Identification of a major determinant of mouse neurovirulence of dengue virus type 2 using stably cloned genomic-length cDNA

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A genomic-length cDNA clone corresponding to the RNA of dengue virus type 2 (DEN-2) New Guinea C strain (NGC) was constructed in a low copy number vector. The cloned cDNA was stably propagated in Escherichia coli and designated pDVWS501. RNA transcripts produced in vitro from the cDNA using T7 RNA polymerase yielded infectious virus (MON501) upon electroporation into BHK-21 cells. When compared with parental NGC virus, MON501 replicated to similar levels in Aedes albopictus C6/36 cells and showed similar neurovirulence in suckling mice. In contrast, a second genomic-length cDNA clone (pDVWS310) used as an intermediate in the construction of pDVWS501 produced virus (MON310) that replicated well in C6/36 cells but was not neurovirulent in mice. MON310 contained the prM and E genes of the non-neurovirulent PUO-218 strain in an NGC background. There were seven amino acid differences between the prM and E proteins of MON310 and MON501. The differences were generally conservative, with the exception of E residue 126, which was Glu in MON310 and Lys in MON501. To examine the role of this residue in mouse neurovirulence, substitutions of Glu → Lys and Lys → Glu were made in MON310 and MON501, respectively. The properties of these mutants clearly demonstrated that Lys at E residue 126 is a major determinant of DEN-2 mouse neurovirulence.

Introduction

Dengue viruses types 1–4 (DEN-1–DEN-4) belong to the family Flaviviridae (Murphy et al., 1995). The viruses cause dengue fever and the severe diseases of dengue haemorrhagic fever and shock syndrome, which are of increasing concern in tropical and subtropical regions (Monath, 1994). In common with other flaviviruses, dengue viruses have an 11 kb ssRNA genome of positive polarity encoding the proteins C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 in one open reading frame. To construct flaviviruses of known sequence for studies on virus replication and vaccine development, infectious RNA has been transcribed from genomic-length cDNA of yellow fever (YF), DEN-4, Japanese encephalitis (JE), Kunjin (KUN), DEN-2 and tick-borne encephalitis (TBE) viruses. For DEN-4, KUN, TBE (strain Neudoerfl) and DEN-2 (strain 16681), the full-length cDNA was stably cloned in Escherichia coli (Lai et al., 1991; Khromykh & Westaway, 1994; Kinney et al., 1997; Mandl et al., 1997), whereas for other viruses the template for transcription was cDNA either ligated in vitro (Rice et al., 1989; Sumiyoshi et al., 1992; Gritsun & Gould, 1995; Kapoor et al., 1995) or cloned in yeast (Polo et al., 1997).

At present, no dengue vaccine is commercially available, although vaccines for YF, JE and TBE are in current use (Monath & Heinz, 1996). One major difficulty hindering the development of a dengue vaccine has been the lack of a suitable animal model of infection. Dengue viruses were originally isolated by passaging in mice, and the testing of the mouse neurovirulence of dengue viruses has a long history (reviewed in Schlesinger, 1977). The neurovirulence test is widely accepted for human encephalitic flaviviruses such as JE and TBE because of the similarity in symptoms in humans and mice, but is not considered ideal for the dengue viruses, which cause encephalitis only rarely in humans (Lum et al., 1996). However, early studies with mouse-adapted dengue viruses inoculated into human volunteers indicated that mouse neurotropic viruses were attenuated in humans (Sabin & Schlesinger, 1945; Schlesinger et al., 1956; Schlesinger, 1977), and mutant viruses are still assessed for mouse neurovirulence (Bray & Lai, 1991; Chen et al., 1995; Kinney et al., 1997). Thus
an improved understanding of the molecular basis of mouse neurovirulence is important in the development of live dengue vaccines.

The preparation of genomic-length DEN-2 cDNA stably cloned in E. coli is of high priority in the study of DEN-2 replication and pathogenesis, and in the production of recombinant viruses with vaccine potential. Here we describe the features of the first such clone for the mouse-adapted and prototypic DEN-2 strain, New Guinea C (NGC). Furthermore, a comparison of the properties of the virus produced from this clone with those of a virus containing the E glycoprotein of the non mouse-adapted strain PUO-218 (Gruenberg et al., 1988) has enabled the identification of a major determinant of DEN-2 neurovirulence in suckling mice.

