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Stable Rescue of a Glycoprotein gII Deletion Mutant of Pseudorabies Virus by Glycoprotein gI of Bovine Herpesvirus 1

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Glycoproteins homologous to glycoprotein B (gB) of herpes simplex virus constitute the most highly conserved group of herpesvirus glycoproteins. This strong conservation of amino acid sequences might be indicative of a common functional role. Indeed, gB homologs have been implicated in the processes of viral entry and virus-mediated cell-cell fusion. Recently, we showed that pseudorabies virus (PrV) lacking the essential gB-homologous glycoprotein gII could be propagated on a cell line expressing the gB homolog of bovine herpesvirus 1, gI(BHV-1), leading to a phenotypic complementation of the gII defect (I. Rauh, F. Weiland, F. Fehler, G. Keil, and T. C. Mettenleiter, J. Virol. 65:621–631, 1991). However, this pseudotypic virus could still replicate only on complementing cell lines, thereby limiting experimental approaches to analyze the effects of the gB exchange in detail. We describe here the construction and isolation of a PrV recombinant, 9112C2, that lacks gII(PrV) but instead stably carries and expresses the gene encoding gI(BHV-1). The recombinant is able to replicate on noncomplementing cells with growth kinetics and final titers similar to those of its gII-positive wild-type PrV parent. Neutralization tests and immunoprecipitation analyses demonstrated incorporation of gI(BHV-1) into 9112C2 virions with concomitant absence of gII(PrV). Analysis of in vitro host ranges of wild-type PrV, BHV-1, and recombinant 9112C2 showed that in cells of pig, rabbit, canine, monkey, or human origin, the plating efficiency of 9112C2 was similar to that of its PrV parent. Exchange of gII(PrV) for gI(BHV-1) in recombinant 9112C2 or by phenotypic complementation of gII$^-$ PrV propagated on gI(BHV-1)-expressing cell lines resulted in penetration kinetics intermediate between those of wild-type PrV and BHV-1. In conclusion, we report the first isolation of a viral recombinant in which a lethal glycoprotein mutation has been rescued by a homologous glycoprotein of a different herpesvirus. Our data show that in gII$^-$ PrV, gI(BHV-1) in vitro fully complements the lethal defect associated with lack of gII(PrV). These results conclusively demonstrate that gI(BHV-1) in a PrV background can execute all essential functions normally provided by gII(PrV). They also indicate that the origin of gB-homologous glycoproteins influences the penetration kinetics of herpesviruses.

Herpesvirus glycoproteins that are present in the virus envelope are known to mediate several important steps in virus-host cell interaction (44). They are involved in virus adsorption to target cells (15, 33, 36, 43), are essential for penetration (6, 7, 9, 11, 16, 23, 26, 38), and also influence virus release from infected cells (32, 43, 48, 51). Most notably, all essential herpesvirus glycoproteins analyzed so far are involved in fusion events between either virus envelope and cellular cytoplasmic membrane or between cytoplasmic membranes of infected and adjacent uninfected cells (10, 26, 45). By virtue of their involvement in the first steps of virus infection, glycoproteins appear important in determining whether virions are able to enter target cells and initiate a productive infection.

In herpes simplex virus (HSV), 10 glycoproteins have been identified. Glycoproteins gB, gD, gH, and probably gK and gL, appear essential for virus replication since virus mutants deficient in any of these glycoproteins are unable to replicate (6, 11, 17, 23). Essential glycoproteins homologous to gB, gD, and gH have also been characterized in pseudorabies virus (PrV) (gII, gp50, and gH) (14, 20, 25, 30, 37, 41, 46) and bovine herpesvirus type 1 (BHV-1) (gI, gIV, and gH) (9, 13, 21, 34, 50). The conservation of amino acid sequence and probably also higher-order structure might be indicative of common functions of these glycoproteins in their respective viruses. Indeed, it has been shown that the gB-homologous glycoproteins which exhibit the highest degree of sequence conservation are essential for virus penetration and cell-cell spread (6, 7, 38). Glycoproteins homologous to gD(HSV) are also involved in viral entry and cell-cell fusion (9, 16, 23, 38). The gC-homologous glycoproteins gIII(PrV), gC(HSV), and gIII(BHV-1) all bind to a cellular heparinlike receptor, thereby mediating adsorption of the virus to target cells (15, 33, 36). In initial studies aimed at analyzing whether homologous glycoproteins also function in a heterologous viral background, the nonessential gIII(PrV) gene was replaced by the gene encoding its homolog gC(HSV). Although expression of gC from the recombinant PrV genome could be shown in infected cells, expression levels were low and mature gC could not be found in recombinant pseudorabies virions. In addition, complementation of defects associated with the lack of gIII was not observed (49).

Our goal is to elucidate whether essential homologous glycoproteins can function in a heterologous herpesvirus background and whether the exchange of glycoproteins by their homologs from a different herpesvirus influences viral growth characteristics in vitro and in vivo. For our studies, we focused on two important animal pathogens, PrV and BHV-1. Both, like HSV, belong to the alphaherpesvirus subfamily of the *Herpesviridae* family. On the basis of cross-hybridization analyses (5) and antigenic cross-reactivity (1, 2), they appear closely related. However, they differ strikingly in several biological properties, e.g., host range. Whereas PrV can productively and in most cases lethally infect a wide range of mammals except probably horses and

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higher primates, including humans (28), the host range of BHV-1 is naturally restricted to ruminants (24).

Since both viruses are not pathogenic for humans, they represent safe tools with which to study the influence of specific gene exchanges on the biological parameters of each virus. Recently, a recombinant BHV-1 strain that lacked the nonessential gC-homologous glycoprotein gIII(BHV-1) and instead expressed gIII(PrV) was described (22). As judged by initial in vitro growth analyses, gIII(PrV) was able to provide at least some of the functions normally executed by gIII(BHV-1). However, since both glycoproteins are nonessential, complete functional complementarity is difficult to assess.

Previously, we showed phenotypic *trans* complementation of a PrV mutant lacking the essential glycoprotein gII by the homologous gI(BHV-1) provided in a gI(BHV-1)-expressing cell line (39). Both gII(PrV) and gI(BHV-1) are homologous to gB(HSV), exhibit an amino acid identity of 63%, and, in contrast to gB(HSV), are proteolytically processed into two disulfide-linked subunits (10, 14, 25). The observed phenotypic complementation, however, is dependent on virus replication in complementing cell lines. Upon passage in noncomplementing cells, noninfectious virions lacking either glycoprotein are produced (38, 39). Analysis of possible effects of the glycoprotein exchange is therefore restricted to complementing cells. To overcome these limitations and to analyze functional complementarity in more detail, we attempted to insert the gI(BHV-1) gene into the genome of gII⁻ PrV and to express it in a way leading to stable complementation of the defect associated with lack of gII. In this report, we describe the construction and isolation of an infectious gII⁻ PrV recombinant that carries and expresses the gI(BHV-1) gene. In vitro analyses showed that the growth characteristics of the recombinant were not significantly altered compared with those of the wild-type PrV parent, proving that gI(BHV-1) is functionally equivalent to gII(PrV) in a PrV background. However, exchange of gII(PrV) for gI(BHV-1) led to a delay in penetration, resulting in penetration kinetics intermediate between those of the parental PrV and BHV-1 strains.

