



# The virulence of mouse hepatitis virus strain A59 is not dependent on efficient spike protein cleavage and cell-to-cell fusion

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**The cleavage and fusion properties of recombinant murine hepatitis viruses (MHV) were examined to assess the role of the cleavage signal in determining the extent of S protein cleavage, and the correlation between cleavage and induction of cell-to-cell fusion. Targeted recombination was used to introduce amino acid substitutions into the cleavage signal of the fusion glycoprotein (spike or S protein) of MHV strain A59. The recombinants were then used to address the question of the importance of S protein cleavage and viral-mediated cell-to-cell fusion on pathogenicity. Our data indicate that cleavage of spike is not solely determined by the amino acid sequence at the cleavage site, but may also depend on sequences removed from the cleavage site. In addition, efficient cell-to-cell fusion is not necessary for virulence. *Journal of NeuroVirology* (2002) 8, 400–410.**

**Keywords:** cell-to-cell fusion; coronavirus; fusion protein; murine hepatitis virus; pathogenicity; targeted recombination

## Introduction

Murine hepatitis viruses (MHV) are coronaviruses that can be hepatotropic and/or neurotropic (Hirano *et al*, 1981). For example, strains MHV-A59 and MHV-4 are both neurotropic, and can produce meningitis, encephalitis, and demyelinating disease. In addition, MHV-A59, along with another strain, MHV-2, can cause severe hepatitis. MHV-4, although considerably more neurovirulent than MHV-A59, causes minimal hepatitis, whereas MHV-2 causes only minimal disease in the central nervous system (CNS).

An important determinant of pathogenicity for MHV is the spike, or S, glycoprotein. This protein is

responsible for both viral attachment to the host cell receptor and virus-induced membrane fusion. The S protein of all MHV strains mediates fusion between the viral envelop and the eukaryotic cell membrane, an event that is necessary for entry into a host cell. The S protein of some strains of MHV can also induce cell-to-cell fusion, producing syncytia. In this report, we consider viruses capable of syncytia formation to have a fusion-positive phenotype, whereas those that do not produce syncytia are referred to as nonfusogenic.

The S protein is a 180-kDa glycoprotein that is cleaved into two 90-kDa subunits, the aminoterminal S1 and carboxyterminal S2 subunits (Luytjes *et al*, 1987). Although cleavage of the S protein correlates with more efficient syncytia formation *in vitro* (Bos *et al*, 1996; Gombold *et al*, 1993; Stauber *et al*, 1993; Taguchi *et al*, 1992), the relationship between fusogenicity *in vitro* and pathogenicity *in vivo* has not been clearly established for the different strains of MHV. This is in contrast to several other virus systems, where cleavage of the fusion glycoprotein is required for fusion activity of this protein, and cleavage has a direct affect on virulence (Maisner *et al*, 2000; Rott *et al*, 1995; Stadler *et al*, 1997; Volchkov *et al*, 1998).

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This work was supported by Public Health Service grants NS-21954 and NS-30606 (SRW).

Received 28 May 2002; revised 16 July 2002; accepted 19 July 2002.

Cleavage of MHV S proteins occurs following a basic-X-basic-amino acid sequence motif that is recognized by furin or furin-like proteases (Barr, 1991; Nakayama, 1997). The furin-like proteases are cellular enzymes that are responsible for processing hormone precursors into mature, active hormones. These proteases have also been shown to cleave a variety of viral fusion glycoproteins (Bolt and Pedersen, 1998; Bolt *et al*, 2000; Hallenberger *et al*, 1997; Vey *et al*, 1995; Volchkov *et al*, 2000). The fusogenic strains MHV-A59 and MHV-4 both have a cleaved S protein and have cleavage signals of RRAHR and RRARR, respectively. MHV-2, on the other hand, is nonfusogenic, and expresses an uncleaved S protein having the sequence HRARS at the region homologous to the MHV cleavage signal. In order to evaluate the role of the cleavage signal in determining fusion phenotype, and potentially pathogenicity, we wanted to generate viruses with different mutations in the cleavage signal of their S proteins.

Our previous studies using mutants of MHV-A59 derived from persistently infected glial cells indicate that a wild-type fusion phenotype is not required for replication in the CNS (Hingley *et al*, 1994). These mutants, however, encode two amino acid substitutions in the S protein. One mutation lies within a putative receptor binding region of S (Kubo *et al*, 1994), and the second, at amino acid codon 716 (H716D), is within the cleavage signal of S (Luytjes *et al*, 1987). Previous data suggested that the H716D substitution prevents cleavage of the S protein, which would account for the delayed fusion phenotype of these mutants, because several cleavage and fusion revertant viruses replaced the aspartic acid residue (Gombold *et al*, 1993). The mutant cleavage and fusion phenotypes were seen only when the H716D substitution was present (Hingley *et al*, 1995; Leparco-Goffart *et al*, 1997, 1998); however, none of the glial cell mutants had the H716D mutation alone, so it was necessary to isolate recombinant viruses expressing the H716D mutation alone in order to assess the potential affect of this particular mutation on cleavage of S, fusogenicity, and virulence.

Targeted recombination has been used to isolate murine hepatitis viruses with a defined spike gene placed into an MHV-A59 background (Fischer *et al*, 1997; Koetzner *et al*, 1992). We have used this technology to select viruses that contain either the H716D point mutation, or the corresponding sequence from the MHV-2 S gene, in the cleavage signal of the MHV-A59 spike protein. These cleavage site recombinants indicate that the cleavage signal alone is not the sole determinant of S protein cleavage and viral fusion phenotype.

