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# Antigenically Distinct G Glycoproteins of BRSV Strains Share a High Degree of Genetic Homogeneity

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Bovine respiratory syncytial (BRS) virus can be divided into antigenic subgroups based on the reactivity of monoclonal antibodies (mAbs) to the attachment glycoprotein, G. Further, the polyclonal antibody response of calves vaccinated with recombinant vaccinia viruses expressing the G protein of a particular subgroup is also subgroup-specific. To investigate the genetic basis for the antigenic heterogeneity of the BRS virus G protein, the genes for the G protein from 6 BRS virus strains representative of the antigenic subgroups were cloned, sequenced, and compared with the prototype subgroup A strain, 391-2. There was only 10% nucleic acid difference and 15% amino acid difference between strains from different subgroups. These findings are in sharp contrast to the situation with human RS virus, where there is a 45% difference in amino acid identity between subgroups. In fact, the extent of amino acid difference between BRS virus subgroups is similar to the level of heterogeneity observed within human subgroups. Analysis of the reactivity of mAbs with peptides from the cysteine-rich region (174–188) of the G protein representing each antigenic subgroup indicated that amino acids at positions 180, 183, and possibly 184 are important in subgroup distinction. Taken together, these data suggest that although the genetic variation responsible for the antigenic differences determining subgroups among BRS viruses is more limited than that observed among human RS virus subgroups, the amino acid differences that exist have a profound effect upon antibody recognition. 

## INTRODUCTION

Bovine respiratory syncytial (BRS) virus is an economically significant respiratory pathogen of cattle which shares many pathological and epidemiological characteristics with the related human pathogen, human respiratory syncytial (HRS) virus (Stott and Taylor 1985; Baker 1991). Both of these viruses cause bronchiolitis and pneumonia, which are particularly severe in the young. Both viruses are distributed throughout the world and cause annual winter epidemics and recurrent infections. BRS virus infection in cattle is common and serological studies indicate that greater than 95% of cattle over the age of 3 years have been infected and that 70% of calves are infected within the first year of life (Stott and Taylor, 1985).

These viruses are classified as pneumoviruses within the family *Paramyxoviridae.* The protein composition of both viruses appears to be identical (Lerch *et al.,* 1989; Cash *et al.,* 1977) and many BRS virus proteins cross-react antigenically with their HRSV homologs, most notably the structural proteins, fusion (F), phosphoprotein (P), matrix (M), and nucleocapsid (N) (Lerch *et al.,* 1989; Orvell *et al.,* 1987). However, the attachment glycoprotein G of BRS virus does not cross-react with antiserum to the HRS virus G glycoprotein (Lerch *et al.,* 1989).

Both BRS virus and HRS virus strains possess antigenically heterogenous G proteins. Numerous studies based on the antigenic reactivity of HRS virus strains with monoclonal antibodies (mAbs) to the G protein have demonstrated two antigenic subgroups, A and B (Anderson *et al.,* 1985; Mufson *et al.,* 1985). Sequence comparison of the G genes of representative strains of these two human RS virus subgroups indicates that the G proteins can vary by as much as 47% between subgroups (Johnson *et al.,* 1987). In addition, within subgroup A, the G proteins can vary by as much as 20% (Cane *et al.,* 1991), while within the B subgroup they can vary by as much as 9% (Sullender *et al.,* 1991). Seroepidemiological studies have identified multiple lineages within each subgroup and the cocirculation of more than one lineage during an epidemic (Cane and Pringle, 1991; 1995; Hendry *et al.,* 1986; Garcia *et al.,* 1994).

Although there have been fewer studies of BRS virus strains, available data suggest that there are distinct antigenic subgroups of this virus as well. Initial analysis of the BRS virus strain, 391-2, demonstrated antigenic differences between the G protein of this strain and that of the BRS virus strain 127 (Lerch *et al.,* 1989). Recently, the antigenic characterization of BRS virus strains was extended to 17 additional strains using polyclonal sera and mAbs raised against the 2 strains 391-2 and 127

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TABLE 1

BRS Virus Strains Used in This Study

| Designation | Country of origin | Year of isolation | Antigenic subgroup |
|---|---|---|---|
| 391-2[a] | USA | 1985 | A |
| Snook[a] | UK | 1976 | A |
| 220-69[a] | Belgium | 1969 | Intermediate |
| Rispoval[b] | SKB vaccine | 1969 | Intermediate |
| Lelystad[c] | Holland | NK | Intermediate |
| 127[a] | UK | 1973 | B |
| 4642[a] | UK | 1976 | B |
| 220-60[a] | NK | NK | B |
| WBH[a] | Holland | 1986 | Untyped |

*Note.* NK, Not known.

[a] Analysis of antigenic subgroups and origin of strains has been described previously (Furze *et al.,* 1994).

[b] Rispoval vaccine strain of BRS virus was derived from strain 220-69 (Dr. N. Zygraich, personal communication).

[c] Westenbrink and Kimman (1987).

(Furze *et al.,* 1994). These results showed the presence of at least 2 antigenic subgroups of BRS virus. The G protein of 13 strains reacted with the polyclonal serum and panel of mAbs raised against the 391-2 strain, while the G protein of 5 strains reacted with the serum and a single mAb raised against the 127 strain. One strain, 220-69, exhibited intermediate reactivity, while another, WBH, was unreactive with any sera or mAb. In addition, structural differences were noted in the F proteins of the two subgroups. These antigenic analyses showed the occurrence of distinct BRS virus subgroups based on variation in the G protein. In contrast, a report comparing the G gene sequence from only two BRSV strains with that of the 391-2 G gene demonstrated at least 90% homology between these strains, leading the authors to suggest that BRS virus is monotypic (Mallipeddi and Samal, 1993).

To better understand the extent of variation in the G gene and its encoded protein sequence responsible for the antigenic differences reported by Furze *et al.* (1994), we have cloned and sequenced the G genes from three subgroup B strains, a second subgroup A strain, the intermediate strain 220-69, and the untyped strain WBH. These studies show that while these proteins are antigenically distinct, the amino acid sequences of the G proteins differed by only 15%. This variation is much lower than that observed between HRS virus antigenic subgroups and is more similar to that reported for HRS virus strains from within the same subgroup.

