JOURNAL OF VIROLOGY, Feb. 1992, p. 831–839
0022-538X/92/020831-09$02.00/0

Vol. 66, No. 2

Glycoprotein IV of Bovine Herpesvirus 1-Expressing Cell Line Complements and Rescues a Conditionally Lethal Viral Mutant

FRANK FEHLER, JOHANNES M. HERRMANN, ARMIN SAALMÜLLER, THOMAS C. METTENLEITER, AND GÜNTHER M. KEIL*

Federal Research Center for Virus Diseases of Animals, P.O. Box 1149, D-7400 Tübingen, Germany

Received 5 July 1991/Accepted 17 October 1991

Glycoprotein IV (gIV) of bovine herpesvirus 1 (BHV-1), a homolog of herpes simplex virus glycoprotein D, represents a major component of the viral envelope and a dominant immunogen. To analyze the functional role of gIV during BHV-1 replication, cell line BUIV3-7, which constitutively expresses gIV, was constructed and used for the isolation of gIV$^-$ BHV-1 mutant 80-221, in which the gIV gene was replaced by a *lacZ* expression cassette. On complementing gIV-expressing cells, the gIV$^-$ BHV-1 replicated normally but was unable to form plaques and infectious progeny on noncomplementing cells. Further analysis showed that gIV is essential for BHV-1 entry into target cells, whereas viral gene expression, DNA replication, and envelopment appear unchanged in both noncomplementing and complementing cells infected with phenotypically complemented gIV$^-$ BHV-1. The block in entry could be overcome by polyethylene glycol-induced membrane fusion. After passaging of gIV$^-$ BHV-1 on complementing cells, a rescued variant, BHV-1res, was isolated and shown to underexpress gIV in comparison with its wild-type parent. Comparison of the penetration kinetics of BHV-1 wild type, phenotypically complemented gIV$^-$ BHV-1, and BHV-1res indicated that penetration efficiency correlated with the amount of gIV present in virus particles. In conclusion, we show that gIV of BHV-1 is an essential component of the virion involved in virus entry and that the amount of gIV in the viral envelope modulates the penetration efficiency of the virus.

Bovine herpesvirus 1 (BHV-1) belongs to the subfamily *Alphaherpesvirinae* of the herpesviruses. It is a natural pathogen in cattle and causes respiratory and genital diseases, abortions, and encephalitis (for reviews, see references 15, 28, and 48). Mature herpes virions are composed of an icosahedral capsid which encloses the linear double-stranded DNA genome. The capsid is in turn surrounded by a lipid envelope containing virus-encoded glycoproteins. These glycoproteins not only are predominant targets for the infected host's immune response (13, 20, 34, 35, 43) but also mediate important interactions between virus and target cells. In the BHV-1 genome, seven genes that encode polypeptides with significant homologies to glycoproteins found in herpes simplex virus (HSV) and pseudorabies virus (PrV) (12, 24a, 40, 45) have been localized (summarized in reference 46) and sequenced. Glycoproteins gI, gII, gIII, and gIV have been demonstrated to be structural components of virions (42, 44). No protein products have been found so far for the genes encoding gH, gi, and gx. Whereas functional analysis of HSV and PrV glycoproteins is well advanced, studies aimed at elucidating the functions of BHV-1 glycoproteins have only recently been started.

Glycoprotein gIII has been shown to be involved in virus attachment to a heparin-containing cellular receptor as are the homologous proteins gC(HSV) and gIII(PrV) (26, 32, 47). In addition, gI, which is homologous to gB(HSV) and gII(PrV) (36, 45), as well as gIV, which is a homolog of gD(HSV) and gp50(PrV) (40), have been implicated in adsorption and penetration (3, 5, 14, 18, 19, 26). Whereas gIII is nonessential for BHV-1 replication (26) as is gIII in PrV (30) and gC in HSV (17), gI and gIV, like their HSV and PrV counterparts, most likely constitute essential virion components. It has recently been shown that gI(BHV-1) is able to complement functionally the defect in a PrV mutant lacking the essential glycoprotein gII (36). Thus, there is growing evidence that herpesviral glycoproteins exhibiting sequence homology also share functional properties.

To analyze the function of gIV of BHV-1, we started by constructing a cell line that constitutively expresses gIV of BHV-1 strain Schönböken. This cell line was then used to isolate a BHV-1 mutant lacking the gene encoding gIV. Our results show that gIV is essential for penetration of BHV-1 and that the amount of gIV in the viral envelope modulates penetration of the virion.

MATERIALS AND METHODS

Cells and viruses. BHV-1 strains Schönböken and Aus12 were obtained from O. C. Straub (Tübingen, Germany) and propagated on Madin-Darby bovine kidney cell clone Bu100 (MDBK-Bu100; kindly provided by W. Lawrence and L. Bello, Philadelphia, Pa.). 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). For selection of recombinant cell clones, geneticin (700 μg/ml; Sigma, Munich, Germany) was added. To induce the metallothionein (MT) promoter, cells were incubated in medium containing 100 μM zinc sulfate for 12 to 15 h.

Antibodies and sera. Monospecific rabbit antiserum against affinity-purified gIV of strain Schönböken was prepared essentially as described previously (41). Monoclonal antibodies (MAbs) against gIV (21/3/3), gI (72/14/6), and gIII (118/2/4) of strain Schönböken will be described in detail elsewhere (24a). Rabbit anti-BHV-1 serum was collected after immunization of rabbits with sucrose gradient-purified virions of strain Schönböken.

* Corresponding author.

