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Structural proteins of bovine coronavirus strain L 9: effects of the host cell and trypsin treatment

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Summary. The polypeptide profile of the cell-adapted strain of bovine coronavirus (Mebus BCV-L 9) is remarkably affected by the host cell and trypsin. We compared the structural proteins of virus purified from different cell lines and found cell-dependent differences in the virus structure. BCV was purified from four clones of human rectal tumour cells (HRT-18): 3 F3, D 2, 3 E 3, and 4 B 3. The structural profiles of BCV propagated in clones 3 E 3 and 3 F 3 were identical, consisting of proteins with molecular weights of 185, 160, 140, 125, 110, 100, 52, 46, 37, 31 – 34, and 26 – 28 kilodaltons (kd). BCV purified from clone D2 lacked the 100 kd species, and clone 4 B 3 yielded virus lacking the 46 kd protein. We compared the structures of BCV propagated in HRT-18 cells [BCV(HRT-18)] and virus raised in bovine fetal spleen cells [BCV(D 2 BFS)]. The concentration of the 185 kd protein was higher in BCV (D 2BFS), and it also contained a 200 kd species. Protein profiles of in vitro trypsin treated and untreated BCV(HRT-18) differed only under reducing conditions, suggesting that trypsin cleavage sites are located within disulfide-linked regions of affected proteins. Propagation of BCV in D 2 BFS cells in the presence of trypsin resulted in cleavage of the 185 kd protein and a concommitant increase of the 100 kd protein. Activation of the fusion function probably depends on this cleavage process because fusion of BCV-infected D 2 BFS cells is trypsin dependent.

Introduction

Proteolytic cleavage of inactive precursor polypeptides of assembled virions into an active form is necessary for inducing the infectivity of a number of viruses. Notably, orthomyxoviruses and paramyxoviruses require cleavage of envelope proteins for the viruses to become infectious. Processing of the viral proteins is accomplished either by cellular proteases or by exogenous proteases such as trypsin [2, 3, 7, 9, 10, 11, 18, 19]. Such findings are emerging for

coronaviruses as well. Frana et al. [6] found that processing of the 180 kd glycoprotein of mouse hepatitis virus strain A 59 (MHV-A 59) was host cell-dependent and affected the functional capacity of the virus. Those cells which fully cleaved the 180 kd protein to its 90 kd subunits activated rapid cell fusion. Cells which cleaved the protein incompletely required trypsin to activate rapid cell fusion.

Trypsin enhances the replication and cytopathic expression of bovine coronaviruses. We demonstrated trypsin-dependent cell fusion of bovine fetal spleen cells infected with BCV-L9 [20]. Storz and coworkers [21] reported enhanced cell fusion of BCV-L9 infected bovine fetal brain and bovine fetal thyroid cells in the presence of trypsin. The protein responsible for cell fusion has not been determined. BCV virions with shortened surface projections were produced in trypsin-treated bovine fetal thyroid cell cultures [21]. Williams [31] detected two phenotypes of virus when BCV-L9 was purified from human rectal tumour-18 (HRT-18) cells by isopycnic cetrifugation in sucrose or CsCl gradients. The dense and light forms of the virus had densities of 1.255 and 1.233 g/cm^3, respectively. The two virus forms had distinct polypeptide profiles, and the light form was converted to the dense form by in vitro trypsin treatment.

One objective of this study was to assess putative cell-dependent differences in the protein composition of BCV-L9. The virus was propagated in HRT-18 parent cells, four different HRT-18 clones [St. Cyr-Coats et al., submitted], and bovine fetal spleen cells (D 2BFS). These cells display different cythopathic effects when infected with the virus. A second objective was to compare the protein profiles in vitro trypsin-treated or untreated BCV-L 9 propagated in HRT-18 and D 2BFS cells. The profiles were examined under reducing and non-reducing conditions to reveal the effect of the enzyme on the viral polypeptides. D 2BFS cells infected with BCV require trypsin to activate cell fusion, which provided conditions to identify the viral protein(s) responsible for cell fusion.

