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Deduced Sequence of the Bovine Coronavirus Spike Protein and Identification of the Internal Proteolytic Cleavage Site

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The sequence of the spike (also called peplomer or E2) protein gene of the Mebus strain of bovine coronavirus (BCV) was obtained from cDNA clones of genomic RNA. The gene sequence predicts a 150,825 mol wt apoprotein of 1363 amino acids having an N-terminal hydrophobic signal sequence of 17 amino acids, 19 potential N-linked glycosylation sites, a hydrophobic anchor sequence of approximately 17 amino acids near the C terminus, and a hydrophilic cysteine-rich C terminus of 35 amino acids. An internal Lys-Arg-Arg-Ser-Arg-Arg sequence predicts a protease cleavage site between amino acids 768 and 769 that would separate the S apoprotein into S1 and S2 segments of 85690 and 65153 mol wt, respectively. Amino terminal amino acid sequencing of the virion-derived gpl00 spike subunit confirmed the location of the predicted cleavage site, and established that gpl20 and gp100 are the glycosylated virion forms of the S1 and S2 subunits, respectively. Sequence comparisons between BCV and the antigenically related mouse hepatitis coronavirus revealed more sequence divergence in the putative knob region of the spike protein (S1) than in the stem region (S2).

The bovine coronavirus (BCV) is an important cause of neonatal calf diarrhea (14, 26) and may also be the cause of winter dysentery in adult cattle (30). The mechanisms by which BCV causes disease and persistent infection are not understood, nor are current vaccines universally regarded as effective. Toward these ends, we have begun a detailed study of the BCV protein and genome structure.

BCV is comprised of four major structural proteins (17). These are (i) a 200-kDa spike (peplomer) glycoprotein (S), that exists on the virion as cleaved subunits of approximately 120 and 100 kDa, (ii) a 140-kDa glycoprotein (HE) that has both hemagglutinating (18) and esterase (37) activities, and which is comprised of two identical, disulfide-linked 65-kDa subunits (10, 12, 16, 28), (iii) a 26-kDa integral membrane glycoprotein (M) (21), and (iv) an internal phosphorylated nucleocapsid protein (N) (21). Of these, the S protein is presumed to be the major structure by which coronaviruses attach to cells and initiate infection (reviewed by Spaan et al. (34)). The HE protein, however, may also bind to cells to initiate infection, and for BCV, the relative importance of these two proteins in initiating infection is not known. Rnth S and HF are probably important in inducing immunity since antibodies to each are known to neutralize virus infectivity in cell culture and in calves (8, 9). S and HE, therefore, may both be useful in developing effective engineered vaccines against BCV.

cDNA cloning of BCV genomic RNA was accomplished essentially as previously described (11, 21) except that random 5-mer oligodeoxynucleotides (Pharmacia) and 17-mer oligodeoxynucleotides of specific sequences were used as primers for first-strand synthesis. Clones were mapped relative to one another and to the 3' end of the genome using a matrix spot hybridization technique. Some clones were sequenced by the chemical method of Maxam and Gilbert (25) and some by the dideoxynucleotide induced chain termination method of Sanger (31) as described by Kraft et al. (19) using Sequenase enzyme (United States Biochemicals). For much of the sequencing, restriction endonuclease fragments were subcloned into the pGEM4Z vector (Promega) and forward and reverse sequencing

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primers for the pGEM vectors were used. Sequence-specific oligodeoxynucleotides were also synthesized and used for sequencing within certain regions of the large clones.

The amino-terminal ends of purified gp120 and gp100 subunits were subjected to sequencing by the method of Matsudaira (24). Unlabeled BCV was purified by isopycnic sedimentation in sucrose gradients and the proteins were electrophoretically separated after reduction in 2-mercaptoethanol (17) and electroblotted (13) onto polyvinylidene difluoride membrane (24). Proteins were visualized by staining with Coomassie brilliant blue and the gp120 and gp100 bands were excised and shipped to Dr. Matsudaira for analysis.

Complete sequencing of clone MA7 which extends 4.2 kilobases from the 3' end of the genome (Fig. 1) revealed a continuous open reading frame located on the 5' side of the ORF for a potential 4.9-kDa protein (Abraham et al., to be published elsewhere). The deduced amino acid sequence of the extended ORF demonstrated high sequence similarity to the C-terminal end of the antigenically related MHV-A59 (22) and MHV-JHM (32) S proteins, both antigenic homologs of the BCV S protein (13). These data suggested that the S protein gene of BCV lies in the same relative position on the genome as does the spike protein gene of MHV. To complete the sequencing of the S gene, both strands of three clones, I1, HPA2, and G6, generated by random priming, and three clones, LK5, LP6 and 29, generated by specific priming, were sequenced (Fig. 1).

The total sequence for the putative S ORF extended to a position 7.4 kb from the 3' end of the genome and contained 4089 bases (Fig. 2). We conclude this ORF to be the S gene since it potentially encodes a 1363 amino acid protein of 150,625 Da, the approximate size of the unglycosylated spike precursor (10), and because its deduced amino acid sequence shows extensive sequence similarity throughout with the S proteins of both strains of MHV. Five other open reading frames ranging in size from 34 to 66 amino acids were also found within the S gene sequence in the plus one reading frame, but their significance is not known at this time. The putative S ORF is preceded immediately upstream (beginning at base 12 in Fig. 2) by the consensus CYAAC sequence thought to play a role in leader priming of coronavirus transcription. The sequence is also found three times within the S ORF, beginning at positions 817, 1667, and 3776, but it is not established that transcripts initiate at any of these sites.