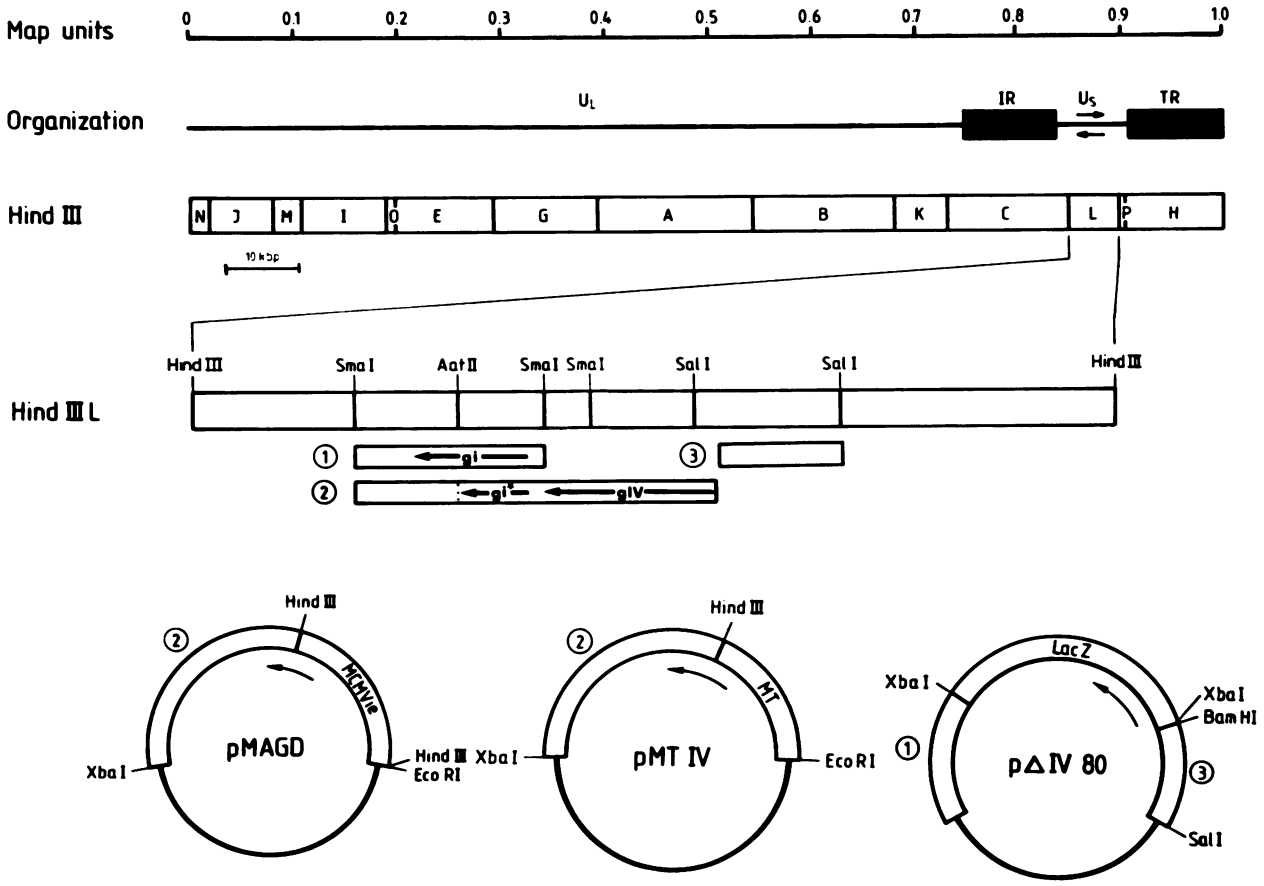

FIG. 1. Plasmid constructs used for the establishment of cell line BUIV3-7 and generation of gIV$^-$ BHV-1 mutant 80-221. The *Hin*dIII restriction fragment map is shown below a schematic diagram of the prototype orientation of the genome of BHV-1 strain Schönböken (11, 29). The *Hin*dIII fragment encompassing the gIV gene is enlarged. Locations of important restriction enzyme cleavage sites are indicated, as are subfragments used for the construction of plasmids shown below. Direction of transcription of the gIV and gi genes is marked by arrows. In fragment 2, the gi gene was interrupted by a 4-bp deletion and concomitant frameshift at the unique *Aat*II cleavage site, as indicated by an asterisk and a dotted vertical line. Plasmid pMAGD contains fragment 2 and the MCMV immediate-early enhancer/promoter complex (MCMVie). In plasmid pMTIV, the MCMV sequences are replaced by the murine MT promoter (MT). In pΔIV80, the *lacZ* expression cassette (LacZ) was flanked by fragments 1 and 3 to provide homologous regions for recombination. Direction of transcription from the plasmids is indicated by arrows.

Plasmid constructions. Cloning procedures were carried out according to established methods (38). The pUC12-based plasmid pCLD3 carries the genes encoding gIV and gi, a polyadenylation consensus sequence after the gi gene, and the first 300 nucleotides of the gene encoding gII of BHV-1 strain Schönböken. Plasmid DNA was cleaved at a *Hin*dIII site generated by linker insertion immediately upstream of the putative gIV mRNA start site. The strong constitutive immediate-early promoter/enhancer complex of murine cytomegalovirus (MCMV) (9) flanked by *Hin*dIII cleavage sites (23) was isolated after cleavage with *Hin*dIII and inserted in front of the gIV open reading frame (ORF). At a unique *Aat*II cleavage site within the gi ORF, a 4-bp deletion was introduced to cause a frameshift in the gi ORF and thereby eliminate potential gi expression. Transient expression of gIV from the resulting plasmid, pMAGD (Fig. 1), was monitored by indirect immunofluorescence. To construct pMTIV, the gIV transcription unit was isolated from pCLD3 after digestion with *Hin*dIII and *Xba*I and inserted into the corresponding sites of plasmid pMTα, which contains the mouse MT promoter in front of a polylinker sequence (2). In transient assays, gIV expression was detectable only after induction of the MT promoter with zinc sulfate. To delete the gIV gene in BHV-1, the 3.4-kbp gX(PrV)-*lacZ* expression cassette (31) was flanked by fragments 1, containing the gene coding for gi, and 3, containing the gIV promoter and upstream sequences (Fig. 1), after the addition of *Xba*I linkers. Expression of β-galactosidase (β-Gal) from the resulting plasmid, pΔIV80, was analyzed in transient expression assays (31, 36) after cotransfection with pAMB25 (25) to provide herpesviral immediate-early functions required for transactivation of the PrV gX promoter.

Isolation of recombinant cell lines. Subconfluent MDBK-Bu100 monolayers in 60-mm dishes were cotransfected by the $Ca_3(PO_4)_2$ coprecipitation technique (16) with pMTIV or pMAGD, respectively, and pAG60 encoding neomycin phosphotransferase (8). After 2 days, cells were trypsinized and reseeded on 100-mm dishes; after an additional 24 h, 700 μg of geneticin per ml was added. Medium was changed every 4 days. Individual geneticin-resistant colonies were picked 10 to 14 days after transfection (25) and screened for gIV expression by indirect immunofluorescence with MAb

21/3/3. One recombinant cell line (BUIV3-7) exhibiting bright fluorescence was selected for further experiments. To analyze surface expression of gIV, cells were briefly trypsinized, washed twice in phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum, and incubated for 30 min with MAb 21/3/3. Cells were washed three times in PBS and incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (IgG) (Dianova, Hamburg, Germany). The cells were then washed again three times in PBS, and surface fluorescence was analyzed in a fluorescence-activated cell sorter (FACStar Plus; Becton Dickinson, Mountain View, Calif.).

Isolation of gIV$^-$ virus. DNA of BHV-1 strain Aus12 was cotransfected with pΔIV80 into MDBK-Bu100 cells as described previously (25). After complete cytopathic effect had developed, the supernatant was plated onto BUIV3-7 monolayers in serial dilutions. Two hours postinfection (p.i.), the cells where overlaid with DMEM containing 0.7% methylcellulose (Methocel; Fluka, Neu-Ulm, Germany). After plaques became visible, medium was replaced by an overlay containing 1% agarose and 300 μg of Bluo-Gal (GIBCO-BRL, Dreieich, Germany) per ml in DMEM. Blue staining plaques were picked and further plaque purified three times on BUIV3-7 cells.

Immunoprecipitation and immunoblotting. Confluent monolayers of MDBK cells grown in six-well tissue culture dishes (Greiner, Nürtingen, Germany) were infected at a multiplicity of infection (MOI) of 5. After 1 h of adsorption, the inoculum was replaced by 500 μl of DMEM lacking methionine. After addition of [^{35}S]methionine (50 μCi/ml), cells were incubated for the times indicated and immunoprecipitations were carried out as described previously (24). Labeled proteins were visualized after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by fluorography (24). Immunoblotting was performed as described previously (24).

Whole cell DNA isolation and Southern hybridization. Cells from one 35-mm dish were harvested 20 h after infection with wild-type or recombinant BHV-1, pelleted by low-speed centrifugation, and resuspended in 450 μl of 10 mM Tris-HCl–10 mM EDTA, pH 7.5. Sarcosyl and RNase A (Sigma) were added to final concentrations of 2% and 200 μg/ml, respectively. After incubation at 37°C for 30 min, proteinase K (Boehringer, Mannheim, Germany) was added (final concentration, 200 μg/ml). After 1 h at 56°C, proteins were extracted by phenol, and DNA was ethanol precipitated, pelleted by centrifugation, and resuspended in 500 μl of 10 mM Tris-HCl–1 mM EDTA, pH 7.5. Aliquots were digested with restriction enzymes, and resultant DNA fragments were separated in 0.6% agarose gels, transferred to nitrocellulose filters, and hybridized (10). ^{32}P-labeled DNA probes were prepared by using a random-primed DNA-labeling kit (Boehringer).

