# Fusion Formation by the Uncleaved Spike Protein of Murine Coronavirus JHMV Variant cl-2

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The fusogenic properties of the uncleaved spike (S) protein of murine coronavirus JHMV variant cl-2 were studied by expressing the S protein with a deleted putative cleavage site. The amino acid sequence of the putative cleavage site, Arg-Arg-Ala-Arg-Arg, was replaced by Arg-Thr-Ala-Leu-Glu by in vitro mutagenesis of the cl-2 S protein cDNA. Recombinant vaccinia viruses containing the cl-2 S cDNA [RVV t(+)] or the mutated cDNA [RVV t(−)] were constructed and monitored for fusion formation and cleavage of the expressed S proteins. When cultured DBT cells were infected with RVV t(+) at a multiplicity of infection of 0.5, fusion formation was first observed at 10 to 12 h postinoculation and spread throughout the whole culture by 20 to 24 h postinoculation. In cells infected with RVV t(−) under the same conditions, fusion formation appeared by 12 to 14 h. This result represented a 2- to 4-h delay in the onset of fusion, compared with its appearance in cells expressing the wild-type S protein. By 25 to 30 h, most of the cells infected by RVV t(−) had fused. By immunoprecipitation and Western blotting (immunoblotting), the 170-kDa S protein was detected in DBT cells expressing the wild-type S protein and the mutated S protein. However, interestingly, the cleavage products of the S protein, S1 and S2, were not detected in RVV t(−)-infected cells, producing the mutated S protein, even though fusion was clearly visible. Both products were, of course, detected in RVV t(+)-infected DBT cells, producing the wild-type S protein. The same results concerning the fusion formation and cleavage properties of the S proteins were reproduced by the transiently expressed S proteins. These results suggest that the cleavage event in the S protein of murine coronavirus JHMV is not a prerequisite for fusion formation but that it does facilitate fusion formation.

Coronaviruses are enveloped viruses with single-stranded, positive-sense genomic RNA that is 26 to 31 kb in length (15, 29). They are important respiratory, neurologic, and enteric pathogens for humans and domestic animals. The virions, 60 to 170 nm in size, contain two species of transmembrane glycoprotein, the spike (S) protein and the integral membrane (M) protein. In some coronaviruses, a third membrane glycoprotein, the hemagglutinin-esterase (HE) protein, is also found. Inside the virion envelope is the nucleocapsid, which is composed of genomic RNA and multiple monomers of the nucleocapsid (N) protein (28, 29).

The S protein, comprising the peplomer on the virion surface, is 150 to 180 kDa, depending on the virus (21, 28, 29, 32). This protein is multifunctional (6, 11, 28, 29). It is known that the S protein is involved in an initial step of virus infection by binding to the receptor on susceptible cells (5, 41). Fusion of infected cells into polykaryocytes has also been shown to be dependent on the S protein (1, 29, 36, 37). In addition, the major neutralizing epitopes exist on the S protein, and it is recognized by cytotoxic lymphocytes (13). Furthermore, speculation is that the S protein is, if not totally, at least mostly involved in the virulence of the virus (2, 6, 7, 19, 38). However, correlation of these biological activities with the S protein structure has not been clarified, except for some neutralizing epitopes (17, 23).

Fusion formation occurs in cells infected with orthmyxoviruses (14, 40), paramyxoviruses (12, 24), retroviruses (16, 20), and other enveloped RNA viruses (40). In these viruses, glycoproteins protruding from the envelope are responsible for fusion activity (40). These glycoproteins on the virion surface are cleaved by a host cell-derived proteolytic enzyme that activates the fusogenic potential of the proteins (12, 16, 40). Proteolytic cleavage of the proteins places the

hydrophobic amino acid cluster at the N terminus of the membrane-anchoring chain of the cleavage product (40). The hydrophobic amino acid cluster is designated the fusion peptide and may work as the fusion active site (40).

Cells infected with murine coronaviruses are usually fused (28, 29), and it is clear that the S protein is not only important but also sufficient for fusion formation, since the S protein expressed by various vectors can induce fusion formation (10, 33, 36). In addition, it has been reported that a cleavage event is also important for the manifestation of the fusogenic properties of the S protein (8, 30, 31), as in the cases of orthomyxoviruses and paramyxoviruses (40). Cleavage of the S protein is supposed to be carried out by a host cell-derived trypsin-like proteolytic enzyme. In the case of murine coronavirus JHMV, the putative cleavage site is composed of the basic amino acid cluster Arg-Arg-Ala-Arg-Arg (22, 25, 33). On the other hand, it has been reported that cleavage product S2 has no hydrophobic amino acid cluster that might function as a putative fusion peptide (22, 25, 29, 33). This situation differs from those of other viruses with fusion activity (40). This finding suggests that the mechanisms of coronavirus fusion formation may be different from those of the orthomyxoviruses and paramyxoviruses. For delineation of the coronavirus fusion mechanism, it is important to determine whether S protein cleavage is required for S protein fusion formation.

