

Short
CommunicationSecondary structure of dengue virus type 4
3' untranslated region: impact of deletion and
substitution mutationsTammy A. Romero,¹ Ebenezer Tumban,¹ Jeongwon Jun,¹ William B. Lott²
and Kathryn A. Hanley³

Correspondence

Kathryn A. Hanley
khanley@nmsu.edu^{1,3}Molecular Biology Program¹ and Department of Biology³, New Mexico State University
(NMSU), Las Cruces, NM 88003, USA²School of Life Sciences, Queensland University of Technology, Brisbane, QLD 4001, Australia

Several studies have generated computer-based predictions of secondary structure of the 3' untranslated region (UTR) of *Dengue virus* (DEN); however, experimental verification of the formation of these structures *in vitro* is lacking. This study assessed the congruence of Mfold predictions of secondary structure of the core region of the DEN type 4 3' UTR with nuclease maps of this region. Maps and predictions were largely consistent. Maps supported the existence of previously predicted pseudoknots and identified putative regions of dynamic folding. Additionally, this study investigated previously identified conserved elements in the flavivirus 3' UTR that differ among viruses with different modes of transmission. Specific regions of mosquito-borne DEN type 4 were either deleted or replaced with homologous sequences from tick-borne *Langat virus*. All of these mutations caused substantial distortion of secondary structure, yet viruses carrying these mutations were viable.

Received 5 May 2006

Accepted 26 June 2006

The four serotypes of mosquito-borne *Dengue virus* (DEN; genus *Flavivirus*, family *Flaviviridae*) are the aetiological agents of dengue fever, dengue haemorrhagic fever and dengue shock syndrome. Dengue disease is reemerging worldwide; currently, an estimated 100 million DEN infections occur each year (Mackenzie *et al.*, 2004; Weaver & Barrett, 2004). Despite the urgent need to control this escalating pandemic, neither antiviral therapies nor a licensed vaccine for DEN are available (Halstead & Deen, 2002). Rational vaccine design, which employs targeted mutations in the DEN genome to generate vaccine candidates, is being actively pursued (Blaney *et al.*, 2006; Pugachev *et al.*, 2005). However, the success of this strategy depends upon detailed knowledge of both structure and function of the region to which mutations are added (Alvarez *et al.*, 2005b; Martin, 1994; Proutski *et al.*, 1997).

The current study investigates the secondary structure of the DEN4 3' untranslated region (UTR). DEN possesses a single-stranded, positive-sense RNA genome that encodes a single polypeptide and is organized as follows: 5' UTR–C–prM–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5–3' UTR (C, capsid protein; prM, membrane precursor protein; E, envelope protein; NS, non-structural protein) (Rice, 1996).

The 3' UTR is approximately 450 nt long and encompasses three regions: (i) a variable region (VR) immediately 3' of the stop codon, (ii) a core region downstream of the VR and (iii) a 3'-terminal region, which contains the cyclization sequence (CS) and a terminal long stable hairpin (LSH) (Markoff, 2003). The 3' UTR is involved in circularization of the genome through interactions with the 5' UTR (Alvarez *et al.*, 2005a).

The function of the flavivirus 3' UTR depends on both primary sequence and secondary structure (Alvarez *et al.*, 2005b; Elghonemy *et al.*, 2005; Markoff, 2003; Thurner *et al.*, 2004; Tilgner & Shi, 2004; Tilgner *et al.*, 2005; Yu & Markoff, 2005), thus structural analysis of the DEN 3' UTR may contribute to understanding of several aspects of flavivirus biology. The nearly 80 flavivirus species group into three lineages: (i) mosquito-borne, (ii) tick-borne and (iii) those with no known vector (Cook & Holmes, 2005). Conserved regions of both primary sequence and secondary structure that vary among these three lineages have been identified in both the core and 3'-terminal regions of the 3' UTR (Leyssen *et al.*, 2002; Markoff, 2003). Such conserved elements may potentially influence a variety of virus properties, including virus replication, host-cell tropism, vector specificity, pathogenicity and virulence (Alvarez *et al.*, 2005b; Blaney *et al.*, 2006; Markoff, 2003; Yu & Markoff, 2005; Zeng *et al.*, 1998).

