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登革病毒外套膜蛋白質基因之研究：蚊媒與宿主之序列變異及形成病毒顆粒之功能

Study of the Envelope Gene of Dengue Virus: Sequence Variation in Mosquito and Human Host and Functional Role in Particle Formation

林素如

Su-Ru Lin

指導教授：王維恭　博士

Advisor: Wei-Kung Wang, M.D., Sc.D.

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摘要

登革病毒為黃病毒科，黃病毒屬之一員，有血清型 1～4 型。登革病毒感染為在熱帶及亞熱帶地區流行的蟲媒病毒疾病之首位，可引起自限性的登革熱以及顏重致死的登革出血熱／登革休克症候群。官方估計全球每年約有 5 千萬～1 億人口感染登革病毒，其中有 25 萬～50 萬是登革出血熱病例。登革病毒之基因體為一正向、單股之核醣核酸，可在轉譯成 3 個結構性蛋白質，包含殼蛋白質、前驅膜蛋白質、外套膜蛋白質，以及 7 個非結構性蛋白質，包含非結構性蛋白質 1、2A、2B、3、4A、4B、5。

目前已有許多致力於了解登革病毒感染及其致病機轉之臨床與流行病學方面的研究工作正在進行中，但對於登革病毒的傳播及複製仍有許多問題尚待解決。登革病毒的外套膜蛋白質是其細胞向性與病毒毒力之主要決定蛋白質，而且此蛋白質在登革病毒生活史中扮演許多重要的角色。因此本研究之整體目標為探就外套膜蛋白質於登革病毒傳播與複製時之功能。登革病毒可以在埃及斑蚊及宿主間交替複製。有研究報告指出登革病毒在被感染個體中以類種的形式存在，但其在病媒蚊體內之序列變異程度以及其類種結構在病媒蚊與人類宿主間傳播時之變化目前則未明。最近有流行病學研究報告指出登革出血熱的病例數在登革病毒流行的晚期有逐漸增多的趨勢，顯示登革病毒在同次流行中持續演化，然而其毒力決定區及其與疾病越趨嚴重的演化關係則尚缺定論。有研究指出在細胞中一起表現外套膜蛋白質與前驅膜蛋白質可以產生類病毒顆粒，其結構與抗原性質與具有感染力的病毒顆粒十分相似。外套膜蛋白質由 N 端外區（具有 395 個胺基酸）及 C 端柄與嵌入區（具有 100 個胺基酸）組成。由蜱媒介性腦炎病毒的外套膜蛋白質之連續截斷蛋白質研究顯示其柄區域對於類病毒顆粒之形成極為重要，然而影響此功能之重要殘基為何則尚待研究。

本研究的第一個目標為研究登革病毒在病媒蚊體內之序列變異程度與在傳播時其類種結構之變化。我們分析來自同一次登革病毒流行時，取樣自自然感染的病媒蚊及八位登革病人血漿中的登革病毒血清型第三型之核酸序列。以外套膜基因為例，在登革病毒病媒蚊體內的平均歧異度為（0.21％）較在登革病人體內（0.38%）為低。分析殼蛋白基因也有類似的分析結果，病媒蚊體內的殼蛋白基因平均歧異度為 0.09％，低於在登革病人體內的 0.23%。我們更進一步分析五隻用實驗感染登革病毒的蚊子體內的登革病毒外套膜基因（平均歧異度為 0.09％）與殼蛋白基因（平均歧異度為 0.1％）證實這項推論。我們的實驗結果顯示登革病毒在病媒蚊體內之序列變異程度普遍低於在病人體內。同時我們也以在病媒蚊與病人體內的登革病毒外套膜基因變異度分析其類種結構，結果顯示二者的主要變種序列相同，於是我們推測這個主要變種在病媒蚊與病人之間傳播。總而言之，我們的實驗結果支持「病媒蚊對於登革病毒演化之保守性有所貢獻」的假說，也就是登革病毒在病媒蚊體內保持序列較為相近的病毒族群及以主要變種傳播。

本研究的第二個目標為探討登革病毒毒力決定區及其與疾病越趨嚴重的演化關係。我們分析了來自 2001 至 2002 年台灣南部連續的兩次登革病毒血清型第二型流行時所採樣的 31 位病人（14 個登革熱病例及 17 個登革出血熱病例）血漿中的登革病毒血清型第二型序列。結果顯示有五個分別在外套膜基因、非結構性蛋白基因 1、4A、5 的核酸變異。此結果與 1997 年在古巴流行的登革病毒血清型第二型之分析報告並無重複，所以我們推測與嚴重流行相關之病毒毒力決定區與造成流行之基因型有關。與其他研究兩次間隔數年的登革病毒流行之譜系交替的報告相較之下，我們的分析結果顯示 2002 年的高雄登革病毒來自於 2001 年的小部分病毒變種，而這中間只經過不到 6 個月的時間。我們的發現可能也代表著一種登革病毒在流行期之間的演化機制。

本研究的第三個目標為在一個可以同時表現登革病毒血清型第四型外套膜蛋白質與膜蛋白質前驅蛋白質的質體系統中，以點突變的方法尋找外套膜蛋白質柄區域中對於類病毒顆粒形成之重要殘基。我們發現位於外套膜蛋白質柄區域的第一螺旋區域（H1）之胺基酸殘基位置 398、401、405、408，第二螺旋區域（H2）之胺基酸殘基位置 429、436、439、446 以及其間胺基酸殘基位置 422 若以脯胺酸取代原本胺基酸會造成類病毒顆粒之產量減少。共免疫沉澱實驗結果顯示位於 H1 之點突變影響外套膜蛋白質與膜蛋白質前驅蛋白質之交互作用，位於 H2 之點突變則未然。脂質膜結合能力實驗結果則顯示帶有造成降低類病毒顆粒形成的 H2 之點突變亦損害其與脂質膜之結合能力，而帶有造成降低類病毒顆粒形成的 H1 之點突變則未然。綜合以上實驗結果，我們發現外套膜蛋白質柄區域之 H1 殘基藉參與其與膜蛋白質前驅蛋白質間之交互作用而影響類病毒顆粒之形成，而 H2 則因具有與脂質膜結合之能力而影響類病毒顆粒之形成。

總而言之，在本研究中顯示登革病毒在病媒蚊體內以類種的形式存在，且藉著在病媒蚊體內中相似度較高的病毒族群維持其演化上的保守性，此外我們也發現了登革病毒以主要變種傳播。同時在本研究中亦藉著大量且持續的外套膜蛋白基因以及基因體全長之序列分析釐清登革病毒從一個只有少數嚴重病例的流行到一個越來越多嚴重病例的流行是如何演化。本研究中亦更進一步探討外套膜蛋白質之柄區域如何參與類病毒顆粒之形成與其在登革病毒生活史中病毒組裝的步驟之可能功能。推而廣之，本研究提供登革病毒傳播與複製之更深入的了解以及預防與控治登革病毒之新策略。

關鍵詞：登革病毒、序列變異、病毒演化、外套膜蛋白質、類病毒顆粒

Abstract

Dengue virus (DENV) belongs to the genus *flavivirus* in the family flavivirus. There are four serotypes of DENV (DENV1 to 4), which are the leading cause of the arboviral diseases in the tropical and subtropical areas, including a self-limiting disease, dengue fever (DF) and a severe and life-threatening disease, dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS). It has been estimated that approximately 50-100 million of DENV infection and 250-500 thousands cases of DHF occur annually throughout the world. DENV contains a positive-sense single stranded RNA genome, which encodes three structural proteins, including capsid (C), precursor membrane (PrM) and envelope (E) at the N-terminus, and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, at the C-terminus.

While substantial efforts have been made to study the clinical and epidemiological aspects of dengue disease and pathogenesis, several fundamental questions of the transmission and replication of DENV remain unclear. The E gene of DENV is the major determinant of tropism and virulence, and is known to play important roles in the life cycles of DENV. The overall objective of this study is to focus on the E gene and investigate its roles in some poorly understood areas in the DENV transmission and replication. DENV replicates alternately in the mosquito vectors and human hosts. It has been reported that DENV is present as quasispecies in plasma of infected individuals. However, the extent of sequence variation of DENV in the mosquito vector and the quasispecies structure changes during transmission between human and mosquitoes remain unknown. Several epidemiological studies have reported recently that the proportion of severe cases, DHF, to total cases increases toward the end of an outbreak, suggesting DENV evolves during the same outbreak. However, the viral determinants and evolution linked to outbreak with increased severity remain unclear. Co-expression of PrM and E proteins is sufficient to produce virus-like particles (VLPs), which structurally and antigenically similar to infectious particles. The E protein contains 395 amino acids at the N-terminal ectodomain and 100 amino acids at its C-terminus, which consists of the stem and anchor regions. Deletional study of the E protein of the tick-borne encephalitis virus (TBEV) suggested the importance of the stem region in the formation of VLPs, however, the critical residues involved remain unknown.

In the first specific aim, we studied the extent of sequences variation of DENV in the mosquitoes and the changes of quasispecies during transmission by examining DENV3 sequences in naturally infected mosquitoes in comparison with those in eight patients from the same outbreak. For the E gene, the mean diversity in naturally infected mosquitoes was 0.21%, lower than that in patients (0.38%). Similarly, the mean diversity of C gene in naturally infected mosquitoes was 0.09%, lower than 0.23% in patients. This was further verified with five experimentally infected mosquitoes (mean diversity, 0.09 and 0.10% for the E and C genes, respectively). Our findings suggested that the extent of sequence variation in the mosquitoes was generally lower than that in the patients. Examination of the quasispecies structures of the DENV3 E sequences in the mosquitoes and patients revealed that the sequences of the major variants were the same, suggesting that the major variant was transmitted. These findings support our hypothesis that mosquitoes contribute to the evolutionary conservation of dengue virus by maintaining a more homogenous viral population and a dominant variant during transmission.

In the second specific aim, we investigated viral determinants and evolution linked to outbreak with increased severity by examining DENV2 sequences from plasma of 31 patients (14 DF, 17 DHF) continuously during two consecutive DENV2 outbreaks in southern Taiwan in 2001-2002, in which both the total cases and proportion of DHF cases increased. Analysis of E gene and full-genome sequences between viruses of the two outbreaks revealed 5 nucleotide changes in E, NS1, NS4A, and NS5 genes. None was identical to those reported in the DENV2 outbreak in Cuba in 1997, suggesting viral determinants linked to severe outbreak were genotype dependent. Compared with previous reports of lineage turnover years apart, our findings that the 2002 viruses descended from a minor variant of the 2001 viruses in less than 6 months were novel, and may represent a mechanism of evolution of DENV from one outbreak to another.

In the third specific aim, we investigated critical residues in the stem region of E protein involved in the formation of VLPs by employing site-directed muatgenesis in the context of DENV4 PrM/E expression construct. We found that proline substitutions introduced to residues 398, 401, 405, and 408 within the first helix (H1) of the stem, residues 429, 436, 439, and 446 within the second helix (H2) of the stem, and residue 422 between H1 and H2 impaired the production of VLPs. Co-immunoprecipitation experiment revealed that mutants in the H1 affected the PrM-E interaction, whereas mutants in the H2 did not. Membrane binding assay of chimeric β-gal constructs containing the stem or mutants suggested while the H1mutants did not

affect the ability of the stem to bind membrane, the H2 mutants that impaired VLP production affected such membrane-binding ability. Taken together, our findings suggest that the H1 residues of the stem region are involved in the production of VLPs by participating in the interaction with PrM protein, whereas the H2 residues are involved in the production of VLPs by contributing to the membrane association of the stem region.

In summary, we showed that DENV exists as quasispecies in the mosquito vectors, and they contribute to the evolutionary conservation of DENV by maintaining a more homogenous viral population and a dominant variant during transmission. By extensive and continuous sequencing analysis of the E gene as well as full-genome, we demonstrated how DENV evolved from an outbreak with fewer severe cases to an outbreak with more severe cases. Moreover, we showed that how the stem region of DENV E protein is involved in the VLP production and presumably the assembly of DENV replication cycle. Information derived from this study would provide new insights into our understanding of the transmission and replication of DENV, as well as strategies for prevention and control of dengue diseases.

Keyword: dengue virus, sequence variation, virus evolution, envelope protein, virus like particle.

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Chapter 1 Introduction

1.1 The importance of studying dengue virus

Dengue viruses (DENV) belong to the genus *Flavivirus* of the family *Flaviviridae*. In the genus *Flavivirus*, there are more than 70 members including several arthropod-borne viruses, such as DENV, tick-borne encephalitis virus (TBEV), yellow fever virus (YFV), West Nile virus (WNV), Kunjin virus (KUNV) and Japanese encephalitis virus (JEV). DENV consists of four genetically related but antigenically distinct serotypes, DENV1 to DENV4, which are the cause of the most important arboviral diseases and continue to threaten more than 2.5 billion people living in the areas at risk for epidemic transmission of DENV (Gubler & Clark, 1995; Lindenbach *et al.*, 2007). Each of the four serotypes causes a spectrum of illness, ranging from asymptomatic infection to dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). Since 1970s, DHF has been reported in more than 60 countries and DENV has become endemic in more than 100 countries, including most of the tropical and subtropical areas (Gubler, 2002; Guzman & Kouri, 2002). It has been estimated that there are 100 million DENV infections and 250,000 to 500,000 cases of DHF annually throughout the world, with an average fatality rate of 5%

(WHO, 1997). This rapid spread of DENV infection has posed a public health concern globally and needs intensified efforts to prevent infection.

1.2 Clinical manifestations and pathogenesis of DENV infection

The majority of DENV infections are asymptomatic or self-limited (Balmaseda *et al.*, 2006; Burke *et al.*, 1988; Endy *et al.*, 2002; Thein *et al.*, 1997), however, the infection has caused more symptomatic presentation in Tahiti in 1971 (Moreau *et al.*, 1973).

Classic DF

The clinic features of classic DF were described previously (Eram *et al.*, 1979; Halstead *et al.*, 1969; Sabin, 1952; Siler *et al.*, 1926; Simmons *et al.*, 1931). After an incubation period of 2 to 14 days (4 to 7 days in most cases), there is an onset of fever which lasts for 1 to 2 days and may persist for 6 to 7 days accompanied by headache, retroocular pain, abdominal pain, mylagias, arthralgias and nausea. About 50% of DF patients present rash on the trunk with evanescent and polymorphic appearance (Hayes & Gubler, 1992; Waterman & Gubler, 1989). Clinical laboratory findings associated with DF include leucopenia, thrombocytopenia and elevation of liver enzyme in serum (Gubler, 1998).

DHF and DSS

Similar to patients with DF, DHF/DSS patients present a sudden onset of fever, which usually lasts for 2 to 7 days. However, DHF and DSS are characterized by thrombocytopenia with platelet count $\leqq 100{,}000/mm^3$, hemorrhagic manifestations and plasma leakage leading to shock during or after the defervescence (Gubler, 1998). According to the severity of illness, WHO has classified DHF/DSS to four grades, Ⅰ to Ⅳ (WHO, 1997). Grade Ⅰ has scattered petechiae or a positive tourniquet test. Grade Ⅱ is more severe with overt hemorrhagic manifestations. Grade Ⅲ is featured by hypovolemia, weak pulse, hypotension and coagulopathy. Grade Ⅳ is the most severe form of DSS with characterized by severe bleeding, disseminated intravascular coagulopathy and profound shock.

The pathogenesis of DHF/DSS

The pathogenesis of DHF/DSS has been an area of great interest and investigations. Among the risk factors that have been reported to associate with DHF/DSS, including age, nutrition status, HLA typing and underlying diseases factors, two controversial factors, the viral strain and the host immune status, have been extensively studied (Gubler, 1998; Halstead, 1997).

Virus factor

It was reported that the patients with primary infections from an outbreak of DENV2 on a Pacific island, Tonga, had great variation in both severity and duration of viremia (Gubler *et al.*, 1978). Most of the infected individuals had undetectable viremia whereas some had detectable viremia with low titers and short duration in the same outbreak. However, the same DENV2 virus caused severe disease resulting in an explosive epidemic in neighboring islands 3 years ago. These observations suggested that the virus had changed genetically from an epidemic strain to a silently transmitted one.

There have been several studies examining the full genome sequences of DENV to identify the viral determinants linked to severe diseases, DHF/DSS. A study investigated the sequences of the American (Am) genotype of DENV2 which caused only DF, and those of the Southeast Asian (SEA) genotype which caused both DF and DHF, and found that there were 55 amino acids differences in the coding region as well as structural differences at the 5' and 3' non-translated region (NTR) (Leitmeyer *et al.*, 1999). Moreover, the residue at position 390 of E protein in SEA genotype was

reported to contribute to high replication of DENV in macrophages (Pryor, 2001). It has also been demonstrated that the SEA genotype has selective advantages over the Am genotype in human cells and mosquitoes (Cologna *et al.*, 2005).

Taken together, these studies suggested that viral factor plays an important role in the pathogenesis of DHF/DSS.

Host immune status

Based on the epidemic observations that DHF/DSS was associated with secondary infections in individuals at the age over one years-old (Halstead *et al.*, 1970; Halstead *et al.*, 1967) and with primary infections in infants between the ages of 6 to 12 months in endemic areas (Halstead *et al.*, 2002; Kliks *et al.*, 1988), it was suggested a phenomenon, called antibody dependent enhancement (ADE), occurred in 85 to 90% of DHF/DSS cases with secondary infections (Halstead *et al.*, 1970). Heterotypic, nonneutralizing antibodies, produced by a secondary infection with a different DENV serotype, is thought to bind DENV and facilitate the entry of DENV into cells bearing Fc-γ receptor, thus enhancing the total amount of both virus and cytokines produced and leading to severe disease. This was supported by in vitro studies of ADE (Halstead &

O'Rourke, 1977; Morens *et al.*, 1987) and by several cohort studies (Burke *et al.*, 1988; Guzman *et al.*, 1990; Sangkawibha *et al.*, 1984).

1.3 Transmission cycles of DENV

There are evidences suggesting that the ancestral forms of DENV maintained in sylvatic cycles and circulated among nonhuman primates by transmission of several arboreal canopy-dwelling *Aedes* spp. Mosquitoes (Vasilakis & Weaver, 2008). With the advanced technologies of virus isolation and phyologenetic analysis, several sylvatic strains of DENV were discovered, sequenced and analyzed (Rico-Hesse, 1990; Wang *et al.*, 2000). Of note, the principal transmission of DENV is in the urban cycle that involves humans and the major vector, *Aedes aegypti*, and the minor vectors, such as *Aedes albopictus* and *Aedes polynesiensis* (Gubler, 2002; Kuno, 1997; Rodhain & Rosen, 1997).

