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Communication between S1N330 and a Region in S2 of Murine Coronavirus Spike Protein Is Important for Virus Entry into Cells Expressing CEACAM1b Receptor

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The soluble receptor-resistant (srr) mutants, srr7 and srr11, isolated from a murine coronavirus, mouse hepatitis virus (MHV) JHMV, have an amino acid mutation at positions 1114 (Leu to Phe) and 65 (Leu to His), respectively, in the spike (S) protein. These mutants failed to efficiently infect BHK cells expressing CEACAM1b (BHK-R2), due to their low entry into this cell line, although they infected cells expressing CEACAM1a (BHK-R1) in a manner similar to that of wild-type (wt) JHMV cl-2 (Matsuyama and Taguchi, *Virology* 273, 80–89, 2000). Following the repeated passage of these mutants through BHK-R2 cells, viruses were no longer isolated from srr11-infected cells, while two distinct mutants, srr7A and srr7B, were obtained from srr7-infected cells. Srr7A and srr7B grew 2 log10 higher than srr7 and induced fusion in BHK-R2 cells, being similar to wt virus. In addition to the amino acid change at position 1114 that stemmed from parental srr7, srr7A and srr7B had mutations around position 280, corresponding to the third region of the S1N330 receptor-binding site (S1N330-III) common to all MHV strains examined thus far. Srr7A and srr7B S proteins showed high fusogenicity in both BHK-R1 and BHK-R2 cells, like the wt virus, while srr7Aa and srr7Ba S proteins, which had mutations in S1N330-III but not at amino acid 1114, exhibited profoundly reduced fusion activity in these cell lines. These findings suggest that communication between S1N330-III and the amino acid at position 1114 is important for efficient fusion activity in BHK-R2 cells. S1N330-III is a possible region in the S1 involved in viral entry into cells.

INTRODUCTION

Mouse hepatitis virus (MHV), a member of the coronavirus family, causes a variety of diseases with different organ tropisms and virulence, depending upon MHV strains. The major target organs of MHV are the liver, central nervous system, and intestines. The MHV virion has a genome of single-stranded, positive-sense RNA of about 31 kb in length which is packaged by a nucleocapsid (N) protein of about 50 kDa within the viral envelope. The virion envelope contains an integral membrane (M) protein of 20–23 kDa, envelope (E) protein of about 8 kDa, and spike (S) protein of 170–200 kDa. Some strains of MHV have an optional protein, hemagglutinin-esterase protein (60–65 kDa) on the envelope as well.

The S protein constitutes the projection or peplomer on the virion surface. After being synthesized and modified by glycosylation, the S protein is cleaved into N terminal S1 and C terminal S2 subunits by host cell-derived proteases (Sturman *et al.,* 1985). S1 comprises the outermost, knob-like structure of the projection, and the membrane-anchored S2, the stem-like structure beneath the knob (De Groot *et al.,* 1987). In the middle of S1, there is a highly variable region spanning about 150 amino acids, called the hypervariable region (HVR). A number of viruses and mutants have amino acid mutations or deletions of variable lengths in this region relative to the largest S protein of MHV-JHMV (La Monica *et al.,* 1991; Parker *et al.,* 1989; Taguchi *et al.,* 1985; Wang *et al.,* 1992).

S protein has several important biological functions. The N terminal region, consisting of 330 amino acids of S1 (S1N330), is responsible for the binding to the receptor, and the receptor-binding site is formed by a conformational structure (Kubo *et al.,* 1994). In S1N330, there are three regions common to all MHV strains, S1N330-I, II, and III, of which I and II are involved in receptor-binding activity (Suzuki and Taguchi, 1996), while the function of S1N330-III is not yet clarified. Amino acid Thr at position 62, located in S1N330-I, and its neighboring residues, are assumed to be particularly important for receptor-binding activity (Suzuki and Taguchi, 1996). S protein is also involved in the viral entry into cells via fusion of the viral envelope and cell membrane (Collins *et al.,* 1982; Taguchi *et al.,* 1992). Several different regions in S2 are considered critical for this fusion activity (Gallagher, 1996; Luo and Weiss, 1998; Taguchi and Shimazaki, 2000). Cleavage events of S are not absolutely necessary for fusion activity (Stauber *et al.,* 1993; Taguchi, 1993), and the S protein has epitopes for neutralizing antibodies and cytotoxic T cells (Castro and

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Perlman, 1995; Flory *et al.,* 1993). The key to determining MHV virulence is believed to lie in the S protein (Dalziel *et al.,* 1986; Fleming *et al.,* 1986; Matsuyama *et al.,* 2001; Phillip *et al.,* 1999), and thus, it is critical to the early events of virus infection, as well as for the host animals to protect against MHV infection (Taguchi, 1999).

Several different proteins work as MHV receptors (Chen *et al.,* 1995; Dveksler *et al.,* 1991; Nedellec *et al.,* 1994; Yokomori and Lai, 1992), among which CEACAM1 is the most functional receptor. Two allelic forms are known for CEACAM1, CEACAM1a (MHVR1) and CEACAM1b (MHVR2) (Beauchemin *et al.,* 1999; Dveksler *et al.,* 1993; Yokomori and Lai, 1992). MHVR1 is expressed in most mouse strains with a high susceptibility to MHV, while MHVR2 is derived from the SJL mouse strain resistant to MHV (Dveksler *et al.,* 1993; Ohtsuka and Taguchi, 1997; Smith *et al.,* 1984; Yokomori and Lai, 1992). MHVR1 is a 10- to 100-fold more functional receptor for MHV than is MHVR2 (Ohtsuka *et al.,* 1996; Rao *et al.,* 1997).

Mutant viruses resistant to neutralization by a soluble receptor (srr) have been isolated from various viruses. Generally, srr mutants fail to bind to a soluble receptor as efficiently as they bind to the membrane-anchored receptor (Colston and Racaniello, 1994; Kaplan *et al.,* 1990; McKeating *et al.,* 1991). However, some MHV srr mutants isolated in our lab bound to both soluble and membrane-anchored receptors as efficiently as the wt virus (Saeki *et al.,* 1997). Thus, the mechanism of soluble receptor-resistance of those MHV mutants appeared to be different from that of other viruses.

