Synthesis and processing of structural and intracellular proteins of two enteric coronaviruses
by Lisa Marie Sardinia

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
Microbiology
Montana State University
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Abstract:
The synthesis and processing of virus-specific proteins of two economically important enteric
coronaviruses, bovine enteric coronavirus (BCV) and transmissible gastroenteritis virus (TGEV), were
studied at the molecular level. To determine the time of appearance of virus-specific proteins,
virus-infected cells were labeled with 35S-methionine at various times during infection, immunoprecipitated with specific hyperimmune ascitic fluid, and analyzed by SDS-polyacrylamide gel
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postinfection (hpi). The appearance of all virus-specific proteins was coordinated. In contrast, the peak
of TGEV protein synthesis was at 8 hpi, but the nucleocapsid protein was present as early as 4 hpi.

Virus-infected cells were treated with tunicamycin to ascertain the types of glycosidic linkages of the
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indicating that they possessed N-linked carbohydrates. The matrix protein of TGEV was similarly
affected. The matrix protein of BCV, however, was resistant to tunicamycin treatment and therefore
has CD-linked carbohydrates. Only the nucleocapsid protein of both viruses is phosphorylated as
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while the TGEV nucleocapsid protein appears to be the cleavage product of a slightly larger species.
Both the peplomer and matrix proteins of BCV and TGEV are produced by the glycosylation of
polypeptide precursors. A panel of monoclonal antibodies (MAbs) against each virus was prepared
using hybridoma technology. These MAbs were used to precipitate intracellular and virion proteins. A
number of virus-specific proteins which do not show any precursor-product relationship do appear to
share common determinants. Limited digest peptide mapping to compare structural relationships
among virus-specific proteins indicated that each virus coded for a set of two proteins related to the
nucleocapsid protein. The peplomer proteins of each virus produced a set of peptides that were
identical to a smaller protein found in virions. All other structural proteins were found to have unique
peptide maps.
SYNTHESIS AND PROCESSING OF STRUCTURAL AND INTRACELLULAR PROTEINS OF TWO ENTERIC CORONAVIRUSES

by

Lisa Marie Sardinia

A thesis submitted in partial fulfillment of the requirements for the degree of
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APPROVAL

of a thesis submitted by

Lisa Marie Sardinia

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

The synthesis and processing of virus-specific proteins of two economically important enteric coronaviruses, bovine enteric coronavirus (BCV) and transmissible gastroenteritis virus (TGEV), were studied at the molecular level. To determine the time of appearance of virus-specific proteins, virus-infected cells were labeled with 35S-methionine at various times during infection, immunoprecipitated with specific hyperimmune ascitic fluid, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The peak of BCV protein synthesis was found to be at 12 hours post-infection (hpi). The appearance of all virus-specific proteins was coordinated. In contrast, the peak of TGEV protein synthesis was at 8 hpi, but the nucleocapsid protein was present as early as 4 hpi. Virus-infected cells were treated with tunicamycin to ascertain the types of glycosidic linkages of the glycoproteins. The peplomer proteins of both viruses were sensitive to inhibition by tunicamycin indicating that they possessed N-linked carbohydrates. The matrix protein of TGEV was similarly affected. The matrix protein of BCV, however, was resistant to tunicamycin treatment and therefore has O-linked carbohydrates. Only the nucleocapsid protein of both viruses is phosphorylated as detected by radiolabeling with 32P-orthophosphate. Pulse-chase studies and comparison of intracellular and virion proteins were done to detect precursor-product relationships. The nucleocapsid protein of BCV may be the product of phosphorylation of a protein which migrates slightly faster in SDS-PAGE, while the TGEV nucleocapsid protein appears to be the cleavage product of a slightly larger species. Both the peplomer and matrix proteins of BCV and TGEV are produced by the glycosylation of polypeptide precursors. A panel of monoclonal antibodies (MAbs) against each virus was prepared using hybridoma technology. These MAbs were used to precipitate intracellular and virion proteins. A number of virus-specific proteins which do not show any precursor-product relationship do appear to share common determinants. Limited digest peptide mapping to compare structural relationships among virus-specific proteins indicated that each virus coded for a set of two proteins related to the nucleocapsid protein. The peplomer proteins of each virus produced a set of peptides that were identical to a smaller protein found in virions. All other structural proteins were found to have unique peptide maps.
INTRODUCTION

The development of vaccines which provide protection against viral diseases is an ongoing research concern. Preparation of monoclonal antibodies with prophylactic and therapeutic value against these diseases is also an important goal. In order to develop truly effective and safe products, the basic biology and biochemistry of the virus must be studied. Since it is the virus-specific proteins which are immunogenic, the study of the synthesis and processing of these proteins will provide valuable information that can then be used in the development of anti-viral vaccines and treatments. The purpose of my research was to study at the molecular level the synthesis and processing of virus-specific proteins of two economically important enteric coronaviruses, bovine enteric coronavirus (BCV) and transmissible gastroenteritis virus (TGEV).

Biology and Pathogenesis of Coronaviruses

The Coronaviridae are a heterogeneous family of pathogenic viruses which naturally infect many animal species including humans and cause a variety of diseases involving a number of different organ systems. Coronavirions are
spherical, moderately pleomorphic particles 60-220 nanometers in diameter which bear characteristic widely-spaced club-shaped surface projections. This "corona" of peplomers which is clearly visible in electron micrographs prompted the International Committee on the Taxonomy of Viruses to approve the creation of a new family, Coronaviridae, in 1975 (63). The genome is a large (6-8 megadaltons) single-stranded, poly-adenylated RNA molecule which is infectious and of positive polarity. The outer shell of the virus consists of a cell-derived lipid bilayer which is necessary for infectivity. All coronavirus species multiply exclusively in the cytoplasm of infected cells. They bud internally into vesicles derived from the endoplasmic reticulum and the Golgi apparatus. Virions are released from intact cells by fusion of post-Golgi vesicles with the plasma membrane (39,49,59,63,68,70).

These viruses are widespread in nature and are associated with a wide variety of diseases with an acute or subacute disease process, as well as subclinical infections. For most coronaviruses causing enteric diseases, the disease is caused by virus multiplication and cytopathogenicity. The target cells for these infections are the epithelial cells of the intestines.

The mammalian coronaviruses can be divided into two groups on the basis of cross-reactivity in serological
tests. BCV and TGEV do not cross-react with one another serologically although they cause very similar diseases in their respective hosts.

Bovine enteric coronavirus was first characterized in 1972 by Mebus and co-workers (40,51) as a coronavirus-like agent causing diarrhea in calves. Since it caused diarrhea in gnotobiotic calves, it is considered to be a primary pathogen. Symptoms begin 24-30 hours after inoculation, last 4-5 days, and can be lethal in newborn calves. The most severe lesions develop in the small intestine where destruction of the intestinal epithelium leads to pathophysiological changes followed by an extensive loss of water and electrolytes (68). The finding by Storz et al. (56) that trypsin treatment in vitro of BCV accelerated cytopathic functions and improved infectivity yields suggests that the initiation of infection might be promoted by the action of proteolytic enzymes in the intestinal tract. Maternal antibodies (IgA and IgM) are transmitted via the colostrum to calves and reduce the severeness of the disease (68).

The biochemical and molecular characterization of BCV has been hindered in the past by the difficulty of propagating and assaying the virus in vitro. Most of the earlier work was done in primary or non-continuous cell lines. Laporte et al. (34) reported in 1980 the use of HRT-18 cells, a human adenocarcinoma cell line, for
cultivation of high titers of BCV. The next year, Vautherot (64) developed a plaque assay using this same cell line. These developments have greatly facilitated BCV studies.

Transmissible gastroenteritis is an acute disease affecting pigs of all ages. In a herd, morbidity approaches 100%. Mortality is usually restricted to piglets under 3 weeks of age but often reaches 100% in this age group. The disease is characterized by vomiting, diarrhea, and dehydration within 24 hours of infection. In addition, there is congestion of mesenteric lymph nodes and blood vessels and occasionally hemorrhages in the kidneys. TGEV replicates rapidly in the jejunum, duodenum, and ileum, but not in the stomach or colon. The virus is resistant to low pH and trypsin and is moderately resistant to bile. These factors contribute to its ability to survive passage in the alimentary tract. Replication of virus takes place only in the columnar cells of the villi with cell desquamation accompanied by shortening of the villi. Replacement of the villous epithelial cells begins 18-72 h post-infection by migration of undifferentiated cells from the crypts. No reinfection occurs although released virus is present. This may be due either to the presence of IgA in the alimentary tract or to the production of a new population of epithelial cells that are resistant to the virus.
Passive resistance to the virus in the form of neutralizing antibody is transferred to piglets through the colostrum (3,8,23,68,71). There has been much research into the pathogenesis of TGEV and a number of vaccines have been introduced, but these have been only partially successful (19).

**Coronavirus Proteins**

The majority of the studies on coronavirus proteins have involved different strains of infectious bronchitis virus (IBV), which is an avian virus, and murine hepatitis virus (MHV). In general, coronavirus virions contain from 3 to 7 structural proteins which seem to fall into three classes. The nucleocapsid (N) protein is a phosphorylated protein of 50-60 kilodaltons (kd) that is associated with the viral genome (9,37,49,59,69). N protein in intact virions is resistant to treatment with bromelain (9) and pronase (69) indicating that it is located internally. Siddell et al. (48) have detected a protein kinase activity associated with the virion which, in the absence of exogenous protein substrates, specifically phosphorylates the virion nucleocapsid protein *in vitro*. The same protein is the only virion protein that is significantly phosphorylated in infected cells.

The second species of structural proteins is a large (125-200 kd) glycoprotein, P, which appears to comprise
the peplomers (5,9,11,19,30,37,67,69). Treatment with bromelain removes this protein from the virion (12). Smaller glycopeptides (65-120 kd) which are often detected may be the result of cleavage of the larger glycoprotein by proteases (55,59,97). In vitro cleavage of the P protein by trypsin is often associated with a twofold increase in infectivity (58) and a ten-fold increase in yield of infectious virus (56). MHV usually replicates and produces plaques better in transformed cells. This may be due to the fact that transformed cells show an increased level of protease (72). Biological activities associated with this protein include binding of virions to membrane receptors of susceptible cells (27,44,59), induction of neutralizing antibody (22,25,44), and cell fusion (15,27,60).

Some coronaviruses may have more than one peplomeric glycoprotein. BCV virions possess another smaller glycoprotein which is also associated with the surface projections and is about 100 to 120 kd (24,26,30,66). It appears that this protein is a dimer of lower molecular weight (65 kd) glycopeptides held together by disulfide linkages (26,30). This structural protein elicits neutralizing antibodies. Monoclonal antibodies to this glycoprotein suggest that it is responsible for the hemagglutinating activity of BCV (65,66).
The third family of structural proteins is a heterogeneous glycoprotein species, M, (matrix) of 20 to 30 kd which often appears as several bands on SDS-polyacrylamide gels. The bands generally reflect differing degrees of glycosylation (2,5,9,37,49,67,69). The matrix glycoprotein appears to possess three domains: a glycosylated hydrophilic region which projects outside the viral envelope, a hydrophobic portion which extends across the membrane, and a third domain which is probably associated with the viral RNA on the inner side of the viral envelope. Treatment of coronavirus virions with proteolytic enzymes such as pronase or bromelain produces a 20% reduction in the apparent size of the M polypeptide which corresponds to about 5 kd (2,58). This segment which is degraded contains all of the carbohydrate on the molecule. A hydrophobic domain resides within the lipid bilayer. Heating at 100°C in the presence of reducing agents induces aggregation of the M protein (57) indicating that reduction of disulfide bonds exposes a highly hydrophobic region (59). The third domain of M resides on the inner surface of the envelope and is presumably associated with the nucleocapsid. The M proteins can be separated from the nucleocapsid on gradients after solubilization of the viral envelope with NP-40 at 4°C, but will reassociate with the nucleocapsid at 37°C (61).
Most viruses that have been studied share a common pathway of glycosylation. This cotranslational event is dolichol-mediated and is sensitive to tunicamycin. Tunicamycin is an analog of UDP-N-acetylglucosamine which interferes with the formation of dolichol-linked intermediates that transfer oligosaccharides to asparagine residues on glycoproteins. These carbohydrate moieties are termed N-linked sugars. In contrast, the matrix protein of MHV has an O-glycosidic linkage.

