Expression of the S1 and S2 subunits of murine coronavirus JHMV spike protein by a vaccinia virus transient expression system

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The spike (S) protein of murine coronavirus JHMV, variant cl-2, comprises two polypeptides, N-terminal S1 (with an N-terminal signal peptide) and C-terminal S2 (with a C-terminal transmembrane domain). In order to express these subunits, we constructed three different vaccinia virus transfer vectors (VV-TVs) containing cDNAs encoding the S1 protein without a transmembrane domain (pSFS1utt), the S1 protein with a Cterminal transmembrane domain derived from S2 (pSFS1tmd) or the S2 protein with an N-terminal signal peptide derived from S1 (pSFssS2). The S1 and S2 proteins were expressed in DBT cells by infection with vaccinia virus and transfection of these VV-TVs. In cells transfected with the pSFS1utt and pSFS1tmd, 96K and 106K proteins, respectively, were detected by Western blotting. The ssS2 protein expressed by pSFssS2 was 96K, which was slightly larger than the authentic S2

Introduction

Coronaviruses are enveloped viruses with a positivestrand genomic RNA and are responsible for a variety of acute and chronic diseases of the respiratory, gastrointestinal and neurological systems (Wege *et al.*, 1982; Spaan *et al.*, 1990). The neurotropic strain of murine coronavirus (mouse hepatitis virus, MHV), JHMV, has been studied as an animal model of virus-induced diseases of the central nervous system (ter Meulen *et al.*, 1989).

MHV contains four major structural proteins: the nucleocapsid protein of 50K to 60K, the integral membrane protein of 23K to 25K, the haemagglutinin-esterase protein of 65K and the spike (S) protein (Siddell *et al.*, 1983; Sturman & Holmes, 1983; Spaan *et al.*, 1988). The S protein, which forms the projecting spike on the surface of the virion (Tyrrell *et al.*, 1968; Siddell *et al.*, 1983) is 150K to 180K and is glycosylated cotranslationally in the endoplasmic reticulum (Niemann *et al.*, 1982; Sturman & Holmes, 1985). During virus maturation the *N*-glycosylated oligosaccharides of the S protein undergo specific modifications in the Golgi

protein. The S1utt and S1tmd proteins were shown by binding studies using a panel of monoclonal antibodies to be antigenically indistinguishable from the authentic S1 protein. The S1tmd and ssS2 proteins were detected on the cell surface by immunofluorescence, whereas the S1utt protein was not. However, when the S1utt protein was expressed together with the ssS2 protein, the S1utt was detected on the cell membrane. This suggested that the S1utt was associated with ssS2 on the cell membrane. These observations indicate that the expressed S1 and S2 proteins associated in a similar manner to the authentic S1 and S2 proteins produced in DBT cells infected with cl-2. However, cell fusion was not observed in cells expressing either S1 or S2 nor in cells coexpressing both S1 and S2, although the whole S protein expressed by VV-TV did induce fusion.

apparatus (Niemann *et al.*, 1982). Recent studies have suggested that the coronavirus spike is a homotrimer of the S protein and that this trimerization occurs before transport of the S protein to the medial Golgi apparatus (de Groot *et al.*, 1987; Delmas & Laude, 1990). An additional post-translational modification of the S protein is the proteolytic cleavage into the N-terminal S1 and C-terminal S2 subunits by a host cell protease (Frana *et al.*, 1985; Luytjes *et al.*, 1987; Schmidt *et al.*, 1987). This proteolytic cleavage occurs in the late stage of virus maturation, after transport from the Golgi apparatus (Niemann *et al.*, 1982; Frana *et al.*, 1985; Sturman *et al.*, 1985). The S1 subunit forms the globular head and the S2 subunit forms the stalk region of the corona-like peplomers (de Groot *et al.*, 1987).

