

Bovine Coronavirus Structural Proteins

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The tissue culture-adapted strain (Mebus) of bovine coronavirus was grown in the presence of isotopically labeled amino acids, glucosamine, or orthophosphate for the purpose of analyzing the virion structural proteins. Five species of polypeptides were identified when purified virions were solubilized in urea and sodium dodecyl sulfate and resolved by polyacrylamide gel electrophoresis. Four species were glycosylated and had apparent molecular weights of 140,000, 120,000, 100,000, and 26,000. The glycoproteins were susceptible to proteolytic cleavage and enzymatic iodination when intact virions were studied and are thus at least partially external to the virion envelope. The 140,000-molecular-weight glycoprotein is apparently a dimer of 65,000-molecular-weight glycopolypeptides held together by disulfide linkages. Species 5 was phosphorylated and had an apparent molecular weight of 52,000. In the intact virion, it was unaffected by protease and was not enzymatically iodinated. It is therefore apparently an internal protein.

The most characterized members of the coronavirus family are positive-stranded RNA viruses which possess a single-stranded, nonsegmented, polyadenylated, infectious RNA genome (2, 12, 15, 16, 22). These properties together are shared by only picornaviruses and togaviruses (1), yet certain other structural features of coronaviruses make them unique among the positive-stranded viruses. (i) The virions are large (80 to 120 nm in diameter) and are often pleomorphic (21, 32). (ii) The surface peplomers are long (12 to 20 nm) and characteristically petal shaped (21, 32). (iii) The nucleocapsid appears to be helical rather than icosahedral (10, 17). (iv) They possess a large genome (5×10^6 to 8×10^6 molecular weight) which, if the larger number is used, is roughly twice the size of a togavirus genome, or three times the size of a picornavirus genome (2, 12, 16).

Analyses of the coronavirus structural polypeptides from several members reveal diverse patterns, suggesting that coronaviruses are the most complex of the positive-stranded RNA viruses (4, 5, 8, 13, 18, 28, 33). The number of major polypeptide species in the coronavirus range from three for the mouse hepatitis virus (28, 29) to seven for the avian infectious bronchitis virus (18). Surface polypeptides are glycoproteins with molecular weights ranging from 15,000 to 200,000, but the sizes found are unique for each species of virus. An internal protein of around 50,000 molecular weight appears to be a common feature of all coronaviruses. Although the avian coronaviruses apparently do not share antigenic relatedness with the mammalian cor-

onaviral species, two groups of mammalian coronaviruses are recognized on the basis of shared antigenicity as detected by immunofluorescence (19). The exact structural interrelatedness among coronaviruses is therefore poorly understood.

This report describes the identification and partial characterization of the structural proteins of one member of this family, the Mebus strain of the bovine coronavirus (BCV). Purified virus was resolved into five polypeptide species by gel electrophoresis. Four of the species were glycosylated and at least partially external to the virion envelope and had apparent molecular weights of 140,000, 120,000, 100,000, and 26,000. One species was internal and phosphorylated and had an apparent molecular weight of 52,000. The 140,000-molecular-weight glycoprotein is apparently a disulfide-linked dimer of 65,000-molecular-weight subunits.

MATERIALS AND METHODS

Cells. The embryonic bovine kidney cell line (BEK-1) as described by Inaba et al. (9) was grown as monolayers in Dulbecco modified eagle medium containing 50 μ g of gentamicin per ml and 10% donor calf serum (Flow Laboratories, Rockville, Md.) or 10% newborn calf serum (KC Biologicals, Inc., Lenexa, Kans.).

Virus. The Mebus strain of the BCV (24, 26), which had undergone approximately 40 tissue culture passages, was obtained from J. Black, Kord Animal Disease Laboratory, Nashville, Tenn. The virus was carried an additional 20 passages by us and then cloned by three successive isolations from single plaques. Cloned virus was passaged twice at a multi-

plicity of <0.1 PFU per cell, and then viral stocks were prepared from passages 3 through 18 by infecting cells at a multiplicity of approximately 1 PFU per cell. Viral titers ranging from 10^7 to 10^8 PFU per ml were obtained in stock virus preparations.