In this report, fusion formation by cleaved and uncleaved S proteins, expressed in mouse DBT cells by recombinant vaccinia viruses (RVVs) or in a transient expression system, was studied. The results suggested that cleavage of the S protein is not a prerequisite for fusion formation but that it does somehow enhance or facilitate fusion formation.

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        R   R   A   R   R ▾ S   V                    Cla I
   GTCA CGC AGA GCC CGC CGA TCA GTT TCTACTGGCTATCGATTAACCACA

          ↓       ↓   ↓
   ---- --- -C- --- -T- -A- --- --- ------------------------
          R   T   A   L   E   S   V

   3' CAGT GCG TGT CGG GAG GTT AGT CAA AGATGACCGATAGCTAATTGGTGT 5' : JHM-TRY(-)
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FIG. 1. Mutating oligonucleotide [JHM-Try(−)] used to replace the putative proteolytic cleavage site in the wild-type S protein. The amino acid sequence and nucleotide sequence in the vicinity of the cleavage site in the wild-type S protein are shown in the first and second lines from the top, respectively. The third line shows the three substituted nucleotides. The fourth line shows the amino acid sequence of the mutant S protein generated by PCR mutagenesis; this protein was expected not to be proteolytically cleaved. The fifth line shows the negative-sense mutating oligonucleotide sequence. JHM-Try(−), which was used to obtain the mutant S protein. Note that at the 5′ end of the oligonucleotide there is a ClaI site.

## MATERIALS AND METHODS

**Viruses and assay of infectivity.** Murine coronavirus JHMV variant cl-2, originally isolated from the brain of a rat (34), was propagated in DBT cells and used as a wild-type virus. The infectivity of cl-2 was determined by a plaque assay on DBT cells as previously reported (35). A. Kojima of the National Institute of Health, Tokyo, Japan, provided the WR strain of vaccinia virus with a hemagglutinin (HA)-positive phenotype [VV-HA(+)]; this virus was used to obtain RVVs. A vaccinia virus with an HA-negative phenotype [VV-HA(−)], provided by H. Shida, Kyoto University, was also used. These viruses were tested in a plaque assay to determine infectivity on RK 13 cells. DBT cells and RK 13 cells were grown in Dulbecco's modified minimal essential medium supplemented with 7 to 10% fetal calf serum.

**Oligonucleotide-directed site-specific mutagenesis and preparation of RVVs.** The cDNA of the cl-2 S gene, obtained as previously reported (33), was used as a target for site-specific mutagenesis by use of the polymerase chain reaction (PCR). To replace the nucleotide sequence for the putative cleavage site, Arg-Arg-Ala-Arg-Arg, we prepared by using a gene assembler (Pharmacia) a 49-mer synthetic oligonucleotide, JHM-Try(−), 5′TGTGGTTAATCGATAGCCAGTAG AAACTGATTGGAGGGCTGTGCGTGAC3′, which is complementary to the genome sense. JHM-Try(−) has a nucleotide sequence that encodes Arg-Thr-Ala-Leu-Glu in place of the putative cleavage site and that has a ClaI site at the 5′ end (Fig. 1). Another oligonucleotide, the complement of JHM-Try(−) in PCR, was S-P (1592), 5′TACACGTGT GAGTGCACTTG3′, which is genomic sense and corresponds to the nucleotide sequence from 1592 to 1611, located about 650 nucleotides upstream from the region of the JHM-Try(−) sequence. After amplification with this pair of primers and cl-2 S cDNA as a template, the fragment was cut with ClaI and BalI, and the resulting 443-nucleotide fragment was inserted into pUC19 containing cl-2 S cDNA (pUC19-cl2S) with a deletion of the ClaI-BalI fragment. The cDNA with the mutated fragment, pUC19-cl2S-t(−), was selected by hybridization with oligonucleotide t(−) 20m, 5′AAACTGATTGGAGGGCTGTG3′, which specifically bound to the mutated sequence, and finally confirmed by nucleotide sequencing by dideoxynucleotide chain termination. pUC19-cl2S-t(−) was then cut with BamHI, and cl2S-t(−) was isolated and treated with T4 DNA polymerase to yield blunt ends. The blunt-ended fragment was inserted into transfer vector pSF7.5EB1-B5-12 (pSF) (9), provided by H. Shida, and cut with SmaI to make RVVs. An RVV with cl-2

S cDNA containing the putative cleavage site, RVV t(+) (33), and that with mutated S cDNA, RVV t(−), were prepared as previously reported (9, 33). The nucleotide sequence of the putative cleavage site of RVV t(+) and the mutated site of RVV t(−) were amplified by PCR with a pair of primers, P8, 5′ATTTCCCGTGAGGAAAAACCC3′, which corresponded to nucleotides 2162 to 2181, and S-N (2460), 5′CACCTTGGG GAGCCCTTATC3′, which corresponded to the negative-sense nucleotide sequence from 2479 to 2460. These were prepared on the basis of registered sequence data (33). The fragments were inserted into pUC19, and the areas around the putative cleavage sites were sequenced.