Materials and methods

Cells and virus

The human adenocarcinoma cell line (HRT-18) and four HRT-18 clones, 3 F3, D 2, 3 E 3, and 4 B 3, established by limiting dilution, were grown in 850 cm^2 Corning roller bottles or in 150 cm^2 flasks. When seeded in roller bottles, 25 mM HEPES and 12 mM $NaHCO_3$ were supplemented to stabilize pH in Dulbecco's Modified Minimum Essential Medium (DMEM) (Gibco). After 1 day incubation at 37° C, this medium was replaced with DMEM containing 44 mM $NaHCO_3$, penicillin (100 units/ml), streptomycin (100 µg/ml), and 5% fetal calf serum. D 2BFS cells are a heterogenous subpopulation derived from bovine fetal spleen cells which overcame precrisis to multiply past the 30th passage. These cells were grown in Eagles Minimum Essential Medium (MEM) containing 25 mM HEPES, penicillin and streptomycin, and 10% fetal calf serum.

BCV-L 9 is a bovine coronavirus strain adapted to cell culture by Mebus et al. [15]. This virus was plaque purified twice before use in these investigations.

Virus growth and purification from HRT-18 cells

HRT-18 cells were infected with BCV-L 9 at an MOI of 0.01-0.1 PFU/cell. Following adsorption for 1 h at 37° C, excess inoculum was removed and cells were washed three times with Dulbecco's PBS. Medium was added, and cells were incubated at 37° C. When CPE affected approximately 80% of the monolayer, cells were frozen at −70 °C. Bottles were thawed; the infected material was pooled into 250 ml centrifuge bottles, sonicated, and refrozen.

Virus was purified from thawed cell lysate by isopycnic centrifugation in linear sucrose gradients according to the procedure described by Wege et al. [22]. Gradient fractions were collected by puncturing the bottom of the centrifuge tubes. Virus-containing fractions were identified by hemagglutinating activity (HA). Fractions containing the highest HA titers were pooled and concentrated by sedimentation through a 5 ml 20% sucrose cushion for 2 h at 90 000 x **g**. Virus was resuspended in TNE buffer (0.01 M tris-HCL, 0.01 M NaCl, 0.001 M EDTA), pH 7.4. This virus preparation was considered partially purified. For further purification the virus suspension was layered onto a preformed CsCl-TNE gradient (1.0606–1.2886 g/cm^3) and centrifuged at 55,000 × **g** for 20 h. Bands were collected as described above, concentrated, and resuspended in TNE buffer.

Propagation and concentration of virus from D2BFS cells

To minimize loss of virus, BCV-L 9 was directly concentrated from D2BFS cells, which produce low yields of virus in the absence of trypsin. D2BFS cells in 150 cm^2 flasks were infected with BCV-L 9 at an MOI of approximately 0.1 PFU/cell. After absorption, cells were washed 3 times to remove unadsorbed virus. MEM was added to one set of flasks, and MEM with 5 μg/ml trypsin (Difco; 1 : 250) was added to the other set. Flasks were incubated at 37 °C for 3 days, then frozen at −70°C, thawed, and the cell lysate was pooled. Fluid was clarified at 10,000 × **g** for 20 min to remove cell material, then concentrated as described.

Preparation of uninfected cell antigen for Western blots

Washed cells in 150 cm^2 flasks were frozen at −70°C, thawed, and cell lysate was collected in centrifuge tubes. Cell lysate was sonicated for 1 min, then the suspension was centrifuged at 1,000 × **g** for 15 min to settle nuclei. The supernatant was centrifuged at 90,000 × **g** for 2 h, and the pellet was resuspended in TNE buffer.

In vitro trypsin treatment

Aliquots of partially purified virus preparations were treated with 1 μg/ml trypsin (Sigma, TPCK treated) in Dulbecco's PBS at 37°C for 30 min. The action of trypsin was stopped by the addition of 1 μg/ml soybean trypsin inhibitor (Sigma) and incubation at 4° C for 30 min. The virus suspension was centrifuged through a 2 ml 20% sucrose-TNE cushion at 90,000 × **g** for 2 h. Pellets were resuspended to original volume in TNE buffer.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate − polyacrylamide gel electrophoresis (SDS-PAGE) of virus samples was done using the Laemmli buffer system [14] in the presence or absence of the reducing agent, β-mercaptoethanol. Gels containing 10 or 12% bis-acrylamide were prepared in 1 mm thickness, and run at a constant power of 4 watts/gel (approximately

25 mA/gel) for 3–4 h. BioRad low and high molecular weight standards ranging from 14,400 to 200,000 daltons were used. Proteins were visualized using the BioRad silver stain kit developed according to the procedure of Merrill et al. [16] or by Western blotting.

