Chapter C13

MOLECULAR DETERMINANTS OF CORONAVIRUS MHV- INDUCED DEMYELINATION

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Abstract: Mouse hepatitis virus (MHV) is a member of the coronavirus family of the *nidovirales* order. MHV is an enveloped virus with single-stranded, positive genomic RNA of about 31kb. Infection of susceptible strains of mice with the MHV-JHM and A59 strains results in acute encephalomyelitis and chronic demyelinating disease with features similar to the human demyelination disease multiple sclerosis (MS). Because the mechanism of demyelination in MS is not completely understood, various experimental models, including MHV infection in mice, have been used to study the pathogenesis of inflammatory autoimmune demyelination. The spike (S) glycoprotein of MHV has been implicated as the most critical genomic determinant of MHV pathogenesis and demyelination. However, other genes and proteins are likely to contribute to MHV pathogenesis as well.

Key words: Mouse hepatitis virus (MHV), Coronaviruses, Nidoviruses, Molecular determinants, Demyelination, Multiple Sclerosis (MS).

THE MHV GENOME

The MHV genome is approximately 31 kb of single-stranded RNA of positive polarity. The genomic organization consists of 7 functional genes, 5 of which encode structural proteins (Fig 1). Genes 2b, 3, 5b, 6 and 7 encode the structural proteins hemagglutinin-esterase (HE) in some strains of MHV, spike (S), membrane (M), envelope (E) and nucleocapsid (N) [1]. The remaining genes encode non-structural proteins. The nucleotide sequences have been determined for 3 strains of MHV: A59, JHM and MHV-2. The

structural protein genes are clustered at the 3' of the genome, whereas the 5' third of the genome consists of a single gene encoding a large polyprotein comprising of viral polymerase, proteases and associated nonstructural proteins. The nucleotide sequence at the 5' end contains a 65 to 85 nucleotide-long leader RNA, followed by 200 to 500 non-translated nucleotides. The first 60% of the length of the genome, roughly 20 Kb from the 5' end, consists of two overlapping open reading frames, ORF 1a and 1b, that encode the viral RNA- dependent RNA polymerase, proteases and helicase. At the overlapping region between ORF 1a and 1b there is a 7-nucleotide slippery sequence and a pseudoknot structure, essential for ribosomal frame shifting. Several of the non-structural proteins are not essential for viral replication *in vitro* but may play a role in viral pathogenesis. An important aspect of MHV biology is the high frequency of RNA-RNA recombination between different, but closely related MHVs, when mixed in the same culture. RNA recombination is likely an important mechanism of viral evolution and may contribute to viral pathogenesis.
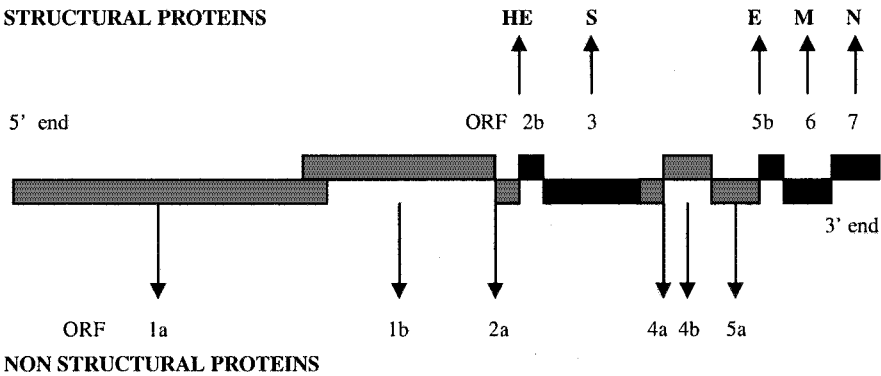
Figure 1 . Schematic diagram of the MHV genomic organization.

STRUCTURAL-FUNCTIONAL RELATIONSHIP OF MHV

The virions of MHV contain a nucleocapsid protein (N), which binds to the viral RNA to form a helically symmetric nucleocapsid. Surrounding the nucleocapsid is a membrane envelope, which contains 3 or 4 major glycoproteins: the spike (S), the membrane (M), the small envelope protein (E), and in some strains such as MHV-JHM, the hemagglutinin-esterase protein (HE) [1-4].

