Association of the Infectious Bronchitis Virus 3c Protein with the Virion Envelope

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A highly purified radiolabeled preparation of the coronavirus infectious bronchitis virus (IBV) was analyzed, by immunoprecipitation with monospecific antisera, for the presence of a series of small virus proteins recently identified as the products of IBV mRNAs 3 and 5. One of these, 3c, a 12.4K protein encoded by the third open reading frame of the tricistronic mRNA3 was clearly detectable and was found to cofractionate with virion envelope proteins on detergent disruption of virus particles. These results, together with the hydrophobic nature of 3c and its previously demonstrated association with the membranes of infected cells, suggest strongly that 3c represents a new virion envelope protein, which may have counterparts in other coronaviruses. © 1991 Academic Press, Inc.

Infectious bronchitis virus (IBV) is the prototype virus of the Coronaviridae, a family of viruses containing a large single-stranded RNA genome of positive sense. The morphology of the virion is characterised by a "corona" of widely spaced bulbous projections which consist of the virus spike glycoprotein (S) (21). This S protein is composed of two glycopolypeptides, gp90 and gp84, which are produced by post-translational cleavage of a precursor, gp155 (7, 8, 25). In addition to the spike protein, only two other major virion structural proteins are currently recognized. These are the nucleocapsid protein (N), which is a 51K phosphoprotein found in close association with the genomic RNA (26), and the membrane protein (M), an integral envelope protein which appears as a heterogeneous collection of molecules differing in their extent of glycosylation (p23, gp28, gp31, and gp36) (6, 24).

Boursnell and colleagues (4), have deduced the complete sequence of IBV genomic RNA through cloning of cDNA. Their results indicated that the IBV genome is 27.6 kb in length with at least ten open reading frames (ORFs), of which only three were known certainly to encode virus proteins (the S, N, and M genes). The seven unassigned ORFs were clustered in three groups on the genome—two very large ORFs at the 5′ end of the RNA (1a and 1b), three small ones between the S and N genes (3a, 3b, and 3c), and two small ones between the M and the N genes (5a and 5b). Recently through expression of nucleotide sequences from these unassigned ORFs as bacterial fusion proteins, and production of monospecific antisera against the bacterial products, we have been able to establish posi-

tively the coding function of several of these ORFs. It now appears that the 3a, 3b, and 3c ORFs encode polypeptides in virus-infected cells (17, 23) and that the 5a and 5b ORFs also represent functional genes (18). It was therefore of interest to examine whether any of these newly identified proteins are associated with the virus particle. Although their function is as yet unknown, certain of the polypeptides, particularly the 12.4K 3c and 7.5K 5a polypeptides, possess hydrophobic sequences characteristic of integral membrane proteins (2, 3) and we have shown previously that 3c can be found in association with IBV-infected cell membranes (23). We were therefore particularly interested in the possibility that these proteins might represent previously unidentified virion envelope proteins. We report here that the 3c protein is indeed virion-associated and that it cofractionates with other virion envelope proteins on detergent disruption of virus particles.

Confluent monolayers of primary chicken kidney cells were prepared from 2- to 3-week-old birds as previously described (23). The cells were infected with infectious bronchitis virus strain Beaudette (a kind gift from Dr. D. Cavanagh, AFRC Institute for Animal Health, Houghton Laboratory, UK) at a multiplicity of infection (m.o.i.) of approximately 2 plaque-forming unit (PFU)/cell. After infection, the cells were incubated in serum-free Glasgow modified MEM at 37° for 4 hr and then labeled with [35S]methionine by replacing the medium with methionine-free MEM supplemented with 30 μci/ml [35S]methionine and incubating at 37° for 15 hr. The virus was then harvested and purified by successive cycles of differential centrifugation essentially as described by Stern et al. (24), as follows: Forty milliliters of tissue culture supernatant from 20 dishes (100

