JOURNAL OF VIROLOGY, Nov. 1990, p. 5559–5569
0022-538X/90/115559-11$02.00/0

Vol. 64, No. 11

Nucleotide Sequence Analysis and Expression from Recombinant Vectors Demonstrate That the Attachment Protein G of Bovine Respiratory Syncytial Virus Is Distinct from that of Human Respiratory Syncytial Virus

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Received 17 May 1990/Accepted 4 August 1990

Bovine respiratory syncytial (BRS) virus causes a severe lower respiratory tract disease in calves similar to the disease in children caused by human respiratory syncytial (HRS) virus. While there is antigenic cross-reactivity among the other major viral structural proteins, the major glycoprotein, G, of BRS virus and that of HRS virus are antigenically distinct. The G glycoprotein has been implicated as the attachment protein for HRS virus. We have carried out a molecular comparison of the glycoprotein G of BRS virus with the HRS virus counterparts. cDNA clones corresponding to the BRS virus G glycoprotein mRNA were isolated and analyzed by dideoxynucleotide sequencing. The BRS virus G mRNA contained 838 nucleotides exclusive of poly(A) and had a major open reading frame coding for a polypeptide of 257 amino acid residues. The deduced amino acid sequence of the BRS virus G polypeptide showed only 29 to 30% amino acid identity with the G protein of either the subgroup A or B HRS virus. However, despite this low level of identity, there were strong similarities in the predicted hydropathy profiles of the BRS virus and HRS virus G proteins. A cDNA molecule containing the complete BRS virus G major open reading frame was inserted into the thymidine kinase gene of vaccinia virus by homologous recombination, and a recombinant virus containing the BRS virus G protein gene was isolated. This recombinant virus expressed the BRS virus G protein, as demonstrated by Western immunoblot analysis and immunofluorescence of infected cells. The BRS virus G protein expressed from the recombinant vector was transported to and expressed on the surface of infected cells. Antisera to the BRS virus G protein made by using the recombinant vector to immunize animals recognized the BRS virus attachment protein but not the HRS virus G protein and vice versa, confirming the lack of antigenic cross-reactivity between the BRS and HRS virus attachment proteins. On the basis of the data presented here, we conclude that BRS virus should be classified within the genus *Pneumovirus* in a group separate from HRS virus and that it is no more closely related to HRS virus subgroup A than it is to HRS virus subgroup B.

Bovine respiratory syncytial (BRS) virus is a respiratory tract pathogen of cattle. The virus causes bronchiolitis and pneumonia, and annual winter outbreaks are economically important to the cattle industry (5, 42, 43). The disease caused by BRS virus in cattle is similar to that caused by human respiratory syncytial (HRS) virus in children in pathology and in the reoccurrence of infections. In order to increase our understanding of the molecular biology and pathogenesis of BRS virus and to gain insight into its relationship to HRS virus, we have initiated a molecular analysis of BRS virus. Previously, we identified 9 proteins and 10 mRNAs specific to BRS virus-infected cells corresponding to 9 of the 10 HRS virus proteins and the 10 HRS virus mRNAs. We also identified the BRS virus genomic RNA and generated and identified cDNA clones to 9 of the 10 BRS virus mRNAs (25).

Previous work has shown that there is no cross-reactivity between the attachment surface glycoprotein G of BRS virus and that of HRS virus, whereas there is antigenic cross-reactivity between the fusion (F) transmembrane glycoprotein and the major structural proteins, N, P, and M (25, 35). Available evidence indicates that BRS virus has a narrow host range, infecting only cattle and bovine cells in culture, in comparison with HRS virus which can infect a variety of cell types and experimental animals (17, 31, 36). Since the G protein of HRS virus has been characterized as the viral attachment protein (26), it is possible that the differences in the BRS virus and HRS virus G proteins may be responsible for the differences in attachment and host range observed between BRS virus and HRS virus.

On the basis of sequence analysis of the HRS virus G mRNA, the G protein is proposed to have three domains: an internal or cytoplasmic domain, a transmembrane domain, and an external domain which comprises three-fourths of the polypeptide (40, 47). Evidence suggests that the respiratory syncytial virus G protein is oriented with its amino terminus on the internal side of the viral membrane and its carboxy terminus exposed to the external domain (34, 40, 45, 47). Unlike the other paramyxovirus attachment proteins, the respiratory syncytial virus G protein lacks both neuraminidase and hemagglutinating activities (12, 38). The mature G protein, found in virions and infected cells, has an estimated molecular mass of 80 to 90 kilodaltons (kDa) on the basis of migration in sodium dodecyl sulfate (SDS)-polyacrylamide gels (10, 12, 23, 37). In contrast, the G mRNA sequence predicts a protein with a molecular mass of 32 kDa (40, 47), and when the G mRNA is translated in vitro, it directs synthesis of a 36-kDa protein that is specifically immunopre-

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cipitated by anti-G serum (47). It has been shown that there is N-linked and extensive O-linked glycosylation of the polypeptide backbone (22, 48). Experiments with glycosidases, inhibitors of sugar addition, and a cell line defective in O-linked glycosylation estimate that 55% of the molecular weight of the mature G protein may be due to O-linked glycosylation and that 3% may be due to N-linked glycosylation (22, 48). However, these estimates were made on the basis of migration in SDS-polyacrylamide gels and at best are only approximate values. Consistent with the evidence for extensive O-linked glycosylation is a high content (30%) of threonine and serine residues in the predicted amino acid sequence of the G protein (40, 47). Threonine and serine are amino acid residues that serve as sites for O-linked oligosaccharide attachment (19), and in the HRS virus G protein 85% of the threonine and serine residues are in the extracellular domain (47). The high content of proline (10%), serine and threonine (30%), and the extensive O-linked glycosylation of the G protein are features similar to those of a group of cellular glycoproteins known as the mucinous proteins (47) but unusual among viral glycoproteins.

Isolates of HRS virus have been divided into two subgroups, A and B, on the basis of the antigenic variations observed among G proteins by using panels of monoclonal antibodies (2, 32). However, a few monoclonal antibodies exist which recognize the G protein of both subgroups (32, 35). Sequence analysis of the G mRNA of HRS viruses from the two subgroups showed a 54% overall amino acid identity between the predicted G proteins, with 44% amino acid identity in the extracellular domain of the protein (18).

Because of the differences in species specificity of BRS virus and HRS virus and the antigenic differences observed among the G proteins of the two viruses (25, 35), we have undertaken a molecular study of the BRS virus G protein to determine its structure and ultimately to determine whether it has a role in the species specificity of BRS virus. We constructed cDNA clones to the BRS virus G mRNA and compared the nucleotide and predicted amino acid sequences of the BRS virus G mRNA to the G mRNA and amino acid sequences of HRS viruses from subgroups A and B. There were similar levels of nucleic acid identity among the mRNA sequences of the different viruses; however, the predicted amino acid sequence of the BRS virus G protein had much less identity with either of the HRS virus G proteins than the G proteins of HRS virus subgroups A and B have with each other. Despite the low level of amino acid identity, there was overall similarity among the hydropathy profiles of the various G proteins.