Recently, we found that our srr mutants failed to efficiently infect cells expressing MHVR2 (BHK-R2), while they infected cells expressing MHVR1 (BHK-R1) in a fashion similar to the wt virus (Matsuyama and Taguchi, 2000). Inefficient srr infection in BHK-R2 cells was demonstrated to be due to low fusionability, i.e., inefficient entry into cell (Matsuyama and Taguchi, 2000). From those srr mutants, we tried to isolate viruses that grow well in BHK-R2, revertants in terms of growth capability in BHK-R2 cells, to see whether any other region in the S protein compensates for the inefficient fusogenicity caused by the mutations in srr mutants. In the present study, we show revertants successfully isolated from srr7. Their characterization suggests the importance of the communication between S1N330-III and a region in S2 for the fusogenicity on BHK-R2 cells.

RESULTS

Isolation of mutant viruses from srr7

Srr7 and srr11 with a mutation at amino acid position 1114 (Leu to Phe) and 65 (Leu to His), respectively, failed to grow as efficiently as the wt virus in BHK-R2 cells that constitutively express MHVR2 receptor (Matsuyama and Taguchi, 2000). To see whether some other amino acids can compensate for the defects caused by these mutations, we tried to isolate viruses that grow in BHK-R2 cells as efficiently as the wt virus by repeated passage of these mutants through the cell line. These mutants, $5–10 \times 10^5$ PFU as examined using DBT cells, were inoculated onto BHK-R2 cells and the cells were incubated for 24 h after infection. Viruses grown in cells were subjected to a subsequent cycle of infection onto BHK-R2 cells. We repeated this passage 20 times. As for srr11, syncytia ceased to form after three to four passages, and no infectious viruses were recovered from cells in the fifth to sixth passage in two independent experiments. In contrast, srr7 was successfully adapted to grow in BHK-R2 cells. During the first two to four passage levels, no remarkable syncytia were observed; however, with repeated passages, syncytia became visible that were similar to those of wt virus. After 20 passages, we isolated viruses by plaque purification using DBT cells. In two independent experiments, we obtained two different viruses, srr7A and srr7B, that grew in BHK-R2 cells more efficiently and produced larger syncytia than did the parental srr7. These viruses were detected after the 10th to 15th passage in BHK-R2 cells.

Biological characterization of srr7A and srr7B

Two viruses, srr7A and srr7B, isolated after the 20th passage, were compared with the parental srr7, as well as wt cl-2 in terms of the growth in BHK-R2 and BHK-R1 cells. Following infection at a multiplicity of infection (m.o.i.) of 0.1 in BHK-R2 cells, progeny appeared in the cells at 12 h postinoculation (p.i.) irrespective of the type of viruses used. However, the titers of srr7A and srr7B grown in this cell line were about 2 log10 higher than that of parental srr7 throughout the course of infection, but slightly lower (1 in log10) than the wt cl-2 titer. All of these viruses reached a plateau at 18 to 24 h p.i. There was no significant difference in the growth kinetics in BHK-R1 cells among these viruses (Fig. 1). We compared the size of the syncytia produced by these viruses. In BHK-R1 and BHK-R2 cells infected and incubated at 37°C for 12 to 15 h, we analyzed syncytia formed with NIH image software. As shown in Fig. 2A and B, the syncytia formed on BHK-R2 cells by srr7A and srr7B were significantly larger ($P < 0.001$) than those produced by srr7, but significantly smaller ($P < 0.001$) than those of wt virus. In contrast, there was no size difference in syncytia produced in BHK-R1 cells by srr7, srr7A, and srr7B, although these syncytia were revealed to be slightly, but significantly smaller than those produced by the wt virus ($P < 0.001$) (Fig. 2B). These findings indicated that the fusogenicity of srr7A and srr7B reverted, if not perfectly, into the wt-like feature in BHK-R2 cells, but not in BHK-R1 cells, and correlated well with the growth capacity in BHK-R2 cells as shown in Fig. 1.

To compare the efficiency of infection in BHK-R2 cells

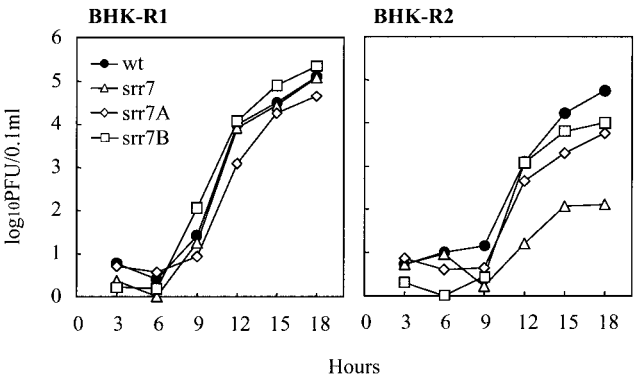

FIG. 1. Growth of wt cl-2 and srr mutants in BHK-R1 and BHK-R2 cells. Cells were infected with wt cl-2 or srr mutants at an m.o.i. of 0.1 and cells, as well as culture fluids, were collected together at intervals after infection. After three rounds of freeze–thawing and spinning at 3000 rpm for 10 min, virus titers in the supernatants were determined. The titer is a mean value of three independent samples.

as well as BHK-R1 cells, we examined the infectious centers and plaquing efficiencies of wt and mutant viruses. An infectious center assay demonstrated that srr7A and srr7B infected BHK-R2 cells with an efficiency similar to that of the wt virus rather than to srr7, whereas there was no remarkable difference in infection of BHK-R1 cells among these four viruses (Fig. 3). Also, the plaquing efficiency of those two mutants was similar to that of wt virus, but far higher than srr7 in BHK-R2 cells, although there was no difference in BHK-R1 cells (data not shown). These results demonstrated that srr7A and srr7B were more similar to the wt virus than to srr7 in terms of growth and cytopathic effects on BHK-R2 cells. Thus, those mutants were defined as phenotypical revertants of srr7, and those revertants were revealed by a virus overlay protein blot assay to bind to MHVR1 or MHVR2 as efficiently as wt cl-2 and srr7 (data not shown).