mm diameter) of IBV-infected cells was layered onto two tubes each containing 10 ml of 20% (w/v) TNE-buffered sucrose solution (TNE buffer: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA) and centrifuged at 75,000 g for 3 hr at 4°. The resulting virus pellets from the two tubes were resuspended in 10 ml of TNE buffer, layered onto a 20-ml 20–55% linear sucrose gradient prepared with TNE buffer, and centrifuged at 75,000 g for 18 hr at 4°. The gradient was fractionated (22 samples) and the radioactivity present in each fraction determined directly by liquid scintillation. Three adjacent fractions which contained most (70%) of the radioactivity were pooled, diluted three times in TNE buffer, and applied to a second density gradient consisting of 20 ml of 10–50% Renografin (meglumine diatrizoate, MW 809.1, Sigma) prepared with TNE buffer. This gradient was centrifuged and fractionated as before. Aliquots of 13 fractions starting from the bottom of the gradient were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 1a). Prominent radiolabeled bands corresponding to the known structural proteins of IBV (6, 24) were observed in fractions 7–10 (lanes 10–14). The sedimentation rate of these proteins corresponded with that reported originally for IBV virions by Stern et al. (24), whose basic protocol was used for the purification carried out here. We also observed that a polypeptide of approximately 12K, with an electrophoretic mobility similar to that of in vitro-translated 3c (23), cofractionated exactly on the gradient with the virus major structural proteins, suggesting that it was associated with virus particles. Polypeptides corresponding to in vitro-translated 5a, 5b, 3a, and 3b (17, 18), however, were not detectable. A radiolabeled protein with an apparent molecular weight of 44K, appearing in fractions 1–6 (lanes 4–9), probably represents cellular actin as previously reported (19).

To confirm further the association of the putative 12K polypeptide with virions, fractions 7, 8, and 9 (lanes 10, 11, and 12), which contained most of the radioactive virions, were diluted threefold with TNE buffer, pooled, and fractionated on a second 20–55% linear sucrose gradient under the same conditions as before. Twenty-two fractions were collected, of which 16 (starting from the bottom of the gradient) were analyzed by SDS–PAGE (Fig. 1b). Once again the 12K polypeptide was clearly present and cofractionated exactly with the major virion structural proteins, appearing mostly in fractions 5–9 (lanes 6–10). There was considerably less evidence of contaminating cellular proteins in this gradient, and the relative proportion of the 12K protein to the other virion proteins appeared similar to that observed at the previous stage in purification, strongly supporting the idea of a specific association between the 12K protein and virus particles. A single

band migrating at the position expected of one of the cleaved portions of the spike protein, gp90, was observed in the upper part of the gradient, which may represent the degraded product of the spikes; morphological examination of unlabeled equivalent preparations at this stage by electron microscopy after negative staining showed that a quarter of the virions had lost their spike structures (data not shown).

In order to establish the identity of the 12K polypeptide as the product of the 3c gene, and to examine the possibility that IBV virions might contain other new virus polypeptides in amounts too small to detect directly, we next carried out immunoprecipitation experiments using monospecific antisera directed against the predicted products of the 3a, 3b, 3c, 5a, and 5b ORFs (17, 18, 23). For this purpose, fractions 5, 6, and 7 from the second sucrose gradient (lanes 6, 7, and 8, Fig. 1b) were pooled, diluted three times in TNE buffer, and centrifuged at 65,000 g for 3 hr at 4° to pellet the virus. The virus pellet was then resuspended in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 10% sodium deoxycholate) and aliquots were immunoprecipitated with a series of specific and control antisera as described (5). The result of this experiment (Fig. 2a) showed clearly that the 12K polypeptide was immunoprecipitated by anti-3c antiserum, but not by any of the other antisera. Further evidence for the specificity of the association between 3c and purified virions comes from analysis of the relative amount of the 12K protein in virus-infected cells before virion assembly (Fig. 2b); in this case the 3c protein is completely undetectable except by immunoprecipitation. Thus the likelihood of the 3c protein appearing in virions by chance from contaminating cellular material which has survived three cycles of gradient purification is remote. These experiments indicate therefore that 3c is indeed a virion-associated polypeptide. No evidence was obtained, however, for the presence of the other small virus polypeptides.

