Short Communication

Replacement of the 3′ untranslated variable region of mosquito-borne dengue virus with that of tick-borne Langat virus does not alter vector specificity

Ebenezer Tumban,[1]† Dana N. Mitzel,[2] Nyree E. Maes,[1]‡ Christopher T. Hanson,[3] Stephen S. Whitehead[3] and Kathryn A. Hanley[1,4]

Correspondence
Kathryn A. Hanley
khanley@nmsu.edu

[1]Molecular Biology Program, New Mexico State University, Las Cruces, NM 88003, USA

[2]Laboratory of Virology, National Institute of Allergy and Infectious Disease, National Institutes of Health, Hamilton, MT 59840, USA

[3]Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD 20892, USA

[4]Department of Biology, New Mexico State University, Las Cruces, NM 88003, USA

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The four major flavivirus clades are transmitted by mosquitoes, ticks, directly between vertebrates or directly between arthropods, respectively, but the molecular determinants of mode of transmission in flaviviruses are unknown. To assess the role of the UTRs in transmission, we generated chimeric genomes in which the 5′ UTR, capsid and/or 3′ UTR of mosquito-borne dengue virus serotype 4 (rDENV-4) were replaced, separately or in combination, with those of tick-borne Langat virus (rLGTV). None of the chimeric genomes yielded detectable virus following transfection. Replacement of the variable region (VR) in the rDENV-4 3′ UTR with that of rLGTV generated virus rDENV-4-rLGTswapVR, which showed lower replication than its wild-type parents in mammalian but not mosquito cells in culture and was able to infect mosquitoes *in vivo*. Neither rDENV-4 nor rDENV-4-rLGTswapVR could infect larval *Ixodes scapularis* ticks immersed in virus, while rLGTV was highly infectious via this route.

The four major clades of the genus *Flavivirus* utilize four distinct modes of transmission (Cook *et al.*, 2009; Farfan-Ale *et al.*, 2009): (i) mosquito transmission between vertebrates (Gould *et al.*, 2003; Gubler, 1998, 2002; Mackenzie *et al.*, 2004), (ii) tick transmission between vertebrates (Gaunt *et al.*, 2001; Gould *et al.*, 2003; Kuno & Chang, 2005), (iii) no known vector (NKV), believed to be directly transmitted between vertebrates (Charlier *et al.*, 2002; Fairbrother & Yuill, 1987; Gould *et al.*, 2003; Johnson, 1967; Leyssen *et al.*, 2002), and (iv) arthropod-specific (AS) viruses which are transmitted directly between mosquitoes (Cammisa-Parks *et al.*, 1992; Cook *et al.*, 2006, 2009; Crabtree *et al.*, 2003; Hoshino *et al.*, 2009; Kent *et al.*, 2010; Kim *et al.*, 2009; Sang *et al.*, 2003) and other arthropods (Moureau *et al.*, 2010) without a vertebrate host. The vector-borne flaviviruses include some of the most significant emerging threats to global health, such as dengue virus (DENV) (Kyle & Harris, 2008), Japanese encephalitis virus (van den Hurk *et al.*, 2009) and tick-borne encephalitis virus (Mansfield *et al.*, 2009), while none of the NKV or AS flaviviruses cause significant disease in humans. Although mode of transmission is intimately linked to disease potential, neither the host nor viral determinants of this trait have been identified (Charlier *et al.*, 2010).

The current study sought to test the impact of specific regions of the flavivirus genome on use of ticks versus mosquitoes as vectors by generating chimeras between mosquito-borne DENV and tick-borne Langat virus (LGTV) (Fig. 1). This task was facilitated by conservation of the organization of the flavivirus genome, which comprises a single segment of positive-sense RNA that encodes a single polyprotein that is co- and post-translationally processed into three structural proteins [capsid (C), pre-membrane (prM) and envelope (E)] and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Chambers *et al.*, 1990; Murray *et al.*, 2008; Padmanabhan & Strongin, 2010). The coding sequence is flanked by 5′ and 3′ UTRs that fold into complex secondary structures (Charlier *et al.*, 2002; Friebe & Harris, 2010; Khromykh *et al.*, 2001; Lodeiro *et al.*, 2009;

†Present address: Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, NM 87131, USA.

