

## Growth restriction of dengue virus type 2 by site-specific mutagenesis of virus-encoded glycoproteins

Melinda J. Pryor, Rosa C. Gualano, Bo Lin,† Andrew D. Davidson and Peter J. Wright

Department of Microbiology, Monash University, Clayton 3168, Victoria, Australia

The three flavivirus glycoproteins prM, E and NS1 are formed by post-translational cleavage and are glycosylated by the addition of *N*-linked glycans. NS1 may form homodimers, whereas E may form homodimers, homotrimers or heterodimers (prM–E). Modification of these processes by mutagenesis of the proteins has the potential to generate viruses that are restricted in growth and are possible vaccine candidates. Using an SV40-based expression system, we previously analysed dimerization and secretion of the NS1 protein of dengue virus type 2 (DEN-2) with mutations in the conserved Cys residues, or within hydrophilic or hydrophobic regions, or at glycosylation sites. In this study, mutations which reduce cleavage at the DEN-2 prM/E signalase cleavage site are described. On the

basis of earlier and current results with transient expression, six mutations which reduced NS1 dimerization and two mutations which inhibited prM/E cleavage were analysed individually for their effects on virus growth using a genomic length cDNA clone. Two viruses were obtained that showed reduced growth in cell culture and attenuation of neurovirulence when inoculated into 3-day-old mice. One of these viruses encoded NS1 that lacked the second glycosylation site, the other encoded a Ser → Ile change at the -3 position of the prM/E cleavage site. A third virus encoding a mutation in NS1 within a hydrophilic region grew as well as the parental virus. No virus was detected for the remaining five mutations.

### Introduction

The four serotypes of dengue virus (DEN-1 to DEN-4) form a single antigenic complex within the family *Flaviviridae* (Calisher *et al.*, 1989). Dengue fever (DF) and the more severe dengue haemorrhagic fever (DHF) are serious arthropod-borne human diseases. The *Aedes aegypti* vector is found throughout tropical and subtropical regions, where more than half the world's human population lives (Halstead, 1988). It has been estimated that 80–100 million dengue infections occur annually (Halstead, 1988; Monath, 1994). As there is no effective vaccine and mosquito control programs are difficult to implement and sustain, the incidence and geographic distribution of DF/DHF is increasing (Monath, 1994).

The genome of dengue virus and other flaviviruses consists of a single strand of positive-sense RNA, which is 10·5–11 kb in length. The viral genome encodes three structural and seven nonstructural proteins which are cleaved from a polyprotein

precursor by host- and virus-encoded proteases (reviewed by Rice, 1996). The three viral proteins that have *N*-linked glycans occur consecutively in the polyprotein. These are prM (precursor to the membrane protein M), E (the envelope protein) and the nonstructural protein NS1. The cleavages that produce the N termini of these proteins are mediated by the host signalase enzyme. The C termini of prM and E are also generated by signalase cleavage. Cleavage at the C terminus of NS1 occurs at a site that meets the requirements for signalase cleavage, but lacks an upstream membrane anchor (Wright *et al.*, 1989). It appears that this cleavage is effected by a host protease, which may actually be signalase, in the endoplasmic reticulum (Falgout & Markoff, 1995).

Details on the further maturation and function of the three flavivirus glycoproteins are accumulating. The prM glycoprotein is cleaved to produce the mature M protein just before or at the time of release of virions from cells (Murray *et al.*, 1993). This internal cleavage of prM is effected by the host protease furin (Stadler *et al.*, 1997). The prM and E proteins form heterodimers in cell-associated virions (Wengler & Wengler, 1989). This is proposed to prevent premature acid-induced conformational changes in the E protein during virion maturation in the *trans*-Golgi network and secretory vesicles

**Author for correspondence:** Peter Wright.

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

† **Present address:** Macfarlane Burnet Centre for Medical Research, Yarra Bend Road, Fairfield 3078, Victoria, Australia.

(Heinz *et al.*, 1994). The E protein mediates binding of virus to host cell receptor(s) (Anderson *et al.*, 1992; Chen *et al.*, 1996). The three-dimensional structure of a soluble fragment of the E protein of tick-borne encephalitis (TBE) virus has been determined (Rey *et al.*, 1995). Single mutations in the E protein can have significant effects on the tropism and growth of flaviviruses (Holzmann *et al.*, 1990; Gualano *et al.*, 1998). The NS1 glycoprotein is associated with both intracellular membranes and the plasma membrane (Stohlman *et al.*, 1975; Westaway & Goodman, 1987). Newly synthesized monomeric NS1 is converted to a dimeric form, which may facilitate membrane association of NS1 (Winkler *et al.*, 1989). NS1 dimers are secreted from cells infected with DEN-2 (Winkler *et al.*, 1989) or transiently expressing the NS1 gene of DEN-2 (Pryor & Wright, 1993). Results obtained by immunoelectron microscopy of DEN-2-infected cells (Mackenzie *et al.*, 1996) and studies with a temperature-sensitive mutant of yellow fever (YF) virus (Muylaert *et al.*, 1997) suggest a possible role for NS1 in viral RNA replication.

For a number of flaviviruses, including the dengue viruses, genomic length cDNA clones have been constructed which can be transcribed to yield infectious RNA. This has greatly facilitated the study of flavivirus replication and protein function in infected cells. Genomic length dengue virus cDNA yielding infectious RNA is available for the New Guinea C (NGC) strain of DEN-2 (Kapoor *et al.*, 1995; Polo *et al.*, 1997; Gualano *et al.*, 1998), the DEN-2 16681 strain and its PDK-53 vaccine derivative (Kinney *et al.*, 1997), and the 814669 strain of DEN-4 (Lai *et al.*, 1991). It is now feasible to consider the production of genetically defined and growth-impaired viruses, which may be useful as live vaccines. Post-translational cleavage and *N*-linked glycosylation of the flavivirus polyprotein results in the formation of the glycoproteins prM, E and NS1 which may then associate as prM-E heterodimers and NS1 homodimers. These processes are potential targets for modification by mutagenesis with the aim of restricting, but not preventing, virus growth.

