Short Communication

Replacement of conserved or variable sequences of the mosquito-borne dengue virus 3′ UTR with homologous sequences from Modoc virus does not change infectivity for mosquitoes

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The genus *Flavivirus* includes both vector-borne and no known vector (NKV) species, but the molecular determinants of transmission mode are not known. Conserved sequence differences between the two groups occur in 5′ and 3′ UTRs. To investigate the impact of these differences on transmission, chimeric genomes were generated, in which UTRs, UTRs + capsid, or the upper 3′ UTR stem–loop of mosquito-borne dengue virus (DENV) were replaced with homologous regions from NKV Modoc virus (MODV); the conserved pentanucleotide sequence (CPS) was also deleted from the DENV genome. Virus was not recovered following transfection of these genomes in three different cell types. However, DENV genomes in which the CPS or variable region (VR) of the 3′ UTR were replaced with MODV sequences were recovered and infected *Aedes aegypti* mosquitoes with similar efficiencies to DENV. These results demonstrate that neither vector-borne CPS nor VR is required for vector-borne transmission.

The genus *Flavivirus* includes both vector-borne viruses and those with no known vector (NKV) (Billoir *et al.*, 2000; Chambers *et al.*, 1990; Cook & Holmes, 2006; Gaunt *et al.*, 2001; Gritsun & Gould, 2006; Markoff, 2003). Attempts to infect ticks and mosquitoes as cultured cell lines or *in vivo* with NKV viruses have been unsuccessful, suggesting that this group is transmitted horizontally among vertebrates (Charlier *et al.*, 2010; Fairbrother & Yuill, 1987; Hendricks *et al.*, 1983; Johnson, 1967; Kuno, 2007; Lawrie *et al.*, 2004). Vector-borne flaviviruses include important human pathogens such as dengue virus (DENV) (serotypes 1–4), yellow fever virus (YFV), tick-borne encephalitis virus (TBEV) and West Nile virus (WNV). Despite striking variation in the mechanisms of transmission within a single genus, the molecular determinants of transmission mode in the flaviviruses have not yet been identified.

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Two supplementary tables are available with the online version of this paper.

Comparison of vector-borne and NKV flavivivirus genomes is facilitated by conservation of genome size, proteolytic cleavage site location and genome organization. Flaviviruses are positive-sense ssRNA viruses with a genome size of about 11 kb. The genome codes for a single ORF, which is processed by both cellular and viral proteases into three structural proteins and at least seven non-structural proteins (Chambers *et al.*, 1990; Leyssen *et al.*, 2002; Rice, 1996). The ORF is flanked by 5′ and the 3′ UTRs; both UTRs fold into complex stem–loop structures required for replication.

Charlier *et al.* (2010) investigated the role of the structural proteins in flavivirus infection of arthropod cells by generating chimeric genomes composed of the genomic backbone of one of two mosquito-borne flaviviruses, YFV or DENV, in which the structural genes were replaced with those of NKV Modoc virus (MODV). Transfection of mosquito C6/36 cells with the parent YFV or DENV genomes or the chimeric genomes yielded viable viruses, whereas MODV was incapable of infecting mosquito cells. Similarly Engel *et al.* (2011) have demonstrated that a

chimeric DENV genome carrying the structural genes from TBEV retained the ability to infect mosquito cells in culture. Moreover, this chimeric construct failed to infect tick cells or larvae. These results suggest that the ability of a flavivirus to infect an arthropod vector is not determined by its structural proteins.

A sequence comparison suggests that key determinants of the transmission mode may reside in the UTRs of the flavivirus genome. Differences in conserved sequence motifs between vector-borne and NKV flaviviruses have been identified in the highly conserved 3′ stem–loop (3′ SL), the core region and the variable region (VR) of the 3′ UTR. For example, the conserved pentanucleotide sequence (CPS) CACAG located at the top of the 3′ SL is conserved in all vector-borne flaviviruses but differs from the most common sequence (CUCAG) found in NKV flaviviruses (Charlier *et al.*, 2002; Leyssen *et al.*, 2002; Markoff, 2003). Previous studies using WNV replicons have shown that replacement mutations in any but the fourth nucleotide position of the CPS abolishes replication (Tilgner *et al.*, 2005). However, in other studies using WNV and YFV full-length genomic RNA, individual mutations of either the second, third or fourth CPS nucleotide were tolerated (Elghonemy *et al.*, 2005; Silva *et al.*, 2007). Some of these mutations mimicked the CPS of NKV flaviviruses but their impact on replication was tested only in mammalian cell lines. Within the VR, NKV flaviviruses have a conserved motif of 22 nt which is not present in vector-borne flaviviruses (Charlier *et al.*, 2002). Finally, NKV and vector-borne flaviviruses each possess unique and complementary conserved sequences that circularize the genome during replication (Alvarez *et al.*, 2005; Khromykh *et al.*, 2001; Kofler *et al.*, 2006). In order to investigate whether these conserved motifs in the UTRs dictate the mode of transmission, we generated chimeric constructs in which designated regions of the mosquito-borne DENV-4 genome were replaced with homologous regions of NKV MODV.