A supplementary table showing post-transfection titres of wild-type DEN (rDEN4) and viruses carrying mutations in the 3' untranslated region is available in JGV Online.

Although the secondary structure of the DEN 3' UTR has been predicted by using a variety of computer models (Olsthoorn & Bol, 2001; Proutski *et al.*, 1997; Shurtleff *et al.*, 2001), experimental verification of the formation of these structures *in vitro* (e.g. Chen & Frey, 1999; Tuplin *et al.*, 2004) is lacking. Thus, the current study had three objectives: to (i) generate computer predictions of the DEN4 3' UTR core region; (ii) compare these predictions with patterns generated by nuclease maps of this region; and (iii) assess the secondary structure and viability of DEN4 viruses in which conserved elements in the 3' UTR have been either deleted or replaced with homologous sequence from a tick-borne virus.

Mfold (Zuker, 2003) was used to predict the secondary structure of the core region of the wild-type DEN4 3' UTR (isolate Dominica 1981; GenBank accession no. AF326825). To maintain consistency with previous predictions for this region, Mfold predictions were constrained to maintain the structures of elements upstream and including repeat CS2 (RCS2) and the 3' LSH (stem-loops 6 and 7) (Markoff, 2003; Proutski *et al.*, 1997; Shurtleff *et al.*, 2001; Thurner *et al.*, 2004). The lowest free-energy structure was compared with a nuclease map of this region generated by single- and double-strand-specific RNases (Fig. 1b). This RNase map was generated following methods described by Tuplin *et al.* (2004); briefly, DEN4 cDNA p4 (Durbin *et al.*, 2001) was transcribed as described previously (Hanley *et al.*, 2002) and the transcript was treated with RNases T1 (0.05 U μl^{-1}), A (0.01 U μl^{-1}) and V1 (0.005 U μl^{-1}) (Ambion) to cleave the 3' end of single-stranded guanines (Gs), the 3' end of single-stranded uracils and cytosines (Us and Cs) and double-stranded nucleotides, respectively. Reverse IR-700 primer D4.3'UTR.TR.001 (Li-Cor Biosciences) (Fig. 2) was used for reverse transcription of the digested RNA constructs and sequencing of the undigested cDNA constructs. Primer-extension controls using undigested template were included to detect non-specific cleavage and premature transcriptional termination (intrinsic stops) during reverse transcription. All products were analysed with a Li-Cor Biosciences 4200 DNA sequencer.

Seventy-seven per cent of enzymic cleavages in the nuclease map of the wild-type DEN4 3' UTR core region corresponded to the Mfold-predicted structure. For this construct and all remaining constructs, single-stranded cleavage products observed at nucleotides located on the 3' ends of a helix near internal loops are deemed consistent with the predicted structure because: (i) RNase A has a high affinity for pyrimidine-adenosine linkages, resulting in cleavage of some double-helical regions (Christiansen & Garrett, 1988); and (ii) RNases T1 and A cut at 3' ends of single-stranded nucleotides and could be recognizing these ends as single-stranded.

Three areas of inconsistency between Mfold predictions and the RNase map were identified. First, nuclease cleavages at nt U10420 and G10429 indicated that these were single-stranded when they were predicted to be double-stranded by