In this study, we describe mutagenesis at the prM/E signalase cleavage site, at two Cys residues and one hydrophilic region within NS1, and at both NS1 glycosylation sites. Proteins with these mutations were initially characterized using transient expression systems, followed by analysis of the effects of selected mutations on virus growth using cloned genomic length DEN-2 cDNA.

## Methods

■ **Cells and viruses.** BHK-21 and *Aedes albopictus* C6/36 cells were grown at 37 and 30 °C respectively, in Eagle's basal medium containing 7.5% heat-inactivated foetal calf serum (FCS). COS cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. Stocks of DEN-2 viruses were grown and titred by plaque assay in C6/36 cells as described previously (Gualano *et al.*, 1998). Concentrated stocks of some viruses were produced by precipitation with polyethylene glycol (Della-Porta & Westaway, 1972).

■ **Constructs for transient expression of DEN-2 proteins.** The SV40-based expression vector pcEXV-1 (Miller & Germain, 1986) was used to construct pcEXV-37. The pcEXV-37 construct contains cDNA corresponding to the last 8 nt of the DEN-2 5' UTR, all of the C, prM and E genes, and the first 56 nt of the NS1 gene (Gruenberg & Wright, 1992; Lin *et al.*, 1994). Twelve mutations were introduced into the prM/E signalase cleavage site by overlap extension PCR (OL-PCR) (Ho *et al.*, 1989). PCR fragments encoding these mutations were cloned into pcEXV-37 as 441 nt *Bst*EII<sup>585</sup>-*Bsu*36I<sup>1026</sup> fragments (numbering follows Gruenberg *et al.*, 1988).

Plasmids were transfected into COS cells by the DEAE-dextran method (Pryor & Wright, 1993). Synthesis of prM-E polyproteins was assessed by radioimmunoprecipitation (RIP). Cells were labelled with L-[<sup>35</sup>S]methionine (ICN; specific activity 1175 Ci/mmol) for 30 min at 72 h post transfection (Pryor & Wright, 1993), and proteins were immunoprecipitated using a mixture of anti-E monoclonal antibodies (Gruenberg & Wright, 1992).

■ **Insertion of mutations into genomic length DEN-2 cDNA clones.** The genomic length DEN-2 cDNA clones pDVWS310 and pDVWS501 were described in detail by Gualano *et al.* (1998). Briefly, pDVWS310 contains 1967 nt derived from the PUO-218 strain of DEN-2 (Gruenberg *et al.*, 1988), and the remainder of the genome is from the NGC strain of DEN-2. The PUO-218 region spans the last 36 nt of the C gene, all of the prM gene and all but the last 51 nt of the E gene. The pDVWS501 clone consists entirely of NGC DEN-2 cDNA. A range of mutations was characterized in transient expression systems, and then selected mutations were analysed using genomic length DEN-2 cDNA. cDNA encoding mutations in the prM/E cleavage site (Ser → Ile at the -3 position and Thr → Leu at -1; residues 164 and 166 in prM, respectively) were subcloned from the pcEXV-37 constructs as 724 nt *Bst*EII<sup>660</sup>-*Sph*I<sup>1384</sup> fragments (this and all subsequent numbering follows that of Irie *et al.*, 1989), and ligated into pDVWS310 digested with *Bst*EII<sup>660</sup> and *Sph*I<sup>1384</sup>. A 1212 nt OL-PCR fragment encoding the Ser → Ile change was ligated into pDVWS501 digested with *Bsr*GI<sup>172</sup> and *Sph*I<sup>1384</sup>.

Mutations in the NS1 gene that had been previously characterized in transient expression assays were cloned into pDVWS310 by removing a 1209 nt *Spe*I<sup>2370</sup>-*Spe*I<sup>3579</sup> fragment from the appropriate pSV.SPORT 1 clone (Pryor & Wright, 1993) and ligating this into partially *Spe*I-digested pDVWS310. Mutations in NS1 that were chosen for analysis in the pDVWS310 system were: (i) replacement of the fourth or eighth Cys residues (Cys-143 and Cys-291) by Ala; (ii) replacement of residues Glu-173 and Lys-174 (a hydrophilic region, designated IL2) (Pryor & Wright, 1993) by Ala; and (iii) removal of one or both glycosylation sites by replacement of Asn-130 and Asn-207 with Ala (Pryor & Wright, 1994). The latter constructs are referred to as G<sub>1</sub><sup>-</sup>, G<sub>2</sub><sup>-</sup> or G<sub>1,2</sub><sup>-</sup>, respectively. For insertion of cDNA encoding the IL2 and G<sub>2</sub><sup>-</sup> mutations into pDVWS501, 2273 nt OL-PCR fragments were ligated into pDVWS501 digested with *Nhe*I<sup>2427</sup> and *Nsi*I<sup>4700</sup>. The PCR-derived regions of all clones were sequenced.

■ **Production of virus from genomic length cDNA clones.** Procedures for transcription of RNA, electroporation and immunofluorescence (IF) of BHK-21 cells and passaging of virus in C6/36 cells have been described previously (Gualano *et al.*, 1998). Briefly, genomic length cDNA clones were linearized with *Xba*I and capped transcripts were produced using the Promega RiboMAX kit. Subconfluent BHK-21 cells were trypsinized, washed in PBS, and mixed with 7–10 µg transcribed viral RNA and 50 µg carrier yeast tRNA in a 0.4 cm cuvette. The cells were electroporated using GenePulser I apparatus (BIO-RAD), which was set to deliver a single pulse at 500 µF, 300–350 V. IF of electroporated BHK-21 cells was determined at 4–6 days post-

electroporation with a mixture of anti-E monoclonal antibodies (Gruenberg & Wright, 1992). Cytopathic effects (CPE) were visible in BHK-21 cells at 7 days post-electroporation. At this time, medium from BHK-21 cells was collected and passaged twice in C6/36 cells. Virus stocks were titred by plaque assay in C6/36 cells.

