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Identification and Characterization of the Pseudorabies Virus UL3.5 Protein, Which Is Involved in Virus Egress

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Alphaherpesvirus genomes exhibit a generally collinear gene arrangement, and most of their genes are conserved among the different members of the subfamily. Among the exceptions is the UL3.5 gene of pseudorabies virus (PrV) for which positional homologs have been detected in the genomes of varicella-zoster virus, equine herpesvirus 1, and bovine herpesvirus 1 but not in the genomes of herpes simplex virus types 1 and 2. To identify and characterize the predicted 224 amino acid UL3.5 protein of PrV, a rabbit antiserum was prepared against a UL3.5 fusion protein expressed in *Escherichia coli*. In Western blot (immunoblot) analyses the antiserum detected a 30-kDa protein in the cytoplasm of PrV infected cells which was absent from purified virions. For functional analysis, UL3.5-expressing cell lines were established and virus mutants were isolated after the rescue of defective, glycoprotein B-negative PrV by insertion of the complementing glycoprotein B-encoding gene of bovine herpesvirus 1 at two sites within the UL3.5 locus. A PrV mutant carrying the insertion at codon 159 and expressing a truncated UL3.5 protein was still capable of efficient productive replication in noncomplementing cells. In contrast, a PrV mutant carrying the insertion at codon 10 of the UL3.5 gene did not express detectable UL3.5 protein and exhibited a dramatic growth deficiency on noncomplementing cells with regard to plaque formation and one-step replication. Electron microscopical studies showed an accumulation of unenveloped capsids in the vicinity of the Golgi apparatus. This defect could be compensated by propagation on complementing UL3.5-expressing cell lines. Our results thus demonstrate that the PrV UL3.5 gene encodes a nonstructural protein which plays an important role in virus replication, presumably during virus egress. The functionally relevant domains appear to be located within the N-terminal part of the UL3.5 protein which also comprises the region exhibiting the highest level of homology between the predicted UL3.5 homologous proteins of other alphaherpesviruses.

Pseudorabies virus (PrV), also designated suid herpesvirus 1, is the causative agent of Aujeszky's disease, which inflicts severe losses in pig husbandry. It is also highly pathogenic for most other mammals except higher primates, including humans (29, 43). Together with other animal pathogens such as bovine herpesvirus 1 (BHV-1) and equine herpesvirus 1 (EHV-1) as well as the human varicella-zoster virus (VZV) and herpes simplex virus type 1 (HSV-1) and HSV-2, PrV is grouped into the *Alphaherpesvirinae* subfamily of the *Herpesviridae* (33). The PrV genome consists of a double-stranded DNA of approximately 150 kbp. It possesses characteristics of a class D herpesviral genome consisting of a unique long (U_L) and an invertible unique short (U_S) region which is flanked by inverted repeat sequences (5). Although at present only approximately 60% of the DNA sequence of the PrV genome is known (29), it is clear that gene arrangement is mostly collinear to those found in the completely sequenced genomes of VZV (10), HSV-1 (27), and EHV-1 (36), except for a large inversion affecting a gene cluster within the U_L region (6).

Despite the overall similarity in gene organization of alphaherpesviruses, differences were observed. These nonconserved regions are particularly interesting since they might contribute to distinct host tropism and pathogenicity of each virus. In particular, the gene content in the unique short regions exhibits a certain degree of variability (9, 40). The US6 gene encoding the glycoprotein gD, for example, is absent in the VZV genome, although gD has been shown to be essential for virus replication in HSV-1, PrV, and BHV-1 (13, 24, 30, 31). Also, genes homologous to HSV-1 US11 and US12 have not been detected in PrV, VZV, or EHV-1. A second region of divergence is located at the left-hand termini of the U_L regions of VZV, EHV-1, and PrV, which each correspond to the right-hand terminus of the prototypic HSV-1 U_L region (3, 10, 27, 36). In contrast, the opposite end of the U_L region appears more highly conserved. This part of the PrV genome has been sequenced in strains Indiana-Funkhauser (Ind-FH) (11, 12) and Kaplan (Ka) (19, 37) and has been demonstrated to encompass open reading frames (ORFs) homologous to the UL1 (glycoprotein gL), UL2 (uracil-DNA glycosylase), UL3, UL4, and UL5 (helicase) genes of HSV-1. However, immediately downstream from the UL3 gene, an additional reading frame, designated UL3.5, which is absent in the HSV-1 or HSV-2 genomes (26, 27) was detected. In VZV, EHV-1, and BHV-1, similar additional ORFs with deduced translation products showing limited homology to the predicted amino acid sequence of the PrV UL3.5 protein were detected at homologous genomic locations (10, 18, 36). The PrV UL3.5 gene encodes deduced proteins of 220 or 224 amino acids (aa) in strains Ind-FH or Ka, respectively, and an abundant polyadenylated early-late 1-kb RNA was found to be transcribed from this region (11, 37).

So far, none of the alphaherpesvirus UL3.5 homologous proteins has been identified and functional analyses have not yet been performed. We attempted to address these questions for PrV after preparation of a monospecific antiserum directed

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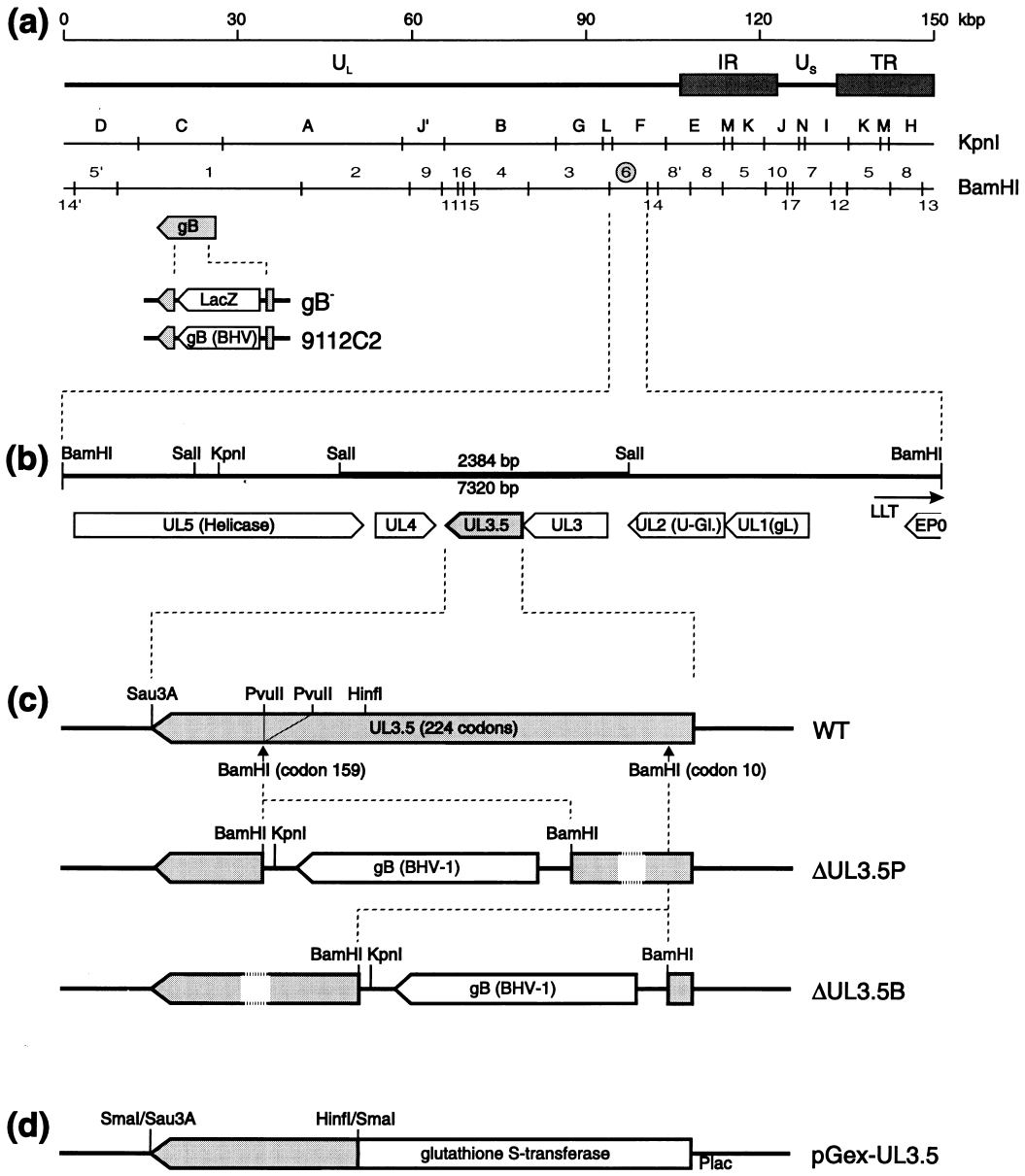