Western blot procedures

Western blots were done according to a modification of the procedure of Burnette [1]. Briefly, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using Tris-glycine transfer buffer (20 mM Tris base, 150 mM glycine), pH 8.3. Electrophoretic transfer was carried out for 18 to 20 h at 35 volts at 10 to 15° C. Transferred proteins were stained with Ponceau S [17], and molecular weight markers were cut off and saved for use in determining molecular weights of viral proteins. Transferred viral proteins were localized by incubating bovine anticoronavirus serum with blotted nitrocellulose membranes for 16 to 18 h. Goat antibovine IgG (H + L chain) peroxidase conjugate (Kirkegaard and Perry Laboratories) was used. Membranes were developed by reacting with substrate consisting of 15 mg 4-chloro 1-naphthol dissolved in 5 ml methanol, followed by addition of 25 ml 0.05 M Tris pH 6.8, and 12 µl H_2O_2.

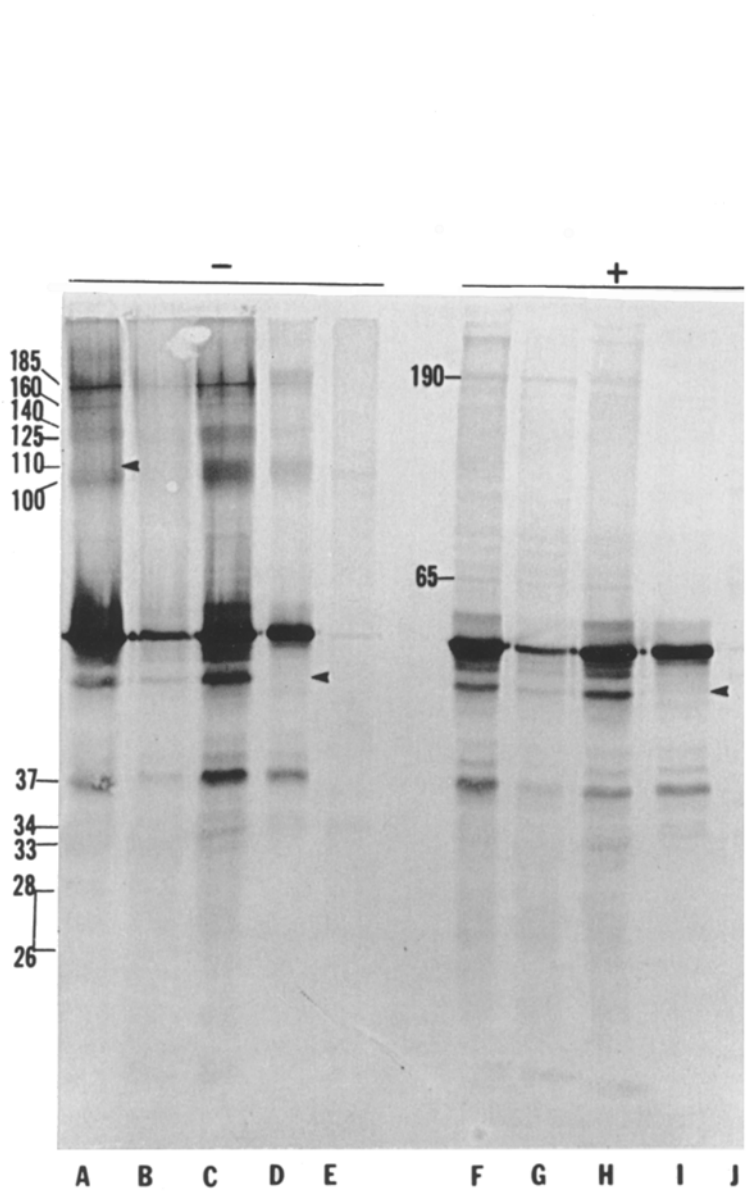
Results

Comparison under reducing and non-reducing conditions of the polypeptide structure of BCV-L 9 propagated in different cells