Penetration kinetics and PEG fusion. Penetration kinetics were determined essentially as described previously (30), using low-pH inactivation of extracellular virus at different times after a shift of infected cells from 4 to 37°C to allow membrane fusion. Polyethylene glycol (PEG)-induced membrane fusion was performed as described by Sarmiento et al. (39).

Determination of β-Gal activity. Cells were collected 16 h p.i. after trypsinization by low-speed centrifugation, washed twice in PBS, resuspended in 50 μl of 0.25 M sucrose in 10 mM Tris–10 mM EDTA, pH 7.4, and lysed by sonication. Cellular debris was removed by centrifugation (40,000 rpm; Beckman TLA45 rotor, Beckman TL100 ultracentrifuge). The supernatant was added to 200 μl of 60 mM Na_2HPO_4–40 mM NaH_2PO_4–10 mM KCl–1 mM $MgSO_4$–50 mM β-mercaptoethanol, pH 7.0, and the reaction was started with 40 μl of ONPG solution (4 mg of *o*-nitrophenyl-β-D-galactopyranoside per ml in 100 mM phosphate buffer, pH 7.0) and incubated at 30°C for appropriate times. The reaction was terminated by the addition of 100 μl of 1 M Na_2CO_3. A_{420} was determined, and β-Gal activity was calculated as A_{420}/(reaction time [minutes] × 0.0002) = units of β-Gal per ml (33).

RESULTS

Isolation and characterization of gIV-expressing cell line BUIV3-7. To isolate a gIV-expressing cell line capable of complementing a BHV-1 mutant lacking the gIV gene, cotransfections of MDBK-Bu100 cells were performed with plasmids pMAGD (Fig. 1), which contains the ORF for gIV under control of the strong immediate-early promoter/enhancer complex of the MCMV, and pAG60, which contains the bacterial neomycin phosphotransferase gene. Examination of parallel cultures at 48 h after transfection for gIV expression by indirect immunofluorescence using MAb 21/3/3 revealed strongly fluorescent cells that were abnormal in size, frequently containing several nuclei. In cell cultures that were trypsinized and reseeded 2 days after transfection, gIV-expressing cells could no longer be detected and no gIV-expressing cells were found among geneticin-resistant cell clones (data not shown). Since this finding indicated that expression of gIV in high amounts caused cytotoxicity, the gIV gene was inserted downstream from the inducible murine MT promoter. The resulting plasmid, pMTIV (Fig. 1), was cotransfected with pAG60 into MDBK-Bu100 cells. Of 288 individual geneticin-resistant cell clones examined for gIV expression, approximately 60% expressed gIV after induction by zinc sulfate. One clone (BUIV3-7) that exhibited bright gIV-specific fluorescence was selected for further experiments. To analyze gIV expression, BUIV3-7 cells were incubated in medium with and without zinc sulfate. After 2 h, [^{35}S]methionine was added, and gIV was assayed by immunoprecipitation with anti-gIV MAb 21/3/3 and SDS-PAGE (Fig. 2a). In induced cells, two polypeptides of 72 and 63 kDa (Fig. 2a, lane 4) were specifically recognized that migrated comparably to immature pgIV (63 kDa) (42) and mature gIV (72 kDa) (42), respectively, immunoprecipitated from cells infected with BHV-1 strain Schönböken (Fig. 2a, lane 2). In noninduced BUIV3-7 cells, only a small amount of labeled protein that migrated as mature gIV could be precipitated (Fig. 2a, lane 3), demonstrating inducible gIV expression in BUIV3-7 cells. However, after 15 cell culture passages, labeled gIV could barely be detected in noninduced and induced cells by immunoprecipitation, whereas indirect immunofluorescence indicated expression of gIV in the cytoplasm and on the surface of BUIV3-7 cells (data not shown). The presence of gIV in noninduced and induced BUIV3-7 cells was therefore tested by immunoblotting with a monospecific rabbit anti-gIV serum (Fig. 2b). Similar amounts of pgIV and gIV were found in BUIV3-7 cells independently of induction of the MT promoter (Fig. 2b, lanes 3 and 4). Thus, after passaging, BUIV3-7 cells express gIV constitutively.

Insertion of gIV into the cytoplasmic membrane was confirmed by cytofluorometry (Fig. 3). For this experiment, BUIV3-7 cells after only 10 passages were grown with or without induction of the MT promoter. After 12 h, cells were

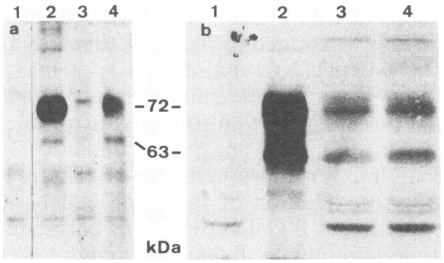

FIG. 2. Analysis of gIV expression in BUIV3-7 cells. (a) Inducible expression of gIV. Immediately after cloning, BUIV3-7 cells were incubated in medium without (lane 3) or with (lane 4) 100 μM zinc sulfate for 2 h and subsequently labeled with [^{35}S]methionine for 12 h. After cell lysis, proteins were immunoprecipitated with gIV-specific MAb 21/3/3 and visualized by fluorography after 10% SDS-PAGE. Also shown are proteins from noninfected (lane 1) and BHV-1 strain Schönböken-infected (lane 2) MDBK-Bu100 cells labeled from 4 to 16 h p.i. and precipitated with MAb 21/3/3. The apparent molecular masses of the proteins are indicated. (b) Loss of inducibility of gIV expression after passaging of BUIV3-7 cells. BUIV3-7 cells after 15 cell culture passages were incubated in medium without (lane 3) or with (lane 4) 100 μM zinc sulfate for 12 h. Cells were lysed, and proteins of about 10^5 cells were separated by 10% SDS-PAGE and transferred to nitrocellulose. Lane 1 shows proteins from MDBK-Bu100 cells; lane 2 shows proteins from 2×10^4 MDBK-Bu100 cells infected with BHV-1 strain Schönböken. The filters were incubated with monospecific rabbit anti-gIV serum, and bound antibodies were visualized after incubation with peroxidase-labeled anti-rabbit IgG by staining with H_2O_2 and chloronaphthol.

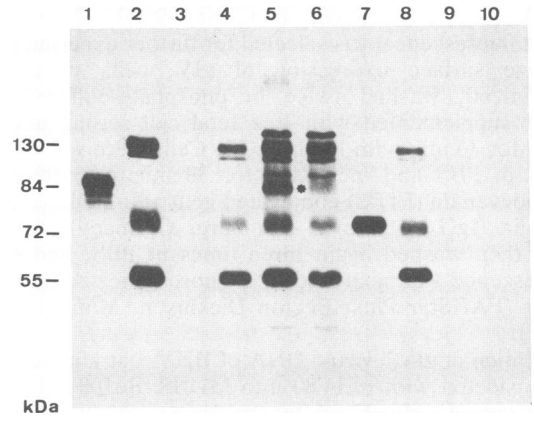

FIG. 4. Lack of gIV expression in strain 80-221. MDBK-Bu100 cells were infected with BHV-1 strain Aus12 (lanes 1, 2, and 5), 80-221 (lanes 3, 4, and 6), or Schönböken (lanes 7 and 8) or mock infected (lanes 9 and 10) and labeled with [^{35}S]methionine from 4 h to 20 h p.i. Thereafter, cells were lysed and immunoprecipitations were carried out with anti-gIV MAb 21/3/3 (lanes 1, 3, 7, and 9), anti-gI MAb 72/14/6 (lanes 2, 4, 8, and 10), or a rabbit antiserum raised against purified virus particles (lanes 5 and 6). Precipitated proteins were separated in a 10% SDS-polyacrylamide gel and visualized by fluorography. The apparent molecular masses of the proteins are indicated. The asterisk denotes the position of the 82-kDa gIV of Aus12 (lane 5).

