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# Molecular analysis of the S1 subunit of the spike glycoprotein of respiratory and enteric bovine coronavirus isolates

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**Abstract**

It is unclear whether respiratory and enteric bovine coronavirus (BoCV) strains are distinctive in biological, antigenic and genetic characteristics. In the present study, we analyzed the nucleotide and amino acid sequence of the S1 subunit of the S glycoprotein, including the cleavage site, of both respiratory ($n = 5$) and enteric ($n = 3$) BoCV isolates including two paired isolates from the same feedlot animals and compared them with the prototype Mebus and two enteric and one respiratory BoCV strains from Quebec. A total of 75 polymorphic nucleotides were identified in the S1 subunit of the spike glycoprotein of BoCV isolates compared with the Mebus strain. These polymorphisms led to 42 amino acid changes at 38 distinct sites. The amino acid changes were distributed throughout the S1 subunit with clustering around residues 40–118, 146–179, and 458–531. Among these variations, only 19 amino acid substitutions altered the charge, hydrophobicity and surface probability of the protein. Based on phylogenetic analysis, our respiratory and enteric isolates clustered into two major groups with two subgroups. Although, there were only a few amino acid changes between the respiratory and enteric paired isolates, the other two respiratory isolates, one isolated from the same farm as a paired strain and the other from a different farm, showed more sequence diversity. Amino acid alterations in residues 113, 115, 118, 146, 148, 501, 510 and 531 of respiratory isolates conferred significant changes in the predicted secondary structure compared with the prototype winter dysentery (WD) and the calf diarrhea (CD) strains of BoCV. In conclusion, the data suggests that respiratory strains of BoCV may differ genetically from the classical calf enteric and adult WD strains. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Respiratory and enteric BoCV; S1 subunit; Sequencing; Allelic variation

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## 1. Introduction

Bovine coronavirus (BoCV), a member of the family *Coronaviridae*, order *Nidovirales*, (Van Regenmortel et al., 2000) possesses a single-stranded, enveloped, non-segmented RNA genome of positive polarity (De Vries et al., 1997). The virion contains five major structural proteins: the nucleocapsid (N) protein, the transmembrane (M) protein, the hemagglutinin/esterase (HE) protein, the spike (S) protein and the small membrane (E) protein (Chouljenko et al., 1998; Lai and Cavanagh, 1997; Saif, 1993). The HE glycoprotein has receptor binding and detachment functions similar to the HE of influenza C virus mediated by an acetylesterase (AE) which hydrolyses an ester bond to liberate acetate linked to the 9-O-position of the sialic acid containing receptors of erythrocytes and susceptible cells. The S glycoprotein also recognizes the 9-O-acetylated sialic acid, and it requires fewer of these receptors on the surface of erythrocytes for agglutination than the HE protein (Schultze et al., 1991). Thus the S glycoprotein is the major hemagglutinin of BoCV (Schultze and Herrler, 1994) and it is also proposed to be responsible for the primary attachment of BoCV to other cell surface receptors (Schultze et al., 1991). The variation in host range and tissue tropism of coronaviruses is largely attributable to variations in the S glycoprotein (Gallagher and Buchmeier, 2001). The S glycoprotein is a type 1 membrane glycoprotein that carries distinct functional domains near the amino (S1) and carboxy (S2) termini (Gallagher and Buchmeier, 2001). The S1 subunit is peripheral and is associated with receptor binding functions whereas the S2 subunit is a transmembrane protein mediating fusion of viral and cellular membranes (Cavanagh, 1995). In general, the S glycoprotein facilitates viral attachment to susceptible cells, causes cell fusion, and induces neutralizing antibodies. Of the two functional subunits, S1 and S2 that contain several antigenic domains, S1 appears to most efficiently elicit monoclonal antibodies (Mabs) with higher neutralizing activity (Vautherot et al., 1992; Yoo et al., 1990, 1991; Yoo and Deregt, 2001).