The polypeptide profiles of BCV-L 9, partially purified from HRT-18 clones D2, 3E3, 3F3, and 4B3 were analyzed by Western blotting (Fig. 1). We observed cell-dependent differences in virus structure. Under non-reducing conditions viral proteins of 185, 160, 140, 125, 110, 100, 52, 46, 37, 31–34, and 26–28 kd were detected from clones 3E3 and 3F3. However, the 46 kd protein was absent in BCV purified from clone 4 B 3. Doublet bands of 110 and 100 kd were found with BCV obtained from clones 3F3, 3E3, and 4B3, but only a single 100 kd band was present in virus from clone D2. To illustrate this finding, the profiles of BCV propagated in clones D2 and 3F3 are compared in Fig. 2. The viral structural proteins thus follow the characteristic pattern of high, intermediate and low molecular weight of coronaviruses.

BCV propagated in HRT-18 cells [BCV(HRT-18)] and D2BFS cells [BCV(D2BFS)] shared common proteins of 185, 160, 140, 100, 52, 37, and 31–34 kd (Fig. 3). Notable structural differences included the following: A > 200 kd protein was found in D2BFS-produced virus, but not in virus obtained from HRT-18 cells. The 185 kd protein was present a high concentration in BCV(D2BFS), but in proportion to the other proteins comprising the profile, it was present at low concentration in BCV(HRT-18).

Silver staining was the technique employed for discerning reduced proteins because we observed diminished binding of antibody to reduced viral proteins (Fig. 1). Viral proteins of 140 and 100 kd, consistently present under non-reducing conditions, were seen as 95 and 65 kd proteins under reducing



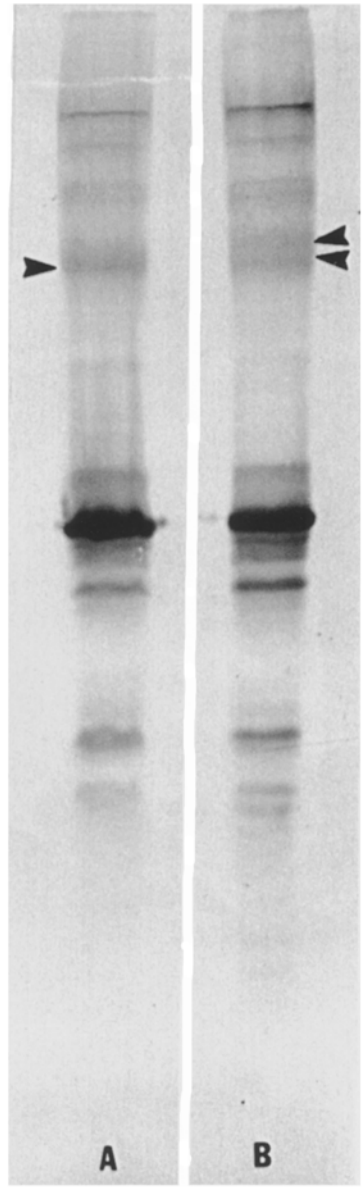


Fig. 1 **Fig. 2**

Fig. 1. Structural proteins of BCV-L 9 propagated in HRT-18 clones D2, 3E3, 3F3, and 4B3 as detected by Western blotting, 10% acrylamide gel. *A-E* Non-reduced virus. *F-J* Reduced virus. *A* and *F* BCV (D2), *B* and *G* BCV (3E3), *C* and *H* BCV (3F3), *D* and *I* BCV (4B3), *E* and *J* uninfected HRT-18 cells. ▶ Missing bands

Fig. 2. Structural proteins of BCV-L 9 propagated in HRT-18 clones D 2 and 3F3 as detected by Western blotting. Non-reduced, 10% acrylamide gel. *A* BCV (D2), *B* BCV (3F3). ▶ Differences in the profiles

