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Molecular Cloning, Sequencing, and Expression of Functional Bovine Herpesvirus 1 Glycoprotein gIV in Transfected Bovine Cells†

SURESH K. TIKOO,[1]* DAVID R. FITZPATRICK,[1] LORNE A. BABIUK,[1,2] AND TIM J. ZAMB[2]

Department of Veterinary Microbiology, University of Saskatchewan,[1] and the Veterinary Infectious Disease Organization,[2] Saskatoon, Saskatchewan, Canada, S7N OWO

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The gene encoding bovine herpesvirus 1 (BHV-1) glycoprotein gIV was mapped, cloned, and sequenced. The gene is situated between map units 0.892 and 0.902 and encodes a predicted protein of 417 amino acids with a signal sequence cleavage site between amino acids 18 and 19. Comparison of the BHV-1 amino acid sequence with the homologous glycoproteins of other alphaherpesviruses, including herpes simplex virus type 1 glycoprotein gD, revealed significant homology in the amino-terminal half of the molecules, including six invariant cysteine residues. The identity of the open reading frame was verified by expression of the authentic recombinant BHV-1 gIV in bovine cells by using eucaryotic expression vectors pRSDneo (strong, constitutive promoter) and pMSG (weak, dexamethasone-inducible promoter). Constitutive expression of gIV proved toxic to cells, since stable cell lines could only be established when the gIV gene was placed under the control of an inducible promoter. Expression of gIV was cell associated and localized predominantly in the perinuclear region, although nuclear and plasma membrane staining was also observed. Radioimmunoprecipitation revealed that the recombinant glycoprotein was efficiently processed and had a molecular weight similar to that of the native form of gIV expressed in BHV-1-infected bovine cells. Recombinant gIV produced in the transfected bovine cells induced cell fusion, polykaryon formation, and nuclear fusion. In addition, expression of gIV interfered with BHV-1 replication in the transfected bovine cells.

Bovine herpesvirus (BHV-1), an alphaherpesvirus (44), is a predominant cause of respiratory disease, abortion, and genital infections in cattle (57). As in other herpesviruses, several glycoproteins are synthesized and incorporated into the BHV-1 envelope. These glycoproteins are thought to play an important role in virus-cell interactions, e.g., by functioning in attachment and penetration of virus into the susceptible cells (21, 23, 31), virus neutralization (19, 21), and immune destruction of virus-infected cells (3, 36, 46, 47).

To date, four major glycoproteins, gI, gII, gIII, and gIV, have been identified in the virus envelope and plasma membrane of BHV-1-infected cells (48, 50). These glycoproteins are also recognized by sera from cattle infected with BHV-1 (49). Of these, genes of glycoproteins gI and gIII have been sequenced (14, 55) and expressed in mammalian cells (5, 16, 17). These cell lines have been useful in determining the role of individual glycoproteins in virus-cell interactions and in immune responses to BHV-1. Expression of gI in mammalian cells was shown to induce cell fusion and polykaryon formation (16, 17) but did not render the cells resistant to BHV-1 infection, although plaque size was reduced (5). The expression of gIII in bovine cells did not affect either the number of viral plaques or the yield of virus when the cells were infected with BHV-1 (5), even though it has been suggested that glycoprotein gIII is important for the attachment of BHV-1 to tissue culture cells (39). These studies indicate that some BHV-1 glycoproteins may be functionally redundant at least in tissue culture cells, since otherwise the recombinant glycoprotein produced in the transfected cells would interfere with the function of the authentic glycoprotein of the incoming virion (24, 42).

BHV-1 gIV, a glycoprotein of 71,000 molecular weight containing both N- and O-linked oligosaccharides, has been identified as a major molecule on the surface of the virion and virus-infected cells (50). Monoclonal antibodies against this glycoprotein exhibit high neutralizing titers, even in the absence of complement (22). Monoclonal antibodies directed against domain I of gIV neutralize the virus after virus adsorption, suggesting that gIV may be involved in penetration of the virus into cells (22). In addition, calves immunized with purified gIV show significant protection against the lethal challenge of BHV-1 and *Pasteurella haemolytica* (2). All these properties of gIV are similar to those reported for herpes simplex virus type 1 (HSV-1) gD (21) and pseudorabies virus (PRV) gp50 (43, 52).

Although the role of this glycoprotein in BHV-1 immune response is well documented, little is known regarding its role in the initiation of BHV-1 infection. Thus, it was of interest to identify the gIV gene sequence and express the glycoprotein in mammalian cells, in order to aid in elucidating its importance in initiating virus infection as well as to further define the specificity and protective role of the host cell-mediated immune response to this glycoprotein.

In this communication, we present the sequence of the gIV glycoprotein gene and describe the derivation of stable bovine cell lines expressing recombinant gIV, a cytotoxic glycoprotein. Preliminary studies showed that the recombinant glycoprotein is correctly processed and transported to the cell surface. Moreover, the expression of gIV in bovine cells induced cell fusion and polykaryon formation and interfered with BHV-1 replication.

* Corresponding author.

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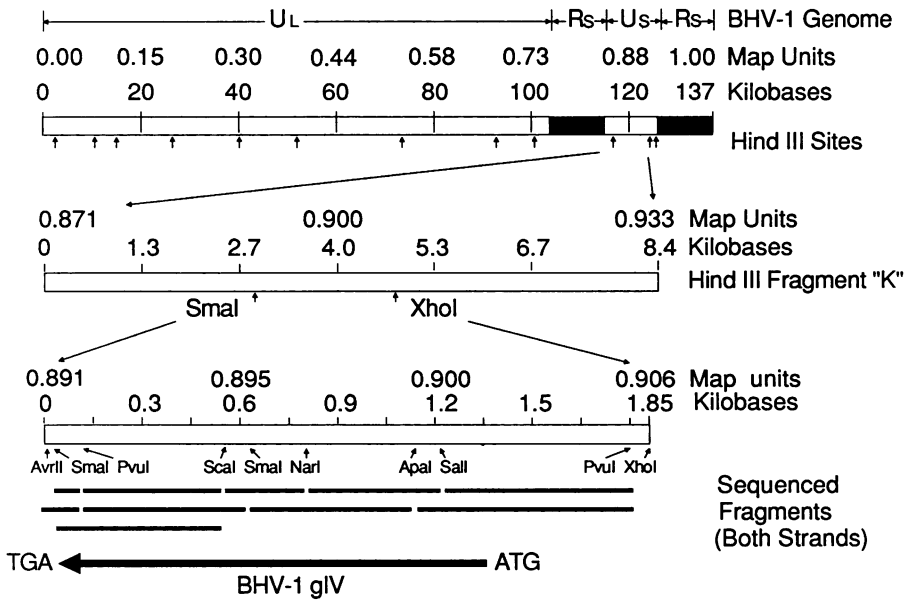