A naïve female *Aedes aegypti* is infected with DENV when it bites infected individuals during viremic stage. Viral replication is initially found in the posterior midgut of the mosquito, then in the proventriculus and in other organ systems including the salivary glands(Chen *et al.*, 1993). After 10 to 14 days of the so-called extrinsic incubation period (EIP), the salivary glands become infected and the *Aedes aegypti*

female is capable of transmitting DENV to another host by injecting saliva (Sabin, 1952; Siler *et al.*, 1926). Humans are infected with DENV by the bite of an infective *Aedes aegypti*, a highly domesticated tropical mosquito that prefers to lay its eggs in artificial containers. The adult female mosquitoes prefer to feed on human during daylight hours with exceptions for feeding all day indoors and on overcast days (Gubler, 1998). The *Aedes aegypti* females disrupt the imbibing process at the slightest movement, thus resulting in feeding on several persons during a single blood meal. Therefore, the infected mosquitoes may transmit DENV to multiple individuals in a short time (Gubler & Casta-Valez, 1991; Platt *et al.*, 1997; Putnam & Scott, 1995; Scott *et al.*, 1997).

1.4 Structure and physical properties of DENV virion

The flavivirus virion sediments between 170 and 210S and has buoyant density of 1.19 to 1.23 g/cm^3 (Russell *et al.*, 1980). Revealed by electron microscopy and cryo-electron microscopic (cryo-EM) studies, the virion of DENV is a spherical, enveloped particle with a diameter of 50 nm and 60 nm in the mature and immature particle, respectively. The virion contains an electron dense core, which consist of capsid (C) proteins and genomic RNA and is about 30 nm in diameter (Kuhn *et al.*, 2002; Murphy, 1980; Zhang *et al.*, 2003a; Zhang *et al.*, 2003b). The core structure is

surrounded by a lipid bilayer derived from endoplasmic reticulum (ER) of host cell during virus budding. The outer shell of glycoprotein consists of 180 copies of each of the envelope (E) and precursor of membrane (PrM) or membrane (M) protein.

In the immature virion, PrM and E proteins form 90 heterodimers with 60 trimeric spikes of E protein extending away from the surface of the virion. During the process of virus maturation, PrM protein is cleaved by furin or furin-like protease in the Golgi to Pr and M protein, which is a transmembrane protein beneath the E protein shell. In contrast to the spiky surface of immature virion, mature virion is smooth and contains 90 homodimers of E proteins lying flat against the lipid bilayer (Mukhopadhyay *et al.*, 2005; Perera & Kuhn, 2008).

1.5 Genome organization and viral proteins

Genome organization

DENV genome contains a positive-sense, single-strand RNA genome of ~10.6 kb in length and a type Ⅰ 5' cap without 3' polyadenylated tail (Cleaves & Dubin, 1979; Wengler & Gross, 1978). Flanked by 5' and 3' noncoding region (NCR), viral genome encodes an open reading frame (ORF), translating into a single polyprotein, which is cleaved by viral and cellular protease, into 3 structural proteins, C, PrM/M, E, and 7

nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 (Lindenbach *et al.*, 2007).

Viral proteins

Capsid protein

The C protein is a highly basic protein containing 120 amino acids with a molecular weight (MW) of 11 kDa. Based on the structure determined by nuclear magnetic resonace (NMR) (Jones *et al.*, 2003; Ma *et al.*, 2004) and X-ray crystallography of the homologous, KUNV C protein (Dokland *et al.*, 2004), the C protein of DNEV folds into a compact dimer with each monomer consisting of four α helices, α1 to α4. Based on the deletional studies of the TBEV C protein, the α1 and α2 were not required for assembly and α4 might play a role in dimerization of C proteins (Kofler *et al.*, 2002; Kofler *et al.*, 2003). Based on the asymmetric distribution of positively charge and hydrophobic residues observed in NMR structure, it is believed that C protein is involved in packaging of RNA genome, forming the core structure (nucleocapsid core), and membrane association (Ma *et al.*, 2004).

Precursor membrane (PrM) glycoprotein and membrane (M) glycoprotein

The PrM protein has a MW of 26 kDa and contains 166 amino acids with one N-linked glycosylation site at residue 69 and six conserved cysteine residues. With the C-terminal hydrophobic region of C protein as its signal sequence, PrM protein is translocated into the ER lumen and cleaved by signal peptidase, which has been shown to occur simultaneously with the coordinated cleavage of the C protein at amino acid position 101 by by viral protease (Amberg *et al.*, 1994; Lobigs, 1993; Yamshchikov & Compans, 1994). After cleavage of the PrM protein by the Golgi-resident protease, furin, the Pr protein (residues 1 to 91) is released, and the M protein which consists of the ectodomain (residues 92 to 130) and C-terminal transmembrane (TM) region (residues 131 to 166) composed of two helices (M-T1 and M-T2) remains in the virion (Stadler *et al.*, 1997). The TM region of PrM protein was reported to contain an ER-retention signal and is involved in forming heterodimers with E protein and virus assembly (Lin & Wu, 2005; Op De Beeck *et al.*, 2003; Op De Beeck *et al.*, 2004). Based on previous studies, the PrM protein serves as a molecular chaperone to assist the proper folding of E protein (Konishi & Mason, 1993; Lorenz *et al.*, 2002) and prevents E protein from undergoing conformational change into fusogenic state in low pH

environment during its transport through the secretory pathway (Li *et al.*, 2008; Yu *et al.*, 2008).

Envelope (E) glycoprotein

The E protein is the major antigenic determinant on virus particles and is a type Ⅰ membrane protein. It contains 495 amino acids (MW of 53 kDa) with two N-linked glycosylation sites at residues 67 and 153 and twelve conserved cysteine residues (Nowak & Wengler, 1987; Winkler *et al.*, 1987). After the X-ray crystal structure of the trypsin-cleaved ectodomain of TBEV E protein was determined (Rey *et al.*, 1995), a lot of progress has been made in resolving the structure of the ectodomain of DENV E protein either in the pre-fusion or post-fusion state (Modis *et al.*, 2003; 2004; 2005; Nayak *et al.*, 2009; Zhang *et al.*, 2004). E protein contains an ectodomain (residues 1-395) at the N-terminus, followed by a stem region (residues 396 to 450) and a transmembrane (TM) region (residues 451 to 495) at the C-terminus. E protein forms head-to tail homodimers paralleling with the virus envelope at neutral pH and the ectodomain contains three domains which are composed mostly of β sheets in secondary structure. Domain Ⅰ, the central domain, contains the N-terminus. Domain Ⅱ is an elongated domain with the fusion loop at its distal end. Domain Ⅲ,

an immunoglobulin (Ig)-like domain, is involved in receptor binding (Crill & Roehrig, 2001; Hung *et al.*, 1999). Revealed by the cryo-EM study of the E protein in the mature virion, the stem region consists of two conserved α helices, E-H1 (residues 398 to 420) and E-H2 (residues 426 to 448), separated by a small conserved segment, and the TM region consists of two helices, E-T1 (residues 452 to 467) and E-T2 (residues 474 to 491) and a stretch of non-hydrophobic residues in between (Kuhn *et al.*, 2002; Zhang *et al.*, 2003a). Studies of TBEV E protein, either the full-length or ectodomain plus the stem region, have shown that E dimmers dissociate into monomers and reassociate irreversibly to form trimers on acidification (Allison *et al.*, 1995; Stiasny *et al.*, 1996; Stiasny *et al.*, 2004). After the resolution of the X-ray crystallographic structure of DENV E protein and Semliki Forest virus (SFV, a member of alphavirus genus) E1 protein, it was proposed that the E proteins of flavivirus and SFV belong to the type II fusion proteins, whose mechanism of fusion different from that of the type I fusion proteins, such as the gp41 of human immunodeficiency virus (HIV) and the hemagglutin (HA) of influenza virus (Gibbons *et al.*, 2004; Jardetzky & Lamb, 2004; Modis *et al.*, 2004; Nayak *et al.*, 2009). It is proposed the fusion peptide is exposed at the tip of the trimer in acidic environment of the endosome. After the penetration of the fusion peptide into host cell endosomal membrane, there is a major conformational

change in the positioning of domain III, which folds back towards domain II and forms a hydrophobic channel in between the trimer. It is predicted that the stem region of E protein extends itself along the groove and towards the fusion peptide, and therefore brings the TM region into close proximity with the endosomal membrane resulting in fusion of viral membranes and host membrane (Bressanelli *et al.*, 2004; Modis *et al.*, 2004).

Nonstructural protein (NS) 1 protein

The NS1 protein is a glycoprotein of 46 kDa and contains 12 conserved cycteins (Mason, 1989; Smith *et al.*, 1970). NS1 protein is translocated into the ER lumen during synthesis and cleaved by host signal peptidase and an unknown ER-resident enzyme for releasing from the polyprotein in progress (Falgout *et al.*, 1989; Falgout & Markoff, 1995). While largely retained within infected cells, NS1 protein was also found on the cell surface and secreted from the cell (Noisakran *et al.*, 2007; Schlesinger *et al.*, 1990). In spite of the hydrophilic nature of the amino acid content and the lack of TM domain, NS1 protein forms stable oligomers (dimers and hexamers) and is capable of membrane association (Flamand *et al.*, 1999; Winkler *et al.*, 1989; Winkler *et al.*, 1988). Although the function of the secreted, oligomeric NS1 protein remains to

be clarified, it has been reported the extracellular NS1 protein participates in the viral invasion through inhibition of complement activation (Brandt *et al.*, 1970; Chung *et al.*, 2006a; Chung *et al.*, 2006b). The intracellular NS1 protein localizes to the site of RNA replication and forms complex with NS4A protein involving in RNA replication (Lindenbach & Rice, 1997; 1999; Mackenzie *et al.*, 1996; Westaway *et al.*, 1999; Westaway *et al.*, 1997).

NS2A and NS2B proteins

NS2A protein has a MW of 22kDa and is a hydrophobic protein with a predicted transmembrane topology. After the cleavage by an unknown ER-resident protein at the N-terminus in the ER luminal side and by NS2B-3 viral protease in the cytosolic side, two forms of NS2A protein, NS2A and NS2Aα are generated (Chambers *et al.*, 1990a; Falgout & Markoff, 1995; Nestorowicz *et al.*, 1994). Both forms are involved in RNA replication complex and important for virus production (Kummerer & Rice, 2002; Liu *et al.*, 2003; Mackenzie *et al.*, 1998). Based on the previous studies of DENV2, WNV and KUNV, it was also demonstrated that NS2A protein inhibits interferon (IFN) signaling pathway by acting as an IFN antagonist (Liu *et al.*, 2004; Liu *et al.*, 2006; Munoz-Jordan *et al.*, 2003).

The NS2B protein a membrane-associated protein, has a MW of 14kDa and forms a stable complex with NS3 protein by acting as a cofactor for the viral serine protease (Arias *et al.*, 1993; Chambers *et al.*, 1991; Chambers *et al.*, 1993; Clum *et al.*, 1997; Falgout *et al.*, 1991; Jan *et al.*, 1995; Yusof *et al.*, 2000). Based on the crystal structure of WNV NS2B-NS3pro and the mutagenesis study of DENV NS2B protein, it is implicated that the residues 67 to 80 of NS2B protein (Trp-62, Leu-75, Ile-79) are critical for the protease activity of NS3 protein (Aleshin *et al.*, 2007; Niyomrattanakit *et al.*, 2004).

NS3 protein

The NS3 protein has a MW of 70 kDa and is a multifunctional protein of 618 amino acids. The N-terminal one third of NS3 protein (residues 1 to 180) is the protease domain of the NS2B-NS3 viral protease, a chymotrypsin-like serine protease complex responsible for polyprotein processing (Bazan & Fletterick, 1989; Chambers *et al.*, 1990b; Gorbalenya *et al.*, 1989a). Based on the crystal structure of DENV-2 NS3 protease domain with or without a substrate inhibitor, it consists of six β-strands that form two β-barrels with a catalytic triad (His-51, Asp-75, Ser-135) in between (Murthy *et al.*, 1999; Murthy *et al.*, 2000). The consensus cleavage site of NS2B-NS3 protease

requires a dibasic (Arg/Lys)-Arg motif at the P2 and P1 positions respectively and a small amino acid (Gly) at the P1' position (Chambers *et al.*, 1995).

Because of the structural homology, the C-terminal helicase domain of NS3 protein (residues 180 to 618) belongs to the supergroup 2 RNA helicases (Gorbalenya *et al.*, 1989b). It has been demonstrated that NS3 helicase contains the functions of RNA-stimulated nucleoside triphosphatase (NTPase) and RNA unwinding and plays an essential role in viral replication (Matusan *et al.*, 2001; Warrener *et al.*, 1993; Wengler *et al.*, 1991). The C-terminus of NS3 protein also has an RNA triphosphatase (RTPase) activity which is proposed to dephophorylate the 5' end of genomic RNA before cap addition (Wengler, 1993). Based on the crystal structures of the NS3 helicase domains of YFV, JEV, and DENV, it can be divided into three subdomains (Wu *et al.*, 2005a; Xu *et al.*, 2005; Yamashita *et al.*, 2008). Both subdomains Ⅰ (residues 181 to 326) and Ⅱ (residues 327 to 481) are composed of a central six-stranded parallel β-sheet, which is flanked by four α-helices. Subdomain Ⅲ (residues 482 to 618) has four parallel α-helices surrounded by three shorter α-helices and two solvent-exposed antiparallel β-strands. The crystal structure of the full-length NS3 protein of DENV-4 has shown that NS3 protein is an extended molecule with protease domain spatially

oriented on top of subdomains Ⅰ and Ⅱ of the helicase (Luo *et al.*, 2008). There were evidences suggesting that NS3 protein bound to 3' stem loop (3' SL) within 3' NCR in association to NS5 protein and its NTPase activity was enhanced in the presence of NS5 protein (Chen *et al.*, 1997; Cui *et al.*, 1998).

NS4A and NS4B proteins

The NS4A and NS4B proteins are intergral membrane proteins and have MW of 16 kDa and 27 kDa, respectively. Due to the hydrophobic nature, structural analyses of NS4A and NS4B proteins still remain to be resolved. However, the membrane topology of NS4A and NS4B proteins has been predicted through biochemical analyses (Miller *et al.*, 2007; Miller *et al.*, 2006). NS4A protein is localized in replication complex with NS1 protien and proposed to induce membrane alterations critical for viral RNA replication (Mackenzie *et al.*, 1998; Roosendaal *et al.*, 2006). NS4B protein colocalizes with NS3 protein and vial double stranded RNA in ER-derived membrane and thus implicates in assisting RNA replication (Miller *et al.*, 2006; Westaway *et al.*, 2002). It was also reported that NS4A and NS4B proteins of DENV can block IFN α/β-induced signal transduction (Munoz-Jordan *et al.*, 2005; Munoz-Jordan *et al.*, 2003).

NS5 protein

The NS5 protein has a MW of 103 kDa and contains 900 residues, which is the largest and the most conserved protein of DENV (69% sequence identity among four serotypes of DENV) (Kuno *et al.*, 1998). It possesses a methyltransferase (MTase) domain within its N-terminus (residues 1 to 296) and a RNA-dependent RNA polymerase (RdRP) at its C-terminus end (residues 320 to 900). Based on the homology with S-adenosyl-methionine (SAM)-dependent MTase and the functional study of the purified N-terminal domain of DENV2 NS5 protien, it is also suggested that this protein has MTase activity (Egloff *et al.*, 2002; Koonin, 1993). In the crystal structure of the MTase domain, it was shown that guanosine triphosphate (GTP) was bound with high specificity via an unusual set of contacts, suggesting an attractive drug target (Benarroch *et al.*, 2004; Egloff *et al.*, 2002; Zhou *et al.*, 2007).

The C-terminus of NS5 protein is homologous to RdRP of other positive-stranded RNA viruses (Koonin & Dolja, 1993; Rice *et al.*, 1985) and the polymerase activity was confirmed with recombinant NS5 protein thereafter (Ackermann & Padmanabhan, 2001; Guyatt *et al.*, 2001; Tan *et al.*, 1996). The crystal structures of both DENV and WNV polymerase have been solved (Malet *et al.*, 2007; Yap *et al.*, 2007). Based on the

X-ray crystal geographic study, the polymerase domain of NS5 protein consists of a canonical right hand conformation with a well-known GDD motif and conserved aspartic acid residues for the incorporation of nucleotides. As an RdRP, NS5 protein binds to the 3' SL of genomic RNA and forms a complex with NS3 protein to stimulate its NTPase and RTPase activities (Cui *et al.*, 1998; Johansson *et al.*, 2001; Kapoor *et al.*, 1995; Yon *et al.*, 2005).

Interestingly, it was reported that NS5 protein localize to the nucleus (Buckley *et al.*, 1992; Kapoor *et al.*, 1995). Residues 320 to 405 of NS5 protein are predicted as the nuclear localization signal (NLS), especially, residues 320 to 368, which are strictly conserved among the flaviviruses and implicated to interact with NS3 protein and bind β-importin (Brooks *et al.*, 2002; Johansson *et al.*, 2001; Kapoor *et al.*, 1995; Malet *et al.*, 2007; Yap *et al.*, 2007). It is suggested that a new role of NS5 protein, apart from its enzymatic functions, is to engage in virus-host interactions by targeting cellular factors in nucleus.

1.6 Replication cycle of DENV and formation of virus-like particles

Receptors for DENV

Several cellular proteins and carbohydrate molecules have been reported to be the attachment factors of DENV and mediate viral entry (Mukhopadhyay *et al.*, 2005). Dendritic-cell-specific ICAM-grabbing non-integrin (DC-SIGN) binds to glycans on adjacent monomers of the E dimer on DENV2 virion, as revealed by cryo-EM reconstruction (Pokidysheva *et al.*, 2006). It has been reported that the internalization of DC-SIGN is not necessary for DENV entry suggesting that DC-SIGN is an attachment factor (Lozach *et al.*, 2005; Navarro-Sanchez *et al.*, 2003; Tassaneetrithep *et al.*, 2003). Glucose-regulating protein 78 (GRP78/Bip) and CD14-associated molecules have also been reported as primary receptors for DENV infection of hepato- and monocytic cell linage, respectively (Chen *et al.*, 1999; Jindadamrongwech *et al.*, 2004). Recently, CLEC5A, a member of C-type lectins, was identified as an attachment factor for DENV. Blockage of its interaction with DENV suppressed the secretion of proinflammatory cytokines. Moreover, anti-CLEC5A monoclonal antibodies inhibit plasma leakage and hemorrhage in STAT1-deficient mice (Chen *et al.*,

2008). Unfortunately, a specific cellular receptor for DENV, which is required for inducing clathrin-mediated endocytosis, has not yet been identified.

Replication cycle

After the binding of domain III of E protein to the putative receptor, DENV is internalized via clathrin-coated pits and trafficked to a prelysosomal ecdocytic compartment (Acosta *et al.*, 2008; Peng *et al.*, 2009). On acidification, the conformational change of E protein leads to the exposure of the fusion peptide resulting in membrane fusion and the release of the viral nucleocapsid into cytosol, where translation processing of polyprotein and replication of genomic RNA take place (Gollins & Porterfield, 1985; 1986; Lindenbach *et al.*, 2007; Modis *et al.*, 2004).

