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Analysis of the S Spike (Peplomer) Glycoprotein of Bovine Coronavirus Synthesized in Insect Cells

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The bovine coronavirus (BCV) spike glycoprotein precursor (S, formerly termed peplomer) and its two subunit polypeptides (S1 and S2) were individually expressed in *Spodoptera frugiperda* (Sf9) insect cells. Each recombinant baculovirus expressed both glycosylated (S, 170K; S1, 95K; S2, 80K) and unglycosylated (S_0, 140K; $S1_0$, 75K; and $S2_0$, 65K) forms of BCV spike polypeptides in Sf9 cells. The mature 95K S1 polypeptide was secreted whereas the S and S2 polypeptides remained cell-associated. The S precursor was partially cleaved in Sf9 cells, and the resulting S1 was also released into the medium. Neutralizing monoclonal antibodies representing two antigenic domains bound to recombinant S and S1 but not the S2 polypeptides, indicating that two major epitopes for BCV neutralization are located on the S1 subunit.

INTRODUCTION

Bovine coronavirus (BCV) is an enteropathogenic coronavirus causing severe diarrhea in newborn calves. BCV contains four major structural proteins: the nucleocapsid protein (N; 50K–54K), the matrix protein (M; 23K–26K), the hemagglutinin/esterase (HE; 124K), and the spike protein (S; 180K) (King and Brian, 1982; Deregt *et al.*, 1987; Cry-Coats *et al.*, 1988). The HE glycoprotein is a disulfide-linked dimer, which can be reduced to two identical subunits of 60K polypeptide, and exhibits hemagglutinating and receptor-destroying activities (Vlasak *et al.*, 1988a,b; Parker *et al.*, 1990b; Kienzie *et al.*, 1990).

The S glycoprotein forms large surface projections characteristic of coronaviruses (Spaan *et al.*, 1988). The S polypeptide is 1363 amino acids long, as deduced from the BCV S nucleotide sequence, and exhibits a typical class I glycoprotein profile with a N-terminal signal sequence and a transmembrane sequence near the C-terminal end (Parker *et al.*, 1990a). Amino acid sequence comparison of the BCV S glycoprotein revealed a sequence homology to murine hepatitis coronavirus (MHV) strains JHM and A-59 with a notable difference of a 49 and 138 amino acid insertion in the S1 region, respectively (Schmidt *et al.*, 1987, Luytjes *et al.*, 1987; Parker *et al.* 1990a). Proteolytic cleavage of the S glycoprotein yields two subunit proteins; the N-terminal half as the S1 subunit and the C-terminal half as the S2 subunit (Spaan *et al.*, 1988). Cleavage of the coronavirus S precursor is a prerequisite for the fusion activity (Storz *et al.*, 1981; Sturman *et al.*, 1985). Similar to other coronaviruses, cleavage of the BCV S glycoprotein is predicted to occur at the sequence RR-SRR in positions 764–768 (Parker *et al.*, 1990a).

The S glycoprotein functions in virus attachment to permissive cells (Collins *et al.*, 1982; Cavanagh and Davis, 1986), cell fusion (Storz *et al.*, 1981; Sturman *et al.*, 1985; Yoo *et al.*, manuscript in preparation), and induction of neutralizing antibodies (Cavanagh *et al.*, 1988; Spaan *et al.*, 1988). For avian infectious bronchitis virus (IBV), the most relevant region for interaction with and induction of neutralizing antibodies are found near the N-terminus of the S1 subunit (Cavanagh *et al.*, 1988). In contrast, for MHV, the S2 subunit has been reported to contain neutralizing epitopes (Makino *et al.*, 1987; Talbot *et al.*, 1988; Luytjes *et al.*, 1989). Two nonoverlapping groups of monoclonal antibodies (A and B) have been identified which interact with the BCV S glycoprotein (Deregt and Babiuk, 1987). These monoclonal antibodies neutralize BCV infectivity *in vivo* (Deregt *et al.*, 1989b) and *in vitro* (Deregt and Babiuk, 1987). However, the location of the neutralizing epitopes on the BCV S glycoprotein have not yet been determined.

