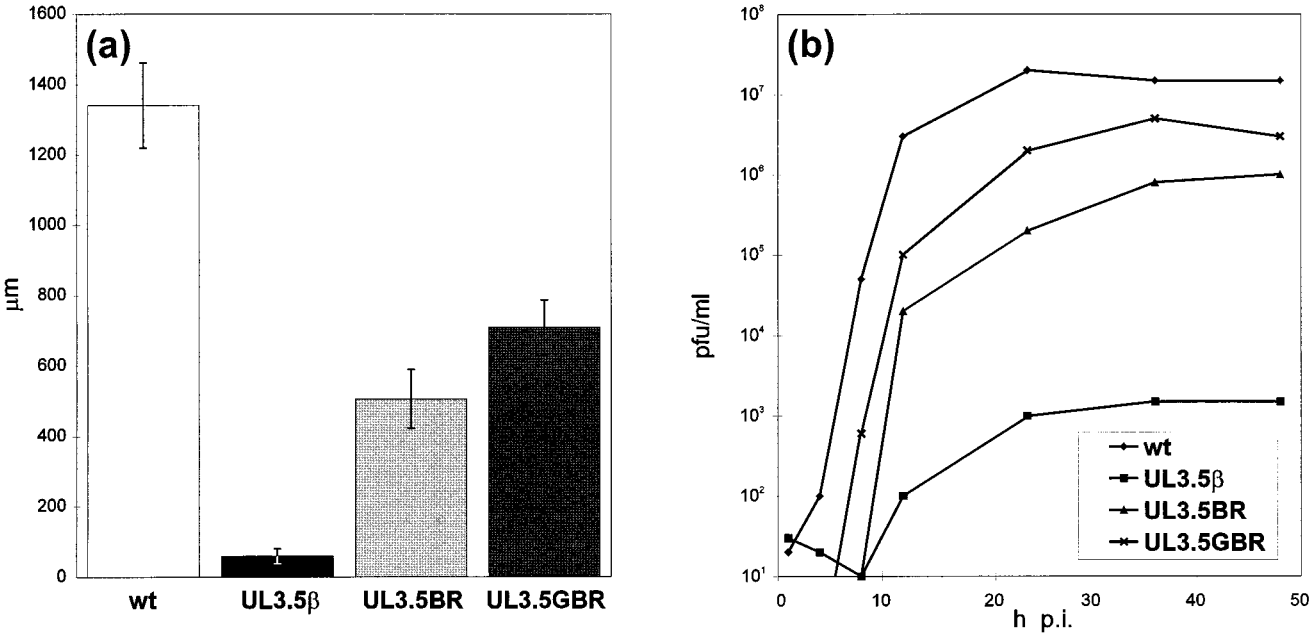

FIG. 3. Growth properties of PrV UL3.5 mutants. (a) Plaque sizes. Diameters of thirty plaques each of wt PrV, PrV-UL3.5BR, and PrV-UL3.5GBR were determined on monolayers of Vero cells after staining with crystal violet 3 days after infection. Sizes of infected foci produced by PrV-UL3.5β were determined after X-Gal staining. Average sizes are shown as bars, and standard deviations are indicated by vertical lines. (b) One-step growth kinetics. Vero cells were infected with either wt PrV, PrV-UL3.5β, PrV-UL3.5BR, or PrV-UL3.5GBR at an MOI of 5. After 1 h at 4°C the inoculum was replaced by prewarmed medium, and cultures were incubated at 37°C. After an additional hour, the remaining extracellular virus was inactivated by low-pH treatment. Immediately thereafter, and after the indicated times of incubation at 37°C, total virus titers were determined on complementing VnB6 cells.

also migrates with an apparent molecular mass approximately 6 kDa higher than that predicted (8).

As expected, the 17-kDa BHV-1 UL3.5 protein was not detected in wt PrV- or PrV-UL3.5β-infected cells (Fig. 2a). In contrast, the 17-kDa protein was present in cells infected with chimeric PrV-UL3.5BR or PrV-UL3.5GBR. Signal strength indicated a significantly lower expression level of the BHV-1 UL3.5 protein in PrV-UL3.5BR- than in PrV-UL3.5GBR-infected cells. Obviously, this is not due to different infection conditions, since similar amounts of the 33-kDa viral dUTPase originating from the UL50 gene (13) were detectable in all PrV-infected cell lysates (Fig. 2b). The previously described 30-kDa UL3.5 protein of PrV and its putative degradation products (8) were found only in cells infected with wild-type PrV but not in any PrV UL3.5 mutant-infected cell lysate (Fig. 2c).