Plaque assays. BCV was assayed on BEK-1 cell monolayers in 35-mm dishes (Costar, Cambridge, Mass.). Virus was adsorbed for 2 h at 37°C before the monolayers were fed with medium containing 4% fetal calf serum and 0.5% agarose. Plaques were counted 4 days after infection. Plaques were 1 mm in diameter and consisted of rounded cells and syncytia.

Hemagglutination assay. The hemagglutination assay was performed as described previously (6).

Isotopic labeling of viral polypeptides. For *in vivo* labeling, confluent monolayers of cells grown in 700-cm² glass roller bottles were drained and rinsed three times with Earl balanced salt solution. Cells were then infected with virus at a multiplicity of approximately 5 PFU per cell. After 2 h of adsorption at 37°C, the inoculum was removed, and 18 ml of the appropriate medium containing 4% fetal calf serum and the isotope was added. All radioisotopes were obtained from Amersham Corp., Arlington Heights, Ill. Viral polypeptides were labeled by adding 400 μ Ci of ³H-labeled essential amino acid mixture (70 to 100 Ci/mmol) per bottle in medium containing 10% normal essential amino acid concentrations. Viral glycopolypeptides were labeled by adding 400 μ Ci of [³H]glucosamine (38 Ci/mmol) per bottle. Viral phosphopolypeptides were labeled by adding 1,000 μ Ci of ³²P_i per bottle in medium containing 10% of normal phosphate concentration.

For *in vitro* labeling, viral polypeptides were iodinated by using the lactoperoxidase method (Enzymobeads) as recommended by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.) and a modification of the method of Sefton et al. (23). Intact virus was iodinated in a 1-ml reaction mixture containing approximately 300 μ g of purified virus taken directly from a sucrose gradient, 100 μ l of Enzymobeads, and 1 mCi of carrier-free sodium [¹²⁵I]iodide (13 to 17 mCi/ μ g). To iodinate disrupted virus, approximately 300 μ g of gradient-purified and pelleted virus was resuspended in 100 μ l of 1% sodium dodecyl sulfate (SDS), diluted to 1 ml, and reacted with 100 μ l of Enzymobeads and 0.5 mCi of carrier-free sodium [¹²⁵I]iodide. All solutions were made in 25 mM Tris (pH 7.2). The iodination reactions were carried out at room temperature for 30 min with constant mixing. The beads were removed by centrifugation at 1,000 \times g for 10 min. The supernatant fluids were dialyzed against buffer and used directly for SDS-polyacrylamide gel electrophoresis.

Virus purification. Infected supernatant fluids were harvested when 50 to 60% of the cells were rounded and detached or were part of detaching syncytia. Harvests were made between 40 and 50 h p.i. Supernatant fluids were clarified by centrifugation at 7,500 \times g for 10 min, layered onto a 20-ml continuous 60 to 20% (wt/wt) sucrose gradient and isopycnicly sedimented at 70,000 \times g for 16 h in a Sorvall AH-627 rotor. The gradient was collected from the top by using an Isco gradient fractionator. Fractions showing peak radioactivity and hemagglutinating activity were diluted with TMN (50 mM Tris acid maleate [pH 6], 0.1 M NaCl), layered onto a 30-ml continuous 40 to 20% (wt/wt)

sucrose gradient, and sedimented at 70,000 \times g for 1.5 h in a Sorvall AH-627 rotor. Fractions containing the virus were used directly for further studies or were pelleted at 150,000 \times g for 2 h in a Sorvall AH-650 rotor. When necessary, viral pellets were stored at -20°C. All sucrose solutions were made in TMN, and all steps were carried out at 4°C.