Methods

**Cells and virus.** BHK-21 and Aedes albopictus C6/36 cells were grown in Eagle’s basal medium containing Earle’s salts (BME) supplemented with 7.5% heat-inactivated foetal calf serum (FCS), 2 mM glutamine and 100 U/ml each of penicillin and streptomycin. The BHK-21 and C6/36 cells were incubated at 37°C and 30°C, respectively. The virus stocks of DEN-2, NGC and PUO-218 strains (Gruenberg et al., 1988) and all recombinant strains produced in this study were prepared in C6/36 cells and titred by plaque assay on C636 cells.

**Synthesis and mutagenesis of DEN-2 cDNA.** Reverse transcription (RT)—PCR was used to synthesize cDNA which was either used in the production of gene constructs or sequenced to verify the identity of recombinant viruses. First strand cDNA synthesis was performed using DEPC-treated RNA extracted from 250 µl culture supernatant fluid using the acid-guanidinium isothiocyanate method of Lewis et al. (1992). One-fifth of the RNA isolated was mixed with a DEN-2 specific oligonucleotide primer and RNA was reverse-transcribed using SuperScript II (Life Technologies). The first strand cDNA was amplified in a PCR reaction using a thermostable DNA polymerase possessing proof-reading ability (Pwo polymerase, Boehringer Mannheim; or Pfu polymerase, Stratagene) to minimize the incorporation of nucleotide mismatches. The resulting RT—PCR products were purified from agarose gels using a QIAEX II gel extraction kit (QIAGEN) and used for cloning or analysed by DNA sequencing. Nucleotide sequencing was performed using DEN-2 specific oligonucleotide primers and an automated DNA sequencer (ABI-Perkin Elmer) following the protocols of the manufacturer. Specific base changes were introduced into cDNA using the overlap extension PCR (OL—PCR) protocol of Ho et al. (1989).

**Construction of genomic-length DEN-2 cDNA clones.** The chimeric DEN-2 genomic-length cDNA clone pDVWS310 is composed of 1967 nt DEN-2 PUO-218 sequence (nt 403–2370) in an NGC DEN-2 cDNA background. This cDNA clone served as the basis for the construction of the complete NGC DEN-2 genomic cDNA clone pDVWS501. The cDNA clones used for the construction of pDVWS310 and pDVWS501 and the strategies used to assemble the genomic-length cDNA clones are described below and shown in Fig. 1(a). The nucleotide numbering system used corresponds to that of the pDVWS501 cDNA sequence (GenBank accession no. AF038403).

**Construction of pDVWS310.** Four of the cDNA clones used for the construction of pDVWS310 had been isolated in our laboratory during previous investigations. These clones were pDS419, a chimeric DEN-2 construct which had been produced by ligation of cDNA derived from NGC DEN-2 (nt 31–402) and PUO-218 DEN-2 (nt 403–2477) (Gruenberg & Wright, 1992), and pD1046, pDEN34 and pDEN75, corresponding to nt 2341–6345, 6859–8060 and 7881–9236 of NGC DEN-2, respectively. RT—PCR was used to synthesize cDNA fragments corresponding to regions of the NGC DEN-2 genome not represented by the pre-existing cDNA clones. The RT—PCR fragments A, B and C (Fig. 1a) were cloned into Smal-cut pUC18 to yield the clones pDVUC300, pDVUC301 and pDVUC304, respectively. Primers used for the cloning of the 5’ and 3’ termini of the DEN-2 genome were designed to facilitate the production of run-off transcription products from genomic-length DEN-2 cDNA. The 5’ terminal primer contained a bacteriophage T7 promoter sequence fused to the first 24 nt of the DEN-2 genome sequence (as deduced from the DEN-2 Jamaica strain sequence; Deubel et al., 1988) whilst the 3’ terminal primer introduced a Xbal site at the end of the DEN-2 genome (Fig. 1b).