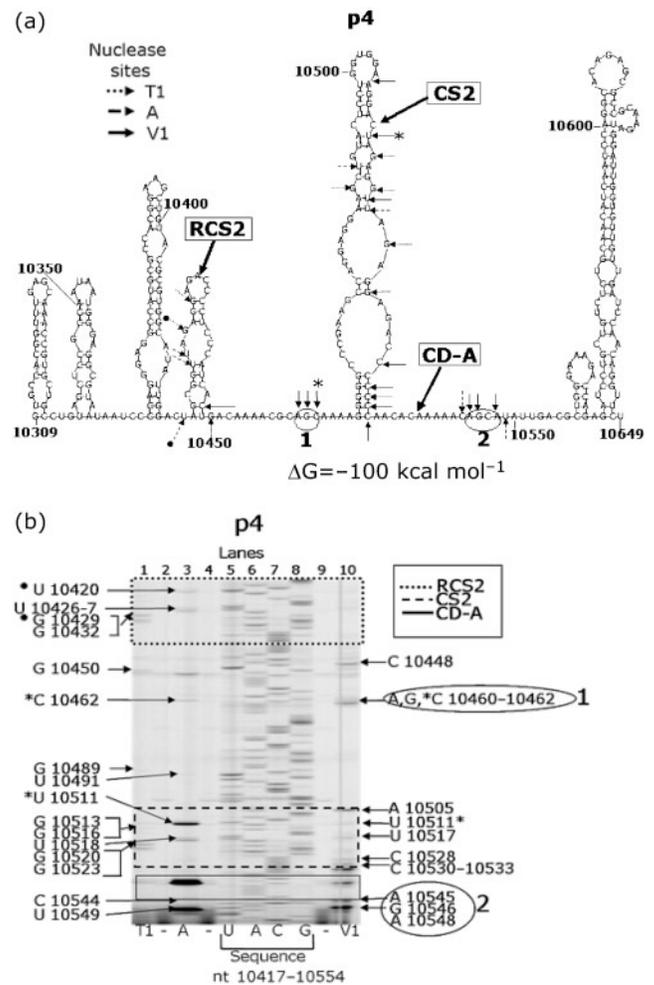


Fig. 1. (a) Mfold secondary-structure prediction for the p4 3' UTR. Single-stranded regions 1 and 2 (circled) in the Mfold prediction were found to be double-stranded in the nuclease map. RCS2, CS2 and CD-A are indicated. (b) RNase map for the p4 3' UTR. Lanes 1, 3 and 10, RNases T1, A and V1, respectively; lanes 2, 4 and 9, primer-extension controls. Nuclease cleavages were compared with the DNA template by sequencing (lanes 5–8). Asterisks are placed on nucleotides with ambiguous cleavages seen in both RNase A and V1 lanes. Closed circles (●) indicate cleavages used to revise the Mfold prediction in (a).

Mfold. RNase V1 cleavage between nt A10505 and A10506 indicated double-stranded nature in this region and RNase T1 cleavage of nt G10516 showed it to be single-stranded. The initial Mfold prediction was revised accordingly. Moreover, nt C10473 and C10474 were constrained to be single-stranded in this revised structure to achieve a more favourable free-energy value. The revised Mfold prediction is depicted in Fig. 1(a). Second, predictions at nt C10462 and U10511 were ambiguous, as cleavages occurred in both RNase A and V1 lanes. Bands were absent in the nuclease-free controls, indicating that these were not intrinsic stops.

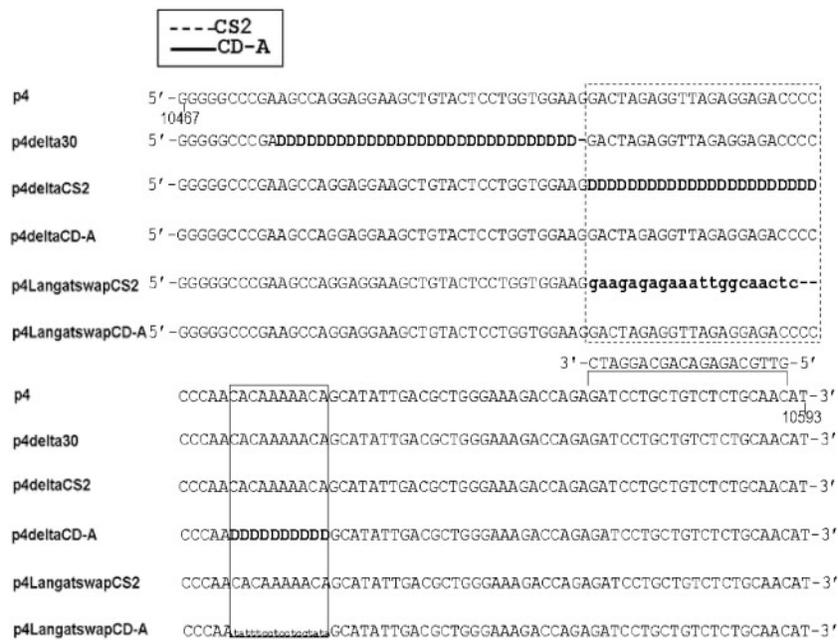


Fig. 2. Partial sequence (nt 10467–10593) of the 3' UTR of wild-type DEN4 cDNA p4 (top row) and p4 carrying mutations inserted into this region. This sequence focuses on the region into which mutations were added and represents only a subsection of the sequence used for Mfold analysis. Mutations consisted of deletions (denoted by a bold D) in p4delta30, p4deltaCS2 and p4deltaCD-A, and substitution of homologous sequence from LGT (denoted by bold lower-case letters) in p4LangatswapCS2 and p4LangatswapCD-A. Sequence of the primer (D4.3'UTR.TR.001) used for sequencing in conjunction with nuclease mapping is indicated above the p4 sequence.