We also report the construction of a recombinant vaccinia virus (VV) containing a cDNA copy of the BRS virus G protein gene, the demonstration of expression of the BRS virus G protein in cells infected with the recombinant virus, and the use of the recombinant virus to generate monospecific polyclonal antibody. The antibody recognizes only the BRS virus attachment protein but not the HRS virus counterpart and therefore confirms the antigenic specificity of the G proteins (35).

(This work was done by R. A. Lerch in partial fulfillment of the requirements for the Ph.D. degree from The University of North Carolina at Chapel Hill, 1989.)

MATERIALS AND METHODS

Viruses and cells. The growth and propagation of BRS virus isolate 391-2, wild-type (Copenhagen strain) and recombinant VVs, bovine nasal turbinate (BT) cells, HEp-2 cells, and thymidine kinase-negative (tk^-) 143B cells were as described previously (15, 25, 41).

cDNA synthesis, molecular cloning, and identification of BRS virus G-specific cDNA clones. cDNAs were synthesized by using the strand replacement method of Gubler and Hoffman (13), as previously described. T4 DNA polymerase (Bethesda Research Laboratories) was used to make the ends of the cDNAs blunt (28). The cDNAs were ligated into M13mp19 replicative-form (RF) DNA, which had been digested with *Sma*I and treated with calf intestinal alkaline phosphatase (28) and transfected into competent *Escherichia coli* DH5α F′ cells (Bethesda Research Laboratories) (14). M13mp19 phage containing BRS virus G-specific inserts were identified by dot blot hybridization of phage DNA (8) probed with a previously identified BRS virus G clone (25) that had been labeled by nick translation (28, 39). Growth and manipulations of M13mp19 and recombinant phage were as previously described by Messing (30).

Nucleotide sequencing and primer extension on RNA. Dideoxynucleotide sequencing with the Klenow fragment of *E. coli* DNA polymerase I (Pharmacia) or a modified T7 DNA polymerase (Sequenase; U.S. Biochemicals) was done as previously described (27, 44) with an M13 sequencing primer (New England BioLabs). Extension of a synthetic DNA primer, complementary to bases 154 to 166 of the BRS virus G mRNA, on BRS virus mRNA with avian myeloblastosis virus reverse transcriptase (Molecular Genetic Resources) was done to determine the 5′ sequence of the BRS virus G mRNA (1). BRS virus mRNA used as a template in the primer extension on RNA was harvested as previously described for mRNA used for cDNA synthesis (25). Nucleotide sequencing and primer extensions were done by using [α-^{35}S]dATP (Amersham) and polyacrylamide-urea gradient gel electrophoresis, as previously described by Biggin et al. (4). The nucleotide sequence was analyzed by using the University of Wisconsin Genetics Computer Group software package (9).

Synthesis and cloning of a complete cDNA to the BRS virus G mRNA. A cDNA containing the complete open reading frame of the BRS virus G mRNA was synthesized by using a specific synthetic oligonucleotide for second-strand synthesis. The nucleotide sequence for the oligonucleotide was determined from sequence analysis of BRS virus mRNAs. First-strand synthesis occurred as described previously (7). After synthesis, the RNA template was digested with RNase A (1 μg/μl) for 30 min at 37°C. The resulting single-stranded cDNAs were isolated by phenol extraction and ethanol precipitation. The oligonucleotide used for second-strand synthesis had the sequence 5′-CACGGATCCACAAGT ATGTCCAACC-3′ with the 5′ first nine bases of the oligonucleotide containing a *Bam*HI restriction enzyme site in the gene. The single-stranded cDNAs were mixed with the oligonucleotides (50 μg/ml), heated to 100°C for 1 min, and placed on ice. Avian myeloblastosis virus reverse transcriptase was used to synthesize the second strand of the cDNAs in a reaction (50 mM Tris hydrochloride [pH 8.0], 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 1.6 mM dNTP, 100 U of avian myeloblastosis virus reverse transcriptase) that was incubated for 1 h at 50°C. The cDNAs were separated from protein by phenol extraction and recovered by ethanol precipitation, and the ends were made blunt with T4 DNA polymerase (28). Blunt-ended cDNAs were then digested with the restriction enzyme *Bam*HI (Bethesda Research Laboratories) and separated from protein by phenol extraction. M13mp18 RF DNA that was digested with *Bam*HI and *Sma*I and treated with calf intestinal phos-

phatase was mixed in solution with the cDNAs and recovered by ethanol precipitation. The cDNAs and vector were ligated and transfected into competent DH5α F′ cells (Bethesda Research Laboratories) as described previously (14).

Construction and isolation of recombinant VV vectors. A complete cDNA clone, G4, corresponding to the BRS virus G mRNA was subcloned into the recombination plasmid pIBI76-192 by digestion with *Bam*HI and *Kpn*I, treatment with T4 DNA polymerase to make the ends of the cDNA blunt, and ligation into the unique *Sma*I site of pIBI76-192 (28). The plasmid pIBI76-192 is similar to recombination plasmids described previously (3). pIBI76-192 contains base pairs 1 to 1710 of the *Hin*dIII J fragment of VV inserted between the *Hin*dIII and *Sma*I sites of pIBI76 (International Biotechnology Inc.). Inserted into the *Eco*RI site (base pair 670 of the *Hin*dIII J fragment) of the VV *tk* gene (base pairs 502 to 1070 of the *Hin*dIII J fragment [46]) is a 280-base-pair fragment of DNA that contains the 7.5K promoter of VV. The orientation of this promoter fragment was such that it directed transcription from right to left on the conventional VV map, opposite to the direction of transcription of the VV *tk* gene. The unique *Sma*I site in pIBI76-192 is downstream from the major transcriptional start site of the 7.5K promoter.

The isolation of recombinant VVs containing the BRS virus G mRNA sequence was as described previously (41) except that the recombinant viruses were identified with the blot procedure of Lavi and Ektin (24).

Characterization of recombinant VV vectors. VV core DNA from wild-type and recombinant viruses was prepared for Southern blot analysis, as described previously by Esposito et al. (11). Restriction enzyme digestion, Southern blot analysis, and radioactive labeling of DNA by nick translation were as described previously (28, 39). Metabolic labeling of the proteins from BRS virus and wild-type and recombinant VV-infected cells was as described previously (25) except that proteins in VV-infected cells were labeled for 3 h, starting at 3 h postinfection. SDS-polyacrylamide gel electrophoresis of proteins was done by using standard procedures (21). For Western immunoblot analysis, proteins from infected and uninfected cells were harvested as described previously (25) at 30 h postinfection for BRS virus-infected BT cells and 6 h postinfection for VV-infected BT cells. Anti-BRS virus 391-2 serum (25) was used in the Western blot analysis. Immunofluorescence was done on HEp-2 cells grown on glass cover slips. HEp-2 cells were infected with wild-type or recombinant VV (multiplicity of infection [MOI] = 10). At 48 h postinfection, the cells were stained for surface immunofluorescence as described previously (48) by using anti-BRS virus 391-2 serum as a first antibody followed by fluorescein-conjugated anti-bovine immunoglobulin G (heavy chain + light chain). Fluorescence was observed through a Nikon fluorescence microscope.