Glycosylation is a post-translational event and is resistant to tunicamycin (27,41). Not all coronaviruses, however, possess this unique glycoprotein. Glycosylation of the M protein of IBV is inhibited by tunicamycin (52).

The M protein appears to be the only protein required for coronavirus budding. In MHV-infected, tunicamycin-treated cells, P protein is made in markedly reduced amounts. MHV virions are nevertheless formed and released normally (59). Apparently glycosylation is not required for particle formation as virions are released from IBV-infected cells treated with tunicamycin (52). In either case, however, the particles are not infectious indicating that the P protein is indispensable for viral infectivity (49,52).

In MHV-infected cells, two additional virus-specific proteins are often found (4,5,47). These proteins are 14 kd and 35 kd. They are not found in virions and are
therefore designated as non-structural proteins. They have been shown to be primary gene products by cell-free translation (36,47). Tryptic peptide maps indicate that these proteins are not related to each other or to any of the other MHV-specific proteins (4). The functions of these proteins are not known.

Bovine Enteric Coronavirus Proteins

There has been some disagreement as to the number and character of the structural proteins of BCV. Workers from different labs have reported from 4 to 7 polypeptides in purified virions. The molecular weights of these proteins range from 23 to 190 kd (24,26,30,33,55). The peplomer glycoprotein appears to be a 190 kd protein that is normally present as two smaller subunits of 120 and 100 kd (26,30). A second peplomer protein of 105 kd (65,66) or 140 (26,30) is responsible for the hemagglutinating activity of the virus. The two groups who reported on this second peplomer protein, Vautherot et al. (65,66) and the workers in Brian's laboratory (26,30), each work with a different strain of BCV which may account for the striking difference in size of this protein. 140 kd glycoprotein is apparently a disulfide-linked dimer of 2-65 kd subunits. Antibodies against the 105 kd glycoprotein defined at least two functional domains, one outlined by neutralizing monoclonal antibodies and the
other recognized by monoclonal antibodies with neutralizing and hemagglutination inhibition activity (65,66). Trypsin treatment produces BCV virions with distinctly shorter, more regularly arranged surface projections. Cytopathic functions and cell fusion are accelerated and hemagglutination activity is improved when trypsin is added (56).

The matrix protein is a heterogeneous group of closely migrating glycoproteins of 23 to 26 kd (26,30,55). These bands may represent different degrees of glycosylation. In contrast to the peplomer protein, the M protein could not be metabolically labeled with mannose or fucose indicating that it represents an O-linked glycoprotein (55).

An internal phosphoprotein of 50 to 52 kd, corresponding to the nucleocapsid protein has been reported (26,30,33,55). The intracellular non-structural proteins of BCV have not been described.

Transmissible Gastroenteritis Virus Proteins

Purified particles of TGEV are reported to have three major polypeptide species. The largest polypeptide contains carbohydrate and is located in the virus surface projections (19,21). This 195-200 kd glycoprotein elicits a neutralizing antibody response (22). Only one serotype of TGEV is known so far, and this is determined by the
peplomer protein. When in vivo glycosylation of the P protein is inhibited by tunicamycin, synthesis of the protein is either prevented or greatly reduced (21). Cleavage of most of the sugar moiety by endoglycosidase H generates a 145 kd protein (28). The nucleocapsid protein is rich in arginine residues (21) and has a molecular weight of 47,000 to 50,000 (19,21). This protein may be partially degraded to a 42 kd protein (19). The matrix glycoprotein is 28 to 30 kd. Glycosylation of the 26 kd precursor is prevented by tunicamycin (19,21). TGEV-specified intracellular proteins have not been described.

**Synthesis and Processing of Virus-Specific Proteins**

Almost all of the studies on the biogenesis of virus-specific proteins and their subsequent processing have been done using either IBV or strains of MHV. A number of techniques including pulse-chase labeling, analysis of glycosylation, in vitro translation, and tryptic peptide mapping have been used in these studies. The results obtained will be discussed below.

Radiolabeling of virus-specific proteins at various times during infection indicates that, for MHV, protein synthesis is moderately coordinated. Virus-specific proteins begin to appear by 3 to 4 hours after infection (49). In some cases, a large (150 kd) protein which is probably the peplomer precursor is detected 1 hour before
other virus-specific proteins (5), although in cells which show minimum cytopathic effect, the synthesis of N was detected much earlier than that of P or M (59).

The nucleocapsid protein has been shown to be a phosphorylated protein of 50 to 60 kd. Since this protein can be detected after a very short (2 min) pulse-labeling period (13), and is produced in an in vitro translation system (36), it has been assumed that it is a primary translation product and is not post-translationally modified. Recently, however, Stohlman et al. (54) detected a slightly smaller (57 kd) protein after a 2 min pulse-labeling period. After a 10 min chase, the larger (60 kd) phosphoprotein was found. As the chase continued, the amount of 57 kd protein decreased and the amount of 60 kd protein increased. The 57 kd protein was not phosphorylated. It was detected by two monoclonal antibodies that precipitate the 60 kd protein but was not recognized by two other monoclonal antibodies to the 60 kd protein. The investigators theorized that additional determinants may be present on the 60 kd protein due to conformational changes induced by phosphorylation.

It has been noted by a number of workers (1,13,52) that after chases of long duration (1-2 h), a protein that is smaller than N appears. This protein, which is smaller by 6 to 10 kd, has a tryptic peptide map which is identical to that of N (4,13,52). This smaller protein
has been presumed to be the product of proteolytic cleavage of N. Its appearance is variable according to the cell type and virus strain. It is also found as a product of in vitro translation (36). Premature termination of translation or, less likely, internal initiation, have been hypothesized to account for its presence in the translation system.

Both the intracellular and virion forms of the matrix protein are often seen as a broad band or group of bands on SDS-polyacrylamide gel electrophoresis. All of these proteins have identical tryptic peptide maps (4,13,46,52). The smallest of these proteins ranges in size from 18 to 23 kd and is not glycosylated (1,10,13,46,50,52). In MHV, glycosylation increases the size of the M protein by about 2 kd (13,46,50) whereas in IBV, the size is increased by 5 to 11 kd (10,52). The MHV M protein is unique among viral glycoproteins in that it contains only O-glycosidic linkages (27,41,42). In contrast, the M protein of IBV possesses the more common N-linked oligosaccharides (52). Glycosylated forms of IBV M protein can be detected after only a 5 min pulse (52) which indicates that glycosylation is a cotranslational event. This is consistent with the concept of dolichol-mediated, tunicamycin-sensitive glycosylation (31). M glycoproteins of MHV are not labeled during a short pulse and appear only after a
longer chase (13,41,50). This suggests that the glycosylation of M is a post-translational event.

The pathway of synthesis and processing of the peplomer protein has been much debated. The difficulty in sizing large glycoproteins has contributed to the confusion. Workers disagree as to the sizes of precursors and products, but several patterns of processing have been hypothesized.

The peplomers of IBV appear to be composed of two glycoproteins, 90 kd and 84 kd (9,11,12,52). A model by Cavanaugh (11,12) suggests that there are two molecules of each size in the peplomer. In this "ice cream cone" model the smaller glycoproteins are embedded in the membrane with a dimer of the larger glycoprotein forming the bulb at the end of the peplomer.

In IBV-infected cells, a large glycoprotein of 155 kd is detected which does not comigrate with virion proteins. This protein is sensitive to digestion with endoglycosidase H and is not detectable in cells treated with tunicamycin It is therefore an N-linked glycoprotein. The tryptic peptide map of this glycoprotein contains all of the peptides in the maps of both the 84 kd protein and the 90 kd protein, which are different from each other. In pulse-labeled cells, the 155 kd glycoprotein was evident but neither the 84 kd nor the 90 kd glycoprotein could be detected. These proteins appeared only after a
60 min chase. This indicated that these two proteins were derived from a precursor. Since the 155 kd glycoprotein includes the sequences of the smaller glycoproteins and is the only virus-specific protein larger than these two proteins, it must be the precursor (52).

The synthetic pathway of MHV P protein is somewhat more obscure. MHV virion peplomers have two glycoproteins associated with them. One is 180 kd (1,6,13,58) and one is 90 kd (5,50,58,60). These two proteins have identical tryptic peptide maps (4,46,58) and the large glycoprotein can be converted to the 90 kd glycoprotein by treatment of virions with trypsin in vitro (58). In vitro translation studies have shown that the primary translation product which is precipitated by antiserum specific for the P protein is 120 kd (36). This corresponds to the polypeptide core of P glycoprotein which has been found to be 120 kd (46). Pulse-chase studies, however, reveal a 150 kd glycoprotein after a short pulse, which is then chased into a 180 kd glycoprotein (6,50). The tryptic peptides of the 150 kd glycoprotein are also identical to those of the 180 kd and 90 kd glycoprotein (4,46). Recently, Sturman and Holmes (60) showed that there are actually two species of 90 kd protein. These have been designated 90A and 90B and were isolated by exploiting the fact that 90A is acylated. Taking all the information into consideration, the following pathway has been
proposed by Siddell (46). The primary translation product in vivo is a 150 kd glycoprotein which has a 120 kd polypeptide core and is cotranslationally glycosylated. The conversion of this primary product to the larger virion peplomer protein possibly involves further modification of the carbohydrate side chains. The 90 kd glycoprotein has not been found by all workers. Trypsin mediated cleavage of the 180 kd glycoprotein may not occur in all cell lines used for propagating MHV. The evidence for the role of proteolytic cleavage of P in activation of cell fusion suggests, however, that this step may have importance in natural coronavirus infection and pathogenesis.

Goals and Experimental Design

The goal of my research was to analyze the synthesis and processing of virus-specific proteins of two economically important enteric coronaviruses. Although these viruses cause a similar disease in their respective hosts, they belong to different serological groups and appear to have differences in their structural proteins.

A number of methods were used in the analysis of proteins of BCV and TGEV. To determine the time of appearance of virus-specific proteins, virus-infected cells were radiolabeled with $^{35}$S-methionine at various times during infection, radioimmunoprecipitated with
specific hyperimmune ascitic fluid, and analyzed by SDS-polyacrylamide gel electrophoresis. Pulse-chase studies and comparison of intracellular and virion proteins were done to detect precursor-product relationships. Virus-infected cells were treated with tunicamycin to ascertain the type of glycosidic linkages of the glycoproteins. Phosphorylation of virus-specific proteins was detected by radiolabeling with $^{32}$P-orthophosphate. A panel of monoclonal antibodies prepared against each virus was used to precipitate proteins and discover common determinants on viral proteins. Limited digest mapping was performed on intracellular and virion proteins and the patterns compared. Finally, a model was prepared describing the pathway of synthesis and processing of BCV- and TGEV-specific proteins.
MATERIALS AND METHODS

Chemicals and Media

Reagent grade liquid organic chemicals were obtained from J. T. Baker Chemical Co. Other chemicals and reagents were obtained from Sigma Chemical Co. unless otherwise stated in the text. Radioisotopes were obtained from New England Nuclear Corp. Cell culture media were purchased from Irvine Scientific (Santa Ana, CA), and sera were purchased from Sterile Systems (Logan, UT).

Most cell cultures were maintained in Dulbecco's Modified Eagle's (DME) medium that was supplemented with 200 Units/ml penicillin G, 25 ug/ml streptomycin, and 10% (vol/vol) calf serum. DME 20 was prepared as above except that 20% (vol/vol) fetal bovine serum was added instead of calf serum. Infection of cells with virus was done in DME 2. Instead of calf serum, 2% (vol/vol) fetal bovine serum was added to DME and the medium was supplemented with 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 10 mM N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES).

