Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

VIROLOGY 190, 378–392 (1992)

Structural, Functional, and Immunological Characterization of Bovine Herpesvirus-1 Glycoprotein gI Expressed by Recombinant Baculovirus[1]

S. VAN DRUNEN LITTEL-VAN DEN HURK,*,[2] M. D. PARKER,*,[3] D. R. FITZPATRICK,*,[4]
J. V. VAN DEN HURK,* M. CAMPOS,* L. A. BABIUK,*,† AND T. ZAMB*

*Veterinary Infectious Disease Organization and †Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0

Received April 2, 1992; accepted June 1, 1992

The major glycoprotein complex gI of bovine herpesvirus-1 was expressed at high levels (36 μg per 1×10^6 cells) in insect cells using a recombinant baculovirus. The recombinant gI had an apparent molecular weight of 116 kDa and was partially cleaved to yield 63-kDa (gIb) and 52-kDa (gIc) subunits. This processing step was significantly less efficient in insect cells than the analogous step in mammalian cells, even though the cleavage sites of authentic and recombinant gI were shown to be identical. The oligosaccharide linkages were mostly endoglycosidase-H-sensitive, in contrast to those of authentic gI, which has mostly endoglycosidase-H-resistant linkages and an apparent molecular weight of 130/74/55 kDa. Despite the reduced cleavage and altered glycosylation, the recombinant glycoprotein was transported and expressed on the surface of infected insect cells. These surface molecules were biologically active as demonstrated by their ability to induce cell–cell fusion. Fusion was inhibited by three monoclonal antibodies specific for antigenic domains I and IV on gI. Domain I maps to the extracellular region of the carboxy terminal fragment gIc and domain IV to the very amino terminus of the gIb fragment, indicating that domains mapping in two distinct regions of gI function in cell fusion. Monoclonal antibodies specific for eight different epitopes recognized recombinant gI, indicating that the antigenic characteristics of the recombinant and authentic glycoproteins are similar. In addition, the recombinant gI was as immunogenic as the authentic gI, resulting in the induction of gI-specific antibodies in cattle.

INTRODUCTION

Bovine herpesvirus-1 (BHV-1) gI belongs to a group of homologous glycoproteins that have been detected in all herpesviruses analyzed to date. The prototype for this family of glycoproteins is gB of herpes simplex virus (HSV) (Pellett *et al.*, 1985b; Bzik *et al.*, 1986). Glycoproteins with homology to gB have been described for human cytomegalovirus (HCMV) (Cranage *et al.*, 1986), varicella zoster virus (VZV) (Keller *et al.*, 1986), Epstein–Barr virus (EBV) (Pellett *et al.*, 1985a), pseudorabies virus (PRV) (Robbins *et al.*, 1987), BHV-1 (Whitbeck *et al.*, 1988; Misra *et al.*, 1988), BHV-2 (Hammerschmidt *et al.*, 1988), equine herpesvirus-1 (EHV-1) (Whalley *et al.*, 1989), EHV-4 (Riggio *et al.*, 1989), Marek's disease virus (Ross *et al.*, 1989), herpesvirus of turkeys (Buckmaster *et al.*, 1988), and herpesvirus saimiri (Albrecht and Fleckenstein, 1990). These glycoproteins show a high degree of homology at the DNA, protein, and structural level, which suggests that they play a central role in the biology of herpesviruses. This is underscored by the observation that HSV gB and PRV gII are essential for viral replication (Cai *et al.*, 1987; Rauh *et al.*, 1991). On the basis of the high degree of homology between the gB homologs, it is very likely that BHV-1 gI is an indispensible glycoprotein. A number of functions have been ascribed to this glycoprotein, among which are a role in attachment and penetration into susceptible cells (Liang *et al.*, 1991), induction of cell–cell fusion (Fitzpatrick *et al.*, 1988, 1990b), and the induction of neutralizing antibodies (van Drunen Littel-van den Hurk and Babiuk, 1985). It is also a major target for the immune response of the host during BHV-1 infection (Collins *et al.*, 1985; van Drunen Littel-van den Hurk and Babiuk, 1986a) and it confers protection from BHV-1 challenge in cattle (Babiuk *et al.*, 1987; van Drunen Littel-van den Hurk *et al.*, 1990a), which makes it a suitable subunit vaccine candidate.

Mature gI consists of three related polypeptides that are derived from a common 105-kDa primary translation product. After glycosylation the precursor is cleaved by a cellular protease into two smaller glycoproteins of 74 and 55 kDa that are covalently linked by disulfide bonds (Marshall *et al.*, 1986; van Drunen Lit-

[1] Veterinary Infectious Disease Organization Journal Series No. 135.

[2] To whom reprint requests should be addressed.

[3] Present address: USAMRIID, Fort Detrick, MD 21702.

[4] Present address: Department of Medicine, University of Western Australia, Nedlands, Perth, Western Australia 6009.

0042-6822/92 $5.00

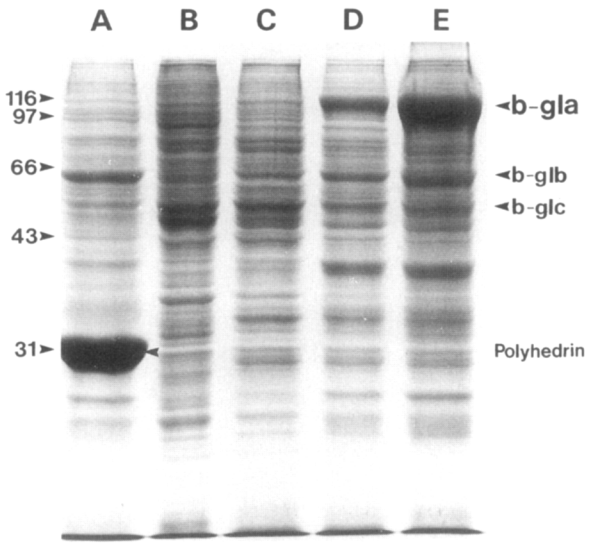

FIG. 1. SDS–PAGE analysis of Bac-gI-infected Sf9 cells at 0 (B), 24 (C), 48 (D), and 72 (E) hr after infection, and of AcNPV-infected Sf9 cells at 48 hr (A) postinfection. Approximately 1.5×10^5 cells were applied per well. The polypeptides were separated on 10% polyacrylamide gels and stained with Coomassie brilliant blue. Molecular weight markers $\times 10^{-3}$ are indicated in the left margin. Recombinant glycoproteins are indicated as bgla, bglb, and bglc.

tel-van den Hurk and Babiuk, 1986b). The proteolytic cleavage process, however, is not complete, which results in the presence of a 130-kDa uncleaved glycoprotein in BHV-1 infected cells. The homologs of other herpesviruses, like VZV gpII (Montalvo and Grose, 1987), HCMV gB (Britt and Vugler, 1989; Spaete *et al.*, 1990), EBV gB (Pellett *et al.*, 1985a), PRV gII (Lukacs *et al.*, 1985; Mettenleiter *et al.*, 1986), and EHV-1 and EHV-4 gB (Whalley *et al.*, 1989; Meredith *et al.*, 1989), show similar proteolytic processing of the primary translation product. Interestingly, the HSV homolog gB is not processed proteolytically. Nevertheless, the gB homologs are highly conserved among the herpesvirus family. As such, they are the only glycoproteins that exhibit a significant degree of structural conservation and immunological cross-reactivity (Davison and Taylor, 1987; Misra *et al.*, 1988).

Our objective was to synthesize enough gI for structural, functional, and immunological analyses of this essential and highly conserved glycoprotein. In recent years the cloned BHV-1 gI gene has been expressed in a number of different vectors, including *Escherichia coli* bacteria (Zamb, unpublished data) and vaccinia virus (van Drunen Littel-van den Hurk *et al.*, 1989). Disadvantages of these systems include loss of immunogenicity of *E. coli*-produced gI and low yields of gI in vaccinia-virus-infected cells. Among other expression systems, those derived from the insect virus *Autographa californica* nuclear polyhedrosis virus (AcNPV) have proven to be valuable for foreign gene expression both in terms of the yields of the expressed protein and in the conservation of the biological properties of the derived protein (Luckow and Summers, 1988). Unfortunately, insect cells do not always authentically glycosylate and process the heterologous protein, resulting in aberrant forms with altered immunogenicity. Expression of the gI gene of BHV-1 in baculovirus resulted in the production of very high levels of gI. Although the recombinant gI was neither glycosylated nor cleaved as efficiently as the authentic gI, several of its functional, antigenic, and immunogenic properties were similar to those of authentic gI. It was transported to the cell surface and it mediated cell fusion. Affinity-purified recombinant gI induced the same level of antibodies in cattle as authentic gI. The availability of large amounts of gI will facilitate further studies on its function and potential as a subunit vaccine to BHV-1.

MATERIALS AND METHODS

Cells, viruses, and antibodies

Madin Darby bovine kidney (MDBK) cells were cultured in Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO). Virus

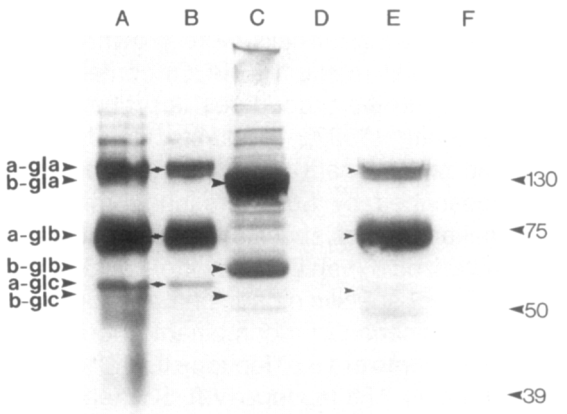

FIG. 2. Immunoblot analysis of gI synthesized in Sf9 cells infected with Bac-gI. Total proteins were harvested at 48 hr postinfection and separated on 8.5% polyacrylamide gels. Glycoprotein I was identified in affinity-purified form (lanes A [2.5 μg] and B [0.5 μg]) in Bac-gI infected Sf9 cells (lane C) and in BHV-1 infected MDBK cells (lane E) by a gI-specific monoclonal antibody mixture. No reaction was observed with mock-infected Sf9 cells (lane D) or mock-infected MDBK cells (lane F). Recombinant gI is indicated as b-gI and authentic gI as a-gI. Authentic gIc (55 kDa) and recombinant gIc (52 kDa) are weakly visible.

