JOURNAL OF VIROLOGY, Feb. 1991, p. 621–631
0022-538X/91/020621-11$02.00/0

Vol. 65, No. 2

Pseudorabies Virus Mutants Lacking the Essential Glycoprotein gII Can Be Complemented by Glycoprotein gI of Bovine Herpesvirus 1

ISABELLA RAUH, FRANK WEILAND, FRANK FEHLER, GÜNTHER M. KEIL, AND THOMAS C. METTENLEITER*

Federal Research Centre for Virus Diseases of Animals, P.O. Box 11 49, D-7400 Tübingen, Federal Republic of Germany

Received 3 August 1990/Accepted 23 October 1990

The genome of pseudorabies virus (PrV) encodes at least seven glycoproteins. The glycoprotein complex gII consists of three related polypeptides, two of them derived by proteolytic cleavage from a common precursor and linked via disulfide bonds. It is homologous to herpes simplex virus (HSV) gB and is therefore thought to be essential for PrV replication, as is gB for HSV replication. To isolate PrV mutants deficient in gII expression, we established cell lines that stably carry the PrV gII gene. Line N7, of Vero cell origin, contains the gII gene under its own promoter and expresses gII after transactivation by herpesviral functions after infection. MDBK-derived line MT3 contains the gII gene under control of the mouse metallothionein promoter. However, it has essentially lost inducibility and constitutively produces high amounts of correctly processed glycoprotein gII. We used a β-galactosidase expression cassette inserted into a partially deleted cloned copy of the gII gene for cotransfection with PrV DNA. gII$^-$ PrV mutants were isolated from viral progeny by taking advantage of their blue-plaque phenotype when incubated under an agarose overlay containing a chromogenic substrate. Analysis of these mutants proved that gII is indeed essential for PrV replication, since the gII$^-$ mutants grew normally on gII-complementing cells but were unable to produce plaques on noncomplementing cells. Surprisingly the PrV gII$^-$ mutants were also able to grow on a cell line constitutively expressing the gB-homologous glycoprotein gI from bovine herpesvirus 1 (BHV-1) to the same extent as on cells expressing PrV gII. gII$^-$ PrV propagated on cells expressing BHV-1 gI became susceptible to neutralization by anti-BHV-1 gI monoclonal antibodies. We also found that BHV-1 gI is present in the envelope of purified gII$^-$ pseudorabies virions grown on cells expressing BHV-1 gI, as judged by radioimmunoprecipitation and immunoelectron microscopy. These results prove that BHV-1 gI is integrated into the PrV envelope and can functionally replace glycoprotein gII of PrV.

Pseudorabies virus (PrV) belongs to the herpesvirus group of enveloped animal viruses. It possesses a linear double-stranded DNA genome of approximately 150 kb which encodes at least seven glycoproteins (13, 23). All known PrV glycoproteins share homologies with the respective glycoproteins of herpes simplex virus (HSV). The homologous pairs are as follows (the virus expressing the named protein is given in parentheses): gI(PrV)-gE(HSV) (37, 57), gII(PrV)-gB(HSV) (42), gIII(PrV)-gC(HSV) (41), gp63(PrV)-gI(HSV) (37), gp50(PrV)-gD(HSV) (49), gH(PrV)-gH(HSV) (19a, 35a), and gX(PrV)-gG(HSV) (38). gI, gIII, gp63, and gX as well as their HSV homologs are not required for viral growth in tissue culture and are therefore designated nonessential. In contrast, gII, gp50, and gH are thought to be essential for PrV replication, as are the respective HSV glycoproteins (5, 11, 21).

Although variable degrees of homology based on sequence comparison between these glycoproteins have been found, it is still largely unknown whether the respective glycoproteins exert similar functions in the different viruses. Some of the functions of the nonessential glycoproteins of PrV have been elucidated. It has been shown that gI and gp63, which are present in the form of a noncovalently linked glycoprotein complex (57), are involved in virus release from infected cells (56). Interestingly, the homologous HSV glycoproteins gE and gI also form a complex that has been demonstrated to bind the Fc part of monomeric immunoglobulin G (1, 9, 16). No such activity has been observed for the PrV complex (57).

gIII(PrV), although nonessential for viral replication, is important in the initial interaction between PrV and its host cell. Adsorption of wild-type PrV is mediated by the interaction of gIII with a heparinlike cell surface receptor (29). A heparinlike molecule has also been demonstrated as primary receptor for HSV (55). In the absence of either gIII or the heparin receptor, PrV adsorption is considerably impaired (29, 43, 58) and penetration of the virus into the cell is delayed (26, 58). However, adsorption and penetration can occur even in the absence of gIII.

The essential glycoproteins gB, gD, and gH in HSV have all been shown to be involved in the second step of virus infection, i.e., penetration of the virus particle into the target cell (5, 11, 21). Initial experiments using either partial neutralization with complement-independent monoclonal antibodies (MAbs) (14, 15) or temperature-sensitive mutant viruses (8, 22) were complemented by the analysis of virus mutants defective in the synthesis of essential glycoproteins (6). Genetic engineering of these viruses was made feasible after cell lines that carry the essential gene to be deleted from the viral DNA were established. These cell lines are able to provide in *trans* the essential protein and to support replication of the defective virus mutant.

To gain insight into the functions of PrV essential glycoproteins, we started by constructing and isolating cell lines that express the essential glycoprotein gII. Mature gII consists of three related polypeptides that are derived from a common 110-kDa primary translation product (27). After

* Corresponding author.

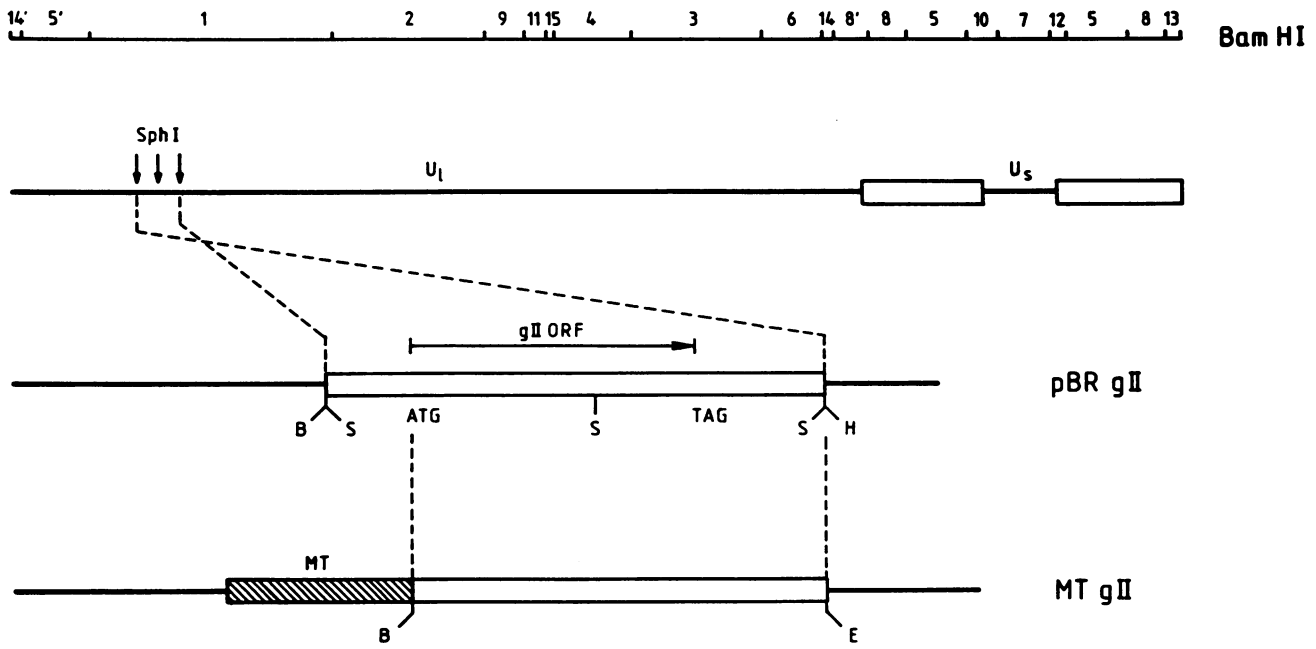

FIG. 1. Plasmids used for establishment of cell lines. A *Bam*HI restriction map is shown above a schematic drawing of the PrV genome. Open boxes represent inverted repeat sequences that bracket the unique short sequence and separate it from the unique long region. The fragment used for construction of pBR-gII was obtained after incomplete *Sph*I digestion and consists of two fused *Sph*I fragments. Site-directed mutagenesis created a *Bam*HI site immediately in front of the gII ORF (43a). This was used to insert the gII gene under control of the mouse metallothionein promoter in plasmid MT-gII. Cleavage sites: B, *Bam*HI; S, *Sph*I; H, *Hin*dIII; E, *Eco*RI. The *Hin*dIII and *Eco*RI sites originate from vector sequences.

glycosylation, the precursor is cleaved by a cellular protease located in the Golgi apparatus (52) into two smaller glycoproteins of 67 and 58 kDa that are covalently linked by disulfide bonds (13, 23, 27, 54). Under nonreducing conditions, the gII-protein complex exhibits an apparent size of 155 kDa (13, 23). The gB-homologous proteins of other herpesviruses show similar proteolytic processing of the primary translation product. The gB of bovine herpesvirus 1 (BHV-1), named gI and referred to here as gI(BHV-1), is cleaved into two subunits of 75 and 55 kDa that also remain linked by disulfide bonds (30, 53). The gB homologs of varicella-zoster virus (31), human cytomegalovirus (4, 45), and equine herpesvirus (25, 50) are also represented by disulfide-linked complexes of two glycopolypeptides. It is therefore interesting that gB(HSV), although present in the viral envelope in higher-molecular-weight structures, probably dimers (7), is not processed proteolytically as are the other gB proteins. However, the high conservation of the gB sequence among the herpesvirus family might indicate that these glycoproteins fulfill a common important function.

