Key words: bovine coronavirus/structural proteins/glycoprotein processing

Structural Proteins of Bovine Coronavirus and Their Intracellular Processing

By DIRK DEREGT,¹ MARTA SABARA^{1,2} and LORNE A. BABIUK^{1,2*}

¹Veterinary Microbiology, University of Saskatchewan, and the ²Veterinary Infectious Disease Organization, 124 Veterinary Road, Saskatoon, Saskatchewan, Canada, S7N 0W0

(Accepted 14 July 1987)

SUMMARY

The Quebec isolate of bovine coronavirus (BCV) was found to contain four unique major structural proteins. These proteins consisted of the peplomeric protein (gp190/E2, gp100/E2), the nucleocapsid protein (p53/N) and its apparent trimer (p160/N), a family of small matrix glycoproteins (gp26/E1, gp25/E1 and p23/E1) and the putative haemagglutinin (gp124/E3). Pulse-chase experiments utilizing polyclonal antiserum and monoclonal antibodies indicated that the unique BCV E3 protein had as its primary precursor an N-linked glycoprotein with an M_r of 59000 (gp59) which underwent rapid dimerization by disulphide bond formation to yield gp118. Further glycosylation of gp118 produced gp124/E3 which incorporated fucose. Thus gp124/E3 was probably a homodimer. The processing of the E2 and E1 proteins of BCV was similar to that shown previously for mouse hepatitis virus. A large N-linked precursor glycoprotein, gp170, underwent further glycosylation to yield gp190/E2 before subsequent proteolytic cleavage to yield gp100/E2. The glycosylated E1 (gp26, gp25) proteins arose as a result of O-linked glycosylation of p23/E1 as indicated by the resistance of these species to tunicamycin.

INTRODUCTION

Most coronaviruses can be described as having three or four unique major structural proteins. These proteins consist of the peplomer protein, the nucleocapsid protein and a small matrix glycoprotein. An additional protein, identified as the haemagglutinin, is observed in the group of haemagglutinating mammalian coronaviruses of which bovine coronavirus (BCV) is a member (Siddell *et al.*, 1983; King *et al.*, 1985; Hogue *et al.*, 1984).

The peplomeric protein, often designated E2, is the glycoprotein that makes up the large surface projections of the virus and can be found in an uncleaved form with M_r of approximately 170K to 200K and/or cleaved forms of approximately 85K to 100K (Sturman *et al.*, 1985; Cavanagh *et al.*, 1986). The cleaved forms may either comigrate or migrate as separate entities on polyacrylamide gels. For mouse hepatitis virus (MHV), pulse-chase studies have identified the precursor to this protein as a 150K intracellular glycoprotein (Siddell *et al.*, 1981; Rottier *et al.*, 1981). The E2 glycoprotein is responsible for virus attachment and cell membrane fusion and elicits the production of virus-neutralizing antibodies (Collins *et al.*, 1982; Sturman *et al.*, 1985; Wege *et al.*, 1984).

The nucleocapsid protein together with the RNA of the virus forms the helical nucleocapsid. This protein is phosphorylated, has M_r of 50K to 60K and is the most abundant protein of the virion (Stohlman & Lai, 1979; King & Brian, 1982).

The small matrix glycoprotein, designated E1, is often found as a family of differently glycosylated proteins including its unglycosylated precursor (Siddell *et al.*, 1981; Rottier *et al.*, 1981; Stern & Sefton, 1982*b*). These proteins span a range of M_r 20K to 35K. For MHV, pulsechase experiments have identified the precursor protein as a 20K to 23K non-glycosylated protein which undergoes *O*-linked glycosylation (Niemann & Klenk, 1981; Holmes *et al.*, 1981; Cheley & Anderson, 1981; Siddell *et al.*, 1981; Rottier *et al.*, 1981). This protein is involved in the budding of virus into the membranes of the endoplasmic reticulum and Golgi apparatus (Holmes *et al.*, 1981; Tooze *et al.*, 1984).

The additional protein present on haemagglutinating mammalian coronaviruses is a disulphide-linked dimer of approximately 130K to 140K, reducible to comigrating subunits of 65K (King & Brian, 1982; Hogue & Brian, 1986). Little else is known about this protein and the mRNA coding for it remains to be identified. Furthermore, the function of this protein in virus-cell interactions is unknown.

In this report the structural proteins of the Quebec isolate of BCV were identified and were found to conform to the general pattern described above. Although, in all, eight structural polypeptides were identified, several were found to be related, and thus the profile of BCV could be described as consisting of four unique major proteins. Further, intracellular precursor proteins to all three BCV structural glycoproteins were identified and their processing is described.

METHODS

Virus and cells. The Quebec isolate of BCV, an enteric virus, (obtained from S. Dea, Department of Pathology and Microbiology, Faculty of Veterinary Medicine, University of Montreal, St. Hyacinthe, Quebec, Canada) was used in these studies (Dea *et al.*, 1980). The virus was plaque-purified three times and propagated at an m.o.i. of 0.5 p.f.u./cell in confluent monolayers of Madin–Darby bovine kidney (MDBK) cells grown in Eagle's MEM (Gibco) supplemented with 10% foetal bovine serum (FBS) at 37 °C.

Plaque assays. BCV was assayed on MDBK cells in six-well (35 mm) dishes (Linbro). Cells were washed with MEM and virus was then adsorbed for 1 h at 37 °C. After adsorption the virus inoculum was removed and cells were overlaid with 0.8% agarose (Bio-Rad) in MEM. After 3 days of incubation, the cells were stained with neutral red and the plaques were counted.

Virus purification. Purified virions were obtained by a modified version of the method described by Spaan et al. (1981). Supernatants from BCV-infected cells were clarified by centrifugation (GS-3 rotor, Sorvall) at 10000 g at 4 °C for 20 min. Phenylmethylsulphonyl fluoride (PMSF, Sigma) at 0·1 mM final concentration was added to the supernatant. This was followed by the addition of 100 g/l polyethylene glycol (PEG) 6000 (Sigma) and the slow addition of 23·3 g/l NaCl with gentle stirring. After continued slow stirring for 3 h at 4 °C, the virus suspension was centrifuged at 10000 g for 20 min at 4 °C. The pellet was resuspended in TNE buffer (20 mM-Tris-HCl pH 7·2, 100 mM-NaCl, 1 mM-EDTA), and clarified by centrifugation (SS-34 rotor, Sorvall) at 10000 g for 5 min at 4 °C. The resulting suspension was layered over 13 ml linear 20 to 55% (w/v) sucrose gradients and centrifuged overnight (SW27·1 rotor, Beckman) at 25000 r.p.m. at 4 °C. The visible virus band at a density of 1·18 g/ml was recovered and diluted in 5 to 10 vol. of TNE buffer containing 0·1 mM-PMSF and again centrifuged (SW27·1 rotor, Beckman) at 25000 r.p.m. for 3 h at 4 °C. Finally, pelleted virus was resuspended in a small volume of TNE buffer. This suspension was then prepared for polypeptide analysis on polyacrylamide gels by the addition of an appropriate sample buffer.