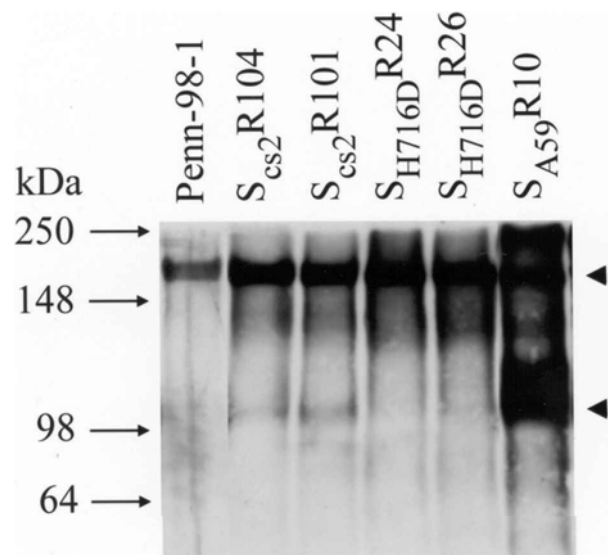
To assess the *in vivo* and *in vitro* phenotypes of the recombinants, it was important to include as a control wild-type recombinants in which the wild-type S genes of MHV-A59 or MHV-2 are (re)introduced into the parent MHV-A59 background. These recombinants, described in previous studies (Das Sarma

*et al*, 2000; Leparco-Goffart *et al*, 1998; Phillips *et al*, 1999), would control for possible effects of mutations outside of the S gene, because all recombinants have the same background genes and therefore differ only by the defined mutation near the cleavage site. This is the first time that pairs of viruses have been available for study that differ only by defined mutations known to disrupt cleavage and fusion properties of the S protein.

## Results

### *Cleavage of S protein in recombinant viruses*

Several laboratories have shown that lack of cleavage of S correlates with less efficient induction of cell-to-cell fusion (Bos *et al*, 1996; Gombold *et al*, 1993; Stauber *et al*, 1993; Taguchi *et al*, 1992). Previous studies from this laboratory using mutants isolated from persistently infected glial cells (Gombold *et al*, 1993) have suggested that an H716D mutation, which changes the RRAHR cleavage signal to an RRADR sequence, is associated with an uncleaved S protein, but did not directly assess the affect of that mutation on virulence. As one would predict, and as seen in Figure 1, the H716D mutation did dramatically reduce the amount of S protein cleavage compared to cleavage of the wild-type protein ( $S_{H716D}R24$  and  $S_{H716D}R26$  versus  $S_{A59}R10$ ). However, upon examining multiple blots of  $S_{H716D}R24$  and  $S_{H716D}R26$  lysate and virion samples, it was also evident that, although cleavage was inhibited, it rarely appeared to be completely prevented.



**Figure 1** Cleavage of S protein in recombinant viruses. Virions derived from infected L2 cells were analyzed by Western blot as described in Materials and methods. On the left is shown the migration of molecular weight markers, in kDa, while on the right is shown the migration of cleaved and uncleaved forms of S protein (arrowheads).

**Table 1** Percent cleavage of the S protein

Virus	% cleavage of S <sup>a</sup>
S <sub>A59</sub> R10 <sup>b</sup>	61 ± 7
S <sub>H716D</sub> R24 <sup>b</sup>	8 ± 8
S <sub>H716D</sub> R26 <sup>b</sup>	7 ± 7
S <sub>CS2</sub> R104 <sup>c</sup>	18 ± 14
S <sub>CS2</sub> R101 <sup>c</sup>	12 ± 11
Penn-98-1 <sup>c</sup>	3 ± 2

<sup>a</sup>% cleavage =  $(OD_{90\text{kDa}}/OD_{90\text{kDa}} + OD_{180\text{kDa}}) \times 100$ .

<sup>b</sup>% cleavage was calculated from optical density measurements of six different sample preparations run on eight different Western blots.

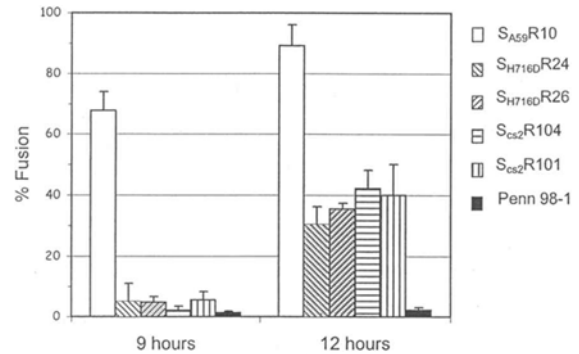
<sup>c</sup>% cleavage was calculated from optical density measurements of three different sample preparations run on five different Western blots.

Image analysis of either viral pellets or infected cell lysates offered a semiquantitative measure of the relative amounts of cleaved and uncleaved S protein. The measure of S cleavage was based on the relative intensity of protein bands migrating at either 180 kDa (uncleaved S) or 90 kDa (cleaved S). It does not take into account oligomeric, unglycosylated, or alternatively processed forms of S. These data indicate that for S protein derived from S<sub>H716D</sub> recombinants, less than 10% of the material in the two bands migrated as cleaved S, whereas approximately 60% of the S<sub>A59</sub>R10 S protein migrated as cleaved S (Table 1).

To further examine the relationship between cleavage of the S protein and the sequence of the cleavage signal, we isolated a second pair of recombinant viruses (S<sub>CS2</sub> recombinants) that contain the MHV-2 cleavage signal (TSHRARS) in an MHV-A59 spike background. Because the S protein of MHV-2 is not cleaved, it was anticipated that this change would create a variant of MHV-A59 with an uncleaved S protein. However, cleavage did occur in the two S<sub>CS2</sub> recombinants, S<sub>CS2</sub>R101 and S<sub>CS2</sub>R104, albeit at reduced levels relative to wild-type MHV-A59 S protein. The amount of cleaved S, or 90-kDa protein, observed with the S<sub>CS2</sub> recombinants (10% to 20%) was typically slightly greater than that of the S<sub>H716D</sub> recombinants, but markedly less than that seen with S<sub>A59</sub>R10 (Table 1). In contrast, S protein expressed by Penn-98-1, a recombinant virus with the entire MHV-2 S gene placed into the MHV-A59 background, appeared uncleaved in Western blots (Figure 1). The calculated % cleaved S of Penn-98-1, based on image analysis, was <5% (Table 1), indicating that the optical density of the portion of the lane corresponding to the migration of a 90-kDa protein was minimally above background levels.

