An amino acid substitution (V3I) in the Japanese encephalitis virus NS4A protein increases its virulence in mice, but not its growth rate in vitro

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Our previous studies have shown that the Japanese encephalitis virus (JEV) strain Mie/40/2004 is the most virulent of the strains isolated by us in Japan from 2002 to 2004. Comparison of the amino acid sequence of Mie/40/2004 with those of low-virulence strains revealed that an isoleucine residue at position 3 of the Mie/40/2004 NS4A protein may increase viral pathogenicity. A recombinant virus with a single valine-to-isoleucine substitution (V3I) at position 3 in the low-virulence Mie/41/2002 background (rJEV-Mie41-NS4A V3I) exhibited increased virulence in mice compared with the Mie/41/2002 parent strain. The V3I mutation did not affect virus growth in several cell lines. These results demonstrate that the isoleucine at position 3 in the NS4A protein of Mie/40/2004 is responsible for its high virulence in vivo. This is the first report to show that an amino acid substitution in a flavivirus NS4A protein alters viral pathogenicity in mice.

Japanese encephalitis virus (JEV), which is transmitted to humans by mosquitoes, causes the serious neurological disorder Japanese encephalitis (JE). The fatality rate of JE is approximately 30% and each year 30 000–50 000 clinical cases of JE and 10 000 deaths are reported, mainly in China, South East Asian countries, and India (Tsai, 2000). Most cases of JE occur in South, East and South East Asia; however, JE cases have been reported in northern areas of Australia in recent decades (Hanna et al., 1996). JEV belongs to the genus Flavivirus within the family Flaviviridae and is now classified into five genotypes (genotypes I–V) based on genomic RNA homology (Uchil et al., 2003). JEV has a single-stranded, positive-sense RNA genome. The approximately 11 kb genome contains one ORF encoding three structural proteins (capsid (C), premembrane (prM) and envelope (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), as well as 5’ and 3’ NTRs (Lindenbach et al., 2007). The E protein, the main structural protein, has a putative receptor-binding domain and neutralization epitopes. The E protein is thought to play major roles in determining viral pathogenicity by defining cell tropism and mediating entry into susceptible cells (Lindenbach et al., 2007; Gubler et al., 2007). Since the early 1990s, various attempts have been made to clarify the molecular basis of JEV virulence by comparing the nucleotide sequences of virus strains with different degrees of virulence (Nitayaphan et al., 1990; Aihara et al., 1991; Cecilia & Gould, 1991; Hasegawa et al., 1992; Sumiyoshi et al., 1995; Chen et al., 1996; Ni & Barrett, 1996, 1998; Arroyo et al., 2001; Wu et al., 2003; Lee et al., 2004; Chambers et al., 2007; Pujhari et al., 2011; Wu et al. 2011). Some of these reports have suggested that nucleotide substitutions in the E protein may alter the virulence of JEV. Studies using an infectious clone of JEV demonstrated that a single glutamic acid-to-lysine substitution at position 138 of the E protein attenuated the JEV strain in which it was made (Sumiyoshi et al., 1995; Zhao et al., 2005; Liang et al., 2009). It has also been reported that a single methionine-to-lysine substitution at position 279 of
Table 1. Comparison of amino acid residues between Mie/41/2002 and Mie/40/2004

Complete genome sequences of Mie/41/2002 and Mie/40/2004 were determined and predicted amino acid sequences were compared between the two strains.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virulence in mice*</th>
<th>Protein and amino acid position therein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Mie/41/2002</td>
<td>Low</td>
<td>R</td>
</tr>
<tr>
<td>Hiroshima/25/2002†</td>
<td>Low</td>
<td>K</td>
</tr>
<tr>
<td>Kagawa/35/2004†</td>
<td>Low</td>
<td>K</td>
</tr>
<tr>
<td>Mie/40/2004</td>
<td>High</td>
<td>K</td>
</tr>
</tbody>
</table>

*Cited from Nerome et al. (2007).
†Only partial sequences of the C, NS3, NS4A and NS5 regions of the genomes of Hiroshima/25/2002 and Kagawa/35/2004 were determined.