To confirm that the desired mutation was still present, total RNA was extracted from infected C6/36 cells and mouse brain (where appropriate) by the acid-guanidium isothiocyanate method of Lewis *et al.* (1992). RT-PCR of viral RNA was performed (Gualano *et al.*, 1998) and the products were directly sequenced over the region spanning the mutation.

■ **Neurovirulence tests of mutant DEN-2 viruses.** The neurovirulence of recombinant DEN-2 viruses was tested by intracerebral inoculation of 3-day-old BALB/c mice (Gualano *et al.*, 1998). Mice were checked daily for 21 days for signs of encephalitis, and severely paralysed mice were killed by cervical dislocation.

■ **Detection of NS1 dimers by immunoblotting.** For analysis of the dimerization properties of NS1 mutants, BHK-21 cells were infected with MON501, 501NS1-G<sub>2</sub><sup>-</sup> or 501NS1-IL2 at an m.o.i. of approximately 20. The cells were harvested at 48 h post-infection, when CPE had just become apparent. The stability of mutant dimers was determined by incubating the cell lysate at 37 °C for 1 h, followed by immunoblotting as previously described (Pryor & Wright, 1993), except that ECL detection reagents (Amersham) were used.

■ **Radiolabelling of infected cells.** BHK-21 cells were infected with MON501 or 501prM-S164I at an m.o.i. of approximately 20. At 48 h post-infection, cells were washed in PBS and incubated for 3 h in DMEM containing 1/100th the normal concentration of methionine and 1 µg/ml actinomycin D. The cells were labelled for 7 min at 37 °C with 200 µCi/ml L-[<sup>35</sup>S]methionine, after which the cells were immediately harvested or chased for 10 min at 37 °C in medium containing a 10-fold excess of cold methionine. Radiolabelled cell lysates were immunoprecipitated with anti-E monoclonal antibodies (Gruenberg & Wright, 1992).

## Results

### Mutagenesis of the prM/E cleavage site and selection of mutations for inclusion in recombinant virus

The prM/E cleavage is mediated by a host signalase enzyme (Ruiz-Linares *et al.*, 1989; Gruenberg & Wright, 1992). The signalase recognition site has been well characterized (von Heijne, 1986). The most critical positions are those at -3 and -1 relative to the site of cleavage. For sites that follow the (-3, -1) model, the residue at -3 is not aromatic, charged, large or polar, whereas the residue at -1 is generally small (von Heijne, 1986).

In this study, mutations predicted to impair signalase cleavage (and hence virus production) were analysed in a transient expression system. Fig. 1 shows the amino acids found at the -4 to +2 positions of flavivirus prM/E cleavage sites, as well as the 12 mutations made to three positions of the DEN-2 prM/E cleavage site. We predicted that the majority of the changes would modify cleavage for the following reasons. The Pro residue at -4 is strictly conserved amongst all flaviviruses. The Ser at -3 is conserved in DEN viruses of all serotypes but not in other flaviviruses, where Ala or Val are usually found. The residue at -1 in flaviviruses is small, and in DEN viruses it is Thr, Ala or Gly.

	-4	-3	-2	-1		+1	+2
WN	P	A	Y	S	↓	F	N
MVE	.	.	.	.	↓	.	.
JE	.	.	.	.	↓	.	.
KUN	.	.	.	.	↓	.	.
YF	.	.	.	.	↓	A	H
TBE	.	V	Y	A	↓	S	R
DEN-1	.	S	M	A	↓	M	R
DEN-3	.	S	M	T	↓	M	R
DEN-4	.	S	Y	G	↓	M	R
<b>DEN-2</b>	.	S	M	T	↓	M	R
	↓	↓		↓			
	I	I		L			
	T	T		P			
	N	K		Q			
	S	R		R			

Fig. 1. Flavivirus prM/E cleavage sites. The sequence reads from the -4 to the +2 position; the arrow denotes the site of cleavage. Virus abbreviations are as follows (GenBank accession numbers are given in parentheses): WN, West Nile virus (M10103); MVE, Murray Valley encephalitis virus (M24220); JE, Japanese encephalitis virus (M18370); KUN, Kunjin virus (D00246); YF, yellow fever virus (K02749); TBE, tick-borne encephalitis virus (M33668); DEN-1, dengue 1 virus (M23027); DEN-3, dengue 3 virus (M93130); DEN-4, dengue 4 virus (M17255); and DEN-2, dengue 2 virus (M19727). The twelve mutations made to the -4, -3 and -1 positions of the DEN-2 prM/E cleavage site are shown.

Mutations were introduced into the pcEXV-37 construct, which contains the DEN-2 C, prM and E genes. COS cells were transiently transfected with the mutagenized constructs and proteins were radiolabelled for 30 min as described by Pryor & Wright (1993). The extent of prM/E signalase cleavage was assessed by RIP of COS cell lysates. The results of a typical experiment are shown in Fig. 2; the anti-E monoclonal antibodies detected both the E protein and the uncleaved C-prM-E polyprotein. No prM-E was detected with this expression system because of the lack of efficient cleavage between C and prM in the absence of the viral NS2B/3 protease (Rice, 1996). The protein C-prM, but not prM, was detected by RIP with antiserum directed against prM (not shown).

For eight of the mutants, cleavage was severely inhibited, as determined by the accumulation of C-prM-E and the presence of little or no E (Fig. 2, lanes 1, 2, 6, 7, 9-12). Intermediate levels of cleavage were detected for four mutants (Fig. 2, lanes 3, 4, 5 and 8), which is similar to that observed for the parental construct (Fig. 2, lane 13).

