FUNCTIONAL ANALYSIS OF THE CORONAVIRUS MHV-JHM SURFACE GLYCOPROTEINS IN VACCINIA VIRUS RECOMBINANTS

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INTRODUCTION

Coronavirus MHV-JHM has two surface glycoproteins. The S protein is a heterodimer comprised of two non-covalently bound, subunits of about 90,000 molecular weight (mol.wt.) (S_1 and S_2) which are derived by proteolytic processing of the 180,000 mol.wt. precursor S. Multimers of the heterodimer assemble together to produce the characteristic peplomer structures at the surface of the virion. The second surface projection is smaller and is comprised of disulphide-linked homodimer(s) of the HE protein. The reduced HE monomer has a mol.wt. of 65,000 (Siddell et al., 1981).

In spite of an increasing amount of structural data regarding these proteins there is still relatively little known about their functions. Indirect evidence using S protein specific monoclonal antibodies (Collins et al., 1982; Wege et al., 1984) suggests that the MHV S protein mediates binding of the virus to a cellular receptor and the fusion of viral and cellular membranes. A function for the MHV-JHM HE protein has not been shown. Earlier results from King et al. (1985) correlated the haemagglutinating activity of bovine coronavirus (BCV) with the HE protein and recently Vlasak et al. (1988a) have demonstrated that BCV and the human coronavirus HCV OC43 can recognize and destroy a receptor on the cell surface which is similar or identical to the influenza C virus receptor. Moreover, Vlasak et al. (1988b) could demonstrate that in the case of BCV the receptor destroying activity associated with the HE protein had the specificity of an acetylesterase.

Luytjes et al. (1988) have recently cloned and sequenced the unique region of the MHV-A59 mRNA 2. They found two large open reading frames (ORFs) of which the downstream ORF lacks an amino-terminal initiation codon. These authors interpreted this ORF as a pseudogene but noted a striking similarity with the HEF_1 subunit of the Influenza C surface glycoprotein (Nakada et al., 1984).

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The aim of the experiments described in this paper were

1) to determine directly whether the MHV-JHM S protein was able to mediate cell fusion
2) to determine the primary sequence of the MHV-JHM HE protein and
3) to determine whether the MHV-JHM HE protein has receptor binding and receptor destroying activities.

RESULTS

I Analysis of the fusion activity of the MHV-JHM S protein

In order to demonstrate that the MHV-JHM S protein is responsible for the fusogenic activity of the virus, we have cloned complete copies of the S gene ORF into the unique Bam HI site of the Vaccinia virus cloning vector pTF7.5 (Fuerst et al., 1987). The isolation and characterization of the S gene cDNA clones will be described in detail elsewhere. The construct, pTF7.5/S+ was then used to transfect DBT cells which had been infected with Vaccinia virus recombinant vTF7.3, which expresses the T7 RNA polymerase gene. Fig. 1 shows the cytopathic effect (c.p.e.) 10 hours after infection and transfection with the Vaccinia virus recombinant vTF7.3 and the recombinant construct pTF7.5/S+. It is evident that the infected/transfected DBT cells display extensive syncytia formation, the characteristic cytopathic result of the MHV-JHM fusogenic activity. This result shows that the MHV-JHM S protein alone is sufficient to mediate the membrane fusion of infected cells. No further component on the surface of the virion is required for this effect.

II Cloning and sequencing of the MHV-JHM HE gene

A cDNA encompassing the unique region of the MHV-JHM mRNA 2-1 was made using a specific oligonucleotide primer, essentially according to the method of Gubler and Hoffman (1983). Within this region, which is 5' proximal to the S gene, is a large ORF of 428 aminoacids with the potential to encode a polypeptide of 47,000 mol.wt. (Fig. 2). Within the sequence are 8 potential N-glycosylation sites which would increase the apparent molecular weight of the protein to approximately 65,000 (the size of the HE protein). At the amino and carboxy-termini of the polypeptide are stretches of hydrophobic aminoacids which could represent a signal recognition sequence and a membrane anchor region respectively, both typical features for membrane bound proteins.