trypsinized briefly and incubated sequentially with MAb 21/3/3 and an FITC-conjugated second antibody. It is obvious that both noninduced (Fig. 3c) and induced (Fig. 3d) BUIV3-7 cells express gIV on the cellular surface and that the amount of membrane-bound gIV is only slightly enhanced after induction. After additional passages of the cells, induction did not lead to any increase in fluorescence intensity (not shown). Comparison of the fluorescence intensities obtained with MAb 21/3/3 from BUIV3-7 cells and from MDBK-Bu100 cells infected with strain Schönböken showed that infected cells contained about 50- to 100-fold more gIV on the cell surface (Fig. 3b) than did BUIV3-7 cells (Fig. 3c). This ratio of gIV content was similar to that seen in the immunoblot analysis (Fig. 2b; compare lanes 2 and 3). Taken together, these results show that gIV is constitutively expressed in BUIV3-7 cells, that it is integrated into the cytoplasmic membrane, and that the binding domain for MAb 21/3/3 is exposed on the surface. No significant induction was observed after more than 15 cell culture passages. Since zinc sulfate is cytotoxic, BUIV3-7 cells were used without treatment for further experiments. Southern blot experiments showed that BUIV3-7 cells contained about 150 gene equivalents of pMTIV per cell (data not shown).

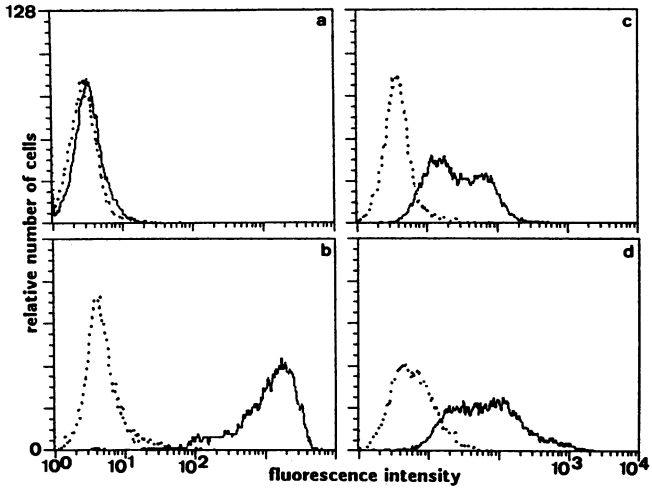

FIG. 3. Expression of gIV on the surface of BUIV3-7 cells. Uninfected MDBK-Bu100 cells (a), MDBK-Bu100 cells infected with BHV-1 strain Schönböken (b), and BUIV3-7 cells after 10 cell culture passages without (c) or with (d) zinc sulfate induction were incubated with a negative control antibody (dotted lines) or anti-gIV MAb 21/3/3 (solid lines) and subsequently treated with FITC-labeled goat anti-mouse IgG. Surface localization of gIV was analyzed by using a FACStar Plus with a 2-W Argonion laser at 200 mW. Dead cells were excluded from the analyses by gating on viable cells. Fluorescence intensities of 10^4 cells are depicted on a logarithmic scale.

Isolation of a gIV$^-$ BHV-1 mutant. BHV-1 strain Aus12 was chosen for deletion of the gIV gene because it encodes a gIV with an apparent molecular mass of 84 kDa (24a) which can easily be differentiated from the 72-kDa gIV of BHV-1 strain Schönböken (Fig. 4, lanes 1 and 7). Progeny virus from cotransfections of MDBK-Bu100 cells with Aus12 DNA and pΔIV80 was serially diluted and plated onto BUIV3-7 cells. Plaques that stained blue under a Bluo-Gal agarose overlay were picked and titrated on MDBK-Bu100 and BUIV3-7 cells. One isolate, 80-221, which produced blue plaques only on complementing cells, was plaque purified two additional times and further characterized. Plaques on BUIV3-7 cells during the purification procedure always stained blue. Plaques could never be detected on MDBK-Bu100 cells, which however exhibited pronounced cytopathic effect when infected at an MOI of >0.1, indicating virus-induced cell lysis. To confirm the lack of gIV expression in strain 80-221, MDBK-Bu100 cells were in-

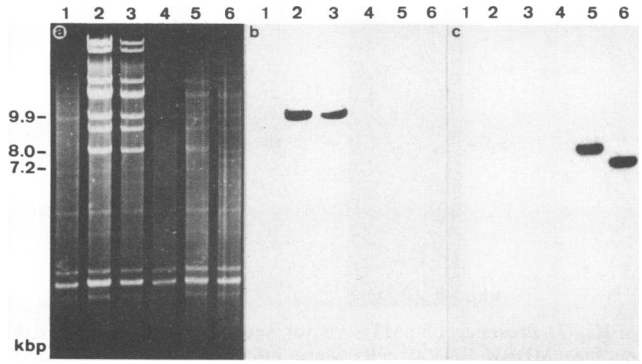

FIG. 5. Evidence that strain 80-221 carries the *lacZ* expression cassette and lacks the gIV gene. MDBK-Bu100 cells (lanes 1, 2, 5, and 6) and BUIV3-7 cells (lanes 3 and 4) were mock infected (lanes 1 and 4) or infected with BHV-1 strain 80-221 (lanes 2 and 3), Aus12 (lane 5), or Schönböken (lane 6). Whole cell DNA was prepared 20 h p.i. and cleaved with *Hin*dIII. After separation in a 0.6% agarose gel, fragments were transferred to nitrocellulose and hybridized with the ^{32}P-labeled 337-bp *Sma*I fragment (Fig. 1) from the body of the gIV gene (c) or ^{32}P-labeled *lacZ* DNA (b). Panel a shows the ethidium bromide-stained DNA fragments. Fragment sizes are indicated.

fected with strains Aus12, Schönböken, and 80-221. Proteins were labeled with [^{35}S]methionine and immunoprecipitated with either MAb 21/3/3 (Fig. 4, lanes 1, 3, and 7), gI-specific MAb 72/14/6 (Fig. 4, lanes 2, 4, and 8), or a polyspecific rabbit anti-BHV-1 serum (Fig. 4, lanes 5 and 6). MAb 21/3/3 precipitated the 84-kDa gIV of Aus12 (Fig. 4, lane 1) and the 72-kDa gIV of Schönböken (Fig. 4, lane 7) but did not recognize any protein from 80-221-infected cells (Fig. 4, lane 3). In contrast, MAb 72/14/6 reacted with gI in Aus12-, Schönböken-, and 80-221-infected cells (Fig. 4, lanes 2, 4, and 8). Comparison of the proteins precipitated by the polyclonal anti-BHV-1 serum also showed that after infection with 80-221, gIV is missing among these polypeptides (Fig. 4, lanes 5 and 6; marked by an asterisk). Immunoprecipitation analysis also revealed the presence of gIII in all three virus strains (data not shown). Thus, after infection of MDBK-Bu100 cells, 80-221 does not express gIV but synthesizes other viral antigens, comparable to its Aus12 parent.