Recently, the baculovirus *Autographa californica* (AcMNPV) has been widely used as an helper-independent expression vector for high level foreign gene expression. Recombinant proteins appear to undergo proper post-translational modification and transport in insect cells (Luckow and Summers, 1988; Miller, 1988; Cameron *et al.*, 1989). We have introduced the peplomeric glycoprotein gene of BCV into the baculovirus genome. In this paper we report the characteriza-

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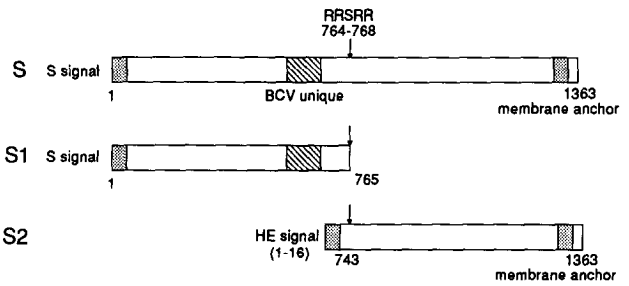

FIG. 1. Schematic representation of primary structure of the recombinant spike polypeptides of BCV. For construction of the S1-coding sequence, the S sequence was cleaved within the sequence encoding a putative proteolytic cleavage site, and a translation termination sequence was attached. The S2-coding sequence was fused with the 5′ terminal sequence of the BCV HE glycoprotein gene to contain a 16 amino acid HE membrane translocational signal. RRSRR indicates a putative proteolytic cleavage site. Shaded areas indicate hydrophobic domains. Numbers indicate amino acid positions. BCV unique region is regarded as an additional sequence when compared to that of the MHV-JHM S protein.

tion of the recombinant BCV peplomeric polypeptides produced in Sf9 cells, and demonstrate that the major neutralizing epitopes are located on the S1 subunit.

MATERIALS AND METHODS

Cells and viruses

S. frugiperda cells (Sf9, ATCC CRL 1711) were grown in suspension in TNM-FH medium containing 50 μg/ml of gentamycin supplemented with 10% fetal bovine serum (GIBCO) at 28° (Summers and Smith, 1987). The E2 strain of *A. californica* (AcMNPV) and the recombinant baculoviruses were propagated and titrated on a monolayer of Sf9 cells.

Cloning of recombinant plasmids

Restriction enzymes and DNA modifying enzymes were purchased from Pharmacia. Figure 1 illustrates the primary structure of the recombinant spike proteins of BCV. The cloned S gene of BCV (Quebec strain) was obtained from plasmid pCVE2 (Parker *et al.*, 1990a). For cloning of the S1-coding sequence, pCVE2 was digested with *Tth*111 I, which cleaved nucleotides 2294–2295 within the sequences encoding a putative proteolytic cleavage site of the S protein precursor, and asymmetric ends were blunted by Klenow fill-in reaction followed by digestion with *Bam*HI. The 2.3-kb fragment was purified and ligated into the *Bam*HI and blunt-ended *Xba* I sites of the pTZ18R. A universal translation termination sequence (5′-GCTTAATTAATTAAGC-3′) was subsequently inserted at the *Hin*cII site of the polylinker region and the resulting plasmid was termed pCVS1. pCVS1 was digested with *Sph*I and repaired with T_4 DNA polymerase. A *Bam*HI linker was then attached to the blunted *Sph*I end followed by subsequent *Bam*HI digestion and ligation into the *Bam*HI site of the baculovirus transfer vector pAcYM1 (Matsuura *et al.*, 1987; a gift from Dr. C. Y. Kang, University of Ottawa, Canada). Construction of the S2 gene will be described elsewhere. Briefly, a *Sph*I–*Sty*I fragment of pCVE3 (Parker *et al.*, 1989a) was fused to a *Sca*I–*Sph*I fragment from pCVE2 so that the resulting clone contained the entire S2-coding sequence proceeded by the sequence encoding 16 amino acids of the HE membrane translocational signal.

Antisera

Anti-BCV polyclonal rabbit sera and the BCV S-specific monoclonal antibodies were used as mouse ascitic fluids. Production and properties of the S-specific monoclonal antibodies, BB7-14, HF8-8, JB5-6, and HE7-3, have been previously described (Deregt and Babiuk, 1987).

Construction of recombinant viruses

A monolayer of *S. frugiperda* cells (1.2×10^6 cells per 35-mm dish) was transfected with a mixture of 1 μg of AcMNPV genomic DNA and 2–4 μg of plasmid DNA in 20 m*M* Hepes, pH 7.05, 1 m*M* Na_2HPO_4, 5 m*M* KCl, 150 m*M* NaCl, and 100 m*M* glucose. Prior to inoculation, the above mixture was precipitated by incubation with $CaCl_2$ (final concentration 125 m*M*) at room temperature for 30 min. The transfection mix was removed after 1 hr and the cells were then further incubated with 2 ml of fresh medium. The supernatant was harvested after 4 days, and recombinant viruses were screened by plaque hybridization followed by several rounds of plaque purification (Summers and Smith, 1987). Construction of the recombinant baculoviruses containing the S precursor and S2 subunit genes (termed pAcS and pAcS2, respectively) are described in detail elsewhere (Parker *et al.*, 1990a; Yoo *et al.*, manuscript in preparation).