Some important biological features of MHV are associated with the S protein. It mediates virus attachment to receptors on susceptible cells (Collins *et al.*, 1982; Williams *et al.*, 1991). It is also the major protein eliciting neutralizing antibodies in animals infected with MHV (Holmes *et al.*, 1981; Collins *et al.*, 1982; Fleming *et al.*, 1983; Wege *et al.*, 1984; Spaan *et al.*, 1988). One of the prominent biological activities of the S protein in





Fig. 1. The structure of the cl-2 S gene (4.3 kb; a), VV-TVs pSFS1utt and pSFS1tmd (b), pSFssS2 (c) and nucleotide and amino acid sequences in the junction region of the manipulated S1 and S2 genes (d). The strategy used to construct these plasmids is described in detail in Methods. utt, Universal translation terminator; ss, signal sequence; tmd, transmembrane domain; cd, cytoplasmic domain; \uparrow , cleavage site.

cultured cells is the induction of polykaryocyte formation by cell fusion (Collins et al., 1982; Sturman & Holmes, 1985). It has been considered that cleavage of the S protein is an important step in fusion activity (Frana et al., 1985; Sturman et al., 1985), which is very similar to the mechanisms of fusion-activation observed in other fusogenic RNA viruses (White, 1990). However, it has recently been found that by expressing uncleaved S proteins the cleavage event is not an absolute requirement for fusion activity (Stauber et al., 1993; Taguchi, 1993). The S protein has been shown to be directly or indirectly implicated in the pathogenicity of MHV (Fleming et al., 1983; Dalziel et al., 1986). However, structural analysis of these biological functions of the S protein, i.e. the active site of these biological activities, is still not complete. In order to investigate the relationship between this function and structure, we have expressed both the entire and a part of the S protein and observed their biological activities.

Methods

Viruses and cells. Murine coronavirus JHM variant cl-2 (Taguchi et al., 1985) and vaccinia virus (VV) strain WR were propagated and assayed using DBT cells and RK13 cells, respectively, as previously described (Taguchi et al., 1992). The growth and maintenance of these cell lines were described elsewhere (Taguchi et al., 1992).

Construction of recombinant plasmids containing the genes encoding the S1 and S2 proteins. In order to express the S1 and S2 proteins, we used cDNA to the S gene of MHV variant cl-2 (Taguchi *et al.*, 1992) shown in Fig. 1(*a*). The plasmid containing the entire cl-2 S gene,

pUC19cl2-S(+), was cut with BamHI and ClaI and a 2.3 kb fragment covering the entire S1 gene was excised from an agarose gel. After Klenow polymerase treatment, this fragment was ligated into the SmaI site of a pUC19 derivative which contains a universal translation terminator (5' GCTTAATTAATTAAGC 3') at the BamHI site in a multiple cloning site (pUC19S1utt). An EcoRI-HindIII fragment (2.3 kb) encoding the S1 gene with a termination codon was cut out from pUC19S1utt and ligated after Klenow fragment treatment into the SmaI site of pSF7.5EB1-B5-12 (pSF) (Funahashi et al., 1991; kindly provided by Dr H. Shida). The resulting plasmid was designated pSFS1utt (Fig. 1b and d). This S1 gene encoded an additional 12 amino acids at the C terminus compared with the authentic S protein as shown in Fig. 1(d). A second S1 gene was constructed to express the S1 protein with a transmembrane domain. From the S gene cDNA, inserted into the pUC119 BamHI site (pUC119c12-S), a 1.6 kb fragment covering approximately 75% of the S2 gene was removed after cutting with ClaI and NdeI. The truncated pUC119c12-S was ligated with a 9 bp DNA fragment composed of synthetic oligonucleotides (5' CGAGGCACA 3' and its complement 5' TATGTGCCT 3') with ClaI and NdeI sites at each end. A plasmid containing the whole S1 gene and nucleotides encoding the transmembrane and intracytoplasmic domains of the S protein (pUC119S1tmd) was obtained. The 2.7 kb S1tmd gene was excised by SmaI digestion from pUC119S1tmd and inserted into the SmaI site of pSF. The resulting plasmid was designated pSFS1tmd (Fig. 1b and d).