Based on these results, mutants with the Ser → Ile change at the -3 position (Fig. 2, lane 8) and the Thr → Leu change at the -1 position (Fig. 2, lane 2) were chosen for analysis using genomic length cDNA. These two changes yielded intermediate and low levels of cleavage. Densitometer traces

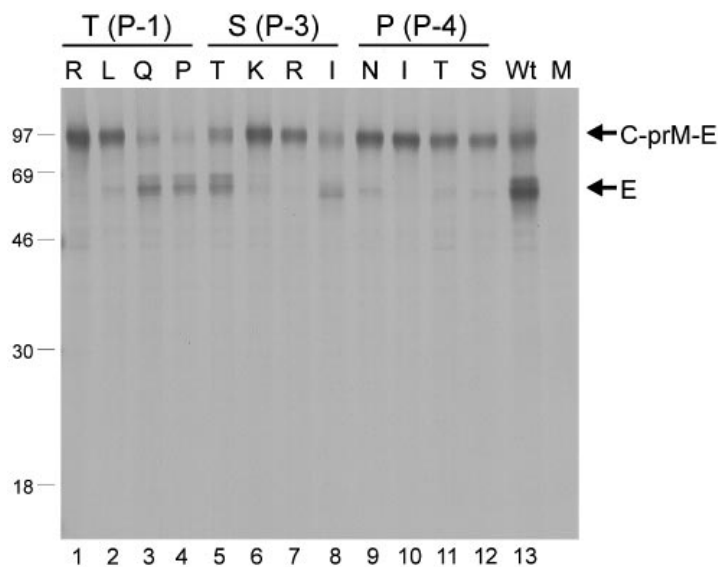


Fig. 2

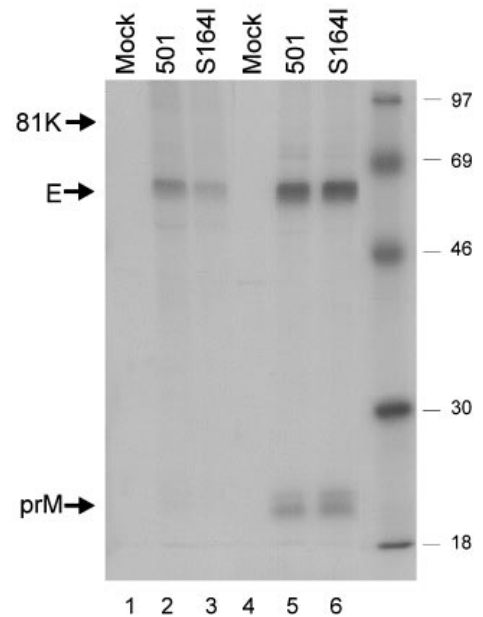


Fig. 3

Fig. 2. Analyses by gel electrophoresis of immunoprecipitates of  $^{35}\text{S}$ -labelled COS cells transfected with plasmid pcEXV-37 (containing C-prM-E genes) (lane 13) or derived constructs (lanes 1 to 12). The prM/E cleavage site was mutagenized at positions  $-1$  (lanes 1–4),  $-3$  (lanes 5–8), or  $-4$  (lanes 9–12). The amino acids encoded by the mutant constructs at each position are shown at the top of the lanes. Lane 14 shows an immunoprecipitate of mock-transfected cells. A mixture of anti-E monoclonal antibodies was used for the RIPs. Molecular mass markers (kDa) and the positions of proteins of interest are indicated.

Fig. 3. Analysis by gel electrophoresis of immunoprecipitates of  $^{35}\text{S}$ -labelled BHK-21 cells infected with parental virus MON501 (lanes 2 and 5), 501prM-S164I (lanes 3 and 6), or mock-infected (lanes 1 and 4). Cells were pulse-labelled for 7 min (lanes 1–3), or pulsed and then chased for 10 min (lanes 4–6). A mixture of anti-E monoclonal antibodies was used for the RIPs. Molecular mass markers (kDa) and the positions of proteins of interest are indicated.

of the autoradiograms (not shown) demonstrated that the respective levels of cleavage were 81 and 8% of those of the wild-type.

#### Selection of NS1 mutations for inclusion in recombinant virus

We have previously examined the formation of stable dimers and secretion of the DEN-2 NS1 protein following mutagenesis and transient expression of the NS1 gene in COS cells (Pryor & Wright, 1993, 1994). In these earlier studies, the sites of NS1 chosen for mutagenesis included: (i) one or both of the two glycosylation sites (Asn  $\rightarrow$  Ala); (ii) the 12 Cys residues that are conserved among the flaviviruses (Cys  $\rightarrow$  Ala); and (iii) the two most hydrophobic and three most hydrophilic areas of the protein. Briefly, glycosylation of DEN-2 NS1 was shown not to be essential for NS1 dimerization. However, a decrease in the proportion of dimer detected was observed for all three glycosylation mutants, particularly in the absence of the second N-linked glycan (Pryor & Wright, 1994). For NS1 Cys residues, we demonstrated that the substitution of Ala for any one of the fifth to the twelfth Cys residues (Cys<sub>5</sub> to Cys<sub>12</sub>) prevented dimer formation, whereas the substitution of Ala for the third and fourth Cys residues (Cys<sub>3</sub> and Cys<sub>4</sub>)

allowed partial dimerization (Pryor & Wright, 1993; M. J. Pryor & P. J. Wright, unpublished results). In the study of hydrophobicity and hydrophilicity, one mutation (designated OB2: W330A, Y331A) prevented dimerization and one (IL2: E173A, K174A) allowed partial dimerization (Pryor & Wright, 1993).

Based on the transient expression results, six mutations were chosen for incorporation into genomic length cDNA in order to test their effects on virus growth, i.e. the three glycosylation mutants, Cys<sub>4</sub> and IL2 (all partial dimerization), and Cys<sub>8</sub> (no dimerization).

#### Analysis of the effects of selected mutations on virus yields

Mutations that affected a range of properties in transient expression systems were analysed for their effects on virus growth using genomic length DEN-2 cDNA. Initially, all mutations were introduced into the plasmid pDVWS310, which comprises cDNA from the closely related NGC and PUO-218 strains of DEN-2 (Gualano *et al.*, 1998). However, viruses derived from pDVWS310 are not neurovirulent in mice. To study the effect of mutations on mouse neurovirulence, those mutations that yielded virus in the

**Table 1.** Yields of recombinant viruses

Yields were calculated after electroporation of BHK-21 cells and two passages of virus in C6/36 cells.