Infection and protein analysis

Sf9 cells, at a density of 2×10^6 cells per 35-mm dish, were infected with baculoviruses at a m.o.i. of 10 to 20. The inoculum was removed after 1 hr adsorption and replaced with TNM-FH for further incubation. For *in vivo* radiolabeling, the medium was replaced with methionine-free Grace's medium for 1 hr. A total of 120 μCi/ml of [^{35}S]methionine (800 Ci/mmol, Amersham) was added and the cells were incubated for 4 hr. For pulse–chase studies, the cells were radiolabeled for 30 min, followed by addition of methionine-enriched TNM-FH for the indicated chase times. The cells were then

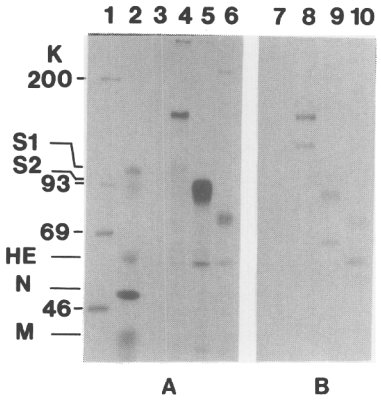

FIG. 2. Expression of the S, S1, and S2 polypeptides in SF9 cells by recombinant baculoviruses. *Spodoptera frugiperda* cells were infected with recombinant baculoviruses and labeled at 24 (A) or 48 hr postinfection (B) for 1 hr with 120 μCi/ml of [^{35}S]methionine. Cell lysates were prepared and immunoprecipitated using anti-BCV polyclonal rabbit sera and resolved by 7.5% SDS–PAGE. Lanes: 1, ^{14}C-labeled protein mol wt marker; 2, BCV proteins (S1, S2, HE, N, and M); 3 and 7, wild-type AcNPV-infected cell lysates; 4 and 8, vAcS-infected cell lysates; 5 and 9, vAcS1-infected cell lysates; 6 and 10, vAcS2-infected cell lysates.

washed with cold phosphate-buffered solution and lysed with 200 μl of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 150 m*M* NaCl, 50 m*M* Tris–HCl, pH 7.4, 10 m*M* EDTA, 0.1% SDS). For the radioimmunoprecipitation, 1 μl of the appropriate antibody was incubated with a suspension of 10 mg protein A–Sepharose CL-4B beads (Pharmacia). The beads were washed three times with RIPA buffer and 20 μl of cell lysate was added to the beads for further incubation. The immune complexes were dissociated with sample buffer (10% SDS, 25% glycerol, 10% 2-mercaptoethanol, 10 m*M* Tris–HCl, pH 6.8, 0.02% bromphenol blue) and subjected to electrophoresis on a 7.5% discontinuous SDS–polyacrylamide gel followed by autoradiography.

RESULTS

Synthesis of the S, S1, and S2 polypeptides in Sf9 cells

The synthesis of the BCV S polypeptides in insect cells infected with recombinant baculoviruses was measured by immunoprecipitation at 24 and 48 hr postinfection (Fig. 2A). Polypeptides of 170K (lane 4), 95K (lane 5), and 80K (lane 6) were produced 24 hr postinfection from the cells infected with vAcS, vAcS1, and vAcS2 recombinants, respectively. The 170K polypeptide in vAcS-infected cells was similar in migration rate to the uncleaved S precursor protein found in BCV-infected MDBK cells (Deregt *et al.*, 1987). The 95K and 80K polypeptides from the cells infected with vAcS1 and vAcS2 represented the S1 and S2 polypeptides, respectively. The recombinant S1 and S2 polypeptides were smaller in molecular weight than those of 100K and 95K found in the S2 polypeptides were smaller in molecular weight than those of 100K and 95K found in the BCV virion (Fig. 2A, lane 2).

When the vAcS-infected cell lysate was analyzed 48 hr postinfection (Fig. 2B), in addition to the 170K polypeptide a 140K polypeptide (S_0) was immunoprecipitated (lane 8). Cells infected with vAcS1 and vAcS2 also produced two forms of recombinant polypeptides at 48 hr postinfection (Fig. 2B, lanes 9 and 10); the 95K (S1) and 75K ($S1_0$) polypeptides and the 80K (S2) and 65K ($S2_0$) polypeptides, respectively.