Translation of polyprotein is cap dependent and is initiated by ribosomal scanning. Followed by co- and post-translational processing of the polyprotein, genomic RNA replicates within the ER-derived internal membranous structure. It has been demonstrated that cellular factors, EF1A and La, interact with 5' NCR or 3' NCR and facilitate the replication of genomic RNA (Blackwell & Brinton, 1997; De Nova-Ocampo *et al.*, 2002; Garcia-Montalvo *et al.*, 2004). RNA replication begins with the synthesis of a negative-stranded RNA serving as a template. Negative-stranded

templates continue to accumulate throughout the infection, but the amount is not proportional to that of the positive-stranded RNA, which is with 10 to 100-fold in excess (Cleaves *et al.*, 1981; Muylaert *et al.*, 1996). Ultrastructural study of the flaviviruses infected cells by EM has shown that virions are within the lumen of a compartment thought to be the ER (Lindenbach *et al.*, 2007; Murphy, 1980). It is believed that flaviviruses bud from ER, however, the budding intermediates have not been frequently observed, suggesting a very rapid process of assembly. The newly synthesized virions, which contain the genomic RNA and nucleocapsid core surrounded by lipid bilayer membrane with PrM/M and E proteins, appear to be transported through the secretory pathway along with proteolytic maturation (Mackenzie & Westaway, 2001). A model of the flavivirus maturation pathway has been proposed based on the crystal structure of PrM-E protein complex at neutral pH, cryo-EM reconstruction of immature DENV virion at low pH and biochemical analysis of furin-cleaved immature virions (Li *et al.*, 2008; Yu *et al.*, 2008). The immature, non-infectious virus travels from ER to Trans-Golgi network (TGN) whereas furin functions. Interestingly, the Pr peptide remains associated with E after cleavage in the acidic environment. It is suggested that the Pr peptide functions as a cap-like structure and keeps viral membrane from undergoing premature fusion with TGN. The virions become mature until they

are released into the neutral pH of the extracellular environment, whereas the Pr peptide dissociates with E protein (Perera & Kuhn, 2008).

Virus-like particles (VLPs)

VLPs contain the E and PrM/M proteins and a lipid bilayer membrane. VLPs are frequently observed during flavivirus replication (Russell *et al.*, 1980; Smith *et al.*, 1970). Previously, these particles were termed slowly sedimenting (70S) hemagglutinin (SHA), because they can agglutinate red blood cells at low pH. Compared with virus particles, VLPs are smaller with an average diameter of 315 Å but have a similar buoyant density (Allison *et al.*, 2003; Ferlenghi *et al.*, 2001). It has been demonstrated that cells expressing plasmid DNA containing PrM and E alone can produce VLPs in culture supernatant, suggesting that PrM and E protein are involved in flavivirus particle formation (Allison *et al.*, 1995; Chang *et al.*, 2003; Lorenz *et al.*, 2003). VLPs have also been shown to have the acid-catalyzed fusion activity similar to virus particles (Schalich *et al.*, 1996). Therefore, VLPs can be applied to study the functions of PrM and E in particle formation and membrane fusion. Approved by USDA, a West Nile DNA vaccine containing the PrM and E genes as equine vaccine is now available for public use. This DNA vaccine is also effective in several

disease-target hosts and now undergoes phase Ⅰ clinical trial (Bunning *et al.*, 2007; Chang *et al.*, 2007; Davis *et al.*, 2001; Martin *et al.*, 2007). DNA vaccine consisting of PrM and E genes from DENV1, delivered in three doses by Navy Medical Research Center is underway to phase Ⅰ clinical trial (Kochel *et al.*, 2000; Raviprakash *et al.*, 2003; Raviprakash *et al.*, 2000). It has also been demonstrated that DNA vaccines consisting of PrM and E genes from DENV2 and 3 elicit immune responses to protect mice from DENV challenge (Blair *et al.*, 2006; De Paula *et al.*, 2008; Konishi *et al.*, 2000; Putnak *et al.*, 2003; Simmons *et al.*, 2006).

1.7 Specific aims

Although the clinical and epidemiological aspects of dengue disease and pathogenesis have been extensively studied in the past, several fundamental questions regarding the transmission and replication of DENV remain unclear. The E gene of DENV is the major determinant of tropism and virulence, and is known to play important roles in the life cycles of DENV. The overall objective of this study is to focus on the E gene and investigate its roles in some poorly understood areas in the DENV transmission and replication.

RNA viruses is known to exist as a population of closely related sequences, the quasispecies (Domingo *et al.*, 1988; Farci *et al.*, 2000; Holland *et al.*, 1992; Martell *et al.*, 1992; Steinhauer & Holland, 1987). A study of the PrM and E genes from 23 geographically and temporally distinct DENV3 isolates has been reported that the amino acid similarity of PrM and E proteins was more than 95% over a 36-year period (Lanciotti *et al.*, 1994). Previous studies of other arboviruses have suggested that alternate replication of arboviruses in the vertebrate host and arthropod vectors may contribute to the genetic stability of arbovirus (Weaver *et al.*, 1999; Weaver *et al.*, 1992). By investigating the sequence variation of the C, E, NS2B genes of DENV3 in plasma from dengue patients, we reported that DENV exists as quasispecies in the human hosts (Wang *et al.*, 2002a; Wang *et al.*, 2002b). However, the extent of sequence variation of DENV in the mosquito vector and how the quasispecies structure changes during transmission between human and mosquitoes remain unknown. **The first specific aim** of this study is to study the extent of sequence variation of DENV in mosquitoes and the changes of quasispecies during transmission. We hypothesized that mosquitoes may contribute to the genetic stability of DENV by maintaining a more homogenous viral population and/or selecting a dominant variant during transmission. We investigated sequence variation of both the C and E genes in the field-captured mosquitoes, *Aedes*

aegypti, and eight patients from a DENV3 outbreak in southern Taiwan in 1998 (King *et al.*, 2000). Moreover, sequence variation in five experimentally infected mosquitoes was also examined.

Several studies have attempted to identify the molecular determinants of DENV associated with severe disease at full-genome level and reported different sequences associated with virulence (Leitmeyer *et al.*, 1999; Mangada & Igarashi, 1998; Rodriguez-Roche *et al.*, 2005). Since the viruses analyzed in these studies have been passaged in tissue culture, the possibility that sequence changes introduced by *in vitro* selection might mask the differences of viral sequences *in vivo* cannot be completely ruled out. On the other hand, recent epidemiological studies have shown that the proportion of severe cases, DHF, to total cases increases toward the end of an outbreak (Chao *et al.*, 2004; Guzman *et al.*, 2000; Guzman *et al.*, 2002; Rodriguez-Roche *et al.*, 2005; Wu *et al.*, 2005b). Because frequent sampling and sequencing of DENV during and between outbreaks were lacking, how DENV evolved during an outbreak or from one outbreak to another remain unclear. **The second specific aim** of this study is to investigate the viral determinants and evolution linked to outbreak with increased severity. In 2001 and 2002, there were two consecutive DENV2 outbreaks in

Kaohsiung area (Wu *et al.*, 2005b). The first one was from September to December in 2001 with a total of 194 DF and 13 DHF cases confirmed. With few sporadic cases reported during the winter and spring, the second one started in June to early December in 2002 with a total of 5,039 DF and 422 DHF cases confirmed. We carried out a comprehensive study to examine viral sequences associated with severe disease and to investigate how viruses evolve from a smaller outbreak with relatively fewer severe cases to a larger outbreak with more severe cases. In this part of study, we analyzed E sequences and full-genome directly from plasma of DF and DHF patients sequentially, two to three patients per month, during these two outbreaks.

More and more evidence suggests that the stem region of E protein is involved in assembly step of the life cycle of DENV. Based on the study of series of C-terminal deletional mutants of TBEV E protein, it was reported that E-H2 is involved in the heterodimerization of prM-E proteins, whereas E-H1 is involved in the trimerization of soluble E protein under low pH environment (Allison *et al.*, 1999). Substitutions of three residues in the stem region of DENV2 E protein were reported to enhance the production of VLPs (Purdy & Chang, 2005). However, the critical stem residues involved in the assembly remain unclear. **The third specific aim** of this study is to

investigate the roles of the stem region of E protein in the formation of VLPs. We introduced several proline substitutions to residues of the stem region in the content of plasmid expressing the PrM and E genes of DENV4 and investigated their effects on VLPs production, as well as the effects on the PrM-E heterodimerization and membrane association.

Chapter 2 Materials and methods

2.1 Study participants and samples

During the DENV3 outbreak in southern Taiwan in 1998 (King *et al.*, 2000) and the DENV2 outbreak in Kaohsiung in 2001 and 2002 (Wu *et al.*, 2005b), patients who were diagnosed with DF and DHF/DSS according to the WHO clinical definitions (WHO, 1997) and admitted to three hospitals in Tainan area (Chi-Mei Foundation Medical Center, Kuo General Hospital, and Sin-Lau Christian Hospital) and two in Kaohsiung area (Yuan General Hospital and Huei-Te Hospital) were included in this study (Table 1 and 2). With patients' consent, acute blood samples between day 2 and day 8 after onset of fever were collected in EDTA-containing tubes and plasma was prepared within 6 hours of collection and stored at -80℃ until used. All patients were confirmed to be DENV2 or DENV3 cases by a nested RT-PCR assay (Harris *et al.*, 1998; Lanciotti *et al.*, 1992). Primary or secondary infection was determined by a previously described NS1-specific IgM and IgG capture enzyme-linked immunosorbent assay (Shu *et al.*, 2003).

2.2 Viral RNA extraction from patients and mosquitoes

Viral RNA extraction from plasma

Dengue viral RNA was extracted directly from plasma, 140 μl, by using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

Viral RNA extraction from experimentally infected mosquitoes

Five mosquitoes, *Aedes aegypti*, infected with a DENV3 isolate by intrathoracic inoculation were kindly provided by Dr. Wei-June Chen (Chen *et al.*, 1993; Rosen & Gubler, 1974). Briefly, mosquitoes were inoculated with 0.17 μl of DENV (10^6 PFU/ml) into the membrane area of the thoracic pleura and maintained in a growth chamber at 32℃, 85% RH, and a photoperiod of 12:12 (Light: Dark). Tissues or organs were examined for DENV infection by a direct immunofluorescent assay (IFA) with a fluorescein isothiocyanate (FITC)-conjugated antibody, prepared by human hyperimmune serum (Chen *et al.*, 1993; Kuberski & Rosen, 1977). The inoculated DENV3 virus was isolated from plasma of patient, ID23, by using C6/36 cells (Singh & Paul, 1969; Tesh, 1979) and the titer was determined by a plaque assay using BHK-21 cells (Morens *et al.*, 1985). Each IFA positive mosquito was macerated in 100 μl of

minimal essential medium (Invitrogen, Carlsbad, CA), triturated on ice, centrifuged, and filtered through a 0.22-μm filter. The filtrate was subjected to RNA extraction.

Viral RNA extraction from field captured mosquitoes

During the DENV3 outbreak in Tainan in 1998, attempts were made to capture mosquitoes in pools from different districts for virus isolation. Each pool of mosquitoes was macerated in 100 μl of minimal essential medium (Invitrogen, Carlsbad, CA), triturated on ice, centrifuged, and filtered through a 0.22-μm filter and an aliquot of the filtrate was inoculated into C6/36 cells and monitored by an indirect IFA for 7 to 10 days. One pool, which was derived from 22 female *Aedes aegypti* captured at the central district of Tainan city, the major area of the outbreak, yielded DENV3. An aliquot of the filtrate was subjected to viral RNA extraction for subsequent analyses.

2.3 Reverse transcription, polymerase chain reaction, and sequencing

Reverse transcription

The eluted RNA was subjected to reverse transcription (RT) by cDNA synthesis kit using random hexamers as primer (Invitrogen, Carlsbad, CA) and the Superscript II RT enzyme.

Polymerase chain reaction and direct sequencing

Based on the DENV3 sequences available in the Genebank, we designed a nested polymerase chain reaction (PCR) to amplify a 360-nucleotide region in C gene and a 430-nucleotide region in E gene (Wang *et al.*, 2002a; Wang *et al.*, 2002b) (Fig. 1). The PCR conditions for amplifying the region in C or E gene were 95℃ for 5 min, followed by 30 cycles of 95℃ for 1 min, 58℃ (C region) or 62℃ (E region) for 1 min and 72℃ for 1 min, and then 72℃ for 5 min. The PCR product was purified by GFX PCR purification kit (GE Healthcare, Chalfont St. Giles, United Kingdom) and subjected to clonal sequencing.

For the RNA derived from sera of patients during the DENV2 outbreak in Kaohsiung in 2001 and 2002, we also strategically amplified 8 overlapping fragments of DENV2 genome (Table 3). The PCR product was purified by GFX PCR purification kit (GE Healthcare, Chalfont St. Giles, United Kingdom) and subjected to sequencing directly using the primers covering the whole genome (Table 4).

Clonal Sequencing

Each PCR product was cloned to the TA cloning vector, pCRⅡ-TOPO (Invitrogen, Carlsbad, CA) and was transformed to TOP10 competent cells. For each plasma

sample from infected individual, at least 10 clones derived from two separate PCR were picked up and sequenced by using the BigDye terminator cycle sequencing kit and the ABI 377 automated sequencer or ABI 3100 DNA sequencer (Invitrogen, Carlsbad, CA).

Sequence analysis

The sequencing results were converted by the program Chromas version 1.45 (Griffith university, Australia) and aligned by the program Dnaman version 4.15 (Lynnon Biosoft, Canada). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007).

2.4 Cells and antibodies

293T cells, a human embryonic kidney cell line transformed with SV40 large T gene, were maintained in Dulbecco's Modified Eagle Medium (DMEM) (JRH Biosciences, Lenexa, KS) containing 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 2% HEPES (Invitrogen, Carlsbad, CA). 293T cells were incubated at 37℃ and 5% CO_2.

Flavivirus group-reactive, anti-E mouse monoclonal antibody, FL0232, was purchased from Chance biotechnology (Taipei, Taiwan). Mouse anti-β-galactosidase monoclonal antibody was purchased from Promega (Madison, Wis.). Horseradish peroxidase-conjugated anti-human and anti-mouse IgG antibodies were purchased from Pierce (Rockford, IL).

2.5 Constructs and PCR mutagenesis

We used the plasmid expressing the PrM/E proteins of DENV4 (814669 strain), pCB-D4, as the template to generate proline substitution mutants at selected positions in the stem region of E protein by two-step PCR mutagenesis method as previously described (Hu *et al.*, 2007).

To construct pcDNA3-β-gal plasmids that encode b-galactosidase gene, from *Escherichia coli*, fused with the stem region derived from wild type or mutated E gene, d4E396AEcoRI (5'-CCCGAATTCAGTTCCATTGGCAAG-3') or d4E398IPEcoRI (5'-CCCGAATTCAGTTCCCCTGGCAAG-3') and d4E450BXbaI (5' –TAGTCTAGATTATCCTCCAAACAT-3') were used as the primer pair to amplify the stem region with the restriction enzyme sites, *Eco*RI and *Xba*I introduced. The PCR product was digested with *Eco*RI and *Xba*I, purified and cloned into the vector,

pcDNA3-β-gal-706-856, (kindly provided by Dr. Chen S.S.) (Chen *et al.*, 2001) by the EcoR Ⅰ and Xba Ⅰ site (Fig. 9A).

2.6 Transfection and collection of VLPs

Transfection

293T cells (1×10^5 cells) in 10 cm^2 dish were transfected with 10 μg DNA by calcium phosphate method (Chen & Okayama, 1987). Briefly, 10 μl DNA at concentration of 1μg/μl was mixed with 260 μl nuclease-free water (Promega, Madison, Wis.), 30 μl 2.5 M $CaCl_2$, and 300 μl 2×BBS (50 mM BES, pH 6.9, 280 mM NaCl, and 1.5 mM Na_2HPO_4), incubated at room temperature for 15 min and then added to 293T cells. After incubation at 35℃ and 3% CO_2 overnight, the transfected cells were replaced with growth medium and incubated at 37℃ and 5% CO_2 for 48 h. Cell lysates were prepared with lysis buffer containing 1% NP40, 50 mMTris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche, Germany) and subjected to Western blot analysis.

Collection of VLPs

Culture supernatants were collected at 48 h after transfection and subjected to centrifuge at 2,500 rpm for 20 min and flew through a 0.22-μm-pore-sized filter to deplete residual cell debris. The filtered culture supernatants were subjected to 20% sucrose cushion ultracentrifugation as previously described (Hu *et al.*, 2007). Briefly, the filtered culture supernatants were adjusted to 35 ml with 1×phosphate buffered saline (PBS) in a ultracentrifugation tube and added 3.5 ml 20% sucrose (Sigma, St. Louis, MO) to the bottom, following by ultracentrifugation at 19,000 rpm (47,770×g) with accurate weighting in rotor SW28 rotor (Beckman, Fullerton, CA). After ultracentrifugation at 4℃ for 5 h, the pellets were resuspended in 15 μl TAN buffer, pH 8.0 (50 mM triethanolamine, 0.1 M NaCl) and then subjected to Western blot analysis.

2.7 Western blot analysis

An aliquot of cell lysates or pellets were mixed with equal volume of 2×sample buffer (250 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, 0.4% bromophenol blue), followed by boiling at 95℃ for 2 min, subjected to SDS-12% polyacrylamide gel electrophoresis (PAGE), and then transferred to nitrocellulose membrane (Hybond-C, GE Healthcare, Chalfont St. Giles, United Kingdom) with an electrotransfer apparatus

(Bio-Rad, Hercules, CA). Membranes were blocked with 4% milk, prepared in wash buffer (1×PBS containing 0.2% Tween-20), and incubated with primary antibody at room temperature for 1 h. After washing for 3 times, a horseradish peroxidase-conjugated secondary antibody was added and the incubated at room temperature for 1 h. After washing for 3 time, the signals were detected by enhanced chemiluminescence reagents (Perkin Elmer life sciences, Boston, MA) (Wang *et al.*, 2006). The intensities of E and PrM bands were further analyzed by the program ImageQuant™ TL (GE Healthcare, Chalfont St. Giles, United Kingdom) and the ratio of the intensity of E or PrM band in pellets to that in cell lysates was also determined.