Hybridomas were maintained in DME 20 supplemented with 1 ug/ml amphotericin B, and 1% (vol/vol) of 10 mM
hypoxanthine, 1.6 mM thymidine (HT) and 0.04 mM aminopterin (A) and was designated as HAT medium.

Virus Strains and Cell Lines

Bovine enteric coronavirus (BCV) was obtained from ATCC (ATCC VR-874) as was the Miller strain of transmissible gastroenteritis virus (TGEV) (ATCC VR-743). BCV was cloned by plaque purification and propagated in a continuous human adenocarcinoma cell line (HRT-18) (34) obtained from Dr. David Brian. A continuous swine testicle cell line, ST, established by McClurkin and Norman (38) and obtained from Dr. Brian, was used for the multiplication of TGEV. TGEV was also cloned by plaque purification. BCV was passaged 3 to 5 times in HRT 18 cells before use in experiments. TGEV was passaged 11 to 13 times in ST cells. These cell lines were maintained in DME 10.

A murine sarcoma cell line (S180) (ATCC TIB-66) was derived from Swiss Webster mouse ascitic tumor cells. NS-1 cells are a BALB/c myeloma line (P3/NS1/1-Ag4-1) that is a non-secreting clone of P3X63Ag8. NS-1 cells were obtained from the Salk Institute. Baby hamster kidney (BHK) cells were obtained from Dr. John Holland. S180 cells and BHK cells were maintained in DME 10, while DME 20 was used for NS-1 cells.
Virus Stocks

Monolayers of cells in 10 cm plastic dishes (Nunc) were infected with virus at a multiplicity of infection (MOI) of 1 in DME 2. The virus was allowed to adsorb at room temperature for 1 h. The inoculum was removed and replaced with 7 ml DME 2. The infected cells were incubated at 37°C until greater than 75% of the cells were lysed. The plates were frozen at -70°C, and freeze-thawed twice and the resulting lysates were scraped with a rubber policeman and sonicated for 105 s in a Heat Systems Sonicator (model W-225R) using a cup probe at 70% power. The lysates were clarified by centrifugation (1200 x g) for 5 min, aliquoted and stored at -70°C.

Plaque Assay

Virus stocks were titered by plaque assay on the appropriate cell monolayers. Cells were seeded into plastic six-well dishes (Nunc) in DME 10 and incubated at 37°C overnight. Monolayers were infected with 0.5 ml of serial 10-fold dilutions of virus in DME 2. Dilutions of virus were allowed to adsorb at room temperature for 1 h. The inoculum was removed and the monolayers were overlaid with 3 ml of DME 2 containing 0.75% (wt/vol) agarose (type II, Sigma), and incubated at 37°C for 3 days. Cells were fixed by adding 0.5 ml of 2% (vol/vol) glutaraldehyde to
each well and incubating at room temperature for several hours. The agarose overlays were removed, the plates dried at room temperature and the plaques counted. Data were expressed as plaque-forming units per ml (PFU/ml).

**Infection and Purification**

Monolayers of cells were infected with virus at an MOI of 5 in DME 2. The virus was allowed to adsorb at room temperature for 1 h. The inoculum was then removed and replaced with DME 2. The virus-infected cells were incubated at 37°C for 18 h (TGEV) or 56 h (BCV) and then frozen at -70°C.

To purify virus, an adaptation of the method of Leibowitz, et al. (35) was used. The cell-associated virus was released by two cycles of freeze-thawing and the resulting lysate clarified by centrifugation in an HB-4 rotor at 10,000 x g for 30 min at 4°C. Virus was concentrated by centrifugation at 35,000 rpm for 60 min in an SW 41 rotor through a pad of 15% (w/w) potassium tartrate (KT) in MSE buffer (0.01 M morpholinopropane-sulfonic acid, 0.15 M NaCl, 0.001 M EDTA, pH 6.8) onto a pad of 40% (w/w) KT in MSE buffer. The visible band was removed, diluted to less than 10% KT in MSE buffer, layered onto a 9 ml 10-40% KT gradient, and centrifuged in an SW 41 rotor at 37,000 rpm for 4 h at 4°C. The visible band was removed, diluted in MSE buffer and pelleted at
45,000 rpm for 1 h at 4°C in an SW 50.1 rotor. The pellet was resuspended in 0.1-0.2 ml B10 [50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.02% (wt/vol) sodium azide, 0.05% (vol/vol) NP40, 1% (vol/vol) Aprotinin, 0.1% (wt/vol) bovine serum albumin], transferred to a 1.5 ml Eppendorf tube and stored at -20°C.

**Immunization of Mice**

Eight-to-twelve week old BALB/c mice were inoculated intraperitoneally (ip) three times at weekly intervals with 0.15 ml of mock- or virus-infected cell lysates emulsified with an equal volume of complete Freund’s adjuvant. A final booster inoculation of 0.3 ml cell lysate without adjuvant was given one week later. Each inoculation contained $10^5$ to $10^8$ PFU of virus.

**Production of Hyperimmune Ascitic Fluid**

Ascitic fluid was induced by ip inoculation of immunized mice with $10^6$ S180 cells in 0.3 ml phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 9.4 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 0.9 mM CaCl$_2$, 0.5 mM MgCl$_2$ pH 7.2) one day after the final booster inoculation (62). Ascitic fluid was harvested by abdominal paracentesis using an 18-gauge needle, stored at 4°C overnight and centrifuged at 800 x g for 5 min to remove cells and debris.
Prior to use, the ascitic fluid was absorbed twice at 4°C for 24 h with methanol-fixed cell monolayers to remove antibodies directed against non-viral components. The absorbed ascitic fluid was stored at -70°C and was used in the detection of virus-specified proteins by immunoprecipitation.

**Preparation of Monoclonal Antibodies**

Mice were immunized as described above. Spleens were removed from immunized mice 4 days after the final booster injection. Immune spleen cells (3 x 10^7) were fused with 5 x 10^7 NS-1 cells using 50% polyethylene glycol (mol. wt. 1000, Sigma cat. no. P3515) in DME 0. Fused cells were diluted in HAT medium and seeded into 96-well plates (Nunc) containing mouse peritoneal macrophages and irradiated baby hamster kidney cells as feeder cells. Incubation and maintenance of the hybridomas was carried out according to the microculture protocol of de St. Groth and Scheidegger (16). Culture fluids from growing colonies were screened for anti-viral antibodies by immunofluorescence as described below. Cells from positive wells were cloned twice by limiting dilution in 96-well plates. Ascitic fluids containing high concentrations of anti-viral monoclonal antibody were prepared by injecting 1.5 x 10^6 hybridoma cells into Pristane (2,6,10,14-tetramethylpentadecane) treated mice.
The mice were injected ip with 0.5 ml of Pristane at least one week prior to the injection of hybridoma cells.

**Immunofluorescence Assay**

Mock- and virus-infected cells were seeded (10 ul/well) into 60-well Terasaki plates (Nunc), incubated at 37°C for 8 h (TGEV) or 12 h (BCV), washed with PBS and fixed with methanol as previously described (43). For indirect immunofluorescence staining, the plates were rinsed once with PBS and 30 ul of hybridoma supernatant fluid was added to each well. After 30 min at 37°C the plates were washed 4 times with PBS and 10 ul of fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse immunoglogin (Antibodies Inc. cat. no. 2146) was added to each well. The plates were incubated at 37°C for an additional 30 min and washed 4 times with PBS. Immunofluorescence was observed with an Olympus IMT inverted microscope equipped with reflected fluorescence accessories.

**Plaque Reduction Test**

A plaque reduction test was used to determine the virus neutralizing activity of the hyperimmune ascitic fluid. Virus suspensions were diluted to approximately 300 PFU/ml and mixed with an equal volume of ascitic fluid containing monoclonal antibodies. After incubation for 30
min at 37°C, 0.5 ml of the virus/antibody mixture was inoculated onto cell monolayers and the plaque assay was completed as described. Monoclonal antibodies were considered to be neutralizing if the plaque reduction was greater than 90%.

**Determination of Isotype of Monoclonal Antibodies**

The Ouchterlony method of gel diffusion (18) was used to determine the isotypes of monoclonal antibodies. One percent agarose was prepared in 0.15 M NaCl, 0.05% NaN₃ and poured into 50 mm plastic dishes. Wells were punched into the agarose. The center well was filled with 18 ul of class-specific goat anti-mouse immunoglobulin. The outer wells were filled with 18 ul of one of the following: supernatant fluid from hybridomas, ascitic fluid containing monoclonal antibodies, normal mouse serum, or normal mouse ascitic fluid. The plates were incubated at room temperature in a humidified chamber for 24-48 h until precipitin bands formed.

**Radiolabeling of Virus-Specified Proteins**

Purified virions radiolabeled with L-[³⁵S]-methionine (NEG-009A) were prepared as follows. Monolayers of cells in 10 cm plastic dishes were infected with virus at an MOI of 5 in DME 2 and incubated at 37°C. At 6 h post-infection (hpi) for TGEV and 8 hpi for BCV, the medium was
removed and replaced with labeling medium (80% methionine-free DME 2, 20% DME 2, 20-100 uCi/ml $^{35}$S-methionine). The virus-infected cells were incubated at 37°C and harvested at 18 hpi for TGEV and 56 hpi for BCV. The virions were purified from infected cell lysates as described above.

Virus-specified intracellular proteins were labeled with L-$[^{35}$S]-methionine using the following procedure. Confluent cell monolayers in 35 mm plastic dishes (Nunc) were mock-infected or inoculated with stock virus at an MOI of 5 in DME 2. After 1 h at room temperature, the inoculum was removed and replaced with 1 ml DME 2. At various times post-infection the medium was removed and replaced with 0.3 ml of methionine-deficient DME 2. Twenty min later, $^{35}$S-methionine was added to a final concentration of 200-400 uCi/ml. After a 2-4 h labeling period, the medium was removed and the cells were lysed with 0.1 ml B10 for 5 min on ice. The cytoplasmic lysates were harvested and stored at -20°C.

The same method was used to label infected cells with $^{32}$P-orthophosphate (NEX-054) except that phosphate-deficient medium was used during the labeling period.

**Immunoprecipitation of Virus-Specified Proteins**

Cytoplasmic lysates were immunoprecipitated with hyperimmune ascitic fluid by a modification of the procedure of Bond et al. (6). Fifteen ul samples of cell
lysates were diluted ten-fold in RIP buffer [50 mM Tris-
HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.2% (vol/vol) NP40,
0.05% (vol/vol) SDS, 1% (vol/vol) Aprotinin, 0.02%
(wt/vol) sodium azide]. Five ul of hyperimmune ascitic
fluid were added and incubated at 0°C for 1 h. Immune
complexes were precipitated with 50 ul of 10% (vol/vol)
formalin-fixed *Staphylococcus aureus* (Cowan) (29) by
incubation at 0°C for 1 h and pelleted by centrifugation
at 6,500 x g for 15 s. The pellets were washed 4 times
with RIP buffer. The proteins were eluted with 30 ul of
20 mM dithiothreitol (DTT), 1% (wt/vol) SDS for 15 min at
room temperature and 5 min at 60°C. Bacteria were removed
by centrifugation at 6,500 x g for 5 min. The supernatant
fluids containing virus-specified proteins were removed,
mixed with an equal volume of 1-D PAGE diluent [120 mM
Tris-PO₄ (pH 6.7), 1% (wt/vol) SDS, 40% (vol/vol)
glycerol, 0.02% (wt/vol) phenol red] and stored at -20°C.

**SDS-Polyacrylamide Gel Electrophoresis**

Virion proteins were prepared for SDS-polyacrylamide
gel electrophoresis (SDS-PAGE) by adjusting the lysate to
0.02 M DTT, 1% (wt/vol) SDS, heating for 5 min at 60°C and
then adding an equal volume of 1-D PAGE diluent.
Immunoprecipitated proteins were used directly.