MATERIALS AND METHODS

Viruses and cells. PrV strain Ka (18) and BHV-1 strain Schönböken (provided by O. C. Straub, Tübingen, Germany) were used as parental strains. Isolation and characterization of a gII-deletion mutant of PrV carrying a β-galactosidase expression cassette (31) has been described previously (39). Viruses were propagated in Madin-Darby bovine kidney (MDBK) cells unless indicated otherwise. Vero cells were transfected by the calcium phosphate coprecipitation technique (12). Cells of pig (PSEK and PK-15), rabbit (RK-13), canine (MDCK), primate (CV-1 and Vero), or human (143TK⁻ and HeLa) origin were grown in Eagle's minimum essential medium (MEM) or Dulbecco's modification of MEM supplemented by 10% fetal calf serum.

MAbs. Monoclonal antibodies (MAbs) 5/14 [anti-gII(PrV) (25)] and 3/6 [anti-gI(PrV) (25)] were provided by H.-J. Rziha. Anti-gI(BHV-1) MAb 42/18/7 and anti-gIV(BHV-1) MAb 21/3/3 were obtained from G. Keil. Anti-gp50(PrV) antibody MCA 50-1 was provided by M. Wathen (46).

Plasmids. Plasmid pBR-gII Δ2/Bam has been described recently (39). Large-scale amplification and cloning procedures were performed according to published protocols (42).

DNA isolation and Southern blot hybridization. DNA was isolated by phenol extraction from virions purified by centrifugation through a 30% sucrose cushion as described previously (3). After restriction endonuclease digestion, fragments were separated in 0.8% agarose gels and transferred to nylon membranes (Zeta-Probe; Bio-Rad). ^{32}P-labeled probes were prepared by nick translation (40) or oligonucleotide-primed synthesis (Oligo-labelling kit; Amersham, Braunschweig, Germany). Hybridization was performed in 50% deionized formamide–0.5% nonfat milk–1% sodium dodecyl sulfate (SDS)–0.8M NaCl–1 mM EDTA–0.5 mg of heat-denatured salmon sperm DNA per ml for 24 h at 56°C. After hybridization, filters were washed for 30 min at room temperature in 2× SSC–0.5% SDS, 30 min at 72°C in 2× SSC–0.5% SDS, and 30 min at 72°C in 0.1× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The filters were then briefly dried, wrapped in Saran Wrap, and exposed to Kodak XAR-5 films.

Immunoprecipitation of ^{35}S-labelled viral proteins. MDBK monolayer cells were infected with the respective virus at a multiplicity of 2. Five hours postinfection (p.i.), the medium was replaced by medium without methionine; after an additional hour, [^{35}S]methionine (Amersham-Buchler, Braunschweig, Germany) was added to a final concentration of 50 μCi/ml. After clear cytopathic effect had been observed, cells were harvested, lysed, and processed as described previously (25).

To obtain purified virions, supernatants from labelled cell cultures exhibiting complete cytopathic effect were harvested and cleared by low-speed centrifugation. Virions were purified by centrifugation on a discontinuous gradient of 1.5 ml of 50% sucrose and 1 ml each of 40 and 30% sucrose in 10 mM Tris-HCl (pH 7.4)–0.2 mM EDTA in a Beckman SW41 rotor for 1.5 h at 22,000 rpm. Virions that accumulated at the interphase between the 40 and 50% sucrose layers were collected by aspiration, diluted 10-fold in phosphate-buffered saline (PBS), and pelleted in the same rotor at 22,000 rpm for 1 h. Pelleted virions were resuspended in 1 ml of 10 mM Tris-HCl (pH 7.4)–1 mM EDTA–150 mM NaCl, lysed, and processed for immunoprecipitation as described previously (25).

Immunoprecipitations with MAbs were performed as reported previously (25). Precipitates were separated in SDS–10% polyacrylamide gels, and fluorographic images were obtained on Kodak XAR-5 films.

Neutralization tests. Neutralization was assessed in plaque reduction assays (39) and recorded as percent plaque reduction compared with values for controls treated with either rabbit preimmune serum or non-herpesvirus-specific control MAbs.

One-step growth curve. MDBK cells were infected at a multiplicity of 5 for 1 h at 4°C. Immediately after a temperature shift to 37°C and at various times thereafter, cells were scraped into the medium, frozen at −70°C, thawed at 37°C, and titrated on MDBK monolayers. Titers given indicate PFU per milliliter.

Determination of plating efficiency. Monolayer cells of various origin in six-well tissue culture dishes were infected with 1 ml each of serial 10-fold dilutions of virus suspension. After 1 h at 37°C, the inoculum was removed and the cells were overlaid with MEM containing 0.5% methylcellulose. After 2 to 4 days, cells were fixed in 5% formaldehyde and stained with crystal violet, and plaques were counted. The number of plaques found on MDBK cells was arbitrarily taken as 100%, and the percentage of plaques on the other cell lines was calculated accordingly.