#### Fusion phenotype of recombinant viruses in vitro

Previous studies indicated that the H716D mutation in S was necessary for a delayed fusion phenotype, but did not prove that the mutation alone



**Figure 2** Fusion phenotype of recombinant viruses. Confluent L2 monolayers were infected with virus at an MOI of 1. The percentage of nuclei involved in syncytia was quantified as described in Materials and methods. The results shown are representative of three separate fusion assays. The mean viral titers of duplicate infected wells at 9 or 12 hours post infection are listed in Table 1.

was responsible for this phenotype (Gombold *et al*, 1993). Figure 2 compares the fusion phenotype in L2 cells of S<sub>H716D</sub> recombinants and that of a wild-type recombinant (S<sub>A59</sub>R10) that contains the wild-type MHV-A59 S sequence. As shown in Figure 2, the fusion phenotype of the S<sub>H716D</sub> recombinants was delayed relative to the wild-type control, and therefore this mutation was sufficient to inhibit cell-to-cell fusion.

Because the MHV-2 S protein is known to be non-fusogenic, it was predictable that Penn-98-1, which expresses the MHV-2 S protein, would have a similar cleavage and fusion phenotype, as indeed was the case (Figure 2). We had originally expected the S<sub>CS2</sub> recombinants, S<sub>CS2</sub>R101 and S<sub>CS2</sub>R104, to be nonfusogenic, but instead they displayed a delayed fusion phenotype similar to that of the S<sub>H716D</sub> recombinants, and consistent with the low level of cleavage observed with these viruses. Any observed differences in the amount of cell-to-cell fusion produced by the various recombinant viruses were not due to inhibition of viral replication in cell culture. As seen in Table 2, all viruses had similar titers at 9 and 12 h post infection.

**Table 2** Viral titers obtained at 9 and 12 hours post infection

Virus	PFU/ml <sup>a</sup>	
	9 hours	12 hours
S <sub>A59</sub> R10	$2.6 \times 10^7$	$6.4 \times 10^7$
S <sub>H716D</sub> R24	$3.6 \times 10^7$	$5.5 \times 10^7$
S <sub>H716D</sub> R26	$3.9 \times 10^7$	$1.8 \times 10^7$
S <sub>CS2</sub> R104	$1.8 \times 10^7$	$4.0 \times 10^7$
S <sub>CS2</sub> R101	$2.7 \times 10^7$	$6.5 \times 10^7$
Penn-98-1	$3.0 \times 10^7$	$2.7 \times 10^7$

<sup>a</sup>PFU/ml indicates the mean viral titer of supernates from duplicate wells infected for the fusion experiment presented in Figure 2.

**Table 3** Virulence of recombinant viruses

Virus	LD <sub>50</sub>
S <sub>A59</sub> R10	5.0 × 10 <sup>3</sup>
S <sub>H716D</sub> R24	1.9 × 10 <sup>4</sup>
S <sub>H716D</sub> R26	2.7 × 10 <sup>4</sup>
S <sub>cs2</sub> R101	>2 × 10 <sup>6</sup>
S <sub>cs2</sub> R104	>2 × 10 <sup>6</sup>

#### *Replication and virulence of recombinant viruses in the brain and liver*

In some viral systems, cleavage and subsequent activation of the fusion protein correlates with viral virulence and tropism (Maisner *et al*, 2000; McCune *et al*, 1988; Rott *et al*, 1995; Stadler *et al*, 1997). The cleavage site recombinants provide a means to examine the importance of viral-induced cell-to-cell fusion on pathogenicity for MHV. The virulence of these viruses, as well as their ability to replicate *in vivo*, was assessed following intracerebral inoculation of C57BI/6 mice with S<sub>A59</sub>R10, S<sub>H716D</sub>R24, S<sub>H716D</sub>R26, S<sub>cs2</sub>R101, and S<sub>cs2</sub>R104.

The LD<sub>50</sub> of the wild-type recombinant is approximately 5 × 10<sup>3</sup> PFU following intracerebral (IC) inoculation, similar to that of wild-type MHV-A59 (Hingley *et al*, 1994; Leparc-Goffart *et al*, 1998) (Table 3). The LD<sub>50</sub> values of S<sub>H716D</sub>R24 and S<sub>H716D</sub>R26 are four- to fivefold higher, at 1.9 × 10<sup>4</sup> and 2.7 × 10<sup>4</sup>, respectively, indicating that these viruses are only slightly attenuated relative to wild-type virus. On the other hand, the LD<sub>50</sub> values of the S<sub>cs2</sub> recombinants (>2 × 10<sup>6</sup>; Table 3) are considerably higher than both wild type and S<sub>H716D</sub> recombinants.

As seen in Figure 3, the S<sub>H716D</sub> recombinants replicated in the brains of infected animals as well as or better than wild-type recombinants, although liver titers tended to be more variable, and were more likely to be reduced in mice infected with the S<sub>H716D</sub> recombinants. In contrast, replication of the S<sub>cs2</sub> recombinants was significantly impaired, in both liver and brain, compared to that of S<sub>A59</sub>R10 (Figure 3). Brain titers in mice infected with the S<sub>cs2</sub> recombinants peaked anywhere from 3 to 5 days post infection, at which point they were typically more than 10-fold lower than those in mice infected with S<sub>A59</sub>R10 or the S<sub>H716D</sub> recombinants. Liver titers were often undetectable in mice infected with the S<sub>cs2</sub> recombinants, though low titers were seen occasionally, especially at 3 days post infection.

Immunohistochemistry was performed to examine the distribution of viral antigen in brain and liver sections from infected mice. Positive staining for viral antigen was observed in similar regions of the brains of mice infected with S<sub>A59</sub>R10, S<sub>H716D</sub> recombinants, or S<sub>cs2</sub> recombinants (Figure 4). However, consistent with the low titers detected in tissue from animals infected with S<sub>cs2</sub> recombinants, viral antigen staining was much less prevalent in these mice. Positive staining for viral antigen was often observed in the

following regions: (1) at the level of the hippocampus and habenular nucleus; (Figure 4; A, C, E) (2) in the frontal cortex just off the midline, at the level of the accumbens nucleus, and including the cingulate and infralimbic cortex; (Figure 4; B, D, F) and (3) in the vicinity of the hippocampus and along the lateral ventricle. Viral antigen was associated predominately with cells that morphologically resembled glial cells, neurons, and choroid plexus epithelia. Inflammation was often observed in regions staining positive for viral antigen.