FIG. 1. Map location and sequencing strategy for the BHV-1 gIV gene. The 137-kb BHV-1 (Cooper strain) genome consists of a long unique region (U_L), a short region which contains a unique segment (U_S), and two repeat segments (R_S). The *Hin*dIII map of the prototype orientation of the genome is shown, as adapted from Mayfield et al. (34). The BHV-1 gIV gene was mapped to the *Hin*dIII K fragment between map units 0.871 and 0.933 and then to the 1.85-kb *Xho*I-*Sma*I partial fragment between map units 0.892 and 0.906 by Southern hybridization of the cloned genomic DNA fragments with radiolabeled PRV gp50 DNA probes (43). This fragment was then physically mapped by combination restriction endonuclease digestion. Relevant restriction endonuclease subfragments were subcloned and then sequenced by chemical method (33). Both strands of each subfragment were sequenced. The start codon (90 bp to the right of the *Sal*I site), stop codon (the first triplet following the *Sma*I site), and the direction of transcription of the gene (see Fig. 2) are indicated.

MATERIALS AND METHODS

Cells and virus. Madin-Darby bovine kidney (MDBK) cells were cultured in Eagle minimum essential medium (MEM) with 5% fetal bovine serum. Virus stocks of BHV-1 (P8-2 strain) were prepared in MDBK cells and quantitated by plaquing in microtiter plates with an antibody overlay, as previously described (45). The working stocks of HSV-1 and equine herpesvirus 1 were made in MDBK cells.

Reagents and media. Restriction enzymes, T4 DNA ligase, calf intestinal alkaline phosphatase, deoxynucleoside triphosphates, and protein A Sepharose were purchased from Pharmacia, Dorval, Quebec, Canada, and used as recommended by the manufacturer. Dexamethasone, xanthine, hypoxanthine, and other reagents for DNA manipulations, hybridizations, and protein analysis were purchased from Sigma Chemical Co., St. Louis, Mo., and used according to standard methods, as described by Maniatis et al. (32) except as indicated below. Cell culture media, fetal bovine serum (FBS), dialyzed FBS, G418, mycophenolic acid, the lipofectin transfection kit, and other cell culture reagents were obtained from GIBCO/Bethesda Research Laboratories, Burlington, Ontario, Canada. Antibodies, avidin-biotin immunoperoxidase staining kits, and other reagents for staining were purchased from Dimension Laboratories, Mississuaga, Ontario, Canada, and used as recommended by the manufacturers. Radioisotopically labeled compounds and reagents for fluorography were purchased from Amersham Corp., Oakville, Ontario, Canada. The panel of monoclonal antibodies used in this study has been previously described (22, 50).

Plasmid library construction. The construction and restriction mapping of pBR322-based plasmid library containing fragments of the BHV-1 Cooper strain genomes have been described in detail (34). The *Hin*dIII C fragment (not present in the above library) has now been cloned into the *Hin*dIII site of pBR322. All plasmids were grown in *Escherichia coli* MC1000 (9, 32).

Cloning methods, Southern hybridization, and DNA sequencing. Plasmid DNA was prepared and manipulated with DNA-modifying enzymes by using standard methods (32). Previously, we had mapped, subcloned, and sequenced the gp50 gene from PRV (unpublished data), which has a map location and sequence identical to those described earlier (43). An 1,130-base-pair (bp) *Pvu*II-*Sau*I subfragment from the *Bam*HI 7 PRV genomic clone (43) carrying the bulk of the coding sequence for the gp50 gene was labeled with ^{32}P by nick translation (32) and was used as a probe. The DNA fragments were separated, transferred to nitrocellulose, and hybridized with the probe as previously described (14).

BHV-1 DNA fragments to be sequenced were mapped (Fig. 1) as previously described (14) and sequenced by the chemical method (33). Both strands of each fragment were sequenced, and the identity of each nucleotide was thus verified at least four times (Fig. 1).

DNA sequence analysis. Sequence data were analyzed by using the IBI-Pustell sequence analysis software (International Biotechnologies Inc., New Haven, Conn.). Hydropathic analysis was conducted by using an algorithm of Kyte and Doolittle (28) contained in the software, with a nine-amino-acid window. Amino acid homology analysis was conducted by using a version of the FASTP program (30) contained within the above software, with a search parameter setting of gap = −6, ktup = 1 and use of a PAM 250 amino acid substitution matrix (10). Data base searches were conducted on NBRF protein identification resource (release 19.0) and GenBank (release 55.0) data bases.

Plasmid construction. Two types of mammalian cell expression vectors were used to express gIV in bovine cells: pRSDneo (15), which contains the Tn*5* neomycin resistance gene as a selectable marker and in which the gIV coding sequence is under the control of a strong constitutive enhancer-promoter of the Rous sarcoma virus (56), and pMSG (Pharmacia), which contains the *E. coli* xanthine-guanine phosphoribosyl transferase gene as a selectable marker and in which the gIV gene is under the control of the mouse mammary tumor virus long terminal repeat (25), a weak promoter that can be induced by the addition of dexamethasone to the cell culture media (29).

The complete coding sequence of BHV-1 gIV was excised from a subclone of plasmid pSD98 (34) as a 1.3-kilobase (kb) *Mae*I fragment (Fig. 2), treated with T4 DNA polymerase, and ligated to the *Bgl*II-cut and T4 DNA polymerase-treated pRSDneo, such that the *Bgl*II sites were retained. These manipulations removed the normal viral promoter upstream of the gIV gene and placed the start codon of the gIV gene approximately 105 bp downstream of the Rous sarcoma virus promoter (56) and approximately 75 bp downstream of transcriptional start site associated with the promoter. Approximately 58 bp lay between the gIV stop codon and the simian virus 40 polyadenylation signals of pRSDneo. The gIV gene was cloned into *Sma*I-digested pMSG as a 1.3-kb blunt end repaired *Bgl*II fragment from pRSDneo, thus placing the gIV start codon approximately 340 bp downstream of the mouse mammary tumor virus long terminal repeat promoter and approximately 310 bp downstream of the transcriptional start site (25). Approximately 25 bp existed between the BHV-1 gIV and the simian virus 40 sequences of pMSG.

Transfection and selection. Plasmid DNA was prepared for transfection by equilibrium banding in cesium chloride-ethidium bromide gradients and sterilized by ethanol precipitation (32). MDBK cells were transfected by the lipofection method (13). The cells, at about 50 to 60% confluency in 100-mm plates, were rinsed and incubated at 37°C in fresh Opti-MEM I containing 2% FBS for 3 to 4 h. Immediately prior to transfection, the cells were washed twice with Opti-MEM I and covered with Opti-MEM I medium without serum. The lipid-DNA complexes were made by mixing 20 μg of DNA and 30 μg of lipofection reagent, each diluted to 100 μl with water, and incubating the mixture for 15 min at room temperature before adding it to the cells. The DNA complexes were adsorbed at 37°C for 5 h in a 4% CO_2 atmosphere (16) before replacing the medium with MEM containing 10% FBS. After 16 to 18 h, the medium was again replaced with MEM containing 10% FBS and the cells were incubated further for 36 to 48 h. The cells were then passaged and grown either in MEM containing 10% FBS and 400 μg of G418 per ml for selection of neomycin-resistant cells (16) or in MEM containing 10% dialyzed FBS, 25 μg of mycophenolic acid, 250 μg of xanthine, and 15 μg of hypoxanthine per ml for selection of mycophenolic acid-resistant cells (35). The selection medium was changed every 3 to 5 days. G418-resistant colonies appeared in 28 to 33 days, while the mycophenolic acid-resistant colonies appeared in 10 to 15 days. The colonies derived from transfection with one type of plasmid were pooled and screened for expression of gIV by immunocytochemistry before cloning by limiting dilution.

Immunocytochemistry assay. To detect the distribution of the gIV antigen in the cells, an immunoperoxidase staining procedure described in detail previously (16) was used.

In vivo radiolabeling and immunoprecipitation. Cells were grown to approximately 80% confluency before being incubated in methionine-free MEM supplemented with 2% FBS at 37°C. After 6 h of incubation, [^{35}S]methionine was added to a final concentration of 50 μCi/ml, and the cells were further incubated for 16 h with or without 1 μM dexamethasone. BHV-1 infected MDBK cells were similarly radiolabeled, as previously described. The cell lysates were made and immunoprecipitated with gIV-specific monoclonal antibodies (22, 50), as described in detail elsewhere (16).

RESULTS

Map location and sequence of BHV-1 gIV gene. Southern hybridization of the BHV-1 genomic library with the radiolabeled PRV gp50 gene probe identified one 8.4-kb *Hin*dIII fragment from map unit 0.871 to 0.933 with a partially complementary DNA sequence (Fig. 1). Further subcloning, mapping, and hybridization studies of this region placed the homologous region in a 1,850-bp *Xho*I-*Sma*I partial fragment between map units 0.892 and 0.906 (Fig. 1). In addition, the same subfragment was found to bind a ^{32}P-end-labeled (32) 256-fold degenerate DNA probe with the following sequence: CCNCCNGCNTACCCNATGCC (where N is equal to each of the four nucleotides: G, C, A, and T). This degenerate nucleotide sequence was patterned from the amino-terminal amino acid sequence (residues 14 through 20) established for the monoclonal antibody affinity-purified mature BHV-1 gIV glycoprotein (Table 1).

Within the nucleotide sequence presented in Fig. 2, a single open reading frame large enough to code for gIV was identified. This open reading frame is 1,251 nucleotides long and starts from the first ATG in the sequence at nucleotide 85 and terminates at nucleotide 1335. Upstream from the ATG is the sequence TATATA which may function as the TATA box (7) of the endogenous BHV-1 gIV promoter. Our nucleotide sequence analysis did not extend further upstream to locate other transcriptional regulatory elements, such as potential CAAT boxes, or further downstream of the stop codon for the location of potential consensus polyadenylation signals. The nucleotide composition of the open reading frame was calculated to be 16.7% A, 13.5% T, 35.4% C, and 34.2% G.

A hydropathic analysis (Fig. 3) of the protein encoded by this open reading frame demonstrates the presence of two prominent hydrophobic peaks characteristic of a glycoprotein. The amino-terminal hydrophobic peak represents the signal sequence, since amino acids 4 through 18 have the length, relative hydrophobicity, and consensus cleavage site that are characteristic of signal sequences (Fig. 2 and 3) (51). The N-terminal amino acid analysis (11) of the affinity-purified mature gIV confirmed and identified residue 19 as the amino terminus of the mature molecule (Table 1). The

FIG. 2. DNA sequence and deduced amino acid sequence of BHV-1 gIV. The DNA sequence of the gene described in the legend to Fig. 1 is shown. TATA box and selected restriction endonuclease cleavage sites are indicated above the sequence and underlined. Below the DNA sequence is the deduced amino acid sequence of the open reading frame encoding BHV-1 gIV in standard three letter code. The putative signal sequence is underlined with a single line, and the putative transmembrane sequence is underlined with a double line. *, Potential N-linked glycosylation sites; +++, cysteine residues.