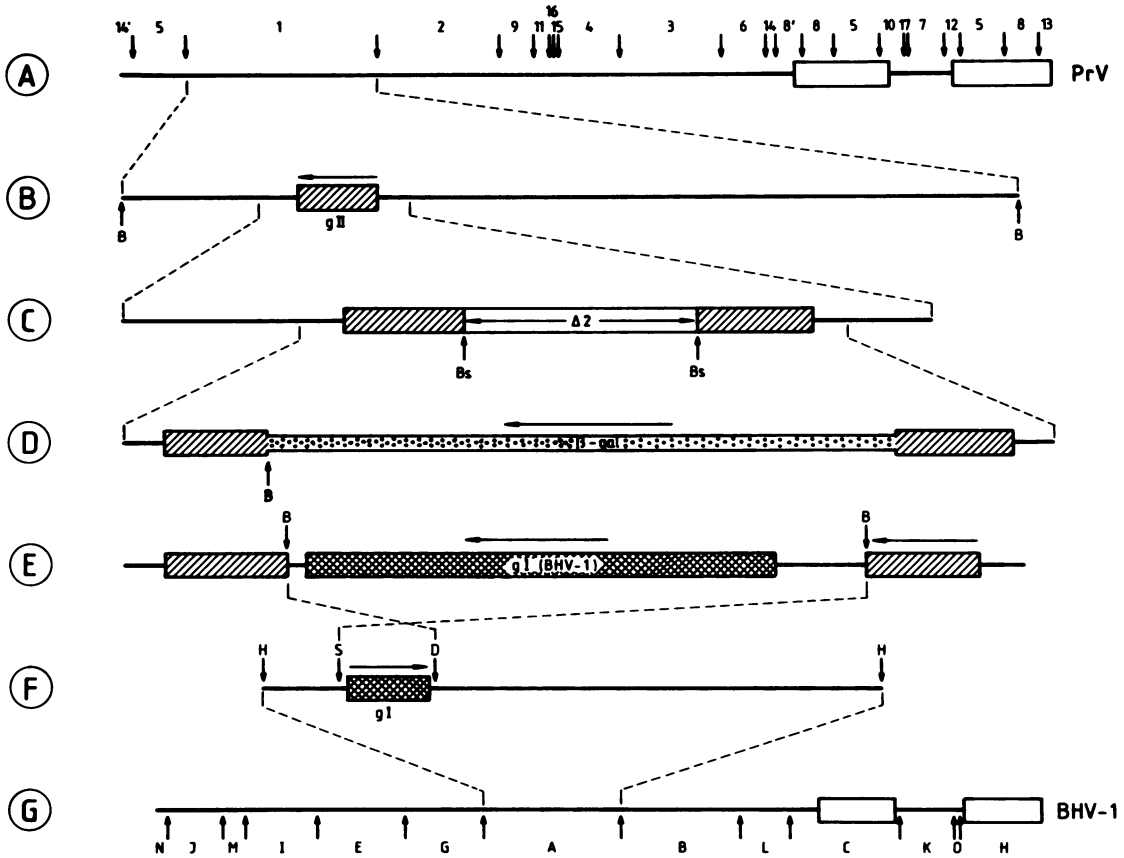

FIG. 1. Construction of recombinant 9112C2. (A) *Bam*HI restriction fragment map of the PrV genome. Open rectangles denote inverted repeat sequences that bracket the unique short region. (B) Enlargement of *Bam*HI fragment 1 encompassing the gII gene. Transcriptional direction is indicated by the horizontal arrow. (C) Deletion Δ2 (39) that had been introduced into the gII gene after cleavage with *Bst*EII prior to insertion of a *Bam*HI linker. After *Bam*HI cleavage, the β-galactosidase expression cassette (31) was inserted (D) and used as selectable marker for isolation of a gII$^-$ PrV mutant (39). (G) *Hin*dIII restriction fragment map of the BHV-1 genome (8, 27). *Hin*dIII fragment A encompassing the gI(BHV-1) gene is enlarged in panel F; the horizontal arrow indicates transcriptional direction. (E) Genomic map in the region of interest of plasmid pBBgI/2, which was used for isolation of recombinant 9112C2, depicting the 3.3-kb gI(BHV-1) insert. The gI(BHV-1) gene had been inserted in the same transcriptional orientation (indicated by horizontal arrows) into the PrV gII gene after addition of *Bam*HI linkers. Transcriptional direction of the gI(BHV-1) gene is inverted in panel E versus panel F, since in the prototypic genomic orientation (shown in panels A and G) gB homologs in PrV and BHV-1 are transcribed in opposite directions. Relevant cleavage sites: B, *Bam*HI; Bs, *Bst*EII; D, *Dra*III; H, *Hin*dIII; S, *Sal*I.

Penetration kinetics. Kinetics of virus penetration were analyzed by using low-pH inactivation of extracellular virus. Briefly, MDBK cells were infected with approximately 500 PFU per well at 4°C, a temperature at which adsorption can occur but penetration is blocked. After 1 h, plates were overlaid with prewarmed medium and shifted to 37°C. Immediately after the temperature shift and at different times thereafter, the medium was removed and the monolayer was overlaid for 2 min with 40 mM citric acid–10 mM KCl–135 mM NaCl (pH 3.0). The cells were then washed twice with PBS and overlaid with methylcellulose medium, and plaques were counted after 2 days. To determine penetration kinetics into gII-expressing MT-3 cells, virus was adsorbed for 20 min at 4°C, and the monolayers were washed once with ice-cold PBS before temperature shift and low-citrate treatment. Values were calculated with reference to those for control plates that had been incubated with PBS only and are given as percentage of PFU surviving the low-pH treatment; they represent averages of at least three independent experiments.

RESULTS

Isolation of gII$^-$ PrV carrying the gI(BHV-1) gene. The complete gI(BHV-1) expression unit containing the open reading frame encoding gI(BHV-1), 432 bp of upstream sequences, and 55 bp of downstream sequences, including the polyadenylation signal AATAAA, is contained in a 3.3-kb *Dra*III-*Sal*I fragment (Fig. 1F). After addition of *Bam*HI linkers, this fragment was cloned into the *Bam*HI site of plasmid pBR-gII Δ2/Bam, which encompasses the gII(PrV) gene carrying a 1.4-kb deletion (Fig. 1C) and concomitant insertion of a unique *Bam*HI restriction site. The gI(BHV-1) gene was inserted into the residual gII gene in both transcriptional orientations, yielding plasmids pBBgI/1, in which transcription of the gII(PrV) gene is in opposite orientation to transcription of the gI(BHV-1) gene, and pBBgI/2, in which the genes are transcribed in parallel (Fig. 1E).

Cotransfections were then performed into noncomplementing Vero cells by using plasmid pBBgI/1 or pBBgI/2 and

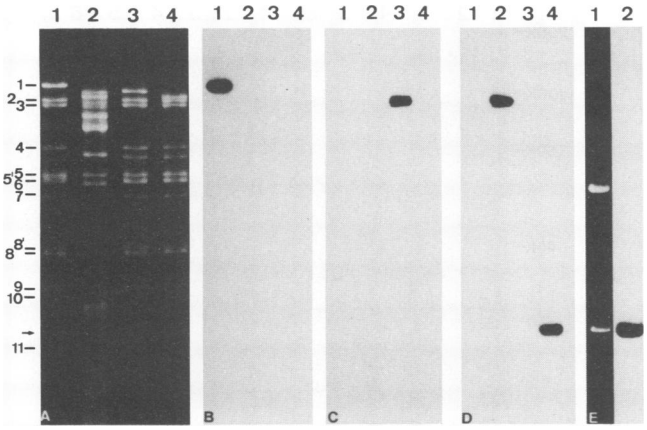

FIG. 2. Genomic characterization of recombinant 9112C2. DNA was isolated from purified virions of either wild-type PrV (lanes 1), wild-type BHV-1 (lanes 2), β-galactosidase-expressing gII⁻ PrV (lanes 3), or recombinant 9112C2 (lanes 4). After cleavage with either *Bam*HI (lanes 1, 3, and 4) or *Hin*dIII (lanes 2), fragments were separated in a 0.8% agarose gel. After transfer to nylon membranes, hybridizations were performed with probes specific for gII(PrV) (B), β-galactosidase (C), or gI(BHV-1) (D). (E) *Bam*HI cleavage of plasmid pBBgI/2 (lane 1) and hybridization with a gI(BHV-1)-specific probe (lane 2). Numbers in panel A denote *Bam*HI fragments of wild-type PrV DNA. The arrow points to the 3.3-kb gI(BHV-1) fragment appearing in DNA from recombinant 9112C2 (lane 4).

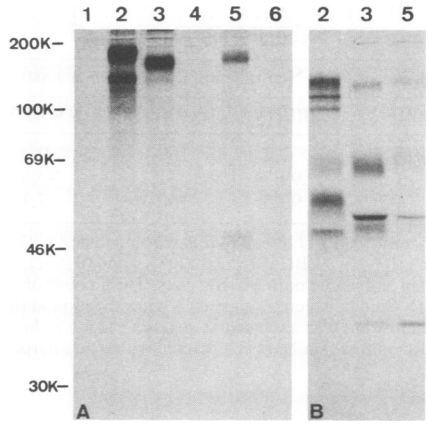

FIG. 3. Immunoprecipitation of infected cell lysates. MDBK cells were infected with either wild-type PrV (lanes 1 and 2), wild-type BHV-1 (lanes 3 and 4), or recombinant 9112C2 (lanes 5 and 6), proteins were labelled with [^{35}S]methionine, and infected cell lysates were analyzed by electrophoresis in SDS–10% polyacrylamide gels after immunoprecipitation with anti-gI(BHV-1) MAb 42/18/7 (lanes 1, 3, and 5) or anti-gII(PrV) MAb 5/14 (lanes 2, 4, and 6). Positions of molecular weight markers are indicated at the left. (A) Nonreducing conditions; (B) reducing conditions.