We replaced the 3′ UTR, alone or in combination with the 5′ UTR and/or capsid (C), of a full-length DENV-4 infectious clone with that of NKV MODV (Table S1, available in JGV Online). The 5′ UTR, C and 3′ UTR of MODV, synthesized by Blue Heron Biotechnology, were digested and ligated to plasmid p4, which contains the full-length cDNA genome of DENV-4 Dominica strain 814669 (GenBank accession no. AY648301) (Durbin *et al.*, 2001). All plasmid constructs were sequenced up to and across cloning junctions to verify the authenticity of the replacement mutations.

First, we replaced the 5′ and 3′ UTRs of DENV-4 singly with those of MODV to generate p4-MODswap5′ UTR and p4MODswap3′ UTR, and then we replaced both DENV-4 UTRs together to generate p4-MODswap5′3′UTRs (Table S1). All constructs were linearized with *Acc65I*, which generates an authentic terminus of the rDENV-4 3′ UTR but leaves an extra G at the end of the MODV 3′ UTR. Capped 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 RNeasy kit (Qiagen) and transfection was conducted in duplicate in *Aedes albopictus* mosquito C6/36 cells, African green monkey kidney Vero cells or baby hamster kidney BHK-21 cells, essentially as previously described (Durbin *et al.*, 2001; Hanley *et al.*, 2002). MODV strain M544 (GenBank accession no. NC_003635), obtained from the World Arbovirus Reference Center, was passaged four times in Vero cells. Titre was determined by serial dilution and immunostaining (Durbin *et al.*, 2001) on the same cell types as used for transfection; hyperimmune mouse ascites fluids against DENV-4 and MODV were used to detect recombinant DENV-4 (rDENV-4) and MODV, respectively. From a single C6/36 transfection pool of each virus, separate clones were isolated by terminal dilution as previously described (Blaney *et al.*, 2001) in C6/36, Vero or BHK cells and working pools were prepared by amplifying terminally diluted viruses in the same cell type in which they were terminally diluted, yielding C6/36, BHK and Vero stocks of each virus.

As expected, wild-type rDENV-4 was recovered in all three cell lines, while MODV infected only the two mammalian cell lines (Charlier *et al.*, 2010) (Table S1). No detectable virus was recovered from any of the chimeric genomes in any of the three cell types (Table S1). We hypothesized that the failure of these genomes to produce viable virus may have resulted from: (i) a mismatch in hypothetical packaging sequences in the UTRs and recognition sites in the C protein (Mandl *et al.*, 1998; Men *et al.*, 1996; Proutski *et al.*, 1997) or (ii) a lack of a complementarity of the 5′ MODV cyclization sequence (5′ **AUG**UGCGAAAAUAACAGG 3′, which extends into C) with the 3′ UTR cyclization sequence (3′ UUAAACCUUUUAUUGUCC 5′) (Leyssen *et al.*, 2002) (the start codon is in bold, the C is in grey and base-paired nucleotides are not underlined).

To test these hypotheses, we next replaced the C gene in the rDENV-4-MODswap5′3′UTRs chimera with that of MODV, thus generating rDENV-4-MODswap5′C3′UTRs, which carried the complete C (including the circularization sequence above) and anchored C (C_{anch}) sequence of MODV as well as complete C/pre-membrane (prM) signalase cleavage sites from both MODV and rDENV4 (Charlier *et al.*, 2004). This construct also yielded no detectable virus following transfection. Finally, since previous research on chimeric flaviviruses (Huang *et al.*, 2005; Pletnev *et al.*, 2002) has suggested that homology between C_{anch}, which helps to translocate the prM into the endoplasmic reticulum for post-translational maturation, and prM sequences may be critical for viability, we generated rDENV-4-MODswap5′C3′UTRs-dAn, which contained the C_{anch} and the C/prM signallase cleavage site from rDENV-4. This construct also failed to generate viable virus (Table S1). Transfections that produced no detectable virus were repeated three times and all but MODswap5′C3′UTRs-dAn were further attempted by