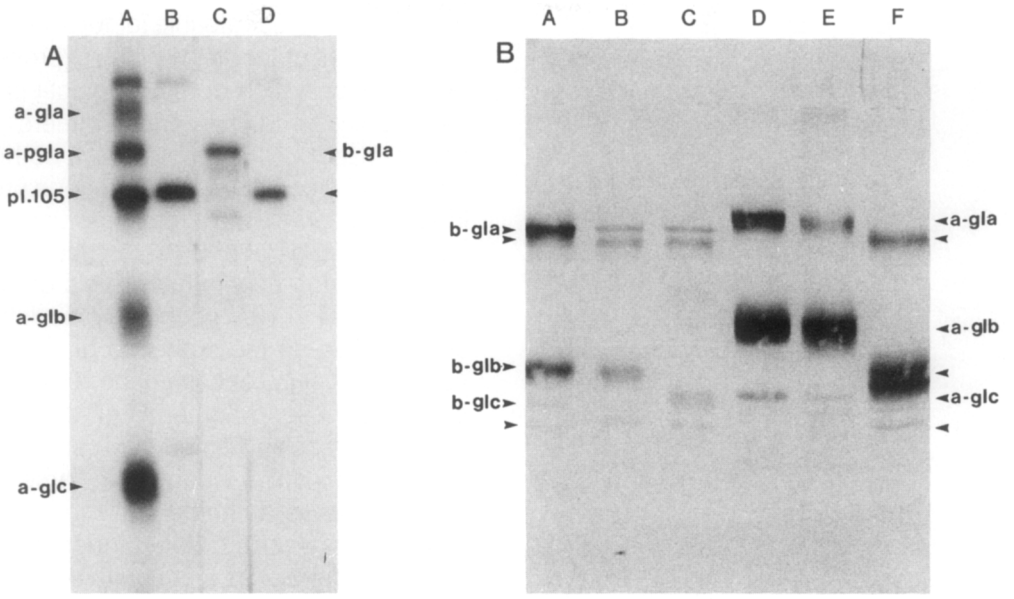

FIG. 3. Processing of recombinant and authentic gI. (A) Tunicamycin treatment. BHV-1-infected MDBK cells (lanes A and B) and Bac-gI-infected Sf9 cells (lanes C and D) were labeled with L-[^{35}S]methionine and treated with tunicamycin (lanes B and D) or left untreated (lanes A and C). Cell lysates were precipitated with a gI-specific monoclonal antibody cocktail and analyzed on 7.5% polyacrylamide gels. (B) Endoglycosidase treatment. Bac-gI-infected Sf9 cells (lanes A, B, and C) and BHV-1-infected MDBK cells (lanes D, E, and F) were treated with endo H (lanes B and E), endo F (lanes C and F) or left untreated (lanes A and D). Cell lysates were separated on 8.5% polyacrylamide gels and analyzed by immunoblotting with a gI-specific monoclonal antibody cocktail. The positions of authentic gI (a-gI) and recombinant gI (b-gI) and the molecular weight shifts due to the various treatments are indicated with arrowheads.

stocks of BHV-1 strain Cooper were grown in MDBK cells as previously described (Babiuk *et al.*, 1975). *Spodoptera frugiperda* (Sf9) cells were grown and maintained in TNM-FH medium (GIBCO) containing 10% FBS according to the procedures described by Summers and Smith (1987). Virus stocks of wild-type AcNPV and recombinant virus were prepared in Sf9 cells as described by Summers and Smith (1987). Monoclonal antibodies specific for gI were developed and characterized by van Drunen Littel-van den Hurk *et al.* (1984). The gI-specific monoclonal antibody mixture used for identification of recombinant gI consisted of equivalent amounts of 1B10 (epitope I), 3F3 (epitope II), 1E11 (epitope III), 1F8 (epitope IVa), 5G2 (epitope IVb), 3G11 (epitope IVb), 5G11 (epitope IVc), 6G11 (epitope IVc), 1F10 (epitope V), and 2C5 (epitope V).

Insertion of BHV-1 gI DNA into the transfer vector

A cassette of the gI glycoprotein gene has been prepared in plasmid pSV2Neo as previously described (Fitzpatrick *et al.*, 1988). The plasmid was digested with restriction endonuclease *Bgl*II and the fragment representing the gI gene was purified by agarose gel electrophoresis and ligated into the *Bam*HI site of the baculovirus transfer vector pVL941 to form the expression vector pVLgB. The pVLgB plasmid was purified for transfection by CsCl gradient centrifugation and two cycles of ethanol precipitation.

Transfection and selection of recombinant viruses

The purified plasmid was mixed with an equal amount of *A. californica* viral DNA and used to transfect subconfluent monolayers of Sf9 cells as outlined by Summers and Smith (1987). Recombinant baculoviruses were identified by plaque hybridization essentially as outlined by Summers and Smith (1987). The polyhedrin-negative recombinants were plaque purified three to four times on Sf9 cells to remove contaminating wild-type virus.

Preparation of cell lysates and immunoprecipitation

To analyze expression of recombinant gI, confluent monolayers of Sf9 cells on 35-mm petri dishes were infected with individual polyhedrin-negative recombinants at a m.o.i. of 5 and incubated for 48 hr at 28°. The cells were scraped into PBS, pelleted at 150 *g* for 1 min, and resuspended in 50 μl of RIPA buffer (0.02 *M* Tris–hydrochloride [pH 8.0], 0.15 *M* NaCl, 1% sodium deoxycholate, 1% nonidet P-40, 10 m*M* ethylene diamine tetraacetic acid [EDTA], 10 m*M* phenylmethylsulfonylfluoride [PMSF]). The supernatant was collected and 5 μl was combined with reducing electro-

```
1    MAARGGAERA AGAGDGRRGQ RRHLRPGRVL AALRGPAAPG AGGARAAHAA ALLWATWALL
61   LAAPAAGRPA TTPPAPPPEE AASPAPPASP SPPGPDGDDA ASPDNSTDVR AALRLAQAAG
          RPA  TTPPAPPPEE  AASPAPP
       Signal↑
121  ENSRFFVCPP PSGATVVRLA PARPCPEYGL GRNYTEGIGV IYKENIAPYT FKAYIYYKNV
181  IVTTTWAGST YAAITNQYTD RVPVGMGEIT DLVDKKWRCL SKAEYLRSGR KVVAFDRDDD
241  PWEAPLKPAR LSAPGVRGWH TTDDVYTALG SAGLYRTGTS VNCIVEEVEA RSVYPYDSFA
301  LSTGDIIYMS PFYGLREGAH REHTSYSPER FQQIEGYYKR DMATGRRLKE PVSRNFLRTQ
361  HVTVAWDWVP KRKNVCSLAK WREADEMLRD ESRGNFRFTA RSLSATFVSD SHTFALQNVP
421  LSDCVIEEAE AAVERVYRER YNGTHVLSGS LETYLARGGF VVAFRPMLSN ELAKLYLQEL
481  ARSNGTLEGL FAAAAPKPGP RRARRAAPSA PGGPGAANGP AGDGDAGGRV TTVSSAEFAA
                          ↑AAPSA PGGPGAA
                    gIb    gIc
                     ←      →
541  LQFTYDHIQD HVNTMFSRLA TSWCLLQNKE RALWAEAAKL NPSAAASAAL DRRAAARMLG
601  DAMAVTYCHE LGEGRVFIEN SMRAPGGVCY SRPPVSFAFG NESEPVEGQL GEDNELLPGR
661  ELVEPCTANH KRYFRFGADY VYYENYAYVR RVPLAELEVI STFVDLNLTV LEDREFLPLE
721  VYTRAELADT GLLDYSEIQR RNQLHELRFY DIDRVVKTDG NMAIMRGLAN FFQGLGAVGQ
781  AVGTVVLGAA GAALSTVSGI ASFIANPFGA LATGLLVLAG LVAAFLAYRY ISRLRSNPMK
841  ALYPITTRAL KDDARGATAP GEEEEEFDAA KLEQAREMIK YMSLVSAVER QEHKAKKSNK
901  GGPLLATRLT QLALRRRAPP EYQQLPMADV GGA
```

FIG. 4. Predicted amino acid sequence of BHV-1 gI and N-terminal amino acid sequence of the gIb and gIc subunits (bold). Cleavage sites are indicated by arrows and labeled.

phoresis sample buffer and boiled for 2 min for analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting. For immunoprecipitation, BHV-1-infected MDBK cells were overlaid with methionine-free MEM containing 1% FBS, labeled at 6 hr postinfection with 50 μCi per ml of [L-^{35}S]methionine (Amersham, Oakville, Ontario) and harvested at 24 hr post-infection. Bac-gI infected cells were overlaid at 16 hr postinfection with methionine-free TNM-FH medium containing 10% FBS, labeled with 100 μCi per ml of [L-^{35}S]methionine 1 hr later, and harvested at 19 hr postinfection. Tunicamycin at 10 μg per ml was added with the methionine-free medium. Immunoprecipitation with a gI-specific monoclonal antibody cocktail was performed as described previously (van Drunen Littel-van den Hurk *et al.*, 1984).

