

# From Essential to Beneficial: Glycoprotein D Loses Importance for Replication of Bovine Herpesvirus 1 in Cell Culture

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**Glycoprotein D (gD) of bovine herpesvirus 1 (BHV-1) has been shown to be an essential component of virions involved in virus entry. gD expression in infected cells is also required for direct cell-to-cell spread. Therefore, BHV-1 gD functions are identical in these aspects to those of herpes simplex virus 1 (HSV-1) gD. In contrast, the gD homolog of pseudorabies virus (PrV), although essential for penetration, is not necessary for direct cell-to-cell spread. Cocultivation of cells infected with phenotypically gD-complemented gD<sup>-</sup> mutant BHV-1/80-221 with noncomplementing cells resulted in the isolation of the cell-to-cell-spreading gD-negative mutant ctc<sup>+</sup>BHV-1/80-221, which was present in the gD-null BHV-1 stocks. ctc<sup>+</sup>BHV-1/80-221 could be propagated only by mixing infected with uninfected cells, and virions released into the culture medium were noninfectious. Marker rescue experiments revealed that a single point mutation in the first position of codon 450 of the glycoprotein H open reading frame, resulting in a glycine-to-tryptophan exchange, enabled complementation of the gD function for cell-to-cell spread. After about 40 continuous passages of ctc<sup>+</sup>BHV-1/80-221-infected cells with noninfected cells, the plaque morphology in the cultures started to change from roundish to comet shaped. Cells from such plaques produced infectious gD<sup>-</sup> virus, named gD<sup>-</sup>infBHV-1, which entered cells much more slowly than wild-type BHV-1. In contrast, integration of the gD gene into the genomes of gD<sup>-</sup>infBHV-1 and ctc<sup>+</sup>BHV-1/80-221 resulted in recombinants with accelerated penetration in comparison to wild-type virions. In summary, our results demonstrate that under selective conditions, the function of BHV-1 gD for direct cell-to-cell spread and entry into cells can be compensated for by mutations in other viral (glyco)proteins, leading to the hypothesis that gD is involved in formation of penetration-mediating complexes in the viral envelope of which gH is a component. Together with results for PrV, varicella-zoster virus, which lacks a gD homolog, and Marek's disease virus, whose gD homolog is not essential for infectivity, our data may open new insights into the evolution of alphaherpesviruses.**

A series of recent reports dealing with the entry of herpes simplex virus type 1 (HSV-1), pseudorabies virus (PrV), and bovine herpesvirus 1 (BHV-1) showed that infection of target cells is a complicated process involving a cascade of events. Following initial interaction between glycoprotein C (gC) and heparan sulfate on the cell surface (8, 12, 13, 23, 24, 30, 31, 35, 51), gD presumably interacts with cellular receptors, resulting in stable attachment (15, 46). Subsequent pH-independent fusion between the target cell membrane and the viral envelope results in release of the viral nucleocapsid into the cytoplasm of the host cell (50). gB, gD, gH, and gL, which might act separately or, more likely, in combination, are essential for the entry process (17, 47). Although this course of events appears to be generally accepted (21, 38), it should be noted that the gB homologs of HSV-1 and BHV-1 also bind heparin (6, 21, 22), that the gD homolog of Marek's disease virus (MDV) is not essential for infectivity (39), and that the genome of varicella-zoster virus (VZV) even lacks a gD homolog (48), indicating differences in the molecular mechanisms of the entry processes among alphaherpesviruses.

The existence of functional differences among homologous glycoproteins was demonstrated by the findings that BHV-1 gB can complement the lethal defect in gB<sup>-</sup> PrV and that PrV gB functionally substitutes for HSV-1 gB, whereas the respective reciprocal complementations were not achieved (34, 42). In

addition, compared to PrV gD, gD of HSV-1 and that of BHV-1 appear to have accessory functions, because in the latter viruses gD is essential for cell-to-cell spread (10, 26), whereas in PrV-infected cells gD is not required for direct spreading (40, 41). Recently, Liang et al. (25) reported that BHV-1 gD was not essential for but facilitated intercellular spreading of gD<sup>-</sup> mutant BHV-1/80-221 and concluded that the cell-to-cell spread that they observed does not result from a further mutation of the virus but represents a general property of gD-null BHV-1. This result contrasts the data published by Fehler et al. (10), who demonstrated that gD is essential for direct cell-to-cell spread of BHV-1/80-221.

In this report, we show that a single point mutation within the BHV-1 gH open reading frame (ORF) compensates for the gD function for cell-to-cell spread, demonstrating that gD-independent spreading is not inherent in BHV-1. We further show that after continuous passaging of the cell-to-cell-spreading BHV-1/80-221 mutant, at least one additional mutation results in the appearance of infectious gD-negative virions in the cell culture supernatants, suggesting that also the function of gD for virus entry into the target cells can be taken over by some other constituent(s) of BHV-1 particles. These results indicate that the major role of gD for BHV-1 is to ensure a penetration-competent conformation of other (glyco)proteins in the viral envelope rather than directly causing membrane fusion.

## MATERIALS AND METHODS

**Cells and viruses.** BHV-1 strains Schönböken (BHV-1/Schö) and Aus12 (BHV-1/Aus12) were obtained from O. C. Straub (Tübingen, Germany) and

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propagated on Madin-Darby bovine kidney (MDBK) cell clone Bu100 (MDBK-Bu100; kindly provided by W. Lawrence and L. Bello, Philadelphia, Pa.), as were the *lacZ*<sup>+</sup> mutants BHV-1res, which underexpresses gD (10), and BHV-1/e1lacZ, in which the gI ORF is replaced by the *lacZ* ORF under control of the murine cytomegalovirus early 1 promoter (4, 19). BHV-1/Aus12 encodes a gD with an apparent molecular mass of 84 kDa (10) and was used to construct the gD-negative mutant BHV-1/80-221, in which the ORF encoding gD was replaced by a *lacZ* cassette (10, 33). Relevant characteristics of virus strains used are summarized in Table 1. BHV-1/80-221 was propagated on the constitutively gD-expressing cell line BU-Dorf, isolated as described for cell line BUIV3-7 (10) after cotransfection of MDBK-Bu100 cells with plasmids pAG60 (7) and pMT Dorf, which contains the ORF coding for the 72-kDa gD of BHV-1/Schö. Cell line MDBK-C20 was obtained after cotransfection of plasmids pAG60 and pbal5, which, in addition to the sequences encompassed by plasmid pbal10 (see Fig. 4), contains the putative promoter of the gH gene of ctc<sup>+</sup>BHV-1/80-221. Cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 5% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (0.35 mg/ml).

**Passaging of ctc<sup>+</sup>BHV-1/80-221.** Cultures containing ctc<sup>+</sup>BHV-1/80-221-induced plaques were trypsinized, and cells were mixed 1:10 to 1:100 with trypsinized uninfected MDBK-Bu100 cells.