Upon infection of susceptible cells the MHV genomic RNA serves as a template for the negative strand RNA synthesis, which, in turn, is used for the synthesis of genomic and subgenomic mRNAs [2]. Each mRNA has a leader RNA of about 70 nucleotides at the 5' end [3]. The exchange of leader RNA is a common phenomenon during coronavirus replication and mRNA 1 can be synthesized during MHV infection with or without a 9-nucleotide sequence (UUUAUAAAC) located immediately downstream of the leader RNA of the 5' terminus of MHV [4]. Studies of naturally occurring and artificially constructed defective interfering (DI) RNAs have shown that both 5'- and 3'-terminal portions of the MHV genome are required for making the DIs functional [5, 6]. More specifically, the 5' cis-acting elements essential for MHV-JHM and A59 DI RNA replication have been mapped to 5' 474 nucleotides of which at least 446 nucleotides are required [6]. At the 3' end of the MHV genome 417 to 463 nucleotides is the minimal number of nucleotides required for replication [5]. Using interspecies-targeted RNA recombination, MHV-JHM gene 4 was genetically inactivated. Interestingly, the virus lacking gene 4 replicated in tissue culture cells with similar kinetics as the wild type virus. Thus ORF 4 is not essential for viral growth in tissue culture or in the CNS of the infected host [7]. Likewise, the deletion of the 2a/HE gene cluster did not affect viral growth in vitro; deletion of the 4/5a gene cluster merely decreased viral replication rate by 10 fold [8].

The function of the M protein has been recognized as the predominant constituent of virion architecture. However, the function of HE protein has yet to be established other than the hemagglutinating and acetyl-esterase activities [9]. The HE protein forms disulfide-linked homodimers and strains of viruses that express this protein show a second, shorter fringe of peplomers on their virions. The small envelope protein E, has been shown to be translated both in vivo and in vitro. The E and M proteins are both necessary and sufficient for the formation and extracellular release of particles appearing structurally identical to MHV virions, thus suggesting that the E and M proteins are important for virion assembly [10, 11].

The N protein of MHV is a 50-60 kDa phosphoproteins with overall high basic amino acid content and 3 structural domains [12]. The functions of the N protein involve interactions between N and viral RNAs [13, 14]. The internal gene I, a large open reading frame embedded entirely within the 5' half of N gene, has been proved not essential for the replication of MHV either in tissue culture or in its natural host [15]. The middle domain of N is responsible for RNA binding. N binds certain regions of coronavirus RNA more efficiently than nonviral RNA and the binding specificity for encapsidation and packaging in the context of virus-infected cells may be

influenced by interactions with the membrane protein (M) or possibly other viral proteins [16].

THE SPIKE GLYCOPROTEIN (S)

Forming the spikes on the virion surface, the spike protein (S) of coronavirus MHV has a variety of important biological activities. The S protein is a transmembrane glycoprotein of about 180 kDa with 4139 nucleotides in length, encoding two posttranslational subunits: S1, predicted to form the globular head, and S2, predicted to form the stalk of the glycoprotein. The MHV S protein plays a critical role in viral pathogenesis, including functions in both viral entry and spread within the host [17, 18]. The S1 portion may independently bind to cellular receptors and the S2 portion is responsible for fusion of viral and cellular membranes [19]. The S protein is also the target for both humoral and cellular immune responses [20-22]. The S protein contains determinants of neurovirulence and demyelination [23-26].

Studies to determine molecular determinates of MHV pathogenesis have been limited by the large size of the MHV genome. For many years the size of the MHV genome presented an obstacle for the construction of a full-length cDNA clone, which would be amenable for genetic manipulations. The construction of a plasmid vector, pMH54, containing the entire 3' of the MHV-A59 genome downstream from the hemagglutinin esterase gene and two marker restriction sites *avrII* and *sbfI,* marked a new stage in pathogenesis studies. The availability of this construct allowed the introduction of different S genes into the background of MHV-A59 and the introduction of site directed mutatation into S or other genes [27, 28]. Using targeted RNA recombination between the trandfected and manipulated pMH54 plasmid and an infectious virus, various properties of the S protein were studied in the past few years.

Replacing the S gene of MHV-A59 with the S gene of the highly neurovirulent MHV-4, increased the virulence of the new recombinant virus substantially [23, 29] Alterations in both S1 and S2 resulted in reduced viral replication in the brain, associated with decrease in neurovirulence [25]. Mutations and deletions in the hypervariable region (HVR) suggested that the HVR was important for neurovirulence [25, 30-32]. In addition, the H716D amino acid substitution correlated with a delayed fusion phenotype, but without loss of hepatotropism [33, 34]. Q159L amino acid substitution affected hepatotropism and demyelination [35, 36] and L1114R (within the S2 region) has been speculated to play a role in the conformation of spike and in the dynamic interaction between S1 and S2 [37, 38]. The S gene was also found to contain determinants of hepatotropism [39].

Infection of MHV-A59 in 4-week-old C57BL/6 mice produces a biphasic disease with acute hepatitis and meningoencephalitis followed by chronic CNS demyelination [40-42]. Thus infection of mice with MHV-A59 has been used as an animal model for the human demyelinating disease multiple sclerosis (MS) [43-46]. MHV-2, previously recognized as mainly hepatotropic virus with only weakly neurotropic properties [47-50], was shown to produce severe hepatitis and meningitis, without encephalitis and demyelination [26, 51]. The weak neurotropic properties of MHV-2 made this strain ideal for a comparative study with the closely related, neurotropic, demyelinating strain MHV-A59 [52]. We first sequenced the entire genome of MHV-2 and compared it with that of MHV-A59 [52]. We then studied the role of the S gene in demyelination by replacing the S gene of MHV-A59 with that of MHV-2, using targeted RNA recombination. The new recombinant viruses (Penn98-1, 2) were more virulent than A59, but unable to induce demyelination in C57BL/6 mice [26]. Thus we hypothesized that demyelination determinants map to the S gene of MHV-A59.