Analysis of carbohydrates

Proteins were digested with endoglycosidase H or glycopeptidase F as described by Ronin *et al.* (1987). Infected cells were collected by centrifugation and 2 × 10^5 cells were resuspended in 10 μl of appropriate enzyme incubation buffer. Digestion with glycopeptidase F (Boehringer-Mannheim, Laval, Quebec, Canada) was performed in 50 mM Tris-hydrochloride (pH 8.6), 25 mM EDTA, 1% Triton X100, 1% 2-mercaptoethanol, 0.2% SDS and 1.5 U of enzyme. Digestion with endo H (Boehringer-Mannheim) was performed in 0.1 M sodium acetate (pH 5), 0.15 M sodium chloride, 1% Triton X100, 1% 2-mercaptoethanol, 0.2% SDS, and 1.5 mU of enzyme. The cells were incubated for 18 hr at 37°C. Proteins were precipitated by adding 1 ml of ice-cold acetone and centrifugation and subjected to SDS–PAGE followed by immunoblot analysis.

SDS–PAGE, Immunoblot, and ELISA

SDS–PAGE was performed under reducing conditions in 7.5, 8.5, or 10% polyacrylamide discontinuous gels as previously described (van Drunen Littel-van den Hurk *et al.*, 1984). Protein bands were visualized by staining with coomassie brilliant blue.

In order to identify recombinant gI produced by baculovirus, immunoblot assays were performed as previously described (van Drunen Littel-van den Hurk *et al.*,

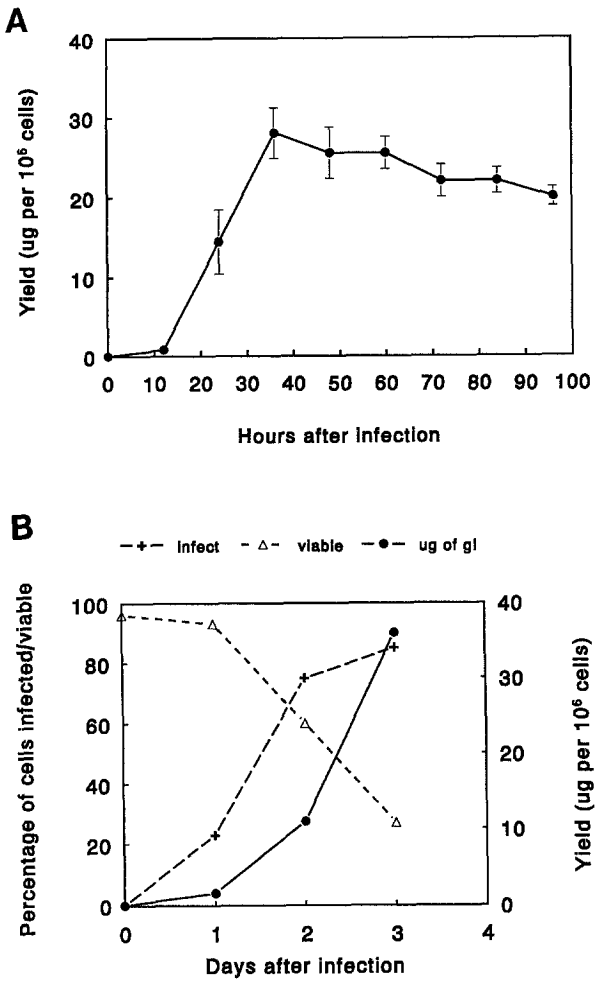

FIG. 5. Kinetics of Bac-gl infection and gl expression in Sf9 cells. (A) Temporal expression of gl in Sf9 cells as measured by ELISA. The amounts of recombinant glycoprotein at each time point were quantitated by direct comparison of the optical density readings of Bac-gl-infected cell samples to the OD values of a standard curve of a known amount of affinity-purified recombinant gl. Background values from mock- and wild-type-AcNPV-infected cells were substracted. The range of glycoprotein detected in different infections is shown. (B) Comparison of percentage of viable cells, determined by trypan blue exclusion and counted with a haemocytometer (Δ), percentage of virus-infected cells, determined by flow cytometry (+) and amount of gl detected by ELISA (●).

1984). Briefly, after electrophoresis cell lysates were electrophoretically transferred to nitrocellulose sheets. Subsequently, the instructions for use of the Bio-Rad (Mississauga, Ontario) immunoblot assay kit were followed. One gl-positive recombinant baculovirus, named Bac-gl, was amplified by growth on Sf9 cells. The supernatants from this infection were stored at 4° and used in all subsequent experiments.

Sandwich ELISAs were used to determine the yields of glycoprotein gl in recombinant baculovirus-infected Sf9 cells. Sf9 cells in monolayers or suspension cultures were infected with recombinant virus at a m.o.i. of 1. Suspension cultures were grown in 500-ml spinner flasks at a cell density of 1×10^6 cells/ml. The cells were harvested at various times postinfection, washed with PBS, and resuspended in RIPA buffer at 1×10^7 cells/ml for analysis by ELISA. Equivalent samples from uninfected cells and/or cells infected with the parental virus were always included as controls. Microtiter plates were coated with the IgG fraction of bovine hyperimmune serum as the capture antibody and then incubated with serial dilutions of lysates from recombinant-virus-infected and control cells, or affinity-purified standard gl. Indirect ELISAs were used to analyze the antigenic properties of recombinant gl as compared to those of authentic gl. In the indirect assay, the cell lysates and glycoproteins were adsorbed to the microtiter plates. Mixtures or individual gl-specific monoclonal antibodies, followed by horseradish peroxidase (HRPO) conjugated goat anti-mouse IgG (Boehringer-Mannheim) were used for detection as previously described (van Drunen Littel-van den Hurk *et al.*, 1984). The reaction was visualized using 0.8 mg/ml of 5-aminosalicylic acid and 0.006% H_2O_2 as described.

Protein sequencing

N-terminal sequencing of the glb and glc components of affinity-purified authentic and recombinant gl was performed by Edman degradation with an Applied Biosystems Model 470A gas phase sequenator equipped with an on-line model 120 PTH amino acid analyzer.

Immunofluorescence and flow cytometry

The expression of glycoprotein gl in recombinant-baculovirus-infected Sf9 cells was determined at 24, 48, and 72 hr postinfection. Briefly, cells were washed in PBS and cytospin smears were prepared and fixed in methanol. They were incubated for 30 min at 37° with a 1:100 dilution of a gl-specific monoclonal antibody mixture and washed in PBS and ddH_2O. They were stained with fluorescein-isothiocyanate-conjugated (FITC) rabbit anti-mouse IgG (Boehringer-Mannheim) for 30 min at 37° and washed again before being mounted in PBS–glycerol for examination. For surface staining and flow-cytometric analysis, cells were suspended in PBS containing 0.2% gelatin and 0.03% NaN_3 (PBSG) at 4×10^7 cells/ml. They were plated in microtiter plates at 2×10^6 cells per well and incubated with serial dilutions of monoclonal antibody mixtures for 30 min on ice. Subsequently, they were washed in PBSG and then incubated with FITC rabbit anti-mouse

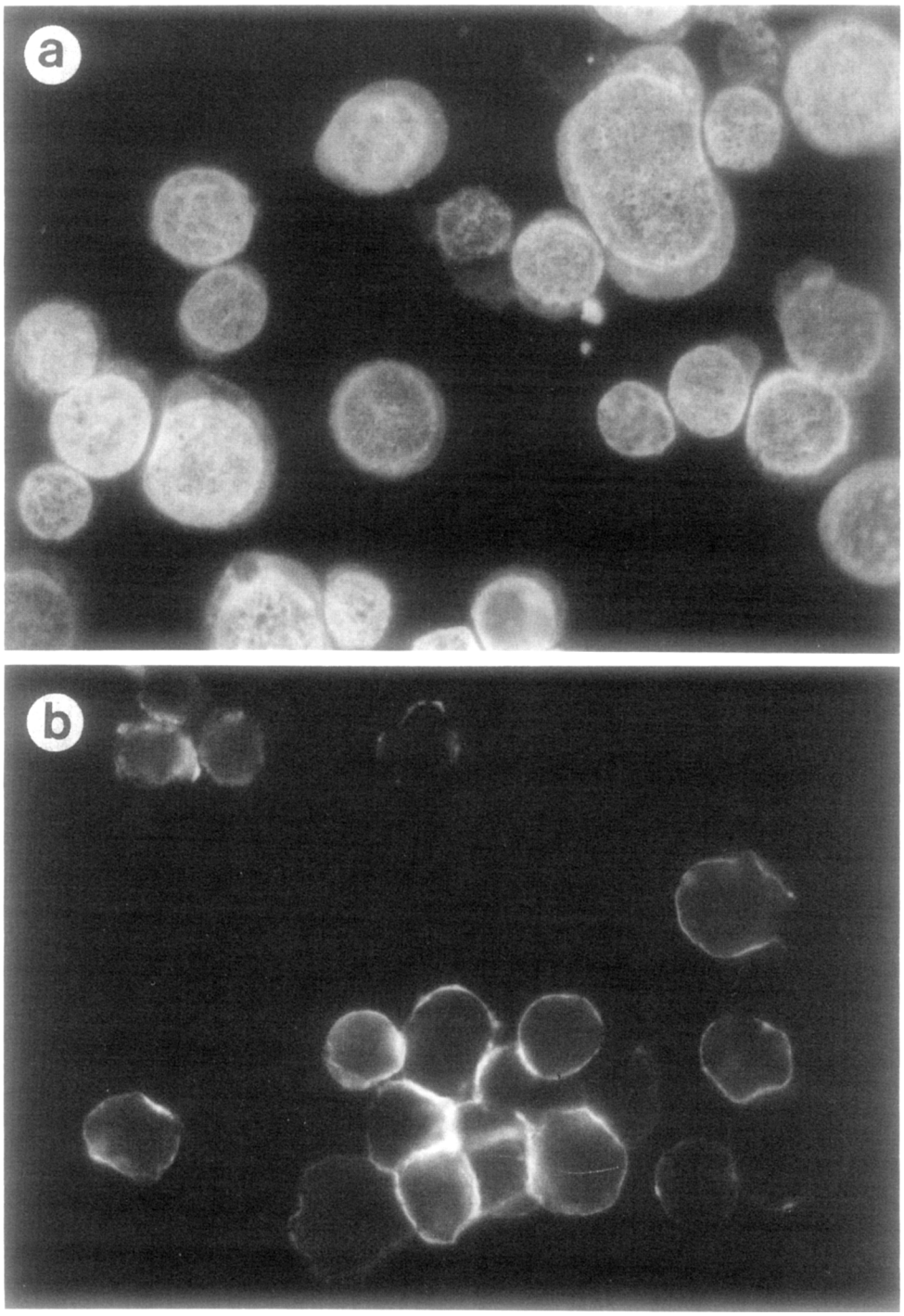

FIG. 6. Immunofluorescence of Sf9 cells infected with Bac-gI. Cells were incubated with a gI-specific monoclonal antibody mixture, washed with cold PBS, and stained for fluorescence microscopy with FITC-labeled goat anti-mouse IgG. (a) Methanol-fixed Bac-gI-infected Sf9 cells. (b) Unfixed Bac-gI-infected Sf9 cells. Phase-contrast views of the corresponding cells are also shown (c and d). Magnification: ×1000.