**Passaging of gD<sup>-</sup>infBHV-1.** After development of full cytopathic effect (CPE), supernatants from cultures infected with gD<sup>-</sup>infBHV-1 were frozen at -70°C, thawed, sonicated twice for 10 s at 100 W in a Branson B15 ultrasonic water bath, clarified by low-speed centrifugation, and filtered through a 0.2-µm-pore-size filter unit (Schleicher & Schüll, Dassel, Germany) prior to inoculation of subconfluent MDBK-Bu100 cultures.

**DNA sequencing and PCR.** Sequencing of the gH genes of BHV-1/80-221 and ctc<sup>+</sup>BHV-1/80-221 and cloned PCR products was performed with a T7 sequencing kit (Pharmacia, Freiburg, Germany) on double-stranded plasmid DNA, using appropriate subclones inserted into plasmid vector pSP73 (Promega, Heidelberg, Germany) and the primers recommended by the manufacturer or specific internal oligonucleotides derived from established sequences. To analyze BHV-1 gH sequences containing codon 450 (36), a 396-bp fragment was amplified by using primers P1 (TAGTCTAGAGCACCATCGCGCAGCTGGCC, encompassing gH codons 428 to 434) and P2 (GTAGAATTCATACCTCCAGGTCCAGC, complementary to gH codons 553 to 547). To facilitate subsequent cloning, cleavage sites for *Xba*I and *Eco*RI (boldfaced in the corresponding sequences) were added 5' of the gH sequences of P1 and P2, respectively. PCR was performed in 100 µl of a reaction mixture containing 10 µl of 10× *Taq* buffer supplied with the enzyme and 1 U of *Taq* polymerase (Boehringer, Mannheim, Germany), 0.5 mM MgCl<sub>2</sub>, 5% dimethyl sulfoxide, 0.1 mM deoxynucleoside triphosphates (Boehringer), 100 ng of template DNA, and 0.1 µM each primer. Mixtures were overlaid with paraffin oil and incubated for 1 min at 97°C, and amplification was achieved by 30 repeated cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, using a Perkin-Elmer GENE-AMP 9600 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). PCR products were purified by phenol extraction followed by ethanol precipitation, cleaved with *Eco*RI and *Xba*I, and then ligated into the *Eco*RI- and *Xba*I-cleaved plasmid vector pSP73.

**Plasmid constructions.** All cloning procedures were carried out according to standard methods (43). The *Hind*III A fragment of ctc<sup>+</sup>BHV-1/80-221 (see Fig. 4 for genomic location), inserted into plasmid vector pACYC177 (9), was cleaved with *Bgl*II, and a 8.5-kbp fragment was ligated into the *Bgl*II-cleaved plasmid vector pSP73. Orientation of the insert in the resulting plasmid, pBB8.5 (see Fig. 4), was analyzed by sequencing from both ends. Sequence analysis demonstrated that a *Bgl*II site located within the thymidine kinase (*tk*) gene (3) at codon 156 (37) was placed adjacent to the residual polylinker of pSP73 and that the second

*Bgl*II recognition sequence was within the BHV-1 UL19 ORF (49). Thus, pBB8.5 encompasses the entire BHV-1 gH (UL22), UL21, and UL20 genes (49). Plasmid pBB8.5 was linearized with *Xba*I within the polylinker and incubated with *Bal* 31 for 5 and 10 min. Digestion products were treated with Klenow polymerase to create blunt ends followed by *Bgl*II cleavage. Truncated fragments were isolated after agarose gel electrophoresis and ligated into pSP73 cleaved with *Bam*HI and *Eco*RV. The extent of the truncations in the resulting plasmids pbal5 and pbal10 (see Fig. 4) was determined by sequencing using SP6 promoter-specific oligonucleotides. Results showed that in pbal5 and pbal10, BHV-1 sequences started 362 nucleotides upstream and 39 nucleotides downstream, respectively, from the putative gH TATA box.

To obtain pbal10/15, plasmid pbal10 was cleaved with *Tth*1111 0.7 kbp downstream from the gH stop codon, incubated with *Bal* 31 for 15 min, cleaved with *Xba*I, and treated with Klenow polymerase. Reaction products were separated in a 0.6% agarose gel, and DNA fragments with the expected size of the gH ORF together with the plasmid vector were isolated and religated. Several of the resulting clones were sequenced by using T7 promoter-specific oligonucleotides; this analysis revealed that in plasmid pbal10/15 (see Fig. 4), BHV-1 sequences ended 126 nucleotides downstream from the gH polyadenylation signal.

To isolate the BHV-1/80-221 gH gene, the respective 8.5-kbp fragment was isolated after cleavage of the *Hind*III A fragment of BHV-1/80-221 with *Bgl*II and inserted into the *Bgl*II-cleaved plasmid vector pSP73. The resulting plasmid, p80BB8.5, was cleaved with *Sly*I 11 nucleotides upstream from the gH TATA box and 86 nucleotides downstream from the gH polyadenylation signal and blunt ended with Klenow polymerase, and the 2.8-kbp fragment encompassing gH was inserted into pSP73 cleaved with *Xho*I and *Pvu*II and blunt ended with Klenow polymerase. The resulting plasmid was named p80gH. To replace codon 450 of BHV-1/80-221 gH by codon 450 of ctc<sup>+</sup>BHV-1/80-221 gH, pbal10/15 was cleaved with *Pvu*II and *Xho*I within codons 423 and 453, respectively. The 61-bp DNA fragment was isolated after electrophoresis in a 1.5% agarose gel and used to replace the corresponding 61-bp fragment in p80gH. The resulting plasmid was named pRep61gH.

Plasmid pMTDorf was constructed by inserting the gD ORF isolated from plasmid pROME after cleavage with *Xba*I (19) into *Xba*I-cleaved pMTMα, a derivative of pMTα (2) which contains the mouse metallothionein promoter in front of a polylinker sequence followed by the murine cytomegalovirus immediate-early 2 polyadenylation signal (29). Expression of gD by pMTDorf was demonstrated by transient expression experiments after induction of the metallothionein promoter with zinc sulfate (10).

**Isolation of recombinant cells.** Subconfluent MDBK-Bu100 cells were cotransfected with plasmids pbal5 and pAG60 encoding neomycin phosphotransferase (7). Individual geneticin-resistant colonies were picked 10 to 14 days after transfection (10, 18) and screened for the presence of the gH gene by dot blot hybridization (43). One recombinant cell line (MDBK-C20) hybridizing strongly to the <sup>32</sup>P-labeled gH gene-specific probe was selected for further experiments.