## MATERIALS AND METHODS

### Virus isolates

The BRS virus strains included in this study were described previously and grouped by reactivity with hyperimmune sera and mAbs (Table 1) (Furze *et al.,* 1994). The Lelystad strain of BRS virus (Westenbrink and Kimman, 1987) was obtained from Dr. J. van Oirschot, Lelystad, The Netherlands.

### Antibodies

Thirteen murine anti-G mAbs raised against the 391-2 isolate of BRS virus and one murine anti-G mAb 70 raised against the 127 isolate of BRS virus have been described previously (Furze *et al.,* 1994). mAbs 75 to 79, 86 and 87 were prepared from mice inoculated intraperitoneally with a recombinant vaccinia virus (rVV) expressing the G protein of the 127 strain of BRS virus (Furze *et al.,* unpublished results), followed by 0.2 ml of 10-fold concentrated serum-free supernatant from CV-1 cells infected with the 127 strain of BRS virus, as described previously (Furze *et al.,* 1994).

Polyclonal serum M117, which has a neutralizing titer of 1:16 and an ELISA titer of $\log_{10}$ 4.5 using the 127 strain of BRS virus as antigen, was obtained from a gnotobiotic calf immunized subcutaneously on two occasions with an immunoprecipitation band derived from a lysate of CK cells infected with the 127 strain of BRS virus and a hyperimmune calf serum, in Freund's incomplete adjuvant. Polyclonal serum 2106, which has a neutralizing titer of 1:10,000 and an ELISA titer of $\log_{10}$ 5.1 using the Snook strain of BRS virus as antigen, was obtained from a gnotobiotic calf infected by intranasal and intratracheal inoculation with the 391-2 strain of BRS virus on two occasions, 3 weeks apart, followed by a lysate of CK cells infected with the 391-2 strain, in Freund's incomplete adjuvant administered subcutaneously on two occasions 3 weeks apart. Sera was also obtained from calves vaccinated 6 weeks previously with $2 \times 10^8$ PFU of rVV expressing the G or F protein of the 391-2 strain of BRS virus (Lerch *et al.,* 1990, 1991).

### Isolation of viral RNA, cloning, and sequencing

Two synthetic oligonucleotides were used in cDNA synthesis and amplification. Oligonucleotide 5′G (5′ CACGGATCCACAAGTATGTCCAACC 3′) corresponds to nucleotides 10-25 of the 391-2 mRNA and is complementary to virion sense RNA and contains a *Bam*HI site. The second oligonucleotide 3785 (5′ CACGGATCCGGCTGTTGCCGCCATCC 3′) is complementary to nucleotides 12-28 of the F protein open reading frame of message sense RNA and also contains a *Bam*HI site.

Total cytoplasmic RNA was prepared from infected-cell lysates by extraction with hot phenol (Penman, 1969). First-strand cDNA was synthesized using a single primer and either Moloney murine leukaemia or avian myeloblastosis virus reverse transcriptase (Gibco BRL). Subsequently, the second strand was synthesized with *Taq* polymerase (Gibco BRL) and the addition of the 5′ primer. The cDNAs of the expected size were isolated on low-melt agarose gels, extracted, and ligated into pGEM3

downstream of the T7 promoter using the *Bam*HI site. Plasmid DNA was sequenced using a Sequenase kit (U.S. Biochemicals Corp.) and $^{35}S$ or the Applied Biosystems *Taq* DyeDeoxy Terminator Cycle Sequencing kit and a 373A DNA sequencer. Both strands from each of two independant clones were sequenced for each of the BRS virus strains. DNA and amino acid sequence analyses were performed with a VAX computer and the University of Wisconsin Genetics Computer Group programs (Devereux *et al.,* 1984).

The cloning, sequencing, and expression from recombinant rVV of the 391-2 G gene has been reported previously (Lerch *et al.,* 1990). For expression using vTF7-3, this gene was subcloned into pGEM4 downstream of the T7 promoter.

### Expression and immunoprecipitation of the G proteins from cDNA

The G proteins from each clone were expressed in cells using the rVV expressing T7 polymerase (vTF7-3) (Fuerst *et al.,* 1986). Semiconfluent monolayers of HuTK$^-$143B cells in 60-mm dishes were infected with vTF7-3 at an m.o.i. of 5 PFU per cell. One hour later the cells were transfected with 5 $\mu$g of plasmid DNA using 20 $\mu$l of lipofectin (Gibco BRL) in minimal essential medium (MEM) per dish. Five hours postinfection, the medium was aspirated and replaced with MEM containing 50 $\mu$Ci/ml [$^3$H]glucosamine (40 Ci/mmol; ICN). The monolayers were incubated at 37° for 5 hr, at which time cytoplasmic extracts were prepared as described previously (Wertz *et al.,* 1989). For immunoprecipitation, aliquots equivalent to one-half of the lysate per dish were incubated with hyperimmune sera or mAb for 90 min at room temperature. The antigen–antibody complexes were then precipitated as described previously (Roberts *et al.,* 1994), with the exception that 20 $\mu$l of Protein G–Sepharose (Pharmacia) was used to precipitate complexes containing mAbs. After washing, the samples were resuspended in SDS–PAGE sample buffer and analyzed by SDS–PAGE on 10% polyacrylamide gels. Following electrophoresis, the gels were fixed and soaked in 1 *M* sodium salicylate for 30 min before drying. Dried gels were exposed to Fuji RX film at −70°.

