Multiple regions of the murine coronavirus spike glycoprotein influence neurovirulence

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The spike (S) glycoprotein of mouse hepatitis virus (MHV) is a major determinant of neurovirulence. Using targeted recombination we previously demonstrated that the S gene of the highly neurovirulent MHV-4 conferred a dramatic increase in neurovirulence to the mildly neurovirulent MHV-A59. To identify the genetic determinants of neurovirulence within the MHV-4 spike, we generated isogenic recombinant viruses containing various MHV-4/MHV-A59 chimeric spike genes, and studied their phenotypes *in vivo*. The MHV-4/MHV-A59 chimeric spike genes consisted of either reciprocal exchanges between the S1 and S2 spike subunits, or smaller exchanges specifically in the hypervariable region (HVR) of S1. The chimeric spike gene containing recombinants all exhibited efficient replication *in vitro*, yet many were severely attenuated for virulence *in vivo*. Furthermore, these attenuated recombinants exhibited decreased titers of infectious virus in the brain relative to the parental recombinant viruses containing the full-length MHV-4 or MHV-A59 spike genes. This is the first report that compares the neurovirulence and pathogenesis of isogenic viruses with defined alterations in the MHV spike protein. From these studies, it appears that the interactions of multiple regions of the MHV spike, including the HVR, act in concert to allow for efficient infection of and virulence in the murine central nervous system. *Journal of NeuroVirology* (2001) 7, 421–431.

Keywords: mouse hepatitis virus; encephalitis; recombinant coronaviruses; neurovirulence

Introduction

Murine coronaviruses, or mouse hepatitis viruses (MHV), are enveloped positive-stranded RNA viruses that can induce a variety of respiratory, gastrointestinal, and neurologic diseases in rodents. As with a number of viral pathogens, the severity and organ tropism of the disease depend, in part, on the viral strain. Infection with the highly neurovirulent MHV type 4 (MHV-4, a strain JHM isolate) results in a potentially fatal acute encephalomyelitis (Dalziel *et al*, 1986). Infection with a similar dose of the mildly neurovirulent MHV type A59 (MHV-A59) results in a much less severe disease with only mild

encephalomyelitis and virtually no mortality (Lavi *et al*, 1986; Lavi *et al*, 1988). Recently, using targeted recombination, we have demonstrated that the spike gene of MHV-4 is sufficient to confer this highly neurovirulent phenotype to MHV-A59 (Phillips *et al*, 1999).

The MHV spike or S glycoprotein, expressed on the virion envelope and on the plasma membrane of infected cells, plays a vital role in viral entry, viral spread, and in the immune response to infection (Collins *et al*, 1982; Buchmeier *et al*, 1984; Dveksler *et al*, 1991; Castro and Perlman, 1995; Bergmann *et al*, 1996). Depending both on the viral strain and the host cell the spike is posttranslationally cleaved dividing the spike into an amino-terminal, S1, subunit and a carboxy-terminal, S2, subunit (Sturman *et al*, 1990). S1, thought to form the globular head of the spike, mediates binding to the viral receptors (Collins *et al*, 1982; DeGroot *et al*, 1987; Kubo *et al*, 1994) that are members of the CEACAM (previously Bgp (Beauchemin *et al*, 1999)) subclass of the

large carcinoembryonic antigen (CEA) gene family (Dveksler et al, 1991; Williams et al, 1991). Subsequent to receptor-binding, there is thought to be a conformational change in spike which allows S2, the stalk portion of the peplomer, to induce virus-cell membrane fusion (Collins et al, 1982; DeGroot et al, 1987; Sturman et al, 1990; Weismiller et al, 1990). Subsequent to infection, S expressed on the host cell membrane can also mediate cell–cell fusion. During an in vivo infection, the MHV spike is also an important target for both humoral and cellular immune responses (Collins et al, 1982; Fleming et al, 1983; Castro and Perlman, 1995; Bergmann et al, 1996).
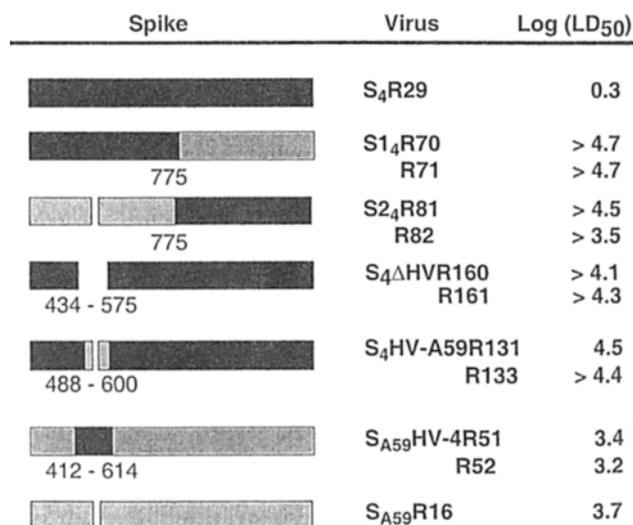
Despite the dramatic differences in neurovirulence conferred by spike proteins of MHV-4 and MHV-A59 they are highly homologous proteins, sharing 95% amino acid identity in S2 and 90% identity in S1. Within the S1 subunit of the less neurovirulent MHV-A59 spike, however, there is deletion of 52 amino acids in a region termed the hypervariable region (HVR) (Luytjes et al, 1987). The spike HVR, loosely defined between amino acids 400 and 600, exhibits a great deal of variation in length from strain to strain (Parker et al, 1989; Banner et al, 1990). The highly neurovirulent MHV-4 has the longest known HVR, but deletions in this region of up to 159 amino acids have been reported (Parker et al, 1989). The specific role of the HVR in pathogenesis appears to be complex and multifunctional. Deletions or mutations in this region have been found to alter viral fusion, eliminate neutralizing monoclonal antibody epitopes, and abrogate CD8^{+} T-cell epitopes (Dalziel et al, 1986; Fleming et al, 1986; Wege et al, 1988; Parker et al, 1989; Taguchi and Fleming, 1989; Gallagher et al, 1990; Pewe et al, 1996; Tsai et al, 1999; Wang et al, 1992). In addition, deletions or mutations in the HVR have been associated with neuroattenuation. In one such study, a 142-amino acid deletion in the HVR was associated with decreased neurovirulence and decreased viral spread in the CNS (Fazakerley et al, 1992).