‡Present address: Department of Internal Medicine, Section of Infectious Diseases, Yale University School of Medicine, New Haven, CT 06520, USA

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

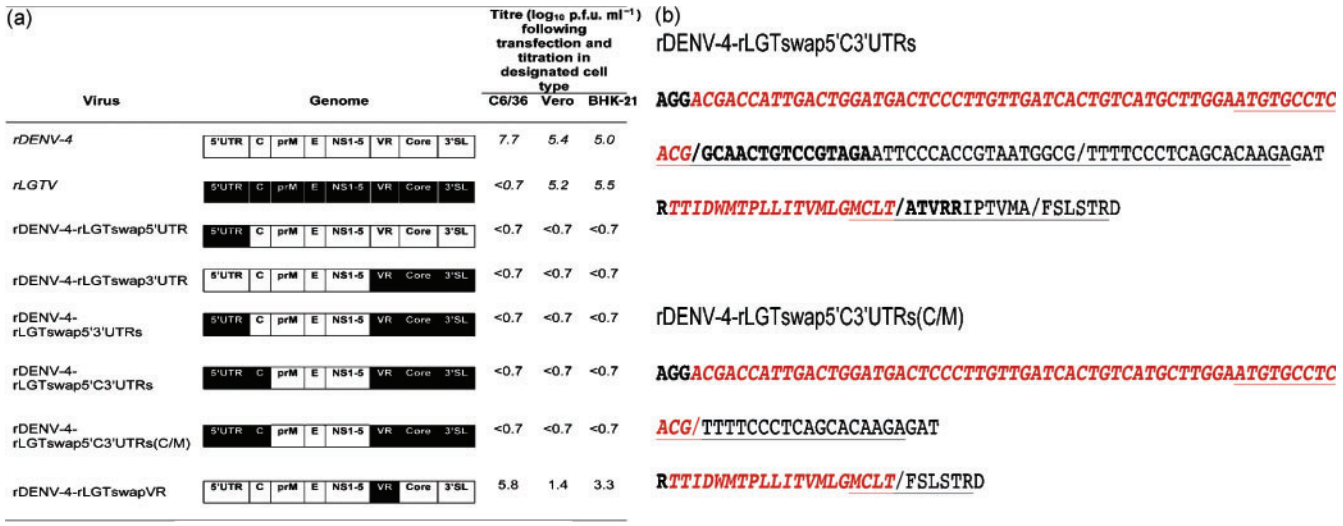

Fig. 1. (a) Titre of each wild-type parent virus (in italics), chimeric virus and mutant virus following transfection into the designated cell type. Titres are the mean of duplicate transfections; the limit of detection for an individual assay is 0.7 log_{10} p.f.u. ml^{-1}. Genomic schematics show rDENV genomic regions in white background with black text, rLGTV genome regions in black background with white text. The 3′ UTR is subdivided into the VR, core region and 3′ SL. (b) Two chimeric genomes carrying the LGTV C gene were generated with alternative cleavage sites: the complete cleavage site from both genomes, shown at the top, and a chimeric cleavage site, shown at the bottom. In the nucleotide and amino acid sequence of each cleavage site, rLGTV sequence is shown in bold, rDENV-4 sequence in plain text, the signalase recognition sequence is underlined, and the signal peptide sequence (C anchor) is in red, italic text.

Proutski *et al.*, 1997a, b; Romero *et al.*, 2006; Thurner *et al.*, 2004). The 3′ UTR consists of the variable region (VR), which is heterogeneous in size and sequence, the core region, which contains conserved secondary structural elements and the 3′ stem–loop (3′ SL) (Gritsun & Gould, 2006; Markoff, 2003).