**Transient expression.** DBT cells cultured in six-well plates (Falcon) were infected with VV-HA(+) at a multiplicity of infection (MOI) of 0.3 to 0.5, and 10 to 12 h later, 1 μg of pSF containing cl2S-t(+) [pSFt(+)] or cl2S-t(−) [pSFt(−)] or vector pSF was transfected by calcium phosphate coprecipitation (18). Three to 4 h later, the cells were treated with a glycerol solution (15% glycerol in N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid [HEPES]-buffered saline) and cultured with Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum.

**Immunoprecipitation.** DBT cells were infected with RVV t(+), RVV t(−), or VV-HA(−) at an MOI of 0.3 to 0.5 and incubated for 14 h. The medium was then changed to Dulbecco's modified minimal essential medium without methionine, and the culture was incubated for 30 min. For 30 min of pulse-labeling, 20 to 50 μCi of [$^{35}$S]methionine (EXPRE$^{35}$S$^{35}$S-protein labeling mix; NEN) was added. The cells were collected from petri dishes, lysed with lysing buffer containing 0.65% Nonidet P-40 and 2 mM phenylmethylsulfonyl fluoride (Sigma) in phosphate-buffered saline (pH 7.2) (PBS), and immunoprecipitated. For chase-labeling, [$^{35}$S]methionine was removed from the medium after 30 min of incubation, and the cells were further cultured for 1, 2, or 3 h, collected, and lysed. The lysates were immunoprecipitated with a mixture of monoclonal antibodies (MAbs) specific for cl-2 S protein (unpublished data), and the precipitates were analyzed by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (27, 34).

**Western blotting (immunoblotting) analysis.** DBT cells cultured in six-well plates were transfected with pSFt(+), pSFt(−), or vector pSF after VV-HA(+) infection as mentioned above and collected at intervals. DBT cells infected with RVV t(+), RVV t(−), or VV-HA(−) at an MOI of 0.3 to 0.5 were also collected at intervals after infection. These cells were washed once with chilled PBS and lysed with lysing buffer. The lysed samples were centrifuged at 15,000 rpm for 10 min at 4°C in Eppendorf tubes, and the supernatants were used for Western blotting analysis. For Western blotting, the lysates were boiled in sample buffer (60 mM Tris-HCl [pH 6.8], 0.1 M dithiothreitol [DTT], 2% SDS, 10% glycerol, 0.01% bromophenol blue) and electrophoresed in a 10% SDS-polyacrylamide gel as previously reported (27, 34). Alternatively, under milder denaturing conditions, the lysates were treated with sample buffer lacking DTT at room temperature for 3 min and electrophoresed. The gel was then treated with a buffer containing 0.1 M Tris, 0.192 M glycine, and 20% methanol for 15 to 20 min, and the gel proteins were electrically transferred to nitrocellulose paper by use of a horizontal blotting apparatus (Atto, Tokyo, Japan). After blotting was done, the paper was blocked with Block Ace (Yukijirushi, Tokyo, Japan) for 1 h at room temperature and treated with MAbs specific for JHMV S protein (23); these MAbs were provided by S. G. Siddell. After being washed

with PBS containing 0.1% Tween 20, the paper was treated with anti-mouse immunoglobulin G labeled with horseradish peroxidase (Bioyeda, Rehovot, Israel) and then analyzed by enhanced chemiluminescence (Amersham).

**Fusion.** DBT cells were infected with different RVVs at an MOI of 0.3 to 0.5, and at intervals cells were fixed with ethanol. After cells were stained with Giemsa stain, the rate of cell fusion was calculated by counting the number of cells relative to the number of nuclei.

## RESULTS

**Removal of the endoproteolytic cleavage site of the S protein.** The amino acid sequence predicted to be involved in cleavage by the host cell-derived proteolytic enzyme is Arg-Arg-Ala-Arg-Arg. This sequence encompasses amino acids 765 to 769, and the cleavage could occur between amino acids 769 and 770 (Fig. 1). This basic amino acid cluster is very sensitive to cleavage by trypsin-like enzymes. Replacing it with Arg-Thr-Ala-Leu-Glu is predicted to confer resistance to trypsin-like enzymes. Therefore, a mutant with this uncleavable signal was created to test the effect of S protein cleavage on cell fusion. The strategy for site-directed mutagenesis, which made use of PCR, is described in detail in Materials and Methods. In brief, two oligonucleotides were used to insert the mutation; one was a complementary sequence encoding a mutated amino acid sequence with a three-nucleotide substitution (Fig. 1). After amplification by PCR, a 443-nucleotide fragment was obtained by cutting with ClaI and BalI. This was fragment inserted in the wild-type S gene where the corresponding wild-type BalI-ClaI 443-nucleotide fragment had been. The mutated S gene, expected to encode the uncleavable S protein, was then inserted into transfer vector pSF for observation of transient expression as well as to obtain RVVs. Finally, RVVs with the wild-type S gene [RVV t(+)] and the mutated S gene [RVV t(−)] were isolated. The nucleotide sequences of the mutated site of the mutant S gene as well as the corresponding site of the wild-type S gene in RVVs were confirmed by sequencing after PCR amplification.