With the recent development of targeted RNA recombination the structural genes of MHV have become accessible, for the first time, to genetic manipulation (Fischer et al, 1997; Leparc-Goffart et al, 1998). Using this technique we have definitively demonstrated that the S gene is a major determinant of MHV neurovirulence (Phillips et al, 1999). To further identify genetic determinants of neurovirulence within the MHV spike, we generated a series of recombinants containing MHV-4/MHV-A59 chimeric spike genes, and examined the pathogenesis of these isogenic viruses that differed exclusively in spike. From these studies, we conclude that multiple regions of the MHV-4 spike, including the hypervariable region, are required for efficient infection and virulence in the mouse CNS.

Results

Pathogenesis of recombinants expressing S1/S2 chimeric spike genes

To begin to identify specific regions of the MHV spike which play a role in neurovirulence, we generated recombinant viruses containing exchanges between the S1 and S2 subunits of the highly neurovirulent MHV-4 spike and the much less neurovirulent MHV-A59 spike. The recombinants were generated using a modification of the targeted recombination strategy as recently described by Kuo et al (2000) (see Materials and methods). To reduce the risk of secondary site mutations influencing the viral phenotype, two independently selected recombinants (R), as indicated by their numbering, were isolated and characterized for each desired recombinant virus. As shown in Figure 1, the recombinants containing the S1 of MHV-4 and the S2 of MHV-A59 were named S1$_4$R70 and S1$_4$R71, and the recombinants containing the S1 of MHV-A59 and the S2 of MHV-4 were called S2$_4$R81 and S2$_4$R82. The S1/S2 junction was located just 3' to the cleavage site at amino acid 775 (see Materials and

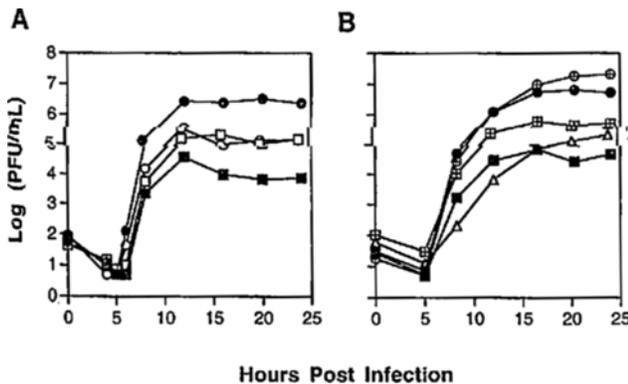| Spike	Virus	Log (LD$_{50}$)
	S$_4$R29	0.3
775	S1$_4$R70 R71	> 4.7 > 4.7
775	S2$_4$R81 R82	> 4.5 > 3.5
434 - 575	S$_4$ΔHVR160 R161	> 4.1 > 4.3
488 - 600	S$_4$HV-A59R131 R133	4.5 > 4.4
412 - 614	S$_{A59}$HV-4R51 R52	3.4 3.2
	S$_{A59}$R16	3.7

Figure 1 Schematic diagram of the spike gene from the recombinant viruses and their corresponding virulence following intracranial inoculation. LD$_{50}$ assays were conducted as described previously (Hingley et al, 1994). The recombinant viruses are represented as indicated. S$_{A59}$R16 and S$_4$R29 contain the MHV-A59 spike and the MHV-4 spike, respectively. S1$_4$R70 and 71 contain the S1 of the MHV-4 spike with the S2 of the MHV-A59 spike, and S2$_4$R81 and 82 contains the S1 of MHV-A59 and the S2 of MHV-4. S$_4$ΔHVR160 and 61 represent recombinants that have a 142-amino acid deletion in the MHV-4 hypervariable region (HVR), while S$_4$HV-A59R131 and R133 contain an exchange of the MHV-4 HVR for the corresponding MHV-A59 region. S$_{A59}$HV-4R51 and R52 contain the MHV-A59 spike with the HVR of MHV-4. The amino acid residues at the exchange sites are indicated by their position in the MHV-4 spike. Recombinant S2$_4$R81 contained a single coding mutation L661Q, and recombinants S$_4$HV-A59R131 and R133 each contained a single coding mutation S359I and N374Y, respectively. Grey bars indicate the MHV-A59 sequence, and closed bars indicate the MHV-4 sequence. The 52-amino acid deletion in the MHV-A59 HVR is indicated.

Methods). As controls, recombinants containing the full-length spike gene of MHV-4, S_4R29, or MHV-A59, $S_{A59}R16$, were isolated and characterized in parallel. To further decrease the impact of secondary site mutations in the recombinant viruses, we sequenced the entire S gene from at least one of each pair of recombinants. S_4R29, $S_{A59}R16$, and $S1_4R71$ contained no secondary site mutations in spike. $S2_4R81$ contained a single point mutation in the S1 subunit that resulted in a codon change L661Q. An independently isolated recombinant virus, $S2_4R82$, contained no secondary site mutations in S.

To examine the efficiency of replication of the chimeric spike gene recombinants we performed growth curves on L2 cell monolayers with $S1_4R70$, $S2_4R81$, S_4R29, and $S_{A59}R16$. The results are shown in Figure 2A. Consistent with previous reports, recombinants containing the MHV-A59 spike, $S_{A59}R$, replicated to higher titer in cell culture than recombinants containing the MHV-4 spike, S_4R (Phillips et al, 1999). Both chimeric spike gene recombinants, $S1_4R70$ and $S2_4R81$, replicated in cell culture, and exhibited similar kinetics as the two parental recombinants, $S_{A59}R$ and S_4R. The titers of infectious virus, however, were intermediate between $S_{A59}R$ and S_4R.
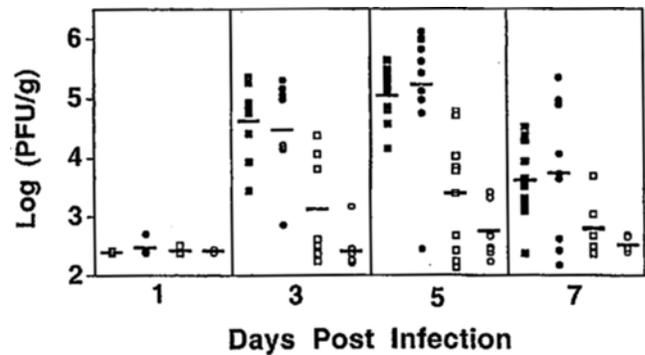
The plaque morphology of the chimeric spike gene recombinants was also intermediate between $S_{A59}R$ and S_4R. At 48 h after infection of L2 cell monolayers, the $S1_4$ recombinants and the $S2_4$ recombinants exhibited a medium-plaque phenotype (0.75 mm and 0.5 mm, respectively) as compared to the large-plaque phenotype produced by $S_{A59}R$ (1.0 mm) and the small-plaque phenotype produced by S_4R (0.25 mm). Thus, the S1/S2 chimeric spike gene recombinants replicated efficiently in cell culture, but exhibited a slightly different in vitro phenotype from either of the parental MHV-4 or MHV-A59 spike containing recombinants.