### Peptide synthesis and ELISAs

Four peptides corresponding to amino acids 174–188 of the G protein of the 391-2, 220-69, 127, and WBH strains of BRS virus were synthesized on an ABI 430A peptide synthesizer using f-moc chemistry and purified by reverse-phase HPLC (Table 2). Peptides were dissolved in sterile distilled water, diluted in 0.1 *M* carbonate buffer, pH 8.2, and coated onto 96-well immunosorbent plates (Nunc Maxisorb Flow) at 1 $\mu$g/well in 100 $\mu$l buffer. The plates were blocked for 1 hr using 5% porcine sera in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PS/PBS/Tw). After washing in PBS containing 0.05% Tween 20 (PBS/Tw), antibodies were titrated in PS/PBS/Tw. After a 1-hr incubation, the plates were washed in PBS/Tw and peroxidase-labeled conjugated anti-mouse IgG (KPL) was added and the plates were again incubated for 1 hr. After washing, bound conjugate was detected using 3,3′,5,5′-tetramethylbenzidine (TMB) (ICN Pharmaceuticals) as substrate. ELISAs using BRS virus antigens prepared from CK cells infected with different BRS virus strains were carried out as previously described (Furze *et al.,* 1994).

## RESULTS

### Characterization of mAbs to subgroup B BRS viruses

Previous studies characterizing the antigenic heterogeneity of BRS viruses utilized a panel of 22 mAbs specific for subgroup A strains but only a single mAb specific for subgroup B strains (Furze *et al.,* 1994). In order to expand our analyses of the subgroup B viruses, a new panel of mAbs raised against the subgroup B strain 127 was prepared. Hybridoma cultures secreting antibodies that reacted with only the 127 strain and not the Snook strain of the BRS virus were selected for cloning. The culture fluids of all seven of the resulting hybridoma

TABLE 2

Sequences of Synthetic Peptides

| Subgroup | BRSV strain | Peptide sequence | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 174 | | | | | | 180 | | | 183 | 184 | | | | 187 | 188 |
| A | 391-2 | S | T | C | E | G | N | L | A | C | L | S | L | C | H | I |
| Int[a] | 220-69 | S | T | C | E | G | N | L | A | C | S | S | L | C | Q | I |
| B | 127 | S | T | C | E | G | N | P | A | C | S | P | L | C | Q | I |
| U[b] | WBH | S | T | C | E | G | N | P | A | C | S | S | L | C | Q | I |

[a] Intermediate strain.
[b] Untyped strain.

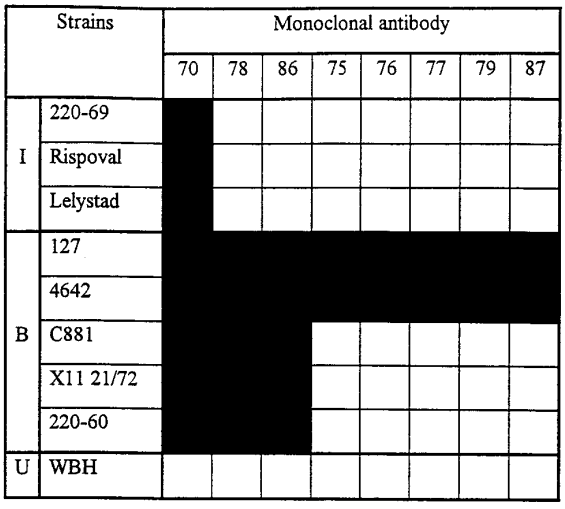



FIG. 1. Binding of subgroup B G mAbs to BRS virus strains. The strains of BRS virus were tested for their reactivity in an ELISA against subgroup B G mAbs. I, intermediate strains; U, untyped strain. Patterns: More than 50% (■) and less than 25% (□) of the values obtained for each antibody against the 127 strain of BRS virus.

clones recognized the G protein of the 127 strain of BRS virus by radioimmunoprecipitation or Western blotting (not shown). Although all seven mAbs, 75–79 and 86–87, recognized both the 127 and the 4642 strains of BRS virus in ELISA, only two mAbs, 78 and 86, recognized all the subgroup B strains studied (Fig. 1). None of the mAbs reacted with any of the strains previously designated as subgroup A, with intermediate strains, or with the WBH strain (Fig. 1), confirming previous studies of the antigenic heterogeneity of the G protein of BRS virus.

## Antibody response of calves vaccinated with rVV-G

Antigenic differences in the G protein of BRS virus strains have also been demonstrated previously using two hyperimmune polyclonal bovine sera raised against the 127 and 391-2 strains (Furze *et al.,* 1994). In order to examine the specificity of the polyclonal antibody response to the G protein further, sera from calves vaccinated with a rVV expressing the G protein of the 391-2 strain of BRS virus were examined for their ability to react with four antigenically distinct strains of BRS virus by ELISA (Table 3). Sera from these vaccinated calves recognized the Snook strain, another subgroup A strain, and also the 220-69 strain, an intermediate strain, although the titer was 10-fold lower. However, the sera failed to recognize either the 127, subgroup B, or the WBH, untyped strain. Nevertheless, mAbs recognizing the G protein of the Snook, 127, or 220-69 strains all had ELISA titers greater than $\log_{10}$ 6.0 against the respective antigens. Furthermore, all BRS virus strains were recognized by serum from calves vaccinated with a rVV expressing the F protein from the 391-2 isolate of BRS virus. Thus, polyclonal sera from calves vaccinated with rVV expressing the G protein of BRS virus are subgroup-specific.

## Nucleic acid sequence analysis

In order to determine the genetic basis for the antigenic differences in the G proteins identified by mAbs and polyclonal sera, the nucleotide and amino acid sequences of the attachment glycoprotein from the BRS virus strains 127, 4642, and 220-60 (subgroup B strains), 220-69 (an intermediate strain), Snook (a subtype A strain), and WBH (an untyped strain) (Furze *et al.,* 1994) were determined. Figure 2 shows the sequences of these strains in comparison with that of the 391-2 strain (Lerch *et al.,* 1990), the original subtype A strain. The first nine nucleotides of the 5′ sequence of the G mRNA were not determined because the 5′G primer annealed to nucleotides 10–25 of this region (Fig. 2). Alignment of the nucleotide sequences for the six strains determined in this study with the nucleotide sequence of the 391-2 G ORF did not introduce any gaps or insertions. There were three nucleotide deletions in the 3′ noncoding region of all BRS virus strains compared to the corresponding region of strain 391-2. Strains 220-69, 391-2, and Snook had the first termination codon (UAA, UAG, and UAA respectively) at position 787–789, while strains 127, 4642, 220-60, and WBH had the termination codon (UAA) at position 805–807. Correspondingly the 3′ noncoding region of the G mRNAs of strains 220-69, 391-2, and Snook were 18 nucleotides shorter than those of strains 127, 4642, 220-60, and WBH. Variation in the position of the termination codons has been described for HRS virus (Sullender, 1991) and for other strains of BRS virus (Mallipeddi and Samal, 1993).

