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Primary Structures of Hemagglutinin-esterase and Spike Glycoproteins of Murine Coronavirus DVIM

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Abstract. Diarrhea virus of infant mice (DVIM) is a member of murine hepatitis viruses (MHVs). The nucleotide sequences of the genes encoding the hemagglutinin-esterase (HE) and the spike (S) glycoproteins from DVIM were determined and compared with those of other MHVs. The deduced amino acid sequence of the HE protein was most similar to that of MHV-S strain (94% identity), and the S protein sequence was most similar to that of MHV-Y strain (90% identity). The DVIM HE protein has a unique N-linked glycosylation site in addition to other glycosylation sites common to many MHV strains. Unlike in some typical MHV strain, such as MHV-A59 and MHV-JHM, the vast majority of the S glycoprotein molecules in DVIM exist an uncleaved form probably due to several amino acid substitutions around the cleavage site.

Key words: MHV, DVIM, hemagglutinin-esterase glycoprotein, spike glycoprotein, cleavage of surface glycoprotein, N-linked glycosylation site

Murine hepatitis virus (MHV) is a member of the *Coronavirudae* that causes respiratory, neurologic and enteric disease in rats and mice (1–3). DVIM (diarrhea virus of infant mice) belongs to the group of enteric murine coronaviruses that possess both hemagglutinating and acetylesterase activities (4, 5). It infects the intestinal tracts and causes diarrhea in new-born mice (4–8).

Like other MHVs, DVIM possesses a single-strand, nonsegmented positive sense RNA genome of about 30 kb, and DVIM virion contains five major structural proteins encoded in the genomic RNA: the nucleocapsid protein (N), the transmembrane glycoprotein (M), the small membrane glycoprotein (sM), the spike glycoprotein (S) and the hemagglutinin-esterase glycoprotein (HE) (9). Among these proteins, the S and HE glycoproteins form projections anchored in the virus envelope, and these projections are major determinants for neutralizing antibodies (3). It has been shown that the S glycoprotein for MHV-A59 or MHV-JHM plays an important role in attachment of the virus to the cellular receptor and that it induces membrane fusion for virus penetration (10). In addition, the S glycoproteins of these strains cause cell fusion between the target cells infected by viruses (3,10). The HE glycoprotein of DVIM possesses a hemagglutinating activity for binding to sialic acids on erythrocytes and an acetylesterase activity as a receptor-destroying enzyme as in the case of the HEF glycoprotein of influenza C virus (13). However, the role of the HE glycoprotein in virus infection is not yet known. Some strains of MHVs, such as DVIM and MHV-S, express HE glycoproteins (HE positive), while others, such as MHV-A59 and MHV-2, do not (HE negative) (1,2). It has been shown that both HE positive and negative strains use a common CEA (carcino embryonic antigen) family protein as a functional receptor on the target cells (17,18).

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The nucleotide sequence data reported in this paper have been submitted to the Genbank nucleotide sequence database and have been assigned the accession numbers AB008939 and AB008940.

