Chapter 1. Introduction

1.1 Dengue Virus

Dengue virus (DV) belongs to the genus Flavivirus within the family of *Flaviviridae*. Members of the genus are typically transmitted to vertebrates by mosquitoes or ticks and frequently cause significant human morbidity and mortality. In addition to dengue virus, other human pathogens such as Japanese encephalitis virus, tick borne encephalitis virus, West Nile virus, yellow fever virus, and Kunjin virus are classified to this family (Beeck *et al.*, 2004).

There are four serotypes of dengue virus, all transmitted by *Aedes* mosquitoes. They cause dramatic variations in clinical symptom varying from asymptomatic to dengue fever (DF), or even more severe infection with bleeding and shock, known as the dengue haemorrhagic fever (DHF), and dengue shock syndrome (DSS) (McBride *et al.*, 2000). Each year, tens of millions of cases of DF occur and, depending on the year, up to hundreds of thousands of cases of DHF/DSS. The case-fatality rate of DHF/DSS in most countries is about 5% (CDC, USA, 2005).

In 2005, dengue is one of the most important mosquito-borne viral diseases affecting humans. In the 1950s, there were only nine countries reporting the clinical manifestations of dengue. Today the geographic distribution includes more than 100 countries worldwide (Fig. 1.1). Many of these countries had no dengue reported for over 20 years and several countries have no known history of the diseases. The World Health Organization estimates that more than 2.5 billion people are at risk of dengue infection. First recognized in the 1950s, DHF/DSS has become a leading cause of child mortality in several Asian and South American countries (Guha-Sapir and Schimmer, 2005).

Currently, no dengue vaccine is available commercially. Recently, however,

attenuated candidate vaccine viruses have been developed. Efficacy trials in human volunteers have not completed yet (Solomon and Mallewa, 2001). On the other hand, new dengue virus strains and serotypes will likely continue to be introduced into many areas where the population densities of *Aedes aegypti* are at high levels. