

# The Class II Membrane Glycoprotein G of Bovine Respiratory Syncytial Virus, Expressed from a Synthetic Open Reading Frame, Is Incorporated into Virions of Recombinant Bovine Herpesvirus 1

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**The bovine herpesvirus 1 (BHV-1) recombinants BHV-1/eG<sub>ori</sub> and BHV-1/eG<sub>syn</sub> were isolated after insertion of expression cassettes which contained either a genomic RNA-derived cDNA fragment (BHV-1/eG<sub>ori</sub>) or a modified, chemically synthesized open reading frame (ORF) (BHV-1/eG<sub>syn</sub>), which both encode the attachment glycoprotein G of bovine respiratory syncytial virus (BRSV), a class II membrane glycoprotein. Northern blot analyses and nuclear runoff transcription experiments indicated that transcripts encompassing the authentic BRSV G ORF were unstable in the nucleus of BHV-1/eG<sub>ori</sub>-infected cells. In contrast, high levels of BRSV G RNA were detected in BHV-1/eG<sub>syn</sub>-infected cells. Immunoblots showed that the BHV-1/eG<sub>syn</sub>-expressed BRSV G glycoprotein contains N- and O-linked carbohydrates and that it is incorporated into the membrane of infected cells and into the envelope of BHV-1/eG<sub>syn</sub> virions. The latter was also demonstrated by neutralization of BHV-1/eG<sub>syn</sub> infectivity by monoclonal antibodies or polyclonal anti-BRSV G antisera and complement. Our results show that expression of the BRSV G glycoprotein by BHV-1 was dependent on the modification of the BRSV G ORF and indicate that incorporation of class II membrane glycoproteins into BHV-1 virions does not necessarily require BHV-1-specific signals. This raises the possibility of targeting heterologous polypeptides to the viral envelope, which might enable the construction of BHV-1 recombinants with new biological properties and the development of improved BHV-1-based live and inactivated vector vaccines.**

Bovine herpesvirus 1 (BHV-1), a member of the subfamily *Alphaherpesvirinae* with a double-stranded DNA genome of approximately 136 kbp, causes infectious rhinotracheitis and infectious pustular vulvovaginitis as the most common clinical symptoms in cattle (27, 34, 39). Vaccination with attenuated live viruses or inactivated virions is widely used to control the disease and to reduce the concomitant financial losses. As with other large DNA viruses, interest exists in the use of recombinant BHV-1 as an improved live vaccine against BHV-1 infection (1, 21, 42) or as a vector for bi- or multivalent vaccines against BHV-1 and additional bovine pathogens (17, 18). To date, incorporation of heterologous genes into the genome of BHV-1 has concentrated mainly on the expression of the procaryotic *lacZ* gene to identify essential and nonessential genes or as a reporter gene for analytical studies (3, 8, 12, 15, 20, 29, 37, 38, 45). Recently, BHV-1 has been used to express biologically active bovine interleukins (21, 32) and glycoproteins of pseudorabiesvirus (19, 31). However, expression of RNA virus-encoded proteins by BHV-1 has not been published so far. Remarkably, expression of genes from cytoplasm-replicating viruses by other herpesviruses of mammals has only rarely been reported (5, 43).

Attempts to express the fusion glycoprotein F and the at-

tachment glycoprotein G of bovine respiratory syncytial virus (BRSV), a pneumovirus of the family *Paramyxoviridae* which is also prevalent worldwide and causes severe respiratory disease in young calves similar to the disease caused by human respiratory syncytial virus in children (4), were not successful (13, 33). Although the cDNA fragments encoding the respective glycoproteins were flanked by transcription control elements that are active in the genomic context of BHV-1 (21), no BRSV-specific transcripts were detected in cells infected with the BHV-1 recombinants (13) (see below). We therefore assumed that RNAs containing the authentic BRSV sequences were unstable in the nuclei of infected cells. To test this assumption, the BHV-1 glycoprotein D (gD) codon usage preferences (13, 40) were used to construct a modified open reading frame (ORF) encoding the BRSV G glycoprotein by chemically synthesized oligonucleotides.

In this report, we show that expression of the attachment glycoprotein G of BRSV (BRSV G glycoprotein), a type II membrane glycoprotein (36, 44), by BHV-1 was dependent on the modification of the base composition of the ORF encoding BRSV G glycoprotein, that virions produced by the recombinant contained the BRSV G glycoprotein, and that the presence of this protein in the viral envelope does not significantly interfere with the infectivity of BHV-1. Our findings suggest that RNA viruses which replicate in the cytoplasm can contain sequences or sequence elements that lead to instability of transcripts within the nucleus.

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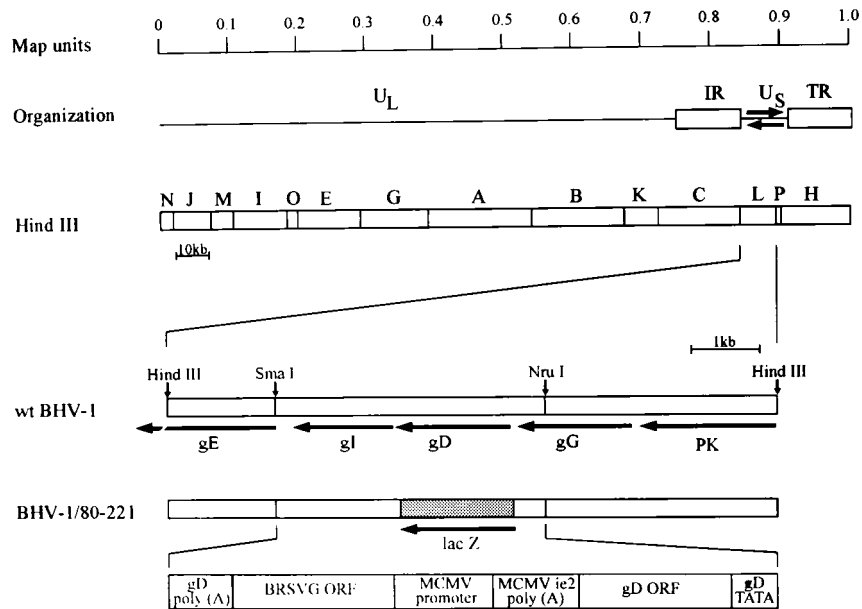


FIG. 1. Construction of BHV-1 recombinants. The schematic representation of the prototype orientation of the BHV-1 genome is shown above the *Hind*III restriction fragment map of BHV-1 strain Schönböken (7, 27). The wild-type *Hind*III L fragment is enlarged, and the location and direction of transcription of genes encoding the putative protein kinase (PK) and glycoproteins G (gG), D (gD), I (gI), and E (gE) are indicated by arrows (15, 25, 40, 45). Relevant restriction enzyme cleavage sites are marked. The respective *Hind*III fragment of the  $gD^{-} lacZ^{+}$  mutant BHV-1/80-221 is depicted below. The location and direction of transcription of the *lacZ* cassette (dotted area, not drawn to scale) that replaces the gD ORF is indicated by an arrow. Underneath, a diagram of the integration fragment contained in the recombination vectors is shown. The gD TATA and gD poly(A) segments indicate BHV-1 sequences representing the gD promoter and the gD polyadenylation signal, respectively, which provide homologous regions for recombination. In this study, transcription of the BRSV  $G_{ori}$  or  $G_{syn}$  ORF (BRSVG ORF) was directed by the MCMV *ie1* enhancer-promoter element (6) in transient-expression experiments or by the MCMV *e1* promoter (2) in recombinant BHV-1 infected cells. Transcription of the BHV-1 gD ORF which is followed by the MCMV *ie2* poly(A) signal (28) is under the control of the authentic gD promoter.

## RESULTS

**Rationale for synthesis of a modified BRSV G glycoprotein ORF.** To express the BRSV G glycoprotein by recombinant BHV-1, recombination plasmid pROME $G_{ori}$ , which contains a cDNA fragment encompassing the BRSV G glycoprotein ORF under control of the murine cytomegalovirus (MCMV) early 1 promoter (Fig. 1), was cotransfected with purified BHV-1/80-221 DNA into MDBK cells and the progeny virus was serially diluted and plated on MDBK cells. Since the ORF encoding the essential gD is replaced by a *lacZ* expression cassette in BHV-1/80-221 (8), only viruses which have acquired the gD ORF by replacement of the *lacZ* cassette by the insert of the recombination plasmid should be able to replicate on non-complementing cells (21). Virus from plaques that did not stain blue under a Bluo-Gal-containing agarose overlay were again subjected to titer determination on MDBK cells, and isolates which produced only "white" plaques were plaque purified once more and characterized further (see Fig. 4). Analysis of MDBK cells infected with the resulting recombinant BHV-1/ $eG_{ori}$  gave no indication of BRSV G glycoprotein expression by indirect immunofluorescence (see Fig. 7), and no stable transcripts were detected within cytoplasmic RNA (data not shown) or whole-cell RNA (see Fig. 5). BRSV G glycoprotein expression was also not detected in transient-expression experiments with plasmid pROMie $G_{ori}$  (Fig. 1) in which the BRSV G glycoprotein ORF is under the control of the MCMV immediate-early 1 enhancer/promoter element (data not shown). In vitro translation of mRNA, transcribed in vitro from pSPG $_{ori}$ , resulted in synthesis of a polypeptide whose apparent molecular mass of 31 kDa (Fig. 2, lane 2) was in good agreement with the calculated size of 29 kDa (24). This result indicated that mRNA from the BRSV  $G_{ori}$  ORF contained

within pROME $G_{ori}$  has the potential to direct protein synthesis, and we assumed that the failure to detect BRSV G glycoprotein-specific gene products was due to instability of BRSV G glycoprotein transcripts in the nuclei of BHV-1/ $eG_{ori}$ -infected cells. Therefore, a modified ORF encoding the BRSV G glycoprotein (Fig. 3) was chemically synthesized, isolated from plasmid pBRSVG $_{syn}$ , and inserted into pROME and pSP73. The resulting plasmids were named pROME $G_{syn}$  and pSPG $_{syn}$ , respectively, and RNA transcribed from pSPG $_{syn}$  directed the synthesis of a 31-kDa protein in a rabbit reticulocyte lysate (Fig. 2, lane 3) that comigrated with the polypeptide synthesized in vitro from the  $G_{ori}$  ORF, supporting the conclusion

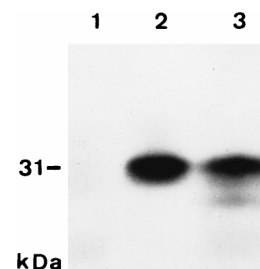


FIG. 2. Cell-free translation of cRNAs encompassing the BRSV  $G_{ori}$  or  $G_{syn}$  ORF.  $^{35}$ S-labeled proteins translated in vitro from RNA transcribed in vitro from plasmids pSPG $_{ori}$  (lane 2) or pSPG $_{syn}$  (lane 3) were size separated in SDS-10% polyacrylamide gels and visualized by fluorography. In lane 1, proteins synthesized from RNA transcribed from pSPG $_{ori}$  antisense to the BRSV G glycoprotein ORF were separated. The apparent molecular mass of the translation products, indicated in kilodaltons on the left, was calculated from the migration of  $^{14}$ C-methylated protein molecular mass standards run on the same gel.

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1  ATG TCC AAC CAT ACC CAT CAT CTT AAA TTC AAG ACA TTA AAG AGG GCT TGG AAA GCC TCA
1  ATG AGC AAC CAC ACG CAC CAC CTG AAG TTC AAG ACG CTG AAG CGC GCG TGG AAG GCT AGC
1  M S N H T H H L K F K T L K R A W K A S
61  AAA TAC TTT ATA GTA GGA TTA TCA TGT TTA TAT AAG TTC AAT TTA AAA TCC CTT GTC CAA
21  AAG TAC TTC ATC GTC GGC CTG AGC TGC CTG TAC AAG TTC AAC CTG AAG AGC CTG GTC CAG
21  K Y F I V G L S C L Y K F N L K S L V Q
121  ACG GCT TTG TCC ACC CTA GCA ATG ATA ACC TTG ACA TCA CTC GTC ATC ACA GCC ATT ATT
41  ACG GCG CTG AGC ACG CTC GCG ATG ATC ACG CTG ACG AGC CTG GTC ATC ACG ACG ATC ATT
41  T A L S T L A M I T L T S L V I T A I I
181  TAC ATT AGT GTG GGA AAT GCT AAA GCC AAG CCC ACA TCC AAA CCA ACC ATC CAA CAA ACA
61  TAC ATC TCC GTG GGC AAC GCG AAG GCG AAG CCG ACG TCC AAG CCG ACG ATC CAG CAG AGC
61  Y I S V G N A K A K P T S K P T I Q Q T
241  CAA CAG CCC CAA AAC CAT ACC TCA CCA TTT TTC ACA GAG CAC AAC TAC AAA TCA ACT CAC
81  CAG CAG CCG CAG AAC CAC ACG ACG CCG TTC TTC ACG GAG CAC AAC TAC AAA TCA ACT CAC
81  Q Q P Q N H T S P F F T E H N Y K S T H
301  ACA TCA ATT CAA AGC ACC ACA CTG TCC CAA CTA CTA AAC ATA GAC ACT ACT AGA GGA ATT
101  ACG AGC ATC CAG AGC ACG ACC TTA AGC CAG CTG CTG AAG ATC GAC ACT ACT AGA GGA ATT
101  T S I Q S T T L S Q L L N I D T T R G I
361  ACA TAT GGT CAC TCA ACC AAC GAA ACC CAA AAC AGA AAA ATC AAA GGC CAA TCC ACT CTA
121  ACG TAT GGC CAC AGC ACG AAC GAG ACG CAG AAC CGC AAG ATC AAA GGC CAG AGC AGC CTG
121  T Y G H S T N E T Q N R K I K G Q S T T
421  CCC GCC ACC AGA AAA CCA CCA ATC AAT CCA TCG GGA AGC ATC CCC CCT GAA AAC CAT CAA
141  CCG GCG ACG CGC AAG CCG CCG ATC AAC CCG AGC GGA TCC ATA CCG CCG GAG AAC CAC CAG
141  P A T R K P P I N P S G S I P P E N H Q
481  GAC CAC AAC AAC TTC CAA ACA CTC CCC TAT GTG CCT TGC AGT ACA TGT GAA GGT AAT CTT
161  GAC CAC AAC AAC TTC CAG ACG CTG CCG TAC GTG CCT TGC AGC ACG TGC GAG GGT AAT CTT
161  D H N N F Q T L P Y V P C S T E G N L
541  GCT TGC TTA TCA CTC TGC CAT ATT GAG ACG GAG AGA GCA CCA AGC AGA GCC CCT ACA ATC
181  GCG TGC CTG AGC CTG TGC CAC ATC GAG ACG GAG CGC GCG CCG AGC CCG GCC CCG ACG ATC
181  A C L S L C H I E T E R A P S R A P T I
601  ACC CTC AAA AAG ACT CCA AAA CCC AAA ACC ACT AAA AAG CCA ACC AAG ACA ACA ATC CAC
201  ACG CTG AAG AAG ACG CCG AAG CCG AAG ACG ACG AAG AAG CCG ACG AAG ACG ACG ATC CAC
201  T L K K T P K P K T T K K P T K T T I H
661  CAC AGA ACC AGC CCT GAA ACC AAA CTG CAA CCT AAA AAC AAC ACA GCA ACT CCA CAA CAA
221  CAC CGC ACT AGT CCG GAG ACG AAG CTC CAG CCG AAG AAC AAC ACG GCG ACG CCG CAG CAA
221  H R T S P E T K L Q P K N N T A T P Q Q
721  GGC ATC CTC TCT TCA ACA GAA CAT CAC ACA AAT CAA TCA ACT ACA CAG ATC TAG
GGC ATC CTG AGC AGC ACG GAG CAC CAC ACG AAC CAG AGC ACG ACG CAG ATC TGA
241  G I L S S T E H H T N Q S T T Q I *
    
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FIG. 3. Comparison of the nucleotide sequences of the BRSV G glycoprotein cDNA ORF (upper sequence) and the G<sub>syn</sub> ORF (lower sequence). Exchanged nucleotides are in boldface type, and the deduced BRSV G glycoprotein amino acid sequence is shown in the single-letter code.

that the 31-kDa protein represents the primary translation product encoded by the BRSV G glycoprotein ORF.

**Integration of the BRSV-G<sub>syn</sub> ORF into the genome of BHV-1/80-221.** Recombination plasmid pROME<sub>G<sub>syn</sub></sub> was cotransfected with purified BHV-1/80-221 DNA into MDBK cells, and the recombinant virus, BHV-1/eG<sub>syn</sub>, was isolated as described above for BHV-1/eG<sub>ori</sub>. To demonstrate integration of the expression cassettes into the genomes of the respective viruses, MDBK cells were infected with BHV-1/eG<sub>ori</sub>, BHV-1/eG<sub>syn</sub>, and BHV-1/80-221, phenotypically complemented by propagation on gD-expressing cells (8). Whole-cell DNA was prepared 20 h postinfection (p.i.), cleaved with *Hind*III, transferred to nitrocellulose filters after size separation in 0.6% agarose gels, and hybridized to G<sub>syn</sub><sup>-</sup>, G<sub>ori</sub><sup>+</sup>, and *lacZ*-specific <sup>32</sup>P-labeled DNA fragments. The sizes of the fragments that hybridized to the respective probes were as expected (data not shown). We concluded that BHV-1/eG<sub>ori</sub> and BHV-1/eG<sub>syn</sub> were generated by homologous recombination as envisaged.

**Transcription of the BRSV G<sub>ori</sub> and BRSV G<sub>syn</sub> ORFs in recombinant BHV-1-infected cells.** To test for transcription from the recombinant BRSV G genes, MDBK cells were infected with BHV-1/eG<sub>syn</sub> (Fig. 4, lanes 1, 3, 5, and 7) and BHV-1/eG<sub>ori</sub> (lanes 2, 4, 6, and 8). Cytoplasmic RNA was isolated at 6 h p.i., size separated by agarose gel electrophoresis, and transferred to nitrocellulose filters. A <sup>32</sup>P-labeled DNA probe representing the BRSV G<sub>syn</sub> ORF detected an RNA of 1.3 kb after infection with BHV-1/eG<sub>syn</sub> (lane 1) and, after extended exposure, a transcript of 1.8 kb (lane 5) whose synthesis is initiated approximately 500 bp upstream from the

MCMV e1 promoter (13). Even after a longer exposure, G<sub>ori</sub> ORF-specific transcripts of the expected size were not detected (lanes 2 and 6). The faint smear in lane 6 might indicate the presence of fragmented G<sub>ori</sub> RNA. Hybridization of the filters with <sup>32</sup>P-labeled DNA encompassing the BHV-1 gD ORF showed that comparable amounts of gD mRNA were detected in BHV-1/eG<sub>syn</sub>-infected cells (lanes 3 and 7) and BHV-1/eG<sub>ori</sub>-infected cells (lanes 4 and 8), demonstrating that the

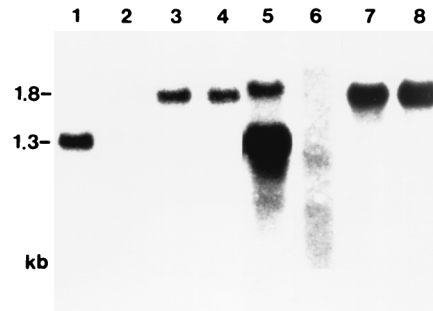


FIG. 4. Identification of transcripts encompassing the G<sub>syn</sub> ORF. Whole-cell RNA from cells infected with BHV-1/eG<sub>syn</sub> (lanes 1, 3, 5, and 7) and BHV-1/eG<sub>ori</sub> (lanes 2, 4, 6, and 8) was prepared at 6 h p.i., and 5-μg samples were transferred to nitrocellulose after 1% agarose gel electrophoresis. The filters were hybridized to <sup>32</sup>P-labeled DNA from the BRSV-G<sub>syn</sub> ORF (lanes 1 and 5), the BRSV-G<sub>ori</sub> ORF (lanes 2 and 6), and the BHV-1 gD ORF (lanes 3, 4, 7, and 8). Bound radioactivity was visualized by autoradiography. Lanes 5 to 8 are longer exposures of lanes 1 to 4. Transcript sizes are indicated in kilobases.

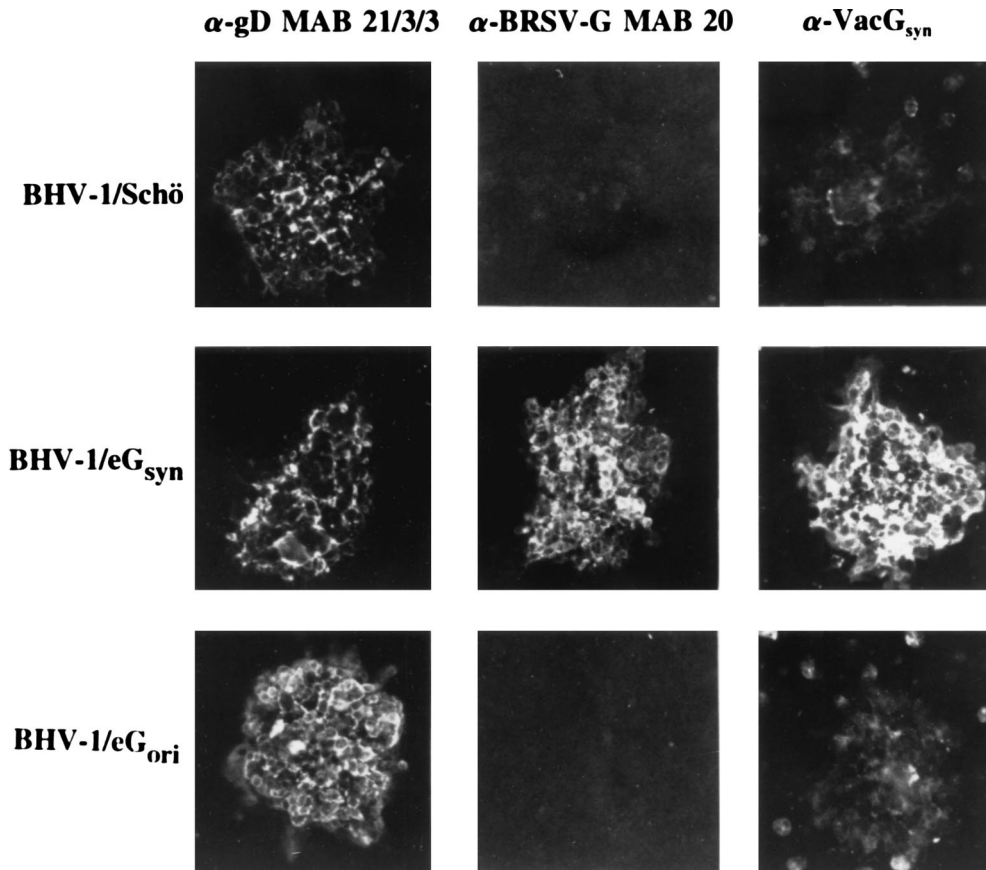


FIG. 5. BHV-1-encoded BRSV G glycoprotein is expressed on the cell surface. MDCK cell cultures were infected with approximately 100 PFU of BHV-1/Schö, BHV-1/eG<sub>syn</sub>, and BHV-1/eG<sub>ori</sub>, fixed after the development of plaques, and used for indirect immunofluorescence with BHV-1 gD-specific MAb 21/3/3, BRSV G glycoprotein-specific MAb 20, and the anti-VacG<sub>syn</sub> serum. Bound antibodies were visualized by staining with DTAF-conjugated goat anti-species immunoglobulin G.

absence of stable transcripts from the BRSV G<sub>ori</sub> ORF was not due to degradation of the RNA from BHV-1/eG<sub>ori</sub>-infected cells. Substantially the same results were obtained with RNAs isolated 16 h after infection with BHV-1/eG<sub>syn</sub> and BHV-1/eG<sub>ori</sub> in the absence or presence of phosphonoacetic acid (data not shown).

To support the assumption that transcripts from the BRSV G<sub>ori</sub> ORF are unstable in BHV-1/eG<sub>ori</sub>-infected cells, elongation of initiated transcripts in BHV-1/eG<sub>ori</sub>- and BHV-1/eG<sub>syn</sub>-infected MDCK cells was analyzed by nuclear runoff transcription assays. <sup>32</sup>P-labeled RNA, synthesized in nuclei isolated at 6 h p.i., was hybridized to DNA from the plasmid vector pSP73 or from plasmids pSPG<sub>ori</sub>, pSPG<sub>syn</sub>, or pSPGD dotted onto nitrocellulose membranes. Elongation of RNA from both BRSV G ORFs was detected (data not shown). The ratio of bound radioactivity between the respective BRSV G ORFs and the corresponding BHV-1 gD ORFs was 1.7 with RNA from BHV-1/eG<sub>ori</sub>-infected cells and 2.1 with RNA from BHV-1/eG<sub>syn</sub>-infected cells, demonstrating that the G<sub>ori</sub> ORF was transcribed in BHV-1/eG<sub>ori</sub>-infected cells. These results are in accordance with the conclusion that BHV-1-expressed BRSV G<sub>ori</sub> RNAs are unstable in the nuclei of infected cells.

**Identification and characterization of BHV-1-expressed BRSV G glycoprotein.** To test for the expression of the BRSV G glycoprotein in BHV-1/eG<sub>syn</sub>-infected cells, BRSV G glycoprotein-specific MAb 20 and a polyclonal antiserum (anti-VacG<sub>syn</sub>), raised in rabbits after infection with G<sub>syn</sub>-expressing recombinant vaccinia virus, were used to stain wild-type

BHV-1 strain Schönböken (BHV-1/Schö)-, BHV-1/eG<sub>syn</sub>- or BHV-1/eG<sub>ori</sub>-induced plaques by indirect immunofluorescence. The cultures were fixed 2 days after infection and incubated with the BHV-1 gD-specific MAb 21/3/3, MAb 20, or the anti-VacG<sub>syn</sub> serum. Antibodies bound on the cell surface were visualized by using DTAF-conjugated anti-species immunoglobulin G and fluorescence microscopy. As shown in Fig. 5, MAb 20 and the anti-VacG<sub>syn</sub> serum bound only to cells in plaques generated by BHV-1/eG<sub>syn</sub> whereas MAb 21/3/3 reacted with gD on the surface of cells infected with BHV-1/Schö, BHV-1/eG<sub>syn</sub>, and BHV-1/eG<sub>ori</sub>. The conclusion that the BRSV G<sub>syn</sub> ORF-encoded protein is expressed on the surface of BHV-1/eG<sub>syn</sub>-infected cells was also demonstrated by flow cytometry with living cells and the same antibodies as above (data not shown).

To further characterize the BRSV G<sub>syn</sub> ORF-encoded gene product, proteins from infected cells (Fig. 6, lanes 1 to 4), purified virions (lanes 5 to 7), and the cell culture medium from BHV-1/eG<sub>syn</sub>-infected cells (lane 8) were analyzed by immunoblotting because MAb 20 and the anti-VacG<sub>syn</sub> serum did not work for immunoprecipitation. MAb 20 did not specifically bind to proteins from cells infected with BHV-1/Schö (Fig. 6, lane 1) and BHV-1/eG<sub>ori</sub> (lane 2) or to proteins from purified BHV-1/Schö and BHV-1/eG<sub>ori</sub> virions (lanes 5 and 6) but reacted strongly with proteins with apparent molecular masses of 38 and 43 kDa from BHV-1/eG<sub>syn</sub>-infected cells (lane 3). In addition, several weaker-staining bands ranging in size from 70 to about 100 kDa were detected. To elucidate

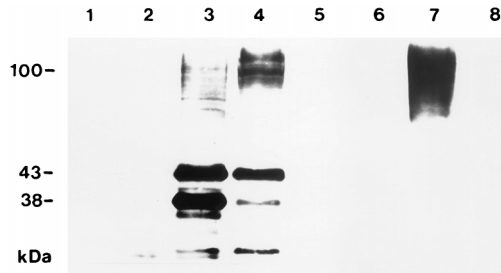


FIG. 6. Identification of the BHV-1/eG<sub>syn</sub>-expressed BRSV G glycoprotein. MDBK cells were infected with BHV-1/Schö (lanes 1 and 5), BHV-1/eG<sub>ori</sub> (lanes 2 and 6), and BHV-1/eG<sub>syn</sub> (lanes 3, 4, 7, and 8). Proteins from infected cells, harvested at 10 h p.i. (lanes 1 to 4), from purified virions (lanes 5 to 7), and from cell culture medium (lane 8), were analyzed by immunoblotting with BRSV G glycoprotein-specific MAb 20. Proteins shown in lane 4 were from cells incubated with cycloheximide (100 µg/ml) for 2 h before lysis. Apparent molecular masses are indicated in kilodaltons.

possible precursor-product relationships among the MAb 20-reactive polypeptides, BHV-1/eG<sub>syn</sub>-infected cells were incubated for 2 h with cycloheximide (final concentration, 100 µg per ml) before lysis to inhibit de novo protein synthesis. This treatment resulted in a considerable decrease in the signal at 43 kDa and a slight reduction in the band intensity of the signal at 38 kDa, whereas the bands corresponding to larger proteins increased in size and abundance (Fig. 6, lane 4). Prolongation of the cycloheximide incubation time did not significantly alter this pattern but resulted in a decrease in the intensities of all bands (data not shown). Among proteins from purified virus particles, MAb 20 bound only to polypeptides from BHV-1/eG<sub>syn</sub> virions which migrated as a diffuse band with an apparent molecular mass between 70 and 120 kDa (lane 7). No MAb 20-reactive proteins were detected in the medium of BHV-1/eG<sub>syn</sub>-infected cells (lane 8). Identical results were obtained with the anti-VacG<sub>syn</sub> serum and BRSV G glycoprotein-specific MAb 57 (9). The latter immunoprecipitated the large molecules but not the 38- and 43-kDa proteins from cells infected with BHV-1/eG<sub>syn</sub> in the presence of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine. From these results, we conclude that the 38- and 43-kDa proteins represent BRSV G glycoprotein precursor molecules which are subsequently modified to the large form, which is also associated with BHV-1/eG<sub>syn</sub> virions.

The carbohydrate composition of virus particle-associated BRSV G glycoprotein was analyzed by incubation of purified BHV-1/eG<sub>syn</sub> virions with glycosidase F, neuraminidase, and neuraminidase together with *O*-glycosidase followed by immunoblotting after SDS-PAGE with MAb 20 (Fig. 7a) or a polyclonal rabbit serum raised against purified BHV-1 gD (Fig. 7b) to monitor the reactions. According to the enzymes used, the electrophoretic mobilities of virion-associated gD increased as expected (41), and no differences were detected in comparison to the reaction products of immunoprecipitated virion gD (Fig. 7b and c), indicating the presence of sufficient enzymatic activity for complete deglycosylation within the respective probes. The BRSV G glycoprotein molecules appeared unaffected after incubation with neuraminidase (Fig. 7a, lane 2) and exhibited an increased electrophoretic mobility following digestion with neuraminidase and *O*-glycosidase (lane 3) or *N*-glycosidase F (lane 4), which demonstrates the presence of *O*- and *N*-linked carbohydrates in the virion-associated BRSV G glycoprotein. However, digestion products obtained after incubation of the virion proteins with neuraminidase and *O*-glycosidase migrated slower and were more diffuse, as expected for the only *N*-glycosylated form. Whether this discrep-