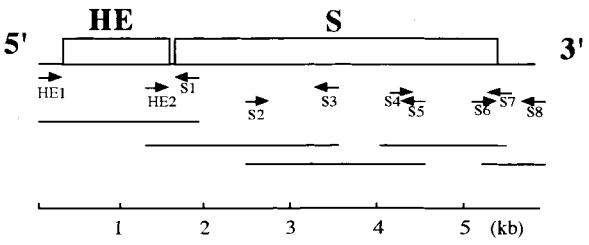


Fig. 1. Cloning strategy of the HE and S glycoprotein genes. Five cDNA (complementary DNA) were prepared by RT-PCR (reverse transcription-polymerase chain reaction) from total RNA fraction of DBT cells infected with MHV-DVIM. Arrow indicates the position and direction of primer. First strand cDNAs were synthesized with reverse transcriptase at 42°C using the anti-sense primers (S1: 5′TAACGTCGACCCCARCCCWTTRGWAACATC3′, S3: 5′GTCAGTCGACCCGTTTAAGTTACCATTAACATC3′, S5: 5′ACAAGTCGACTCGCTAACTTCTTGACCTCCAGTGCAATT 3′, S7: 5′CTGTGTCGACCAATCCTCATGGGATGAAATATTATGTATC3′ and S8: 5′ATCAGTCGACGGATTATTATATCATCCACC3′), which are complementary to the sequences of the regions conserved among MHV-JHM, MHV-S and MHV-A59 (where R: A or G, W: A or T). The cDNAs were mixed with the sense primers (HE1: 5′CCTCGAATTCCTAAAACTCTTGTAGTTT3′, HE2: 5′ATGAGAATTCTCCGGTCATACTGCTAGGTGTATTATTGGG3′, S2: 5′CAATGAATTCAACCCCTCCTCTTGGAACAGGCGGTATGG3′, S4: 5′ATTTGAATTCGTCAAACTCTCTGATGTTGGCTTTGTCGA3′ and S6: 5′CAGGGAATTCCTCATGTTGTTTTAAGAAGT3′) corresponding to the sequences conserved among MHV strains. The reaction mixture (50 µl) containing 1 unit of LA-*Taq* polymerase, 0.2 µM dNTP mixture, and 2 pmol each of two primers was incubated at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 90 sec for 30 cycles. The amplified cDNA fragment indicated by solid line was digested with *Eco*RI and *Sal*I, and was ligated into the *Eco*RI-*Sal*I site of pUC119. The recombinant plasmid DNA was transformed into *E. coli* strain DH10B, and the plasmid DNA was prepared by the alkaline SDS method for DNA sequencing. The single-strand DNA fragment from each plasmid DNA was sequenced using two Texas-red labeled universal primers, M13F and M13R, corresponding to the sequences at both sides of the multiple cloning site of pUC119. Three clones from dependently amplified PCR products were sequenced. Sequencing was performed according to the dideoxy termination method using a HITACHI DNA Sequencer SQ-5500. All enzymes were purchased from Takara Co., Ltd (Kyoto, Japan). The sequences determined will appear in the Nucleotide Sequence Databases under the accession number [AB008939] and [AB008940].

DVIM is the only strain of MHVs in which the HE glycoprotein has both of the above activities (1,4,5).

As the first step to analyze the functions of HE and S glycoproteins in the primary process of DVIM infection, we attempted to determine the structural features of these proteins, and determined the nucleotide sequence of the genes for these two proteins.

DVIM was propagated in DBT cell, and RNA was extracted from the infected cells by the acid guanidinium-phenol-chloroform method. The cDNAs including the coding regions for HE and S proteins in the DVIM genomic RNA were made by reverse transcription-polymerase chain reaction (RT-PCR) using synthetic DNA primers, which have been designed based on the conserved sequences among other MHVs (Fig. 1). The total 5500 nucleotides determined include the reading frames for HE (440 amino acids) and for S (1361 amino acids) glycoproteins.

Fig. 2 shows the deduced amino acid sequence of the HE glycoprotein of DVIM aligned with those of other coronaviruses. The amino acid sequence has a high similarity to that of the HE glycoprotein of MHV-S (94.10%) and MHV-JHM (92.06%). It has been reported that all the HE proteins of MHVs sequenced contain seven common potential N-linked glycosylation sites (NXS or NXT: where $X \neq P$) (14). The HE protein of DVIM has all of these sites at the corresponding positions as well, although with one additional potential glycosylation site at N124 position (see Fig. 2).

The amino acid sequence of the DVIM S glycoprotein deduced from its gene sequence is aligned with those of five other MHVs (Fig. 3). According to the S glycoprotein sequences, MHVs can be divided into two subgroups: one having high sequence similarity to the DVIM S glycoprotein, including MHV-Y (90.04%) and MHV-2 (87.56%); and the other, including MHV-JHM (72.70%), MHV-RI (78.79%) and MHV-A59 (76.28%), having low sequence similarity to DVIM. This appears inconsistent with the pathological view that MHV-RI belongs to the enterotropic group as do MHV-Y and DVIM (1,6–8). These results indicate that there is no apparent relevance between the phylogenetic relationship of glycoproteins and the organ tropism of MHV strains.

The S glycoprotein of MHV-A59 or MHV-JHM is cleaved into two subunits, S1 and S2 (molecular weight of about 90 kDa each) by a host-dependent trypsin-like protease (14). This protease recognizes a basic amino acid-rich sequence around position 770, (773) RRAHR (777) in MHV-A59 and (773) RRARR (777) in MHV-JHM, and cuts the peptide bound between 777 and 778. On the other hand, MHV-2 S glycoprotein, which has an amino acid substitution at position 777 (R to S), is not cleaved by protease (15).