**Immunoprecipitation.** Confluent monolayers of MDBK cells grown in six-well tissue culture dishes (Greiner, Nürtingen, Germany) were infected at a multiplicity of infection of 10. After 1 h of adsorption, the inoculum was replaced by 500 µl of DMEM lacking methionine. After addition of [<sup>35</sup>S]methionine (500 µCi/ml), cells were incubated for the times indicated and immunoprecipitation of proteins from cell lysates was carried out as described previously (16). Labeled proteins were visualized after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by fluorography (16).

**Southern blot hybridizations.** Whole-cell DNA, isolated as described previously (45), or purified virus DNA was digested with *Hind*III, size fractionated in a 0.6% agarose gel, transferred to nitrocellulose filters, and hybridized by using

TABLE 1. Characteristics of BHV-1 strains and recombinants

Strain or recombinant	Genetic background	Genotype	Cell-to-cell spread	Infectious progeny released from noncomplementing cells
BHV-1/Schö <sup>a</sup>	Wild type	Wild type	+	+
BHV-1/Aus12 <sup>b</sup>	Wild type	Wild type	+	+
BHV-1/e1lacZ <sup>c</sup>	BHV-1/Aus12	gI <sup>-</sup> <i>lacZ</i> <sup>+</sup>	+	+
BHV-1res <sup>d</sup>	BHV-1/Aus12	<i>lacZ</i> <sup>+</sup>	+	+
BHV-1/80-221	BHV-1/Aus12	<i>lacZ</i> <sup>+</sup> gD <sup>-</sup>	-	-
ctc <sup>+</sup> BHV-1/80-221	BHV-1/80-221	<i>lacZ</i> <sup>+</sup> gD <sup>-</sup>	+	-
gD <sup>-</sup> infBHV-1	ctc <sup>+</sup> BHV-1/80-221	<i>lacZ</i> <sup>+</sup> gD <sup>-</sup>	+	+
ctc <sup>+</sup> BHV-1/CV27	ctc <sup>+</sup> BHV-1/80-221	gD <sup>+</sup>	+	+
BHV-1*/CV27	gD <sup>-</sup> infBHV-1	gD <sup>+</sup>	+	+

<sup>a</sup> Encodes 72-kDa gD which is expressed in cell lines BUIV3-7 and BU-Dorf.

<sup>b</sup> Encodes 84-kDa gD and was used to construct BHV-1/80-221.

<sup>c</sup> *lacZ* expression cassette replaces the gI ORF and penetrates as does wild-type BHV-1.

<sup>d</sup> Underexpresses gD resulting in retarded penetration.

standard procedures (43). DNA probes used for hybridization were labeled with [ $\alpha$ - $^{32}$ P]dCTP, using a Ready To Go DNA labeling kit (Pharmacia).

**Penetration kinetics.** Penetration kinetics were determined essentially as described previously (10, 30), using low-pH inactivation of extracellular virions at different times after a shift of infected cells from 4 to 37°C.

**Staining of cells for *lacZ* expression and determination of  $\beta$ -Gal activity.** To demonstrate *lacZ* expression in fixed cells, cultures were incubated for 5 min at 4°C with 2% paraformaldehyde in phosphate-buffered saline (PBS), washed with PBS, and incubated at 37°C for up to 8 h with PBS containing 1 mg of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) per ml, 2 mM MgCl<sub>2</sub>, 5 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>], and 5 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>]. For the isolation and plaque purification of recombinant viruses, cells were overlaid with 0.6% agarose and 300  $\mu$ g of Bluo-Gal (Gibco-BRL, Dreieich, Germany) per ml in DMEM (33). Quantitative determination of  $\beta$ -galactosidase ( $\beta$ -Gal) activity in infected cells was determined as described previously (10).

**Marker rescue experiments and isolation of gD<sup>+</sup> revertants.** DNA from gD-null mutants BHV-1/80-221, ctc<sup>+</sup>BHV-1/80-221, and gD<sup>-</sup>infBHV-1 was isolated from purified virions as described previously (9). All transfection experiments were done by using MDBK-Bu100 cells and a Stratagene (La Jolla, Calif.) mammalian transfection kit according to the manufacturer's protocol. For marker rescue, 1  $\mu$ g of purified BHV-1/80-221 DNA was cotransfected either with gel-purified fragments of ctc<sup>+</sup>BHV-1/80-221 DNA after cleavage of 5  $\mu$ g of viral DNA with *Hind*III and agarose gel electrophoresis or with 5  $\mu$ g of purified plasmid DNA. As controls, ctc<sup>+</sup>BHV-1/80-221 DNA and BHV-1/80-221 DNAs (1  $\mu$ g of each) were transfected into parallel cultures. Cells were fixed 3 days after transfection and stained for *lacZ* expression. Marker transfer was regarded as positive when at least four blue-staining plaques had developed.

For integration of the gD gene into the genomes of ctc<sup>+</sup>BHV-1/80-221 and gD<sup>-</sup>infBHV-1, 1  $\mu$ g of each of the respective viral DNAs was cotransfected with 5  $\mu$ g of plasmid pCV27 containing the wild-type *Hind*III L fragment, which encompasses the entire gD gene. Virus progeny was titrated on MDBK-Bu100 cells. Virus from plaques that did not stain blue under a Bluo-Gal agarose overlay was picked and plaque purified. Replacement of the *lacZ* cassette by the gD gene in the isolates was demonstrated by Southern blot hybridizations.

## RESULTS

**Isolation of a BHV-1/80-221 mutant which spreads from cell to cell.** In the mutant BHV-1/80-221, the gD ORF was replaced by a *lacZ* cassette (10). BHV-1/80-221 replicates normally on BHV-1 gD-expressing cells but is unable to form plaques and infectious progeny on noncomplementing cells, demonstrating that gD is essential for virus entry into cells and direct cell-to-cell spread. After propagation of BHV-1/80-221 on complementing BUIV3-7 cells, gD-expressing viruses appear at a frequency of approximately 10<sup>-4</sup> and have been rescued by integration of the gD gene from the complementing cell into their genomes (10). Since this is most likely due to homologous sequences derived from the gI gene which are present in both cell line BUIV3-7 and virus mutant BHV-1/80-221 (10), we constructed a cell line which contained only the gD ORF and no additional BHV-1 sequences. This cell line, designated as BU-Dorf, exhibited no qualitative differences from BUIV3-7 cells regarding, e.g., complementation of gD<sup>-</sup> BHV-1. However, the frequency of rescued virus proved to be lower than 10<sup>-7</sup>.