Proteins were electrophoresed on 8%, 10%, or 12%
(wt/vol) polyacrylamide slab gels as described by Laemmlı
and Favre (32). Preparative gels were dried directly onto Whatman 3 MM paper and exposed to preflashed Kodak XAR-2 x-ray film. Analytical gels were fixed overnight in 5% trichloroacetic acid (TCA).

Standard proteins were detected by staining with Coomassie brilliant blue G (17). Labeled proteins were detected by impregnating the gels with 10% (wt/vol) 2,5-diphenyloxazole (PPO) in dimethyl sulfoxide (DMSO) followed by drying and exposure to preflashed x-ray film at -70°C (7).

The molecular weights of virus-specified proteins were determined from their distance of migration in slab gels relative to those of standard proteins of known molecular weight (45). The following proteins were used as standards in molecular weight determinations: thyroglobulin (200 kd), beta-galactosidase (115 kd), phosphorylase B (97.4 kd), bovine serum albumin (66 kd), ovalbumin (45 kd), carbonic anhydrase (29 kd).

Limited Digest Peptide Mapping

Virus-specific intracellular and virion proteins labeled with $^{35}$S-methionine were electrophoresed on a 10% polyacrylamide gel. Bands corresponding to virus-specified proteins were identified by exposure of dried preparative gels to x-ray film. Using the x-ray film as a template, the appropriate bands were excised and
rehydrated in enzyme buffer [125 mM Tris (pH 6.8), 0.1% SDS, 1 mM EDTA, 10% glycerol]. A 16% acrylamide gel with a 3% stacking gel of 4 cm in height was prepared. Enzyme buffer with enzyme (5 µg of Staphylococcus aureus V8 protease or TLCK-chymotrypsin or 10 µg of TPCK-trypsin) and indicator dye was added to each well. Gel pieces were transferred to the wells. Electrophoresis was carried out at 120 V until the dye front was 1 cm above the resolving gel. The current was then turned off for 30 min to allow for digestion of the proteins by the enzyme. Electrophoresis was continued at 120 V until the dye front reached the bottom of the gel (14). The gel was then treated in the same manner as other SDS-polyacrylamide gels as described above.
RESULTS

Time Course of Virus Multiplication

The kinetics of BCV and TGEV multiplication at 37°C were determined (Figure 1). Cultures infected with BCV began yielding virus by 8 hpi and reached their maximum titer by 56 hpi. The maximum yield of BCV was approximately $9 \times 10^7$ PFU/ml. BCV-infected HRT 18 cells did not begin to lyse until 40 to 48 hpi. The concomitant rise of cell-associated and released virus may be due to released virus readsorbing to cell membranes.

The course of infection of TGEV was markedly faster than that observed for BCV. Cultures infected with TGEV reached their maximum yield of virus by 18 hpi. The maximum yield of TGEV was approximately $6 \times 10^6$ PFU/ml. TGEV-infected ST cells were almost completely lysed by 24 hpi, although cell remnants remained attached to the dish. The relatively low titers of released virus late in infection may be due to the instability of the virus for prolonged periods at 37°C.

Time Course of Virus-Specific Protein Synthesis

The kinetics of virus-specific protein synthesis in BCV- and TGEV-infected cells were determined. HRT 18 and
Figure 1. Time course of production of infectious virus from infected cells. Cells were infected at an MOI of 1.0. At the indicated times, the supernatant fluids from monolayers of infected cells were removed and the amount of infectious virus in the supernatant fluid was determined by plaque assay [released virus (□)]. DME 2 was added to the monolayers which were then frozen at -70°C. The lysates were clarified as described in Materials and Methods and titered by plaque assay [cell-associated virus (△)]. Data are expressed as plaque-forming units (PFU) per ml.
ST cells were infected with BCV and TGEV, respectively, at an MOI of 5.0. They were labeled for 2 h (TGEV) or 4 h (BCV) with $^{35}$S-methionine at various times post-infection. At the times indicated in Figure 2, the labeling medium was removed and the cells were lysed with B10. The cell lysates were immunoprecipitated and a 2 μl sample was removed for liquid scintillation counting. TGEV-specific protein synthesis reached a peak at 6 to 8 hpi and dropped off sharply by 12 hpi. In contrast, BCV-specific protein synthesis peaked at 12 hpi but remained relatively high throughout infection. These results should be expected considering that BCV-infected HRT 18 cells retain their integrity and therefore their ability to support virus-specific protein synthesis for a much longer time than TGEV-infected ST cells.

The immunoprecipitated lysates of virus- and mock-infected cells were analyzed by SDS-PAGE. The results are shown in Figure 3 (TGEV) and Figure 4 (BCV). The positions and molecular weights of virus-specific polypeptides are indicated in the figures.

The first TGEV-specific protein to be detected was the nucleocapsid protein N (48 kd). This protein appeared faintly as early as 4 hpi. The majority of TGEV-specific proteins were first apparent by 6 hpi and the maximum rate of synthesis was between 6 and 8 hpi. Proteins corresponding to P (190 kd), N, and M (29 kd) were
Figure 2. Time course of viral protein synthesis in cells infected with BCV and TGEV. Virus- (△) and mock- (□) infected cells were labeled with 20 uCi/ml $^{35}$S-methionine at various times post-infection as described in Materials and Methods. Cell lysates were immunoprecipitated with homologous anti-viral ascitic fluid, and the amount of labeled protein determined by liquid scintillation counting. Data are expressed as counts per minute (CPM) per 2 ul sample.
Figure 3. Time course of TGEV-specific protein synthesis. Virus- and mock-infected cells were labeled at various times post-infection for 2 h with 200 uCi/ml $^{35}$S-methionine. Cell lysates were immunoprecipitated with anti-viral ascitic fluid and analyzed on a 10% gel. Viral proteins are indicated with their approximate sizes in kd. The number above each lane indicates the hour post-infection that the labeling period ended and the cells were lysed. The first 4 lanes show mock-infected cell lysates. Lanes 5-11 show TGEV-infected cell lysates.
Figure 4. Time course of BCV-specific protein synthesis. Virus- and mock-infected cells were labeled at various times post-infection for 4 h with 200 uCi/ml $^{35}$S-methionine. Cell lysates were immunoprecipitated with anti-viral ascitic fluid and analyzed on an 8% gel. The number above each lane indicates the hour post-infection that the labeling period ended and the cells were lysed. The first 3 lanes show mock-infected cell lysates. Lanes 4-13 show BCV-infected cell lysates.
present, as well as proteins of 91 kd, 84 kd, 54 kd, and 42 kd. This was the only labeling time during which a faint band of 14 kd could be detected.

By 8 hpi, three BCV-specific proteins could be detected corresponding to the peplomer protein (160 kd), the nucleocapsid protein (60 kd), and the matrix protein (23 kd). Protein synthesis peaked from 8 to 12 hpi when a 102 kd protein appeared. The hemagglutinin protein (116 kd) was seen faintly at 12 hpi, but never attained the intensity of other virus-specific bands. Whereas TGEV-specific protein synthesis fell off sharply by 12 hpi, BCV-specific proteins were apparent throughout infection.

In subsequent experiments, labeling was done at the peak of virus-specific protein synthesis. For TGEV-infected ST cells labeling was at 6 to 8 hpi and for BCV-infected HRT 18 cells, at 8 to 10 hpi.

**Comparison of Virion and Intracellular Virus-Specific Proteins**

HRT 18 and ST cells were infected with BCV and TGEV, respectively, and radiolabeled with $^{35}$S-methionine. The virions were purified as described in Materials and Methods, and the purified virions analyzed by SDS-PAGE. Radiolabeled intracellular proteins from virus-infected cell lysates were immunoprecipitated using hyperimmune ascitic fluid. These immunoprecipitates of virus- and
mock-infected cells were compared with the virions. Lane 5 of Figure 5 shows eight TGEV proteins with molecular weights of 190K, 140K, 91K, 84K, 54K, 48K, 42K, and 29K. The lowest molecular weight protein (14 kd) of TGEV appeared faintly in the original autoradiogram and is not visible in the figure. BCV-infected HRT 18 cells contained nine viral proteins of 160 kd, 116 kd, 102 kd, 60 kd, 53 kd, 48 kd, 23 kd, 18 kd, and 14 kd (lane 2).

These intracellular proteins were reproducibly detected in several experiments using different cell lysates. The polypeptide profiles were essentially the same in each experiment. The estimated sizes of the proteins varied slightly in different gels. The range of sizes and the average size of each protein is given in Table 1.

BCV virions appear to be comprised of six proteins (Figure 5, lane 3). The background in this lane was fairly high and did not photograph well. The major proteins are, however, apparent. The sizes of these proteins are indicated in the figure and are included in Table 1. The two smallest intracellular proteins as well as the 53 kd protein were not found in virions. The 160 kd peplomer protein was absent, but the intensity of the 102 kd protein band was greatly increased over the same band found in an immunoprecipitate of virus-infected cell lysate. The hemagglutinin band (116 kd) was, however,
Figure 5. Comparison of virion and intracellular virus-specific proteins. Virions (BCV, lane 3 and TGEV, lane 4) were labeled and purified as described in Materials and Methods. Intracellular proteins were labeled for 2 h from 6-8 hpi (TGEV, lane 5) or 8-10 hpi (BCV, lane 2). Samples were analyzed on 10% gels. Lane 1 shows mock-infected HRT 18 cell lysates. Lane 6 shows mock-infected ST cell lysates.
Table 1. BCV and TGEV virus-specific proteins found in virions or in infected cells.

<table>
<thead>
<tr>
<th>TGEV Virion</th>
<th>IC(^a)</th>
<th>BCV Virion</th>
<th>IC</th>
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<tr>
<td>190(^b)</td>
<td>190 (178-200)(^c)</td>
<td>160 (132-160)</td>
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<td>14</td>
<td>14 (14-16)</td>
<td>18 (16-18)</td>
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</table>

\(^a\) Intracellular

\(^b\) Size of protein in kilodaltons.

\(^c\) Range of size of proteins from different gels.

much fainter in the virion lane than in the intracellular protein lane. The 48 kd protein was not seen in virions, but a protein of 46 kd was found. A 33 kd protein was found that was unique to virions. The matrix protein appeared as a heterogeneous band from 23-25 kd.

There are six TGEV virion proteins (Figure 5, lane 4). The peplomer protein was found to be 190 kd. The 140 kd and 91 kd intracellular proteins were not found in virions. An 84 kd virion protein was not seen in an immunoprecipitate of virus-infected cell lysate. The 48 kd nucleocapsid protein was found in virions, along with
the 42 kd and 54 kd species. The matrix protein (29 kd) was found to be the same size in virions and infected cells.

Phosphorylation of Virus-Specific Proteins

Phosphorylation of virus-specific proteins was determined by labeling infected cells with $^{32}\text{P}$-orthophosphate for 2 h beginning at 6 hpi for TGEV and 8 hpi for BCV. As shown in Figure 6, the only protein that is significantly phosphorylated is the nucleocapsid protein. The molecular weights of the phosphorylated proteins are indicated in the figure. Phosphate adheres non-specifically to aggregates of proteins. The radioactivity present at the top and bottom of lanes 4-8 may represent $^{32}\text{P}$-orthophosphate that adhered to these aggregates. In addition, Siddell et al. (48) showed that the nucleocapsid protein of mouse hepatitis virus JHM had kinase activity. The high molecular weight bands in lane 6 may represent cellular proteins which were phosphorylated by the kinase activity of the TGEV nucleocapsid protein. Finally, the nucleocapsid protein is closely associated with the viral genome. Using the labeling procedure described, the RNA as well as protein would be labeled with $^{32}\text{P}$-orthophosphate. The close association of N with the genome could inhibit the activity of RNase
Figure 6. Phosphorylation of virus-specific proteins. Virus- and mock-infected cells were labeled with $^{35}$S-methionine (lanes 1-4) or $^{32}$P-orthophosphate (lanes 5-8) and the cell lysates immunoprecipitated with appropriate ascitic fluid. The immunoprecipitates were analyzed by SDS-PAGE on a 10% gel. Phosphorylated viral proteins are indicated with their approximate sizes in kd. Lanes 1 and 5 show mock-infected ST cells. Lanes 2 and 6 show TGEV-infected ST cells. Lanes 3 and 7 show BCV-infected HRT 18 cells. Lanes 4 and 8 show mock-infected HRT 18 cells.
during the immunoprecipitation. The protein-nucleic acid complex would be expected to remain at the top of the gel. In repeated experiments, the amount of radioactivity found in the band corresponding to the nucleocapsid protein was highly consistent, while the amounts of extraneous radioactivity was highly variable.