Clade-specific sequence motifs occur in the flavivirus UTRs (Gritsun & Gould, 2006; Markoff, 2003). To assess the role of the UTRs in determining mode of transmission, we first generated six chimeric genomes (Fig. 1) composed of the coding sequence of recombinant dengue virus serotype 4 (rDENV-4) and one or more of the 5′ UTR, 3′ UTR and C from recombinant tick-borne Langat virus (rLGTV). All constructs were generated by PCR amplification of designated fragments of plasmid pE5, which contains the full-length cDNA of LGTV strain E5 [GenBank accession no. AF253420 (Campbell & Pletnev, 2000)] , using the primers listed in Supplementary Table S1 (available in JGV Online). Fragments were then digested and ligated into plasmid p4, which contains the full-length cDNA of rDENV-4 Dominica strain 814669 [GenBank accession no. AY648301 (Durbin *et al.*, 2001)], using standard cloning techniques. All constructs were validated by sequencing across cloning junctions and the entire insert.

Initially, the rDENV-4 5′ UTR was replaced with that of rLGTV, to produce p4-rLGTswap5′UTR and the rDENV-4 3′ UTR was replaced with that of rLGTV, to produce p4-rLGTswap3′UTR. The two chimeras were then combined to generate p4-rLGTswap5′3′UTRs, in which both the 5′ and 3′ UTRs of rDENV-4 were replaced with those of rLGTV. Plasmid p4 and all chimeric constructs were linearized with *Acc*65I, which generates an authentic terminus of the rDENV-4 3′ UTR but leaves an extra G at the end of the rLGTV 3′ UTR; plasmid pE5 was linearized with *Eco*RV, which generates an authentic terminus of the rLGTV 3′ UTR. mRNAs were synthesized from each linearized plasmid using Amplicap High Yield Message Maker kit (Epicentre Technologies) according to the manufacturer's instructions. Full-length RNA transcripts were purified using the RNeasy kit (Qiagen) and 1 μg of each RNA transcript was transfected in duplicate onto monolayers of African green monkey kidney (Vero), baby hamster kidney (BHK-21) and *Aedes albopictus* (C6/36) cells as described previously (Durbin *et al.*, 2001; Hanley *et al.*, 2002). Five days post-transfection, cell supernatant was collected from each cell type and frozen in 1× SPG (218 mM sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4 and 6 mM L-glutamic acid, pH 7.2) at −80 °C. Titre was determined by plaque titration and immunostaining on the same cell type used for transfection as described previously (Blaney *et al.*, 2001; Durbin *et al.*, 2001). Transfections that produced no detectable virus were repeated three times in each cell type.

Since complementary cyclization sequences critical for replication of rLGTV reside in the 5′ and 3′ UTRs (Khromykh *et al.*, 2001; Kofler *et al.*, 2006; Markoff, 2003), we anticipated that constructs carrying only one of the two

rLGTV UTRs would not be viable, and in fact both failed to yield detectable virus following transfection (Fig. 1). However, even when both UTRs of rDENV-4 were replaced with those of rLGTV, the construct still failed to yield viable virus (Fig. 1). We hypothesized that the UTRs of rLGTV might require a homologous C protein, and we therefore replaced C in the rDENV-4 chimera carrying the rLGTV UTRs with that of rLGTV. Replacement of genes within the coding sequence was complicated by variation in the signalase recognition sequence between rDENV-4 and rLGTV (Chambers *et al.*, 1990). Thus, two constructs were generated, one (p4-rLGTswap5′C3′UTRs) with the intact signalase recognition sequences from both rDENV-4 and rLGTV (Charlier *et al.*, 2004) and the other [p4-rLGTswap5′C3′UTRs(C/M)] in which the rLGTV 5′ UTR-C gene was directly abutted to the prM gene of rDENV-4. Nonetheless both constructs failed to yield viable virus, even after three passages following transfection (Fig. 1).