In summary, several lines of evidence point to common functions of homologous herpesvirus glycoproteins. However, direct functional homology based on the ability of one glycoprotein to complement a deficiency in a homologous glycoprotein in a different herpesvirus has not been demonstrated.

We isolated PrV mutants lacking the gB homolog gII and found that these mutants were able to grow only on cell lines that provide the gII in *trans*. This finding demonstrates that gII is essential for PrV replication. In addition, we found that cell lines expressing gI(BHV-1) can functionally complement PrV mutants that lack the essential gII glycoprotein. This result clearly proves functional homology between these gB-homologous glycoproteins in two different herpesviruses.

MATERIALS AND METHODS

Viruses and cells. PrV strains Ka (18) and Phylaxia and BHV-1 strain Schönböken were used. They were propagated on Madin-Darby bovine kidney (MDBK) cells.

Plasmids. An *Sph*I fragment containing the complete gII expression unit of PrV strain Phylaxia was cloned into pBR322, yielding plasmid pBR-gII (Fig. 1). Site-directed mutagenesis created a *Bam*HI site immediately in front of the gII open reading frame (ORF) (43a), and the resulting fragment was cloned into the *Bam*HI-*Sph*I sites of the multiple cloning site of plasmid pUC18. A *Bam*HI-*Eco*RI fragment encompassing the gII ORF and gII mRNA polyadenylation signal was excised and cloned behind the mouse metallothionein promoter (3). This plasmid was called MT-gII (Fig. 1). Plasmid pMT-gI contained the ORF for gI(BHV-1) placed under control of the mouse metallothionein promoter.

To obtain a gII-deleted PrV mutant, two plasmids were constructed (Fig. 2). To delete almost all of the gII gene, plasmid pBR-gII was cleaved with *Sma*I, thereby deleting 2,540 bp of gII sequences (Δ1). After addition of *Bam*HI linkers (Bethesda Research Laboratories, Eggenstein, Federal Republic of Germany), a gX–β-galactosidase (β-gal) expression cassette was inserted as a selectable insertional marker (28). To obtain a smaller deletion in the gII gene, pBR-gII was cleaved with *Bst*EII, which resulted in deletion of 1,395 bp (Δ2; Fig. 2). After blunt ending with Klenow polymerase and *Bam*HI linker insertion, the gX–β-gal expression cassette was introduced as described above.

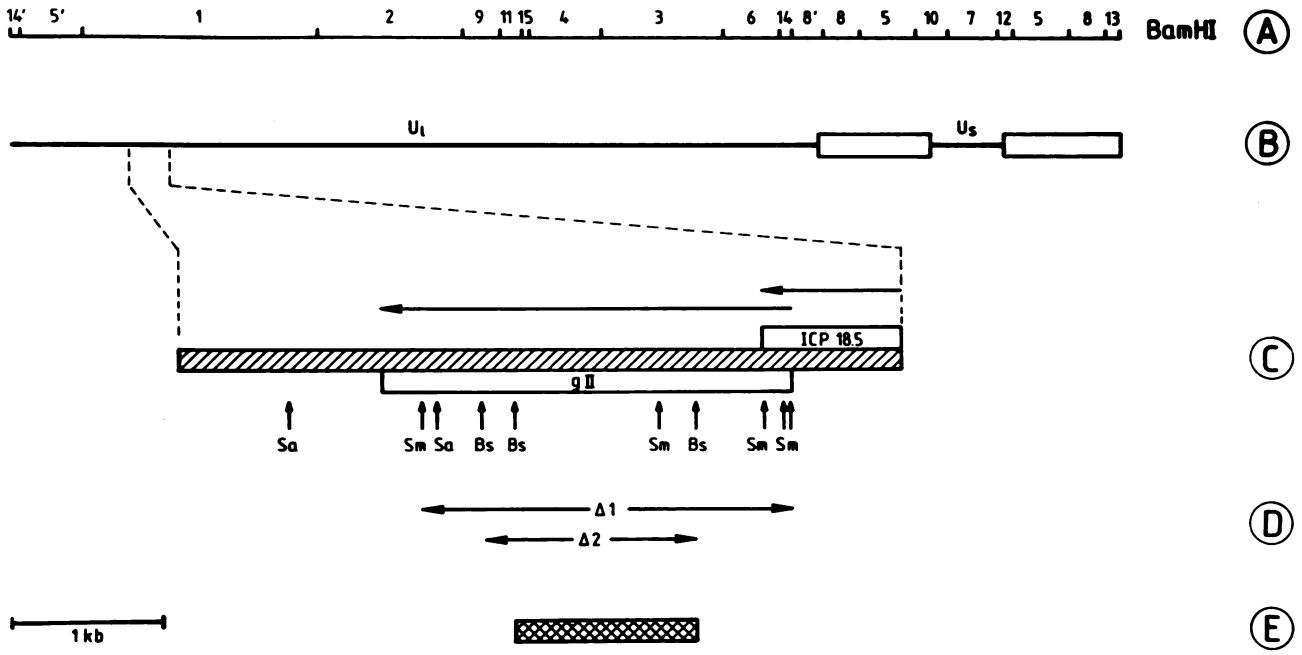

FIG. 2. Constructs used for isolation of mutant viruses. The *Bam*HI restriction map (A) of the PrV genome (B) is shown. The enlarged region (C) encompasses the gII gene and the carboxy-terminal part of the ICP18.5 homolog gene that overlaps the gII gene by 127 bp. Transcriptional direction is indicated by arrows. Δ1 and Δ2 (D) indicate deletions introduced into pBR-gII to obtain gII⁻ viruses. The stippled box in (E) indicates the *Bst*EII fragment used for hybridization (see Fig. 5) that is lacking in both Δ1 and Δ2. Cleavage sites: Bs, *Bst*EII; Sa, *Sal*I; Sm, *Sma*I.

Both plasmids were tested for β-gal expression in transient assays.

Isolation of stable cell lines. To isolate a cell line that expresses gII(PrV) in a transactivatable fashion, subconfluent Vero cells on 5-cm tissue culture plates were cotransfected with plasmids pBR-gII and pSV2neo (44), using the Lipofectin method (Bethesda Research Laboratories). Two days after transfection, cells were trypsinized and reseeded onto 10-cm plates. Three days after transfection, the medium was changed to selection medium containing geneticin (900 μg/ml; Sigma). The selection medium was replaced daily. Eleven days after transfection, cell colonies became visible. They were picked and seeded into 24-well plates. Seven days after colony isolation, replica wells were analyzed for gII expression. One cell line, N7, was selected and further maintained in medium containing 500 μg of geneticin per ml.

A similar procedure was used for isolation of MDBK cell lines MT3, containing the gII gene, and G1, containing the gI(BHV-1) gene under control of the metallothionein promoter (3) except that the calcium phosphate coprecipitation technique (12) was used for cotransfection of plasmids MT-gII or pMT-gI and pSV2-neo. Cells were maintained in medium containing 700 μg of geneticin per ml. Cell clones were tested for gII expression as follows. Vero cell transformants cotransfected with pSV2neo and pBR-gII were superinfected with HSV-1 at a multiplicity of infection (MOI) of 5. After 24 h, monolayers were fixed in 3% paraformaldehyde for 20 min and 3% paraformaldehyde–0.1% Triton X-100 for 10 min, and immunofluorescence analyses were performed by using gII(PrV)-specific antibody 5/14 (23). As a control, an anti-gB(HSV-1) antibody II-105-2 (kindly provided by R. Braun, Heidelberg, Germany) was used. Clones that were clearly positive for gII expression were selected for further experiments.

MDBK cells transfected with MT-gII or pMT-gI(BHV-1) were analyzed with or without induction of the metallothionein promoter with 100 μM $ZnSO_4$. Seven hours after induction, the $ZnSO_4$-containing medium was replaced with medium supplemented with 10% fetal bovine serum. After an additional 20 to 24 h, immunofluorescence assays were performed.

Isolation of gII⁻ PrV. Plasmid pBRgII-Δ1 or pBRgII-Δ2 (Fig. 2) were cotransfected with purified PrV DNA by calcium phosphate coprecipitation into Vero line N7 or MDBK line MT3. After cells showed complete cytophatic effect (CPE), supernatants were harvested. Viral progeny was serially diluted, plated onto N7 or MT3 monolayers, and incubated under a methylcellulose overlay for 2 days until plaques became clearly visible. The methylcellulose overlay was then removed, and cells were overlaid with 1% agarose in Eagle minimal essential medium containing Bluo-Gal (300 μg/ml; Bethesda Research Laboratories). After 24 h blue plaques became visible. They were picked by aspiration of the agar plug above the plaque and either used to inoculate fresh complementing cells at once or incubated in 0.6 ml of Eagle minimal essential medium at 4°C for 4 h and replated after serial dilution. Four plaque purifications were performed until all plaques stained blue under the Bluo-Gal overlay.

