The four dengue virus (DENV) serotypes are mosquito-borne members of the genus Flavivirus (family Flaviviridae) and are endemic in the tropical and subtropical regions of the world. Transmission to humans is by peridomestic Aedes spp. mosquitoes, primarily Aedes aegypti. Manifestations of a DENV infection range from subclinical to a self-limited fever and rash [dengue fever (DF)] to a severe and sometimes deadly illness characterized by capillary leakage, thrombocytopenia and hypovolaemic shock [dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS)]. Taken together, these viruses are estimated each year to cause 100 million cases of DF, 500,000 cases of DHF/DSS and 25,000 deaths, and 40% of the world population (i.e. 2.5 billion people) is at risk of infection (Month, 1994).

Epidemiological studies have identified dengue virus type 2 (DENV-2) as the most important dengue virus serotype worldwide. The DENV-2 genome is a single-stranded, positive-sense RNA molecule of approximately 10,700 nt that contains a type I 5′-cap structure, but lacks a 3′ poly(A) tail. The genome is translated directly as a single polyprotein containing the three structural genes (NH2–Capsid–PrM–Envelope) fused to the seven non-structural genes (NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5–COOH) that is processed co- and post-translationally (Chambers et al., 1990).

Molecular analysis of the envelope gene from DENV-2 field isolates originally identified five major DENV-2 genotypes: Caribbean (American), South Pacific, Middle East and Indonesia, Vietnam and Thailand (Asian), and West African (Lewis et al., 1993; Rico-Hesse, 1990; Rico-Hesse et al., 1997; Wang et al., 2000). Additional analysis of more DENV-2 samples has revealed a total of six DENV-2 genotypes: American, American/Asian, Asian I, Asian II, Sylvatic and Cosmopolitan (Twiddy et al., 2002). The new DENV-2 American/Asian genotype, which includes the DENV-2 Jamaican 1409 isolate (1409), is an Asian genotype DENV-2 introduced into the Americas, with subsequent proliferation and circulation of the virus in this region (Loron˜o-Pino et al., 2004; Twiddy et al., 2002; Uzcategui et al., 2001). Introduction of viruses from this genotype, of which 1409 is speculated to be an early isolate, was linked to a significant increase in the number of DHF cases across Latin America (Uzcategui et al., 2001). DENV-2 of the Asian and American/Asian genotypes infects North American A. aegypti mosquitoes more readily than do the DENV-2 American genotypes (Armstrong & Rico-Hesse, 2001). The American/Asian genotype viruses are also believed to have supplanted the American genotype in many areas, leaving a more virulent and readily transmitted DENV-2 in its place.

The 1409 virus was originally isolated in 1983 from a human DF patient in Jamaica. The virus was previously plaque-purified on LLC-MK2 cells, stocks were prepared in C6/36 cells and the genome was sequenced (Deubel et al., 1986, 1988). For our studies, both a low-passage (C6/36-passage 2) 1409 virus (1409lp), obtained from the Centers for Disease Control and Prevention [Fort Collins, CO, USA (CDC)], and high-passage 1409 virus (1409hp), which has been passaged...
routinely (> 25 passages) in C6/36 cells, were used (Bennett et al., 2002).

For genome sequencing and infectious clone (ic) construction, five cDNA fragments (f1–f5) representing the entire 1409hp genome were generated by RT-PCR (Titan RT-PCR kit; Roche Biochemicals), each of the fragments was inserted into a modified pBR322 plasmid (Kinney et al., 1997) and the five plasmids (p1–p5; Fig. 1a) were transfected by electroporation into competent *Escherichia coli* XL-1 Blue cells (Stratagene). Three individual bacterial colonies for each cloned fragment were cultured, plasmids were isolated and sequenced and the data were aligned against previously published DENV-2 1409 (1409pub) data (GenBank accession no. M20558). A significant nucleotide change in the genome between 1409hp and the 1409pub sequences was identified as a nucleotide substitution that occurred at the same position in all three colonies. In no cases were variations found in only two of three colonies. Nucleotide substitutions that occurred in only one of three colonies are not reported here.