## Deduced protein sequence analysis

The deduced protein sequence was determined from the nucleotide sequence for each gene and then aligned in comparison with the 391-2 strain (Fig. 3). The G mRNA of strains 127, 4642, 220-60, and WBH encoded a major open reading frame of 263 amino acids, 6 amino acids longer than the major open reading frame encoded by the G mRNA of strains 391-2, 220-69, and Snook. All

TABLE 3

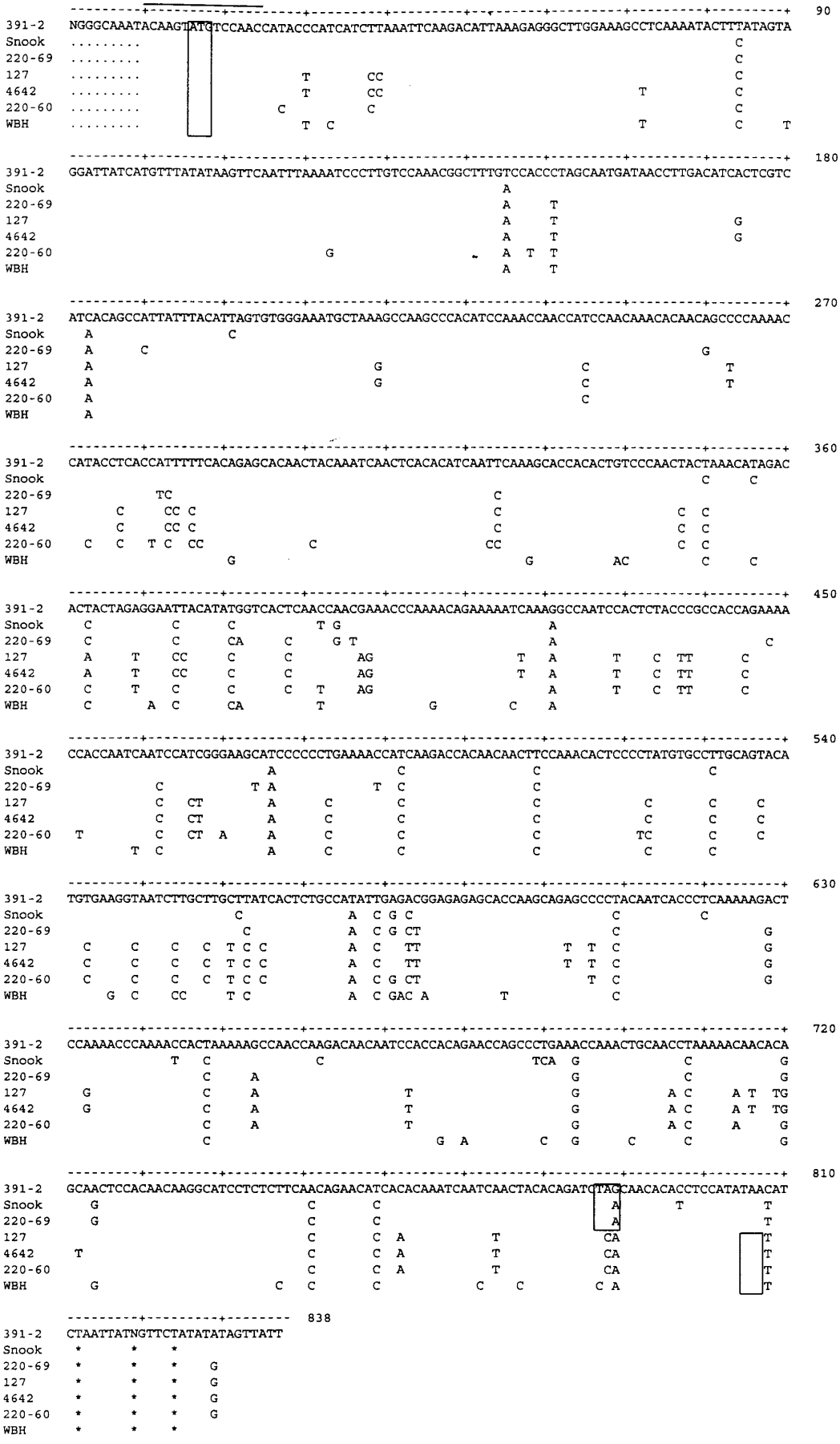
Recognition of Bovine RSV Strains by Sera from Calves Vaccinated with rVV Expressing the F or G Proteins of the 391-2 Strain of BRS Virus

| Vaccinia virus | Bovine RSV strains | | | |
|---|---|---|---|---|
| | Snook (A) | 220-69 (Int)[a] | 127 (B) | WBH (U)[b] |
| rV VF | 3.6 ± 0.2 | 3.4 ± 0.2 | 3.7 ± 0.3 | 3.3 ± 0.3 |
| rV VG | 3.0 ± 0.4 | 2.1 ± 0.3 | <1.5 | <1.5 |

*Note.* $\log_{10}$ ELISA antibody titer, mean ± standard deviation of groups of three to five calves.

[a] Intermediate.

[b] Untyped.