To express the S2 protein, we constructed a cDNA with the signal sequence of the S gene positioned at the 5' end of the S2 gene. The plasmid containing the cl-2 S gene in pUC19 (reverse orientation compared with the orientation described above), pUC19cl2-S(-), was cut with *Hin*dIII and *Cla*I and a 4.7 kb fragment containing the S2 gene lacking 18 bases at the 5' end was obtained by agarose gel electrophoresis. The 53-mer oligonucleotides (5' AGCTTATGCTGTT CGTCTTTATTTACTATTACCAGTAGCAGTAGAAACTGAGGGTAA TAGTAAAATAAAGACGAACAGCAATA 3') were synthesized to encode 10 hydrophobic amino acids at the N terminus of the S protein

and six amino acids constituting the N terminus of the S2 protein, which is deleted in the S2 gene constructed in the 4.7 kb fragment. The synthetic DNA had *Hin*dIII and *Cla*I sites at each end. The 4.7 kb fragment and 53-mer DNA were ligated (pUC19ssS2). The pUC19ssS2 was then cut with *Hin*dIII and *Bam*HI and a 2.0 kb fragment containing the ssS2 gene was isolated, treated with Klenow polymerase and inserted into the pSF *Sma*I site. The resulting plasmid was designated pSFssS2 (Fig. 1c and d). All of these plasmids were confirmed to be correctly constructed for the expected S protein-coding regions by dideoxynucleotide chain termination sequencing (Sanger *et al.*, 1977). For expression of the entire S protein of cl-2, we have used the vaccinia virus transfer vector (VV-TV), pSFcl2-S whose construction has been previously described (Taguchi *et al.*, 1992).

Transfection. The VV-TVs, containing the S1 and S2 genes as well as the entire S gene were transfected onto DBT cells using Lipofectin (Gibco BRL) as described previously (Felgner *et al.*, 1987) with some modifications. Briefly, 80% confluent DBT cells in six-well plates or 3 cm Petri dishes (Falcon) were inoculated with VV strain WR at an m.o.i. of 1 and adsorbed at 37 °C for 1 h. Cells were then washed three times with PBS pH 7·2, and cultured with 1 ml serum-free Dulbecco's modified Eagle's medium (DMEM, Nissui). The VV-TVs DNA, usually 2 µg in 12 µl of distilled water, was mixed gently with an equal volume of Lipofectin in a polystyrene tube (Falcon) and left at room temperature for 15 min. This mixture was added to DBT cells cultured in serum-free DMEM and further incubated at 37 °C for 4 h. Then, 1 ml of DMEM supplemented with 20% fetal calf serum (Gibco) was added to the culture. After a further 20 to 25 h culture, the cells were analysed.

Mild-denaturing Western blotting. Proteins expressed by the transfection of VV-TVs in DBT cells were analysed using a mild-denaturing Western blotting as described previously (Cohen et al., 1986; Taguchi, 1993). Cell lysates and medium of DBT cells transfected with the recombinant plasmids were used for the analyses. DBT cells were lysed in PBS containing 0.65 % Nonidet P40 and 2 тмphenylmethylsulphonyl fluoride (Sigma) and the lysates were centrifuged at 12000 r.p.m. for 15 min at 4 °C. The supernatants were used for analysis. Medium from transfected cells (2 ml) was clarified by centrifugation at 3000 r.p.m. for 10 min, and concentrated 10-fold by lyophilization. A portion of these samples was then mixed with an equal volume of sample buffer without DTT (50 mM-Tris-HCl pH 6.8, 1% SDS, 5% glycerol, 0.01% bromophenol blue) and incubated at room temperature for 5 min. These lysates were electrophoresed in a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose paper (Millipore) which was then treated with 25% Block ace (Yukijirushi) at 4 °C overnight. The nitrocellulose papers were incubated with PBS containing monoclonal antibodies (MAbs) 47 and 10G, specific for the S1 and the S2 subunits, respectively (Routledge et al., 1991; Kubo et al., 1993). After washing three times with PBS supplemented with 0.5% Tween 20, the paper was incubated with horseradish peroxidase-labelled anti-mouse IgG (Bio-Yeda) at room temperature for 1 h. After washing four times with the above buffer, the specific binding of MAbs to the expressed proteins was examined by enhanced chemiluminescence (Amersham).