The phenotypic effects of the UL3.5 gene substitution in PrV-UL3.5BR and -UL3.5GBR were monitored by in vitro replication studies. As shown before, the UL3.5 gene of PrV plays an important role in virus replication in vitro (8), a role which was confirmed by observation of the dependence of novel mutant PrV-UL3.5β on complementing cells for productive replication. On normal Vero cells, PrV-UL3.5β is unable to produce plaques, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining at various times after infection revealed only single, or small foci of, blue infected cells (data not shown). In contrast, the BHV-1 UL3.5-expressing mutants PrV-UL3.5BR and -UL3.5GBR regained plaque-forming capacity on Vero cells. For exact quantitation, infected Vero and PrV UL3.5-expressing VnB6 cell monolayers were overlaid with semisolid medium containing 0.8% methylcellulose, fixed 3 days after infection, and stained with crystal violet, since wt PrV and mutant PrV-UL3.5BR do not express β-galactosidase. A calculation of the average diameters of 30 randomly selected plaques of each virus showed that on complementing VnB6 cells, the plaques formed by mutants PrV-UL3.5β, PrV-UL3.5BR, and PrV-UL3.5GBR were nearly of wt size (data not shown). On Vero cells no plaques of PrV-UL3.5β were detectable after crystal violet staining, and diameters of infected foci (Fig. 3a) had to be determined on X-Gal-stained plates. In contrast, plaques of both PrV mutants containing the BHV-1 UL3.5 gene were clearly visible and their average diameters reached 40% (for PrV-UL3.5BR) or 60% (PrV-UL3.5GBR) of wt size (Fig. 3a). Plaque assays performed on MDBK cells revealed similar effects of PrV UL3.5 gene deletion and heterologous *cis* complementation by the BHV-1 UL3.5 gene (data not shown).

One-step growth kinetics of wt PrV, PrV-UL3.5β, PrV-UL3.5BR, and PrV-UL3.5GBR were also monitored. Progeny virus titers in total cell lysates harvested at the indicated times (Fig. 3b) after infection at an MOI of 5 were determined on VnB6 cells to provide equal growth conditions for all PrV mutants. Whereas on PrV UL3.5-expressing VnB6 cells the growth kinetics of all viruses were similar (data not shown), the final titers on Vero cells differed significantly among the PrV mutants (Fig. 3b). The yield of PrV-UL3.5β was decreased nearly 10,000-fold compared to that of wt PrV. This finding is in good agreement with results obtained with a different UL3.5-negative PrV mutant (8). Heterologous rescue of the UL3.5 gene in PrV-UL3.5BR and PrV-UL3.5GBR increased final virus titers on Vero cells ca. 1,000-fold compared to those of PrV-UL3.5β. Again, the observed complementation appears to be more pronounced in PrV-UL3.5GBR than in PrV-UL3.5BR, correlating with the different expression levels of the BHV-1 UL3.5 protein (Fig. 2a). These findings strongly indicate that the complementation of the PrV UL3.5 gene defect observed with PrV-UL3.5BR and -UL3.5GBR is indeed mediated by the UL3.5 gene product of BHV-1. However,