To determine if either the S1 or the S2 subunit of the MHV-4 spike was sufficient to confer an increase in neurovirulence, we infected mice intracranially with $S1_4R70$, $S1_4R71$, $S2_4R81$, $S2_4R82$, S_4R29, and $S_{A59}R16$, monitored for lethality, and calculated LD_{50} values. The neurovirulence of each recombinant is shown in Figure 1. The LD_{50}s for the S_4 and S_{A59} recombinants were similar to those reported previously (Phillips et al, 1999). The virulence of the chimeric S gene recombinants, however, was very different from either of the parental viruses. Both $S1_4$ and $S2_4$ recombinants were highly attenuated for virulence. Consistent with this decrease in virulence, mice exhibited clinical signs such as hunched posture, ruffled fur, and abnormal gait only at high doses of virus (>1000 PFU).

To determine if the reduced virulence of the chimeric spike gene recombinants was attributable to decreased viral replication in the brain, we titered brain homogenates for infectious virus at various days after intracranial inoculation with 10 PFU of virus (see Materials and Methods). The results are shown in Figure 3. As we have shown previously, the titers of infectious virus in the brain were similar following inoculation with S_4R and $S_{A59}R$ despite the dramatic difference in virulence exhibited by the two recombinant viruses (Phillips et al, 1999). The two chimeric S gene recombinants, however, exhibited markedly reduced virus titers. $S1_4R70$ exhibited low levels of virus while the amount of infectious $S2_4R81$ virus was at or below the level of detection (200 PFU/g). Thus, despite the high amino acid identity between the MHV-4 and MHV-A59 spike proteins they could not substitute functionally for one another in an in vivo infection. This

Hours Post Infection

Figure 2 Time course of recombinant virus production on L2 cell monolayers. Each point represents the mean titer of duplicate samples. (A) Cells were infected at a multiplicity of infection of 1.8 PFU/cell with $S_{A59}R16$ (●), $S1_4R70$ (□), $S2_4R81$ (O), and S_4R29 (■). (B) Cells were infected at a multiplicity of 0.4 PFU/cell with $S_{A59}R16$ (●), $S_{A59}HV$-4R51 (cross in O), S_4HV-A59R131 (cross in □), $S_4\Delta HVR160$ (△), and S_4R29 (■).

Days Post Infection

Figure 3 Titers of infectious virus in the brains of animals inoculated with viral recombinants containing chimeric MHV-4/MHV-A59 S genes. Weanling B6 mice were inoculated intracranially with 10 PFU of S_4R29 (■), $S_{A59}R16$ (●), $S1_4R70$ (□), or $S2_4R81$ (O). Animals were sacrificed at the indicated times, and virus titers in the brain were determined by plaque assay. The titers of infectious virus in the brain are given for each mouse and the mean virus titers are shown as bars (n = 4–10 mice per time point, data for days 5 and 7 are from three independent experiments). On day 5 p.i., the mean virus titers in S_4R29-infected mice was significantly greater than in $S2_4R81$-infected mice (two-tailed t-test, P > 0.0001). The limit of detection was 200 PFU/g of tissue.

result suggests that the high neurovirulence conferred by the MHV-4 spike protein requires specific homotypic interactions between regions in S1 and S2, and that disruption of these interactions has a major impact on the pathogenesis of the virus.

Role of the HVR in pathogenesis

Within the S1 subunit there is a region, termed the hypervariable region (HVR), which can tolerate extraordinary variation particularly with respect to its length (Banner et al, 1990; Parker et al, 1989). It has been proposed that the HVR has a vital function in MHV pathogenesis. Numerous variant viruses have been identified in which mutations or deletions in this region have been associated with alterations in neuropathogenesis (Dalziel et al, 1986; Fleming et al, 1986; Wege et al, 1988; Parker et al, 1989; Taguchi and Fleming, 1989; Gallagher et al, 1990; Wang et al, 1992). To definitively assess the role of the hypervariable region (HVR) in neurovirulence, we used targeted recombination to generate a series of recombinant viruses with small exchanges in the HVR between MHV-4 and MHV-A59. To determine if the HVR of the highly neurovirulent MHV-4 spike was necessary to confer an increase in neurovirulence, we generated recombinants in which the MHV-4 HVR was deleted or replaced with the MHV-A59 HVR (see Figure 1). Deletion of the MHV-4 HVR was based on a previously identified neutralizing monoclonal antibody escape mutant with a deletion in the HVR, and the two independently isolated recombinants containing this 142-amino acid deletion were named $S_4 \Delta HVR160$ and $S_4 \Delta HVR161$ (Dalziel et al, 1986; Parker et al, 1989). Recombinants $S_4 HV$-A59R131 and $S_4 HV$-A59R133 contained the MHV-4 S gene with replacement of a 112-amino acid region in the HVR with the corresponding 60-amino acid region from the MHV-A59 S gene. In addition, to examine if the MHV-4 HVR was sufficient to confer some degree of increased neurovirulence we replaced the MHV-A59 HVR with the MHV-4 HVR (see Figure 1). Thus, recombinants $S_{A59}HV$-4R51 and $S_{A59}HV$-4R52 contained the MHV-A59 spike with replacement of a 150-amino acid region in the HVR with the corresponding 202-amino acid region from the MHV-4 spike. Once again, we sequenced the entire S gene from at least one recombinant of each pair of recombinants. Recombinants $S_4 \Delta HVR160$ and $S_{A59}HV$-4R51 contained no secondary site mutations. Sequencing of recombinant $S_4 HV$-A59R131, containing the MHV-4 spike with the MHV-A59 spike HVR, revealed a single coding mutation S359I. Interestingly we were unable to select a $S_4 HV$-A59R recombinant without a secondary site mutation. $S_4 HV$-A59R133, an independently isolated recombinant, contained a single coding mutation N374Y, and two additional recombinants, $S_4 HV$-A59R132 and R134, were partially sequenced and found to contain single coding mutations (L1114F and L660Q, respectively). These two

recombinants were not further characterized. The inability to select $S_4 HV$-A59 recombinants without secondary site mutations in S suggests that these mutations were selected for during replication in cell culture.

