

Mutagenesis of the dengue virus type 2 NS3 proteinase and the production of growth-restricted virus

Anita E. Matusan,¹ Peter G. Kelley,¹ Melinda J. Pryor,¹ James C. Whisstock,² Andrew D. Davidson¹ and Peter J. Wright¹

Department of Microbiology¹ and Department of Biochemistry and Molecular Biology², Monash University, PO Box 53, Victoria 3800, Australia

The N-terminal one-third of the NS3 protein of *Dengue virus type 2* (DEN-2) complexes with co-factor NS2B to form an active serine proteinase which cleaves the viral polyprotein. To identify sites within NS3 that may interact with NS2B, seven regions within the NS3 proteinase outside the conserved flavivirus enzyme motifs were mutated by alanine replacement. Five sites contained clusters of charged residues and were hydrophilic. Two sites were hydrophobic and highly conserved among flaviviruses. The effects of five mutations on NS2B/3 processing were examined using a COS cell expression system. Four retained significant proteinase activity. Three of these mutations and two more were introduced into genomic-length cDNA and tested for their effects on virus replication. The five mutant viruses showed reduced plaque size and two of the five showed significantly reduced titres. All seven mutations were mapped on the X-ray crystal structure of the DEN-2 NS3 proteinase: three were located at the N terminus and two at the C terminus of the NS2B-binding cleft. Two mutations were at the C terminus of the proteinase domain and one was solvent-exposed. The study demonstrated that charged-to-alanine mutagenesis in the viral proteinase can be used to produce growth-restricted flaviviruses that may be useful in the production of attenuated vaccine strains.

Introduction

There are four serotypes of dengue virus that are transmitted by the *Aedes* mosquito in tropical and subtropical regions (Halstead, 1988; van Regenmortel *et al.*, 2000). The viruses are classified in the genus *Flavivirus* of the family *Flaviviridae* and are responsible for dengue fever, haemorrhagic fever and shock syndrome (Monath, 1994). No commercial dengue virus vaccine is currently available and mosquito control programs are difficult to implement and maintain, making the development of new antiviral drugs and a safe vaccine imperative.

The flavivirus genome is a positive-sense RNA molecule of approximately 11000 nucleotides encoding the proteins C–prM–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5 in a single long open reading frame. Co- and post-translational polyprotein processing by host and viral proteinases generate three structural proteins, namely C (capsid), M (membrane) and

E (envelope), and seven nonstructural (NS) proteins, namely NS1 through to NS5 (reviewed by Rice, 1996).

The focus of this paper is the viral NS2B/3 proteinase of *Dengue virus type 2* (DEN-2). NS2B/3 cleaves at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 junctions. It also cleaves within C, NS2A, NS4A and NS3, in the latter case producing NS3' and NS3'' (Arias *et al.*, 1993; Teo & Wright, 1997). Proteolysis occurs following a pair of basic amino acids or Gln–Arg and preceding either Gly, Ser or Ala (Rice, 1996). The motifs and catalytic triad typical of a trypsin-like serine proteinase are located in the N-terminal one-third of NS3 (Bazan & Fletterick, 1989); the X-ray crystal structure for this part of NS3 was described recently (Murthy *et al.*, 1999). The active form of the viral proteinase is a complex between NS3 and NS2B (Preugschat *et al.*, 1990; Falgout *et al.*, 1991). A hydrophilic region of 40 amino acids in NS2B containing a short central hydrophobic segment is required for the association of NS2B with NS3 and for enzyme activity (Falgout *et al.*, 1993; Chambers *et al.*, 1993; Yusoff *et al.*, 2000). Similarly, an NS3-containing complex is an active proteinase of *Hepatitis C virus* (HCV), which also belongs to the family

Author for correspondence: Peter Wright.

Fax +61 3 9905 4811. e-mail Peter.Wright@med.monash.edu.au

Flaviviridae, but to the genus *Hepacivirus*. In this case the complex is formed between NS3 and NS4A (Failla *et al.*, 1995; Lin *et al.*, 1995).

Initial studies on the cleavage of the flavivirus polyprotein targeted either the four regions of homology shared between serine proteinases and the flavivirus NS3 protein, or the cleavage sites in the polyprotein (Chambers *et al.*, 1990; Valle & Falgout, 1998). However, in this study, seven locations in NS3 outside these regions and sites were chosen for mutagenesis. By avoiding motifs containing the catalytic triad and residues known to be involved in substrate binding, it was reasoned that sites involved in NS2B–NS3 interaction may be mutated and that suitable modification at such sites had the potential to reduce, without abolishing, proteinase activity and virus replication. Virus mutants of this type are candidates for incorporation into live vaccine strains of DEN-2 and other flaviviruses. Mutations were tested for their effects on NS2B/3 proteinase activity by transient expression of the NS2B/3 genes in COS cells, virus replication by the incorporation into genomic-length DEN-2 cDNA or both. Results are interpreted below with reference to the location of the mutations mapped on the X-ray crystal structure of the DEN-2 NS3 proteinase (Murthy *et al.*, 1999) and a model of the NS2B/3 complex.

Methods

Cells, virus and antisera. Growth of BHK-21, *A. albopictus* C6/36 and COS cells, the preparation of stocks of DEN-2 viruses and plaque assays in C6/36 cells at 28 °C have all been described previously (Gualano *et al.*, 1998; Pryor *et al.*, 1998). For plaque assays, virus inoculum was removed from the monolayers and cells were overlaid with medium containing 1% SeaPlaque agarose (FMC BioProducts). At 5 days after infection, cells were overlaid with the same mixture containing 0.018% neutral red. Plaques were then counted 7 days after infection. Concentrated stocks of some viruses were produced by precipitation with polyethylene glycol (PEG) (Della-Porta & Westaway, 1972). The preparation of rabbit polyclonal antiserum directed against DEN-2 NS3 (residues 355–593) has been described previously (Teo & Wright, 1997).

Transient expression of DEN-2 genes in COS cells. The vector pSV.SPORT 1 (Gibco BRL) was used to express DEN-2 cDNA (strain New Guinea C) encoding NS2B/NS3. The construction of plasmid pSV.NS2B/3 (S2, the parental NS2B/NS3 construct) (Fig. 1) was described previously (Teo & Wright, 1997). The nucleotide numbering used to describe the mutants below follows that of Irie *et al.* (1989). Four mutant constructs derived from pSV.NS2B/3 (S2) were prepared by replacing the *EcoRV*⁴³³¹–*BstBI*⁵⁰⁷⁰ fragment (Fig. 1) with a mutated fragment prepared by overlap extension PCR (Ho *et al.*, 1989), i.e. constructs pSV.NS2B/3_{17–20} (S_{17–20}), pSV.NS2B/3_{32–36} (S_{32–36}), pSV.NS2B/3_{63–66} (S_{63–66}) and pSV.NS2B/3_{95–96} (S_{95–96}); the plasmid pSV.NS2B/3_{179–181} (S_{179–181}) was prepared by conventional PCR using a mutagenic primer overlapping the *BstBI*⁵⁰⁷⁰ site. The PCR-derived regions of all clones were sequenced. The plasmid pSV.NS2B/3_{32–36} was initially designed to encode only the substitutions G₃₂A and Y₃₃A. However, an additional I₃₆V change was introduced during PCR.

Constructs were electroporated into COS cells as described by Teo & Wright (1997). Cells were maintained at 31 °C or 37 °C for 48 h and then starved of methionine for 2 h prior to radiolabelling with

[³⁵S]methionine for either 3 h at 31 °C or 1 h at 37 °C (Pryor & Wright, 1993). Cell lysates were analysed by radioimmunoprecipitation, gel electrophoresis and fluorography (Pryor & Wright, 1993; Teo & Wright, 1997).