BoCV is associated with severe diarrhea in newborn calves (CD), winter dysentery (WD) in adult cattle and respiratory tract infections in calves and feedlot cattle (Cho et al., 2000; Clark, 1993; Lathrop et al., 2000; Saif et al., 1991; Storz et al., 2000). Respiratory BoCV strains have been detected by ELISA and isolated from nasal swab samples of feedlot cattle with respiratory tract disease after shipping (Cho et al., 2001a,b; Hasoksuz et al., 2001; Lathrop et al., 2000; Silva et al., 1999; Storz et al., 2000). It is still unclear whether respiratory and enteric BoCV strains are distinctive in biological, antigenic and genetic characteristics. Previous researchers demonstrated that some BoCV strains isolated from the respiratory tract had different biological, antigenic (Hasoksuz et al., 1999a,b; Lin et al., 2000) and genetic (Chouljenko et al., 1998; Gelinas et al., 2001) properties compared with enteric BoCV strains, whereas others (Reynolds et al., 1985; Zhang et al., 1994) did not detect any consistent differences. Interpretation of these results is difficult because the strains were isolated several years apart and the studies did not compare isolates from a single farm or from the same animal. Furthermore, there are few studies of cross-protection between enteric and respiratory BoCV strains to confirm the impact of the antigenic or genetic differences identified.

To examine if enteric and respiratory BoCV strains differ genetically, we analyzed the nucleotide and the deduced amino acid sequences of the S1 subunit of the S glycoprotein from: (a) concurrent respiratory and enteric isolates from the same animals; (b) isolates from the same farm collected at different time points and in different animals; and (c) strains from different farms, but isolated during the same year.

## 2. Materials and methods

### 2.1. Viruses

The DBA was a previously characterized enteric BoCV strain isolated in 1990 from feces of an adult dairy cow with winter dysentery (Benfield and Saif, 1990; Tsunemitsu and Saif,

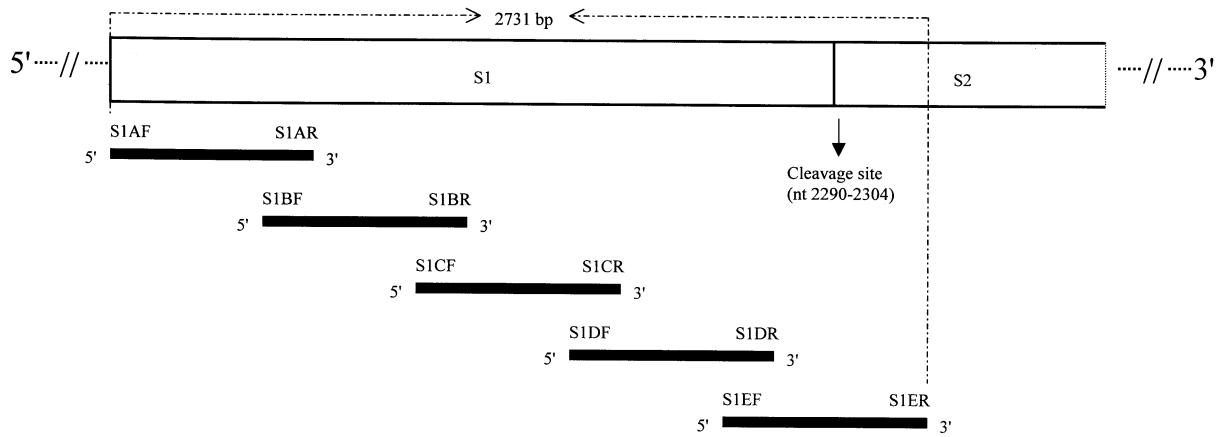



Fig. 1. S glycoprotein gene of BoCV and sequencing strategy. The figure depicts the location and direction of the oligonucleotide primers used to sequence a 2731 bp region.