In order to identify the protein product of the MHV-JHM mRNA 2-1 ORF and to analyse its biological functions we have expressed this ORF using a Vaccinia virus expression system. The construction of the clone BSPMHV2-1/1 which contained the mRNA 2-1 ORF and 7 additional nucleotides upstream of the ATG start codon will be described in detail elsewhere. This cDNA was cloned into the unique BamHI site of the pTF7-5 vector with the ATG start codon next to the T7 RNA polymerase promoter. This construct, pTF7-5/MHV2-1, was used to transfect DBT cells which

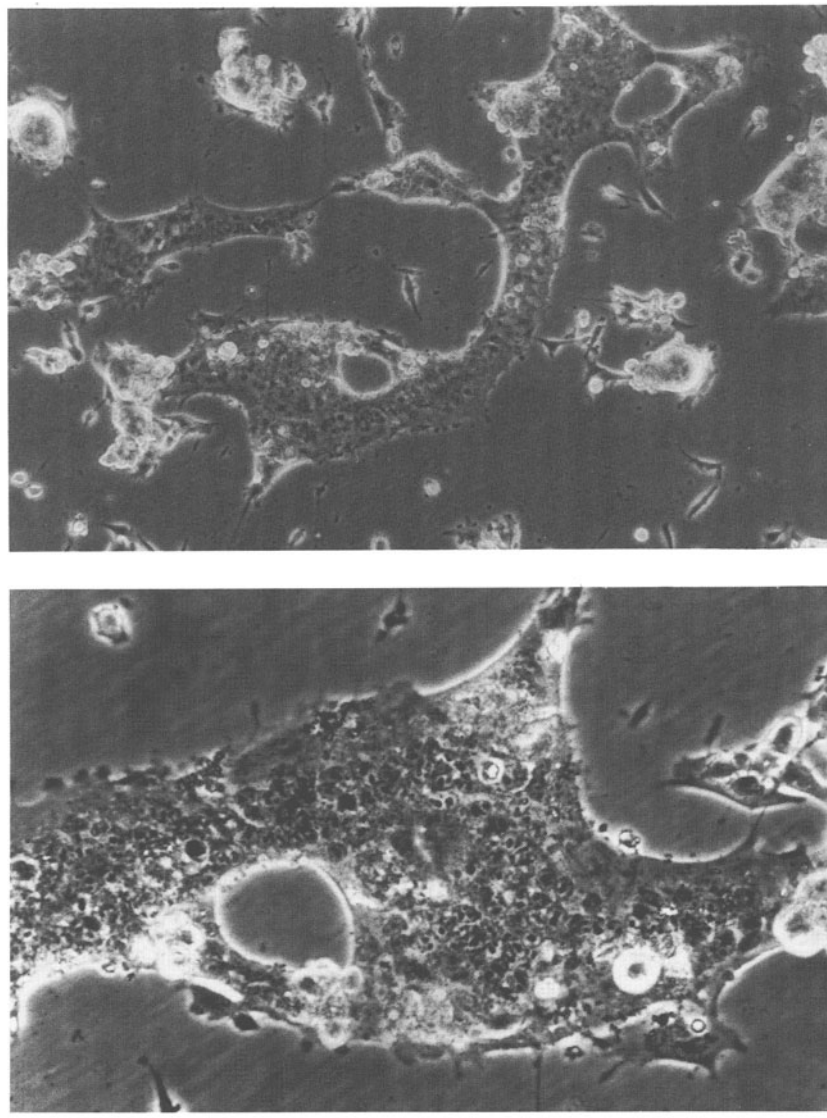

Fig. 1. Fusion activity of the MHV-JHM S protein
2 x 10^6 DBT cells in a 6 cm petri dish were infected with the recombinant Vaccinia Virus vTF7-3 with a moi of 30 pfu/cell. Two hours p.i. the virus was removed and replaced by calciumphosphate coprecipitated pTF7-5/S+ plasmid DNA (20 µg). After 30 min at room temperature medium was added and incubation was continued at 37°C. The monolayers were photographed 10 hours post transfection.