Extensive labeling for viral antigen was observed in livers of mice infected with the three types of recombinant viruses (Figure 5), even though viral titers were often undetectable in mice infected with S<sub>cs2</sub> recombinants. However, necrotic foci were more prevalent and larger in animals infected with A<sub>A59</sub>R10 or S<sub>H716D</sub> recombinants than in mice infected with the S<sub>cs2</sub> recombinants.

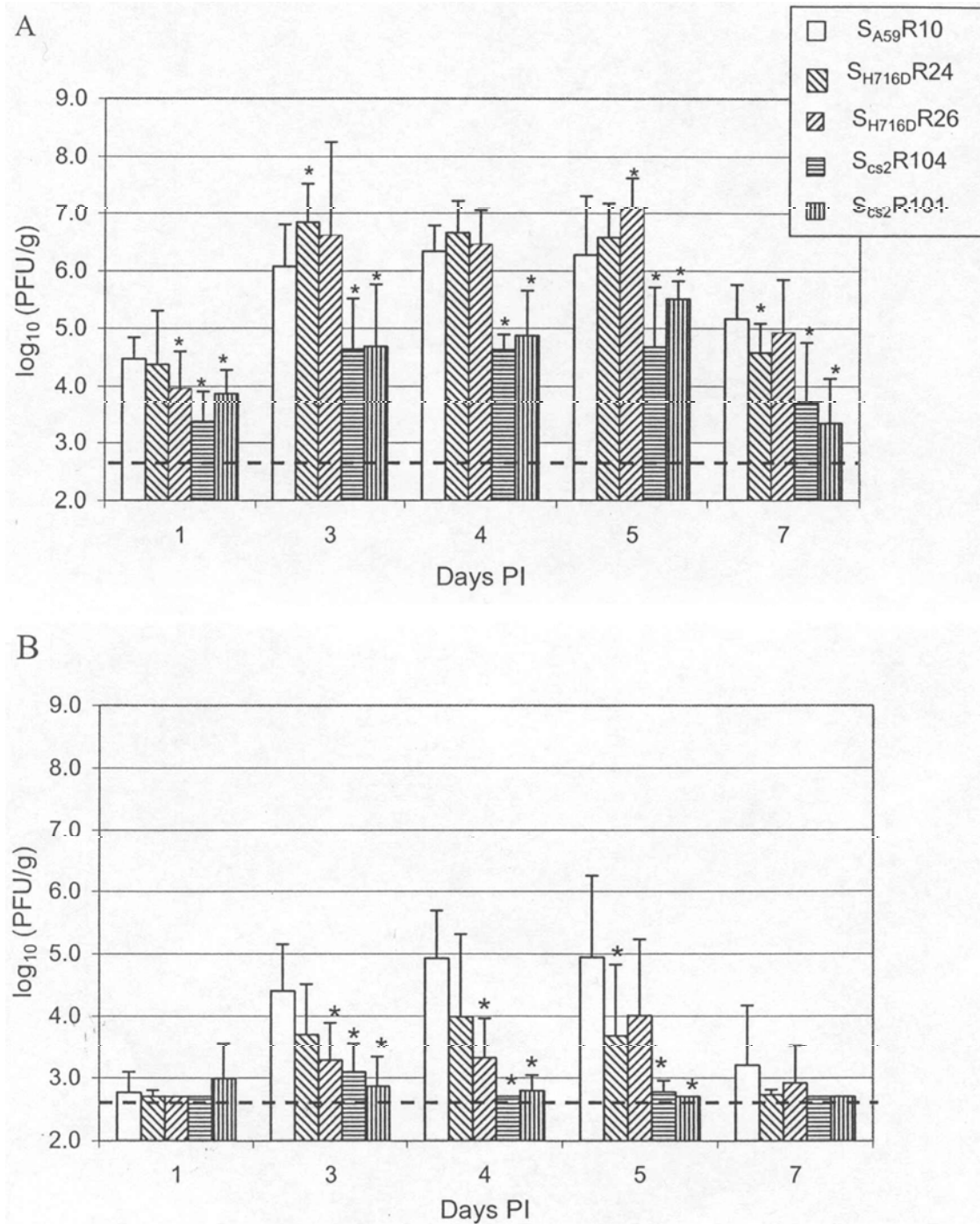
## Discussion

Our initial studies on the pathogenesis of MHV-A59 focused on the role of the S protein in determining pathogenicity, because this protein mediates both viral attachment and cell-to-cell fusion. A mutation located near the cleavage site of S, H716D, correlated with lack of cleavage of the S protein and a delayed fusion phenotype (Gombold *et al*, 1993). However, it was not established that this mutation alone was sufficient to inhibit cleavage and fusion.

To assess the relationship between cleavage of the S protein, fusogenicity and virulence, targeted recombination was used to generate recombinant MHV viruses whose spike proteins contained altered cleavage signals, including the H716D amino acid substitution. A pair of recombinant viruses that replaced the MHV-A59 cleavage signal with the corresponding MHV-2 sequence (S<sub>cs2</sub> recombinants) was also isolated. MHV-2 has a noncleaved, nonfusogenic S protein, so it was thought that the S<sub>cs2</sub> recombinants would have similar cleavage and fusion phenotypes. Interestingly, cleavage was only inhibited in the S<sub>cs2</sub> recombinants, not abolished. The low level of S cleavage observed in both the S<sub>H716D</sub> and S<sub>cs2</sub> recombinants could account for the delayed fusion phenotype of these viruses.

The MHV-A59 S protein is cleaved following a basic-X-basic-basic motif (RAHR). By substituting the MHV-2 sequence (TSHRARS) for the MHV-A59 sequence (KSRRRAHR) at the cleavage site, we disrupt two consecutive basic-X-basic-basic motifs (RAHR and KSRR). We do not know if the KSRR sequence is used as an alternate cleavage site in S; however, eliminating both potential cleavage motifs would prevent the possible use of the KSRR sequence as a cleavage site in the absence of the RAHR signal.