These regions may participate in dynamic folding, as discussed in detail below. Third, regions 1 and 2 (Fig. 1a, b) were predicted to be single-stranded by Mfold, but were shown to be double-stranded by RNase mapping. Both regions have previously been predicted to contribute to RNA pseudoknots, designated *pk2* and *pk1*, that pair with regions designated *pk2'* and *pk1'* (Olsthoorn & Bol, 2001). Mfold is unable to predict pseudoknots (Zuker, 2003). Thus, seven nuclease cleavages out of 30 total cleavages were not predicted by Mfold, resulting in 23 % inconsistent cleavages.

To expand the relevance of this study for vaccine design, an Mfold prediction and nuclease map were also generated for p4delta30 (GenBank accession no. AY376438), the construct used to generate vaccine candidate rDEN4delta30 (GenBank accession no. AF326827), which carries a deletion of nt 10477–10507 in the 3' UTR (Fig. 2) (Durbin *et al.*, 2001; Men *et al.*, 1996). For this construct and all remaining constructs, Mfold predictions utilized constraints to maintain previously predicted structure in RCS2 and elements upstream of RCS2 because cleavages in these two areas were identical to p4. The 3' LSH was also constrained in all structures. The remainder of the predicted region was unconstrained (Fig. 3a). Seventy-one per cent of enzymic cleavages in the nuclease map of p4delta30 corresponded to the Mfold prediction. Ambiguous cleavages were detected at nt C10462 and U10480. Regions 1 and 2 (Fig. 3a) identify the same putative pseudoknots as seen in Fig. 1(b). Nucleotide U10487, detected as single-stranded by RNase mapping, was located in an area predicted to be double-stranded by Mfold.

To investigate the impact of altering conserved elements in the DEN4 3' UTR on secondary structure, two classes of mutation were generated (Fig. 2): (i) deletion of conserved element CS2 (Hahn *et al.*, 1987; Mandl *et al.*, 1993), which

includes nt 10508–10530 of DEN4, as well as a second conserved element termed conserved difference A (CD-A), which includes nt 10536–10545 of DEN4 and which was identified by alignment of flavivirus 3' UTR sequences [K. A. Hanley and others, unpublished data; this region was not identified as being homologous by Olsthoorn & Bol (2001)]; or (ii) replacement of these regions with homologous sequence from tick-borne *Langat virus* (LGT) (GenBank accession no. AF253419; Pletnev *et al.*, 2001). DEN4 CS2 was replaced with LGT nt 10508–10528 and DEN4 CD-A was replaced with LGT nt 10536–10551. Mutations were generated via Kunkel mutagenesis and inserted individually into p4 by using previously described methods (Hanley *et al.*, 2002) to create the following constructs: p4deltaCS2, p4deltaCD-A, p4LangatswapCS2 and p4LangatswapCD-A. Mfold predictions and nuclease maps were generated for each construct (Fig. 3b–e).

Nuclease maps were highly consistent with Mfold predictions for all four constructs (64, 83, 75 and 75 %, respectively). p4deltaCS2 had the lowest consistency, due to a relatively low number of cleavages; a low number of cleavages may be attributable to steric radii in the nucleases that sometimes block access to solvated nucleotides (Christiansen & Garrett, 1988). Moreover, the two putative pseudoknots identified in p4 (regions 1 and 2) were also identified in each of the four constructs (p4LangatswapCD-A had only region 2). In p4deltaCS2, secondary structure at nt C10462 was ambiguous. In p4deltaCD-A, secondary structure at nt U10511 was ambiguous. In p4LangatswapCD-A, nt 10543 and 10546 (region A) were predicted to be single-stranded by Mfold, but were shown to be double-stranded in the nuclease map.