In order to determine if the 140K, 75K, and 65K bands represented nonglycosylated forms of the corresponding mature polypeptide, tunicamycin treatment was carried out. Tunicamycin, an inhibitor of N-linked glycosylation, has been demonstrated to be effective in insect cells (Charlton and Volkman, 1986). The vAcS-infected cells were radiolabeled at 24 postinfection in the presence of various concentrations of tunicamycin. Only the 170K polypeptide is synthesized at this time of infection in the absence of tunicamycin as shown in Fig. 3A (lane 3). Tunicamycin at 15 μg/ml inhibited the production of the 170K polypeptide and only the 140K polypeptide was detected (Fig. 3B, lane 4). This result suggested that the 140K polypeptide was the nonglycosylated immature form of the mature 170K S polypeptide. Similar results were obtained with vAcS1 (Fig. 3C, lane 3) and vAcS2 (Fig. 3D, lane 6), demonstrating that the 75K and 65K polypeptides were the nonglycosylated S1 and S2 polypeptides, respectively. Molecular weights of the nonglycosylated S, S1, and S2 polypeptides are consistent with those of predicted polypeptides deduced from the nucleotide sequences (Parker *et al.*, 1990a). Cleavage of the S glycoprotein precursor in vAcS-infected cells was not detected either in the absence or presence of tunicamycin.

Neutralizing epitopes of the S1 subunit

Two antigenic domains responsible for BCV neutralization have been identified on the BCV S protein (Deregt and Babiuk, 1987). In order to approximate the location of these domains, the cell lysates prepared with vAcS1 or vAcS2 were reacted with monoclonal antibodies specific for each domain. The S (Fig. 4A) and S1 (Fig. 4C) polypeptides were immunoprecipitated by both group A, HE7-3 (lane 1), JB5-6 (lane 2), and HF8-8 (lane 3), and group B, BB7-14 (lane 4), monoclonal antibodies. None of monoclonal antibodies precipitated the S2 polypeptide (Fig. 4B, lanes 1–4) whereas

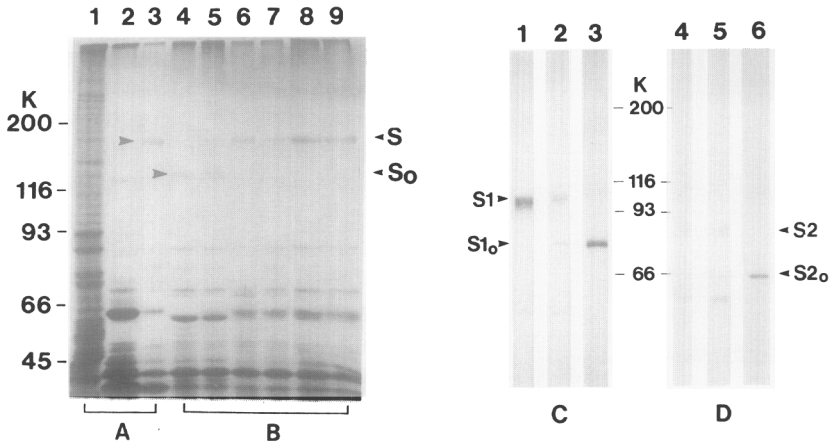

FIG. 3. Synthesis of the recombinant S (A and B), S1 (C), and S2 (D) polypeptides in the absence and presence of tunicamycin. Sf9 cells were infected with recombinant baculoviruses and incubated for 24 hr. The cells were pretreated with tunicamycin for 30 min and radiolabeled with 120 μCi/ml of [^{35}S]methionine for 1 hr in the presence of tunicamycin. Cells were disrupted with RIPA buffer and resolved on a 7.5% polyacrylamide gel directly (A and B) or after immunoprecipitation (C and D) as described under Materials and Methods. The gel was fluorographed and exposed at −70°. (A) Cell lysates in the absence of tunicamycin; lanes: 1, mock-infected; 2, wild AcNPV-infected; 3, vAcS-infected. (B), vAcS-infected cell lysates in the presence of tunycamycin. Concentrations of tunicamycin from lanes 4 to 9 are 15, 7.5, 2.5, 0.1, 0.01, and 0.001 μg/ml, respectively. (C) vAcS1-infected and (D) vAcS2-infected; lanes: 1 and 4, no tunicamycin; 2 and 5, 7.5 μg/ml of tunicamycin; lanes 3 and 6, 15 μg/ml of tunicamycin.

the polyclonal antibody did (Fig. 4B, lane 5). These results indicate that two major neutralizing epitopes of BCV are located on the S1 subunit. The unglycosylated S and S1 polypeptides were also bound by these monoclonal antibodies (Figs. 4A and 4C), suggesting that monoclonal antibodies HE7-3, JB5-6, HF8-8, and BB7-14 were all glycosylation-independent. Furthermore, these results also indicate that the recombinant S and S1 polypeptides produced in Sf9 cells assume the proper conformation in the vicinity of these epitopes, since these monoclonal antibodies have been shown to recognize conformation-dependent epitopes (Deregt and Babiuk, 1987).