To further explore which specific regions of the S gene are directly responsible for the demyelinating phenotype, we studied a set of random recombinant viruses (ML-7, ML-8, ML-10, and ML-11) that were produced by co-infection of cultures with MHV-2 and LA-7, a ts mutant of MHV-A59 [53]. All of these recombinant viruses were unable to induce demyelination *in vivo*. *In vitro* study ascertained that this group of recombinant viruses was able to replicate to the same titers as their parental viruses in L2 cells tissue cultures. Morphologically, the viruses ML-7, ML-8 and ML-10 produced large plaques and syncytia (similar to MHV-A59), whereas ML-11 produced small plaques as MHV-2. Sequencing of the S genes of ML-7, ML-8 and ML-10 revealed that they contained three common mutations, I375M, L652I, and T1087N [54]. The I375M mutation is located downstream from the receptor-binding site, the L652I mutation is located in the Hypervariable (HVR) region of the S gene and the T1087N mutation is located between the two-heptad repeat regions. Since these viruses were derived from a recombination between LA-7 and MHV-2, we also sequenced the S gene of LA-7 and we confirmed that the S gene of LA-7 contained the exact same three mutations implicating LA-7 as the source of these mutations. In addition, the sequence results of the S gene of ML-11 confirmed that the S gene of ML-11 was identical and was derived from to the S gene of MHV-2 [51].

We then investigated the role of the three amino acid substitutions in MHV-A59-induced demyelination. We first produced viruses with all 3 amino acid substitutions by targeted recombination. The virulence of Penn2K-1 and 2 was dramatically reduced (LD50>50,000pfu) although Penn2K-1 and 2 exhibit similar kinetics of replication as their parental virus

MHV-A59 in L2 cell cultures. Pathogenesis studies of Penn2K-1 and 2 showed only mild encephalitis and hepatitis when high doses were used for infection (50,000pfu I.C). No demyelination was found with any dose of virus used for infection. Thus we hypothesized that one of the 3 amino acid substitutions or a combination of two or three of these mutations marked genomic determinants of CNS demyelination.

Using site directed mutagenesis and targeted recombination; we then made a series of recombinant viruses containing each one of the 3 points mutation and each of the combined 2 point mutations. In vitro studies showed that all of these recombinant viruses exhibited similar kinetics of replication in L2 cell culture. The virulence of all of these recombinant viruses was reduced compared to wtR-A59. The recombinant viruses with the T1087N mutation (Penn99-1 and 2) were the most virulent ($LD50=20,000pfu$) among these recombinant viruses. The virulence of all other recombinant viruses, including the I375M point mutation (Penn01-1 and 2), the L652I point mutation (Penn01-3 and 4) and I375M-L652I two point mutation (Penn2K3 and 4), I375M, and T1087N two point mutation (Penn01-5 and 6), L652I, and T1087N two point mutation (Penn01-7 and 8), were all significantly attenuated ($LD50>50,000pfu$). The reduced virulence correlated well with the reduced in severity of hepatitis and encephalitis. Interestingly, demyelination was not influenced by the reduction of virulence because most of the recombinant viruses with one or two combined amino acid mutations were significantly less virulent but demyelination-positive. It further confirmed that the induction of demyelination is a separate pathologic property that does not depend on the intensity of prior encephalitis or hepatitis during the acute phase [26]. Penn2K-3 and 4, the combination of mutations in S1 (I375-M, L652I) did not produce demyelination in C57BL/6 mice even when mice were infected with the highest dose (50,000pfu, I.C.). We then searched for additional mutations around amino acid 375 and eventually found that N345S was a single point mutation that abolished demyelination (Fu et al, manuscript in preparation). Thus we identified a region in the S1 gene downstream from the receptor-binding domain that controls demyelination.

SUMMARY.

The S gene controls a variety of biologic functions many of them are important for pathogenesis. Our laboratory identified a region around amino acid 345 of the S gene, outside and downstream from the receptor-binding domain that appears to control demyelination. Further studies are underway to define the functions of this region in virus-host interactions. Crystallization studies will determine whether the three

dimensional folding of this S1 region of the molecule may enable a spatial interaction between this site and the other two mutation sites which may affect viral host interaction and subsequently pathogenesis. More detailed mutagenesis, coupled with functional studies such as studies of receptor binding, S1-S2 separation, and fusion properties, will also be helpful in elucidating the mechanism of MHV neuropathogenesis.

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