Phenol extraction of viral proteins. Viral proteins were separated from viral nucleic acids by phenol extraction. ³²P-labeled virus was purified, pelleted, dissolved in 1% SDS in 0.5 ml of TNE-9 (50 mM Tris [pH 9.0], 0.1 M NaCl, 1 mM EDTA), and mixed in a Vortex blender for 5 min at 20°C with an equal volume of TNE-9-saturated phenol. The phenol phase was separated by centrifugation at 1,000 \times g for 3 min, and reextracted twice with an equal volume of TNE-9. Viral proteins were precipitated from the phenol by adding 5 volumes of chilled methanol and 100 μ g of bovine serum albumin as carrier and storing overnight at -20°C. The precipitate was collected by centrifugation and washed twice with methanol. The pellet was then suspended in 0.5 ml of TMN by gentle bath sonication and used directly for enzyme digestion studies.

Polyacrylamide gel electrophoresis. The discontinuous buffer gel system of Laemmli was used (11). Samples were dissolved in 50 mM Tris-hydrochloride (pH 7)-2% SDS-5.0 M urea and treated at 100°C for 2 min immediately before electrophoresis. Electrophoresis was performed in slab gels (0.75 by 100 by 160 mm), using concentrations of 7.5%, 9%, or 12% polyacrylamide and 0.5% bisacrylamide. A 1-cm stacking gel of 3% polyacrylamide-0.5% bisacrylamide was used. Electrophoresis was carried out by using a current of 12 mA per slab gel. Gels were analyzed by autoradiography or fluorography after Enhance (New England Nuclear Corp., Boston, Mass.) treatment. Dried gel slabs were exposed to preflashed Kodak X-Omat R film by the method of Laskey and Mills (14). For molar ratio determination, viral bands were excised from dried gels, dissolved overnight at 37°C in 0.2 ml of HClO₄-0.4 ml of H₂O₂, and counted in 10 ml of Aquasol (New England Nuclear Corp.).

Electron microscopy. Aliquots (5 μ l) of virus from sucrose gradients were diluted with an equal volume of distilled water, spotted onto Formvar-film, carbon-coated, glow-discharged copper grids, and stained with 2% phosphotungstic acid. Electron microscopy was done on a Philips 201.

Materials. Protein molecular weight standards which included myosin (200,000), galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400), were obtained from Bio-Rad. Pronase P, type VI, RNase A, type IA, and 2-mercaptoethanol were obtained from Sigma Chemical Co., St. Louis, Mo. RNase T₁ was obtained from Calbiochem, La Jolla, Calif.

RESULTS

Identification of virion polypeptides. BCV labeled with either ³H-amino acids, [³H]glucosamine, or ³²P_i was routinely located in sucrose gradients by assaying hemagglutinating activity and trichloroacetic acid-precipitable radioactiv-

ity. After isopycnic sedimentation, peaks of hemagglutinating activity and radioactivity coincided and represented an average buoyant density of 1.20 g/cm³. Peak infectivity titers, when tested, coincided with this buoyant density. Fractions showing peak activities in the rate-zonal gradient were pooled, pelleted, and used for electrophoretic analysis. In several experiments, differentially labeled uninfected cellular material consisting of supernatant fluids and disrupted cells was mixed with the virus harvest before purification to detect cellular contaminants in the purified virus. At no time was any cellular material detected in the purified virus (data not shown). When purified ³H-amino acid-labeled BCV was dissolved in 2% SDS-5.0 M urea and analyzed by electrophoresis in 9% polyacrylamide gels, five distinct polypeptide bands were resolved (Fig. 1). These results were essentially the same when [³⁵S]methionine-labeled virus was analyzed (data not shown). The molecular weights of the virion polypeptides were determined by comparing their electrophoretic mobilities with those of commercially available standards in gel concentrations of 7.5%, 9% and 12% polyacrylamide (data not shown).

