

## Structural Analysis of the Conformational Domains Involved in Neutralization of Bovine Coronavirus Using Deletion Mutants of the Spike Glycoprotein S1 Subunit Expressed by Recombinant Baculoviruses

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Two conformation-dependent neutralizing epitopes, A and B, have been mapped to the S1 subunit of the S spike glycoprotein of bovine coronavirus (BCV). In order to characterize the structure of these antigenic sites, we constructed a series of cDNA clones encoding deleted or truncated S1 derivatives and expressed the modified genes in insect cells using recombinant baculoviruses. Monoclonal antibodies directed against epitopes A and B recognized only the mutant S1 polypeptides containing amino acids 324-720, as demonstrated by immunoprecipitation and Western blot analysis in the absence of  $\beta$ -mercaptoethanol. In addition, two domains within this region were identified and only mutants containing both domains were immunoreactive, indicating that both were critical in the formation of the antigenic determinants. One domain was localized between residues 324 and 403 and the other at residues 517-720. Deletion of either domain inhibited extracellular secretion of the mutant proteins whereas mutants containing both or none of the domains were secreted efficiently. This observation suggests a vital function of the native conformation of the S1 protein in both antigenic structure and intracellular transport. Antigenic determinants A and B were not distinguished, but these determinants appeared to require both domains for epitope formation. Our results suggest that the antigenic determinants formed by two domains are likely associated with the probable polymorphic region of the BCV S1 subunit. © 1991 Academic Press, Inc.

### INTRODUCTION

Bovine coronavirus (BCV) is an enteropathogenic coronavirus that causes severe diarrhea in neonatal calves. The genome of BCV is a single-stranded RNA with positive polarity of approximately 30 kb in length and encodes four major structural proteins, which are the nucleocapsid protein (N; 52K), the matrix protein (M; 25K), the spike protein (S; 180K) and the hemagglutinin/esterase (HE; 65K) (King and Brian, 1982; Deregts *et al.*, 1987; Cry-Coat *et al.*, 1988).

The S glycoprotein is the major viral component possessing functions responsible for cell binding (Collins *et al.*, 1982), cell fusion (Sturman *et al.*, 1985; Yoo *et al.*, 1991), and induction of neutralizing antibody response (Deregts and Babiuk, 1987; for a review, see Spaan *et al.*, 1988). The BCV S glycoprotein is post-translationally cleaved into two subunits at amino acids 768-769 (Abraham *et al.*, 1990). Accumulated information suggests that the carboxy-terminal S2 subunit is an integral membrane protein comprising the stalk

portion of the peplomer whereas the amino-terminal S1 subunit constitutes the bulbous part of the peplomer. Recently, the nucleotide sequence of the BCV S glycoprotein gene has been determined (Parker *et al.*, 1990; Abraham *et al.*, 1990; Boireau *et al.*, 1990). When the deduced amino acid sequence of the BCV S glycoprotein was compared to that of mouse hepatitis coronavirus (MHV) (Luytjes *et al.*, 1987; Schmidt *et al.*, 1987), a large additional sequence of 49 and 138 amino acids was identified in the BCV S1 subunit that was not present in MHV strains JHM and A59, respectively. The function of this additional sequence present in the BCV S1 subunit is not yet clearly defined. However, recent studies have identified a region of 142 to 159 amino acids similar to the BCV S1 additional sequence in the N-terminal half (S1 counterpart) of the spike glycoprotein of wild type MHV-4 coronavirus (Parker *et al.*, 1989). This region is highly polymorphic in different neutralization resistant MHV-4 variants, suggesting its important role in MHV-4 pathogenicity. Considerable homology in the amino acid sequences between the S1 subunits of MHV-4 and BCV suggests that the additional sequence of amino acid residues 456-592 within the BCV S1 subunit may be a polymorphic domain similar to that found in MHV-4. Recently, individual subunits of the BCV S glycoprotein have

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been expressed in insect cells using recombinant baculoviruses, and two major BCV neutralizing epitopes were localized to the S1 subunit (Yoo *et al.*, 1990). Thus, in order to examine if the major BCV neutralizing epitopes were associated with the probable BCV polymorphic region, we constructed a series of S1 deletion mutants and expressed them in insect cells. Here we describe the location of two antigenic determinants and the identification of two domains in the S1 subunit critical for the formation of these antigenic determinants, and discuss the possible involvement of the probable polymorphic region of the S1 subunit in BCV antigenicity.