The replication efficiency of these recombinants on L2 cell monolayers is shown in Figure 2B. The recombinants all exhibited similar kinetics of viral replication; however, the extent to which they replicated differed. In general, the growth phenotype of the recombinants segregated with the spike gene. Recombinants with primarily MHV-4 spike sequence, $S_4 \Delta HVR$ and $S_4 HV$-A59R, replicated to a similar extent as $S_4 R$, and the HVR recombinant with primarily MHV-A59 spike sequence, $S_{A59}HV$-4R, replicated to a similar extent as $S_{A59}R$. The plaque morphology of the recombinants was also dependent on the spike gene. 48 h p.i. of L2 cell monolayers, $S_{A59}R$, and $S_{A59}HV$-4R exhibited a large-plaque morphology (1.0 mm). The recombinants with primarily the MHV-4 spike exhibited smaller plaque-phenotypes. $S_4 HV$-A59R exhibited an intermediate-plaque size (0.75 mm), and $S_4 \Delta HVR$ exhibited a delayed plaque-phenotype as plaques were not yet visible at 48 h p.i. The delay in plaque-formation observed with $S_4 \Delta HVR$ was consistent with previous reports associating deletions in the MHV-4 HVR with delayed fusion and decreased cytopathicity (Gallagher et al, 1990). In general, recombinants with alterations in the hypervariable region exhibited similar properties in cell culture as the parental spike from which they were derived.

To determine the effect of mutations in the HVR on virulence, we inoculated animals intracranially with serial dilutions of the recombinant viruses, observed them for lethality, and then calculated LD_{50} doses. The results are shown in Figure 1. Removal of the MHV-4 HVR, either by deletion, $S_4 \Delta HVR$, or by replacement with the MHV-A59 HVR, $S_4 HV$-A59R, resulted in recombinant viruses with attenuated virulence. Thus the MHV-4 HVR was necessary for the highly neurovirulent phenotype conferred by the MHV-4 spike. In contrast, insertion of the MHV-4 HVR into the MHV-A59 spike, $S_{A59}HV$-4, did not alter the virulence of the recombinant viruses indicating that the MHV- 4 HVR was not sufficient to confer an increase in virulence to the MHV-A59 spike. In addition, the similar virulence exhibited by $S_{A59}R16$ and the two recombinants $S_{A59}HV$- 4R51 and R52 indicate that an insertion of 52 amino acids is well tolerated in the MHV-A59 HVR.

To determine the effect of alterations in the HVR on the efficiency of viral replication in the brain, we inoculated mice intracranially with the HVR recombinants, and determined the titers of infectious virus in the brain at various times p.i. (Figure 4). Consistent with their poor virulence, the MHV-4 S gene containing recombinants with deletions or exchanges in the HVR exhibited dramatically reduced virus titers in the brain which were at or below the level of

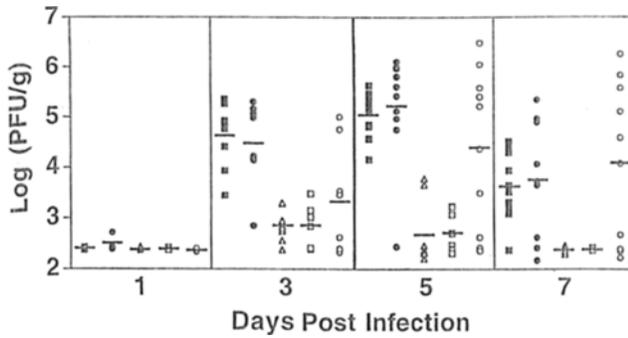Multiple regions of spike influence neurovirulence
JJ Phillips et al
425

Figure 4 Titers of infectious virus in the brains of animals inoculated with recombinant viruses with alterations in the HVR. As described in Figure 3, animals were inoculated intracranially with 10 PFU of S_4R29 (■), $S_{A59}R16$ (●), $S_{A59}HV-4R51$ (○), $S_4HV-A59R131$ (□), or $S_4\Delta HVR160$ (△), and viral titers were determined at various days post infection. Each data point represents the titer of infectious virus from a single mouse and the mean virus titers are shown as bars ($n = 4-10$ mice per time point, data for days 5 and 7 are from three independent experiments). The data for the two parental recombinant viruses, S_4R29 and $S_{A59}R16$, is the same as in Figure 3, and is shown here for comparison. The limit of detection was 200 PFU/g of tissue. On day 5 p.i., the mean titers of infectious virus in S_4R29 infected mice was significantly greater than in $S_4HV-A59R131$- or $S_4\Delta HVR160$-infected mice (two-tailed t-test, $P > 0.0001$ for both).

detection (200 PFU/g). On days 3 and 5 p.i. with $S_4\Delta HVR$ or $S_4HV-A59R$ the titers of infectious virus were significantly different from the titers following infection with S_4R29 (two-tailed t-test, $P > 0.0001$). In contrast, replacement of the MHV-A59 HVR with the corresponding region from MHV-4 did not result in a dramatic reduction in the amount of infectious virus recovered from the brain. Although $S_{A59}HV-4R$ exhibited similar average titers of infectious virus as $S_{A59}R$, there was a large degree of animal to animal variation as indicated in Figure 4. On day 7 p.i., some animals exhibited relatively high titers of infectious virus. To demonstrate that these high titers late in infection were not attributable to the replication of variant viruses with secondary site mutations in the spike, we amplified virus from the brain at 7 days p.i., and sequenced the spike gene to generate a consensus sequence. No secondary site mutations were found in the spike gene.