DNA isolation and Southern blot hybridization. To analyze copy numbers of the introduced gII genes in cell lines N7 and MT3, whole-cell DNA was obtained by phenol extraction, using standard procedures (24). PrV DNA was isolated from purified virions as described previously (2).

DNA was digested with *Bam*HI, *Sal*I, or *Bst*EII, and fragments were separated in a 0.8% agarose gel. After transfer onto nitrocellulose filters, hybridizations were performed in 50% formamide–1% sodium dodecyl sulfate

(SDS)–500 μg of denatured salmon sperm DNA per ml–0.5% low-fat milk–4× SSPE (0.18 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) at 52°C for 24 h with ^{32}P-labeled probes obtained by either nick translation or multipriming (Amersham, Braunschweig, Federal Republic of Germany). For detection of gII sequences in the cell lines, 7% dextran sulfate was added to the hybridization solution. The blots were then washed for 30 min at room temperature in 2× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS, for 45 min at 72°C in the same buffer, and for 30 min in 0.1× SSC–0.1% SDS at 72°C. Filters were dried briefly, covered in Saran Wrap, and exposed to Kodak XAR-5 X-ray film.

^{35}S labeling of viral proteins and immunoprecipitation. To label viral proteins, monolayers were infected at an MOI of 5 and incubated for 1 h at 37°C. Thereafter, the inoculum was removed and the cells were overlaid with fresh medium. Five hours postinfection, the medium was replaced by medium without methionine. After additional 1 h of incubation at 37°C, [^{35}S]methionine (50 μCi/ml; Amersham) was added, and the cells were incubated for 24 to 36 h at 37°C. Cell extracts were prepared (23), sonicated, and clarified by centrifugation, and aliquots were reacted with the respective antibodies. Labeled virions were purified after infected cell supernatants were harvested and clarified by low-speed centrifugation. Virions were then purified by centrifugation through a 30% sucrose cushion (2); pelleted virions were resuspended in 200 μl of Eagle minimal essential medium and subsequently lysed (23). Immunoprecipitations were done essentially as described elsewhere (23), using fixed *Staphylococcus aureus* cells (19). MAbs against different glycoproteins were kindly provided by H.-J. Rziha, Tübingen, Federal Republic of Germany (anti-gI 3/6 and anti-gII 5/14; 23), T. Ben-Porat, Nashville, Tenn. (anti-gIII M1; 13), and M. Wathen, Kalamazoo, Mich. (anti-gp50 MCA50-1; 49).

Precipitates were separated on discontinuous 10 or 7.5% SDS–polyacrylamide gels (20, 23). Gels were incubated with En^{3}Hance (DuPont Biochemicals), dried, and exposed to Kodak XAR-5 X-ray film.

Neutralization tests. Virus (approximately 500 PFU per well) was mixed with appropriate dilutions of ascitic fluid of the MAbs inactivated at 56°C for 30 min before use. After the addition of 5% normal rabbit serum as a source of complement, the mixture was incubated for 1 h at 37°C and used to inoculate monolayer cells in six-well tissue culture plates.

After 1 h at 37°C, the inoculum was aspirated and the cells were overlaid with semisolid methylcellulose medium. After 2 to 3 days, monolayers were fixed with 5% formaldehyde and stained with 1% crystal violet. Neutralization was assessed as percent plaque reduction compared with that of controls incubated with similar concentrations of a non-PrV-specific control MAb.

Electron microscopy. Confluent monolayers were infected with the respective viruses (MOI = 10), and the supernatant was harvested after complete CPE had been induced. Virions were purified by centrifugation through a 30% sucrose cushion (2), and the pellet was resuspended and recentrifuged in a linear 12 to 52% sucrose gradient. The opalescent virus band was removed by aspiration with a syringe and either used for electron microscopy at once or diluted with 5 volumes of phosphate-buffered saline (PBS), and virions were pelleted by ultracentrifugation. The resuspended virus pellet was then used for electron microscopy. Virus particles were adsorbed to carbon-coated Pioloform films on 400-mesh copper grids for approximately 3 min. The grids were thoroughly washed four times with PBS containing 0.5% bovine serum albumin and 0.2% gelatin (PBG; 47), floated on drops of MAbs (ascitic fluid diluted 1:1,000 in PBG), and incubated for 45 min at 20°C. After further washings with PBG, they were then immersed in a drop of gold-tagged goat anti-mouse immunoglobulins G plus M (15-nm gold; GAF 15 BioCell, Cardiff, United Kingdom) for 45 min at 20°C. Grids were again washed and finally negatively stained with a 1% unbuffered uranyl acetate solution.

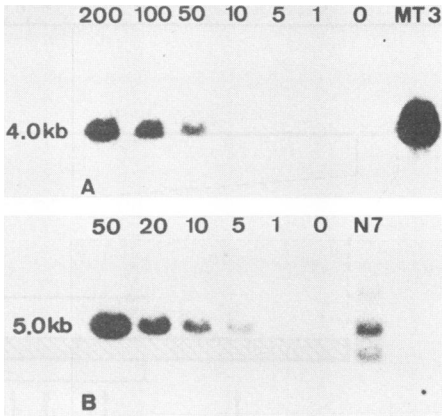

FIG. 3. Analysis of the genomic gII gene in cell lines MT3 and N7. DNA was isolated from cell lines MT3 (A) and N7 (B) and cleaved with either *Bam*HI-*Eco*RI (A) or *Bam*HI-*Hin*dIII (B) to release gII inserts of 4.0 and 5.0 kb, respectively (See Fig. 1). For comparison, either MDBK (A) or Vero (B) cell DNA was mixed with the indicated amounts of genome copies of the respective plasmids and cleaved accordingly. After separation in a 0.8% agarose gel, Southern hybridization with a gII-specific probe was performed.

RESULTS

Construction of cell lines expressing gII(PrV). Since the homolog of gII(PrV) in HSV, gB, has been shown to be essential for viral growth (5, 6, 8, 14, 15, 22), we started by constructing cell lines that express gII and should be able to complement gII$^-$ PrV mutants. Vero cells were cotransfected with plasmid pBR-gII, which contains a genomic PrV DNA fragment encompassing the complete gII transcription unit (Fig. 1) and plasmid pSV2neo containing the bacterial neomycin resistance gene under control of the simian virus 40 early promoter (44). Transformants were selected by their ability to grow in medium containing geneticin at 900 μg/ml. Expression of the gII gene was monitored in immunofluorescence analyses, using a gII-specific antibody, after infection of selected clones with HSV-1 which transactivates the resident gII gene. One clone, designated N7, that showed the brightest fluorescence was chosen for further characterization. Southern analyses showed that N7 cells contained approximately 10 copies of intact gII gene per genome (Fig. 3B). These cells expressed gII only when transactivated after infection with HSV, as demonstrated in immunofluorescence assays, Northern (RNA) blot experiments, and radioimmunoprecipitations (data not shown).

In addition, we attempted to establish a cell line that expresses gII under control of an inducible promoter that is not dependent on viral transactivation. To this end, the gII gene was cloned behind the mouse metallothionein promoter in plasmid MT-gII (Fig. 1). Cotransfection of MT-gII and

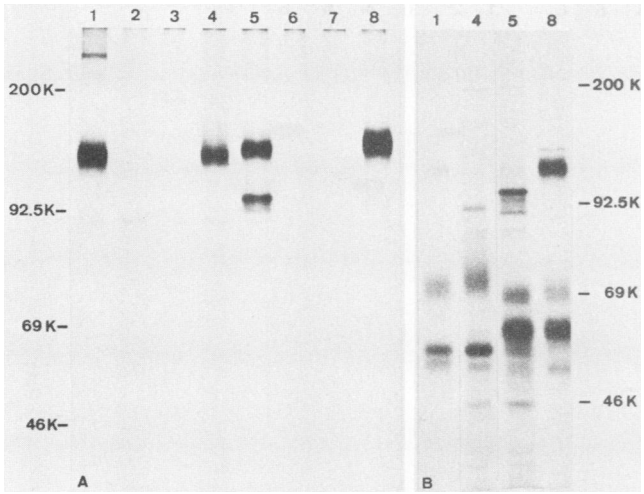

FIG. 4. Analysis of gII(PrV) and gI(BHV-1) expression in cell lines. Proteins of cell lines MT3 (lanes 3 and 5) or G1 (lanes 4 and 6) were labeled with [^{35}S]methionine, and cell extracts were precipitated with either MAb 5/14, directed against gII(PrV) (lanes 5 to 8), or MAb 42-18-7, directed against gI(BHV-1) (lanes 1 to 4). Proteins from a BHV-1-infected cell lysate (lanes 1 and 7) and from a PrV strain Phylaxia-infected cell lysate (lanes 2 and 8) were also precipitated. The ca. 120-kDa band seen in lane 5 most likely represents the gII(PrV) precursor protein (13, 23). Panel A was run under nonreducing conditions, and panel B was run under reducing conditions. The glycoprotein complex was not completely processed in both PrV- and BHV-1-infected cell lysates, as can be seen by the continued presence of a ca. 150-kDa band under reducing conditions (B). K, Kilodaltons.

pSV2neo followed by selection in medium containing 700 μg of geneticin per ml led to the isolation of cell clones that were tested in immunofluorescence with a gII-specific MAb with and without Zn^{2+} induction. Surprisingly, several clones showed similar bright fluorescence under both inducing and noninducing conditions. One of these clones, MT3, was chosen for further investigations. It contains more than 200 copies of the gII gene per cell (Fig. 3A).

gII expression in MT3 cells. To analyze expression of gII(PrV) in the genetically engineered cell line MT3 and of gI(BHV-1) in cell line G1, proteins were labeled metabolically with [^{35}S]methionine, and cell lysates were prepared and immunoprecipitated with either anti-gII MAb 5/14 or anti-gI(BHV-1) MAb 42-18-7 (Fig. 4). MAb 5/14 recognized a protein from MT3 lysates (Fig. 4A, lane 5) that migrated in nonreducing gels similarly to the glycoprotein gII complex precipitated from MDBK cell lysates infected with PrV strain Phylaxia (Fig. 4A, lane 8), the source of the gII gene. The approximately 120-kDa protein band that is prominent in the precipitation from MT3 lysate (Fig. 4A, lane 5) most likely represents the gII precursor protein. MAb 5/14 did not recognize any protein in cell lysates originating from line G1 expressing gI(BHV-1) (Fig. 4A, lane 6) or from lysates of BHV-1-infected MDBK cells (Fig. 4A, lane 7).