Plasmid pDVWS310 was assembled in two halves which were joined at a unique Nsi restriction site to yield the final genomic-length cDNA clone in a low copy number vector as follows. In the following description, the locations of restriction enzyme sites cleaving in DEN-2 cDNA are shown in superscript; sites present in plasmid vectors are not numbered. (i) The Smal—BglI restriction fragment from pDVUC300 was isolated and ligated with Smal—BglII—digested pDS419 to obtain the plasmid pDVUC306. This plasmid was cleaved with NsiI and protruding 5’ ends were filled with Klengow enzyme and further digested with Xbal. The resulting cDNA fragment, containing 2477 bp chimeric DEN-2 sequence, was isolated and inserted into the Smal—Xbal sites of the low copy number vector pWSK29 (Wang & Kushner, 1991), yielding the construct pDVWS306. The SpeI—Xbal fragment from pD1046, containing 3975 bp DEN-2 sequence, was obtained by partial SpeI digestion and ligated in vitro to the SmlI—SpeI fragment isolated from pDVWS306. The resulting in vitro ligation product was inserted into SmlI—Xbal digested pDVWS306, yielding the construct pDVWS307. (ii) The SphI— HindIII fragment from pDVUC301 was isolated and introduced into the Smal—HindIII sites of the low copy number vector pSPORT 1. (Life Technologies) to produce the construct pDVSO301. The AatII—BanII fragment from pDEN34, containing 1168 nt DEN-2 sequence, was isolated and inserted into the AatII—BanII sites of the plasmid pDVSO301, resulting in the clone pDVSO302. Plasmid pDEN75 was digested with NdeI to release the 1066 bp fragment which was isolated and inserted into the NdeI—SpeI site of pDVSO302, yielding pDVSO303. To obtain the 3’ half of the DEN-2 genome in a single construct, the HindIII—AatII fragment from pD1046 was isolated and ligated in vitro with the AatII—BanII—SpeI fragment isolated from pDVOS030; the resulting in vitro ligation product was inserted into HindIII—BamHI—SpeI—Xbal digested pDVUC304 to obtain the clone pDVUC309. (iii) To produce a genomic-length DEN-2 cDNA clone harboured in a low copy number vector, the 6022 bp NotI—Xbal fragment isolated from pDVUC309 was inserted into the corresponding sites of pDVWS307 to obtain pDVWS310. All plasmid clones used in the procedures, including pDVWS310, were propagated in E. coli strain DH5α.

**Construction of pDVWS501.** The entirely NGC DEN-2 genomic-length cDNA clone pDVWS501 was constructed by substitution of the POU-218-derived nucleotides of pDVWS310 with cDNA prepared from NGC DEN-2 by RT—PCR (Fig. 1a). To facilitate the substitution it was first necessary to introduce an Nhel restriction site into pDVWS310. OL—PCR was used to introduce an Nhel site (at DEN-2 nt 2544–2549) into an intermediate plasmid containing nt 2341–4131 of NGC DEN-2 cDNA. A 1209 bp fragment containing the Nhel site was released from this plasmid by SphI digestion (SphI—SphI restriction fragment had been isolated from pDVWS310) and introduced into the corresponding sites of partially SphI-digested pDVWS310 to produce the clone pDVWS310N (Fig. 1b). RT—PCR was used to syn-

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Fig. 1. (a) Construction of the genomic-length DEN-2 cDNA clones pDVWS310 and pDVWS501. The cDNA clones used for the construction of pDVWS310 and the corresponding regions of the DEN-2 genome represented are shown. The cDNA used had either already been cloned in the laboratory (pDS419, pD1046, pDEN34 and pDEN75) or was synthesized from NGC DEN-2 RNA using RT–PCR (A, B and C). A T7 promoter (striped box) and a XbaI site were added to the 5' and 3' termini of DEN-2 cDNA. Plasmid pDVWS310 contained cDNA derived from DEN-2 PUO-218 (open box) and NGC DEN-2 (filled box). Relevant restriction sites employed in the construction of pDVWS310 are shown. Plasmid pDVWS310 was used to construct pDVWS501 by the introduction of an NheI site, followed by insertion of cDNA derived from NGC DEN-2. (b) The 5' and 3' termini of the DEN-2 cDNA contained in pDVWS310, and the resulting in vitro transcribed RNA. The predicted transcriptional start site and the end point after linearization with XbaI are shown. The predicted non-virus nucleotides at the 5' and 3' termini of DEN-2 RNA following in vitro transcription are underlined.

thesize a 2506 bp NGC DEN-2 cDNA fragment for exchange with the PUO-218 sequence contained in pDVWS310. The RT–PCR product was digested with BsrGI and NheI and inserted into the corresponding sites of pDVWS310N to produce the genomic-length NGC DEN-2 cDNA clone pDVWS501.