Isotopic labelling of virion structural proteins. Confluent MDBK cells in flasks (150 cm², Corning) were washed with MEM and then infected with virus at an m.o.i. of 1.0 to 2.0 p.f.u./cell by adsorption for 2 h at 37 °C. The inoculum was then replaced with MEM plus 2% FBS. At 12 to 14 h post-adsorption the medium was removed and 16 ml of the appropriate medium and the isotope were added. All isotopes were obtained from Amersham. Viral proteins were labelled by addition of [³⁵S]methionine or ³H-labelled amino acids (a mixture of leucine, lysine, phenylalanine, proline and tyrosine) at $50 \,\mu$ Ci/ml in MEM containing 10% of the normal concentration of methionine or 10% of the normal concentration of leucine, lysine, phenylalanine and tyrosine respectively, and 2% FBS. Viral glycoproteins were labelled with D-[6-³H]glucosamine hydrochloride at 100 μ Ci/ml in MEM containing 20% of the normal concentration of glucose and 2% FBS. Supernatants containing radiolabelled virus were harvested at 38 to 50 h post-adsorption and were mixed with supernatants containing unlabelled virus at a 1:5 ratio before purification.

Isotopic labelling of intracellular proteins. Confluent monolayers of MDBK cells were washed once with MEM. Virus, at an m.o.i. of 5 to 10 p.f.u./cell, was then adsorbed for 1.0 to 2.0 h at 37 °C. After adsorption the inoculum was removed and replaced with MEM and 2% FBS. For most experiments the medium was removed and replaced with the appropriate medium and isotope at 12 to 14 h post-adsorption as described above for labelling of virion structural proteins. Cells were also labelled with L-[6-3H]fucose (Amersham) at 100 μ Ci/ml in MEM. For experiments involving inhibition by tunicamycin of glycosylation, tunicamycin at a concentration of 0.001 to 1.0 μ g/ml was added with the isotope. At 30 to 36 h post-adsorption the cells were washed and harvested in ice-cold phosphate-buffered saline (PBS) and after pelleting were prepared for radioimmunoprecipitation or electrophoresis.

For pulse-chase experiments the cells were starved of methionine for 2 h and then labelled with $200 \,\mu\text{Ci/ml}$ of [³⁵S]methionine in methionine-deficient medium beginning at 18 to 23 h for different experiments. After the pulse, the cells were washed with MEM or MEM containing ten times the normal concentration of methionine and then further incubated with this same medium for 15 min to 6 h. Pulse-labelled and pulse-chased cells were washed and harvested in ice-cold PBS and after pelleting were prepared for radioimmunoprecipitation or electrophoresis.

Radioimmunoprecipitation. Radiolabelled cells were washed and harvested in ice-cold PBS, pelleted and resuspended in 150 µl of RIPA buffer (0.05 M-Tris-HCl pH 7.0, 0.15 M-NaCl, 1% deoxycholate, 1% Triton X-100) containing 2 to 4% Aprotinin (Sigma) and 0.2 mM-PMSF per 60 mm plate (Corning), vortexed and then sonicated for about 5 s at 100 W (Braunsonic 1510; B. Braun Instruments, San Mateo, Ca., U.S.A.). The cell lysates were clarified by centrifugation at 25000 r.p.m. (SW50-1 rotor, Beckman) for 1 h at 4 °C or in an airfuge at 205 kPa (178000 g) for 15 min and the lysate was used immediately for immunoprecipitation without freezing. Viral antigens were immunoprecipitated overnight at 4 °C by the addition of 150 µl RIPA buffer containing SDS (final concentration of 0.5%) and hybridoma ascites fluid at a dilution of 1 :2000 or 25 µl RIPA containing SDS and 25 µl rabbit polyclonal antisera to 50 µl of lysate. After incubation, 40 µl of rabbit anti-mouse Immunobeads (Bio-Rad), for immunoprecipitations using monoclonal antibody (MAb), or 50 µl RIPA buffer containing 100 mg/ml Protein A-Sepharose (Pharmacia) for immunoprecipitations using polyclonal antiserum, was added. The mixture was then incubated for an additional 2 h on a rocking platform at 4 °C. Each sample was then washed two or three times with ice-cold RIPA buffer containing 0.1% SDS and 0.1 mM-PMSF. After the final wash, the samples were prepared for electrophoresis.

Bromelain digestion of viral structural proteins. The method folowed was essentially that described by Sturman (1977). Purified virus obtained from sucrose density gradients containing TMEN (50 mm-Tris-maleate, 1 mm-EDTA, 0·1 m-NaCl pH 6·0) was resuspended in 4 ml of TMEN and incubated with or without bromelain (pineapple stem, Sigma) at 1 mg/ml for 3 h at 37 °C in the presence of 0·1 mm-2-mercaptoethanol. After incubation the virus was rebanded on a linear 20 to 55% (w/w) sucrose gradient and processed as described for purification of virus above.

Peptide mapping. The procedure followed was essentially that of Cleveland *et al.* (1977). Briefly, L-[³⁵S]methionine-labelled viral proteins were solubilized with a non-reducing sample buffer containing 6 M-urea (see below), and separated by electrophoresis on a 10% preparative polyacrylamide gel. Protein bands were localized by Coomassie Brilliant Blue staining or overnight autoradiography of the wet gel and then excised from the gel. The protein in each gel slice was then digested on an analytical 15% polyacrylamide gel with a 5% stacking gel using various concentrations of *Staphylococcus aureus* V8 protease (Sigma). After electrophoresis, the gel was dried and the resulting peptide bands were visualized by autoradiography.

Production of monoclonal antibodies. BALB/c mice were injected intraperitoneally with purified BCV mixed with an equal volume of Freund's complete adjuvant. Two weeks later the mice were boosted with purified virus emulsified in Freund's incomplete adjuvant. A final intravenous injection of virus in PBS was given 4 days prior to fusion.