## MATERIALS AND METHODS

### Cells, viruses, and antibodies

*Spodoptera frugiperda* cells (Sf9, ATCC CRL 1711) were grown in suspension in Grace's medium supplemented with 0.3% yeastolate, 0.3% lactalbumin, and 10% fetal bovine serum (GIBCO) at 28° (Summers and Smith, 1987). *Autographa californica* nuclear polyhedrosis baculovirus (AcNPV) and recombinant baculoviruses were propagated and titrated on monolayers of Sf9 cells (Summers and Smith, 1987). Polyclonal rabbit anti-BCV antiserum and mouse ascitic fluids of monoclonal antibodies, HB10-4, JB5-6, HF8-8, HE7-3, and BB7-14, were prepared as described (Deregt and Biuk, 1987).

### Construction of DNA clones and recombinant transfer vectors

Restriction enzymes and DNA modifying enzymes were purchased from Pharmacia. Plasmid pCVS1 was used as the source of DNA sequence encoding the S1 subunit of BCV (Quebec strain) (Parker *et al.*, 1990). Strategies for the construction of S1 deletion mutants are illustrated in Fig. 1. Truncated fragments of  $\Delta pu$ ,  $\Delta bx$ , and  $\Delta pt$  were generated by digestion of the S1 gene with restriction enzymes *PvuII*, *BstXI*, and *PstI*, respectively. The 3' terminus of all fragments was blunt-ended by Klenow fragment, and a translational termination sequence (5'-GCTTAATTAATTAAGC-3') was attached. Deletion fragments  $\Delta III$  and  $\Delta V$  were constructed by partial digestion of pCVS1 with *HincII*. For fragment  $\Delta VI$ ,  $\Delta pu$  was digested with *AccI* and *HindIII*, and a 1.3-kb fragment was isolated. The 1.3-kb fragment was then cloned into pCVS1 which was linearized by complete digestion with *HindII* and *HindIII*. Fragments  $\Delta pu$ ,  $\Delta bx$ , and  $\Delta pt$  were subcloned into the *BamHI* site of transfer vector pVL941 (Luckow and Summers, 1989) or pAcYM1 (Matsuura *et al.*, 1987), and fragments  $\Delta III$ ,  $\Delta V$ , and  $\Delta VI$  were subcloned into

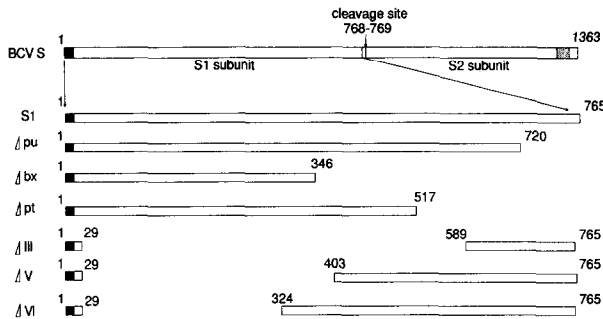
the *NheI* site of transfer vector pJVP10Z (Vialard *et al.*, 1990).

### DNA transfection, screening of recombinant viruses, and plaque assay

Extracellular virions (AcNPV) were purified by linear equilibrium centrifugation in a 25–55% sucrose gradient and the viral DNA was prepared with trypsin and sarkosyl treatment followed by phenol extraction (Summers and Smith, 1987). Plasmid DNA was prepared through CsCl gradients according to standard procedures. Approximately  $2 \times 10^6$  Sf9 cells were co-transfected with 1  $\mu$ g of AcNPV viral DNA and 2  $\mu$ g of transfer vector plasmid DNA by calcium precipitation as described previously (Yoo *et al.*, 1990). Transfected cells were incubated at 28° for 3 days and the culture supernatants were harvested and plated on Sf9 cell monolayers for plaque assay. Plaque assays were performed in 35-mm dishes with 1.5% agarose overlay as described (Summers and Smith, 1987). Recombinant plaques produced with pAcYM1 or pVL941 were screened either by the absence of polyhedrin or by plaque hybridization. For screening of recombinant plaques produced with transfer vector pJVP10Z, 1 ml of medium containing 150  $\mu$ g/ml of Blue-Gal (BRL) was added to the agarose overlay on Day 4 of incubation. Blue plaques were picked and further purified by several rounds of plaque assay. Purified recombinant plaques were amplified and the stocks with titers of approximately  $10^8$  PFU/ml were used in the study.