Table 1. Mutations in wild-type (rDENV-4) and mutant viruses derived from a single transfection in C6/36 cells followed by separate passage in Vero or C6/36 cells

Nucleotide and amino acid sequence numbering is from the start of the genome and the polyprotein, respectively. NA, Not applicable; Y, yes.

Virus	Cell type	Nucleotide mutation	Amino acid mutation	Gene	Replacement of designated DENV sequence with MODV sequence confirmed?
rDENV-4	Vero	None	–	–	NA
rDENV-4-MODswapCPS	Vero	A4560C	T1487P	NS3	Y
rDENV-4-MODswapVR	Vero	G10023A	D3308N	NS5	Y
rDENV-4	C6/36	None	–	–	NA
rDENV-4-MODswapCPS	C6/36	A4560C	T1487P	NS3	Y
rDENV-4-MODswapVR	C6/36	A7485C	N2462H	NS4B	Y
		A8342T	Silent	NS5	Y
		G10023A	D3308N	NS5	Y

electroporation. In no case did a construct that failed to produce detectable virus following transfection produce any detectable virus following electroporation.

In previous work we have shown that chimeric genomes in which the 5′ UTR, C and 3′ UTR of rDENV-4 were replaced with those of tick-borne Langat virus (LGTV) also failed to generate viable virus (Tumban *et al.*, 2011). However numerous studies have shown that structural genes of DENV can be replaced with those of either tick-borne (Lai & Monath, 2003) or NKV viruses (Charlier *et al.*, 2010). Together with the current study, these results point to a fundamental incompatibility between the UTRs of NKV and tick-borne flaviviruses with the replication complex of mosquito-borne flaviviruses. The flavivirus replication complex includes NS1, NS2A, NS3, NS4A, NS4B, NS5, translation elongation factor 1α, La protein and polypyrimidine track-binding protein (Blackwell & Brinton, 1995; De Nova-Ocampo *et al.*, 2002; Lai, 1998; Mackenzie *et al.*, 1998; Umareddy *et al.*, 2006). This replication complex interacts extensively with the 3′ UTR; for instance, NS3 and NS5 in association with NS2A have been shown to bind to the 3′ SL of WNV (Chen *et al.*, 1997; Mackenzie *et al.*, 1998). In addition, translation elongation factor 1α, La protein and polypyrimidine track-binding protein have been shown to bind to both the core region and the 3′ SL of flaviviruses (De Nova-Ocampo *et al.*, 2002). More recently, host proteins such as DDX6, G3BP1 and Caprin1 have been shown to bind to the UTRs of DENV-2 (Ward *et al.*, 2011). Thus, we propose that the failure of rDENV4-MODswap5′C3′UTR and rDENV4-MODswap5′C3′UTR-dAn to produce viable virus may have resulted from the inability of the rDENV-4 replication complex, in association with host proteins, to recognize the UTRs of MODV.