Nucleotide sequence accession number. The nucleotide sequence data reported here have been submitted to GenBank and assigned the accession number M37121.

RESULTS

Nucleic acid sequence and comparison. In order to determine the sequence of the BRS virus G mRNA and deduce the amino acid sequence, we made cDNAs to the BRS virus G mRNA and determined the nucleotide sequence from nine cDNA clones. The nine clones were derived independently from four separate cDNA synthesis reactions. We also carried out direct sequencing of the 5′ end of G mRNA by primer extension of a synthetic DNA primer on BRS virus mRNA. The areas of the BRS virus G mRNA sequence determined from the different clones and from primer extension are shown in Fig. 1. Clone G4 is a full-length BRS virus G clone synthesized by using an oligonucleotide primer specific for second-strand synthesis. Three independent clones, G4 (bases 8 to 838), G10 (bases 19 to 828), and G33 (bases 23 to 808), were excised with *Pst*I and *Kpn*I and subcloned into M13mp18 RF DNA to allow for sequencing from the opposite end of the cDNA. Clones G10 and G33 were sequenced in their entirety. Clones G1, G7, G12, G5, and G3 were all less than 500 nucleotides in length and were only partially sequenced for this reason.

The BRS virus G protein mRNA was 838 nucleotides in length, excluding the poly(A) tail (Fig. 2). The BRS virus G mRNA sequence was compared with the published nucleotide sequences of the G protein mRNAs of HRS viruses A2 and 18537, a subgroup A virus and a subgroup B virus, respectively (Fig. 2) (18, 47). The BRS virus G mRNA was shorter than the HRS virus A2 and 18537 G mRNAs by 81 and 84 bases, respectively. There were some conserved features in common between the BRS virus G mRNA and the HRS virus G mRNA. With the exception of the first nucleotide, which was not demonstrated, the BRS virus G mRNA had the conserved gene start signal 5′-GGGGCA AAU. . .-3′ (6), found at the 5′ end of all HRS virus mRNAs. The 3′ end of the BRS virus G mRNA also conformed to one of the two consensus gene end sequences, 5′. . .AGU(A/U) AU(A/U)U-poly(A)-3′, found at the 3′ end of all HRS virus genes (6). The position of the initiation codon, nucleotides 16 to 18, for the major open reading frame of the BRS virus G mRNA was identical to the position of the initiation codons of the G mRNAs of HRS viruses (18, 40, 47). The noncoding region at the 3′ end of the BRS virus mRNA, however, was 42 bases long compared with 6 bases for the HRS virus A2 G mRNA and 27 bases for the HRS virus 18537 G mRNA (18, 40, 47). This resulted in the termination codon for the BRS virus G protein occurring 119 bases prior to the termination codon for the G protein of HRS virus A2 and 98 bases prior to the termination codon in the 18537 G mRNA. One clone, G4, was missing bases 812, 819, and 824, all of which were after the termination codon in the 3′ noncoding region. The BRS virus G mRNA lacked an upstream ATG found in the HRS virus G mRNAs (18, 40, 47).

The BRS virus G mRNA shared 51.7% sequence identity with the HRS virus A2 G mRNA and 50.8% with the HRS virus 18537 G mRNA when the BRS virus G mRNA was aligned with the HRS virus A2 G mRNA. The HRS virus A2 and 18537 G mRNAs share 67.4% sequence identity (18). A slightly different alignment occurred when the BRS virus G mRNA was aligned with the HRS virus 18537 G mRNA, but the alignment resulted in a change of less than 1% in sequence identity between the G mRNA of BRS virus and the G mRNA of either HRS virus A2 or 18537 (data not shown). Computer analysis was used to determine whether an internal deletion would result in a better alignment, but no internal deletion was found (data not shown).

Amino acid sequence and comparison. The BRS virus G mRNA had a major open reading frame which predicted a polypeptide of 257 amino acids. The predicted molecular mass of this polypeptide was 28.6 kDa. The deduced amino acid sequence of the BRS virus G protein was compared with the published amino acid sequences of the HRS virus A2 and 18537 G proteins (18, 47) (Fig. 3). The BRS virus G protein was similar in overall amino acid composition to the HRS virus G proteins, with a high content (25.7%) of

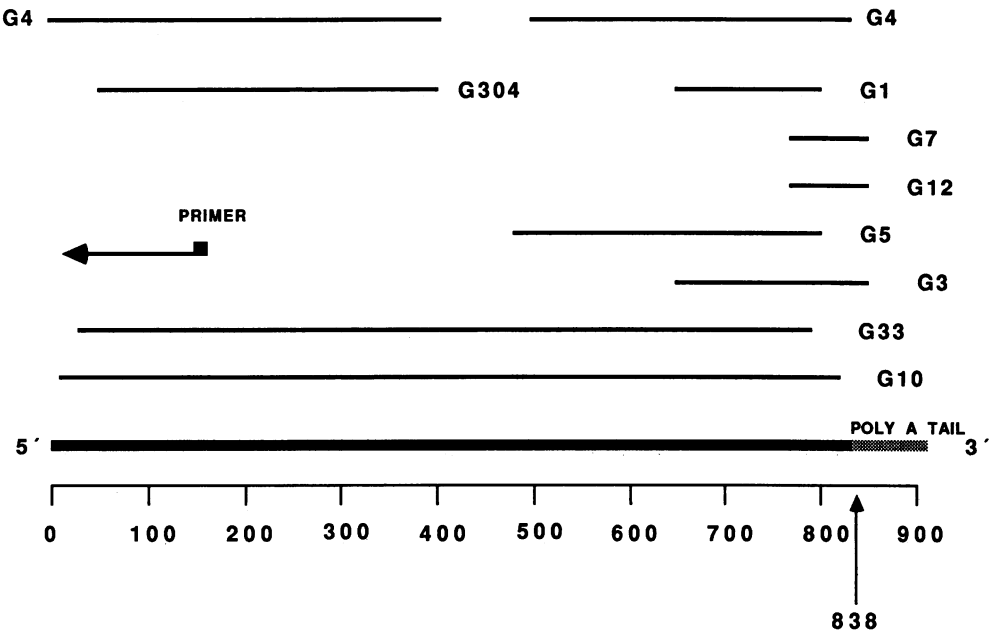

FIG. 1. Sequencing strategy of G cDNAs of BRS virus. The scale at the bottom indicates the number of nucleotides from the 5' end of the BRS virus G mRNA. cDNAs were inserted into M13mp19 RF DNA, and the nucleotide sequences were determined by dideoxynucleotide sequencing with an M13-specific sequencing primer. The arrow indicates the portion of the G mRNA sequence determined by extension of a synthetic oligonucleotide primer on BRS virus mRNA. The sequence of the primer was complementary to bases 154 to 166 of the BRS virus G mRNA sequence. The cDNAs in clones G4, G10, and G33 were also excised with *Pst*I and *Kpn*I and inserted into M13mp18 RF DNA for sequencing of the opposite end of the cDNA. The lines for each clone indicate the sequence of the mRNA determined from that clone. Only clones G10 and G33 were sequenced in their entirety. Clones G1, G7, G12, G5, and G3 were all less than 500 nucleotides in length and were only partially sequenced for this reason.