■ Insertion of mutations into genomic-length DEN-2 cDNA.

The plasmid pDVWS501 containing genomic-length New Guinea C strain DEN-2 cDNA has been described in detail (Gualano *et al.*, 1998). For these experiments, transient expression was used to examine the effects of five mutations in the NS3 proteinase on proteolytic activity. Three mutations were selected and then inserted into genomic-length DEN-2 cDNA to study their effects on virus replication. Two additional charged-to-alanine mutations that were external to proteinase motifs were also inserted into the genomic-length DEN-2 cDNA (Fig. 1).

The plasmid pDVWS501NS_{32–36} was prepared by replacing the *NheI*²⁵⁴⁴–*NsiI*⁴⁷⁰⁰ fragment of pDVWS501 with a mutated fragment prepared by overlap extension PCR (Fig. 1). The other four mutations were initially constructed in the subclone pDVSO8298 prior to ligation into pDVWS501. This strategy was devised following consideration of the available restriction enzyme sites. Plasmid pDVSO8298 contained DEN-2 cDNA corresponding to nucleotides 4494 (upstream of *NsiI*⁴⁷⁰⁰) to 8744 (downstream of *StuI*⁷⁸⁷⁴) cloned into *XbaI/KpnI*-digested pSPORT 1 (Gibco BRL). cDNA encoding mutations KRIE (63–66) (underlined residues changed to alanine) or EDD (179–181) in the NS3 hydrophilic regions was cloned into pDVSO8298 by removing the mutated *NsiI*⁴⁷⁰⁰–*PpuMI*⁵⁸⁵⁴ fragment from the corresponding pSV.NS2B/3 plasmid and ligating this into *NsiI*⁴⁷⁰⁰/*PpuMI*⁵⁸⁵⁴-digested pDVSO8298. The two remaining charged-to-alanine mutations, EGEE (91–94) and EKSIE (169–173), were introduced into *NsiI*⁴⁷⁰⁰/*PpuMI*⁵⁸⁵⁴-digested pDVSO8298 as overlap extension PCR fragments. All four mutants (*NsiI*⁴⁷⁰⁰–*StuI*⁷⁸⁷⁴-mutated fragments) were then removed from the appropriate pDVSO8298 plasmid and ligated into *NsiI*⁴⁷⁰⁰/*StuI*⁷⁸⁷⁴-digested pDVWS501. PCR-derived regions were sequenced.

■ Production of virus from genomic-length cDNA. Procedures for transcription of RNA, electroporation and immunofluorescence of BHK-21 cells and passaging of virus in C6/36 cells have been described previously (Gualano *et al.*, 1998). Briefly, capped transcripts were produced from plasmids containing genomic-length DEN-2 cDNA using the Promega RiboMAX kit. Approximately 7–10 µg of transcript RNA and 50 µg of carrier tRNA were electroporated into BHK-21 cells, which were then incubated at 33 °C or 37 °C. Cells were examined for immunofluorescence 4 to 6 days later using anti-E monoclonal antibodies (Gruenberg & Wright, 1992). After 7 days, the culture medium was used to infect C6/36 cells. The culture medium from these infected C6/36 cells was then used 4 to 5 days later to initiate a second passage. When approximately 50% of the cells exhibited cytopathic effects, or 5 days later if no cytopathic effects were visible, these second passage virus stocks were titred by plaque assay on C6/36 cells.

Each virus was derived at least twice from its parental construct. To confirm that each mutation was present after electroporation and passaging, total RNA was extracted from infected C6/36 cells or supernatant and RT-PCR of viral RNA was performed (Gualano *et al.*, 1998; Pryor *et al.*, 1998). The complete NS2B and NS3 genes were sequenced to confirm the presence of the introduced mutation and the absence of any other changes that may have been introduced during virus passaging.

■ Co-ordinates and calculations. The crystal structures of the DEN-2 NS3 serine proteinase (protein database identifier IBEF; Murthy *et al.*, 1999) and the HCV NS3/NS4A proteinase–co-factor complex (protein database identifier 1NS3; Yan *et al.*, 1998) were obtained from the protein database (Bernstein *et al.*, 1977; Berman *et al.*, 2000).

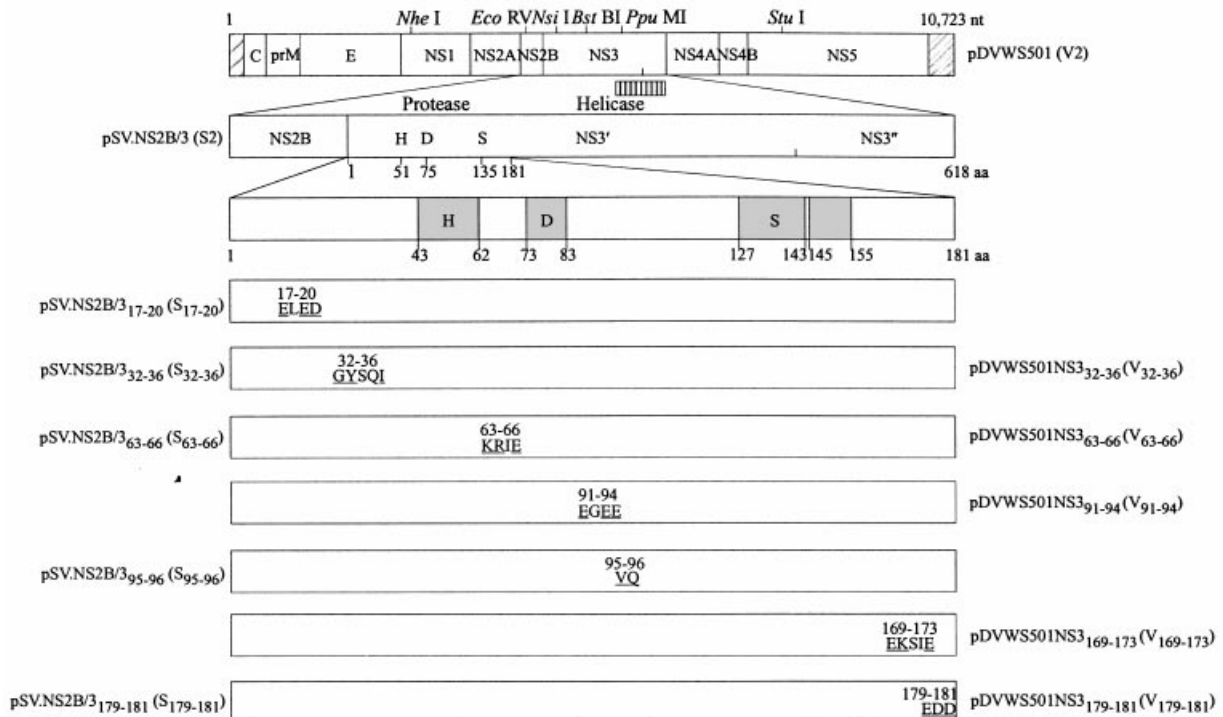


Fig. 1. Genomic map of DEN-2 and NS3 proteins encoded by mutant constructs. The catalytic residues H, D and S and the serine proteinase motifs (shaded) are shown (Bazan & Fletterick, 1989); residue numbers within NS3 are given. The full (pSV) and abbreviated (S) designations of the gene constructs encoding NS2B/3 in pSV.SPORT 1 are shown on the left. S designations are also used for the corresponding encoded mutant proteins where appropriate. The names of the plasmids containing genomic-length cDNA (pDVWS) and derived viruses (V) are shown on the right. Antiserum against a bacterial fusion protein raised in rabbits (Teo & Wright, 1997) was directed against a segment of NS3 (vertical stripes). Underlined residues were mutated to alanine (with the exception of I₃₆V).