FIG. 1. Construction of PrV UL3.5 mutants and UL3.5-GST fusion protein. (a) Diagram of the PrV genome with *Bam*HI and *Kpn*I restriction fragment maps. The genome consists of a U_L and a U_S region which is flanked by inverted repeat sequences (an internal repeat [IR] and a terminal repeat [TR]). Genomic alterations introduced in gB$^-$ PrV and PrV 9112C2 are indicated. (b) Enlarged map of the 7.3-kbp *Bam*HI fragment 6. The locations of genes (open boxes) and designations of gene products (U-Gl., uracil-DNA glycosylase) are given, as is the start of the long latency transcript (LLT). Shown by a bold line is the 2.4-kbp *Sal*I subfragment used for establishing cell line VnS2.4. (c) Construction of PrV UL3.5 mutants. In plasmids which encompass the wild-type (WT) UL3.5 gene (shaded box) *Bam*HI sites were created by oligonucleotide-directed mutagenesis at codon 10 or by deletion of a *Pvu*II fragment and addition of *Bam*HI linkers at codon 159. Into these sites, the gB gene of BHV-1 was inserted, and the resulting recombinant plasmids were used to generate PrV mutants ΔUL3.5B and ΔUL3.5P, respectively, by stable heterologous *cis* complementation of gB$^-$ PrV. The 5′ part of the UL3.5 gene in ΔUL3.5P and the 3′ part of the UL3.5 gene in ΔUL3.5B are not drawn to scale. (d) Procaryotic expression of the UL3.5 gene. In plasmid pGEX-UL3.5 a *Hin*fI-*Sau*3A fragment encoding UL3.5 aa 136 to 224 was 3′-terminally fused to the GST gene. The fusion protein which was expressed under control of the bacterial *lacZ* promoter (Plac) was used for immunization.

against a bacterially expressed UL3.5 fusion protein and by construction and analysis of different PrV UL3.5 insertion mutants.

MATERIALS AND METHODS

Cells and viruses. In this study, a gB-negative PrV mutant (gB$^-$ PrV) derived from wild-type PrV Ka (17) by partial deletion of the gB gene and insertion of a *lacZ* expression cassette (32) was used. This virus mutant was propagated on PrV gB-expressing MDBK cells (MT-3; 32). PrV 9112C2 (22) carries the BHV-1 gB gene in the partially deleted PrV gB locus. Insertion of the BHV-1 gB gene restores replication competence in noncomplementing cells to the gB$^-$ PrV mutant. Wild-type PrV Ka and PrV 9112C2 were propagated on porcine kidney (PSEK) cells. Transfection experiments and plaque assays were performed on Vero cells. Cells were grown in Eagle's minimum essential medium supplemented with 5% fetal calf serum.

Construction of PrV UL3.5 mutants. A 2.4-kbp *Sal*I subfragment of genomic *Bam*HI fragment 6 (Fig. 1) was cloned into plasmid TN-77 (3). After cleavage with *Pvu*II, which removes codons 159 to 179 of the UL3.5 ORF, *Bam*HI linkers were added and a 3.3-kbp *Bam*HI fragment containing the BHV-1 gB gene (22) was inserted. The resulting plasmid was designated pHD-gB. Plasmid pWF41-gB was constructed by insertion of a 6-kbp *Kpn*I-*Bam*HI subfragment of *Bam*HI fragment 6 into phagemid pTZ19R (Pharmacia, Freiburg, Germany) followed by

inactivation of the *Bam*HI site by fill-in reaction with Klenow polymerase and religation. A unique *Bam*HI site was then created at codon 10 of the UL3.5 gene by oligonucleotide-directed mutagenesis, which served for insertion of the 3.3-kbp BHV-1 gB gene fragment. Mutant viruses were isolated after cotransfection of pHD-gB and genomic DNA of the gB$^-$ PrV mutant into normal Vero cells or after cotransfection of pWF41-gB and genomic DNA of the gB$^-$ PrV mutant into complementing UL3.5-expressing cells (see below).

Construction of UL3.5-expressing cell lines. For the construction of UL3.5-expressing cell lines, the 7.3-kbp genomic *Bam*HI fragment 6 of PrV Ka which encompasses the genes UL1 through UL5 (Fig. 1) was inserted into plasmid pSV2-neo (35). In a second approach, a 2.4-kbp *Sal*I subfragment of *Bam*HI fragment 6 containing only the genes UL3, UL3.5, and UL4 (Fig. 1) was inserted into pSV2-neo. Vero cells transfected with either plasmid by calcium phosphate coprecipitation (16) were selected in medium containing 700 μg of G418 (Geneticin; Sigma, Deisenhofen, Germany) per ml as described previously (32). Resistant cell clones were isolated and tested for their ability to allow replication of PrV-ΔUL3.5B (see below). Complementing cell lines were maintained in medium containing 500 μg of G418 per ml. Two cell lines designated VnB6, containing the complete *Bam*HI fragment 6, and VnS2.4, containing the 2.4-kbp *Sal*I subfragment, were further analyzed.

Procaryotic expression and preparation of antiserum. For procaryotic expression of the UL3.5 gene, a 267-bp *Hin*fI-*Sau*3A subfragment of *Bam*HI fragment 6 (Fig. 1) was inserted into expression vector pGEX-4T3 (Pharmacia). The resulting plasmid, pGEX-UL3.5, contains codons 136 to 224 of the UL3.5 gene 3′-terminally fused to the glutathione *S*-transferase (GST) gene. Correct insertion was confirmed by restriction analysis and DNA sequencing. *Escherichia coli* DH5α was transformed with plasmid pGEX-UL3.5 and grown in Luria Bertani medium supplemented with 100 μg of ampicillin per ml and 1% glucose at 37°C to an optical density at 600 nm of approximately 1.0. Bacteria were then sedimented by centrifugation (15 min, 5,000 × *g*, 4°C) and resuspended in fresh medium containing 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After 4 h at 37°C the bacteria were again sedimented, washed with phosphate-buffered saline (PBS), and extracted three times with 50 mM Tris-HCl (pH 8.0)–1 mM EDTA–100 mM NaCl–1% Triton X-100 and once with 70% ethanol. Lysates were sonicated at 100 W three times for 10 s after each step, and insoluble proteins were sedimented by centrifugation. Finally, the insoluble protein fraction was separated by electrophoresis on a preparative sodium dodecyl sulfate (SDS)–12% polyacrylamide gel (PrepCell; Bio-Rad, Munich, Germany). Eluted fractions containing the 38-kDa GST-UL3.5 fusion protein were identified by Western blot (immunoblot) analysis with an antiserum directed against the GST part (Pharmacia). Proteins from positive fractions were concentrated (Centricon-Concentrator; Amicon, Witten, Germany). 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 analyzed.