To compare the amino acid sequences of these revertant S proteins with those of wt and srr7, we directly sequenced the cDNA to the S genes of these viruses amplified by RT-PCR, as described in Materials and Methods. Amino acid sequences of these S proteins, as deduced from nucleotide sequences, contained a mutation at position 286 (Ser to Ile) in srr7B and two mutations at positions 278 (Ile to Leu) and 461 (Ser to Thr) in srr7A (Table 1). Relative to wt, these two revertants had a mutation as well at position 1114 (Leu to Phe), which stemmed from the parent srr7 (Table 1). Mutated amino acids 286 and 278, found in srr7B and srr7A, respectively, were revealed to lie in the third common region of S1N330 (S1N330-III), consisting of amino acids 278 to 288, which was shown to be one of three conserved regions at the receptor-binding site (Suzuki and Taguchi, 1996). The deduced sequence results suggested that amino acid mutations in S1N330-III found in both revertants are important for the phenotypic reversion of srr7 to the wt virus. They also suggested that inefficient infection of srr7 in BHK-R2 cells caused by the mutation at 1114 of the wt virus S protein could be compensated for by the additional mutation in S1N330-III.

Fusogenicity of mutant S proteins in BHK-R2 cells

Wt S protein was demonstrated to induce large syncytia in BHK-R2 cells, while srr7 S protein failed to do so, which resulted in a difference in efficiency of infection between wt virus and srr7 in BHK-R2 cells (Matsuyama and Taguchi, 2000). We have compared the fusion activity of srr7A and srr7B in BHK-R2 cells with that of srr7 and the wt cl-2. Srr7A and srr7B S genes were inserted into a pTarget vector for expression, as described in Materials and Methods. These vectors and those containing wt S and srr7 S genes were transfected into BHK-R2 or BHK-R1 cells, and their fusogenicity was examined by using recombinant vaccinia virus vTF7.3 to express T7 RNA polymerase. Transfection efficiencies of these vectors were assessed by the activity of luciferase expressed from the cotransfected plasmid. As shown in Figs. 4A and 4C, the wt S showed strong fusion activity, while srr7 S failed to induce efficient fusion in BHK-R2 cells, as reported previously (Matsuyama and Taguchi, 2000). However, both srr7A and srr7B S proteins induced fusion in BHK-R2 cells similar to that induced by the wt virus rather than to that induced by srr7 (Figs. 4A and 4C). To see which of two mutations in the srr7A S protein is important for fusion activity, we have prepared vectors containing a single mutation found in srr7A. Srr7A S with a mutation in S1N330-III alone (srr7A-330III) displayed fusion activity similar to that of the original srr7A, while srr7A S protein with a mutation at position 461 (srr7A-HVR) had lost fusion activity (Fig. 4B), indicating that mutation in S1N330-III is responsible for fusion activity. The kinetics of fusion formation of srr7A and srr7B in BHK-R2 cells was also similar to that of wt virus; syncytia started to appear at 4 to 5 h after vTF infection and spread to most cells by 15 h. However, syncytia by srr7 were very restricted even at 15 h after infection. In contrast, all of these S proteins induced fusion extensively in BHK-R1 cells, and most cells were included into syncytia at 15 h after infection (Fig. 4A). These results indicated that the mutation in S1N330-III enhanced the fusogenicity of the S protein, which had been impaired by a mutation at amino acid 1114. This result suggested either that the mutation in S1N330-III enhances the fusion activity independent of amino acid 1114 or that the combination of these two regions located a long distance apart is critical for sufficient fusogenicity in BHK-R2 cells. To see which is correct, we have constructed srr7A and srr7B S genes without a mutation at amino acid 1114, designated them srr7Aa and srr7Ba, respectively, and examined their fusogenicity. Srr7Aa and srr7Ba S proteins were unable to induce fusion in BHK-R2 cells as