The model between the DEN-2 NS3 proteinase and a portion of the NS2B co-factor was generated using the Quanta/CHARMM software (MSI). The peptide sequence G₆₉SSPILSITISE₈₀ within NS2B corresponds to the portion of the NS4A co-factor seen in the structure of the HCV proteinase (Brinkworth *et al.*, 1999). The DEN-2 and HCV proteinases were superimposed and the NS4A peptide within the HCV proteinase structure was used as a template to model the NS2B sequence (GSSPILSITISE) into the DEN-2 NS3 proteinase. The model was then subjected to rounds of CHARMM minimization, initially with constraints that were applied to the proteinase; the co-factor was allowed to move freely. Later rounds were performed to convergence with no constraints. Dihedral constraints were applied to four residues in non-allowed conformations. A Ramachandran plot of the final model indicated that all residues were in allowed conformations.

We examined the positions of the mutations described in Fig. 1 with respect to the interactions seen in the crystal structure of the DEN-2 NS3 proteinase. In addition, the model between the NS3 proteinase and the NS2B co-factor was used to analyse three groups of mutations that mapped to the NS2B-binding cleft.

Results

Mutagenesis of the N-terminal 181 amino acids of NS3

Hydrophilic regions were targeted using clustered charged-to-alanine mutagenesis (Diamond & Kirkegaard, 1994). Charged amino acids probably occupy exposed positions in

the tertiary structure and therefore interact with other proteins. Previous experiments with DEN-4 identified a 40 amino acid segment of NS2B that was, overall, hydrophilic and essential for protease activity (Falgout *et al.*, 1993). This segment also contained a short, central hydrophobic sequence of approximately 12 amino acids. It was possible that changes to the hydrophilicity of NS3 might modify the interaction of NS3 with NS2B and the proteinase activity of the NS2B/3 complex.

The first 181 amino acids of NS3 were scanned for clusters of five residues that contained at least three charged amino acids. Five such clusters outside proteinase motifs were chosen for mutagenesis and the charged residues were changed to alanine (Fig. 1) (Bass *et al.*, 1991). The changes made in DEN-2 NS3 were as follows: E₁₇A, E₁₉A and D₂₀A; K₆₃A, R₆₄A and E₆₆A; E₉₁A, E₉₃A and E₉₄A; E₁₆₉A, K₁₇₀A and E₁₇₃A; and lastly E₁₇₉A, D₁₈₀A and D₁₈₁A. Alanine was chosen as the replacement amino acid since it removes the side chain beyond the β -carbon and also minimizes any steric effects within the polypeptide caused by the replacement (Cunningham & Wells, 1989). In addition, two hydrophobic regions were chosen (Fig. 1) on the basis of hydropathy plot data (Hahn *et al.*, 1988) and conservation of sequence across the flaviviruses (Westaway & Blok, 1997; Chang, 1997). They were G₃₂A and Y₃₃A, and V₉₅A and Q₉₆A. Thus a total of seven sites were mutated.

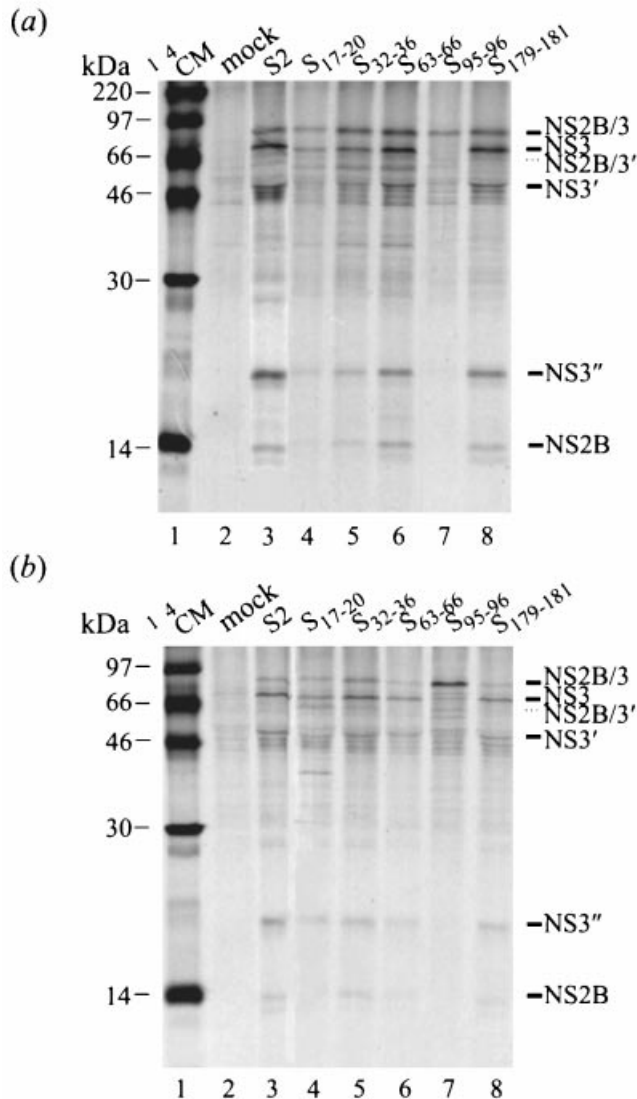


Fig. 2. Analysis of NS2B/3 proteinase activity in transfected COS cells. Cells were transfected with parental S2 (lane 3) or mutant S₁₇₋₂₀ to S₁₇₉₋₁₈₁ (lanes 4–8) constructs and labelled for (a) 1 h at 37 °C or (b) 3 h at 31 °C. Proteins were immunoprecipitated with antiserum directed against NS3 and separated by 14% SDS-PAGE. Size markers are shown on the left.

Transient expression and proteolysis of NS2B/3

The first experiments were designed to test for proteinase activity of mutant NS2B/3 proteins using transient expression of the pSV constructs in COS cells. We did not wish to investigate virus replication with mutations that abolished proteinase activity. Five constructs were tested. COS cells were transfected with the pSV plasmids listed in Fig. 1, radiolabelled and analysed by radioimmunoprecipitation and electrophoresis (Fig. 2). As reported previously (Teo & Wright, 1997), in cells transfected with the parental construct S2 and radiolabelled for 1 h at 37 °C, several polypeptides indicative of proteinase activity were detected using anti-NS3 antiserum (Fig. 2a, lane 3). They were uncleaved NS2B/3 (83 kDa) and

the cleavage products NS3 (69 kDa), NS2B/3' (64 kDa), NS3' (50 kDa) and NS3'' (19 kDa). The bands corresponding to NS2B/3' and NS3' were faint and not well-resolved from host proteins; however, bands of NS2B/3, NS3 and NS3'' were clear and sufficient for the assessment of proteolysis. NS2B (14 kDa) was detected by co-precipitation using anti-NS3 antiserum, as described for dengue virus and other flaviviruses (Arias *et al.*, 1993; Chambers *et al.*, 1993; Jan *et al.*, 1995; Teo & Wright, 1997). The number and sizes of the observed proteins demonstrated that cleavage was occurring at the NS2B/NS3 and NS3'/NS3'' sites. Proteinase activity was also detected in cells transfected by S₁₇₋₂₀, S₃₂₋₃₆, S₆₃₋₆₆ or S₁₇₉₋₁₈₁ and maintained at 37 °C (Fig. 2a, lanes 4–6, 8). However, little or no cleavage occurred in cells transfected with the construct S₉₅₋₉₆; only NS2B/3 was readily detected (Fig. 2a, lane 7).