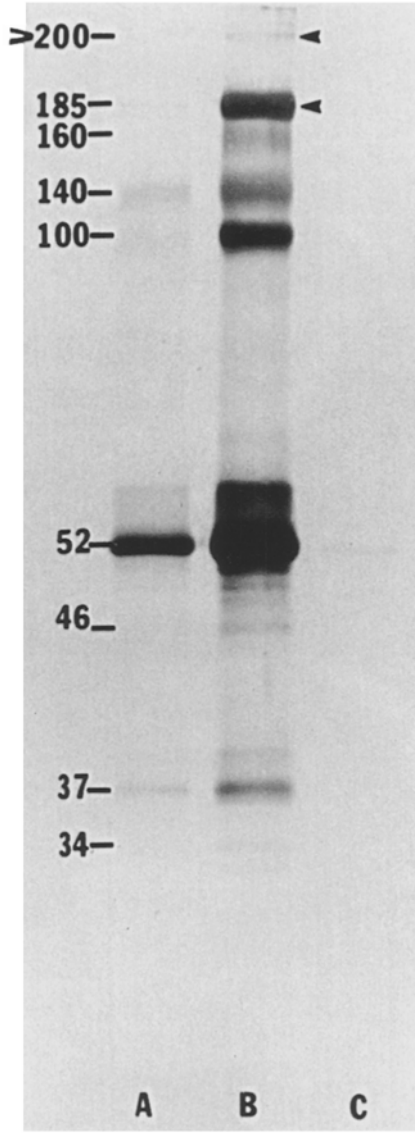


Fig. 3. Polypeptide profiles of BCV-L 9 propagated in D2BFS cells and HRT-18 cells as detected by Western blotting. Non-reduced. 10% acrylamide gel. *A* BCV (HRT-18), *B* BCV (D2BFS), *C* uninfected D2BFS cells. ► Differences in the profiles

conditions. Three distinct bands of 23, 20, and 18 kd were converted to a cluster of bands ranging from 20–23 kd following reduction (Fig. 4). We confidently identified reduced proteins of 190 and 65 kd by Western blot (Fig. 1).

Effect of trypsin treatment on BCV-L 9 polypeptides

Purified, HRT-18-cell-propagated BCV was treated in vitro with trypsin. The profiles of untreated and trypsin-treated BCV were examined under

