Genetic analysis of murine hepatitis virus non-structural protein 16

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MHV-Wüts18 is an RNA-negative, temperature-sensitive mutant of mouse coronavirus, strain murine hepatitis virus (MHV)-A59. We have previously identified the putative causal mutation of MHV-Wüts18 as a C to U transition at codon 2446 in ORF1b, which results in a substitution of proline 12 with serine in non-structural protein 16. Here, we have used a vaccinia virus-based reverse genetic system to produce a recombinant virus, inf-MHV-Wüts18(AGC) that encodes nsp16 serine 12 with AGC rather than UCU; a difference that facilitates the isolation of second-site revertants. Sequence analysis of nine inf-MHV-Wüts18(AGC) revertant viruses suggests that their phenotype is most probably due to the intra-molecular substitution of amino acids in nsp16. However, the revertant viruses displayed different plaque sizes and whole genome sequencing of two revertants showed that they were isogenic apart from a mutation in nsp13. These results are discussed in the context of a model of coronavirus MHV nsp16 structure.

INTRODUCTION

 Coronaviruses are enveloped, positive-stranded RNA viruses with large genomes ranging in size from 27 to 32 kb. Approximately two-thirds of the genome encodes non-structural proteins (nsps) that are involved in viral RNA synthesis. In 2003, the coronavirus nsp16 protein was predicted to be an S-adenosyl-L-methionine-dependent (nucleoside-2′-O)-methyltransferase (2′O-MTase) (Snijder et al., 2003) and subsequently, Decroly et al. (2008) showed that bacterially expressed, alphacoronavirus 1, strain FCoV (FCoV) nsp16, has a 2′O-MTase activity. More recently, the same group (Bouvet et al., 2010; Lugari et al., 2010) extended their studies to investigate Severe acute respiratory syndrome-related coronavirus (SARSr-CoV) and have been able to reconstitute in vitro the coronavirus cap-methyltransferase pathway using recombinant nsp14 [the S-adenosyl-L-methionine-dependent (guanine-N7)-methyltransferase] and nsp16. Intriguingly, these studies also show that efficient coronavirus cap 2′O-methylation requires a third viral nsp, nsp10, which acts as an essential trigger to complete RNA-cap-1 formation.

In addition to biochemical studies, classical and reverse genetics have been used to investigate the structure-function relationships of the coronavirus nsp16 protein. For example, the functional importance of nsp16 was supported by mutagenesis experiments using a SARSr-CoV replicon system (Almazan et al., 2006). In our laboratory, we have focused on the analysis of conditional, temperature-sensitive (ts) mutants that are unable to synthesize viral RNA when the infection is initiated and maintained at the non-permissive temperature (Sawicki et al., 2005; Stokes et al., 2010). Amongst our collection of RNA-negative ts mutants, murine hepatitis virus (MHV)-Wüts18 is one of two mutants that comprise cistron VI and map to nsp16.

In this study, we have taken a genetic approach to investigate the structure-function relationships of the MHV nsp16 protein. This approach is based upon the idea that, depending upon the nature of any given ts mutation, second-site revertants will have the potential to provide information on both intra- and intermolecular interactions within the coronavirus Replication-Transcription complex (RTC). In order to generate these second-site mutants at a higher frequency, we have used a reverse genetic approach to produce a ts mutant virus that has the same phenotype as MHV-Wüts18, i.e. substitution of proline 12 with serine in nsp16, but uses a different serine 12 codon (AGC instead of UCU), a change that would require two nucleotide substitutions to revert to a proline codon.

Supplementary material is available with the online version of this paper.
RESULTS

Characterization of the recombinant inf-MHV-Wüts18(AGC) mutant

We used our MHV reverse genetic system (Coley et al., 2005) to derive the recombinant mutant virus inf-MHV-Wüts18(AGC). After recovery and three-times plaque purification of inf-MHV-Wüts18(AGC), we produced a virus stock with a titre of $1 \times 10^6$ p.f.u. ml$^{-1}$. We then determined the efficiency of plating (EOP) (determined by dividing the titre at 39.5 °C by the titre at 33 °C) of this virus stock compared to a similar stock of inf-MHV-A59. The results showed that inf-MHV-Wüts18(AGC) had an EOP of $5 \times 10^{-2}$ compared with an EOP of $1.25 \times 10^0$ for inf-MHV-A59. These EOPs were almost identical to the values that we obtained previously when comparing the original MHV-Wüts18 mutant virus with our laboratory strain of MHV-A59 (Sawicki et al., 2005). These results confirm that the C to U transition at codon 2446 in ORF1b was solely responsible for the mutant phenotype of MHV-Wüts18(AGC)R8 and R9. Sequencing was done as described previously (Stokes et al., 2010). The nucleotide changes are identical at both 33 and 37 °C although, as expected, the replication cycle is slower at the lower temperature. This shows that the three nucleotide codon mutation in inf-MHV-Wüts18(AGC) has no significant effect upon the replication of the virus at the permissive temperature and that the ts phenotype of the mutant only manifests at temperatures above 37 °C.

Isolation of second-site revertants

In order to isolate second-site revertants of inf-MHV-Wüts18(AGC), we did plaque assays (Stokes et al., 2010) of stock virus at 39.5 °C. It was immediately obvious that the plaque morphologies of the viruses that replicated at this temperature were heterogeneous and the size of the plaques was smaller when compared with the recombinant ts mutant virus replicating at 37 °C (Fig. 1b). We chose to investigate five reventant virus stocks that produced large plaques and five revertant virus stocks that produced small plaques [representatively labelled as A and B in Fig. 1(b), 39.5 °C, $10^{-3}$ dilution]. As we were plaque purifying and producing virus stocks at 39.5 °C, it also became clear that the plaque morphology of the small-plaque revertant viruses was not necessarily stable; specifically, whilst the plaque size of revertant virus inf-MHV-Wüts18(AGC)R9 remained small, the revertant viruses inf-MHV-Wüts18(AGC)R2, R5 and R6 changed to include large-plaque viruses. In these cases, we isolated and used the large-plaque virus to produce virus stocks (Table 1). The titres of the virus stocks were between $1 \times 10^6$ and $1 \times 10^7$ p.f.u. ml$^{-1}$ for the large-plaque revertants and $5 \times 10^4$ and p.f.u. ml$^{-1}$ for the small-plaque revertant, compared to $1 \times 10^9$ p.f.u. ml$^{-1}$ for the parental virus, inf-MHV-A59. The EOP of the revertants were all within a twofold range compared with parental virus.

Sequence analysis of second-site revertants

We sequenced the nsp16 coding region of the revertant viruses inf-MHV-Wüts18(AGC)R1 to R7, and the entire RNA genomes of the revertant viruses inf-MHV-Wüts18(AGC)R8 and R9. Sequencing was done as described previously (Stokes et al., 2010). The nucleotide changes are detailed in Supplementary Table S1 (available in JGV Online) and the results are summarized in Table 1 as amino acid changes relative to the recombinant parental virus inf-MHV-A59. The results show that all of the revertant viruses have retained the AGC codon for nsp16.

![Fig. 1. Phenotypic characterization of inf-MHV-Wüts18(AGC).](http://vir.sgmjournals.org) (a) Replication kinetics following infections at high m.o.i. were determined by plaque assay of culture supernatants for inf-MHV-A59 at 33 (●) and 37 °C (■), and for inf-MHV-Wüts18(AGC) at 33 (○) and 37 °C (△). (b) A stock of inf-MHV-Wüts18(AGC) was plaque assayed at 33, 37 and 39.5 °C. The plaques that formed at various dilutions and temperatures are shown.
Table 1. Sequence analysis of inf-MHV-Wu\textsubscript{18(AGC)} revertants