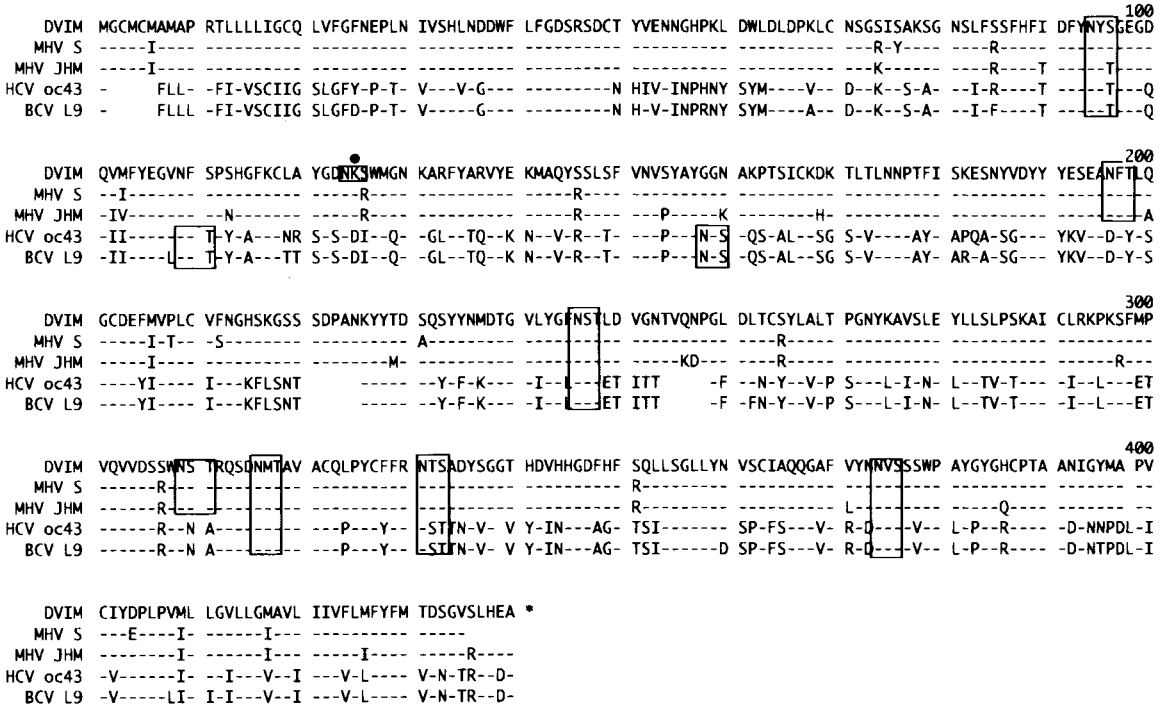
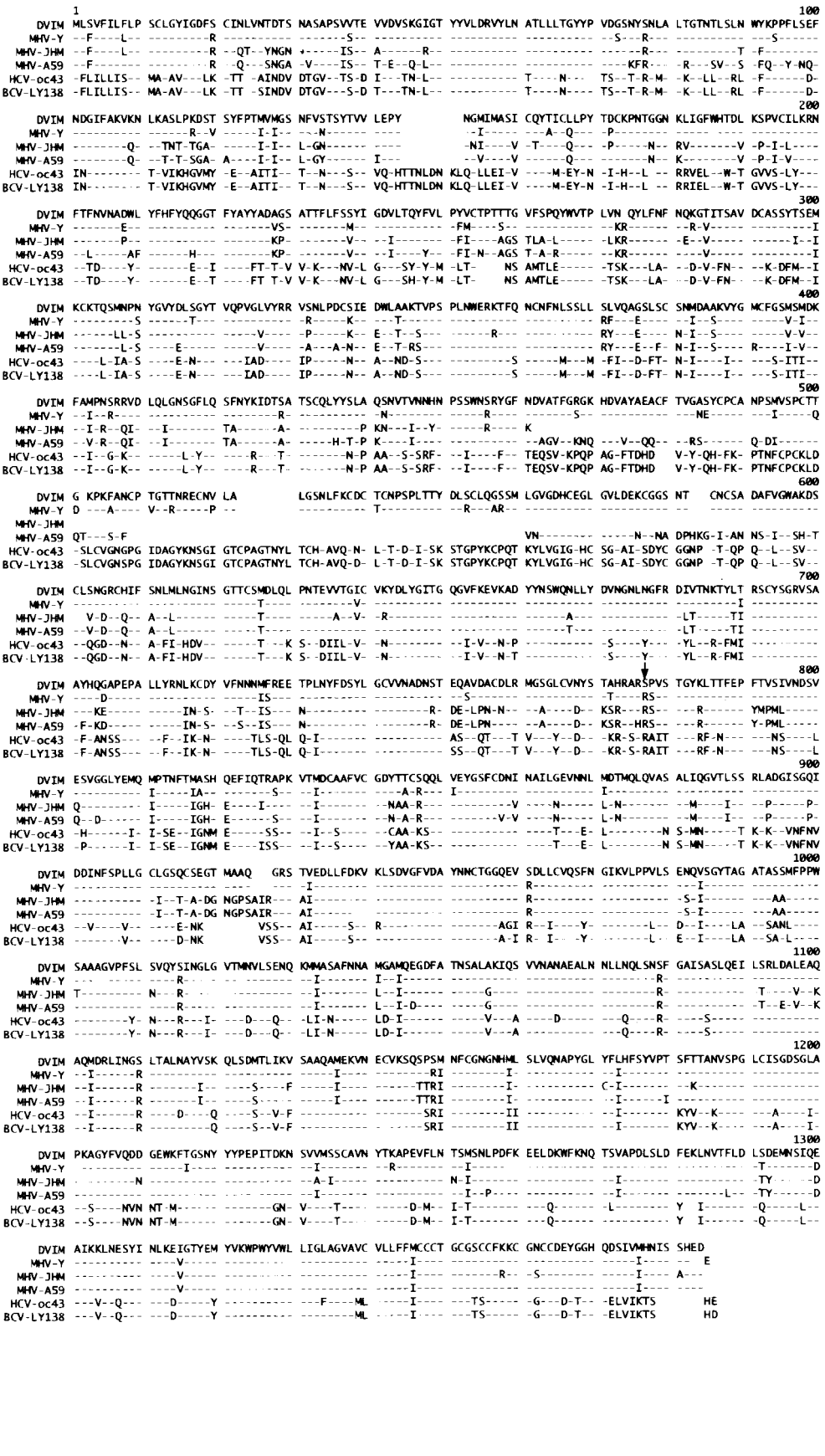