1995). The respiratory strain 220 was isolated from the nasal sample of a feedlot calf in Lucasville, Ohio in 1998 (Hasoksuz et al., 1999b). Respiratory isolates 182 and 232 were from nasal samples of two feedlot cattle and isolates 187FS and 187NS were paired fecal and nasal samples from the same feedlot calf, respectively, at the Ohio Agricultural Research and Development Center (OARDC) feedlot in 2000 (Cho et al., 2001b). Strains 65FS and 65NS were isolated from paired fecal and nasal samples, respectively, from the same calf at the OARDC feedlot in 2001 (Hasoksuz et al., 2001). Isolates originated from samples collected from animals with overt enteric and/or respiratory signs.

### 2.2. RT-PCR

Total RNA was extracted from human rectal tumor cell culture (HRT-18) supernatants of plaque-purified BoCV isolates by using TRIZOL LS (Gibco BRL, Life Tech, Grand Island, NY) reagent according to the manufacturer's instructions. A One-step RT-PCR assay was performed as described by Cho et al. (2001a,b).

The oligonucleotide primers used in the RT-PCR were designed from the published sequence of the S gene of the Mebus strain (GenBank accession No. M31053) including the S1 subunit and cleavage region. The schematic of the location of the primer pairs for the S glycoprotein amplification and sequencing is shown in Fig. 1. The primer sequences and predicted product sizes are shown in Table 1.

Table 1
The oligonucleotide primers designed from the S glycoprotein gene of the BoCV Mebus strain (GenBank accession No. M31053) used for DNA sequencing

| Name | Sequence | Location |
|---|---|---|
| S1AF[a] | 5′-ATG TTT TTG ATA CTT TTA ATT-3′ | 1–21 |
| S1AR[b] | 5′-AGT ACC ACC TTC TTG ATA AA-3′ | 654–635 |
| S1BF | 5′-ATG GCA TTG GGA TAC AG-3′ | 549–565 |
| S1BR | 5′-TAA TGG AGA GGG CAC CGA CTT-3′ | 1039–1018 |
| S1CF | 5′-GGG TTA CAC CTC TCA CTT CT-3′ | 782–801 |
| S1CR | 5′-GCA GGA CAA GTG CCT ATA CC-3′ | 1550–1531 |
| S1DF | 5′-GTC CGT GTA AAT TGG ATG GG-3′ | 1460–1479 |
| S1DR | 5′-TGT AGA GTA ATC CAC ACA GT-3′ | 2286–2267 |
| S1EF | 5′-TTA CAA AAA TCA AAC ACA GAC AT-3′ | 1855–1877 |
| S1ER | 5′-AAA CTT TAT TAC AAT CGC TTC C-3′ | 2731–2710 |

[a] F: upstream primer.
[b] R: downstream primer.

### 2.3. DNA sequencing

The RT-PCR products were purified using a PCR purification kit (Cat. No. 28104, Qiagen, Valencia, CA) according to the manufacturer's instructions. The DNA sequencing was done using an automated DNA sequencer (ABI system 377, Applied Biosystem Inc., Foster City, CA).

Nucleotide sequences of our BoCV isolates were first compared for the S1 subunit sequence of the Mebus calf diarrhea strain (GenBank accession No. M31053), Quebec BCQ 3994 respiratory strain (GenBank accession No. AF 339836), Quebec BCQ 7373 winter dysentery (WD) strain (GenBank accession No. AAG40595), and Quebec BCQ 1523 calf diarrhea (CD) strain (GenBank accession No. AF239307) using the Clustal method of the Lasergene Biocomputing Software (DNASTAR Inc. Madison, WI). The deduced amino acid sequences were then assembled and analyzed on the Protean module of the DNASTAR.