Fusion was performed by a modification of the method of Kennett *et al.* (1978). Briefly, cells of a single spleen were mixed with 10^7 P3/NSI/1-Ag4-1 myeloma cells (Köhler & Milstein, 1976), pelleted and fused by addition of 2 ml of 40% (w/w) PEG1540 (Baker Chemicals, Edmonton, Alberta, Canada) in MEM for 7 min. The hybrid cells were suspended in RPMI-1640 (Gibco) containing 20% FBS and were then incubated for 1 h at 37 °C. They were then pelleted, resuspended in HAT medium [RPMI-1640, supplemented with 20% FBS, 1% glutamine, 25μ g/ml of gentamicin, 10% NCTC-135 (Gibco), 0.45% w/w glucose, 0.1 mM-hypoxanthine, 0.03 mM-aminopterin and 0.4 mM-thymidine] and dispensed into wells of microtitre plates containing feeder layers of mouse macrophages. The cells were incubated at 37 °C and HAT medium was changed twice per week. The supernatants of growing hybridomas were screened for production of BCV-specific antibodies by indirect immunofluorescence, using an equal mixture of infected and uninfected cells fixed with ethanol to a glass slide or by an ELISA. Positive clones were subcloned in microtitre plates by the limiting dilution method.

Ascites fluids were obtained by intraperitoneal injection of 10^6 to 10^7 specific antibody-producing hybridoma cells into BALB/c mice (Charles River Breeding Laboratories Canada, Montreal, Quebec) that had been primed with pristane (2,6,10,14-tetramethylpentadecane; Aldrich) 2 to 6 weeks previously.

Western immunoblotting. Viral proteins were radiolabelled with L-[³⁵S]methionine, solubilized with a nonreducing sample buffer containing 6 M-urea (see below) and fractionated on a 10% polyacrylamide gel. Proteins were then transferred to nitrocellulose paper (Schleicher & Schüll) by electroblotting at 8.75 V/cm for 12 h at 4 °C in Tris-glycine-methanol buffer (20 mM-Tris-HCl pH 8.3, 190 mM-glycine, 20% methanol). Nitrocellulose strips were autoradiographed to determine the efficiency of protein transfer.

Localization of viral proteins was achieved by incubating hybridoma supernatant with the nitrocellulose blots by a slight modification of the method described by Braun *et al.* (1983). Specifically, each nitrocellulose strip was sealed in a plastic bag containing 3 ml of PBS with 3% bovine serum albumin (fraction V, Sigma) and 5μ l of





Fig. 2. BCV intracellular structural proteins. Immunoprecipitation of cell lysates by MAbs and rabbit anti-BCV serum (lanes 1 to 10). Incorporation of radiolabelled sugars in intracellular BCV proteins (lanes 11 to 14). Cells were infected with BCV (odd-numbered lanes) or mock-infected (even-numbered lanes), labelled with ³⁵S]methionine (lanes 1 to 4, 9 and 10), [³H]glucosamine (lanes 5 to 8, 11 and 12) or [³H]fucose (lanes 13 and 14). Cell lysates were then immunoprecipitated with MAb BD9-8C (lanes 1 to 4), MAb BB7-14 (lanes 5 and 6), rabbit anti-BCV serum (lanes 7 and 8) or MAb AE12-4 (lanes 9 and 10) or cell lysates were prepared directly for electrophoresis (lanes 11 to 14). Samples were solubilized with a sample buffer containing urea (final concentration 3 M) without 2-mercaptoethanol (lanes 1, 2, 9 to 12) or sample buffer containing 2-mercaptoethanol (other lanes). Analysis was done in gels ranging from 7 to 12.5% polyacrylamide. Positions of molecular weight standards are shown next to lane 8.

Clone designation	Specificity
BD9-8C	gp124/E3, gp59*, gp118*
BB7- 14	gp190/E2, gp100/E2, gp170*, gp340
AE12-4	gp26/E1
CC7-3	gp26/E1, gp25/E1
MD8, MG6, MC5, NC10	p53/N, p160/N

Table 1. MAbs directed against bovine coronavirus proteins

* Precursor proteins.

horseradish peroxidase-coupled rabbit anti-mouse IgG or horseradish peroxidase-coupled goat anti-rabbit IgG (Jackson ImmunoResearch, Avondale, Pa., U.S.A.). The strips were subsequently developed by reaction with 0.02% dianisidine dihydrochloride (Sigma) substrate overnight at room temperature.

PAGE. Samples were resuspended in electrophoresis sample buffer (Laemmli, 1970), boiled for 2 to 5 min and stored at -20 °C or analysed immediately on various percentages of polyacrylamide gels. Alternatively, samples were resuspended in sample buffer without 2-mercaptoethanol or mixed 1:1 with 2 × urea sample buffer (6 or 10 M-urea, 4% SDS, 0.5% bromophenol blue in 62.5 mM-Tris-HCl pH 6.8). In the latter case samples were incubated at 37 °C for 30 min. Electrophoresis was carried out overnight at 8 mA in 12.5 cm to 15 cm × 1.5 mm gels with a 3% stacking gel in electrophoresis buffer. After electrophoresis, gels were stained with Coomassie Brilliant Blue, destained in 7% acetic acid, and dried on a slab gel dryer. Gels containing tritium-labelled proteins and immunoprecipitates were fluorographed (Amplify; Amersham) before drying. Dried gels were exposed to 3M X-ray film (Picker International, Saskatoon, Saskatchewan, Canada) at room temperature or at -70 °C for fluorographed gels.

RESULTS

BCV structural proteins

To determine the number of BCV structural proteins and to determine which were glycoproteins, virus was grown in the presence of tritiated amino acids or glucosamine, purified and analysed by PAGE (Fig. 1). Eight proteins were resolved under non-reducing conditions and had M_{τ} of 190K, 160K, 124K, 100K, 53K, 26K, 25K and 23K. Based on their incorporation (gp) or lack of incorporation (p) of [³H]glucosamine these proteins were designated gp190, gp124, gp100, gp26, gp25 and p160, p53 and p23 respectively (Fig. 1, lanes 1 and 2). When a reducing agent was used in sample preparation the virus profile changed with reduction of gp124 to 62K subunits (gp62) (Fig. 1, lanes 3 and 4). Occasionally, glycosylated species with higher M_{τ} were detected and these were assumed to be multimers of gp100 and/or gp190. The glycoprotein gp190 was often present in small amounts.

The protein p160 was also often present in small amounts and was not observed when 2mercaptoethanol was used as a reducing agent, suggesting that this was a multimer in which subunits were held together by disulphide bonds. Further evidence in this report indicates that this species is a multimer (probably a trimer) of the nucleocapsid protein p53.

It was noted that there was a tendency for the small glycoproteins gp26 and gp25, as well as p23, to aggregate to a slower migrating form (i.e. species a in Fig. 1). Although three bands were usually obvious, there was some heterogeneity in the size and number of protein bands in this region.