IgG for 30 min at 4°. After washing, the cells were fixed in 2% formaldehyde and analyzed with an EPICS CS (Coulter Electronics, Ltd.) flow cytometer as described elsewhere (Campos *et al.*, 1989). The percentage of positive cells was calculated using the immuno-program (Coulter Electronics, Ltd., MDAPS system) for the analysis of immunofluorescence histograms.

Cell fusion assay

Monolayers of Sf9 cells in 24-well tissue culture plates were infected with recombinant virus at a m.o.i. of 5–10 PFU per cell. At 36 hr post-infection, the medium was replaced with TNM-FH medium, adjusted to a pH ranging from 5.0 to 6.5. Syncytia formation was

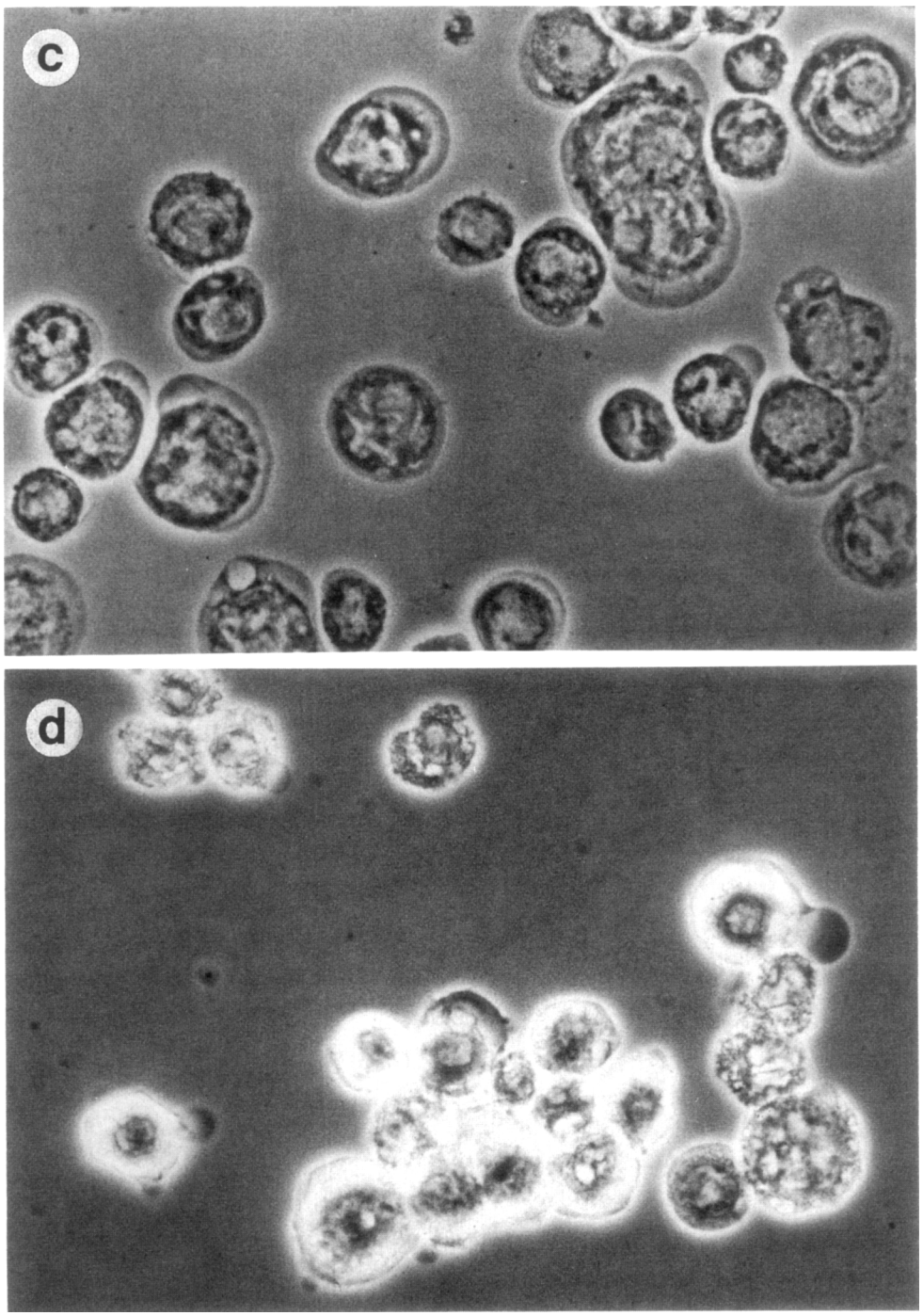

FIG. 6—*Continued*

observed at pH 5.4 and monitored under a phase contrast microscope (Zeiss Model IM35; magnification 200×). Monospecific and monoclonal antibodies were added at a dilution of 1:100 at the time of pH shift.

Immunization of cattle

Glycoprotein gI was purified by immunoadsorbant chromatography from Bac-gI-infected Sf9 cells or BHV-1-infected MDBK cells as described in detail previously (van Drunen Littel-van den Hurk and Babiuk, 1985). Groups of eight animals each were immunized with 100 or 10 μg of affinity-purified recombinant or authentic gI in Emulsigen PLUS at a ratio of 7:3 (v/v) as outlined by the manufacturer (MVP Laboratories, Ralston, NE). The animals were injected intramuscularly and they received a booster immunization 28 days

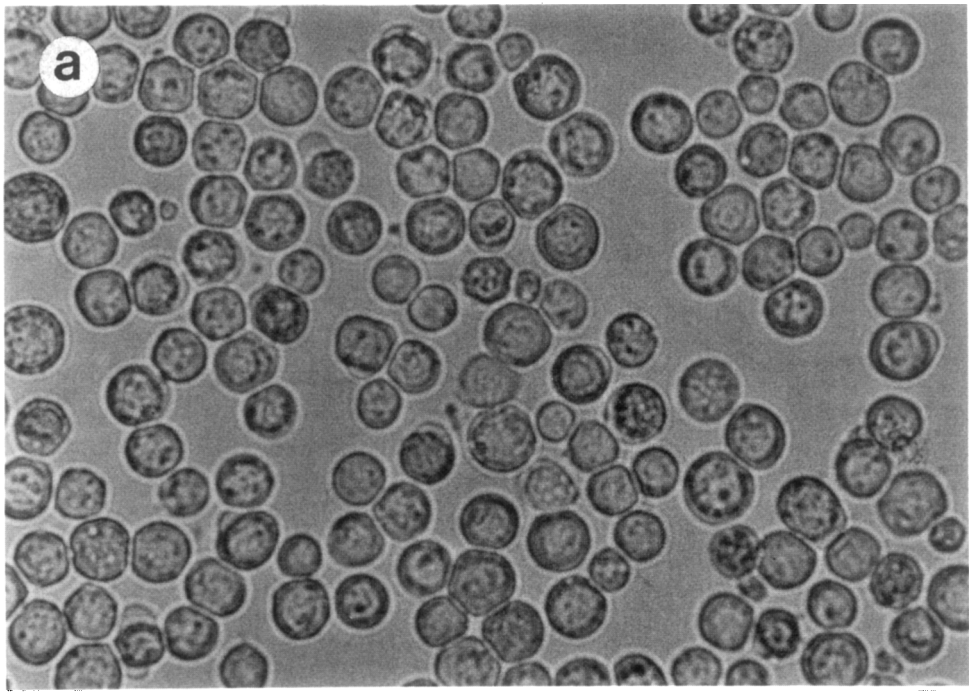

FIG. 7. Cell fusion induced by Bac-gI. At 36 hr postinfection medium was replaced by TNM-FH, pH 5.4. Cell fusion was monitored with an inverted phase contrast microscope before pH shift (a) as well as 2 hr (b) and 8 hr (c) after pH shift. Magnification: ×200.

later. They were bled at the times of immunization and 2 weeks after the second immunization for assessment of antibody responses. The specificity of the antibody response to gI in the vaccinated animals was assayed in an immunoblot assay with purified BHV-1 as the antigen, as described previously (van Drunen Littel-van den Hurk *et al.*, 1990a). The level of the antibody response to gI was measured by ELISA with affinity-purified gI from BHV-1 infected cells as the antigen, as described previously (van Drunen Littel-van den Hurk *et al.*, 1990a).