Fusion phenotype of the recombinant viruses

Despite efficient replication and growth in cell culture most of the chimeric spike containing recombinant viruses exhibited attenuated virulence *in vivo*. Due to the numerous selective pressures present during an *in vivo* infection it is possible that small alterations in viral entry, spread, or replication, which were not readily observable in cell culture, had a profound impact on virulence. Previous studies have found an association between alterations in the ability of the MHV spike to induce fusion and alterations in pathogenesis (Frana *et al*, 1985; Gallagher *et al*, 1990; Gallagher *et al*, 1991; Gallagher *et al*, 1992;

Rao and Gallagher, 1998). To examine the fusion phenotype of the recombinant viruses following virus infection we performed cell–cell fusion assays on L2 cell monolayers (see Materials and Methods). By 24 h p.i., all of the recombinant viruses had induced syncytia formation, but at early times postinfection, there were clearly differences among the recombinant viruses with respect to their ability to induce cell–cell fusion. The percent fusion relative to S_4R29 was found to be similar at 7 and 9 h p.i.; the results at 7 hours are shown in Figure 5. Similar levels of cell–cell fusion were observed in L2 cells infected with the two parental recombinants, S_4R and $S_{A59}R$, and the severely attenuated recombinant $S2_4R$, expressing the S1 of MHV-A59 and the S2 of MHV-4. In contrast, recombinants expressing spikes with alterations in the HVR, $S_4\Delta HVR$, $S_4HV-A59R$, and $S_{A59}HV-4R$, or with pairing of the S1 subunit of MHV-4 with the S2 subunit of MHV-A59, $S1_4R$, all exhibited some degree of delay in cell–cell fusion. Deletion of the MHV-4 HVR resulted in the most dramatic decrease in fusion, while replacement of the MHV-A59 HVR with the MHV-4 HVR resulted in only a moderate decrease in fusion. The delayed fusion formation observed with $S_4\Delta HVR$ is consistent with the observation by Gallagher *et al* (1990) that deletions in the HVR can result in decreased fusion. Thus, the neuroattenuated virulence observed with some but

Virus
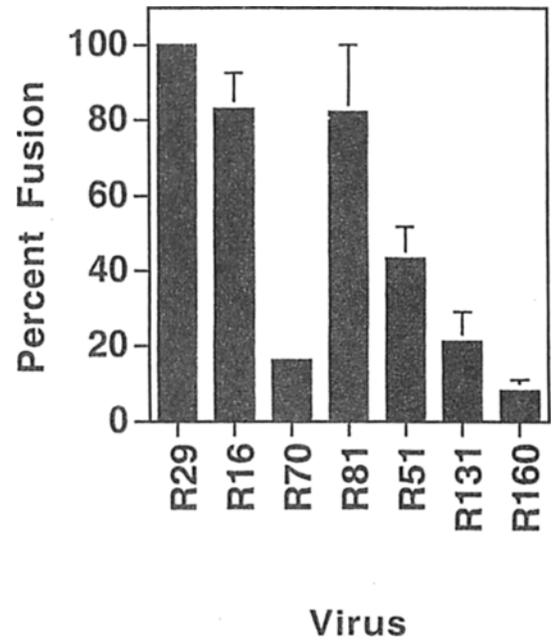
Figure 5 (A) Cell–cell fusion assay on L2 cell monolayers. Cells were infected at a multiplicity of infection of 0.1 or 0.7 PFU/cell with S_4R29, $S_{A59}R16$, $S1_4R70$, $S2_4R81$, $S_{A59}HV-4R51$, $S_4HV-A59R131$, and $S_4\Delta HVR160$. At 7 h p.i., cells were fixed. Using phase-contrast microscopy and a 20×-objective the total number of nuclei in syncytia per field was determined for at least 10 random fields per 6-well plate. The percent fusion relative to that observed with S_4R29 was calculated for each experiment and the means (and standard deviations) are shown.

not all of the recombinants may be associated with decreased cell–cell fusion in cell culture.

Discussion

With the development of targeted recombination the structural genes of MHV have become amenable to genetic manipulation. Using this technique we previously demonstrated that the spike gene of the highly neurovirulent MHV-4 virus can confer a dramatic increase in virulence to the much less virulent MHV-A59 virus (Phillips et al, 1999). Here we report on studies designed to localize this phenotype to a specific region of the spike, and to examine the effect of specific alterations in the spike on pathogenesis.

Using targeted recombination, we generated a series of recombinants containing MHV-4/MHV-A59 chimeric spike genes. We first characterized their in vitro phenotype to ensure that the chimeric spike genes were functional for infection and replication in tissue culture. We then examined the in vivo phenotype of the recombinants. The two parental recombinants, S_4R (containing the MHV-4 spike gene) and $S_{A59}R$ (containing the MHV-A59 spike gene), exhibit similar kinetics of replication in cell culture, but $S_{A59}R$ achieves higher peak titers than S_4R. In cell culture, all of the chimeric spike gene recombinants replicated efficiently. They exhibited similar kinetics of replication as S_4R and $S_{A59}R$, and their peak titers were similar to or intermediate between the high titers observed with $S_{A59}R$ and the lower titers observed with S_4R.

When we infected mice with the recombinant viruses, however, we observed that many of the recombinants displayed a different phenotype from either of the two parental recombinants. Recombinants containing exchanges between the S1 and S2 subunits ($S1_4R$ or $S2_4R$) or deletion or replacement of the MHV-4 spike hypervariable region ($S_4 \Delta HVR$ or S_4HV-A59R, respectively) exhibited a profound decrease in viral replication in the brain as demonstrated by the titers of infectious virus. Furthermore, these recombinants were highly attenuated for neurovirulence as demonstrated by their high intracranial LD_{50} values. The high titers of infectious virus in the brains of mice infected with recombinant $S_{A59}HV$-4R, containing the MHV-A59 spike with the MHV-4 spike HVR, demonstrated that not all of the chimeric spike gene recombinants exhibited attenuated replication. In addition, the virulence of $S_{A59}HV$-4R was not attenuated as it was similar to the parental $S_{A59}R$ recombinant. As illustrated by the two parental recombinants decreased neurovirulence is not always associated with decreased titers of infectious virus in the brain. S_4R and $S_{A59}R$ exhibit a 3 $\log_{10}$ difference in intracranial LD_{50}, yet when mice are infected with the same dose of virus, S_4R and $S_{A59}R$ exhibit similar titers of infectious virus in the brain. The chimeric

spike gene recombinants, however, demonstrate that alterations in both S1 and S2 can result in reduced viral titers in the brain, and that this decrease in viral replication in the brain is associated with decreased neurovirulence.