MAbs to BCV structural proteins

Monoclonal antibodies to BCV structural proteins were produced and were used to elucidate the relationships and identities of the structural proteins (Table 1). These MAbs were useful in confirming tentative protein assignments, establishing relationships between proteins and for understanding their processing in cells.

MAb BD9-8C immunoprecipitated a 124K glycoprotein from BCV-infected cell lysates (Fig. 2, lane 1) and also reacted to virion gp124 in a Western immunoblot (data not shown). When sample buffer containing the reducing agent 2-mercaptoethanol was used in the preparation of the immunoprecipitate, a 62K glycoprotein was observed (Fig. 2, lane 3), thus confirming the dimeric nature of gp124.



Fig. 3. Western immunoblotting of BCV structural proteins. Reactivity of rabbit anti-BCV serum (lane 1) and MAb CC7-3 (lane 2) to Western blots of BCV structural proteins. Differential reactivity to p160 and p53 by hybridoma supernatants: MD8 (lane 3), MG6 (lane 4), MC5 (lane 5), and NC10 (lane 6). Positions of molecular weight standards are shown on the right.

Several MAbs which recognized different epitopes (D. Deregt *et al.*, unpublished results) immunoprecipitated a series of glycoproteins with M_r of 100K, 170K, 190K and 340K (Fig. 2, lane 5) indicating a relationship between these proteins. The 190K glycoprotein was more readily observed in pulse-chase experiments. Both the 100K and 190K intracellular glycoproteins comigrated with the virion gp100 and gp190. Further, the MAb BB7-14 reacted with virion gp100 in Western immunoblots, but reactivity with virion gp190 was not demonstrated presumably because the quantity of antigen was not sufficient for detection (data not shown).

The MAb AE12-4 immunoprecipitated a 26K protein from BCV-infected cells (Fig. 2, lane 9) and reacted with virion gp26 in Western immunoblots (data not shown). Another MAb, CC7-3, reacted with both virion gp26 and gp25 in Western immunoblots (Fig. 3, lane 2) and the presumptive aggregated species a observed in Fig. 1.

Supernatants from several hybridomas reacted with both p53 and a protein of about 160K in a Western immunoblot showing apparent relatedness for these two proteins (Fig. 3, lanes 3 to 7). The latter protein was concluded to be the same as that designated p160 in Fig. 1.

Incorporation of radiolabelled sugars

Both the intracellular proteins gp124/gp62 and gp100 were found to incorporate tritiated glucosamine and fucose (Fig. 2, lanes 7, 11, 13). In contrast, the 26K intracellular protein could be labelled with tritiated glucosamine, although weakly, but not with tritiated fucose (Fig. 2, lanes 11 and 13). Attempts to demonstrate incorporation of tritiated glucosamine into the 25K

Structural proteins of BCV



Fig. 4. Proteolytic digestion of virion gp124, gp100 and p53 (a, b and c, respectively) with S. aureus V8 protease. The amounts of V8 protease used were 0 µg (lanes 1), 1·0 µg (lanes 2), 0·1 µg (lanes 3), 0·01 µg (lanes 4) and 0·001 µg (lanes 5). Analysis was done in a 15% polyacrylamide gel.

intracellular protein were not successful. This may have been due to levels of labelling being insufficient for detection even after long exposure of the gel.

Limited proteolysis of BCV structural proteins

To define further the relationships between proteins, gel slices containing the most abundant virion protein species gp124, gp100, p53, gp26, gp25 and p23 were cut from preparative gels and subjected to limited proteolysis. It was apparent that gp124, gp100, p53 and gp26 exhibited unique digestion patterns indicating an absence of relationships between these proteins (Fig. 4a, b and c and Fig. 5, lane 1). However, proteolysis of gp26, gp25 and p23 (taken from a different virus preparation) produced similar digestion patterns (Fig. 5, lanes 2 to 4). There was apparent identity of the three fastest migrating peptides (small arrows). Generation of the largest of these three peptides appeared to be more efficient for p23 (Fig. 5, lane 4) than for gp26 and gp25 (Fig. 5, lanes 2 and 3 respectively). Further, the largest peptide in each lane migrated progressively more slowly from p23 to gp26 (large arrows) which may have been due to different degrees of glycosylation of this peptide. Thus, these results suggest that these three proteins were probably identical except for their glycosylation.

In BCV-infected cell lysates, polypeptides of 26K, 25K and 23K were also observed although some heterogeneity was noted for polypeptides in this size range, both in number and apparent M_r . Usually two or three bands were noted, the 25K protein appearing variably. It was apparent, as demonstrated by partial proteolysis, that these were also related proteins (Fig. 5, lanes 5 to 7).

Digestion of BCV structural proteins

To determine the location of the structural proteins on the virion, virus was grown in the presence of [³⁵S]methionine, purified, mock-digested or digested with bromelain and analysed by PAGE (Fig. 6).

The glycoproteins gp100, gp26 and gp25 were susceptible to bromelain digestion indicating that at least a portion of each of these proteins was located on the surface of the virus (Fig. 6). The species labelled a, which was also susceptible to bromelain digestion was originally thought to be gp190. However, upon consideration of its M_r (>200K) and upon closer inspection of the gel it was concluded that species a was probably an aggregated form of gp190 (Fig. 6, lane 1) that was at least partially converted to gp190 (open arrow) by bromelain digestion (Fig. 6, lane 2). Sturman & Holmes (1977) also noted aggregated forms of the MHV E2 glycoprotein when virus



Fig. 5. Proteolytic digestion of virion and intracellular gp26, gp25 and p23. The amount of *S. aureus* V8 protease used was 10 μ g in all lanes. Lane 1, virion gp26. Lanes 2 to 4, virion gp26, gp25 and p23 respectively, taken from another virus preparation. Lanes 5 to 7, the intracellular 26K, 25K and 23K species respectively. Peptides that migrated with the same apparent mobility are indicated by small arrows and those that differed in their mobilities by large arrows. Some of the original protein remained undigested (open arrows). Higher M_r bands represent aggregated species. Analysis was done in a 15% polyacrylamide gel.

samples were prepared without boiling and in the absence of a reducing agent; this was the method of sample preparation used here.

The glycoproteins gp26 and gp25 were digested to a product that migrated with the same apparent mobility as p23. This indicated that these proteins were probably integral membrane proteins with only a small portion of the protein exposed to the surface as are the E1 glycoproteins of MHV (Sturman, 1977; Rottier *et al.*, 1984). Based on these results and for other reasons given in this report, the BCV proteins gp26, gp25 and p23 were designated E1 proteins and gp190 and gp100 were designated E2 proteins (E, envelope protein) to be consistent with the nomenclature for MHV (Sturman & Holmes, 1977).