Characterization of the genome of 80-221. To demonstrate that the gIV gene in 80-221 was correctly replaced by the gX(PrV)-*lacZ* expression cassette, MDBK-Bu100 and BUIV3-7 cells were infected at an MOI of 1, and whole cell DNA was prepared 20 h p.i. DNA cleaved with *Hin*dIII was electrophoresed in 0.6% agarose gels, and the resultant fragments were transferred to nitrocellulose. Filters were probed with a labeled 337-bp *Sma*I fragment (Fig. 1) from the body of the gIV gene (Fig. 5c) or with labeled DNA of the *lacZ* cassette (Fig. 5b). The *lacZ* probe hybridized to a 9.9-kbp fragment present in 80-221 DNA (Fig. 5b, lanes 2 and 3). The size of this fragment is as expected, since substitution of the gIV gene of Aus12 by the 3.4-kbp *lacZ* expression unit in the 8-kbp *Hin*dIII fragment should result in an increase in size of about 1.9 kbp. Cleavage of 80-221 DNA with *Xba*I showed that the 3.4-kbp *lacZ* cassette appeared unaltered (see Fig. 6b, lane 4). Viral DNA of strains Schönböken and Aus12 did not hybridize to *lacZ* DNA (Fig. 5b, lanes 5 and 6). As expected, the gIV probe hybridized to the 8- and 7.2-kbp *Hin*dIII fragments of Aus12 and Schönböken, respectively (Fig. 5c, lanes 5 and 6) and not to DNA from 80-221-infected or noninfected cells. We conclude that the gIV gene in 80-221 had been specifically deleted and that sequences upstream and downstream of the mutation were not significantly affected. This conclusion has been confirmed by additional hybridizations (data not shown).

TABLE 1. Restoration by PEG of the infectivity of gIV$^-$ 80-221 virions[a]

Expt	Infectivity of 80-221 grown on MDBK-Bu100					
	PFU/ml on:				β-Gal activity (U) on MDBK-Bu100	
	BUIV3-7		MDBK-Bu100			
	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG
1	21,000	20	0	0	14.9	2.3
2	75,000	40	0	0	49.3	2.3

[a] MDBK-Bu100 cells were infected with 80-221 grown on BUIV3-7 at an MOI of 0.1. Two days p.i., cell culture supernatant (experiment 1) or supernatant plus infected cells (experiment 2) was frozen and thawed. Virus was titrated after low-speed centrifugation on BUIV3-7 cells in the presence or absence of PEG. Plaques were counted 4 days p.i. Infectivity of noncomplemented 80-221 on MDBK-Bu100 cells was analyzed by determination of the β-Gal activity induced by 1 ml of clarified infected cell culture supernatant at 18 h p.i.

PEG restores infectivity of gIV$^-$ BHV-1. The observation that phenotypically complemented 80-221 replicated in MDBK-Bu100 cells but was unable to produce infectious progeny or plaques prompted us to test whether gIV$^-$ 80-221 particles were able to form plaques on BUIV3-7 cells after PEG-mediated fusion between gIV$^-$ virus envelopes and the cytoplasmic membranes. For this purpose, MDBK-Bu100 cells were infected with 80-221 at an MOI of 0.1, and supernatant or whole cell cultures collected at 48 h p.i. were titrated on BUIV3-7 and MDBK-Bu100 cells in the presence or absence of PEG. As shown in Table 1, PEG treatment increased infectivity of 80-221 grown on MDBK-Bu100 cells approximately 1,000-fold. The size of the plaques that all stained blue in the presence of Bluo-Gal was similar to that of plaques induced by 80-221 grown on BUIV3-7 cells. Thus, gIV$^-$ virus after having entered the cells by PEG-mediated membrane fusion was able to spread from cell to cell in complementing but not in noncomplementing cells (Table 1). This observation indicates that gIV is required for both penetration and viral spread by cell-cell fusion. To demonstrate that the PEG-mediated virus entry was not dependent on gIV expressed by the BUIV3-7 cells, the same virus preparations were used to infect MDBK-Bu100 cells in the presence or absence of PEG. Cells were harvested at 18 h p.i. and lysed, and β-Gal activity was determined. Since the level of β-Gal induced by 80-221 in cell cultures correlates with the number of infected cells (data not shown), expression of the *lacZ* gene can be used to monitor PEG-mediated penetration of gIV$^-$ 80-221. Enzymatic activity was detected only in cells infected in the presence of PEG (Table 1). Under these conditions, PEG had no significant effect on the infectivity of phenotypically complemented 80-221. Thus, PEG-induced membrane fusion was not influenced by the target cell.

pMTIV in the genome of BUIV3-7 rescues 80-221. For preparation of virus stocks, BUIV3-7 cells were infected with 80-221 at an MOI of approximately 5. Progeny virus was harvested after complete CPE had developed. To test for presence of wild-type BHV-1, 1 ml of the cell culture

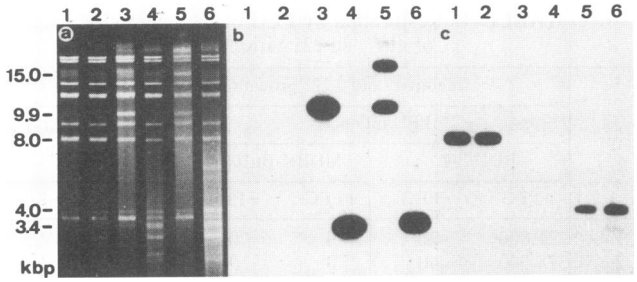

FIG. 6. Integration of the gIV gene into the genome of BHV-1res. MDBK-Bu100 cells were infected with BHV-1 strains Aus12 (lanes 1 and 2), 80-221 (lanes 3 and 4), and BHV-1res (lanes 5 and 6). At 20 h p.i., whole cell DNA was prepared and cleaved with *Hin*dIII (lanes 1, 3, and 5) or *Hin*dIII and *Xba*I (lanes 2, 4, and 6), and fragments were transferred to nitrocellulose after separation in a 0.6% agarose gel. Filters were hybridized to ^{32}P-labeled DNA of the *lacZ* gene (b) or 337-bp *Sma*I fragment from the body of the gIV gene (c). Panel a shows the gel after ethidium bromide staining. Fragment sizes are indicated.

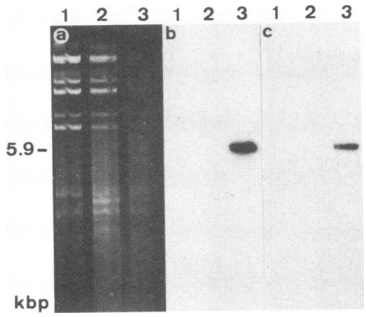

FIG. 7. Presence of pMTα vector sequences in the BHV-1res genome. MDBK-BU100 cells were infected with strains Aus12 (lanes 1), 80-221 (lanes 2), and BHV-1res (lanes 3). At 20 h p.i., whole cell DNA was prepared and cleaved with *Hin*dIII and *Xba*I, and fragments were transferred to nitrocellulose after separation in a 0.6% agarose gel. Filters were hybridized to ^{32}P-labeled DNA of the MT promoter (1.8-kbp *Hin*dIII-*Eco*RI fragment of pMTα) (b) or ^{32}P-labeled pUC12 DNA (c). Bound radioactivity was visualized by autoradiography. Panel a shows the gel after ethidium bromide staining. Fragment sizes are indicated.

supernatant was used to infect MDBK-Bu100 cells, which were harvested 60 h p.i., frozen, thawed, and again used for infection of MDBK-Bu100 cells. Two days later, about 20 slowly growing plaques per ml of inoculum that stained blue under a Bluo-Gal overlay appeared. They were picked and plaque purified twice. No white plaques could be detected on MDBK-Bu100 cells. Progeny from one plaque, designated BHV-1res, was chosen for further characterization. Southern blot hybridization analysis of *Hin*dIII-*Xba*I-cleaved DNA from BHV-1res- and 80-221-infected MDBK-Bu100 cells revealed the presence of the 3.4-kbp *lacZ* cassette (Fig. 6b, lanes 4 and 6). However, after cleavage with *Hin*dIII only, the *lacZ* probe hybridized to two BHV-1res DNA fragments of 15.0 and 9.9 kbp (Fig. 6b, lane 5), the latter having the same size as the *lacZ*-containing *Hin*dIII fragment of 80-221 DNA (Fig. 6b, lane 3). The 9.9- and the 15.0-kbp *Hin*dIII fragments of BHV-1res appeared to be submolar in comparison with the 3.4-kbp *Xba*I fragment containing the *lacZ* cassette, whereas the corresponding fragments of 80-221 show identical signals (Fig. 6b, lanes 3 and 4). In contrast to Aus12, in which the gIV gene is located in an 8-kbp *Hin*dIII fragment, the gIV sequence was contained within a 4-kbp *Hin*dIII fragment in BHV-1res (Fig. 6c, lanes 1, 2, 5, and 6). As expected 80-221 did not hybridize to the gIV probe (Fig. 6c, lanes 3 and 4). A 5.9-kbp *Hin*dIII-*Xba*I fragment of BHV-1res DNA also hybridized to labeled DNA from the MT promoter (Fig. 7b, lane 3) and from the plasmid vector (Fig. 7c, lane 3). We conclude that 80-221 had been rescued by insertion of the intact gIV gene and additional MT and plasmid sequences.