Virus		IF*	Titre (p.f.u. per ml)	NS1 dimer (%)†	
				Transient	Virus
Parental	MON310	+++	$(3.3 \pm 0.3) \times 10^6$		
	MON501	+++	$(4.0 \pm 0.2) \times 10^6$	80	100
prM/E cleavage mutants	310prM-T166L	—	Undetectable		
	310prM-S164I	+	$(6.2 \pm 0.5) \times 10^5$		
	501prM-S164I	++	$(3.4 \pm 0.5) \times 10^5$	NA	NA
	310NS1-G <sub>1,2</sub> <sup>-</sup> (N130A, N207A)	—	Undetectable	46	NA
NS1 glycosylation mutants	310NS1-G <sub>1</sub> <sup>-</sup> (N130A)	—	Undetectable	61	NA
	310NS1-G <sub>2</sub> <sup>-</sup> (N207A)	+	$(3.2 \pm 0.3) \times 10^3$		
	501NS1-G <sub>2</sub> <sup>-</sup>	++	$(1.2 \pm 0.2) \times 10^4$	9	91
	310NS1-Cys <sub>4</sub> (C143A)	—	Undetectable	40	NA
NS1 Cys mutants	310NS1-Cys <sub>8</sub> (C291A)	—	Undetectable	0	NA
	310NS1-IL2 (E173A, K174A)	+++	$(2.0 \pm 0.3) \times 10^6$		
NS1 hydrophilic mutants	501NS1-IL2	+++	$(8.3 \pm 0.3) \times 10^6$	8	78

\* Immunofluorescence in BHK-21 cells at 5 days. IF was scored as — to + + + +, where: —, no positive cells; +, 0–25% positive cells; ++, 25–50% positive cells; + + +, 50–75% positive cells; and + + + +, 75–100% positive cells.

† Determined by immunoblotting after incubation for 1 h at 37 °C. Transient expression data are from Pryor & Wright (1993, 1994). Virus data are from Fig. 4. NA, Not applicable.

pDVWS310 system were introduced into pDVWS501. This plasmid contains only cDNA derived from the NGC strain and yields mouse-neurovirulent virus.

Viruses derived from the parental plasmids pDVWS310 and pDVWS501 were named MON310 and MON501, respectively. Mutant viruses were named according to the parental construct, the mutated gene and the specific mutation. Virus was produced from genomic length cDNA by established procedures (Gualano *et al.*, 1998) as follows. RNA was transcribed and electroporated into BHK-21 cells. The BHK-21 cells were tested for IF with anti-E antibodies. Medium from BHK-21 cells was passaged twice in C6/36 cells, and the titre of virus was determined by plaque assay in C6/36 cells.

These procedures were completed at least twice for each construct, and the results are summarized in Table 1. The Ser → Ile mutation at the -3 position (prM residue 164) of the prM/E cleavage site slightly reduced cleavage in the transient expression system (Fig. 2, lane 8), and virus that contained this change showed a 5- to 10-fold reduction in titre. The Thr → Leu mutation at the -1 position of the prM/E cleavage site strongly inhibited cleavage in the transient expression system (Fig. 2, lane 2), and no virus was obtained from this mutated construct.

Virus was detected for only two of the six mutated NS1 constructs, those with the G<sub>2</sub><sup>-</sup> and IL2 mutations. However, there was no correlation between the formation of stable dimers in the transient system and virus yield. For example, the yields of viruses G<sub>2</sub><sup>-</sup> and IL2 differed by 1000-fold, with the latter equivalent to that of parental virus, but both had similar

levels of dimers at 9 and 8%, respectively, in the transient system. Neither of the constructs encoding changes at Cys residues yielded detectable virus, although the Cys<sub>4</sub> protein was capable of forming dimers (40%). For the glycosylation mutants, the presence of the first N-linked glycan was a determinant of virus production rather than dimerization; constructs encoding NS1 which lacked this glycan (G<sub>1,2</sub><sup>-</sup> and G<sub>1</sub><sup>-</sup>) did not produce any virus that was detectable by our methods, and yet the corresponding proteins did form stable dimers (46 and 61%).

### Proteins encoded by mutant viruses

Since the mutations incorporated into the recombinant viruses were selected on the basis of results obtained by transient expression of subgenomic lengths of viral cDNA, it was of interest to examine the post-translational processing of the mutant proteins in virus-infected cells.

Cleavage at the prM/E site was examined by RIP of cell lysates from BHK-21 cells infected with MON501 or 501prM-S164I which had been pulse-labelled for 7 min with [<sup>35</sup>S]methionine. After the pulse, the E glycoprotein (*M<sub>r</sub>* 61 000) was observed for both viruses (Fig. 3, lanes 2 and 3). A protein corresponding to prM-E (predicted *M<sub>r</sub>* 81 000) was not detected, and neither were any other precursor proteins. Thus, cleavage at the prM/E site was rapid and more efficient than in the transient expression system (Fig. 2). E was more heavily labelled during the chase of 10 min (Fig. 3, lanes 5 and 6) following the completion of nascent E molecules, but again no prM/E was detected and no difference was observed

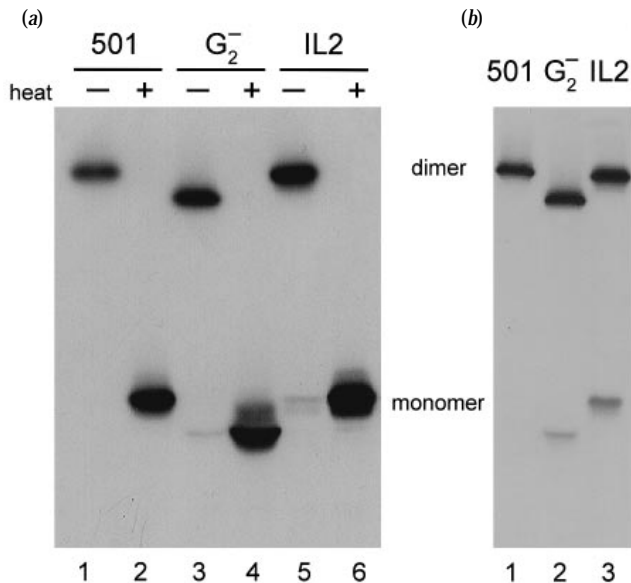


Fig. 4. Analysis by gel electrophoresis and immunoblotting (anti-NS1 antibodies) of lysates of BHK-21 cells infected with the parental virus MON501, 501NS1- $G_2^-$  or 501NS1-IL2. (a) Lysates were heated at 100 °C for 2 min before electrophoresis (lanes 2, 4, and 6) or remained unheated (lanes 1, 3, and 5). (b) Lysates were incubated at 37 °C for 1 h before electrophoresis.

between the parental and mutant viruses. Two additional bands corresponding to polypeptides of  $M_r$  21 500 and 19 500 were detected following the chase. Presumably these are glycosylated and unglycosylated forms of prM (Smith & Wright, 1985) which have co-precipitated with E following the formation of E-prM heterodimers (Wengler & Wengler, 1989).