Construction of pDVWS319 and pDVWS505. OL–PCR mutagenesis coupled with subgenomic fragment exchange was used to introduce mutations into the genomic-length DEN-2 cDNA clones pDVWS310 and pDVWS501 to produce the clones pDVWS319 and pDVWS505, respectively (Fig. 2). The strategies used for introduction of the mutations were as follows.

For the construction of pDVWS319, a 1284 bp subgenomic fragment containing G → A substitutions at DEN-2 PUO-218 nt 1312 and 1314 resulting in the codon change GAG → AAA (Glu → Lys) was produced.
Fig. 2. Construction of the genomic-length DEN-2 cDNA clones pDVWS319 and pDVWS505. OL–PCR mutagenesis was used to produce a subgenomic cDNA fragment from pDVWS310 containing the codon change at E126 GAG (Glu) to AAA (Lys). The mutated cDNA was inserted into pDVWS310 using the enzyme sites BstEII and SphI to produce pDVWS319. In a similar procedure, OL–PCR mutagenesis was used to produce a subgenomic fragment from pDVWS501 containing the codon change at E126 AAA (Lys) to GAG (Glu). The mutated cDNA was inserted into pDVWS501 using the sites BsrGI and SphI to obtain pDVWS505. The cDNA derived from DEN-2 PUO-218 is indicated by the open box as in Fig. 1. Plasmid pDVWS319 contained an additional silent mutation in codon 130.

Production of genomic-length RNA transcripts and recombinant viruses. Genomic-length DEN-2 clones were linearized by digestion with XbaI, and the DNA was purified by phenol extraction and ethanol precipitation. Transcription was carried out using a RiboMAX kit (Promega) according to the manufacturer’s instructions with 2.5 µg template DNA and an incubation time of 2 h. The RNA cap structure analogue m7G(5'ppp)5'G (New England Biolabs) was included at a concentration of 1 mM in the transcription mix. The yield and approximate size of the products were analysed by non-denaturing agarose gel electrophoresis. The RNA was precipitated with sodium acetate and ethanol, and stored at −70 °C. RNA for electroporation into cells was pelleted and resuspended in water immediately before use.

BHK-21 cells were grown until the monolayers were 60–80% confluent. Cells were trypsinized, pooled, and washed twice in cold PBS. The cells were resuspended in cold PBS to a concentration of 1 × 10^7 cells/ml, and 0.5 ml aliquots were dispensed into 0.4 cm Gene Pulser cuvettes (Bio-Rad). Approximately 50 µg yeast tRNA and 7–10 µg transcribed virus RNA were added, and the cuvettes were held on ice for 10 min. The samples were pulsed using a Gene Pulser I apparatus (Bio-Rad) at 500 µF and 300–350 V. After electroporation, the cells were kept for 10 min on ice, replated in growth medium, and then incubated at 37 °C.

The medium was changed at 16–24 h, and when the monolayers were confluent (approx. 48 h), replaced with maintenance medium (BME containing 2% FCS, 2 mM glutamine, 100 U/ml penicillin and streptomycin). Seven days after electroporation, the culture medium collected from BHK-21 cells was used to inoculum to infect C6/36 cells. Three to five days later, the culture medium from the C6/36 cells was used to initiate a second passage in C6/36 cells. The culture medium from the
second C6/36 passage was harvested when the majority of cells showed cytopathic effects (2–4 days) and used for further experiments.

To determine the percentage of cells infected by the RNA transcripts, an aliquot of each sample of electroporated BHK-21 cells was replated in a chamber slide, and medium changes were done as described above. At 4–6 days post-electroporation, virus replication was assayed by monitoring the production of E using a mix of anti-E monoclonal antibodies by indirect immunofluorescence as described previously (Gruenberg & Wright, 1992).

