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Human Coronavirus OC43 RNA 4 Lacks Two Open Reading Frames Located Downstream of the S Gene of Bovine Coronavirus¹

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Nucleotide sequences between the spike (S) and membrane (M) protein genes of the OC43 strain of human coronavirus were obtained from PCR-amplified viral mRNAs. Sequence analysis of this region revealed the presence of two ORFs encoding proteins of 12.9 and 9.5 kDa. These two proteins were identified as putatively nonstructural (ns) due to their homology to the corresponding BCV ns gene products. Northern blot analysis indicated that each of these two genes was present on a separate mRNA (5 and 5-1, respectively). *In vitro* translation analyses demonstrated that the HCV-OC43 9.5-kDa protein, like its BCV counterpart, is poorly translated when situated downstream of the 12.9-kDa ORF, although immunofluorescence studies did confirm its presence in infected cells. Sequence analysis showed that a large portion of the 3'-end of the leader sequence is present within the viral genome, upstream of the 12.9-kDa ORF. In addition, two ORFs encoding potential 4.9- and 4.8-kDa ns proteins in BCV are absent in HCV-OC43, although a corresponding mRNA 4 was found at a very low level. These results demonstrate that these two putative ns proteins are not essential for virus replication, at least in HRT-18 cells. © 1993 Academic Press, Inc.

Human coronaviruses (HCV) are recognized as the causative agents of respiratory diseases: they are responsible for 15–35% of common colds (1). Other disease associations have been suggested, such as the involvement of HCV in severe diarrhea (2) or neurologic diseases such as multiple sclerosis (3–5). Recently, some primates were shown to develop neurologic disease after inoculation with a murine-related coronavirus (6). Indeed, some strains of murine hepatitis virus (MHV) in rodents have been used as model systems to study chronic and acute hepatic and neurologic diseases (7).

Coronaviruses contain a single-stranded, capped, and polyadenylated positive-sense RNA molecule of 27 to 31 kb, which directs the synthesis, by leader-primed transcription, of a nested set of six to eight sub-genomic mRNAs with common 3'-ends but extending to different lengths toward the 5'-end of the genome. Each mRNA possesses a common 5'-end leader of about 72 nucleotides, derived from the 5'-end of genomic RNA. Only the 5'-most open reading frame (ORF) of each mRNA is usually translated, although these mRNAs contain multiple ORFs. The intergenic regions between ORFs contain a stretch of sequence varying from 7 to 18 nucleotides that is homologous to the 3'-end of the leader RNA. This region is presumably

involved in regulation of the transcription of viral mRNAs (8).

Along with the genes for structural proteins (HE, S, M, and N), coronavirus genomes contain a number of ORFs potentially encoding nonstructural proteins. The number and positions of these genes differ among coronavirus species. In avian infectious bronchitis virus (IBV), there are five ORFs, three located between the S and M genes and two located between the M and N genes (9, 10). In porcine transmissible gastroenteritis virus (TGEV), there are four ORFs, three located between the S and M genes and one located at the 3' side of the N gene (11–13). In MHV, there are five ORFs, three located between the S and M genes (14–16), one at the 5'-side of the S gene (17, 18) and one located within the N gene (19). In bovine coronavirus (BCV), there are six ORFs, four located between the S and M genes (20), one at the 5' side of the HE gene (21) and one located within the N gene (22).

HCV-OC43 and BCV show remarkable similarity, as shown by oligonucleotide fingerprinting analysis of their genomic RNAs (23, 24), by immunoprecipitation of viral proteins with specific antisera (25) and from the sequences of the N, M, and HE genes of HCV-OC43 (26–28). These studies suggest that HCV-OC43 and BCV may have diverged fairly recently.

In this report, we present the genome sequence between the S and M genes of HCV-OC43. Only two ORFs, encoding potential proteins of 12.9 kDa (mRNA 5) and 9.5 kDa (mRNA 5-1), were found, revealing a

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major difference between putative nonstructural proteins of BCV and HCV-OC43. We also found about 47 nucleotides of the leader sequence within the genomic RNA.