### Radiolabeling and immunoprecipitation

Sf9 cells were infected at an m.o.i. of 5–10 PFU/cell. At 24 hr postinfection, cells were starved for 1 hr in cysteine-free Grace's medium followed by labeling for 2 hr with 50  $\mu$ Ci/ml of [<sup>35</sup>S]cysteine (Amersham; specific activity 3000 Ci/mmol). For glycosylation studies, virus-infected cells were treated with 10  $\mu$ g/ml of tunicamycin (Sigma) for 1 hr and labeled in the presence of tunicamycin. Cells were scraped and harvested by centrifugation at 2000 rpm for 10 min. The cells were lysed with 0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and the cytoplasmic fraction was used for immunoprecipitation. For secretion experiments, cells were labeled for 12 hr at 24 hr postinfection, and the culture media were immunoprecipitated. For immunoprecipitation, samples were incubated with antibody at room temperature for 2 hr, and 10 mg of Protein A-Sepharose beads (Pharmacia) were added. The mixtures in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA) containing 0.5% SDS were incubated overnight at 4° with continuous shaking. Immune complexes



**FIG. 1.** Schematic presentation of the BCV S1 deletion mutants. Location of the mutant proteins to the spike glycoprotein is indicated by amino acid position (number). Darkened areas indicate the N-terminal signal sequence (amino acid positions 1–17), and shaded area indicates the C-terminal hydrophobic transmembrane sequence (positions 1312–1328).

were washed three times with RIPA buffer and dissociated by boiling for 5 min in 10% SDS, 25% glycerol, 10% mercaptoethanol, 0.02% bromophenol blue, 10 mM Tris-HCl, pH 6.8. The polypeptides were analysed on 12% SDS-polyacrylamide gels followed by autoradiography.

**Western blot analysis**

Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuel) by electroblotting in Tris-glycine buffer (20 mM Tris-HCl, pH 8.3, 190 mM glycine) containing 20% methanol. Membranes were blocked with 3% skim milk powder in 10 mM PBS overnight at 4°. Membranes were then incubated with monoclonal antibodies (1:200 dilution) in PBS containing 0.05% Tween 20 (PBST) and 1% skim milk powder (PBSTS) for 2 hr at room temperature. Blots were washed with PBST for 2 hr and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG in PBSTS for 90 min at room temperature. Membranes were developed by reaction with hydrogen peroxide and 0.05% 4-chloro-1-naphthol substrate (Bio-Rad) for 20 min or more.

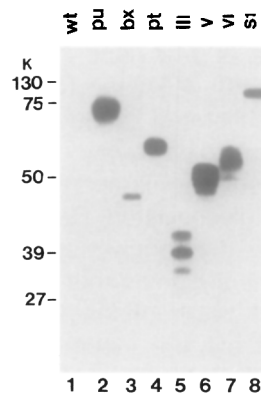
**RESULTS**

**Expression of the S1 deletion mutant polypeptides in insect cells**

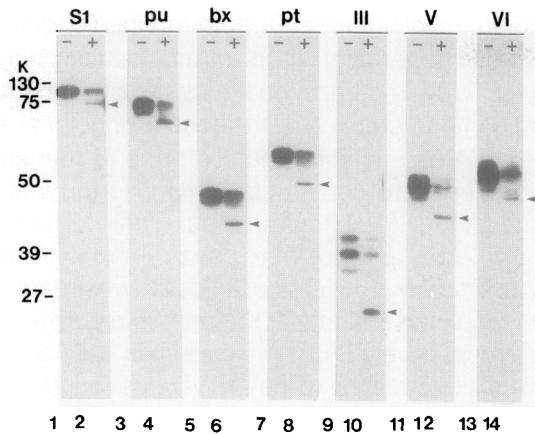
A series of neutralizing monoclonal antibodies specific for the BCV S glycoprotein were previously developed and classified by competitive antibody binding assays into two nonoverlapping groups, A and B (Deregt and Babiuk, 1987). These monoclonal antibodies were reactive with the S1 subunit, and the reactions were sensitive to reducing agents but resistant to ionic

detergents, indicating that both antigenic determinants were dependent upon intramolecular disulfide linkages. To characterize the structure of the antigenic determinants interacting with these monoclonal antibodies, cDNAs encoding the six forms of mutant S1 polypeptides were constructed and inserted into the genome of AcNPV baculoviruses. Three S1 derivatives, Δpu, Δbx, and Δpt were constructed to truncate at approximately 100 amino acids downstream, upstream, and in the middle of the probable polymorphic region, respectively (Fig. 1) Derivatives ΔIII, ΔV, and ΔVI were constructed to delete various lengths from the N-terminus of the S1, but the first 29 N-terminal amino acids were included so as to retain a membrane translocational signal. A translational termination codon was attached at the 3' terminus of each derivative.