**Fusion formation by wild-type and mutated S proteins expressed by RVVs.** DBT cells were infected with RVV t(+) to produce the wild-type S protein or RVV t(−) to produce the mutated S protein at an MOI of 0.3 to 0.5 and examined for the presence or absence of fusion. Fused cells first appeared at about 8 to 10 h postinoculation (p.i.) with RVV t(+). About 50% of cells were fused at 12 to 14 h p.i. By 18 to 20 h p.i., more than 90% of cells were involved in fusion. In DBT cells infected with RVV t(−) under the same conditions, fusion was first observed at 10 to 12 h p.i. Thereafter, fusion developed in the same way as for RVV t(+) (Fig. 2), except for a 2- to 4-h delay in appearance and development. This result is clearly shown in a comparison of RVV t(−) and RVV t(+) kinetics of fusion formation in Fig. 3. The fusion produced by RVV t(+) and RVV t(−) was due to S protein expression, on the basis of the fact that fusion formation was prevented by MAbs specific for the cl-2 S protein (data not shown). Moreover, the fused cells were revealed to express S proteins by immunofluorescence. These data clearly showed that the wild-type S protein as well as the mutated S protein containing Arg-Thr-Ala-Leu-Glu in place of Arg-Arg-Ala-Arg-Arg induced fusion in DBT cells.

Fusion was observed previously in some cell lines infected with VV-HA(−) (26). This result suggests that the fusion observed in DBT cells infected with RVVs was due to the

intrinsic fusion activity of VV-HA(−) and not to the fusogenic properties of the S protein, because all of the RVVs had been selected on the basis of the HA-negative phenotype (9, 33). However, fusion was hardly detectable in DBT cells infected with VV-HA(−). For exclusion of the possibility that VV-HA(−) influences fusion, S proteins were transiently expressed by the transfection of pSFt(+) or pSFt(−) together with infection by VV-HA(+). DBT cells were infected with VV-HA(+) at an MOI of 0.5, and at 10 h p.i., the cells were transfected with pSFt(+) or pSFt(−). Thereafter, fusion formation was checked every 6 h for 24 h. In this system, DBT cells producing the wild-type S protein as well as those producing the mutated S protein were shown to be fused. This fusion was the same as that during RVV infection; fusion by the wild-type S protein was first observed at about 6 h posttransfection, while that by the mutated S protein was detected at 8 to 9 h posttransfection. The appearance and development of fusion by the expressed, mutated S protein was delayed 3 to 4 h compared with that by the wild-type S protein (Fig. 4).

**Analysis by immunoprecipitation and Western blotting of wild-type and mutated S proteins expressed by RVVs and the transient expression system.** RVV t(+) and RVV t(−) as well as VV-HA(−) as a control were used to infect DBT cells at an MOI of 0.5. At 14 h after infection, when about 50% of RVV t(+)-infected cells and 20% of RVV t(−)-infected cells were fused, the cells were pulse-labeled with [$^{35}$S]methionine for 30 min. This is enough time to detect the precursor of the S protein, which has an approximate molecular weight of 170,000. Thereafter, the labeled proteins were chased for 1 to 3 h to detect S protein cleavage products. MAbs specific for the cl-2 S protein precipitated only one band of about 170 kDa from the lysates of pulse-labeled DBT cells infected with either RVV t(+) or RVV t(−) (Fig. 5). S1 and S2 cleavage products were detected in the chase-labeled samples from RVV t(+)-infected cells, but no such bands were found in the chase-labeled samples from RVV t(−)-infected cells. The infected cells were labeled for only 30 min, a time that may have been too short for the sufficient incorporation of [$^{35}$S]methionine. Therefore, the presence of S1 and S2 was detected by the more sensitive technique of Western blotting with enhanced chemiluminescence. At intervals after RVV infection, lysates were prepared and analyzed by Western blotting. As shown in Fig. 6, both the 170-kDa S protein and its cleavage product S2 were observed in RVV t(+)-infected DBT cells. In contrast, only the 170-kDa S protein was detected in RVV t(−)-infected DBT cells. The S1 subunit was faintly detected in cells infected with RVV t(+). The low level of detection of S1 compared with that of S2 may have been due to the MAbs used for the Western blots. To improve detection, we ran Western blots after mild denaturation of the lysates at room temperature without DTT. After this treatment, the MAbs recognizing the conformational epitopes were still able to react with the protein. By this method, the S protein as well as its cleavage products were clearly shown in DBT cells infected with RVV t(+), while no cleavage products were detected in DBT cells infected with RVV t(−). In cells infected with RVV t(−), a strong band with an approximate molecular weight of 200,000 was visible. At present, little is known about this protein, except that it consists of uncleaved S protein, including epitopes existing on S1 and S2 subunits. It may be a differentially glycosylated S protein, because of the lack of a cleavage event.