The proteins p53 and p160 were both found to be resistant to bromelain digestion. This was indicative of an internal location for these proteins and was consistent with their presumptive identities as the nucleocapsid protein (p53) and its multimer (p160). These were designated N proteins.

The glycoprotein gp124 was also found to be resistant to bromelain digestion. The analogous protein on the Mebus strain of BCV was found to be bromelain-resistant but sensitive to pronase, indicative of an external location for this protein (King & Brian, 1982). Since gp124 was clearly analogous to this protein, it was concluded to be the third envelope protein and designated E3.

Effect of tunicamycin on intracellular viral protein synthesis

To determine the type of glycosylation possessed by each BCV glycoprotein, infected and mock-infected cells were subjected to different concentrations of tunicamycin during [³⁵S]methionine labelling (Heifetz *et al.*, 1979). Tunicamycin depressed overall intracellular protein synthesis as detected by the intensity of bands of cell lysates after electrophoresis and



Fig. 6. Bromelain digestion of BCV structural proteins. Purified virus was mock-digested (lane 1) or digested (lane 2) with bromelain at a concentration of 1 mg/ml. Proteins were solubilized with a sample buffer containing urea (final concentration 5 M) without 2-mercaptoethanol. Analysis was done in a 10% polyacrylamide gel.

Fig. 7. The effect of tunicamycin on intracellular viral protein synthesis. BCV-infected cells (lanes 1 to 4) and mock-infected cells (lanes 5 and 6) were labelled with [35 S]methionine in the presence of tunicamycin at concentrations of 0 µg/ml (lanes 1 and 5), 0.01 µg/ml (lane 2), 0.1 µg/ml (lane 3) and 1.0 µg/ml (lanes 4 and 6). Cell lysates were immunoprecipitated with rabbit anti-BCV serum. Proteins were solubilized in sample buffer containing 2-mercaptoethanol. Analysis was done in an 11% polyacrylamide gel.

fluorography (data not shown). However, tunicamycin differentially affected the synthesis of BCV glycoproteins. Tunicamycin at levels of 0·1 and 1·0 μ g/ml prevented the appearance of gp170, gp124 and gp100 (Fig. 7, lanes 3 and 4). Even at 0·01 μ g/ml, synthesis of these proteins was greatly affected (Fig. 7, lane 2). However, synthesis of gp26 and the 25K polypeptide was not affected by tunicamycin relative to overall protein synthesis. Thus, the carbohydrate moieties of these proteins were evidently not linked via *N*-glycosidic bonds as indicated for gp170, gp124 and gp100.

No new viral polypeptides corresponding to the unglycosylated versions of gp170, gp124 and gp100 were observed in tunicamycin-treated cells, presumably because these polypeptides were quickly degraded or synthesis was arrested by abnormal processing of these glycoproteins. In a further attempt to observe these unglycosylated proteins, cells were incubated with tunicamycin for 4 h and then pulse-labelled rather than being labelled for an extended period. This method



Fig. 8. Processing of BCV glycoproteins. BCV-infected cells (lanes 2 to 7) and mock-infected cells (lanes 1 and 8) were pulse-labelled with [35 S]methionine at 18 h (*a*) or 23 h (*b*, *c* and *d*) for 15 min and chased in MEM for various times. Cell lysates were then immunoprecipitated with polyclonal rabbit anti-BCV serum (*a*), MAb BB7-14 (E2-specific) (*b*) and MAb BD9-8C (E3-specific) (*c* and *d*). Lanes 1 and 2 (all panels) represent pulse-labelled cells. Lanes 3 to 8 in (*a*) represent cells that were pulsed and chased for 15 (lane 3), 30 (lane 4), 60 (lane 5), 120 (lane 6) and 240 (lanes 7 and 8) min. Lanes 3 to 8 in (*b*) to (*d*) represent cells that were pulsed and chased for 15 (lanes 7 and 8) min. Proteins were solubilized with sample buffer in the absence (*a* to *c*) or presence (*d*) of 10% 2-mercaptoethanol. Analysis was done on 8% (*b* and *c*) or 10% (*a* and *d*) polyacrylamide gels. Positions of molecular weight standards are on the right. The lower portion of the gel in (*a*) represents a longer exposure.

had been successful for detection of nascent coronavirus proteins by others (Rottier *et al.*, 1981; Stern & Sefton, 1982*b*). However, initial attempts by this method also failed to reveal nascent BCV proteins.



Fig. 9. Pulse-labelling of intracellular viral proteins. BCV-infected cells (lanes 1 and 3) and mockinfected cells (lanes 2 and 4) were labelled with 200 μ Ci/ml of [³⁵S]methionine for 15 min (lanes 1 and 2) or 300 μ Ci/ml of [³H]glucosamine for 75 min (lanes 3 and 4). Proteins were solubilized in sample buffer containing 2-mercaptoethanol. The protein, p32.5, which could not be immunoprecipitated with anti-BVC serum, was considered to be a putative non-structural viral protein. Analysis was done in a 10% polyacrylamide gel.

Fig. 10. A model describing the genesis of BCV structural glycoproteins. The following processing steps were concluded to occur: for E3, (a) dimerization by disulphide bonds of the primary precursor, gp59, to yield gp118, (b) glycosylation by addition of complex sugars to *N*-linked E3 and E2 precursors (gp118 and gp170, respectively) and addition of *O*-linked sugars to p23/E1 and (d) proteolytic cleavage (E2). Reduction of disulphide bonds, (c). Two comigrating E2 species (gp100 A,B) are presumed to be the result of proteolytic cleavage of gp190.

Processing of BCV structural proteins

To study the processing of BCV proteins, pulse-chase experiments were conducted with polyclonal and monoclonal antibodies. After a short pulse with [^{35}S]methionine several virus-specific proteins were immunoprecipitated with polyclonal antiserum (Fig. 8*a*, lane 2). These proteins had M_r of 170K, 118K, 59K, 53K, 26K and 23K. During the chase period the intensity of the 170K, 118K, 59K and 23K protein bands diminished and proteins of 190K, 124K and 100K appeared (Fig. 8*a*, lanes 3 to 7). The 26K protein band appeared to increase in intensity concomitant with a decrease in the 23K protein band presumably due to glycosylation of the 23K protein to form the 26K species. The 26K glycoprotein diminished in intensity at later times, probably due to virus maturation and release from the cell.