DNA isolation and Southern blot hybridization. PrV DNA was isolated as described previously (4). After cleavage with restriction endonucleases fragments were separated on 0.7% agarose gels, transferred to nylon membranes (Hybond N$^+$; Amersham, Braunschweig, Germany), and hybridized to ^{32}P-labeled probes prepared from cloned PrV and BHV-1 DNA fragments (RediPrime kit; Amersham). Hybridization and wash conditions were as described previously (22).

Determination of plaque sizes and one-step growth curves. To quantitate the sizes of plaques produced by wild-type and mutant viruses on complementing and noncomplementing cells, serial virus dilutions were plated onto Vero, VnB6, or VnS2.4 monolayer cells, overlaid with medium containing 0.8% methylcellulose, and incubated for 3 days at 37°C. Thereafter, cells were fixed and stained with either 1% crystal violet or X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as described previously (34). Thirty plaques per virus strain and cell line were measured microscopically, and average plaque sizes were determined. For analysis of one-step growth, Vero or VnB6 cells were infected with the respective virus at a multiplicity of infection (MOI) of 5. After 1 h at 4°C prewarmed medium was added, and cells were further incubated for 1 h at 37°C to allow virus penetration. The inoculum was then removed, and the remaining extracellular virus was inactivated by low-pH treatment (28). Cells were washed twice with PBS and overlaid with fresh medium. Immediately thereafter, and after 4, 8, 12, 24, and 48 h of incubation at 37°C, cells were scraped into the medium and lysed by freezing at −70°C and thawing at 37°C. Titers of progeny virus were determined by plaque assays on VnB6 cells.

Western blot analyses of virions, cell lysates, and subcellular fractions. PrV virions were isolated as described previously (20). Infected and noninfected cells were collected by centrifugation after scraping into the medium. Cells were fractionated according to established protocols (14). Briefly, sedimented cells were resuspended in 10 mM Tris-HCl (pH 8.5)–250 mM sucrose–0.2 mM phenylmethylsulfonyl fluoride and homogenized in a Wheaton homogenizer. Nuclei were separated by centrifugation for 10 min at 1,000 × *g* and further purified by ultracentrifugation through a 2.5 M sucrose cushion for 30 min at 30,000 × *g*. The nuclear fraction was characterized by analytical estimation of DNA content (7). The remaining supernatant was divided into cytosolic and membrane fractions by centrifugation at 100,000 × *g* for 30 min and 5′ nucleosidase activity was assayed as a marker for membrane proteins (1). Ten micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis (PAGE) (23), electrotransferred to nitrocellulose filters (39), and reacted for 1 h with the UL3.5-specific rabbit antiserum at a dilution of 1:5,000. After incubation with peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany), bound antibody was visualized by luminescence (ECL Western blot detection system; Amersham) recorded on X-ray films.

Electron microscopy. Vero and VnB6 cells were infected with the respective virus at an MOI of 5. Infected cells were fixed 14 h postinfection (hpi) with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Cells were gently scraped off the plate, collected by centrifugation and preembedded in 2% low-melting-point agarose dissolved in PBS without divalent cations. Agarose pieces with cell pellets were postfixed with 1% osmium tetroxide, briefly washed, stained en bloc with 0.5% uranyl acetate, dehydrated stepwise in ethanol, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined with an electron microscope (EM 400T; Philips, Eindhoven, The Netherlands).

RESULTS

Isolation of PrV UL3.5 mutants. The UL3.5 ORF of PrV Ind-FH encodes a deduced protein of 220 aa (11). Sequence determination of the UL3.5 gene of PrV Ka revealed the presence of five amino acid exchanges in the predicted protein product and an addition of 4 aa compared with the UL3.5 gene of PrV Ind-FH. Thus, the deduced UL3.5 protein of PrV Ka comprises 224 aa (37). To analyze the importance of the PrV UL3.5 protein for virus replication, we sought to mutate the UL3.5 ORF by deletions and insertions of foreign DNA sequences into the gene. For this, we devised a novel mutagenesis procedure designated stable heterologous *cis* complementation. It has previously been shown that PrV gB is essential for virus penetration and cell-to-cell spread (30, 31), as is true for gB homologs of other herpesviruses (for a review, see reference 29). PrV mutants lacking gB are, therefore, dependent on transcomplementing PrV gB-expressing cell lines for productive replication. The defect in gB$^-$ PrV can, however, also be compensated by the gB protein of BHV-1, and a corresponding replication-competent PrV recombinant lacking PrV gB but expressing BHV-1 gB has been isolated and characterized (21, 22). Transfection of DNA from gB$^-$ PrV into normal cells does not lead to productive virus replication. However, after cotransfection of normal cells with DNA from gB$^-$ PrV and plasmids containing the BHV-1 gB gene within distinct nonessential PrV gene sequences, after homologous recombination only the desired virus recombinants should be able to produce infectious progeny, since the defective gB locus of the parental virus itself should not be rescued by the heterologous gB gene because of diversity in the DNA sequence. This proved to be critical, since a complete elimination of the PrV gB gene would also impair the partially overlapping essential UL28 gene (32). The remaining gB gene sequences contained in the overlap between the gB and UL28 genes could thus serve as targets for site-specific recombination if PrV gB were used for insertional mutagenesis. In contrast, use of a heterologous gB gene avoids this rescue of the gB locus. By this approach, a 3.3-kbp *Bam*HI fragment containing the BHV-1 gB gene under the control of its own promoter and including the mRNA polyadenylation signal (22) was inserted at two positions within the cloned UL3.5 gene of PrV Ka (Fig. 1c). For the 3′-terminal insertion, a 60-bp *Pvu*II fragment encompassing codons 159 to 179 of the UL3.5 ORF was replaced by the BHV-1 gB gene in plasmid pHD-gB. For a second, 5′-terminally located mutation, the BHV-1 gB gene was inserted at UL3.5 codon 10 after oligonucleotide-directed creation of a *Bam*HI site at this position, yielding plasmid pWF41-gB. In both cases the inserted BHV-1 gB gene should be transcribed parallel to the UL1 to UL3.5 gene cluster (Fig. 1). After cotransfection of Vero cells with gB$^-$ PrV DNA and pHD-gB infectious virus progeny appeared and was further plaque purified to obtain a homogeneous virus preparation. One plaque isolate, designated PrV-ΔUL3.5P, was further analyzed.

After cotransfection of normal Vero cells with gB$^-$ PrV DNA and plasmid pWF41-gB, infectious recombinant virus could not be plaque purified. In all plaques analyzed, a heterogeneous population of parental gB$^-$ PrV and the expected recombinant virus was present. We reasoned that in this situation, the expected recombinant virus provides in *trans* the BHV-1 gB protein to complement gB$^-$ PrV, whereas gB$^-$ PrV provides in *trans* the UL3.5 gene product to complement the UL3.5 insertion mutant. Since this indicated that the 5′-terminal insertion in UL3.5 could be lethal for virus replication, we established complementing cell lines harboring the viral UL3.5 gene. Two Vero cell lines with stable genomic insertions of the PrV UL3.5 gene were established. Cell line VnB6 was obtained after transfection of Vero cells with the 7.3-kbp genomic viral *Bam*HI fragment 6, which encompasses the UL1 to UL5 genes (11, 12) as well as the promoter for the latency-associated transcripts and the 3′-terminal part of the EP0 gene (8) (Fig. 1b). The cell line VnS2.4 harbors the 2.4-kbp *Sal*I fragment containing only the UL3, UL3.5, and UL4 genes. The presence of the intact 7.3-kbp *Bam*HI fragment 6 or 2.4-kbp *Sal*I subfragment in cell lines VnB6 and VnS2.4 was ascertained by Southern blot hybridization of appropriately cleaved cellular DNA (data not shown). On both cell lines mutant PrV-ΔUL3.5B could easily be plaque purified to homogeneity, indicating that they express functional UL3.5 protein. Indeed, Western blot analysis of cell lysates with the αUL3.5 serum (see below) showed the presence of the UL3.5 protein after infection of VnB6 and VnS2.4 cells with PrV-ΔUL3.5B, whereas it was undetectable in noninfected VnB6 or VnS2.4 cells. Normal Vero cells did not express a UL3.5 protein after infection with PrV-ΔUL3.5B. This shows that after virus infection, the expression of the UL3.5 gene resident in the recombinant cell lines was activated and UL3.5 protein accumulated to levels which were similar to those found after infection of Vero cells with wild-type PrV (data not shown).