The source and cultivation of the HRT-18 human rectal tumor cells and the OC43 strain of HCV, as well as the preparation, reverse transcription, polymerase chain reaction (PCR) amplification, cloning, and sequencing of viral RNA (both mRNA and genomic RNA) were performed as described elsewhere (27). For Northern blot analysis, poly(A)-containing RNA was selected with the PolyATract mRNA isolation system (Promega, Fisher Scientific, Montréal, Québec, Canada) according to the manufacturer's instructions, was size fractionated by electrophoresis in 1% (w/v) agarose gels containing 5.3% formaldehyde, and was transferred onto Hybond-C extra (Amersham Canada Ltd., Oakville, Ontario, Canada) nitrocellulose filters (29). Blots were hybridized with the random-primed ³²P-labeled (ICN Biomedicals Canada Ltd., Mississauga, Ontario, Canada) DNA probes at 42° as described previously (30).

The nucleotide sequence of the region between the S and M genes of HCV-OC43 and the predicted amino acid sequences of open reading frames are shown in Fig. 1, together with the potential glycosylation site and intergenic consensus sequences. This region contains ORFs for two proteins of 12.9 and 9.5 kDa, in that order. An 11-amino acid ORF with the potential to encode a very small polypeptide of 1.33 kDa is also observed upstream of the 12.9-kDa ORF.

The 12.9-kDa ORF (nucleotides 132 to 458) potentially encodes a 109-amino acid protein. This protein has an amino acid sequence identity of 96.3% with the 12.7-kDa protein of BCV (20). As in BCV, there is one potential N-linked glycosylation site at amino acid position 18 (Fig. 1). The single putative initiation codon for the translation of this protein is in a context not frequently used for initiation of protein synthesis (31; Table 1). The UCAAAC consensus intergenic sequence is present about 107 nucleotides upstream of the initiation codon, within the 3'-terminus of the S gene (Fig. 1).

The 9.5-kDa ORF begins at nucleotide 448 and ends at nucleotide 699. It predicts an 84-amino acid protein. This protein has amino acid sequence identities of 96.4% with the 9.5-kDa protein of BCV (20). As in BCV, there are two methionine residues at the putative first and third codons of the protein. The first putative initiation codon is not in favorable context for initiation of protein synthesis, whereas the presence of a G residue at position +4 of the second putative initiation codon would presumably improve the situation (31; Table 1). The 9.5-kDa protein contains one large hydrophobic domain that comprises more than 50% of the molecule, which is potentially transmembranous (data not shown). The UCAAAC consensus sequence is pres-

ent about 120 nucleotides upstream of the first potential initiation codon, within the 12.9-kDa protein gene (Fig. 1).

The 11-residue ORF (nucleotides 34 to 66) predicts a peptide of 1.33 kDa, which shows 82% identity (9/11 amino acids) with the N-terminus of the BCV 4.9-kDa nonstructural protein (20). A consensus intergenic sequence UCAAAC is found within the S gene (Mounir and Talbot, manuscript in preparation). This small ORF is followed by a 47-nucleotide stretch that has striking resemblance with the 3'-half of the 82-nucleotide HCV-OC43 leader sequence (26), as shown by 37 identical nucleotides (Fig. 2).

To identify the subgenomic RNAs that encode the structural and nonstructural proteins, we performed Northern blot analysis using DNA probes (the localization of these probes is schematized on the right of Fig. 3). The M probe, which encompasses the M protein gene plus the first 80 nt of the N protein gene, detected nine HCV-OC43-specific RNAs that have been numbered 1, 2, 2-1, 3, 4, 5, 5-1, 6, and 7 in order of decreasing sizes. The 9.5-kDa probe hybridized to RNA 1 to 5-1, but not RNAs 6 and 7. The 12.9-kDa probe hybridized to RNA 1 to 5, but not RNAs 5-1, 6 and 7. The S2 probe, which extends into the region upstream of the 12.9-kDa ORF, hybridized to RNA 1 to 4 only (Fig. 3). These results lead us to propose that RNAs 5 and 5-1 encode the nonstructural proteins 12.9 and 9.5 kDa, respectively, a situation similar to that for BCV homologues (20) and different from that for MHV, where only one transcript (mRNA 5) is utilized for the synthesis of both the 13- and 9.6-kDa ns proteins (14, 32). Since the initiation codon for the BCV 12.7-kDa ns protein is in a more favorable context for initiation of translation than the HCV-OC43 12.9-kDa ns protein (20; G instead of U in position -3), we wanted to test the translatability of both ORFs, either independently or in tandem. In BCV, *in vitro* translation experiments using a synthetic transcript containing both the 12.7- and 9.5-kDa ORFs in tandem demonstrated that the majority of the protein synthesized was the upstream 12.7-kDa protein (20). On the contrary, when the same experiment was performed on a MHV RNA containing both ORFs (13 and 9.6 kDa), the downstream ORF (9.5 kDa) was preferentially synthesized (14). When we performed a similar translation experiment with a synthetic HCV-OC43 RNA containing both ORFs (see clone A, Fig. 4), we observed that, like in BCV, the upstream 12.9-kDa protein was preferentially synthesized (Fig. 4, lane 2). However, when synthetic RNA containing only one of the two ORFs (clones B and C; Fig. 4) was *in vitro* translated, both proteins were efficiently synthesized (Fig. 4, lanes 1 and 3, respectively), although small amounts of unexpected products were observed. The latter polypeptides are most likely non-specific products, because all constructs were shown