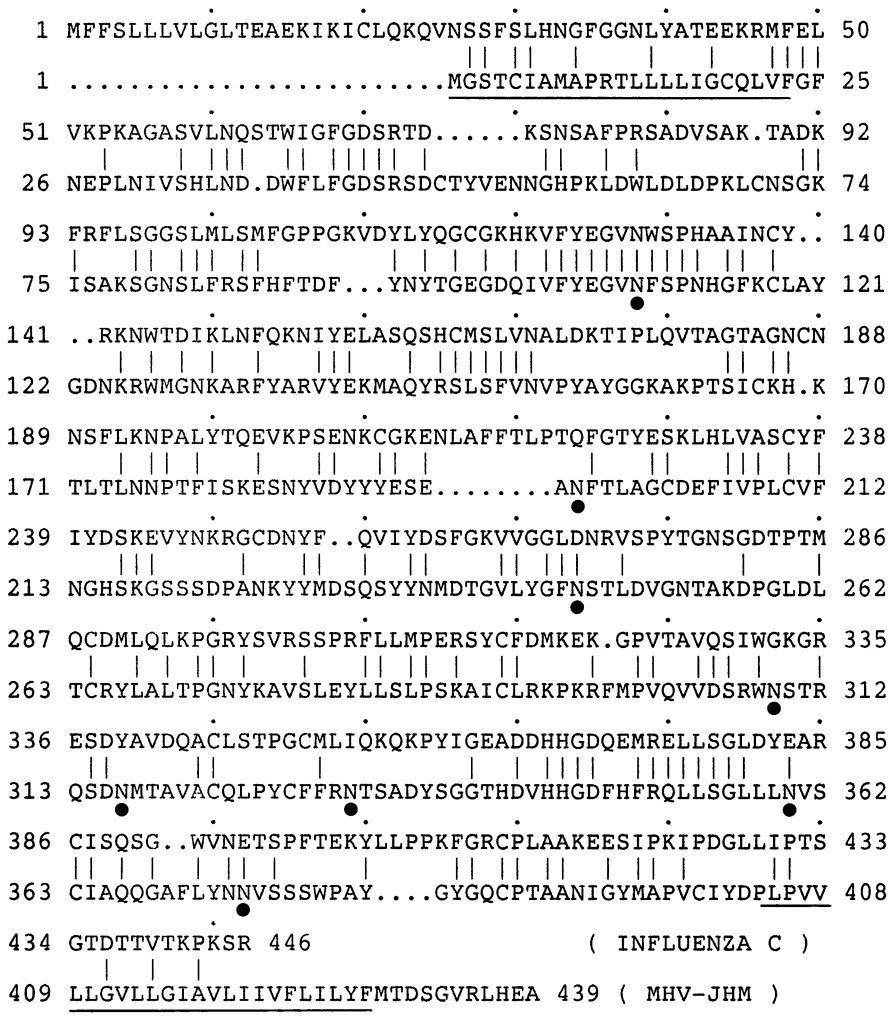

Fig. 2. Nucleotide sequence of the unique region of the MHV-JHM mRNA 2-1.
The figure shows the nucleotide sequence and the deduced amino acid sequence of the MHV-JHM mRNA 2-1 unique region in comparison to the HEF_1 subunit of the Influenza C virus HEF protein (Pfeifer and Compans, 1984). The putative signal recognition sequence and the membrane anchor region of the MHV-JHM polypeptide are underlined. The potential glycosylation sites are marked.

