EFFECTS OF MUTAGENESIS OF MURINE HEPATITIS VIRUS NSP1 AND NSP14 ON REPLICATION IN CULTURE

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1. INTRODUCTION

The 32-kb positive-strand RNA genome of murine hepatitis virus (MHV) contains a replicase gene (gene 1) that comprises two-thirds (22kb) of the genome, is the 5' most gene, and is translated from two overlapping open reading frames (ORFs 1a and 1b) and processed to yield intermediate and mature nonstructural proteins (nsps). For the group 2 coronaviruses such as MHV, as well as for SARS-CoV, at least 16 mature nsps are processed co- and post-translationally from the gene 1 polyprotein by two or three proteinases expressed as part of the polyprotein.1,2 The intermediate and mature nsps are thought to be essential for replication. These include demonstrated or predicted functions such as RNA polymerase and RNA helicase (nsp12 and nsp13), as well as recently predicted functions in RNA synthesis, modification, or processing such as ADP ribosylation, exonuclease, endoribonuclease, and RNA methyltransferase (nsp3, nsp14, nsp15, and nsp16, respectively). Finally, there are several nsps with no demonstrated or predicted functions, such as nsp1 and nsp2. To test the requirements for nsp1 and nsp14 in replication and to probe their functions, deletions or mutations were engineered into the viral genome in nsp1 and nsp14 and mutant viruses were analyzed for virus viability, replication, protein expression, and RNA synthesis. The results demonstrate that deletions and substitutions in nsp1 are tolerated in viable mutants, including deletion of the carboxy-terminal half of nsp1. Nsp14 appears to be essential for replication in culture but can tolerate substitution of tyrosine 414, as well as deletion of flanking cleavage sites. Together, these results show the ability to generate mutations in each of these proteins and recover viable mutants.

2. MATERIALS AND METHODS

2.1. Generation of Recombinant Viruses

Mutations and deletions of nsp1 and nsp14 were introduced into MHV as previously described.3,4 Briefly, mutations were engineered into cloned MHV genome fragments at

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locations shown in Table 1, and the cloned and mutated cDNA fragments were digested, assembled into full-length genome cDNA in vitro, and transcribed into full-length genome RNA that was electroporated into replication-permissive BHK cells expressing the MHV receptor (BHK-R cells). Electroporated cells were monitored for cytopathic effect (CPE, syncytia) beginning at 24 h p.i., and both supernatant media and cells were passed onto new monolayers of BHK-R or murine delayed brain tumor (DBT) cells.

2.2. Virus Infection and Growth Experiments

Virus stocks were obtained from infected cell supernatants and titer determined on DBT cells. For growth experiments, DBT cells were infected with wild-type virus and with nsp1 and nsp14 mutants at an MOI of 5 pfu/cell (high MOI-single cycle) or 0.01 pfu/cell (low MOI-multiple cycle). Media supernatant was obtained at intervals from 0

| Nsp1 Mutations | Amino Acid | Supernatant Virus |
|----------------|------------|-----------------|
| VUSB 1         | K4A, K6A   | Yes             |
| VUSB 3         | E26A, K27A | Yes             |
| VUSB 4         | E46A, K48A | Yes             |
| VUSB 5         | H57A       | Yes             |
| VUSB 6         | R64A, E69A | No              |
| VUSB 7         | R78A, E69A | No              |
| VUSB 8         | K88A, E90A | Yes             |
| VUSB 9         | K132A, R133A | Yes       |
| VUSB 10        | R207A, R208A | No         |
| VUSB 11        | E220A, D221A | No         |
| VUSB 12        | K231A, R233A | Yes       |
| Δ124-242       | Δ124 - 242 | Yes             |

| Nsp14 Mutations | Amino Acid | Supernatant Virus |
|-----------------|------------|-----------------|
| VUSS 6          | nsp13, ΔQ600 | Yes             |
| VUSS 17         | nsp14, ΔQ521 | Yes             |
| VUSS 8          | D90A, E92A  | Yes             |
| VUSS 11         | C205A, C208A | No              |
| VUSS 9          | D243A       | No              |
| VUSS 10         | D272A       | No              |
| VUSS 3          | Y414 (wt)   | Yes             |
| VUSS 13         | Y414S       | Yes             |
| VUSS 14         | Y414A       | Yes             |
| VUSS 16         | S412Y, Y414H | Yes            |
| VUSS 20         | Y414T       | Yes             |
| VUSS 21         | Y414K       | No              |
| VUSS 22         | ΔY414       | No              |
to 48 h p.i. and used for plaque assay on DBT cells. Extent of CPE was assessed by cell loss and percent of monolayer involved in virus-induced syncytia formation.