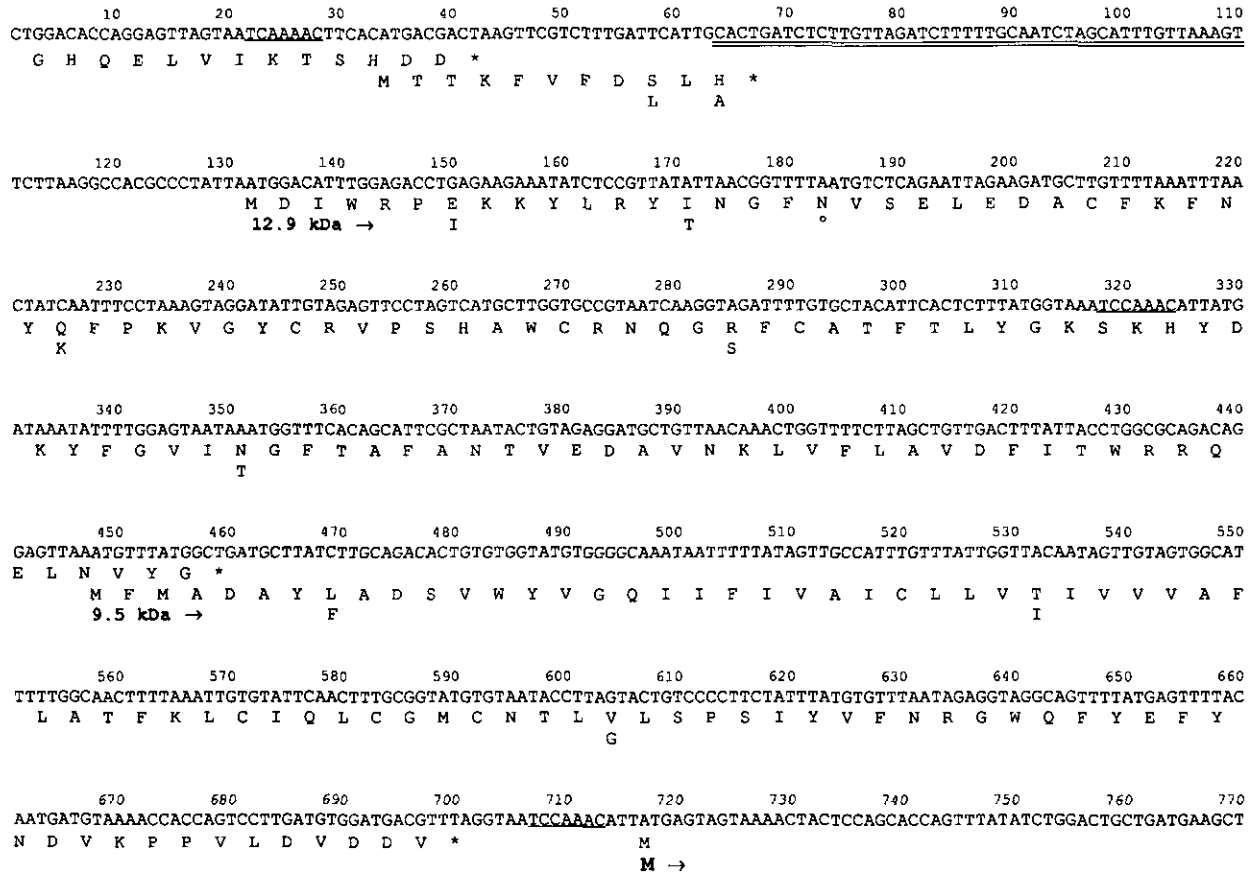


Fig. 1. Nucleotide and predicted amino acid sequence between the S and M genes of HCV-OC43. The leader sequence is doubly underlined. The potential *N*-glycosylation site (°) is indicated. Asterisks indicate stop codons. Consensus intergenic sequences are underlined. Individual amino acids correspond to amino acid differences in the Mebus strain of BCV (20), as compared to the HCV-OC43 strain.

to be correct by sequencing. Indeed, such non-specific products were also observed, albeit at lower levels, when no mRNA was added (Fig. 4, lane 4). We also showed that the HCV-OC43 9.5-kDa protein is made during infection using antibody directed against the MHV 9.6-kDa protein (32), which specifically stained

HCV-OC43-infected HRT-18 cells in an immunofluorescence assay (data not shown).