In order to assess the approximate amount of the 3c polypeptide present in the purified virion preparation, the autoradiograph shown in Fig. 2a, lane "total V", was analyzed by scanning densitometry (Fig. 2c), and the relative peak areas for the virion proteins NP, M, S, and 3c were measured. Using these data, and taking account of the methionine content of these proteins (3c has only one aside from its initiator, and the S, NP, and M proteins have 17, 4, and 4, respectively), we calculated the relative molar proportion of the four proteins to be approximately 1.0:11.0:10.1:1.7 (S:NP:M:3c). These relative values for the major structural proteins are broadly similar to those reported previously for IBV (6, 7) except that the proportion of envelope proteins to NP is rather lower. This calculation also assumes that
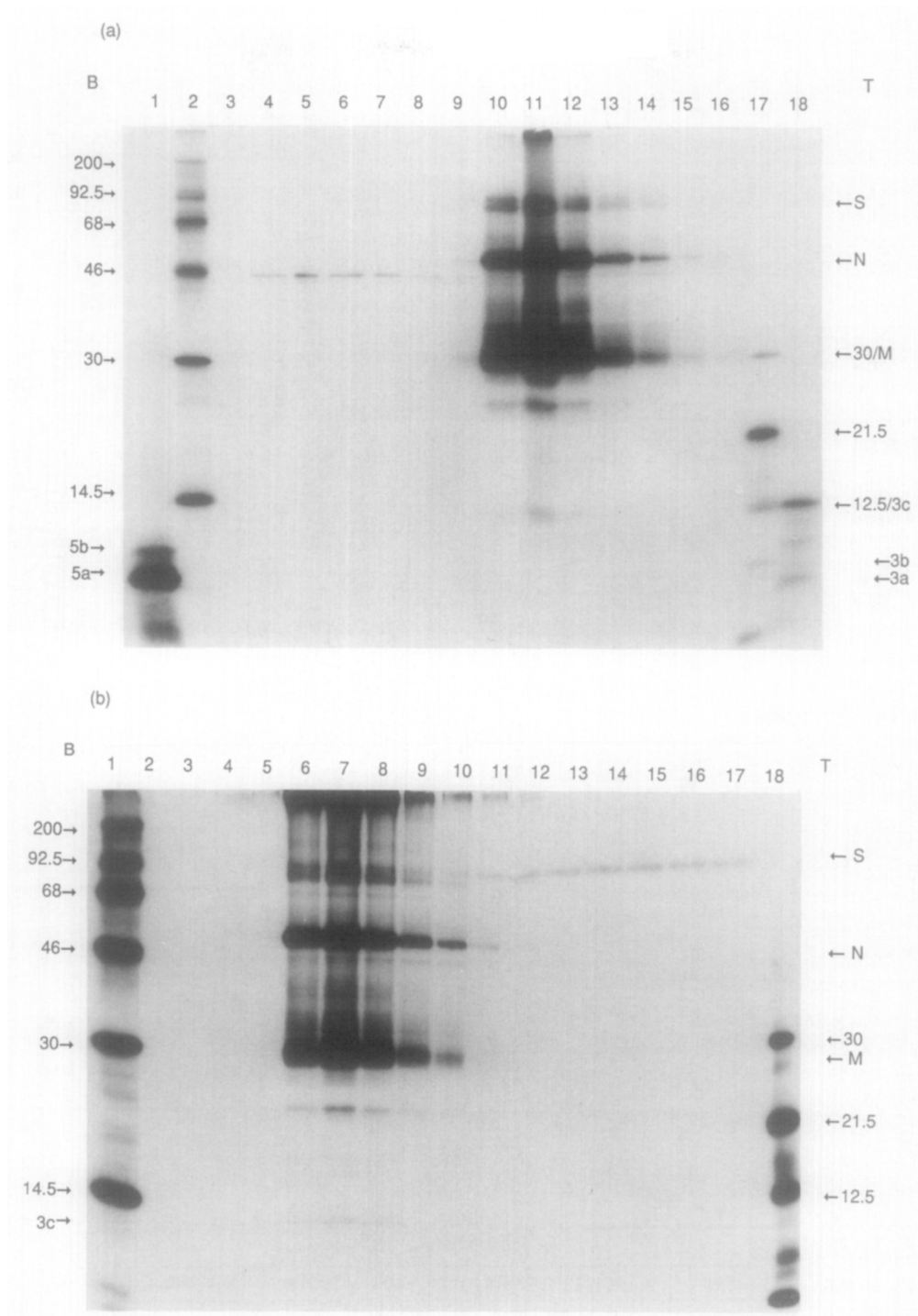
FIG. 1. SDS–PAGE analysis of IBV virion polypeptides. IBV strain Beaudette was grown on primary chicken kidney cells, labeled with [^{35}S]methionine, and purified as described in the text. Polypeptides were detected by fluorography of the dried gel. (a) Twenty-two fractions were collected from the second gradient (consisting of 10–50% Renografin), of which 14 fractions (starting from the bottom) were analyzed by electrophoresis on a 22% gel (lanes 4–16, with lane 4 corresponding to the bottom fraction 1). Radiolabeled markers for the 5a and 5b polypeptides (lane 1) and for the 3a, 3b, and 3c polypeptides (lane 18) were prepared by *in vitro* translation of synthetic RNAs in the wheat germ cell-free system in the presence of [^{35}S]methionine. These RNAs were produced by *in vitro* transcription of pIBB4 (giving an mRNA encoding both the 5a and the 5b polypeptides) and pIBT3 (giving an mRNA encoding each of the 3a, b, and c polypeptides) as described elsewhere (*17, 18*). [^{14}C]-Labeled low (lane 17) and high (lane 2) molecular weight radiolabeled protein markers were also included. (b) Fractions 7, 8, and 9 from the gradient shown in (a) (corresponding to lanes 10, 11, and 12) were pooled and subjected to further purification by sucrose density gradient centrifugation as described in the text. Twenty-two fractions were collected, of which 16 (starting from the bottom) were analyzed by SDS–PAGE under the same conditions as described above (lanes 2–17, with lane 2 corresponding to the bottom fraction 1). Also shown are high (lane 1) and low (lane 18) molecular weight radiolabeled protein markers.