391-2 NGGGCAAATACAAGTATGTCCAACCATACCCATCATCTTAAATTCAAGACATTAAAGAGGGCTTGGAAAGCCTCAAAATACTTTATAGTA
Snook
220-69
127
4642
220-60
WBH
391-2 GGATTATCATGTTTATATAAGTTCAATTTAAAATCCCTTGTCCAAACGGCTTTGTCCACCCTAGCAATGATAACCTTGACATCACTCGTC
391-2 ATCACAGCCATTATTTACATTAGTGTGGGAAATGCTAAAGCCAAGCCCACATCCAAACCAACCATCCAACAAACACAACAGCCCCAAAAC
391-2 CATACCTCACCATTTTTCACAGAGCACAACTACAAATCAACTCACACATCAATTCAAAGCACCACACTGTCCCAACTACTAAACATAGAC
391-2 ACTACTAGAGGAATTACATATGGTCACTCAACCAACGAAACCCAAAACAGAAAAATCAAAGGCCAATCCACTCTACCCGCCACCAGAAAA
391-2 CCACCAATCAATCCATCGGGAAGCATCCCCCCTGAAAACCATCAAGACCACAACAACTTCCAAACACTCCCCTATGTGCCTTGCAGTACA
391-2 TGTGAAGGTAATCTTGCTTGCTTATCACTCTGCCATATTGAGACGGAGAGAGCACCAAGCAGAGCCCCTACAATCACCCTCAAAAAGACT
391-2 CCAAAACCCAAAACCACTAAAAAGCCAACCAAGACAACAATCCACCACAGAACCAGCCCTGAAACCAAACTGCAACCTAAAAACAACACA
391-2 GCAACTCCACAACAAGGCATCCTCTCTTCAACAGAACATCACACAAATCAATCAACTACACAGATCTAGCAACACACCTCCATATAACAT
391-2 CTAATTATNGTTCTATATATAGTTATT
Snook
220-69
127
4642
220-60
WBH

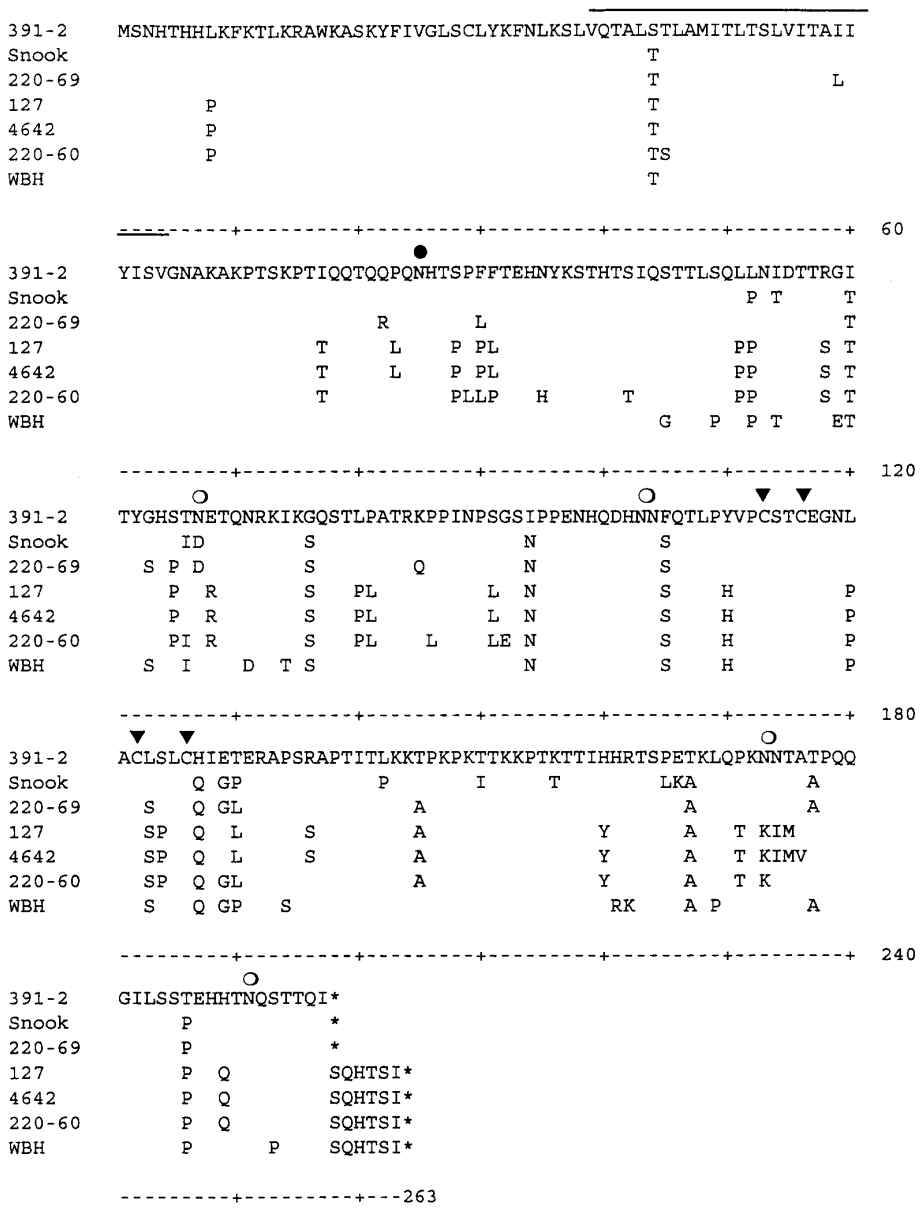



FIG. 3. Comparison of the deduced amino acid sequences among the G proteins of BRS virus strains. The sequence for the 391-2 is strain is shown; for the other strains only residues that differ from the 391-2 strain are shown. The crosses below the sequences are spaced every 10 amino acid residues and the number of the last residue for each line is shown to the right of the line. The horizontal line above the sequence indicates the signal/anchor domain. The four conserved cysteine residues are indicated (▼). The potential N-linked glycosylation sites are also indicated; (●) conserved between isolates; (○) nonconserved between isolates.

changes in the deduced protein sequences involved single amino acid changes and no insertions, deletions, or frameshifts were observed.