Recently, it was reported that BCV is closely related to HCV-OC43 at both the protein and RNA levels (20, 26–28). In the present study, we found a major difference in the human coronavirus, represented by the absence of the genes potentially encoding two non-structural proteins of 4.9 and 4.8 kDa in BCV (20). No attempt has yet been reported on the detection of these two small proteins in BCV-infected cells or virions. Their absence in HCV-OC43 indicates that they are not essential for virus replication, at least in HRT-18 cells. Similarly, the ns2, ns4 and ns5a nonstructural proteins of MHV were found not to be essential for virus replication in transformed cells (33, 34). The apparent presence of low levels of mRNA 4 in HCV-OC43-infected cells, in the absence, most likely, of an associated protein product suggests that this mutation may be a recent event. However, it remains possible, although unlikely, that a putative 11-residue peptide is expressed from this mRNA.

The absence of two putative nonstructural proteins in HCV-OC43, if they are found to be expressed in BCV-infected cells, as well as amino acid differences

TABLE 1

Gene product	Sequences surrounding AUG initiation codon	Intergenic initiation sequence of downstream gene product
HE ^a	AAA <u>AUGU</u>	ACUAAAC
S ^b	AACA <u>UGU</u>	UCUAAAC
12.9 kDa ORF	UUA <u>AUGG</u>	UCAAAAC
9.5 kDa ORF	UAA <u>AUGU</u>	UCCAAAC
M ^c	AUU <u>AUGA</u>	UCCAAAC
N ^d	AGGA <u>UGU</u>	UCUAAAU
Consensus ^e	A <u>CCAUGG</u> G	

^a Zhang *et al.*, 1992 (28).

^b Mounir and Talbot (manuscript in preparation).

^c Mounir and Talbot, 1992 (27).

^d Kamahora *et al.*, 1989 (26).

^e Kozak *et al.*, 1989 (31).

BCV	GATTGCGAGCGA-TTTGCGTGGCGTGCATCCCGCTTCA-CTGATCTCTTGTAGATCTTTTATAATCTAAAC-----	70
	*** *****	
OC43 ^a	--TTGTGAGCGAAGTTGGCGTGGCGTGCATCCCGCTTCACCTGATCTCTTGTAGATCTTTTCTAATCTAAATTTAAGG	82
	*** *****	
OC43 ^b	-----CA-CTGATCTCTTGTAGATCTTTTGAATCTA-GCATTGTGTTAAAGT	47

Fig. 2. Comparison of leader sequences between HCV-OC43 (26), BCV (Hoffmann *et al.*, 1991, GenBank Accession No. M62375), and the 47 nucleotides upstream the 12.9-kDa ORF. Identical nucleotides are indicated with asterisks. ^a Leader sequence of HCV-OC43 (26). ^b Sequences found upstream of the HCV-OC43 12.9-kDa ns protein gene (doubly underlined in Fig. 1).

within the S protein (Mounir and Talbot, manuscript in preparation), could be involved in the apparently preferential respiratory tropism of HCV-OC43, which contrasts with the presumed preferential enterotropism of BCV. Indeed, the S proteins of MHV and TGEV were suggested to be important in tissue tropism (35, 36). However, replication of BCV in the respiratory tract has been reported (28) and OC43-like human enteric coronaviruses have been isolated (37). Thus, it is premature to conclude on differential tropism of these two viruses.

The sites surrounding the putative protein synthesis initiation codon in mRNAs 5 and 5-1, UUAAUGG for the 12.9-kDa ORF and UAAAUGU for the 9.5-kDa ORF (Table 1), are not those usually used for initiation of eukaryotic protein synthesis, whereas the AUG initiation codons for the HE, S, M, and N genes are in the context AAAAUGU (28), AACAAUGU (Mounir and Talbot, manuscript in preparation), AUUAAUGA (27), and AGGAAUGU (26), respectively, all of them frequently found as functional initiation sites where A is present at the -3 position relative to the A of AUG (31). The second potential initiation codon for the 9.5-kDa ORF would be in a more favorable context for initiation of protein synthesis, given the presence of a G residue at position +4 (Fig. 1 and Table 1). The actual initiation site cannot be inferred before the N-terminus sequence of the 9.5-kDa

protein can be determined. Interestingly, the initiation codon for the 12.9-kDa protein is in a less favorable context than the one for the BCV 12.7-kDa protein, which has a G instead of a U at the -3 position (20) and is nevertheless preferentially used when in tandem with the 9.5-kDa ORF.