threonine and serine residues compared with that observed for the G proteins of the HRS viruses 18537 (28.4%) and A2 (30.6%) (18, 47). Serine and threonine residues are potential sites for the addition of O-linked carbohydrate side chains (19). Of 66 threonine and serine residues in the BRS virus protein, 52 (79%) were in the proposed extracellular domain. The positions of only nine threonine residues (amino acids 12, 52, 72, 92, 129, 139, 199, 210, 211, and 235) and eight serine residues (amino acids 2, 28, 37, 44, 53, 102, 109, and 174) were conserved between predicted amino acid sequences of all the G proteins examined (Fig. 3). In addition to the potential O-linked carbohydrate addition sites, there were four potential sites for N-linked carbohydrate addition in the BRS virus G protein. The potential N-linked addition sites were not conserved between the BRS virus and the subtype B HRS virus; two of the four potential sites were the same in HRS virus A2 and BRS virus G.

The BRS virus G protein had a high proline content (7.8%) similar to that observed for the G proteins of the HRS viruses A2 (10%) and 18537 (8.6%) (18, 40, 47). Six proline residues were conserved among all RS virus G proteins sequenced to date. These proline residues were amino acids 146, 155, 156, 172, 194, and 206 (Fig. 3).

There were four cysteine residues in the proposed extracellular domain of the BRS virus G protein. These four residues were exactly conserved in position, relative to the amino terminus of the protein, with the four cysteine residues conserved among the HRS virus A2, Long (data not shown), and 18537 G proteins (Fig. 3) (18, 40, 47). In addition, the G proteins of BRS virus, HRS virus A2, and HRS virus 18537 all shared a cysteine residue in the proposed cytoplasmic domain (Fig. 3). However, this cysteine residue is not present in the HRS virus Long G protein (18).

Although the cysteine residues were conserved in the BRS virus G protein, a 13-amino-acid region reported previously to be exactly conserved in the G proteins of subgroup A and B HRS viruses was not conserved in the BRS virus G protein (18). Only 6 of the 13 amino acids in this region of the BRS virus G protein were conserved, and 2 of the 6 were the cysteine residues (Fig. 3).

The amino acid identity among the BRS virus G protein and the HRS virus proteins was significantly lower than the amino acid identity observed between the G proteins of the HRS virus subgroups A and B (Fig. 4). The overall amino acid identity between the HRS virus A2 and 18537 G proteins is 53% (18). The BRS virus G protein shared only 29% amino acid identity with the HRS virus A2 G protein and 30% amino acid identity with the HRS virus 18537 G protein. Comparison of amino acid identity within each of the three postulated domains of the G proteins showed distinct differences in the levels of identity in the three domains. The identity between the proposed extracellular domain of the BRS virus and HRS virus G proteins of either subgroup was significantly lower than the overall amino acid identity and lower than the identity between extracellular domains of the two HRS virus G proteins (Fig. 4). The proposed cytoplasmic and transmembrane domains of the BRS virus G protein were more conserved than the extracellular domain, with 43 and 55% amino acid identity, respectively, observed among the corresponding domains of either HRS virus G protein (Fig. 4).

Although the overall identity of the BRS virus G protein to either HRS virus G protein was lower than that between the HRS virus G proteins, there were similarities in the hydropathy profiles of the different G proteins (Fig. 5) (18, 40, 47). There was an initial hydrophilic region followed by a hydro-