The virion polypeptides had molecular weights of 140,000, 120,000, 100,000, 52,000, and 26,000. The 26,000-molecular-weight polypeptide was characteristically observed as a broad band or as a group of three to four closely migrating bands. Until further resolution is accomplished, the 26,000-molecular-weight band is presumed to represent a single polypeptide with perhaps various levels of glycosylation (see below). Three minor protein species were variably present. The largest, with a molecular weight in excess of 200,000, is identified as species a in Fig. 1. It was not always present in virus preparations and may represent an aggregate of other viral species. Two polypeptide species migrating with molecular weights of about 38,000 and 22,000 were frequently seen with various intensities. In some preparations, they were totally missing. These polypeptides were always present in greater amounts after protease treatment of the virions (see below) and probably, therefore, represent cleavage products of other structural proteins.

Since murine coronavirus polypeptide profiles are dependent upon the method of virion disruption (28, 29), we investigated the effects of boiling and the addition of a sulfhydryl agent on BCV polypeptides. No difference in the polypeptide profiles could be detected when virus disrupted at 37°C for 30 min and virus disrupted at 100°C for 2 min were compared (data not shown). A marked change in the electrophoretic profile was observed, however, when 2-mercaptoethanol was added to the solubilization buffer.

In the presence of 2-mercaptoethanol, the 140,000-molecular-weight species was no longer visible and a 65,000-molecular-weight species became apparent. Although more rigorous analyses are needed for proof, it appears that the 140,000-molecular-weight species is a disulfide-linked oligomer of probably two smaller polypeptides.

Identification of virion glycopolypeptides. For the purpose of identifying glycosylated polypeptides, virus labeled with [³H]glucosamine was purified and analyzed by gel electrophoresis (Fig. 1). These analyses revealed that the 140,000, 120,000, 100,000, and 26,000-molecular-weight species were each glycosylated, and they are therefore named gp140, gp120, gp100, and gp26, where gp designates a glycosylated polypeptide, and the number designates the molecular weight ($\times 10^3$). The polypeptide designated as species a was also glycosylated. When the glucosamine-labeled virus was disrupted in the presence of 2-mercaptoethanol, gp140 was not present, and a glycosylated polypeptide with a molecular weight of 65,000 (designated gp65) became apparent. The fact that both the 65,000- and the 140,000-molecular-weight species were glycosylated is consistent with the hypothesis that the smaller polypeptide is a subunit of the larger.

Identification of a virion phosphopolypeptide. Stohlman and Lai (27) have reported that the nucleocapsid protein of four strains of murine hepatitis virus were phosphorylated. Recently, a protein kinase was shown to be associated with the virion of the JHM murine coronavirus (25). It was therefore of interest to determine whether

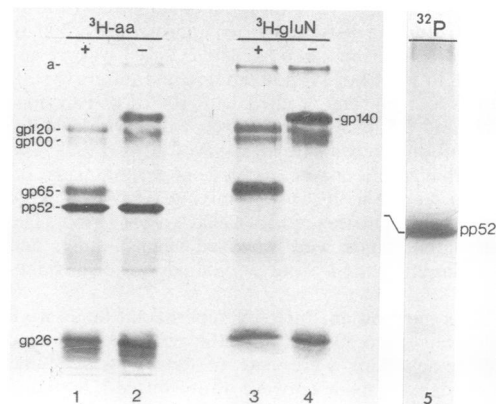


FIG. 1. Electrophoretic analysis of BCV polypeptides. BCV labeled with either ³H-amino acids or [³H]glucosamine was electrophoresed after solubilization in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 2% 2-mercaptoethanol. BCV labeled with ³²P_i was treated as described in the text to remove RNA and electrophoresed separately (lane 5). The acrylamide concentration in the gel was 9%.