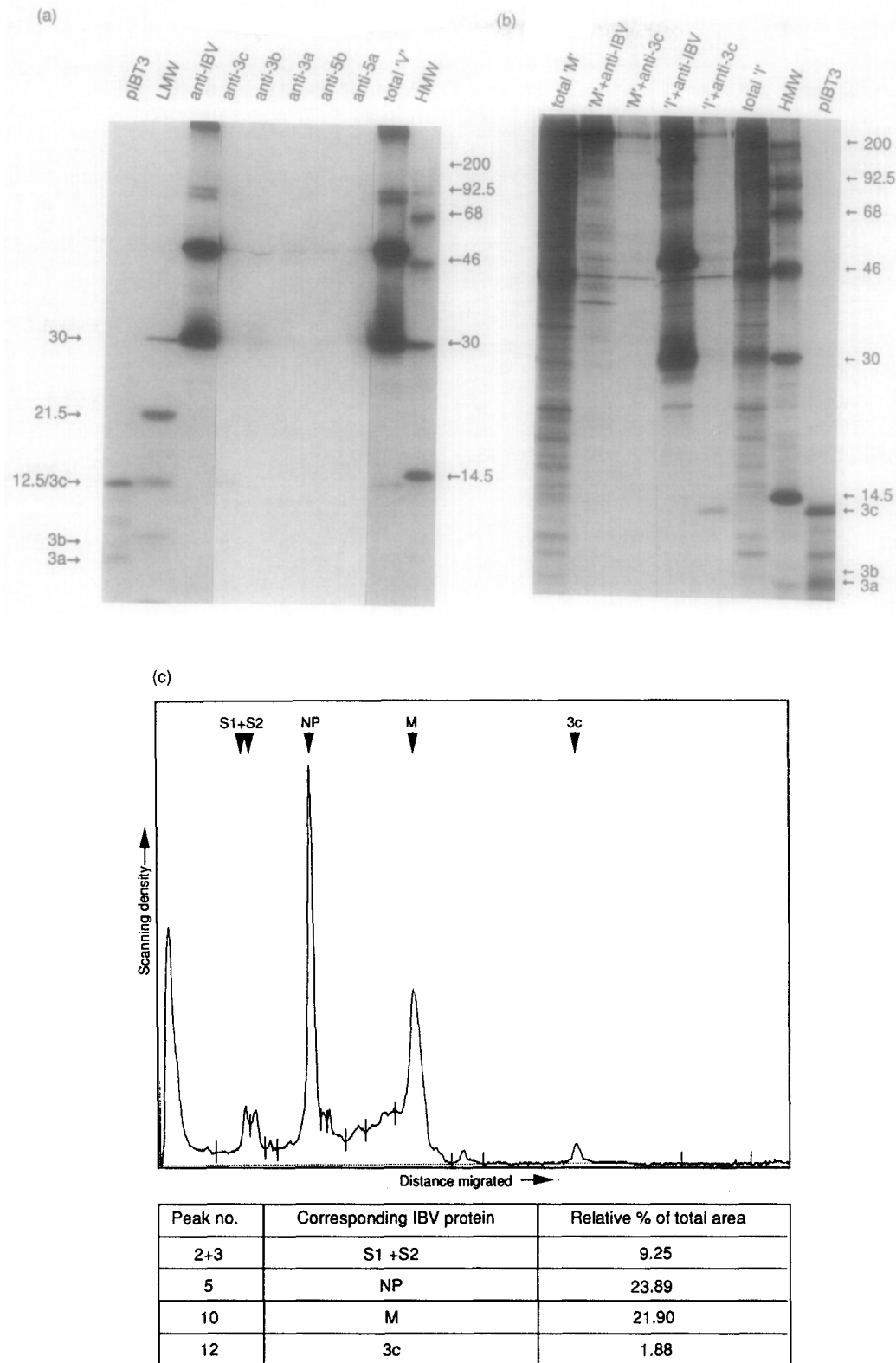| Peak no.	Corresponding IBV protein	Relative % of total area
2+3	S1 +S2	9.25
5	NP	23.89
10	M	21.90
12	3c	1.88