To investigate the ability of the mutant NS1 proteins  $G_2^-$  and IL2 to form dimers in infected cells, lysates of cells infected with the appropriate virus were analysed by SDS-PAGE either without heating or following 2 min at 100 °C. NS1 was detected by immunoblotting (Fig. 4). Both mutant proteins

formed dimers, with the size of the  $G_2^-$  protein reduced because of the lack of one *N*-linked glycan (Fig. 4*a*, lanes 3 and 4). In order to compare the stability of these dimers with those formed following transient expression of NS1 cDNA, the cell lysates were held at the standard conditions of 37 °C for 1 h before electrophoresis and immunoblotting. The results of this experiment are shown in Fig. 4(*b*). The proportions of NS1 in the dimeric form were determined from densitometer traces and are listed in the final column of Table 1. The levels of dimer formation and stability were much higher for NS1 synthesized in infected cells than in cells transiently expressing cDNA. However, there was still no direct correlation between dimerization (91 and 78% for  $G_2^-$  and IL2 viruses, respectively) and virus yield (1000-fold difference).

#### Mouse neurovirulence of mutant viruses

The three pDVWS501-based mutant viruses were tested for neurovirulence in suckling mice. The results are summarized in Table 2. At a dose of 100 p.f.u., mortality was high for all viruses (88–100%). At 10 p.f.u., no mice were killed by the 501NS1- $G_2^-$  virus, whereas the mortality was 44–83% for the other three viruses. The number of days to death for mice infected by each of the four viruses were also compared and the significance of the differences determined by Student's *t*-test. A value of  $P < 0.05$  was considered significant. The differences between MON501 and 501NS1-IL2 at both doses were not significant. However, for 501prM-S164I at 100 and 10 p.f.u., the time to death was longer than for MON501 and was significant. Similarly for 501NS1- $G_2^-$  at 100 p.f.u., the increased time to death was significant. Thus, the results demonstrate that 501NS1- $G_2^-$  was attenuated in mice, based on mortality rates and time to death, and that 501prM-S164I was also attenuated, based on time to death only. RNA was extracted from mouse brains, RT-PCR was performed and the products were sequenced across the region containing the mutation. In all cases, the desired mutation was retained.

Table 2. Mouse neurovirulence of recombinant viruses

Virus	p.f.u. inoculated	Mice dead at 21 days	Days to death [days $\pm$ (1 $\times$ SD)]
MON501	100	15/15 (100%)	10.1 $\pm$ 2.1
501prM-S164I	100	8/8 (100%)	12.3 $\pm$ 1.7
501NS1- $G_2^-$ (N207A)	100	7/8 (88%)	12.7 $\pm$ 2.5
501NS1-IL2 (E173A, K174A)	100	8/8 (100%)	8.5 $\pm$ 1.2
MON501	10	12/16 (75%)	10.5 $\pm$ 1.4
501prM-S164I	10	5/6 (83%)*	17.4 $\pm$ 1.9
501NS1- $G_2^-$	10	0/8	–
501NS1-IL2	10	4/9 (44%)	11.8 $\pm$ 2.2

\* This group originally had eight mice, but two died from causes other than dengue infection.

## Discussion

We have used transient expression systems and a genomic length cDNA clone to analyse the effects of mutations in DEN-2 glycoproteins on their processing and virus production. As expected, cleavage at the prM/E junction was modified by either the replacement of a conserved amino acid (Pro at -4) or by changing residues at -3 or -1 to those that did not fit the (-3, -1) model. The 501prM-SI64I virus showed a small reduction in growth in cell culture (5- to 10-fold; Table 1) and mouse neurovirulence (based on time to death), which corresponded to the decrease in efficiency of cleavage in the transient expression system. Virus was not recovered from the construct encoding a Thr → Leu change at the -1 position of the prM/E signalase site, and this mutation greatly reduced cleavage in the transient expression system (Fig. 2, lane 2). It was not possible to detect uncleaved prM-E polyproteins, or other E polyprotein precursors, in pulse-chase experiments of cells infected with the MON501 or 501prM-SI64I viruses. Thus, signalase cleavage between prM and E in the infected cells was rapid and more efficient than following transient expression. Yet it was perhaps surprising that no prM-E was observed in cells infected with 501prM-SI64I, considering the reduced virus yield (Table 1). Similarly, precursors to the E glycoprotein have not been detected previously in pulse-chase experiments of cells infected with wild-type YF virus (Chambers *et al.*, 1990) and DEN-2 viruses (Smith & Wright, 1985).

Suboptimal cleavage of the flavivirus polyproteins and impairment of virus growth has been studied by others, but has focused on either the NS2B/3 viral protease or the furin-like activity that cleaves within prM, rather than on signalase. Mutations in the YF virus NS2A/2B and NS2B/3 viral protease cleavage sites were characterized in cell-free translation systems and transient expression systems, followed by analysis of the effects of selected mutations on virus growth using genomic length YF virus cDNA (Nestorowicz *et al.*, 1994; Chambers *et al.*, 1995). Mutations that had little or no effect on protein processing caused only a small reduction in virus titre, as observed here. Mutations which significantly impaired cleavage had variable effects on virus yield, and virus was not recovered using RNA transcripts containing mutations that abolished cleavage.