Genome analysis of PrV UL3.5 mutants. To analyze genetic homogeneity of the isolated virus populations and to ascertain correct insertion of the BHV-1 gB gene in the two locations within the UL3.5 gene of PrV recombinants ΔUL3.5P and ΔUL3.5B, virion DNA was isolated and compared with wild-type PrV DNA and DNA from gB$^-$ PrV by restriction analysis (Fig. 2a) and Southern blot hybridization (Fig. 2b and c). Cleavage with *Kpn*I showed that the 14-kbp *Kpn*I fragment C of wild-type PrV was enlarged to 16 kbp in PrV mutants gB$^-$, ΔUL3.5P, and ΔUL3.5B (Fig. 2a) as a consequence of the deletion and *lacZ* gene insertion in the PrV gB locus (32). This confirms that the UL3.5 mutants are derived from gB$^-$ PrV as expected. Blot hybridization of the same gel with the labeled 9.2-kbp *Kpn*I fragment F of wild-type PrV, which encompasses *Bam*HI fragment 6 with the area of interest (Fig. 1), shows two fragments of 2.1 and 10.3 kbp in PrV-ΔUL3.5P and two fragments of 2.6 and 9.9 kbp in PrV-ΔUL3.5B. This is due to the introduction of an additional *Kpn*I site contained at the left-hand terminus of the inserted BHV-1 gB sequences (Fig. 2b; compare Fig. 1). In contrast, the 9.2 kbp *Kpn*I fragment F was detected in wild-type and gB$^-$ PrV DNA (Fig. 2b). Hybridization with the labeled gB gene of BHV-1 (Fig. 2c) demonstrates the presence of this gene in the 10.3 and 9.9 kbp *Kpn*I fragments of PrV-ΔUL3.5P and PrV-ΔUL3.5B, respectively. The observed fragment sizes agree with those predicted from correct insertion of the BHV-1 gB gene in the two different locations within the UL3.5 gene of PrV. The results also show that both UL3.5 mutant preparations appeared genetically homogeneous. Even after overexposure of the blot shown in Fig. 2b, a wild-type-sized *Kpn*I fragment F was not detectable in the DNA of mutants PrV-ΔUL3.5P and PrV-ΔUL3.5B (data not shown).

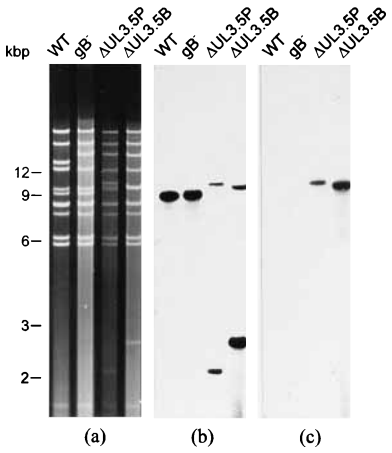

FIG. 2. DNA analysis of PrV UL3.5 mutants. Virion DNA of wild-type PrV (WT) and PrV mutants gB$^-$, ΔUL3.5P, and ΔUL3.5B were cleaved with *Kpn*I; resulting fragments were separated on a 0.7% agarose gel and transferred to nylon membranes. (a) The ethidium bromide-stained gel is depicted. Results from hybridization with ^{32}P-labeled wild-type genomic *Kpn*I fragment F (b) or with the BHV-1 gB gene (c) are also shown. Locations of molecular weight standards are indicated on the left.

Growth characteristics of PrV UL3.5 mutants. The fact that mutant PrV-ΔUL3.5P could be isolated on normal cells, whereas mutant PrV-ΔUL3.5B required complementing cells for replication, already indicated that insertion of the BHV-1 gB gene at the 3′-terminal site within UL3.5 did not abolish replicative ability of the virus, whereas insertion at the 5′-terminal site obviously interfered with productive virus replication. To assay in more detail the growth properties of the UL3.5 mutants, in vitro replication was analyzed by plaque assays and one-step growth kinetics on complementing and noncomplementing cells. As controls, wild-type PrV Ka as well as BHV-1 gB recombinant PrV 9112C2 (22) was used. PrV 9112C2 expresses BHV-1 gB from the PrV gB locus (Fig. 1a) and, therefore, should allow the discrimination of effects solely attributable to the gB substitution.

Plaque sizes were monitored 3 days after infection of cell monolayers by staining with either crystal violet (for wild-type PrV and recombinant PrV 9112C2) or X-Gal (for the UL3.5 mutants). As shown in Fig. 3, substitution of PrV gB by BHV-1 gB decreases plaque sizes by about 40% as has been described before (21). On noncomplementing Vero cells, mutant PrV-ΔUL3.5P formed plaques with sizes similar to those induced by PrV 9112C2. This indicates that insertion of the BHV-1 gB gene at the 3′-terminal site within the UL3.5 gene had no influence on plaque size. In contrast, only tiny accumulations of maximally three to five cells, and for the most part only single infected cells, were observed after infection of noncomplementing Vero cells with PrV-ΔUL3.5B (Fig. 4). This growth defect could be fully compensated on complementing VnB6 cells, on which all three BHV-1 gB-expressing virus recombinants induced plaques of identical size. We conclude that insertion of the BHV-1 gB gene at the 3′ site within the UL3.5 gene did not impair the ability of the virus to induce plaques whereas insertion at the 5′ site abolished plaque forming capacity. However, this defect could be corrected in *trans* in the complementing cell line VnB6. The cell line VnS2.4 yielded identical results (data not shown).

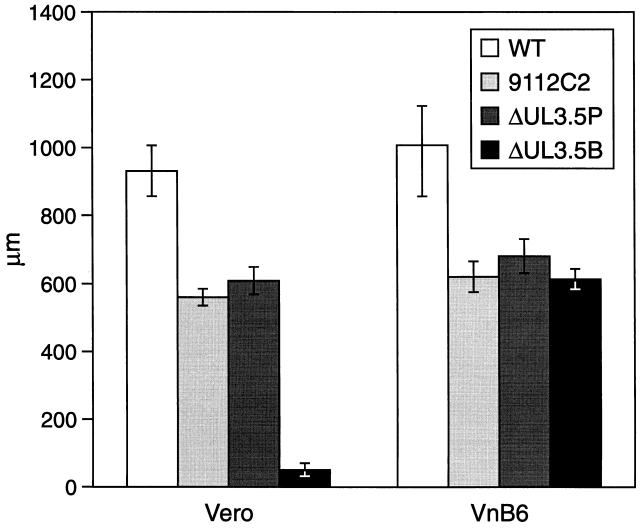

FIG. 3. Analysis of plaque sizes of PrV UL3.5 mutants. Normal Vero and complementing VnB6 cells were infected with wild-type PrV (WT) and PrV recombinants 9112C2, ΔUL3.5P, and ΔUL3.5B under plaque assay conditions. Three days after infection cells were stained with crystal violet (WT, PrV 9112C2) or X-Gal (PrV-ΔUL3.5P, PrV-ΔUL3.5B) and the diameters of 30 plaques per assay were determined microscopically. Vertical lines indicate standard deviations.