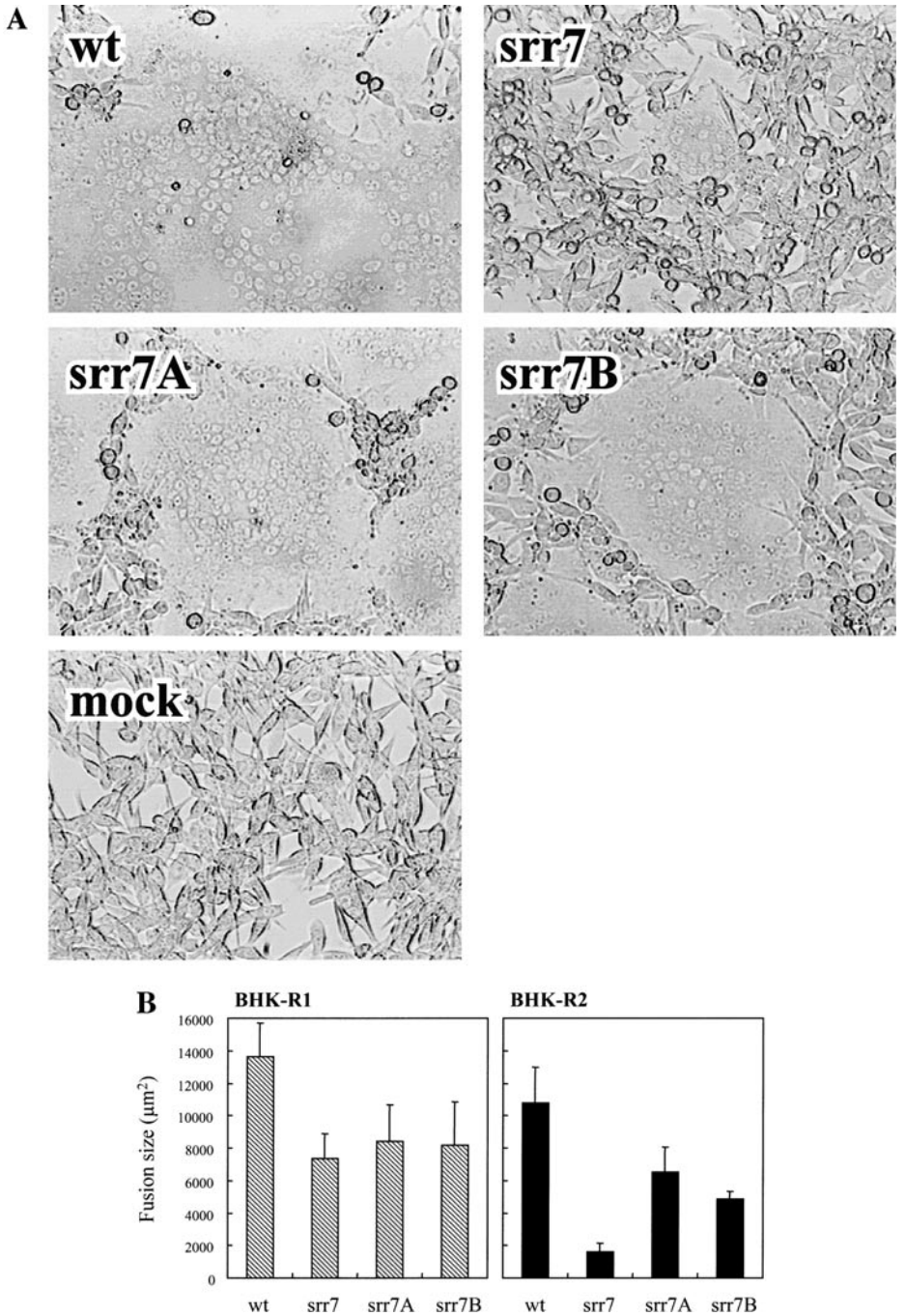

FIG. 2. Syncytia formed by wt cl-2 and srr mutants. (A) Syncytia produced by wt cl-2 and srr mutants in BHK-R2 cells. BHK-R2 cells prepared in 24-well plates were infected with about 100 PFU of either wt cl-2 or srr mutants and microscopically observed at 15 h after infection. (B) BHK-R1 and BHK-R2 cells cultured in 24-well plates were infected with about 100 PFU of virus, and syncytia were observed at 15 h after infection by microscopy. The size of more than 20 syncytia for each virus was analyzed by NIH image. The vertical line extending above each bar indicates the standard deviation.

well as in BHK-R1 cells (Fig. 4A). Inability of srr7Aa and srr7Ba S proteins to induce syncytia was revealed to be due to defects in oligomerization (data not shown). The difference in fusogenicity in BHK-R1 and BHK-R2 cells shown in Fig. 4 did not appear to result from the difference in the amounts of S proteins expressed, because, as shown in Fig. 5, there was no apparent difference in the amounts expressed. It was noted that S2 with a mutation at amino acid 1114, as seen in srr7, srr7A, and srr7B S proteins, moved slightly faster than the S2 without mutation, although these S2 proteins did not differ in molecular weight (Fig. 5). All of these S proteins, irrespective of their fusogenicity, were similarly oligomerized and transported onto the cytoplasmic membrane (data

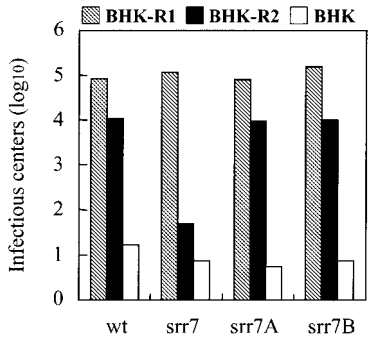

FIG. 3. Infectious centers produced by wt cl-2 and srr mutants on BHK-R1 and BHK-R2 cells. BHK-R1, BHK-R2, and BHK cells were infected either with wt or mutant viruses (m.o.i. = 1) and cultured 3 h. After washing three times with PBS containing 0.5% BSA and 0.05% Tween 20, cells were treated with trypsin. Then, 10-fold serial dilutions of the cells were overlaid onto DBT cell monolayers and cultured in DMEM containing 0.5% methylcellulose for 15 h. The plaques were counted after staining with neutral red. The result is representative of multiple independent experiments.

not shown). These results suggested that the combination of these two regions played a critical role in fusion formation rather than that the mutation in S1N330-III enhanced the fusion activity irrespective of amino acid at 1114.

Taken together, the present study, which was summarized in Fig. 6, indicated that additional mutations in S1N330-III found in srr7A and srr7B enhanced the fusion activity of srr7 S protein to the level of the wt S protein. However, this enhancement was not simply caused by a mutation in S1N330-III alone, but was dependent on the combination of amino acids in S1N330-III and at 1114.

DISCUSSION

Two srr mutants, srr7 and srr11, showed heavily reduced growth in BHK-R2 cells, which originated from their reduced entry into the cell (Matsuyama and Taguchi, 2000). The former has a mutation in S2 and the latter in S1 (Saeki *et al.,* 1997). To further analyze the region in the S protein important for fusion in BHK-R2 cells, we tried to isolate the revertant viruses from those two srr mutants in terms of growth in BHK-R2. After several passages through BHK-R2 cells, srr11 could no longer be detected. Srr11 could not adapt to grow in BHK-R2 cells, which implies that Leu at position 65 mutated to His in srr11 is critical for efficient infection in BHK-R2 cells. Because this amino acid is located within S1N330-I, one of the two most important regions for receptor-binding activity in S1N330 (Kubo *et al.,* 1994; Suzuki and Taguchi, 1996), it could play a vital role in receptor binding. As expected, the binding of srr11 to both BHK-R1 and BHK-R2 cells was slightly reduced relative to wt and srr7 (Matsuyama and Taguchi, 2000; Saeki *et al.,* 1997). The inability of srr11 to adapt to BHK-R2 cells might be due to this low receptor-binding capacity, or to double defects in fusogenicity and receptor binding, as shown previously (Matsuyama and Taguchi, 2000; Saeki *et al.,* 1997).