Surprisingly, when 10<sup>6</sup> MDBK-Bu100 cells were infected at a multiplicity of infection of 10 with BHV-1/80-221 transcomplemented by propagation on BU-Dorf cells, trypsinized 6 h postinfection (p.i.), and reseeded with a 10-fold excess of noninfected MDBK-Bu100 cells, a total of about 100 plaques appeared in the cultures at 2 days p.i. These plaques were smaller than those induced by wild-type or gD-rescued BHV-1, and they stained blue under a Bluo-Gal agarose overlay. When these plaques were picked by aspiration, frozen, thawed, and used to infect either MDBK-Bu100 or BU-Dorf cells, no CPE developed and staining for *lacZ* proved to be negative. In contrast, after trypsinization and cocultivation with noninfected MDBK-Bu100 or BU-Dorf cells, plaques appeared, indicating that infectivity was transmitted by direct cell-to-cell spread requiring living virus-infected cells. To test for the presence of free infectious virus, supernatants of MDBK-Bu100 and BU-Dorf cells were clarified by low-speed

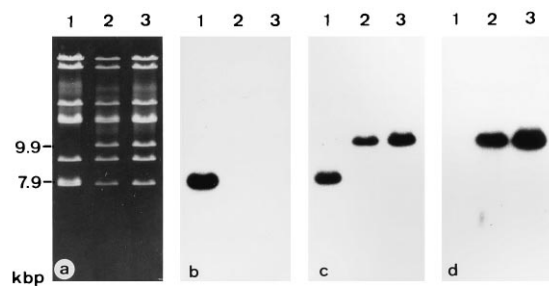


FIG. 1. ctc<sup>+</sup>BHV-1/80-221 lacks the gD ORF. DNA isolated from virions released into the supernatants of MDBK-Bu100 cells infected with BHV-1/Aus12 (lanes 1), BHV-1/80-221 (lanes 2), and ctc<sup>+</sup>BHV-1/80-221 (lanes 3) was cleaved with *Hind*III, and fragments were transferred to nitrocellulose after separation in a 0.6% agarose gel and photography of the ethidium bromide-stained gel. Filters were hybridized to <sup>32</sup>P-labeled DNA encompassing the gD ORF (b) or the gI ORF (c) or from the *lacZ* ORF (d). Bound radioactivity was visualized by autoradiography.

centrifugation, filtered through 0.2- $\mu$ m-pore-size filters, and titrated on MDBK-Bu100 cells. Cell-free infectivity was detected in supernatants from gD-expressing BU-Dorf cells but was absent in cleared supernatants from MDBK-Bu100 cells. However, after ultracentrifugation, typical herpesvirus particles could be observed by electron microscopic examination in both preparations, indicating that virions released from MDBK-Bu100 cells were noninfectious. Since it thus appeared that the original gD<sup>-</sup> mutant BHV-1/80-221 had acquired the propensity to directly spread from primary infected target cells to neighboring noninfected cells, we designated this virus ctc<sup>+</sup>BHV-1/80-221.

To check the genotype of ctc<sup>+</sup>BHV-1/80-221 and to ascertain continuous absence of the gD gene, Southern blot hybridizations were performed after cleavage of virion DNA with *Hind*III. As shown in Fig. 1a, the fragment patterns of DNA from ctc<sup>+</sup>BHV-1/80-221 and BHV-1/80-221 were identical after ethidium bromide staining. In addition, no differences were observed after hybridization with a gI-specific (Fig. 1c) or *lacZ*-specific (Fig. 1d) probe, and a gD-specific probe failed to hybridize with either DNA (Fig. 1b). In contrast, after cleavage of DNA from wild-type BHV-1/Aus12, the gD and gI probes hybridized to a 7.9-kbp fragment, whereas the *lacZ* probe did not specifically react, as expected (Fig. 1b to d).

To directly test for lack of gD expression, MDBK cells were infected with wild-type BHV-1/Schö, BHV-1/80-221 grown on BU-Dorf, and ctc<sup>+</sup>BHV-1/80-221 grown on BU-Dorf. Proteins were labeled with [<sup>35</sup>S]methionine and immunoprecipitated with either gD-specific monoclonal antibody (MAb) 21/3/3 (Fig. 2, lanes 1 to 3) or gC-specific MAb 118/2/4 (Fig. 2,

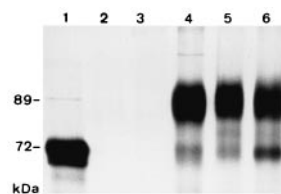


FIG. 2. Evidence that gD is not expressed by ctc<sup>+</sup>BHV-1/80-221. MDBK-Bu100 cells were infected with BHV-1/Schö (lanes 1 and 4) or phenotypically gD-complemented BHV-1/80-221 (lanes 2 and 5) and ctc<sup>+</sup>BHV-1/80-221 (lanes 3 and 6). [<sup>35</sup>S]methionine was added at 2 h p.i., and cells were lysed at 16 h p.i. Lysates were incubated with gD-specific MAb 21/3/3 (lanes 1 to 3) or gC-specific MAb 118/2/4 (lanes 4 to 6). Bound proteins were visualized by fluorography after immunoprecipitation and SDS-PAGE.



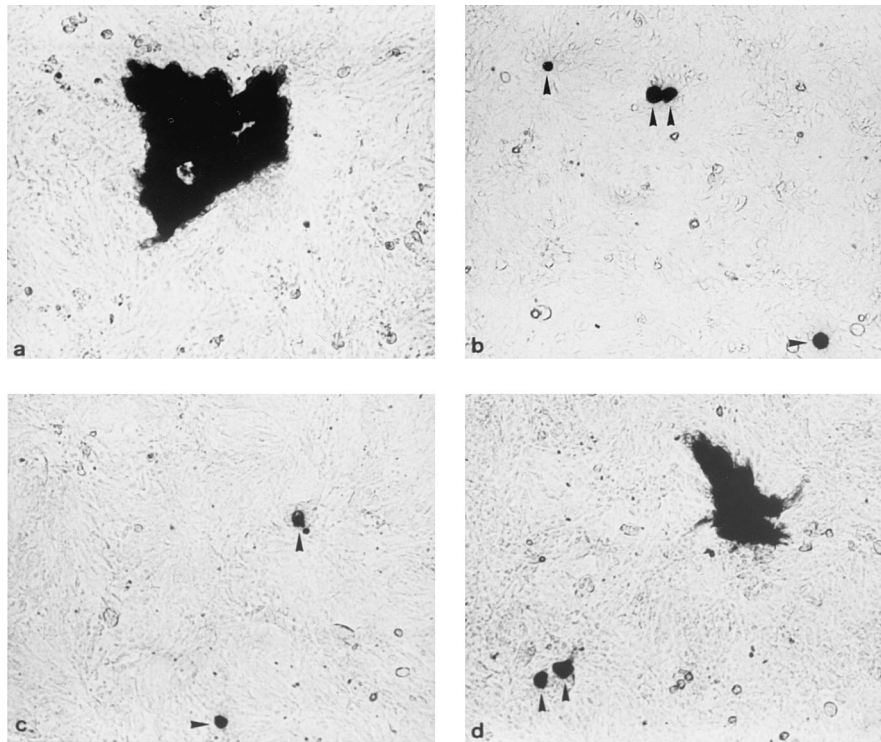


FIG. 3. Localization of the locus involved in cell-to-cell spreading by marker rescue experiments. MDBK-Bu100 cells were transfected with purified DNA of *ctcs*<sup>+</sup>BHV-1/80-221 (a), purified DNA of BHV-1/80-221 (b), purified DNA of BHV-1/80-221 and the gel-purified *Hind*III B fragment of *ctcs*<sup>+</sup>BHV-1/80-221 DNA (c), or DNA of BHV-1/80-221 and the gel-purified *Hind*III A fragment of *ctcs*<sup>+</sup>BHV-1/80-221 DNA (d). Cultures were fixed and stained for *lacZ* expression 3 days after transfection.  $\beta$ -Gal-expressing single cells are indicated by arrowheads.