Effect of Tunicamycin on Yield of Infectious Virus

The peplomer protein of coronaviruses is required for infectivity as discussed in the Introduction. P is a glycosylated protein and this glycosylation is sensitive to inhibition by tunicamycin in other coronaviruses that have been studied. To determine the effect of tunicamycin on the yield of infectious virus from TGEV- and BCV-infected cells, the following experiment was done. Monolayers of cells were inoculated with virus at an MOI of 5 and allowed to incubate at room temperature for 1 h. The inoculum was removed and replaced with DME 2 containing various amounts of tunicamycin. At 18 hpi (TGEV) or 56 hpi (BCV), the plates were frozen at -70°C, freeze-thawed twice, and the resulting lysates clarified as described in Material and Methods. The amount of infectious virus in each cell lysate was determined by plaque assay. The results are shown in Figure 7. Concentrations of tunicamycin greater than 1 ug/ml
Figure 7. Effect of tunicamycin on the production of infectious virus. Various amounts of tunicamycin were added to virus-infected cells at 1 hpi. At 18 hpi for TGEV and 56 hpi for BCV, the infected cell monolayers were frozen at -70°C and the resulting lysates clarified. Lysates were titered by plaque assay as described in Materials and Methods. Data are expressed as plaque-forming units (PFU) per ml.
inhibited the yield of infectious virus from both TGEV- and BCV-infected cells.

**Effect of Tunicamycin on Virus-Specific Protein Synthesis**

Intracellular proteins were radiolabeled as described in Materials and Methods except that tunicamycin was added at 1 hpi. Proteins from virus-infected cell lysates were immunoprecipitated with hyperimmune ascitic fluid and analyzed by SDS-PAGE. Figure 8 (TGEV) and Figure 9 (BCV) show the results of this study. In the case of both BCV and TGEV, non-glycosylated proteins such as N were unaffected by the addition of tunicamycin. The 42 kd, 91 kd, and 140 kd intracellular proteins of TGEV were also unchanged.

The amount of the large glycoprotein of TGEV corresponding to P was reduced when tunicamycin was added to a concentration of 0.1 ug/ml and was undetectable at a concentration of 1.0 ug/ml. It is not known if the protein was not made or if it was undetected because it no longer possessed the determinants recognized by antibodies in the hyperimmune ascitic fluid used for immunoprecipitation. The 84 kd TGEV structural protein appeared to be converted to 80 kd upon the addition of tunicamycin.

In lane 6 of Figure 9, a unique band of 127 kd appeared which could possibly be the non-glycosylated
Figure 8. Effect of tunicamycin on TGEV-specific protein synthesis. Various amounts of tunicamycin were added to mock- (lanes 1-3) and virus- (lanes 4-9) infected cells at 1 hpi. The samples in lanes 1-6 were analyzed on a 12% gel. The samples in lanes 7-9 were analyzed on an 8% gel. The infected cells were labeled for 2 h at 6 hpi. The cells were lysed at the end of the labeling period and immunoprecipitated with anti-viral ascitic fluid. Lanes 1, 4, and 7 had no tunicamycin added. Lanes 2, 5, and 8 had tunicamycin added to 0.1 μg/ml. Lanes 3, 6, and 9 had tunicamycin added to 1.0 μg/ml.
Figure 9. Effect of tunicamycin on BCV-specific protein synthesis. Various amounts of tunicamycin were added to mock- (lanes 1-3) and virus- (lanes 4-6) infected cells at 1 hpi. The infected cells were labeled for 2 h at 8 hpi. The cells were lysed at the end of the labeling period, immunoprecipitated with anti-viral ascitic fluid and analyzed on a 10% gel. Lanes 1 and 4 had no tunicamycin added. Lanes 2 and 5 had tunicamycin added to 0.1 ug/ml. Lanes 3 and 6 had tunicamycin added to 1.0 ug/ml.
form of the BCV P protein. The 102 kd BCV protein appeared to be reduced to a protein of 100 kd with the addition of tunicamycin.

The presumptive hemagglutinin of BCV of 116 kd was apparently not labeled sufficiently to appear even in the absence of tunicamycin. The work of others (24,30) suggests that H is a glycoprotein with N-linked carbohydrates.

The M protein (23 kd) of BCV was unaffected by the addition of tunicamycin (Figure 9, lanes 4, 5, and 6). This indicated that BCV M protein possesses O-linked carbohydrates. In contrast, the TGEV M protein is converted from a 29 kd protein to a 24 kd species (Figure 8, lanes 4, 5, and 6), suggesting that it contains the more common N-linked carbohydrates. A 14 kd protein, which is a non-structural virus-specific protein, was detected in the absence of tunicamycin (lane 4 in each figure). In TGEV-infected cells it disappeared at an increased concentration of tunicamycin (lanes 5 and 6). It may be that this protein also possesses N-linked carbohydrates. The non-glycosylated polypeptide core may be so small that it migrates off the end of the gel. It may be, however, that this protein is not glycosylated, but that the synthesis of the protein itself is inhibited. Commercial preparations of tunicamycin may not be pure and contaminants may inhibit protein synthesis and be
responsible for the disappearance of this protein. The corresponding BCV-specific protein was not inhibited by tunicamycin. If it is glycosylated, the carbohydrates must be attached through O-linkages.

Pulse-Chase Labeling of Virus-Specific Proteins

Pulse-chase labeling was used to determine precursor-product relationships among virus-specific intracellular proteins. Virus-infected cells were labeled with $^{35}$-S methionine for 5 min (TGEV) or 10 min (BCV). The labeling medium was then replaced with complete medium. If proteins labeled during the pulse period are posttranslationally modified, new species of proteins will appear during the chase period, with a concomitant decrease in the amount of precursor.

HRT 18 and ST cells were infected with BCV and TGEV, respectively, at an MOI of 5.0. Three dishes of TGEV-infected cells were labeled at 6 hpi for 5 min with $^{35}$S-methionine. The labeling medium was then removed. The cells in one dish were lysed immediately with B10 as described in Materials and Methods. The intracellular proteins from this dish were designated as pulse-labeled. The other two plates were washed and DME 2 was added. One dish was incubated (chased) to 6.5 hpi, and the cells lysed, and one was chased to 7.5 hpi, and the cells lysed. BCV-infected cells were labeled similarly except that the
labeling was done at 8 hpi for 10 min. The results of the pulse-chase labeling experiments are shown in Figure 10.

The M protein of TGEV has a molecular weight of 29K. This protein appears only faintly in the pulse (lane 4), but is the same size as the protein found in the chase (lanes 5 and 6). Glycosylation of this protein was inhibited by tunicamycin and the carbohydrates are therefore N-linked. As discussed in the Introduction, N-linked glycoproteins are added cotranslationally and therefore no non-glycosylated precursor is found.

The 48 kd species of TGEV N protein normally found in infected ST cells did not appear in pulse-labeled cells. A 54 kd protein was present in the pulse-labeled cells. Over the subsequent chase period, the autoradiographic intensity of the 54 kd protein decreased, apparently in favor of the 48 kd species. Other investigators (19) have reported the degradation of the N protein by 5 to 8 kd after prolonged chases. It must be a specific cleavage since only one new species is generated.

The probable precursor to the P protein of TGEV is a 173 kd protein seen in pulse-labeled cells. After a 30 min chase, a 176 kd protein appeared and after 90 min, a 190 kd protein was found in addition to the 176 kd protein. The intensity of the 176 kd protein band was greatly decreased at this time indicating that it is probably the precursor to the mature 190 kd P protein.
Figure 10. Pulse-chase labeling of virus-specific proteins. Mock- and virus-infected cells were labeled as described in the text. Immunoprecipitates of pulse-chase labeled cells were analyzed by SDS-PAGE on 10% gels. Lanes 1-3 show mock-infected ST cell lysates. Lanes 4-6 show TGEV-infected ST cell lysates. Lanes 7-9 show mock-infected HRT 18 cell lysates. Lanes 10-12 show BCV-infected HRT 18 cell lysates. The first lane of each set shows pulse-labeled cell lysates. The second lane shows a 30-min chase. The third lane shows a 90-min chase.
Another protein of 145 kd was also found after pulse-labeling. The band disappeared during the chase period as a 133 kd protein appeared. A protein in the range of 133-165 kd appears in TGEV-infected cell lysates. It is not found in TGEV virions. The possibility that this protein is the precursor to P cannot be ruled out completely. As discussed in the Introduction, cleavage of most of the sugar moiety from P by endoglycosidase H generates a 145 kd protein (28). However, a protein of this size was normally found in TGEV-infected cells, while a 173 kd protein was not, indicating that this larger protein was quickly modified to the peplomer protein.

The 91 kd intracellular protein appeared only during the chase periods. It may be processed from the 145 kd protein, as the intensity of the 91 kd and 133 kd protein bands as determined by spectrophotometric scanning of the autoradiogram together do not exceed that of the 145 kd protein band.

Lane 10 shows pulse-labeled BCV-infected cell lysates. There was very high background on the autoradiogram and the photograph of this autoradiogram is therefore quite dark. The BCV-specific bands on the autoradiogram were, however, unambiguous. Glycosylation of M protein (23 kd) of BCV is resistant to inhibition by tunicamycin and therefore has O-linked carbohydrates. A 23 kd protein was present, however, in pulse-labeled cells
(lane 10) as well as during the chase period (lanes 11 and 12). This may be due to the longer pulse time used for BCV, allowing for more posttranslational modification. Shorter pulse times resulted in little if any labeling of virus-specific proteins. It is possible that the precursor to the glycosylated form of the matrix protein was also present in pulse-labeled cells but was undetected due to the inability of the antibodies in the ascitic fluid to recognize the nonglycosylated species during immunoprecipitation. The amount of carbohydrate added may also be relatively small and the two bands may not have been resolved on this gel.

The N protein of BCV does not appear to be processed. It appeared as a 60 kd protein in pulse-labeled cells and remained unchanged throughout the chase. Two proteins of 53 kd and 48 kd were also seen. These two proteins are often detected in infected cells. It may be that these are precursors rather than degradation products as for TGEV. Stohlman et al (54) detected a protein of MHV-JHM that was slightly smaller than the mature nucleocapsid after a 2 min pulse which was quickly chased into the nucleocapsid. The 10 min pulse period may have been too long to detect only the unmodified precursors. They are probably detected in continuously labeled infected cells because of the continuous production of both precursor and mature N protein throughout infection.
A 102 kd protein was found in pulse-labeled, BCV-infected cells. This protein disappeared during the chase period concomitant with the appearance of a 160 kd protein. This second protein corresponds to P. In BCV-infected cells labeled continuously for 2-4 h, proteins of approximately these sizes were found. The 102 kd protein may be synthesized continuously and then posttranslationally glycosylated or may be dimerized to produce the larger protein.

A small protein (14 kd) was found in both the pulse-labeling and subsequent chase of BCV-infected cells. This small protein was found in preparations of virus-specific intracellular proteins but was not found in BCV virions. It is not known if it is a glycoprotein. It was unaffected by the addition of tunicamycin so, if it is glycosylated, the carbohydrates are posttranslationally O-linked.