Passaging of BHV-1res in MDBK-Bu100 cells after 10 rounds of propagation resulted in the appearance of white plaques, which were faster developing than the blue plaques induced by BHV-1res. The resulting white-plaque isolate BHV-1resW lacked the *lacZ* gene and pMT vector sequences, indicating insertion of the gIV gene downstream from the authentic gIV promoter (data not shown). The rearrangements resulting in the generation of BHV-1resW and BHV-1res had dramatic effects on gIV expression. In BHV-1res-infected cells and virions, gIV was significantly underrepresented, as judged after immunoprecipitation analysis (Fig. 8, lanes 3 and 9), whereas BHV-1resW expressed gIV in amounts similar to those in strain Schönböken (Fig. 8, lanes 1 and 7 and lanes 2 and 8, respectively). Underexpression of gIV in BHV-1res was especially striking when compared with the expression of gI, which was nearly identical in BHV-1resW, Schönböken, and BHV-1res (Fig. 8, lanes 4 to 6 and 10 to 12).

Penetration kinetics correlate with the level of gIV in BHV-1 virions. After the isolation of recombinant BHV-1 strains exhibiting different gIV expression levels, we tested whether the amount of gIV would influence the penetration behavior of the respective viruses. Penetration kinetics were identical for strains Aus12 and Schönböken as well as the revertant BHV-1resW. About 50% of infectious virions were protected from inactivation after 30 min, and penetration was complete after 2 h (Fig. 9, asterisks). In contrast, BHV-1res (Fig. 9, triangles) started to penetrate only after about 30 min, required 2 h to reach 50% penetration, and required more than 5 h to completely enter the cells. As shown in Fig. 8, BHV-1res underexpresses gIV, and virions contain only a small amount of the mature glycoprotein. Strain 80-221, possessing the gIV provided by BUIV3-7 cells, which is significantly less than produced during wild-type BHV-1

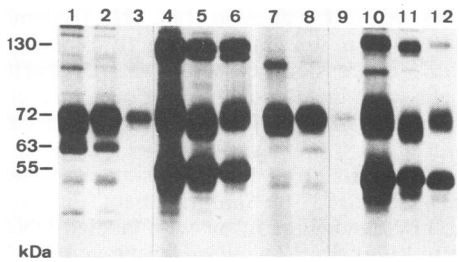

FIG. 8. Underexpression of gIV by BHV-1res. MDBK-Bu100 cells were infected with BHV-1resW (lanes 1, 4, 7, and 10), Schönböken (lanes 2, 5, 8, and 11), and BHV-1res (lanes 3, 6, 9, and 12). At 2 h p.i., [^{35}S]methionine was added and labeled proteins were immunoprecipitated from lysed cells (lanes 1 to 6) or from virions isolated from the cell culture supernatant 24 h p.i. (lanes 7 to 12), using gIV-specific MAb 21/3/3 (lanes 1 to 3 and 7 to 9) or gI-specific MAb 74/14/6 (lanes 4 to 6 and 9 to 12). Precipitated proteins were separated on a 10% SDS-polyacrylamide gel and visualized by fluorography. The apparent molecular masses of the proteins are indicated.

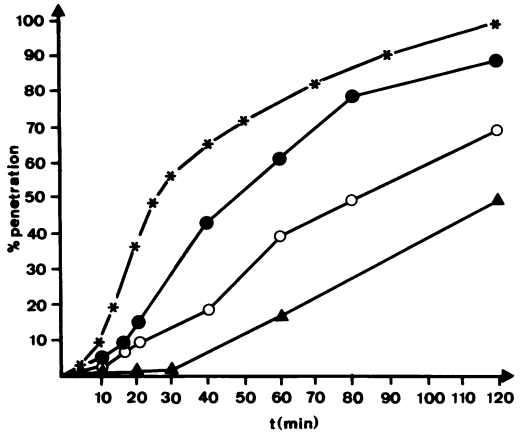

FIG. 9. Correlation of the penetration kinetics of BHV-1 with gIV expression. Stocks of strains BHV-1res (triangles), BHV-1resW, Aus12, and Schönböken were diluted to yield approximately 400 PFU per cell culture dish. Cells were preincubated at 4°C for 15 min, and the respective viruses were allowed to adsorb for 2 h at 4°C. After a temperature shift to 37°C, duplicate cultures were washed with PBS and incubated with sodium citrate buffer, pH 3.0, or PBS at room temperature for 2 min. Cells were washed twice with PBS and overlaid with 0.7% methylcellulose medium, and plaques were counted 2 days later. Penetration of 80-221 (open circles) and of progeny from MDBK-Bu100 cells infected with Aus12 at an MOI of 10 and 80-221 at an MOI of 1 (closed circles) was determined by measuring β-Gal activity at 18 h p.i. Percent penetration was calculated from the respective values obtained with or without sodium citrate treatment. Penetration kinetics for Aus12, Schönböken, and BHV-1resW were essentially identical and therefore are all represented in one curve (asterisks).

infection (Fig. 2 and 3), exhibited kinetics intermediate between those of Aus12, Schönböken, and BHV-1resW and BHV-1res, respectively (Fig. 9, open circles). These results indicate that the amount of gIV in the virion influences penetration kinetics. To analyze further the correlation between the amount of gIV expression and penetration efficiency, MDBK-Bu100 cells were infected with Aus12 at an MOI of 10 and with 80-221 at an MOI of 1. Viral progeny was harvested after 2 days, and the penetration kinetics of 80-221 virions containing the gIV provided by Aus12 was determined by measuring the β-Gal activity in the infected cell cultures 24 h after a temperature shift from 4 to 37°C. In comparison with 80-221 grown on BUIV3-7, penetration of the 80-221 progeny from the mixed infection was accelerated (Fig. 9, closed circles). Since MDBK-Bu100 cells infected with wtBHV-1 contain approximately 50- to 100-fold more gIV than did BUIV3-7 cells (Fig. 2b and 3), it appears that penetration efficiency is dependent on the amount of gIV in the virion.