lanes 4 to 6). MAb 21/3/3 precipitated the 72-kDa gD of BHV-1/Schö (Fig. 2, lane 1) but did not specifically react with any protein from BHV-1/80-221- and *ctcs*<sup>+</sup>BHV-1/80-221-infected cells (Fig. 2, lanes 2 and 3). In contrast, MAb 118/2/4 precipitated the 89-kDa gC from cells infected with BHV-1/Schö, BHV-1/80-221, and *ctcs*<sup>+</sup>BHV-1/80-221 (Fig. 2, lanes 4 to 6), demonstrating comparable levels of infection of the various cell cultures. From these results, it was concluded that a variant of BHV-1/80-221, designated *ctcs*<sup>+</sup>BHV-1/80-221, which is capable of spreading from cell to cell in the absence of gD had been isolated.

**Marker rescue experiments.** The mutation(s) that compensates for the gD function for cell-to-cell spread was delineated by marker rescue experiments as shown in Fig. 3. MDBK-Bu100 cells were transfected with purified DNA from *ctcs*<sup>+</sup>BHV-1/80-221 as a positive control (Fig. 3a) or BHV-1/80-221 DNA as a negative control (Fig. 3b) or were cotransfected with BHV-1/80-221 DNA and DNA from a genomic, gel-purified *ctcs*<sup>+</sup>BHV-1/80-221 *Hind*III B (Fig. 3c) or *Hind*III A (Fig. 3d) fragment. For map locations of *Hind*III fragments, see Fig. 4. Cultures were fixed and stained for *lacZ* expression 3 days after transfection. About 20 blue-staining plaques were always observed in cells transfected with *ctcs*<sup>+</sup>BHV-1/80-221 DNA only (Fig. 3a). After cotransfection of BHV-1/80-221 DNA with DNA from the *ctcs*<sup>+</sup>BHV-1/80-221 *Hind*III A fragment, typically four to six blue plaques were detected besides stained single cells (Fig. 3d). In cultures transfected with BHV-1/80-221 DNA alone (Fig. 3b) or together with the *Hind*III B fragment of *ctcs*<sup>+</sup>BHV-1/80-221 (Fig. 3c),  $\beta$ -Gal activity was consistently detected only in single cells. The latter result was also obtained when the other gel-purified genomic *ctcs*<sup>+</sup>BHV-1/80-221 *Hind*III fragments were used (not shown). To further

pinpoint the locus containing the mutation(s), the *Hind*III A fragment of *ctcs*<sup>+</sup>BHV-1/80-221 was inserted into the plasmid vector pACYC177, and a 14-kbp *Hind*III-*Bgl*II subfragment and an 8.5-kbp *Bgl*II subfragment were integrated into the plasmid vector pSP73 to obtain pHB14 and pBB8.5. From these, only pBB8.5, which contains the 3' end of the BHV-1 *tk* gene (37), the BHV-1 gH gene (36), and sequences homologous to HSV-1 ORFs UL21, UL20, and UL19 (49) rescued the cell-to-cell spreading capacity in BHV-1/80-221 (Fig. 4). To more precisely define the sequences required, pBB8.5 was incubated with *Bal* 31 after linearization within the *tk* gene. Truncated fragments were isolated after size fractionation in agarose gels, integrated into pSP73, and tested for rescue of cell-to-cell spread in BHV-1/80-221. From these truncations, plasmid pbal10 contained the shortest fragment tested which resulted in plaque formation after cotransfection with BHV-1/80-221 DNA (Fig. 4). This result led to the assumption that gH of *ctcs*<sup>+</sup>BHV-1/80-221 enables gD-independent cell-to-cell spread of *ctcs*<sup>+</sup>BHV-1/80-221. This assumption was supported by induction of plaque formation after cotransfection of plasmid pbal10/15 (Fig. 4) with BHV-1/80-221 DNA into MDBK-Bu100 cells.

To corroborate the results obtained by the marker rescue experiments, the gene encoding gH of *ctcs*<sup>+</sup>BHV-1/80-221 was integrated into the genome of MDBK-Bu100 cells, resulting in transgenic cell line MDBK-C20. Phenotypically gD-complemented BHV-1/80-221 and *ctcs*<sup>+</sup>BHV-1/80-221 were titrated on MDBK-Bu100 and MDBK-C20 cells. In contrast to *ctcs*<sup>+</sup>BHV-1/80-221, which formed plaques in MDBK-Bu100 and MDBK-C20 cultures at appropriate dilutions, BHV-1/80-221 induced plaques only on MDBK-C20 cells (Fig. 5), sup-