One-step growth curves of wild-type PrV and PrV mutants 9112C2, ΔUL3.5P, and ΔUL3.5B were also determined on Vero and VnB6 cells (Fig. 5). At the indicated times after infection at a MOI of 5, cells were scraped into the medium and lysed by freeze-thawing and progeny virus was titrated on complementing VnB6 cells to provide equal growth conditions for all PrV mutants. As shown in Fig. 5, growth kinetics of PrV 9112C2 on both cell lines were identical and appeared only slightly delayed compared with those of wild-type PrV. Growth curves of PrV-ΔUL3.5P were indistinguishable from those of PrV 9112C2 on both complementing and noncomplementing cells. Final titers for these viruses were approximately 10^6 PFU/ml. In contrast, mutant PrV-ΔUL3.5B exhibited a strong growth deficiency on noncomplementing Vero cells. Although the duration of the eclipse phase was similar to that of the other PrV mutants and a significant increase in virus titer was observed between 8 and 12 h after infection, final titers were decreased by more than 99%, not exceeding 3×10^3 PFU/ml. This growth deficiency was fully compensated on complementing VnB6 cells (Fig. 5).

Identification of PrV UL3.5 protein. The C-terminal part (aa 136 to 224) of the UL3.5 ORF of PrV was expressed in *E. coli* as a fusion protein with GST (Fig. 1d). With the isolated 38-kDa fusion protein a UL3.5 specific rabbit antiserum was generated. With this serum, purified wild-type PrV virions and PrV infected Vero cell lysates were investigated by Western

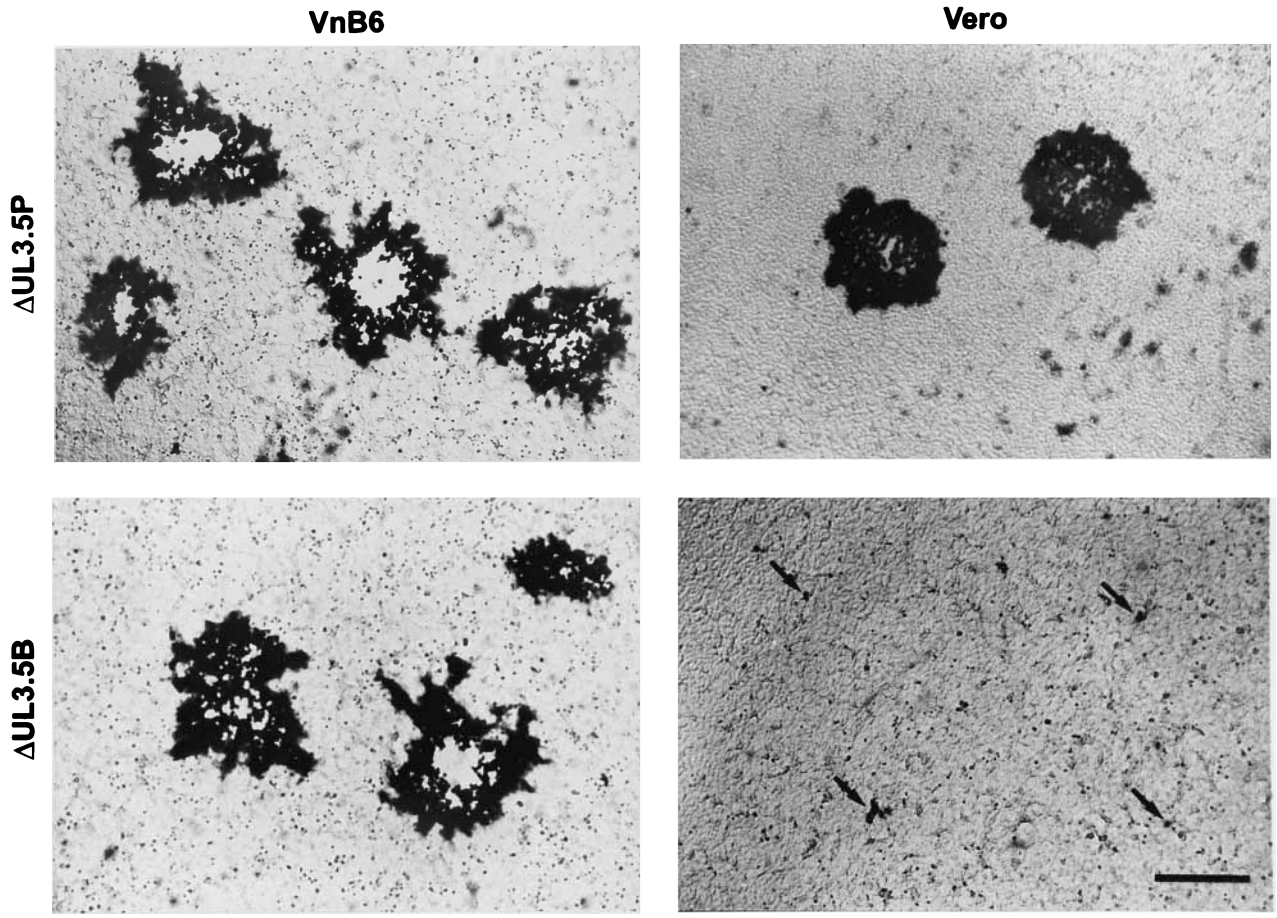

FIG. 4. Plaque morphology of PrV UL3.5 mutants. Noncomplementing Vero or complementing VnB6 cells were infected with PrV-ΔUL3.5P or PrV-ΔUL3.5B under plaque assay conditions. Three days after infection virus-infected cells were stained with X-Gal. Arrows denote tiny accumulations of few infected cells or single infected cells. Bar = 500 µm.

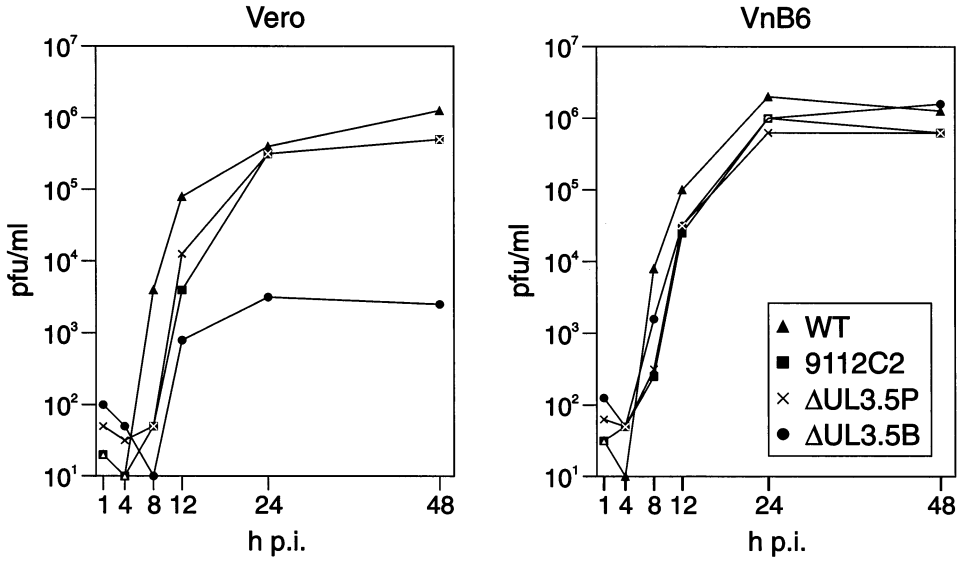

FIG. 5. One-step growth curves. Normal Vero or complementing VnB6 cells were infected with wild-type PrV (WT) or PrV recombinants 9112C2, ΔUL3.5P, and ΔUL3.5B at an MOI of 5 for 1 h at 4°C. After an additional hour at 37°C nonpenetrated virus was inactivated by low-pH treatment. Immediately thereafter, and after different periods of incubation at 37°C, cells were scraped into medium and lysed by freeze-thawing, and virus progeny was titrated on VnB6 cells.