These data are consistent with the existence of RNA pseudoknots predicted by Olsthoorn & Bol (2001) in regions

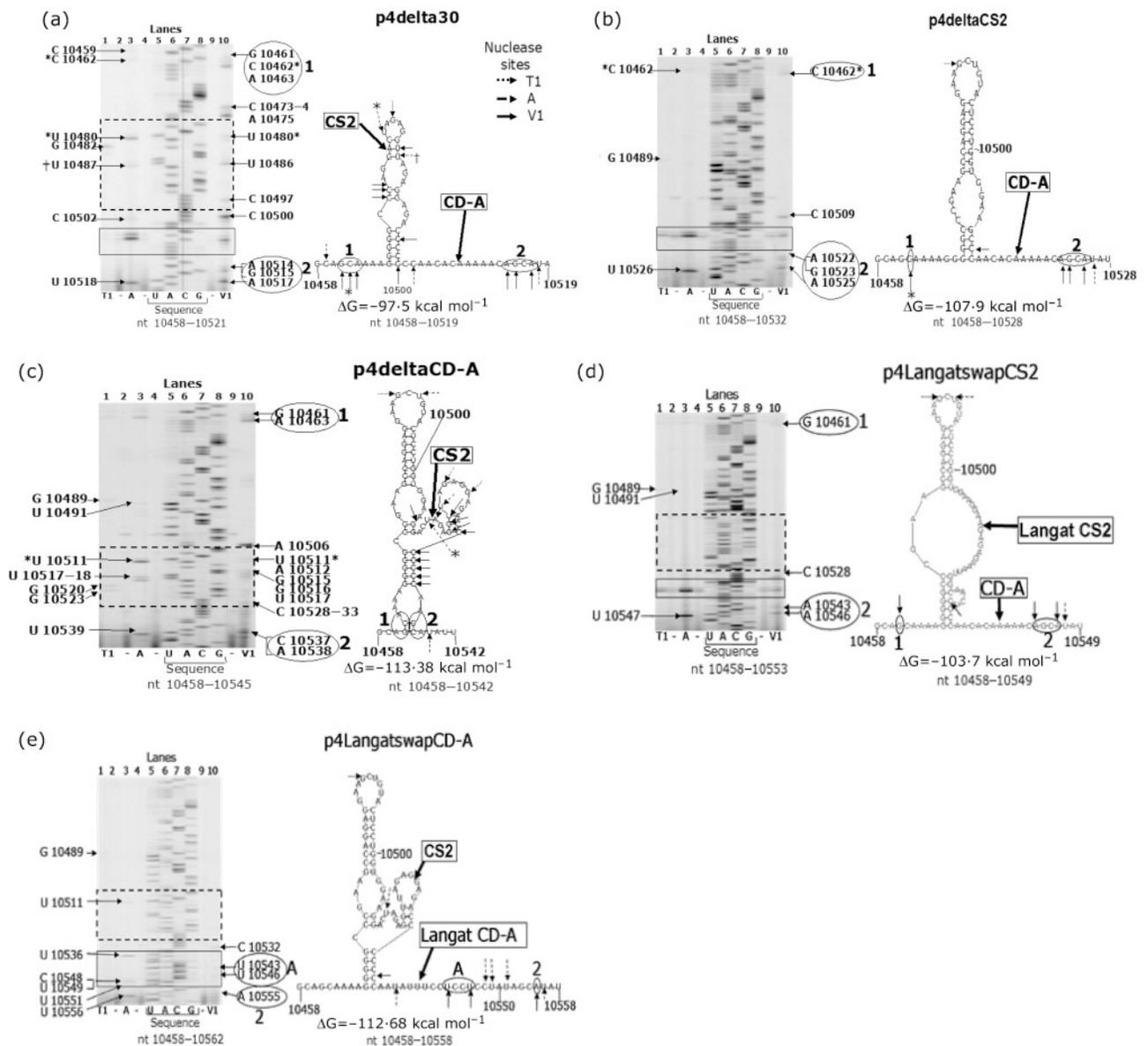


Fig. 3. RNase map and Mfold secondary-structure prediction for DEN4 3' UTR carrying designated mutations: (a) p4delta30; (b) p4deltaCS2; (c) p4deltaCD-A; (d) p4LangatswapCS2; (e) p4LangatswapCD-A. Note that mutations result in variation in length of the 3' UTR. Lanes 1, 3 and 10, RNases T1, A and V1, respectively; lanes 2, 4 and 9, primer-extension controls (indicated by -). Nuclease cleavages were compared with the DNA template by sequencing (lanes 5–8). Asterisks are placed on nucleotides with ambiguous cleavages seen in both RNase A and V1 lanes. Daggers (†) indicate a nucleotide found to be single-stranded in the RNase map and double-stranded in the Mfold prediction. Single-stranded regions 1 and 2 (circled) in the Mfold prediction were found to be double-stranded in the nuclease map. In RNase maps, CS2 and CD-A are indicated with a dashed and a solid box, respectively. RNase maps and Mfold structures were cropped to focus on regions affected by mutations.

1 and 2. The observation that *pk1'*, the *pk1* complement, has single-stranded cleavages at nt G10489 and U10491 must therefore be explained. These band intensities were exceptionally low, suggesting that some transcripts were folded incompletely. The secondary-structure predictions of Proutski *et al.* (1999) for the DEN4 3' UTR did not identify pseudoknots *pk1* and *pk2*, but did identify pseudoknots at the 3' terminus of the region. An additional pseudoknot

may occur in region A (Fig. 3e) of p4LangatswapCD-A. Interestingly, nt 10542–10547 (CUCCUC) in this construct complement nt 10520–10525 (GAGGAG), the latter located in a loop of a branched internal stem-loop. Positive-sense RNA viruses tend to fold into extensive quaternary structures (Simmonds *et al.*, 2004), thus discrepancies between Mfold and nuclease maps could represent genome-scale ordered RNA structure (GORS) rather than

pseudoknots and reflect long-range interactions in the genome that could not be characterized fully with the techniques used in this study. Based on the work of Alvarez *et al.* (2005a), interactions between the 3' and 5' UTRs seem a likely possibility for such long-range interactions.