In recent years, we have isolated JEV from pigs and characterized the JEV strains prevalent in Japan (Nerome et al., 2007). Among these new isolates, isolate Mie/40/2004 showed high virulence relative to other genotype I isolates (Nerome et al., 2007) (Table 1). Genetic analysis showed no differences in the deduced amino acid sequences of the E proteins among the four genotype I isolates, indicating that the E region is not responsible for the higher virulence of Mie/40/2004 (Nerome et al., 2007). To determine the amino acid differences among the high- and low-virulence strains, we first compared the full amino acid sequence of Mie/40/2004 (GenBank accession no. AB241118) to that of Mie/41/2002 (GenBank accession no. AB241119), the least virulent of the new genotype I isolates (Nerome et al., 2007) (Table 1). Seven amino acid differences were detected between these two strains, four of which were found in the NS5 protein (positions 20, 429, 526 and 860) and one each in the C (position 10), NS3 (position 467) and NS4A (position 3) proteins. We then compared the amino acid residues at the seven divergent positions to those in two additional low-virulence strains, Hiroshima/25/2002 and Kagawa/35/2004 (Table 1). While Hiroshima/25/2002 and Kagawa/35/2004 were identical to Mie/40/2004 rather than to Mie/41/2002 at six of these positions, all three low-virulence strains had valine at position 3 of the NS4A protein, whereas the high-virulence Mie/40/2004 had isoleucine at this position. This result suggests that the amino acid at position 3 of the NS4A protein may influence the virulence of JEV in mice. Molecular epidemiological analysis revealed that JEV strains with the Ile-3 NS4A protein were a minor group, while the majority of JEV strains belong to the group having the Val-3 NS4A protein (data not shown).

To investigate whether the amino acid at position 3 of the NS4A protein of Mie/40/2004 determines its high-virulence phenotype in mice, we first constructed a recombinant intertypic JEV clone, rJEV-Mie41–NS3 4A\textsuperscript{Mie40}/pMW119, which has the NS3–NS4A region of Mie/40/2004 in the Mie/41/2002 background of the parent clone rJEV(Mie/41/2002)/pMW119, described previously (Tajima et al., 2010) (Fig. 1a). Recombinant virus rJEV-Mie41-NS3–4A\textsuperscript{Mie40} was recovered by transfection of in vitro-transcribed viral RNA into Vero cells, as previously described (Tajima et al., 2006). Groups of 3-week-old female C3H/He mice (n=6) were infected intraperitoneally with 1 \times 10^2 p.f.u. of Mie/41/2002, Mie/40/2004 or rJEV-Mie41-NS3–4A\textsuperscript{Mie40} and were observed for 2 weeks (Table 2, experiment 1). Mouse challenge experiments were performed in accordance with the fundamental rules for animal experiments of our
institute. We assessed the neuroinvasiveness of these viruses by determining their ability to replicate in peripheral tissues, invade the central nervous system and cause encephalitis. Five of the six mice in each group infected with Mie/40/2004 or rJEV-Mie41-NS3–4A\textsuperscript{Mies0}, but only two of the six mice infected with Mie/41/
2002, died within the observation period. This result suggests that the NS3–4A region may determine the difference in virulence observed between Mie/41/2002 and Mie/40/2004 in vivo. In addition to NS4A Ile-3, the NS3–4A region of Mie/40/2004 has another amino acid residue, NS3 Asn-467, which is different from that of Mie/41/2002. To determine which of the polymorphic amino acids in the NS3–4A region are responsible for the higher virulence phenotype of rJEV-Mie41-NS3–4AMie40, we produced two additional substitution recombinant viruses, rJEV-Mie41-NS3S467N and rJEV-Mie41-NS4AV3I, which have, respectively, a serine-to-asparagine mutation at position 467 (S467N) in the NS3 region of Mie/41/2002 and a valine-to-isoleucine mutation at position three (V3I) in the NS4A region of Mie/41/2002 (Fig. 1a). Groups of mice (n=10) were inoculated with 1×10^5 p.f.u. of recombinant virus (Table 2, experiment 2). Nine and seven mice infected with Mie/40/2004 and rJEV-Mie41-NS4AV3I, respectively, died within 2 weeks of the challenge, whereas only two and one mice infected with Mie/41/2002 and rJEV-Mie41-NS4AV3I, respectively, died within the same period. We repeated the mouse challenge experiment using Mie/41/2002, Mie/40/2004 and rJEV-Mie41-NS4A V3I, and obtained similar results (Table 2, experiment 3). To investigate the contribution of NS4A Ile-3 to JEV virulence further, groups of mice (n=10) were inoculated with 1×10^5, 1×10^4 and 1×10^3 p.f.u. of Mie/41/2002, Mie/40/2004 and rJEV-Mie41-NS4AV3I viruses, respectively (Table 2, experiment 4). At least 70% of the mice challenged with Mie/40/2004 or rJEV-Mie41-NS4AV3I died at every dose compared with fewer than 40% of mice challenged with the Mie/41/2002 strain. These results indicate that the single V3I substitution mutation of NS4A increased the pathogenicity of JEV in mice. The survival curve for rJEV-Mie41-NS4AV3I-infected mice was similar to that for Mie/40/2004 (data not shown); however, statistical analyses (log-rank test) indicated that rJEV-Mie41-NS4AV3I was slightly less virulent than Mie/40/2004 (Table 2). These data suggest that the amino acid at position 3 of NS4A is largely, but not entirely, responsible for the difference in pathogenicity between Mie/41/2002 and Mie/40/2004.