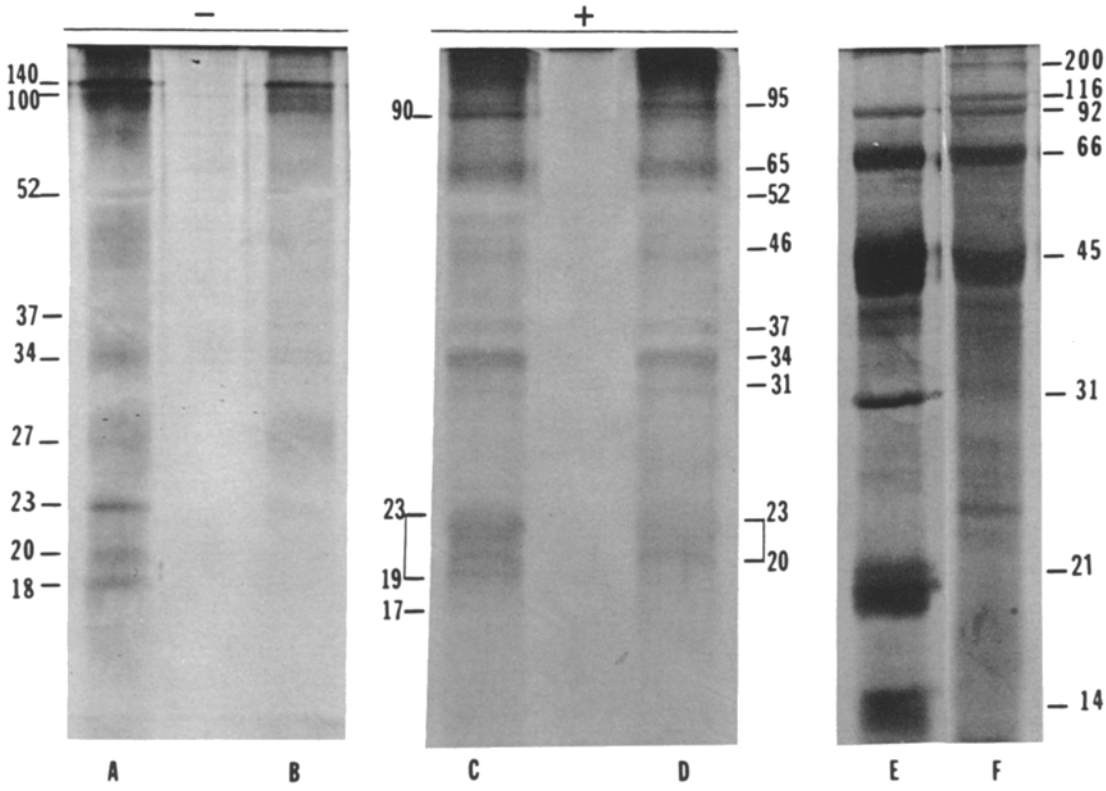


Fig. 4. Polypeptide profiles of in vitro trypsin-treated and untreated BCV-L 9 propagated in HRT-18 cells as detected by silver staining. 12% acrylamide gel. *A* and *B* Non-reduced virus. *C* and *D* Virus prepared with reducing agent. *A* and *C* Trypsin-treated virus, *B* and *D* untreated virus, *E* and *F* molecular weight standards

non-reducing and reducing conditions (Fig. 4). In the absence of reducing agent, the profiles were identical. Detectable bands had molecular weights of 140, 100, 52, 27, 23, 20, and 18 kd. Differences in the electrophoretic migration of trypsin-treated and untreated viral proteins were evident only under reducing conditions. A 95 kd protein was found in non-trypsinized virions, while a 90 kd protein was found in trypsin-treated virions. Additionally, a cluster of 20–23 kd proteins was found in untreated virions, but this cluster changed in trypsin-treated virions to 19–23 kd with the emergence of an additional 17 kd protein.

BCV-L 9, propagated in D2BFS cells in the presence and absence of trypsin, was analyzed under non-reducing conditions by Western blotting (Fig. 5). Virus propagated without trypsin had the following profile: >200, 185, 140, 100, 52, 46, 37, and a 31–34 kd cluster. In the presence of trypsin, the 185 kd protein was significantly diminished in concentration with a concommitant increase of the 100 kd protein. An additional difference between the profiles was the absence of the 31–34 kd cluster in trypsin-treated virus.

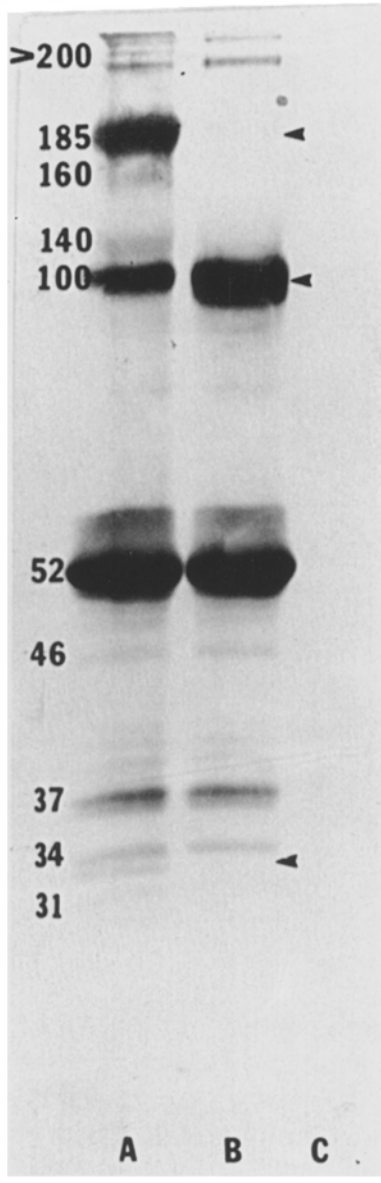


Fig. 5. Polypeptide profiles of BCV-L9 propagated in D2BFS cells in the presence and absence of trypsin as detected by Western blotting. Non-reduced. 10% acrylamide gel. *A* Without trypsin, *B* with trypsin, *C* uninfected D2BFS cells. ► Profile differences