2.8 Radioimmunoprecipitation

293T cells prepared in 6-well plate were transfected with 4μg DNA by calcium phosphate method as described previously. The transfected cells were washed with 1×PBS twice and incubated with culture medium at 37℃ for 2 h. After washing with 1×PBS twice and Met-free DMEM (Sigma, St. Louis, MO) once, cells were incubated with starvation medium (Met-free and FBS-free DMEM containing 1% penicillin/streptomycin) at 37℃ for 2 h. After washing with 1×PBS twice and Met-free DMEM once, cells were labeled with 60 μCi [^{35}S]methonine (GE Healthcare,

Chalfont St. Giles, United Kingdom) and then incubated at 37 °C for 6 h. Cell lysates were prepared as previously described by adding 50 μl lysis buffer per well. To avoid non-specific binding of antigens and protein A beads, cell lysates were incubated with protein A sepharose beads (GE Healthcare, Chalfont St. Giles, United Kingdom) at 4℃ for 2 h. The pre-clear cell lysates were incubated with mouse anti-E monoclonal antibody (Mab), FL0232 (Chance Biotechnology, Taipei, Taiwan), which was pre-incubated with protein A beads, at 4℃ overnight. After washing with RIPA buffer (50 mM Tris, pH7.5, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS) at 4℃ for 5 min each for 4 times. The supernatants were discarded and the beads were added with 15 μl 2×sample buffer, boiled at 95℃ for 5 min. An aliquot was loaded and subjected to SDS-12% PAGE. The gel was then dried with the gel dryer equipment (Bio-Rad, Hercules, CA). After exposure of the X-ray film, the intensities of E and PrM bands were further analyzed by the program ImageQuant^{TM} TL. The ratio of the intensity of PrM band to E band was also determined.

2.9 Membrane flotation assay

293T cells (1×10^5 cells) in 10 cm^2 dish were transfected by 10 μg DNA by calcium phosphate method. Membrane flotation assay was performed as described previously

(Chen *et al.*, 2001; Kiernan *et al.*, 1999). Briefly, the transfected cells were washed twice with buffer A (10 mM Tris-HCl, PH 7.4, 1 mM EDTA and 1 mM EGTA) and lysed with 0.5 ml buffer B (10 mM Tris-HCl, PH 7.4, 1mM EDTA, 10% sucrose, and complete protease inhibitor cocktail). The cell lysates were sonicated twice on ice and then centrifuged at 1,000×g at 4℃ for 10 min to discard the nuclear debris. An aliquot of 0.25 ml postnuclear supernatants were mixed with 85.5% sucrose and then overlaid with 65% and 6% sucrose followed by ultracentrifugation at 24,000 rpm (98768×g) at 4℃ for 18 h (SW41 rotor, Beckman, Fullerton, CA). Twelve fractions were collected from top to bottom and then concentrated by trichloroacetic acid (TCA) precipitation method (Lowry *et al.*, 1951). Briefly, 300 μl of each fraction was added in 240 μl of 1×PBS, 60 μl of TCA at concentration of 100%, and 20 μg of BSA. After vigorously mixing and chill on ice for 30 min and centrifugation at 12,000 rpm at 4℃ for 30 min (Eppendorf, Westbury, NY), the pellets were resuspended with 24 μl of 1×sample buffer, followed by adding NaOH at concentration of 5N until neutralized. An aliquot of the precipitated sample was added with dithiothreitol (DTT, Bio-Rad, Hercules, CA) to a final concentration of 100 mM and then subjected to Western blot analysis.

2.10 Subcellular fractionation assay

293T cells (1×10^5 cells) in 10 cm^2 dish were transfected with 10 μg DNA by calcium phosphate method. Subcellular fractionation assay was performed as described previously (Xu *et al.*, 1997). Briefly, the transfected cells were washed 3 times with 1× PBS after 48h and then resuspended in 1 ml modified buffer B containing 10% sucrose, 20 mM Tris, 150 mM NaCl, 10 mM magnesium acetate, 1 mM EGTA, pH 7.6. After freeze-thaw for 8 times using dry ice bath containing ethanol and thawed at 37℃, the cell lysates were passed through 25-gauge needle for 10 times on ice and subjected to centrifugation at 1,000×g for 10 min to discard the nuclear debris. The supernatants of the cleaned cell lysates were subjected to centrifugation at 14,000 rpm for 30 min at 4 ℃ to pellet down the membrane fractions of cell lysates. The resulting supernatants were subjected to 20% sucrose cushion ultracentrifugation at 22,000 rpm (45,264×g) (SW50.1 rotor, Beckman, Fullerton, CA) at 4℃ for 1.5 h to collect the pellets of the soluble fractions. The membrane fractions and soluble fractions of cell lysates were then subjected to Western blot analysis.

Chapter 3 Results

3.1 Sequence variation of dengue type 3 virus in naturally infected mosquitoes and human hosts

Sequence variation of E and C genes of DENV in naturally infected mosquitoes

To investigate sequence variation of DENV in the naturally infected mosquitoes, we studied DENV3 sequences in a pool of mosquitoes consisting of 22 female *Aedes aegypti* mosquitoes, captured at the central district of Tainan City, the major area of the outbreak, during the DENV3 epidemic in Taiwan in 1998. We analyzed 430-nucleotide region covering the domain Ⅲ of the E gene as well as 360-nucleotide region covering the entire C gene as described previously (Fig. 1) (Wang *et al.*, 2002a; Wang *et al.*, 2002b).

There were 17 nucleotides substitutions, of which 9 were silent and 8 were nonsilent, in the analyzed region of E gene derived from 21 clonal sequences of the pool of naturally infected mosquitoes (Table 5). The mean diversity, which is the number of substitutions divided by the total number of nucleotides sequenced, was 0.21%. Pairwise comparison of amino acid sequences of individual clones by program MEGA

revealed that the pairwise p-distance ranged from 0 to 1.53% with mean of 0.58%. Among these 21 clones examined, there were six nucleotide substitutions within C gene with a mean diversity of 0.09% (Table 5). Pairwise comparison of amino acid sequences of C gene examined shown a mean p-distance of 0.27% ranging from 0 to 2.83%. These findings suggested that DENV3 virus is present as quasispecies in the mosquitoes, albeit as a relative homogenous population.

Sequence variation of DENV in mosquitoes and human hosts

To compare the extent of sequence variation in mosquitoes with that in human hosts, dengue viral RNA, which were derived from acute plasma samples of eight confirmed DENV-3 patients who lived in the same district or in nearby districts, was subjected to RT-PCR and clonal sequencing as previously described (Wang *et al.*, 2002a; Wang *et al.*, 2002b). For the E gene, ten to fifteen clones from each patient were sequenced and analyzed. The mean diversity ranged from 0.15 to 0.59% with the overall mean diversity of 0.38%, which was higher than that determined for the mosquitoes (Table 5). For the C gene, ten clones from each patient were analyzed as reported previously (Wang *et al.*, 2002b). The mean diversity ranged from 0.13 to 0.41% with the overall mean diversity of 0.23%, which was higher than that in the

mosquitoes (Table 5). Both of the genetic diversity in E and C genes determined in patients was higher than that in mosquitoes. These findings suggested that the extent of sequence variation in the naturally infected mosquitoes was generally lower than that in dengue patients.

Analysis of amino acid sequences of DENV in mosquitoes and patients

The deduced amino acid sequences of E gene from the field-captured mosquitoes were aligned. Within the 131-amino acid region analyzed, there were 13 clones with identical sequences and the other 8 clones with eight amino acid substitutions, respectively (Fig. 2A). In the representative case, ID18, there were identical sequences in 4 clones and several substitutions with an in-frame stop codon in the other 11 clones (Fig. 2B). The analysis of the quasispecies structures of the E gene from the field-captured mosquitoes and the patients, suggested that the consensus sequences of 21 clones from the mosquitoes and of multiple clones from each patient were the same (Fig. 2 and data not shown). Moreover, the consensus sequence corresponded to the sequences of the major variants of the quasispecies suggesting that the major variant is transmitted between mosquitoes and humans (Fig. 2).

To further verify this, RNA derived from the mosquitoes and four of the eight patients were subjected to RT-PCR for E gene and direct sequencing, the sequence derived from which is believed to represent the dominant sequence. The results revealed that the full-length E sequences of the mosquitoes and of the four patients were the same (data not shown), supporting the notion that DENV with an E sequence of the major variant were transmitted.

Sequence variation of E and C genes of DENV in experimentally infected mosquitoes

The mean diversity observed from the homogenates of 22 field-captured mosquitoes may represent the average of the results for the greater part of infected mosquitoes rather than for a single one. To investigate the extent of sequence variation in a single mosquito, we infected *Aedes aegypti* with 0.17 μl (10^6 PFU/ml) of a DENV3 isolated from plasma of patient ID23 through intrathoracic inoculation, identified each infected mosquito by a direct immunofluorescent antibody test (Chen *et al.*, 1993; Gubler & Rosen, 1976; Rosen & Gubler, 1974). There were 5 infected mosquitoes subjected to extract viral RNA and following clonal sequence analysis as previously described. The overall mean diversity of the E gene, 0.09% ranging from

0.05 to 0.14% (Table 6), was slightly lower than that seen with the patients (Table 5). For C gene, the overall mean diversity, 0.1% ranging from 0 to 0.19%, was also slightly lower than that seen with the patients (Table 5 and Table 6). Taken together, these data indicated that the extent of sequence variation in mosquitoes was lower than that in dengue patients.

Pairwise comparison of amino acid sequences of DENV E protein in naturally and experimentally infected mosquitoes and in human hosts

To examine the extent of quasispecies in mosquitoes and human hosts, pairwise comparison of amino acid sequences of E protein was performed using the program MEGA (Tamura *et al.*, 2007). A more homogenous population in the mosquitoes than in the patients was noted (Figs. 3A and 3C). Similarly, a more homogenous population in each experimentally infected mosquito than in the patients was also observed (Figs. 3B and 3C). The results were consistent with that findings that the mean diversity, the proportion of nucleotide substitutions to total, was lower in mosquitoes than in the patients.

3.2 Evolution of dengue virus type 2 during two consecutive outbreaks in Southern Taiwan in 2001-2002

Sequence analysis of E gene

It was recently reported that the proportion of severe cases (DHF) to total cases increased toward the end of an outbreak in more than three occasions (Chao *et al.*, 2004; Guzman *et al.*, 2000; Guzman *et al.*, 2002; Rodriguez-Roche *et al.*, 2005; Wu *et al.*, 2005b). In 2001 and 2002, there were two consecutive DENV2 outbreaks in Kaohsiung city and county, a metropolitan area in southern Taiwan. As the total cases increased from 2001 to 2002 outbreaks, the proportion of DHF to total cases was also increased from 6.3% (13 DHF out of 207 total cases) to 7.7% (422 DHF out of 5,461 total cases). To investigate the viral sequences associated with DHF patients and how viruses evolve from a small outbreak with relatively fewer severe cases to a large outbreak with relatively more severe cases, we collected plasma from patients with different disease severities sequentially and 2 to 3 patients per month during these two outbreaks (Fig. 4 and Table 2). Because the E protein is known to be the major determinant of tropism and the major target of neutralizing antibodies, we first examined the entire E gene derived directly from plasma of 8 patients (3 DHF, 5 DF)

during 2001 outbreak and 23 patients (11 DHF, 12 DF) during 2002 outbreak. The sequences of E gene from the 2001 viruses were very similar to those from the 2002 viruses with a mean *p*-distance of 0.27%, ranging from 0.14 to 0.52%, calculated by the program MEGA (Tamura *et al.*, 2007). There was no consistent nucleotide or amino acid change between E sequences of viruses from patients with different disease severities when comparing either outbreak or two outbreaks together (Table 7).

To investigate the origin of the DENV2 of the 2001-2002 outbreaks in Kaohsiung, we performed phylogenetic analysis of E sequences including 31 E sequences studied, 27 available E sequences from countries, where there were cases imported to Taiwan during this period, and 4 to 7 E sequences from each of the 5 genotypes of DENV2 (Salda *et al.*, 2005; Taiwan, 2004; Twiddy *et al.*, 2002). Based on the phylogenetic relationship depicted by Maximum likelihood tree, the Kaohsiung viruses belonged to the cosmopolitan genotype and formed two clusters corresponding to the year of outbreak, the 2001 and 2002 viruses clusters (Fig. 5). Similar results were also shown by Neighbor joining tree and the Bayesian analysis (data not shown) (Felsenstein, 2005; Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003).

Interestingly, two isolates from Philippines during this period, 00U18 (in 2000) and 01RBD1 (in 2001), were closely related to the 2001 cluster of the Kaohsiung viruses (Fig. 5). Pairwise comparison of the E sequences from two Philippines isolates, 00U18 (in 2000) and 01RBD1 (in 2001), with the 2001 cluster of the Kaohsiung viruses revealed a mean *p*-distance of 0.29% ranging from 0.21 to 0.46% and 0.41% ranging from 0.36 to 0.46%, respectively. By further examining the E sequences, three unique genome positions of the Kaohsiung viruses, A at genome position 1088, T at genome position 1509 and 2057, were shared by the Philippines isolates rather than other 50 DENV-2 of the 5 genotypes. These findings suggested that the Kaohsiung viruses of the 2001 and 2002 outbreaks originated from the Philippines (data not shown).

Sequence analysis of full genome

To investigate viral sequences linked to disease severity in other regions of the genome, we sequenced 17 full-genome sequences from 5 patients (2 DHF and 3 DF) during the 2001 outbreak and 12 patients (5 DHF and 7 DF) during the 2002 outbreak. Compared with the sequence of the earliest 2001 virus examined, several nucleotide substitutions were found in different regions of the genome (Table 8). With proportional to the relative size of structural and nonstructural genes, there were 26.5%

nucleotide substitutions (62 nucleotide substitutions out of total 234 substitutions observed) in the structural genes and 73.5% (172 out of total 234 nucleotide substitutions) in the nonstructural genes. Most (184 out of total 234 substitutions, 78.6%) of the nucleotide substitutions were silent. There was no consistent nucleotide or amino acid change found between viruses from patients with different disease severity, when comparing viruses from DHF patients with DF patients of either outbreak or two outbreaks together (Table 8).

Comparison of the 17 full-genome sequences revealed 5 consistent nucleotide differences between the 2001 and the 2002 viruses (Table 8). Of the five, three were in the nonstructural genes, including one each in NS1, NS4A, and NS5, and two were in the structural genes, the E gene. There was only one nucleotide substitution resulting in amino acid change from threonine to isoleucine at residue 46 of the E protein (Table 8). A closer examination of the full-genome sequences of the 2001 and 2002 viruses revealed that 4 nucleotide substitutions, A at genome position 1220 and 8774, T at genome position 8342, C at the genome position 8374, which were conserved by all the 2002 viruses, were present in 915-DF01 virus, one of the five (20%) 2001 viruses studied (Table 8). Phylogenetic relationship of the 2001 and 2002 viruses and viruses

of the 5 genotypes of DENV2 was determined by the maximum likelihood method and by the program Phylip (Felsenstein, 2005) (Fig. 6). Among the 2001 viruses, 915-DF01 was the one most closely related to the 2002 viruses. Our findings suggested that the 2002 viruses probably evolved from a minor variant, 915-DF01, of the 2001 viruses.

We further investigated the overall and condon-specific selection pressure in the entire conding region of 17 full-genome sequences by using the SLAC, EFL, and REL methods in the Datamonkey facility (Pond & Frost, 2005). Consistent with previous reports, the ratio of d_N to d_S, d_N/d_S, was 0.108, which was lower than 1, for the entire conding region. There was no evidence of positive selection (Klungthong *et al.*, 2004; Myat Thu *et al.*, 2005; Zhang *et al.*, 2006). Moreover, there was no positively selected site in the entire coding region by the SLAC and FEK methods, except that 15 positively selected sites at the C, PrM, E, NS2A, NS3, NS4B and NS5 genes were found by the REL method (Table 9). One negatively selected site at codon 366 of NS5 gene was consistently determined by all three methods. There were 16 negatively selected sites at the E gene and the 7 nonstructural genes found by the FEL method.

3.3 The involvement of the stem region of DENV E protein on particle formation

Production of VLPs

It was reported previously that noninfectious VLPs are produced during natural flavivirus infections (Russell *et al.*, 1980), and expression of flaviviral PrM and E genes are capable of producing VLPs (Chang *et al.*, 2000; Davis *et al.*, 2001; Hunt *et al.*, 2001). Our lab has reported recently that a strong ER retention signal was in the stem and anchor regions of DENV2 E protein, which affected the production of VLPs (Hsieh *et al.*, 2008). To investigate the critical residues in the stem region of E protein which are involved in VLPs production, we performed site-directed mutagenesis within the stem region in the context of DENV4 PrM/E expressing plasmid, pCB-D4 (Fig. 7A). Based on the helical wheel analysis of the two consecutive, amphipathic α-helixes, H1 and H2, of the stem region of E protein, we introduced proline substitution, an α-breaker, at *a* and *d* positions of these heptad repeats by site-directed mutagenesis. We collected cell lysates and pellets from the culture supernatants of the transfected cells and examined the PrM and E proteins by Western blot analysis using sera from confirmed DENV patients as primary antibody (Fig. 7B). The expression of PrM and

E proteins in cell lysates of each of the mutants were generally comparable to that of the wild type (WT, pCB-D4), except mutant 412MP of which the E protein seemed to degrade rapidly. We then quantified the intensities of PrM protein bands and calculated the ratio of the intensity of mutant PrM band in pellet to that in cells, relative to the ratio of WT as the VLP production index. Thus, the VLP production index = [intensity of mutant PrM band (pellets) / mutant PrM band (cells)] × [intensity of WT PrM band (pellets) / WT PrM band (cells)]. We found that the production of VLP was moderately affected by proline substitutions at residues 415, 418, 422, 429, 436 and 446 and severely decreased by proline substitutions at residues 398, 401, 405, 408 and 439 (Fig. 7B). Our findings suggested that residues at 398, 401, 405, 408, 415, and 418 in the H1 of the stem region of E protein were involved in the formation of VLP.

PrM-E heterodimerization

It was reported previously that proper PrM-E interaction was important for the production of VLP (Allison *et al.*, 1999). We conducted a radioimmunoprecipitation assay to investigate the interaction between PrM and E protein of these proline mutants. The 293T cells transfected with WT and mutant plasmids were metabolically labeled with [^{35}S]methonine, then lysed and immunoprecipitated with a mouse anti-E Mab,

FL0232. The intensities of PrM bands relative to those of E bands were measured, and the PrM-E interaction index = [intensity of mutant PrM band / mutant E band] × [intensity of WT PrM band / WT E band] was calculated (Fig. 8). As shown in Fig. 8, interestingly, the proline mutants in the H1 (such as 398, 410, 405, 408, and 415) had lower PrM-E interaction index when compared with that of the WT, suggesting that H1 residues are involved in PrM-E interaction, and that the decreased PrM-E interaction of the H1 residues may contribute to their impaired VLP production. On the other hand, while the proline mutants in the H2 did not affect the PrM-E interaction, some H2 proline mutants (such as 436, 439, and 446) had impaired VLP production, suggesting that these H2 residues are involved in the VLP production by mechanisms other than affecting the PrM-E interaction.