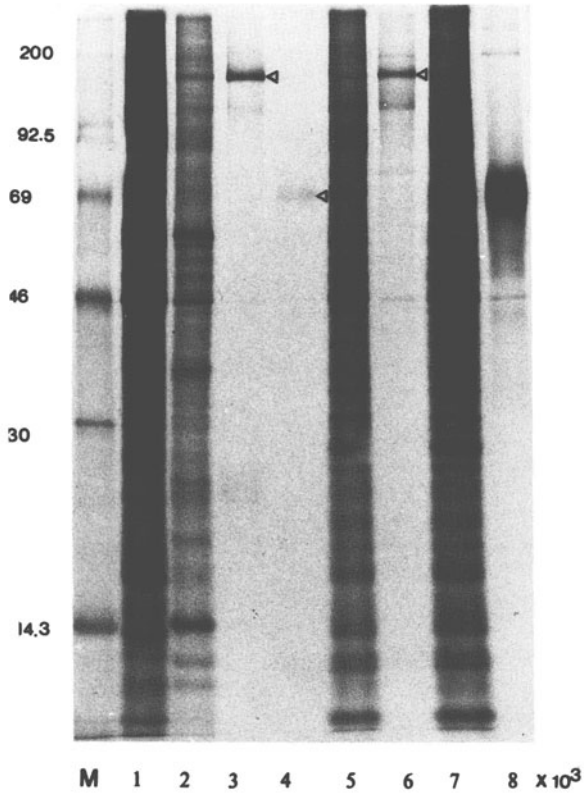

Fig. 3. Expression of the MHV-JHM HE protein using a Vaccinia virus expression system
2×10^6 DBT cells in a 6 cm petri dish were infected with the recombinant Vaccinia virus vTF7-3 with a moi of 30 pfu/cell and transfected with either a pTF7-5/S gene plasmid DNA, or pTF7-5/MHV2-1 plasmid DNA. Six hours after transfection the cells were metabolically labelled with ^{35}S methionine for 60 minutes and then lysed with 200 µl of lysis buffer (Siddell et al., 1981). Alternatively 2×10^6 DBT cells were infected with MHV-JHM with a moi of 5 and lysed 12 hours p.i. 5 µl of lysate were directly loaded on a 15 % SDS polyacrylamide gel, or 50 µl were used for the immunoprecipitation with either HE or S specific monoclonal antibodies (Wege et al., 1984). M: MW marker; lane 1: lysate from uninfected DBT cells; lane 2: lysate from MHV-JHM infected DBT cells; lanes 3 and 4: immunoprecipitation of the lysate from lane 2 with S specific or HE specific monoclonal antibodies; lane 5: lysate from vTF7-3 infected/pTF7-5/S transfected DBT cells; lane 6: immunoprecipitation of the lysate from lane 5 with S specific monoclonal antibodies; lane 7: lysate from vTF7-3 infected/pTF7-5/MHV2-1 transfected DBT cells; lane 8: immunoprecipitation of the lysate from lane 7 with a HE specific monoclonal antibody.

had been infected previously with the recombinant Vaccinia virus vTF7-3. Lysates were made from the infected/transfected cells and immunoprecipitated with an MHV-JHM HE specific monoclonal antibody (kindly provided by Dr. H. Wege). Figure 3, lane 8, shows the immunoprecipitate from vTF7-3 infected cells transfected with the pTF7-5/MHV2-1 plasmid DNA. Comparison with the immunoprecipitate from MHV-JHM infected DBT cells demonstrates that the protein product of the MHV-JHM mRNA 2-1 unique region, expressed via a Vaccinia virus expression system, has the same electrophoretic and antigenic properties as the HE protein from MHV-JHM infected cells.

This result shows that the mRNA 2-1 ORF is the gene for the MHV-JHM HE protein. In addition, the Vaccinia virus system produces far more HE protein than MHV-JHM infected cells. This overexpression allowed the analysis of the biological functions of the protein.

III Analysis of the biological function of the MHV-JHM HE protein

The HEF_1 subunit of the Influenza C HEF protein has an acetylesterase activity which specifically cleaves off the acetate moiety on position 9 from the substrate N-acetyl-9-0-acetyl-neuraminic acid (Herrler et al., 1985). This activity can be analysed using the organic substrate p-nitrophenylacetate (pNPA). Esterases specifically cleave off the acetate moiety from this substrate leaving the product p-nitrophenol. In order to demonstrate the esterase activity of the MHV-JHM HE protein we immunoprecipitated the HE expressed in the Vaccinia virus system with a specific monoclonal antibody and analysed the activity of the purified immunocomplexes. Figure 4 shows that the HE, but not the S protein, has an esterase activity. HE protein immunocomplexes from MHV-JHM infected cells also show esterase activity which is consistent with the low level of expression shown in Figure 3, lane 4.

Since we could demonstrate an HE specific acetylesterase activity, the next question was whether the protein can also recognize and bind to sialic acid containing receptors on the cell surface. Thus we investigated the ability of the HE protein to bind rat erythrocytes, which are known to contain the receptor of the Influenza C virus in large amounts on their surface (G. Herrler, personal communication). DBT cells were infected with the recombinant Vaccinia virus vTF7-3, transfected with either pTF7-5/S plasmid DNA or pTF7-5/MHV2-1 plasmid DNA and assayed for hemadsorption activity.

Fig. 5 clearly demonstrates that only DBT cells which express the MHV-JHM HE protein (2), specifically bind erythrocytes. Uninfected DBT cells (3), or DBT cells which express the MHV-JHM S protein (1) do not have such an activity. This result shows that MHV-JHM has a second glycoprotein on its surface which binds to a specific receptor on the host cell. This second receptor is very similar or identical to that used by the Influenza C virus.