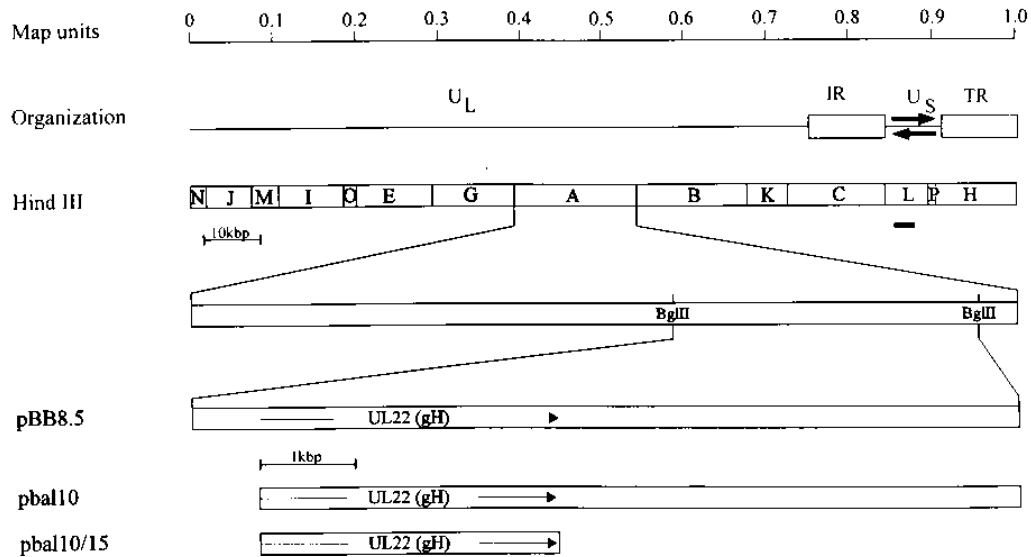


FIG. 4. The gH ORF of *ctcs*<sup>+</sup>BHV-1/80-221 complements the gD function for cell-to-cell spread. The *Hind*III restriction fragment map is shown below a schematic diagram of the prototype orientation of the genome of BHV-1 (27). Location of the gD gene in wild-type BHV-1 or the *lacZ* cassette in BHV-1/80-221 is indicated by a black bar below the *Hind*III L fragment. The *Hind*III A fragment is enlarged, and *Bgl*II cleavage sites used for construction of plasmid pBB8.5 are marked. The latter is expanded, and the position and direction of transcription of the BHV-1 gH gene are depicted. Below, locations of truncated fragments contained within plasmids pbal10 and pbal10/15 are indicated. For simplicity, only fragments which rescued direct spreading in BHV-1/80-221 are depicted. IR, internal repeat; TR, terminal repeat.

porting the conclusion that gH of *ctcs*<sup>+</sup>BHV-1/80-221 confers cell-to-cell spreading of BHV-1/80-221 *in trans*.

**A single point mutation within the gH ORF is necessary and sufficient for conversion of BHV-1/80-221 to *ctcs*<sup>+</sup>BHV-1/80-221.** To precisely determine the mutation(s) resulting in cell-to-cell spread of gD<sup>-</sup> BHV-1, the gH genes of BHV-1/80-221, its parent strain BHV-1/Aus12, and *ctcs*<sup>+</sup>BHV-1/80-221 were sequenced. The comparison revealed only one nucleotide exchange, from G to T at the first position of codon 450 of the gH ORF, resulting in an amino acid exchange from glycine in gH of BHV-1/Aus12 and BHV-1/80-221 to tryptophan in *ctcs*<sup>+</sup>BHV-1/80-221 gH (Fig. 6). This point mutation is located within a 61-bp *Pvu*II-*Xho*I fragment of the *ctcs*<sup>+</sup>BHV-1/80-221 gH ORF. This fragment was isolated from pbal10/15 and used

to replace the corresponding sequence within plasmid p80gH, which contains the BHV-1/80-221 gH ORF, to confirm that the exchange from glycine to tryptophan mediates gD-independent cell-to-cell spread. The resulting plasmid, pRep61gH, was cotransfected with BHV-1/80-221 into MDBK-Bu100 cells. Plaques were detected after 3 days. Whole-cell DNA from cells infected with the resulting recombinant, BHV-1/80-221gHW<sub>450</sub> was isolated, and introduction of the mutation was confirmed by sequencing of a PCR-amplified DNA segment encompassing codon 450 (Fig. 6). Thus, the transition of glycine<sub>450</sub> to tryptophan is sufficient to confer the ability for direct cell-to-cell spread to gD<sup>-</sup> BHV-1/80-221. This mutation appears not to affect the overall structure of gH because no difference in the migration of the gH variants in SDS-gels,

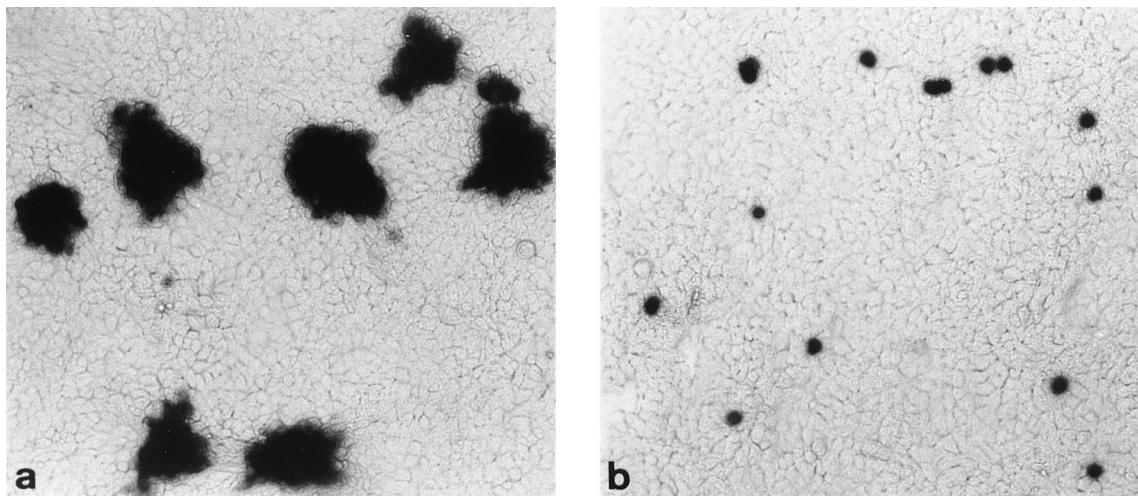


FIG. 5. Transcomplementation of the cell-to-cell spread defect in BHV-1/80-221. MDBK-C20 cells (a) and MDBK-Bu100 cells (b) were infected with diluted, phenotypically gD-complemented BHV-1/80-221, fixed 2 days p.i., and stained for *lacZ* expression.





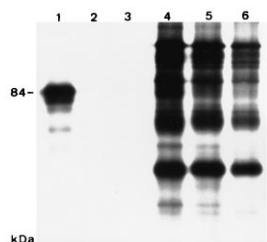


FIG. 8.  $gD^-$ infBHV-1 does not express gD. MDBK-Bu100 cells were infected with BHV-1/Aus12 (lanes 1 and 4), phenotypically gD-complemented  $ctcs^+$ BHV-1/80-221 (lanes 2 and 5), and phenotypically gD-complemented  $gD^-$ infBHV-1 (lanes 3 and 6). At 2 h p.i., [ $^{35}S$ ]methionine was added, and labeled proteins were immunoprecipitated from cells lysed at 16 h p.i., using gD-specific MAb 21/3/3 (lanes 1 to 3) or a rabbit anti-BHV-1 serum (lanes 4 to 6) to monitor infection. Precipitated proteins were separated on an SDS-10% polyacrylamide gel and visualized by fluorography. The 84-kDa gD of BHV-1/Aus 12 is indicated.