Thirteen significant nucleotide differences between 1409hp and 1409pub were detected (Table 1). Genomic 1409lp was then sequenced at these specific positions to corroborate the published sequence, and seven nucleotide variations between the 1409pub sequence and the 1409lp were subsequently identified: 3934–3935, 7086–7087, 8241–8242 and 10089 (Table 1). Nucleotide substitutions specific to our 1409hp laboratory strain and the 1409lp strain occurred at six positions: 954, 1101, 4107, 4596, 8562 and 10403. This resulted in only one amino acid change, located at envelope protein position 6 (E6) (Ile→Met), and one untranslated region (UTR) change, located at 3' UTR-143 (T→G), between 1409lp and 1409hp (Table 1). As our goal was to produce an ic representing the early 1409 isolate, we included these two 1409lp sequence sites in our ic. Thus, the 1409ic has an Ile at E6 and a thymine at 3' UTR-143. Engineering of the clone also required an intentional silent nucleotide substitution of t/6651/c in the 1409ic genome via site-directed mutagenesis PCR to produce an Nhel restriction site used in full-length clone construction. Additionally, unique PCR primers attached the T7 RNA polymerase recognition sequence and the linearization SphI restriction site, each of which are required for the A-cap analogue-dependent *in vitro* transcription of the clone (Fig. 1b).

For ic construction, subclones p1–2–3 (nt 1–6650) and p4–5 (6651–10724) were first constructed (Fig. 1a). Next, f4–5 (extracted from p4–5) was ligated to p1–2–3 to generate p1–2–3–4–5 (i.e. p1409ic). Unfortunately, a pBR322 plasmid containing the full-length 1409 cDNA was never recovered from electroporated (BTX ECM 630; 2–5 kV, 25 μF and 200 Ω) XL-1 Blue *E. coli* cells. Attempts to modify growth temperature, medium type, vector construction and *E. coli*
Table 1. Nucleotide and amino acid variation among DENV-2 1409 genomes

Bold type represents nucleotides or amino acids unique to the previously published sequence (GenBank accession no. M20558). –, 3’ UTR.

<table>
<thead>
<tr>
<th>Genome nucleotide position</th>
<th>Nucleotide</th>
<th>Amino acid</th>
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<tr>
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*pub, lp, hp and ic represent the DEN2 published, low-passage, high-passage and infectious clone residues, respectively.
†Unique sequence identifier for DEN2 1409ic.

cell type all failed to yield full-length p1409ic. We observed that transformants containing either p1–2–3 or p4–5 produced a small colony size in E. coli host cells on standard YT plates [8 g tryptone, 5 g yeast, 5 g NaCl, 15 g agar (1 H2O)–1], indicating potential stress to the bacterial host. The inability of E. coli cells to stably accept full-length ic plasmids of other flaviviruses has been observed previously (Kapoor et al., 1995; Mandl et al., 1997; Rice et al., 1989; Sumiyoshi et al., 1992; Yamshchikov et al., 2001). To minimize the toxicity associated with the 1409 cDNA, we changed the plasmid vector from pBR322 (~20 copies per cell) to the single-copy pBeloBac11 vector (New England Biolabs) for construction of the pBAC1–2–3 subclone [via the 5’ AgeI site of f1–2–3, which was blunted by mung bean nuclease (Fig. 1b) prior to ligation to pBeloBac’s SfoI site and via interligated Sphi sites of f1–2–3 and vector]. f4–5 was then inserted successfully into the pBAC1–2–3 plasmid to create the full-length pBAC1409ic (Fig. 1a). Site-directed mutagenesis of pBAC1–2–3 inserted a guanosine at position 954, which translates into a Met at E6, and a full-length clone was then constructed (pBAC1409-I/E6/M) as described above.