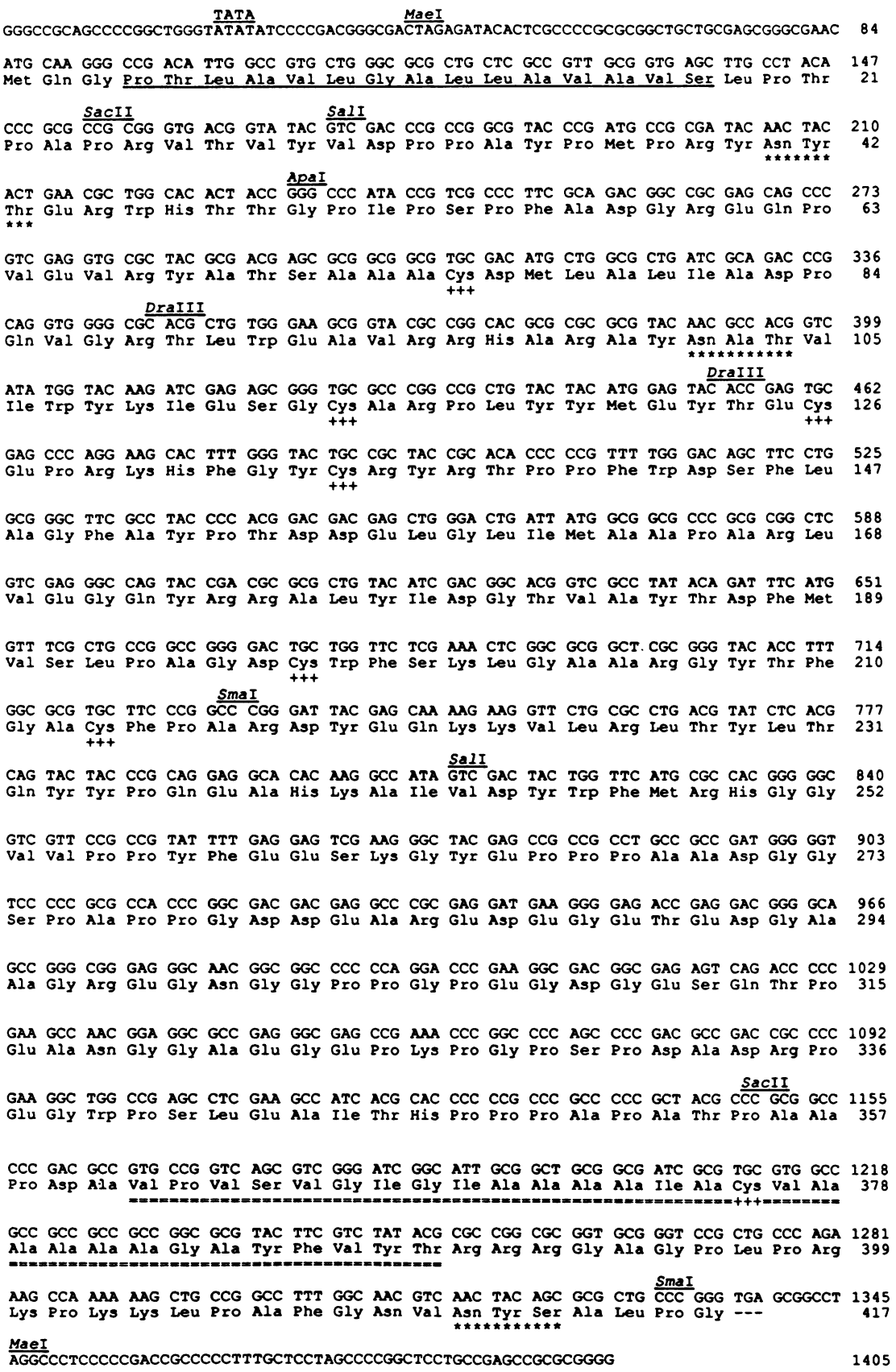
TATA
MaeI
SacII
SalI
ApaI
DraIII
DraIII
SmaI
SalI
SacII
SmaI
MaeI

TABLE 1. N-terminal amino acid sequence of affinity-purified BHV-1 gIV

Technique	Amino acid sequence[a]					
	1 (19)	7 (25)	13 (31)	19 (37)	25 (43)	30 (48)
Edman degradation	LPXPAPRVTVYVDPPAYPMPXYXYTEXWXT					
Gene sequence	LPTPAPRVTVYVDPPAYPMPRYNYTERWHT					

[a] The numbers outside the parentheses refer to residue numbers obtained from amino acid sequencing of mature gIV (15). The numbers in the parentheses refer to the residue numbers determined by gene sequencing (Fig. 2). X, Ambiguous amino acid.

second hydrophobic peak, occurring near the carboxy terminus of the glycoprotein between amino acids 361 and 389, has the position, length, and relative hydrophobicity that are characteristic of transmembrane anchor sequences (Fig. 2 and 3). There are seven cysteine residues in the predicted protein, six of which are present in the putative external domain and one which is present in the putative transmembrane domain (Fig. 2). There are three potential sites for the addition of N-linked oligosaccharides (27); two are present in the amino-terminal half of the predicted protein (Fig. 2) (both are associated with hydrophilic peaks [Fig. 3]), while the third is present in the cytoplasmic domain (Fig. 2).

Comparison of the BHV-1 gIV amino acid sequence with those of the homologous glycoproteins of other alphaherpesviruses. In order to estimate the conservation of BHV-1 gIV-related glycoproteins among alphaherpesviruses, we compared the amino acid sequence of gIV to those of the related glycoproteins of HSV-1 (54), HSV-1 (53), and PRV (43). We employed a sensitive amino acid homology program, FASTP (30), which incorporates conservative amino acid substitutions according to the PAM 250 matrix (10) into its search and alignment operations. The results are shown in Fig. 4. The amino-terminal half is relatively well conserved in the alphaherpesviruses. Of the 45 identical residues, 36 are located in the amino-terminal half of the gIV-like molecules of alphaherpesviruses. BHV-1 gIV is more homologous to gp50 of PRV (56%) than to HSV-2 gD (46%). Overall, the four proteins have an amino acid homology of 27%.

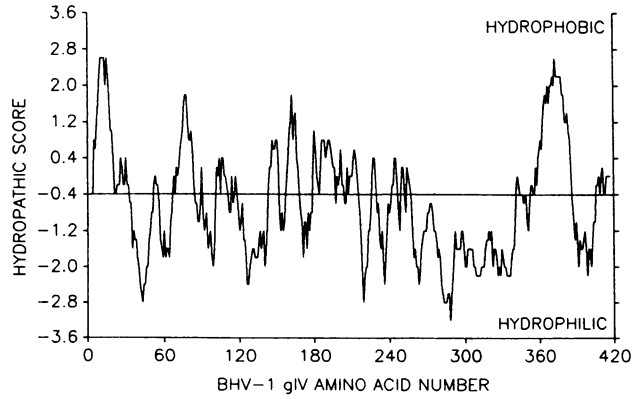

FIG. 3. Hydropathic analysis of the amino acid sequence of BHV-1 gIV. The BHV-1 gIV amino acid sequence was analyzed for hydrophobicity and hydrophilicity characteristics by using the Kyte-Doolittle algorithm (28) and a nine-amino-acid window. The vertical axis represents a relative hydropathic score in which positive values are hydrophobic and negative values are hydrophilic. The horizontal axis represents the amino acid number of the gIV sequence, as discussed in the legend to Fig. 2.

All but one of the cysteine residues are conserved and are readily aligned, without significant insertions and deletions, in all of the gIV-like molecules (Fig. 4). The non-aligned seventh cysteines present in BHV-1 gIV, HSV-1 gD, and PRV gp50 occur in the putative transmembrane domains of these proteins. There is remarkable variation in the occurrence of potential N-linked glycosylation sites. The BHV-1 gIV, HSV-1 gD, and HSV-2 gD have three sites (Fig. 2) (53, 54), while PRV gp50 has none (43). One potential N-linked glycosylation site (at position 102 of BHV-1 gIV) is conserved among HSV-1, HSV-2, and BHV-1 (Fig. 4). The BHV-1 gIV has an unusual stretch of amino acids (280 to 292) which is rich in negatively charged residues (Fig. 4), while the PRV gp50 has repeats of Arg-Pro from amino acids 267 to 278 (43) (Fig. 4).