Ambiguous cleavages were observed at either C10462 or U10511 (U10480 in p4delta30) or both in most transcripts. Ambiguous cleavages often occur where a sequence may be sensitive to both V1 and a single-strand-specific nuclease (Christiansen & Garrett, 1988; Lowman & Draper, 1986; Ziehler & Engelke, 2000). C10462 is the 3' nucleotide of the *pk2* pseudoknot (Olsthoorn & Bol, 2001), thus a double-stranded cleavage is expected. Single-stranded cleavage at this nucleotide may be due to a lack of complete folding or to recognition of the 3' end of C10462 as single-stranded. Alternatively, this may be a dynamic region where pseudoknots form transiently. In p4, U10511 is located in a 2 bp stem within a large stem-loop structure and is predicted by Mfold to be double-stranded. It is cleaved by both V1 and RNase A; as mentioned previously, the latter sometimes cuts at double-stranded regions. In p4delta30, U10480 is located at the top of a stem-loop structure at the 5' end of the loop. It is predicted to be single-stranded by Mfold and is cleaved by both RNases A and V1 in the RNase map. RNase V1 can cleave adjacent to, rather than within, helices (Lowman & Draper, 1986), which may account for this ambiguous cleavage. In p4deltaCD-A, U10511 is predicted to be double-stranded by Mfold, but the structure is kinked between the major stem-loop structure and an internal stem-loop branching off it. Such a kink may also produce an ambiguous cleavage.

Each of the six transcripts described above was transfected in duplicate into both simian (Vero) and mosquito (C6/36) cells and the presence of virus in the cell supernatant was detected as described previously (Hanley *et al.*, 2002). All six constructs produced viable virus in at least one of the two cell types. Titres following transfection ranged from 2.2 to 5.4 log₁₀(p.f.u. ml⁻¹) in Vero cells and from <0.7 to 7.9 log₁₀(p.f.u. ml⁻¹) in C6/36 cells (see Supplementary Table S1, available in JGV Online). Thus, despite their substantial impacts on secondary structure, neither the deletions nor the substitutions described in this study abolished viability completely. Future research will assess the impacts of these mutations on the phenotypes, particularly host-cell tropism and vector specificity, of the viruses carrying them.