Expression of the S1 deletion products in Sf9 cells was determined at 24 hr postinfection by immunoprecipitation of the baculovirus-infected cell lysates. (Fig. 2). [<sup>35</sup>S]Cysteine was chosen for radiolabeling due to the limited number of methionine residues in some of the mutant polypeptides. No protein was immunoprecipitated from wild-type AcNPV infected cells (lane 1). Anti-BCV polyclonal antisera immunoprecipitated single polypeptides of 75K (Δpu, lane 2), 45K (Δbx, lane 3), 60K (Δpt, lane 4), 50K (ΔV, lane 6), or 55K (ΔVI, lane 7) from cells infected with corresponding recombinant baculoviruses whereas ΔIII produced three species of immunoprecipitable polypeptides, 43K, 39K, and 35K (lane 5). The immunoprecipitable mutant proteins including the intact S1 (lane 8) were all larger in molecular weights than those of the predicted polypeptides



**FIG. 2.** Synthesis of the BCV deletion S1 mutants in insect cells. Sf9 cells were infected with recombinant baculoviruses and radiolabeled with [<sup>35</sup>S]cysteine for 2 hr at 24 hr postinfection. Cell lysates were prepared, immunoprecipitated with polyclonal rabbit anti-BCV antiserum and resolved on 12% SDS polyacrylamide gel as described under Materials and Methods. Each lane represents cell lysates prepared from 5 × 10<sup>5</sup> cells infected with, Lane 1: wild-type baculovirus. Lane 2: Δpu, Lane 3: Δbx. Lane 4: Δpt. Lane 5: ΔIII. Lane 6: ΔV. Lane 7: ΔVI. Lane 8: S1.



**Fig. 3.** Effect of tunicamycin on migration of BCV S1 protein and its derivatives. Virus-infected cells were treated with tunicamycin (10  $\mu\text{g}/\text{ml}$ ) for 30 min prior to radiolabeling, and labeled with [ $^{35}\text{S}$ ]cysteine (100  $\mu\text{Ci}/\text{ml}$ ) for 2 hr in the presence of tunicamycin. Cell lysates were immunoprecipitated and analyzed by SDS-PAGE. Designation above each lane indicate the specific mutants with + and - referring to the presence or the absence of tunicamycin. Arrowheads indicate the unglycosylated mutant polypeptides.

deduced from nucleotide sequences, suggesting that the mutant polypeptides synthesized in Sf9 cells were glycosylated.

#### Glycosylation and extracellular transport of the S1 deletion products

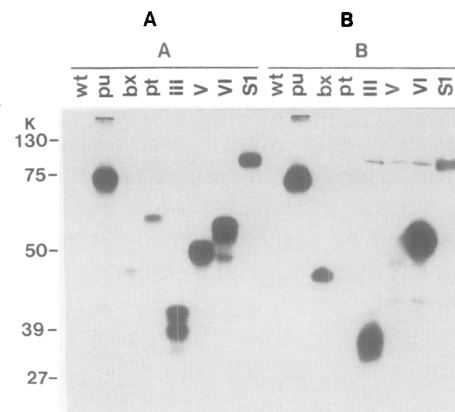
In order to confirm that the immunoprecipitable mutant polypeptides were glycosylated, the mutant polypeptides were radiolabeled in the presence of tunicamycin (Fig. 3). As shown previously (Yoo *et al.*, 1990), 10  $\mu\text{g}/\text{ml}$  of tunicamycin moderately inhibited glycosylation in Sf9 cell, and lower molecular-weight polypeptides of 75K (lane 2), 65K (lane 4), 42K (lane 6), 50K (lane 8), 25K (lane 10), 46K (lane 12), and 48K (lane 14) were immunoprecipitated in addition to the corresponding glycosylated polypeptides (lanes 1, 3, 5, 7, 9, 11, 13). These results confirmed that the mutant polypeptides were all glycosylated. Deletion  $\Delta\text{III}$  produced a single species of the nonglycosylated, 25K polypeptide (lane 10). This suggests that the immunoprecipitable 39K and 35K polypeptides produced by deletion  $\Delta\text{III}$  (lanes 9–10) are the incompletely glycosylated forms of the 43K mutant polypeptide.