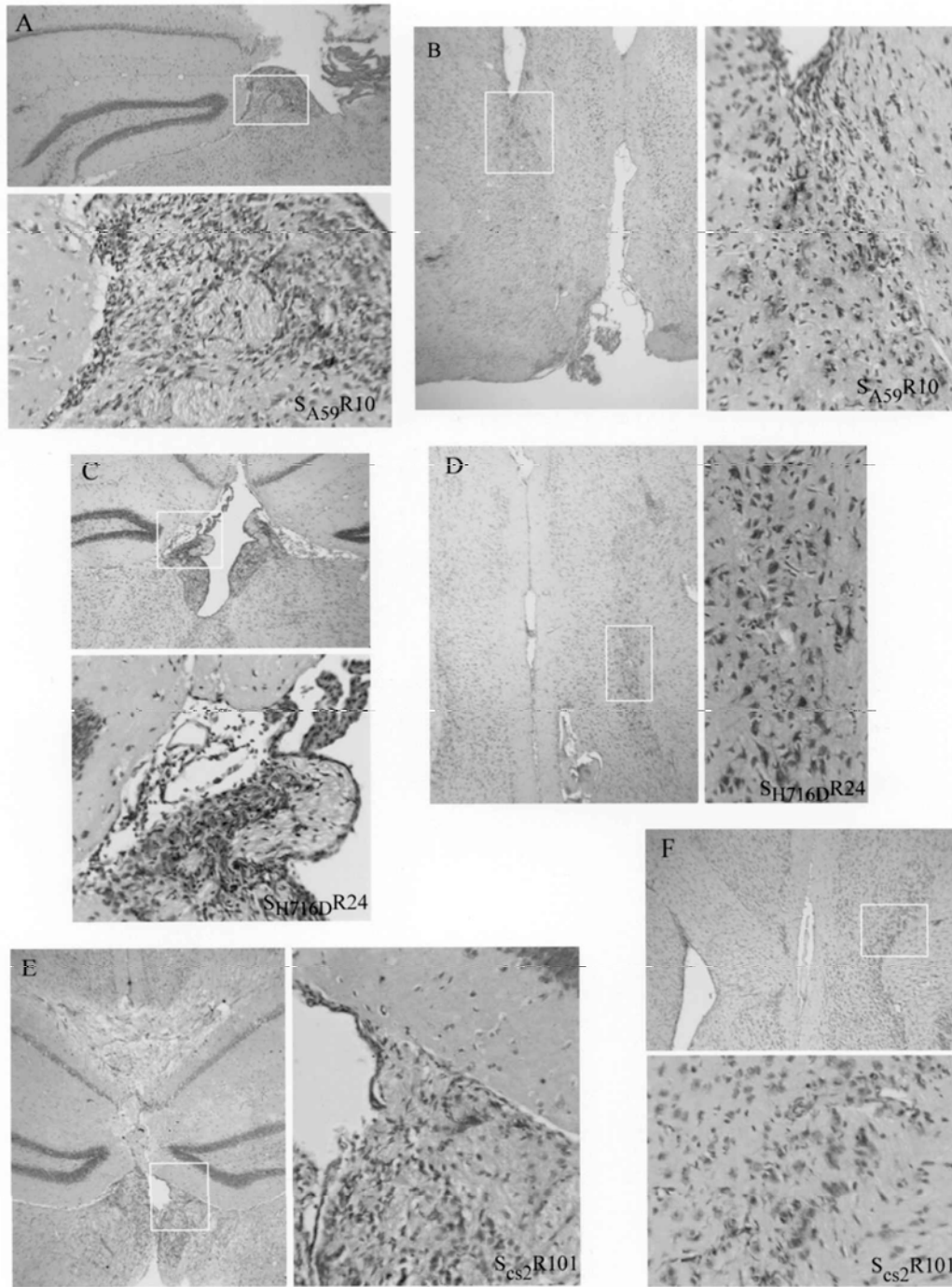
As shown in this study, disruption of the RAHR motif inhibits, but does not abolish, both cleavage of S and S-mediated cell-to-cell fusion. This observation



**Figure 3** Replication of wild-type and recombinant viruses in the brains and livers of infected animals. C57Bl/6 mice were infected IC with 5000 PFU of virus. Animals were sacrificed at the indicated times, and virus present in brain (A) or liver (B) homogenates was titered by plaque assay on L2 cells. Data are expressed as the  $\log_{10}$  PFU/g of tissue; shown is the mean  $\pm$  standard deviation of the titers from 6 to 10 animals/virus. Titers that are significantly different from  $S_{A59R10}$  are indicated (\*). The limit of detection was  $2.7 \log_{10}$  PFU/g.

is consistent with the results from several laboratories that use a vaccinia expression system to demonstrate that mutating the basic amino acid residues at the cleavage site disrupts both cleavage and fusion properties of S, but does not completely prevent cell-to-cell fusion (Bos *et al*, 1996; Stauber *et al*, 1993; Taguchi *et al*, 1992). However, the levels of S protein

present on the cell surface by the vaccinia expression system may be artificially high, and not accurately represent the relationship between cleavage of S and fusogenicity for intact virus. Therefore, the level of cell-to-cell fusion observed following infection with the  $S_{H716D}$  and  $S_{CS2}$  recombinant viruses would be a more precise measure of the importance of cleavage