Expression of BHV-1 gIV in bovine cells. The complete gIV open reading frame was cloned into two eucaryotic expression vectors, pMSG (inducible promoter) and pRSDneo (strong constitutive promoter), and MDBK cells were transfected with either plasmid. The cells surviving the selection for the covalently linked positive selection markers were pooled and examined for gIV expression by immunocytochemistry. Antigen was detected in cells transfected with both plasmids; however, the cells transfected with pMSG-gIV plasmid were positive only after the dexamethasone induction (1 μM) overnight. In contrast, the cells transfected with pRSDneogIV were positive throughout the growth, showed a punctate type of staining, and assumed abnormal shapes (Fig. 5G and H). Despite repeated attempts, no stable cell line could be established. However, stable cell lines could be established from the pMSGgIV-transfected cells, suggesting that the constitutive expression of gIV was toxic to cells. The clone PgIV-1 was chosen for further studies.

Preliminary experiments regarding the time course of appearance of gIV in the cloned PgIV-1 cell line revealed that gIV was detectable between 3 and 6 h after dexamethasone (1 μM) induction and reached maximum levels (0.9 μg per 10^6 cells) at approximately 16 h postinduction (data not shown). In subsequent experiments, the cells were induced with dexamethasone (1 μM) for 16 h.

The gIV-expressing cells were examined by immunocytochemistry for cellular distribution of the glycoprotein. Following induction, 100% of the cells produced gIV, which was predominantly localized in the perinuclear region, probably corresponding to the rough endoplasmic reticulum and golgi apparatus (Fig. 5F), and in the nuclear membrane, as rings surrounding the nuclei (Fig. 5C). In addition, gIV was also visible on the surface of the cells (Fig. 5B). In contrast, no expression was detectable in cells transfected with control plasmid (Fig. 5A and D). The expression of gIV in MDBK cells was associated with cell fusion, polykaryon formation, and nuclear fusion (Fig. 5E).

Antigenic structure of recombinant gIV. Radioimmunoprecipitation of BHV-1-infected MDBK cells revealed a major protein band of approximately 71,000 molecular weight (Fig. 6, lane A) which represents the mature form of gIV (48). A similar sized protein band could also be immunoprecipitated from the pMSGgIV-transfected PgIV-1 cell clone (Fig. 6, lane B). The level of expression in PgIV-1 cells was greatly enhanced if the clone was induced for 16 h with 1 μM dexamethasone (Fig. 6, lane C). The lower-molecular-weight bands observed in Fig. 6 are probably nonspecific bands, since they appear in all the lanes. To further confirm the authenticity of the protein produced in PgIV-1 cells, the