No protein corresponding to the hemagglutinin of BCV was found in the pulse or the chase. The hemagglutinin band was faint even in cells labeled continuously for 4 h. Ten minutes is apparently not sufficient time for adequate incorporation of radiolabeled methionine into this polypeptide.
Analysis of Virus-Specific Proteins Using Monoclonal Antibodies

The interpretation of the immunoprecipitation data using polyclonal ascitic fluid was difficult. To partially alleviate these difficulties, monoclonal antibodies specific for the proteins of BCV and TGEV were prepared using hybridoma technology. Several clones which were positive by IFA for anti-viral specificity were chosen for analysis. None of the MAbs from these clones showed any activity against mock-infected cells by IFA or by immunoprecipitation. Ten clones which produced anti-BCV MAbs were amplified in mice. The ascitic fluid which was obtained was used for immunoprecipitation of virus-specific proteins. The same was done for seven clones which produced MAbs against TGEV. These seven TGEV hybridomas were prepared by Dr. Susan Goss in our laboratory.

The Ouchterlony method of gel immunodiffusion was used to determine the isotype of each of the MAbs. Figure 11 shows the results. All of the MAbs were found to be IgG. Each MAb produced only a single precipitin band. There was no difference in the results obtained when hybridoma supernatant fluid or ascitic fluid was used. Several of the MAbs precipitated more than one polypeptide. In each case, however, only one or two
Figure 11. Determination of isotype of monoclonal antibodies. The central well of each plate was filled with the indicated goat anti-mouse immunoglobulin. The outer wells were filled with the indicated monoclonal antibodies or normal mouse serum (NMS) or ascitic fluid (NMA). The plates were incubated as described in Materials and Methods. Supe: supernatant fluids from hybridomas. Asc: ascitic fluid.
structural proteins were dominant. A summary of these results is shown in Table 2. A plaque reduction test was performed on each of the MAbs to determine if any of them exhibited virus neutralization activity. These results are also included in Table 2.

Table 2. Characteristics of monoclonal antibodies.

<table>
<thead>
<tr>
<th>Polypeptide Specificities</th>
<th>MAba Structural</th>
<th>Intracellular</th>
<th>VNb</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 N (48) C</td>
<td>14,42,48,91,140,190</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>T6 N (48)</td>
<td>14,42,48,91,140,190</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>T7 N (48)</td>
<td>14,48,91,140,190</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>T18 N (48)</td>
<td>48,140,190</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>T22 P (190)</td>
<td>84,91,140,190</td>
<td>+</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>T39 N (48)</td>
<td>48,84,91,140,190</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>T41 M (29), N (48)</td>
<td>29,54,84,140,190</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>B1 H (116)</td>
<td>14,18,60,116</td>
<td>+</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>B2 M (23), N (60)</td>
<td>14,18,23,48,53,60,116</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>B3 P (160)</td>
<td>60,160</td>
<td>+</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>B4 P (160)</td>
<td>160</td>
<td>+</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>B5 H (116)</td>
<td>60,116</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>B6 H (116)</td>
<td>60,116</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>B7 M (23), N (60)</td>
<td>14,18,23,48,53,60</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>B8 M (23), N (60)</td>
<td>14,18,23,48,53,60</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>B9 P (102)</td>
<td>102,160</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>B10 P (102)</td>
<td>102,160</td>
<td>-</td>
<td>IgG</td>
<td></td>
</tr>
</tbody>
</table>

aMonoclonal antibodies were designated with a number preceded by a letter identifying the immunizing virus. T-TGEV, B-BCV.

bVirus neutralization activity as determined by plaque reduction.

cSize of polypeptides in kilodaltons.
Figure 12 shows the results of immunoprecipitation of radiolabeled TGEV intracellular proteins using both polyvalent and monoclonal antibodies. The polyvalent antibodies in hyperimmune ascitic fluid (lane 2) precipitated all the usual TGEV-specific proteins. MAbs T1 and T6 precipitated all of the TGEV intracellular proteins except M. They bound the 14 kd protein to a much greater extent than did the polyvalent ascitic fluid. MAbs T7, T18, and T39 were directed primarily against N but also reacted with both the 91 kd protein found in infected cells and the 84 kd protein found in virions. Although it is difficult to see in the figure, MAb T22 showed a much stronger reaction to the peplomer protein than did any of the other MAbs. This is the only TGEV-specific MAb in this library with virus neutralization activity. T41 reacted almost exclusively with N (54 kd) and M (29 kd). As discussed in the Introduction, M is closely associated with N in virions and is required for budding. This MAb may be directed toward the determinant formed at the point of interaction, but may be able to precipitate the separated proteins. It is interesting that the N protein precipitated by this MAb has the same molecular weight as the proposed precursor to the mature nucleocapsid protein.

Figure 13 shows the results of immunoprecipitation of TGEV virion proteins with polyvalent and monoclonal
Figure 12. Immunoprecipitation of TGEV-specific intracellular proteins using monoclonal antibodies. Mock- and TGEV-infected cells were labeled for 2 h at 6 hpi. Lane 1 shows mock-infected cell lysates immunoprecipitated with polyclonal ascitic fluid. The TGEV-infected cell lysates were immunoprecipitated with polyvalent hyperimmune ascitic fluid (lane 2) or monoclonal antibodies (lanes 3-9) and analyzed on a 10% gel. The numbers above lanes 3-9 indicate the number of the monoclonal antibody used for immunoprecipitation.
Figure 13. Immunoprecipitation of TGEV virion proteins by monoclonal antibodies. TGEV virions were labeled and purified as described in Material and Methods. Virion proteins were immunoprecipitated with polyvalent hyperimmune ascitic fluid (lane 1) or monoclonal antibodies (lanes 2-8) and analyzed on a 10% gel.
antibodies. Polyvalent ascitic fluid precipitated virion proteins of 190 kd, 84 kd, 54 kd, 48 kd, 42 kd, and 29 kd. Only MAb T22 precipitated the peplomer protein. MAb T22 was shown to have neutralizing activity against TGEV in a plaque reduction assay. This was the MAb which did not react at all with the nucleocapsid protein. MAbs T7, T18, and T39 precipitated only N. MAbs T1 and T6 reacted with N, but also precipitated the 84 kd protein. MAb T41 coprecipitated the 54 kd species of N and the matrix protein as it did when used for immunoprecipitation of intracellular proteins. The 14 kd, 91 kd, and the 140 kd proteins were not found in virions by MAbs.

The immunoprecipitation of BCV-specific intracellular proteins is shown in Figure 14. Polyvalent ascitic fluid (lane 2) precipitated nine BCV-specific proteins. The 14 kd and 18 kd proteins were very faint on the original autoradiogram and do not appear in lane 2 of the figure. MAbs B1, B3, and B4 neutralized the infectivity of BCV. MAb B1 was directed primarily against the hemagglutinin (116 kd), while MAbs B3 and B4 reacted only with the peplomer (160 kd). All of the MAbs which reacted with H (B1, B5, and B6) also reacted with N. This reaction was not reciprocal, however (MAbs B7 and B8). All of the MAbs which precipitated M (23 kd) (B2, B7, and B8) also precipitated N (60 kd). Again, the reaction was not reciprocal (MAbs B5 and B6). The two MAbs which
Figure 14. Immunoprecipitation of BCV-specific intracellular proteins using monoclonal antibodies. Mock- and BCV-infected HRT 18 cells were labeled for 2 h at 8 hpi. Lane 1 shows mock-infected cell lysates immunoprecipitated with polyvalent ascitic fluid. The BCV-infected cell lysates were immunoprecipitated with polyvalent hyperimmune ascitic fluid (lane 2) or monoclonal antibodies (lanes 3-12) and analyzed on a 10% gel.
recognized the 102 kd intracellular protein also precipitated P (160 kd).

The immunoprecipitation of BCV virion proteins is shown in Figure 15. The pattern of protein bands is quite similar to that found in Figure 14. When BCV virion proteins were precipitated with polyvalent ascitic fluid (lane 2), the 116 kd hemagglutinin protein band was much more prominent than in lane 1 which shows disrupted, non-immunoprecipitated virion proteins. The samples that were loaded onto lanes 1 and 2 contained equal amounts of acid-insoluble radioactivity and do not represent equal volumes of purified virions. Therefore, the distribution of radioactivity would not necessarily be equal in the two lanes. The presence of a high proportion of antibodies in the polyvalent ascitic fluid which were specific for the hemagglutinin protein would account for the greater intensity of this band in lane 2.

The 14 kd intracellular protein which was precipitated from infected cells lysates by MAbs B1, B2, B7, and B8 was not detected in any of the lanes of Figure 15 indicating that the 14 kd protein is not a structural protein.

MAbs B3 and B4 were found to neutralize virus. They both precipitated the 160 kd peplomer protein from infected cell lysates. When virion proteins were subjected to immunoprecipitation with these two MAbs, the peplomer
Figure 15. Immunoprecipitation of BCV virion proteins by monoclonal antibodies. BCV virions were labeled and purified as described in Material and Methods. Lane 1 shows non-immunoprecipitated virion proteins. Virion proteins were immunoprecipitated with polyvalent hyperimmune ascitic fluid (lane 2) or monoclonal antibodies (lanes 3-12) and analyzed on a 10% gel.
protein did not appear. This is surprising since the test to determine the neutralizing activity of a MAb is done by adding the MAb to stock virus prior to infection. The MAb therefore must recognize the peplomer protein. The immunoprecipitation procedure is carried out under mildly reducing conditions. Under these conditions, disulfide bonds would be broken. If the 160 kd protein is actually a dimer of the 102 kd protein, some lack of secondary structure could be responsible for the inability of MAbs B3 and B4 to precipitate P from a preparation of purified virions.

**Limited Digest Peptide Mapping**

Comparison of virion and intracellular protein and pulse-chase experiments suggested precursor-product relationships, and immunoprecipitation of viral proteins with monoclonal antibodies indicated antigenic relationships. Analysis of the structural relationships among viral proteins by limited digest peptide mapping was used to support or refute the results obtained from other experiments.

Maps of peptides of virus-specific proteins generated by digestion with TPCK-trypsin, TLCK-chymotrypsin, or Staphylococcus aureus V8 protease were compared. Trypsin cleaves proteins on the carboxyl side of lysine and arginine residues; chymotrypsin cleaves proteins on the
carboxyl side of aromatic amino acids; *S. aureus* V8 protease cleaves proteins on the carboxyl side of aspartate and glutamate residues. Proteins with identical amino acid sequences will yield identical peptides after enzyme digestion. By utilizing all three enzymes, it is possible to detect minor as well as major differences in the primary structures of related proteins.

Limited digest peptide mapping of TGEV virion and intracellular proteins is shown in Figures 16-18. The 190 kd and 84 kd proteins appear to have identical maps, as do the 140 kd and 91 kd proteins. This indicates that there is extensive sequence homology between the 190 kd and 84 kd proteins and also between the 140 kd and 91 kd proteins. This is consistent with the theory that the smaller polypeptide of each pair is a cleavage product of the larger protein. The maps of all four of these proteins are quite similar. It may be that all four proteins are the product of a single viral gene.

The nucleocapsid set of proteins (54 kd, 48 kd, and 42 kd) generated nearly identical peptide maps. While this does not clarify the precursor-product relationship among these proteins, it does indicate that they are all products of the same viral gene.