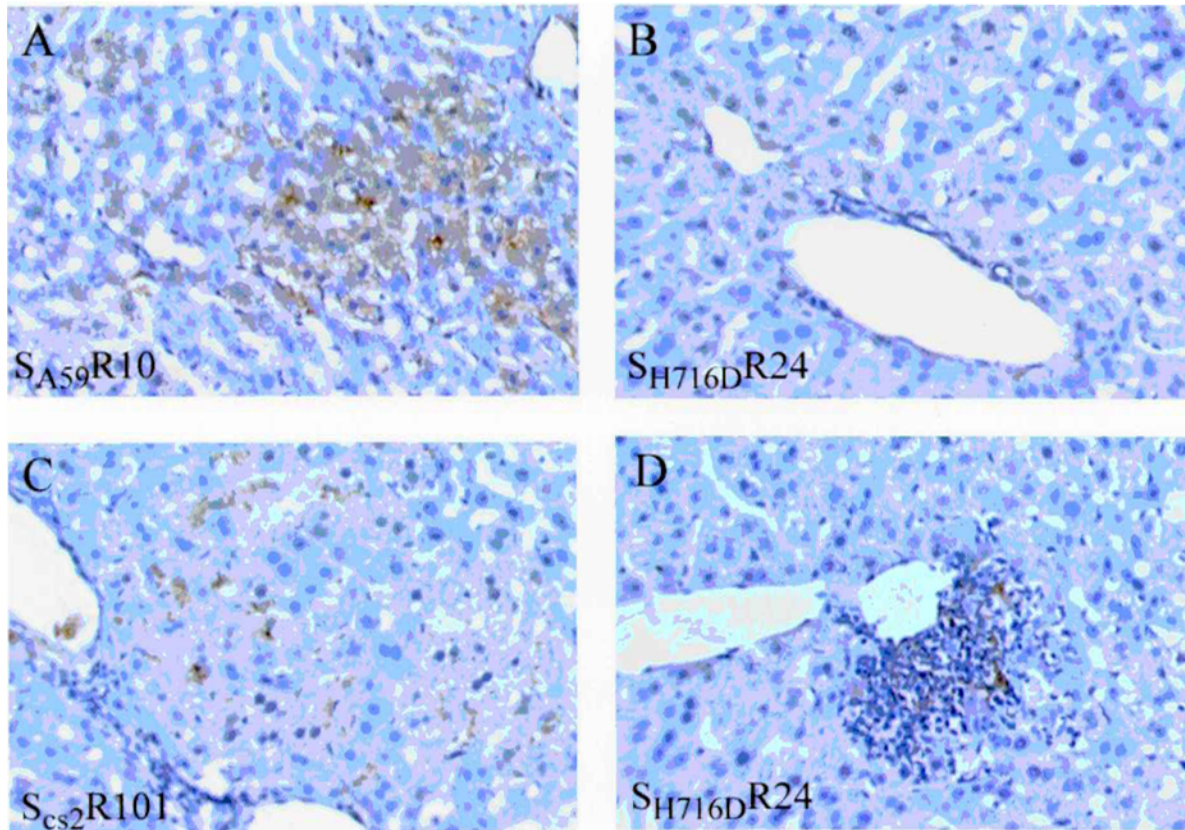


**Figure 4** Viral antigen staining in the brains of infected mice. Brains from mice infected with  $S_{A59}R10$  (A, B),  $S_{H716D}R24$  (C, D), or  $S_{cs2}R101$  (E, F) were removed at 3 days post infection, processed for histology, and stained for the presence of viral antigen. Positive staining for viral antigen is shown here at the level of the hippocampus and habenular nucleus (A, C, E) and off the midline of the frontal cortex (B, D, F). (Original magnification: 60 $\times$  for low-power micrographs and 300 $\times$  for high-power micrographs.)

of S for induction of cell-to-cell fusion. These data confirm, using intact virus instead of a vaccinia expression system, that mutations altering the basic-X-basic-basic motif of the cleavage signal of S are sufficient to disrupt both cleavage of S and viral-mediated cell-to-cell fusion.

Sequences outside of the cleavage signal must influence both cleavage and fusogenicity. Previous studies showed that fusogenicity is influenced by sequences away from the cleavage site, in viruses in which spike protein is cleaved. For example Tsai *et al* (1999) described a 12-amino acid deletion in the





**Figure 5** Viral antigen staining in the livers of infected mice. Livers from mice infected with  $S_{A59}R10$  (A),  $S_{H716RD}R24$  (B, D), or  $S_{CS2}R101$  (C) were removed at 3 days post infection, processed for histology, and stained for the presence of viral antigen. Positive staining for viral antigen was seen in animals infected with all three recombinants (A–C), whereas necrotic foci (D) were more evident in mice infected with either  $S_{A59}R10$  or  $S_{H716RD}R24$ . (Original magnification 300 $\times$ ).

hypervariable region of S1 that converts a cleaved, fusogenic S protein into an S protein that is still cleaved, but nonfusogenic. There are additional examples of mutations either in the hypervariable region of S1 (Phillips *et al*, 2001), or in the heptad repeat region of S2 (Gallagher *et al*, 1991; Luo and Weiss, 1998), that disrupt S-mediated cell-to-cell fusion independently of cleavage. The data reported here are consistent with the observation that cleavage of S correlates with more efficient induction of cell-to-cell fusion. However, our data differ from these other studies in that they are the first observation of viruses with the same cleavage signal sequence (Penn-98-1 versus  $S_{CS2}$  recombinants), yet different levels of cleavage and fusion. Thus, the cleavage signal alone does not determine cleavage of S.

Krueger *et al* (2001) suggest that S-mediated cell-to-cell fusion correlates with a dissociation of the S1 and S2 subunits of the MHV spike protein. Consequently, a more stable interaction between S1 and S2 would be associated with a delayed fusion phenotype. Inhibition of S protein cleavage would be associated with a more stable interaction between S1 and S2 and correlate with a loss of fusogenicity. This could account for the delayed fusion phenotype observed with the  $S_{H716D}$  and  $S_{CS2}$  recombinants.

For coronaviruses, the relationship between induction of cell-to-cell fusion and virulence is complex. The data on the  $S_{H716D}$  and  $S_{CS2}$  recombinants suggest that the ability to produce syncytia *in vitro* is not a predictor of pathogenicity. The  $S_{CS2}$  recombinants were considerably more attenuated than the  $S_{H716D}$  recombinants, even though both sets of recombinants had the same cleavage and fusion phenotypes. Similarly, the  $S_{CS2}$  recombinants did not replicate *in vivo* as well as the  $S_{H716D}$  recombinants, although the regions of the brain that were infected by the different recombinants was similar. For the viruses examined in this study, there was no correlation between ability to induce cell-to-cell fusion *in vitro* and ability to cause disease *in vivo*.