Since the completely denatured S glycoprotein of BCV did not react with conformational monoclonal antibodies (Deregt and Babiuk, 1987), we were interested in the conformation of the mutant fragments of the BCV S1 protein. To determine the conformational changes of the mutant polypeptides, we attempted to measure the relative rates of secretion of the mutant S1 polypeptides. It has been established that native conformation

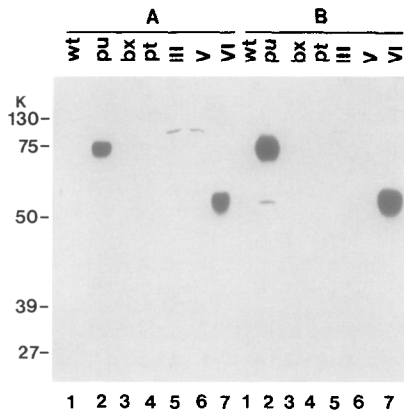
is essential for transport of proteins through intracellular secretory pathways (for a review, see Rose and Doms, 1988). Secretion of the BCV S1 subunit protein from insect cells has been previously demonstrated (Yoo *et al.*, 1990). Insect cells producing the mutant S1 polypeptides were labeled for 12 hr beginning at 24 hr postinfection and the secreted polypeptides in the medium were immunoprecipitated (Fig. 4). At 36 hr postinfection, no  $\Delta\text{pt}$  product was detected in the medium (Fig. 4B, lane 4) even though the  $\Delta\text{pt}$  product was present in the cell pellet (Fig. 4A, lane 4). Similarly, deletion  $\Delta\text{V}$  was present in the cell lysate in large quantities (Fig. 4A, lane 6), but only a trace amount was detected in the medium (Fig. 4B, lane 6). In contrast, other polypeptides,  $\Delta\text{pu}$ ,  $\Delta\text{III}$ , and  $\Delta\text{VI}$  and the intact S1 protein were efficiently secreted into the media (Fig. 4B, lanes 2, 5, 7, 8). A significant amount of the truncated  $\Delta\text{bx}$  polypeptide also accumulated in the medium (Fig. 4B, lane 3) even though the amount of intracellular  $\Delta\text{bx}$  was small (Fig. 4A, lane 3). These observations indicate that extracellular transport of the  $\Delta\text{pt}$  and  $\Delta\text{V}$  was significantly inhibited, suggesting the altered conformation of mutants  $\Delta\text{pt}$  and  $\Delta\text{V}$ .

#### Antigenic location and identification of domains essential for the antigenic determinants

Antigenic structure of the S1 subunit was evaluated by determining the immunoreactivities of the derivatives of the S1 subunit with pooled monoclonal antibodies representing group A or with monoclonal antibody



**Fig. 4.** Extracellular secretion of the S1 mutants. Cells were radiolabeled with [ $^{35}\text{S}$ ]cysteine for 12 hr from 24 to 36 hr p.i. Culture media were collected and subjected to immunoprecipitation with polyclonal rabbit anti-BCV antibody. The corresponding cell lysates were also prepared and immunoprecipitated. Proteins were analysed on a 12% polyacrylamide gel and visualized by autoradiography. A: cell lysates of respective mutants. B: cell culture media. Lane 1: wild type AcNPV. Lane 2:  $\Delta\text{pu}$ , Lane 3:  $\Delta\text{bx}$ . Lane 4:  $\Delta\text{pt}$ , Lane 5:  $\Delta\text{III}$ . Lane 6:  $\Delta\text{V}$ . Lane 7:  $\Delta\text{VI}$ .



**Fig. 5.** Immunoprecipitations of the deletion mutants with BCV S1 monoclonal antibodies. At 24 hr p.i., cells were labeled with 100  $\mu$ Ci of [ $^{35}$ S]cysteine for 2 hr. The lysates were prepared and immunoprecipitated with monoclonal antibodies followed by electrophoresis through 12% SDS-PAGE gels and autoradiography. A: a pool of the antigenic group A monoclonal antibodies HB10-4, JB5-6, and HF8-8. B: antigenic group B monoclonal antibody BB7-14. Lane 1: wild-type AcNPV. Lane 2:  $\Delta$ pu, Lane 3:  $\Delta$ bx. Lane 4:  $\Delta$ pt. Lane 5:  $\Delta$ III. Lane 6:  $\Delta$ V. Lane 7:  $\Delta$ VI.