It is noteworthy that we found 47 nucleotides of the leader of HCV-OC43 in the sequence between S and M genes (Fig. 2). This situation is not predicted by the proposed models of leader-primed transcription (8), because part of the leader sequence (47 nt of 82 nt) is found within the genomic RNA instead of at the 5'-end of the transcript. It may be a result of recombination, whereby HCV-OC43 lost the sequence coding for the 4.9- and 4.8-kDa ns proteins and developed a mechanism for conservation of the ORF encoding the 12.9-kDa protein. This sequence does not contain one of the novel transcription initiation signals reported recently (38). We are currently investigating the role of this sequence in virus infection.

The functions of the putative 12.9- and 9.5-kDa proteins in infected cells are not known. These small coronavirus proteins, which have both hydrophobic and charged domains, have been suggested to act as a membrane-anchoring region for structural proteins during virus assembly, or to play a role in membrane association of the viral polymerase during replication

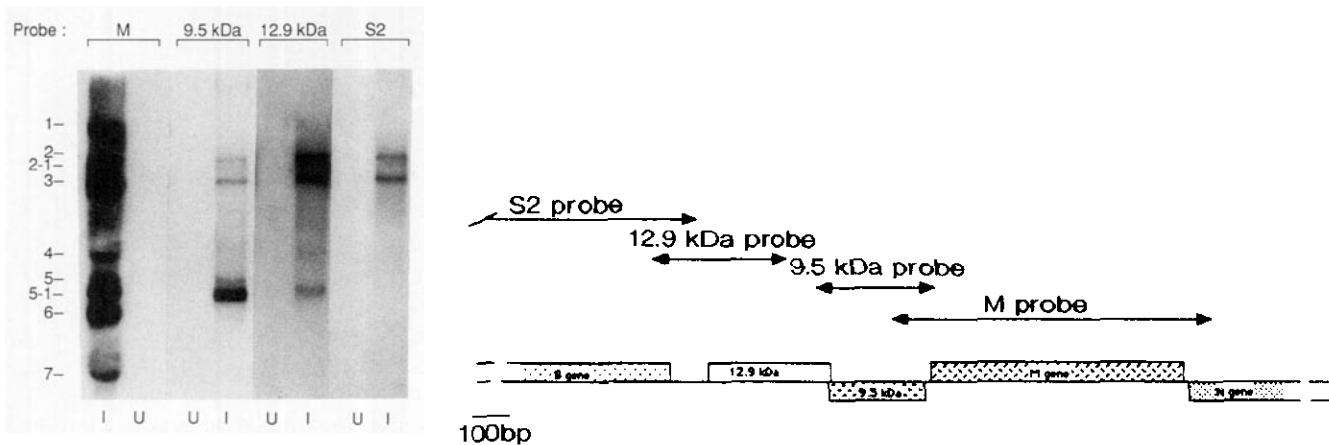


Fig. 3. Northern blot analysis of RNA from uninfected cells (U) and infected cells (I). RNAs were revealed with the probes schematized to scale on the right (base numbers are from Fig. 1, except for 1501, which corresponds to base 737 in Ref. 27): a 900-bp *EcoRI-ScaI* fragment containing the M region and the first 80 nucleotides of the N region, bases 605 through 1501 was designated the M probe; a 327-bp *HincII-BamHI* fragment containing bases 416 through 743 was designated the 9.5-kDa probe; a 362-bp *EcoRI-BsmI* fragment containing bases 1 through 362 was designated the 12.9-kDa probe; a 1.8-kb *EcoRI-EcoRI* fragment containing the S2 region (Mounir and Talbot, manuscript in preparation) and bases 1 through 126 was designated the S2 probe. Subgenomic mRNAs are indicated on the left. Total RNA was used for hybridization with the M probe, whereas poly(A)-mRNA was used with the other probes.