Fig. 2. Deduced amino acid sequences of the hemagglutinin-esterase (HE) glycoproteins of DVIM, MHV-S, MHV-JHM, BCV-L9 and HCV-oc43. Amino acid sequences were aligned using the clustal method (9). Dash (-) indicates the amino acid identical to that in the DVIM sequence. Space denotes the gap introduced to the sequence for maximum alignment. Potential N-linked glycosylation sites (NXS or NST, where X ≠ P) are boxed. Dot indicates the DVIM HE glycoprotein-specific potential N-linked glycosylation site. The sequences are taken from the GenBank database. The GenBank accession numbers: [M64316] MHV-S, [D00764] MHV-JHM, [M76372] BCV-L9 and [M76373] HCV-oc43.

The MHV-Y S glycoprotein is also uncleaved, presumably due to the presence of an extra N-linked glycosylation site at position 768 and/or sequence alteration around the protease recognition site ((768) DYSKSRRAHR (777) in MHV-A59 to (768) NYSTTHRARR (777) in MHV-Y) (Fig. 3) (16). The DVIM S protein contains an amino acid substitution at position 777 identical to that for the MHV-2 S glycoprotein and an extra N-linked glycosylation site at position 768 (Fig. 3). It is thus expected that the DVIM S protein exists as an uncleaved form in the virion.

To examine whether the DVIM S glycoprotein is cleaved into two subunits or not, the ^{35}S-labeled virion proteins were analyzed by SDS-PAGE in the presence or absence of 1% 2-mercaptoethanol. As shown in Fig. 4, four major bands, which correspond in size to the uncleaved S (180 kDa), HE monomer (69 kDa) in the reduced or HE dimer (140 kDa) in the unreduced preparation, N (58 kDa) and M (25–30 kDa), were detected. Several faint bands were also observed. One of them is the band of about 90 kDa in the reduced preparation, which corresponds in size to the cleaved S glycoprotein. Using a density-meter, we were able to estimate the intensity of the 90 kDa band was less than 5% of that of the 180 kDa band. These results suggest that a small portion of DVIM S glycoproteins exists as a cleaved form in the virion, although the vast majority as an uncleaved form. Gagneten et al. also detected a 90-kDa band from DVIM virion proteins in their gel electrophoresis which interacts with the antibody raised against the S glycoprotein of MHV-A59 (17).