HRS virus G proteins and the 391-2 G protein are highly modified by the addition of N- and O-linked carbohydrates (Gruber and Levine, 1985; Wertz *et al.,* 1985). The deduced amino acid sequences of all of the BRS virus strains showed a high level (25%) of serine and threonine residues, the potential O-linked carbohydrate acceptor sites. There were also four potential N-linked glycosylation sites present in the extracellular domain from each strain, although only the site at amino acid residue 85

FIG. 2. Nucleotide sequence comparisons among the attachment protein G mRNAs of BRS virus strains as determined from cDNA sequence analysis. The sequence of the 391-2 G cDNA is shown (Lerch *et al.,* 1990); for other viruses only differences relative to 391-2 are shown. The line above the sequence indicates the nucleotides covered by the 5′ PCR primer. The consensus initiation codon and the termination codons for the different virus G mRNAs are boxed. The asterisks indicate the deletion mutations. + above the sequences are spaced every 10 nucleotides and the number of the last nucleotide on the line is indicated to the right of the sequence.

TABLE 4

Percentages of Nucleotide and Amino Acid Identities for G Genes and Proteins of BRS Virus

| Antigenic subgroups | | 391-2 | Snook | 220-69 | 127 | 4642 | 220-60 | WBH |
|---|---|---|---|---|---|---|---|---|
| A | 391-2 | | 95.0 | 94.5 | 90.2 | 90.2 | 90.2 | 92.3 |
| A | Snook | 92.2 (90.0) | | 96.3 | 91.6 | 91.4 | 92.0 | 94.8 |
| Int[a] | 220-69 | 99.2 (90.6) | 93.3 (91.6) | | 92.8 | 92.8 | 93.0 | 94.2 |
| B | 127 | 86.0 (82.2) | 85.6 (81.1) | 87.9 (84.8) | | 99.6 | 96.7 | 90.9 |
| B | 4642 | 86.0 (82.2) | 85.6 (81.1) | 87.9 (84.8) | 99.2 (99.0) | | 96.5 | 90.9 |
| B | 220-60 | 84.8 (81.3) | 85.9 (82.2) | 87.9 (85.4) | 94.6 (93.4) | 94.3 (92.3) | | 91.3 |
| U[b] | WBH | 89.5 (86.4) | 91.8 (89.0) | 91.4 (89.0) | 85.2 (80.7) | 85.2 (80.7) | 85.2 (81.3) | |

*Note.* Nucleotide identities (%) appear above the diagonal rule; amino acid identities (%) appear below the diagonal rule. Data in parentheses represent amino acid identity in the extracellular domain.

[a] Intermediate.

[b] Untyped.

was conserved among all seven strains (Fig. 3). There was a high level of proline residues (13%) as noted previously for both human and bovine G protein sequences.

There were four cysteine residues in the extracellular domain of the seven BRS virus strains. The spacing of these residues was identical to that observed with other BRS virus and HRS virus G glycoproteins (Cane *et al.,* 1991; Mallipeddi and Samal, 1993). The 13-amino-acid sequence preceding the first cysteine residue which is conserved among both HRS virus subgroups (Johnson *et al.,* 1987) was not conserved among the BRS virus strains.

Other features that were conserved between the G proteins of the BRS virus strains and the HRS virus strains include a methionine at amino acid residue 48, which is the initiation site for the shed form of the G protein (Roberts *et al.,* 1994), a highly conserved amino terminal cytoplasmic domain, and an extended hydrophobic domain from residues 38 to 66, which is the signal/anchor domain (Lichtenstein *et al.,* 1996) (Fig. 3).

## Sequence comparison

The nucleotide and deduced amino acid sequences for the seven BRSV strains were compared to determine the extent of sequence identity. The seven G mRNA sequences varied from only 1 to 10% (Table 4), with the greatest divergence seen between subgroup A and subgroup B strains and between subgroup B strains and the untyped WBH strain.

The levels of identity among the G proteins of the seven BRS virus strains were 85–99%, with the greatest divergence seen between subgroup A and subgroup B strains and between the 127 strain and the WBH strain. Two subgroup B strains, 127 and 4642, which were isolated on different farms in the United Kingdom, 3 years apart, differed from each other by only one amino acid. Most of the variation between the deduced protein sequences was limited to the extracellular domain. Only four amino acid changes were detected in the cytoplasmic or the transmembrane domains among the six BRS virus strains and 391-2, and the extracellular domains of the G protein varied by less than 20% between strains. The amino acid variability is not evenly distributed through the protein. The first 76 amino acids at the amino terminus, which are conserved, are followed by amino acids 77–91, which are fairly variable. There is another small conserved area, followed by highly variable domains between amino acids 105 and 136, amino acids 181 and 196, and amino acids 220 and 247.

## Protein expression

One of the eventual goals of our studies of BRS virus G proteins is an improved understanding of the structural features responsible for the antigenic variation. To confirm that the initial antigenic characterization made using virus did map to the G protein and to validate the cloned G genes, the G proteins expressed from the cDNAs of each gene were analyzed with subgroup-specific hyperimmune sera and mAbs (Fig. 4). Immunoprecipitation of cytoplasmic extracts prepared from cells transfected with 391-2 or the two group B strains, 127 and 4642, demonstrated that these clones had antigenic reactivity identical to that of the G proteins produced during infection (Furze *et al.,* 1994). As expected, the G proteins from the 127 and 4642 clones reacted with M117 serum (Fig. 4, lanes 2 and 3), and with mAb 70 (Fig. 4, lanes 10 and 11), while the 391-2 G protein was not recognized by M117 serum (Fig. 4, lane 4). The G protein expressed from the 391-2 clone reacted with 2106 serum (Fig. 4, lane 8) and with mAb57 (Fig. 4, lane 13). Both of the subgroup B G proteins cross-reacted weakly with the 2106 serum (Fig. 4, lanes 6 and 7), as had been reported previously for the G proteins produced during infections with these two virus strains (Furze *et al.,* 1994).