BB7-14 representing group B (Fig. 5) The  $\Delta$ bx and  $\Delta$ pt polypeptides were not immunoprecipitated by any of the monoclonal antibodies (Fig. 5A, lanes 3, 4; Fig. 5B, lanes 3, 4) while the  $\Delta$ pu product, extending 203 amino acids from the C-terminus of the  $\Delta$ pt, was immunoprecipitated by both group A and B monoclonal antibodies (Fig. 5A, lane 2; Fig. 5B, lane 2). These results initially demonstrated that a region of 203 amino acids between residues 517–720 constituted a domain important for S1 antigenicity. Deletions  $\Delta$ III and  $\Delta$ V were not immunoprecipitated by any of the monoclonal antibodies (Fig. 5A, lanes 5, 6; Fig. 5B, lanes 5, 6). This observation led us to conclude that the domain 517–720 was important for antigenic determinants A and B; however, another domain located upstream from residue 403 was also required for the formation of both antigenic determinants. The nonspecific, high molecular weight bands in lanes 5 and 6 represented  $\beta$ -galactosidase overexpressed by baculovirus transfer vector pJVP10Z (Vialard *et al.*, 1990).

In order to identify the upstream domain involved in the completion of the antigenicity, deletion  $\Delta$ VI was constructed. Deletion  $\Delta$ VI overlapped with deletion  $\Delta$ V but extended 79 amino acids towards the N-terminus (Fig. 1). The  $\Delta$ VI mutant polypeptide was immunoprecipitated well by both group A and B monoclonal antibodies (Fig. 5A, lane 7; Fig. 5B, lane 7). These observations, together with the results from deletions  $\Delta$ pu and  $\Delta$ pt, demonstrate that antigenic determinants A and B are both located on a region between residues 324 and 720, and that a short region composed of residues

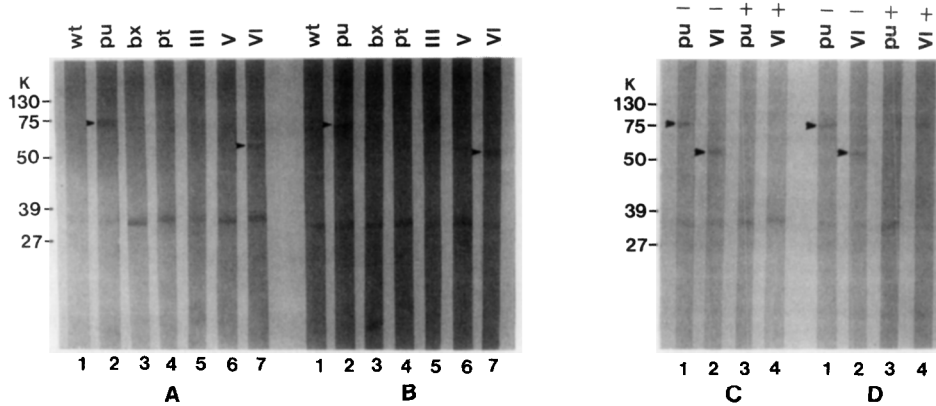
324–403 contained a second domain necessary for the formation of BCV S1 conformational epitopes.

Immunoprecipitation results of the mutant polypeptides obtained with conformational monoclonal antibodies were confirmed by Western blot analysis (Fig. 6), since the previous data characterizing these monoclonal antibodies indicated that, in the absence of  $\beta$ -mercaptoethanol, the antigens transferred to a membrane retained sufficient conformation for monoclonal antibody recognition (Deregt and Babiuk, 1987). As with the results of immunoprecipitation, only the  $\Delta$ pu and  $\Delta$ VI constructs were recognized by groups A and B monoclonal antibodies (Fig. 6A, lanes 2, 7; Fig. 6B, lanes 2, 7), indicating that only deletions  $\Delta$ pu and  $\Delta$ VI contained both domains necessary for forming proper conformation of the S1 protein. The contention of two domains on the  $\Delta$ pu and  $\Delta$ VI polypeptides was further confirmed by comparing Western blots in the absence and presence of  $\beta$ -mercaptoethanol. When  $\beta$ -mercaptoethanol was included in the sample buffer, deletions  $\Delta$ pu, and  $\Delta$ VI were no longer recognized by monoclonal antibodies A and B (Fig. 6C, lanes 3, 4; Fig. 6D, lanes 3, 4), demonstrating the role of two domains in antibody recognition.

## DISCUSSION

Continuous epitopes are generally mapped by measuring reactivities of the short peptides with specific antibodies. This can be achieved by either synthetic peptide technology (Geysen *et al.*, 1984) or alternatively by expressing DNA fragments generated by DNase or restriction enzymes in *Escherichia coli* (Mehra *et al.*, 1984; Nunberg *et al.*, 1986). Such an approach has been utilized for the identification of continuous epitopes of the spike glycoproteins of mouse hepatitis coronavirus (Talbot *et al.*, 1988; Luytjes *et al.*, 1989), infectious bronchitis virus (Lenstra *et al.*, 1989), and transmissible gastroenteritis virus (Delmas *et al.*, 1990; Correa *et al.*, 1990). However, attempts to study conformational epitopes using synthetic peptides or prokaryotic expression systems often result in the inability of antibodies to recognize these recombinant proteins or peptides (Delmas *et al.*, 1990). This problem is most probably due to the lack of proper post-translational modification and consequently resulting in altered protein conformation.