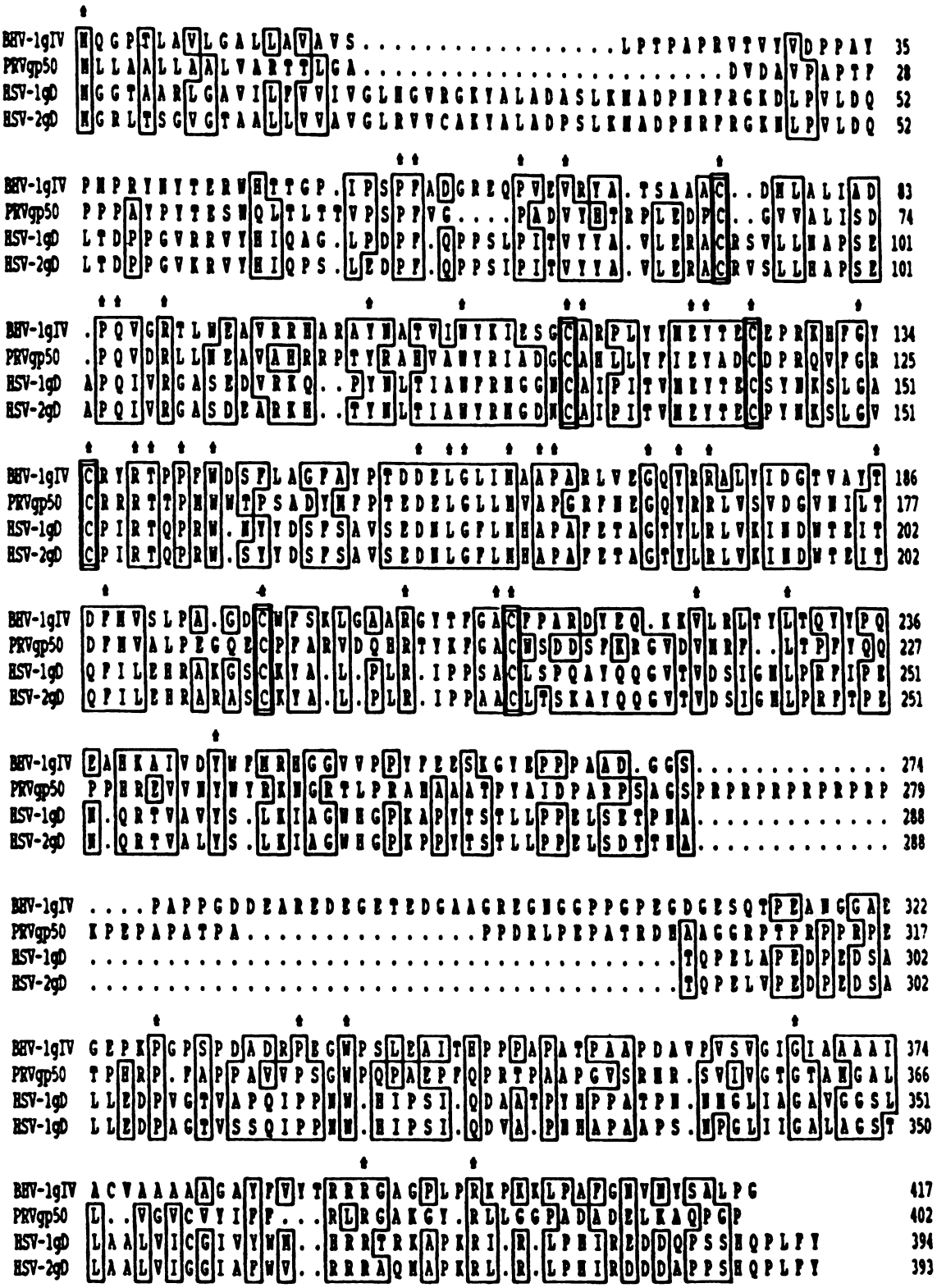

FIG. 4. Amino acid homology of alphaherpesvirus gIV-like glycoproteins. The amino acid sequences of the four alphaherpesvirus gIV-like glycoproteins reported at present were compared by using the FASTP program (30) (incorporating amino acid substitutions according to the PAM 250 matrix (10) and with an alignment setting of gap = −6 and ktup = 1). Sequence data and amino acid numbering were derived from the following references: PRV gp50 (42), HSV-1 gD (54), and HSV-2 gD (53). Dots indicate gaps introduced by the FASTP program and by inspection to maximize homology, particularly of residues conserved in all alphaherpesvirus gIV-like glycoprotein sequences. Aligned identical or conservatively substituted (PAM 250 matrix score of ≥1) amino acids present in at least three of the four alphaherpesvirus gIV-like glycoprotein sequences are indicated by surrounding boxes. Aligned residues which are identical in all four alphaherpesvirus gIV-like glycoproteins are indicated by an asterisk. Aligned identical cysteine residues are indicated by double boxes.

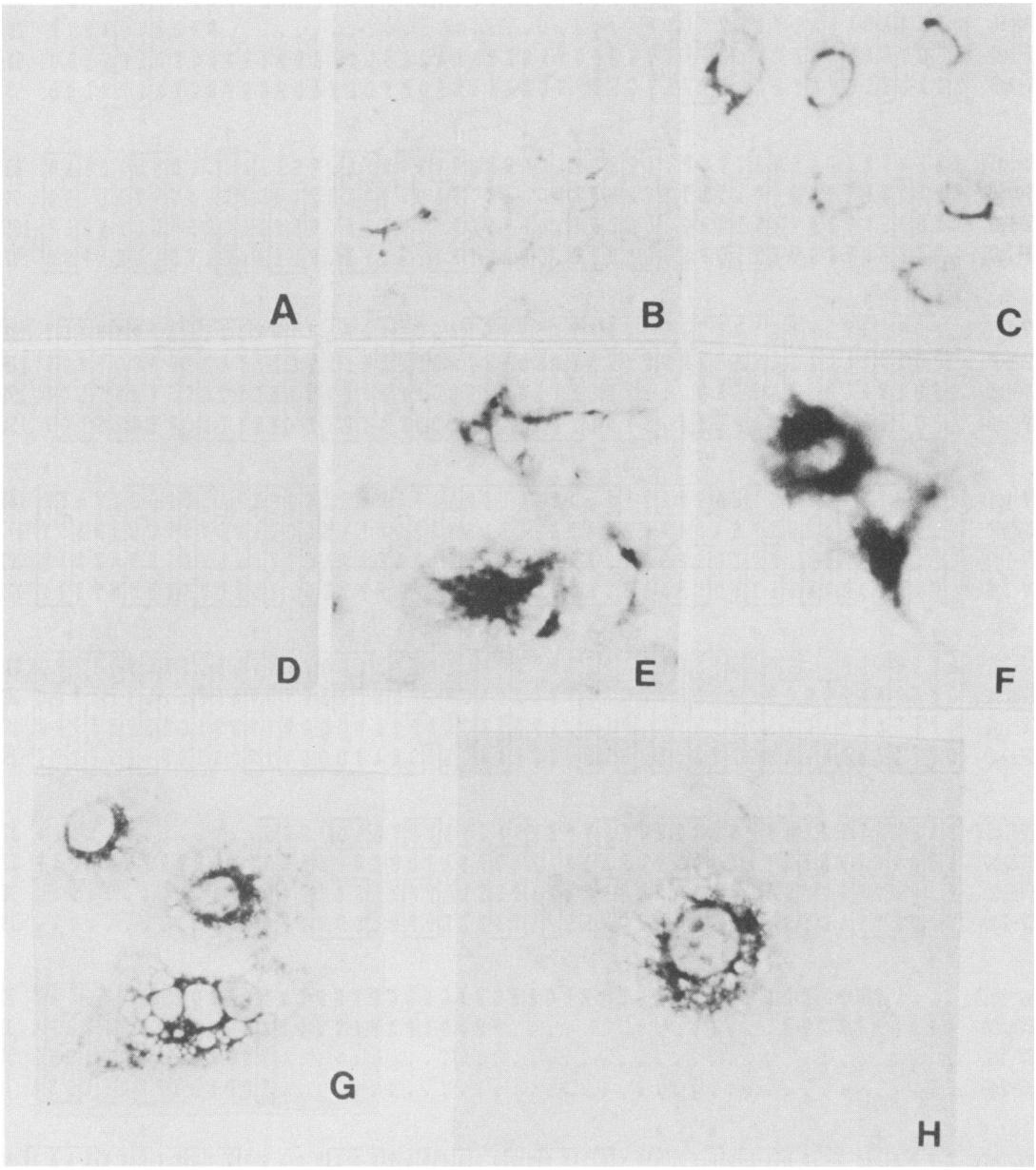

FIG. 5. Immunocytochemistry of transfected bovine cells expressing BHV-1 gIV. MDBK cells were transfected with pMSG (A and D), pMSGgIV (B, C, E, and F), or pRSDneogIV (G and H). Live unfixed cells (A and B) or methanol-fixed and permeabilized cells (C to H) were treated with monoclonal antibodies for gIV (C to H) or with polyclonal rabbit anti-gIV (A and B) and then subjected to an immunoperoxidase staining procedure (16). The cells transfected with pMSGgIV (B to F) were induced with 1 μM dexamethasone for 16 h before being stained. The staining of cells transfected with pRSDneo and pMSGgIV (uninduced) was similar to that of cells shown in panel A.

antigenic structure of the recombinant gIV was analyzed with a panel of gIV-specific monoclonal antibodies which have been mapped by competitive binding studies to different epitopes (22, 50). The reactivity patterns of seven monoclonal antibodies, five of which recognize conformation-dependent epitopes, were identical for authentic gIV produced in BHV-1-infected and transfected bovine cells when tested by immunocytochemistry and immunoprecipitation assays, suggesting that the conformation of the recombinant glycoprotein was similar to the gIV produced in the BHV-1-infected cells, at least in the vicinity of the epitopes probed by the monoclonal antibody panel (data not shown).