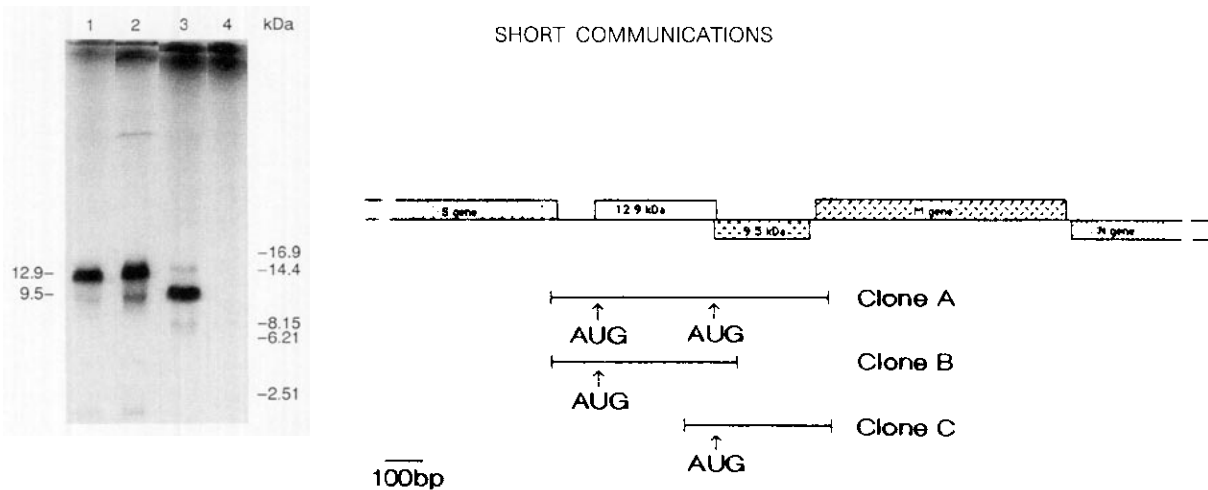


Fig. 4. *In vitro* translation of transcripts from clones schematized to scale on the right: clone A, lane 2; clone B, lane 1; clone C, lane 3. Lane 4, *in vitro* translation with no mRNA added. The migration of molecular weight markers (LKB, Canlab, Pointe-Claire, Québec, Canada) is shown on the right and that of the two viral proteins on the left. Plasmid constructs 5'-12.9-kDa ORF-9.5-kDa ORF-3' (clone A) and 5'-12.9-kDa ORF-3' (clone B) were created by unidirectional deletion of the 1.5-kb insert in pBluescript II SK(+) using exonuclease III and mung bean nuclease (Stratagene, La Jolla, CA). A construct 5'-9.5-kDa ORF-3' (clone C) was prepared by removing from clone A a *BsmI*-*Bam*HI fragment that begins in the polylinker region of the pBluescript II SK(+) vector and ends 86 nucleotides upstream from the 9.5-kDa ORF. Recombinant plasmids were linearized with *Bss*HI (clones A and C) or with *Nae*I (clone B) and transcribed *in vitro* with T3 RNA polymerase (42). Approximately 1 μ g of *in vitro*-transcribed RNA was translated in a wheat germ cell-free extract (Promega, Fisher Scientific, Montréal, Québec, Canada) using 1 mCi/ml of L-[³⁵S]cysteine (1064 Ci/mmol; Amersham) as the radiolabeled precursor. The conditions for translation were those recommended by the manufacturer, except that potassium acetate was optimized at 40 mM. Translation products were analyzed under reducing conditions on a 20% SDS-polyacrylamide gel (43) and revealed by fluorography with EnLightning (NEN Dupont, Montréal, Québec, Canada).

(9, 10, 15, 16, 20, 32). Recently, proteins analogous to the HCV-OC43 and BCV 9.5-kDa molecules were shown to be present in IBV and TGEV virions and were termed small membrane (sM) proteins (39, 40). We have shown that the HCV-OC43 9.5-kDa ns protein, like its BCV and MHV counterparts, is expressed in virus-infected cells. It will also most likely be found in virions. The conservation of the 12.9-kDa protein also suggests that it fulfills an important function in coronavirus biology.

The biological importance of the various nonstructural proteins encoded by coronaviruses remains to be investigated and some of them may turn out to be structural components. So far, only the proteins encoded by mRNAs 2, 4, and 5b of MHV (17, 18, 32, 41) and mRNAs 5-1 of BCV (20) and HCV-OC43 (this study) were shown to be produced in infected cells. Our study emphasizes the importance of further work on the biological functions of coronavirus nonstructural and novel structural proteins.

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