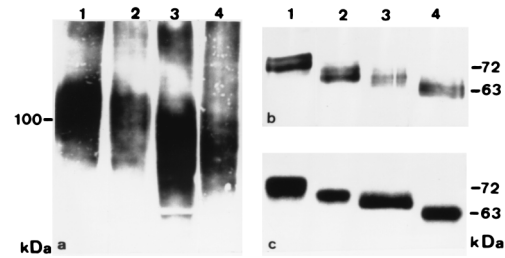


FIG. 7. Evidence that BHV-1-expressed G glycoprotein contains *N*- and *O*-linked sugars. (a and b) Purified BHV-1/eG<sub>syn</sub> virions were resuspended in PBS plus 0.5% NP-40 and digested overnight at 37°C with neuraminidase (lanes 2), neuraminidase and *O*-glycosidase (lanes 3), *N*-glycosidase F (lanes 4), and without enzyme (lanes 1). Digestion products were analyzed by immunoblotting with G glycoprotein-specific MAb 20 (a) or a polyclonal, BHV-1 gD-specific rabbit antiserum (b). (c) Purified, [<sup>35</sup>S]methionine-labeled BHV-1/Schö virions were lysed and incubated with gD-specific MAb 21/3/3. Immunoprecipitated proteins were incubated as described above, separated on SDS-10% polyacrylamide gels, and visualized by fluorography. The apparent molecular masses, indicated in kilodaltons on the left, were calculated from the migration of prestained (a and b) or <sup>14</sup>C-methylated protein molecular mass standards (c) run on the respective gel.

ancy is due to additional modifications, the presence of *O*-glycosidase-resistant *O*-linked carbohydrates (22), or incomplete removal of *O*-linked glycans due to specifics of the BRSV G glycoprotein has to be analyzed.

**Virus neutralization assays.** The immunoblot analysis in Fig. 6 indicated that BHV-1/eG<sub>syn</sub> virus particles contain the BRSV G glycoprotein and therefore should be susceptible to inactivation by BRSV G glycoprotein-specific antibodies. BHV-1/eG<sub>ori</sub> and BHV-1/eG<sub>syn</sub> virions were tested for complement-dependent and -independent neutralization by MAb 57, the anti-VacG<sub>syn</sub> serum, and the anti-BRSV serum 2106 (9). With complement, MAb 57, which had no effect on the infectivity of BHV-1/eG<sub>ori</sub> (data not shown), reduced the number of BHV-1/eG<sub>syn</sub>-induced plaques to 52% at a final dilution of 1:25. The anti-VacG<sub>syn</sub> serum specifically neutralized BHV-1/eG<sub>syn</sub> in the presence of complement to 50% neutralization at a dilution of about 1:1,000 (Fig. 8). No activity was detectable without complement (data not shown). The BHV-1/eG<sub>syn</sub>-neutralizing activity of anti-BRSV serum 2106 was weak in the absence of complement and more pronounced after the addition of complement. Both polyclonal sera had no significant effect on the infectivity of BHV-1/eG<sub>ori</sub> (Fig. 8) and BHV-1/Schö (data not shown).

## DISCUSSION

Analysis of the expression from the authentic BRSV G glycoprotein ORF after infection of cells with BHV-1/eG<sub>ori</sub> indicated that the transcripts are unstable in the nucleus. We assumed that this was due to the nucleotide sequence of the RNA, which might contain signals that do not interfere with the stability of the RNA in the cytoplasm of cells infected with BRSV or recombinant vaccinia virus expressing the BRSV G glycoprotein (24) but result in degradation of the transcripts in the nucleus. Therefore, the complete BRSV G glycoprotein ORF was reconstructed by using synthetic oligonucleotides. The codon usage of the modified ORF was adjusted to that of BHV-1 gD, resulting in an increase of the G+C content from 42 to 62%, and the introduction of putative splice donor signals (30) was avoided. The synthetic ORF was inserted into the genome of BHV-1 under the control of the MCMV e1 promoter (2, 21), and analysis of the gene products in BHV-1/eG<sub>syn</sub>-infected cells demonstrated transcription of mRNA,

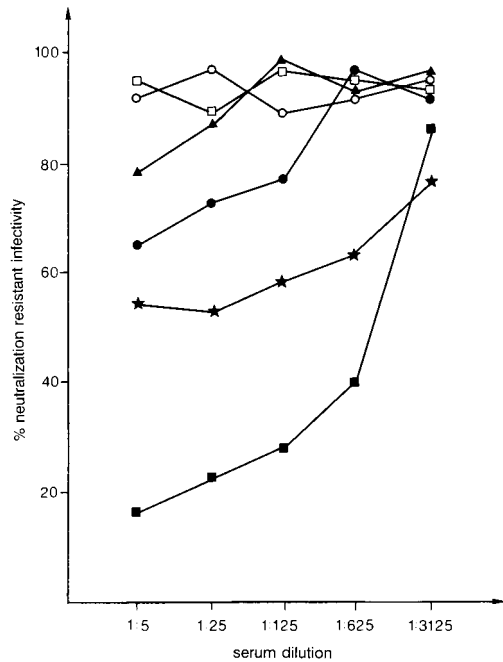


FIG. 8. BHV-1/eG<sub>syn</sub> virions are susceptible to neutralization by antibodies against the BRSV G glycoprotein. Approximately 200 PFU of BHV-1/eG<sub>ori</sub> was incubated with 20% fetal calf serum plus complement or serial dilutions of anti-VacG<sub>syn</sub> with complement (open squares) or the anti-BRSV hyperimmune serum with complement (open circles), and about 160 PFU of BHV-1/eG<sub>syn</sub> was incubated with 20% FCS and complement or with serial dilutions of anti-VacG<sub>syn</sub> and complement (solid squares), MAb 57 with complement (stars), anti-BRSV serum 2106 without complement (solid triangles, no complement added to the FCS control), or anti-BRSV serum 2106 with complement (solid circles). After 60 min at 37°C, the virions were plated on MDBK cells and the cultures were overlaid with semisolid medium. Plaques were counted 3 days later. The plaque count of each FCS control was defined as 100% neutralization-resistant infectivity. Serum dilutions are indicated.