For inf-MHV-A59 and inf-MHV-Wu\textsubscript{18(AGC)} revertants 8 and 9, the entire genome was sequenced. For the remaining revertants, only the region of the genome encoding nsp16 was sequenced. Variant codons are shown as the encoded amino acid. –, Sequence was not determined; +, sequence was determined and was identical to inf-MHV-A59.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plaque size*</th>
<th>nsp16 (codon)</th>
<th>ORF2b (nt)</th>
<th>nsp5 (codon)</th>
<th>nsp8 (codon)</th>
<th>nsp12 (codon)</th>
<th>nsp13 (codon)</th>
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<tbody>
<tr>
<td></td>
<td>Original</td>
<td>Stock</td>
<td>12</td>
<td>43</td>
<td>76</td>
<td>100</td>
<td>114</td>
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<tr>
<td>inf-MHV-A59</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>Ser</td>
<td>Pro</td>
<td>Asn</td>
<td>Lys</td>
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<tr>
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<td>Large</td>
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<td>Asn</td>
<td>Glu</td>
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<td>Large</td>
<td>Large</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>inf-MHV-Wu\textsubscript{18(AGC)\textsubscript{R3}}</td>
<td>Large</td>
<td>Large</td>
<td>Large</td>
<td>Ser</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<td>Large</td>
<td>Ser</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>Ser</td>
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<td>Large</td>
<td>Ser</td>
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<td>+</td>
</tr>
<tr>
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<td>Large</td>
<td>Small</td>
<td>Ser</td>
<td>+</td>
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<tr>
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<td>Small</td>
<td>Ser</td>
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<tr>
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<td>Small</td>
<td>Small</td>
<td>Ser</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*After 2 days at 39.5 °C, wild-type (wt) plaques were 7 mm in diameter, large plaques were between 5 and 6 mm in diameter and small plaques were between 2 and 3 mm in diameter. Revertant viruses that had large-plaque morphology typically produced stocks with titres of between $1 \times 10^6$ and $1 \times 10^7$ p.f.u. ml$^{-1}$. Revertant virus that had small-plaque morphology produced a stock with a titre of $5 \times 10^4$ p.f.u. ml$^{-1}$. 
serine 12, the substitution that confers a ts phenotype. Next, we found that all of the revertant viruses had second-site mutations in nsp16. In the majority of cases [inf-MHV-Wüstts18(AGG)R9, R4 to R9], we found the substitution of nsp16 asparagine 43 with serine. In two revertant viruses, we found a different substitution; namely lysine 76 with glutamic acid in inf-MHV-Wüstts18(AGG)R2 and aspartic acid 130 with asparagine in inf-MHV-Wüstts18(AGG)R3. In four revertants, we also found a second substitution in nsp16 (Table 1).

The complete genome sequences of inf-MHV-Wüstts18(AGG)R8 (large plaque) and inf-MHV-Wüstts18(AGG)R9 (small plaque) showed that both revertants had identical sequences over the entire genome, with the exception of a unique substitution of threonine 115 with isoleucine in the nsp13 coding region of inf-MHV-Wüstts18(AGG)R9. Compared with inf-MHV-A59, we also found that both revertants had three amino acid substitutions and a C to U transition at nt 22999.

These results lead us to four conclusions. First, the reversion of inf-MHV-Wüstts18(AGG) to a non-ts phenotype, is most probably due to the intra-molecular substitution of amino acids in nsp16; often the substitution of nsp16 asparagine 43 with serine, but also possibly the substitution of lysine 76 with glutamic acid or aspartic acid 130 with asparagine. Second, in addition to these substitutions, there may be further substitutions in nsp16, but there is no reason to conclude that these are related to the reversion phenotype. Third, the genomic sequencing of inf-MHV-W üstts18(AGG)R8 and inf-MHV-W üstts18(AGG)R9 suggests that the large- and small-plaque phenotype of the revertants can be dictated by nucleotide changes outside the nsp16 coding region. In the case of inf-MHV-W üstts18(AGG)R8, the large-plaque phenotype (which is still smaller than the parental inf-MHV-A59 virus) could be attributed to any one or more of three mutations, which result in amino acid substitutions (Table 1). We consider it unlikely that the C to U transition at nt 22999 is involved in the plaque-size phenotype as it is located within the haemagglutinin-esterase pseudogene (Luytjes et al., 1988). Fourth, in the case of inf-MHV-W üstts18(AGG)R9, the results show that a mutation in nsp13, which results in the substitution of threonine 115 with isoleucine, has a profound effect upon the plaque size. Compared with the isogenic inf-MHV-Wüstts18(AGG)R8, inf-MHV-Wüstts18(AGG)R9 has a smaller plaque size, which also manifests in a lower titre of stock virus.

**DISCUSSION**

In this study, we have combined classical and reverse genetics to probe the structure–function relationships of the MHV-A59 nsp16 protein. Unfortunately, the structure of the coronavirus nsp16 protein has not yet been solved and we and others have to rely on mutagenesis and modelling to help interpret our data. In the case of the FCoV nsp16 protein, this approach has led Decroly and colleagues (Decroly et al., 2008) to identify residues lysine 45, aspartic acid 129, lysine 169 and glutamic acid 202 as the putative K-D-K-E catalytic tetrad of the enzyme. The same study also implicated various other residues in different aspects of the methylation reaction, although it is often difficult to link a unique function to a specific residue.

Using the same modelling approach, we have analysed the MHV nsp16 protein. A linear alignment analysis (Supplementary Fig. S1, available in JGV Online) shows that the canonical active site residues are strictly conserved across representatives of alphacoronaviruses (FCoV, strain FIPV), betacoronaviruses (MHV and SARSr-CoV) and gammacoronaviruses (avian coronavirus, strain IBV). In this analysis, it is also interesting to note that the aromatic residue corresponding to FIPV tryptophan 175 is conserved across all of the representative coronaviruses. This leads us to suggest that MHV nsp16 residue tryptophan 176 is likely to represent the second of two aromatic residues that are proposed to be necessary for the recognition of type-0 cap structures (Hu et al., 1999).