blot analysis (Fig. 6). The rabbit antiserum (αUL3.5 [Fig. 6, upper panel]) detected a protein with an apparent molecular mass of 30 kDa in wild-type PrV-infected cell lysates at 4 and 8 hpi, as well as in lysates of Vero cells infected with PrV 9112C2. This protein was absent in lysates from noninfected cells and was also not detectable in purified PrV virions. In lysates from cells infected with PrV-ΔUL3.5P, a 25-kDa protein was detected. Cells infected with PrV-ΔUL3.5B did not express a protein recognizable by the αUL3.5 serum. Specificity of the reaction was confirmed by comparison with the preimmune serum as well as by competition experiments using the bacterial fusion protein (data not shown).

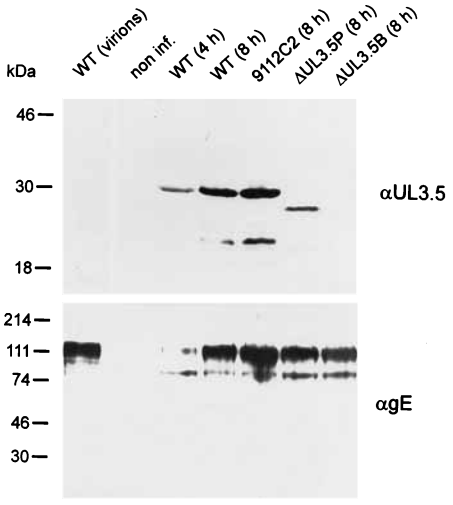

FIG. 6. Identification of PrV UL3.5 protein. Lysates of cells at 4 or 8 h after infection with wild-type PrV (WT) or 8 h after infection with PrV recombinants 9112C2, ΔUL3.5P, or ΔUL3.5B or noninfected (non inf.) cell lysates were separated by gel electrophoresis, electrophoretically transferred to nitrocellulose, and probed with a polyclonal rabbit serum prepared by immunization with a UL3.5-GST fusion protein (αUL3.5) or a monoclonal antibody directed against viral glycoprotein gE (αgE). In the first lane, purified wild-type PrV virions were analyzed. Identical amounts of protein were separated in each lane of the SDS-polyacrylamide gels (12 and 7.5% polyacrylamide for the upper and lower panels, respectively). Bound antibody was visualized by luminescence after incubation with peroxidase-conjugated secondary antibodies. Locations of molecular mass markers are indicated on the left.

The UL3.5 protein migrated with an apparent molecular mass of 30 kDa in SDS-PAGE. However, its molecular mass predicted from the deduced amino acid sequence amounts to only 24 kDa. It remains to be investigated whether this discrepancy is a consequence of posttranslational modifications or whether it is caused by the very basic character of the protein (isoelectric point of 12.4), which could lead to an abnormal migration behavior in SDS-PAGE. A second protein detected by the antiserum at 8 hpi in cells infected with wild-type PrV and PrV 9112C2 most likely constitutes a degradation product of the UL3.5 protein, since it was not detectable at earlier times (4 hpi). The expression of a truncated but obviously still functional UL3.5 protein by PrV-ΔUL3.5P and the absence of this protein in cells infected with PrV-ΔUL3.5B correlate with the different growth characteristics of the two viruses. As a control, a parallel blot was also incubated with a monoclonal antibody directed against PrV glycoprotein gE. gE was present in all samples analyzed except for the noninfected cell lysate (Fig. 6, bottom panel).

Subcellular localization of UL3.5 protein. Since the UL3.5 protein was not detectable in purified PrV virions we next analyzed its subcellular location during virus infection. Indirect immunofluorescence analysis of wild-type PrV-infected cells with the αUL3.5 serum indicated an extranuclear localization of the protein (data not shown). This finding could be verified by cell fractionation studies (Fig. 7). In these studies, equivalent amounts of protein from cell fractions obtained from homogenized wild-type PrV-infected Vero cells were investigated by Western blot analysis with the αUL3.5 serum. Within the nuclear fraction (Fig. 7, lane N) which was separated from the total homogenate (lane VP) by low-speed centrifugation, the UL3.5 protein was not detected. Separation of the postnuclear supernatant into cytosolic (lanes S) and membrane-plus-microsome (lanes P) fractions by ultracentrifugation revealed that the UL3.5 protein was enriched in the latter fraction. Pretreat-

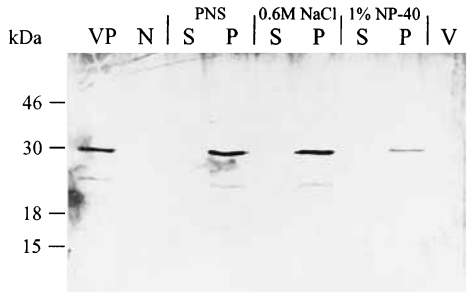

FIG. 7. Detection of PrV UL3.5 protein in subcellular fractions. Eight hours after infection with wild-type PrV at an MOI of 5, Vero cells were homogenized (lane VP) and nuclei (lane N) were separated by centrifugation at 1,000 × *g* and purified by ultracentrifugation through a 2.5 M sucrose cushion. The postnuclear supernatant was separated into cytosolic (lanes S) and membrane (lanes P) fractions by centrifugation at 100,000 × *g* without preincubation (PNS) or after prior incubation in 0.6 M NaCl or 1% Nonidet P-40 (NP-40). Equivalent amounts of all fractions as well as of noninfected cell lysates (lane V) were separated by SDS-PAGE, electroblotted onto nitrocellulose filters, and reacted with a rabbit αUL3.5 serum.

ment of the postnuclear supernatant with 0.6 M NaCl, which solubilizes peripheral membrane proteins, or with 1% Nonidet P-40, which solubilizes integral membrane proteins (14), did not lead to detection of the UL3.5 protein in the supernatant, although after treatment with 1% Nonidet P-40 the signal obtained from the membranous pellet decreased. In summary, the PrV UL3.5 gene product was detected as a 30-kDa protein in the membrane-plus-microsome fraction of infected cells.

Electron microscopy. To further analyze the replication defect, ultrastructural studies were performed. As shown in Fig. 8, after infection of normal Vero (Fig. 8A and B) and complementing VnB6 (Fig. 8C and D) cells with PrV-ΔUL3.5P (Fig. 8A and C) and PrV-ΔUL3.5B (Fig. 8B and D) at an MOI of 5, at 14 hpi typical stages of maturation of viral particles in the nucleus, including empty and full capsids as well as particles which had acquired an envelope by budding through the inner nuclear membrane, were observed. No differences between the two mutant viruses and the two cell lines were observed. Secondary envelopment in the Golgi region was readily observed after infection of complementing VnB6 cells by both viruses (Fig. 9C and D), as was the presence of extracellular mature virions (Fig. 9C and data not shown). However, whereas in noncomplementing Vero cells infected by PrV-ΔUL3.5P maturation stages (Fig. 9A) comparable to those found in wild-type PrV infected Vero cells (data not shown) were detected, in noncomplementing Vero cells infected by PrV-ΔUL3.5B naked capsids accumulated in the Golgi region and envelopment processes were not observed (Fig. 9B). This resulted in a lack of extracellular enveloped viral particles (Fig. 9B). We conclude that late in infection the absence of functional UL3.5 protein leads to a block in the final envelopment and release of virions because of a retention of naked capsids in the Golgi area.