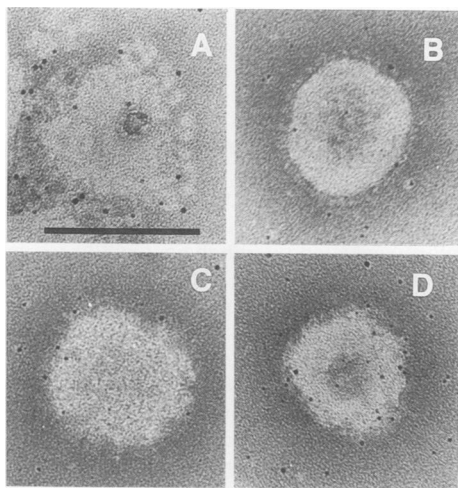


FIG. 2. Electron micrographs of repurified virions after protease treatment. Aliquots of purified virus were treated with pronase at a final concentration of 1 mg/ml at 37°C for the times indicated and then repurified by isopycnic sedimentation as described in the text. Virus was incubated for 30 min without pronase (A), for 30 min with pronase (B), for 60 min with pronase (C), and for 120 min with pronase (D). The bar represents a length of 100 nm.

any of the BCV structural proteins are phosphorylated. ^{32}P -labeled BCV proteins were extracted with phenol, subjected to RNase treatment to remove contaminating RNA, and analyzed by gel electrophoresis. After treatment with RNase, only one species, the 52,000-molecular-weight polypeptide was seen (Fig. 1). This species was completely destroyed by pronase and was therefore designated pp52.

Location of virion polypeptides. Two approaches were used to locate the virion polypeptides within the virion. In the first approach, virus radiolabeled *in vivo* was studied. In the second, virus was purified, and viral polypeptides were labeled *in vitro* before analysis. To determine the location of BCV glycoproteins with the first approach, purified ^3H -amino acid-labeled virus was treated with protease for different periods of time, repurified by isopycnic sedimentation, and analyzed by electron microscopy (Fig. 2) and gel electrophoresis (Fig. 3). Virions treated with protease lost all hemagglutinating activity by 30 min and decreased in average buoyant density from 1.200 to 1.178 g/cm^3 after 120 min of digestion. Direct examination of repurified particles showed that after 30 min of protease digestion, the large bulbous peplomers on the virion surface had disappeared coincidentally with the disappearance of the high-molecular-weight glycoproteins (gp120, gp100, and gp65) from the gel (Fig. 2 and 3),

suggesting that one or more of these glycoproteins is the hemagglutinin. After 30 min of digestion, a fringe of smaller peplomers was still present on the virion, and we speculate that these are the structural counterparts of the smaller glycoprotein gp26 still seen on the gel and possibly also of the 38,000- and 22,000-dalton proteins which have appeared or greatly increased. Peplomers of two distinct sizes have previously been observed electron microscopically on preparations of BCV (3). After 120 min of digestion, pp52 and the 38,000- and 22,000-molecular-weight polypeptides were the predominant species remaining (Fig. 3). The particles at this time appeared relatively smooth (Fig. 2). It appears, therefore, that only a portion of some of the glycoproteins are susceptible to proteolysis. The 38,000- and 22,000-molecular-weight species may represent portions of the glycoproteins which are within the virion envelope and are thus protected. Alternatively, they may represent external portions of the proteins that are otherwise protease resistant or possibly aggregates of the type described by Sturman (28). Since pp52 was unaffected by protease treatment, it is apparently protected by and therefore located internal to the virion envelope.

In the second approach, virion polypeptides were radiolabeled *in vitro* by enzymatic iodination of whole or disrupted virions. When whole virions were iodinated, only the glycoprotein species became radiolabeled (Fig. 4). pp52 became radiolabeled only after first disrupting the virion with detergent (Fig. 4). Detergent treat-

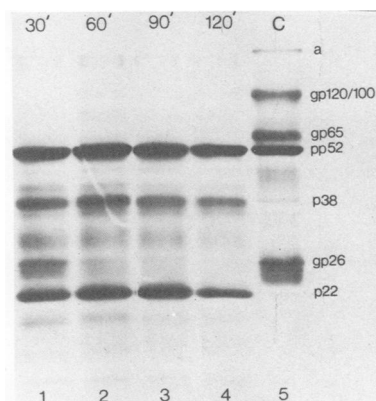


FIG. 3. Polypeptide analysis of BCV after protease treatment. Aliquots of purified virus were treated with pronase at a final concentration of 1 mg/ml at 37°C for the times indicated, repurified by isopycnic sedimentation, and electrophoresed as described in the text. Control virus was incubated for 30 min without pronase (C). Electrophoresis was performed in the presence of 2-mercaptoethanol. The concentration of acrylamide in the gel was 10%.