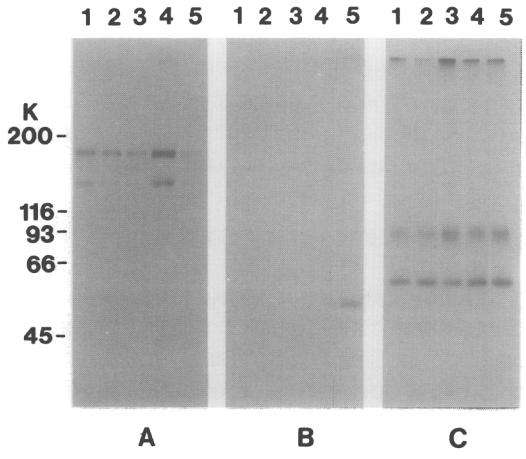

FIG. 4. Specific reactivity of the neutralizing monoclonal antibodies with the recombinant S1 polypeptide. (A) vAcS-infected; (B) vAcS2-infected; (C) vAcS1-infected cell lysates. Immunuoprecipitation with group A monoclonal antibodies, HE7-3 (lane 1), JB5-6 (lane 2), or HF8-8 (lane 3); with group B monoclonal antibody BB7-14 (lane 4); or with anti-BCV polyclonal rabbit sera (lane 5).

Secretion of the S1 subunit and partial cleavage of the S polypeptide

Since the S1 subunit has been found to contain major BCV neutralizing epitopes and because the sequences encoding the S1 subunit have been constructed to retain a membrane translocation signal, but not a membrane anchor, we were interested in the cellular transport of the S1 polypeptide in insect cells. In order to determine if the S1 polypeptide was secreted, cell culture supernatants were immunoprecipitated with S1-specific monoclonal antibodies and analyzed by SDS–PAGE. Cell labeling was done early in the infection period (20 hr p.i.) prior to the appearance of cytopathology. As shown in Fig. 5, a 90K polypeptide was released into the culture medium (lanes 3 and 5) and had a slightly faster migration rate than the 95K intracellular counterpart (lanes 8 and 10). The decrease in the size of the 90K polypeptide was probably due to the further processing of the S1 polypeptide during the extracellular transport. When the culture medium from cells infected with vAcS was examined, the uncleaved S precursor polypeptide was not detected (lane 2), confirming that the presence of the S1 in the culture medium was not due to cell destruction. Previously, the recombinant S polypeptide was shown to be associ-

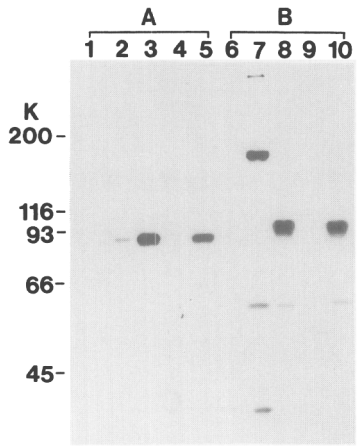

FIG. 5. Extracellular secretion of the recombinant S1 polypeptide. A monolayer of SF9 cells (2×10^6 cells per 35-mm dish) were infected with recombinant viruses and incubated at 28° with TNM-FH medium. At 20 hr postinfection, the medium was replaced with 800 μl of methionine-free Grace's medium containing 800 μCi/ml of [^{35}S] methionine (800 Ci/mmol, Amersham), and the cells were further incubated. After 12 hr incubation, the culture media was harvested and the cell debris were clarified two times by centrifugation at 14,000 rpm for 5 min in a microcentrifuge. The supernatants were then subjected to immunoprecipitation with a pool of the S1-specific group A monoclonal antibodies. The corresponding cell lysates were also prepared and analyzed by immunoprecipitation. (A) extracellular culture media; (B) intracellular lysates corresponding to the lanes in A; lanes: 1 and 6, wild-type AcNPV; 2 and 7, vAcS; 3 and 8, vAcS1; 4 and 9, vAcS2; 5 and 10, co-infection of vAcS1 and vAcS2.