FIG. 2. (a) Immunoprecipitation of radiolabeled virion proteins with specific antisera. Virions were recovered from fractions 5, 6, and 7 of the sucrose density gradient shown in Fig. 1b as described in the text and immunoprecipitated with different antisera as indicated above each track. The monospecific test antisera were prepared as described elsewhere (17, 18, 23), and control anti-whole IBV serum was a gift from Dr. D. Cavanagh (AFRC Institute for Animal Health, Houghton Laboratory, UK). pIBT3-radiolabeled 3a, b, and c polypeptide markers were prepared by *in vitro* transcription and translation as described for Fig. 1a. Total 'V'-unprecipitated virions. Polypeptides were separated on a 22% polyacrylamide gel and detected by fluorography. Lanes containing anti-3a, b, and c and anti-5a and b antisera were exposed for 10 days, and other lanes for 2 days. (b) Synthesis of 3c protein in IBV-infected cells. CK cells were mock-infected or infected with IBV and labeled with [^{35}S]methionine from 6 to 7.5 hr p.i. as described before (23). Cells were harvested immediately after labeling and analyzed on a 22% SDS–polyacrylamide gel

the initiator methionine is not removed from any of the proteins; if one were to assume that cleavage does occur, the calculated relative proportion of 3c would increase. Figures are not available for the number of molecules of each structural protein present in IBV virions, but using those reported for human coronavirus OC43 as a guide (NP—726; M—726; S—88) (12), we suggest that each virion may contain in excess of 100 molecules of 3c, as many or perhaps even more than the number of spike proteins. This relatively large proportion of 3c further supports the idea that the 3c protein is a genuine virion structural protein which may play an important role in virion assembly or infectivity.

Since the product of the 3c ORF is known to be associated with the membranes of infected cells (23), it seemed likely that it could be located in the virion envelope. To test this possibility, a sample of purified radiolabeled virions purified by sucrose and then Renografin density gradient sedimentations as described above was pelleted by centrifugation and disrupted according to the procedure of Sturman et al. (27) by treatment with 1 ml of TNE buffer containing 0.25% NP-40. The ribonucleocapsid (RNP) and envelope components of the virus were then separated by centrifugation, at 180,000 g for 18 hr at 4°, through a 10-ml 10–55% linear sucrose gradient containing 0.1% NP-40, layered on top of a 1-ml cushion of 75% Renografin also containing 0.1% NP-40. Eighteen fractions were collected from the gradient (of which the first two correspond to the Renografin cushion), and samples were analyzed by SDS–PAGE (Fig. 3). It can be seen clearly that the major virion envelope proteins S1, S2, and M cofractionate with each other, appearing maximally in fractions 15–18, but are well separated from the nucleocapsid protein (fractions 1–10), indicating successful separation of the virion components. It is also evident that the 12K virion-associated protein cofractionates with the spike and membrane proteins rather than the nucleocapsids, suggesting strongly that it is indeed associated with the virion envelope. Immunoprecipitation of the pooled RNP and envelope fractions confirmed that the 12K protein present in the envelope fraction was indeed 3c and further indicated the absence of the protein in the RNP fraction (data not shown).