ment of the virion also affected the degree to which viral glycoproteins incorporated the ^{125}I label. Before detergent treatment, distribution of ^{125}I among gp120 to gp100, gp65, and gp26 was 6%, 54%, and 40%, respectively, as determined from densitometric tracings of the gel shown in Fig. 4. After detergent treatment, the distribution was 10%, 15%, and 75%, respectively, exclusive of the label in pp52 and other minor proteins. Detergent treatment, therefore, appears to expose proportionately more tyrosine residues in the 26,000-molecular-weight glycoprotein than in the other glycoprotein species.

Taken together, these data indicate that the glycoproteins are located, at least in part, on the virion surface, external to the virion envelope, and the phosphoprotein is located entirely internal to the virion envelope.

Molar ratios of virion polypeptides. The molar ratios of virion polypeptides were determined as described in Table 1.

DISCUSSION

The growth of BCV on BEK-1 cells with marked cytopathic effect has allowed us to clone the virus by plaque selection and to quantitate the virus routinely by plaque titration. These are definite advantages over the primary cells used in earlier studies (6, 24).

The structural proteins of BCV correlated with the general polypeptide profile of coronaviruses as summarized by Pensaert and Calle-

baut (20). Since there is little agreement to date on the exact number or size of coronaviral structural proteins from the many species, precise correlations are difficult to make. Nevertheless, some fundamental similarities and differences can be noted between BCV and other coronavirus species. BCV has three large glycoproteins of 140,000, 120,000, and 100,000 molecular weight, a small glycoprotein of 26,000 molecular weight, and a nonglycosylated protein of 52,000 molecular weight. The largest virion glycoprotein of BCV appears to differ fundamentally from the largest glycoprotein of the well-characterized mouse hepatitis virus A59. Whereas the largest peplomeric glycoprotein of the mouse hepatitis virus (molecular weight, 180,000) is not changed by the action of reducing agents such as 2-mercaptoethanol (28, 29), the largest peplomeric glycoprotein of BCV (molecular weight, 140,000) is reduced to a presumptive subunit (molecular weight, 65,000) by 2-mercaptoethanol. This suggests the presence of one or more disulfide linkages in an oligomeric structure of the BCV glycoprotein. The 180,000-molecular-weight mouse hepatitis glycoprotein is cleaved to a subunit (molecular weight, 90,000) by mild proteolysis (29), however, but we have not observed this with BCV. We reported previously that gp140 is apparently the virion hemagglutinin (B. King and D. Brian, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, T96, p. 251). This is based on our observation that bromelain treatment removes all glycoproteins from the virion, except for gp140, and at the same time fails to destroy virion hemagglutinating activity. Recently, a similar 140,000-molecular-weight glycoprotein, a disulfide-linked dimer, was described for the porcine hemagglutinating encephalomyelitis virus that is also believed to be the virion hemagglutinin (4). The hemagglutinating coronaviruses, therefore, which include BCV and the porcine hemagglu-

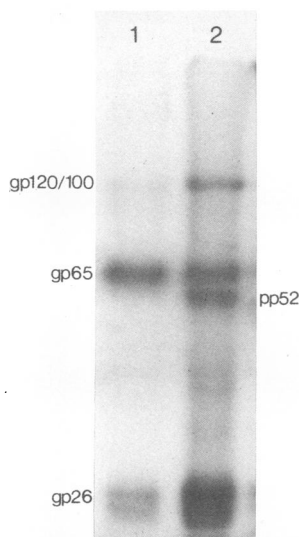


FIG. 4. Identification of viral polypeptides labeled in vitro with ^{125}I . BCV was labeled in vitro with ^{125}I either as intact virus particles (lane 1) or as SDS-disrupted particles (lane 2). Electrophoresis was performed in the presence of 2-mercaptoethanol.