The only difference between the  $S_{H716D}$  and  $S_{CS2}$  recombinants is the amino acid sequence at the carboxy-terminus of the S1 subunit. It is surprising that these two different cleavage signals, which produce the same fusion/cleavage phenotypes *in vitro*, produce virus with a greater than 2  $\log_{10}$  difference in  $LD_{50}$ , and a significant difference in ability to replicate *in vivo*. The different *in vivo* properties of these two sets of recombinants indicate that disrupting the cleavage signal of S affects more than just cleavage and fusion phenotypes. This observation, along with

evidence indicating that mutations removed from the cleavage signal disrupt cleavage and fusogenicity of the S protein, implies that the conformation of S is an important determinant of function. Mutations in one region of S may alter the conformation of this protein as a whole, and subsequently disrupt protein functions mediated by other regions of S.

Given the role of the S protein in viral attachment and induction of immune response, protein functions that might be altered by mutations in S include viral affinity for host cell receptors, as well as induction of neutralizing antibody and T cells. It is possible that the S<sub>cs2</sub> recombinants have lower affinity for cellular receptors in the brain and/or liver, which could account for their impaired ability to replicate in these tissues. Alternatively, conformational differences between the S<sub>H716D</sub> and the S<sub>cs2</sub> recombinants could produce differences in the epitopes recognized by the immune response, which could in turn influence viral clearance. Thus, the mutations introduced into the cleavage signal of the S protein, by altering the conformation of S, might indirectly affect the *in vivo* phenotype of the recombinant viruses, even though their *in vitro* fusion phenotypes are similar.

## Materials and methods

### Virus and cells

MHV-A59 was obtained from Lawrence Sturman (Albany, NY). Alb4, obtained from Paul Masters, is a temperature-sensitive variant of MHV-A59 that contains an 87-nucleotide deletion (resulting in a 29-amino acid in-frame deletion) in a nonessential spacer region in the N gene (Koetzner *et al*, 1992).

The recombinant viruses S<sub>A59</sub>R10 and Penn-98-1 have been described previously (Das Sarma *et al*, 2000; Leparac-Goffart *et al*, 1998). S<sub>A59</sub>R10 and Penn-98-1 contain the MHV-A59 and MHV-2 S gene, respectively, in the MHV-A59 background. The recombinant viruses S<sub>cs2</sub>R101 and S<sub>cs2</sub>R104 contain an MHV-A59 S gene with the MHV-2 cleavage site (TSHRARS replaces KSRRAGR). The S<sub>H716D</sub> recombinant viruses, S<sub>H716D</sub>R24 and S<sub>H716D</sub>R26, contain a single point mutation (D replaces H) at amino acid codon 716. This mutation results in a cleavage signal of RRADR, mimicking the mutation observed in the fusion-delayed glial cell mutants previously charac-

terized (Gombold *et al*, 1993; Hingley *et al*, 1994). The isolation of the S<sub>H716D</sub> recombinants and the S<sub>cs2</sub> recombinants is described below.

Murine L2 cells and 17Cl-1 cells were maintained on plastic tissue culture dishes in Dulbecco's minimal essential medium (MEM) with 10% fetal bovine serum (FBS). Spinner cultures of L2 cells were maintained in Joklik's MEM with 10% FBS at densities between  $2 \times 10^5$  and  $2 \times 10^6$  cells per milliliter.

### Plasmids and PCR mutagenesis

The construction of pFV1 (obtained from Paul Masters) was described previously (Fischer *et al*, 1997). This plasmid contains the entire MHV-A59 S gene (gene 3), and all the structural genes 3' of S, including a wild-type N gene sequence. The sequence of the S gene in pFV1 is the same as that of our wild-type MHV-A59 strain, except for silent changes in codons 173 and 174, resulting in the loss of a *HindIII* site and the concomitant addition of an *AseI* site. As described previously (Leparac-Goffart *et al*, 1998), these changes enabled a *HindIII* site following the polyA tail to be used for linearization of the plasmid for RNA transcription, as well as providing a way to distinguish the S gene of the parent virus from that derived from pFV1 in the recombinant viruses.

Polymerase chain reaction (PCR) mutagenesis, with Vent polymerase, was used to introduce the H716D mutation into the S gene of pFV1. A fragment containing the H716D mutation was generated by amplification of two shorter fragments. The 5' end fragment was generated using primers FS413 and RSH716D, and the 3' end using primers WZL47 and RS830 (Table 4). Primers RSH716D and WZL47 introduced a histidine to aspartic acid nucleotide substitution in amino acid codon 716. The 928-bp and 387-bp PCR fragments were gel purified and used as templates for a third PCR reaction with primers FS413 and RS830. The resulting 1315-bp fragment was gel purified, digested with restriction enzymes *DraIII* and *XhoI*, and then cloned into the corresponding sites in pFV1. This clone was designed pFV1-H716D. The entire *DraIII/XhoI* fragment of pFV1-H716D was sequenced to verify the presence of the H716D mutation, as well as the absence of other mutations.

PCR mutagenesis was similarly used to alter the cleavage signal of MHV-A59 S gene so that it

**Table 4** Primers used for targeted recombination

Primer	Sequence (5' → 3')	Location in S (nucleotide no.)
FS413	CTGCTAATTATAAGATTGATACAGC	1238–1262
RSH716D	GCCAGTAGAAACTGATCGGTGAGCCCTGCCGTGATTTTGAATA	2124–2166
WZL47	TATTCAAATCACCGAGGGCTGACCGATCAGTTTCTACTGGC	2124–2126
CSR	TGAGCTGCGAGCCCTGTGTGATGTTGA	2128–2154
CSF	TCAACATCACACAGGGCTCGCAGCTCA	2128–2154
RS830	CTGAGATGCCGTCTGGCAGTCTCG	2489–2512
WZL60B	CCGGTCTCAAG	2684–2695



resembled that of MHV-2 (cs2 mutation). Primers FS413 and WZL60A were used to amplify a 1520-bp fragment from two smaller overlapping templates generated using primers FS413 and CSR, and CSF and WZL60B (Table 4). The 1520-bp fragment was digested with *DraIII* and *XhoI* and cloned into the corresponding sites in the plasmid pMH54, creating the plasmid pMH54-cs2. The vector pMH54 is similar to pFV1, but contains a 5' extension of the S gene that includes the 3' end of the HE gene (Fischer *et al*, 1997). The *DraIII/XhoI* fragment of pMH54-cs2 was sequenced to confirm the presence of the cleavage signal mutations and the absence of additional mutations.