In contrast, anti-gI(BHV-1) MAb 42-18-7 recognized the mature gI(BHV-1) complex in BHV-1-infected MDBK cells (Fig. 4A, lane 1) and gI(BHV-1)-expressing cell line G1 (lane 4). As expected, it did not recognize any protein from PrV-infected cells (lane 2) or from line MT3 expressing gII(PrV) (lane 3).

Analysis of precipitates under reducing conditions (Fig. 4B) demonstrates presence of the disulfide-bonded proteolytically processed constituents of the respective complexes. gII(PrV) was cleaved into two subunits of 67 and 58 kDa (Fig. 4B, lanes 5 and 8), whereas gI(BHV-1) consisted of two subunits of 75 and 55 kDa (Fig. 4B, lanes 1 and 4). This result proves that proteolytic processing of gII(PrV) does not involve an essential virus-encoded or virus-induced factor but is achieved by the cellular machinery. The same is true for gI(BHV-1).

Isolation of a gII⁻ PrV mutant. Cotransfections were performed with PrV DNA and plasmid pBRgII-Δ1 or pBRgII-Δ2 (Fig. 2). Progeny was serially diluted, and plaques that stained blue under a Bluo-Gal overlay were picked. Progeny of cotransfections with plasmid pBRgII-Δ1 gave rise to blue-staining plaques that could be enriched until they comprised approximately 30% of the virus suspension. However, they could not be purified to homogeneity despite considerable efforts. We interpret this result in the light of recent findings that a reading frame coding for a protein homologous to a polypeptide called ICP18.5 in HSV overlaps the gII(PrV) ORF for 127 bp (Fig. 2C; 33–35). Deletion Δ1, which encompasses the very 3′ end of this reading frame (Fig. 2C and D), therefore might produce mutants that are impaired in ICP18.5 expression and cannot be complemented by gII alone, since they also lack a functional ICP18.5. This indicates that the ICP18.5 homolog in PrV may be essential for viral growth.

To overcome this problem, cotransfection of PrV DNA and plasmid pBRgII-Δ2 carrying a deletion that removes approximately 1,400 bp of the gII gene but leaves the ICP18.5 gene intact was performed (Fig. 2C and D). Mutant viruses were purified by making use of their blue-plaque phenotype under Bluo-Gal. In this case, the virus could be purified to homogeneity after four rounds of plaque purification. The virus mutant could be propagated on both lines N7 and MT3, indicating that both are able to complement the gII⁻ PrV mutant by providing gII in *trans*.

Genomic characterization of gII⁻ PrV. Homologous recombination of the in vitro-manipulated plasmid carrying a deletion of ca. a 1.4-kb *Bst*EII fragment in the gII gene and a concomitant insertion of the β-gal expression cassette with viral DNA should result in the replacement of the wild-type gII gene with the engineered gII gene. To check whether correct recombination at the expected position had taken place, we isolated DNA from wild-type PrV and gII⁻ PrV. DNA was cleaved with either *Bam*HI, *Sal*I, or *Bst*EII, and fragments were separated in an agarose gel (Fig. 5A) and transferred to nitrocellulose. Replica filters were hybridized with a labeled β-gal-specific probe (Fig. 5C) and labeled 1.2-kb *Bst*EII fragment that should be deleted in gII⁻ PrV (Fig. 5B). As expected, fragment *Bam*HI-1 appeared smaller in the gII⁻ mutant (Fig. 5A, lane 2) than in the PrV wild type (lane 1), and an additional band appeared since the introduction of a *Bam*HI site into *Bam*HI-1 should give rise to two cleavage products (marked by asterisks in Fig. 5A). The labeled *Bst*EII fragment deleted during the cloning reacted only with PrV wild-type DNA, not with the gII⁻ mutant DNA, proving absence of this fragment from the mutant genome (Fig. 5B). Hybridization with the β-gal probe yielded signals only with DNA derived from the gII⁻ mutant (Fig. 5C). These data show the presence of the correct deletion of gII sequences and insertion of the β-gal gene as predicted.

Growth of gII⁻ PrV on different host cells. To determine whether gII is indeed essential for PrV infectivity, we titrated the gII⁻ mutant virus stock on different host cells (Table 1) in two independent experiments. Titers on line

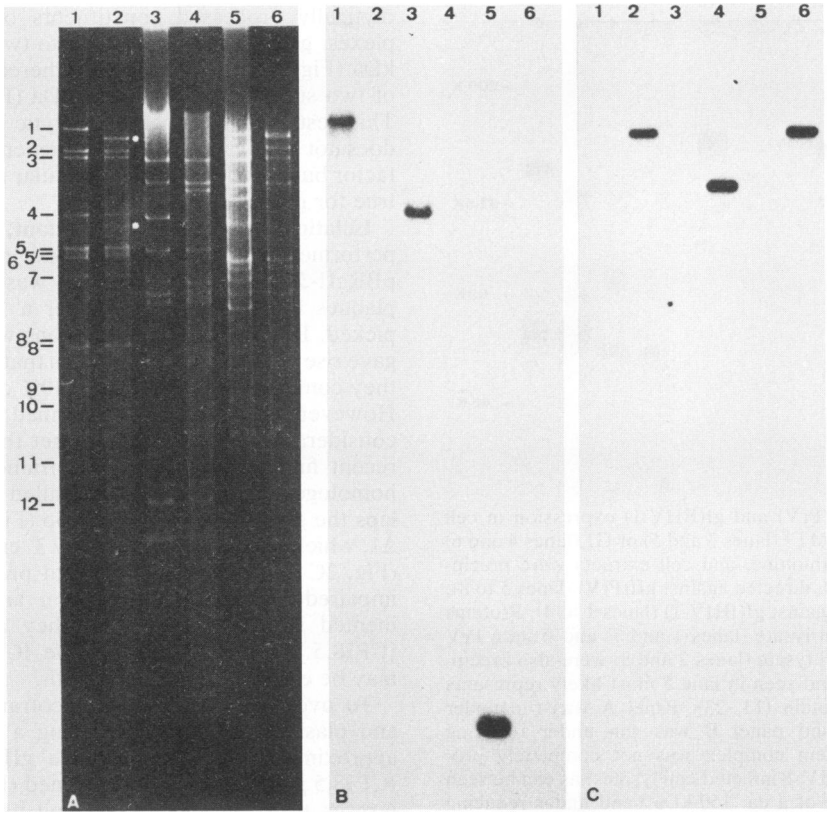

FIG. 5. DNA analysis of gII⁻ PrV. DNA was isolated from wild-type (lanes 1, 3, and 5) or gII⁻ (lanes 2, 4, 6) pseudorabies virions, cleaved with either *Bam*HI (lanes 1 and 2), *Sal*I (lanes 3 and 4), or *Bst*EII (lanes 5 and 6), run in a 0.8% agarose gel, and transferred to nitrocellulose filters. (A) Ethidium bromide-stained gel. The asterisks mark the two bands originating from wild-type fragment *Bam*HI-1 that appear in the gII⁻ mutant as a result of the introduction of a *Bam*HI site into *Bam*HI-1. (B) Result of hybridization with a labeled *Bst*EII 1.2-kb fragment (see Fig. 2). (C) Hybridization with a β-gal-specific probe.

MT3 constitutively expressing gII were in the range of 1×10^7 to 2×10^7 PFU/ml. Titers on line N7 were somewhat lower (ca. 5×10^6 PFU/ml) reflecting the lower plating efficiency of PrV on Vero cells. Titers on noncomplementing MDBK and Vero cells were approximately 10^5-fold lower. On MDBK cells, single plaques could be found in the lowest dilution tested, i.e., 10^{-2}. These plaques stained white under a Bluo-Gal overlay and most likely represent rare recombinants between the gII⁻ viral genome and one of the resident gII genes in the cellular genome. On Vero cells, no viral plaques could be found at the 10^{-2} dilution. These data prove that gII is indeed essential for viral replication and that gII⁻ PrV mutants are able to produce infectious progeny and form plaques only on cell lines providing gII in *trans*.