TABLE 1. Molar ratios of BCV structural proteins^a

Protein	% of label incorporated ^b	Molar ratio
gp120	9.0 ± 3	2.1
gp100	3.5 ± 3.5	1
gp65	19.7 ± 3	8.7
pp52	45.5 ± 6	25
gp26	22.3 ± 12	24.5

^a Determined by counting the radioactivity in excised gel bands as described in the text. Electrophoresis was performed in the presence of 2-mercaptoethanol.

^b An average of three separate experiments. The total number of counts per minute recovered from a gel lane, which includes all five protein bands, ranged from 3433 to 63,627.

inating encephalomyelitis virus, may share this oligomeric protein as a distinctive structural feature which sets them apart from nonhemagglutinating coronaviruses. The 120,000- and 100,000-molecular-weight glycoproteins apparently have no analog in mouse hepatitis virus. The low-molecular-weight glycoprotein gp26 is apparently a common feature of all coronaviruses since a similar glycoprotein in the molecular weight range of 20,000 to 29,000 is invariably reported. The nonglycosylated 52,000-molecular-weight protein was demonstrated to be an internal phosphorylated species. An internal nonglycosylated protein of 48,000 to 52,000 molecular weight is a common feature of all characterized coronaviruses, and phosphorylation of this protein may be a common feature. Among coronaviruses studied to date, phosphorylation and phosphokinase activities have been described for only the internal protein of the mouse hepatitis virus (25, 27).

BCV strain LY-138 purified from the small intestines of infected calves was resolved into four glycosylated and three nonglycosylated structural proteins by Hajer and Storz (7). The proteins ranged in molecular weight from 36,000 to 110,000. The differences observed in our studies may be a reflection on our purification procedures or perhaps the strain of virus used.

Figure 5 is our schematic picture of BCV. gp120 and gp100 are depicted as the longest peplomers projecting from the virion surface. This is based on our observation that they are

inapparent by both electron microscopy and gel electrophoresis after treatment with pronase (Fig. 3) or bromelain (King and Brian, unpublished data). gp140, however, does remain on the virion particle after bromelain treatment, as determined by gel electrophoresis, and since short surface projections are visible by electron microscopy after bromelain treatment, some of the projections must apparently represent gp140. It is interesting that a shorter peplomer should have the larger molecular weight, but this is explained by the oligomeric structure of the gp140. We are unable to say exactly how much if any of the large glycoproteins span the virion envelope. The fact that two distinct protease-resistant fragments of 38,000 and 22,000 molecular weight exist after pronase digestion suggests that perhaps large portions of some of the glycoproteins are protected by the virion envelope. At present, we presume that the high-molecular-weight glycoproteins do not span the total distance of the virion envelope. This is based on electron microscopic observations that the large peplomers appear at times to clump or undergo a "capping" phenomenon (6), suggesting that they are not well anchored. A similar phenomenon is reported for the large peplomers of the mouse hepatitis virus (L. S. Sturman and K. V. Holmes, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, S231, p. 251). The low-molecular-weight glycoprotein, gp26, on the other hand, may span the virion envelope and have an association with virion internal structures. This