Srr7 could successfully be adapted to grow in BHK-R2 cells by repeated passage through this cell line. This biological change was accompanied by an amino acid change in S1. Srr7B had only one additional mutation in S1N330-III; however, srr7A contained two, one in S1N330-III and the other in HVR of S1. The mutation in the S1N330-III, but not that in the HVR, was revealed to be responsible for the acquisition of fusion activity by srr7A. Thus, the mutation in S1N330-III must be critical for conversion from the srr7-type virus, which is inefficient in infecting in BHK-R2 cells into the wt-like virus with efficient infection and fusion activity. However, the amino acid changes in S1N330-III alone did not enhance fusion activity, as seen for srr7Aa or srr7Ba, but a simultaneous mutation at position 1114 was inevitable. These findings strongly suggest that the combination of S1N330-III and the amino acid at 1114, presumably 1114 and its neighbors common to various MHV strains (Kunita *et al.,* 1995; Luytjes *et al.,* 1987; Parker *et al.,* 1989; Taguchi *et al.,* 1992; Yamada *et al.,* 1997; Yamada and Yabe, 2000), determines the fusogenic feature of the S protein. It is not evident at present how S1 and S2

TABLE 1

Identification of S Protein Mutations in Adapted srr7

	Substitution at the following position[a]							
	Nucleotide				Amino acid			
Virus	832	857	1382	3340	278	286	461	1114
wt (JHMV cl-2)	A	G	G	C	Ile	Ser	Ser	Leu
srr7	—[b]	—	—	T	—	—	—	Phe
srr7A	C	—	C	T	Leu	—	Thr	Phe
srr7B	—	T	—	T	—	Ile	—	Phe

[a] Nucleotide and amino acid positions were numbered from the first ATG codon and methionine, respectively.
[b] —, Identity with the wild-type sequence.

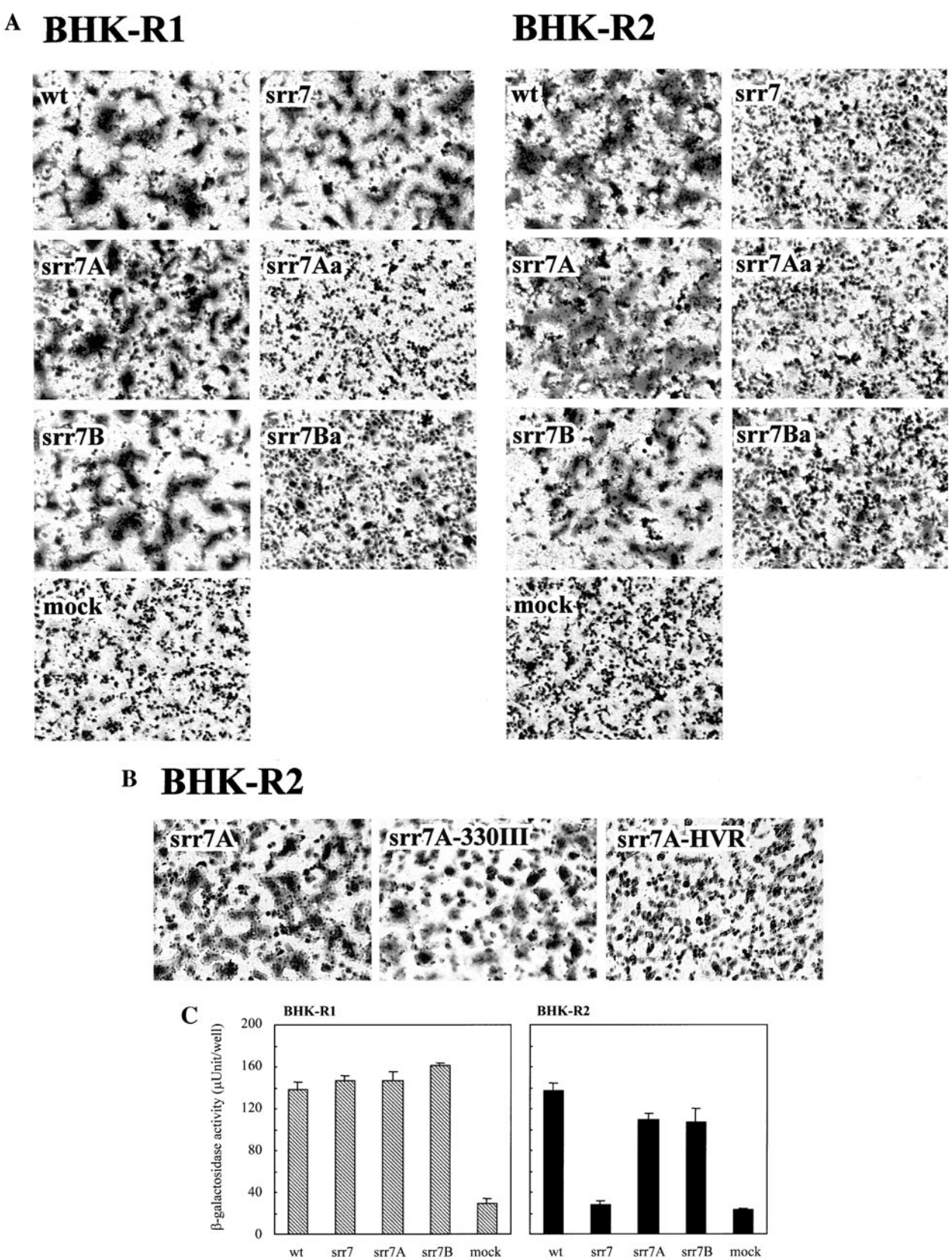

FIG. 4. (A) and (B) Fusion activity of wt cl-2 and srr mutant S proteins in BHK-R1 and BHK-R2 cells. BHK-R1 and BHK-R2 cells were transfected with plasmids containing various S genes or plasmid alone and infected with vTF7.3. After 15 h incubation, cells were fixed with formaldehyde and stained with hematoxylin and eosin. (C) Fusion activity of the various S proteins as accessed by β-galactosidase activity. Target cells, either BHK-R1 or BHK-R2 cells, were infected with wt vaccinia virus, then transfected with plasmid pG1NT7β-gal and cultured in 96-well plates for 15 h. Onto these cells, effector cells, from the same cell line infected with vTF7.3 and transfected with pTarget vector containing either wt or mutant S genes, were overlaid and cultured for 7 h. The β-galactosidase activity expressed as a result of fusion of effector and target cells was measured.