The S protein and its cleavage products were also examined in a transient expression system (Fig. 7). As was
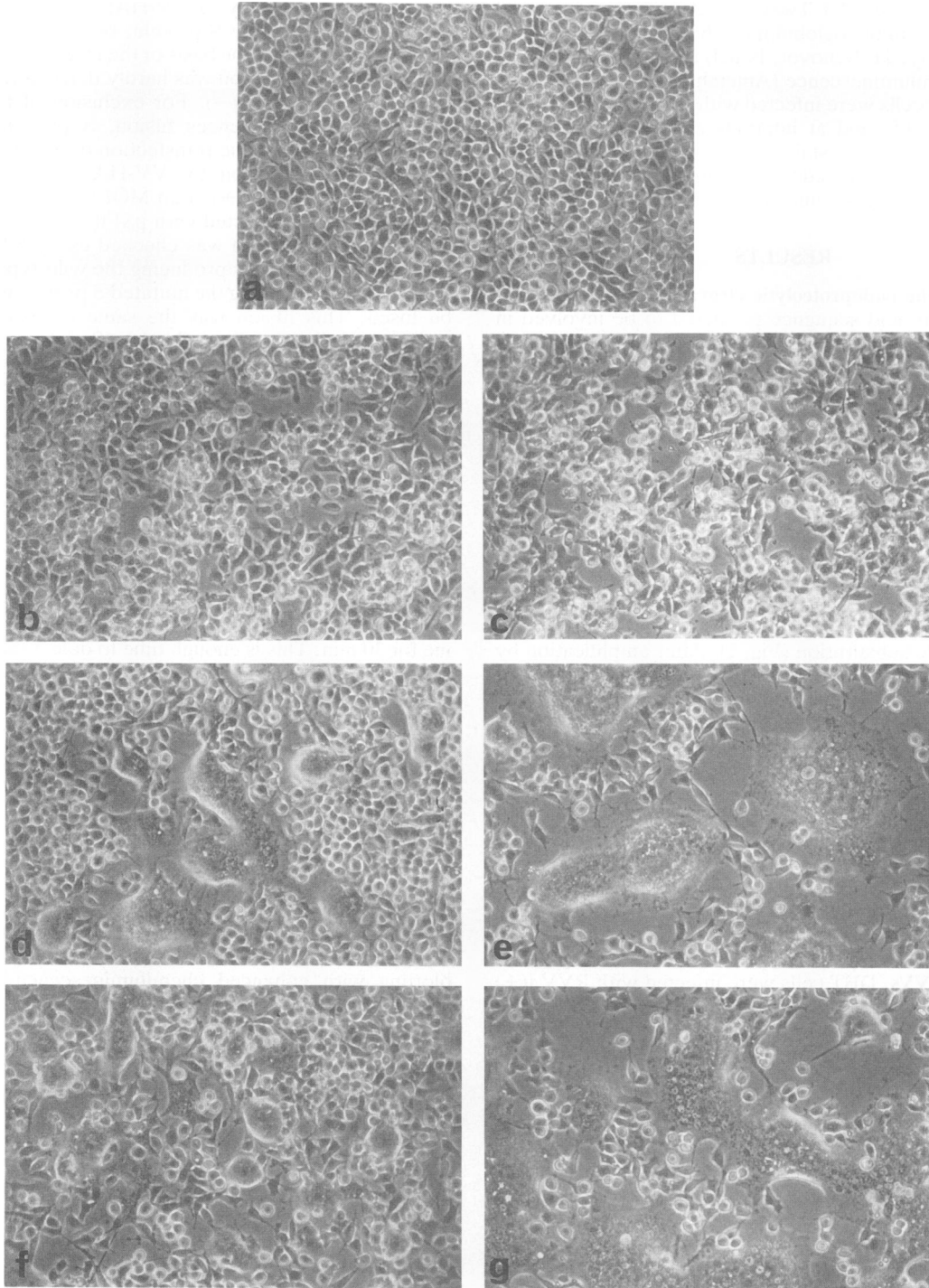
FIG. 2. Fusion formation by RVV t(+), RVV t(−), or VV-HA(−). DBT cells were mock infected (a) or infected with VV-HA(−) (b and c), RVV t(+) (d and e), or RVV t(−) (f and g) at an MOI of 0.5 and observed microscopically at 11 h (a, b, d, and f) or 15 h (c, e, and g) p.i.

observed in the infection with RVVs, cleavage of the S protein was detected in cells producing the wild-type S protein but not in cells expressing the mutated S protein. These results indicated that the mutated S protein was much more resistant to proteolytic cleavage by host cell-derived enzymatic activity than the wild-type S protein, with its predicted cleavage site.