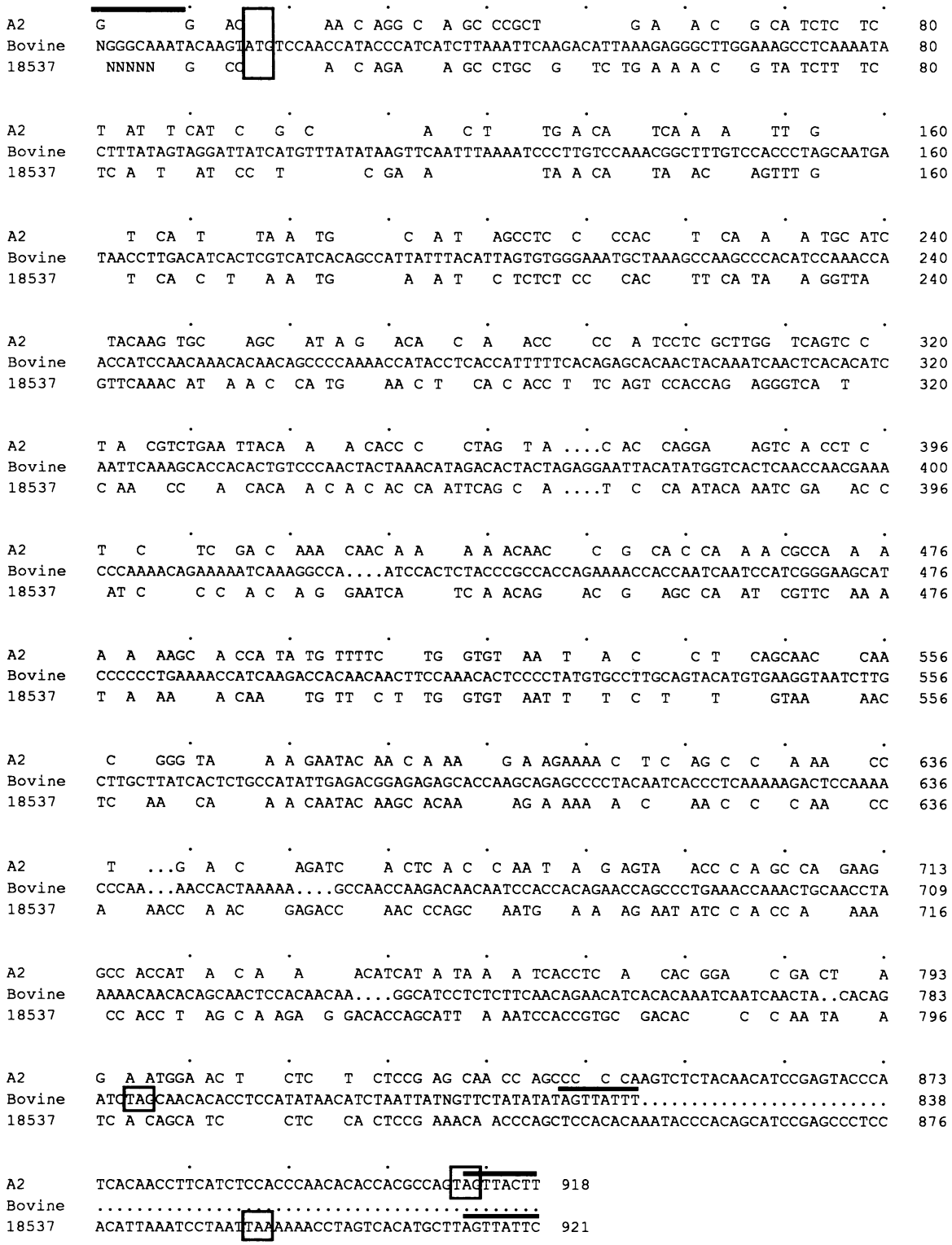

FIG. 2. Alignment of the complete BRS virus G mRNA sequence with those of the HRS virus A2 and 18537 G mRNAs. Alignment was done by the method of Needleman and Wunsch (33), comparing the BRS virus G (Bovine) sequence against the HRS virus A2 G sequence. Only nonidentical bases are shown for the G mRNA sequences of the HRS viruses A2 and 18537. Gaps, shown by the dotted lines, were used to maximize sequence identity of the HRS virus A2 G sequence to the BRS virus G sequence. The HRS virus 18537 G sequence was aligned with the HRS virus A2 G sequence as determined by Johnson et al. (18). The HRS virus consensus gene start and gene stop signals are overlined. The consensus initiation codon and the stop codons for the different virus G mRNAs are boxed. The dots above the sequences are spaced every 10 nucleotides and the number of the last nucleotide on a line is indicated to the right of the sequence.

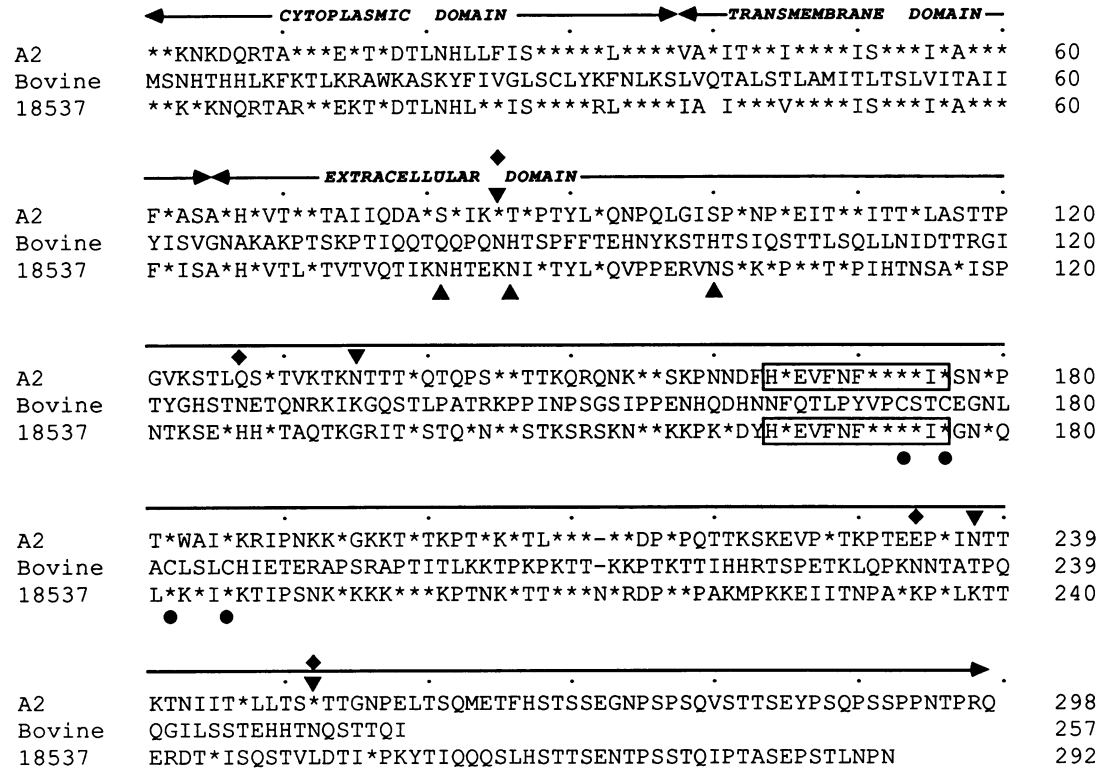

FIG. 3. Alignment of the predicted amino acid sequence of the BRS virus G protein and the G proteins of the HRS viruses A2 and 18537. Alignment was done by the method of Needleman and Wunsch (33). Identical amino acids for the HRS virus A2 and 18537 G proteins are indicated by an asterisk. The proposed domains are indicated above the sequence. Potential N-linked carbohydrate addition sites are indicated for the BRS virus G protein (Bovine) (▼), for the HRS virus A2 G protein (◆), and for the HRS virus 18537 G protein (▲). The four conserved cysteine residues are also indicated (●). The conserved 13-amino-acid regions of the HRS virus A2 and 18537 G proteins are boxed. A gap in the HRS virus A2 G protein sequence compared with the HRS virus 18537 G protein sequence as described by Johnson et al. (18) is shown by a dash. The dots above the sequences are spaced every 10 amino acid residues, and the number of the last amino acid residue on a line is indicated to the right of the sequence.

	CYTO-PLASMIC	TRANS-MEMBRANE	EXTRACELLULAR	TOTAL
BOVINE vs HUMAN A2	43%	59%	21%	29%
BOVINE vs HUMAN 18537	43%	59%	22%	30%
HUMAN A2 vs HUMAN 18537	81%	86%	44%	53%

FIG. 4. Amino acid identity between the BRS (BOVINE) and HRS (HUMAN) virus G proteins. The overall identity and identity in the different proposed domains between the various G proteins are shown and are based on the alignment shown in Fig. 3.