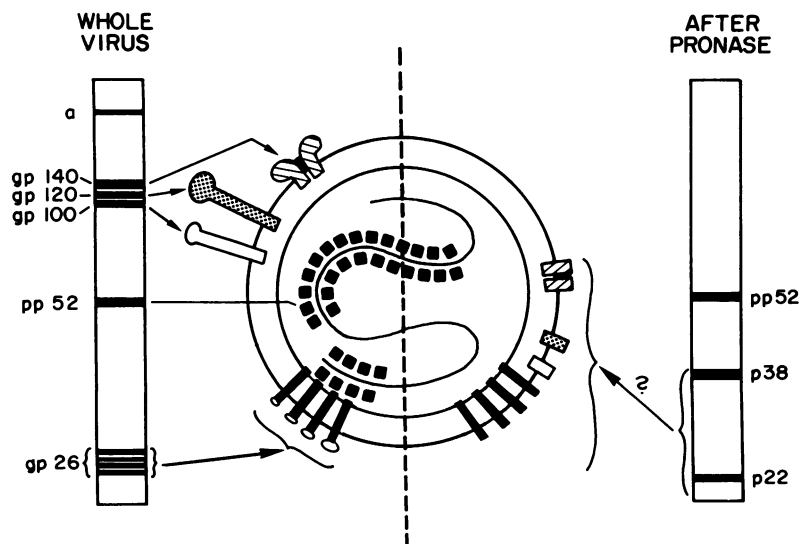


FIG. 5. Schematic model of BCV. The polypeptide profile on the left is that observed when no 2-mercaptoethanol is used in the sample treatment buffer. The polypeptide profile on the right is that observed from particles recovered by sedimentation after pronase treatment of purified virus. The model is discussed in the text.

is based on our observation that subviral particles prepared by Nonidet P-40 treatments of BCV and purified by differential sedimentation are composed almost entirely of pp52 and gp26 (data not shown). Similar observations have been made for the analogous protein of mouse hepatitis virus (30, 33), porcine hemagglutinating encephalitis virus (4), and avian infectious bronchitis virus (13). Whether gp26 interacts directly with the RNA, as suggested by Sturman for the analogous protein in mouse hepatitis virus (30), or whether it interacts directly with the internal protein pp50 remains to be shown. The presence of pp50 and gp26 in essentially equimolar amounts (Table 1) suggests that perhaps there is a direct association of one molecule of pp50 with one molecule of gp26. We have drawn gp26 as a transmembrane protein with the glycosylated portion external on the virion and an internal portion interacting with the ribonucleoprotein. gp26 consistently appears as a series of three to four closely migrating bands, and we suspect that these are actually one protein species differing only in the degree of glycosylation. Tryptic peptide analyses will be needed to prove this point. The phosphorylated pp52 protein is apparently in close association with the RNA since both enzymatic digestion studies and *in vitro* labeling studies with radiolabeled iodine show this protein to be totally internal to the virion envelope. Whether this protein functions as a kinase and can phosphorylate or undergo phosphorylation *in vitro* as does the analogous protein in the mouse hepatitis virus (25) remains to be determined.

We have recently grown BCV in the human adenocarcinoma cell line HRT-18 (31) to compare polypeptide structures with virus grown in BEK-1 cells. No difference in the sizes and species of polypeptides were observed (data not shown). No new polypeptides were visible. The only difference discernible was in the gp26 glycoprotein region. Whereas the band within the gp26 glycoprotein region having the highest apparent molecular weight was more heavily labeled with ^3H -amino acids in virus grown on the bovine cells, the second band of the gp26 series was more heavily labeled in virus grown on the human cells. Whether this reflects differences in the glycosylating properties of the two kinds of cells remains to be shown.

We have reported that the genome for BCV is a single-stranded, nonsegmented, polyadenylated RNA molecule of approximately 3.8×10^6 molecular weight when measured by electrophoresis on polyacrylamide-agarose gels (6). Recent measurements on denaturing formaldehyde gels of 1% agarose, however, reveal the molecular weight to be closer to 6.8×10^6 (data not shown). If we assume that the larger size is

correct and also that there is no redundancy in the coding capacity of the genome, then approximately 4.5×10^5 daltons of proteins could be coded for by the genome. We have accounted for approximately 3.63×10^5 daltons in virion structural proteins, assuming that each protein species possesses a unique polypeptide chain and overlooking the effect of glycosylated moieties on migration in polyacrylamide gels. Genetic information, therefore, apparently exists to code for essential nonstructural proteins such as an RNA polymerase. Studies are currently in progress to detect BCV nonstructural proteins in infected cells.

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