1/80-221 and  $gD^-$ infBHV-1 to test whether the respective mutations influence virus entry after coexpression with gD. For that analysis, purified viral DNA was cotransfected with plasmid pCV27, which contains the *Hind*III L fragment in which the gD gene is flanked by genes encoding gG and gI (10, 20), which were not affected by deleting the gD ORF in BHV-1/80-221 (10). Infectious progeny from the transfected cultures was titrated on MDBK-Bu100 cells, and plaques that did not stain blue under an agarose overlay containing Blu-Gal were picked and plaque purified twice. Replacement of the  $\beta$ -Gal cassette by the gD gene was confirmed by Southern blot hy-

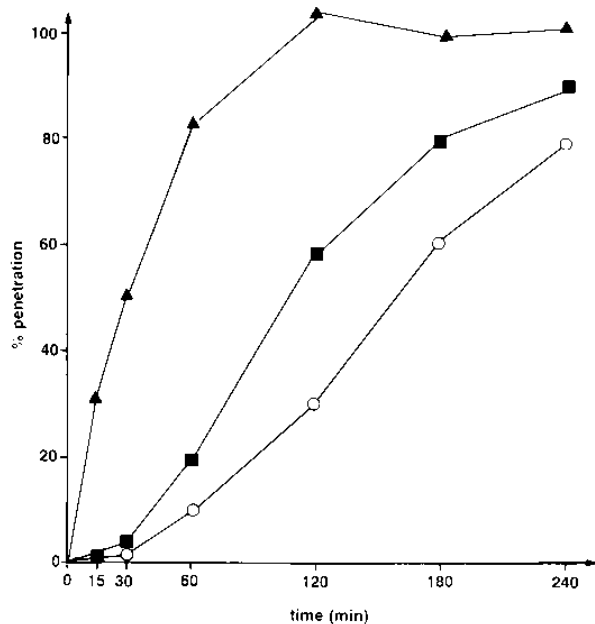


FIG. 9. Penetration kinetics of  $gD^-$ infBHV-1. MDBK-Bu100 cells were preincubated at 4°C for 15 min, and strains BHV-1/e1lacZ (triangles), BHV-1/res (squares), and  $gD^-$ infBHV-1 (circles) (approximately 10,000 PFU of each per cell culture dish) were allowed to adsorb for 2 h at 4°C. After a temperature shift to 37°C, either duplicate cultures were washed with PBS or nonpenetrated virions were inactivated at the times indicated by incubation with sodium citrate buffer (pH 3.0) for 2 min. After washing with PBS, cultures were incubated for 16 h with normal cell culture medium. Cells were harvested, and  $\beta$ -Gal activity was measured. Percent penetration was calculated from the value obtained with or without low pH treatment.

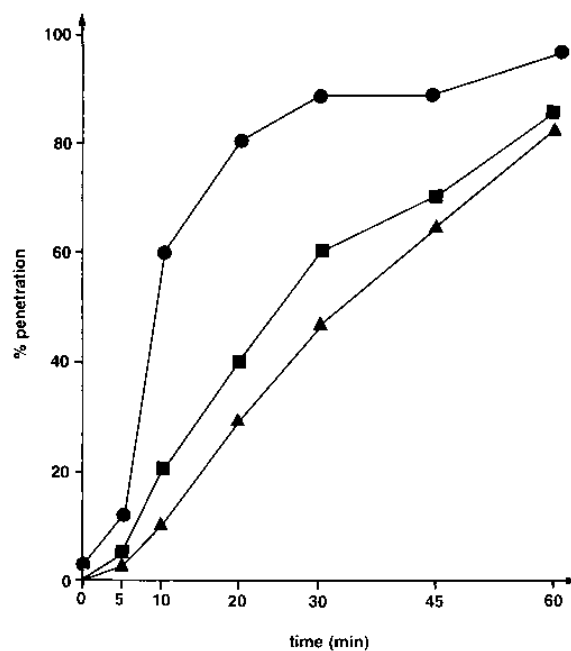


FIG. 10. Integration of the gD gene into the genome of  $gD^-$ infBHV-1 results in enhanced penetration. Stocks of strains BHV-1/Aus12 (triangles), BHV-1\*/CV27 (circles), and  $H_{W450}$ BHV-1/CV27 (squares) were diluted to yield approximately 200 PFU per cell culture dish. Penetration kinetics were determined as described previously (10).

bridizations, and gD expression was demonstrated by immunoprecipitations using MAb 21/3/3 (data not shown).

The resulting viruses were named  $H_{W450}$ BHV-1/CV27 (genetic background of  $ctcs^+$ BHV-1/80-221) and BHV-1\*/CV27 (genetic background of  $gD^-$ infBHV-1), and their penetration kinetics were determined by using wild-type BHV-1/Aus12 as a control. As shown in Fig. 10, BHV-1/Aus12 penetrated with a kinetics well established for several wild-type BHV-1 strains (10) and required about 30 min to reach 50% penetration, whereas half of the  $H_{W450}$ BHV-1/CV27 virions were protected against low-pH inactivation after 25 min. In contrast to the slightly improved penetration behavior of  $H_{W450}$ BHV-1/CV27 compared to BHV-1/Aus12, BHV-1\*/CV27 rapidly entered the cells after adsorption and needed only about 8 min for 50% penetration. Thus, the modification in gH of  $ctcs^+$ BHV-1/80-221 seems to have only a small effect on gD-dependent entry, whereas the mutation(s) in  $gD^-$ infBHV-1 apparently results in formation of penetration-mediating structures whose efficiency is dramatically improved by gD, underlining the beneficial role of gD for BHV-1 infectivity.

## DISCUSSION

In studies using gD deletion mutant BHV-1/80-221, it has been shown that gD is essential for penetration into cells as was reported for the gD homologs of HSV-1 and PrV (10, 26, 41). In addition, HSV-1 and BHV-1 require gD for direct cell-to-cell spread (10, 26), whereas PrV gD is dispensable for direct spreading (41). Here we report the isolation of  $ctcs^+$ BHV-1/80-221, which is able to spread directly from cell to cell even in the absence of gD, and demonstrate that a single point mutation within the gene encoding gH compensates the gD function in this process.

$ctcs^+$ BHV-1/80-221 was detected after cocultivation of MDBK-Bu100 cells infected with BHV-1/80-221, phenotypi-

cally complemented by propagation on BU-Dorf cells, which, in contrast to the previously used gD-expressing cell line BUIV3-7, do not contain sequences homologous to BHV-1/80-221 DNA. Cell line BU-Dorf was engineered to decrease the risk for generation of gD-rescued viruses which have been invariably observed in BHV-1/80-221 stocks grown in BUIV3-7 cells (10).