RESULTS

Production of recombinant gI glycoprotein in Sf9 cells

Recombinants containing the gI gene inserts were tested for their ability to produce BHV-1 glycoprotein I after infection of Sf9 cells. All of the gI recombinants directed the synthesis of a polypeptide with an apparent molecular weight of 116 kDa, which was visible on a Coomassie brilliant blue stained gel at 48 hr postinfection. This protein was missing in uninfected cells and cells infected with the parental baculovirus and probably represented recombinant glycoprotein gIa. Three additional polypeptides appeared during infection with the gI recombinants. The 63- and 52-kDa polypeptides probably corresponded to the gIb and gIc components of the recombinant glycoprotein (Fig. 1). In order to confirm the identity of these glycoproteins, immunoblot analyses were performed on Bac-gI-infected Sf9 cells and BHV-1-infected MDBK cells (Fig. 2). A gI-specific monoclonal antibody mixture that recognized the 130k, 74k, and 55k components of authentic gI in BHV-1-infected MDBK cells, reacted with three polypeptides with apparent molecular weights of 116, 63, and 52 kDa in Bac-gI-infected Sf9 cells. The 38-kDa polypeptide, observed in Fig. 1, was either a gI fragment not detected by gI-specific monoclonal antibodies, or a baculovirus-specified polypeptide. These data suggest that terminal glycosylation of gI has not occurred in the recombinant virus-infected Sf9 cells. Although recombinant gI was cleaved in infected Sf9 cells, the efficiency of cleavage was lower than that of authentic gI.

Processing of gI in mammalian and insect cells

To further analyze the observed difference in molecular weight of the recombinant and authentic gI, Bac-gI-infected Sf9 cells, and BHV-1-infected MDBK cells were treated with tunicamycin, an inhibitor of N-linked glycosylation. In these cells only one polypeptide with an apparent molecular weight of 105k was observed (Fig. 3A), which corresponds to the previously identi-

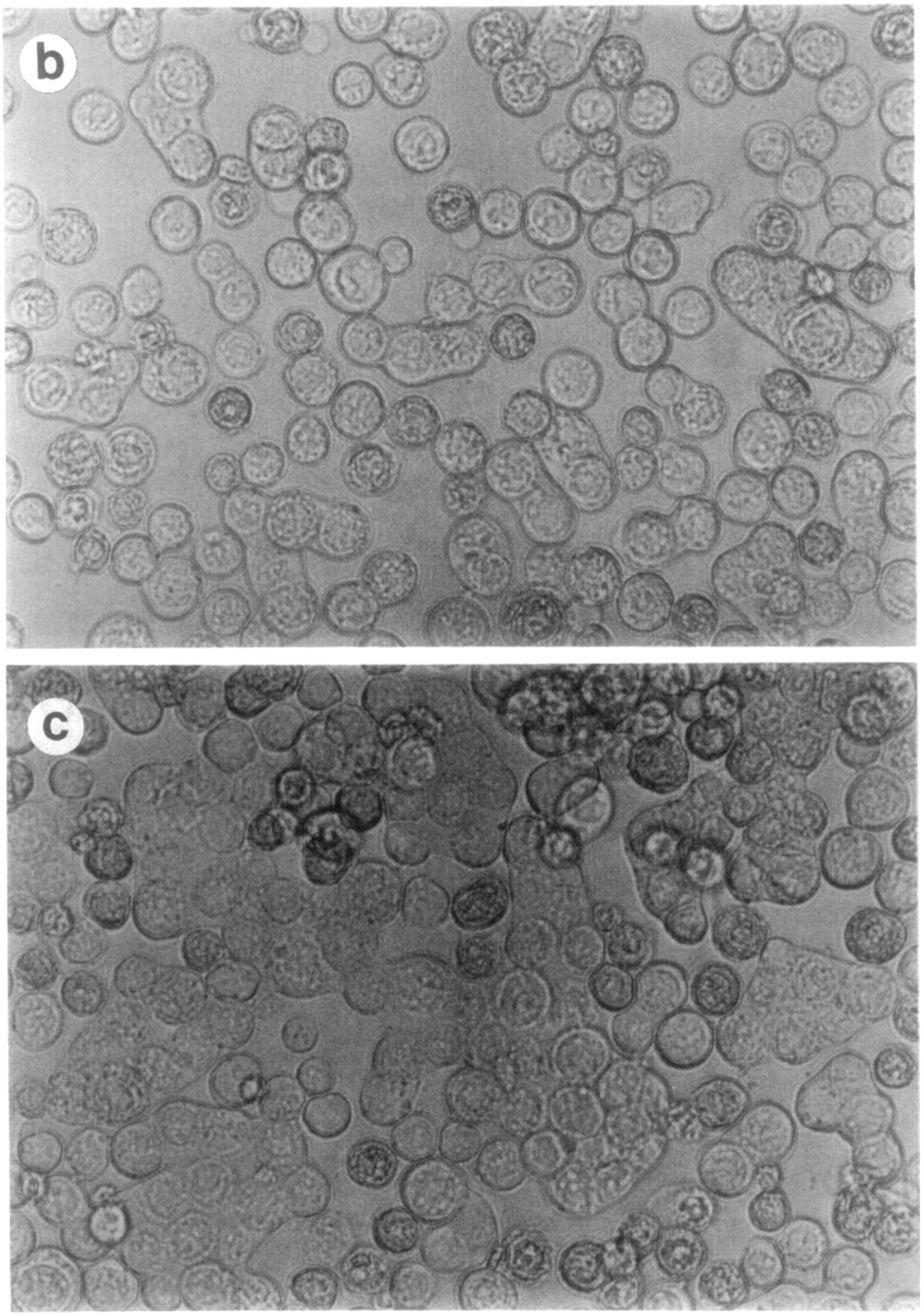

FIG. 7—*Continued*

fied polypeptide backbone of authentic gl (van Drunen Littel-van den Hurk and Babiuk, 1986b). This experiment proved that the decreased molecular weight of gl produced in insect cells was due to altered glycosylation. Due to the short labeling period, the recombinant glb and glc were not observed on this gel. To compare the type of carbohydrate attached to recombinant and authentic gl, both glycoproteins were subjected to digestion with endo H or endo F. Digestion with endo H resulted in a slight decrease in apparent molecular weight of authentic gla and glc, but had no effect on glb, which confirms previous studies (van Drunen Littel-van den Hurk *et al.*, 1986b). The greater portion of recombinant gla and glc was sensitive to endo H, indicating the presence of high-mannose type oligosaccharides. In contrast, the recombinant glb was not

TABLE 1

INHIBITION OF FUSION ACTIVITY MEDIATED BY gI EXPRESSED IN BACULOVIRUS

Treatment[a]	Fusion activity (%)[b]
TNM-FH, pH 5.4	80
Trypsin	80
Normal Rabs	80
gI-specific Rabs	0
Control Mab	80
gI-specific Mabs mixture	0
1B10 Mab (I)	5
3F3 Mab (II)	80
1E11 Mab (III)	80
1F8 Mab (IVa)	80
5G2 Mab (IVb)	10
5G11 Mab (IVc)	60
1F10 Mab (V)	80

[a] Cell fusion was induced at 36 hr postinfection by replacing the cell culture medium with TNM-FH, pH 5.4. At the time of pH shift a final dilution of 1:100 of Rabs (rabbit serum) or Mabs (monoclonal antibodies) was added to the medium. The control Mab was specfic for an unrelated, bovine rotavirus antigen. Treatment with 20 μg trypsin was carried out for 10 min, just before pH shift at 36 hr.

[b] The cells were counted 8 hr after the pH shift. The percentage of fused cells was calculated on a total of 400 cells and rounded to the nearest decimal.

sensitive to endo H, suggesting that these oligosaccharides were trimmed. All of the recombinant and authentic forms of gI were endo-F-sensitive, showing precursor molecules with similar apparent molecular weights in BHV-1 and Bac-gI-infected cells (Fig. 3B).

Authentic and recombinant gI are both cleaved during processing to the mature polypeptide. However, the cleavage process is incomplete in mammalian cells and even less efficient in insect cells. It has been proposed that the Arg–Arg–Ala–Arg–Arg sequence (501–505), which occurs in the region of nonsimilarity with HSV-1, may be the processing site for BHV-1 gI (Whitbeck *et al.*, 1988). To confirm the position of the cleavage site of authentic as well as recombinant gI, we sequenced the N-terminus of the gIc glycoprotein from infected MDBK and Sf9 cells. This analysis confirmed that the first 12 N-terminal amino acids of authentic and recombinant gIc correspond to positions 506–517 (Fig. 4). Since recombinant gI was cleaved at the same site as authentic gI, the reduced cleavage efficiency is probably due to the presence of relatively low amounts of enzyme in baculovirus-infected cells, as compared to the large amounts of gI produced in these cells. N-terminal sequencing of the gIb glycoprotein demonstrated that the same signal is cleaved in MDBK and Sf9 cells and that the amino terminal residue of authentic as well as recombinant gI is Arg-68.

Kinetics and level of expression of the recombinant gI glycoprotein

The amount of gI synthesized in recombinant baculovirus-infected Sf9 cells was quantitated by ELISA standardized with affinity-purified recombinant gI. Sf9 cells grown as monolayers in 35-mm petri dishes were infected with Bac-gI at a m.o.i. of 5, and aliquots of 1×10^6 cells were harvested at various times postinfection. Immunoreactive gI could be detected as early as 24 hr after infection and maximal expression was observed between 36 and 48 hr, thereafter, a slight decrease in measurable glycoprotein occurred. This decline presumably reflects cell lysis and subsequent degradation of the glycoprotein. This analysis showed that, at maximal levels of expression, 28 μg of gI were produced per 10^6 cells (Fig. 5A). In order to analyze the possibility of producing recombinant gI on a larger scale, Sf9 cells were grown in suspension cultures and infected with the recombinant baculovirus at a m.o.i. of 1. In addition to yield by ELISA, the viability of the cells and percentage of infected cells were determined. Figure 5B shows that the percentage of infected cells increased gradually, reaching peak levels of 85% at 72 hr after infection, when the viability of the cells was down to 25%. The viability of the cells was too low for flow

TABLE 2

REACTIVITY OF MONOCLONAL ANTIBODIES WITH AUTHENTIC AND RECOMBINANT gI

Monoclone Designation[a]	Epitope Specificity[b]	Neutralizing Activity[c]	ELISA titer[d] BHV-1 gI	ELISA titer[d] AcNPV gI
1B10	I	−	100	6400
3F3	II	±	6400	25600
1E11	III	++	1600	6400
1F8	IVa	+	25600	6400
5G2	IVb	+	6400	6400
3G11	IVb	+	1600	1600
5G11	IVc	+	1600	100
6G11	IVc	++	400	100
1F10	V	±	1600	1600
2C5	V	±	6400	6400

[a] Monoclonal antibodies developed by van Drunen Littel *et al.* (1984).