#### Targeted RNA recombination

Targeted RNA recombination was carried out between parent virus having an MHV-A59 background, and RNAs transcribed from either wild-type pFV1 or pFV1-H716D. Infection of L2 cells with parent virus, transfection with the synthetic RNA, and isolation and plaque purification of recombinant virus were carried out as described previously (Leparc-Goffart *et al*, 1998; Masters *et al*, 1994). Targeted recombination using the pMH54-cs2 plasmid was performed in a similar manner, except the parent virus contained the feline S protein necessitating a different screening protocol for recombinant virus. This protocol has also been previously described (Kuo *et al*, 2000; Phillips *et al*, 1999). The presence of the desired mutation, H716D or cs2, was confirmed by sequencing.

#### Sequencing

Templates for sequencing were derived by reverse transcriptase-polymerase chain reaction amplification (RT-PCR) of cytoplasmic RNA extracted from virus-infected L2 cell monolayers. PCR products were purified (Wizard prep; Promega) and analyzed by automated sequencing using the Taq dye terminator procedure according to the manufacturer's protocol (Taq DyeDeoxy Terminator Cycle Sequencing kit; Applied Biosystems, Foster City, CA). Each fragment was sequenced in both directions.

#### Viral fusion assay

L2 cell monolayers were prepared in 6-well plates in Dubelcco's MEM with 10% FBS. Confluent monolayers were infected with each virus at a multiplicity infection (MOI) of 1 and incubated for 1 h at 37°C. Following adsorption, the cells were washed with Hanks balanced salt solution (Gibco) two times and then fed with 1 ml of DMEM-10% FBS. At the times indicated, the supernatants were removed and titered by plaque assay on L2 cells as previously described (Gombold *et al*, 1993). The infected cells were fixed with 4% paraformaldehyde, stained with hematoxylin, and fusion quantified by counting the number of nuclei involved in syncytia, and

expressing this as a percent of total nuclei in a field. Two fields of approximately 300 nuclei each were counted, in duplicate wells, for each virus. The mean  $\pm$  SD of percent fusion was then calculated from the four fields (approximately 1200 nuclei) examined.

#### Western blot analysis of S

L2 cell monolayers in 6-well plates or T25 flasks were infected with 100 or 200  $\mu$ l virus, respectively, at an MOI of 1 to 5 PFU/cell. After the virus was allowed to attach to the cells for 1 h, additional media was added and the infection continued for 9 to 16 h. Virions present in culture supernates were obtained by ultracentrifugation at 126,000  $\times$  g for 2 h. Viral lysates were prepared by lysing infected cells with 200  $\mu$ l lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40). Viral pellets and lysates were electrophoresed into 7% sodium dodecyl sulfate (SDS)-polyacrylamide gels in a Tris Acetate buffer system (Novex) and transferred to nitrocellulose or PVDF membrane. MHV S protein was detected using a polyclonal goat anti-S antibody (AO4; provided by K Holmes, University of Colorado HSC, Denver, CO) and a chemiluminescence detection system (Amersham).

Image analysis of Western blots was performed using Gel-Pro Analyzer software from Media Cybernetics. Protein bands migrating at a molecular weight of 180 and 90 kDa were selected, and the analysis program generated an optical density measurement for these bands after filtering out background. The relative amount of material migrating at 90 kDa, corresponding to cleaved S, compared to protein migrating at 180 kDa, or uncleaved S, was calculated using the following formula:  $OD_{90\text{ kDa}} / (OD_{90\text{ kDa}} + OD_{180\text{ kDa}})$ , and expressing the results as a percentage. This number is defined as the percent cleaved S. A limitation of this type of quantification is that it does not take into account oligomeric, alternatively processed, or immature forms of S. Thus, the numbers obtained in this manner must be considered to be a semiquantitative measure of S cleavage.

#### Viral replication in vivo

All animal experiments used four week old MHV-free C57BI/6 male mice (Jackson Laboratories, Bar Harbor, ME). Viruses were diluted into phosphate-buffered saline containing 0.75% bovine serum albumin (PBS/BSA). Mice were anesthetized with methoxyflurane (Metofane, Pittman-Moore, Mundelein, IL), and inoculated intracerebrally with 20  $\mu$ l of diluted virus (5000 pfu) injected into the right cerebral hemisphere.

For measurement of virus replication in the brain and the liver, at selected times post infection, mice were sacrificed and brains and livers removed. Brains were placed directly into 4 ml of isotonic saline with 0.167% gelatin (gel saline), whereas livers were first

rinsed with PBS and then placed in 10 ml of gel saline. All organs were weighed and stored frozen at  $-80^{\circ}\text{C}$  until titered for virus. Organs were homogenized and virus titered by plaque assay on L2 cell monolayers all as previously described (Hingley *et al*, 1994). *In vivo* replication of the different viruses was compared using Wilcoxon's rank sum test.

#### Virulence assay

Mice were inoculated intracerebrally with either 5-fold or 10-fold serial dilutions of wild-type or recombinant virus, five mice per dilution. Mice were examined for signs of disease or death on a daily basis up to 21 days post infection. LD<sub>50</sub> values were

calculated by the Reed-Meunch method (Lavi *et al*, 1984; Reed and Muench, 1938).

#### Immunohistochemistry

Mice inoculated intracerebrally with 5000 pfu of virus were sacrificed at 3 or 4 days post infection. Mice were perfused with 4% paraformaldehyde, and tissue was processed and embedded in paraffin. Immunohistochemistry was performed using a rabbit anti-MHV-A59 polyclonal primary antibody (UV13) and horseradish peroxidase (HRP)-conjugated secondary antibody. Diaminobenzadine tetrahydrochloride was used as a substrate, and slides were counterstained with hematoxylin.

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