Our previous findings suggest that the virulence of JEV in mice correlates with its growth properties in vitro (Tajima et al., 2010). Therefore, we examined the growth properties of Mie/41/2002, Mie/40/2004 and rJEV-Mie41-NS4AV3I in cultured cells. We first compared the plaque sizes of the strains as previously described (Tajima et al., 2010). The plaque morphologies of all three viruses were similar (Fig. 1b), indicating that the V3I mutation does not affect plaque formation and that the difference in virulence between Mie/41/2002 and Mie/40/2004 strains does not correlate with their plaque sizes in Vero cells. We next compared the growth rates of the three strains in four cell lines as previously described (Tajima et al., 2010). In Vero cells, the growth curves were very similar in all three viruses (Fig. 1c). No difference was observed among the growth curves in human lung adenocarcinoma A549 cells (Fig. 1d), which are often used to study the type 1 interferon (IFN) response to flavivirus infection (Muñoz-Jordan et al., 2003), or in mouse hepatoma Hepa 1-6 cells (Fig. 1e), which can also respond to type 1 IFN (data not shown). We also examined the growth kinetics of the three viruses in IFN-β-pretreated A549 cells, and no significant difference was observed among the growth curves of the viruses (Supplementary Fig. S1, available in JGV Online). As we have shown previously that there is a good correlation between virulence in vivo and the growth characteristics of JEV in mouse neuroblastoma-derived N18 cells (Tajima et al., 2010), we analysed the growth kinetics of our viruses in N18 cells. However, even in N18 cells, the growth rates of Mie/40/2004 and rJEV-Mie41-NS4AV3I resembled that of Mie/41/2002 (Fig. 1f). These results indicate that there is no significant difference between the growth properties of the high-virulence Mie/40/2004 and rJEV-Mie41-NS4AV3I viruses and those of the low-virulence Mie/41/2002 virus.

In the present paper, we have attempted to identify the amino acid in Mie/40/2004 that is responsible for its higher pathogenicity in mice. Amino acid sequence comparisons between the highly virulent Mie/40/2004 and other less virulent JEV strains suggested that an amino acid at position 3 of the NS4A protein is a determinant of pathogenicity in the mouse model. Reverse-genetics

### Table 2. Neuroinvasiveness in mice

<table>
<thead>
<tr>
<th>Experiment no. and virus used</th>
<th>Dose*</th>
<th>Mortality</th>
<th>P value‡</th>
<th>Ratio†</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Mie/41/2002</td>
<td>2</td>
<td>2/6</td>
<td>33</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Mie/40/2004</td>
<td>2</td>
<td>5/6</td>
<td>83</td>
<td>0.059</td>
<td>0.047§</td>
</tr>
<tr>
<td>rJEV-Mie41-NS3–4AMie40</td>
<td>2</td>
<td>5/6</td>
<td>83</td>
<td>0.047§</td>
<td></td>
</tr>
<tr>
<td>2 Mie/41/2002</td>
<td>3</td>
<td>2/10</td>
<td>20</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Mie/40/2004</td>
<td>3</td>
<td>9/10</td>
<td>90</td>
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<td></td>
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<tr>
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<td>70</td>
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<td>1/10</td>
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</tr>
<tr>
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<td>3/12</td>
<td>25</td>
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<td>–</td>
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<tr>
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</tr>
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<td>3</td>
<td>7/12</td>
<td>58</td>
<td>0.065</td>
<td></td>
</tr>
<tr>
<td>4 Mie/41/2002</td>
<td>2</td>
<td>2/10</td>
<td>20</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Mie/40/2004</td>
<td>2</td>
<td>2/10</td>
<td>20</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>rJEV-Mie41-NS4AV3I</td>
<td>2</td>
<td>8/10</td>
<td>80</td>
<td>0.097</td>
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<tr>
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<td>8/10</td>
<td>80</td>
<td>0.016§</td>
<td></td>
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<tr>
<td>rJEV-Mie41-NS4AV3I</td>
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<td>7/10</td>
<td>70</td>
<td>0.022§</td>
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<tr>
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<td>4</td>
<td>8/10</td>
<td>80</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>