DISCUSSION

gIV of BHV-1 exhibits significant amino acid homology to gD of HSV, with strict conservation of the spacing of the first six cysteine residues (40). Both gIV and gD are major constituents of the viral envelope and play an important role in the induction of the immune response in their respective hosts (1, 7, 37). gD(HSV) is essential for viral infectivity (14, 21, 22, 27), as are other gD-homologous proteins such as gp50(PrV) (35a) and gIV(BHV-1) (this report). Recently it has been reported that gIV of BHV-1 strain Cooper is exposed on the cell surface of transfected MDBK cells and that gIV expression is cytotoxic and induces spontaneous cell fusions (40), as has been shown for gD(HSV) (4). In contrast, Chase et al. (6) found no indication for cell fusion in gIV-expressing bovine skin fibroblasts. However, they failed to detect gIV on the cell surface. Our results with cell line BUIV3-7, which constitutively expresses gIV of BHV-1 strain Schönböken, and transient expression assays using pMAGD show that gIV is cytotoxic only when expressed at high levels and that a substantial amount of the glycoprotein is tolerated by MDBK cells. Cell line BUIV3-7 can be propagated for several months without any signs of degeneration or fusion; thus, under our cell culture conditions, gIV of BHV-1 strain Schönböken did not appear to induce spontaneous cell fusions. Identical results have been obtained in a murine LTK^- cell line constitutively expressing gIV on the cellular surface (11a). Although it is difficult to compare the levels of gIV expressed in the different systems, we assume that BUIV3-7 expresses at least the same amount of gIV as the cell line used by Tikoo et al. (PgIV-1 [40]). This assumption is based on the degree of resistance to BHV-1 superinfection, which correlates with the amount of gIV in the transgenic cells and is 10- to 100-fold higher in BUIV3-7 (data not shown) than in induced PgIV-1 cells (40). Differences in the properties of the gIV-expressing cell lines might be due to the different parental cells or gIV genes from different BHV-1 strains.

The observation that 80-221 replicates on BUIV3-7 cells but does not produce infectious particles in MDBK-Bu100 cells clearly shows that gIV is essential for BHV-1 infectivity. Phenotypically complemented gIV^- BHV-1 is able to enter MDBK-Bu100 cells, as shown by the expression of early- and late-regulated glycoproteins, by the replication of viral DNA, and by the release of enveloped viral particles as detected by electron microscopy (44a). Whereas 80-221 particles isolated from single plaques on BUIV3-7 cells were unable to produce infectious progeny on MDBK-Bu100 cells, passaging of 80-221 invariably resulted in the appearance of infectious BHV-1. On noncomplementing cells, these rescued viruses under a Bluo-Gal overlay formed blue-staining plaques that were significantly smaller than wild-type plaques. The gIV expressed by the rescued variant BHV-1res appears identical to gIV of BHV-1 strain Schönböken, the source for the gIV gene for establishment of the BUIV3-7 cell line. This finding demonstrates that these viruses did not constitute contaminants due to the continuous presence of the parental strain Aus12. The only possible source for the gIV gene could have been the BUIV3-7 cell line, which contains about 150 copies of pMTIV. Since pMTIV contains no BHV-1 sequences upstream from the gIV ORF, rescue of the gIV deletion has probably been mediated by a tandem repeat of pMTIV in the cellular genome rather than by illegitimate recombination at the 5′ end of the cellular pMTIV sequences. Genome analysis of the rescued isolate BHV-1res showed that integration of the gIV gene, MT promoter and plasmid vector sequences, as well as about 1.2 kbp of unidentified cellular sequences (not shown) into the viral genome occurred downstream from the *lacZ* cassette within the gi gene. The only functional BHV-1 gene introduced into BHV-1res by this recombination is that encoding gIV, which shows that gIV alone is able to rescue 80-221. Because of the complex alterations within the unique short region, the reasons for the underexpression of gIV by BHV-1res are currently unknown. Experiments are in progress to characterize in more detail the recombination events leading to the emergence of BHV-1res.

The regular generation of infectious viruses after propa-

gation of conditionally lethal mutants in complementing cell lines must also be regarded with respect to the proposed strategy of using these mutants as replication-deficient viral vaccines. According to our results, these vaccines might be problematic because of the potential shedding of infectious virus after vaccination or reactivation from latency.

The isolation of BHV-1res, which underexpresses gIV and exhibited only small amounts of gIV in both infected cells and virions, allowed the analysis of possible influences of the amount of gIV in virions on viral penetration into cells. Penetration kinetics revealed that the efficiency of BHV-1 to enter cells is correlated with the amount of gIV in the virion. This conclusion is based on the inefficient penetration of BHV-1res compared with that of wtBHV-1 and BHV-1resW. Furthermore, 80-221 grown on BUIV3-7 cells contains more gIV and penetrates faster than BHV-1res, which exhibits less gIV. Both viruses, as expected, contain identical amounts of gI. In addition, penetration of 80-221 complemented with gIV of Aus12 after double infection accelerated, supporting the conclusion that under the conditions used, the penetration of the different BHV-1 strains is modulated by the amount of gIV and not by its size or any other strain-specific interactions between viral and cellular functions. This conclusion is also supported by the penetration kinetics of BHV-1resW, which proved to be identical to that of wtBHV-1. In summary, our results show that gIV of BHV-1 is essential for viral infectivity, that functional gIV is not necessarily toxic for cells, and that it does not inevitably induce cell fusion. The gIV negative BHV-1 mutant 80-221 forms the basis to efficiently integrate mutated gIV genes into the viral genome, which should help in further analyses of the function of gIV and in characterization of essential and nonessential domains within this glycoprotein.

ACKNOWLEDGMENTS

We thank Angela Schroeder and Sabine Maurer for expert technical assistance, Isabella Rauh for helpful advice, Frank Weiland for electron microscopic studies, and Heiner Niemann for critical reading of the manuscript.

This work was financially supported by grant 0319131A from the Bundesministerium für Forschung und Technologie, by contract BIOT-CT90-0191-C(EDB) from the Commission of the European Communities, and by grant Me 854/2-2 from the Deutsche Forschungsgemeinschaft.

REFERENCES

1. **Babiuk, L. A., J. L'Italien, S. van Drunen Littel-van den Hurk, T. Zamb, M. J. P. Lawman, G. Hughes, and G. A. Gifford.** 1987. Protection of cattle from bovine herpesvirus type I (BHV-1) infection by immunization with individual viral glycoproteins. Virology **159**:57–66.
2. **Brinster, R. L., H. Y. Chen, M. Trumbauer, A. W. Senear, R. Warren, and A. D. Palmiter.** 1981. Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. Cell **27**:223–231.
3. **Campadelli-Fiume, G., M. Arsenakis, F. Farabegoli, and B. Roizman.** 1988. Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. J. Virol. **62**:159–167.
4. **Campadelli-Fiume, G., E. Avitabile, S. Fini, D. Stripe, M. Arsenakis, and B. Roizman.** 1988. Herpes simplex virus glycoprotein D is sufficient to induce spontaneous pH-independent fusion in a cell line that constitutively expresses the glycoprotein. Virology **166**:598–602.
5. **Campadelli-Fiume, G., S. Qi, E. Avitabile, L. Foà-Tomasi, R. Brandimarti, and B. Roizman.** 1990. Glycoprotein D of herpes simplex virus encodes a domain which precludes penetration of cells expressing the glycoprotein by superinfecting herpes simplex virus. J. Virol. **64**:6070–6079.
6. **Chase, C. C. L., K. Charter-Allen, C. Lohoff, and G. J. Letchworth.** 1990. Bovine cells expressing bovine herpesvirus 1 (BHV-1) glycoprotein IV resist infection by BHV-1, herpes simplex virus, and pseudorabies virus. J. Virol. **64**:4866–4872.
7. **Cohen, G. H., M. Ponce de Leon, and C. Nichols.** 1972. Isolation of a herpes simplex virus-specific antigenic fraction which stimulates the production of neutralizing antibody. J. Virol. **27**:172–181.
8. **Colbere-Garapin, F., F. Herodniceanu, R. Kourilsky, and A. Garapin.** 1981. A new dominant hybrid selective marker for higher eukaryotic cells. J. Mol. Biol. **150**:1–14.
9. **Dorsch-Häsler, K., G. M. Keil, F. Weber, M. Jasin, W. Schaffner, and U. H. Koszinowski.** 1987. A long and complex enhancer activates transcription of the gene coding for the highly abundant immediate early mRNA in murine cytomegalovirus. Proc. Natl. Acad. Sci. USA **82**:8325–8329.
10. **Ebeling, A., G. M. Keil, E. Knust, and U. H. Koszinowski.** 1983. Molecular cloning and physical mapping of murine cytomegalovirus DNA. J. Virol. **47**:421–433.
11. **Engels, M., C. Giuliani, P. Wild, T. M. Beck, E. Loepfe, and R. Wyler.** 1986/87. The genome of bovine herpesvirus 1 (BHV-1) strains exhibiting a neutropathogenic potential compared to known BHV-1 strains by restriction site mapping and cross hybridization. Vir. Res. **6**:57–73.