- **Mouse neurovirulence assays.** Groups of eight (in some experiments four), 3-day-old BALB/c mice were injected intracerebrally (i.c.) with 10 µl virus diluted in PBS. The virus inoculum was immediately re-titrated by plaque assay to confirm that the mice received the calculated dose of virus. Mice were observed daily for 21 days for signs of encephalitis, which was seen as loss of condition, hunching and paralysis. Severely paralysed mice were killed by cervical dislocation.

When necessary, the brains of infected mice were removed and stored at −70 °C. For analysis, brains were thawed and homogenized in 0.5 ml PBS by gentle passage through 18 and 21 gauge needles. The suspension was clarified by low-speed centrifugation for 5 min. The homogenate was used for plaque assays, or the RNA was extracted from the supernatant fluid and its sequence determined as described above.

**Results**

**Construction and properties of parental recombinant viruses**

The first construct of genomic-length DEN-2 cDNA was assembled with cDNA derived from the NGC and PUO-218 strains (Fig. 1a). The genome was assembled in two halves; the 5' section contained nt 1–6345 and was cloned in the low copy number vector pWSK29, while the 3' section was cloned in pUC18 and contained nt 3235–10724. The two halves were joined at a unique NsiI site (nt 4700) to form genomic-length cDNA inserted into plasmid pWSK29. This plasmid was designated pDVWS310. The cDNA originally derived from PUO-218 (nt 403–2370) encoded 11% of C, all of prM and 95% of E (Gruenberg et al., 1988). The complete sequence of pDVWS310 was determined and submitted to GenBank (accession no. AF038402).

RNA was transcribed from the T7 promoter after cleavage of pDVWS310 with XbaI. As can be seen in Fig. 1(b), it was expected that the transcription products would contain an extra nucleotide (G) at the DEN-2 5' terminus, and an additional two nucleotides (AG) at the 3' terminus. It was not ascertained whether the non-virus nucleotides were subsequently retained after virus RNA replication. The transcribed RNA was electroporated into BHK-21 cells. At 4–5 days, approximately 80% of cells showed immunofluorescence using antibody directed against the E glycoprotein. Cytotoxic effects were routinely observed at 5–7 days. To increase virus titres to a suitable level for further infection experiments, the culture medium was passaged twice in C6/36 cells. The final titres of the virus obtained, designated MON310, were determined by plaque assay in C6/36 cells. The titres were in the range of 1–5 × 10⁶ p.f.u./ml. The sizes of plaques were marginally smaller than those of NGC and PUO-218 (approximately 1.5 mm in diameter). However, the growth curves in C6/36 cells of the NGC, PUO-218 and MON310 viruses, and the final titres achieved, were similar (Fig. 3). The authenticity of the recovered MON310 virus was determined by RT–PCR and sequencing of a 646 nt segment that contained 592 nt of the E gene and the first 54 nt of the NS1 gene. This segment included the junction of the NGC and PUO-218 sequences and contained 25 nucleotide differences between the two viruses, enabling unambiguous recognition of NGC, PUO-218 or MON310 viruses. In later experiments, MON501 virus derived entirely from NGC cDNA (pDVWS501) was also prepared (Fig. 1a).

The MON501 and MON310 viruses derived from their respective plasmids replicated to similar titres during the standard procedures of RNA transcription, electroporation into BHK-21 cells and two passages in C6/36 cells. Progeny MON501 virus was authenticated by confirming the presence of the Nhel site and sequencing nt 1270–1840 of the E gene. The sequence of pDVWS501 was assembled in conjunction with that of pDVWS310 and submitted to GenBank (accession no. AF038403). The differences in sequence between the previously sequenced NGC strain (Irie et al., 1989) and pDVWS501 are given in Table 1. There were 22 nucleotide differences: six led to amino acid changes and two were located in the 3' untranslated region (UTR). Comparisons of the amino acid and 3' UTR nucleotide changes of pDVWS501 with the corresponding amino acid and 3' UTR nucleotide sequences of other DEN-2 isolates (Deubel et al., 1988; Hahn et al., 1988) revealed that the substitution at residue 2081 was the only amino acid change unique to pDVWS501, whereas both the 3' UTR changes were unique to pDVWS501. The change at amino acid 2081 (T → A) is conservative and has no significance with respect to known sequence motifs. The 3' UTR changes are located well upstream of conserved RNA sequences (Hahn et al., 1987; Shi et al., 1996) and had no apparent effects on virus replication (Fig. 3)
Table 1. Comparison of NGC DEN-2 nucleotide and amino acid sequence changes