## 3. Results

### 3.1. Patterns of allelic variation in the S1 subunit of the BoCV spike glycoprotein

The S1 subunit of the S glycoprotein of coronaviruses is a 100 kDa protein with 768 amino acid residues. Nucleotide and amino acid sequence analysis of this entire S1-glycoprotein region of respiratory and fecal isolates revealed several changes. Among all isolates analyzed, a total of 75 polymorphic nucleotides were identified in the S1 subunit of the spike glycoproteins of the BoCV isolates compared with the Mebus strain (data not shown). These polymorphisms led to 42 amino acid changes at 38 distinct sites (Table 2). The amino acid differences were throughout the protein with clustering around residues 40–118, 146–179 and 458–531 (Table 2). Based on the total number of amino acid substitutions, a phylogenetic tree was constructed using the Clustal method (MegALIGN, DNASTAR, Madison, WI) (Fig. 2). Phylogenetic data showed that all isolates from the present study including WD strain DBA clustered with the respiratory strain BCQ 3994, while the BCQ enteric strains clustered on a separate major branch (Table 2, Fig. 2). Thus isolates were classified into two major groups with two subgroups (Groups I, Ia, II and IIa) compared with the Mebus strain (Fig. 2). Group I consisted of enteric and respiratory BoCV isolates collected concurrently from the two different calves (65FS/65NS; 2001, 187NS/187FS; 2000) in the same feedlot at different times. This group also included the respiratory strain 232NS, isolated from the same feedlot in the year 2000. Groups Ia and II included 182NS and 220NS, respectively, and Group IIa included DBA, a virulent WD BoCV strain. Interestingly the enteric and respiratory BoCV pairs collected concurrently from two feedlot calves were identical to each other except at amino acid 447 (T → I) for the 65FS/65NS pair and amino acids 49 (T → I) and 204 (S → N) for the 187FS/187NS pair (Table 2). None of these changes altered the predicted secondary structure. However, variations in amino acids 113, 118, 146, and/or 510 conferred properties that would potentially alter the predicted secondary structure.

### 3.2. Group characteristics of the amino acid substitutions in the BoCV S1 protein

Group I isolates did not differ from the Mebus and BCQ strains sequences at amino acid residue 118 while Group II and Group IIa had an M → K substitution (Table 2). Isolates in Groups I, Ia, II and BCQ.3994 (a respiratory strain) differed from Mebus, BCQ 7373 and BCQ 1523 strains at amino acid 113 (I → V) and 510 (S → T) (except Group Ia) that led to a reduced charge and hydrophilicity. Amino acid 115 (K → D) substitution in Group I, Ia isolates and BCQ strains, led to the loss of a hydrophobic region and reduced the total charge. This amino acid change (115, K → N) was unique to the Group II isolate and led to a lower surface probability and hydrophilicity, while the change (115, K → Y) in Group IIa led to a slight increase in hydrophilicity. Groups I, Ia, II isolates and BCQ 3994 had identical amino acid substitutions at residues 113, while I, II and BCQ3994 were identical at residues 510 and 531.

Table 2
The amino acid differences between a.a. 34 and 779 of the S glycoprotein of Mebus and respiratory and enteric BoCV isolates

| A.A. # | Mebus | Group I[a] | | | | | Group Ia | Group II | Group IIa |
|---|---|---|---|---|---|---|---|---|---|
| | | 65FS[b] | 65NS[c] | 187FS | 187NS | 232NS | 182NS | 220NS | DBA |
| 40 | I | T | T | T | T | T | T | T | T |
| 49 | T | * | * | * | I | * | * | * | * |
| 88 | R | T | T | T | T | Ts | T | T | T |
| 100 | I | T | T | T | T | T | T | T | T |
| 113 | I | V | V | V | V | V | V | V | V |
| 115 | K | D | D | D | D | D | D | N | Y |
| 118 | M | * | * | * | * | * | * | K | K |
| 146 | N | I | I | I | I | I | I | * | * |
| 148 | D | G | G | G | G | G | G | * | * |
| 154 | L | F | F | F | F | F | F | * | * |
| 169 | H | N | N | N | N | N | N | N | N |
| 173 | H | N | N | N | N | N | N | * | * |
| 175 | N | * | * | * | * | * | * | T | T |
| 179 | K | Q | Q | Q | Q | Q | Q | Q | Q |
| 204 | N | * | * | S | * | * | * | * | * |
| 225 | D | * | * | H | H | * | * | * | * |
| 248 | L | M | M | M | M | M | M | M | M |
| 253 | S | N | N | N | N | N | N | N | G |
| 256 | M | L | L | L | L | L | L | L | * |
| 365 | S | * | * | * | * | * | * | * | F |
| 447 | T | * | I | * | * | * | * | * | I |
| 458 | F | S | S | S | S | S | S | S | S |
| 465 | V | A | A | A | A | A | A | A | A |
| 470 | H | D | D | D | D | D | D | D | D |
| 484 | S | T | T | T | T | T | T | T | T |
| 499 | N | S | S | S | S | S | * | S | S |
| 501 | P | S | S | S | S | S | S | S | F |
| 509 | N | * | * | * | * | * | T | * | * |
| 510 | S | T | T | T | T | T | * | T | * |
| 531 | N | D | D | D | D | D | * | D | * |
| 543 | S | A | A | A | A | A | A | A | A |
| 571 | Y | H | H | H | H | H | * | * | * |
| 578 | T | S | S | S | S | S | S | S | S |
| 716 | T | 8 | * | * | * | * | * | I | I |
| 743 | S | * | * | * | * | * | I | * | * |
| 754 | S | * | * | * | * | * | N | * | * |
| 769 | A | S | S | S | S | S | S | S | S |
| 778 | T | N | N | N | N | N | N | N | N |