The neuroattenuated phenotype of recombinants containing S1/S2 exchanges between the MHV-4 and MHV-A59 S genes suggest that under the stringent conditions of an in vivo infection homotypic S1/S2 interactions are required for efficient replication in the CNS. Moreover, in combination with previous studies associating mutations in S1 and S2 with neuroattenuation, these data suggest that regions within both subunits of the MHV-4 spike are required to confer the highly neurovirulent phenotype (Parker et al, 1989; Wang et al, 1992; Grosse and Siddell, 1994; Saeki et al, 1997). These regions of spike, which when mutated, deleted, or replaced influence neuropathogenesis, may interact structurally in the tertiary conformation of the protein, or cooperate functionally for efficient functioning of spike. There is experimental evidence to support the idea that regions of S1 and S2 closely interact. Variant viruses have been identified in which mutations in S1, responsible for receptor binding, alter fusion, and mutations in S2, responsible for viral fusion, alter the conformation of S1 or confer resistance to neutralization by soluble receptor (Gallagher et al, 1990; Grosse and Siddell, 1994; Saeki et al, 1997). Although the spike proteins of MHV-4 and MHV-A59 share a high degree of amino acid homology, the severely attenuated phenotype of the recombinants with chimeric spike genes indicate that there are critical differences between the two spike genes in both S1 and S2. As has been demonstrated with Theiler's virus (Pritchard et al, 1993; Zhang et al, 1993), the in vivo attenuation observed with viruses containing chimeric viral proteins between similar strains demonstrates the complexity and sequence specificity of viral protein interactions, and attests to the stringent constraints imposed on a virus in vivo.

Additional recombinants containing alterations exclusively in the MHV-4 hypervariable region (HVR) demonstrated that this region of S1 is required for the high neurovirulence of the MHV-4 spike. Deletion or replacement of the MHV-4 HVR resulted in neuroattenuation of the recombinant viruses. These results definitively confirm the data from previous studies suggesting an association between mutations or deletions in the MHV-4 HVR and neuroattenuation (Dalziel et al, 1986; Fleming et al, 1986; Wege et al, 1988; Parker et al, 1989; Taguchi and Fleming, 1989; Gallagher et al, 1990; Wang et al, 1992). Although no single function has been attributed to the HVR, studies of variant viruses suggest that alterations in this region can influence S1-S2 interactions and fusion (Gallagher et al, 1990; Gallagher, 1997). In addition, the MHV-4 spike HVR contains neutralizing antibody epitopes and T-cell epitopes (Dalziel et al,

Multiple regions of spike influence neurovirulence
JJ Phillips et al
427

1986; Taguchi and Fleming, 1989; Gallagher et al, 1990; Castro and Perlman, 1995), and thus alterations in this region could also influence the immune response to infection. Due to the severely attenuated in vivo replication and virulence exhibited by recombinants with deletion or replacement of the MHV-4 spike HVR, however, it appears that this region is critical for the in vivo functioning of the MHV-4 spike.

In contrast, replacement of the MHV-A59 spike HVR with the corresponding region from the MHV-4 spike demonstrated that the HVR of MHV-4 was not sufficient to confer an increase in neurovirulence. Interestingly, the presence of the MHV-4 HVR in the MHV-A59 spike did not appear to alter the LD_{50} of the resulting recombinant virus. Thus, not only could the MHV-4 HVR grossly substitute for the corresponding region of MHV-A59, but the MHV-A59 spike HVR was able to tolerate an insertion of 52 amino acids with no obvious effect on neurovirulence.

To identify more subtle alterations or defects in the MHV-4/MHV-A59 chimeric spike proteins we tested a number of their in vitro properties. Previous studies have suggested that the fusogenicity of the MHV spike is an important factor in both viral spread in the host and in virus-induced cytotoxicity (Gallagher et al, 1990; Gallagher et al, 1992; Rao and Gallagher, 1998). To determine if the spike proteins of the attenuated recombinants exhibited an altered ability to induce cell–cell fusion we performed cell–cell fusion assays. Four of the recombinants ($S1_4R70$, $S_4\Delta HVR160$, S_4HV-A59R131, and $S_{A59}HV$-4R51) exhibited some degree of delayed fusion. Although not all of the attenuated recombinants exhibited decreased cell–cell fusion, it appeared that a severe reduction in cell–cell fusion was correlated with neuroattenuation. Interestingly, the slight decrease in cell–cell fusion observed with the recombinants containing a replacement of the MHV-A59 HVR with the MHV-4 HVR did not result in a corresponding decrease in virulence. This result suggests that MHV infection and replication in the CNS may require a certain threshold level of fusion. Although not all viruses with fusion defects exhibit neuroattenuation (ST Hingley, unpublished data), decreased fusogenicity of the MHV spike has previously been found to associate with decreased spread in the CNS (Gallagher et al, 1990; Fazakerley et al, 1992). Thus for some of the recombinant viruses decreased fusion resulting in decreased spread and dissemination may contribute to the decreased replication observed in the CNS.

In addition to cell–cell fusion we also examined the temperature sensitivity and the thermostability of the recombinant viruses. The temperature sensitivity of the recombinants, as determined by the plating efficiency at 39.5°C compared to at 37.0°C, revealed no differences between the highly virulent and highly attenuated recombinant viruses (data not shown). The thermostability of the recombinant viruses was also examined by determining the decrease in viral stock titers over time at 40°C, pH 7.3 (data not shown). The neuroattenuated recombinants fell into a range of thermostabilities, however, they were bounded by the highly thermostabile $S_{A59}R16$ and much less thermostabile S_4R29 (data not shown). Thus, there was no clear correlation between thermostability and neurovirulence.

Due to the many selective pressures inherent in an in vivo infection, small differences in the kinetics of an infection can dramatically affect pathogenesis. Such differences may explain why many of the recombinants with alterations in the spike displayed efficient replication in cell culture, yet highly inefficient replication in vivo. By creating recombinant viruses with heterotypic S1/S2 interactions we may have inadvertently altered either structural or functional interactions between the S1 and S2 subunits. These perturbations in spike may not have been apparent in cell culture due to differences between in vitro and in vivo infection such as receptor density on the host cell, the availability of free virus, the virion density required for an efficient infection, and the fusogenicity of the host cell. It is also possible that there were slight alterations in the processing or expression of the chimeric spike proteins that were not apparent in vitro. The lack of a correlation between replication in cell culture and replication in vivo is not unique to the chimeric spike gene recombinants as it has been observed previously for other MHV strains and for other viruses (Gallagher et al, 1990; Pritchard et al, 1993).