Discussion

We identified host cell-dependent differences in the polypeptide profile of BCV-L9. Notably, the 185 kd protein was present in high concentration in virus propagated in D2BFS cells, but it was present in low concentration in virus obtained from HRT-18 parent cells and clones. These findings are in agreement with those of Deregt et al. [5] who reported that gp 190 was present in low concentration in the Quebec BCV isolate propagated in Madin-Darby bovine

kidney cells. A 100–110 kd doublet band was detected in BCV propagated in clones 3F3, 3E3, and 4B3, but only the 100 kd species was evident in BCV from clone D2. This difference may reflect a variation in processing or in glycosylation of the protein. Other investigators reported that HRT-18-propagated BCV (Mebus strain) contains predominantly 120 and 100 kd proteins which are cleaved forms of gp 190 [8, 12]. Our demonstration of a doublet band of 100–110 kd concurs with the results of Deregt et al. [5]. These scientists reported that cleavage of gp 190 yields two comigrating gp 100 species. Glycoprotein staining of these blots using concanavalin A and peroxidase [4] indicated that the 100 kd protein was glycosylated. A 46 kd protein reacting with antibody was evident in virus raised in all cell lines except clone 4B3 and D2BFS. It was not detected in Western blots of uninfected cells.

Silver-stained gels revealed that the 140 and 100 kd proteins, present under non-reducing conditions, disappeared under reducing conditions. At the same time, proteins of 95 kd and 65 kd appeared in the presence of a reducing agent. Similar findings were reported by Deregt et al. [5]. Hogue and coworkers [7] found that the 140 kd species consists of disulfide-linked subunits of 65 kd, but they did not observe a change in the electrophoretic migration of the 100 kd species under reducing conditions. The 18, 20, and 23 kd group of proteins was converted to a 20–23 kd cluster by reduction. Western blotting also revealed a change in the electrophoretic mobility of the 185 kd to a 190 kd protein under reducing conditions. Rather than consisting of disulfide-linked subunits, the 185 and 100 kd molecules and the 18–23 kd group of proteins possibly contain intrachain disulfide bonding. Reduction could alter the migration pattern of the proteins by breaking the intrachain bonds under this assumption.

Silver staining was a superior method to Western blotting for the detection of reduced viral proteins. Due to a change in antibody binding affinity of reduced proteins, an extended reaction time in the substrate was required to visualize reduced proteins. This condition led to a high background, making it difficult to discern the viral protein bands.

The native form of the BCV is the disease-causing agent in natural infections of calves. Consequently, it was considered more important to focus on the non-reduced profile of BCV. The use of antiserum obtained from a BCV-infected calf in Western blot identified the antigenically active components of the virus.

We observed differences in the number of detectable viral proteins and their relative migration patterns between preparations that were silver stained or Western blotted. These differences emphasize the relevance of the techniques chosen for detection of viral proteins. The use of different detection methods may explain discrepancies in the reported profiles of this virus [5, 8, 12, 13, 23].

We demonstrated that trypsin has an enhancing effect on the cytopathic expression and infectivity of BCV in cultures of HRT-18 and D2BFS cells [20; St. Cyr-Coats et al., submitted]. Therefore, we examined the effect of the enzyme on the structure of this virus. Interestingly, the action of trypsin on the virus

structure was evident by silver stain only under reducing conditions. This finding indicates that trypsin-cleaved peptides were held intact by disulfide bonds. When reduced by β-mercaptoethanol, cleavage products were separable. Trypsin-dependent bands of 90 kd and 17 kd were detected in HRT-18 cell-propagated virus and the 20–23 kd cluster found in untreated virions was converted to a 19–23 kd cluster by trypsin.

We examined the polypeptide structure of BCV-L 9 propagated in D2BFS cells with and without trypsin because these infected cells fuse only in the presence of trypsin [20]. This system provided a potential model for determining the viral protein(s) responsible for cell fusion. We found that the 185 kd protein was present in high concentration in the absence of trypsin, but only a faint band was present in virus produced with trypsin. Concommitantly, the concentration of the 100 kd species was much higher in the presence of trypsin. These results agree with those reported by Deregt et al. [5] who demonstrated that gp 100 is a proteolytic cleavage product of gp 190. We conclude that cleavage of the 185 kd protein to the 100 kd form is required for activation of cell fusion. Trypsin was required to cleave this protein in D2BFS cells, but our data indicate that cellular enzymes cleave the 185 kd species in HRT-18 parent cells and the four HRT-18 clones. This finding is analogous to those reported by Frana et al. [6] who found that cleavage of the 180 kd protein of MHV-A 59 by a cellular protease or by trypsin activates the fusing capacity of that virus.

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