TABLE 1. Titrations of gII⁻ PrV on different host cells[a]

Expt no.	Titer	
	gII⁻ PrV MT3	gII⁻ PrV G1
1		
MDBK	$<1.0 \times 10^{2}$[b]	$<1.0 \times 10^{2}$[b]
G1	2.7×10^7	1.2×10^7
MT3	2.3×10^7	4.5×10^7
N7	7.7×10^6	2.3×10^6
Vero	$<1.0 \times 10^{2}$[b]	$<1.0 \times 10^{2}$[b]
2		
MDBK	3.0×10^2	$<1.0 \times 10^{2}$[b]
G1	3.0×10^7	1.5×10^7
MT3	1.2×10^7	1.0×10^7
N7	4.6×10^6	4.0×10^6
Vero	$<1.0 \times 10^{2}$[b]	$<1.0 \times 10^{2}$[b]

[a] gII⁻ PrV was propagated on either gII(PrV)-expressing MT3 cells or gI(BHV-1)-expressing G1 cells. Virus stocks were then titrated on MDBK, G1, MT3, N7, or Vero cells. The results of two independent experiments using different virus stocks are presented.

[b] No plaques were found at a 10^{-2} dilution.

At the lowest dilutions, representing an MOI of ca. 2 noncomplementing cells exhibited a pronounced CPE indicating that viral infection occurred, leading to cell death. The supernatant from complementing and noncomplementing cells was titrated on either MT3 or MDBK cells (Table 2). Whereas the titer of gII⁻ PrV grown on MT3 cells and plated onto MT3 cells was as expected, titers of gII⁻ PrV grown on normal MDBK cells were ca. 10^5-fold lower. This result indicates that virus released from noncomplementing cells (see below) and therefore lacking gII is not able to productively infect either complementing or noncomplementing cells. After infection of noncomplementing cells with gII⁻ PrV grown on line MT3, the cells stained uniformly blue under a Bluo-Gal overlay (not shown). Taken together, these data show that gII⁻ PrV grown on complementing cells is able to infect noncomplementing cells, leading to expression of the β-galactosidase gene (under control of the early gX promoter; 28, 38) and resulting in cell death. The released gII⁻ virus particles are, however, noninfectious (Table 2).

TABLE 2. Influence of gII on viral titers on complementing and noncomplementing cells[a]

Expt no.	Titer	
	gII⁻ PrV MT3	gII⁻ PrV MDBK
1		
MT3	1.7×10^7	3.7×10^2
MDBK	2.0×10^2	1.0×10^2
2		
MT3	1.1×10^7	6.0×10^2
MDBK	7.0×10^2	6.0×10^2

[a] Either gII(PrV)-expressing MT3 cells or noncomplementing MDBK cells were infected with gII⁻ PrV grown on MT3 cells at an MOI of 10. After complete CPE was observed, virus supernatants were titrated on either MT3 or MDBK cells. Results of two independent experiments using different virus stocks are presented. gII⁻ PrV grown on noncomplementing cells (gII⁻ PrV MDBK), although containing similar amounts of virions (see Fig. 6 and 8), showed viral titers on either complementing MT3 or noncomplementing MDBK cells that were ca. 10^5-fold lower than titers of gII⁻ PrV grown on complementing cells (gII⁻ PrV MT3).

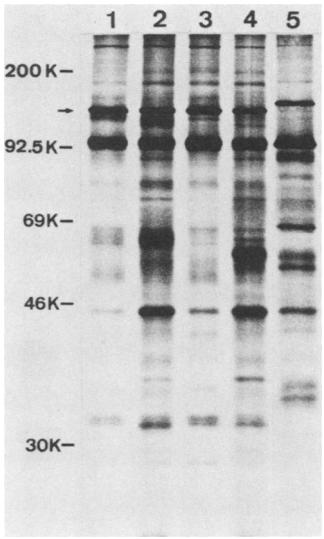

FIG. 6. Analysis of gII⁻ virions grown on different host cells. gII⁻ PrV was grown in [^{35}S]methionine-labeling medium on either MT3 (lane 2), G1 (lane 4), or noncomplementing MDBK (lane 3) cells. As a control, wild-type PrV-infected MDBK cells (lane 1) and BHV-1-infected MDBK cells (lane 5) were used. Extracellular virions were harvested, purified by centrifugation through a 30% sucrose cushion, lysed, and analyzed by 10% SDS-PAGE. The arrow points to the 142-kDa major capsid protein of PrV. K, Kilodaltons.

gI(BHV-1) can complement the gII⁻ PrV mutant. gI(BHV-1) is the homolog of gII(PrV) (30, 53). The two glycoproteins are 73 and 61% homologous on the DNA and amino acid levels, respectively (18a, 30, 40, 53). To analyze whether this sequence homology also reflects functional homology, i.e., whether gI(BHV-1) provided in *trans* could complement gII⁻ PrV mutants, we tried to grow such mutants on the MDBK cell line G1, which constitutively expresses gI(BHV-1). Surprisingly, this cell line also complemented the gII defect in the gII⁻ PrV mutants. When titers of gII⁻ PrV on line MT3 expressing gII(PrV) and line G1 expressing gI(BHV-1) were compared, we found essentially no difference in numbers of plaques; titers were 1×10^7 to 3×10^7 PFU/ml on both MT3 and G1 cells (Table 1). Similar results were obtained with gII⁻ mutant virus stocks grown on either line G1 or line MT3 (Table 1). In conclusion, both cell lines yielded gII⁻ PrV progeny exhibiting similar titers in both MT3 and G1 cells. The gB homolog gI(BHV-1) therefore is able to fully complement the gII defect in the PrV mutants.

gI(BHV-1) is incorporated into the envelope of gII⁻ PrV. Both glycoproteins gI(BHV-1) and gII(PrV) are major constituents of the envelope of the respective viruses (13, 23, 48). Since gI(BHV-1) was able to complement gII⁻ PrV functionally, it should also be incorporated into the PrV envelope. To analyze this, complement-dependent neutralization assays were performed using a panel of antibodies specific for either PrV or BHV-1 (Table 3). PrV was neutralized by MAbs against gI(PrV), gII(PrV), gIII(PrV), and gp50(PrV) but was resistant to neutralization by MAbs against gI(BHV-1) and gIV(BHV-1). In contrast, BHV-1 was resistant to antibodies directed against PrV glycoproteins but was neutralized by anti-gI(BHV-1) and anti-gIV(BHV-1) antibodies. gII⁻ PrV grown on gII-expressing line MT3 behaved exactly like wild-type PrV. It was resistant to anti-BHV-1 antibodies but sensitive to all anti-PrV antibodies. When the gII⁻ PrV mutant was passaged once on gI(BHV-1)-expressing line G1, it lost sensitivity to the anti-gII(PrV) MAb completely. However, it became fully susceptible to neutralization by the anti-gI(BHV-1) antibody. Similar results were obtained irrespective of whether virus titers after neutralization were determined on MT3 or on G1 cells, therefore eliminating the possibility of complement-mediated lysis of virus-infected cells as the factor leading to plaque reduction.

TABLE 3. Neutralization of PrV, gII⁻ PrV, and BHV-1[a]

Virus	MAb specificity						
	C	gI(P)	gII(P)	gIII(P)	gp50(P)	gI(B)	gIV(B)
PrV	0	99	99	99	100	0	0
BHV-1	0	15	0	0	0	100	100
gII⁻ PrV [gII(P)]	0	99	99	99	100	10	0
gII⁻ PrV [gI(B)]	0	99	0	94	100	100	0

[a] Appropriate dilutions (ca. 500 PFU per well) of either PrV, BHV-1, gII⁻ PrV grown on gII(PrV)-expressing line MT3 [gII(P)], and gII⁻ PrV grown on gI(BHV-1)-expressing line G1 [gI(B)] were incubated with ascitic fluid of MAbs against gI(PrV), gII(PrV), gIII(PrV), gp50(PrV), gI(BHV-1), and gIV(BHV-1) and 5% rabbit serum and plated onto MT3 or G1 cells. The amount of antibody added was adjusted to give between 95 and 100% neutralization in the homologous system. Neutralization was assessed as percent plaque reduction compared with that of controls that had been reacted with a non-PrV-specific control MAb (C). The values found after reaction of BHV-1 with anti-gI(PrV) MAb (15%) and gII⁻ PrV [gII(P)] with anti-gI(BHV-1) MAb (10%) are not significant.

Protein analysis of gII⁻ PrV mutants. To analyze the protein composition of gII⁻ PrV grown on either gII(PrV)- or gI(BHV-1)-expressing cell lines or on noncomplementing cell lines, cells were infected at an MOI of 10 and proteins were labeled with [^{35}S]methionine. After cell lysis was complete, virions were purified from the supernatant by centrifugation through a 30% sucrose cushion. Radioactively labeled virions were lysed and subjected to gel electrophoresis. Noncomplementing cell lines released virus particles into the supernatant (Fig. 6, lane 3), similar to cells infected with wild-type PrV (lane 1) or complementing lines MT3 (lane 2) and G1 (lane 4) after infection with gII⁻ PrV. Proteins from purified BHV-1 virions are shown in lane 5.

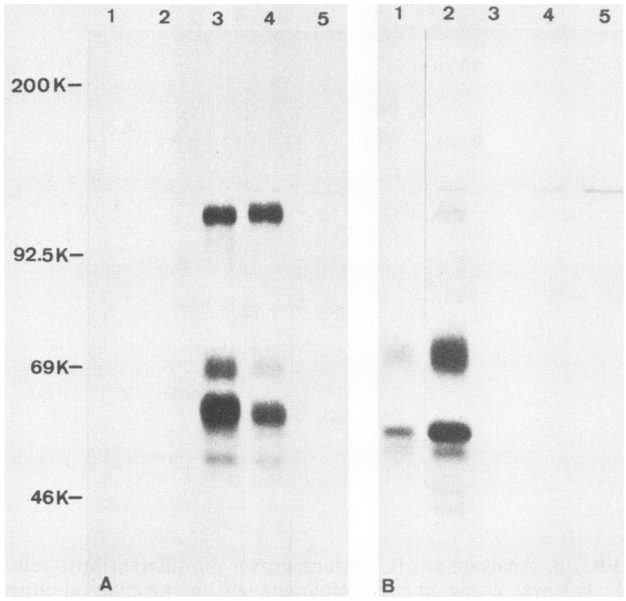

FIG. 7. gII(PrV) or gI(BHV-1) in purified gII$^-$ virions. gII$^-$ PrV obtained as described in the legend to Fig. 6 was immunoprecipitated with either anti-gII(PrV) MAb 5/14 (A) or anti-gI(BHV-1) MAb 42-18-7 (B). Lanes: 1, BHV-1 grown on MDBK cells; 2, gII$^-$ PrV grown on line G1; 3, gII$^-$ PrV grown on line MT3; 4, PrV grown on MDBK cells; 5, gII$^-$ PrV grown on noncomplementing MDBK cells. The precipitates were separated by 7.5% SDS-PAGE under reducing conditions.

When the same material was analyzed in immunoprecipitation using anti-gII or anti-gI(BHV-1) MAbs (Fig. 7), it was found that the virions released from noncomplementing cells lacked gII (Fig. 7A, lane 5) and gI(BHV-1) (Fig. 7B, lane 5). Virions released from wild-type PrV-infected cells (Fig. 7A, lane 4) or MT3 cells (lane 3) exhibited the normal gII. Pseudorabies virions released from G1 cells did not carry gII (Fig. 7A, lane 2) but showed the presence of gI(BHV-1) (Fig. 7B, lane 2). BHV-1 virions, as expected, show the presence of gI(BHV-1) (Fig. 7B, lane 1).

The block in infectivity in the gII$^-$ mutant is therefore apparently due to lack of ability of the mutant virus to further infect target cells and is not accounted for by a defect in virus maturation and release in the primary target cell. These results also lend further evidence for the incorporation of gI(BHV-1) into the envelope of gII$^-$ PrV mutants when grown on gI(BHV-1)-providing target cells.

Electron microscopy of gII$^-$ PrV. To obtain direct proof that gI(BHV-1) is found in the envelope of gII$^-$ PrV grown on gI(BHV-1)-expressing cells, immunoelectron microscopic studies were performed. Sucrose gradient-purified virions were reacted with MAbs against either gII(PrV), gI(PrV), or gI(BHV-1) and subsequently negatively stained with uranyl acetate. gII could be demonstrated in the envelope of wild-type PrV and gII$^-$ PrV grown on gII-expressing line MT3 (Fig. 8, A1 and C1). Reaction with the anti-gI(BHV-1) MAb was not observed (Fig. 8, A3 and C3). In contrast, PrV gII$^-$ grown on gI(BHV-1)-expressing line G1 showed the presence of gI(BHV-1) in the envelope (Fig. 8, D3). No gII(PrV) could be demonstrated (Fig. 8, D1). PrV gII$^-$ mutants grown on noncomplementing MDBK cells did not react with either the anti-gII(PrV) (Fig. 8, B1) or the anti-gI(BHV-1) (Fig. 8, B3) antibody. However, as expected, they stained positive with the anti-gI(PrV) MAb (Fig. 8, B2). As expected, all PrV isolates were found positive when tested with MAb against gI(PrV) (Fig. 8, row 2).

DISCUSSION

The gII(PrV) complex belongs to a group of homologous glycoproteins that have been found in all herpesviruses studied thus far (4, 8, 22, 23, 25, 30, 31, 39, 40, 42, 45, 46, 50, 53). These glycoproteins show similarities in DNA and amino acid sequences to gB(HSV). All gB-homologous glycoproteins except gB(HSV) consist of disulfide-linked heterodimeric complexes (4, 10, 13, 23, 25, 31, 45, 48). The two smaller subunits of the complex have been shown to be derived by proteolytic cleavage from a common primary translation product (13, 23, 27, 51). The gB homologs are major constituents of the viral envelope in the respective viruses and have been shown to induce protective immunity (48).

Mature gB(HSV) is found in the viral envelope in the form of disulfide-bonded homodimers (7). In contrast to many gB-homologous glycoproteins in other herpesviruses, however, gB(HSV) is not processed proteolytically. The enzymatic machinery executing the proteolytic cleavage of most of the gB homologs is still largely unknown. Recently, it has been shown in studies on gII(PrV) that a protease located in the Golgi apparatus is responsible for gII processing (52). However, these studies were performed on gII expressed during PrV infection, and possible influences of viral infection on the host cell properties cannot be excluded. Our results with cell line MT3 clearly show that processing of gII is not dependent on PrV infection but can correctly be performed by cellular enzymes. We found no difference in either proteolytic processing or carbohydrate addition, as judged by the identical appearance of the different gII proteins after radioimmunoprecipitation in SDS-polyacrylamide gel electrophoresis (PAGE). Line MT3 therefore appears to be useful for additional studies of gII processing in the absence of a viral infection, i.e., without possible CPE or interference by viral host cell shutoff.

Line N7, which contains the gII gene in a transactivatable fashion, was constructed to minimize possible cytotoxic effects of the expressed glycoprotein. These effects have been described after expression of glycoproteins of other herpesviruses, e.g., Epstein-Barr virus (32). However, both line MT3 constitutively expressing gII(PrV) as well as line G1 constitutively expressing gI(BHV-1) grew normally and did not exhibit signs of CPE.

To obtain a gII$^-$ PrV mutant, two plasmids carrying two different deletions were constructed. Only cotransfections with plasmid pBRgII-Δ2 yielded virus progeny that could be purified to homogeneity. Deletion Δ2 resides exclusively in the gII gene and does not impair other ORFs. Cotransfections with pBRgII-Δ1 yielded blue plaques under a Bluo-Gal overlay during the first cycle of plaque purification. However, these mutant viruses could not be purified to homogeneity. The most likely explanation for this phenomenon is that deletion Δ1 or insertion of the β-gal cassette impairs expression of an essential PrV gene that is not complemented by the gII-expressing cell lines. It has recently been shown that an ORF coding for a protein that has been designated ICP18.5 in HSV overlaps at its 3′ end the 5′ part of the gII gene by 127 bases (33–35). Deletion Δ1 removes the C-terminal 31 codons of this reading frame. The data indicate that Δ1 or insertion of the β-gal cassette leads to

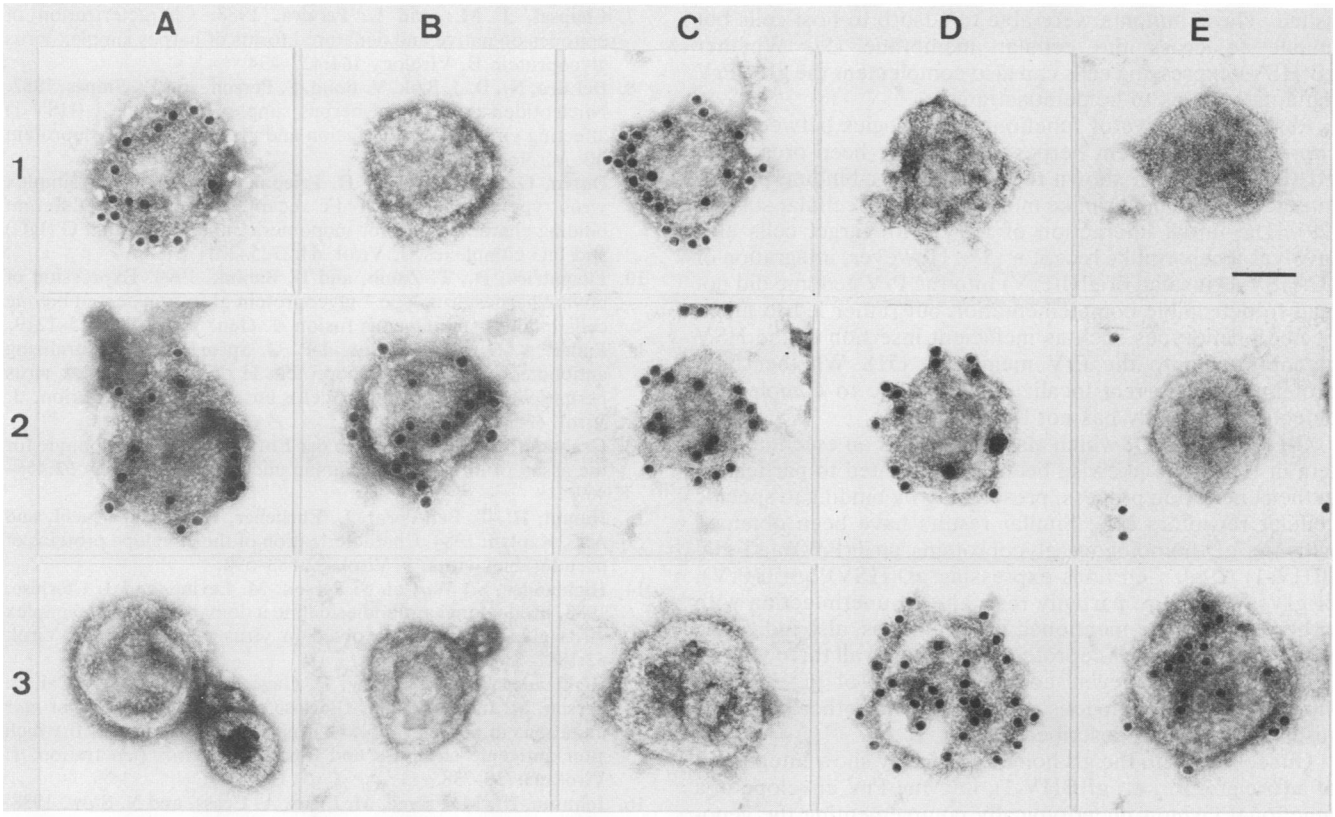

FIG. 8. Immunoelectron microscopy of gII⁻ PrV virions. Wild-type PrV grown on MDBK cells (A), gII⁻ PrV grown on MDBK cells (B), gII⁻ PrV grown on MT3 cells (C), gII⁻ PrV grown on G1 cells (D), and BHV-1 grown on MDBK cells (E) were purified by sucrose gradient centrifugation and reacted with MAbs against gII(PrV) (row 1), gI(PrV) (row 2), and gI(BHV-1) (row 3). After incubation with a secondary gold-labeled antibody, virions were negatively stained with uranyl acetate. Bar, 100 nm.