ated with the plasma membrane of Sf9 cells (Parker *et al.*, 1990a). In addition, the S1-specific antibodies also precipitated a 90K polypeptide from the media of vAcS-infected cells (lane 2). This observation indicates that the S precursor is partially cleaved in Sf9 cells and the resulting S1 subunit is released to the medium. Taken together, all of these findings indicate that the signal sequence of the BCV S polypeptide is efficiently recognized in insect cells, and the S1 polypeptide is post-translationally processed and transported through the secretory pathway. Since the cells were labeled at an early stage of infection, neither the nonglycosylated S nor S1 were detected in the cells.

In order to evaluate the kinetics of S1 secretion, pulse–chase experiments were performed. To examine whether the unglycosylated S1 was also secreted, labeling was carried out at 36 hr postinfection, at which time both glycosylated and unglycosylated forms were present. The cells were labeled for 30 min and chased for up to 60 hr postinfection. The secretion of the S1 polypeptide increased as a function of time up to 7 hr. After 7 hr, there was no apparent increase in the amount of secreted polypeptides (Fig. 6A). In vAcS-infected cells, the 90K polypeptide was again identified in extracellular fluids with a secretion rate similar to that in vAcS1-infected cells (Fig. 6B). This result confirms partial cleavage of the S precursor and release of the S1 subunit. Nonglycosylated S1 was not detected extracellularly in either case despite the presence of both glycosylated and nonglycosylated forms of the S1 and S polypeptides in intracellular fractions (Fig. 6C). No apparent changes were observed in the intracellular amounts of glycosylated or unglycosylated S1 as a function of chase time (Fig. 6C).

DISCUSSION

The S peplomeric precursor of BCV and its two subunit glycoproteins were expressed in Sf9 cells using a recombinant baculovirus vector. The S1 subunit coding sequence contained the entire upstream sequence, including the membrane translocation signal, from the putative cleavage site of the S precursor. Since the S2 coding sequence includes a membrane anchor but lacks a membrane translocational signal, the BCV HE signal sequence was attached to the N-terminus of the S2 gene (Fig. 1). All of the recombinant S, S1, and S2 polypeptides were glycosylated and immunoreactive with anti-BCV polyclonal antisera. Nonglycosylated forms were also detected at 36 hr postinfection. The lack of glycosylation at later stages of baculovirus infection may be due to the saturation of the surface of rough endoplasmic reticulum by polyribosomes, the depletion of the *N*-glycosyl transferase, and/or other effects of baculovirus infection.

Molecular weights of the recombinant S1 and S2 polypeptides were estimated to be 95K and 80K, respectively, while the polypeptides produced in BCV-infected mammalian cells generally comigrate as 100K–110K (King and Brian, 1982; Deregt *et al.*, 1987). Insect cells have been reported to be incapable of processing complex type oligosaccharides in *N*-glycosylation, and to convert high mannose-type oligosaccharides to trimannosyl cores (Hsieh and Robbins, 1984). Recently accumulating information has indicated that the glycosylation of baculovirus-produced recombinant proteins is somewhat different from that of the proteins produced in mammalian cells (Miller, 1988; Cameron *et al.*, 1989; Jarvis and Summers, 1989). Although we cannot rule out the possibility of internal or terminal deletions in the polypeptide sequences produced in insect cells, it seems likely that the molecular weight differences of the authentic versus recombinant S1 and S2 are due to the differences in the nature of glycosylation (Fig. 2). Attempts to directly compare the nonglycosylated-recombinant S1 and S2 with the tunicamycin-treated authentic S1 and S2 were unsuccessful.

Secretion of the S1 polypeptide confirmed that the signal sequences of BCV S and BCV HE glycoproteins