Immunofluorescence assay. DBT cells cultured on cover glasses in Petri dishes were infected with VV and transfected with VV-TVs DNA. About 24 h later, the cells were washed with PBS and fixed with acetone at room temperature for 3 min. The cells were incubated at 37 °C for 1 h with MAb 47 and 10G diluted 1:100 in PBS. After washing three times with PBS, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Cappel) at 37 °C for 1 h and examined under u.v. illumination. For the membrane immunofluorescence, cells infected with VV and transfected with VV-TVs DNA were washed with chilled PBS and reacted with MAbs 47 or 10G at 4 °C for 1 h. After washing three times with chilled PBS, the cells were stained with FITC-conjugated goat anti-mouse immunoglobulin at 4 °C for 1 h and examined as described above.

Results

Analyses of expressed proteins by Western blotting

DBT cells infected with VV and transfected with VV-TVs DNA were lysed after 24 h and the lysates were analysed by Western blotting as described in Methods. For the analyses of the expressed S protein, we used the MAbs 47 and 10G to detect the S1 and S2 subunits, respectively. Since 47 failed to react by standard Western blotting (Kubo *et al.*, 1993), we have analysed the expressed protein by mild-denaturing Western blotting. As shown in Fig. 2(*a*), MAb 47 reacted with proteins in the lysates from DBT cells transfected with pSFcl2-S, pSFS1utt and pSFS1tmd, whose M_r s were 96K, 96K and 106K, respectively. The S1 proteins produced by pSFcl2-S and pSFS1utt were electrophoretically indistinguishable from the authentic S1 protein produced by the cleavage of the S protein in DBT cells infected with MHV variant cl-2.



Fig. 2. Analysis of the expressed S proteins by mild-denaturing Western blotting. DBT cells infected with VV and transfected with pSFcl2-S (lane 2), pSFS1utt (lane 3), pSFS1tmd (lane 4), pSFssS2 (lane 5) or pSF (lane 6) were lysed. DBT cells infected with cl-2 were also lysed (lane 1). The lysates were denatured without DTT in sample buffer at room temperature and electrophoresed in a 10% SDS-polyacrylamide gel. After being blotted onto nitrocellulose paper, the S proteins were detected with S1-specific MAb 47 (a) or S2-specific MAb 10G (b).



Fig. 3. Western blot analysis of the S proteins expressed by cotransfection. DBT cells infected with VV and transfected with pSFcl2-S (lane 1), cotransfected with pSFS1utt and pSFssS2 (lane 2) or cotransfected with pSFS1tmd and pSFssS2 (lane 3) were lysed and the expressed S proteins were detected by MAbs 47 (a) or 10G (b) as described in the legend of Fig. 2.

The S1 protein expressed by the transfection with pSFS1tmd was approximately 10K larger than the authentic S1 protein. This could be accounted for by the 0.4 kb of DNA tagged onto the 3' end of the S1 gene as the transmembrane and intracytoplasmic sequence. In cells transfected with pSFS1tmd, a strong band representing an M_r of about 200K was detected (Fig. 2*a*, lane 4). Because this band was not detectable by standard Western blotting, it is probably a dimer of the expressed S1tmd protein (data not shown). MAb 10G reacted with the proteins produced by transfection with pSFcl2-S and pSFssS2 as shown in Fig. 2(b). The ssS2 protein was slightly larger than the authentic S2 protein. The ssS2 gene was expected to encode an additional 10 amino acid signal peptide at the N terminus as shown in Fig. 1(d), and this peptide is considered to be removed posttranslationally. According to von Heijne's algorithm (von Heijne, 1983, 1986), there is a possibility that the first serine or first serine-valine-serine at the N terminus of the authentic S2 protein is removed. At present, we have no data to explain the larger size of the expressed ssS2 protein.