The matrix protein (29 kd) and the 14 kd non-structural protein generated a unique set of peptides
Figure 16. Limited digest peptide mapping of TGEV virion (top) and intracellular (bottom) proteins by *Staphylococcus aureus* V8 protease. Limited digest peptide mapping was performed as described in Materials and Methods. The number above each lane indicates the size in kilodaltons of the TGEV-specific protein which was digested in that lane.
Figure 17. Limited digest peptide mapping of TGEV virion (top) and intracellular (bottom) proteins by TPCK-trypsin. Limited digest peptide mapping was performed as described in Materials and Methods. The number above each lane indicates the size in kilodaltons of the TGEV-specific protein which was digested in that lane.
Figure 18. Limited digest peptide mapping of TGEV virion (top) and intracellular (bottom) proteins by TLCK-chymotrypsin. Limited digest peptide mapping was performed as described in Materials and Methods. The number above each lane indicates the size in kilodaltons of the TGEV-specific protein which was digested in that lane.
suggesting that they are not structurally related to each other or to any other TGEV-specific proteins.

Limited digest peptide mapping of BCV virion and intracellular proteins is shown in Figures 19-21. The 160 kd and 102 kd proteins have identical maps, indicating that there is extensive sequence homology between these two proteins. This is consistent with the theory that the 102 kd protein is dimerized to form the 160 kd peplomer protein. Digestion of the second peplomer protein, the hemagglutinin, produced a unique set of peptides, indicating that this protein is not structurally related to any other BCV-specific proteins.

The maps of the nucleocapsid set of proteins (60 kd, 53 kd, and 48 kd) showed a great deal of similarity, although some differences were seen. The 46 kd protein found in virions exhibited less homology with the mature 60 kd nucleocapsid protein. These proteins all appear to have at least some sequence homology which provides evidence that they are all products of the same viral gene.

Digestion of both the 33 kd virion protein and the 14 kd intracellular protein resulted in unique peptide patterns. It is unlikely, therefore, that these two proteins are related to one another or to any other BCV-specific protein.
Figure 19. Limited digest peptide mapping of BCV virion (top) and intracellular (bottom) proteins by Staphylococcus aureus V8 protease. Limited digest peptide mapping was performed as described in Materials and Methods. The number above each lane indicates the size in kilodaltons of the BCV-specific protein which was digested in that lane.
Figure 20. Limited digest peptide mapping of BCV virion (top) and intracellular (bottom) proteins by TPCK-trypsin. Limited digest peptide mapping was performed as described in Materials and Methods. The number above each lane indicates the size in kilodaltons of the BCV-specific protein which was digested in that lane.
Figure 21. Limited digest peptide mapping of BCV virion (top) and intracellular (bottom) proteins by TLCK-chymotrypsin. Limited digest peptide mapping was performed as described in Materials and Methods. The number above each lane indicates the size in kilodaltons of the BCV-specific protein which was digested in that lane.
The peptides generated by digestion of the 18 kd and 23 kd intracellular proteins and the 23-25 kd virion protein appear to have some similarity. The 18 kd protein was always coprecipitated with the 23 kd protein by monoclonal antibodies and may represent either a non-glycosylated form of the matrix protein, or a degradation product.
DISCUSSION

Studies of bovine enteric coronavirus (BCV) (24,26,30,33,55,65,66) and transmissible gastroenteritis virus (TGEV) (19,20,22,28) have focused on the structural proteins. None of these studies have proposed a model for the synthesis and posttranslational processing of BCV- and TGEV-specific proteins. There is disagreement in the literature as to the number and size of virus-specific proteins. In addition, the structural and antigenic relationships among these proteins have not been examined. The data presented in this thesis provide evidence for the development of models of protein processing for these two viruses. A reproducible spectrum of virus-specific proteins was obtained from infected cells and molecular weights were determined. The relatedness of intracellular and virion proteins was examined and the information obtained was integrated into the models.

Synthesis of Virus-Specific Proteins

The synthesis of TGEV- and BCV-specific proteins was found to be a coordinated process. The nucleocapsid (N) protein was always detected earliest and remained the predominant protein throughout infection (Figures 3 and
Virus-specific protein synthesis in TGEV-infected cells reached a peak at 8 hpi and dropped off sharply by 12 hpi. This is consistent with the time course of multiplication of infectious virus. Peak production is found between 12 and 18 hpi (Figure 1) followed by cell lysis and therefore a decrease in viral protein synthesis.

Nine TGEV-specific proteins were found in infected cells. The sizes of these proteins in kilodaltons (kd) were: 190, 140, 91, 84, 54, 48, 42, 29, and 14 (Table 1, Figure 5). The 14 kd, 91 kd, and 140 kd proteins are non-structural proteins. They were not found in virions, but were immunoprecipitated from infected cell lysates by hyperimmune ascitic fluid. Non-structural TGEV-specific proteins have not been reported by others. An 84 kd protein was found in virions, and rarely in infected cells. The 190 kd and 84 kd proteins correspond to the peplomer (P) proteins. The size of the peplomer protein has been reported as 195-200 kd by others (19,20,28) and Garwes et al. (19) demonstrated an 85 kd protein in purified virions. N is 48 kd and the matrix (M) protein is 29 kd. This is in agreement with the work of others (19,20). The 42 kd and 54 kd proteins are related to the nucleocapsid. A 42 kd protein was reported by Garwes, et al. (20), but the 54 kd protein has not been previously described.
BCV-specific protein synthesis in infected cells was significantly more protracted. The peak of synthesis occurred at 12 hpi, but the rate of protein synthesis remained high throughout infection (Figure 4). Synthesis of the 23 kd protein diminished as the infection progressed, while the 116 kd protein was not produced in appreciable amounts until relatively late in infection. The rate of infectious BCV multiplication rose early and remained high until quite late in infection (Figure 1). Cell lysis was not apparent until 40 to 48 hpi, thus the HRT 18 cells were capable of supporting viral protein synthesis for a much longer period than were TGEV-infected ST cells.

Lysates of BCV-infected cells contained nine virus-specific proteins. Structural proteins of 160 kd, 116 kd, 102 kd, 60 kd, 53 kd, 48 kd, 33 kd, and 23 kd were detected as well as non-structural proteins of 18 kd and 14 kd (Table 1, Figure 5). The 23 kd protein corresponds to M. The 53 kd and 48 kd proteins are related to the 60 kd nucleocapsid protein. The peplomer proteins are 160 kd and 102 kd and the hemagglutinin protein is 116 kd.

Researchers in Europe (33,65,66) work with European BCV isolates which appear to differ significantly from the BCV isolates used in the United States. Therefore, their work will not be compared with the results of my studies. For this work, I used the BCV strain isolated by Mebus et
al. in 1973 (40). This isolate was also used by Storz et al. (55) and the workers in the laboratory of Brian (26,30). Both labs reported a peplomer protein of 180-190 kd which was normally present as sub-units of 90-120 kd. King and Brian (30) described a 140 kd hemagglutinin that behaves as a disulfide-linked dimer of 65 kd sub-units. Storz et al. (55) reported only the 65 kd glycoprotein. The sizes of the nucleocapsid and matrix proteins agree with the literature except that N was always seen as a single band of 50-55 kd by others. The 33 kd protein has not been described by others and may represent the degradation product of another structural protein. Non-structural BCV-specific proteins have not been described previously.

By analogy to other coronaviruses (19,20,24,28,30) and a reduced molecular size in the presence of tunicamycin, the 29 kd, 84 kd, and 190 kd proteins of TGEV are glycosylated. On the same basis BCV glycoproteins are 23 kd, 102 kd, 116 kd, and 160 kd.

**Antigenic Relationships**

Monoclonal antibodies were employed in immunoprecipitations to determine the antigenic relationships among virus-specific proteins. Several of the MAbs precipitated more than one viral polypeptide (Figures 12-15). One reason for this could be the failure of the cloning
procedure to separate hybridomas with different specificities. However, hybridomas were cloned twice by limiting dilution, and only single colonies were selected for testing and subcloning. Some TGEV hybridomas were cloned twice more and retained the same specificity (Susan Goss, personal communication). In addition, Ouchterlony gel diffusion resulted in a single precipitin band corresponding to a single immunoglobulin type (Figure 11). Therefore the presence of mixed clones is unlikely.

A second explanation is that breakdown or aggregation of viral polypeptides could form antigens which migrate anomalously in SDS-PAGE gels but maintain the same specificity. Multiple bands which actually represent one protein can sometimes be produced by different preparation conditions of samples during immunoprecipitation and gel electrophoresis. This is especially true of coronavirus glycoproteins (57). However, variations in the reducing and denaturing conditions of immunoprecipitation and gel electrophoresis produced no changes in the profiles obtained (Susan Goss, personal communication).

Non-specific binding of antibody or Staphylococcus aureus to viral proteins could account for multiple bands. These reactions, however, are unpredictable and irreproducible. The profiles obtained by immunoprecipitation of virus-specific proteins were reproducible in independent experiments. Furthermore,
control experiments were performed in our laboratory by Dr. Susan Goss to detect the presence of artifacts. These included the immunoprecipitation of virus-infected cells lysates with normal mouse ascites or *S. aureus* in the absence of antibody. Viral proteins occasionally bound non-specifically to *S. aureus*, but these results were not reproducible.

The most likely explanation for recognition of multiple proteins by monoclonal antibodies appears to be shared epitopes on the polypeptides because of primary sequence homologies. Most of the anti-BCV MAbs and one of the anti-TGEV MAbs which reacted with the nucleocapsid protein also recognized the matrix protein. Rather than shared amino acid sequences, these two proteins may form a unique antigen at the point of their interaction in virions. When the proteins were separated during the immunoprecipitation procedure, enough of this epitope must be available on each protein to be recognized by the MAb directed against the interacting proteins.

None of the anti-TGEV MAbs precipitated all of the virus-specific proteins. There did not seem to be any clear subsets of antigens. All of the MAbs showed at least some reactivity with P, although only MAb T22 precipitated P from virions; MAbs that precipitated N did not necessarily react with M; and the non-structural proteins were precipitated by MAbs directed against both P
and N. One consistent response was that of the M protein. It coprecipitated only with the 54 kd form of the nucleocapsid protein. The only other pattern that was apparent concerned the 84 kd and 91 kd proteins. The 84 kd protein coprecipitated with the 190 kd protein, although this did not occur in the immunoprecipitation of virion proteins. The 91 kd protein was always precipitated with the 140 kd protein; this reaction was not reciprocal.

The anti-BCV MAbs did seem to follow a pattern. The matrix protein always coprecipitated with the nucleocapsid protein, as well as the 14 kd and 18 kd non-structural proteins. The hemagglutinin protein was always found in conjunction with N, although this did not always occur when virion proteins were precipitated. Only MAb B2 reacted with all three proteins. MAbs which recognized P (B3, B4, B9, and B10) did not precipitate any other proteins besides the 102 kd protein. Therefore it appears that the peplomer proteins possess some unique antigenic determinants, while all of the other BCV-specific proteins share some antigenic similarity.

Structural Relationships

The structural relationships among virus-specific proteins of BCV and TGEV were analyzed by limited digest peptide mapping. The patterns of peptides produced by
digestion of isolated proteins were compared for similarity.

Digestion of BCV-specific proteins yielded several sets of peptide patterns. The 14 kd non-structural and 33 kd and 116 kd structural proteins all displayed unique peptide maps indicating that they did not share much if any sequence homology with one another or with other BCV-specific proteins. The 18 kd intracellular protein peptide bands corresponded partially to those of the intracellular and virion matrix proteins. Relatively minor changes in the primary sequence of a protein can produce a significantly altered peptide map, so these three proteins may in fact be fairly closely related. The 46 kd, 48 kd, 53 kd and 60 kd proteins which comprise the nucleocapsid set of proteins all had very closely related maps. Only minor differences were seen among these maps indicating that all these proteins are products of the same viral gene. The 102 kd and 160 kd proteins displayed identical peptide maps which is evidence that the 160 kd protein is indeed the product of dimerization of the 102 kd protein.

Digestion of TGEV-specific proteins yielded some results that were very different than from those obtained by digestion of BCV-specific proteins. Like BCV, the 14 kd non-structural protein generated a unique set of peptides indicating a lack of structural similarity with
other virus-specific proteins. The maps of M were also unique.