[b] gI epitopes assigned by competitive binding assays (van Drunen Littel-van den Hurk *et al.*, 1985).

[c] Neutralizing titers were determined for ascites fluids in the presence of guinea pig serum as a source of complement. −, titer < 4; ±, titer < 100; +, titer > 100; ++, titer > 10,000 (van Drunen Littel-van den Hurk *et al.*, 1985).

[d] Antigen titer was expressed as the reciprocal of the highest dilution of infected cells giving a reading of at least 0.05 OD (492 nm). A 1:100 dilution corresponds to 2×10^4 cells.

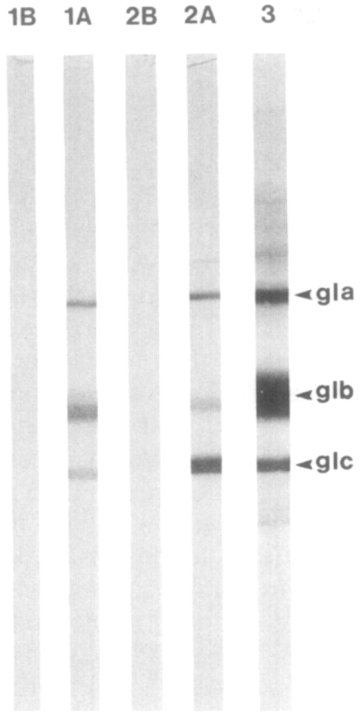

FIG. 8. Immune response of calves to recombinant and authentic affinity-purified gI in Emulsigen PLUS. Western blots of BHV-1 were probed with bovine sera before (1B) and after (1A) immunization with recombinant gI; with bovine sera before (2B) and after (2A) immunization with authentic gI and with a gI-specific monoclonal antibody mixture (3).

cytometric analysis beyond this time point. Analysis by ELISA demonstrated that up to 36 μg of gI were produced per 10^6 cells. This demonstrates the feasability of growing the recombinant baculovirus on a larger scale and obtaining high yields of the glycoprotein.

Intracellular localization of recombinant gI in Sf9 cells

The intracellular distribution of the recombinant gI glycoprotein was examined by an indirect immunofluorescence assay. At 48 hr postinfection, recombinant gI was primarily localized in the perinuclear membranes of the infected Sf9 cells (Fig. 6a). To determine whether the recombinant gI was present on the surface of infected cells, immunofluorescence analysis was carried out on unfixed cells. Localization of gI on the cell surface was demonstrated by bright surface fluorescence (Fig. 6b). Wild-type AcNPV-infected control cells did not show any fluorescence with the gI-specific monoclonal antibody panel (not shown).

Fusogenic properties of recombinant gI in insect cells

It has been shown previously that one of the functional characteristics of gI is its ability to induce cell fusion in absence of other viral proteins (Fitzpatrick *et al.*, 1988, 1990b). To determine whether the recombinant protein retained this functional property, Sf9 cells were infected with Bac-gI. Fusion of the insect cells was not evident under standard culture conditions (Fig. 7a), but after a shift to pH 5.4 fusion was apparent in Bac-gI-infected Sf9 cells within 2 hr (Fig. 7b). The syncytia formation observed in these cells continued to increase over 8 hr of observation (Fig. 7c). Fusion was not detected in cultures infected with wild-type AcNPV over the pH range examined (not shown). Inclusion of gI-specific rabbit serum or a mixture of gI-specific monoclonal antibodies completely inhibited fusion by gI (Table 1). When individual monoclonal antibodies were included in the media, fusion was almost completely inhibited by the monoclonal antibodies 1B10 (epitope I) and 5G2 (epitope IVb) and partially inhibited by 5G11 (epitope IVc). Inclusion of trypsin at the time of pH shift did not affect the fusion activity.

Antigenic and immunogenic properties of gI expressed in Sf9 cells

The antigenic properties of recombinant gI were evaluated using a panel of gI-specific monoclonal antibodies. The epitopes recognized by these monoclonal antibodies have been identified and characterized previously (van Drunen Littel-van den Hurk *et al.*, 1985; Fitzpatrick *et al.*, 1990a). The reactivity of these monoclonal antibodies in an ELISA (Table 2) indicated that all of the epitopes identified on the authentic glycoprotein were also present on the recombinant gI glycoprotein. The reaction between the monoclonal antibodies and two carbohydrate-dependent epitopes (IVa and IVc; van Drunen Littel-van den Hurk *et al.*, 1990b) was weaker on recombinant gI than on authentic gI, which is in agreement with lack of terminal glycosylation of gI in Sf9 cells. Epitopes I, II, and III, however, appeared to

TABLE 3

IMMUNE RESPONSE TO AUTHENTIC AND RECOMBINANT GI

Immunogen[a]	Dose (μg)	Antibody titer[b]
Authentic gI	100	8,125
Authentic gI	10	2,560
Recombinant gI	100	10,240
Recombinant gI	10	1,280
Placebo	N.A.	10

[a] Animals received two intramuscular immunizations of authentic gI, recombinant gI, or PBS (placebo) in Emulsigen PLUS.

[b] ELISA titers were determined against affinity-purified gI and expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the negative control value.

be more reactive on recombinant gI than on its authentic counterpart.

To compare the immunogenicity of recombinant and authentic gI, calves were immunized with affinity-purified glycoprotein from recombinant Bac-gI-infected Sf9 cells or BHV-1-infected MDBK cells. Two immunizations of recombinant or authentic gI in Emulsigen PLUS elicited antibodies that were reactive with gI from BHV-1 in an immunoblot assay (Fig. 8). The antibody titers induced by recombinant and authentic gI were very similar (Table 3).

DISCUSSION

The goal of this study was to determine whether the baculovirus system would produce gI in an authentic form and in sufficient quantities for further structural, functional, and immunological analyses. The level of gI synthesis attained by using the baculovirus expression vector was extremely high, about 36 μg per 10^6 cells by 48–72 hr postinfection. This amount is at least 10-fold greater than the quantity of gI glycoprotein found in BHV-1-infected cells (van Drunen Littel-van den Hurk and Babiuk, 1985), which makes this system the most efficient means for producing gI.

BHV-1 gI has six potential receptor sites for N-linked glycosylation of which one is probably not used (Whitbeck *et al.*, 1988). The apparent molecular weight of the gI complex was 116/63/52 kDa in insect cells, which corresponds in size to the cotranslationally glycosylated gI produced in BHV-1-infected MDBK cells (van Drunen Littel-van den Hurk and Babiuk, 1986b). Authentic gI is processed post-translationally into the mature 130/74/55-kDa complex. The polypeptide backbones of recombinant and authentic gI were both 105 kDa, proving that indeed the lower apparent molecular weight of the recombinant form was due to altered processing of the carbohydrates in insect cells. A portion of the recombinant gI was resistant to endo H cleavage which confirms earlier reports that N-glycans can be trimmed in insect cells to trimannosyl cores, without further elongation (Butters *et al.*, 1981; Hsieh and Robbins, 1984). The majority of the oligosaccharide side chains on the recombinant gI, however, contained endo-H-sensitive linkages, which was in agreement with studies on HCMV gB (Wells *et al.*, 1990), influenza virus hemagglutinin (Kuroda *et al.*, 1986, 1990), human immunodeficiency virus gp160 (Wells and Compans, 1990), and hepatitis B virus surface antigen (Lanford *et al.*, 1989), which also contained mostly endo-H-sensitive carbohydrates when produced in insect cells.

In spite of being incompletely glycosylated, recombinant gI was proteolytically cleaved into its two subunits, albeit less efficiently than authentic gI. The enzymatic machinery executing cleavage of most of the gB homologs is still largely unknown. Recently it has been shown that a protease located in the Golgi apparatus is responsible for PRV gII processing (Whealy *et al.*, 1990). As the exact cleavage site for BHV-1 gI has not been determined yet, we sequenced the N-terminal portion of gIc from insect cells and mammalian cells. The cleavage site was, as anticipated, between residues 505 and 506, just after the Arg–Arg–Ala–Arg–Arg motif proposed to be the processing site (Whitbeck *et al.*, 1988). This consensus cleavage signal is identical in PRV gII (Wolfer *et al.*, 1990), although the N-terminal sequences of BHV-1 gIc and PRV gIIc show considerable heterogeneity (Misra *et al.*, 1988). Thus, the inefficiency with which recombinant gI is cleaved is not due to a difference in the processing site. The slow rate of cleavage of the gI glycoprotein observed in insect cells is in agreement with observations made on other glycoproteins like the influenza hemagglutinin (Kuroda *et al.*, 1986, 1990), the E2 protein of mouse hepatitis virus (Yoden *et al.*, 1989), the gp160 of human immunodeficiency virus (Wells and Compans, 1990), the F glycoprotein of measles virus (Vialard *et al.*, 1990) and the gB glycoprotein of HCMV (Wells *et al.*, 1990). This may reflect the presence in insect cells of low levels of a host cell protease involved in processing of these glycoproteins, or alternatively, the presence of normal levels of the protease which, however, is saturable and unable to process the large amounts of glycoprotein produced in these cells. Another explanation is based on the observation that a portion of recombinant gI remains endo-H-sensitive and thus may not migrate from the ER to the Golgi (Lanford *et al.*, 1989), where cleavage probably occurs.