To assess the effects of lower temperature on proteolysis, transfected cells were radiolabelled at 31 °C for 3 h. The results obtained at 31 °C (Fig. 2b) were similar to those observed at 37 °C (Fig. 2a). The presence of NS3, NS3', NS3'' and NS2B indicated that cleavage occurred at the NS2B/NS3 and NS3'/NS3'' sites for the parental construct (Fig. 2b, lane 3) and mutants S₁₇₋₂₀, S₃₂₋₃₆, S₆₃₋₆₆ and S₁₇₉₋₁₈₁ (Fig. 2b, lanes 4–6, 8). Again no significant proteolysis was detected for the mutant construct S₉₅₋₉₆ (Fig. 2b, lane 7).

For all mutants shown in Fig. 2, with the exception of S₉₅₋₉₆, NS2B was co-precipitated with NS3 by anti-NS3 antiserum. The band corresponding to this protein was faint for S₁₇₋₂₀ (Fig. 2, lanes 4), but was readily seen on longer exposure. Thus for these mutants, interaction between NS2B and NS3 was retained, consistent with the retention of proteinase activity.

Analysis of virus replication

Previous experiments showed that mutations that abolished or strongly reduced NS2B/3 proteinase activity usually prevented or greatly reduced virus replication (Nestorowicz *et al.*, 1994; Chambers *et al.*, 1993; Amberg & Rice, 1999), whereas mutations that retained activity generally allowed the recovery of infectious virus, albeit with reduced plaque titres and small plaque phenotypes (Nestorowicz *et al.*, 1994; Chambers *et al.*, 1995; Amberg & Rice, 1999). Hence five mutations were chosen for incorporation into genomic-length cDNA and examination of their effects on virus replication. All three of the charged-to-alanine mutants tested (S₁₇₋₂₀, S₆₃₋₆₆ and S₁₇₉₋₁₈₁) did not show severe inhibition of proteinase activity in COS cells. We selected the mutations in two of these (S₆₃₋₆₆ and S₁₇₉₋₁₈₁) for incorporation into genomic-length cDNA and added a further two of the charged-to-alanine-type mutations (Fig. 1) without prior testing in COS cells. For the mutations within hydrophobic regions (S₃₂₋₃₆ and S₉₅₋₉₆), only the changes of S₃₂₋₃₆ (cleavage of NS2B/3 detected) were incorporated into genomic-length cDNA.

Virus was produced from genomic-length cDNA by established procedures (Gualano *et al.*, 1998). RNA was

Table 1. Yields of mutant viruses

RNA was transcribed from genomic-length cDNA and electroporated into BHK-21 cells maintained at 33 °C or 37 °C. Virus produced at each temperature was passaged twice in C6/36 cells at 28 °C. Immunofluorescence in BHK-21 cells at 5–6 days post-electroporation was scored as 0 (–), 1–25% (+), 26–50% (++) , 51–75% (+++) or 76–100% (++++) positive cells. Plaque titres after passaging in C6/36 cells are expressed in p.f.u./ml \pm SD. Each virus was derived at least twice from RNA transcripts; therefore, the result shown for each virus is the average of two or more experiments. All samples testing positive by RT-PCR retained the required mutation and had no other changes in the NS2B/3 genes.

Mutation site	Virus*	Immunofluorescence	Virus titre (p.f.u./ml)	Approximate plaque size (mm)	RT-PCR
Parental	V2 33 °C	++++	$(1.1 \pm 0.1) \times 10^6$	4	Yes
	37 °C	++++	$(7.3 \pm 0.8) \times 10^6$	4	Yes
<u>GYSQI</u>	V ₃₂₋₃₆ 33 °C	+	$(3.0 \pm 0.9) \times 10^1$	1	Not
	37 °C	–	ND	–	Not
<u>KRIE</u>	V ₆₃₋₆₆ 33 °C	++++	$(4.6 \pm 0.9) \times 10^5$	1	Yes
	37 °C	+++	$(7.0 \pm 1.5) \times 10^5$	1	Yes
<u>EGEE</u>	V ₉₁₋₉₄ 33 °C	+	$(2.4 \pm 0.3) \times 10^3$	1	Yes
	37 °C	–	ND	–	Not
<u>EKSIE</u>	V ₁₆₉₋₁₇₃ 33 °C	++++	$(2.7 \pm 0.2) \times 10^6$	3	Yes
	37 °C	++++	$(2.2 \pm 0.5) \times 10^6$	3	Yes
<u>EDD</u>	V ₁₇₉₋₁₈₁ 33 °C	+++	$(4.9 \pm 1.0) \times 10^5$	1	Yes
	37 °C	+++	$(1.4 \pm 0.2) \times 10^6$	1	Yes

* BHK-21 cells were incubated at either 33 °C or 37 °C immediately after electroporation.

† RT-PCR product was not obtained due to low titre.

ND, Not detected.

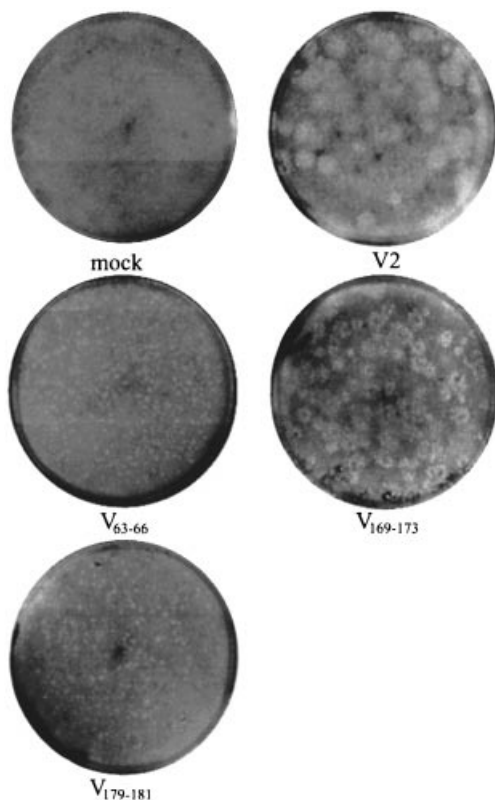


Fig. 3. Plaques of mutant viruses in C6/36 cells at 28 °C. The plaque morphologies of three mutant viruses compared with the parental virus V2 are shown.