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<th>Nucleotide</th>
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<td></td>
<td>10415</td>
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* Nucleotide present in the pDVWS501 sequence (GenBank accession no. AF038403).  
† Nucleotide present in the DEN-2 NGC sequence of Irie et al. (1989) (GenBank accession no. M29095).  
‡ Site-specific change leading to the introduction of the NdeI site in pDVWS501.

Table 2. Mouse neurovirulence of NGC, PUO-218 and MON310 viruses

<table>
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<th>MON310</th>
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<td>10⁰</td>
<td>16/16</td>
<td>0/16</td>
<td>1/16*</td>
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<tr>
<td>10¹</td>
<td>15/16</td>
<td>0/8</td>
<td>1/24*</td>
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* Death not from dengue virus infection.

Table 3. Amino acid differences between the NGC and PUO-218 strains in prM and E

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<td>I</td>
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<tr>
<td>E71</td>
<td>D</td>
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<tr>
<td>E484†</td>
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* Gruenberg et al. (1988).  
† This change not encoded by MON310.

Mouse neurovirulence of MON310

The neurovirulence of the first recombinant virus obtained, MON310, was tested by i.c. inoculation of 3-day-old BALB/c mice and compared with the neurovirulence of NGC and PUO-218 viruses. The mice were held for 21 days or killed when showing terminal paralysis. A portion of each inoculum was re-titrated by plaque assay immediately following the mouse injections to confirm the dose administered. The cumulative results are shown in Table 2. As expected, NGC was neurovirulent. However, both PUO-218 and MON310 were not. Unlike NGC, which has been extensively passaged in mouse brain, PUO-218 (Gruenberg et al., 1988) has received only limited passaging in C6/36 cells since its initial isolation using Toxorhynchites splendens mosquitoes and LLC-MK2 cells. MON310 had the prM and E genes of PUO-218 and was also not neurovirulent.

In the PUO-218 fragment in MON310, there were 76 differences in nucleotide sequence between PUO-218 and NGC (Gruenberg et al., 1988). The NGC virus sequence used in this comparison was determined from NGC virus stocks in our laboratory (Gruenberg et al., 1988). Seven of the differences lead to coding changes (Table 3). The majority of amino acid substitutions were conservative, with the exception of that at residue 126 in the E glycoprotein, where NGC contains Lys while PUO-218 (and MON310) contains Glu (we shall refer to this residue as E126, with the E signifying envelope glycoprotein and not glutamic acid). A similar substitution at this position was found in other DEN-2 mouse-passaged viruses (Lewis et al., 1993), and also conferred neurovirulence to a DEN-3–DEN-4 hybrid (Chen et al., 1995).

The neurovirulence of MON501 virus was also tested in BALB/c mice and MON501 was shown to have properties similar to NGC (see later).

Construction of viruses MON319 and MON505

To confirm the significance of Lys at E126, the residue at this position was changed by site-directed mutagenesis in the hybrid virus MON310 and in MON501. For MON310, the Glu at E126 was replaced with Lys to produce MON319, whereas for MON501 the substitution was reversed: the Lys at E126 was replaced with Glu to produce MON505. OL–PCR was used to produce fragments of 724 and 1851 nt, respectively, for MON310 and MON501 (Fig. 2). These fragments encoded the desired changes at E126 and were inserted into cloned genomic-length cDNA using unique restriction enzyme sites (Fig. 2). The inserted cDNA fragments
were sequenced to confirm the presence of the desired mutations and to detect any PCR-induced errors. Plasmid pDVWS319 contained an additional silent mutation (G → A) 12 nt downstream of E126. The titres of the viruses MON319 and MON505 produced following electroporation of RNA into BHK-21 cells and passaging in C6/36 cells were similar to those of the parental viruses, i.e. of the order of $10^8$ p.f.u./ml. Before proceeding with mouse studies, the sequences of the new viruses at E126 (and the silent mutation for MON319) were confirmed by RT–PCR and sequencing of a cDNA fragment (approximately 400 bp) which contained the codon.