DNA from purified gII⁻ pseudorabies virions. Transfection of gII⁻ PrV DNA alone did not give rise to infectious progeny, as was expected since the gII defect could not be complemented by the host cells. Also, cotransfections between gII⁻ PrV DNA and plasmid pBBgI/1 did not result in the appearance of infectious virus. In contrast, cotransfections between gII⁻ PrV DNA and plasmid pBBgI/2 yielded infectious virus progeny.

Dot spot hybridizations using a gI(BHV-1)-specific probe indicated that gI(BHV-1) sequences had been incorporated into the genome of the cotransfection progeny. Hybridization-positive plaques were purified three times and reanalyzed, and one isolate, 9112C2, was selected for further studies.

Genomic characterization of recombinant 9112C2. For analysis of correct insertion of the gI(BHV-1) gene into the gII⁻ PrV genome, virion DNA from wild-type PrV (Fig. 2, lanes 1), wild-type BHV-1 (lanes 2), gII⁻ PrV (lanes 3), and recombinant 9112C2 (lanes 4) was isolated and cleaved with *Bam*HI (lanes 1, 3, and 4) or *Hin*dIII (lanes 2). Resulting fragments can be seen in Fig. 2A after electrophoresis through a 0.8% agarose gel and ethidium bromide staining. In Fig. 2E, lane 1, plasmid pBBgI/2 was cleaved with *Bam*HI, thereby excising the gI(BHV-1) insert. After hybridization with a gII-specific probe (Fig. 2B; for location, see reference 39), as expected only wild-type PrV DNA was recognized (Fig. 2B, lane 1), thereby eliminating the possibility that the infectious progeny seen after transfection might have resulted from DNA of gII⁻ Pr viruses that had been rescued after propagation on complementing gII-expressing cells. Hybridization with a β-galactosidase-specific probe (Fig. 2C) recognized only the gII⁻ PrV DNA in which a β-galactosidase expression cassette had been inserted into the genetically engineered unique *Bam*HI site in the partially deleted copy of the gII gene (39). After hybridization with a gI(BHV-1)-specific probe (Fig. 2D), wild-type BHV-1 DNA, as well as a fragment of 3.3 kb in DNA from recombinant 9112C2, is recognized. This band corresponds in size to that seen after cleavage of plasmid pBBgI/2 (Fig. 2E). Under our hybridization and wash conditions, cross-hybridization between gII(PrV) and gI(BHV-1) sequences was not observed (Fig. 2).

These results confirm that in recombinant 9112C2, resulting from cotransfection of gII⁻ PrV DNA and plasmid pBBgI/2, the gII gene is still partially deleted and therefore gII cannot be synthesized. Instead, the gI(BHV-1) gene had been integrated by homologous recombination, thereby eliminating the β-galactosidase expression cassette.

Analysis of gI(BHV-1) expression from recombinant 9112C2. To analyze whether gI(BHV-1) is expressed in cells infected with recombinant 9112C2, radioimmunoprecipitations were performed (Fig. 3). To this end, cells were infected with either wild-type PrV (Fig. 3, lanes 1 and 2), wild-type BHV-1 (lanes 3 and 4), or recombinant 9112C2 (lanes 5 and 6), and proteins were labelled with [^{35}S]methionine from 6 to 24 h after infection. Cells were then harvested and lysed, and proteins were separated in nonreducing (Fig. 3A) or reducing (Fig. 3B) SDS–10% polyacrylamide gels after immunoprecipitation with either anti-gI(BHV-1) MAb 42/18/7 (Fig. 3, lanes 1, 3, and 5) or anti-gII(PrV) MAb 5/14 (lanes 2, 4, and 6). Whereas the anti-gII(PrV) antibody recognizes gII in wild-type PrV-infected cells (lanes 2), no protein could be precipitated by MAb 5/14 from either wild-type BHV-1-infected cells (lane 4) or recombinant 9112C2-infected cells (lane 6). In contrast, gI(BHV-1) could be demonstrated in wild-type BHV-1-infected cells (lanes 3) and in recombinant 9112C2-infected cells (lanes 5) but not in PrV-infected cells (lane 1). This result proves that the gI(BHV-1) gene in recombinant 9112C2 is expressed.

Upon reduction, the 155-kDa gII(PrV) complex dissociated into subunits of approximately 70 and 58 kDa (Fig. 3B, lane 2) (14, 25), whereas reduction of the 130-kDa gI(BHV-1) precipitated from recombinant 9112C2-infected (lane 5) or BHV-1-infected (lane 3) cells resulted in the appearance of

TABLE 1. Neutralization of PrV, BHV-1, and recombinant 9112C2[a]

Virus	Neutralization with MAb against:				
	gII(PrV)	gI(BHV)	gp50(PrV)	gIV(BHV)	gI(PrV)
PrV	99.7	2.2	100	0	96.8
BHV-1	6.3	99.7	1.1	99.9	4.2
9112C2	0.8	99.5	100	7.1	98.9

[a] Approximately 500 PFU of each virus was incubated with ascitic fluid of MAbs against gII(PrV), gI(BHV-1), gp50(PrV), gIV(BHV-1), or gI(PrV) and 5% rabbit serum and plated onto MDBK cells. The amount of antibody added was adjusted to yield between 90 and 100% neutralization in the homologous system. Neutralization was assessed as percent plaque reduction compared with values for controls that had been reacted with a non-herpesvirus-specific MAb.

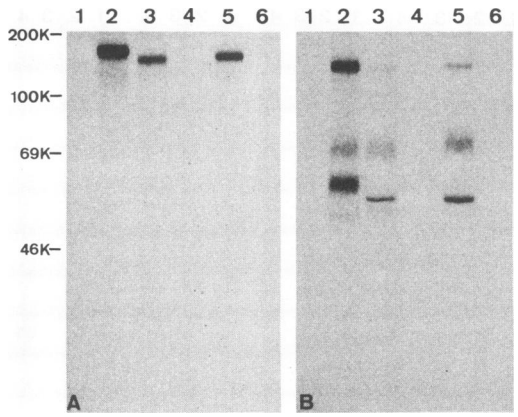

FIG. 4. Immunoprecipitation of purified virions. After infection of MDBK cells and labelling with [^{35}S]methionine, PrV (lanes 1 and 2), BHV-1 (lanes 3 and 4), and recombinant 9112C2 (lanes 5 and 6) virions were purified and lysed, and lysates were precipitated with either anti-gI(BHV-1) MAb 42/18/7 (lanes 1, 3, and 5) or anti-gII(PrV) MAb 5/14 (lanes 2, 4, and 6). Precipitates were analyzed under nonreducing (A) or reducing (B) conditions to show proteolytic processing of the glycoprotein precursor after reduction of the disulfide bonds linking the two smaller proteolytic cleavage products.

approximately 70- and 55-kDa subunits (10). In addition, variable amounts of the uncleaved glycosylated monomer (ca. 120 kDa) were also observed upon reduction (Fig. 3B) (10, 39). The other protein bands most likely constitute differentially glycosylated intracellular forms. The amount of gI(BHV-1) precipitated from recombinant 9112C2-infected cells was consistently only approximately 30 to 40% of that found after precipitation of BHV-1-infected cell lysates (Fig. 3; compare lanes 3 and 5).