Membrane association of the stem region

Based on cryo-EM structure of mature DENV2 particles, the H1 of the stem region of E protein is angled in the outer lipid leaflet, whereas the H2 is partially buried in the outer leaflet (Zhang *et al.*, 2003a). To investigate whether H1 and H2 of the stem region is capable of binding to membrane, we carried out a membrane flotation assay as

described previously (Chen *et al.*, 2001). The plasmid, pCDNA3-β-gal (a gift from Dr. Chen S.S.), expressing β-galactosidase (β-gal) in cytosol was fused with the cytoplasmic tail of HIV-1 gp41, a previously reported membrane associated fragment (Chen *et al.*, 2001), or with the stem region of DENV E protein including WT stem or the H1 or H2 mutant (Fig. 9A). As shown in Fig. 9B, the β-gal alone was found mainly in the bottom fractions, whereas the β-gal/gp41 was found predominantly in the fraction 9, suggesting that cytoplasmic tail of HIV-1 gp41 can bind to membrane. Interestingly, the β-gal/stem (WT) was also found primarily in the fraction 9, suggesting that the stem of DENV E protein can bind to membrane. For the two H1 mutants (398 and 405) tested, both (β-gal/398IP and β-gal/405TP) were found mainly in the fraction 9, suggesting that H1 mutations do not affect the membrane binding ability of the stem region. In contrast, for the 7 mutants in the H2 (429, 436, 439, 443, 446) or between H1 and H2 (422, 425), a considerable proportion of the chimeric β-gal has shifted from the fraction 9 to bottom fractions (β-gal/429FP, β-gal/436VP, and β-gal/443VP) or spreading to several fractions (β-gal/422FP, β-gal/439VP, and β-gal/446TP), whereas β-gal/425VP remains primarily in the fraction 9. Interestingly, of the 7 mutants, 425VP was the only mutant that did not affect VLP production. Taken together, these

findings suggested that for mutants in the H2 or between H1 and H2, decreased membrane binding ability correlate with their impaired VLP production.

Subcellular localization of PrM, E proteins and VLPs

To investigate whether intracellular PrM and E proteins retain in the membrane-bound fraction or form intracellular VLPs in the soluble fraction of cellular compartments, we carried out a subcellular fractionation assay as previously described (Xu *et al.*, 1997). As shown in Fig. 10, the amounts of PrM and E proteins of mutants 398, 401, 422, and 439 were decreased, when compared with those of the WT, in both the membrane-bound fraction and the pellets of the soluble fractions, suggesting that these mutant PrM and E proteins have defects in the secretion of VLPs from the membrane structure, most likely the ER, to the lumen and they do not accumulate in the membrane probably due to degradation of these mutant proteins. As a control, similar amounts of calnexin, an intergral ER membrane protein, were found in the membrane-bound fraction.

Chapter 4 Discussion

4.1 Sequence variation of DENV3 virus in mosquitoes and human hosts

To our knowledge, this is the first study that directly examined dengue viral sequences in the naturally infected mosquitoes. Based on the analysis of both the E and C gens, we demonstrated that the extent of sequence variation was generally lower in the naturally infected mosquitoes than in the human hosts. This was further verified with by examining the five experimentally infected mosquitoes. However, the possibility that the sequence variation observed was due to *in vitro* artifacts needs to be addressed. The error rate of RT was estimated to be around 10^{-4} on a complex template (Smith *et al.*, 1997). The error rate of *Taq* polymerase was reported to be between 3.9×10^{-6} and 9.1×10^{-6}/nucleotide/cycle under most PCR conditions (Lu *et al.*, 1995; Martell *et al.*, 1992; Meyerhans *et al.*, 1989). Together with the RT error rate and after 60 cycles of PCR amplification, the error frequencies ranging from 0.02 to 0.05% was lower than the mean diversity observed with most of our samples. We carried out a control experiment in which a previously sequenced E or C clone was used as template and subjected to *in vitro* transcription, RT, nested PCR, and clonal sequencing. The nucleotide substitution frequencies of control experiments ranging

from 0.03 to 0.04% were also lower than the mean diversity observed with most of our samples (Wang *et al.*, 2002a; Wang *et al.*, 2002b). Therefore, the sequence variation oberved in this study was unlikely to be due to *in vitro* artifacts, though a small proportion of the substitutions might have been introduced by RT or *Taq* polymerase. Of note, for the two experimentally infected mosquitoes (M2 and M3), the mean diversities for the C gene were 0 to 0.03% (Table 6), suggesting a very homogenous population. A study of eastern equine encephalitis virus, an arbovirus, has shown that persistent infection in the mosquito cell line was associated with a lower rate of genetic change (Weaver *et al.*, 1999). It was likely that persistent infection of DENV in mosquitoes would contribute to a lower extent of sequence variation in the mosquitoes than in the patients observed in our study.

This is also the first report showing that the major variant of the dengue viral quasispecies was transmitted between the mosquito and its human hosts. This was different from what had been reported in the transmission study of HIV-1. For transmission of HIV-1, a minor variant was demonstrated to be transmitted and a mechanism of selective transmission or selective amplification was proposed (Wolinsky *et al.*, 1992; Zhu *et al.*, 1993). Although the infection rate of mosquitoes varies with

the titers of the viremia in dengue patients, it has been reported that an amount equal to as much as 10^4 mosquito 50% infectious doses of virus was transmitted (Kuno, 1997; Rodhain & Rosen, 1997; Rosen & Gubler, 1974). Based on previous study, skin Langerhans cells were the initial targets of DENV infection after a bite of an infected mosquito (Wu *et al.*, 2000). Our findings suggested that selective transmission or amplification of a minor variant is unlikely to have occurred before DENV enters the circulation. A study of the structural proteins of several serological subgroups of flaviviruses has shown a high degree of genetic conservation within each subgroup that includes DENV (Mandl *et al.*, 1988). Continuous adaptation of DENV between mosquitoes and humans has been suggested to account for the genetic conservation (Lanciotti *et al.*, 1994). By investigating the quasispecies structures within these two hosts, we demonstrated that only the major variant was transmitted between the mosquitoes and humans. Moreover, DENV tends to maintain a more homogenous population in the infected mosquitoes, which has a life-long infection. These facts would contribute to the evolutionary conservation of DENV.

It has been reported that the genetic stringency imposed on DENV and other arthropod-borne viruses that replicate in both vertebrate and arthropod hosts would

contribute to the sequence conservation (Lanciotti *et al.*, 1994; Weaver *et al.*, 1999; Weaver *et al.*, 1992). We analyzed the analyses of the differences in the number of synonymous nucleotide substitutions per site (dS) and the differences in the number of nonsynonymous nucleotide substitutions per site (dN) by the program MEGA (Tamura *et al.*, 2007). The ratio of dS to dN (dS/dN) was determined. In the infected mosquitoes, the mean dS/dN of the E gene was 3.92 implicated the presence of negative (purifying) selection. Similarly, the mean dS/dN of the E gene, ranging from 1.13 to 4.79, was higher than 1 in seven out of the eight dengue patients, suggesting negative selection in human hosts as well. The negative selection probably results from certain structural or functional conservation of the virus, though the nature of the genetic constraints remains to be determined.

4.2 Sequence signatures correlated with disease severity

The pathogenesis of DHF has been one of the major issues in dengue research (Green & Rothman, 2006; Gubler, 2002; Guzman & Kouri, 2002; Halstead, 1988; Rosen, 1977). One basic question is whether the viral strains found in DHF patients differ from those in DF patients. To address this and to avoid potential mutations introduced by *in vitro* passage of viruses, we examined full-length DENV-2 sequences directly from plasma of 17 patients (10 DF and 7 DHF) during the 2001 and 2002 outbreaks in Kaohsiung. We found no consistent nucleotide or amino acid difference between viruses from DHF patients and those from DF patients. Because the immune status of the host, primary or secondary infection, was known to be an important risk factor for DHF, we excluded one case with primary infection and four cases with unknown status and reanalyzed the 12 full-genome sequences from 6 DF and 6 DHF patients with secondary infection (Table 2). Among these, there was also no consistent nucleotide or amino acid difference between viruses from DF and DHF patients (Table 8). Our findings suggested that host factors, such as age, nutritional status, and underlying diseases probably play a critical role in determining disease severity in the same outbreak. It was reported that underlying diseases, including diabetes and

hypertension, were associated with a complicated clinical course in DHF patient in this outbreak (Lee *et al.*, 2005; Wu *et al.*, 2005b), supporting this interpretation.

A recent study of the DENV2 outbreak in Cuba in 1997 reported that the proportion of DHF cases increased during the peak of the outbreak, from 5.2% in May to 7.4% in June and 11.9% in July (Guzman *et al.*, 2000; Guzman *et al.*, 2002). Analysis of 6 full-genome sequences identified 5 consistent nucleotide changes in NS1, NS2A, and NS5 genes between the early viruses and the late viruses during this outbreak in Cuba in 1997 (Rodriguez-Roche *et al.*, 2005). We also observed a gradual increase in the proportion of DHF cases during the peak of the 2002 outbreak in Kaohsiung, from 5.7% in July-August to 7.8% in September-October and 10.2 in November-December (Wu *et al.*, 2005b). Based on the analysis of 17 full-genome sequences, there was no consistent nucleotide change between viruses of the three indicated periods of the outbreak in Kaohsiung in 2002 (Table 8). Our findings suggested that the observation of increase in disease severity could not be attributed to changes in viral sequences in this outbreak. Interestingly, 5 consistent nucleotide changes in the E, NS1, NS4A, and NS5 genes were found between the early viruses in 2001 and the late viruses in 2002. The observations of no identical change associated

with the late viruses from Cuba outbreak in 1997 and Kaohsiung outbreak in 2002 were summarized in Fig. 11. The DENV2 of the Cuba outbreak in 1997 belonged to the American/Asian genotype, however, those of the Kaohsuing outbreak in 2002 was the cosmopolitan genotype, suggesting that sequence signatures of the presumably more virulent late viruses were different depending on the genotype or strain of DENV.

One of the 5 consistent substitutions between the 2001 and 2002 viruses resulted in threonine to isoleucine change at residue 46 of E protein. Based on the crystallographic structure of DENV E protein, residue 46 was in the D_0 strand of domain Ⅰ and located on the surface (Modis *et al.*, 2003). This residue has been reported to be in one of the antigenic motifs defined by peptide ELISA with human sera (Innis *et al.*, 1989). Peptide containing this residue can elicit neutralizing antibody and has also been identified as one of the T-helper cell epitopes in animals (Roehrig *et al.*, 1990; Roehrig *et al.*, 1994). It is possible that substitution of threonine to isoleucine at this position may affect the binding of neutralizing antibody or recognition by T-helper cells. It is worth noting that residue 46 of E protein was under positive selection pressure identified by the REL method, meanwhile, three of the other 4 consistent substitutions (codon 376 of E, codon 261 of NS1, and codon 529 of NS5) were found

to be negative selection sites by the FEL method (Table 9). In addition, the 5 consistent nucleotide substitutions may have effects on the RNA secondary structure and codon usage, resulting in differences in the efficiency of virus replication. Whether and how each of the 5 substitutions identified in this study or their particular combinations contributes to a higher level of replication in monocytes or dendrictic cells remain to be investigated experimentally in the context of infectious clone. It has been reported that DENV in plasma as quasispecies (Wang *et al.*, 2002a). Thus, it is possible that certain phenotypic differences were attributed to mutations present at a low frequency in the quasispecies and could not be delineated by the 5 consistent nucleotide substitutions observed, which were presumably the dominate variants from each patient.

Because the 2002 viruses shown a high degree of similarity to the 2001 viruses with 99.73% mean *p*-distance of E gene and there was no evidence of re-introduction of new DENV-2 to Kaohsiung in 2002, the 2002 viruses appeared to evolve from the 2001 viruses (Taiwan, 2004). By phylogenetic analysis of the sequences from the viruses collected during the outbreaks sequentially, we demonstrated that the 2002 viruses descended from a minor variant of the 2001 viruses (Fig. 6). To our knowledge, this is the first study examining how DENV evolves during two consecutive outbreaks by

studying the viral sequences continuously at different time points. In agreement with the notion that evolution of DENV is generally undergo a strong negative selection, the analysis of the d_N/d_S ration for the entire coding region of the Kaohsiung viruses revealed no evidence of positive selection overall (Holmes, 2003; Holmes & Twiddy, 2003; Twiddy *et al.*, 2002; Zhang *et al.*, 2006). The 2002 viruses most likely resulted from a genetic bottleneck during the interepidemic period between January and May 2002, in which the temperature was decreased and only sporadic cases were reported. It has also been observed in other studies that lineage extinction and replacement of DENV were attributed to stochastic events during the interepidemic period (Myat Thu *et al.*, 2005; Sittisombut *et al.*, 1997; Wittke *et al.*, 2002). Compared with a recent study of lineage turnover, in which the dominant clade at a given year descended from a minor variant 4 or 5 year ago, it was surprising to observe that the 2002 viruses descended from a minor variant of the 2001 viruses during a short interepidemic period, less than 6 months. More importantly, these findings supported that epidemic strains of DENV evolve via drift from existing minor variants in the population. This may represent a mechanism of evolution of DENV from one outbreak to another, which was not appreciated previously.

4.3 The function of the stem region of E protein

We utilized the approach of site0directed mutagenesis to identify critical residues of the stem region of DENV E protein for particle formation and virus assembly. We found that mutations introduced to residues 398, 401, 405, and 408 in the H1 of the stem region impaired the interaction between PrM and E proteins and the production of VLPs. We also found that mutations introduced to residues 429, 436, 439, and 446 in the H2 of the stem region diminished the membrane binding ability of the stem and resulted in decreased VLP production. These findings suggested that the H1 residues of the stem region in E protein are involved in the PrM-E interaction and the H2 residues of the stem region are involved in association with membrane.

It has demonstrated previously that replacement of the entire stem and anchor region of DENV2 with the corresponding sequence of JEV enhances the biosynthesis and secretion of VLP of DENV2 (Chang *et al.*, 2003). However, replacement of partial H2 of the stem plus the anchor region with that of JEV did not enhace the VLP production of DENV2 (Purdy & Chang, 2005), suggesting that H1 of the stem region also plays an important role in VLP production. In agreement with this, our results suggested that H1 residues of the stem region are involved in the interaction between

PrM and E proteins, and that impaired PrM-E interaction of the H1 proline mutants contribute to the decreased production of VLP.

Based on the C-terminal deletional study of TBEV E protein, it was suggested that H2 and T1 of the stem and anchor regions are important for the PrM-E heterodimerization and the interaction between PrM and the ectodomain of E protein are involved in PrM-mediated transport and secretion of E protein (Allison *et al.*, 1999). Interestingly, our single proline substitution introduced to some of the H1 residues resulted in reduced PrM-E interaction, suggesting that H1 residues are also involved in PrM-E heterodimerization, which was not observed in the deletional study of TBEV. However, our study do not exclude the possibility that the interaction between PrM protein and the ectodomain of E protein also contribute to the PrM-E heterodimerization.

This is the first study showing that the H2 of the stem region is associated with membrane. We identified critical residues at positions 429, 436, 439, and 446 of H2 helix, in the presence of H1 helix, can bind to membrane and are involved in VLP production. Based on the cryo-EM study of DENV virus particles, the stem region contacts with the side of the E protein closest to the lipid, possibly by neutralizing

electrostatic repulsion between the outer phospholipid bilayer head group and the membrane-facing side of the E protein (Mukhopadhyay *et al.*, 2005). Thus, our results suggested that the H2 helix of the stem region plays an important role in VLP production by associating with the membrane, localizing the E protein and curving the membrane for particle formation.

To our surprise, proline substitution at residue 422 in the small conserved sequence (CS) between H1 and H2 of the stem region decreased VLP production, slightly impaired PrM-E heterodimerization and also diminished the ability of membrane association. Based the amino acid sequences analysis, phenylalanie at residue 422 was highly conserved among flaviviruses (data not shown). Thus, residue 422 of E protein may represent a novel target of anti-flavivirus drug.

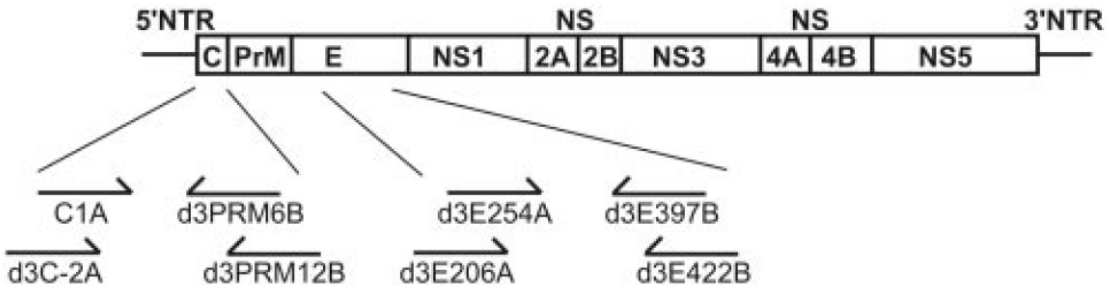
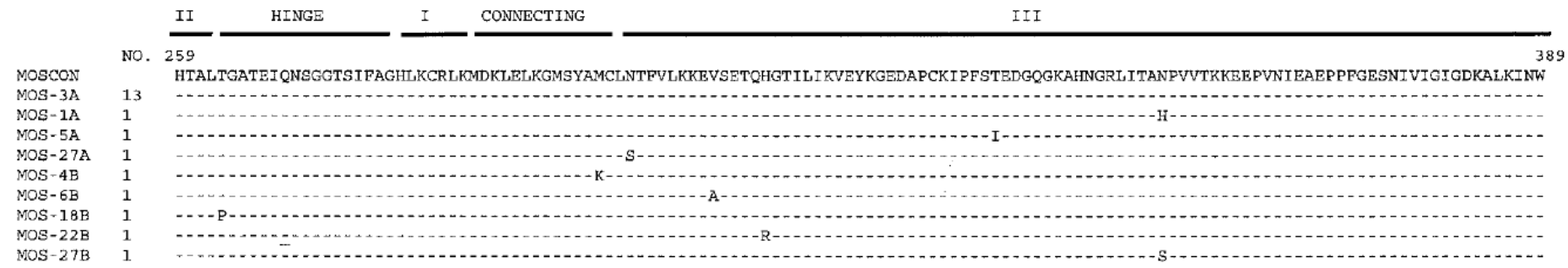


FIG. 1 Schematic diagram of the DENV genome and the C and E regions analyzed in this study. The genome positions corresponds to the DENV-3 H87 strain (Osatomi & Sumiyoshi, 1990). The primer pairs and their sequences are as follows: d3C-2A, 5'-TCTCTGATGAACAACCAACGG-3' (position at 89 to 109); d3PRM12B, 5'-GCGCGGCTCTCCATCTCGTG-3' (position at 469 to 450); C1A, 5'-ATGAACAACCAACGGAAAAAG-3' (position at 95 to 115); d3PRM6B, 5'-ATGAACAACCAACGGAAAAAG-3' (position at 95 to 115); d3E206A, 5'-TGGATGGTACATAGICAATGG-3' (position at 1543 to 1563; I stands for deoxyinosine); d3E422B, 5'-AGTCCCAGGCTGTGTCTCC-3' (position at 2191 to 2173); d3E254A, 5'-ATCICAAGAGGGAGCAATGC-3' (position at 1689 to 1710); d3E397B, 5'- CGAGCTTCCCTTCCTGTACC-3' (position at 2118 to 2099). NTR, nontranslated region; C, capsid; PrM, precursor membrane; NS, nonstructural.