11a. **Fehler, F.** Unpublished data.

12. **Fitzpatrick, D. R., L. A. Babiuk, and T. J. Zamp.** 1989. Nucleotide sequence of bovine herpesvirus type 1 glycoprotein gIII, a structural model for gIII as a new member of the immunoglobulin superfamily, and implications for the homologous glycoproteins of other herpesviruses. Virology **173**:46–57.
13. **Fitzpatrick, D. R., M. J. Redmond, S. K. Attah-Poku, S. van Drunen Littel-van den Hurk, L. A. Babiuk, and T. J. Zamp.** 1990. Mapping of 10 epitopes on bovine herpesvirus type 1 glycoproteins gI and gIII. Virology **176**:145–157.
14. **Fuller, A. O., and P. G. Spear.** 1987. Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus 1 prevent virion-cell fusion at the cell surface. Proc. Natl. Acad. Sci. USA **84**:5454–5458.
15. **Gibbs, E. P. J., and M. M. Rweyemamu.** 1977. Bovine herpes viruses. Part I. Bovine herpesvirus 1. Vet. Bull. **47**:317–343.
16. **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.
17. **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 cells and in infectivity. J. Virol. **65**:1090–1098.
18. **Highlander, S. L., S. L. Sutherland, P. J. Gage, D. C. Johnson, M. Levine, and J. C. Glorioso.** 1987. Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. J. Virol. **61**:3356–3364.
19. **Hughes, G., L. A. Babiuk, and S. van Drunen Littel-van den Hurk.** 1988. Functional and topographical analyses on bovine herpesvirus type 1 glycoprotein IV. Arch. Virol. **103**:47–60.
20. **Hutchings, D. L., S. van Drunen Littel-van den Hurk, and L. A. Babiuk.** 1990. Lymphocyte proliferative responses to separated bovine herpesvirus 1 proteins in immune cells. J. Virol. **64**: 5114–5122.
21. **Johnson, D. C., and M. W. Ligas.** 1988. Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. J. Virol. **62**:4605–4612.
22. **Johnson, R. M., and P. G. Spear.** 1989. Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. J. Virol. **63**:819–827.
23. **Keil, G. M., A. Ebeling-Keil, and U. H. Koszinowski.** 1987. Immediate-early genes of murine cytomegalovirus: location, transcripts, and translation products. J. Virol. **61**:526–533.
24. **Keil, G. M., M. R. Fibi, and U. H. Koszinowski.** 1985. Characterization of the major immediate-early polypeptides encoded by murine cytomegalovirus. J. Virol. **54**:422–428.

24a. **Keil, G. M., et al.** Unpublished data.

25. **Koszinowski, U. H., G. M. Keil, H. Volkmer, M. R. Fibi, A. Ebeling-Keil, and K. Münch.** 1986. The 89,000-M_r murine cytomegalovirus immediate-early protein activates gene transcription. J. Virol. **58**:59–66.
26. **Liang, X., L. A. Babiuk, S. van Drunen Littel-van den Hurk, D. R. Fitzpatrick, and T. J. Zamb.** 1991. Bovine herpesvirus 1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII, and gIV. J. Virol. **65**:1124–1132.
27. **Ligas, M. W., and D. C. 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.
28. **Ludwig, H.** 1983. Bovine herpesviruses, p. 135–214. *In* B. Roizman (ed.), The herpesviruses, vol. 2. Plenum Publishing Corp., New York.
29. **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.
30. **Mettenleiter, T.** 1989. Glycoprotein gIII deletion mutants of pseudorabies virus are impaired in virus entry. Virology **171**: 623–625.
31. **Mettenleiter, T., 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., L. Zsak, F. Zuckermann, N. Sugg, H. Kern, and T. Ben-Porat.** 1990. Interaction of glycoprotein III with a cellular heparinlike substance mediates adsorption of pseudorabies virus. J. Virol. **64**:278–286.
33. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Okazaki, K., E. Honda, T. Minetoma, and T. Kumaagai.** 1986. Mechanisms of neutralization by monoclonal antibodies to different antigenic sites on the bovine herpesvirus type 1 glycoproteins. Virology **150**:260–264.
35. **Okazaki, K., H. Kawakura, M. Okada, E. Honda, T. Minetoma, and T. Kumagai.** 1987. Intracellular localization and transport of three different bovine herpesvirus type 1 glycoproteins involved in neutralization. Arch. Virol. **92**:7–26.
35a. **Rauh, I., and T. C. Mettenleiter.** 1991. Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. J. Virol. **65**:5348–5356.
36. **Rauh, I., F. Weiland, F. Fehler, G. M. 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.
37. **Rouse, B., and L. A. Babiuk.** 1974. Host defense mechanisms against infectious bovine rhinotracheitis virus: in vitro stimulation of sensitized lymphocytes by virus antigen. Infect. Immun. **10**:681–687.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoproteins VP7(B_2) in virion infectivity. J. Virol. **29**:1149–1158.
40. **Tikoo, S. K., D. R. Fitzpatrick, L. A. Babiuk, and T. J. Zamb.** 1990. Molecular cloning, sequencing, and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. J. Virol. **64**:5132–5142.
41. **van Drunen Littel-van den Hurk, S., and L. A. Babiuk.** 1985. Antigenic and immunogenic characterization of bovine herpesvirus type 1 glycoproteins GVP 3/9 and GVP 6/11 a/16, purified by immunoadsorbent chromatography. Virology **144**:204–215.
42. **van Drunen Littel-van den Hurk, S., and L. A. Babiuk.** 1986. Synthesis and processing of bovine herpesvirus 1 glycoproteins. J. Virol. **59**:401–410.
43. **van Drunen Littel-van den Hurk, S., M. D. Parker, D. R. Fitzpatrick, T. J. Zamb, J. V. van den Hurk, M. Campos, R. Harland, and L. A. Babiuk.** 1991. Expression of bovine herpesvirus 1 glycoprotein gIV by recombinant baculovirus and analysis of its immunogenic properties. J. Virol. **65**:263–271.
44. **van Drunen Littel-van den Hurk, S., J. V. van den Hurk, J. E. Gilchrist, V. Misra, and L. A. Babiuk.** 1984. Interactions of monoclonal antibodies and bovine herpesvirus type 1 (BHV-1) glycoproteins: characterization of their biochemical and immunological properties. Virology **135**:466–479.
44a. **Weiland, F.** Unpublished data.
45. **Whitbeck, J. C., L. J. Bello, and W. C. Lawrence.** 1988. Comparison of the bovine herpesvirus type 1 gI gene and the herpes simplex virus type 1 gB gene. J. Virol. **62**:3319–3327.
46. **Wirth, U. V., K. Gunkel, M. Engels, and M. Schwyzer.** 1989. Spatial and temporal distribution of bovine herpesvirus 1 transcripts. J. Virol. **63**:4882–4889.
47. **WuDunn, D., and P. G. Spear.** 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. **63**:52–58.
48. **Wyler, R., M. Engels, and M. Schwyzer.** 1989. Infectious bovine rhinotracheitis/vulvovaginitis (BHV-1), p. 1–72. *In* G. Wittmann (ed.), Herpesvirus diseases of cattle, horses, and pigs. Developments in veterinary virology. Kluwer Academic Publishers, Boston.