DBT cells were cotransfected with pSFssS2 together with either pSFS1utt or pSFS1tmd and the expressed proteins were analysed by Western blotting. As shown in Fig. 3, there was no substantial difference in the size of S1 and S2 proteins when they were expressed individually or simultaneously. However, the amount of S1utt protein expressed by itself (Fig. 2*a*, lane 3) was significantly lower than when expressed together with the ssS2 protein (Fig. 3*a*, lane 2). This suggests that most of the S1utt protein synthesized was excreted from the cells, since the S1utt protein lacked a transmembrane domain. The large amounts of S1utt protein detected in cells when expressed together with the ssS2 protein could be explained by the association of the S1utt and ssS2 proteins, which is similar to the association of the S1 and S2 proteins detected in cells infected with cl-2 (Kubo *et al.*, 1993).

The plasmid pSFS1utt was constructed for the expression of the S1 protein without the transmembrane domain and therefore the product was expected to be excreted from cells. The S1tmd protein, in contrast, was expected to associate with membranes, because of its transmembrane domain. However, there was also a possibility that the S1tmd protein would be cleaved at the authentic S protein cleavage site left in the C-terminal region of the protein and that the S1 protein would be excreted. To examine this, 2 ml of culture medium was collected from cells 24 h after transfection with VV-TVs DNAs and the concentrated materials were examined by Western blotting. As shown in Fig. 4, large amounts of S1utt protein were detected in the medium (lane 3)



Fig. 4. Western blot analysis of the S protein secreted into culture media. Cell lysates were prepared from DBT cells infected with VV and transfected with pSFcl2-S (lane 1), pSFS1utt (lane 2) or pSFS1tmd (lane 4). Media were also taken from cultures of cells transfected with pSFS1utt (lane 3) or pSFS1tmd (lane 5). These lysates and media were analysed for S protein by mild-denaturing Western blotting as described in the legend of Fig. 2.

compared with the cell lysate (lane 2), indicating that the majority of S1utt protein was excreted from cells. The S1tmd protein was more abundant in the lysate than in the medium. The size of the S1tmd protein detected in the medium was identical to that in the lysate, 106K, which indicated that the S1tmd protein in the medium was not produced by cleavage. Two major bands of more than 170K found in Fig. 4 are presumably the dimer and trimer of the S1 protein, respectively, since they could not be detected by standard Western blotting (data not shown).

Expression of the S1 and S2 proteins on the cell membrane

The S protein synthesized in MHV infection was shown to be transported onto the cytoplasmic membrane. Hence, we have examined whether the S1 and S2 proteins expressed in our VV transient expression system were distributed on the cell membrane. DBT cells transfected with VV-TVs DNA containing the S1 or S2 gene were incubated for 24 h and examined by indirect membrane immunofluorescence to locate the S protein. DBT cells transfected with pSFS1tmd or pSFssS2, both of which encode proteins with a transmembrane domain, were shown to have S1 and S2 proteins on the cytoplasmic membrane, respectively (Fig. 5*a*, *b*). Cells transfected with pSFS1utt DNA did not show membrane immuno-



Fig. 5. Detection of the S proteins on cell membranes by immunofluorescence. DBT cells infected with VV and transfected with pSFS1tmd (a), pSFssS2 (b), pSFS1utt (c) or cotransfected with pSFS1utt and pSFssS2 (d) were examined for the cell surface S protein as described in Methods with S1-specific MAb 47 (a, c, d) or S2-specific MAb 10G (b).

pSF vector with S gene	MAb†														
	A			В					С						D
	2	7	19	3	6	13	71	93	8	12	47	63	78	85	18
pSFcl2-S pSFS1utt pSFS1tmd pSFssS2	+ + +	++++	+ + +	+ + +	- + + +	+ + +	+ + +	+++++	+++++	+ + + -	++++	- + + -	+ + +	+ + +	+ - - +

Table 1. Reactivities of cl-2 S protein-specific MAbs with the expressed S proteins*

* The various S proteins were expressed in DBT cells infected with VV after transfection with pSF vectors containing various S genes. The reactivities were examined by indirect immuno-fluorescence.