Our approach to express a series of deletion mutants in eukaryotic insect cells has proven useful for the study of conformational epitopes within the BCV S1 spike glycoprotein. All of the S1 derivatives were constructed to include a membrane translocational signal of the BCV S glycoprotein so as to be properly glycosylated and transported through the secretory pathway.

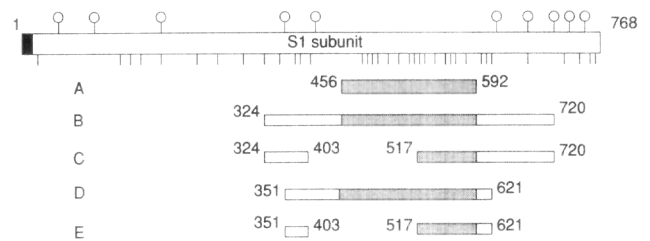


**FIG. 6.** Western blot of the deletion mutants of the BCV S1 polypeptide. Cell lysates representing approximately  $1.5 \times 10^6$  cells were prepared and mixed with protein sample buffer with or without  $\beta$ -mercaptoethanol. Samples were boiled for 2 min and resolved by SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes and the membranes were incubated with a pool of the antigenic group A monoclonal antibodies HB10-4, JB5-6, and HF8-8 (A, C) or with antigenic group B monoclonal antibody BB7-14 (B, D). Reactions were detected using HRP-conjugated goat anti-mouse IgG antibody. Substrate used was 4-chloro-1-naphthol and color was developed for 20–60 min. A, B: without  $\beta$ -mercaptoethanol. C, D: lanes 1, 2; without  $\beta$ -mercaptoethanol (–); lanes 3, 4; with  $\beta$ -mercaptoethanol (+).

The intracellular S1 mutant polypeptides in Sf9 cells were all glycosylated (Fig. 3). Deletions  $\Delta pu$ ,  $\Delta VI$ ,  $\Delta III$ , and  $\Delta V$  were secreted efficiently whereas secretion of deletions  $\Delta pt$  and  $\Delta V$  was significantly inhibited (Fig. 4). Since addition of N-linked oligosaccharides is required for the secretion of the BCV S1 glycoprotein in insect cells (Yoo *et al.*, 1990; Jarvis *et al.*, 1990), the secreted S1 deletion products are likely all glycosylated. Although the  $\Delta pt$  and  $\Delta V$  polypeptides were not secreted, the intracellular  $\Delta pt$  and  $\Delta V$  were glycosylated like other mutant polypeptides (Fig. 3, lanes 7, 11). Therefore, inhibition of the  $\Delta pt$  and  $\Delta V$  polypeptides in their intracellular transport seems to occur after glycosylation.

The results established that the BCV antigenic determinants A and B are located in a segment between residues 324 and 720 (Fig. 7, B). Formation of the determinants A and B appeared to be dependent upon two separate domains. One of the domains was localized within residues 325–403, approximately 50 amino acids upstream from the probable BCV polymorphic region. The other domain was identified within residues 517–720, which included the carboxyl half of the probable BCV polymorphic region (Fig. 7, C). Earlier studies mapped BCV antigenic determinants A and B to a 37K trypsin fragment generated by proteolysis of antigen-antibody complexes (Deregt *et al.*, 1989). Based upon the potential hydrophilic trypsin cleavage sites of the amino acid sequence, the proteolytic cleavage patterns generated with three specific proteolytic enzymes, and other considerations such as the number of glycosylation sites, 37K fragment was tentatively suggested to extend from residues 351 (after Arg at 350) to 621 (Lys) of the S1 subunit (Fig. 7, D) (Deregt,

1988). Part of the rationals for this location was the fact that all three enzymes generated fragments with similar size and other characteristics which suggested that the unique sequence Glu–Arg–Lys (349–351), in a hydrophilic locale, was cleaved by all three enzymes. Given the above, the lysine at 621, also in a hydrophilic locale, was indicated as the only lysine that could generate a 37K fragment. Among our deletions, polypeptides  $\Delta pu$  and  $\Delta VI$  included residues 351–621 were immunoreactive (Figs. 5 and 6). This observation supports the previous suggestion for the location of sites A and B within the 37K fragment. When the first domain was aligned with the 37K fragment, residues 351–403 overlapped (Fig. 7, E). This overlapping region (domain