The function of the 3c protein is not yet clear. However, we are intrigued by an overall similarity in organization between 3c and the influenza A virus M2 protein

(13, 14). The IBV 3c protein is of a similar size and, like M2, has a stretch of hydrophobic amino acids near its N-terminus which could span the membrane. By analogy with M2, which is known to be an integral membrane protein present in small amounts in the virion (15, 31), this would leave the N-terminal 11 amino acids exposed on the external surface of the membrane with an internal C-terminal tail of 76 residues. Recently it has been proposed that the M2 protein forms an ion channel (28) which serves to allow both uncoating of incoming virions and proper assembly of progeny virus, and this hypothesis is consistent with the observation that the M2 protein is the target for the action of the anti-viral agent amantadine (10). M2 appears to be present in the membrane as a homotetramer composed of two disulfide-linked dimers held together by noncovalent interactions, which provides a structural basis for its proposed function in proton translocation (28). Comparison of the predicted amino acid sequence of 3c for five strains of IBV (17) indicates that the hydrophobic character of the proposed transmembrane region near the N-terminus of the protein is preserved, although the actual amino acid sequences show slight differences. There are also two conserved cysteine residues 45 and 46 amino acids from the N-terminus of the predicted sequence of the 3c products in all five strains examined, which might serve the same function as cys17 and cys19 of the M2 protein. It will be of considerable interest to explore this possible parallel further.

Are 3c-like proteins a general feature of coronaviruses? On the basis of currently available information, this seems likely. Messenger RNA 4 of mouse hepatitis virus (MHV) is known to encode a 15K polypeptide, the predicted amino acid sequence of which contains a highly hydrophobic region from residues 8 to 41 (9, 22), although the protein has not so far been found in virions. Wesley and Woods (29) have identified a 17K polypeptide in transmissible gastroenteritis virus (TGEV)-infected cells, which could be a minor viral structural protein. Intriguingly, sequence data deduced from the genomic RNA of several species of coronaviruses have indicated that the ORF immediately upstream of the membrane protein gene, in the equivalent position to that of 3c, has the potential to encode a hydrophobic protein with similar characteristics. For example the second ORF (5b) of MHV mRNA 5 can encode a 10.2K protein with a hydrophobic amino terminal region (16,

directly or after immunoprecipitation with anti-3c antiserum. pIBT3-radiolabeled 3a, b, and c polypeptide markers were prepared by in vitro transcription and translation as described for Fig. 1a. HMW, high molecular weight markers. (c) Densitometer scanning of the "total V" lane from the autoradiograph shown in a. Peaks corresponding to the major virion structural proteins are arrowed. Numbers immediately underneath represent the percentage contribution of that peak to the total peak area for the scan calculated by integration. Scanning and peak measurement were carried out using a Joyce-Loebl Chromoscan 3 scanning densitometer.
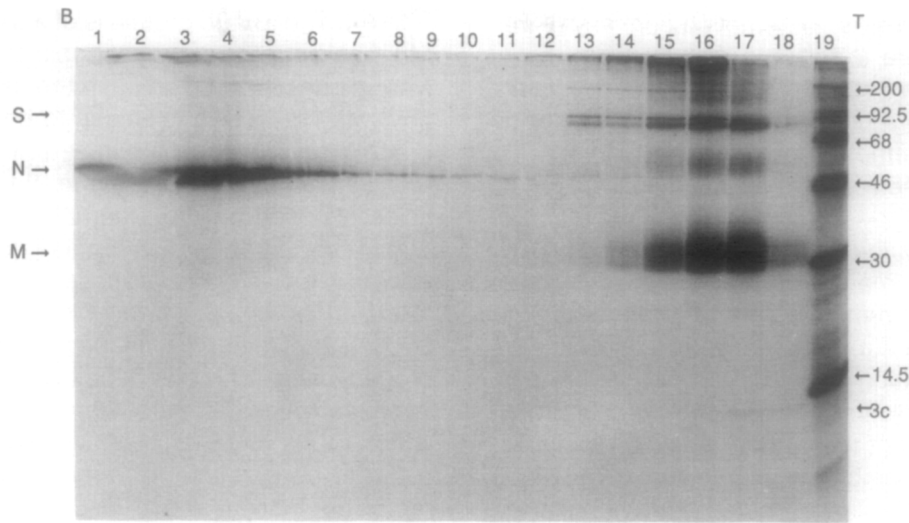
FIG. 3. Fractionation of radiolabeled virions into RNP and envelope components. The purified virions were disrupted and applied to a sucrose density gradient as described in the text. Eighteen fractions were collected (starting from the bottom) and aliquots analyzed by electrophoresis on a 22% gel (lanes 1–18, with lane 1 corresponding to fraction 1). Also shown are radiolabeled high molecular weight protein markers (lane 19). Polypeptides were detected by fluorography.