3. RESULTS AND DISCUSSION

3.1. Deletions in nsp1 Demonstrate Requirement for Amino-Terminal Two-Thirds of nsp1 for Replication and Carboxy-Terminal Third for Processing of nsp1

Deletions were introduced into the nsp1 coding sequence at the amino terminal, middle and carboxy-terminal third of the protein domain. In addition, GFP coding sequence was substituted for the nsp1 coding sequence in cloned cDNA. When infectious genome RNA containing these changes was electroporated into permissive cells, only the carboxy-terminal nsp1 deletion allowed recovery of an infectious mutant virus (nsp1 Δ124-242). The recovered virus had slightly impaired peak titers and viral RNA synthesis compared with parental wild-type virus, but otherwise was indistinguishable in growth kinetics and RNA species generated. The deletion was engineered to retain the proximal (P5-P1) residues of the nsp1-nsp2 cleavage site, and the mutant virus had similar timing and extent of cleavage at the cleavage site between nsp1 and nsp2 as wild type. These results suggested that RNA or protein determinants in the amino-terminal two-thirds of nsp1 are essential for replication in culture, and that the amino-terminal protein determinants are important for the timing or extent of cleavage at the nsp1-nsp2 cleavage site.

3.2. Mutations in nsp1 Demonstrate Requirements for Specific Residues in Virus Viability in Culture

Based on the above results, systematic mutagenesis of clustered-charged residues was performed, both within the putative essential amino-terminal two-thirds of nsp1 as

![Figure 1](image)

**Figure. 1.** Nsp1 mutations and deletions. The organization of nsp1 is shown. Top schematic shows the deletion of aa 124 to 242. The bottom schematic shows clustered-charge to alanine mutations. Mutants from Table 1 are shown by VUSB number inside the box.

well as the dispensable carboxy-terminal third of the nsp1 protein domain. Residues were prioritized for substitution based on clustering of charge and across the protein sequence. Most alanine substitutions were tolerated for virus viability and replication in culture (Table 1 and Fig. 1). These included multiple substitutions in the amino-terminal half of
The exceptions were alanine substitutions VUSB5 (R64A, E69A) and VUSB6 (R78A, D79A), which did not allow recovery of infectious virus from supernatants of infected cells. Interestingly, we identified substitutions in the carboxy-terminal half of nsp1 that also were lethal for recovery of infectious mutant viruses, VUSB13 (R207A, R208A) and VUSB15 (E220A, D221A). This was surprising in light of the ability to delete this portion of the protein domain in viable mutants. Together, these results demonstrate significant flexibility in the amino acid sequence and lack of critical functions for a majority of charged residues. However, the results also suggest that specific residues may be critical for virus survival in culture, possibly due to protein interactions, folding or function. Finally, the results suggest that within apparently dispensable protein domains there may exist protein structure or function determinants that in the context of the intact protein may dramatically impact virus replication.

3.3. Amino Acid Substitutions or Deletions in nsp14 Putative Catalytic Residues or Zn Finger are Highly Deleterious or Lethal for Replication in Culture

The nsp14 protein is conserved in location, size, and significantly in amino acid sequence among all groups of coronaviruses, and it is predicted to be an exoribonuclease of the DE-D-D superfamily of exonucleases. We have previously demonstrated that substitution of nsp14 Tyr414 by His (Y414H) does not affect virus viability or replication in culture but abolishes virulence in mice. Significantly, the Tyr414 residue is 100% conserved in all sequenced coronaviruses. We sought to determine if nsp14 is required for virus viability in culture and if it affects virus growth. We performed systematic mutagenesis of the cloned cDNA genome Fragment F containing the nsp14 coding sequence and used the mutated clones to assemble genome cDNA. We targeted the flanking cleavage sites, predicted catalytic residues and the putative Zn finger motif within nsp14 with substitutions. In addition, multiple substitutions at Tyr414 were introduced to determine if this residue was tolerant of all changes and if different substitutions had distinct effects on replication.