**FIG. 7.** Schematic diagram of the antigenic structure of the S1 subunit involved in BCV neutralization. Primary structure of the S1 subunit protein is also depicted (Parker *et al.*, 1990). Circles with vertical bar (top) indicate the potential N-linked glycosylation sites and vertical lines (bottom) indicate the location of cysteine residues. Darkened area at the N terminus indicates membrane translocational signal, and shaded areas indicate the probable BCV polymorphic region. Numbers indicate amino acid positions of the S1 protein: A, probable BCV polymorphic region; B, location of the antigenic sites A and B; C, two identified domains important for the formation of antigenic sites; D, The 37K trypsin fragment (Deregt *et al.*, 1989); E, predicted location of the two actual domains.

l) appears to include three cysteines at positions 356, 374, and 386. One or more of these three cysteines may be involved in the formation of the antigenic determinants by forming disulfide linkages. Similarly, residues 517–621 of the second domain overlapped with the 37K fragment. Thus, we propose that domain II is located within a region spanned by amino acids 517–621 (Fig. 7, E).

Polypeptides  $\Delta$ pu and  $\Delta$ VI contained both domains I and II, and polypeptides  $\Delta$ III and  $\Delta$ bx contained neither of the domains. In contrast, polypeptides  $\Delta$ pt and  $\Delta$ V contained only one of the domains. It has been documented that correct folding and oligomeric assembly is required for transport of proteins from the endoplasmic reticulum to the cell surface (Kreis and Lodish, 1986; Gething *et al.*, 1986; Rose and Doms, 1988). Glycosylation indirectly promotes intracellular transport by influencing protein folding or oligomerization (Pitta *et al.*, 1989). Therefore, the secretion inhibition of mutants  $\Delta$ pt and  $\Delta$ V seems likely due to the unfavorable conformation of these mutant polypeptides, implicating an important association between these two domains. Polypeptides  $\Delta$ III and  $\Delta$ bx contained neither domain and were secreted efficiently. A possible explanation for this is that, since the  $\Delta$ bx and  $\Delta$ III polypeptides represent the N-terminal and C-terminal portion of the S1 protein, respectively, these portions may be insignificantly linked with either domains, forming a relatively independent conformation. Thus, the folding of the fragmented polypeptides representing these portions may easily mimic the native conformation, resulting in efficient secretion.

Recently, Parker *et al.* (1989) have identified a polymorphic region on the S1 subunit in MHV-4. This region has been demonstrated to undergo deletions or more frequently, point mutations. Furthermore, these point mutations were selectable by neutralizing monoclonal antibodies, and the mutant viruses displayed decreased virulence, indicating that the polymorphic region is directly involved in the MHV-4 pathogenicity (Gallagher *et al.*, 1990). We have mapped BCV antigenic determinants A and B to residues 324–720, and this segment includes the probable BCV polymorphic region (Fig. 7, B). Even though it is not clear whether determinants A and B reside on domains I and/or II, or on the third region, the probable BCV polymorphic region (Fig. 7, A) seems to be associated with these antigenic determinants since the probable polymorphic region is comprised of the most part of domain II and the region between two domains (Fig. 7, B, C, E). Sequence homology with perfectly conserved cysteine residues in the polymorphic region between BCV and MHV-4 (Yoo *et al.*, 1990) and the involvement of this region in BCV antigenicity (Fig. 7, B, D) further support

the association of BCV antigenic determinants with the probable polymorphic region. Since no potential glycosylation sites are found in this region (Fig. 7), glycosylation of the S1 protein does not seem to be directly involved in the proper formation of the antigenic determinants. This is in agreement with the previous finding that unglycosylated forms of the S1 protein were recognized by both groups of monoclonal antibodies (Yoo *et al.*, 1990). This portion of the S1 subunit contains 15 cysteine residues (Fig. 7) and many  $\beta$ -turns as revealed by secondary structure analysis (data not shown, Chou and Fasman, 1978), suggesting that the BCV polymorphic region forms an extremely complex bulbous structure. This structural characteristic may represent an important *in vivo* function of the spike protein in coronavirus pathogenesis. Minor factors may easily direct conformational changes of the S1 polymorphic region, and facilitate the escape of BCV from immunological selective pressure. Fine mapping within the probable polymorphic region and analysis of neutralization resistant BCV mutants will help to better understand the antigenic structure of the BCV S glycoprotein. Such studies are presently in progress.

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