The recombinant gI glycoprotein was transported to and expressed on the surface of infected cells, and as such, was capable of inducing cell-to-cell fusion. This indicates that complete glycosylation is not needed for fusion activity. Although it is not known whether the gI molecules expressed on the surface of the infected cells are cleaved or not, addition of trypsin did not enhance syncytia formation, indicating that the fusion activity is not totally dependent upon cleavage of gI. Optimal conditions for fusion appeared to be more acidic in insect cells than in mammalian cells (Fitzpatrick *et al.*, 1990b). This phenomenon has been observed for different fusion proteins expressed in insect cells, like the F protein of measles virus and the S glycoprotein of bovine coronavirus (Vialard *et al.*, 1990; Yoo *et al.*, 1990). The glycosylation differences observed in all cases may be responsible for conformational changes resulting in the altered optimum pH for fusogenicity. In addition, the membranes of insect and mammalian

cells may differ as targets for fusion. That fusion was mediated by gI was confirmed by total inhibition in the presence of gI-specific polyclonal or a cocktail of monoclonal antibodies. Monoclonal antibody 1B10, specific for epitope I, inhibited fusion almost completely. Epitope I maps to a segment between residues 744–763, a highly conserved portion of the extracellular region of the carboxy terminal fragment (gIc) of gI (Fitzpatrick *et al.,* 1990a). This implies that one domain responsible for cell fusion is located on the gIc subunit, between or in the vicinity of residues 744–763. Monoclonal antibody 5G2, specific for epitope IVb, and monoclonal antibody 5G11, specific for epitope IVc, partially inhibited fusion activity. These epitopes map to the amino terminus of gI between residues 68 and 119 (Fitzpatrick *et al.,* 1990a). This suggests that domain IV is also involved in the fusion process. In analogy to the predicted secondary structure determined for gB (HSV) by Pellett *et al.* (1985), gI might also fold back, thereby positioning the amino terminus in close proximity to the membrane. Alternatively, fusion may be a complex process which requires the sequential or coordinated activity of at least two separate domains of gI. To exclude the possibility that the monoclonal antibodies specific for epitopes I and IV inhibit fusion indirectly, both fusogenic regions need to be confirmed by mutagenic analysis. This is the first report implying domains I and IV of BHV-1 gI in the fusion process. Recently, three domains involved in fusion activity of HSV-1 gB were identified (Navarro *et al.,* 1992). Two of these, D1 and D5a, correspond to the amino terminal domain IV and the carboxy terminal domain I of BHV-1 gI, respectively.

In addition to the structural and functional analyses of recombinant gI, we studied the antigenicity by ELISA. BHV-1 gI has at least four neutralizing and three weakly or nonneutralizing epitopes, some of which are conformational in nature (van Drunen Littel-van den Hurk *et al.,* 1985; Fitzpatrick *et al.,* 1990a). All of these epitopes were recognized on recombinant gI. The reaction with two carbohydrate-dependent epitopes, IVa and IVc, appeared weaker and the recognition of epitopes I, II, and III seemed stronger, but in general the antigenicity was not significantly affected by altered processing and glycosylation. This is in contrast to the situation observed in the absence of carbohydrate, which does affect the antigenicity of gI (van Drunen Littel-van den Hurk *et al.,* 1990b). Altered processing and glycosylation of gI in insect cells did not affect the immunogenicity of gI. Immunization of cattle with purified recombinant or authentic gI led to the induction of similar levels of gI-specific antibodies in cattle.

This work has shown that BHV-1 gI produced by an AcNPV recombinant in insect cells is biologically, antigenically, and immunogenically active, despite the fact that it is incompletely cleaved and glycosylated. These properties indicate that recombinant gI has not undergone conformational rearrangements and should be an appropriate candidate for further structural, functional, and immunological analyses.

ACKNOWLEDGMENTS

The authors are grateful to Dr. D. Yoo for helpful discussions, Tammy Wolfe and Terry Beskorwayne for technical assistance, the animal support staff at VIDO for care and handling of the animals, and Marilee Hagen for preparing the manuscript. Financial support was provided by the Medical Research Council and the Natural Science and Engineering Council of Canada. D.R.F. was a recipient of a Commonwealth fellowship.

REFERENCES

ALBRECHT, J.-C., and FLECKENSTEIN, B. (1990). Structural organization of the conserved gene block of herpesvirus saimiri coding for DNA polymerase, glycoprotein B and major DNA binding protein. *Virology* **174,** 533–542.

BABIUK, L. A., WARDLEY, R. C., and ROUSE, B. T. (1975). Defense mechanisms against bovine herpesvirus: Relationship of virus–host cell events to susceptibility to antibody-complement lysis. *Infect. Immun.* **12,** 958–963.

BABIUK, L. A., L'ITALIEN, J., VAN DRUNEN LITTEL-VAN DEN HURK, S., ZAMB, T. J., LAWMAN, M. J. P., HUGHES, G., and GIFFORD, G. A. (1987). Protection of cattle from bovine herpesvirus type-1 (BHV-1) infection by immunization with individual viral glycoproteins. *Virology* **159,** 57–66.

BRITT, W. J., and VUGLER, L. (1989). Processing of the gp55-116 envelope glycoprotein complex (gB) of human cytomegalovirus. *J. Virol.* **63,** 403–410.

BUCKMASTER, A. E., SCOTT, S. D., SANDERSON, M. J., BOURSNELL, M. E. G., ROSS, N. L. J., and BINNS, M. M. (1988). Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: Implications for herpesvirus classification. *J. Gen. Virol.* **69,** 2033–2042.

BUTTERS, T. D., HUGHES, R. C., and VISCHER, P. (1981). Steps in the biosynthesis of mosquito cell membrane glycoproteins and the effects of tunicamycin. *Biochim. Biophys. Acta* **640,** 672–686.

BZIK, D. J., DEBROY, C., FOX, B., PEDERSON, N. E., and PERSON, S. (1986). The nucleotide sequence of the gB glycoprotein gene of HSV-2 and comparison with the corresponding gene of HSV-1. *Virology* **155,** 322–333.

CAI, W., PERSON, S., WARNER, S. C., ZHOU, J., and DELUCA, N. A. (1987). Linker insertion, nonsense, and restriction site deletion mutations of the gB glycoprotein gene of herpes simplex virus-1. *J. Virol.* **61,** 714–721.

CAMPOS, M., BIELEFELDT-OHMANN, H., HUTCHINGS, D., RAPIN, N., BABIUK, L. A., and LAWMAN, M. P. J. (1989). Role of interferon-gamma in inducing cytotoxicity of peripheral blood mononuclear leukocytes to bovine herpesvirus type-1 (BHV-1) infected cells. *Cell. Immunol.* **120,** 259–269.

COLLINS, J. K., BUTCHER, A. C., and RIEGEL, C. A. (1985). Immune response to bovine herpesvirus type-1 infections: Virus-specific antibodies in sera from infected animals. *J. Clin. Microbiol.* **21,** 546–552.

CRANAGE, M. P., KOUZARIDES, T., BANKIER, A. T., SATCHWELL, S., WESTON, K., TOMLINSON, P., BARRELL, B., HART, H., BELL, S. E., MINSON, A. C., and SMITH, G. L. (1986). Identification of the human cytomeg-

alovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus. *EMBO J.* **5,** 3057–3062.

DAVISON, A. J., and TAYLOR, P. (1987). Genetic relations between varicella zoster virus and Epstein–Barr virus. *J. Gen. Virol.* **68,** 1067–1079.

DELUCA, N., BZIK, D. J., BOND, V., PERSON, S., and SNIPES, W. (1982). Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion and production of glycoprotein gB. *Virology* **122,** 411–423.

FITZPATRICK, D. R., ZAMB, T., PARKER, M. D., VAN DRUNEN LITTEL-VAN DEN HURK, S., BABIUK, L. A., and LAWMAN, M. J. P. (1988). Expression of bovine herpesvirus-1 glycoproteins gI and gIII in transfected murine cells. *J. Virol.* **62,** 4239–4248.

FITZPATRICK, D. R., REDMOND, M. J., ATTAH-POKU, S. K., VAN DRUNEN LITTEL-VAN DEN HURK, S., BABIUK, L. A., and ZAMB, T. J. (1990a). Mapping of 10 epitopes on bovine herpesvirus type-1 glycoproteins gI and gIII. *Virology* **176,** 145–157.

FITZPATRICK, D. R., ZAMB, T. J., and BABIUK, L. A. (1990b). Expression of bovine herpesvirus type 1 glycoprotein gI in transfected cells induces spontaneous cell fusion. *J. Gen. Virol.* **71,** 1215–1219.

HAMMERSCHMIDT, W., CONRATHS, F., MANKERTZ, J., PAULI, G., LUDWIG, H., and BUHK, H.-J. (1988). Conservation of a gene cluster including glycoprotein B in bovine herpesvirus type 2 (BHV-2) and herpes simplex virus type 1 (HSV-1). *Virology* **165,** 388–405.

HSIEH, P., and ROBBINS, P. W. (1984). Regulation of asparagine-linked oligosaccharide processing: Oligosaccharide processing in *Aedes albopictus* mosquito cells. *J. Biol. Chem.* **259,** 2375–2382.

KELLER, P. M., DAVISON, A. J., LOWE, R. S., BENNETT, C. D., and ELLIS, R. W. (1986). Identification and structure of the gene encoding gpII, a major glycoprotein of varicella zoster virus. *Virology* **152,** 181–191.

KURODA, K., HAUSER, C., ROTT, R., KLENK, H.-D., and DOERFLER, W. (1986). Expression of the influenza haemagglutinin in insect cells by a baculovirus vector. *EMBO J.* **5,** 1359–1365.

KURODA, K., GEYER, H., GEYER, R., DOERFLER, W., and KLENK, H.-D. (1990). The oligosaccharides of influenza virus hemagglutinin expressed in insect cells by a baculovirus vector. *Virology* **174,** 418–429.