One of two independent clones obtained from the 127 strain was not recognized by mAb 70, which was raised

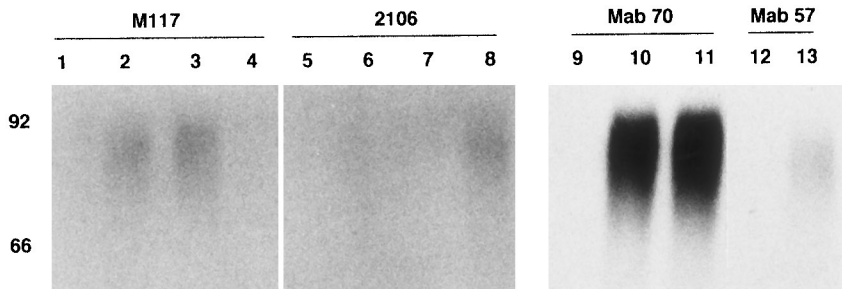



FIG. 4. Expression of the BRSV G proteins from cDNA clones. Semiconfluent monolayers of HuTK⁻143B cells were infected with vTF7-3 and 1 hr later transfected with the cDNA of each of the BRSV G gene clones. The control cells were infected with vTF7-3 and treated with lipofectin, but did not receive any DNA. Medium containing [$^3$H]glucosamine was added 5 hr after transfection. Cell extracts were immunoprecipitated with the polyclonal sera M117 (lanes 1–4) or 2106 (lanes 5–8) or with mAbs 70 (lanes 9–11) or 57 (lanes 12 and 13). The precipitated proteins were analyzed by SDS–PAGE on 10% polyacrylamide gels. Mock-transfected cells (lanes 1, 5, 9, and 12); cells transfected with BRS virus strain 127 (lanes 2, 6, and 10); cells transfected with strain 4642 (lanes 3, 7, and 11) and cells transfected with strain 391-2 (lanes 4, 8, and 13).

against the 127 strain, although the protein was recognized by the polyclonal serum M117 (data not shown). Comparison of the nucleotide sequence of this clone with the nucleotide sequence of a second 127 clone that expressed a G protein that was recognized by mAb 70 revealed a difference at nucleotide 631. This nucleotide difference results in an amino acid change from a lysine at amino acid residue 209 in the G protein recognized by mAb 70 to a gluramic acid in the unreactive protein.

At present there are no polyclonal or monoclonal antibodies which react with the G protein from the WBH strain. Therefore to confirm that a G protein was expressed from this clone, total cytoplasmic extracts from untransfected cells and from cells transfected with the 4642 clone and the WBH clone labeled in the presence of [$^3$H]glucosamine were compared. A highly glycosylated protein was detected in the cytoplasmic extracts prepared from cells transfected with the WBH clone which comigrated with the G protein from the cells transfected with the 4642 clone (data not shown). No glycosylated protein was detected at this molecular weight in the extract from the mock-transfected cells. Additionally, this protein could not be immunoprecipitated with either the M117 or the 2106 sera (data not shown).

### Binding of antibodies to synthetic peptides

Akerlind-Stopner *et al.* (1990) identified amino acids 174–188, which comprises the cysteine-rich region, as representing an important HRS virus subgroup-specific site. Therefore, we examined mAbs that distinguish strains of BRS virus for their ability to react with peptides corresponding to amino acid 174 to 188 of the G protein from four BRS virus strains. In this region, 11 of the residues are conserved, as is the spacing of the cysteine residues among the four strains. However, amino acids at four positions, residues 180, 183, 184, and 187, vary (see Table 2).

The 15 mAbs that react with both the 391-2 and the 220-69 strains reacted with a peptide corresponding to the region 174–188 of the 391-2 G protein as well as to the peptide corresponding to this region of the 220-69 G protein (Fig. 5). Of the 7 mAbs that react only with subgroup A viruses and not with the intermediate strains, two, mAbs 47 and 57, failed to react with the peptide from either strain, while four, mAbs 62, 46, 53, and 69, recognized the 391-2 peptide but not the 220-69 peptide, and one mAb, 61, reacted weakly with peptides from both 391-2 and 220-69. None of the mAbs raised against the 127 G protein reacted with any of the peptides and the WBH peptide was not recognized by any of the mAbs. These studies highlight the importance of the cysteine-rich region in recognition of BRS virus subgroups by mAbs.

## DISCUSSION

The genetic variability of the two antigenically distinct subgroups of HRS virus has been well characterized (Cane and Pringle, 1991; Johnson *et al.,* 1987; Sullender *et al.,* 1991). However, the limited genetic analysis of the G genes of BRS virus strains to date has suggested that this virus is monotypic (Mallipeddi and Samal, 1993). Recently, an analysis of the antigenic relatedness of a large number of BRS virus strains has demonstrated the presence of at least two BRS virus subgroups (Furze *et al.,* 1994). In the work reported here, we have examined the genetic basis for the antigenic heterogeneity of the BRS virus G protein. The divergence among the G proteins of the seven BRS virus strains analyzed here, which are representative of the antigenic groups, was much lower in comparison to the variability observed between the two HRS virus subgroups. Thus, there was up to 15% variability in the deduced amino acid sequence between BRS virus G proteins, whereas there is 47% variability in the G proteins between the prototype strains of the two subgroups of HRS virus (Johnson *et al.,* 1987). In fact the extent of amino acid difference between BRS virus subgroups is similar to that seen within subgroups of HRS virus (Cane *et al.,* 1991; Sullender *et al.,* 1991).