*, same as Mebus.
[a] Groupings based on Fig. 2.
[b] FS: isolates from fecal samples.
[c] NS: isolates from nasal samples.

Groups II and IIa had similar changes at amino acids 118 and 175. Groups I and Ia differed from the others (except BCQ 1523) at amino acid residues 146 (N→I) and 148 (D→G) which exposed a hydrophobic region, increased surface probability and total charge of that region of the protein. Amino acid 253 (S→N) change did not alter the physicochemical properties of any of the isolates and reference strains. The change in amino acid residues 253 (S→G) and 501 (P→F)

were unique to Group IIa and led to the loss of an antigenic site (1-A region) and reduced hydrophobicity.

Although all groups had similar changes at amino acid 769, which is located immediately downstream of the cleavage site, no change in predicted secondary structure was identified using Hopp–Woods analysis (PROTEAN module, DNASTAR, Madison, WI). All groups had identical structural changes at residue 769 with a loss of a beta region, negative charge of the alpha plot and exposed turn regions, using Chou–Fasman analysis (PROTEAN module, DNASTAR, Madison, WI).

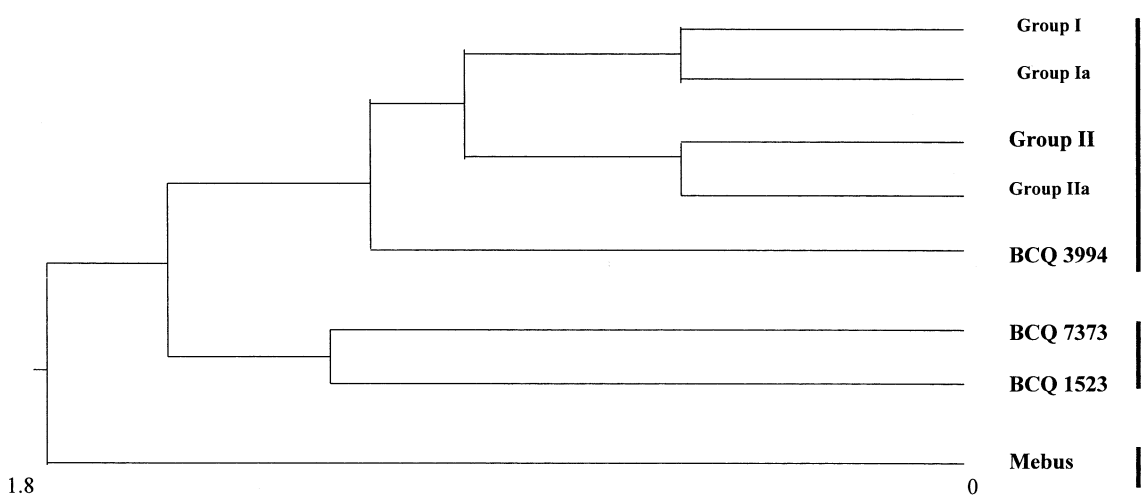
## 4. Discussion

The S protein is the major hemagglutinin of BoCV (Schultze and Herrler, 1994) and is proposed to be responsible for the primary attachment of virus to cell surface receptors (Schultze et al., 1991). The variation in host range and tissue tropism of coronaviruses is largely attributable to variations in the S glycoprotein (Gallagher and Buchmeier, 2001). Previous work from our laboratory has also identified differences in viral neutralization and hemagglutinaton inhibition among respiratory and enteric strains of BoCV but no differences unique to only respiratory or enteric BoCV isolates (Hasoksuz et al., 1999a,b).

Nucleotide and amino acid sequence analysis of the entire S1-glycoprotein region of respiratory and fecal isolates from feedlot cattle revealed several notable changes compared with the Mebus enteric strain. Overall 42 amino acid substitutions were noted in the S1 subunit. Although the importance of such variability in the virulence and tropism of field isolates of BoCV is still unknown, some amino acid changes had significant effects on the charge, hydrophobicity, surface probability and antigenic regions of the protein. These differences may change either the protein folding or physicochemical characteristics.

### *4.1. Clustering of amino acid variations in the S1 glycoprotein and viral tropism*

The amino acid differences identified in the present study were distributed throughout the glycoprotein with clustering around residues 40–118, 146–179, and 458–531. Although several investigators (Rekik and Dea, 1994; Yoo et al., 1990; Chouljenko et al., 1998; Gelinas et al., 2001)