impairment of expression of both ICP18.5 and gII. Since only the gII defect can be overcome on our complementing cell lines, we reason that ICP18.5 is essential for PrV growth and that deletion of the carboxy-terminal 31 amino acids or a possible fusion with the β-gal cassette interferes with its proper function. It should be noted that ICP18.5 is regarded as an essential protein in HSV (33).

The fact that the gII⁻ PrV mutant carrying deletion Δ2 replicated on gII-complementing cells to almost normal titers but was not able to produce infectious progeny and plaques on noncomplementing cells clearly shows that glycoprotein gII of PrV is essential for viral infectivity. Rarely, however, plaques were observed on noncomplementing cells at the lowest dilutions tested. These plaques exhibited a white-plaque phenotype when incubated under Bluo-Gal and therefore most likely are generated by viruses in which the gII mutation has been rescued by the resident gII gene in the host cell. These viruses usually were found at a frequency of less than 10^{-5}.

That gII of PrV is indeed essential for viral infectivity is shown by the fact that after having been propagated on complementing cells, gII⁻ mutants are able to infect noncomplementing target cells. At high MOI, typical CPE is observed and the monolayer turns blue under a Bluo-Gal overlay, indicating that at least the gX–β-gal fusion gene is expressed. Supernatants from those cells contain virus particles that appear normal in the electron microscope. However, they do not contain gII. These viruses are no longer infectious, as shown by the absence of any CPE or blue-staining cells when they were used to infect cells even at high particle/cell ratios. Preliminary data indicate that virions lacking gII are able to adsorb to target cells but are deficient in penetration (37a). Similar results have been obtained for HSV, indicating a common functional role for these two gB homologs (5, 14, 15).

To analyze whether gB-homologous proteins of different herpesviruses not only share a functional role but are functionally homologous in the sense that they can complement defects in the heterologous virus, we used a cell line that constitutively expresses the gB homolog of BHV-1, gI(BHV-1).

Surprisingly, the gII⁻ PrV mutant was able to grow to the same extent on the gI(BHV-1)-expressing line as on the gII(PrV)-expressing line, as assessed by plaque formation and virus production. gII(PrV) and gI(BHV-1) share significant DNA and amino acid homologies of 73 and 61%, respectively. These values are in the range found for other herpesvirus gB proteins. For example, gII(PrV) and gB(HSV-1) are 62% homologous on the DNA level and 50% homologous on the amino acid level (40, 52). Our data indicate either that the nonhomologous regions in these glycoproteins are not involved in the functional role or that amino acid homology in these regions is not required to provide functional complementation. Analysis of temperature-sensitive mutants revealed an essential role of gB in penetration of the virus into cells (22). Genetically engineered virus mutants lacking gB could be isolated after complementing cell lines providing gB in *trans* were estab-

lished. These mutants were able to adsorb to host cells but unable to cross the cellular membrane (5). Whether gB(HSV)-expressing cells can also complement the gII$^-$ PrV mutants remains to be demonstrated.

Results indicative of functional homologies between glycoproteins of different herpesviruses have been presented. gIII(PrV) has been shown to be a receptor-binding protein interacting with heparinlike molecules on the cellular surface (29). The initial interaction of HSV with target cells also involves a heparinlike receptor (55). However, integration of gC(HSV-1) instead of gIII(PrV) into the PrV genome did not lead to detectable complementation but rather led to unexpected phenotypes such as inefficient insertion of the HSV glycoprotein into the PrV membrane (51). Whether both proteins after correct localization are able to complement defects reciprocally has not been shown.

Glycoprotein gD, which also constitutes an essential protein in HSV, has likewise been demonstrated to participate in the penetration process, presumably by binding to specific cellular receptors (21). Similar results have been obtained with the gD-homologous glycoproteins gp50(PrV) and gIV (BHV-1) (29a). Cell lines expressing gD(HSV), gp50(PrV), or gIV(BHV-1) are partially resistant to superinfection with either of the above-mentioned herpesviruses, also indicating a function of these glycoproteins common to all three viruses (17, 18a, 36). However, complementation of gD-deficient viruses by gD-homologous glycoproteins of other herpesviruses has not been described.

Our results with the gB homologs clearly show integration of a foreign gB, i.e., gI(BHV-1), into the PrV envelope in a functional fashion phenotypically complementing the genotypic gII defect in the PrV mutants. We are now attempting to isolate a PrV recombinant carrying the gI(BHV-1) gene instead of the gII gene in the viral genome, i.e., to construct a chimeric virus with the gI(BHV-1) gene replacing the gII(PrV) gene. This recombinant should further help to elucidate the function of both glycoproteins and to assess their roles in in vivo infection.

ACKNOWLEDGMENTS

This investigation was supported by research grant Me 854/2-2 from the Deutsche Forschungsgemeinschaft and by grant 0319131A from the Bundesministerium für Forschung und Technologie. The technical assistance of H. Kern and A. Straubinger is gratefully acknowledged.

REFERENCES

1. **Bell, S., M. Cranage, L. Borysiewicz, and T. Minson.** 1990. Induction of immunoglobulin G Fc receptors by recombinant vaccinia viruses expressing glycoproteins E and I of herpes simplex virus type 1. J. Virol. **64**:2181–2186.
2. **Ben-Porat, T., J. DeMarchi, and A. S. Kaplan.** 1974. Characterization of defective interfering viral particles present in a population of pseudorabies virions. Virology **60**:29–37.
3. **Brinster, R. L., H. Y. Chen, M. Trumbauer, A. W. Senear, R. Warren, and R. D. Palmiter.** 1981. Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. Cell **27**:223–231.
4. **Britt, W. J., and L. Vugler.** 1989. Processing of the gp55-116 envelope glycoprotein complex (gB) of human cytomegalovirus. J. Virol. **63**:403–410.
5. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. **62**:2596–2604.
6. **Cai, W., S. Person, S. Warner, J. Zhou, and N. DeLuca.** 1987. Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. J. Virol. **61**:714–721.
7. **Chapsal, J. M., and L. Pereira.** 1988. Characterization of epitopes on native and denatured forms of herpes simplex virus glycoprotein B. Virology **164**:427–434.
8. **DeLuca, N., D. J. Bzik, V. Bond, S. Person, and W. Snipes.** 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion and production of glycoprotein gB. Virology **122**:411–423.
9. **Dubin, G., I. Frank, and H. Friedman.** 1990. Herpes simplex virus type 1 encodes two Fc receptors which have different binding characteristics for monomeric immunoglobulin G (IgG) and IgG complexes. J. Virol. **64**:2725–2731.
10. **Fitzpatrick, D., T. Zamb, and L. Babiuk.** 1990. Expression of bovine herpesvirus type 1 glycoprotein gI in transfected bovine cells induces spontaneous fusion. J. Gen. Virol. **71**:1215–1219.
11. **Fuller, A. O., R. Santos, and P. G. Spear.** 1989. Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. J. Virol. **63**:3435–3443.
12. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus. Virology **52**:456–467.
13. **Hampl, H., T. Ben-Porat, L. Ehrlicher, K.-O. Habermehl, and A. S. Kaplan.** 1984. Characterization of the envelope proteins of pseudorabies virus. J. Virol. **52**:583–590.
14. **Highlander, S., W. Cai, S. Person, M. Levine, and J. Glorioso.** 1988. Monoclonal antibodies define a domain on herpes simplex virus glycoprotein B involved in virus penetration. J. Virol. **62**:1881–1888.
15. **Highlander, S., D. Dorney, P. Gage, T. Holland, W. Cai, S. Person, M. Levine, and J. Glorioso.** 1989. Identification of *mar* mutations in herpes simplex virus type 1 glycoprotein B which alter antigenic structure and function in virus penetration. J. Virol. **63**:730–738.
16. **Johnson, D., M. Frame, M. Ligas, A. Cross, and N. Stow.** 1988. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J. Virol. **62**:1347–1354.
17. **Johnson, D., and P. G. Spear.** 1989. Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. J. Virol. **63**:819–827.
18. **Kaplan, A. S., and A. E. Vatter.** 1959. A comparison of herpes simplex and pseudorabies viruses. Virology **7**:394–407.
18a. **Keil, G.** Unpublished data.
19. **Kessler, S. W.** 1981. Use of protein A-bearing staphylococci for the immune precipitation and isolation of antigens from cells. Methods Enzymol. **73**:442–459.
19a. **Klupp, B., and T. C. Mettenleiter.** Unpublished data.
20. **Laemmli, U. K.** 1975. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Ligas, M. W., and D. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62**:1486–1494.
22. **Little, S. P., J. Jofre, R. Courtney, and P. A. Schaffer.** 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. Virology **115**:149–160.
23. **Lukács, N., H.-J. Thiel, T. C. Mettenleiter, and H.-J. Rziha.** 1985. Demonstration of three major species of pseudorabies virus glycoproteins and identification of a disulfide-linked glycoprotein complex. J. Virol. **53**:166–173.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Meredith, D. M., J.-M. Stocks, G. R. Whittaker, I. W. Halliburton, B. W. Snowden, and R. A. Killington.** 1989. Identification of the gB homologues of equine herpesvirus types 1 and 4 as disulphide-linked heterodimers and their characterization using monoclonal antibodies. J. Gen. Virol. **70**:1161–1172.
26. **Mettenleiter, T. C.** 1989. Glycoprotein gIII deletion mutants of pseudorabies virus are impaired in virus entry. Virology **171**: 623–625.
27. **Mettenleiter, T. C., N. Lukács, H.-J. Thiel, C. Schreurs, and**