**Neurovirulence of MON319 and MON505**

Three-day-old mice were inoculated i.c. with NGC, MON501, MON319 or MON505 viruses. The cumulative results are given in Table 4. The proportion of mice killed and the mean time to death at each dose are shown. MON501 killed a similar proportion of mice to NGC, but the time to death was longer. The reason for this is unknown, but may reflect minor sequence differences between the population of viruses constituting the extensively passaged NGC virus and the MON501 virus, with only three passages from cloned cDNA. The effect of the additional nucleotides at the 5' and 3' ends of initial transcripts of virus RNA is also unknown.

MON319 caused paralysis and death; the replacement of Glu at E126 in MON310 by Lys generated a neurovirulent virus. In the reverse experiment, the replacement of Lys in MON501 by Glu in MON505 did not completely abolish mouse neurovirulence. However, the proportion of surviving mice and the survival time of those that died were considerably increased.

We next examined the brains of mice infected with $10^5$ p.f.u. to compare the replication of MON501 with that of PUO-218 (avirulent) and of MON505 (reduced neurovirulence). One brain for each virus was harvested 6 days after infection. At this time the mouse infected with MON501 had terminal paralysis. The mice infected with MON505 or PUO-218 showed no symptoms of infection. The estimated titres in p.f.u. per brain were $2 \times 10^8$ (MON501), $2 \times 10^6$ (PUO-218) and $9 \times 10^5$ (MON505). Thus restricted replication of PUO-218 and MON505 indeed occurred.

It was possible that the limited replication of PUO-218 and MON505 arose by the mutation and subsequent selection of viruses no longer retaining Glu at E126. However, by RT–PCR and the sequencing of a 400 nt fragment containing the E126 codon, this possibility was eliminated. No changes at E126 were detected for either virus. Thus viruses containing Glu at E126 were capable of limited replication in mice and in the case of MON505 showed neurovirulence. We also demonstrated by RT–PCR and sequencing of mouse brain extracts that the neurovirulent viruses MON501 and MON319 retained a Lys at E126.

**Discussion**

The results clearly showed that Lys at E126 is a primary determinant of DEN-2 neurovirulence in suckling mice. The change of Glu (MON310) to Lys (MON319) at E126 produced a neurovirulent virus, and the replacement of Lys (MON501) by Glu (MON505) sharply reduced neurovirulence (Table 4). Additional support for the importance of DEN-2 E126 comes from the comparison of 33 published sequences of the E gene of DEN-2 (Lewis et al., 1993). Only five viruses have Lys at E126 and all were passaged in mice: NGC (38 passages in mice) (Irie et al., 1989); Trinidad-1751 (1 number of passages unknown) (Anderson & Downs, 1956); TH-36 (17 passages in mice) (Shiu et al., 1992); and SL1050 and SL77/69 (two to four passages) (Blok et al., 1989). Furthermore, a hybrid virus of DEN-4 containing the C-prM-E genes of DEN-3 (strain CH53489) was constructed by Chen et al. (1995). The hybrid virus became neurovirulent when Glu was replaced with Lys in DEN-3 at the

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**Table 4. Mouse neurovirulence of NGC and recombinant viruses**

<table>
<thead>
<tr>
<th>p.f.u. inoculated</th>
<th>No. of mice dead at 21 days</th>
<th>Time to death (days)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NGC</td>
<td>MON501</td>
</tr>
<tr>
<td>$10^1$</td>
<td>16/16</td>
<td>20/20</td>
</tr>
<tr>
<td>$10^3$</td>
<td>15/16</td>
<td>14/16</td>
</tr>
<tr>
<td>$10^2$</td>
<td>13/15</td>
<td>15/15</td>
</tr>
<tr>
<td>10</td>
<td>12/16</td>
<td>12/16</td>
</tr>
<tr>
<td>1</td>
<td>4/8</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Mean ± sd.
† All mice died 5 days after inoculation.
‡ Pairwise comparisons of survival times were made between mice receiving the same dose of two different viruses. Using Student’s t-test, differences were significant at the 5% level with the exception of this pair.
ND, Not determined.
position corresponding to DEN-2 E126, suggesting that a Lys residue at this position may be of general importance for the neurovirulence of dengue serotypes in addition to DEN-2. However, analyses (not shown) of E sequences for DEN-3 (Lanciotti et al., 1994) and DEN-1 (Fu et al., 1992; Shiu et al., 1992) indicate that this is not necessarily the case.