**Group I:** 65NS / 65FS, 187NS / 187FS, 232NS. **Group Ia:** 182NS. **Group II:** 220NS. **Group IIa:** DBA, winter dysentery strain.

**BCQ 3994:** respiratory BCV strain; **BCQ 7373:** winter dysentery strain; **BCQ 1523:** calf diarrhea strain.

Fig. 2. The Phylogenetic tree of respiratory and enteric isolates of BoCV and prototype strains using Clustal method.

reported similar clustering of variations in the S1 region, the polymorphic regions were limited to only two major domains between amino acid residues 146–179 and 458–531. Rekik and Dea, (1994) reported that the region including amino acid residues 56–192 was conserved among their enteric isolates, but we identified eight amino acid substitutions in respiratory and enteric strains in this region of S1 protein compared with the Mebus strain. All the amino acid changes in this region had significant structural consequences in that they reduced the total charge to neutrality, altered hydrophobicity, and surface probability. The N-terminal region of the S1 subunit, consisting of amino acid residues 1–330, has been shown to function as a receptor binding domain in mouse hepatitis virus, MHV, (Chouljenko et al., 1998). By BLAST database analysis we identified a 55% homology between MHV and BoCV (Mebus) in this region. In addition, Wesley et al. (1991) reported that the first 200 amino acids of the S1 subunit of the porcine respiratory coronavirus (PRCV) were associated with respiratory tropism and reduced enteropathogenicity. The slightly reduced hydrophobicity between the Mebus and DBA enteric BoCV isolates induced by the amino acid change at residue 115 may enhance their ability to survive the enteric microenvironment during viral invasion.

### *4.2. Common theme of substitutions in a designated hypervariable region*

The region spanning amino acid residues 452–593 of the S1subunit has been identified as a hypervariable region (Rekik and Dea, 1994; Yoo et al., 1990). Chouljenko et al. (1998), Gelinas et al. (2001) reported that respiratory strains had alterations in 3 amino acids at residues 510, 531 and 578 in the hypervariable site (a.a. 456–592) of the S glycoprotein similar to our findings in the present study for sites 510 and 531 for Mebus and DBA enteric strains compared with the feedlot respiratory and enteric BoCV strains. Further characterization identified that the changes in amino acids 510 and 531 reduced hydrophilicity and rendered a negative charge to this region, respectively. Based on those findings, Chouljenko et al., (1998) proposed that Mabs to the hypervariable region could be useful in differentiating respiratory and enteric strains. The 200 amino acids immediately upstream and 140 amino acids immediately down stream of the hypervariable site did not show any variations. The implications of this finding could be twofold: (1) the regions surrounding the hypervariable site are functionally important to the virus or (2) the regions are not subject to selective pressure such as host immune responses so that variation in the sequence is not important.