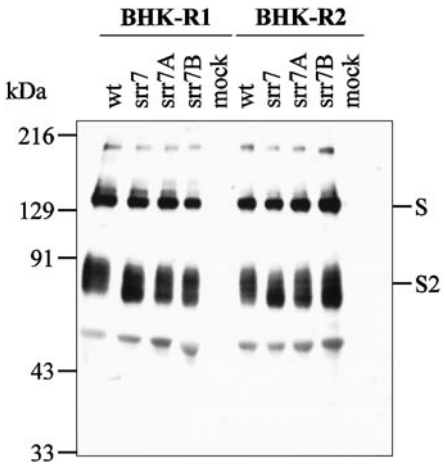

FIG. 5. Western blot analysis of the expressed S proteins. BHK-R1 and BHK-R2 cells transfected with plasmids harboring various S genes and infected with vTF7.3 as described above were lysed at 15 h after transfection. The amounts of S proteins in each lysate were examined by Western blotting using MAb specific to the S2 subunit and ECL.

interact to execute fusion, but it is not surprising in coronavirus S protein that two regions a long distance apart in primary structure cooperatively interact to execute an important biological function. Interaction or proximal localization in the tertiary structure of S1 and S2 in the native S protein was suggested by Grosse and Siddell (1994), who isolated a MAb-resistant mutant with a mutation in S2 after neutralization by S1-specific MAb.

The present study demonstrated that a combination of S1N330-III and amino acid 1114 is important for MHV entry to cells expressing MHVR2. However, such a strict combination was not necessary for entry into cells expressing MHVR1. This difference could be accounted for by the difference in virus-binding ability between MHVR1 and MHVR2. The former has more than at least 100-fold higher binding ability than the latter, as examined by virus overlay protein blot assay and neutralization tests using soluble forms of these MHV receptors (Ohtsuka *et al.,* 1996; Zelus *et al.,* 1998). Tight binding of MHV to the receptor could compensate for the combination undesirable for efficient entry into cells found in srr7 S protein.

S1N330-III, consisting of 11 amino acids at positions 278 to 288 in S1, has been demonstrated to be perfectly conserved among the seven MHV strains examined (Suzuki and Taguchi, 1996). Furthermore, this region is also conserved in three newly isolated MHVs (Kunita *et al.,* 1995; Yamada and Yabe, 2000), and such conservation is suggestive that S1N330-III retains some biological function critical for MHV replication. We suspected it to be involved in receptor binding, because it was located in S1N330, the receptor-binding domain. Site-directed mutagenesis analysis showed, however, that S1N330-III was the least involved in receptor-binding activity in the three conserved regions in S1N330 (Suzuki and Taguchi, 1996). S1N330-III of srr7A and srr7B contained a mutation at positions 278 and 286, respectively, relative to the wt or srr7 S protein; nevertheless, those S proteins equally bound and infected both BHK-R1 and BHK-R2 cells. This is also suggestive that S1N330-III is not important for receptor-binding activity. One may argue that srr7A and srr7B with a mutation in S1N330-III could have

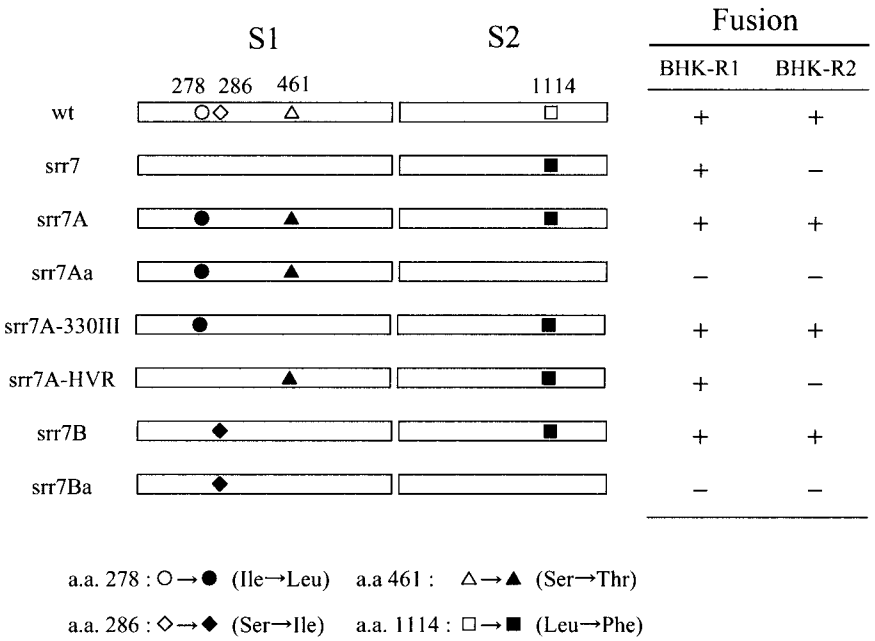

FIG. 6. Summary of JHMV S protein structure and their fusion activity in BHK-R1 and BHK-R2 cells. Mutated amino acids in mutant S proteins were shown by closed marks, while original amino acids in the wt virus were displayed by open ones.

receptor-binding activity due to a second mutation at 1114 in S2, suggesting the possibility that S1N330-III is important for receptor binding, but the latter is unlikely, because the binding of the S protein to the receptor takes place with S1N330 alone, without the cooperation of the S2 subunit or other parts of S1 (Kubo *et al.,* 1994). All of these factors could suggest that S1N330-III plays an important role not in receptor binding, but rather in viral entry into cells.

Several regions or amino acids in the S protein have been reported to be important for fusion activity (Gallagher, 1996; Gallagher *et al.,* 1991; Luo and Weiss, 1998; Luo *et al.,* 1999; Taguchi and Shimazaki, 2000). All of those, such as heptad repeats and amino acid position 1114, are located in the S2 subunit. Alterations from Leu to Arg at position 1114 resulted in the reduced fusion activity in DBT cells expressing MHVR1, suggesting the importance of this amino acid for fusion activity (Gallagher *et al.,* 1991). We also described in the present study that srr7 with an alteration of the same amino acid from Leu to His has reduced fusion activity in BHK-R1, which strengthened the importance of 1114 Leu for fusion. A variant virus S protein of MHV-4 (JHMV) containing a mutation at position 1114, as well as two other mutations, showed pH-dependent fusion activity (Gallagher *et al.,* 1991). The pH-independent fusion activity of srr7 and its revertants suggests no or minor involvement of 1114 Leu in the pH dependence of fusion activity.