When MAb was used to immunoprecipitate viral proteins, it became clear that the 170K glycoprotein was the precursor of gp190 and gp100 (Fig. 8b). A protein of intermediate size (180K, open arrow) was observed in the chase period preceding the appearance of gp190 (Fig.

8b, lane 3). With the appearance of gp190, gp100 was observed and increased in intensity concomitant with a decrease in intensity of gp190 (and gp170). This pattern of processing was consistent with the interpretation that further glycosylation of gp170 produced gp190 and that subsequent proteolytic cleavage of gp190 produced gp100. Two different but comigrating species of gp100 were presumed to be the result of cleavage of gp190. The glycoprotein gp340 which appeared to increase and diminish in intensity with gp170 was assumed to be an aggregate dimer of gp170. It was also stable in the presence of SDS and 2-mercaptoethanol and preliminary data suggested that its formation may occur by association of hydrophobic anchoring sequences after dissociation of membranes in sample preparation.

The 59K (gp59) and 118K (gp118) proteins were found to be precursors to gp124 when a MAb to this protein was used to immunoprecipitate pulse-labelled lysates (Fig. 8c). These were glycoproteins as had been inferred from the apparent N-linkage of sugars in gp124, and later demonstrated by incorporation of tritiated glucosamine (Fig. 9). As both proteins appeared in pulse-labelled samples under non-reducing conditions it was not immediately clear which was the primary precursor, as gp59 could possibly have been derived by cleavage of the gp118 before a presumptive dimerization (Fig. 8c). However, when 2-mercaptoethanol was added to the sample, gp118 was completely converted to gp59 (Fig. 8d). This indicated that gp59 was the primary precursor protein to gp124 and rapid dimerization of gp59 produced gp118. Subsequent processing (probably further glycosylation) produced the final product, gp124. In some experiments a small amount of gp118 appeared under reducing conditions but this was attributed to insufficient reduction or re-formation of disulphide bonds perhaps during electrophoresis. Thus gp124 was very probably a homodimer. Based on these observations the synthesis and processing of E1, E2 and E3 glycoproteins are summarized in Fig. 10.

DISCUSSION

The Quebec isolate of BCV can be described as having four unique major structural proteins. These proteins are the peplomeric E2 protein (gp190/gp100), the nucleocapsid N protein (p53) and its apparent trimer (p160), a family of small matrix E1 glycoproteins (gp26, gp25 and p23) and the putative haemagglutinin, E3 glycoprotein (gp124/gp62). These proteins and the assignments which were made are consistent with those of other coronaviruses (Siddell *et al.*, 1983; Storz *et al.*, 1981; King & Brian, 1982).

Results presented in this manuscript indicate that p160 is a trimer of p53 and that disulphide bonds are involved in the formation of this protein. Hogue *et al.* (1984) noted that antisera to the nucleocapsid also reacted with a putative trimer (a 160K protein) of BCV and human respiratory coronavirus (HCV) OC43. This protein was also observed only when a reducing agent was excluded from the dissolution buffer. Recently, Robbins *et al.* (1986) also observed a putative trimer, a 140K structural protein of MHV that could bind RNA, was antigenically related to the 50K nucleocapsid protein and upon reduction yielded only the 50K nucleocapsid protein. Thus, several lines of evidence have been reported including those given here, that the coronavirus nucleocapsid protein can exist as disulphide-linked oligomeric units. It has been speculated that these oligomeric units may play a role in stabilizing the helical structure of the nucleocapsid (Robbins *et al.*, 1986).

This is the first report describing the processing of BCV glycoproteins. Precursor proteins have previously been identified for the E1 and E2 glycoproteins of MHV, the analogous proteins of infectious bronchitis virus (IBV), transmissible gastroenteritis virus (TGEV) and recently feline infectious peritonitis virus (Siddell *et al.*, 1981; Holmes *et al.*, 1981; Cheley & Anderson, 1981; Niemann & Klenk, 1981; Rottier *et al.*, 1981; Stern & Sefton, 1982*a*; Laude *et al.*, 1986; de Groot *et al.*, 1987). For MHV, to which BCV is closely related, a glycoprotein of 150000 M_r has been identified as the precursor of E2 (180K/90K) (Siddell *et al.*, 1981: Rottier *et al.*, 1981). This is similar in size to the BCV E2 precursor glycoprotein (170K) identified in this report. The MHV E2 apoprotein has an M_r of 110000 to 120000 and therefore, the mature forms of this protein must be heavily glycosylated (Rottier *et al.*, 1981; Leibowitz *et al.*, 1982; Siddell, 1983).

Tunicamycin inhibited formation of the BCV E2 glycoprotein, which is consistent with Nlinked glycosylation of this protein. Addition of complex sugars to the presumptive coreglycosylated gp170 probably accounts for the increase in M_r to yield gp190 (Klenk & Rott, 1980). The ability of gp100 to incorporate tritiated fucose was indicative of further glycosylation. Subsequent proteolytic cleavage of gp190 to yield two presumptive comigrating gp100 species appears to occur quite efficiently, as gp100 is the predominant form of E2 for BCV. This appears to be true also for the Mebus strain of BCV grown in HRT-18 cells as this strain contains predominantly the presumptive cleaved forms (King & Brian, 1982; Hogue *et al.*, 1984). These proteins migrate as separate species (120K and 100K) in contrast to the Quebec isolate.

Thus processing of the BCV E2 glycoprotein is similar to that indicated for MHV and IBV. Originally it was not certain from pulse-chase studies whether gp90/E2 of MHV was a product of proteolytic cleavage of gp180/E2 or whether the latter protein arose from dimerization of gp90/E2 (Siddell *et al.*, 1981; Rottier *et al.*, 1981). It is now apparent that cleavage of gp180/E2 occurs to give two comigrating gp90/E2 species, one of which is acylated (Sturman *et al.*, 1985). For IBV, the two spike (E2) glycoproteins (84K and 90K) are also derived from cleavage of a larger precursor (Stern & Sefton, 1982*a*). Recent sequencing of the IBV gene for the spike (E2) protein and partial amino acid sequencing have identified Arg-Arg-Phe-Arg-Arg as the cleavage sequence (Binns *et al.*, 1985; Cavanagh *et al.*, 1986; Niesters *et al.*, 1986). A very similar sequence occurs on the MHV-JHM E2 gene (Schmidt *et al.*, 1987).