Sphi-linearized pBAC1409ic and pBAC1409-I/E6/M were used as templates for in vitro transcription to generate full-length genomic RNA. Approximately 0.5 μg RNA was electroporated into approximately 5·0 × 10^6 BHK, Vero (BTX ECM630; 450 V, 1200 Ω, 150 μF) and C6/36 (250 V, 25 Ω, 550 μF) cells and, 10 days later, the cells were analysed for the presence of DENV-2 envelope antigen by indirect fluorescent assay (IFA) using the anti-DENV-2 envelope antibody (1:100 dilution) (Mandl et al., 1997). Production of DENV-2 envelope antigen in all cell types (m.o.i. of 0.01) was observed in C6/36 cells (2–10 %), which is counter-intuitive to our DENV-2 observations. Viral titres were collected over time for P-0 viruses as P-1) to each of the LLC-MK2, BHK, Vero and C6/36 cell lines (m.o.i. of 0.01), resulting in DENV-2 envelope antigen IFA-positive cells and production of IVs in all cell types. In contrast, electroporation of in vitro-transcribed 1409-I/E6/M RNA resulted in DENV-2 envelope-positive cells, as well as P-0 IVs from all three cell types. The influence of cell type-specific electroporation efficiencies on IV production is an obvious consideration; however, efficiencies are known to be higher for mammalian cells (30–40 %) than for C6/36 cells (2–10 %), which is counter-intuitive to our DENV-2 results. Viral titres were collected over time for P-0 viruses and the growth-curve patterns (data not shown) resembled that of the standardized growth curve described below, as well as that of other DENV-2 viruses (Kinney et al., 1997).

Standardized growth-curve analysis in LLC-MK2 and C6/36 cells (m.o.i. of 0·01, incubated in 10 % fetal bovine serum/ minimal essential medium, ~5 % CO2 at 37 or 28°C, respectively; titre determined by plaque titration on LLC-MK2 cells) showed similar temporal patterns of replication for all four viruses (Fig. 2a). C6/36 cells infected with 1409ic and 1409lp viruses, but not 1409hp or 1409-I/E6/M viruses, exhibited mild to moderate syncytium formation (Fig. 2b). DENV-2 plaques on LLC-MK2 monolayers 9 days post-infection were all small-sized, with the mean plaque diameter for each of the four viruses ranging from 1·07 to 1·19 mm. Unidirectional sequencing of the P-0 1409ic or 1409-I/E6/M viral genomes revealed no unexpected changes from the parental plasmids.
The mosquito midgut-infection rate (MIR) and dissemination rate (MDR) of DENV-2 were determined by oral challenge of IVs in an infectious blood meal (6.5–6.9 p.f.u. ml$^{-1}$) to A. aegypti strain Rex D mosquitoes (RexD; Rexville D, Puerto Rico, obtained from the CDC) and after an 8 day extrinsic incubation period as described previously (Bosio et al., 1998; Myles et al., 2004). Of the mosquitoes ingesting a blood meal (n = 24, two replicates) containing the 1409ic virus, 66%–6% had DENV-2 envelope antigen (Ab 4G2) in their midguts (i.e. MIR = 66%–6%) 8 days post-infection. The MIRs observed after mosquitoes ingested 1409lp, 1409hp or 1409-I/E6/M were 58%, 72% and 75%, respectively, none of which differed significantly from each other or from the value for 1409ic-infected mosquitoes (Fisher’s exact test, α of 0.05). The MDR, as determined by the number of anti-DENV-2 envelope IFA-positive heads over the number of IFA-positive midguts from the MIR group, for all of these viruses ranged between 78%–6% and 88%–5, none of which differed significantly from each other.

Clearly, the E6 residue of DENV-2 conditions viral propagation in cell-culture experiments and mutational pressure may be directed at this site. As our 1409hp stock had been cultured repeatedly in mosquito cells ( > 25 times), cell-adapted selection may have yielded the observed mutation. Previous work has found that repeated passage of DENV-2 (strain Jamaica) in C6/36 cells also resulted in an Ile→Met variation at E6 (Guirakhoo et al., 1993). These authors noted that the DENV-2 with E6-Met was coded in the genome of a fusion-mutant strain of DENV-2, although a direct link between a specific amino acid residue and the fusion phenotype was not established.