*Log p.f.u. per dose.
†Number of mice dead/number of mice inoculated.
‡P value relative to Mie/41/2002 by log-rank test. Signs indicate statistical significance (§, P<0.05; ‖, P<0.01).
analysis revealed that the single V3I substitution in the NS4A protein of Mie/41/2002 increased its virulence in mice. This result demonstrates that Ile-3 of NS4A in Mie/40/2004 is at least partially responsible for the high virulence of this isolate. This is the first report to show that an amino acid substitution in a flavivirus NS4A protein alters viral pathogenicity in mice. Interestingly, the V3I substitution had no effect on the in vitro growth properties of the virus, and we failed to gain insight into the mechanism by which the amino acid substitution alters the virulence of JEV. Flavivirus NS4A is a small hydrophobic protein that localizes to the sites of viral RNA replication and interacts with a member of the replication complex, NS1, suggesting that NS4A is a component of the flaviviral replication complex and plays a role in viral RNA replication (Lindenbach & Rice, 1999; Mackenzie et al., 1998; Westaway et al., 2003; Miller et al., 2007). Previous findings suggest that NS4A induces the membrane rearrangements necessary to form the viral replication complex (Roosendaal et al., 2006; Miller et al., 2007). NS4A consists of an N-terminal hydrophilic region (aa 1–50) and several hydrophobic transmembrane regions (aa 51–150) (Miller et al., 2007). We recently demonstrated that the N-terminal region of dengue type 1 virus (DENV-1) NS4A is indispensable for the replication of DENV-1 (Tajima et al., 2011). The critical position 3 of JEV NS4A is located in the N-terminal region. A recent report showed that, in West Nile virus, the N-terminal region of NS4A functions as a cofactor of NS3 and regulates the ATPase activity of the NS3 helicase, which is required for unwinding viral RNA during viral replication (Shiryaev et al., 2009). This finding raises the possibility that the amino acid variation at position 3 of NS4A may affect the interaction between NS4A and NS3. However, our data showed that the amino acid difference at position 3 did not influence the growth rate of JEV in cultured cells, suggesting that this polymorphism does not influence a basic replication function such as the interaction between NS3 and NS4A. The site of Ile-3 is also part of the NS3–NS4A cleavage-site sequence, which is cleaved by the viral serine protease (NS2B–NS3 complex). However, the V3I mutation did not alter the growth properties of JEV in vitro, indicating that the mutation does not affect the processing of the NS3–NS4A junction. NS4A also weakly inhibits the IFN signalling pathway (Muñoz-Jordan et al., 2003; Lin et al., 2008). DEAD-box RNA helicase DDX42 interacts with JEV NS4A and is able to overcome the JEV-induced antagonism of IFN responses, supporting the idea that NS4A modulates the IFN pathway (Lin et al., 2008). Although the region of NS4A that interacts with DDX42 is unknown, it is possible that the amino acid at position 3 participates in the binding of NS4A to DDX42. However, in the data presented, there was no difference in growth rates among the three strains in IFN-responsive A549 cells, suggesting that the V3I mutation may not affect IFN signalling pathways. Therefore, the mechanism by which the NS4A V3I mutation increases JEV virulence seems to be complex. In vivo studies on the growth kinetics of the high- and low-virulence viruses and the immune responses to viruses are essential to understand the role of the amino acid residue at position 3 of NS4A. The mutant rJEV-Mie41-NS4AV3I exhibited slightly weaker virulence than did Mie/40/2004 (Table 2; experiments 2, 3 and 4), indicating that the V3I mutation, while important, is not sufficient to explain the high virulence of Mie/40/2004. Other differences between Mie/41/2002 and Mie/40/2004 might also be required for the full virulence of Mie/40/2004.

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