The targeted recombination technique is a powerful tool for studying MHV pathogenesis as defined alterations in spike can be introduced into isogenic viruses. As with all strains of MHV, point mutations can arise during viral replication. To decrease the impact of such secondary site mutations in the recombinant viruses we selected and characterized two independent recombinants for each desired recombinant, and sequenced the entire S gene from at least one recombinant. Interestingly, S_4HV-A59R was the only recombinant for which multiple, independent recombinants were found to contain single coding mutations in S after minimal passage in cell culture. The location of one of these compensatory mutations identified in S2 (L1114F) is striking as numerous studies have identified alterations in this exact residue. The 1114 codon has been identified by Gallagher et al (1991) (L1114R) as one of three amino acids responsible for conferring pH-dependent fusion on MHV-4, by Saeki et al (1997) (L1114F) in the spike gene of soluble-receptor resistant mutants, and by Wang et al (1992) (L1114F) in a neuroattenuated monoclonal antibody escape mutant. It is interesting to speculate that this region of S2 plays a prominent role in the conformation of spike and perhaps in the dynamic interaction that occurs between S1 and S2. The identification of compensatory mutations that

arise both *in vivo* and *in vitro* as a result of various alterations in spike may provide valuable clues as to which regions of spike undergo functional and/or structural interactions.

The spike of MHV is a major determinant of neurovirulence. This is the first report using targeted recombination to examine the role of specific regions of the MHV spike in neuropathogenesis. Despite efficient replication in cell culture many of the MHV-4/MHV-A59 chimeric S gene recombinants displayed severely attenuated replication *in vivo*. Thus, the degree of amino acid homology between the MHV-4 and MHV-A59 spike glycoproteins was high enough for the chimeric spike gene recombinants to replicate efficiently *in vitro*, yet not high enough for efficient replication in the murine CNS. In addition, recombinants with alterations in the spike HVR have confirmed the importance of this region in the highly neurovirulent phenotype conferred by MHV-4 spike. Thus the pronounced neurovirulence conferred by the MHV-4 spike appears to depend on the structural and/or functional interaction of multiple regions of spike rather than on discreet independent functional domains within spike.

Materials and methods

Virus and cells

Feline FCWF cells, fMHV, and Alb4 were obtained from Paul S Masters (Albany, NY). The fMHV virus is a recombinant MHV that contains the ectodomain of the S protein of feline infectious peritonitis virus, the Alb4 replicase, and the MHV-A59 3'-end (Kuo *et al*, 2000). Alb4, derived from MHV-A59, is a temperature-sensitive N gene deletion mutant that produces small plaques at the non-permissive temperature (39°C) and is thermolabile (Koetzner *et al*, 1992). Viruses were propagated on either murine 17Cl-1 cells or feline FCWF cells, and plaque assays and purifications were carried out on murine L2 cells. Cells were maintained on plastic tissue culture flasks 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 of between 2×10^5 and 2×10^6 cells per ml.

Plasmids and PCR mutagenesis

The pMH54 plasmid (obtained from Paul S Masters) includes from codon 28 of the HE pseudogene through to the 3' end of the MHV-A59 genome as described by Kuo *et al* (2000). The pGEM4Z plasmid containing the MHV-4 S gene, pSwt, was obtained from Michael J Buchmeier (LaJolla, CA) (Gallagher *et al*, 1991). The pGEM-S4 and pGEM-A59 plasmids were derived from pSwt and contained the MHV-4 and the MHV-A59 spike genes, respectively (Phillips *et al*, 1999). These plasmids also contained silent mutations at codons 12 and 13 of the S gene to create an

AvrII site, and at 12 nt past the S gene stop codon to create a SbfI site. These restriction sites were used for shuttling the various spike genes into the pMH54 vector.

To generate the various exchanges in the *S* gene, coding-silent changes were introduced into the MHV-4 and MHV-A59 spike genes in pGEM-S4 or pGEM-A59, respectively, that created unique and/or common restriction sites. DNA manipulations were carried out using standard methods (Maniatis *et al*, 1982). All cloning sites and regions generated by PCR mutagenesis were verified by sequencing, and the composition of each construct was checked by restriction analysis.

To exchange the MHV-4 and MHV-A59 S1 and S2 subunits an EcoRV site was introduced at codon 775 (MHV-4 sequence). AvrII/EcoRV or EcoRV/SbfI segments were then exchanged between the MHV-4 and MHV-A59 spikes. The resulting plasmids, pG-S1$_4$, containing the S1 of MHV-4 and the S2 of MHV-A59, and pG-S2$_4$, containing the S1 of MHV-A59 and the S2 of MHV-4, were digested with AvrII/SbfI and the chimeric S genes were shuttled into the pMH54 plasmid creating pMH54-S1$_4$ and pMH54-S2$_4$. A S gene with the MHV-A59 spike and the MHV-4 HVR was generated by introducing silent mutations into the MHV-4 spike creating a PstI site at codon 412 and a KpnI site at codon 614. Using these two sites the resulting 606-nt MHV-4 fragment was cloned into the MHV-A59 spike creating plasmid pG-S$_{A59}$HV-4. The AvrII/SbfI segment from this plasmid was then cloned into pMH54 creating plas mid pMH54-S$_{A59}$HV-4. The MHV-A59 HVR was introduced into the MHV-4 spike by introducing silent mutations in the MHV-A59 spike at codons 488 and 548 creating an EcoR47III site and a BglII site, respectively. For cloning purposes a silent mutation creating a BglII site was also introduced into the MHV-4 S gene at codon 600. Using the Eco47III and BglII restriction sites the MHV-A59 HVR was cloned into the corresponding region of the MHV-4 spike creating plasmid pG-S$_4$HV-A59. The AvrII/SbfI segment from this plasmid was then cloned into pMH54 creating pMH54-S$_4$HV-A59. Deletion of the MHV-4 HVR was based on a previously identified deletion in the MHV spike (Dalziel *et al*, 1986; Parker *et al*, 1989). The 142-amino acid deletion between codons 434 and 575 and codon substitution at K433N was created using PCR mutagenesis in pGEM-S4. The resulting plasmid, pG-S$_4$ΔHV, was used to shuttle the AvrII/SbfI region of S into pMH54 creating pMH54-S$_4$ΔHV.