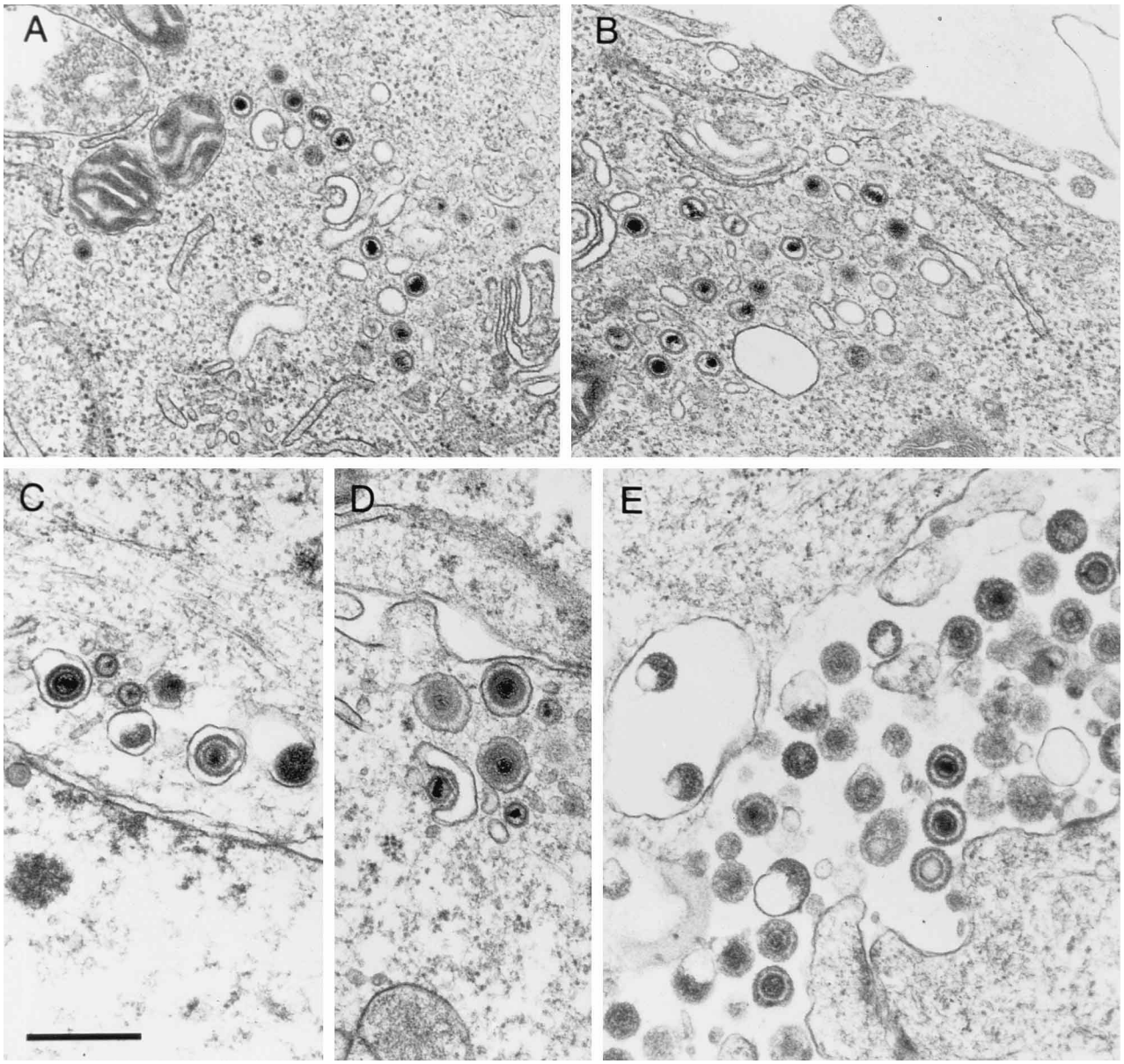

FIG. 4. Electron microscopy of infected Vero cells. Vero cells infected at an MOI of 5 with PrV-UL3.5β (A and B) or PrV-UL3.5BR (C, D, and E) were fixed at 14 h postinfection and stained with uranyl acetate. In cells infected with PrV-UL3.5β, an analysis of ultrathin sections reproducibly revealed an accumulation of naked nucleocapsids in the cytoplasm (A). Neither secondary envelopment in the Golgi region nor release of mature virions (B) was observed. In contrast, insertion of the UL3.5 gene of BHV-1 in PrV-UL3.5BR restored wt-like virus maturation and egress. The budding of nucleocapsids into Golgi vesicles (C and D), as well as the release of enveloped virions from the cells (E), can be detected. Bar = 500 nm.

although the effects of BHV-1 UL3.5 gene insertion on both plaque formation and one-step growth of UL3.5-negative PrV are striking, complete compensation of the growth defects to wt levels was not achieved.

Previously we showed that the growth deficiency of UL3.5-negative PrV correlates with an inhibition of secondary envelopment of virions in the cytoplasm of infected cells (8). Ultrastructural analyses performed 14 h after infection of Vero cells with PrV-UL3.5β also showed an accumulation of naked nucleocapsids in the Golgi regions of the cells (Fig. 4A), which confirms our earlier results. Neither budding of capsids into Golgi region-derived vesicles nor the presence of extracellular virions (Fig. 4B) could be detected. In Vero cells infected under similar conditions with PrV-UL3.5BR, however, budding of virus particles into cytoplasmic vesicles was readily detectable (Fig. 4C and D) and release of mature virions occurred (Fig. 4E). Thus, maturation and egress of PrV-UL3.5BR were indistinguishable at any stage from those of wt PrV (11) or of UL3.5-negative PrV propagated on PrV UL3.5-expressing cells (8). Similar results were obtained with PrV-UL3.5GBR (data not shown).