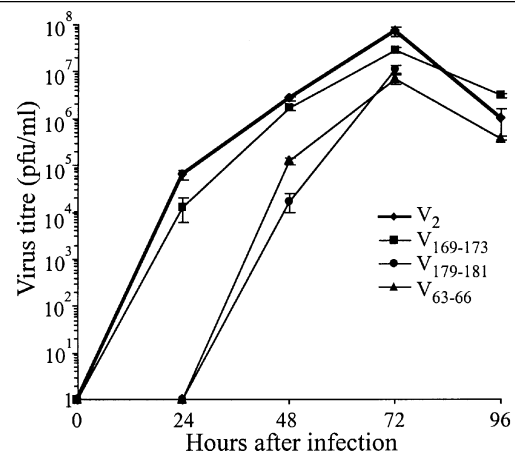


Fig. 4. Replication of mutant viruses in C6/36 cells at 28 °C. Cells were infected at an m.o.i. of 1 and the culture medium was sampled at the times indicated; virus titres were determined by plaque assay in C6/36 cells. The presence of the original mutations in the recovered viruses was confirmed by RT-PCR and sequencing.

transcribed and electroporated into BHK-21 cells and the cells were incubated at 33 °C or 37 °C. BHK-21 cells were tested for immunofluorescence with anti-E antibodies. Medium from the transfected BHK-21 cells was passaged twice in C6/36 cells at 28 °C and virus titre was determined after the second passage by plaque assay in C6/36 cells. Viral RNA was then amplified by RT-PCR and the complete NS2B and NS3 genes were sequenced to check that the mutation was retained during

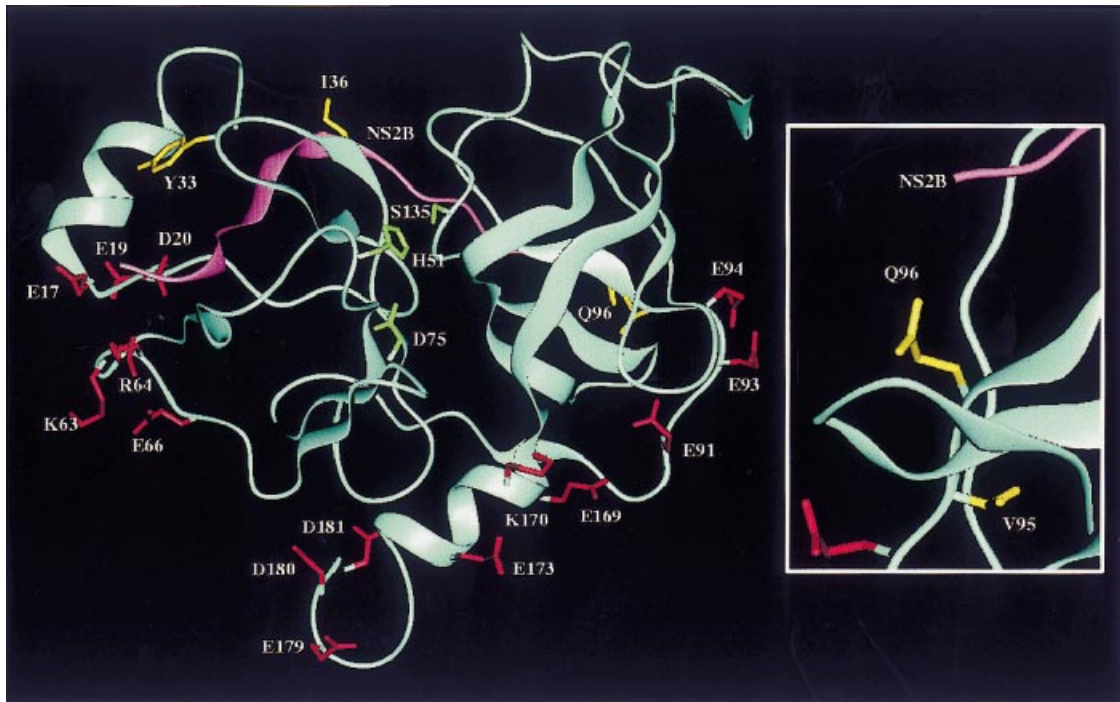


Fig. 5. X-ray crystal structure of the DEN-2 NS3 proteinase (aquamarine) with the NS2B peptide (pink) modelled into the binding cleft. The active site triad, H₅₁, D₇₅ and S₁₃₅, is shown in green stick. Charged regions that were mutated to alanine are labelled and in red. The two conserved hydrophobic regions that were mutated are shown in yellow. The inset shows the proximity of Q₉₆ to the C-terminal end of the NS2B peptide.

passaging and that no other base substitutions were introduced. Virus was derived at least twice for each construct and the results are summarized in Table 1. Virus titres were determined for each experiment and the mean \pm SD corresponding to each construct is shown. Similar results were obtained consistently for a given construct.

The parental (V2) and mutant (V₆₃₋₆₆, V₁₆₉₋₁₇₃ and V₁₇₉₋₁₈₁) viruses grew to comparable titres of 10⁵ to 10⁶ p.f.u./ml following initial electroporation at 33 °C or 37 °C (Table 1). All three mutant viruses showed a reduced plaque size, particularly V₆₃₋₆₆ and V₁₇₉₋₁₈₁ (Table 1, Fig. 3). For viruses V₃₂₋₃₆ and V₉₁₋₉₄, detectable virus was recovered only following electroporation at 33 °C and at low titres (3 \times 10¹ and 2.4 \times 10³ p.f.u./ml respectively). These results suggested that V₃₂₋₃₆ and V₉₁₋₉₄ were severely restricted in replication and were possibly heat-sensitive.

To examine the properties of these viruses further, more concentrated stocks were prepared by PEG precipitation. We wished to obtain sufficiently high titres to enable infection of cells at an m.o.i. of 1. This proved possible only for viruses V2, V₆₃₋₆₆, V₁₆₉₋₁₇₃ and V₁₇₉₋₁₈₁.

Growth of viruses V2, V₆₃₋₆₆, V₁₆₉₋₁₇₃ and V₁₇₉₋₁₈₁ in C6/36 and BHK-21 cells

C6/36 cells were infected with viruses V2, V₆₃₋₆₆, V₁₆₉₋₁₇₃ or V₁₇₉₋₁₈₁ at an m.o.i. of 1, maintained at 28 °C and the medium was sampled at 24 h intervals up to 96 h post-

infection. Virus titres were determined by plaque assay in C6/36 cells. The resulting curves of released virus are shown in Fig. 4. The two viruses with the smallest plaque size (V₆₃₋₆₆ and V₁₇₉₋₁₈₁, Table 1) initially lagged in virus release, although by 48 h after infection, their titres had reached from 1 to 8 \times 10⁴ p.f.u./ml, and by 72 h after infection, the yields of all four viruses were comparable (Fig. 4). The observed delay in virus release for V₆₃₋₆₆ and V₁₇₉₋₁₈₁ was consistent with their very small plaque size. The presence of the respective mutations in the recovered viruses was confirmed by RT-PCR and sequencing.

To analyse the effect of temperature on the replication of V2, V₆₃₋₆₆, V₁₆₉₋₁₇₃ and V₁₇₉₋₁₈₁ in a more rigorous manner than in the experiments summarized in Table 1, BHK-21 cells were infected at an m.o.i. of 1 and cells were incubated at 33 °C or 37 °C. The culture medium was sampled at 72 h after infection and virus titres were determined by plaque assay in C6/36 cells. All four viruses showed no significant temperature sensitivity, as defined by a 100-fold or greater difference in titre between temperatures (data not shown). The integrity of each mutation in recovered virus was reconfirmed by RT-PCR and sequencing.

Modelling the interaction of NS3 and NS2B

The 40 amino acid segment of NS2B required for the activity of the NS2B/3 proteinase is, overall, hydrophilic (L₅₃-E₉₂) and shares no significant similarity with a protein of

known structure. It contains a central hydrophobic region G₆₉SSPILSITISE₈₀ (Falgout *et al.*, 1993; Brinkworth *et al.*, 1999), which was identified as the probable homologue to the HCV NS4A peptide in the HCV NS3/4A proteinase and was used to construct an homology model of the DEN-2 NS3/2B proteinase (Brinkworth *et al.*, 1999). The homology model was based on the structure of the HCV complex of NS3 (N-terminal 179 amino acids) and NS4A (peptide G₂₁-R₃₄) (Yan *et al.*, 1998). However, an improved model for DEN-2 NS3/2B is now possible (Fig. 5) using the coordinates for the crystal structure of the N-terminal 185 amino acids of DEN-2 NS3 (Murthy *et al.*, 1999). This model is useful for analysing the interactions between the NS2B peptide G₆₉-E₈₀ and NS3, but further predictions on the effect of NS2B binding to the substrate-binding cleft or any direct interactions between NS2B and substrate would be overspeculative until the X-ray crystal structure of the NS2B/3 complex is determined.