Acknowledgements

We would like to acknowledge the following: Andrea Bencomo-Garcia (NMSU), Jay Lee (LID/NIH), Barbara Lyons (NMSU), Brian Murphy (LID/NIH), Erin Schirtzinger (NMSU), Sergio Torres (NMSU), Steve Whitehead (LID/NIH) and Michael Zuker (RPI). Funding was provided by an Alfred P. Sloan Fellowship, NIH-NM-INBRE (P20 RR016480-05), NIH-K22 (K22-A164193) and NSF-ADVANCE (SBE-0123690).

References

- Alvarez, D. E., Lodeiro, M. F., Ludueña, S. J., Pietrasanta, L. I. & Gamarnick, A. V. (2005a). Long-range RNA-RNA interactions circularize the dengue virus genome. *J Virol* **79**, 6631–6643.
- Alvarez, D. E., De Lella Ezcurra, A. L., Fucito, S. & Gamarnick, A. V. (2005b). Role of RNA structures present at the 3'UTR of dengue virus on translation, RNA synthesis, and viral replication. *Virology* **339**, 200–212.
- Blaney, J. E., Jr, Durbin, A. P., Murphy, B. R. & Whitehead, S. S. (2006). Development of a live attenuated dengue virus vaccine using reverse genetics. *Viral Immunol* **19**, 10–32.
- Chen, M.-H. & Frey, T. K. (1999). Mutagenic analysis of the 3' *cis*-acting elements of the rubella virus genome. *J Virol* **73**, 3386–3403.
- Christiansen, J. & Garrett, R. (1988). Enzymatic and chemical probing of ribosomal RNA-protein interactions. *Methods Enzymol* **164**, 456–468.
- Cook, S. & Holmes, E. C. (2005). A multigene analysis of the phylogenetic relationships among the flaviviruses (family: *Flaviviridae*) and the evolution of vector transmission. *Arch Virol* **151**, 309–325.
- Durbin, A. P., Karron, R. A., Sun, W. & 10 other authors (2001). Attenuation and immunogenicity in humans of a live dengue virus type-4 vaccine candidate with a 30 nucleotide deletion in its 3'-untranslated region. *Am J Trop Med Hyg* **65**, 405–413.
- Elghonemy, S., Davis, W. G. & Brinton, M. A. (2005). The majority of the nucleotides in the top loop of the genomic 3' terminal stem loop structure are *cis*-acting in a West Nile virus infectious clone. *Virology* **331**, 238–246.
- Hahn, C. S., Hahn, Y. S., Rice, C. M., Lee, E., Dalgarno, L., Strauss, E. G. & Strauss, J. H. (1987). Conserved elements in the 3' untranslated region of flavivirus RNAs and potential cyclization sequences. *J Mol Biol* **198**, 33–41.
- Halstead, S. B. & Deen, J. (2002). The future of dengue vaccines. *Lancet* **360**, 1243–1245.
- Hanley, K. A., Lee, J. J., Blaney, J. E., Jr, Murphy, B. R. & Whitehead, S. S. (2002). Paired charge-to-alanine mutagenesis of dengue virus type 4 NS5 generates mutants with temperature-sensitive, host range, and mouse attenuation phenotypes. *J Virol* **76**, 525–531.
- Leyssen, P., Charlier, N., Lemey, P., Billoir, F., Vandamme, A.-M., De Clercq, E., de Lamballerie, X. & Neyts, J. (2002). Complete genome sequence, taxonomic assignment, and comparative analysis of the untranslated regions of the Modoc virus, a flavivirus with no known vector. *Virology* **293**, 125–140.
- Lowman, H. B. & Draper, D. E. (1986). On the recognition of helical RNA by cobra venom V1 nuclease. *J Biol Chem* **261**, 5396–5403.
- Mackenzie, J. S., Gubler, D. J. & Petersen, L. R. (2004). Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile, and dengue viruses. *Nat Med* **10**, S98–S109.
- Mandl, C. W., Holzmann, H., Kunz, C. & Heinz, F. X. (1993). Complete genomic sequence of Powassan virus: evaluation of genetic elements in tick-borne versus mosquito-borne flaviviruses. *Virology* **194**, 173–184.
- Markoff, L. (2003). 5'- and 3'-noncoding regions in flavivirus RNA. *Adv Virus Res* **59**, 177–228.
- Martin, S. J. (1994). Vaccine design: future possibilities and potentials. *Biotechnol Adv* **12**, 619–624.
- Men, R., Bray, M., Clark, D., Chanock, R. M. & Lai, C.-J. (1996). Dengue type 4 virus mutants containing deletions in the 3' noncoding region of the RNA genome: analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys. *J Virol* **70**, 3930–3937.