Since tunicamycin does not inhibit formation of gp26, processing of the BCV E1 species apparently involves addition of O-linked sugars to the p23 precursor protein. This finding confirms an earlier report on BCV (Mebus strain) which suggested that the virus contained a small O-linked glycoprotein based on the sugars it could incorporate (Storz *et al.*, 1981). Similarly, the E1 protein of MHV has been shown to undergo O-linked glycosylation and the sugars have since been characterized (Niemann & Klenk, 1981; Holmes *et al.*, 1981; Rottier *et al.*, 1981; Niemann *et al.*, 1984). In contrast, the analogous protein of IBV undergoes N-linked glycosylation (Stern & Sefton, 1982b).

The E3 glycoprotein (gp124/gp62), is a disulphide-linked dimer of apparently identical subunits. Formation of the BCV E3 glycoprotein, like that of the BCV E2 glycoprotein, is inhibited by tunicamycin indicating that N-linked glycosylation occurs on this protein as well. Further, the E3 glycoprotein (gp124/gp62) can be labelled with fucose as well as glucosamine. Thus the sugars on gp59, the precursor monomer, probably represent the result of core glycosylation (Klenk & Rott, 1980). Subsequent trimming of core sugars and addition of complex sugars including fucose after dimerization to gp118 probably occurs to yield the mature form of the E3 protein. The increase in M_r from the precursor monomer (gp59) to the mature monomer (gp62) would be consistent with this protein having three or four complex-type sugar chains per monomer if the chains are triantennary as those found for vesicular stomatitis virus (Reading *et al.*, 1978).

The coronaviruses MHV-A59, IBV and TGEV, some of the best studied coronaviruses, all apparently lack a protein analogous to the BCV and HCV OC43 E3 glycoprotein (Sturman & Holmes, 1977; Rottier *et al.*, 1981; Cavanagh, 1981; Stern *et al.*, 1982; Garwes & Pocock, 1975; Hogue *et al.*, 1984; Hogue & Brian, 1986). Whether or not an analogous protein exists on MHV-JHM remains to be determined (Wege *et al.*, 1979; Siddell *et al.*, 1981; Siddell, 1982; Taguchi *et al.*, 1986). We refer to the glycosylated 65K protein, gp65, present as the reduced form of a larger protein species (125K). A notable difference between this protein and the BCV E3 protein is apparent in that the former protein appears rapidly in pulse-labelled cells with no evidence of a smaller precursor protein.

Currently, we are using a panel of MAbs to study the antigenic structure of BCV glycoproteins. Monoclonal antibodies to both BCV E2 and E3 glycoproteins are efficient in neutralizing virus infectivity *in vitro* (D. Deregt *et al.*, unpublished results) which indicates an important role for the E3 protein in BCV-cell interactions.

The authors thank James E. Gilchrist and Elaine Gibbons for their valued assistance with hybridoma production and Irene Kosokowsky for preparation of this manuscript. This work was supported by an NSERC Strategic Grant. Published with the permission of the director as journal series no. 48. A portion of this work was presented at the Third International Coronavirus Symposium at Asilomar, Ca., U.S.A. in 1986.

REFERENCES

- BINNS, M. M., BOURSNELL, M. E. G., CAVANAGH, D., PAPPIN, D. J. C. & BROWN, T. D. K. (1985). Cloning and sequencing of the gene encoding the spike protein of the coronavirus IBV. Journal of General Virology 66, 719–726.
- BRAUN, D. K., PEREIRA, L., NORRILD, B. & ROIZMAN, B. (1983). Application of denatured, electrophoretically separated, and immobilized lysates of herpes simplex virus-infected cells for detection of monoclonal antibodies and for studies of the properties of viral proteins. Journal of Virology 46, 103-112.
- CAVANAGH, D. (1981). Structural polypeptides of coronavirus IBV. Journal of General Virology 53, 93-103.
- CAVANAGH, D., DAVIS, P. J., PAPPIN, D. J. C., BINNS, M. M., BOURSNELL, M. E. G. & BROWN, T. D. K. (1986). Coronavirus IBV: partial amino terminal sequencing of spike polypeptide S2 identifies the sequence Arg-Arg-Phe-Arg-Arg at the cleavage site of the spike precursor propolypeptide of IBV strains Beaudette and M41. Virus Research 4, 133-143.
- CHELEY, S. & ANDERSON, R. (1981). Cellular synthesis and modification of murine hepatitis virus polypeptides. Journal of General Virology 54, 301-311.
- CLEVELAND, D. W., FISCHER, S. G., KIRSCHNER, M. W. & LAEMMLI, U.K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *Journal of Biological Chemistry* 252, 1102-1106.
- COLLINS, A. R., KNOBLER, R. L., POWELL, H. & BUCHMEIER, M. J. (1982). Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. *Virology* **119**, 358-371.
- DEA, S., ROY, R. S. & BEGIN, M. E. (1980). Bovine coronavirus isolation and cultivation in continuous cell lines. American Journal of Veterinary Research 41, 30-38.
- DE GROOT, R. J., TER HAAR, R. J., HORZINEK, M. C. & VAN DER ZEIJST, B. A. M. (1987). Intracellular RNAs of the feline infectious peritonitis coronavirus strain 79-1146. Journal of General Virology 68, 995-1002.
- GARWES, D. J. & POCOCK, D. H. (1975). The polypeptide structure of transmissible gastroenteritis virus. Journal of General Virology 29, 25-34.
- HEIFETZ, A., KEENAN, R. W. & ELBEIN, A. D. (1979). Mechanism of action of tunicamycin on the UDP-GlcNAc:dolichylphosphate GlcNAc-1-phosphate transferase. *Biochemistry* 18, 2186–2192.
- HOGUE, B. G. & BRIAN, D. A. (1986). Structural proteins of human respiratory coronavirus OC43. Virus Research 5, 131-144.
- HOGUE, B. G., KING, B. & BRIAN, D. A. (1984). Antigenic relationships among proteins of bovine coronavirus, human respiratory coronavirus OC43, and mouse hepatitis coronavirus A59. Journal of Virology 51, 384–388.
- HOLMES, K. V., DOLLER, E. W. & STURMAN, L. S. (1981). Tunicamycin resistant glycosylation of a coronavirus glycoprotein: demonstration of a novel type of glycoprotein. *Virology* **115**, 334–344.
- KENNET, R. H., DENIS, K. A., TUNG, A. S. & KLINMAN, R. (1978). Hybrid plasmacytoma production: fusion with adult spleen cells, monoclonal spleen fragments, neonatal spleen cells and human spleen cells. Current Topics in Microbiology and Immunology 81, 77-91.
- KING, B. & BRIAN, D. A. (1982). Bovine coronavirus structural proteins. Journal of Virology 42, 700-707.
- KING, B., POTTS, B. J. & BRIAN, D. A. (1985). Bovine coronavirus hemagglutinin protein. Virus Research 2, 53-59.
- KLENK, H.-D. & ROTT, R. (1980). Cotranslational and posttranslational processing of viral glycoproteins. Current Topics in Microbiology and Immunology 90, 19-48.
- KÖHLER, G. & MILSTEIN, C. (1976). Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. European Journal of Immunology 6, 511–519.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, London 227, 680-685.
- LAUDE, H., CHAPSAL., J.-M., GELFI, J., LABIAU, S. & GROSCLAUDE, J. (1986). Antigenic structure of transmissible gastroenteritis virus. I. Properties of monoclonal antibodies directed against virion proteins. *Journal of General Virology* 67, 119–130.
- LEIBOWITZ, J. L., WEISS, S. R., PAAVOLA, E. & BOND, C. W. (1982). Cell-free translation of murine coronavirus RNA. Journal of Virology 43, 905–913.
- NIEMANN, H. & KLENK, H. D. (1981). Coronavirus glycoprotein E1, a new type of viral glycoprotein. Journal of Molecular Biology 153, 993-1010.
- NEIMANN, H., GEYER, R., KLENK, H. D., LINDER, D., STIRM, S. & WIRTH, M. (1984). The carbohydrates of mouse hepatitis (MHV) A59: structures of the O-glycosidically linked oligosaccharides of glycoprotein E1. EMBO Journal 3, 665–670.
- NIESTERS, H. G. M., LENSTRA, J. A., SPAAN, W. J. M., ZIJDERVELD, A. J., BLEUMINK-PLUYM, N. M. C., HONG, F., VAN SCHARRENBURG, G. F. M., HORZINEK, M. C. & VAN DER ZEIJST, B. A. M. (1986). The peplomer protein of the M41 strain of coronavirus IBV and its comparison with Beaudette strains. Virus Research 5, 253-263.
- READING, C. L., PENHOET, E. E. & BALLOU, C. E. (1978). Carbohydrate structure of vesicular stomatitis virus glycoprotein. Journal of Biological Chemistry 253, 5600-5612.
- ROBBINS, S. G., FRANA, M. F., McGOWAN, J. J., BOYLE, J. F. & HOLMES, K. V. (1986). RNA-binding proteins of coronavirus MHV: detection of monomeric and multimeric N protein with an RNA overlay-protein blot assay. Virology 150, 402-410.
- ROTTIER, P. J. M., HORZINEK, M. C. & VAN DER ZEIJST, B. (1981). Viral protein synthesis in mouse hepatitis virus strain A59-infected cells: effect of tunicamycin. Journal of Virology 40, 350-357.