LANFORD, R. E., LUCKOW, V., KENNEDY, R. C., DREESMAN, G. R., NOTVALL, L., and SUMMERS, M. D. (1989). Expression and characterization of hepatitis B virus surface antigen polypeptides in insect cells with a baculovirus expression system. *J. Virol.* **63,** 1549–1557.

LIANG, X., BABIUK, L. A., VAN DRUNEN LITTEL-VAN DEN HURK, S., FITZPATRICK, D. R., and ZAMB, T. J. (1991). Bovine herpesvirus 1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII, and gIV. *J. Virol.* **65,** 1124–1132.

LUKACS, N., THIEL, H.-J., METTENLEITER, T. C., and RZIHA, H.-J. (1985). Demonstration of three major species of pseudorabies virus glycoproteins and identification of a disulfide-linked glycoprotein complex. *J. Virol.* **53,** 166–173.

LUCKOW, V. A., and SUMMERS, M. D. (1988). Trends in the development of baculovirus expression vectors. *Bio/Technology* **6,** 47–55.

MARSHALL, R. L., RODRIGUEZ, L. L., and LETCHWORTH, G. J., III (1986). Characterization of envelope proteins of infectious bovine rhinotracheitis virus (bovine herpesvirus-1) by biochemical and immunological methods. *J. Virol.* **57,** 745–753.

MEREDITH, D. M., STOCKS, J.-M., WHITTAKER, G. R., HALLIBURTON, I. W., SNOWDEN, B. W., and KILLINGTON, R. A. (1989). Identification of the gB homologues of equine herpesvirus types 1 and 4 as disulphide-linked heterodimers and their characterization using monoclonal antibodies. *J. Gen. Virol.* **70,** 1161–1172.

METTENLEITER, T. C., LUKACS, N., THIEL, H.-J., SCHREURS, C., and RZIHA, H.-J. (1986). Location of the structural gene of pseudorabies virus glycoprotein complex gII. *Virology* **152,** 66–75.

MISRA, V., NELSON, R., and SMITH, M. (1988). Sequence of a bovine herpesvirus-1 glycoprotein gene that is homologous to the herpes simplex gene for the glycoprotein gB. *Virology* **166,** 542–549.

MONTALVO, E. A., and GROSE, C. (1987). Assembly and processing of the disulfide-linked varicella–zoster virus glycoprotein gpII (140). *J. Virol.* **61,** 2877–2884.

NAVARRO, D., PAZ, P., and PEREIRA, L. (1992). Domains of herpes simplex virus-1 glycoprotein B that function in virus penetration, cell-to-cell spread, and cell fusion. *Virology* **186,** 99–112.

PELLETT, P. E., BIGGIN, M. D., BARRELL, B., and ROIZMAN, B. (1985a). Epstein–Barr virus genome may encode a protein showing significant amino acid and predicted secondary structure homology with glycoprotein B of herpes simplex virus 1. *J. Virol.* **56,** 807–813.

PELLETT, P. E., KOUSOULAS, K. G., PEREIRA, L., and ROIZMAN, B. (1985b). Anatomy of herpes simplex virus 1 strain F glycoprotein B gene: Primary sequence and predicted protein structure of the wildtype and of monoclonal antibody resistant mutants. *J. Virol.* **53,** 243–253.

RAUH, I., WEILAND, F., FEHLER, F., KEIL, G., and METTENLEITER, T. C. (1991). Pseudorabies virus mutants lacking the essential glycoprotein II can be complemented by glycoprotein gI of bovine herpesvirus-1. *J. Virol.* **65,** 621–631.

RIGGIO, M. P., CULLINANE, A. A., and ONIONS, D. E. (1989). Identification and nucleotide sequence of the glycoprotein gB gene of equine herpesvirus 4. *J. Virol.* **63,** 1123–1133.

ROBBINS, A. K., DORNEY, D., WATHEN, M. W., WHEALY, M. E., GOLD, C., WATSON, R. J., HOLLAND, L. E., WEED, S. D., LEVINE, M., GLORIOSO, J., and ENQUIST, L. W. (1987). The pseudorabies virus gII gene is closely related to the gB glycoprotein genes of herpes simplex virus. *J. Virol.* **61,** 2691–2701.

RONIN, C., PAPANDREOU, M.-J., CANONNE, C., and WEINTRAUB, B. D. (1987). Carbohydrate chains of human thyrotropin are differentially susceptible to endoglycosidase removal on combined and free polypeptide subunits. *Biochemistry* **26,** 5848–5853.

ROSS, L. N., SANDERSON, M., SCOTT, S. D., BINNS, M. M., DOEL, T., and MILNE. (1989). Nucleotide sequence and characterization of the Marek's disease virus homologue of glycoprotein gB of herpes simplex virus. *J. Gen. Virol.* **70,** 1789–1804.

SPAETE, R., SAXENA, A., SCOTT, P., SONG, G., PROBERT, W., BRITT, W., GIBSON, W., RASMUSSEN, L., and PACHL, C. (1990). Sequence requirements for proteolytic processing of glycoprotein B of human cytomegalovirus strain Towne. *J. Virol.* **64,** 2922–2931.

SUMMERS, M. D., and SMITH, G. E. (1987). "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures." Texas Agricultural Experimental Station Bulletin No. 1555. Texas Agricultural Experimental Station, College Station, TX.

VAN DRUNEN LITTEL-VAN DEN HURK, S., VAN DEN HURK, J. V., GILCHRIST, J. E., MISRA, V., and BABIUK, L. A. (1984). Interactions of monoclonal antibodies and bovine herpesvirus type-1 (BHV-1) glycoproteins: Characterization of their biochemical and immunological properties. *Virology* **135,** 466–479.

VAN DRUNEN LITTEL-VAN DEN HURK, S., and BABIUK, L. A. (1985). Antigenic and immunogenic characteristics of bovine herpesvirus type-1 glycoproteins GVP 3/9 and GVP 6/11/16, purified by immunoadsorbent chromatography. *Virology* **144,** 204–215.

VAN DRUNEN LITTEL-VAN DEN HURK, S., VAN DEN HURK, J. V., and BABIUK, L. A. (1985). Topographical analysis of bovine herpesvirus type-1 glycoproteins: Use of monoclonal antibodies to identify and characterize functional epitopes. *Virology* **144,** 216–227.

VAN DRUNEN LITTEL, S., AND BABIUK, L. A. (1986a). Polypeptide specificity of the antibody response after primary and recurrent infection with bovine herpesvirus-1. *J. Clin. Microbiol.* **23,** 274–282.

VAN DRUNEN LITTEL-VAN DEN HURK, S., and BABIUK, L. A. (1986b). Syn-

thesis and processing of bovine herpesvirus-1 glycoproteins. *J. Virol.* **59,** 401–410.

VAN DRUNEN LITTEL-VAN DEN HURK, S., ZAMB, T., and BABIUK, L. A. (1989). Synthesis, cellular localization, and immunogenicity of bovine herpesvirus-1 glycoproteins gI and gIII expressed by recombinant vaccinia virus. *J. Virol.* **63,** 2159–2168.

VAN DRUNEN LITTEL-VAN DEN HURK, S., GIFFORD, G. A., and BABIUK, L. A. (1990a). Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. *Vaccine* **8,** 358–368.

VAN DRUNEN LITTEL-VAN DEN HURK, S., HUGHES, G., and BABIUK, L. A. (1990b). The role of carbohydrate in the antigenic and immunogenic structure of bovine herpesvirus-1 (BHV-1) glycoproteins gI and gIV. *J. Gen. Virol.* **71,** 2053–2063.

VIALARD, J., LALUMIERE, M., VERNET, T., BRIEDIS, D., ALKHATIB, G., HENNING, D., LEVIN, D., and RICHARDSON, C. (1990). Synthesis of the membrane fusion and hemagglutinin proteins of measles virus, using a novel baculovirus vector containing the beta-galactosidase gene. *J. Virol.* **64,** 37–50.

WELLS, D. E., and COMPANS, R. W. (1990). Expression and characterization of a functional human immunodeficiency virus envelope glycoprotein in insect cells. *Virology* **176,** 575–586.

WELLS, D. E., VUGLER, L. G., and BRITT, W. J. (1990). Structural and immunological characterization of human cytomegalovirus gp55-116 (gB) in insect cells. *J. Gen. Virol.* **71,** 873–880.

WHALLEY, J. M., ROBERTSON, G., SCOTT, N., HUDSON, G., BELL, C., and WOODWORTH, L. (1989). Identification and nucleotide sequence of a gene in equine herpesvirus 1 analogous to the herpes simplex virus gene encoding the major envelope glycoprotein gB. *J. Gen. Virol.* **70,** 383–394.

WHEALY, M. E., ROBBINS, A. K., and ENQUIST, L. W. (1990). The export pathway of the pseudorabiesvirus gB homolog gII involves oligomer formation in the endoplasmic reticulum and protease processing in the Golgi apparatus. *J. Virol.* **64,** 1946–1955.

WHITBECK, J. C., BELLO, L. J., and LAWRENCE, W. C. (1988). Comparison of the bovine herpesvirus 1 gI gene and the herpes simplex virus type-1 gB gene. *J. Virol.* **62,** 3319–3327.

WOLFER, U., KRUFT, V., SAWITZKY, D., HAMPL, H., WITTMAN-LIEBOLD, B., and HABERMEHL, K.-O. (1990). Processing of pseudorabies virus glycoprotein gII. *J. Virol.* **64,** 3122–3125.

YODEN, S., KIKUCHI, T., SIDDELL, S. G., and TAGUCHI, F. (1989). Expression of the peplomer glycoprotein of murine coronavirus JHM using a baculovirus vector. *Virology* **173,** 615–623.

YOO, D., PARKER, M. D., and BABIUK, L. A. (1991). The S2 subunit of the spike glycoprotein of bovine coronavirus mediates fusion in insect cells. *Virology* **180,** 395–399.