DISCUSSION

In this study the protein product of the PrV UL3.5 gene has been analyzed. Positional homologs to PrV UL3.5 have been detected in VZV (gene 57 [10]), EHV-1 (gene 59 [36]), and BHV-1 (18). Initially on the basis of biological criteria (33) which have recently been supplemented by phylogenetic analyses (25), these viruses have been grouped into the *Varicellovirus* genus of the *Alphaherpesvirinae* subfamily. In contrast, in the members of the *Simplexvirus* genus, HSV-1 and HSV-2, no homologous ORF has been detected (26, 27). This is surprising since the other genes located in this region, UL1 through UL5, are present in all alphaherpesviruses analyzed so far. The predicted UL3.5 homologous proteins of different herpesviruses differ in size from 71 aa in VZV to 224 aa in PrV. Amino acid sequence homology is only limited and appears confined to the 50 N-terminal residues (18). A substantial variability could even be detected between the deduced UL3.5 proteins of PrV Ind-FH and Ka, in which nine amino acid differences were detected (11, 37).

The PrV UL3.5 protein apparently represents a nonstructural protein, since it could be detected in infected cells but was absent from purified virions. Increasing amounts of intracellular UL3.5 protein were observed from 4 to 8 h after infection, correlating with the early-late expression kinetics of the UL3.5 mRNA (11). The PrV UL3.5 protein migrated with an apparent molecular mass of 30 kDa in SDS-PAGE, whereas the calculated molecular mass of the primary translation product is 24 kDa. This difference either could be due to posttranslational modifications or could be a consequence of its unusual amino acid composition of 21% basic residues (16% arginine, 5% histidine) with an isoelectric point of 12.4. This basic character is a common feature of all predicted UL3.5 homologs, which prompted speculations about a putative interaction of these proteins with DNA (11, 18). Our immunofluorescence and cell fractionation studies, however, indicate an exclusively cytoplasmic localization of the PrV UL3.5 protein, arguing against a function in DNA binding. Interestingly, the PrV UL3.5 protein proved to be absent from soluble cytosol but could be sedimented from the cytoplasmic fraction by ultracentrifugation, even after solubilization of peripheral and integral membrane proteins with high salt concentrations or detergents. Though puzzling, this finding might be indicative of a more stable interaction of the PrV UL3.5 protein with a subcellular compartment which remains to be characterized. However, the possibility of a temporary association of the PrV UL3.5 protein with immature intracytoplasmic virions should also be considered.

For functional analysis of the PrV UL3.5 protein, we constructed virus recombinants by insertion of the BHV-1 gB gene into the UL3.5 ORF to complement a replication-deficient gB^- PrV mutant (22, 32). This stable heterologous *cis* complementation offers an easy way to construct virus mutants, since only recombinant virus gains the capacity for autonomous productive replication. PrV-ΔUL3.5P carrying the insertion at codon 159 of the UL3.5 gene expresses a smaller UL3.5 protein, which is, however, still detectable by the rabbit antiserum directed against a bacterial fusion protein containing the C-terminal part of the PrV UL3.5 protein starting with aa 136. Since the overlap between the bacterial protein and the truncated protein expressed by the virus mutant is only 23 aa, the reactivity of the antiserum with both proteins indicates the presence of major antigenic epitopes between aa 136 and 158 of the UL3.5 protein. In contrast to PrV-ΔUL3.5P, which replicates on normal cells, PrV-ΔUL3.5B with an insertion at codon 10 of the UL3.5 gene could only be isolated on complementing cell lines and no UL3.5 protein could be found in the lysates of infected normal Vero cells. However, after the infection of complementing cells, UL3.5 protein was detected at levels similar to those found after wild-type PrV infection of noncomplementing cells.

Mutant PrV-ΔUL3.5P exhibited similar one-step growth kinetics and produced plaques identical in size to those of PrV 9112C2 in which the BHV-1 gB gene has been inserted into the defective PrV gB locus (22). Therefore, the C-terminal truncation of the PrV UL3.5 protein did not have a significant

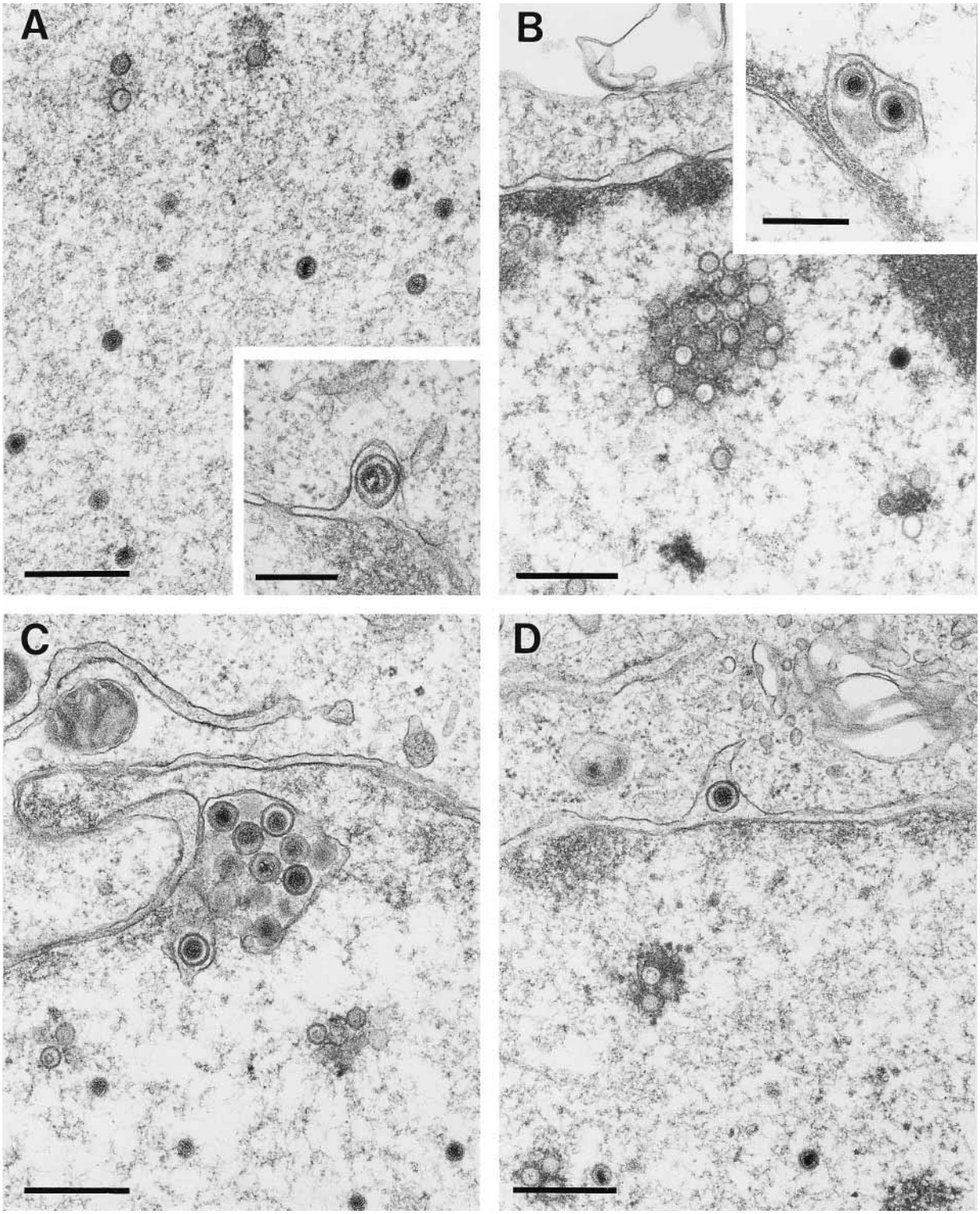

FIG. 8. Electron microscopy: intranuclear capsid formation. Noncomplementing Vero (A and B) and complementing VnB6 (C and D) cells were infected with PrV-ΔUL3.5P (A and C) and PrV-ΔUL3.5B (B and D) at an MOI of 5 and analyzed 14 h after infection. Bar = 500 nm. (Insets) Primary envelopment at the nuclear membrane in Vero cells. Bar = 300 nm.