Whereas a number of reports suggest the importance of S2 for fusion activity for viral entry into cells, there is little to suggest the importance of S1. Tsai *et al.* (1999) reported that a stretch of 12 amino acids in HVR is important for fusion activity. They compared the S protein of fusion-negative MHV-2 with that of fusion-positive MHV-JHM (Schmidt *et al.,* 1987), and 12 amino acids deleted in MHV-2, but not in MHV-JHM, were identified as being responsible for fusion activity. However, this stretch is not important for virus entry into cells, because MHV-2 is able to infect cells. Recently, Krueger *et al.* (2001) reported that mutant JHMVs containing a deletion in the HVR have reduced fusion activity in MHVR-dependent and -independent infections. We also described that JHMV sp-4 virus, with a 141-amino acid deletion in the HVR, has a reduced fusion activity on DBT cells with MHVR1, which was revealed by the slightly smaller plaques relative to wt cl-2 (Matsubara *et al.,* 1991). Sp-4 also showed reduced fusion activity in MHVR-independent infection (Taguchi *et al.,* 1999). However, these viruses can infect and grow in cells expressing MHVR1 with an efficiency higher than or similar to wt virus, in spite of their low fusogenicity (Kreuger *et al.,* 2001; Taguchi *et al.,* 1985; Matsubara *et al.,* 1991). This clearly indicates that HVR is not a vital region in viral entry into cells. Taken together, HVR may influence fusion activity of MHV but it does not affect the potential for virus entry into cells.

In addition to the differing abilities of wt cl-2 and srr7 to infect BHK-R2 cells, we previously revealed another biological difference between them that infection spreads from wt-infected DBT cells to BHK cells deficient in MHVR (called MHVR-independent infection), while srr7 failed to spread in this fashion (Taguchi *et al.,* 1999; Taguchi and Matsuyama, 2002). Srr7A and srr7B, revertants in terms of infective ability to BHK-R2 cells, are able to spread to BHK cells by MHVR-independent infection (data not shown), indicating that srr7A and srr7B are reverted in terms of MHVR-independent infection as well. These findings suggest that the mechanism to infect cells with MHVR2 has some similarity to that of MHVR-independent infection. We are currently studying the mechanism underlying MHVR-independent infection using wt, srr7 as well as two revertants from srr7.

The present study showed that double mutations, one in S1N330-III and the other at amino acid 1114, were important for fusogenicity in BHK-R2 cells. This suggests that these two regions interact with each other and cooperatively execute fusion formation, namely viral entry into cells. How the combination of S1N330-III and amino acid 1114 determines the fusion activity was not addressed in this study. It is reported for retroviruses and other viruses that the receptor-binding protein undergoes conformational change after binding to the receptor (Damico *et al.,* 1998; Ikeda *et al.,* 2000), which is an essential step for virus entry into cells, via virus–cell fusion. MHV S protein supposedly undergoes conformational change after binding to the receptor, which converts the S protein to fusion active phenotype. Reduced fusion activity of srr7 S protein in BHK-R2 cells could be due to the lack of conformational change after binding to the receptor. In contrast, the combination of these two regions in wt virus, srr7A and srr7B, may permit or promote the conformational changes of the S protein. Studies are currently in progress to examine these possibilities.

MATERIALS AND METHODS

Cells and viruses

The highly neurovirulent wt MHV-JHMV cl-2 (Taguchi *et al.,* 1985), as well as srr7 and srr11 derived from cl-2 (Saeki *et al.,* 1997), were propagated on DBT cells (Kumanishi, 1967). Newly isolated mutants, srr7A and srr7B, were propagated on DBT cells as well. DBT cells were also used to estimate the infectivity of MHV. BHK cells as well as BHK cells constitutively expressing MHV receptor MHVR1 (BHK-R1) or MHVR2 (BHK-R2) (Matsuyama and Taguchi, 2000) were used for MHV infection and expression of S proteins. BHK cells lack the MHV receptor and are thus nonpermissive to MHV infection. Recombinant vaccinia virus, vTF7.3, harboring the T7 RNA polymerase gene (Fuerst *et al.,* 1986), kindly provided by B. Moss, was propagated and plaque-assayed on RK 13

cells. All cell lines used in this study were grown in Dulbecco's minimal essential medium (DMEM, Nissui, Tokyo) supplemented with 5% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY).

Isolation of mutant viruses

To select the mutants that grow efficiently in BHK-R2 cells, srr7 and srr11, 5–10 $\times$ 10^5 PFU as examined using DBT cells, were inoculated onto confluent BHK-R2 cells prepared in a 35-mm dish (Costar, Cambridge, MA). Both supernatants and cells were harvested at 24 h p.i. and subjected to three rounds of freeze–thawing. After centrifugation at 3000 rpm for 10 min, the clarified supernatants, 100 μl containing 10^3 to 10^6 PFU of virus, were further inoculated onto BHK-R2 cells. After 20 serial passages under similar conditions, two clones, srr7A and srr7B, were obtained from srr7-infected BHK-R2 cells in independent experiments. These mutants were plaque purified further three times on DBT cells.

Assay of virus infectivity

We measured the MHV infectivity by inoculating 20 μl of virus dilution per well onto DBT cells cultured in 24-well plates and incubating them at 37°C for 1 h (Taguchi and Matsuyama, 2002). Cells were then cultured in DMEM containing 5% FBS and 0.5% methylcellulose (Sigma, St. Louis, MO) at 37°C for 12 to 18 h. Following fixation with formaldehyde, cells were stained with 0.1% crystal violet. The syncytia counted under a microscope (CK30, Olympus, Tokyo) were shown as PFU.