Five features of the deduced BCV S protein reflect the properties of four other coronavirus spike proteins that have been characterized to date from nucleotide sequence data (1, 2, 15, 20, 22, 27, 29, 32). (i) There is an N-terminal hydrophobic stretch of amino acids which predicts a signal peptide with a cleavage site between amino acids 17 and 18 (38). (ii) There are 19 potential asparagine-linked glycosylation sites that could give rise to the only kind of glycosylation demonstrated for this protein (Hogue and Brian, unpublished data; 10). (iii) There is a hydrophobic stretch of 17 amino acids near the C terminus that could serve as a stop-transfer and anchor sequence. (iv) There is a stretch of 8 amino acids on the immediate N-terminal side of the predicted anchor sequence (-K-W-P-W-Y-V-W-L-, beginning with amino acid 1305) that is identical in all coronavirus S proteins sequenced to date. (v) There is a cysteine-rich hydrophilic C-terminus of 35 amino acids that is probably the intravirion domain. In common with MHV- (22, 32) and IBV (1, 2, 20, 27), but not in common with TGEV (15, 29; Tung and Brian, unpublished) and FIPV (19), is also an internal sequence of basic amino acids that, in the case of MHV and IBV, lies on the immediate N terminal side of the protease cleavage site (6, 22). In BCV the sequence is K-R-R-S-R-R beginning.
Fig. 2. Nucleotide sequence of the S gene and its deduced amino acid sequence. The nucleotide sequence shown begins with the TAG termination codon of the HE gene (underlined) 17 bases upstream of the presumed S start site (7407 bases from the poly(A) tail), and ends with the TAA termination codon of the S protein. The first three amino acids of the putative 4.9-kDa protein are shown beginning at base position 4099. Consensus CYAAAC sequences are boxed. The presumed amino-terminal signal peptide and carboxy-terminal anchor sequences are underlined. Potential N-linked glycosylation sites (NXS or NXT, where X ≠ P) are boxed. The proteolytic cleavage site separating S1 and S2 is identified with an arrow. The extended sequence of amino acids missing in MHV JHM is identified by individually underlined amino acids, and that missing in MHV A59, by asterisks.

with amino acid 763, and, on the basis of the pattern in MHV and IBV, predicts a cleavage between amino acids 768 and 769 (note arrow in Fig. 2). Cleavage at this point would divide the unglycosylated S protein into an N-terminal segment of 85,690 Da (S1) and a C-terminal segment of 65,153 Da (S2).

From amino acid sequencing studies, no N-terminal sequence could be obtained from the virion-derived 120-kDa subunit, possibly because of N-terminal blockage. The N-terminal sequence of the 100-kDa subunit could be obtained, however, and was determined to be X-T-T-G-Y-X-F-, identifying the first amino acids downstream from the predicted internal cleavage site. These results confirmed the predicted internal cleavage site and established that the 120-kDa subunit is S1 and the 100-kDa subunit is S2.

The BCV and MHV S proteins show remarkable sequence homology suggesting that these viruses are re-
cently diverged. After aligning sequences for maximal homology, the following points emerge. (i) Relative to BCV, a large deletion appears in the MHV S1 subunits. For JHM it is a contiguous gap of 138 amino acids, and for A59 it is a discontiguous gap of 50 amino acids (Figs. 2 and 3). The function of the additional sequence in the BCV S1 subunit is not known, but it is possibly a structure that interacts in some way with the HE glycoprotein, a structural protein not found on MHV (13, 34) except under certain rare conditions (33). No electron micrographic or chemical data exist, however, to suggest that S and HE do physically interact (3, 17, 18). It is interesting to note that the entire region in the BCV S protein corresponding to the gap region of the JHM S protein is especially rich in cysteine residues and contains 15 (26%) of the 56 total cysteines in the BCV S protein (Figs. 2 and 3). This suggests that this part of the molecule may be important for intramolecular or intermolecular disulfide linkages. (ii) Exclusive of the large gap in the MHV sequences, the S1 subunits of JHM and A59 show 62 and 60% identity, respectively, with BCV, and the S2 subunits show 75 and 74% respectively. Throughout the S protein, 41 of 56 cysteine positions and 13 of 19 potential N-linked glycosylation sites are conserved. The internal proteolytic cleavage position (not yet confirmed for JHM) is also conserved. The pattern of greater amino acid sequence divergence in the S1 subunit is consistent with the model of Cavanagh (4) and De Groot et al. (7) which proposes that the S1 subunit comprises the exposed bulbous structure of the spike and probably contains most (4), but not all (23, 36), of the neutralizable antigenic sites. It is the structure most likely to undergo changes as a result of immunologic selective pressures.

Fusion of cells in culture is one biological activity associated with cleavage of the MHV S protein (35). Despite its extensive sequence similarity with the MHV S protein, however, the BCV S protein shows little fusion activity. In fact, fusion is a behavior we have not observed with the Mebus strain of BCV even though the S protein is primarily in the cleaved form on the virion (13, 17). It is not clear why BCV and MHV behave so differently in their fusogenic properties, but functional evaluation of sequence differences near the cleavage sites of these two viruses may aid in clarifying the mechanisms of fusion by MHV. This is especially interesting since hydrophobic regions, common at the cleavage sites on fusion proteins of paramyxoviruses and myxoviruses, are absent in the MHV S protein (22) and different mechanisms of fusion may be employed.

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