Discussion

Seven sites distributed through the N-terminal proteinase region of DEN-2 NS3 and outside conserved enzyme motifs were mutated in these experiments. Five sites were rich in charged amino acids (Fig. 1) and were considered to be possible sites of interaction with the hydrophilic 40 amino acid fragment of NS2B that is essential for activity (Falgout *et al.*, 1993). The approach of charged-to-alanine mutagenesis was first used successfully with *Poliovirus* to isolate growth-restricted polymerase mutants (Diamond & Kirkegaard, 1994). Ten temperature-sensitive mutants were obtained following mutagenesis of 27 sites and the recovery of 12 viruses. Fewer mutants have been reported for other viruses following charged-to-alanine mutagenesis. Single temperature-sensitive mutants have been described for *Human immunodeficiency virus* with mutations located in the reverse transcriptase and integrase genes (Wiskerchen & Muesing, 1995; Huang *et al.*, 1998), and *Adeno-associated virus type 2* following mutagenesis of the Rep78/68 helicase genes (Gavin *et al.*, 1999). Mutagenesis of the polymerase subunit PB2 of *Influenza A virus* generated three temperature-sensitive mutants after mutagenesis of ten sites (Parkin *et al.*, 1996). The only reported growth-restricted mutant of a flavivirus obtained following charged-to-alanine mutagenesis was a temperature-sensitive *Yellow fever virus* (YFV) mutated in the NS1 protein (Muylaert *et al.*, 1997).

Of the five charged sites mutated to alanine in DEN-2 NS3, three were examined by transient expression in COS cells, four were tested for their effects on virus replication and three were tested by both methods. For no mutant tested was proteinase activity or virus production abolished. Mutations related, but not identical, to those at E₁₇LED₂₀, K₆₃RIE₆₆, E₉₁GEE₉₄ and E₁₆₉KSIE₁₇₃ were tested for their effect on the activity of YFV NS2B/3 proteinase (Droll *et al.*, 2000). No mutation reduced enzyme activity significantly except for those at E₂₁D₂₂ (YFV

numbering). However, for the DEN-2 mutants, the yield of virus V₉₁₋₉₄, and to a lesser extent V₆₃₋₆₆ (Table 1), were reduced compared with V₁₆₉₋₁₇₃ and V₁₇₉₋₁₈₁ and the parental virus V2. All mutant viruses showed reduced plaque size (Table 1, Fig. 3). The locations of the five charged sites were then mapped into a model of NS2B/3 (Fig. 5). The model is based on the crystal structure of DEN-2 NS3 proteinase (Murthy *et al.*, 1999) and a fragment of NS2B corresponding to the fragment of NS4A seen in the structure of HCV proteinase (Brinkworth *et al.*, 1999).

The X-ray crystal structure of DEN-2 NS3 reveals that region E₉₁GEE₉₄, mutated in the low-yielding virus V₉₁₋₉₄, does not form part of the active-site cleft, nor does it interact with the fragment of NS2B in the model. E₉₁ and E₉₃ form salt bridges to R₁₀₇. The loss of two salt bridges in the A₉₁GAA₉₄ mutation would be predicted to have a deleterious effect upon proteinase stability and possibly virus yield. However, the Q₉₆ residue (see below) does form part of the predicted NS2B-binding cleft and we cannot exclude the possibility that E₉₁GEE₉₄ interacts with full-length NS2B. In HCV, the loops equivalent to residues 90-94 and 140-145 in DEN-2 NS3 are linked by interactions with a zinc ion. Interestingly, in the DEN-2 NS3 structure, the primary interaction between these loops is a hydrogen bond between the carbonyl oxygen of E₉₄ and the side chain of K₁₄₂. These data conflict with the prediction of Brinkworth *et al.* (1999) that E₉₃ forms a salt bridge with K₁₄₅. However, this prediction was based on an homology model developed from the HCV NS3 protein X-ray crystal structure before the availability of the DEN-2 NS3 proteinase structure.

The residues K₆₃RIE₆₆ (virus V₆₃₋₆₆) and E₁₇LED₂₀ (mutation not tested in virus) are both located at the N-terminal end of the NS2B-binding cleft. K₆₃, R₆₄ and E₆₆ are solvent-exposed residues located at one end of the NS2B-binding cleft. The model of the NS3-NS2B complex predicts that R₆₄ makes a hydrogen bond to the carbonyl oxygen of the first residue in the NS2B peptide. We predict that more extensive interactions between R₆₄ and E₆₆ may be made with the full-length NS2B protein. The disruption of any one of these interactions, either alone or in combination, may explain the observed reduction in yield of virus V₆₃₋₆₆. The residues E₁₇LED₂₀ line the N-terminal end of the NS2B-binding cleft. We predict that E₁₇ directly interacts with the carbonyl oxygen of G₁ in the NS2B peptide.

The residues E₁₆₉KSIE₁₇₃ and E₁₇₉DD₁₈₁ lie at the C terminus of the proteinase at its junction with the helicase domain of NS3. Both sites are excluded from the minimal proteinase domain, defined as the N-terminal 167 amino acids of NS3 (Li *et al.*, 1999) using *in vitro* transcription and translation. Residues E₁₆₉KSIE₁₇₃ form an α -helix (Fig. 5) and the individual residues form hydrogen bonds with solvent molecules, apart from K₁₇₀, which forms a hydrogen bond to the carbonyl oxygen of E₁₆₇. These residues are located at the end of the substrate-binding cleft (on the P side; Schechter & Berger, 1967) and thus may be important for determining

substrate specificity. For E₁₇₉DD₁₈₁, the structure of NS3 reveals that D₁₈₀ forms a hydrogen bond to the side chain of W₆₉. The interaction with W₆₉ is of particular interest, as this residue is located six residues N-terminal to the catalytic D₇₅. Disruption of this hydrogen bond by the introduction of an alanine at position 180 may affect the conformation of the β -strand containing the catalytic aspartic acid and thus may impair proteinase activity.

In addition to the mutagenesis of the five charged sites, substitutions were made in two hydrophobic regions, G₃₂YSQI₃₆ and V₉₅Q₉₆. The residues G₃₂ and V₉₅Q₉₆ lie outside the enzyme motifs but are highly conserved in members of the genus *Flavivirus* (Chang, 1997). The protein S₃₂₋₃₆ had autocatalytic activity (Fig. 2, lanes 4), but the yield of V₃₂₋₃₆ was the lowest noted. G₃₂ and Y₃₃ line the N-terminal end of the NS2B-binding cleft. Interestingly, the crystal structure of NS3 reveals that Y₃₃ bridges across the cleft, forming a hydrogen bond to the carbonyl oxygen of P₁₀. We would expect a mutation at this position to affect NS2B binding. G₃₂ forms part of a pocket that contains S₃ from the NS2B peptide. We predict that mutation of this residue will affect the size of this pocket.

The mutations at V₉₅Q₉₆ are of particular interest as they are located at the C terminus of the NS2B-binding cleft. Substitution of these residues by alanine severely reduced self-cleavage of the S₉₅₋₉₆ protein (Fig. 2, lanes 7). Examination of the structure of NS3 reveals that V₉₅ is buried in the hydrophobic core of the proteinase and that Q₉₆ is solvent-exposed. In our model of NS3 complexed with the NS2B peptide, Q₉₆ is directly beneath the C terminus of the NS2B peptide (Fig. 5) and forms part of the binding cleft. The inability of the S₉₅₋₉₆ protein to self cleave suggests that mutation of these residues may affect the pre-cleavage interaction between NS3 and the NS2B co-factor and prevent proper processing at the NS2B/NS3 cleavage site.