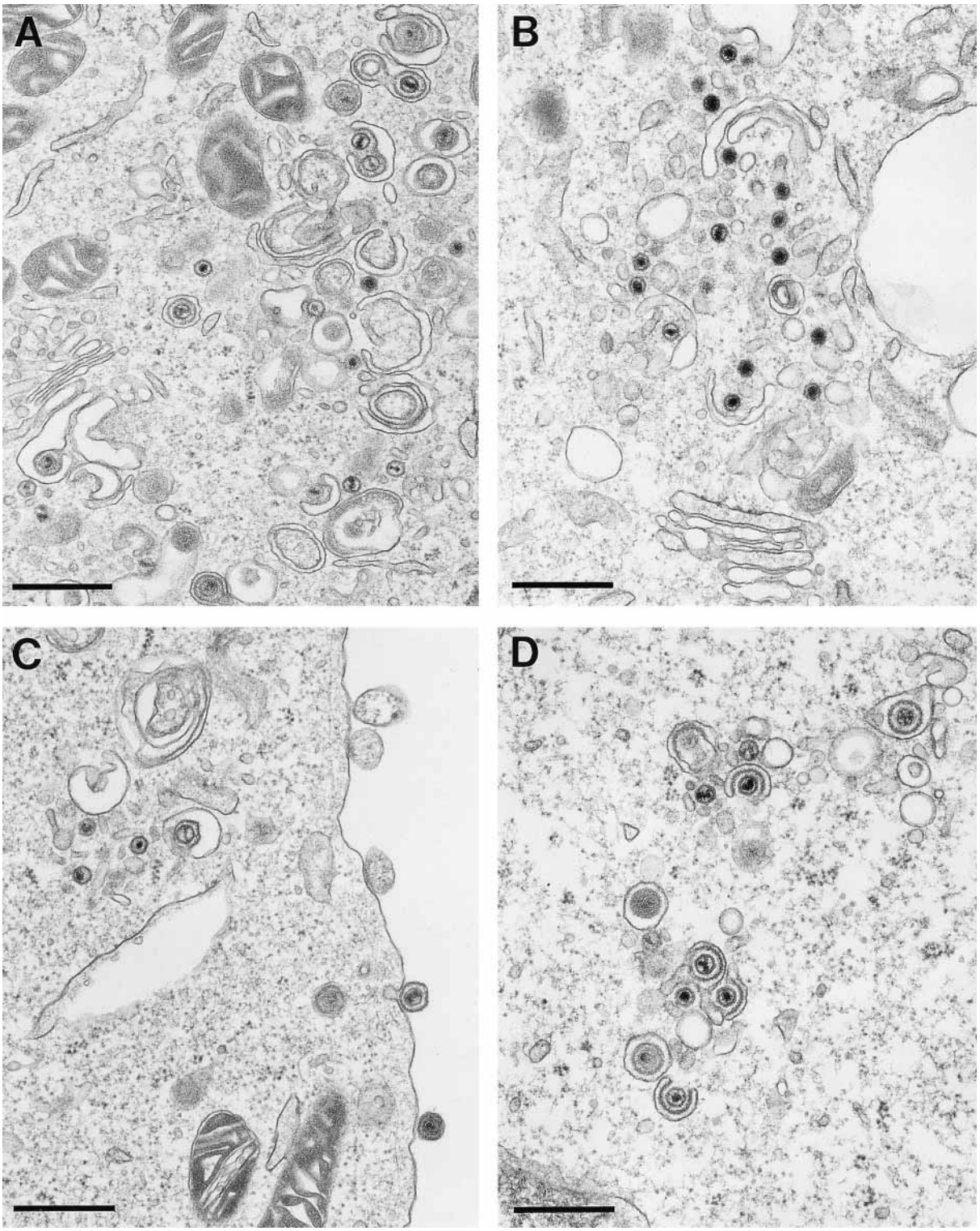

FIG. 9. Electron microscopy: envelopment of intracytoplasmic capsids in Golgi region. Noncomplementing Vero (A and B) and complementing VnB6 (C and D) cells were infected with PrV-ΔUL3.5P (A and C) or PrV-ΔUL3.5B (B and D) at an MOI of 5 and analyzed 14 h after infection. Bar = 500 nm.

effect on PrV replication. This correlates with the continued expression of the conserved N-terminal part of the PrV UL3.5 protein, which obviously harbors the functionally important domains. In contrast, insertional mutagenesis at codon 10 leads to a dramatic replication defect. The capacity to induce plaques in noncomplementing Vero cells is eliminated, and in one-step growth kinetics assays a decrease of final titers by approximately 3 log units compared with those of PrV-

ΔUL3.5P or PrV 9112C2 was observed. Therefore, the 5′-terminal mutation apparently abolishes or severely impairs UL3.5 function. The UL3.5 gene is located at the most downstream position within the 3′-coterminally transcribed gene cluster encompassing UL1, UL2, UL3, and UL3.5 (11) (Fig. 1). Therefore, one could speculate that the insertion of the BHV-1 gB expression unit could interfere with expression of the upstream genes, including the presumably essential glycoprotein gL (19). This, however, is unlikely, because PrV-ΔUL3.5P harbors the same insertion only 450 bp further downstream and still retains its replicative ability. Also, complementation by VnS2.4 cells capable of expressing only UL3, UL3.5, and UL4 shows that the phenotype of PrV-ΔUL3.5B is not attributable to unwanted fortuitous mutations somewhere else in the viral genome. In this context, it is of interest that the UL3 and UL4 genes of HSV-1 have been shown to be nonessential for viral replication (2).

At which stage of virus replication does the UL3.5 protein participate? Since the protein is absent from extracellular mature PrV virions as well as from nuclei of infected cells, it appears unlikely to contribute to virus entry, DNA replication, transcriptional regulation, or encapsidation. Given the localization within membranous cytoplasmic fractions, it might be involved in posttranscriptional regulation of gene expression or posttranslational modification of viral gene products or play a role in virus maturation and egress. Egress of alphaherpesviruses has been discussed as a transit of enveloped virions through the endoplasmic reticulum and Golgi apparatus for HSV-1 (38) or a release of naked nucleocapsids into the cytosol after crossing the nuclear membrane followed by a reenvelopment at the *trans*-Golgi network as shown for PrV and VZV (15, 41, 42). On the basis of the membrane association of the UL3.5 protein in PrV as well as the fact that PrV and VZV specify a UL3.5 homolog which is, however, absent in HSV-1, it could be speculated that the UL3.5 homologous proteins may be involved in this route of virus egress. Electron microscopy indeed showed that in noncomplementing Vero cells infected with PrV-ΔUL3.5B, naked capsids accumulate near the Golgi apparatus or Golgi-derived vesicles, leading to a virtual absence of extracellular virions. In contrast, intranuclear maturation as well as primary envelopment at the nuclear membrane appears to be unperturbed. This implies that the UL3.5 protein plays a role in final envelopment of naked capsids in the Golgi region and substantiates the de-envelopment–reenvelopment scenario for maturation of PrV virions.

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