Effect of gIV expression on BHV-1 infection. Two assays were used to test the ability of BHV-1 to infect and replicate in MDBK cells expressing gIV; first, we compared the control with gIV-expressing PgIV-1 cells for their ability to support the formation of viral plaques. Confluent PgIV-1 and control cell monolayers were induced (for 16 h with 1 μM dexamethansone) or used uninduced in a BHV-1 plaque assay (45). The PgIV-1 cell clone showed a 50 (uninduced) and 95% (induced) reduction in the number of plaques, compared with controls (Table 2). There was no significant difference in the number of plaques between induced and uninduced control cells. This indicated that the PgIV-1 cells

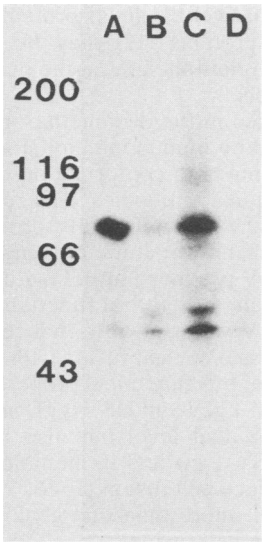

FIG. 6. Immunoprecipitation of gIV from BHV-1-infected and transfected MDBK cells. Lysates from ^{35}S-labeled BHV-1-infected cells (lane A), uninduced (lane B) or dexamethasone-induced (lane C) PgIV-1 cells, and pMSG-transfected cells (lane D) are shown. Radiolabeled cells were immunoprecipitated with a gIV-specific monoclonal antibody (9D6) (22), separated on sodium dodecyl sulfate-polyacrylamide gels, and fluorographed.

were relatively resistant to the cytopathic effects of BHV-1, compared with the control cells. The PgIV-1 cells showed similar resistance to the cytopathic effects of HSV-1 (Table 2). However, both control and PgIV-1 cells showed no significant difference in the number of plaques formed by equine herpesvirus 1 (data not shown).

Secondly, we compared the virus yields from the control and the PgIV-1 cell line with or without dexamethasone induction over a time course of infection. Cells were infected with 0.1 PFU per cell of BHV-1, and the infected cells and culture media were collected at different intervals postinfection and titrated on normal MDBK cells (45). The results are shown in Fig. 7. The total virus yield was lower in uninduced (2-fold) and induced (20-fold) cells than in the control cells at all time points, with maximum differences late in infection (Fig. 7A). Moreover, the amount of intracellular virus was greater in the PgIV-1 cell line (uninduced or induced) than in the control cells over a period of time (Fig. 7B).

TABLE 2. Plaque formation on transfected cells[a]

Cells	Dex induction	BHV-I		HSV-I	
		No. of plaques	% Reduction	No. of plaques	% Reduction
Control[b]	No	1×10^8	0	4×10^6	0
	Yes	9.5×10^7	5	3.7×10^6	7.5
PgIV-1	No	5×10^7	50	5.5×10^5	83
	Yes	5×10^6	95	2×10^5	95

[a] Serial dilutions of virus were plated on cell monolayers and adsorbed for 90 min. The inoculum was replaced with an antibody overlay (47). After plaques had developed (1 to 2 days), the cells were stained with crystal violet. The numbers shown are the averages of duplicate counts. Dex, Dexamethasone.

[b] MDBK cells transfected with pMSG plasmid.

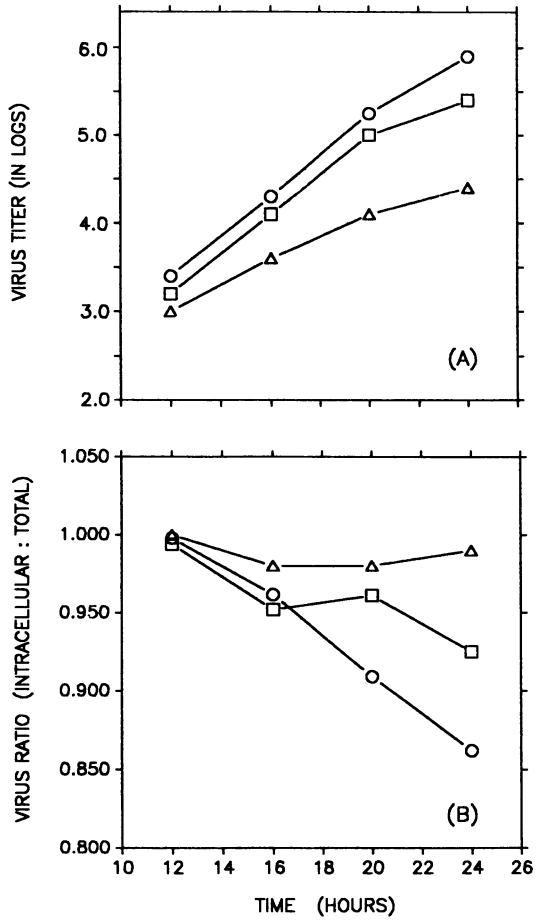

FIG. 7. Total virus yields (A) and ratio of intracellular virus to total virus (B) from BHV-1-infected bovine cell lines. The cell monolayers of pMSG-transfected cells (○), uninduced PgIV-1 cells (□), and dexamethasone-induced PgIV-1 cells (△) were infected with BHV-1 at a multiplicity of infection of 0.1. At various times after infection, the entire culture was collected or culture fluid and infected cells were collected separately. The samples were freeze-thawed and sonicated briefly before the virus yields were determined by titration on normal MDBK cells (45). Each data point represents the average of duplicate samples (from two separate experiments).

DISCUSSION

Our goal in this and previous studies (2, 14–17, 22) has been to precisely dissect the structure and function of different BHV-1 glycoproteins, with respect to both the immune response against BHV-1 and virus-host cell interactions, in an attempt to find a better way of protecting animals from infection. In this report, we describe the structure of BHV-1 gIV, the derivation of stable cell lines expressing recombinant glycoprotein, and their use in determining the possible function of BHV-1 gIV in virus-cell interactions.

The BHV-1 gIV gene maps in the short unique region of the viral genome between map units 0.892 to 0.902 (Fig. 1), which is approximately colinear with PRV gp50 (43) but inverted relative to the map locations of HSV-1 gD and HSV-2 gD in the prototype orientations of the genomes of these viruses (53, 54). Similar to those observed in the gI and gIII gene sequences of BHV-1 (14, 55), the nucleotide content of the gIV gene is extremely biased to the use of G

or C. The GC nucleotide content of the gene is 70%, which is similar to those of 75% of PRV gp50 (43) and 65% of HSV-1 gD (54). The third bases in the BHV-1 gIV triplets are 85% G or C, compared with 96% in PRV gp50 (43) and 85% in HSV-1 gD (54), suggesting that there has been some selective pressure in the evolution of BHV-1 that favors GC-rich genes, as hypothesized for PRV genes (43). The BHV-1 gIV gene codes for a 417-amino-acid protein (including signal sequence) which is the longest of the known gIV-like gene products in alphaherpesviruses (43, 53, 54) (Fig. 4).