Overall, the seven mutated sites were distributed evenly over the primary sequence of the NS3 proteinase and represented distinct regions in the model of NS3 complexed with the NS2B co-factor peptide. Of the mutations located, three (E₁₇LED₂₀, K₆₃RIE₆₆ and G₃₂YSQI₃₆) were at the N terminus of the NS2B-binding cleft, one (V₉₅Q₉₆) was at the C terminus of the cleft, two (E₁₆₉KSIE₁₇₃ and E₁₇₉DD₁₈₁) were at the C terminus of the proteinase domain and one (E₉₁GEE₉₄) was solvent-exposed. Thus, two of the charged regions (E₁₇LED₂₀ and K₆₃RIE₆₆) were adjacent to the NS2B-binding cleft. At present, it is unknown whether any of the other three charged regions interact with full-length NS2B. It is also possible that the basis for their effect on virus replication is unrelated to proteinase activity and may lie, for example, in the interaction of NS3 with other viral proteins such as NS5 (Kapoor *et al.*, 1995; Chen *et al.*, 1997). Substitutions to alanine in conserved hydrophobic regions were more disruptive to self-cleavage (protein S₉₅₋₉₆) and virus production (virus V₃₂₋₃₆) than to changes in charged regions. A total of five

viruses with reduced plaque size on C6/36 cells was obtained; two of these, V₃₂₋₃₆ and V₉₁₋₉₄, were possibly temperature-sensitive but did not grow sufficiently well for adequate testing (Table 1). The remaining three viruses grew to reach good titres (Fig. 4) and displayed small plaques but did not show the temperature-sensitive phenotype that has been observed for some viruses with charged-to-alanine mutations in non-structural genes (Diamond & Kirkegaard, 1994; Parkin *et al.*, 1996; Muylaert *et al.*, 1997; Huang *et al.*, 1998; Gavin *et al.*, 1999). Virus V₃₂₋₃₆ replicated too poorly to be of further use and therefore the viruses of most interest with respect to growth restriction were V₆₃₋₆₆ and V₉₁₋₉₄. Both viruses contained mutations in charged amino acids, showed small plaque phenotypes and replicated less well than parental virus (V₆₃₋₆₆ only marginally less). The mutations contained in these viruses may be suitable for incorporation into growth-restricted vaccine strains. It may be possible to enhance the yield of V₉₁₋₉₄ by reducing the number of charged residues changed to alanine in the sequence E₉₁GEE₉₄ while retaining some growth restriction and a small plaque phenotype.

The results demonstrate that charged-to-alanine mutagenesis may be useful for obtaining growth-restricted viruses of other flavivirus species, either with mutations in the proteinase region or perhaps in other non-structural proteins. In our studies with DEN-2 NS3, we recovered infectious virus for all four of the charged-to-alanine mutants tested and the viruses displayed a useful range of growth restriction. Comparisons of the deduced amino acid sequences of flaviviruses show high conservation of hydrophilicity across the viral polyprotein, regardless of the considerable variation in primary sequence (Westaway & Blok, 1997) and thus it may be possible to extend these results to the other dengue virus serotypes and encephalitic flaviviruses. The mutations that were introduced here required multiple nucleotide and codon changes. In theory, multiple changes reduce the risk of reversion to parental phenotype when introduced into a potential vaccine strain. However, it would be necessary initially to assess each amino acid mutated in a cluster for its contribution to the mutant phenotype. The preferred situation is for each amino acid to make some contribution, rather than for one to be dominant.

The model of the NS3 complexed with an NS2B peptide co-factor enabled the definition of some individual residues important in the interaction between the two proteins. We predict that substitutions of these residues by amino acids other than alanine, both individually and in clusters, will confirm these interactions and expand our understanding of the flavivirus proteinase.

This work was supported by grants from the National Health and Medical Research Council of Australia and the World Health Organization Global Programme for Vaccines and Immunization. J.C. Whisstock is a Peter Doherty Research Fellow of the National Health and Medical Research Council of Australia.