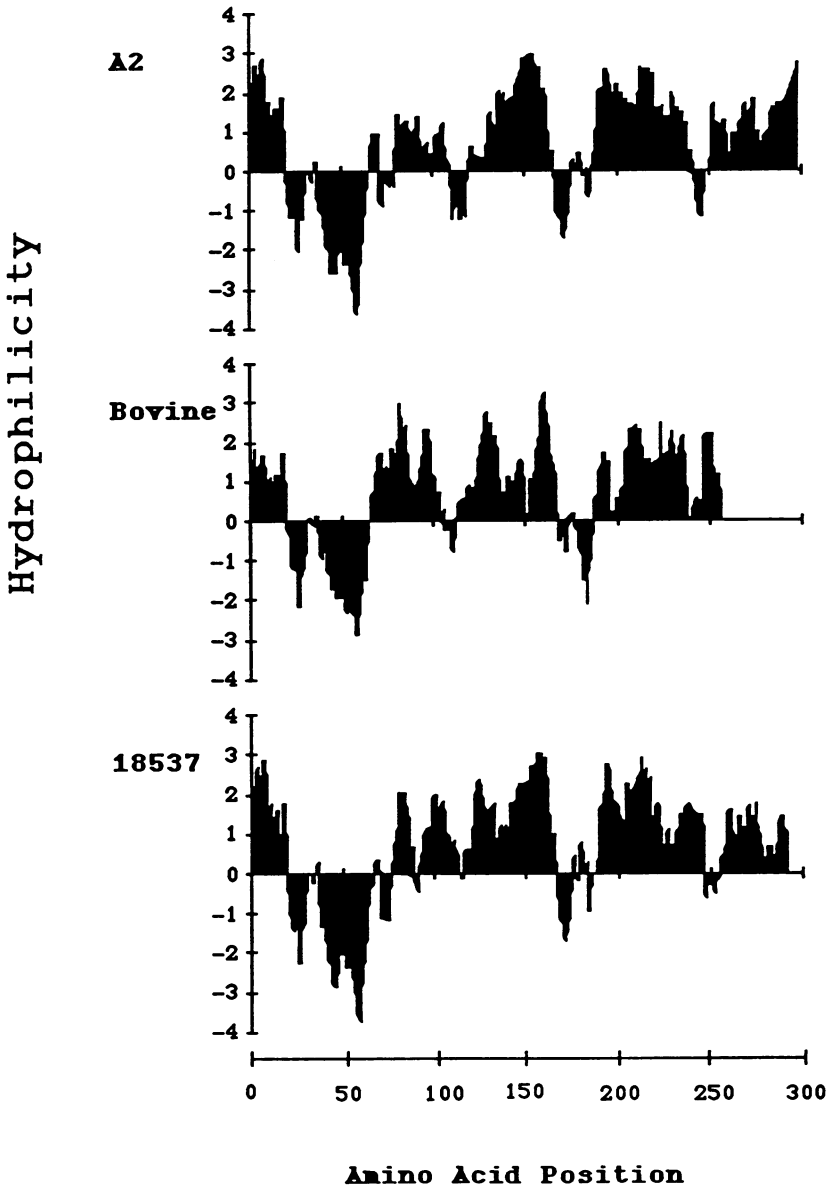

FIG. 5. Hydrophilicity plots for the G protein of BRS virus and the HRS viruses A2 and 18537. The distribution of the hydrophilic and hydrophobic regions along the predicted amino acid sequences of the G proteins was determined by using the algorithm of Kyte and Doolittle (20). The value for each amino acid was calculated over a window of nine amino acids. The bottom scale indicates the amino acid residue starting with the amino-terminal methionine. Hydrophilic regions of the amino acid sequence are shown above the axis, and hydrophobic regions are shown below the axis.

phobic peak in the G proteins of HRS virus A2 and HRS virus 18537 and in the G protein of BRS virus. The two regions together corresponded to the proposed cytoplasmic domain (amino acids 1 to 37). This region was followed by a region of strong hydrophobicity, corresponding to the proposed transmembrane domain (amino acids 38 to 66). The remainder of the protein was mainly hydrophilic, corresponding to the proposed extracellular domain (amino acids 67 to 257, 67 to 292, or 67 to 298, depending on the virus). This hydrophilic extracellular domain was interrupted in all three G proteins by a short region of hydrophobicity (amino acids 166 to 188) which corresponded to the area containing the four conserved cysteine residues.

The BRS virus G mRNA contained two open reading frames in addition to the major open reading frame. One open reading frame began at nucleotide 212, ended at nucleotide 352, and coded for a predicted polypeptide of 46 amino acids. The other and larger of the two was in the same coding frame as the first but began at nucleotide 380 and ended at nucleotide 814. This open reading frame coded for a predicted polypeptide of 144 amino acids.

Construction and characterization of recombinant VV vectors containing the BRS virus G gene. A cDNA containing the complete major open reading frame of the BRS virus G mRNA was inserted into plasmid pIBI76-192, designed for the construction of VV recombinants. The plasmid pIBI76-192 is similar to recombination plasmids described previously (3) which contain a portion of the *Hin*dIII J fragment of

VV with the 7.5K promoter inserted into the *tk* gene. However, in the case of pIBI76-192, the 1,710-base-pair fragment between the *Hin*dIII and *Pvu*II sites of the *Hin*dIII J fragment was inserted between the *Hin*dIII and *Sma*I sites of pIBI76 and the 7.5K promoter directs transcription in the direction opposite of the *tk* promoter. The cDNA of the BRS virus G mRNA was inserted downstream of the major transcriptional start site of the 7.5K promoter. The *Hin*dIII J fragment containing the inserted BRS virus G gene was inserted into the genome of VV (Copenhagen strain) by homologous recombination (41). tk^- recombinant VVs were identified by hybridization of recombinant viral DNA with a probe specific for the BRS virus G gene and were selected by three rounds of plaque purification. Recombinant VVs G642 and G4567 contained the BRS virus G gene in the forward and reverse orientations with respect to the 7.5K promoter, respectively. The genome structures of recombinant VVs were confirmed by Southern blot analysis of digests of VV core DNA to confirm that the BRS virus G gene was inserted within the *tk* gene of the recombinant viruses (data not shown).

Analysis of proteins from cells infected with recombinant VV containing the BRS virus G gene. The ability of the recombinant VV containing the BRS virus G gene to express the BRS virus G protein was examined in BT cells. The proteins from BT cells infected with BRS virus, wild-type VV, or the recombinant VVs containing the BRS virus G gene were analyzed by Western blot analysis with the BRS virus 391-2-specific antiserum because the BRS virus G protein was not readily labeled with [^{35}S]methionine because of the scarcity of this amino acid in the BRS virus G amino acid sequence and because the 391-2 antiserum did not work for immunoprecipitation. The 391-2 antiserum was shown previously to recognize the BRS virus G protein in a Western blot analysis of proteins from BRS virus-infected cells (25). The serum recognized two proteins present in recombinant VV G642 (forward orientation)-infected cells (Fig. 6, lane G642+) but not in recombinant VV G4567 (reverse orientation) or wild-type VV-infected cells (Fig. 6, lanes G4567− and VV, respectively). The two proteins produced in recombinant VV G642-infected cells comigrated with proteins recognized by the antiserum in BRS virus-infected cells. One of the proteins in recombinant VV G642-infected cells comigrated with the mature BRS virus G protein, migrating as a broad band between the 68- and 97-kDa protein markers. The other protein migrated at approximately 43 kDa. Previous work indicates that the 43-kDa band represents a precursor form of the G protein containing only N-linked sugars (22, 48).

Surface expression of BRS virus G protein expressed from recombinant VVs. The G protein is a glycoprotein expressed on the surface of infected cells and incorporated in the membranes of virions (16; Y. T. Huang, Ph.D. dissertation, The University of North Carolina at Chapel Hill, 1983). In order to determine whether the BRS virus G protein expressed in the recombinant VV-infected cells was transported to and expressed on the surface of infected cells, indirect immunofluorescence staining was performed on recombinant virus-infected cells. BT cells were extremely sensitive to VV infection. There was high background fluorescence, and very few cells survived the staining procedure (data not shown). For these reasons immunofluorescence staining was done on recombinant virus-infected HEp-2 cells. HEp-2 cells that were infected with recombinant G642 (Fig. 7, panel rVVG) demonstrated specific surface fluorescence which was not present in either uninfected cells (Fig. 7, panel M) or wild-type VV-infected cells (Fig. 7, panel VV).