The conservation of the first six cysteine residues among the gIV-like molecules suggests that these may be disulfide bond linked and are probably important in maintaining the proper structure and possibly the function of these glycoproteins. Moreover, the presence of these invariant cysteines in the more-conserved amino-terminal half of these molecules suggests that the amino-terminal half of the gIV-like molecules is perhaps important for the different immunological and biological functions. The results reported for HSV-1 gD (M. W. Ligas, V. Feenstra, G. H. Cohen, R. J. Eisenberg, and D. C. Johnson, Abstr. 14th Int. Herpesvirus Workshop, 1989, p. 109) and observations on deletion mutants of BHV-1 gIV (unpublished data) support this view.

An unusual feature in the amino acid sequence of BHV-1 gIV, not reported for BHV-1 gI and gIII amino acid sequences (14, 55), is the presence of a series of negatively charged residues from amino acids 280 to 292 (Fig. 4). As these amino acids fall in the major hydrophilic peak of the glycoprotein (Fig. 3) and at the carboxy-terminal half of the molecule, this region may be interacting with other molecules of the virus or host cell or both through ionic interactions. In contrast, the corresponding region of PRV gp50 protein (43) contains repeats of Arg-Pro (amino acids 267 through 278); it has been speculated that these repeats may produce rigid hydrophilic structures on the exterior surface of the protein.

The identity of the gIV open reading frame was verified by expression of recombinant gIV in bovine cells by using both strong constitutive and weak inducible promoter systems. The following observations on the expression of gIV in bovine cells strongly suggested that the constitutive expression of gIV glycoprotein is toxic to bovine cells. First, the transfected cells expressing gIV, under the control of the strong constitutive enhancer-promoter of Rous sarcoma virus, showed a punctate type of staining and assumed abnormal shape, and despite repeated attempts, no stable gIV-expressing clone could be established. Second, stable cell lines could only be established when cells transfected with pMSGgIV were maintained in an uninduced state. Third, no cell line could be established when dexamethasone-induced (1 μM for 16 h) pMSGgIV-transfected cells were cloned by flow cytometric analysis.

The observations regarding the distribution of recombinant gIV in the transfected cells are in agreement with those described previously for BHV-1-infected cells (40), except that the localization of gIV is concentrated more in the perinuclear region and less in the plasma membrane of the transfected cells than for BHV-1-infected cells. The most plausible explanation for this difference is that gIV is expressed in isolation in transfected cells, whereas in BHV-1-infected cells, a number of different viral proteins are synthesized. It is possible that one or more of these viral proteins may influence the efficient subcellular localization of the gIV. Viral or virus-induced proteins have been shown to affect the subcellular localization of different viral proteins (20, 26, 41). Alternatively, cellular proteins such as "molecular chaperone" proteins (12) may be inhibited in viral infection but may retain the gIV in the perinuclear region in the transfected cells.

The gIV produced in the dexamethasone-induced PgIV-1 cells was analyzed by immunoprecipitation and immunocytochemistry by using both conformation-dependent and -independent monoclonal antibodies. The results indicate that the recombinant gIV was efficiently processed and that it maintained an antigenic structure indistinguishable from that of the authentic gIV produced in BHV-1 infected cells, even in the absence of any other viral function.

Recombinant gIV was shown to induce cell fusion, polykaryon formation, and nuclear fusion in the transfected cells. The conservation of this functional property in HSV-1 gD (4) indicates that BHV-1 gIV and HSV-1 gD are homologous not only at the amino acid level but also in their biological function. Both HSV-1 gB and its homolog BHV-1 gI have been shown to induce cell fusion (1, 16, 17), suggesting that like HSV-1 (6, 20), more than one glycoprotein of BHV-1 may be involved in cell fusion.

Previously, expression of either BHV-1 gI or gIII in bovine cells has been shown not to provide any resistance to BHV-1 infection (5). In contrast, the PgIV-1 cell line expressing BHV-1 gIV showed relative resistance to BHV-1 infection, which varied with the amount of gIV produced in the cells. This resistance in gIV-expressing cells was not due to the dexamethasone, since treatment of the cells with this synthetic glucocorticoid did not significantly affect BHV-1 replication (38), as has been shown for HSV-1 and -2 (8, 37). Moreover, the resistance observed was specific for BHV-1 and HSV-1 infections among the viruses tested. The mechanism of this resistance is presently unknown. Different HSV-1 proteins have been shown to render cells resistant to HSV-1 infection (18, 24), but the mechanism by which these proteins render cells resistant remains unknown.

No direct evidence is available for the role of gIV in in BHV-1 infection except that gIV induces cell fusion (Fig. 5E), and monoclonal antibodies against domain I of gIV neutralize virus after adsorption to the cells (22). The amino acid homology with HSV-1 gD and PRV gp50 suggests that the BHV-1 gIV may also have similar functions.

HSV gD has been shown to be responsible for penetration of virus into the cells, since virus mutants lacking gD adsorb to the cells but fail to penetrate (23). Penetration occurs as a result of fusion of the virion envelope with the plasma membrane, and anti-gD monoclonal antibodies neutralize infectivity by blocking the fusion without inhibiting adsorption (21). This suggested that gD is required for some important event which allows the virus to cross the membrane. It seems likely that the gIV in the BHV-1 virion envelope interacts with unidentified receptors present on the surface of the cells and helps the virus to cross the membrane in a manner similar to that described for HSV-1 (23, 24). Consequently, the endogenously produced gIV in the PgIV-1 cells interacts with these receptor molecules either on the surface or inside the cells, thus blocking the entry of the virus to the cells by preventing the crucial interaction between the virion gIV and the cell receptor molecule.

Although the known function of HSV-1 gD (23, 24) suggests that the lower virus yields from gIV-producing cells are probably because of interference with virus entry, our observations on the ratios of intracellular to total virus produced in control and gIV-expressing cells also indicate that the endogenously produced gIV can interfere directly or indirectly with the release of the virus. A similar observation

has been previously reported for PRV gp50 (42). Whatever the mechanism of resistance, the similarity of the interference data (24, 42) with our results indicates that the BHV-1 gIV is homologous to HSV-1 gD and PRV gp50 both at the amino acid as well as at the functional level.

We are currently carrying out experiments to further investigate this interference mechanism in relation to the different steps during virus replication.

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