## DISCUSSION

Fusion formation observed in cells infected with murine and other coronaviruses (28, 29) is mediated by the S protein, as evidenced by the facts that S protein-specific MAbs are capable of inhibiting fusion formation (1, 37) and that the expression of the S protein in the absence of other
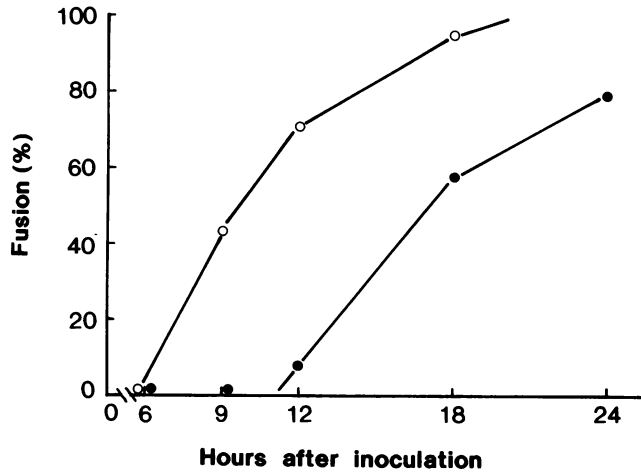
FIG. 3. RVV t(+) and RVV t(−) kinetics of fusion formation in DBT cells. DBT cells were infected with either RVV t(+) (○) or RVV t(−) (●) at an MOI of 0.5. At intervals, the number of cells involved in fusion were calculated after staining with Giemsa stain.



FIG. 5. Analysis by immunoprecipitation of wild-type and mutated S proteins expressed in DBT cells by infection with RVV t(+) or RVV t(−). Lysates were prepared from DBT cells infected with RVV t(+) (lanes 3, 4, 5, and 6), RVV t(−) (lanes 7, 8, 9, and 10), or VV-HA(−) (lanes 11, 12, 13, and 14) at an MOI of 0.5. Cells were pulse-labeled at 14 h p.i. for 30 min (lanes 3, 7, and 11), and the labeled protein was chased for 1 h (lanes 4, 8, and 12), 2 h (lanes 5, 9, and 13), or 3 h (lanes 6, 10, and 14). The lysates were immunoprecipitated with a mixture of MAbs specific for the cl-2 S protein, and immunoprecipitates were electrophoresed in a 10% SDS-polyacrylamide gel. Lanes 1 and 2 show the immunoprecipitates of pulse-labeled (lane 1) or chase-labeled (lane 2) samples of a cl-2-infected DBT cell lysate. M, molecular weight markers. The arrowhead in lane 1 shows the 170-kDa S protein, and the arrowheads in lane 2 indicate S1 (96-kDa) and S2 (86-kDa) cleavage products.

virus-related proteins is able to induce fusion formation (10, 33, 36). The cleavage event has been suggested to be an important step for fusion formation, as indicated by experiments in which mouse hepatitis virus, with a fusion-negative phenotype, was rendered capable of fusion after treatment with proteolytic enzymes (30, 31, 42). Also, it has been reported that a correlation exists between the degree of cleavage of the S protein and the extent of fusion in different virus-cell systems (8). All of these data suggest that cleavage of the S protein is a prerequisite for fusion formation by the
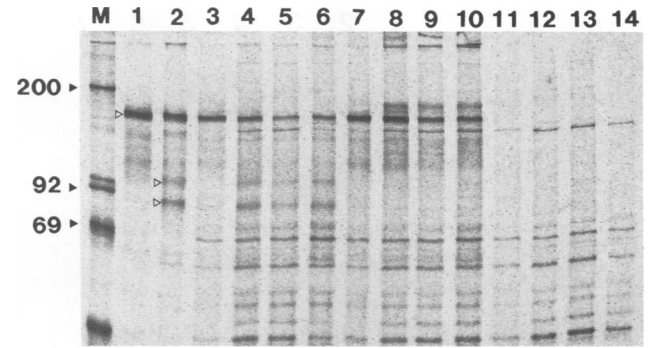
S protein of murine coronaviruses. This mechanism is analogous to the mechanisms of fusion formation in paramyxoviruses (12, 24), orthomyxoviruses (14, 40), and retroviruses (16, 20). The results presented in this paper, however,
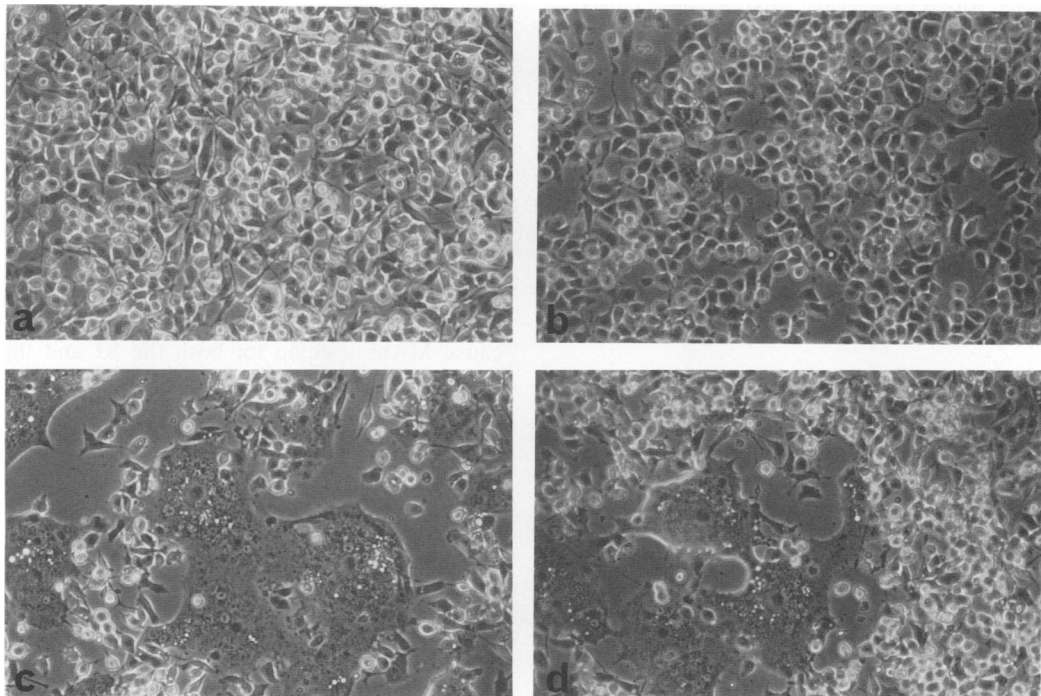


FIG. 4. Fusion formation by transfection of pSFt(+), pSFt(−), or pSF into DBT cells infected with VV-HA(+). DBT cells were infected with VV-HA(+) at an MOI of 0.5 or mock infected (a); 10 h later, 1 μg of pSF (b), pSFt(+) (c), or pSFt(−) (d) was transfected, and cells were microscopically observed at 10 h after transfection.
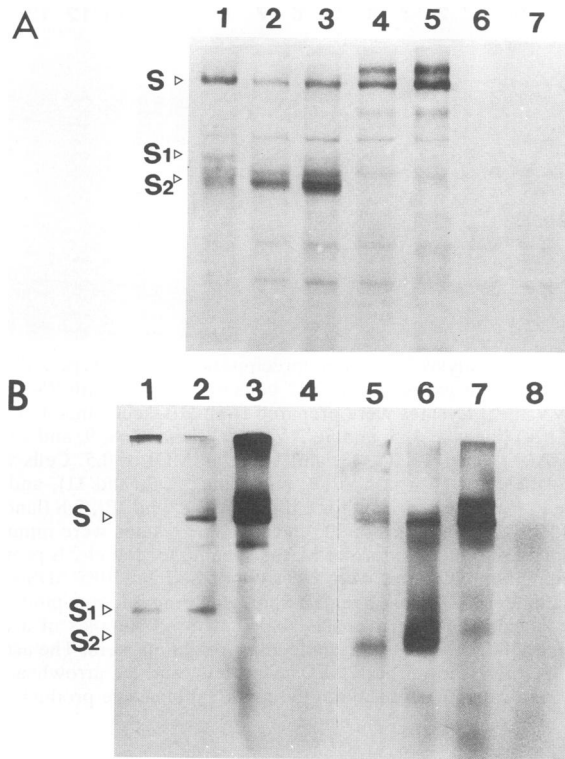
A



B



FIG. 6. (A) Western blot analysis of wild-type and mutated S
proteins expressed in DBT cells infected with RVV t(+) or RVV
t(−). DBT cells infected with RVV t(+) or RVV t(−) at an MOI of
0.5 were lysed, and the lysates were electrophoresed in an SDS–10%
polyacrylamide gel. After being blotted on nitrocellulose paper, S
protein was detected with a mixture of MAbs specific for S1 (11F)
and S2 (10G). Lysates were prepared from DBT cells infected with
cl-2 (lane 1), infected with RVV t(+) and harvested at 12 h (lane 2)
and 15 h (lane 3) p.i., infected with RVV t(−) and harvested at 12 h
(lane 4) and 15 h (lane 5) p.i., or infected with VV-HA(−) and
harvested at 12 h (lane 6) and 15 h (lane 7) p.i. (B) Western blot
analysis of mildly denatured S proteins by use of a MAb specific for
S1 or S2. Lysates were prepared from DBT cells infected with cl-2
(lanes 1 and 5), RVV t(+) (lanes 2 and 6), and RVV t(−) (lanes 3 and
7), or VV-HA(−) (lanes 4 and 8). The lysates were denatured
without DTT in sample buffer at room temperature for 3 min. After
SDS-polyacrylamide gel electrophoresis in a 10% polyacrylamide
gel, the proteins were transferred to a nitrocellulose membrane and
the S proteins were detected with MAb 8, specific for S1 (lanes 1, 2,
3, and 4), or 10G, specific for S2 (lanes 5, 6, 7, and 8).