In the next stage of our modelling, the MHV nsp16 sequence was used to build a profile Hidden Markov model (HMM), which was then compared with a database of HMMs representing protein domains with known structure (http://toolkit.tuebingen.mpg.de/hpred) (Soding et al., 2005). The closest template proposed was the *Escherichia coli* rRNA methyltransferase protein (Supplementary Fig. S2, available in JGV Online). The aligned template was then used to predict a structural model for the MHV nsp16 protein by using MODELLER, which implements comparative protein structure modelling by satisfaction of spatial restraints (Sali & Blundell, 1993). In order to aid the interpretation of this model, we also produced a model of the FIPV nsp16 MTase core domain by using exactly the same software and parameters. The resulting models, which are shown in Fig. 2, can be compared with each other and with the model published by Decroly et al. (2008). Clearly all three models place three of the four catalytic residues (aspartic acid 130, lysine 170 and glutamic acid 203 in MHV) in close proximity. Our models of the MHV and FIPV proteins place lysine 45/46 at some distance from the other three tetrad residues, which, as proposed by Decroly et al. (2008), may suggest that it is involved in RNA binding rather than MTase activity. Also, the MHV model shown here is consistent with the idea that tryptophan 176 is adequately positioned to bind a type-0 cap structure and that, as reported for the equivalent FIPV nsp16 aspartic acid 113 (Decroly et al., 2008), the MHV nsp16 asparagine 114 residue could be involved in AdoMet binding. It is important to emphasize that, within the regions shown in Fig. 2, the structure of the coronavirus nsp16 MTase core domain can be predicted with some confidence (Supplementary Fig. S3) (Luthy et al., 1992).

Our sequence analysis of second-site revertants provides information regarding the genetic basis for both the reversion phenotype and the plaque-size phenotype. First
of all, in five revertants, inf-MHV-Wu¨G.-h. Chang and others flavivirus 2 acid 130 is a critical residue in the catalytic tetrad of phenotype. As earlier studies have suggested that aspartic acid 130 with asparagine can also result in a reversion substitution of lysine 76 with glutamic acid or aspartic acid 130 with asparagine 43 with serine, although the substitution of asparagine 43 with serine, in particular, the sequencing of inf-MHV-Wu¨nsp16. Based upon the frequency of nsp16 mutations and, in particular, the sequencing of inf-MHV-Wu¨AGG, R8 and inf-MHV-Wu¨AGG, R9, we think it very likely that these intra-molecular substitutions account for the reversion phenotype. The most common reversion genotype was the substitution of asparagine 43 with serine, although the substitution of lysine 76 with glutamic acid or aspartic acid 130 with asparagine can also result in a reversion phenotype. As earlier studies have suggested that aspartic acid 130 is a critical residue in the catalytic tetrad of flavivirus 2’O-MTase proteins (Dong et al., 2008), this would imply that substitution of aspartic acid 130 with asparagine does not abrogate MHV nsp16 2’O-MTase activity, which we think is unlikely, or that this activity is not essential for coronavirus replication in cell culture. This conclusion raises an interesting question. If 2’O-MTase activity is not essential, how do we explain the ts phenotype of inf-MHV-Wu¨AGG, R9? One explanation would be that the ts mutation is affecting another essential function of the protein; for example, interactions with viral RNA or viral proteins in the context of the biogenesis or function of the virus RTC.

In addition to the reversion phenotype, we also found that many of the revertants we isolated had different plaque sizes and that this phenotype was not necessarily stable. Therefore, we sequenced the entire genome of two revertants. Both of these revertants had the same substitution of asparagine 43 with serine, although the substitution resulted in the substitution of threonine 115 with isoleucine in nsp13. The coronavirus nsp13 protein has both RNA and DNA duplex-unwinding activities and is commonly referred to as a 5’-to-3’ helicase. It has also been shown to have a number of other activities, including (d)NTPase and RNA 5’-triphosphatase activities (Ivanov et al., 2004; Ivanov & Ziebuhr, 2004). It is worth noting that one of these activities, the RNA 5’-triphosphatase, is presumed to be involved in RNA-cap formation. However, the threonine 115 residue has not been implicated in any enzymic activity and, consequently, we suggest that there is no reason to link this substitution to nsp16 or the synthesis of capped RNA. Instead, we suggest that the phenotype of inf-MHV-Wu¨AGG, R9 may relate to a defect in RNA synthesis.

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