Estimation of syncytium (plaque) size

BHK-R1 and BHK-R2 cells prepared in 24-well plates were infected with the wt and mutant viruses to produce about 50–100 syncytia per well. Infected cells were cultured in DMEM containing 5% FBS and 0.5% methylcellulose for 15 to 18 h. Syncytia observed under a microscope were analyzed for their size by NIH image (a public domain software). Syncytium sizes of viruses were statistically compared using Student's *t* test.

Infectious center assay

Infectious centers were tested using BHK-R1, BHK-R2, and BHK cells as described previously (Matsuyama and Taguchi, 2000). Those cells cultured in 35-mm dishes were infected with wt or mutant viruses (m.o.i. = 1) and incubated at 37°C for 1 h. After washing twice with phosphate-buffered saline (PBS), pH 7.2 containing 0.5% BSA and 0.05% Tween 20, the cells were incubated in DMEM containing 3% FBS at 37°C for 3 h. In some experiments, cells were treated with anti-JHMV MAbs (Kubo *et al.,* 1993) to neutralize remaining infectious viruses. The cells were treated with trypsin and washed three times with DMEM by spinning 1500 rpm for 5 min. Then, the 0.1 ml of 10-fold dilutions of infected cells were overlaid onto confluent DBT cells prepared in 35-mm dishes. After culture for 15 h at 37°C in DMEM supplemented with 0.5% methylcellulose, the cells were stained with neutral red and plaques were counted.

Isolation and expression of S genes

DBT cells prepared in 60-mm dishes were infected with srr7A or srr7B at an m.o.i. of 1. Total RNA was isolated from those cells at 15 h after inoculation with an Isogen RNA extraction kit (Nippon Gene, Tokyo, Japan). Srr7A and srr7B S cDNAs were made by reverse-transcription (RT)-PCR from isolated RNA with a pair of primers as previously described (Matsuyama and Taguchi, 2000), and the PCR products were directly sequenced (Sanger *et al.,* 1977). To obtain the mutant S genes for expression, the amplified DNAs were cloned into a commercial expression vector, pTarget (Promega, Madison, WI). As the S genes integrated into the vector contained some nucleotide differences relative to authentic mutant S genes, we have constructed the mutant S genes by replacing srr7 S gene with DNA fragments containing mutations specific for srr7A or srr7B. Because the wt and srr7 S genes previously constructed had three amino acid mutations (Matsuyama and Taguchi, 2000), compared to the originally reported S genes (Taguchi *et al.,* 1992, 1995), we newly prepared the vectors that contain the authentic S genes, pTargetcl-2S and pTargetsrr7S. The pTargetsrr7S plasmid harboring the srr7 S gene downstream from the T7 promoter (Matsuyama and Taguchi, 2000) was cut with PflM I at nucleotide 749 and Apa I at 1548 calculated from the first nucleotide of the S gene initiation codon. The resulting 800-bp fragment was replaced with the corresponding fragment of srr7A and srr7B. Srr7A and srr7B S genes without a mutation at amino acid 1114 were also constructed by replacing the fragment containing the mutation with the fragment of wt virus as described previously (Matsuyama and Taguchi, 2000). They were named pTargetsrr7Aa and pTargetsrr7Ba. We constructed srr7A containing only one mutation in S1N330-III, srr7A-330III, and that with a mutation at position 465, srr7A-HVR, by exchanging the srr7 and srr7A fragments cut with Sca I. Sca I cuts pTargetsrr7 and pTargetsrr7A at two positions, one in JHMV S gene at position 2183 between two mutations found in srr7A and the other in pTarget vector at position 8412. Thus, digestion by Sca I results in two fragments; one is JHMV S gene 5′ fragment containing a mutated region in S1N330-III alone (3.5 kb) and the other JHMVS 3′ fragment containing mutated HVR alone (6.3 kb). Srr7A-330III S is a chimera consisting of srr7A S 5′ 3.5 kb and srr7 3′ 6.3 kb fragments. The Srr7A-HVR S gene is composed of fragments of srr7 5′ 3.5 kb and srr7A 3′ 6.3 kb. The constructed plasmids were confirmed by sequencing (Sanger *et al.,* 1977). S proteins of wt and mutants were

expressed using recombinant vaccinia virus vTF7.3 harboring the T7 RNA polymerase gene as previously described (Saeki *et al.,* 1997).

Western blot

S proteins expressed in BHK-R1 and BHK-R2 cells after transfection with vectors containing various S genes were analyzed by Western blotting using anti-S MAbs kindly provided by S. G. Siddell (Routledge *et al.,* 1991) by enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL) as previously reported (Matsuyama and Taguchi, 2000).

Fusion activity

Fusion activities of wt and mutant S proteins were examined principally as reported by Nussbaum *et al.* (1994). BHK-R1 and BHK-R2 cells were used for this assay. The same type of cells were used for target and effector cells. Target cells were infected with wt vaccinia virus WR strain (m.o.i. = 10) and incubated at 37°C for 1 h. Those cells (1×10^7 cells) were then transfected with 10 μg of pG1NT7β-gal harboring β-galactosidase gene downstream of the T7 promoter, kindly provided by Dr. E. Berger (Nussbaum *et al.,* 1994), by electroporation as described previously (Ohtsuka *et al.,* 1996). These cells were distributed in collagen-coated, 96-well plates (Iwaki), 5×10^4 cells/well, in DMEM supplemented with 5% FBS and incubated at 37°C for 15 h before being overlaid by effector cells. Effector cells were infected with vTF7.3 (m.o.i. = 10) and incubated at 37°C for 1 h and they (10^6 cells) were transfected with 5 μg of pTarget vectors containing either wt or mutant S genes together with 0.5 μg of plasmid containing firefly luciferase gene pTM-luc (Aoki *et al.,* 1998), kindly provided by Dr. Y. Matsuura, by electroporation. The pTM-luc was used to measure the transfection efficiencies of each vector to express various S proteins. Those treated cells, 5×10^4 cells/well, were overlaid onto target cells prepared in 96-well plates and mixed cells were further cultured at 37°C for 6 to 8 h. The activity of β-galactosidase expressed as a result of fusion of effector and target cells by expressed S protein was measured using a commercial kit (Promega, β-galactosidase enzyme assay system) as recommended by manufacturer.

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