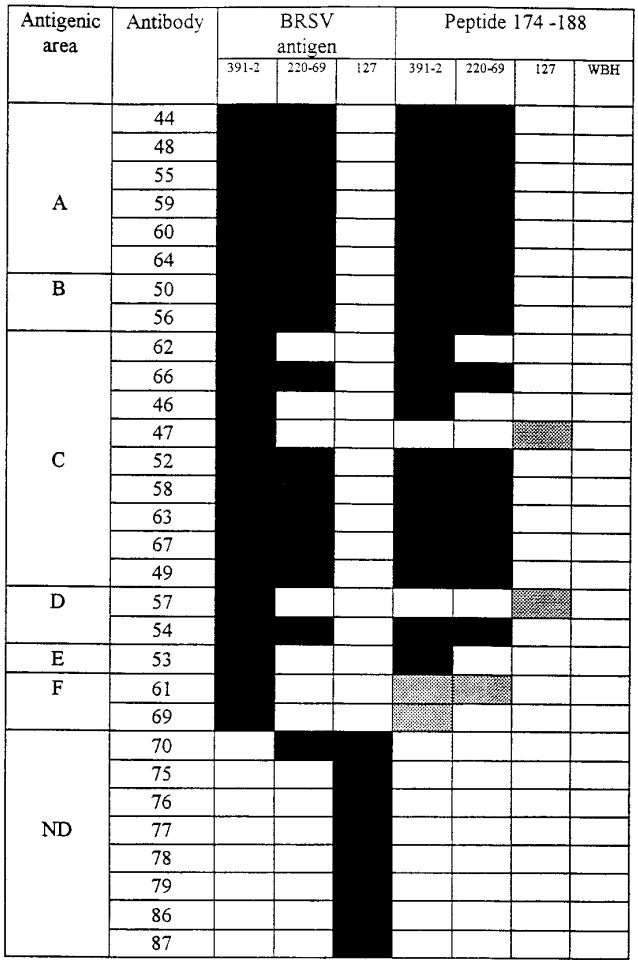



FIG. 5. Binding of antibodies to synthetic peptides. G mAbs were assayed in an ELISA against peptide 174–188 from four BRS virus isolates. Patterns: more than 50% (■), 25–50% (▨), and less than 25% (□) of the values obtained for antibody against BRS virus antigen. A to E, competition groups of the mAbs (Furze *et al.,* 1994). ND, competition group not determined.

The WBH strain, which is not reactive with any of the available mAbs against the G protein or with polyclonal sera of subgroup A or subgroup B BRS viruses, was nevertheless 9 and 15% divergent with the representatives of subgroup A and subgroup B, respectively. Despite the high degree of amino acid identity between BRS virus strains, polyclonal sera from hyperimmune calves recognize only the G protein of strains belonging to the homologous subgroup by immunoprecipitation. Furthermore, sera from calves vaccinated with rVV-G from the 391-2 strain recognizes only subgroup A strains and to a lesser extent the intermediate strain. Thus our genetic analysis combined with the previous antigenic analysis of these strains (Furze *et al.,* 1994) suggests that although the genetic variation responsible for antigenic differences among BRS virus subgroups is more limited than that observed among HRS virus subgroups, the amino acid differences that exist have a profound effect upon antibody recognition.

In the BRS virus strains studied here, there was amino acid variation at only four positions in the cysteine-rich region of the G protein. Three of these, at positions 180, 183, and 184, appear to be important for the antigenic reactivity of these strains. These residues also differ between subgroup A and B HRS virus strains (Akerlind-Stopner *et al.,* 1990) and a synthetic peptide corresponding to amino acids 174–188 of HRS virus reacted with subgroup-specific anti-G mAbs (Norrby *et al.,* 1987). Previous studies showed that the epitopes recognized by mAbs 44 to 69 overlapped extensively (Furze *et al.,* 1994). The close relationship of the epitopes recognized by these mAbs is confirmed by the finding that they all bind to a synthetic peptide corresponding to the region 174–188 of the 391-2 strain G protein. Differences in epitope recognition by the mAbs are probably due to differences in the contributions made by individual amino acids within the peptide to antibody binding.

Binding of antibodies to the region 174–188 of the G protein broadly correlated with their reactivity to strains from the different BRS virus subgroups. Thus, the 15 mAbs that recognized both subgroup A and intermediate viruses reacted with peptide 174–188 of both the 391-2 and the intermediate strain, 220-69, which differ only at amino acids 183 and 187. However mAbs 46, 53, 62, and 69, which recognize only subgroup A viruses, including both 391-2 and Snook, did not bind to the peptide from the 220-69 strain. Since the Snook strain differs from 220-69 only at position 183 in the region 174–188, it is likely that the loss of binding of these three mAbs is due to the substitution of serine for leucine at this position in the intermediate strain. None of the anti-391-2 mAbs which recognized the 174–188 peptide corresponding to the 220-69 G peptide bound to the WBH peptide, although these peptides differ only at amino acid 180. Thus, the replacement of lysine at this position in the 220-69 G peptide by proline in the WBH G protein results in the loss of binding by these mAbs. These findings highlight the importance of amino acids at positions 180 and 183 in determining the antigenic subgrouping of BRS virus strains described previously (Furze *et al.,* 1994).

The G proteins of the three subgroup B strains studied here all have a proline at position 184, whereas the G proteins of all other strains studied have a serine at this position. This is the only difference between the 174–188 peptides of the 127 and WBH G proteins. Although the anti-391-2 mAbs 47 and 57 failed to react with the peptide corresponding to the 391-2 strain, they did react weakly with the corresponding peptide of the 127 strain, but not with that of the WBH peptide, suggesting that the amino acid at position 184 may also be important in recognition by antibody.

Although none of the subgroup B-specific G mAbs recognized any of the peptides corresponding to the region 174–188 of the G protein, a mutation at amino acid 209 of lysine to gluramic acid resulted in the loss of reactivity

of mAb 70 with the G protein, suggesting that this region of the G protein may also be involved in mAb recognition. The region corresponding to residues 204 to 209 has been identified as a linear epitope on the G protein of subgroup A HRS viruses (Garcia *et al.,* 1994). In this region of the G protein of BRS virus, strains that are recognized by mAb 70 differ from those that are not recognized by a replacement of A to T at position 205, suggesting that this substitution may also determine recognition by mAb 70. This suggestion has been confirmed using synthetic peptides (Langedijk *et al.,* in preparation).

In conclusion, although there is a high degree of amino acid identity in the G protein among strains of BRS virus, the amino acid differences that are present have a profound effect on its antigenicity. Furthermore the finding that vaccination with rVV expressing the G protein induces a subgroup-specific antibody response is similar to that observed with rVV expressing G proteins of HRS virus subgroups (Stott *et al.,* 1986; Sullender and Britt, 1996) and may have important consequences for the design of vaccines against BRS virus.

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