- Olsthoorn, R. C. & Bol, J. F. (2001).** Sequence comparison and secondary structure analysis of the 3' noncoding region of flavivirus genomes reveals multiple pseudoknots. *RNA* **7**, 1370–1377.
- Pletnev, A. G., Bray, M., Hanley, K. A., Speicher, J. & Elkins, R. (2001).** Tick-borne Langkat/mosquito-borne dengue flavivirus chimera, a candidate live attenuated vaccine for protection against disease caused by members of the tick-borne encephalitis virus complex: evaluation in rhesus monkeys and in mosquitoes. *J Virol* **75**, 8259–8267.
- Proutski, V., Gould, E. A. & Holmes, E. C. (1997).** Secondary structure of the 3' untranslated region of flaviviruses: similarities and differences. *Nucleic Acids Res* **25**, 1194–1202.
- Proutski, V., Gritsun, T. S., Gould, E. A. & Holmes, E. C. (1999).** Biological consequences of deletions within the 3'-untranslated region of flaviviruses may be due to rearrangements of RNA secondary structure. *Virus Res* **64**, 107–123.
- Pugachev, K. V., Guirakhoo, F. & Monath, T. P. (2005).** New developments in flavivirus vaccines with special attention to yellow fever. *Curr Opin Infect Dis* **18**, 387–394.
- Rice, C. M. (1996).** *Flaviviridae: the viruses and their replication*. In *Fields Virology*, 3rd edn, pp. 931–959. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott–Raven.
- Shurtleff, A. C., Beasley, D. W. C., Chen, J. J. Y. & 9 other authors (2001).** Genetic variation in the 3' non-coding region of dengue viruses. *Virology* **281**, 75–87.
- Simmonds, P., Tuplin, A. & Evans, D. J. (2004).** Detection of genome-scale ordered RNA structure (GORS) in genomes of positive-stranded RNA viruses: implications for virus evolution and host persistence. *RNA* **10**, 1337–1351.
- Thurner, C., Witwer, C., Hofacker, I. L. & Stadler, P. F. (2004).** Conserved RNA secondary structures in *Flaviviridae* genomes. *J Gen Virol* **85**, 1113–1124.
- Tilgner, M. & Shi, P.-Y. (2004).** Structure and function of the 3' terminal six nucleotides of the West Nile virus genome in viral replication. *J Virol* **78**, 8159–8171.
- Tilgner, M., Deas, T. S. & Shi, P.-Y. (2005).** The flavivirus-conserved penta-nucleotide in the 3' stem-loop of the West Nile virus genome requires a specific sequence and structure for RNA synthesis, but not for viral translation. *Virology* **331**, 375–386.
- Tuplin, A., Evans, D. J. & Simmonds, P. (2004).** Detailed mapping of RNA secondary structures in core and NS5B-encoding region sequences of hepatitis C virus by RNase cleavage and novel bioinformatic prediction methods. *J Gen Virol* **85**, 3037–3047.
- Weaver, S. C. & Barrett, A. D. T. (2004).** Transmission cycles, host range, evolution and emergence of arboviral disease. *Nat Rev Microbiol* **2**, 789–801.
- Yu, L. & Markoff, L. (2005).** The topology of bulges in the long stem of the flavivirus 3' stem-loop is a major determinant of RNA replication competence. *J Virol* **79**, 2309–2324.
- Zeng, L., Falgout, B. & Markoff, L. (1998).** Identification of specific nucleotide sequences within the conserved 3'-SL in the dengue type 2 virus genome required for replication. *J Virol* **72**, 7510–7522.
- Ziehler, W. A. & Engelke, D. R. (2000).** Probing RNA structure with chemical reagents and enzymes. In *Current Protocols in Nucleic Acid Chemistry*, pp. 6.1.1–6.1.21. Edited by S. L. Beaucage, D. E. Bergstrom, G. D. Glick & R. A. Jones. New York: Wiley.
- Zuker, M. (2003).** Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31**, 3406–3415.