A



B

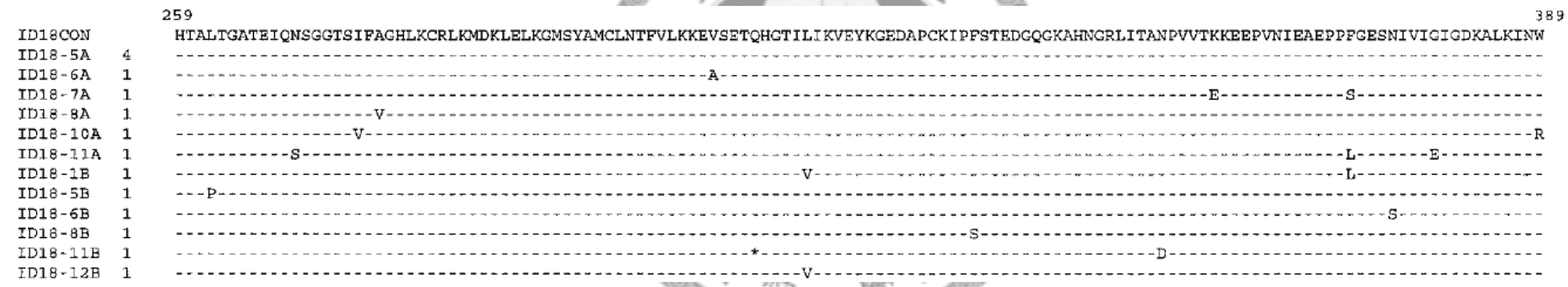


FIG. 2 Alignment of the deduced amino acid sequences of E proteins of multiple clones from the field-captured mosquitoes (A) and patient ID18 (B). The positions of amino acid residues and the corresponding domains and regions within the E protein as determined on the basis of the TBE virus model (Rey *et al.*, 1995) are shown on top. A consensus sequence (CON) was generated for each sample. Dashes indicate sequence identity, an asterisk indicates an in-frame stop codon, and an underline indicates a deletion at that position. Names and numbers of individual clones are shown at the left, with the letters A and B indicating two separate PCRs. For simplicity, only one of the clones with identical sequences is shown.

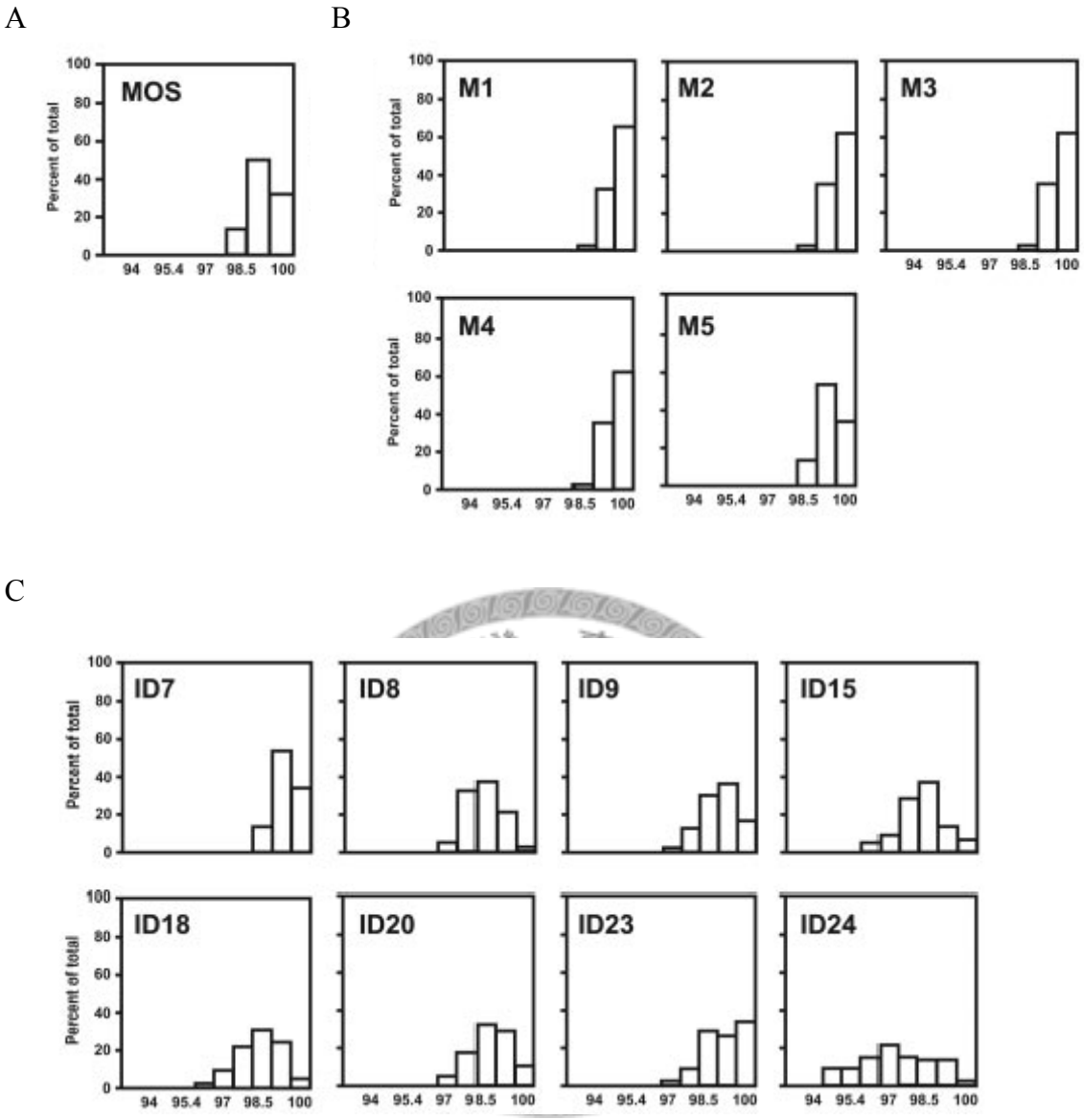


FIG. 3 Amino acid sequence similarity of the E proteins from the field-captured mosquitoes (A), experimentally infected mosquitoes (B), and dengue patients (C). Amino acid sequences of multiple clones from the mosquitoes and patients were subjected to pairwise comparison using the program MEGA (Nei & Gojobori, 1986; Tamura *et al.*, 2007), and the percentages of total comparisons with a given similarity are presented as a histogram for the field-captured (MOS) and experimentally infected (M1 to M5) mosquitoes and for each patient (shown by patient ID number).

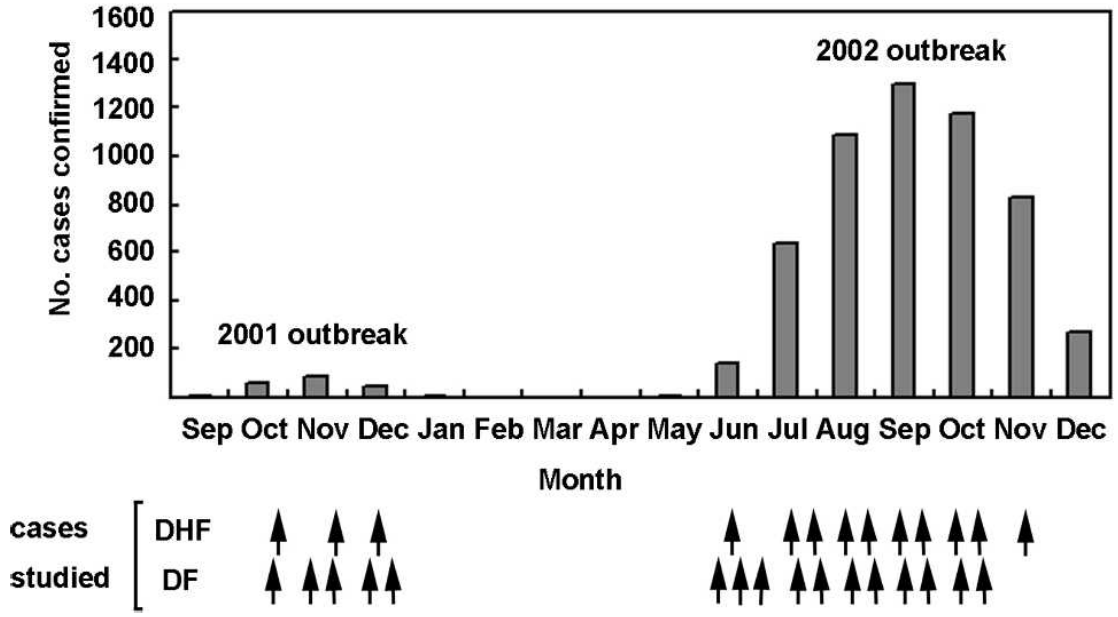


FIG. 4 Numbers of confirmed DENV2 cases in each month during the two consecutive outbreaks in Kaohsiung in 2001 and 2002. The DF and DHF cases studied in each month during the two outbreaks are indicated by arrows.

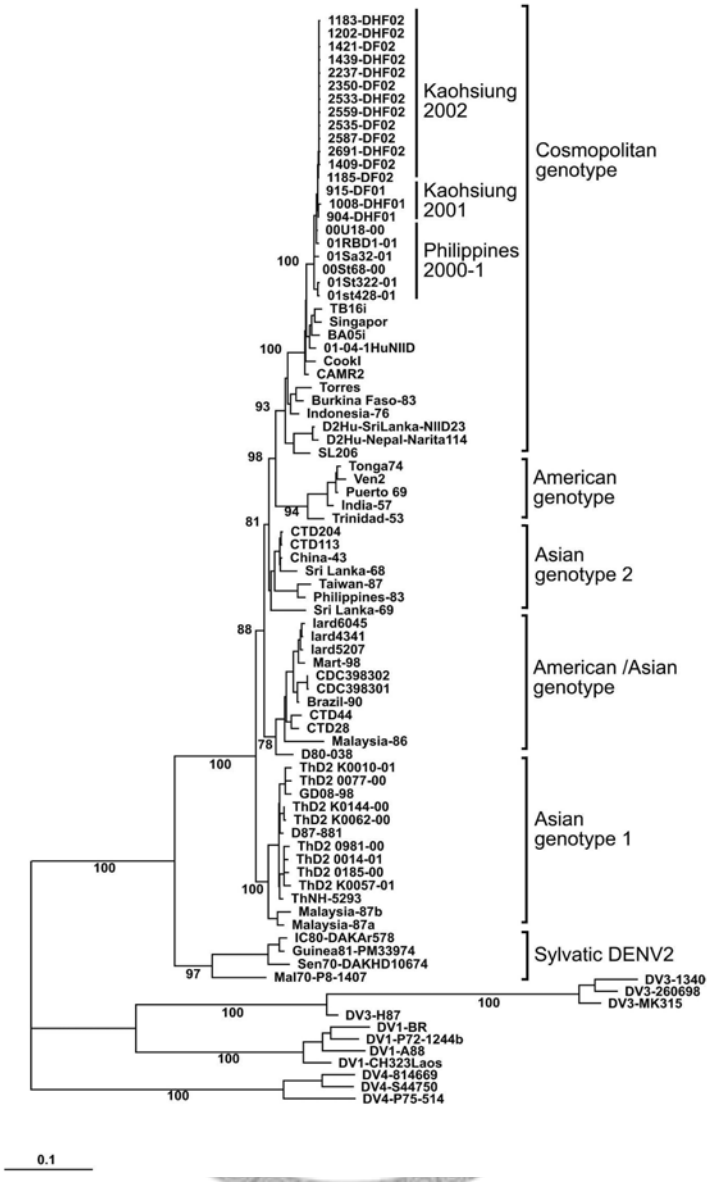


FIG. 5 Maximum likelihood (ML) tree depicting the phylogenetic relationship of 31 DENV2 of the Kaohsiung outbreaks in 2001 and 2002 based on the E gene (1407 nucleotides in length). A total of 85 E sequences, including 16 from the Kaohsiung outbreaks (only one of the identical sequences was included), 23 from countries where there were cases imported to Taiwan during this period, 31 from the 5 known genotypes of DENV2, 4 of the sylvatic DENV2, and E sequence of DENV1, DENV3, and DENV4 as outgroups, were analyzed by the ML method. The 2001 and 2002 Kaohsiung clusters are highlighted by lines, and the 5 genotypes and sylvatic DENV2 by brackets. Similar trees were generated by the NJ method (not shown) with the bootstrap *P* values (1,000 bootstrap samples) shown beside the branches in percentage, which correspond to $P < 0.01$ by the ML method.

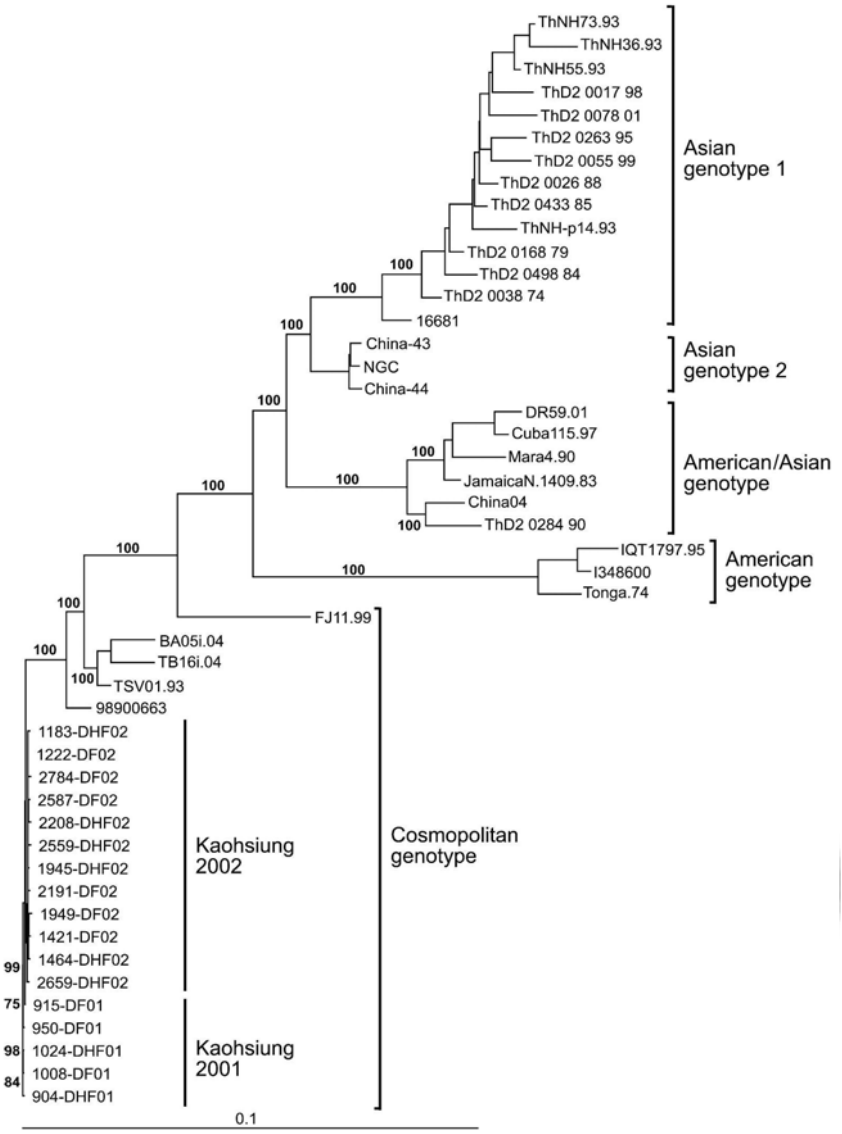
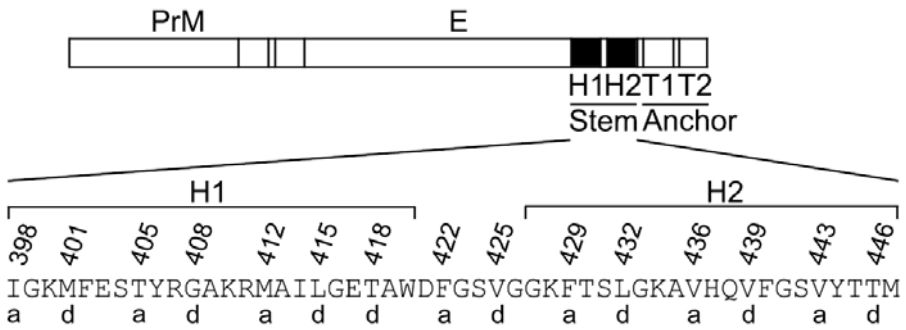


FIG. 6 Maximum likelihood (ML) tree depicting the phylogenetic relationship of 17 DENV2 of the Kaohsiung outbreaks in 2001 and 2002 based on the coding region of full-genome (10176 nucleotides in length). A total of 45 full-genome sequences, including 17 from the Kaohsiung outbreaks and 28 from the 5 known genotypes of DENV-2, were analyzed by the ML method. The evolutionary distances were calculated using the Felsenstein 84 model with empirical transition/transversion ratio and base frequencies including gamma distribution parameter. The 2001 and 2002 Kaohsiung clusters are highlighted by lines, and the 5 genotypes by brackets. A similar tree was generated by the neighboring-joining (NJ) method (not shown), with the bootstrap *P* values (1,000 bootstrap samples) shown beside the branches in percentage. Bootstrap values of 100 correspond to $P < 0.01$ by the ML method.