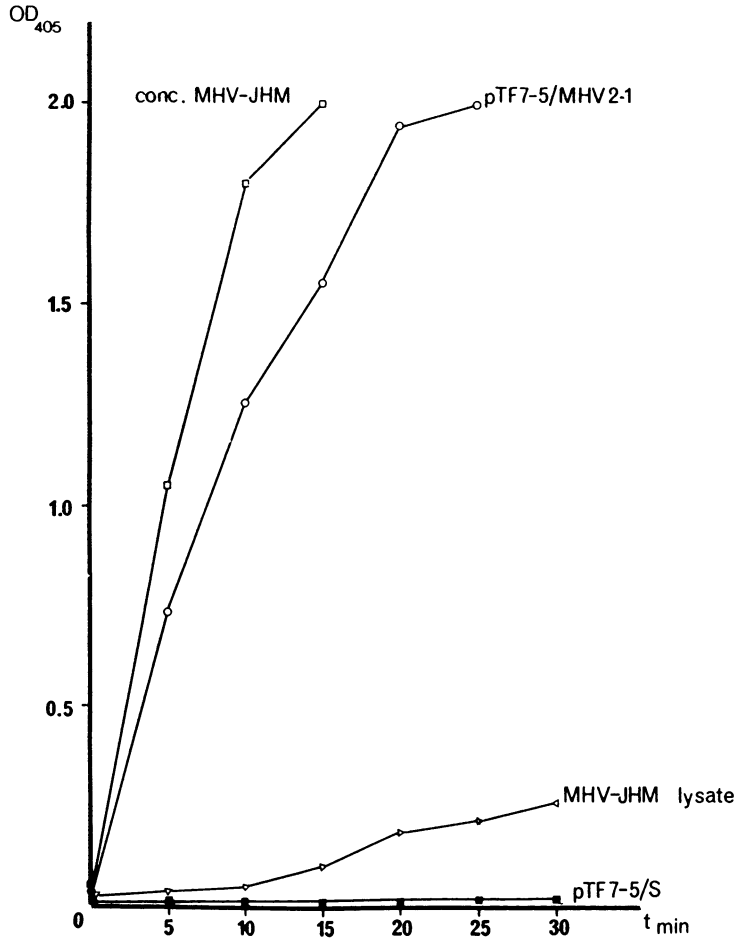

Fig. 4. Esterase activity of the MHV-JHM HE protein
2 x 10^6 DBT cells were infected with the Vaccinia virus recombinant vTF7-3 with a moi of 30 pfu/cell and transfected with either pTF7-5/S plasmid DNA or pTF7-5/MHV2-1 plasmid DNA. 24 hours post transfection cells were lysed with 200 µl of lysis buffer (Siddell et al., 1981). The lysate was immunoprecipitated with a specific monoclonal antibody and 1/10 of the purified immunecomplexes was tested for esterase activity. 10 mg of p-nitrophenylacetate (pNPA) was dissolved in 500 µl ethanol and 100 µl of this solution was mixed with 10 ml PBS (final concentration 200 µg/ml). 20 µl purified MHV-JHM (Siddell et al., 1980) or 20 µl purified HE protein - antibody - protein A Sepharose were mixed with the diluted pNPA solution (1 ml) and the increase of the adsorption at OD_{405} was measured over a period of 30 minutes.