For RNA transcription the designated pMH54 plasmids were linearized just 3' to the poly(A) tail by digestion with PacI.

Targeted RNA recombination and selection of recombinants

Targeted recombination was carried out, as described by Kuo (2000), between the recipient virus fMHV and

Multiple regions of spike influence neurovirulence
JJ Phillips et al
429

synthetic capped RNAs transcribed from pMH54-S4, pMH54, pMH54-S1$_4$, pMH54-S2$_4$, pMH54-S$_4$HV-A59, and pMH54-S$_4\Delta$. Recombination with RNA transcribed from pMH54-S$_{A59}$HV-4 was carried out with the Alb4 recipient virus as described previously (Masters et al, 1994; Leparc-Goffart et al, 1998; Phillips et al, 1999). The potential recombinants were plaque-purified and screened, using PCR amplification and gel electrophoresis, for the presence of the 5′ portion of the recombinant S gene (Fischer et al, 1997; Leparc-Goffart et al, 1998; Phillips et al, 1999; Kuo et al, 2000). The selected recombinants were then plaque-purified once again, and viral stocks were grown on 17Cl-1 cells for further analysis (Fischer et al, 1997). For each desired recombinant virus at least two recombinants derived from independent recombination events were characterized.

S gene sequencing
For sequencing of the S gene, cytoplasmic RNA extracted from virus-infected L2 cells was used as a template for reverse transcriptase-mediated PCR (RT-PCR) amplification. The oligonucleotides described previously (Leparc-Goffart et al, 1998; Phillips et al, 1999) were used for amplification of the designated regions. Double-stranded PCR products were analyzed by automated sequencing by the Taq dye terminator procedure according to the manufacturer's protocol (Taq DyeDeoxy Terminator Cycle Sequencing kit; Applied Biosystems).

The entire S gene was sequenced from at least one member of each pair of recombinant viruses. The sequence of each S gene was identical to the previously published sequences for the MHV-4 and MHV-A59 S genes (Luytjes et al, 1987; Parker et al, 1989; Gallagher et al, 1991; Phillips et al, 1999) except for recombinants containing the S2 of MHV-4, S2$_4$R, and recombinants containing the MHV-4 spike with the MHV-A59 hypervariable region, S$_4$HV-A59R. S2$_4$R81 contained a single point mutation in the S1 subunit that resulted in a codon change L661Q. S2$_4$R82, an independently isolated recombinant virus, contained no secondary site mutations in S. S$_4$HV-A59R131 contained a single coding mutation S359I. An independently isolated recombinant, S$_4$HV-A59R133, also contained a single coding mutation N374Y. Two additional recombinants, S$_4$HV-A59R132 and R134, were partially sequenced and found to contain single coding mutations (L1114F and L660Q, respectively).

Virus growth curves
Confluent L2 cell monolayers in 12-well plates were infected with each virus at the indicated multiplicity of infection. Infections were carried out in duplicate. Following adsorption for 1 h at 37°C, the cells were washed with Tris-buffered saline three times and then fed with 1.5 mL of DMEM-10% FBS. At the specified times, the cells were lysed by three cycles of freeze-thawing, and the supernatants were removed and titered by plaque assay on L2 cells as previously described (Gombold et al, 1993).

Animals
C57Bl/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD). Four-week-old, MHV-free male mice were used in all experiments. Mice were anesthetized with isoflurane (IsoFlo; Abbott Laboratories, North Chicago, IL). The amount of virus designated in each experiment was diluted in PBS containing 0.75% bovine serum albumin, and a total volume of 20 μl was injected into the left cerebral hemisphere. Mock-infected controls were inoculated similarly but with an uninfected 17Cl-1 cell lysate at a comparable dilution.

Virulence assays
Fifty percent lethal dose (LD$_{50}$) assays were carried out as described previously (Hingley et al, 1994). Mice were inoculated intracranially with 4- or 5-fold serial dilutions of recombinant viruses. A total of 5 animals per dilution per virus were analyzed. Mice were examined for signs of disease or death on a daily basis up to 21 days p.i. LD$_{50}$ values were calculated by the Reed-Muench method (Reed and Muench, 1938; Smith and Barthold, 1997).

Infectious virus in the CNS
The levels of infectious virus in the CNS as a function of time following infection with the recombinant viruses was determined in mice inoculated intracranially with 10 PFU of virus. On days 1, 3, 5, and 7 postinfection, mice were sacrificed, perfused with 10 ml of PBS, and the brains were removed. The left-half of the brain was placed directly into 2 ml of isotonic saline with 0.167% gelatin (gel saline) (Ramig, 1982). All organs were weighed and stored frozen at −80°C until titered for virus. Brains were homogenized, and virus titers were determined by plaque-assay on L2 cell monolayers (Hingley et al, 1994). The titers of infectious virus in the brain were determined for all of the recombinant viruses at once in three independent experiments.

Fusion assay
To quantitate cell−cell fusion induced by the recombinant viruses, L2 cell monolayers in 6-well plates were infected with virus in duplicate (MOI 0.1 or 0.7 PFU/cell) and incubated for 1 h at 37°C. Following adsorption, the cells were washed with Tris-buffered saline twice and then fed with 3 ml of DMEM-10% FBS. At 7 and 9 h p.i., the cells were washed with PBS and fixed with 2% paraformaldehyde (Sigma Chemical Co, St. Louis, MO). For each 20×-objective field under phase-contrast microscopy the total number of nuclei in syncytia was counted. At least 10 random fields were counted per well, and the mean

number of nuclei in syncytia per field was calcu-
lated. The percent fusion relative to that observed
with S_4R29 was calculated for each experiment. To
demonstrate that each 20×-objective field had a sim-
ilar number of cells, the total number of cells per
field was counted at random. The results, shown in
Figure 5, represent the means (and standard devia-
tions) of at least three independent experiments ex-
cept $S_4\Delta HVR160$, which is from two independent
experiments.

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Acknowledgements

Multiple regions of spike influence neurovirulence
JJ Phillips et al
431

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