Syncytium formation is the result of fusion of cellular membranes leading to the development of a polykaryocyte. DENV-2 fusion-from-without syncytium development has been demonstrated after 20 min incubation following infection with a high m.o.i. (Summers et al., 1989). Additionally, low-pH cell-culture media have been shown to induce DENV-associated syncytium progression (Randolph & Stollar, 1990). These reports are consistent with our findings that a high m.o.i. and/or a low-pH medium resulted in enhanced syncytium development in C6/36 cells (data not shown).

The flavivirus envelope glycoprotein is considered a class II type fusion protein and has a putative fusion peptide found at the tip of domain II (Allison et al., 2001; Rey et al., 1995). Under natural physiological states, the tip region, including the fusion peptide, is thought to be seated in a protected, hydrophobic pocket between domains I and III of an opposing envelope monomer. As a result of exposure to low-pH conditions in the endocytic vesicles, the entire envelope protein undergoes a conformational change, resulting in the exposure of the fusion peptide to membrane surfaces (Bressanelli et al., 2004; Modis et al., 2004). By analysing previous findings on envelope glycoprotein structure, we determined that the E6 site appears to be located within the domain I–III interface pocket in very close proximity to the fusion peptide (Allison et al., 2001; Modis et al., 2003; Rey et al., 1995). We speculate that the E6 Ile residue alters the domain I–III pocket conformation, which then becomes more sensitized to pH conditions and affects the envelope protein’s function, leading to syncytium formation in C6/36 cells. Potentially, this alteration could cause the premature formation of additional viral particles.
exposure of the fusion peptide, thereby activating fusion between neighbouring cell membranes. This affect may be mosquito cell-type dependent, as we did not observe syncitia in DENV-2 infected A. aegypti midgut cells.

The specific inhibitory effect of E6-Ile on IV production after electroporation of mammalian cells with in vitro-transcribed mRNA points to the critical role of E6 in translation, replication, packaging, release and/or reinfec-
tion of the virus. This was an unexpected result and may be a unique aspect of DENV-2 1409 replication that warrants more investigation. We acknowledge that this may be an effect of in vitro-derived RNA and that this effect may not occur in nature. However, previous studies have found that low-passage strains of DENV-2 show functional differences in their ability to infect human cells productively (Diamond et al., 2000a, b; Edgil et al., 2003). The low-passage DENV-2 strain Thai CO477 failed to produce any infectious viral particles in human foreskin fibroblast cells at an m.o.i. of <0.1. The differences were attributed to low translational efficiency of input viral RNA. Electroporation of in vitro-transcribed 1409ic RNA may mimic a low m.o.i. and an inefficient translational process might prohibit virus production. It should be noted that published reports on other DENV-2 ic constructs have an Ile at E6, but were not inhibited in production of IVs in mammalian cells after electro-
poration (Blaney et al., 2004; Kapoor et al., 1995; Kinney et al., 1997). However, DENV-2 IV production has also been shown previously to be influenced by the unique genetic make-up of the viral genome: specifically, a nucleotide alteration in the envelope gene resulting in an amino acid variation at E-390, together with variations in the 3′ UTR, influenced DENV-2 replication in human cells (Cologna & Rico-Hesse, 2003). Therefore, the overall genetic make-up of the 1409 genome, and not just the E6 locus, may have an influence on translational efficiency and IV production.

The impact of quasispecies generation on IV production from transfected RNA also cannot be excluded. While our P-0 C6/36-generated 1409ic had no unexpected sequence variations, we did find that C6/36 P-3 1409ic virus had a nucleotide change in the genome that produced an Asn→His mutation at E-155 that resulted in the loss of a conserved glycosylation site. The loss of this glycosylation site in dengue viruses due to repeated passage in C6/36 cells has also been described by others (Guirakhoo et al., 1993; Lee et al., 1997). Moreover, these data raise questions about the role and impact of host-cell defences to viral infection (e.g. RNA interference) in mammalian versus insect cells. More analysis of the attributes of the 1409 virus in productive infection of host cells will probably provide insight into viral pathogenicity, host-cell response to DENV-2 infection and genetic determinants of efficient replication.

Acknowledgements

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References