A



B

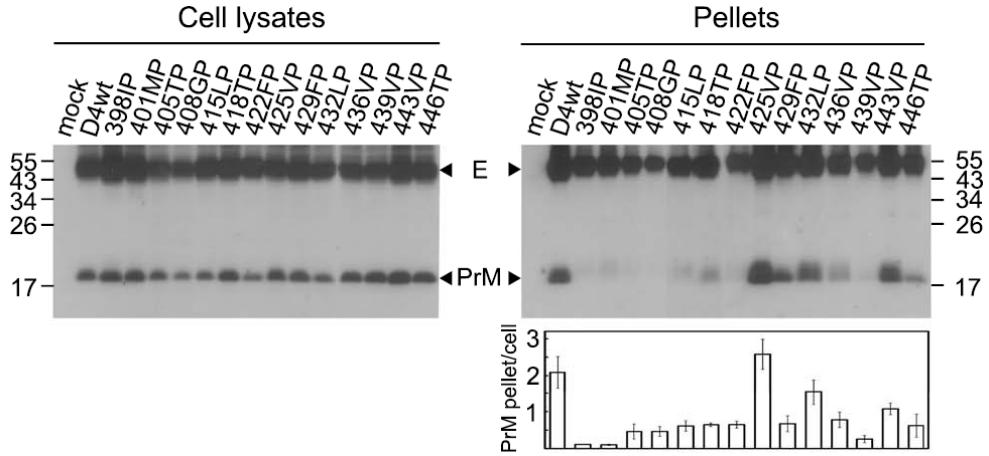


FIG. 7 Schematic diagram of the stem region of E protein and expression of PrM and E proteins in cells and pellets. (A) The stem region consists of two consecutive α helices, H1 and H2, and the anchor region contains two transmembrane α helices, T1 and T2. The numbers as indicated above the amino acid sequence of the stem region of DENV4 E protein were the residues substituted by proline with site-directed mutagenesis. The *a* and *d* positions of these heptad repeats are displayed under the amino acid sequence. (B) 293T (1×10^5) cells were transfected with 10 μg DNA by calcium phosphate method. 48 h later, cell lysates (left panel) and pellets (right panel) were collected as described in the Materials and methods and subjected to Western blot analysis using serum from a confirmed DENV-2 case (Wang *et al.*, 2006). Arrow heads indicate PrM and E proteins recognized by the DENV-2 serum, which did not react with lysates of mock transfected cells. The size of molecular weight markers is shown in kDa. The ratio of the intensity of PrM protein band in pellets to that in cell lysates (PrM pellet/cell) is shown below each lane in the right panel.

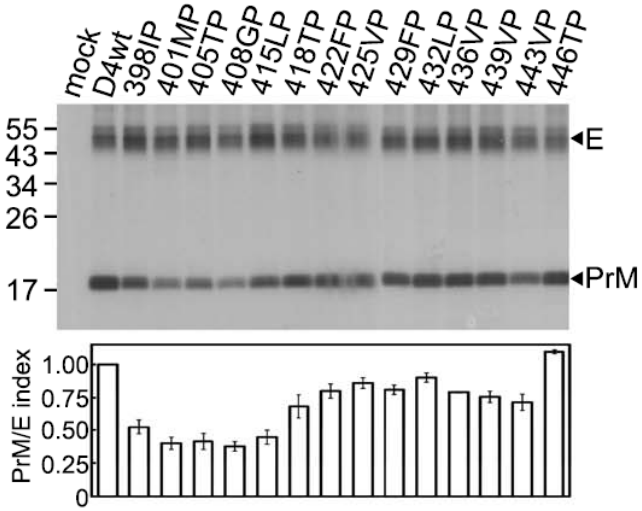
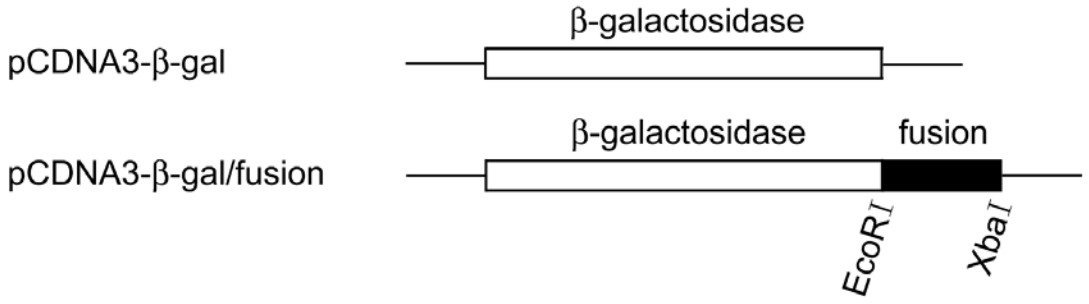


FIG. 8 The interaction between PrM and E proteins. 293T cells prepared in 6-well plate were transfected with 4 μg DNA per well. The transfected cells were metabolically labeled with [^{35}S]methonine at 20 h post-transfection and incubated for 6 h, then lysed and immunoprecipitated with an anti-E Mab, FL0232, and subjected to 12% PAGE as described in the Materials and methods. The ratio of the intensity of PrM protein band relative to that of E band (PrM/E) is shown below each lane.

A


β-galactosidase
pCDNA3-β-gal
β-galactosidase
fusion
pCDNA3-β-gal/fusion
EcoRI
XbaI

B

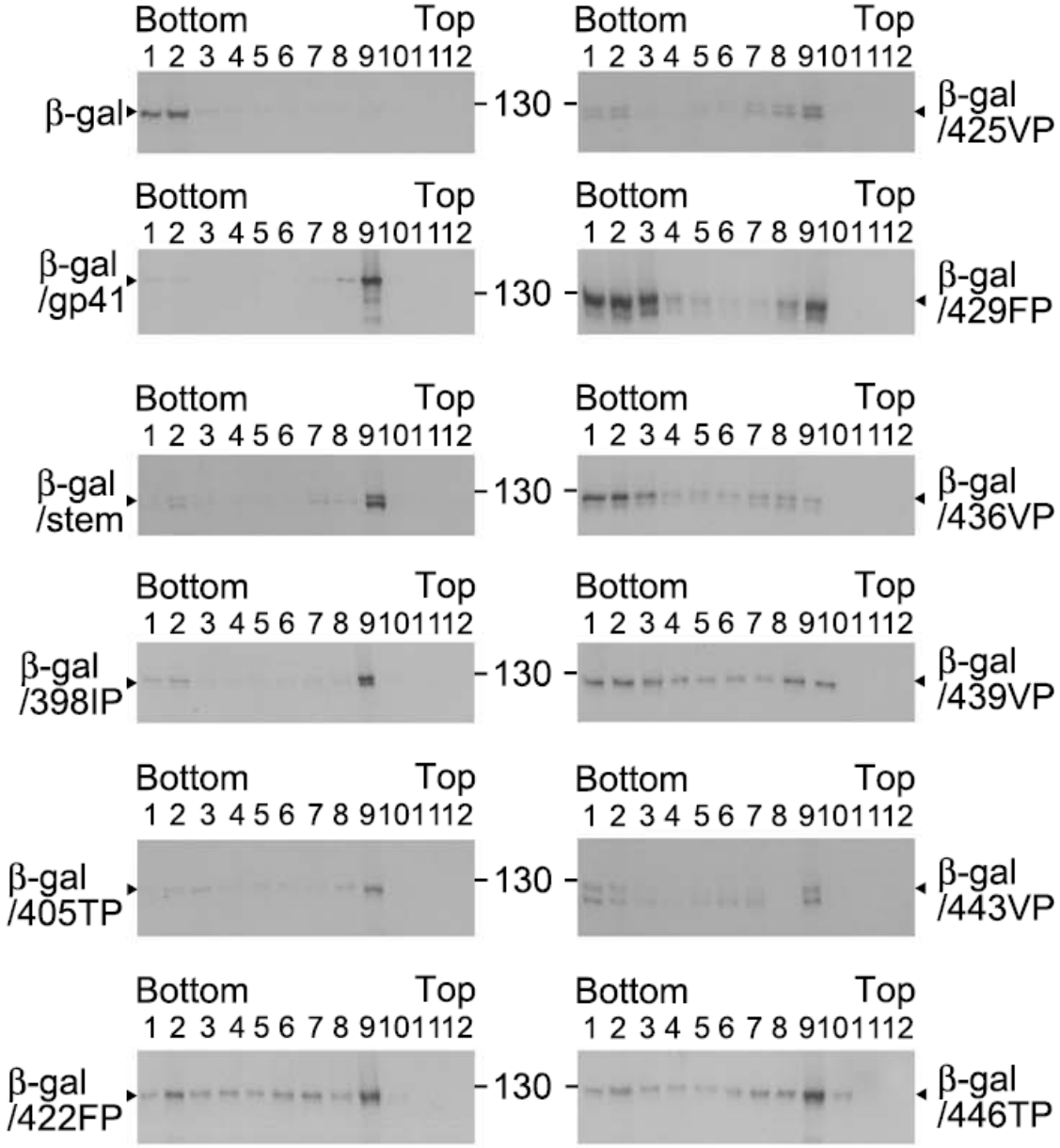

Bottom
Top
1 2 3 4 5 6 7 8 9101112
β-gal
130
β-gal /425VP
β-gal /gp41
β-gal /429FP
β-gal /stem
β-gal /436VP
β-gal /398IP
β-gal /439VP
β-gal /405TP
β-gal /443VP
β-gal /422FP
β-gal /446TP

FIG. 9 The membrane binding ability of the stem region in E protein assayed by membrane flotation assay. (A) Schematic diagrams of pcDNA3-β-gal (kindly provided by Dr. Chen S.S.) and pCDNA3- β-gal/fusion which consists of b-galactosidase expressing gene subsequently linked with a putative membrane association fragment. The plasmid, pCDNA3-β-gal/gp41 (a gift from Dr. Chen S.S.), containing b-gal fused with the C-terminus of gp41, has been demonstrated the ability of membrane binding in the previous report (Chen *et al.*, 2001). PCR products of the stem region (residues 396 to 447) in E gene which were amplified from wild type or indicated PrM and E proteins expressing plasmids with flanking by enzyme sites, *Eco*RI and *Xba*I , were digested and inserted into pCDNA3-β-gal/gp41 previously digested with the same enzymes. (B) 293T cells were transfected with indicated plasmids and subjected to membrane flotation assay as described in Materials and methods. 12 fractions were collected from top to bottom numbered as fraction 12 to 1. Each aliquot was subjected to TCA precipitation, 7.5% PAGE and then Western blot analysis using Mab against β-galactosidase. Arrow heads indicate β-gal or β-gal fused with a putative membrane associated fragment recognized by anti-β-gal Mab. The size of molecular weight markers is shown in kDa.

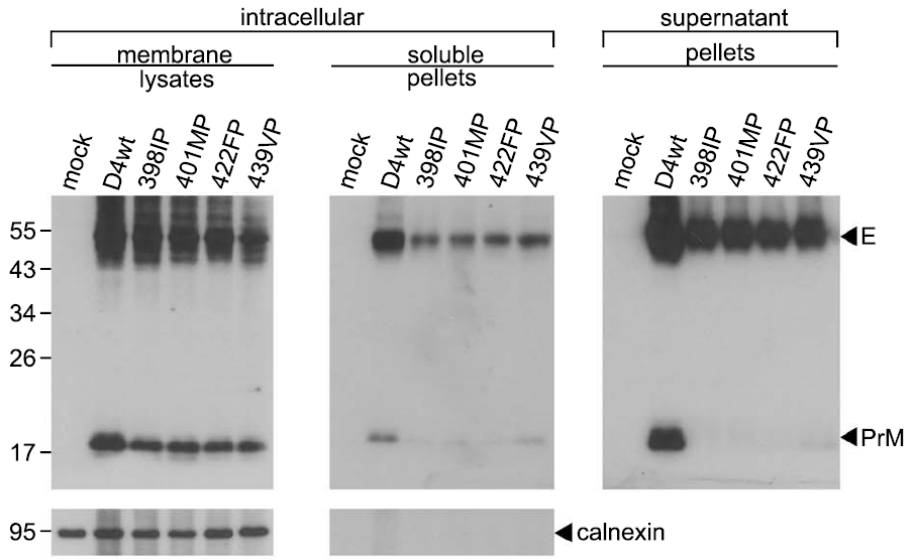


FIG. 10 Subcellular fractionation assay of wild type DENV-4 PrM and E proteins and proline substitutions at the indicated residues of the stem region in E protein. 293T cells were transfected with indicated plasmids and subjected to subcellular fractionation assay as described in the Materials and methods. The culture supernatants of transfected cells were subjected to 20% sucrose cushion to pellet VLP. The obtained membrane-bound lysates, soluble pellets and supernatant pellets were subjected to Western blot analysis by using serum from a confirm DENV-2 case (upper panel) (Wang *et al.*, 2006) and then reprobing with anti-calnexin Mab (lower panel). Arrow heads indicate PrM, E, and calnexin. The size of molecular weight markers is shown in kDa.

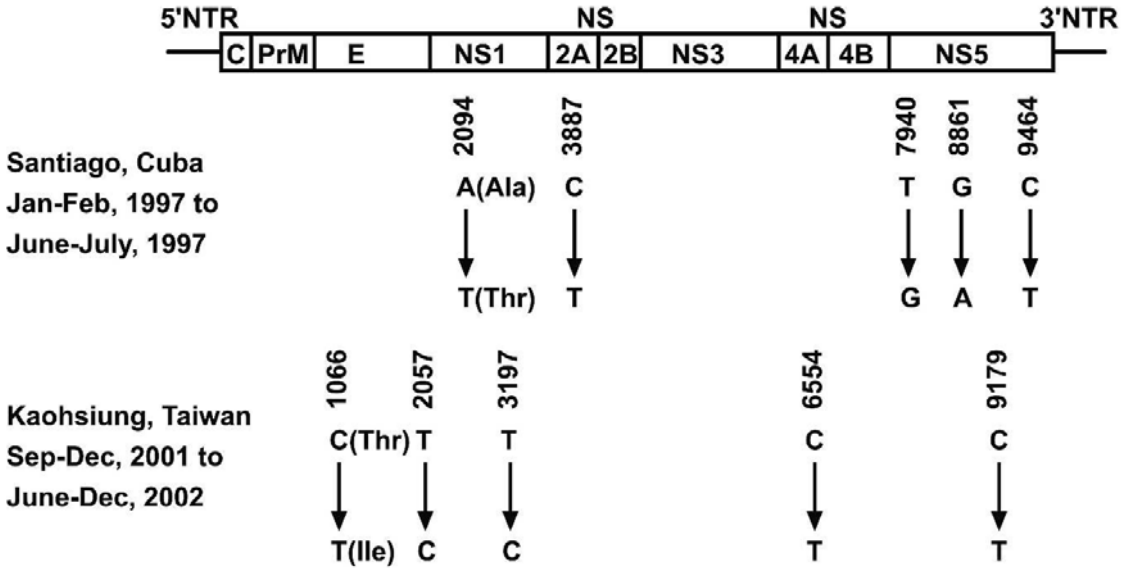


FIG. 11 Comparison of the consistent nucleotide changes between the early and late viruses reported in this and previous studies (Rodriguez-Roche *et al.*, 2005). The consistent nucleotide changes between the early viruses and the late viruses, which were linked to outbreak with more severe cases in the Santiago outbreak in 1997 and in the Kaohsiung outbreaks in 2001–2002, were shown. Parentheses after nucleotides indicate amino acids. The number of genome position was based on the DENV2 strain, NGC (Irie *et al.*, 1989).

Table 1 Information of the selected patients in the DENV3 outbreak in southern Taiwan

Patient	Disease	Age	Gender	Sampling day [a]
ID7	DF	68	M	3
ID8	DF	36	F	7
ID9	DF	49	F	4
ID15	DF	36	F	8
ID18	DHF	57	M	3
ID20	DHF	57	F	4
ID23	DHF	27	M	8
ID24	DHF	63	M	5

[a] Sampling day is the day after onset of fever.

Table 2 Information of the selected patients in the consecutive DENV2 outbreak in Kaohsiung in 2001 and 2002

Patient	Disease	Primary or secondary infection [a]	Sampling day	Virus	Sequence completed[b]	Genbank accession number
904	DHF	S	10/31/2001	904-DHF01	FG	DQ645540
1008	DHF	NT	11/28/2001	1008-DHF01	E	DQ645558
1024	DHF	NT	12/07/2001	1024-DHF01	FG	DQ645544
912	DF	P	10/31/2001	912-DF01	E	DQ645557
915	DF	NT	11/03/2001	915-DF01	FG	DQ645541
950	DF	S	11/12/2001	950-DF01	FG	DQ645542
1018	DF	NT	12/03/2001	1018-DF01	FG	DQ645543
1019	DF	NT	12/03/2001	1019-DF01	E	DQ645559
1202	DHF	S	06/22/2002	1202-DHF02	E	DQ645561
1439	DHF	NT	07/18/2002	1439-DHF02	E	DQ645563
1464	DHF	S	07/20/2002	1464-DHF02	FG	DQ645548
1945	DHF	S	08/18/2002	1945-DHF02	FG	DQ645549
2038	DHF	NT	08/28/2002	2038-DHF02	E	DQ645565
2208	DHF	S	09/13/2002	2208-DHF02	FG	DQ645552
2237	DHF	S	09/14/2002	2237-DHF002	E	DQ645566
2533	DHF	S	10/21/2002	2533-DHF02	E	DQ645568
2559	DHF	S	10/23/2002	2559-DHF02	FG	DQ645553
2659	DHF	S	11/01/2002	2659-DHF02	FG	DQ645555
2691	DHF	NT	11/06/2002	2691-DHF02	E	DQ645570
1183	DF	S	06/17/2002	1183-DF02	FG	DQ645545
1185	DF	NT	06/17/2002	1185-DF02	E	DQ645560
1222	DF	S	06/24/2002	1222-DF02	FG	DQ645546
1409	DF	S	07/16/2002	1409-DF02	E	DQ645562
1421	DF	NT	07/16/2002	1421-DF02	FG	DQ645547
1852	DF	S	08/12/2002	1852-DF02	E	DQ645564
1949	DF	S	08/19/2002	1949-DF02	FG	DQ645550
2191	DF	S	09/12/2002	2191-DF02	FG	DQ645551
2350	DF	NT	09/27/2002	2350-DF02	E	DQ645567
2535	DF	P	10/21/2002	2535-DF02	E	DQ645569
2587	DF	P	10/26/2002	2587-DF02	FG	DQ645554
2784	DF	S	11/18/2002	2784-DF02	FG	DQ645556

[a] Primary (P) or secondary (S) infection was determined as described (Wang *et al.*, 2006). NT, not tested, because convalescent sera were not available.

[b] Envelope (E) or full-length (FG) sequences were completed.