[†] MAbs specific for the S protein of MHV variant cl-2 were classified into four groups (A to D) in terms of their reactivity to various MHV strains (Kubo *et al.*, 1993).

fluorescence (Fig. 5c). When DBT cells were cotransfected with pSFS1utt and pSFssS2 DNA, the S1 protein was detected on the surface (Fig. 5d). This also suggested that the association of the S1utt and ssS2 proteins took place in cotransfected cells.

Antigenicity of the S1 and S2 proteins expressed by VV-TVs

To address the antigenicity of the S1 and S2 proteins transiently expressed by the VV system, we have examined the reactivities towards a panel of MAbs specific for the S protein of cl-2 by indirect immunofluorescence. All 14 MAbs specific for the S1 subunit reacted with the S1utt and S1tmd proteins in a manner similar to the authentic S1 protein. One MAb (no. 18) reacted with the ssS2 protein to the same extent as with the authentic S2 protein (Table 1). These data suggest that the S proteins expressed in our system were antigenically indistinguishable from the authentic S protein.

Cell fusion

DBT cells infected with VV and transfected with VV-TVs DNAs were observed for 48 h after transfection for polykaryocyte formation. The formation of syncytia was seen in cells transfected with pSFcl2-S, appearing at 8 h. Eighty to 90% of cells were involved in the polykaryocytes by 36 h post-transfection. DBT cells transfected with pSFS1utt, pSFS1tmd or pSFssS2 DNA were not fused during the period of observation. Even coexpression of the S1 and S2 proteins by the transfection of pSFssS2 DNA together with pSFS1utt or pSFS1tmd DNA did not induce fusion formation in DBT cells.

Using immunofluorescence analysis, MAb 47 was shown to react with 5 to 10% of DBT cells transfected with pSFS1utt, pSFS1tmd or pSFcl2-S DNA and MAb 10G with 5 to 10% of cells transfected with pSFssS2 or pSFcl2-S DNA. Both MAbs reacted with cells transfected with pSFS1utt or pSFS1tmd DNA together with pSFssS2 DNA. Immunofluorescence analysis also showed that DBT cells transfected with pSFcl2-S DNA were fused, whereas those transfected with other VV-TV DNAs showed no fusion. In such cases cells containing several nuclei were also positively stained (Fig. 6), but the fusion of cells was shown to be due to VV infection, since these were detected amongst DBT cells infected with VV alone.

Discussion

The S protein of coronaviruses has important biological functions (Sturman & Holmes, 1985; Spaan et al., 1988). However, little is known of the relationship between such biological functions and the S protein structure. In order to identify the regions of the S protein structure responsible for its biological functions, it is necessary to express the functional S protein in the absence of other virus-related proteins. Thus, we have previously expressed the entire S protein in cultured mouse cells, using recombinant VV, and showed that the expressed S protein had antigenic and fusogenic properties indistinguishable from those of the authentic S protein (Taguchi et al., 1992; Taguchi, 1993), although the S protein expressed by baculovirus in our laboratory was shown to be similar but not identical to the authentic S protein (Yoden et al., 1989). To study further the regions on the S protein involved in the biological functions, we have expressed the S1 and S2 subunits of the S protein in a system in which they showed no apparent difference in antigenicity and intracellular transport compared with the authentic S proteins. We also found that the S1utt and ssS2 proteins expressed simultaneously were associated with each other on the cell membrane. The S2 protein expressed from the ssS2 construct was slightly