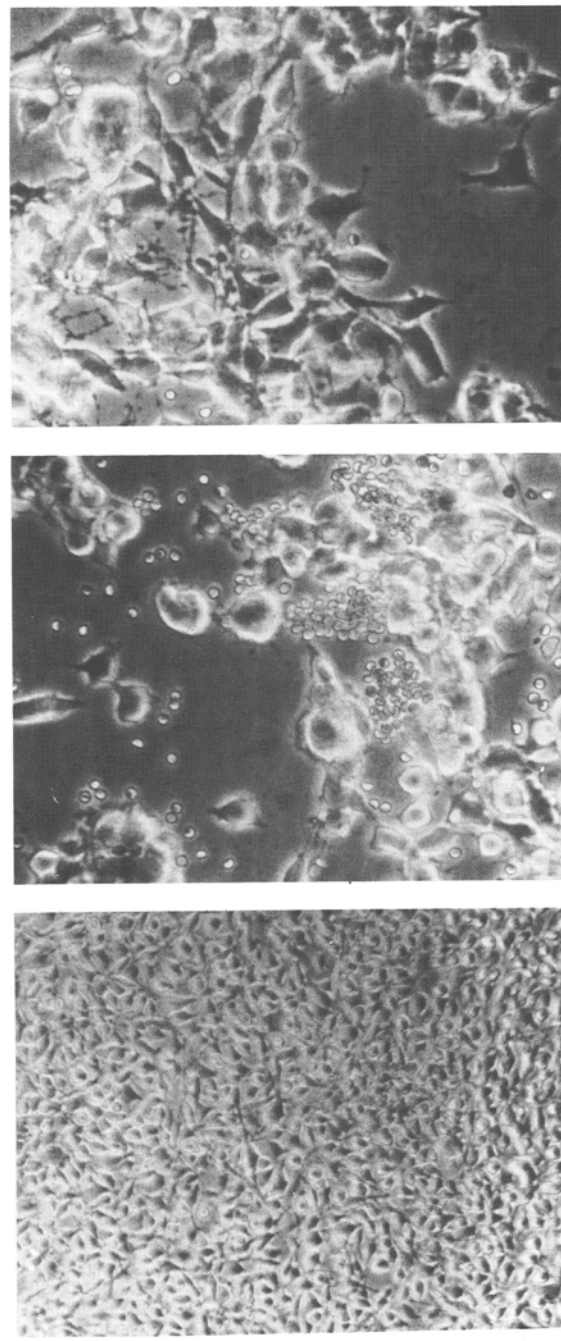

Fig. 5 Hemadsorption activity of the MHV-JHM HE protein
2 x 10^6 DBT cells were infected with the Vaccinia virus recombinant vTF-3 with an moi of 30 pfu/cell and transfected with either pTF7-5/S plasmid DNA or pTF7-5/MHV2-1 plasmid DNA. Monolayers were chilled to 4°C on ice for 30 minutes 6 hours post transfection and incubated at 4°C with 1 ml of a 2 % solution of rat erythrocytes in PBS. Unspecifically bound erythrocytes were washed away with PBS at 4°C

DISCUSSION

Coronavirus MHV-JHM has two surface glycoproteins, the S protein and the HE protein. The experiments reported here, and earlier studies (Wege et al., 1984) suggest that both proteins have the ability to bind to cellular receptor(s). In addition, both proteins have a further biological function which is the fusion activity of the S protein and the esterase activity of the HE protein.

So far three paradigms of fusion have been described. The HA_2 subunit of the Influenza A virus HA protein mediates an acidic pH dependent fusion of viral and cellular membranes. This event is normally restricted to endosomal particles with their acidic pH environment (Wilson et al., 1981). In contrast, a pH independent membrane fusion is mediated by the F_1 subunit of the paramyxoviral F protein (Richardson et al., 1983). Both fusion mechanisms share one common feature, the highly hydrophobic N-terminus of the fusion active subunit.

Some members of the alphavirus group (Sindbis virus and Semliki Forest virus) seem to use a third fusion paradigm. In this case, the fusion event is pH dependent, but the fusion active E1 protein has no hydrophobic N-terminus. A stretch of hydrophobic amino acids, which is found 80 residues away from the N-terminus, implicates an internal rather than an external domain in membrane fusion (Garoff et al., 1980a and b).

It has been shown in this paper, that the MHV-JHM S protein is the fusion active component of the virus. This protein does not require acidic pH conditions to develop its activity (Sturman et al., 1985) and it has no terminal hydrophobic amino acid sequences in its S_2 subunit (Schmidt et al., 1987). Since it has not yet been directly shown that cleavage of the S protein is necessary for MHV-JHM fusion and since some members of the coronavirus family (TGEV and FIPV) (deGroot et al., 1987, 1989) have an uncleaved but fusion active S protein, one can argue that the MHV-JHM S protein mediated fusion activity, like the E1 protein of the alphaviruses, uses one or more internal domains. This idea seems to be supported by recent results obtained by Luytjes et al. (1989) for the closely related MHV-A59.

The second surface glycoprotein of MHV-JHM, the HE protein, has been shown in this paper to have an receptor binding and a receptor destroying (esterase) activity similar to that of the Influenza C virus. The exact role of these two activities for infectivity and pathogenesis of the virus is not yet clear.

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