Insertion of gI(BHV-1) into the 9112C2 envelope. To test for insertion of gI(BHV-1) into the envelope of recombinant 9112C2 virions, complement-dependent neutralization tests with either anti-gII(PrV) MAb 5/14, anti-gI(BHV-1) MAb 42/18/7, anti-gp50(PrV) MAb MCA50-1, anti-gIV(BHV-1) MAb 21/3/3, or anti-gI(PrV) MAb 3/6 were performed. As shown in Table 1, wild-type PrV is susceptible to neutralization by all anti-PrV MAbs, whereas BHV-1 is neutralized by the anti-BHV-1 MAbs. Recombinant 9112C2 proved to be susceptible to anti-PrV MAbs MCA50-1 (anti-gp50) and 3/6 (anti-gI). However, it lost sensitivity against the anti-gII MAb. In contrast, 9112C2 virions became susceptible to neutralization by the anti-gI(BHV-1) MAb but retained resistance against anti-gIV(BHV-1) neutralization.

To further analyze the presence of gI in recombinant 9112C2 virions, virus particles were purified after labelling with [^{35}S]methionine and tested by radioimmunoprecipitation. As shown in Fig. 4, gI(BHV-1) was precipitated from purified recombinant 9112C2 (lanes 5) and wild-type BHV-1 (lanes 3) virions in similar amounts. Lanes 2 of Fig. 4 demonstrate gII(PrV) precipitated from purified pseudorabies virions. In Fig. 4A, separation in SDS–10% polyacrylamide gels was performed under nonreducing conditions. After reduction of the disulfide bonds (Fig. 4B), identical proteolytic cleavage products of gI(BHV-1) from purified recombinant 9112C2 (lane 5) and wild-type BHV-1 (lane 3) virions could be demonstrated. Lane 2 of Fig. 4B shows the subunits of glycoprotein complex gII(PrV) after dissociation of the disulfide bonds. The ca. 120-kDa proteins again represent uncleaved glycosylated gII(PrV) (lane 2) or gI(BHV-1) (lanes 3 and 5) monomers. In summary, these results show insertion of gI(BHV-1) into recombinant 9112C2 virions in amounts similar to those found in purified BHV-1 virions. The lower level of intracellular gI(BHV-1) in 9112C2-infected cells than in wild-type BHV-1-infected cells was therefore not reflected in purified virions. Insertion of gI(BHV-1) into the envelope of recombinant 9112C2 virions could also be visualized by electron microscopy using immunogold labelling (data not shown).

Recombinant 9112C2 therefore stably carries the gI (BHV-1) gene, expresses the gI(BHV-1) protein during its replicative cycle, and integrates the foreign glycoprotein into the PrV envelope.

In vitro growth of recombinant 9112C2. We recently described the phenotypic complementation of gII$^-$ PrV by gI(BHV-1) (39). However, this phenotype is dependent on growth in complementing cells and is lost after one replicative cycle in noncomplementing cells. Therefore, analysis of the influence of the exchange of gB homologs between PrV and BHV-1 on several biological parameters could not be tested. The availability of a stable recombinant thus formed the basis for further studies.

One-step growth curves in MDBK cells comparing PrV, BHV-1, and 9112C2 are shown in Fig. 5. Recombinant 9112C2 exhibited growth characteristics similar to those of PrV. After an initial drop 4 h p.i., there was a sharp increase in the amount of infectious virus at 8 h p.i., reaching near-plateau levels at 24 h p.i. In contrast, in BHV-1 the eclipse phase was prolonged and a rise in titer could not be observed until 12 h p.i. Final titers reached were similar for all three viruses (Fig. 5). Taken together, these results show that gI(BHV-1) appears to fully complement the defect associated with lack of gII(PrV).

As already mentioned, PrV and BHV-1, although closely related, differ significantly in several properties such as growth in different hosts. Our approach of using defined recombinants exchanging single genes should help to identify their importance in determining host range. We first tested plating efficiencies of wild-type PrV, BHV-1, and recombinant 9112C2 on different cell lines (Table 2). Whereas PrV and BHV-1 produced plaques efficiently on MDBK cells, they differed widely in their plating efficiencies on pig (PSEK and PK-15), rabbit (RK-13), canine (MDCK), primate (Vero and CV-1), and human (143TK$^-$ and HeLa) cells. However, in all cases recombinant 9112C2 yielded values similar to those of wild-type PrV, indicating that the lack of gII(PrV) and gain of gI(BHV-1) expression did not alter the plating efficiency of the recombinant in comparison with wild-type PrV.

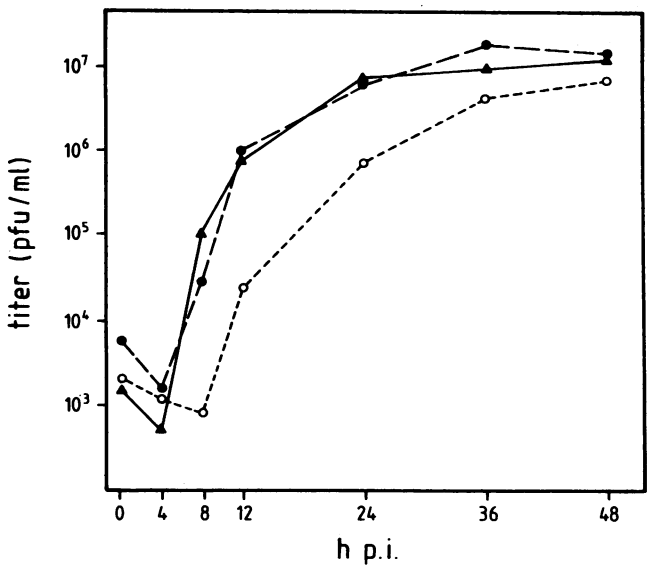

FIG. 5. One-step growth curves. MDBK cells were infected at a multiplicity of 5 with wild-type PrV (●), wild-type BHV-1 (○), or recombinant 9112C2 (▲) for 1 h at 4°C. The inoculum was then removed, and the cells were overlaid with prewarmed (37°C) medium. Immediately after the temperature shift to 37°C and at the indicated times thereafter, cells were scraped into the medium and lysed by freezing (−70°C) and thawing (37°C), cellular debris was removed by centrifugation, and infectivity in the supernatant was determined in plaque assays on MDBK cells. Titers are expressed as PFU per milliliter of suspension.