In summary, our results demonstrate that the UL3.5 proteins of PrV and BHV-1 are functionally related and that a PrV UL3.5 gene mutant could be complemented in *cis* by insertion of the heterologous UL3.5 gene of BHV-1. Since complementation occurred independent of the site of BHV-1 UL3.5 gene insertion in two different chimeric viruses, compensation of the UL3.5 defect by adventitious mutations in

other areas of the PrV genome is highly unlikely. However, additional studies are required to elucidate whether the functions of the UL3.5 homologs during virus replication are indeed identical. This question is of particular interest, since the observed complementation of UL3.5-negative PrV by BHV-1 UL3.5 does not result in wt-like growth properties. On non-complementing cells plaques of PrV-UL3.5BR and PrV-UL3.5GBR had only half the size of those of wt PrV and virus titers were ca. 10-fold lower than that of wt PrV. In contrast, one-step growth of both PrV recombinants as well as of UL3.5-negative PrV was almost completely restored to wt levels on *trans*-complementing PrV UL3.5-expressing cells (data not shown). One possible reason might be a weaker interaction of the heterologous UL3.5 protein with other viral factors required for its function, correlating with the limited (26%) amino acid sequence identity between the BHV-1 and PrV UL3.5 gene products (4, 15).

Another relevant factor may be the expression level of the BHV-1 UL3.5 protein. At 16 h after infection the 17-kDa UL3.5 protein of BHV-1 could be detected in cells infected with either of the PrV recombinants. However, signal strength was considerably lower for cells infected with PrV-UL3.5BR than for cells infected with PrV-UL3.5GBR, indicating a higher expression level of the BHV-1 UL3.5 gene from the PrV gG gene promoter than from its own promoter. Remarkably, the larger amount of BHV-1 UL3.5 protein in PrV-UL3.5GBR-infected cells correlates with slightly increased plaque size and virus titer compared to those for PrV-UL3.5BR infected cells. The BHV-1 UL3.5 protein was not detectable in Western blots earlier than 12 h after infection of cells with either the PrV recombinants or wt BHV-1 (data not shown), whereas the PrV UL3.5 protein was found as early as 4 h after infection (8). Although this discrepancy might partly be due to the different affinities of antisera, RNA analyses also indicated a less abundant and more delayed expression of the BHV-1 gene than of the PrV UL3.5 gene (4, 15). This difference in expression kinetics of the two UL3.5 proteins could also explain the reduced growth of chimeric UL3.5 PrV mutants.

The functional similarity of the UL3.5 proteins of PrV and BHV-1 reported in this study, together with the presence of positionally conserved and structurally related genes in other mammalian and avian alphaherpesviruses, suggests that members of this gene family might contribute to the same general step of alphaherpesvirus maturation and egress. However, HSV apparently lacks a UL3.5 gene (20, 21) but nevertheless is efficiently released from host cells. The mechanism of herpesvirus maturation and egress is a matter of continuing debate. One model, originally developed for PrV (32), proposed primary envelopment of nucleocapsids at the inner nuclear membrane followed by de-envelopment at the outer nuclear membrane or the adjoining endoplasmatic reticulum, a secondary envelopment of cytoplasmic nucleocapsids at the *trans*-Golgi region, and release of mature particles from transport vesicles into the extracellular space. This model was confirmed by ultrastructural analyses of cells infected with wt PrV (11) and PrV mutants blocked at different stages of viral egress (8, 9). A similar model was also proposed for VZV (10). In contrast, for HSV-1 a direct transit of enveloped virions from the perinuclear space through the endoplasmic reticulum to the *trans*-Golgi region was proposed (30). This direct pathway would explain the absence of a UL3.5 protein, which is required for mediating secondary envelopment. However, recent studies provided strong evidence that HSV-1 also undergoes re-envelopment at the Golgi apparatus (2), which then has to proceed independent of UL3.5 function. For a more profound understanding of the general role of UL3.5 proteins during virus maturation and egress, functional investigations of other alphaherpesviral UL3.5 proteins will be required. It might also be interesting to insert a UL3.5 gene into the HSV genome and monitor the effects on the morphogenesis of HSV virions.

This study was supported by a grant from the Deutsche Forschungsgemeinschaft (Me 854/3).

We thank Günther M. Keil for providing BHV-1 virions and cloned DNA fragments, Alice Jöns for the PrV UL50 antiserum, and Egbert Mundt for help with the preparation of the BHV-1 UL3.5-specific antiserum. The technical assistance of C. Ehrlich and C. Möller is greatly appreciated.

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