The allelic variation in the S1 subunit identified in the isolates from this study is consistent when compared with the more recently recovered CD (BCQ 1523), WD (BCQ 7373) and respiratory (BCQ 3994) strains from Quebec. All BCQ strains had 21 additional substitutions in the S1 subunit amino acid sequence (data not shown) but they did not alter the clustering by phylogenetic analysis. Phylogenetic data showed that all isolates from the present study including WD strain DBA clustered with the respiratory strain BCQ 3994, while the BCQ enteric strains clustered on a separate major branch (Fig. 2). All isolates were distinct from Mebus strain suggesting that allelic variation resulted in genetic drift over time. Since our isolates clustered into the same branch with a recent respiratory strain and were significantly distinct from the ancestral enteric strain (Mebus), we propose that the BoCV strains may be diverging from an enteric tropism to a dual (respiratory and enteric) tropism over time via intermediates. A larger scale analysis including isolates from a variety of geographic localities, different time points, and from either enteric or respiratory sources will be required to comprehensively address the divergence theory.

### *4.3. Amino acid variation surrounding the cleavage site of the S glycoprotein*

The cleavage site of the S-glycoprotein is located at amino acid residues 764–768 and is the region that separates the peripheral S1-glycoprotein from the transmembrane domain of the S2 glycoprotein (Spaan et al., 1988). Amino acid residue 769 located immediately downstream of

the cleavage site was altered (A→S) between Mebus and the Group I, Ia, II and IIa isolates. Chouljenko et al. (1998) identified this change exclusively in their respiratory isolates LSU and OK. Further, they speculated that the change in amino acid 769 was associated with extensive cell fusion as proposed by Cyr-Coast et al. (1988) and thus was a potential marker of respiratory tropism. However, Rekik and Dea (1994) reported that this change (769; A→S) did not appear to modulate cleavability, the rate of viral replication or the type of cytopathic changes induced in HRT-18 cells for their enteric BoCV isolates. This is in agreement with Spaan et al. (1988), who did not observe syncytium formation due to a cleaved S protein of feline infectious peritonitis virus (FIPV) and transmissible gastroenteritis virus (TGEV) and subsequently proposed that cleavage is not always a necessary precondition for cell fusion. Since this change was observed in both respiratory and enteric strains, our data does not support the speculation of Chouljenko et al. (1998).

### *4.4. Commonality of variations in respiratory and enteric isolates*

Group I and Ia strains were isolated from nasal and enteric specimens from feedlot cattle, which had shown respiratory disease signs at arrival (Cho et al., 2001b; Hasoksuz et al., 2001). These animals developed diarrhea 4 days after arrival that peaked at 4–7 days post arrival. Therefore, we believe that calves were first infected with a respiratory strain of BoCV that later spread to the intestine leading to diarrhea. This has been observed for gnotobiotic and colostrum-deprived calves inoculated with respiratory isolates of BoCV in that all strains were pneumoenteric and were shed both nasally and rectally and induced diarrhea (Cho et al., 2000). This may explain why there were no variations between the respiratory and enteric isolates in Group I although they differed from the WD strain (DBA, Group IIa) and enteric BCQ strains at several amino acid residues. This difference in sequences of strains isolated in the present study compared with DBA is in agreement with previous variations observed in neutralization and reactivities to Mabs between a respiratory strain R6 and DBA (Hasoksuz et al., 1999a). In conclusion, this data suggests that respiratory strains of BoCV may differ genetically from the classical calf enteric and adult WD strains. However, it is unknown if these differences impact the immunogenicity or cross-protection between respiratory and enteric BoCV strains (Cho et al., 2001a).

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