Chimeric constructs failed to produce viable virus in cells (Vero and BHK-21) that could be transfected by both parental genomes. Thus, the loss of replication in the chimeras is not a product of the failure of the rLGTV UTRs to interact with a necessary cellular protein, but rather a failure to interact properly with the chimeric genome or with an rDENV-4 protein. One obvious candidate is the 14–22 aa signal peptide (SP) located between the C terminus of the capsid protein and the signalase cleavage site. Previous studies have shown that chimeric DENV genomes containing the prM-E genes of either West Nile virus (WNV) (Huang *et al.*, 2005; Pletnev *et al.*, 2002) or rLGTV (Pletnev *et al.*, 1992; Pletnev & Men, 1998) required SP and prM from homologous virus for viability.

We next sought to define which of the three regions of the rLGTV 3′ UTR mediated its incompatibility with the rDENV-4 coding region. We have previously shown that sequences from the core region of rDENV-4 can be replaced with homologous sequences from rLGTV without loss of viability (Romero *et al.*, 2006). In contrast, exchange of the 3′ SL between WNV and DENV2, which share the same mode of transmission, abolishes viability (Elghonemy *et al.*, 2005; Yu & Markoff, 2005; Yu *et al.*, 2008; Zeng *et al.*, 1998). We therefore focused on the VR, and generated a rDENV-4 genome in which the VR was replaced with that of rLGTV. This construct yielded viable virus in C6/36, Vero and BHK-21 cells. Titre of rDENV-4-rLGTswapVR was at least 50-fold lower than either wild-type parent following transfection in all three cell types, except that rLGTV does not replicate in mosquito cells (Fig. 1). The finding that mutant genomes carrying the VR were viable indicates that this region was not responsible for the observed loss of viability in the chimeric genomes and identifies the SL as the critical region for intragenomic interactions.

Clonal populations of rDENV-4-rLGTswapVR and its two parental viruses were purified by terminal dilution in Vero cells as described previously (Blaney *et al.*, 2001) and working pools were prepared by amplifying terminally diluted viruses in all three cell types, except that rLGTV does not replicate in C6/36 cells. To determine whether compensatory mutations may have occurred in rDENV-4-rLGTswapVR during rescue of this virus, the 5′ UTR, C and 3′ UTR were sequenced using standard methods. No mutations from the sequence of cDNA were detected.

Plaque size of rDENV-4, rDENV-4-rLGTswapVR and rLGTV stocks derived from C6/36, Vero and BHK-21 cells were measured as described previously (Blaney *et al.*, 2003) in C6/36, Vero and BHK-21 cell monolayers, respectively. Mean plaque size of rDENV-4-rLGTswapVR did not differ