H.-J. Rziha. 1986. Location of the structural gene of pseudorabies virus glycoprotein complex gII. Virology 152:66–75.
28. Mettenleiter, T. C., and I. Rauh. 1990. A glycoprotein gX-β-galactosidase fusion gene as insertional marker for rapid identification of pseudorabies virus mutants. J. Virol. Methods. 30:55–66.
29. Mettenleiter, T. C., L. Zsak, F. Zuckermann, N. Sugg, H. Kern, and T. Ben-Porat. 1990. Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. J. Virol. 64:278–286.
29a. Misera, S., and T. C. Mettenleiter. Unpublished data.
30. Misra, V., R. Nelson, and M. Smith. 1988. Sequence of a bovine herpesvirus type 1 glycoprotein gene that is homologous to the herpes simplex gene for the glycoprotein gB. Virology 166:542–549.
31. Montalvo, E. A., and C. Grose. 1987. Assembly and processing of the disulfide-linked varicella-zoster virus glycoprotein gpII (140). J. Virol. 61:2877–2884.
32. Motz, M., G. Deby, and H. Wolf. 1987. Truncated versions of the two major Epstein-Barr viral glycoproteins (gp250/350) are secreted by recombinant Chinese hamster ovary cells. Gene 58:148–154.
33. Pancake, B. A., D. Aschman, and P. Schaffer. 1983. Genetic and phenotypic characterization of herpes simplex virus type 1 mutants conditionally resistant to immune cytolysis. J. Virol. 47:568–585.
34. Pederson, N. E., and L. W. Enquist. 1989. The nucleotide sequence of a pseudorabies virus gene similar to ICP18.5 of herpes simplex virus type 1. Nucleic Acids Res. 17:3597.
35. Pellett, P. E., F. Jenkins, M. Ackermann, M. Sarmiento, and B. Roizman. 1986. Transcription initiation sites and nucleotide sequence of a herpes simplex virus 1 gene conserved in the Epstein-Barr virus genome and reported to affect the transport of viral glycoproteins. J. Virol. 60:1134–1140.
35a. Petrovskis, E. A., P. M. Duffus, D. R. Thomsen, A. L. Meyer, and L. E. Post. 1988. Sequence of pseudorabies virus and infectious bovine rhinotracheitis virus glycoprotein gH genes, p. 217. Abstr. 13th Int. Herpesvirus Workshop.
36. Petrovskis, E. A., A. L. Meyer, and L. E. Post. 1988. Reduced yield of infectious pseudorabies virus and herpes simplex virus from cell lines producing viral glycoprotein gp50. J. Virol. 62:2196–2199.
37. Petrovskis, E. A., J. G. Timmins, and L. E. Post. 1986. Use of λgt11 to isolate genes for two pseudorabies virus glycoproteins with homology to herpes simplex virus and varicella-zoster virus glycoproteins. J. Virol. 60:185–193.
37a. Rauh, I., and T. C. Mettenleiter. Unpublished data.
38. Rea, T. J., J. G. Timmins, G. W. Long, and L. E. Post. 1985. Mapping and sequence of the gene for the pseudorabies virus glycoprotein which accumulates in the medium of infected cells. J. Virol. 54:21–29.
39. Riggio, M. P., A. Cullinane, and D. Onions. 1989. Identification and nucleotide sequence of the glycoprotein gB gene of equine herpesvirus 4. J. Virol. 63:1123–1133.
40. Robbins, A. K., D. Dorney, M. W. Wathen, M. E. Whealy, C. Gold, R. J. Watson, L. E. Holland, S. D. Weed, M. Levine, J. Glorioso, and L. W. Enquist. 1987. The pseudorabies virus gII gene is closely related to the gB glycoprotein genes of herpes simplex virus. J. Virol. 61:2691–2701.
41. Robbins, A. K., R. J. Watson, M. E. Whealy, W. W. Hays, and L. W. Enquist. 1986. Characterization of a pseudorabies virus glycoprotein gene with homology to herpes simplex virus type 1 and type 2 glycoprotein C. J. Virol. 58:339–347.
42. Ross, L. N., M. Sanderson, S. D. Scott, M. M. Binns, T. Doel, and B. Milne. 1989. Nucleotide sequence and characterization of the Marek's disease virus homologue of glycoprotein gB of herpes simplex virus. J. Gen. Virol. 70:1789–1804.
43. Schreurs, C., T. C. Mettenleiter, F. Zuckermann, N. Sugg, and T. Ben-Porat. 1988. Glycoprotein gIII of pseudorabies virus is multifunctional. J. Virol. 62:2251–2257.
43a. Simon, A. 1989. Ph.D. thesis. University of Tübingen, Tübingen, Federal Republic of Germany.
44. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 region promoter. J. Mol. Appl. Gen. 1:327–341.
45. Spaete, R., A. Saxena, P. Scott, G. Song, W. Probert, W. Britt, W. Gibson, L. Rasmussen, and C. Pachl. 1990. Sequence requirements for proteolytic processing of glycoprotein B of human cytomegalovirus strain Towne. J. Virol. 64:2922–2931.
46. Stannard, L. M., J. Rider, and G. H. Farrar. 1989. Morphology and distribution of gp52 on extracellular human cytomegalovirus (HCMV) supports biochemical evidence that it represents the HCMV glycoprotein B. J. Gen. Virol. 70:1553–1560.
47. van Bergen en Henegouwen, P. M. P., and J. L. M. Leunissen. 1986. Controlled growth of colloidal gold particles and implications for labelling efficiency. Histochemistry 85:81–87.
48. van Drunen Littel-van den Hurk, S., T. Zamb, and L. Babiuk. 1989. Synthesis, cellular location, and immunogenicity of bovine herpesvirus 1 glycoproteins gI and gIII expressed by recombinant vaccinia virus. J. Virol. 63:2159–2168.
49. Wathen, M. W., and L. M. K. Wathen. 1984. Isolation, characterization, and physical mapping of a pseudorabies virus mutant containing antigenically altered gp50. J. Virol. 51:57–62.
50. Whalley, J. M., G. Robertson, N. Scott, G. Hudson, C. Bell, and L. Woodworth. 1989. Identification and nucleotide sequence of a gene in equine herpesvirus 1 analogous to the herpes simplex virus gene encoding the major envelope glycoprotein gB. J. Gen. Virol. 70:383–394.
51. Whealy, M. E., A. K. Robbins, and L. W. Enquist. 1989. Replacement of the pseudorabies virus glycoprotein gIII gene with its postulated homolog, the glycoprotein gC gene of herpes simplex virus type 1. J. Virol. 63:4055–4059.
52. Whealy, M. E., A. K. Robbins, and L. W. Enquist. 1990. The export pathway of the pseudorabies virus gB homolog gII involves oligomer formation in the endoplasmic reticulum and protease processing in the Golgi apparatus. J. Virol. 64:1946–1955.
53. Whitbeck, J. C., L. Bello, and W. Lawrence. 1988. Comparison of the bovine herpesvirus 1 gI gene and the herpes simplex virus type 1 gB gene. J. Virol. 62:3319–3327.
54. Wölfer, U., V. Kruft, D. Sawitzky, H. Hampl, B. Wittmann-Liebold, and K.-O. Habermehl. 1990. Processing of pseudorabies virus glycoprotein gII. J. Virol. 64:3122–3125.
55. WuDunn, D., and P. G. Spear. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. 63:52–58.
56. Zsak, L., T. C. Mettenleiter, N. Sugg, and T. Ben-Porat. 1989. Release of pseudorabies virus from infected cells is controlled by several viral functions and is modulated by cellular components. J. Virol. 63:5475–5477.
57. Zuckermann, F., T. C. Mettenleiter, C. Schreurs, N. Sugg, and T. Ben-Porat. 1988. Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. J. Virol. 62:4622–4626.
58. Zuckermann, F., L. Zsak, L. Reilly, N. Sugg, and T. Ben-Porat. 1989. Early interactions of pseudorabies virus with host cells: functions of glycoprotein gIII. J. Virol. 63:3323–3329.