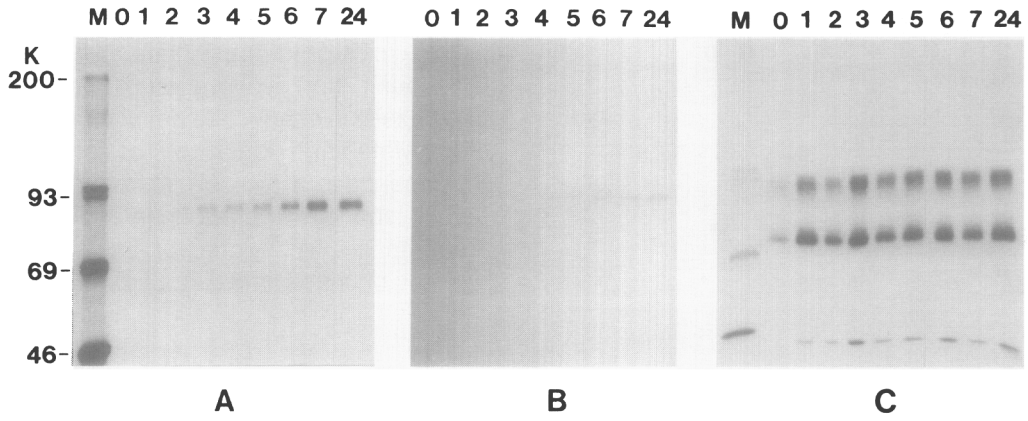

FIG. 6. Pulse–chase kinetics of the secretion of S1 polypeptide. Recombinant virus-infected cells were maintained in TNM-FH media for 24 hr. The media were replaced with methionine-free Grace's media and an intracellular pool of methionine was depleted by incubation for 1 hr. The cells were labeled by addition of a final concentration of 800 μCi/ml of [^{35}S]methionine for 30 min. The label was then immediately replaced with 5× methionine-enriched TNM-FH media and the cells were chased for indicated time. The culture media were harvested, clarified, immunoprecipitated, and analyzed by SDS–PAGE. Corresponding cell lysates were also prepared and immunoprecipitated. (A), vAcS1-infected cell cuture media, 10 days exposure; (B), vAcS-infected cell culture media, 4 weeks exposure; (C), vAcS1-infected cell lysates. Numbers indicate the time of chase in hour. M, protein mol wt markers.

were efficiently recognized in Sf9 cells, and directed membrane translocation and subsequent cellular transport. The recombinant S1 polypeptide was secreted into the culture medium from the cells, while the S precursor and the S2 subunit remained associated with the plasma membrane (Parker *et al.*, 1990a). Even though both glycosylated and unglycosylated S1 polypeptides were present in the cells, only the glycosylated form was secreted (Fig. 6). Similarly, Jarvis and Summers (1989) observed that only glycosylated human tPA was secreted. It has also been shown that secretion of vitellogenin was inhibited in Sf9 cells by tunicamycin treatment (Wojchowski *et al.*, 1986). Thus, these observations suggest that the *N*-glycosylation of the recombinant S1 polypeptide is required for the transport through the secretory pathway in Sf9 cells.

Secretion of the S1 polypeptide was somewhat slower and inefficient in Sf9 cells compared to secretion in mammalian cells. Similar results have been observed for tPA secretion (Jarvis and Summers, 1989). In contrast, the truncated form of the F glycoprotein of respiratory syncytial virus (RSV) was efficiently secreted in Sf9 cells (Wathen *et al.*, 1989). It has been shown with two different viral glycoproteins (influenza hemagglutinin and vesicular stomatitis virus G protein) that folding and assembly of native structures are essential for their transport and subsequent maturation through the exocytotic pathway in mammalian cells (Gething *et al.*, 1986; Kreis and Lodish, 1986). Therefore, it is possible that the unusually high content of cysteine residues in the S1 subunit of BCV (36 residues) may alter the proper folding of the polypeptide synthesized in Sf9 cells, which becomes a limiting factor for efficient secretion (also refer to Pennica *et al.*, 1983; tPA has 35 cysteine residues).

The extracellular form of the S1 polypeptide was slightly smaller by approximately 5K than the intracellular counterpart, as previously noted with a secreted form of the BCV HE polypeptide (Parker *et al.*, 1990b). It remains to be determined whether the difference in the size of the two forms are related to the primary structure of the polypeptide (i.e., cleavage of the signal peptide) or further modification of the oligosaccharides during or after the extracellular transport.

Cleavage of the BCV S precursor in Sf9 cells did not occur to a significant extent. Although the S1 polypeptide was detected in the vAcS-infected cell culture medium, the predominant form was the uncleaved S precursor. We were unable to detect the S2 portion of the cleaved S precursor, probably due to the limited amounts of cleaved products and the lack of monoclonal antibodies specific for the S2 subunit. However, cell fusion was observed in the vAcS-infected Sf9 cells, confirming the cleavage of the S precursor and the presence of the S2 subunit as a cleaved product (Yoo *et al.*, manuscript in preparation). It is noteworthy that the cleavage of the recombinant S has not been previously observed in either IBV peplomer expression in vaccinia virus (Tomley *et al.*, 1987) or MHV-JHM peplomer expression in baculovirus (Yoden *et al.*, 1989).