22), and the corresponding genetic location on the TGEV genome has the potential to encode a 10K polypeptide with striking similarity to 3c (30). Furthermore, the bovine coronavirus genome at this point contains an ORF which could encode a 9.5K hydrophobic protein (1). Comparison of the predicted amino acid sequences of these four proteins (Fig. 4) reveals some interesting common features. First, the amino acids flanking the proposed transmembrane region of the four proteins are negatively charged at the N-terminus and positively charged at the C-terminus. This arrangement could allow them to adopt the orientation of class III integral membrane proteins (11), i.e., with the N-terminus on the outside. It has also been observed that the predicted molecular weight of the 3c protein of IBV, the 5b protein of MHV, and the 9.5K protein of BCV is consistent with their migration rate in SDS–PAGE (1, 16, 17, 22), suggesting that these proteins contain uncleaved signal peptides; this is again consistent with the possibility that they are, like the M2 protein of influenza A virus, class III integral membrane proteins (11,

20). A further common feature is the occurrence of a cysteine residue 34 or 35 nt downstream of the charged amino acid at the N-terminal end of the proposed transmembrane region, which may play a role, as described above for the influenza M2 protein, in forming disulfide linkages. Thus it may be that 3c-like proteins are conserved across the Coronaviridae and may play a similar role in viral replication.

ACKNOWLEDGMENTS

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IBV - Beaudette MMNLLNKSLEENGSFLTALYIIUGFLALYLLGRRLQAFUQRRDACCLFU....60 aas   12.4K (3c)
TGEV - Miller   MTFPRRLTUIDDNGMUISIIFUFLLIIILILLSIRLLNIIKLCMUCCNLG....32 aas   10.2K
MHV - JHM   MFNLFLTDTUUYUGQIIFIURUCLMUTIIUURFLASIKRCIQLCGLC....41 aas   10K (5b)
BCV - Mebus   MFMRDRYFRDTUUYUGQIIFIURICLLUIIUUURFLATFKLCIQLCGMC....35 aas   9.5K
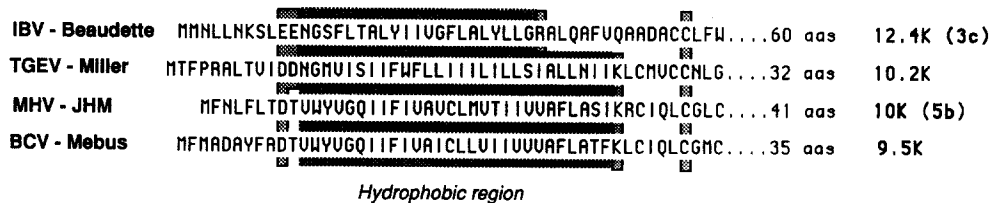
Hydrophobic region

FIG. 4. Comparison of the N-terminal amino acid sequences from four small proteins encoded by various coronaviruses. The shaded areas represent proposed transmembrane domains, and the boxed areas represent suggested conserved features as described in the text. Derivation of the sequence information is also described in the text.

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