Penetration kinetics of PrV, BHV-1, and 9112C2. All gB-homologous glycoproteins analyzed so far have been shown to be involved in viral penetration, i.e., the fusion of viral and cellular membranes initiating infection (6, 10, 38). We therefore tested whether exchange of gII(PrV) by gI(BHV-1) would influence the penetration behavior of the virus. To this end, penetration kinetics were analyzed by using low-pH inactivation of extracellular virus at different times after a shift from the adsorption temperature (4°C) to 37°C. As shown in Fig. 6, whereas PrV entered MDBK cells very quickly, with a half-time of approximately 10 min, BHV-1 showed a longer lag phase and it took approximately 25 min until 50% of adsorbed virions had been internalized. Interestingly, recombinant 9112C2 exhibited an intermediate phenotype, reaching the 50% penetration level after approximately 18 min. Since this result indicated an effect of the exchange of gII(PrV) by gI(BHV-1) on viral penetration kinetics, additional experiments were conducted. In a second experiment, we tested the penetration kinetics of gII⁻ PrV phenotypically complemented by propagation on either gII(PrV)-expressing MT-3 cells or gI(BHV-1)-expressing G1 cells (39). Propagation of gII⁻ PrV on these cells had been shown to result in incorporation of the gB-homologous glycoprotein into the PrV envelope (39). Since phenotypically complemented gII⁻ PrV can produce plaques only in complementing cells, experiments using gII-expressing MT-3 cells were performed (Fig. 7). Whereas gII⁻ PrV propagated on gII(PrV)-expressing cells exhibited penetration kinetics similar to those of wild-type PrV, penetration of gII⁻ PrV propagated on gI(BHV-1)-expressing cells was indistinguishable from that of recombinant 9112C2. In a third experiment, recombinant 9112C2 was propagated on either gII(PrV)-expressing MT-3 or gI(BHV-1)-expressing G1 cells. Penetration of recombinant 9112C2 grown on MT-3 cells was accelerated compared with penetration of 9112C2 grown on

TABLE 2. Plating efficiency of PrV, BHV-1, and recombinant 9112C2[a]

Expt	Cells	Plating efficiency (%)[b]		
		PrV	9112C2	BHV-1
1	MDBK	100 (4.2×10^7)	100 (3.0×10^6)	100 (6.2×10^6)
	PSEK	72 (3.0×10^7)	53 (1.6×10^6)	3 (2.0×10^5)
	Vero	69 (2.9×10^7)	43 (1.3×10^6)	0.7 (4.5×10^4)
	CV-1	13 (5.4×10^6)	8 (2.5×10^5)	—[c]
	143 TK⁻	9 (3.9×10^6)	10 (3.0×10^5)	0.001 (6.0×10^1)
2	MDBK	100 (5.0×10^8)	100 (2.0×10^7)	100 (4.5×10^7)
	MDCK	16 (7.8×10^7)	36 (7.2×10^6)	0.004 (2.0×10^3)
	HeLa	18 (9.0×10^7)	25 (5.0×10^6)	0.05 (2.3×10^4)
	RK-13	82 (4.1×10^8)	105 (2.1×10^7)	0.7 (3.3×10^5)
	PK-15	156 (7.8×10^8)	190 (3.8×10^7)	2.2 (1.0×10^6)

[a] Cells of bovine (MDBK), pig (PSEK and PK-15), canine (MDCK), rabbit (RK-13), primate (Vero and CV-1), or human (143TK⁻ and HeLa) origin grown in monolayers were infected with serial 10-fold dilutions of PrV, BHV-1, or 9112C2. After 2 to 4 days, cells were fixed with 5% formaldehyde and stained with crystal violet, and plaques were counted. Experiments 1 and 2 differ in that different virus stocks were used for infection.

[b] Compared with titer on MDBK cells, which was taken as 100%. Values in parentheses show mean titers derived from two independent titrations.

[c] No plaques visible even after prolonged incubation.

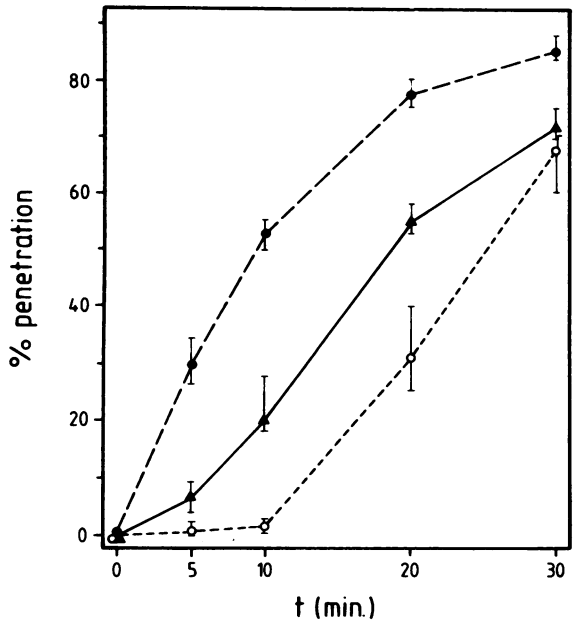

FIG. 6. Penetration kinetics of PrV, BHV-1, and 9112C2 into MDBK cells. MDBK cells in six-well plates were infected with approximately 500 PFU of wild-type PrV (●), wild-type BHV-1 (○), or recombinant 9112C2 (▲) per well for 1 h at 4°C. Thereafter the inoculum was removed, and the cells were overlaid with medium prewarmed at 37°C. Immediately after the temperature shift and after the indicated times, cells were treated with citrate buffer (pH 3.0) to inactivate nonpenetrated viruses. After 2 days of incubation in MEM–0.5% methylcellulose, plaques were counted. The percentage of PFU surviving the low-pH treatment was calculated with reference to the value for PBS-treated control wells and is shown as percent penetration. Values represent averages of at least three independent experiments. Vertical bars indicate minimum and maximum values.

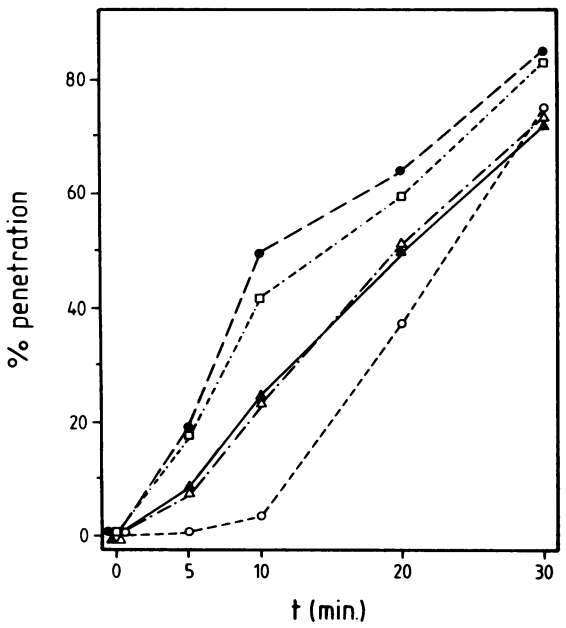

FIG. 7. Penetration kinetics of PrV, BHV-1, 9112C2, and phenotypically complemented gII$^-$ PrV into MT-3 cells. gII-expressing MT-3 cells were infected with wild-type PrV (●), wild-type BHV-1 (○), recombinant 9112C2 (▲), or gII$^-$ PrV propagated on MT-3 cells (□) or on gI(BHV-1)-expressing G1 cells (△). After 20 min of adsorption at 4°C, penetration kinetics were determined by low-pH inactivation of extracellular virus. The percentage of PFU surviving the low-pH treatment was calculated with reference to the value for PBS-treated control wells and is shown as percent penetration. Values represent averages of at least three independent experiments.

normal MDBK cells, whereas there was no difference in the penetration between 9112C2 grown on G1 or on normal MDBK cells (data not shown). Taken together, these results indicate that the exchange of gII(PrV) for gI(BHV-1) slowed penetration of the viruses to a level intermediate between those of wild-type PrV and BHV-1.