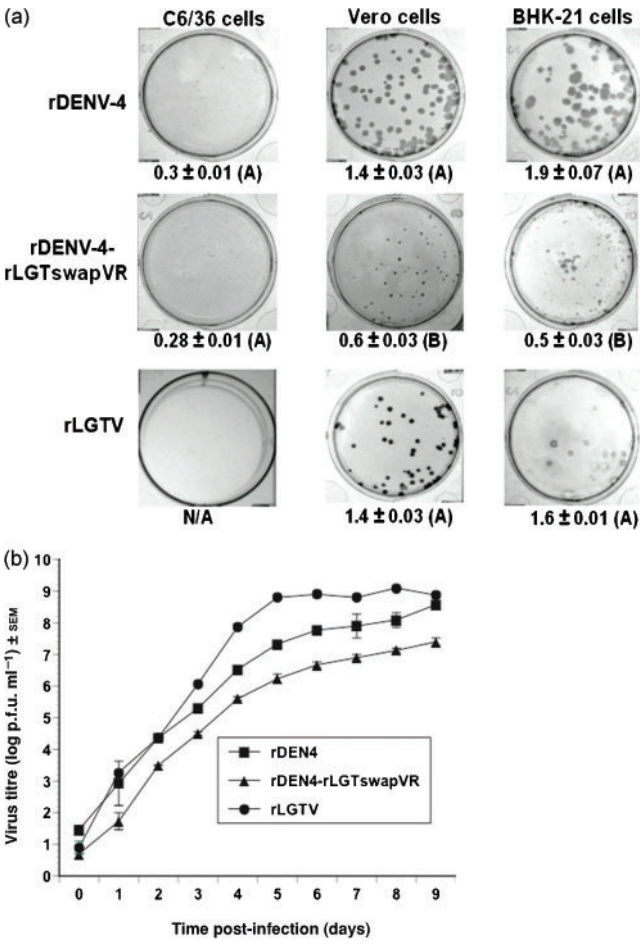

Fig. 2. (a) The mean plaque diameter (mm) of 36 randomly selected plaques of designated viruses in designated cells. For each cell type, viruses that share the same letters (in parentheses) do not differ significantly from each other, while those with different letters have significantly different plaque sizes (Tukey–Kramer post-hoc test, $P<0.05$). (b) Multicycle replication kinetics of rDENV-4, rDENV-4-rLGTswapVR and rLGTV in Vero cells. Values at each time point for each virus represent means from triplicate infections.