which was translated into proteins recognized by BRSV G glycoprotein-specific MAb 20 and the anti-VacG<sub>syn</sub> serum. Although we did not attempt to delineate sequence motifs leading to instability of the G<sub>ori</sub> transcripts in the nucleus, our results strongly suggest their existence, which might also explain the failure to express the authentic ORF encoding BRSV fusion protein F by BHV-1 (13). Introduction of a series of single restriction enzyme cleavage sites into the G<sub>syn</sub> ORF will be helpful in identifying sequence elements that prevent the generation of mRNA from the genomic BRSV G glycoprotein ORF, which may be beneficial for the development of systems for the expression of RNA virus genes in the nuclei of mammalian cells.

The BRSV G glycoprotein is a type II membrane glycoprotein with a 37-amino-acid N-terminal cytoplasmic domain followed by 29 hydrophobic amino acids representing the transmembrane region. The remaining 191 amino acids are mainly hydrophilic and constitute the extracellular domain (24). Among BHV-1/eG<sub>syn</sub>-infected cell proteins, MAb 20, MAb 57, and the anti-VacG<sub>syn</sub> serum detected proteins with apparent molecular masses of 38, 43, and 70 to 120 kDa. Inhibition of de novo protein synthesis by cycloheximide resulted in a decrease of the signals generated by the 38- and 43-kDa proteins and a concomitant increase in intensity and size of the large-protein band. Although the exact precursor-product relationship awaits clarification, we assume that the 38- and 43-kDa proteins represent precursor molecules of the mature BRSV G glycoprotein. This assumption is in accordance with results

reported by Lerch et al. (24), who demonstrated that in cells infected with a BRSV G glycoprotein-expressing recombinant vaccinia virus, two proteins which migrated with an apparent molecular mass of 43 kDa and as a broad band between 68 and 97 kDa specifically reacted with an anti-BRSV serum in immunoblots. They further provided evidence that the 43-kDa form represents a precursor containing only N-linked carbohydrates whereas the 68- to 97-kDa molecules constitute the mature N- and O-glycosylated BRSV G glycoprotein.

Expression of the BRSV G glycoprotein in BHV-1/eG<sub>syn</sub>-infected cells was analyzed only by immunoblotting because the BRSV G glycoprotein-specific MAb 20 and the anti-VacG<sub>syn</sub> serum did not work for immunoprecipitation and MAb 57 precipitated only the mature glycoprotein after synthesis in the presence of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine (not shown). The observation that antibodies against the BRSV G glycoprotein bind in immunoblots but fail to immunoprecipitate the antigen is, although surprising, not without precedent and has also been reported by Lerch et al. (24). The reason for this apparent paradox, however, remains unclear.

Immunofluorescence and flow cytometry (data not shown) with MAb 20 and the anti-VacG<sub>syn</sub> serum demonstrated that BHV-1/eG<sub>syn</sub>-infected cells expressed the BRSV G glycoprotein on the cell surface, and we conclude that it is tightly anchored in the cell membrane since no secreted molecules were found in the medium from BHV-1/eG<sub>syn</sub>-infected cultures. Thus, our results indicate Golgi processing and post-Golgi transport of the BHV-1/eG<sub>syn</sub>-encoded BRSV G glycoprotein to the cell surface, comparable to the situation in BRSV- or recombinant vaccinia virus-infected cells (24).

Since alphaherpesviruses probably do not encode type II glycoproteins, we investigated whether the BRSV G glycoprotein that presumably lacks any herpesvirus-specific targeting signals is incorporated into BHV-1 particles. Western blot analysis with MAb 20 revealed incorporation of the BRSV G glycoprotein into purified virus particles, and this was confirmed by the susceptibility of BHV-1/eG<sub>syn</sub> virions to neutralization by the anti-VacG<sub>syn</sub> serum, the anti-BRSV immune serum, and MAb 57 in the presence of complement. That these antibodies did not neutralize in the absence of complement was not too surprising, since it is unlikely that the BRSV G glycoprotein exhibits a biological function for the infectivity of BHV-1. In addition, flow cytometry demonstrated that MAb 20 and the anti-VacG<sub>syn</sub> serum bound to living cells infected with purified BHV-1/eG<sub>syn</sub> virions in the presence of cycloheximide to prevent de novo protein synthesis (13). This further supports the conclusion that the orientation of the BRSV G glycoprotein within the viral envelope is correct.

To our knowledge, this is the first description of a class II membrane glycoprotein becoming incorporated into alphaherpesvirus virions. The presence of the BRSV G glycoprotein in the given background of BHV-1/eG<sub>syn</sub> exerted only a slight influence on the entry of virions into target cells in comparison to the situation for wild-type BHV-1 (data not shown). Although no repaired viruses were included in this study, we believe that it is reasonable to assume that BRSV G glycoprotein does not significantly interfere with the envelope structures required for the initial steps of the BHV-1 infection.

This study has practical implications for herpesvirus-based vector vaccine development: (i) it indicates that modification of RNA virus genes may be required for efficient expression, and (ii) it raises the possibility of targeting foreign antigens as class II membrane (glyco)proteins into the envelope of herpesvirus virions, which might be important when booster vaccinations with live recombinant viruses are inefficient due to

existing immunity to the vector, a situation observed with recombinant vaccinia viruses (13, 26).

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