A second determinant of mouse neurovirulence is the epitope of DEN-2 E recognized by monoclonal antibody 3H5 (Trirawatanapong et al., 1992; Hiramatsu et al., 1996). In a neuroviral DEN-2(prM-E)-DEN-4 hybrid virus, Hiramatsu et al. (1996) made a number of point mutations in this epitope from E383 to E393. Individual changes at six positions abolished mouse neurovirulence under the conditions tested. Consistent with these observations, sequence analysis of cloned viruses derived from a Mexican DEN-2 strain have also suggested a role for E390 in mouse neurovirulence (Sanchez & Ruiz, 1996).

Two other single amino acid changes in E which significantly alter dengue virus (DEN-4) neurovirulence have been identified using recombinant viruses encoding the structural genes of a mouse-adapted DEN-4 virus, H241-N, or its parent, H241-P, in the genetic background of the DEN-4 strain B14669 (Kawano et al., 1993). The loss of the second site for N-linked glycosylation in E (Asn193-Asp194-Thr195) by the change of Thr to Ile, or the replacement of Phe at E402 (DEN-2) and E401 (DEN-4) (Kawano et al., 1993) and its effect on neurovirulence has already been mentioned. The change at E71 may be significant since neutralization escape mutants of YF have been mapped to E71 and E72 (Lobigs et al., 1987).

For the encephalitic flaviviruses such as JE (Cecilia & Gould, 1991; Hasegawa et al., 1992), TBE (Holzmann et al., 1990, 1997), Murray Valley encephalitis (McMinn et al., 1995) and louping ill (Jiang et al., 1993) viruses, single amino acid changes in the E glycoprotein leading to changes in mouse neuroinvasiveness have also been identified. This was achieved by the nucleotide sequencing of passaged viruses and of monoclonal antibody escape mutants. The neuroinvasiveness of the viruses was usually tested by intraperitoneal or subcutaneous inoculation of mice ranging in age from 2 weeks to adult, a procedure significantly different from the i.c. injection of 3-day-old mice used in dengue experiments. In most cases, the mutant encephalitic flaviviruses showed the same neurovirulence as parental virus when inoculated i.c. Interestingly, Sumiyoshi et al. (1995) were able to demonstrate a reduction in neurovirulence of JE by a change of Glu to Lys at E138 (domain I), the reverse of the substitution noted for DEN-2 E126.

The adaptation of dengue virus to mouse was an important step in dengue research and for many years was the accepted way of virus isolation and propagation before the development of mammalian and mosquito cell culture (Schlesinger, 1977). Thus it is not surprising that mouse-adapted viruses were tested for vaccine potential, although they were known to be neurotropic in mice (Schlesinger et al., 1956). The inoculation of the viruses into human volunteers induced neutralizing antibody, but clinical symptoms of infection, although reduced, were still observed. During passaging in mice, the genetic composition of the virus population presumably changed in two ways. First, there was selection for individual mutations which increased the ability of the virus to replicate in the central nervous system of mice; it would be expected that some enhanced replication more than others. Second, viruses containing these mutations formed an increasing proportion of the virus population as the passage level increased. Thus, while our findings demonstrated that the change at E126 is an important one for mouse neurovirulence of DEN-2, we cannot make a firm link to human attenuation through the early experiments because of the almost certain presence of other mutations and the heterogeneity in the virus populations of the mouse-grown viruses. However, E126 is clearly of sufficient importance for changes at this and neighbouring residues in the dimerization domain II to be tested for possible in-
corporation into candidate human vaccines derived from recombinant viruses.

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