Fig. 6. Detection of the expressed S proteins by immunofluorescence. DBT cells infected with VV were transfected with pSF (a, g), pSFcl2-S (b, h), pSFS1utt (c, i), pSFS1tmd (d, j), pSFssS2 (e, k) or cotransfected with pSFS1utt and pSFssS2 (f, l). These cells were fixed with acetone and examined for the S proteins with S1-specific MAb 47 (a to f) or S2-specific MAb 10G (g to l).

larger than the authentic S2 protein. The larger size of the former may be due to differences in glycosylation compared with the authentic S2 protein.

In the present study, we tried to determine whether the fusion site was on S1 or S2, or situated on both of these subunits. However, the expression of S1 and S2 independently did not induce fusion. Since a lectin, wheatgerm agglutinin (WGA), has been reported to act as a substitute for the haemagglutinin-neuraminidase (HN) protein of paramyxovirus in cell fusion normally induced by the collaboration of the HN and fusion proteins (Hsu et al., 1979), we have tested whether WGA could induce fusion in cells expressing S1 or S2. However, no fusion was found (data not shown). Even coexpression of the S1 and S2 subunits did not induce fusion. It had been expected that fusion might result from co-expression of S1 and S2, since when the entire S protein was expressed fusion was induced in DBT cells and the fusogenic activity of the S protein was enhanced by the cleavage of the S protein (Taguchi, 1993). At present, we have no data to account for the lack of fusion by the expressed S1 and S2. However, one possibility is that differences in the processing of ssS2 compared with that of authentic S2, as revealed by the difference in M_r , account for the non-fusogenic feature of the expressed S proteins. Alternatively, the fusogenic activity of the S protein might occur after processing of the uncleaved S protein and cleavage of the S protein at a late stage of virus maturation only enhances this activity. Cleavage of the S protein is not a prerequisite for fusogenic activity but facilitates it (Stauber *et al.*, 1993; Taguchi, 1993).

The S2 subunit has been suggested to be involved in fusion activity from an analysis of a mutant variant of MHV with pH-dependent fusion induction. A mutation in the hydrophobic domain in the S2 heptad repeat accounted for the difference in fusion activity compared with that of wild-type MHV (Gallagher et al., 1991). MAb mapping revealed domains in the S2 subunit that were associated with the fusion activity (Collins et al., 1982; Luytjes et al., 1987). It has also been reported from studies using MAbs specific for either S1 or S2 that both of these subunits may be involved in the fusion activity (Weismiller et al., 1990; Routledge et al., 1991). Our own studies using MAbs suggested that the S1 subunit may be responsible for fusion (Kubo et al., 1993). Since some of the MAbs prevented fusion without neutralizing the infectivity of recombinant VV containing the cl-2 gene, the epitopes recognized by these MAbs were possibly

directly involved in fusion activity. All of these data are suggestive that both the S1 and S2 subunits are necessary for fusion to occur. An experiment to locate the active site of fusion on the S2 subunit has been done with bovine coronavirus (BCV); the S2 protein expressed in insect cells by recombinant baculovirus was reported to induce fusion in the absence of the S1 subunit (Yoo et al., 1991). This result differs from our own. The fundamental difference observed between these two systems is that the fusion induced by S2 of BCV is pH-dependent, i.e. fusion formation was restricted under acidic conditions, whereas the fusion induced by the S protein of MHV occurred in a wide range of pH (Gallagher et al., 1991). As was reported by Yoo et al. (1991), the considerable difference in glycosylation observed between insect cells and mammalian cells (Yoo et al., 1990) may result in the difference in fusogenicity of the S proteins in these cells.

We thank Dr S. Siddell for providing MAbs and reading the manuscript and Dr H. Shida for providing pSF7.5EB1-B5-12. We also thank Drs A. Yamada, K. Tanabayashi and S. Y. Takase for their helpful suggestions.

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(Received 23 April 1993; Accepted 2 July 1993)