The nucleocapsid set of proteins (54 kd, 48 kd, and 42 kd) appeared to have identical maps indicating a very close relationship among these three proteins.

The 190 kd and 84 kd peplomer proteins appear to have identical maps, as do the 140 kd and 91 kd intracellular proteins. This indicates that there is extensive sequence homology between the 190 kd and 84 kd proteins and also between the 140 kd and 92 kd proteins. This supports the theory that the smaller polypeptide of each pair is a cleavage product of the larger protein. The maps of all four of these proteins are quite similar. This would indicate that all four proteins are the products of a single viral gene.

A large non-structural protein in the range of 140 kd has not been described for any coronavirus. The sizes of the 140 kd and 91 kd proteins remained constant in the presence of tunicamycin. This indicates that they do not possess N-linked carbohydrates as do the 190 kd and 84 kd proteins. Two possibilities exist for the presence of these two proteins in infected cells. The 140 kd protein may be the result of premature termination of translation of the mRNA which encodes the 190 kd protein. It may lack sites for glycosylation and it may lack the signal sequence required for insertion into the membrane. This
would account for the observation that neither the 140 kd nor the 91 kd protein are packaged into virions.

The second possibility is less likely. The 140 kd protein may be a defective analogue of the BCV hemagglutinin. The hemagglutinin protein of BCV, however, generated a unique set of peptides rather than peptides which were closely related to those of the peplomer protein. This observation demonstrates that the 140 kd protein and the H protein are not related.

Cotranslational Processing

Almost all glycosylation of viral proteins has been found to be cotranslational. This dolichol-mediated event is sensitive to inhibition by the antibiotic, tunicamycin. Carbohydrates attached to a protein through a dolichol intermediate are linked to proteins at asparagine residues and are termed N-linked sugars. N-linked glycoproteins synthesized in the presence of tunicamycin will either lack a carbohydrate moiety or will fail to be synthesized entirely.

The peplomer glycoproteins of both TGEV and BCV were undetectable in infected cells radiolabeled with $^{35}$S-methionine in the presence of tunicamycin (Figures 8 and 9). Synthesis of these proteins is either inhibited by tunicamycin, or peplomer proteins which lack carbohydrate are not recognized by antibodies in the hyperimmune
ascitic fluid used for immunoprecipitation. Garwes et al. (20,21) reported that P was not synthesized in the presence of tunicamycin while Hu et al. (28) demonstrated a 145 kd protein when glycosylation was inhibited by tunicamycin.

The 102 kd protein of BCV appears to be a peplomer protein also. The size of the 102 kd protein changes only slightly upon the addition of tunicamycin. This indicates that the protein is not heavily glycosylated and some determinants that are recognizable by polyvalent ascitic fluid remain intact. If this protein is dimerized to form the 160 kd protein, these remaining determinants may become unavailable to antibodies. The lack of carbohydrate may also prevent dimerization of the 102 kd protein. This would explain the absence of any detectable 160 kd protein in cells infected with BCV in the presence of tunicamycin.

The 84 kd structural protein of TGEV appeared to be reduced in size upon the addition of tunicamycin. This protein has a peptide map which is identical to that of the 190 kd peplomer protein. If the 84 kd protein is the cleavage product of the 190 kd protein, determinants for recognition by antibodies in ascitic fluid may become available after cleavage that are absent on the non-glycosylated precursor to the 190 kd protein.
Glycosylation of the TGEV M protein was inhibited by tunicamycin. The non-glycosylated M protein was detectable after immunoprecipitation. About 5 kd of carbohydrate appeared to be added cotranslationally to M.

It was very difficult to label the BCV hemagglutinin protein sufficiently to allow analysis of this protein. The work of others (24,30) indicates that H is a glycoprotein and that the carbohydrate moieties are added cotranslationally.

Posttranslational Modifications

Modifications of virus-specific proteins following synthesis are well-known. Proteins may be cleaved, dimerized, phosphorylated, acylated, or glycosylated. Very few viral proteins have glycosylation initiated posttranslationally, but there are often posttranslational modifications of cotranslationally-added carbohydrate side chains (31). Cleavage or addition of carbohydrates to the side chain may take place following synthesis of the nascent glycoprotein.

The nucleocapsid proteins of both BCV and TGEV are phosphorylated (Figure 6). This is in agreement with the work of others (20,26,30,55). The TGEV phosphoprotein is 48 kd. A non-phosphorylated protein of 54 kd was found as a major band in pulse-labeled cells and was seen faintly in continuously labeled cells and virions (Figures 5 and
A 42 kd protein which also lacked phosphate and had peptide maps which were nearly identical to N was found in infected cells. It was not found in pulse-labeled cells, nor was it found in the subsequent chase. It was only found in cells labeled continuously for 2 h. It would appear that the 54 kd protein is the primary translation product which is then cleaved and phosphorylated to produce the 48 kd N protein. The 42 kd protein appears to be a degradation product of N. This degradative cleavage must include the site of phosphorylation. A 42 kd protein was described by Garwes et al. (20), but was found to be phosphorylated. These workers used a pig kidney cell line for propagating TGEV. The differences in degradation may reflect differences in cellular enzymes.

The nucleocapsid protein of BCV is 60 kd. Limited digest peptide mapping experiments revealed that the two additional BCV-specific proteins of 48 kd and 53 kd had maps that were nearly identical to those of the 60 kd N protein. Neither of these proteins was labeled with \( ^{32} \)P-orthophosphate. This suggests that the 53 kd protein is not a partially phosphorylated intermediate. One possibility is that the 53 kd protein is the primary translation product which is then cleaved to produce the 48 kd protein. This protein may then be phosphorylated to produce the mature 60 kd protein. All three proteins were seen after only 10 min of labeling with \( ^{35} \)S-methionine.
(Figure 10) implying that any modifications must take place very rapidly. Alternatively, the 48 kd and 53 kd proteins may be prematurely terminated translation products of the same gene which encodes the 60 kd protein. If this is true, the site for phosphorylation must either be very near the carboxyl terminus of the protein, or the lack of this end of the protein prevents the creation of secondary structure required for phosphorylation. A 42 kd protein which is structurally related to the 60 kd protein was found in virions. It appeared to have less similarity with the mature nucleocapsid protein than did the 53 kd and 48 kd intracellular proteins. This may be a degradation product which is packaged into virions.

The matrix protein of TGEV does not appear to be posttranslationally modified. The addition of carbohydrate to this protein was inhibited by tunicamycin (Figure 8) and the mature glycoprotein was detected after only a 5 min labeling period (Figure 10). Therefore all of the carbohydrate must be added cotranslationally with no subsequent modification of the side chains. This agrees with the findings of others (19,20).

Glycosylation of the BCV M protein was not inhibited by tunicamycin (Figure 9). This suggests that a mechanism other than the dolichol-mediated linkage of carbohydrates to asparagine residues is utilized to glycosylate this
protein. It has been shown that the glycosylation of the M protein of mouse hepatitis virus (MHV) is resistant to inhibition by tunicamycin (27,41). The carbohydrates of this protein are added posttranslationally through O-glycosidic linkages to serine or threonine residues. Tunicamycin studies of BCV proteins have not been reported. O-linked carbohydrates do not contain mannose or fucose (31). Storz et al. (55) reported that all BCV glycoproteins except M could be metabolically labeled with mannose and fucose indicating that M possesses O-linked carbohydrates.

The non-glycosylated precursor to BCV M protein was not detected in any experiment. Although the carbohydrate moieties are added posttranslationally, it may be a fairly rapid event. A minimum of 10 min was required to sufficiently label BCV-specific proteins. Only the mature M glycoprotein was seen in pulse-labeled cells (Figure 10). The precursor could be glycosylated so rapidly that no precursor is seen. Alternatively, the precursor may not possess antigenic determinants which are recognized by the hyperimmune ascitic fluid used for immunoprecipitation. Finally, the non-glycosylated form of the BCV matrix protein may be the 18 kd intracellular protein which has some structural similarity with the matrix protein.
The initial glycosylation of the TGEV peplomer protein was inhibited by tunicamycin (Figure 8). This modification is therefore a cotranslational event. Pulse-chase studies, however, indicate that further processing of P takes place. After 5 min of labeling, a 173 kd precursor protein was seen (Figure 10). After a 90 min chase period, this protein was no longer seen, but the mature 190 kd protein appeared. It therefore appears that the glycoprotein which is produced initially is 173 kd. Posttranslational glycosylation then increases the size of P to 190 kd. Hu et al. (28) reported a 145 kd protein which was found in the presence of tunicamycin or when most of the sugar moiety of a 195 kd glycoprotein was cleaved off by endoglycosidase H. In vitro translation studies by Garwes et al. (20) demonstrated a 160 kd protein. This group has reported the mature peplomer protein to be 200 kd. No pulse-chase studies have been reported for TGEV-specific proteins. However, the findings of these two groups are consistent with the theory of cotranslational glycosylation of P with further posttranslational modifications of the carbohydrate side-chains.

The 84 kd structural protein was not consistently found in infected cells. It was, however, always found in virions. This protein generated peptide maps which were identical to those from the 190 kd peplomer protein. This
suggests that the 190 kd protein is cleaved to produce the 84 kd protein, but that this cleavage takes place primarily in the assembled virion rather than in the cytoplasm of the infected cell.

In pulse-labeled, BCV-infected cells, the P protein was not seen (Figure 10). The 102 kd protein which has peptide maps that are identical to P was seen after a 10 min pulse. This protein was not detected during the subsequent chase period whereas the 160 kd peplomer was found. The 102 kd protein is found in continuously labeled cells (Figure 5), and in disrupted virions. The 160 kd form of the peplomer protein was not found in SDS-PAGE gels of disrupted virions. Virions are disrupted with SDS and dithiothreitol before gel electrophoresis. This may be sufficient to break disulfide bonds between the subunits of a dimerized protein. These data are consistent with a model wherein the 102 kd protein is synthesized continuously but is subsequently dimerized before incorporation into the virion. The 160 kd peplomer protein is not seen in SDS-PAGE gels of virion proteins because the disulfide bonds linking the 102 kd subunits are disrupted by the sample preparation conditions used. No studies of BCV intracellular proteins have been reported but P is often found in preparations of purified virions in the form of sub-units.
There also appears to be processing of some of the non-structural virus-specific proteins. Pulse-chase studies indicate that the 91 kd protein of TGEV is the cleavage product of a larger protein which is usually found in infected cells with a molecular weight of approximately 140-145K. This conclusion is supported by the similarity of the peptide maps generated by the digestion of these two proteins.

The 14 kd intracellular protein of TGEV is not detected in the presence of tunicamycin suggesting that it is either a glycoprotein with N-linked carbohydrates or that the synthesis of the protein is inhibited by contaminating enzymes in the tunicamycin. The 14 kd intracellular protein of BCV is found in the absence or presence of tunicamycin. If, by analogy with TGEV, it is also a glycoprotein, it must be glycosylated posttranslationally in the same manner as the BCV M protein.

Protein Processing Models

Protein processing models for BCV- and TGEV-specific proteins were prepared using the data accumulated in this study. These models are shown in Figures 22 (TGEV) and 23 (BCV). While not all of the steps have been unambiguously demonstrated, the results described above are consistent with the models proposed in Figures 22 and 23.
Figure 22. Protein processing model for TGEV. Proteins are indicated with their approximate sizes in kilodaltons.
Figure 23. Protein processing model for BCV. Proteins are indicated with their approximate sizes in kilodaltons.
Conclusions

The patterns of protein synthesis and processing of two economically important coronaviruses were studied. Limited digest peptide mapping and immunoprecipitation with monoclonal antibodies were employed to examine structural and antigenic relationships among virus-specific proteins. Determination of structural and non-structural proteins was accomplished by comparison on SDS-polyacrylamide gels of purified virions and immunoprecipitated proteins from virus-infected cell lysates. These data were incorporated into models describing the synthesis and processing of BCV- and TGEV-specific proteins.
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