Some reports have suggested the relationship between the cleavage of S glycoprotein and the virus fusion activity. For example, MHV-2, in which the S glycoprotein is not cleaved, has no fusion activity and an MHV-2 mutant, in which the S glycoprotein is changed to be cleaved, acquires fusogenic activity (15). On the other hand, MHV-Y



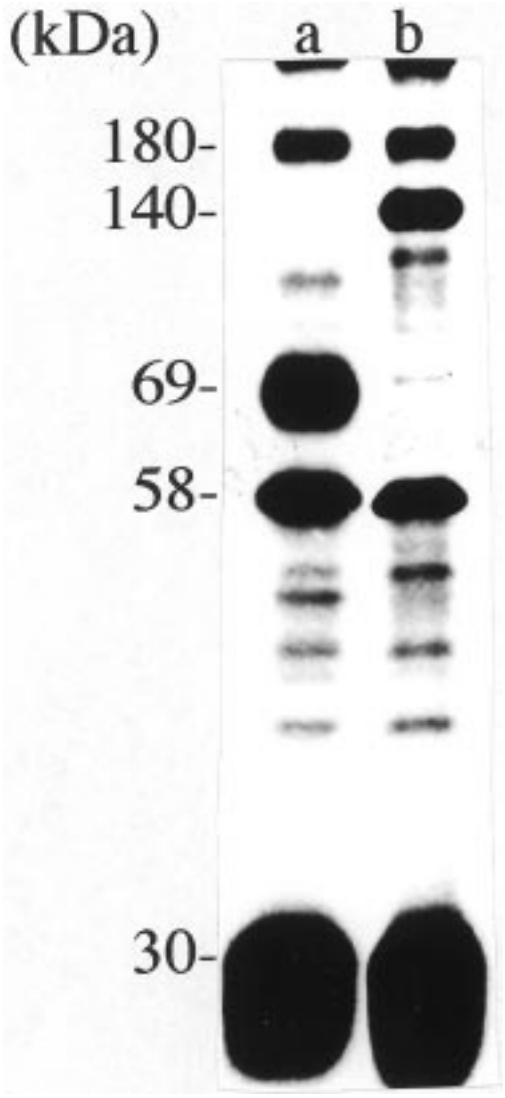


Fig. 4. SDS-polyacrylamide gel electrophoresis of MHV-DVIM structural proteins. Metabolic proteins were labeled with [^{35}S]methionine (Amersham), as described previously (11,12). The labeled virus was purified on a 20 to 60% linear sucrose gradient, and the soluble protein fraction was subjected to SDS-polyacrylamide (12.5%) gel electrophoresis (PAGE) with (lane a) or without (lane b) 1% 2-mercaptoethanol. After electrophoresis, the gel was fixed, soaked in an amplifier (Amersham), dried, and fluorographed at $-70°$C. Positions of viral protein are indicated by approximate molecular weights (kDa) deduced from molecular size maker.

as well as some mutants of MHV-A59 and MHV-JHM, in which the S protein is not cleaved, although the virus is fusogenic (16,19,20). DVIM is apparently in a similar situation, although there remains a possibility that a small portion of the cleaved S glycoprotein may cause the fusogenic activity of DVIM. The relationship between cleavage of the S glycoprotein and fusogenic activity in MHVs remains to be clarified, and DVIM would provide a good system for studying this.

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Fig. 3. Deduced amino acid sequences of the spike (S) protein of DVIM, MHV-Y, MHV-JHM, MHV-A59, HCV-oc43 and BCV-LY138. Amino acid sequences were aligned using the clustal method (9). The arrow at position 777 marks the cleavage site between S1 and S2. The GenBank accession numbers: [U14646] MHV-Y, [D00093] MHV-JHM, [M18379] MHV-A59, [M64667] BCV-L9 and [Z21849] HCV-oc43.

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