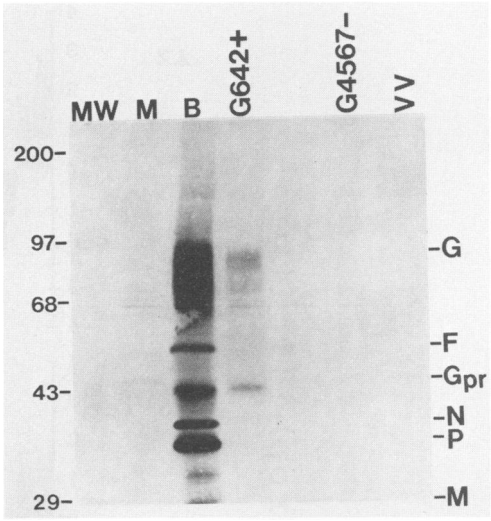

FIG. 6. Western blot analysis of BRS virus and recombinant VV-expressed G proteins. BT cells were infected with recombinant VVs (MOI = 10), wild-type VV (MOI = 10), or BRS virus (MOI = 1). Proteins from BRS virus (lane B), wild-type VV (lane VV), recombinant VV containing the BRS virus G gene in the forward (lane G642+) or reverse (lane G4567−) orientation, and mock (lane M)-infected cells were harvested by lysing the cells at 6 h postinfection for VV-infected cells and 36 h postinfection for BRS virus-infected cells. The proteins were separated by electrophoresis in a 10% polyacrylamide-SDS gel under nonreducing conditions and analyzed by Western blotting by using anti-391-2 serum as a first antibody. Horseradish peroxidase-conjugated anti-bovine immunoglobulin G was used to identify the bound first antibody. The BRS virus proteins are indicated. The 43-kDa N-linked G precursor (G_{pr}) is indicated. The positions of prestained protein molecular weight markers are indicated (lane MW) and labeled according to their molecular masses in kilodaltons.

Production of monospecific, polyclonal antibody to the BRS virus G protein and demonstration of antigenic specificity. To test the biological activity of the BRS virus attachment protein expressed from the recombinant VV vectors and to assess the antigenic cross-reactivity between the BRS and HRS virus G proteins with a polyclonal antiserum, recombinant VV expressing either the BRS virus or HRS virus G protein was used to immunize animals as described previously (41). Sera from animals immunized with the BRS virus G protein specifically immunoprecipitated the BRS virus attachment protein but did not recognize the HRS virus G protein (Fig. 8). Similarly, antiserum raised against the HRS virus G protein was specific for the HRS virus G protein and showed no recognition of the BRS virus G protein, confirming the antigenic distinctness of the two attachment proteins.

DISCUSSION

The G protein of respiratory syncytial virus is an unusual viral glycoprotein for a variety of reasons. Although it has been characterized as the attachment protein for HRS virus (26), it lacks both neuraminidase and hemagglutinating activities observed in the attachment proteins of other viruses in the paramyxovirus family (12, 38). Evidence suggests the HRS virus G protein is extensively glycosylated, with approximately 55% of the mass of the mature protein estimated

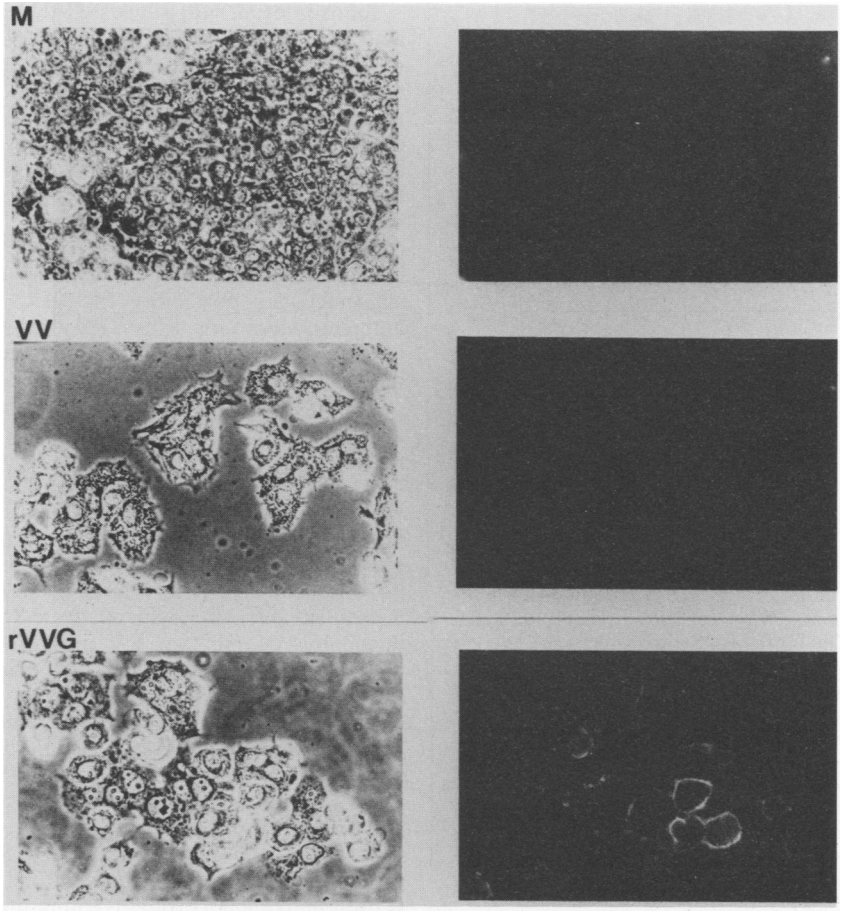

FIG. 7. Surface immunofluorescence of recombinant VV-infected cells. HEp-2 cells, grown on glass cover slips, were either mock infected (M) or infected with wild-type VV (MOI = 10) or recombinant virus G642 (rVVG; MOI = 10). At 24 h postinfection, the live cells were stained with anti-391-2 serum, followed by fluorescein-conjugated anti-bovine immunoglobulin G (heavy chain + light chain). Phase contrast (left panels) and fluorescence (right panels) photographs are shown.

to be due to addition of O-linked oligosaccharide side chains (22, 48). The G protein of BRS virus has been shown to be antigenically distinct from the HRS virus G protein (25, 35). In addition, with the exception of one report (29), BRS virus is unable to productively infect cells of human origin, whereas HRS virus infects both human and bovine cells (17, 31, 36). It is possible that the difference in host range between BRS and HRS viruses may be reflected in the amino acid sequence differences observed in the attachment proteins.

In order to further examine the differences between the BRS virus and HRS virus G proteins, we determined the nucleotide sequences of the BRS virus G protein mRNAs from cDNA clones. The BRS virus G mRNA was smaller than the G mRNAs of the HRS viruses and shared 51% sequence identity with the G mRNAs of the HRS viruses sequenced to date. The consensus viral gene start and end sequences observed in HRS virus genes were conserved in the BRS virus G mRNA, as was the position of the initiation codon with respect to the initiation codon of the HRS virus G mRNA. The BRS virus G mRNA had a larger 3′ noncoding region which, combined with the smaller size of the BRS virus G mRNA, resulted in a major open reading frame which coded for a polypeptide of 257 amino acids having an estimated molecular mass of 28 kDa. This compares with polypeptides of 298 and 292 amino acids coded for by the G mRNAs of HRS virus subgroups A and B, respectively, and estimated molecular masses of about 32 kDa for each. The size of the predicted BRS virus G polypeptide, compared with the estimated size of the mature BRS virus G protein found in infected cells, suggested that there was extensive modification of the BRS virus G polypeptide.