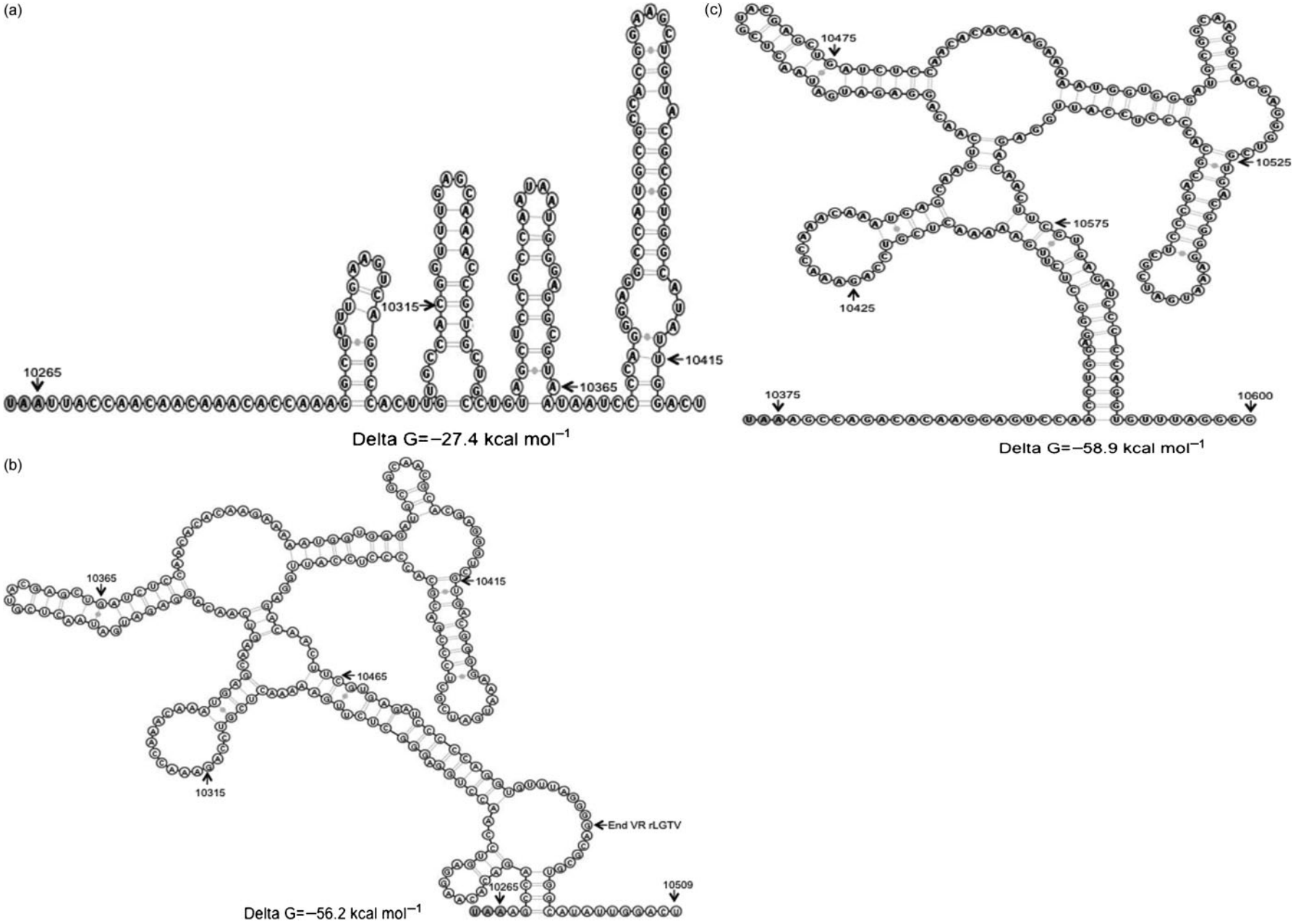

Fig. 3. MFOLD-predicted secondary structures of (a) the VR of the rDENV-4 3′ UTR. Grey-filled circles indicate the stop codon; wobble base pairs are indicated in grey dots. Nucleotides U10310, C10328 and C10398 were constrained to be single-stranded based on results of nuclease mapping. (b) The VR of rDENV-4-rLGTswapVR; G10398 and C10403 were constrained to be single-stranded based on results from nuclease mapping. (c) The VR of rLGTV; structure was not constrained.

significantly from rDENV-4 in C6/36 cells (Fig. 2a). However, in both mammalian cell lines rDENV-4-rLGTswapVR produced significantly smaller plaque sizes than either parent, which did not differ from each other (Fig. 2a). To assess replication kinetics, 80 % confluent monolayers of Vero cells in 25 cm^2 tissue culture flasks were infected in triplicate with either rDENV-4, rLGTV or rDENV-4-rLGTswapVR stocks derived from Vero cells at an m.o.i. of 0.01 as described previously (Blaney *et al.*, 2003; Hanley *et al.*, 2003). One millilitre of cell supernatant was collected daily for 9 days and flasks were refed with 1 ml fresh medium. Virus titre from each supernatant was determined in Vero cells in a single assay as described above. rDENV-4-rLGTswapVR replicated to a significantly lower titre over 10 days than rDENV-4 in Vero cells, which in turn replicated to a lower titre than rLGTV although both achieved a similar peak at day 9 (Fig. 2b); a Tukey–Kramer post-hoc test showed that replication dynamics of all viruses differed significantly from one another ($P<0.05$). These results are consistent with previous studies in DENV documenting that deletions and other mutations in the VR compromise DENV replication in mammalian cells but not mosquito cells (Alvarez *et al.*, 2005; Men *et al.*, 1996; Tajima *et al.*, 2007). This intriguing host-specific effect of the VR merits further study.

The function of the VR is determined not only by primary sequence but by secondary structure. At 136 nt, the VR of rDENV-4 is shorter than the 225 nt VR of rLGTV, with 66 % identity between the two sequences. To gain insight into the impact of replacing the VR on the secondary structure of the rDENV-4 3′ UTR, structures of the VR of rDENV-4 (Fig. 3a) and rDENV-4-rLGTswapVR (Fig. 3b) were generated by MFOLD (Mathews *et al.*, 1999; Zuker, 2003) and constrained by a nuclease map of the same region (data not shown), generated as described previously (Romero *et al.*, 2006). These structures show that the rDENV-4 VR folds into SL configurations that are less complex than those of the rLGTV VR. Despite repeated attempts, nuclease mapping of rLGTV 3′ UTR was not successful. Nonetheless, a structure of the VR of rLGTV generated by MFOLD (Fig. 3c), suggests that insertion of the rLGTV VR into the rDENV-4 UTR did not greatly affect the overall folding of this region.