The mutation leading to gD-independent cell-to-cell spread was localized within the gH gene of *ctcs*<sup>+</sup>BHV-1/80-221 by marker rescue experiments. Sequencing of the gH genes of BHV-1/Aus12, BHV-1/80-221, and *ctcs*<sup>+</sup>BHV-1/80-221 showed that in the latter, only the nucleotide at the first position of codon 450 was changed from G to T, resulting in an amino acid exchange from glycine to tryptophan. Comparison of the BHV-1/Aus12 sequence with the gH sequence of BHV-1 strain Cooper (36) revealed an exchange of threonine<sub>531</sub> (encoded by ACG) in gH of BHV-1/Aus12, BHV-1/80-221, and *ctcs*<sup>+</sup>BHV-1/80-221 to alanine<sub>531</sub> (encoded by GCG) within the Cooper gH (36). Whether this mutation also contributes to gD-independent spreading has to be analyzed. However, the glycine-to-tryptophan exchange alone is sufficient to mediate cell-to-cell spread in the absence of gD, as was demonstrated by introducing this mutation into BHV-1/80-221 and by construction of transgenic cell line MDBK-C20, which expresses gH of *ctcs*<sup>+</sup>BHV-1/80-221 and promotes cell-to-cell spread not only of BHV-1/80-221 (this report) but also of another gD<sup>-</sup>BHV-1 strain (data not shown). It should be noted that MDBK-C20 cells may also express the UL21 and UL20 genes, whose products are clearly not required for direct spreading of BHV-1/80-221. In addition, direct spreading of *ctcs*<sup>+</sup>BHV-1/80-221 was not a unique feature attributable to MDBK-Bu100 cells, since gD-independent cell-to-cell spread was also observed in bovine embryo lung cells, bovine embryonic trachea cells, and owl monkey kidney cells (data not shown).

Our results are to some extent in contrast to recent observations (25) that gD-independent cell-to-cell spread of BHV-1/80-221 grown on BUIV3-7 cells occurred in noncomplementing MDBK cells. The authors concluded that the ability of BHV-1/80-221 to form plaques on normal MDBK cells represents a general property of the virus pool, with no requirement for an additional mutation. A possible explanation for the discrepancy may be that by propagating BHV-1/80-221 on BUIV3-7 cells, *ctcs*<sup>+</sup>BHV-1/80-221 present in the original virus pool was amplified to finally constitute a substantial proportion of the virus stock.

After isolation of *ctcs*<sup>+</sup>BHV-1/80-221, we asked whether this virus would further evolve under the new environmental conditions. Therefore, *ctcs*<sup>+</sup>BHV-1/80-221-infected MDBK-Bu100 cells were continuously passaged, which resulted in the isolation of a further BHV-1 mutant able to penetrate MDBK-Bu100 cells in the absence of gD. Entry of this virus, named gD<sup>-</sup>infBHV-1, was significantly delayed, and titers in the supernatants of infected cell cultures exhibiting full CPE initially did not exceed  $2 \times 10^4$  PFU/ml. The titer increased slightly, to  $3 \times 10^5$  PFU/ml, after 105 continuous passages of infected cell culture supernatants through 0.2- $\mu$ m-pore-size filters, indicating adaptability of the gD<sup>-</sup>infBHV-1 genome. Although the mutation(s) leading to gD-independent infectivity of BHV-1 has not yet been identified, the high number of passages required to compensate for the gD function for penetration in conjunction with the relatively low titers initially achieved leads us to speculate that most likely more than one domain had to be modified to mediate fusion between target cells and virion envelope in the absence of gD. This view is supported by the findings that (i) gD-null virus can enter transgenic cells expressing a glycosylphosphatidylinositol-anchored gD ecto-

main (25) but is unable to infect authentic gD-expressing cells (10, 25) and (ii) by using the approach described here, Schmidt et al. (44) isolated an infectious gD-negative PrV after ca. 10 continuous passages. The rapid conversion from gD-dependent penetration to gD-independent entry in PrV might have been facilitated by the presence of an optimized gD-independent cell-to-cell spread pathway. In addition, attempts to transfer gD-independent infectivity to *ctcs*<sup>+</sup>BHV-1/80-221 by fragmented gD<sup>-</sup>infBHV-1 DNA were not successful. This is in contrast to the marker rescue experiments, in which the ability for gD-independent spreading was efficiently transferred by isolated DNA fragments.

Having shown that mutated virion components were able to take over gD functions, we analyzed the effect of incorporating gD into the envelopes of *ctcs*<sup>+</sup>BHV-1/80-221 and gD<sup>-</sup>infBHV-1. For that analysis, the gD gene was reintegrated into the genomes of these viruses. The resulting viruses, H<sub>W450</sub>BHV-1/CV27 and BHV-1\*/CV27, expressed gD comparably to wild-type BHV-1 and were neutralized by MAb 21/3/3, and in comparison to BHV-1/Aus12, no significant differences in virus yields and growth properties were detected (data not shown), whereas the penetration kinetics demonstrated improved entry of both recombinants.

With the exception of gD, any of the other unique short region (U<sub>s</sub>)-encoded glycoproteins have been shown to be dispensable for BHV-1 replication in cell culture and in vivo (14). The discovery that the essential function(s) of gD for wild-type BHV-1 can be compensated for by other constituents of the viral envelope under appropriate environmental conditions supports the suggestion made by McGeoch (28) that the glycoprotein gene family in the U<sub>s</sub> of alphaherpesviruses has evolved from one precursor gene by duplications and subsequent divergence. It is tempting to speculate that the VZV lineage separated earlier from the lineage leading to HSV, PrV, and BHV-1 than from the lineage leading to MDV. However, it is also possible that MDV and VZV evolved from an ancestor common also to HSV, PrV, and BHV-1 after gD became nonessential, followed by deletions resulting in the VZV lineage. Elucidation of the molecular interactions involved in alphaherpesvirus-cell fusion, to which identification of the mutations leading to gD-independent infectivity certainly will contribute, may also help to shed light on the evolutionary relationships among the alphaherpesviruses.

The ability of gD<sup>-</sup> PrV to directly spread from cell to cell has resulted in the development of efficacious and safe vaccines (11, 40). Identification of the mutation in BHV-1 gH leading to gD-independent cell-to-cell spread now opens the possibility to construct BHV-1 vaccines with similar properties. The efficacy of *ctcs*<sup>+</sup>BHV-1 to protect calves against wild-type BHV-1 infection is currently being analyzed. Due to the apparently high passage number needed for generation of gD-negative infectious variants and their low infectivity, the potential use of such a vaccine should not be counteracted by the possible emergence of infectious gD<sup>-</sup> BHV-1. However, our finding that integration of gD into the genomes of *ctcs*<sup>+</sup>BHV-1/80-221 and gD<sup>-</sup>infBHV-1 resulted in recombinants with faster penetration raises the question of whether recombination between gD<sup>-</sup> vaccines and field viruses might generate viruses with increased virulence. Analysis of the in vivo growth properties and the pathogenicity of gD<sup>-</sup>infBHV-1, H<sub>W450</sub>BHV-1/CV27, and BHV-1\*/CV27 in infected cattle should enable us to evaluate this potential risk.

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