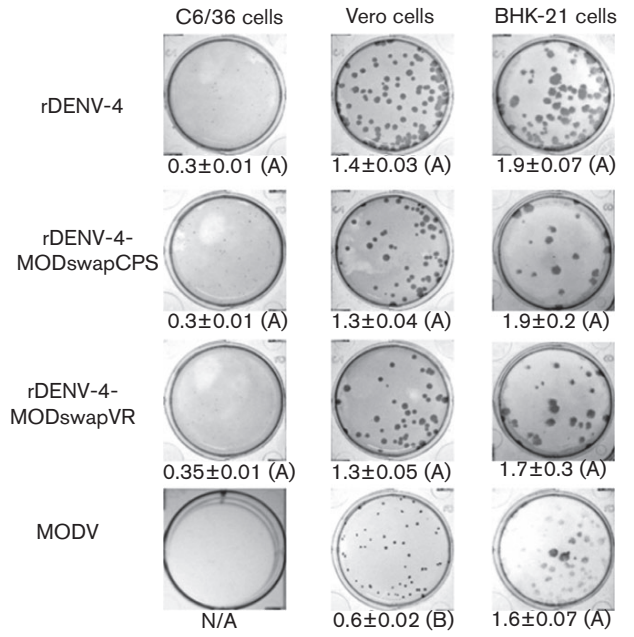

Fig. 1. Plaque sizes of rDENV-4, rDENV-4-MODswapCPS, rDENV-4-MODswapVR and MODV in C6/36, Vero and BHK-21 cells. The mean plaque diameter ± SEM (mm) of 36 randomly selected plaques is shown below each well. For each cell type, viruses that share the same letters (in parentheses) do not differ significantly from each other, while those with different letters have significantly different plaque sizes (Tukey–Kramer post-hoc test, $P<0.05$).

To better define which regions of the UTR may mediate compatibility with the replication complex, and to assess the impact of specific sequence motifs on the ability of DENV to infect mosquitoes, we generated replacement and deletion mutations (by standard PCR mutagenesis using the primers listed in Table S2) in both the 3′ SL and the VR. Consistent with previous studies (Silva *et al.*, 2007), rDENV-4-ΔCPS, in which the CPS (CACAG, located at the top of 3′ SL) was deleted, did not yield detectable viruses in any of the three cell types (Table S1). rDENV-4-MODswapU3′SL containing a replacement of the upper 3′ SL of rDENV-4 with that of MODV also did not yield detectable viruses in any of the three cell types (Table S1). However, both rDENV-4-MODswapCPS (with an A⟶U

Table 2. Infection and dissemination in *A. aegypti* that were fed bloodmeals containing indicated virus

Virus	Derived from	Titre of bloodmeal (log_{10} p.f.u. ml^{-1})	Infection (body)			Dissemination (head)*		
			No.	No. infected (%)	Mean virus titre ± SEM (log_{10} p.f.u. per body)†	No.	No. infected (%)	Mean virus titre ± SEM (log_{10} p.f.u. per head)†
rDENV-4	Vero	7.0	21	10 (47.0)	3.7 ± 0.2	10	8 (80.0)	2.6 ± 0.3
rDENV-4	Vero	6.0	16	2 (12.5)	2.9 ± 0.6	2	1 (50.0)	1.5 ± 0
rDENV-4-MODswapCPS	Vero	6.5	27	3 (11.0)	3.6 ± 0.3	3	3 (100.0)	2.2 ± 0.2
rDENV-4-MODswapVR	Vero	6.6	25	3 (12.0)	3.5 ± 0.1	3	1 (33.0)	2.4 ± 0
MODV	Vero	6.9	20	0	0	–	–	–
rDENV-4	C6/36	8.3	13	9 (69.2)	3.6 ± 0.3	9	7 (77.8)	2.8 ± 0.4
rDENV-4-MODswapCPS	C6/36	7.8	18	7 (38.8)	3.1 ± 0.4	7	3 (42.9)	2.3 ± 0.3
rDENV-4-MODswapVR	C6/36	7.9	14	8 (57.1)	4.0 ± 0.2	8	8 (100)	3.5 ± 0.2

*Only heads from infected bodies were screened.
†Titres from infected samples only.

mutation in the second nucleotide of the CPS) and rDENV-4-MODswapVR, in which the VR of rDENV-4 was replaced with that of MODV, yielded viable viruses following transfection in C6/36, Vero and BHK-21 cells (Table S1). Both constructs had post-transfection titres that were at least 1.3-fold and 100-fold lower than that of rDENV-4 in BHK-21 and Vero cells, respectively; both replicated to similar titres to their rDENV-4 parent in C6/36 cells.

Complete genomes of Vero and C6/36 stocks of rDENV-4, rDENV-4-MODswapCPS and rDENV-4-MODswapVR were sequenced (Table 1); of these, neither rDENV-4 stock possessed any mutations, both rDENV-4-MODswapCPS stocks possessed the same T1487P mutation in NS3, and both rDENV-4-MODswapVR stocks possessed a common D3308N mutation in NS5, although the C6/36-derived rDENV-4-MODswapVR stock also acquired one additional coding and one silent mutation in NS5. Common mutations probably arose during initial transfection in C6/36 cells but were not lost during terminal dilution and passage in the two different cell lines. As these mutations may represent compensatory mutations, it is not possible to unambiguously attribute the phenotypes of the mutant viruses to the MODV or VR replacements alone. However, it is possible to conclude that any phenotypes manifested by the mutant viruses were possible, despite the loss of specific DENV sequences.