Table 3 Primers used for amplification of DENV2 genome

Segment	Primer [a]	Sequence (5'→3')	Genome position [b]	Length (bp)
1	d2-17A	CCCAGACAGATTCTTTGAGGG	17–37	769
	d2-M27B	TTTCCAGGCCCCTTCTGATGACATCCC	759–785	
2	d2-PrM60A	GAICCIGAIGACITIGACTGTTGGTGC	609–635	1909
	d2-E+34B	GGAACTTGTATTGTTCTGTCC	2497–2517	
3	d2-2270A[cd]	GR(A/G)GCTGCY(C/T)TCCAGTGGGG	2263–2280	
	d2-4512B[ce]	ACAGGTACCATGCTGCTGC	4476–4494	2232
	d2-2714A[e]	GGAATCATGCAGGTAGG	2697–2713	
	d2-4137B[d]	GCCAGCTCCTTTTCTTG	4115–4131	
4	d2-4119A	ARCAAGAAAAGGAGCTGGCC	4113–4132	1682
	d2-5812B	CGTCTGGGGTCTATAACCC	5776–5794	
5	d2-5423A[cd]	CAACTCGAGTGGAGATGGG	5416–5434	
	d2-7457B[ce]	CTCCCTGGATTTCCTTCCC	7432–7450	2035
	d2-5708A[e]	AGACTAGAACCAATGATTGGG	5701–5721	
	d2-5876B[d]	GAGTGGGTCACTGGCAT	5853–5869	
6	d2-7240A	GCGGGCATCATGAAAAACCC	7233–7252	1644
	d2-8885B	TTCCTTGTCAACCAGCTCCC	8857–8876	
7	d2-8585A[cd]	ACAGATGGCAATGACAGACAC	8579–8599	
	d2-10370B[cf]	TAACGTCCTTGGACGGGG	10346–10363	
	d2-8786A[e]	AATGCAGCCTTAGGTGCC	8778–8795	1785
	d2-8987B[d]	GCTCTGCTGCCTTTTGC	8964–8980	
	d2-9254A[f]	AGAAACTAGCCGAGGCC	9247–9263	
	d2-9833B[e]	CCCAAACAGGCCGTCTC	9810–9826	
8	d2-10204A	TACACAGATTACATGCCATCC	10197–10214	501
	d2-10704B	CACCATTCCATTTTCTGGCG	10678–10697	

[a] Primers ere named according to the positions of amino acid or nucleotide with A denoting forward primers and B reverse primers.

[b] The number of genome position was based on the DENV-2 NGC strain (Irie *et al.*, 1989).

[C] The primer pairs used in the first round PCR.

[def] The primer pairs used in the second round PCR for the amplification of segments 3, 5, 7 in some samples. The PCR conditions for most segments were 95℃ for 5 min, followed by 30 cycles of 95℃ for 1 min, 56℃ for 1 min, and 72℃ for 2.5 min, and then 72℃ for 5 min except for segments 1 and 8, in which annealing was at 58℃ for 1min and elongation at 72℃ for 1 min.

Table 4 Primers used for directly sequencing of DENV2 genome

Segment	Primer [a]	Sequence (5'→3')
1	d2-17A	CCCAGACAGATTCTTTGAGGG
	d2-C16A	GCTGAAACGCGAGAGAAACC
	d2-C46B	TCCCTGCTGTTGGTGGGATTGTT
	d2-C69B	CCCATCTCITCAIIATCCCTGCTGTTGG[b]
	d2-M27B	TTTCCAGGCCCCTTCTGATGACATCCC
2	d2-PrM60A	GAICCIGAIGACITIGACTGTTGGTGC[b]
	d2E-2A	ATGACAATGCGTTGCATAGG
	d2E-6A	TCGCTCCTTCAATGACAATG
	d2E44A	CTGACAAAAACGGAAGCC
	d2E96A	GGTAGACAGAGGATGGGG
	d2E114A	GGGAAGAGAATGCAGTCGG
	d2E206A	TGGCTIGTICACAIICAATGG[b]
	d2E254A	GIATCCCAIGAIGGIGCCATGC[b]
	d2E261A	ATACAGCACTCACAGGGG
	d2E314A	GAAACACARCATGGRAC[c]
	d2E411A	AGAATGGCCATTYTRGG[c]
	d2E474B	CCAAAATCCCAGGCTGTGTCGCC
	d2E+12B	TTCTTTGTTCTTCCAGCTCAC
	d2-E+34B	GGAACTTGTATTGTTCTGTCC
3	d2-2270A	GRGCTGCYTCCAGTGGGG[cd]
	d2-2714A	GGAATCATGCAGGTAGG
	d2-2992A	AACAGAGCCGTCCATGC
	d2-3785B	TCAATTCCTTGGAGGTCA
	d2-4137B	GCCAGCTCCTTTTCTTG
	d2-4512B	ACAGGTACCATGCTGCTGC
4	d2-4119A	ARCAAGAAAAGGAGCTGGCC[c]
	d2-4297A	GCTGCTGACGTAAGATGGG
	d2-4700A	GGCACGTCACACGCGG
	d2-4996A	GGAGCATATGTGAGTGC
	d2-5812B	CGTCTGGGGTCTATAACCC
5	d2-5423A	CAACTCGAGTGGAGATGGG
	d2-5708A	AGACTAGAACCAATGATTGGG
	d2-6140A	GAGACCTACCAGTCTGG
	d2-6516A	ACTGCCGGAGACCCTGG
	d2-6613B	CCCTATGCCTTTTCCGC
	d2-6884A	CCCAGCAACCCGAGAGC
	d2-7457B	CTCCCTGGATTTCCTTCCC
6	d2-7240A	GCGGGCATCATGAAAAACCC
	d2-7373A	TGATGAGGACTACATGGGC
	d2-7616A	CGATTGAATGCATTGGGG
	d2-7726A	CACGCTGTGTCGCGAGG
	d2-8166A	GGAAATATGGAGGAGCC

Table 4 Primers used for directly sequencing of DENV2 genome (*continued*)

Segment	Primer [a]	Sequence (5'→3')
	d2-8529B	CATGGATGATGCTGATCC
	d2-8885B	TTCCTTGTCAACCAGCTCCC
7	d2-8585A	ACAGATGGCAATGACAGACAC
	d2-8786A[e]	AATGCAGCCTTAGGTGCC
	d2-8987B	GCTCTGCTGCCTTTTGC
	d2-9460A	GATCAGACAGATGGAGGG
	d2-9556B	CATCTCCACTGATGGCC
	d2-9687A	GCCCTTCTGTTCACACC
	d2-10102B	GGTCTTCTCTTTTCCCC
	d2-10370B	TAACGTCCTTGGACGGGG
8	d2-10204A	TACACAGATTACATGCCATCC
	d2-10500A	CGGTTAGAGGAGACCCC
	d2-10704B	CACCATTCCATTTTCTGGCG

[a] Primers ere named according to the positions of amino acid or nucleotide with A denoting forward primers and B reverse primers.

[b] I stands for deoxyinosine.

[c] R represents a mixed primer with A or G at particular position.

[d] Y represents a mixed primer with C or T at particular position.

Table 5 Nucleotide sequence variation of envelope and capsid genes from naturally infected mosquitoes and dengue patients

Source	Envelope gene			Capsid gene		
	No. of clones	No. of substitutions/total no. of nucleotides	Mean [c] diversity (%)	No. of clones	No. of substitutions/total no. of nucleotides	Mean [c] diversity (%)
Mosquitoes [a]	21[e]	17/8,253	0.21	21	6/6,678	0.09
Patients [b]						
ID7	10	6/3,930	0.15	10	6/3,180	0.19
ID8	11	17/4,323	0.39	10	6/3,180	0.19
ID9	11[e]	20/4,323	0.46	10	8/3,180	0.25
ID15	10	17/3,930	0.43	10[d,f]	13/3,180	0.41
ID18	15[d]	21/5,895	0.36	10	6/3,180	0.19
ID20	11[d]	15/4,323	0.35	10	4/3,180	0.13
ID23	10	11/3,930	0.28	10	8/3,180	0.25
ID24	10	23/3,930	0.59	10	7/3,180	0.22
Overall	88	130/34,584	0.38	80	58/25,440	0.23

[a] The dengue virus sequences in mosquitoes were derived from homogenates of a pool of 22 female *Aedes aegypti* mosquitoes.

[b] The patients included four DF (ID7, ID8, ID9, and ID15) and four DHF (ID18, ID20, ID23, and ID24) cases according to the WHO (WHO, 1997).

[c] The mean diversity is the number of substitutions divided by the total number of nucleotides sequenced.

[d] One clone contained a stop codon.

[e] One clone contained a single-nucleotide deletion.

[f] Two clones contained single-nucleotide deletions (one each).

Table 6 Nucleotide sequence variation of envelope and capsid genes from experimentally infected mosquitoes

Source	Envelope gene			Capsid gene		
	No. of clones	No. of substitutions/total no. of nucleotides	Mean [b] diversity (%)	No. of clones	No. of substitutions/total no. of nucleotides	Mean [b] diversity (%)
Mosquitoes [a]						
M1	11	3/4,323	0.07	ND [d]	ND	ND
M2	10	4/3,930	0.10	10	1/3,180	0.03
M3	10 [c]	2/3,930	0.05	10	0/3,180	0
M4	9	5/3,537	0.14	10	6/3,180	0.19
M5	10	4/3,930	0.10	10	6/3,180	0.19
Overall	50	18/19,650	0.09	40	13/12,720	0.10

[a] Dengue virus sequences were derived from each *Aedes aegypti* mosquito after intrathoracic injection of dengue virus and confirmation by an immunofluorescent antibody test (Chen *et al.*, 1993; Rosen & Gubler, 1974).

[b] The mean diversity is the number of substitutions divided by the total number of nucleotides sequenced.

[c] One clone contained a single-nucleotide deletion.

[d] ND, not done.

Table 7 Differenced in E sequences of DENV2 between DF and DHF patients of the 2001 and 2002 outbreaks

Genome position [a]	Virus [b]																															Amino acid position and change [c]	
	904-DHF01	1008-DHF01	1024-DHF01	912-DF01	915-DF01	950-DF01	1018DF01	1019DF01	1202-DHF02	1439-DHF02	1464-DHF02	1945-DHF02	2038-DHF02	2208-DHF02	2237-DHF02	2533-DHF02	2559-DHF02	2659-DHF02	2691-DHF02	1183-DF02	1185-DF02	1222-DF02	1409-DF02	1421-DF02	1852-DF02	1949-DF02	2191-DF02	2350-DF02	2535-DF02	2587-DF02	2784-DF02		
1066	C	–	–	–	–	–	–	–	T	T	T	T	T	T	T	T	T	T	T	T	–	–	T	T	T	T	T	T	T	T	46	T (C)→I (T)
1076	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	49	E (A)→D (C)
1220	T	–	–	A	A	–	–	–	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	97	V No change
1248	T	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	107	L No change
1256	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	109	G No change
1424	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	–	–	–	–	–	–	–	165	T No change
1991	C	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	354	V No change
1994	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	355	N No change
2008	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	360	E (A)→G (G)
2057	T	–	–	–	–	–	–	–	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	376	S No change
2060	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	377	Y No change
2081	G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	384	P No change
2203	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	425	L (T)→P (C)
2216	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	429	F No change
2336	T	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	469	N No change
2339	A	G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	470	S No change
2340	C	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	471	R (C)→S (A)

[a] The number of genome position was based on the DENV2 strain, NGC (Irie *et al.*, 1989).

[b] The viruses were named according to patient ID, disease (DF or DHF) and year (01 or 02).

[c] Parentheses after amino acids indicate the nucleotides.

Table 8 Differences in full-length sequences of DENV2 of the 2001-2002 outbreaks

		Virus [b]																
Genome position [a]	Gene	904-DHF01	1024-DHF01	915-DF01	950-DF01	1008-DF01	1464-DHF02	1945-DHF02	2208-DHF02	2559-DHF02	2659-DHF02	1183-DF02	1222-DF02	1421-DF02	1949-DF02	2191-DF02	2587-DF02	2784-DF02	Amino acid Position and change [c]
40	5'NTR	C	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	
332	C	G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	81 L No change
372	C	T	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	95 L (T)→M (A)
578	PrM	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	49 I No change
771	PrM	G	–	–	–	–	–	–	–	–	C	C	–	–	–	–	–	–	114 E (G)→Q (C)
986	E	C	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	19 S No change
1066	E	C	–	–	–	–	T	T	T	T	T	T	T	T	T	T	T	T	46 T (C)→I (T)
1220	E	T	–	A	–	–	A	A	A	A	A	A	A	A	A	A	A	A	97 V No change
1424	E	A	–	–	–	–	–	–	–	–	–	–	–	G	–	–	–	–	165 T No change
1994	E	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	355 N No change
2008	E	A	–	–	–	–	–	–	–	–	–	G	–	–	–	–	–	–	360 E (A)→G (G)
2057	E	T	–	–	–	–	C	C	C	C	C	C	C	C	C	C	C	C	376 S No change
2203	E	T	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	425 L (T)→P (C)
2981	NS1	A	–	–	G	–	–	–	–	–	–	–	–	–	–	–	–	–.	189 K No change
3123	NS1	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	237 L No change
3197	NS1	T	–	–	–	–	C	C	C	C	C	C	C	C	C	C	C	C	261 H No change
3459	NS1	T	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	349 L No change
3507	NS2A	T	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	13 L No chang e

Genome position [a]	Gene	Virus [b] 904-DHF01	1024-DHF01	915-DF01	950-DF01	1008-DF01	1464-DHF02	1945-DHF02	2208-DHF02	2559-DHF02	2659-DHF02	1183-DF02	1222-DF02	1421-DF02	1949-DF02	2191-DF02	2587-DF02	2784-DF02	Amino acid and change [c]	Position	
3561	NS2A	C	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	31	H (C)→Y (T)	
3785	NS2A	C	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	105	T	No change
3845	NS2A	C	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	125	D	No change
3927	NS2A	T	–	–	–	–	–	–	C	–	–	–	–	–	–	–	C	–	153	L	No change
4136	NS2B	A	–	–	–	–	–	–	–	–	G	–	–	–	–	–	–	–	4	L	No change
4199	NS2B	T	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	25	D	No change
4826	NS3	G	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	104	K	No change
4880	NS3	C	T	–	–	T	–	–	–	–	–	–	–	–	–	T	–	–	122	T	No change
5421	NS3	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	303	R	No change
5837	NS3	G	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	441	V	No change
6008	NS3	C	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	498	I	No change
6113	NS3	T	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	533	F	No change
6186	NS3	G	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	558	A (G)→T (A)	
6524	NS4A	G	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	52	L	No change
6554	NS4A	C	–	–	–	–	T	T	T	T	T	T	T	T	T	T	T	T	62	A	No change
6590	NS4A	C	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	74	S	No change
6615	NS4A	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	83	L	No change
6653	NS4A	A	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	95	L	No change
6788	NS4A	C	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	140	A	No change
6893	NS4B	C	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	25	S	No change

Genome position [a]	Gene	Virus [b] 904-DHF01	1024-DHF01	915-DF01	950-DF01	1008-DF01	1464-DHF02	1945-DHF02	2208-DHF02	2559-DHF02	2659-DHF02	1183-DF02	1222-DF02	1421-DF02	1949-DF02	2191-DF02	2587-DF02	2784-DF02	Amino acid Position and change [c]
6905	NS4B	C	–	–	–	–	T	T	T	T	–	T	T	T	T	T	T	T	29 D No change
7295	NS4B	C	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	159 P No change
7452	NS4B	T	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	212 F (T)→L (C)
7484	NS4B	T	–	–	–	–	C	C	C	C	–	C	C	C	C	C	C	C	222 A No chang e
7814	NS5	G	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	A	84 R No change
8270	NS5	A	–	–	–	–	–	–	–	–	–	G	–	–	–	–	–	–	236 S No change
8342	NS5	C	–	T	–	–	T	T	T	T	T	T	T	T	T	T	T	T	260 S No change
8357	NS5	C	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	265 I No change
8374	NS5	T	–	C	–	–	C	C	C	C	C	C	C	C	C	C	C	C	271 I (T)→T (C)
8632	NS5	T	–	A	A	–	A	–	A	A	A	A	A	A	–	A	A	A	357 V (T)→E (A)
8660	NS5	T	G	G	G	G	–	G	G	G	G	G	G	–	–	G	G	G	366 P No change
8672	NS5	T	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	370 T No change
8738	NS5	G	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	392 R No change
8774	NS5	G	–	A	–	–	A	A	A	A	A	A	A	A	A	A	A	A	404 R No change
9027	NS5	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	490 L No change
9060	NS5	A	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	500 R No change
9164	NS5	C	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	534 D No change
9179	NS5	C	–	–	–	–	T	T	T	T	T	T	T	T	T	T	T	T	539 D No change
9315	NS5	C	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	585 P (C)→S (T)
9454	NS5	G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	631 L (G)→N (A)

Genome position [a]	Gene	Virus [b] 904-DHF01	1024-DHF01	915-DF01	950-DF01	1008-DF01	1464-DHF02	1945-DHF02	2208-DHF02	2559-DHF02	2659-DHF02	1183-DF02	1222-DF02	1421-DF02	1949-DF02	2191-DF02	2587-DF02	2784-DF02	Amino acid Position and change [c]
9594	NS5	C	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	678 L No change
9621	NS5	G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	687 V (G)→I (A)
9633	NS5	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	691 I (A)→V (G)
9993	NS5	A	–	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	811 T (A)→A (G)
10007	NS5	G	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	815 R No change
10076	NS5	T	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	838 Y No change
10283	3'NTR	A	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	
10551	3'NTR	T	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	
10566	3'NTR	A	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	

[a] The number of genome position was based on the DENV2 strain, NGC (Irie *et al.*, 1989).

[b] The viruses were named according to patient ID, disease (dengue fever [DF] or dengue hemorrhagic fever [DHF]), and year (2001 or 2002). The consistent nucleotide changes between the 2001 and 2002 viruses were shaded.

[c] Parentheses after amino acids indicate the nucleotides.

Table 9 The methods and mode of nucleotide substitution used

Method [a]	Nucleotide substitution		Codon model			
	Model [b]	Log likelihood	Log likelihood	Mean dN/dS [c]	Positively selected site	Negatively selected site
SLAC	REV	−14607.1	−14277.5	0.108	0	1 [d]
FEL	REV				0	17 [d]
REL	REV				15 [e]	1 [d]

[a] SLAC, FEL, and REL methods in the Datamonkey facility were used to determine the overall and codon-specific selection pressure in the entire coding region.

[b] General reversible model (REV) was used as recommended by Datamonkey facility.

[c] d_N/d_S, the ratio of the non-synonymous nucleotide substitutions per site (d_N) to the synonymous nucleotide substitutions per site (d_S).

[d] One negatively selected site at codon 366 of NS5 was consistently found by all three methods. Other 16 negatively selected sites found by the FEL method were codons 190, 355, and 376 of E, codons 189 and 261 of NS1, codon 125 of NS2A, codon 25 of NS2B, codons 122 and 533 of NS3, codon 74 of NS4A, codons 25 and 29 of NS4B, codons 260, 534, 539, and 838 of NS5.

[e] The 15 positively selected sites found by the REL method were codon 95 of C, codon 114 of PrM, codons 46, 360, and 425 of E, codon 31 of NS2A, codon 558 of NS3, codon 212 of NS4B, and codons 271, 357, 585, 631, 687, 691, and 811 of NS5.

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