Pletnev *et al.* (1993) used a TBE/DEN-4 chimeric clone to test the effects of mutations in the prM internal cleavage site, which for TBE virus is Arg-Thr-Arg-Arg ↓ Ser. This cleavage releases the mature M protein from the pr protein. Substitution of Val for the Ser residue resulted in virus that showed reduced growth in cell culture and reduced mouse neurovirulence, while in a double mutant, substitution of Val for the Arg residue at the -2 position and the Ser residue abolished virus production.

In seeking stable mutations which modify processing of the viral polyprotein, it may be preferable to change sites cleaved

by a host protease, rather than by a virus-encoded enzyme. Reversion to optimal cleavage and virulence is theoretically less likely in the former case, because reversion must occur in the substrate (probably at or close to the cleavage site), whereas in the latter, compensatory changes could occur either in the substrate or the viral protease.

Our previous studies using a transient expression system in COS cells had identified mutations that prevented or reduced the formation of stable NS1 dimers (Pryor & Wright, 1993, 1994). It was unknown whether dimerization was an absolute requirement for virus growth, but to make and test a large number of dimerization-negative mutants with the aim of finding one or more viruses that were replication-competent was not feasible. Our approach of testing mutants with intermediate levels of dimerization for intermediate levels of virus growth seemed reasonable, since the ultimate goal was the identification of virus that could replicate, albeit at reduced levels. However, stable dimer formation in the transient system was a poor predictor of virus growth, and of dimerization in infected cells (Table 1). In fact, it was intriguing that the two replication-competent viruses with NS1 mutations both showed high levels of dimerization (91 and 78%) in infected cells. Two points follow from this result. Firstly, the results suggest, but fall short of proving until more mutants are analysed, that efficient dimerization may well be one of the functions of NS1 needed for virus growth. Secondly, for these two viruses, stable dimers were formed more efficiently in infected cells than during transient gene expression. In infected cells, NS1 is cleaved from the polyprotein and is flanked by E and NS2A. These polypeptides may contribute to the initial folding of NS1 and the final stability of the dimer, whereas following transient expression of the NS1 gene, only 27 residues of E precede the N terminus of the NS1 protein, and there is no NS2A at the C terminus.

There are several possible explanations for the lack of detectable growth of the  $G_{1,2}^-$ ,  $G_1^-$  and  $Cys_4$  viruses (Table 1). It is possible that these mutations prevent cleavage of NS1 from the viral polyprotein, or correct folding in its presence (the converse of the above). Dimers may not have formed in infected cells, or the mutations may have affected some other dimerization-dependent or independent function of NS1. For example, there is evidence for a role of NS1 in flavivirus RNA replication (Mackenzie *et al.*, 1996; Muylaert *et al.*, 1996, 1997). It has been suggested that NS1 is required for YF virus minus strand synthesis (Lindenbach & Rice, 1997). Since NS1 is found in association with intracellular and plasma membranes, it is also possible that NS1 is involved in virion assembly and maturation (Mason, 1989).

It has also been noted by others that the loss of the second glycan of NS1 ( $G_2^-$ ) has less of an effect on virus growth than loss of the first glycan ( $G_1^-$ ). Pletnev *et al.* (1993) found that mutation of the first NS1 glycosylation site in a chimeric TBE/DEN-4 virus clone yielded a virus that showed reduced titres in cell culture and reduced mouse neurovirulence, while

mutation of the second NSI glycosylation site actually increased mouse neurovirulence. NSI still formed dimers when either one of the two glycans was absent. For YF virus, deletion of the first or both NSI glycosylation sites was not lethal, but titres of virus were reduced and the mutant viruses had impaired growth properties. In contrast, deletion of the second glycosylation site produced a YF virus with growth and mouse neurovirulence properties similar to those of the parental virus (Muylaert *et al.*, 1996).

In summary, two mutations were identified that reduced virus growth in cell culture and decreased neurovirulence in mice, i.e. modification of the prM/E cleavage site and removal of the second glycosylation site in NSI. Our overall aim is to define a group of mutations which together cover a range of virus functions, and whose individual effects on restricting virus replication have been determined. In the long term, the inclusion of mutations selected from this group into viral genomes via genomic length cDNA will generate attenuated strains with potential as human vaccines.

This investigation received financial support from the World Health Organization as part of the Global Program for Vaccines and Immunization, and also from the National Health and Medical Research Council of Australia.

## References

- Anderson, R., King, A. D. & Innis, B. L. (1992). Correlation of E protein binding with cell susceptibility to dengue 4 virus infection. *Journal of General Virology* **73**, 2155–2159.
- Calisher, C. H., Karabatsos, N., Dalrymple, J. M., Shope, R. E., Porterfield, J. S., Westaway, E. G. & Brandt, W. E. (1989). Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *Journal of General Virology* **70**, 37–43.
- Chambers, T. J., McCourt, D. W. & Rice, C. M. (1990). Production of yellow fever virus proteins in infected cells: identification of discrete polyprotein species and analysis of cleavage kinetics using region specific polyclonal antisera. *Virology* **177**, 159–174.
- 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.
- Chen, Y., Maguire, T. & Marks, R. M. (1996). Demonstration of binding of dengue virus envelope protein to target cells. *Journal of Virology* **70**, 8765–8772.
- Della-Porta, A. J. & Westaway, E. G. (1972). Rapid preparation of hemagglutinins of togaviruses from infected cell culture fluids. *Applied Microbiology* **23**, 158–160.
- Falgout, B. & Markoff, L. (1995). Evidence that flavivirus NSI-NS2A cleavage is mediated by a membrane-bound host protease in the endoplasmic reticulum. *Journal of Virology* **69**, 7232–7243.
- 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.
- Gruenberg, A., Woo, W. S., Biedrzycka, A. & Wright, P. J. (1988). Partial nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue virus type 2, New Guinea C and PUO-218 strains. *Journal of General Virology* **69**, 1391–1398.
- Gualano, R. C., Pryor, M. J., Cauchi, M. R., Wright, P. J. & Davidson, A. D. (1998). The 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.
- Halstead, S. B. (1988). Pathogenesis of dengue: challenges to molecular biology. *Science* **239**, 476–481.
- Heinz, F. X., Stiasny, K., Püschner-Auer, G., Holzmann, H., Allison, S. L., Mandl, C. W. & Kunz, C. (1994). Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM. *Virology* **198**, 109–117.
- 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.
- Holzmann, H., Heinz, F. X., Mandl, C. W., Guirakhoo, F. & Kunz, C. (1990). A single amino acid substitution in envelope protein E of tick-borne encephalitis virus leads to attenuation in the mouse model. *Journal of Virology* **64**, 5156–5159.
- 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.
- Kapoor, M., Zhang, L., Mohan, P. M. & Padmanabhan, R. (1995). Synthesis and characterization of an infectious dengue virus type-2 RNA genome (New Guinea C strain). *Gene* **162**, 175–180.
- Kinney, R. M., Butrapet, S., Chang, G.-J. J., Tsuchiya, K. R., Roehrig, J. T., Bhamarapavati, N. & Gubler, D. J. (1997). Construction of infectious cDNA clones for dengue 2 virus: strain 16681 and its attenuated vaccine derivative, strain PDK-53. *Virology* **230**, 300–308.
- Lai, C.-J., Zhao, B., Hori, H. & Bray, M. (1991). Infectious RNA transcribed from stably cloned full-length cDNA of dengue type 4 virus. *Proceedings of the National Academy of Sciences, USA* **88**, 5139–5143.
- Lewis, J. G., Chang, G.-J., Lanciotti, R. S. & Trent, D. W. (1992). Direct sequencing of large flavivirus PCR products for analysis of genome variation and molecular epidemiological investigations. *Journal of Virological Methods* **38**, 11–24.
- Lin, B., Parrish, C. R., Murray, J. M. & Wright, P. J. (1994). Localization of a neutralizing epitope on the envelope protein of dengue virus type 2. *Virology* **202**, 885–890.
- Lindenbach, B. D. & Rice, C. M. (1997). *Trans*-complementation of yellow fever virus NSI reveals a role in early RNA replication. *Journal of Virology* **71**, 9608–9617.
- Mackenzie, J. M., Jones, M. K. & Young, P. R. (1996). Immunolocalization of the dengue virus nonstructural glycoprotein NSI suggests a role in viral RNA replication. *Virology* **220**, 232–240.
- Mason, P. W. (1989). Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology* **169**, 354–364.
- Miller, J. & Germain, R. N. (1986). Efficient cell surface expression of class II MHC molecules in the absence of associated invariant chain. *Journal of Experimental Medicine* **164**, 1478–1489.
- Monath, T. P. (1994). Yellow fever and dengue – the interactions of virus, vector and host in the re-emergence of epidemic disease. *Seminars in Virology* **5**, 133–145.
- Murray, J. M., Aaskov, J. G. & Wright, P. J. (1993). Processing of the dengue virus type 2 proteins prM and C-prM. *Journal of General Virology* **74**, 175–182.
- Muylaert, I. R., Chambers, T. J., Galler, R. & Rice, C. M. (1996). Mutagenesis of the N-linked glycosylation sites of the yellow fever virus NSI protein: effects on virus replication and mouse neurovirulence. *Virology* **222**, 159–168.

- 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.
- Pletnev, A. G., Bray, M. & Lai, C.-J. (1993).** Chimeric tick-borne encephalitis and dengue type 4 viruses: effects of mutations on neurovirulence in mice. *Journal of Virology* **67**, 4956–4963.
- Polo, S., Ketner, G., Levis, R. & Falgout, B. (1997).** Infectious RNA transcripts from full-length dengue virus type 2 cDNA clones made in yeast. *Journal of Virology* **71**, 5366–5374.
- 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. & Wright, P. J. (1994).** Glycosylation mutants of dengue virus NS1 protein. *Journal of General Virology* **75**, 1183–1187.
- Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C. & Harrison, S. C. (1995).** The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* **375**, 291–298.
- 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.
- Ruiz-Linares, A., Cahour, A., Desprès, P., Girard, M. & Bouloy, M. (1989).** Processing of yellow fever virus polyprotein: role of cellular proteases in maturation of the structural proteins. *Journal of Virology* **63**, 4199–4209.
- Smith, G. W. & Wright, P. J. (1985).** Synthesis of proteins and glycoproteins in dengue type 2 virus-infected Vero and *Aedes albopictus* cells. *Journal of General Virology* **66**, 559–571.
- Stadler, K., Allison, S. L., Schalich, J. & Heinz, F. X. (1997).** Proteolytic activation of tick-borne encephalitis virus by furin. *Journal of Virology* **71**, 8475–8481.
- Stohlman, S. A., Wisseman, C. L., Jr, Eylar, O. R. & Silverman, D. J. (1975).** Dengue virus-induced modifications of host cell membranes. *Journal of Virology* **16**, 1017–1026.
- van Heijne, G. (1986).** A new method for predicting signal sequence cleavage sites. *Nucleic Acids Research* **14**, 4683–4690.
- Wengler, G. & Wengler, G. (1989).** Cell-associated West Nile flavivirus is covered with E + prM protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. *Journal of Virology* **63**, 2521–2526.
- Westaway, E. G. & Goodman, M. R. (1987).** Variation in distribution of the three flavivirus-specified glycoproteins detected by immunofluorescence in infected Vero cells. *Archives of Virology* **94**, 215–228.
- Winkler, G., Maxwell, S. E., Ruelmler, C. & Stollar, V. (1989).** Newly synthesized dengue-2 virus nonstructural protein NS1 is a soluble protein but becomes partially hydrophobic and membrane-associated after dimerization. *Virology* **171**, 302–305.
- Wright, P. J., Cauchi, M. R. & Ng, M. L. (1989).** Definition of the carboxy termini of the three glycoproteins specified by dengue virus type 2. *Virology* **171**, 61–67.

---

Received 17 March 1998; Accepted 14 July 1998