The size of 36 randomly chosen plaques (Blaney *et al.*, 2003; Hanley *et al.*, 2008) of Vero stocks of rDENV-4-MODswapCPS and rDENV-4-MODswapVR were compared with those of parent viruses in all three cell lines (Fig. 1). While MODV does not replicate in C6/36 cells, the mean plaque size of the two mutant viruses were similar to that of their rDENV-4 parent in this cell type. In mammalian cells, the mean plaque sizes of the two mutant viruses were similar to that of their rDENV-4 parent in both cell lines and were larger than those of MODV in Vero but not in BHK-21 cells.

The phenotype of rDENV-4-MODswapVR is consistent with some previous studies showing that deletion of the VR of DENV and other flaviviruses did not affect viral replication *in vitro* or *in vivo* (Mandl *et al.*, 1998; Tajima *et al.*, 2006) but contrasts with studies, including a study that we conducted on the same rDENV-4 backbone in which the DENV VR was replaced with that of LGTV (Tumban *et al.*, 2011), reporting that deletion or replacement of the VR decreased replication in mammalian cells but not in insect cells (Alvarez *et al.*, 2005; Men *et al.*, 1996; Pankhong *et al.*, 2009; Tajima *et al.*, 2007). These apparent discrepancies in the effect of the VR may stem from variation in the size of the deletions/replacements used in these studies. For example, the length of the VR replaced in our previous study (Tumban *et al.*, 2011) was almost twice the size of the VR replaced in this study (225 versus 122 nt), which may explain the difference in results of VR replacement.

The ability of each of the mutant viruses to infect mosquitoes was compared with that of their two parental

viruses by feeding *Aedes aegypti*, following previously described methods (Troyer *et al.*, 2001), with bloodmeals containing 6.0–7.0 $\log_{10}$ p.f.u. ml^{-1} of Vero stocks of rDENV-4, MODV, rDENV-4-MODswapVR and rDENV-4-MODswapCPS, as well as C6/36 stocks of rDENV-4, rDENV-4-MODswapVR and rDENV-4-MODswapCPS. As expected, MODV did not infect any *A. aegypti* and both stocks of rDENV-4-infected *A. aegypti* (Table 2). At bloodmeal titres of approximately 6.5 $\log_{10}$ p.f.u. ml^{-1}, Vero-derived rDENV-4-MODswapCPS and rDENV4-MODswapVR infected significantly fewer mosquitoes than rDENV-4 at 7.0 $\log_{10}$ p.f.u. ml^{-1} (Fisher's exact test, $P<0.05$ for both comparisons) but infected a similar percentage compared to rDENV-4 at 6.0 $\log_{10}$ p.f.u. ml^{-1} (Fisher's exact test, $P>0.05$ for both comparisons). Moreover, both mutant viruses disseminated with similar efficiency to that of rDENV-4 (statistical analysis not performed due to small sample sizes). The mean titre of both mutant viruses in the bodies of infected mosquitoes was similar to each other and to that of rDENV-4 at a bloodmeal titre of 7.0 $\log_{10}$ p.f.u. ml^{-1} (ANOVA, df=2, 18, F=1.0, $P=0.39$). Similar patterns were observed for C6/36-derived viruses: neither the per cent of bodies infected (contingency table analysis, Chi-squared=2.96, $P=0.23$) nor the body titre (ANOVA, F=2.39, $P=0.11$) differed among rDENV-4, rDENV-4-MODswapVR and rDENV-4-MODswapCPS. Both rDENV-4-MODswapVR (Fisher's exact test, $P=0.47$) and rDENV-4-MODswapCPS (Fisher's exact test, $P=0.30$) disseminated with similar efficiency as rDENV-4 and head titres were similar among all three viruses (ANOVA, F=2.76, $P=0.10$).

The threat from vector-borne flaviviruses is increasing as the efficacy of traditional vector control measures wane (Gubler, 2006; Mackenzie *et al.*, 2004). The development of a licensed live virus vaccine is challenging, in part, because vector transmission from vaccinees to unvaccinated populations must be prevented. Identifying regions in the genomes of flaviviruses that determine vector transmission can serve as a basis for the design of live attenuated vaccine strains that lack the capacity for vector transmission. The current study has demonstrated that neither the VR nor the CPS of mosquito-borne DENV is necessary for transmission by mosquitoes.

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