Cleavage of the S peplomer appears to be important in coronavirus pathogenesis. Cell fusion is activated by proteolytic cleavage of the S protein (Sturman *et al.*, 1985; Yoo *et al.*, manuscript in preparation). In MHV and IBV, it has been demonstrated that the S protein is

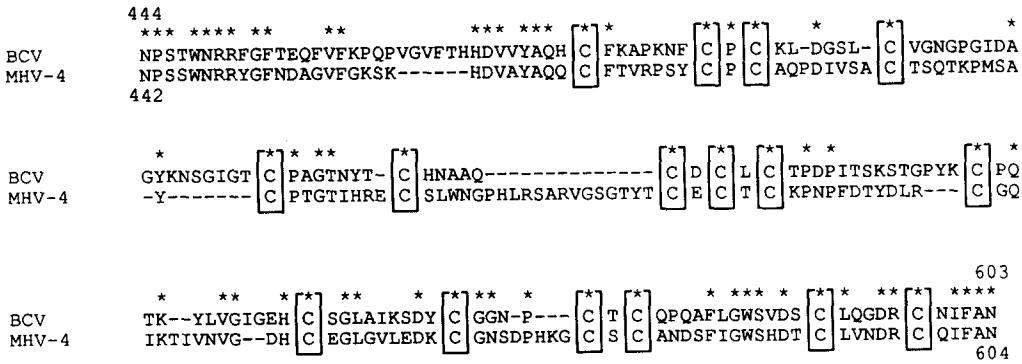

FIG. 7. Conservation of cysteine residues in the BCV S1 region and the wild-type MHV-4 polymorphic domain of the S glycoprotein. Amino acids from 444 through 603 of the BCV S protein (Parker *et al.*, 1990a) were aligned to the comparable amino acid sequences of MHV-4 (Parker *et al.*, 1989b). Dashed lines indicate deletions. Asterisks indicate homologous amino acids. Cysteine residues are boxed.

responsible for the host-cell binding (Collins *et al.*, 1982; Cavanagh and Davis, 1986). While it is not clear whether the S protein of BCV also plays a role for the cell binding, it will be of interest to determine if proteolytic cleavage is related to the cell binding activity of the S protein.

Most of the critical epitopes for neutralization in transmissible gastroenteritis virus (TGE) (Jiménez *et al.*, 1986), IBV (Cavanagh *et al.*, 1988), and MHV (Talbot *et al.*, 1984) appear to be conformation-dependent. The S glycoprotein of BCV also contains at least two major conformation-dependent neutralizing epitopes, which are located on the S1 subunit as shown in Fig. 4. During the preparation of this manuscript, Parker *et al.* (1989b) reported that wild-type MHV-4 contained an additional 142 to 159 amino acids in the S1 region when compared to that of neutralization-resistant, neuroattenuated MHV-4 variants. This polymorphic domain present in wild-type MHV-4 was suggested to be responsible for the neutralization and pathogenesis in MHV (Parker *et al.*, 1989b). A similar situation may exist in the S protein of BCV. Amino acids 456–593 of the BCV S1 subunit were previously thought to be BCV unique by comparison to the S1 sequences of MHV strains JHM and A-59 (Parker *et al.*, 1990a). However, significant sequence homology was observed when this region of the BCV S1 subunit was compared to that of polymorphic domain of wild-type MHV-4 (Fig. 7). Interestingly, all 16 cysteine residues present in this region of BCV were perfectly conserved in the polymorphic domain of wild-type MHV-4 (Fig. 7). This suggests that the region of amino acids 456–593 of the BCV S1 subunit may possess functions similar to the polymorphic domain of the wild-type MHV-4 S protein. Deregt *et al.* (1989a) have tentatively identified a 37K trypsin fragment from the S polypeptide that contains both BCV neutralizing epitopes. The 37K fragment lies upstream to the putative S cleavage site, and partly overlaps with this same region. When we expressed a truncated form of the BCV S1 which extended to the middle (amino acids 1–516) of this region, the polypeptide produced did not react with any of the monoclonal antibodies (unpublished observation). All of these observations imply that amino acids 456–593 of the BCV S1 subunit may be associated with antigenic determinants for both groups of neutralizing antibodies. We have generated several mutants resistant to S1-specific monoclonal antibodies (*mar*) and are currently determining the sequence of the S1 subunit of these *mar* mutants. This will provide valuable information for identifying the neutralizing epitopes and pathogenesis in BCV.

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