- ROTTIER, P., BRANDENBERG, D., ARMSTRONG, J., VAN DER ZEIJST, B. & WARREN, G. (1984). Assembly in vitro of a spanning membrane protein of the endoplasmic reticulum: the E1 glycoprotein of coronavirus mouse hepatitis virus A59. Proceedings of the National Academy of Sciences, U.S.A 81, 1421-1425.
- SCHMIDT, I., SKINNER, M. & SIDDELL, S. (1987). Nucleotide sequence of the gene encoding the surface projection glycoprotein of coronavirus MHV-JHM. Journal of General Virology 68, 47-56.
- SIDDELL, S. G. (1982). Coronavirus JHM: tryptic peptide fingerprinting of virion proteins and intracellular polypeptides. Journal of General Virology 62, 259-269.
- SIDDELL, S. (1983). Coronavirus JHM: coding assignments of subgenomic mRNAs. Journal of General Virology 64, 113–125.
- SIDDELL, S., WEGE, H., BARTHEL, A. & TER MEULEN, V. (1981). Coronavirus JHM: intracellular protein synthesis. Journal of General Virology 53, 145-155.
- SIDDELL, S., WEGE, H. & TER MEULEN, V. (1983). The biology of coronaviruses. Journal of General Virology 64, 761-776.
- SPAAN, W. J. M., ROTTIER, P. J. M., HORZINEK, M. C. & VAN DER ZEIJST, B. A. M. (1981). Isolation and identification of virus-specific mRNAs in cells infected with mouse hepatitis virus (MHV-A59). Virology 108, 424-434.
- STERN, D. F. & SEFTON, B. M. (1982a). Coronavirus proteins: biogenesis of avian infectious bronchitis virus virion proteins. Journal of Virology 44, 794-803.
- STERN, D. F. & SEFTON, B. M. (1982b). Coronavirus proteins: structure and function of the oligosaccharides of the avian infectious bronchitis virus glycoproteins. Journal of Virology 44, 804-812.
- STERN, D. F., BURGESS, L. & SEFTON, B. M. (1982). Structural analysis of virion proteins of the avian coronavirus infectious bronchitis virus. Journal of Virology 42, 208-219.
- STOHLMAN, S. A. & LAI, M. M. C. (1979). Phosphoproteins of murine hepatitis viruses. Journal of Virology 32, 672-675.
- STORZ, J., KALUZA, G., NEIMANN, H. & ROTT, R. (1981). On enteropathogenic bovine coronavirus. Advances in Experimental Medicine and Biology 142, 171–180.
- STURMAN, L. S. (1977). Characterization of a coronavirus. I. Structural proteins: effects of preparative conditions on the migration of protein in polyacrylamide gels. *Virology* **77**, 637–649.
- STURMAN, L. S. & HOLMES, K. V. (1977). Characterization of a coronavirus. II. Glycoproteins of the viral envelope: tryptic peptide analysis. Virology 77, 650–660.
- STURMAN, L. S., RICARD, C. S. & HOLMES, K. V. (1985). Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: activation of cell-fusing activity of virions by trypsin and separation of two different 90k cleavage fragments. Journal of Virology 56, 904–911.
- TAGUCHI, F., MASSA, P. T. & TER MEULEN, V. (1986). Characterization of a variant virus isolated from neural cell culture after infection of mouse coronavirus JHMV. Virology 155, 267–270.
- TOOZE, J., TOOZE, S. & WARREN, G. (1984). Replication of coronavirus MHV-A59 in sac- cells: determination of the first site of budding of progeny virions. *European Journal of Cell Biology* 33, 281-293.
- WEGE, H., WEGE, H., NAGASHIMA, K. & TER MEULEN, V. (1979). Structural polypeptides of the murine coronavirus JHM. Journal of General Virology 42, 37–47.
- WEGE, H., DORRIES, R. & WEGE, H. (1984). Hybridoma antibodies to the murine coronavirus JHM: characterization of epitopes on the peplomer protein (E2). Journal of General Virology 65, 1931–1942.

(Received 13 April 1987)