When alanine was substituted for putative catalytic Asp242 or Asp272 residues, CPE was not detected in electroporated cells, nor was infectious virus recovered. Interestingly, when the Asp89 and Glu91 residues were both substituted with Ala, limited CPE was observed in electroporated cells, but this was not sustained on passage of the entire electroporated cell monolayer nor on overlay of fresh DBT cells. Infectious virus

Figure 2. Nsp14 motifs and mutations. The organization of nsp14 is shown, with putative exonuclease motifs I, II, and III as well as a possible Zn finger motif. Amino- and carboxy-terminal cleavage sites are indicated by arrows. Below schematic are residues and positions substituted as described in Table 1 and text: Q – Gln, D – Asp, E – Glu, C – Cys. Mutants from Table 1 are shown by VUSS number below the bars. * indicates viable mutants.
was recovered from initial electroporated cells and had a profoundly reduced titer of 70 pfu per ml. Although the virus could form plaques, it could not be passaged, suggesting a highly impaired replication machinery. Finally, no substitutions of Ala for Cys residues of the predicted Zn finger structure within nsp14 (Cys206, Cys209) were tolerated for productive virus infection in culture. Together, all of these results support the conclusion that nsp14 performs functions essential in the generation of infectious virus either within the cell or in viral RNA synthesis, and that the Asp242, Asp272, Cys206, and Cys209 residues are indispensable for virus replication. The results also suggest that the Asp89 and Glu91 residues may have some structural or functional flexibility, but that changes are highly deleterious and cannot support survival over multiple rounds of replication. Future experiments will involve Ala substitutions at residues 89 and 91 alone or exchange of Asp89 with Glu and of Glu91 with Asp to retain charge.

3.4. Nsp14 Tolerates Multiple Substitutions at Tyr414, but Not Lysine Substitution or Deletion

Because the substitution at Tyr414 by His had no effect on replication in culture but abolished virulence in mice, we engineered different substitutions or deletion at the Tyr414 residue to determine if it had any requirement in replication. Engineered substitutions for Tyr414 by Ser, Ala, or Thr each resulted in productive infection and infectious virus with growth indistinguishable from wild-type recombinant virus or virus with a Tyr414His substitution, as did a double substitution of Tyr414His and Ser412Tyr. Surprisingly, a Tyr414Lys substitution did not allow recovery of infectious virus from electroporated cells. Deletion of Tyr414 also abolished production of infectious virus in culture. These results demonstrate that while Tyr414 may have great flexibility, it is intolerant of deletion or specific substitutions, suggesting that it may be important for specific viral functions or possibly protein interactions.

3.5. Amino Acid Deletions at Cleavage Sites Flanking nsp14 Allow Productive, but Impaired Virus Growth in Culture

Having shown that nsp14 has essential and dispensable residues and that the protein may serve roles in both replication and pathogenesis, we next sought to determine if intact processing of the protein was required for virus viability or normal replication. Deletions of P1-Gln residues in the flanking cleavage sites between nsp13-14 (VUSS6) and nsp14-15 (VUSS17) were engineered in the infectious clone cDNA and used to generate full-length genome RNA for electroporation into permissive cells. In doing so, the nsp13-14 cleavage site deletion resulted in loss of the carboxy-terminal Gln600 residue in nsp13, while the nsp14-15 cleavage site deletion resulted in loss of the carboxy-terminal Gln521 residue in nsp14. Both cleavage site deletions resulted in syncytia in electroporated cells and recovery of infectious virus from the supernatant media. While both cleavage site deletion mutants were impaired in extent of CPE and virus growth, they differed in the degree of replication impairment. Specifically, the VUSS17 mutant had a slight delay in growth but attained wild-type peak titers, while the VUSS6 mutant showed delays of more than 4 h in peak titer during high MOI infection (5 pfu/cell) and never attained wild-type peak titers. The result suggests that incomplete processing of nsp14 from the flanking proteins alters the replication efficiency of the
viruses. However, it is also possible that the observed effects result from the deletion of the carboxy-terminal glutamine residue of nsp13 (VUSS6) or nsp14 (VUSS17).

4. SUMMARY

For nsp1, the fact that the carboxy-terminal but not the amino-terminal half of the protein can be deleted suggests that there may be specific and distinct domains within the protein or that the entire protein is dispensable but that the RNA encoding the amino-terminal half of nsp1 cannot be deleted. The identification of specific required residues support the conclusion that it is the portion of the protein that is required for replication.

The results of mutagenesis of the nsp14 coding region and flanking cleavage sites also provided important new insights into this protein and its requirements. Our previous study raised the question as to the essential nature of nsp14 in replication. The results of this study show that putative active site residues cannot be substituted without loss of replication in culture. Interestingly, mutagenesis of Tyr414 showed that while this residue can tolerate a number of substitutions, it was intolerant of Lysine or deletion. The results suggest that nsp14 is required for replication. However, whatever functions nsp14 serves appear to be retained by noncleaved or partially processed nsp14, since abolition of either the amino-terminal or carboxy-terminal cleavage site allowed recovery of viable virus.

5. ACKNOWLEDGMENTS

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