Studies with endoglycosidases, inhibitors of carbohydrate addition, and a cell line deficient in O-linked glycosylation have shown that the HRS virus G protein is extensively glycosylated with both N- and O-linked carbohydrate side chains (22, 48). The mature BRS virus G protein from infected cells was shown to be glycosylated and had an electrophoretic mobility similar to that of the 90-kDa HRS virus G protein, whereas the predicted amino acid sequence for the polypeptide indicated a protein of 28 kDa (25, 49). This suggested the BRS virus G protein was also extensively glycosylated, as is the HRS virus G protein. The predicted amino acid sequence of the BRS virus G protein had high levels (25%) of serine and threonine similar to the levels (30 and 28% for subgroups A and B, respectively) for the HRS virus G proteins, although the actual number of potential O-glycosylation sites for BRS virus, 66, is lower than the 91 potential sites found in HRS virus subgroup A (18, 40, 47). The high content of serine and threonine in the deduced BRS virus G amino acid sequence suggests that the BRS virus G

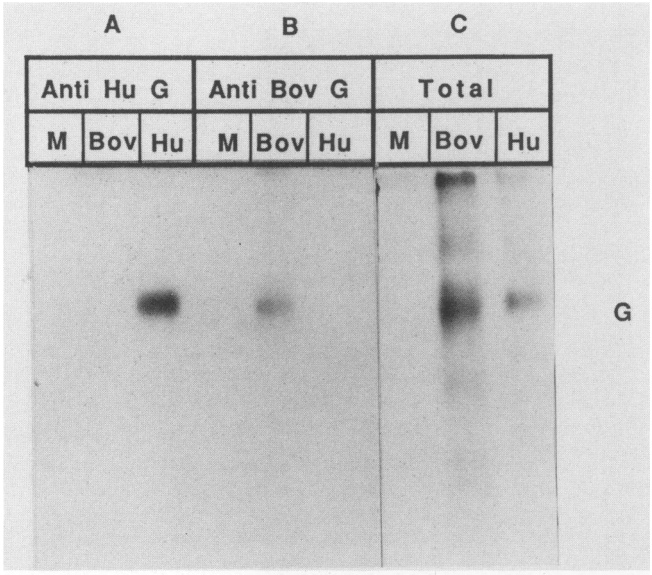

FIG. 8. Immunoprecipitation of [^{3}H]glucosamine-labeled proteins from mock (M)-, BRS virus (Bov)-, or HRS virus (Hu)-infected BT cells. (A) Antisera specific for the HRS virus G protein (Anti Hu G) were prepared by immunizing rabbits with a recombinant VV vector (vvG301) expressing the HRS virus A2 G protein (41) and used to immunoprecipitate the radiolabeled proteins from the mock-, BRS virus-, or HRS virus-infected cells. (B) Antisera specific for the BRS virus G protein were prepared by immunizing mice with a recombinant VV vector (vvG642) expressing the BRS virus G and used to immunoprecipitate proteins from the mock-, BRS virus-, or HRS virus-infected cells. (C) Total [^{3}H]glucosamine-labeled proteins present in cytoplasmic extracts of mock-, BRS virus-, or HRS virus-infected cells.

protein also had the potential for extensive O-linked glycosylation, although this has not been demonstrated directly.

Although the overall amino acid composition of the BRS virus G protein was similar to that of the HRS virus G protein, the BRS virus G amino acid sequence had a low level of overall amino acid identity with the HRS virus G proteins of either the subgroup A or the subgroup B viruses (18). There was only 29 to 30% identity between the BRS virus G protein and the G protein of either subgroup A or subgroup B HRS viruses, whereas there is 53% amino acid identity when the G proteins of the HRS virus subgroup A and subgroup B viruses are compared (18). Higher levels of amino acid identity were present between the BRS virus G protein and the HRS virus G proteins in the proposed cytoplasmic and transmembrane domains, but again this level was not as high as that found when comparing those regions of the G proteins of subgroup A and subgroup B HRS viruses (Fig. 4). The fact that the BRS virus G protein amino acid sequence was not significantly more closely related to the G protein amino acid sequence of HRS virus subgroup A or B suggests that the HRS and BRS viruses diverged prior to the emergence of the HRS virus A and B subgroups and that they should be classified in separate *Pneumovirus* subgroups.

The predicted amino acid sequence of the BRS virus G protein showed that the BRS virus and HRS virus G proteins shared only 29 to 30% amino acid identity. Despite the differences, the hydropathy profiles of the two proteins showed strong similarities, suggesting the possibility of similar overall structural features.

Johnson et al. (18) suggested that a conserved 13-amino-acid region found in the extracellular domain of the G protein of the HRS subgroup A and B viruses could be a candidate for a receptor binding site. The fact that this conserved region was not conserved in the G protein of BRS virus could relate to the host specificity of BRS virus. The four conserved cysteine residues found in the G protein of both the BRS virus and the HRS viruses could result in a similar secondary structure among the G proteins with specific differences in the conserved region changing the host specificity. Preliminary work with convalescent calf serum has suggested the possibility that BRS virus is divided into antigenic subgroups, as is HRS virus (25). If this proves true, it will be of interest to see whether the G proteins from different BRS virus subgroups share only a small conserved region in the extracellular domain, similar to that found by Johnson et al. (18) in HRS virus subgroup A and B G proteins.

Recombinant VVs containing a cDNA insert to the BRS virus G gene expressed the BRS virus G protein. This BRS virus G protein had an electrophoretic mobility in SDS-polyacrylamide gels which was similar to that of the G protein from BRS virus-infected cells. Antiserum specific for BRS virus 391-2 recognized the BRS virus G protein produced by recombinant VV in infected cells as shown by Western blot analysis. The BRS virus G protein expressed from the recombinant VV was transported to and expressed on the surface of infected cells, as shown by indirect surface immunofluorescence. Monospecific polyclonal antisera prepared by immunizing animals with the recombinant vector expressing the BRS virus G protein confirmed that the BRS and HRS virus attachment proteins were antigenically distinct. The recombinant vectors expressing the BRS virus G protein are currently being used to study the abilities of the G protein to elicit a protective immune response in cattle and to protect cattle against live virus challenge.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grants AI R37 12464 and AI 20181 (G.W.W.) from the National Institute of Allergy and Infectious Diseases and received support from the World Health Organization Programme on Vaccine Development. Support for maintenance of the Wisconsin-GCG computer programs utilized here was provided by the Center for AIDS Research grant P30 AI27767. K.A. was supported by training grant T-32-HL 07553 from the National Institutes of Health.

We thank Vicky Amann for excellent technical assistance.

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