To assess the role of the VR in vector infection, both *Aedes aegypti* mosquitoes and *Ixodes scapularis* ticks were exposed to rDENV-4-rLGTswapVR and its two parental viruses, all derived from Vero cells. *Ae. aegypti* were maintained on sucrose and fed on bloodmeals containing designated quantities of virus as described previously (Troyer *et al.*, 2001), incubated for 14 days and frozen. Viral titre in body and head homogenates from mosquito bodies that tested positive for virus was determined in Vero cells as described previously (Hanley *et al.*, 2008). As expected (Pletnev *et al.*, 2001), rLGTV did not infect 15 *Ae. aegypti* that fed on bloodmeal containing 7.5 $\log_{10}$ p.f.u. ml^{-1} LGTV. In contrast, of 16 mosquitoes fed on 6.0 $\log_{10}$ p.f.u. ml^{-1} rDENV-4, two (12.5 %) showed detectable infection in the body and one of these infections (50 % of total infections) disseminated to the head. Similarly, of 31 mosquitoes fed on 5.5 $\log_{10}$ p.f.u. ml^{-1} rDENV-4-rLGTswapVR, the highest titre we were able to obtain, one (3.2 %) showed infection in the body and this one infection disseminated (100 % of total infections). Thus, although overall infection was low, rDENV-4-rLGTswapVR was clearly able to infect and disseminate in *Ae. aegypti*.

I. scapularis adult females with egg sacs (Oklahoma State University, Stillwater, OK) were housed in a relative humidity of 98 % with a 16 : 8 h light : dark cycle for oviposition and larvae were used within 6 months of emergence. Groups of 60 larvae were exposed to designated viruses by immersion as described previously (Mitzel *et al.*, 2007). Infection of ticks was detected using RT-PCR; cultured Vero and *I. scapularis* tick cells (ISE6) (Munderloh *et al.*, 1994) cells infected with rDENV-4 and rLGTV, respectively, were used as positive controls while uninfected cells were used as negative controls. Control cells were lysed in 350 µl Buffer RLT (Qiagen) and homogenized by passing through a QIAshredder spin column (Qiagen). Total RNA was isolated using the RNeasy Mini kit (Qiagen). RNA isolation continued as per manufacturer's instructions and the RNA was eluted in 50 µl RNase-free water. To isolate total RNA from ticks, a group of 25 ticks were frozen in liquid nitrogen and then transferred to Lysing Matrix D tubes (MP Biomedicals) containing 800 µl RLT buffer (Qiagen RNeasy kit). The ticks were homogenized using the Fastprep 24 (MP Biomedicals) set at speed level 6 m s^{-1} for 40 s. The homogenate was cleared by centrifugation at 21 000 ***g*** for 3 min and the RNA from the homogenates was purified using Qiagen's RNeasy Mini kit. Viral RNA was detected with RT-PCR using the primers listed in Supplementary Table S1. As expected, ticks exposed to rLGTV became infected (Supplementary Fig. S1, available in JGV Online). In contrast exposure of ticks to rDENV-4 or rDENV-4-rLGTswapVR did not result in infection (Supplementary Fig. S1).

Thus, rDENV-4-rLGTswapVR shared the vector specificity of its rDENV-4 parent, and the VR did not affect mode of transmission. Whether the remaining regions of the UTRs determine vector specificity is still an open question. Given that the evidence to date suggests that the UTRs cannot be exchanged between tick-borne and mosquito-borne flaviviruses, future studies of this question may need to rely on fine-scale mutagenesis.

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