FIG. 7. Western blot analysis of wild-type and mutated S pro-
teins expressed by transfection with pSF t(+), pSF t(−), or pSF.
DBT cells infected with VV-HA(+) and transfected with pSF t(+)
(lanes 2 and 3), pSF t(−) (lanes 4 and 5), or pSF (lanes 6 and 7) were
lysed at 12 h (lanes 2, 4, and 6) and 15 h (lanes 3, 5, and 7)
posttransfection. Lysates were also prepared from DBT cells in-
fected with cl-2 (lane 1) or mock-infected DBT cells (lane 8).

showed that fusion formation occurred in the presence of
uncleaved S protein containing a mutation in the putative
cleavage site. This result implied that cleavage of the S
protein was not a prerequisite for the fusogenic activity of
the S protein. However, the possibility that trace amounts of
the S protein undetectable even by Western blotting are
responsible for fusion activity cannot completely be ex-
cluded. The data also showed that cleavage of the S protein
enhanced fusion formation by the S protein. Fusion forma-
tion was observed 2 to 4 h earlier in cells producing cleaved
S protein. This result might have been due to the different
efficiency of S protein transport to the cytoplasmic mem-
brane, in which the S protein would fuse to the cytoplasmic
membrane of uninfected neighboring cells.
  The data are compatible with the idea that the mechanism
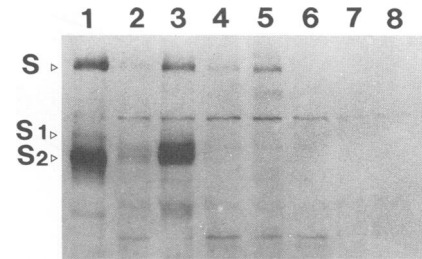of fusion formation by the coronavirus S protein may be

different from those of proteins of other fusogenic RNA
viruses, an idea that has been proposed because the hydro-
phobic amino acid cluster, designated the fusion peptide in
other fusogenic RNA viruses (40), is not found at the N
terminus of the membrane-anchoring chain of coronavirus S
protein cleavage product S2 (10, 22, 25, 29, 33). Semliki
forest virus and Sindbis virus, each capable of inducing
fusion in infected cells after cleavage of the envelope pro-
tein, are known to lack the putative fusion peptide at the N
terminus of the membrane-anchoring chain but internally
have a putative fusion peptide composed of 16 to 17 hydro-
phobic amino acids (40). However, such a large stretch of
hydrophobic amino acids does not exist in the S protein of
JHMV variant cl-2 (33) or in the S proteins of other corona-
viruses (10, 22, 25, 29). Sequence analysis has shown that
some other coronaviruses, i.e., feline infectious peritonitis
virus and porcine transmissible gastroenteritis virus, do not
have the predicted proteolytic cleavage site on the S protein
(3, 29). Nevertheless, fusion was found in cells infected with
such viruses (4, 36). All of these facts, together with the
present findings, suggest that the mechanisms of fusion
induced by coronaviruses must be distinct from those of
other fusogenic RNA viruses.
  Little is known about the mechanism of fusion formation
by coronavirus S protein, and there are only a few reports
analyzing the relationship between the structure and fusion
formation by the S protein in murine coronaviruses (17, 23).
It has been reported that in fusion-negative mutant viruses,
the hydrophobic domain located within the S2 heptad repeat
is associated with pH-dependent fusion formation (10). Be-
cause MAbs specific for both the S1 and the S2 subunits
prevent fusion formation, it has been suggested that these
subunits are important for fusion formation (39). More
precisely, it is assumed that two different domains are
involved, the N terminus of the S1 subunit and the domain
near the heptad repeat in the S2 subunit (23). Also, a region
about 130 amino acids from the N terminus of the S2 subunit
is likely to be involved in fusion formation, as suggested by
MAb mapping (1, 17). Study of the MAbs isolated in our
laboratory revealed that MAbs recognizing highly conforma-
tional epitopes, i.e., epitopes destroyed even by mild dena-
turation, had high fusion-inhibiting activity (unpublished
data), which would indicate that a secondary or tertiary
structure of the S protein may be important for fusion
activity. As for the structure of the fusion active site, both
the S1 and the S2 subunits may be involved, as discussed by
Routledge et al. (23). That MAbs against different epitopes

on the S1 and S2 subunits inhibited fusion formation does not necessarily imply that such epitopes are actually involved in fusion activity, because those MAbs might have inhibited fusion by either neutralization of infectious viruses or steric hindrance. For understanding coronavirus fusion mechanisms, it is essential to locate the fusion active site on the S protein molecule.

## ACKNOWLEDGMENTS

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