References

- Amberg, S. M. & Rice, C. M. (1999).** Mutagenesis of the NS2B–NS3-mediated cleavage site in the flavivirus capsid protein demonstrates a requirement for coordinated processing. *Journal of Virology* **73**, 8083–8094.
- Arias, C. F., Preugschat, F. & Strauss, J. H. (1993).** Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. *Virology* **193**, 888–899.
- Bass, S. H., Mulkerin, M. G. & Wells, J. A. (1991).** A systematic mutational analysis of hormone-binding determinants in the human growth hormone receptor. *Proceedings of the National Academy of Sciences, USA* **88**, 4498–4502.
- Bazan, J. F. & Fletterick, R. J. (1989).** Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses. *Virology* **171**, 637–639.
- Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. & Bourne, P. E. (2000).** The Protein Data Bank. *Nucleic Acids Research* **28**, 235–242.
- Bernstein, F. C., Koetzle, T. F., Williams, G. J., Meyer, E. F., Jr, Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T. & Tasumi, M. (1977).** The Protein Data Bank. A computer-based archival file for macromolecular structures. *European Journal of Biochemistry* **80**, 319–324.
- Brinkworth, R. I., Fairlie, D. P., Leung, D. & Young, P. R. (1999).** Homology model of the dengue 2 virus NS3 protease: putative interactions with both substrate and NS2B cofactor. *Journal of General Virology* **80**, 1167–1177.
- Chambers, T. J., Weir, R. C., Grakoui, A., McCourt, D. W., Bazan, J. F., Fletterick, R. J. & Rice, C. M. (1990).** Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein. *Proceedings of the National Academy of Sciences, USA* **87**, 8898–8902.
- Chambers, T. J., Nestorowicz, A., Amberg, S. M. & Rice, C. M. (1993).** Mutagenesis of the yellow fever virus NS2B protein: effects on proteolytic processing, NS2B–NS3 complex formation, and viral replication. *Journal of Virology* **67**, 6797–6807.
- Chambers, T. J., Nestorowicz, A. & Rice, C. M. (1995).** Mutagenesis of the yellow fever virus NS2B/3 cleavage site: determinants of cleavage site specificity and effects on polyprotein processing and viral replication. *Journal of Virology* **69**, 1600–1605.
- Chang, G.-J. (1997).** Molecular biology of dengue viruses. In *Dengue and Dengue Hemorrhagic Fever*, pp. 175–198. Edited by D. J. Gubler & G. Kuno. Wallingford: CAB International.
- Chen, C. J., Kuo, M. D., Chien, L. J., Hsu, S. L., Wang, Y. M. & Lin, J. H. (1997).** RNA–protein interactions: involvement of NS3, NS5, and 3′ noncoding regions of Japanese encephalitis virus genomic RNA. *Journal of Virology* **71**, 3466–3473.
- Cunningham, B. C. & Wells, J. A. (1989).** High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science* **244**, 1081–1085.
- Della-Porta, A. J. & Westaway, E. G. (1972).** Rapid preparation of hemagglutinins of togaviruses from infected cell culture fluids. *Applied Microbiology* **23**, 158–160.
- Diamond, S. E. & Kirkegaard, K. (1994).** Clustered charged-to-alanine mutagenesis of poliovirus RNA-dependent RNA polymerase yields multiple temperature-sensitive mutants defective in RNA synthesis. *Journal of Virology* **68**, 863–876.
- Droll, D. A., Murthy, H. M. K. & Chambers, T. J. (2000).** Yellow fever virus NS2B–NS3 protease: charged-to-alanine mutagenesis and deletion analysis define regions important for protease complex formation and function. *Virology* **275**, 335–347.
- Failla, C., Tomei, L. & DeFrancesco, R. (1995).** An amino-terminal domain of the hepatitis C virus NS3 protease is essential for interaction with NS4A. *Journal of Virology* **69**, 1769–1777.
- Falgout, B., Pethel, M., Zhang, Y. M. & Lai, C. J. (1991).** Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. *Journal of Virology* **65**, 2467–2475.
- Falgout, B., Miller, R. H. & Lai, C. J. (1993).** Deletion analysis of dengue virus type 4 nonstructural protein NS2B: identification of a domain required for NS2B–NS3 protease activity. *Journal of Virology* **67**, 2034–2042.
- Gavin, D. K., Young, S. M., Jr, Xiao, W., Temple, B., Abernathy, C. R., Pereira, D. J., Muzyczka, N. & Samulski, R. J. (1999).** Charge-to-alanine mutagenesis of the adeno-associated virus type 2 Rep78/68 proteins yields temperature-sensitive and magnesium-dependent variants. *Journal of Virology* **73**, 9433–9445.
- Gruenberg, A. & Wright, P. J. (1992).** Processing of dengue virus type 2 structural proteins containing deletions in hydrophobic domains. *Archives of Virology* **122**, 77–94.
- Gualano, R. C., Pryor, M. J., Cauchi, M. R., Wright, P. J. & Davidson, A. D. (1998).** Identification of a major determinant of mouse neurovirulence of dengue virus type 2 using stably cloned genomic-length cDNA. *Journal of General Virology* **79**, 437–446.
- Hahn, Y. S., Galler, R., Hunkapiller, T., Dalrymple, J. M., Strauss, J. H. & Strauss, E. G. (1988).** Nucleotide sequence of dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. *Virology* **162**, 167–180.
- Halstead, S. B. (1988).** Pathogenesis of dengue: challenges to molecular biology. *Science* **239**, 476–481.
- Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989).** Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51–59.
- Huang, M., Zensen, R., Cho, M. & Martin, M. A. (1998).** Construction and characterization of a temperature-sensitive human immunodeficiency virus type 1 reverse transcriptase mutant. *Journal of Virology* **72**, 2047–2054.
- Irie, K., Mohan, P. M., Sasaguri, Y., Putnak, R. & Padmanabhan, R. (1989).** Sequence analysis of cloned dengue virus type 2 genome (New Guinea-C strain). *Gene* **75**, 197–211.
- Jan, L.-R., Yang, C.-S., Trent, D. W., Falgout, B. & Lai, C.-J. (1995).** Processing of Japanese encephalitis virus non-structural proteins: NS2B–NS3 complex and heterologous proteases. *Journal of General Virology* **76**, 573–580.
- Kapoor, M., Zhang, L. W., Ramachandra, M., Kusukawa, J., Ebner, K. E. & Padmanabhan, R. (1995).** Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5. *Journal of Biological Chemistry* **270**, 19100–19106.
- Li, H. T., Clum, S., You, S. H., Ebner, K. E. & Padmanabhan, R. (1999).** The serine protease and RNA-stimulated nucleoside triphosphatase and RNA helicase functional domains of dengue virus type 2 NS3 converge within a region of 20 amino acids. *Journal of Virology* **73**, 3108–3116.
- Lin, C., Thomson, J. A. & Rice, C. M. (1995).** A central region in the hepatitis C virus NS4A protein allows formation of an active NS3–NS4A serine proteinase complex *in vivo* and *in vitro*. *Journal of Virology* **69**, 4373–4380.
- Monath, T. P. (1994).** Dengue: the risk to developed and developing countries. *Proceedings of the National Academy of Sciences, USA* **91**, 2395–2400.

- Murthy, H., Clum, S. & Padmanabhan, R. (1999).** Dengue virus NS3 serine protease: crystal structure and insights into interaction of the active site with substrates by molecular modeling and structural analysis of mutational effects. *Journal of Biological Chemistry* **274**, 5573–5580.
- Muylaert, I. R., Galler, R. & Rice, C. M. (1997).** Genetic analysis of the yellow fever virus NS1 protein: identification of a temperature-sensitive mutation which blocks RNA accumulation. *Journal of Virology* **71**, 291–298.
- Nestorowicz, A., Chambers, T. J. & Rice, C. M. (1994).** Mutagenesis of the yellow fever virus NS2A/2B cleavage site: effects on proteolytic processing, viral replication, and evidence for alternative processing of the NS2A protein. *Virology* **199**, 114–123.
- Parkin, N. T., Chiu, P. & Coelingh, K. L. (1996).** Temperature-sensitive mutants of influenza A virus generated by reverse genetics and clustered charged to alanine mutagenesis. *Virus Research* **46**, 31–44.
- Preugschat, F., Yao, C. W. & Strauss, J. H. (1990).** *In vitro* processing of dengue virus type 2 nonstructural proteins NS2A, NS2B, and NS3. *Journal of Virology* **64**, 4364–4374.
- Pryor, M. J. & Wright, P. J. (1993).** The effects of site-directed mutagenesis on the dimerization and secretion of the NS1 protein specified by dengue virus. *Virology* **194**, 769–780.
- Pryor, M. J., Gualano, R. C., Lin, B., Davidson, A. D. & Wright, P. J. (1998).** Growth restriction of dengue virus type 2 by site-specific mutagenesis of virus-encoded glycoproteins. *Journal of General Virology* **79**, 2631–2639.
- 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: Lippincott–Raven.
- Schechter, I. & Berger, A. (1967).** On the size of the active site in proteases. I. Papain. *Biochemical and Biophysical Research Communications* **27**, 157–162.
- Teo, K. F. & Wright, P. J. (1997).** Internal proteolysis of the NS3 protein specified by dengue virus 2. *Journal of General Virology* **78**, 337–341.
- Valle, R. & Falgout, B. (1998).** Mutagenesis of the NS3 protease of dengue virus type 2. *Journal of Virology* **72**, 624–632.
- van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeoch, D. J., Pringle, C. R. & Wickner, R. B. (2000).** *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses.* San Diego: Academic Press.
- Westaway, E. G. & Blok, J. (1997).** Taxonomy and evolutionary relationships of flaviviruses. In *Dengue and Dengue Hemorrhagic Fever*, pp. 147–173. Edited by D. J. Gubler & G. Kuno. Wallingford: CAB International.
- Wiskerchen, M. & Muesing, M. A. (1995).** Identification and characterization of a temperature-sensitive mutant of human immunodeficiency virus type 1 by alanine scanning mutagenesis of the integrase gene. *Journal of Virology* **69**, 597–601.
- Yan, Y. W., Li, Y., Munshi, S., Sardana, V., Cole, J. L., Sardana, M., Steinkuehler, C., Tomei, L., Defrancesco, R., Kuo, L. C. & Chen, Z. G. (1998).** Complex of NS3 protease and NS4A peptide of BK strain hepatitis C virus: a 2.2 Å resolution structure in a hexagonal crystal form. *Protein Science* **7**, 837–847.
- Yusoff, R., Clum, S., Wetzel, M., Murthy, H. M. K. & Padmanabhan, R. (2000).** Purified NS2B/NS3 serine proteinase of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids *in vitro*. *Journal of Biological Chemistry* **275**, 9963–9969.

Received 14 December 2000; Accepted 14 March 2001