DISCUSSION

We describe in this report the first isolation of an infectious herpesvirus recombinant lacking an essential glycoprotein whose functions are provided by a homologous glycoprotein from a different herpesvirus. A gII$^-$ PrV mutant that normally is dependent on *trans*-complementing cell lines for replication was rescued by insertion into its genome of the gene encoding the homologous gI(BHV-1). This specific recombinant exhibited growth characteristics similar to those of its wild-type PrV parent. The fact that in vitro infectivity of the gII$^-$ PrV mutant is fully restored by gI(BHV-1), as shown by analysis of growth kinetics as well as plating efficiency on different host cells, conclusively proves that gI(BHV-1) is able to execute all functions necessary in vitro that are normally provided by gII(PrV). Since gII(PrV) has been shown to mediate penetration of PrV into target cells and is also essential for direct cell-cell spread (38), it appears certain that gI(BHV-1) is involved in the same processes in BHV-1. It has also been shown that gII(PrV), in conjunction with gIII(PrV), binds to heparin and might therefore be involved in primary adsorption of PrV to a heparinlike cellular surface receptor (29). Whether gI(BHV-1) in conjunction with gIII(PrV) is also able to bind to heparin is currently under investigation.

To construct the PrV/BHV-1 gB recombinant 9112C2, a BHV-1 genomic DNA fragment encompassing the gI (BHV-1) gene (21, 50) and additional 432 bp of upstream and 55 bp of downstream sequences was inserted into a partially deleted cloned gII gene. The upstream sequences most likely include promoter elements. Whether the gI(BHV-1) gene in recombinant 9112C2 is actually expressed under control of its own promoter remains to be analyzed. It is notable in this context that viable recombinants could be isolated only when the transcriptional orientations of the residual part of the gII gene and the inserted gI(BHV-1) gene were identical (Fig. 1).

Although both the gII$^-$ PrV deletion mutant and recombinant 9112C2 still retain portions of the gII gene and might be able to express at least the amino-terminal 231 amino acids of gII under control of the remaining gII promoter, we were unable to find any such gene product after precipitation of infected cell lysates with two polyclonal rabbit anti-gII sera (29) that had been prepared either against purified nonreduced gII complex or against both subunits after reduction of the disulfide bonds (30).

Previously, a recombinant PrV was constructed that lacked the nonessential glycoprotein gIII gene and instead carried and expressed under control of its own promoter the gene encoding gC(HSV) (49). It was found that gC(HSV) expression in this recombinant was strikingly lower than that of gIII in PrV or gC in HSV. After immunoprecipitation of 9112C2-infected cell lysates, we also found only approximately 30 to 40% of the amount of gI(BHV-1) as in wild-type BHV-1-infected cells. Whether this is due to an inefficient function of the heterologous promoter remains to be analyzed.

Although the PrV gC recombinant did express gC(HSV), as analyzed by immunoprecipitation of infected cell extracts, gC could not be found in the viral envelope, and the defects associated with lack of gIII could not be compensated for (49). In recombinant 9112C2, we were able to demonstrate the presence of gI(BHV-1) in the virion in amounts similar to those found in wild-type BHV-1 virions, leading to efficient complementation of the gII defect. A similar approach has recently been successful in showing complementation of a BHV-1 mutant lacking the nonessential glycoprotein gIII(BHV-1) by the homologous gIII(PrV) (22). In this recombinant, gIII(PrV) was expressed under control of the gIII(BHV-1) promoter in amounts similar to those of gIII (BHV-1) in BHV-1 wild-type strains. Whether the observed lack of complementation between gIII(PrV) and gC(HSV) was therefore due to the low amounts of gC present in infected cells or whether gC(HSV) differs from gIII(PrV), and perhaps gIII(BHV-1), in a way that precludes functional complementation is presently unclear.

Previously, analysis of HSV variants carrying and expressing the gI(BHV-1) gene in addition to their own gB gene indicated at least partial complementation of gB(HSV) function by the homologous gI(BHV-1). In these studies, neutralizing MAbs were used to partially block gB(HSV) function (4, 35). However, since gB(HSV) was still present in the virion, an effect of the remaining gB(HSV) could not be excluded. Our approach, using a gII-deletion mutant of PrV as the parental strain for incorporation of the gI(BHV-1) gene, excludes the possibility of an influence of residual gII.

PrV and BHV-1 differ significantly in biology. PrV, for example, is able to productively and in most cases fatally infect a wide range of mammals, whereas natural infection

by BHV-1 has been reported to occur only in ruminants. In our in vitro studies, we were not able to find a difference between PrV and recombinant 9112C2 in plating efficiency on cells of human, monkey, swine, canine, or rodent origin, on which the plating efficiency of BHV-1 differed widely. At least in cell culture, therefore, the exchange of gII(PrV) for gI(BHV-1) did not affect the efficiency of infection of cells from various species.

Penetration analyses showed that recombinant 9112C2 exhibited kinetics intermediate between those of its PrV and BHV-1 parents. Phenotypically gII(PrV)- or gI(BHV-1)-complemented gII⁻ Pr virions yielded similar results. In addition, recombinant 9112C2 propagated on gII(PrV)-expressing MT-3 cells showed accelerated penetration, whereas propagation on gI(BHV-1)-expressing G1 cells did not influence the penetration behavior of recombinant 9112C2. The hypothesis that gB proteins in herpesviruses not only are essential for viral entry but also modulate penetration kinetics is appealing. Results of experiments using a gB⁻ HSV mutant phenotypically complemented by gII(PrV) support this hypothesis (29). It will be of particular interest to obtain and characterize the reverse recombinant, i.e., a gI⁻ BHV-1 expressing gII(PrV). If our assumption is correct, this virus should exhibit a faster penetration process than does the BHV-1 parental strain.

Availability of the PrV/BHV-1 gB recombinant now also allows studies in vivo to analyze whether the exchange of gII(PrV) for gI(BHV-1) influences pathogenesis in the infected animal. It has been shown that gB proteins from HSV strains that differ in neurovirulence modulate neuropathogenicity of the virus (47). Whether similar effects can be found after exchange of gII(PrV) by gI(BHV-1) is under investigation.

In conclusion, we show that (i) a gII⁻ PrV mutant can be stably rescued by incorporation of the gI(BHV-1) gene into its genome, leading to expression of the BHV-1 glycoprotein and its insertion into the PrV envelope, (ii) the gI(BHV-1) recombinant PrV is similar in in vitro growth characteristics to its PrV parent, proving that necessary functions executed by the essential gII(PrV) in vitro leading to productive infection can be provided by gI(BHV-1), and (iii) exchange of gII(PrV) for gI(BHV-1) alters the penetration kinetics of the recombinant to values intermediate between its PrV and BHV-1 parents.

Future emphasis will be directed on the complementation capabilities of other essential and nonessential herpesvirus glycoproteins in a heterologous background.

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REFERENCES

1. **Aguilar-Setién, A., P. P. Pastoret, G. Burtonboy, F. Coignouil, P. Jetteur, and F. Schoenaers.** 1979. Communaité antigénique entre le virus de la rhinotrachéite infectieuse bovine (bovid herpesvirus 1, BHV-1) et le virus de la maladie d'Aujeszky (suis herpesvirus 1, SHV 1) démontrée, chez le bovin, par un test d'hypersensibilité retardée. Ann. Med. Vet. **123**:55–65.
2. **Aguilar-Setién, A., J. Vandeputte, and P. P. Pastoret.** 1979. Présence concomittante chez les bovins et les porcs d'anticorps neutralisant le virus de la rhinotrachéite infectieuse bovine et celui de la maladie d'Aujeszky après contact avec le virus homologue. Ann. Med. Vet. **123**:275–285.
3. **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.
4. **Blewett, E. L., and V. Misra.** 1991. Cleavage of the bovine herpesvirus glycoprotein B is not essential for its function. J. Gen. Virol. **72**:2083–2090.
5. **Bush, C. E., and R. F. Pritchett.** 1985. A comparison of the genomes of bovine herpesvirus type 1 and pseudorabies virus. J. Gen. Virol. **66**:1811–1817.
6. **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.
7. **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.
8. **Engels, M., C. Giuliani, P. Wild, T. M. Beck, E. Loepfe, and R. Wyler.** 1986. The genome of bovine herpesvirus 1 (BHV-1) strains exhibiting a neuropathogenic potential compared to known BHV-1 strains by restriction site mapping and cross hybridization. Virus Res. **6**:57–73.
9. **Fehler, F., J. Herrmann, A. Saalmüller, T. C. Mettenleiter, and G. M. Keil.** 1992. Glycoprotein IV of bovine herpesvirus 1-expressing cell line complements and rescues a conditionally lethal viral mutant. J. Virol. **66**:831–839.
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. **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.
14. **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.
15. **Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear.** 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cell and in infectivity. J. Virol. **65**:1090–1098.
16. **Highlander, S. L., S. Sutherland, P. Gage, D. Johnson, M. Levine, and J. Glorioso.** 1987. Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. J. Virol. **61**:3356–3364.
17. **Hutchinson, L., K. Goldsmith, S. Primorac, C. Roop, F. L. Graham, and D. C. Johnson.** 1991. Characterization of two novel glycoproteins, gK and gL, involved in membrane fusion, p. 43. Abstr. 16th Int. Herpesvirus Workshop.
18. **Kaplan, A. S., and A. E. Vatter.** 1959. A comparison of herpes simplex and pseudorabies viruses. Virology **7**:394–407.
19. **Keil, G.** Personal communication.
20. **Klupp, B., and T. C. Mettenleiter.** 1991. Sequence and expression of the glycoprotein gH gene of pseudorabies virus. Virology **182**:732–741.
21. **Lawrence, W., R. d'Urso, C. Kundel, C. Whitbeck, and L. Bello.** 1986. Map location of the gene for a 130,000-dalton glycoprotein of bovine herpesvirus 1. J. Virol. **60**:405–414.
22. **Liang, X., L. Babiuk, and T. Zamb.** 1991. Pseudorabies virus gIII and bovine herpesvirus 1 gIII share complementary functions. J. Virol. **65**:5553–5557.
23. **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.

24. **Ludwig, H.** 1983. Bovine herpesviruses, p. 135–214. *In* B. Roizman (ed.), The herpesviruses, vol. II. Plenum Press, New York.
25. **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.
26. **Manservigi, R., P. G. Spear, and A. Buchan.** 1977. Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoproteins. Proc. Natl. Acad. Sci. USA **74:** 3913–3917.
27. **Mayfield, J. E., P. J. Good, H. J. Vanoort, A. R. Campbell, and D. E. Reed.** 1983. Cloning and cleavage site mapping of DNA from bovine herpesvirus 1 (Cooper strain). J. Virol. **47:**259–264.
28. **Mettenleiter, T. C.** 1991. Molecular biology of pseudorabies (Aujeszky's disease) virus. Comp. Immun. Microbiol. Infect. Dis. **14:**151–163.
29. **Mettenleiter, T. C.** Unpublished data.
30. **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.
31. **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.
32. **Mettenleiter, T. C., C. Schreurs, F. Zuckermann, and T. Ben-Porat.** 1987. Role of pseudorabies virus glycoprotein gI in virus release from infected cells. J. Virol. **61:**2764–2769.
33. **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.
34. **Meyer, A., E. Petrovskis, P. Duffus, D. Thomsen, and L. Post.** 1991. Cloning and sequencing of an infectious bovine rhinotracheitis (BHV-1) gene homologous to glycoprotein H of herpes simplex virus. Biochem. Biophys. Acta **1090:**267–269.
35. **Misra, V., and E. Blewett.** 1991. Construction of herpes simplex viruses that are pseudodiploid for the glycoprotein B gene: a strategy for studying the function of an essential herpesvirus gene. J. Gen. Virol. **72:**385–392.
36. **Okazaki, K., T. Matsuzaki, Y. Sugahara, J. Okada, M. Hasebe, Y. Iwamura, M. Ohnishi, T. Kanno, M. Shimizu, E. Honda, and Y. Kono.** 1991. BHV-1 adsorption is mediated by the interaction of glycoprotein gIII with heparinlike moiety on the cell surface. Virology **181:**666–670.
37. **Petrovskis, E. A., J. G. Timmins, M. A. Armentrout, C. C. Marchioli, R. J. Yancey, and L. E. Post.** 1986. DNA sequence of the gene for pseudorabies virus gp50, a glycoprotein without N-linked glycosylation. J. Virol. **59:**216–223.
38. **Rauh, I., and T. C. Mettenleiter.** 1991. Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. J. Virol. **65:**5348–5356.
39. **Rauh, I., F. Weiland, F. Fehler, G. Keil, and T. C. Mettenleiter.** 1991. Pseudorabies virus mutants lacking the essential glycoprotein gII can be complemented by glycoprotein gI of bovine herpesvirus 1. J. Virol. **65:**621–631.
40. **Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **143:**363–393.
41. **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 gene of herpes simplex virus. J. Virol. **61:**2691–2701.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
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.
44. **Spear, P. G.** 1985. Glycoproteins specified by herpes simplex viruses, p. 315–356. *In* B. Roizman (ed.), The herpesviruses, vol. III. Plenum Press, New York.
45. **Tikoo, S. K., D. Fitzpatrick, L. Babiuk, and T. Zamb.** 1990. Molecular cloning, sequencing, and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. J. Virol. **64:**5132–5142.
46. **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.
47. **Weise, K., H. C. Kaerner, J. Glorioso, and C. H. Schröder.** 1987. Replacement of glycoprotein B gene sequences in herpes simplex virus type 1 strain ANG by corresponding sequences of the strain KOS causes changes of plaque morphology and neuropathogenicity. J. Gen. Virol. **68:**1909–1919.
48. **Whealy, M. E., A. K. Robbins, and L. W. Enquist.** 1988. Pseudorabies virus glycoprotein gIII is required for efficient virus growth in tissue culture. J. Virol. **62:**2512–2515.
49. **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.
50. **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.
51. **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.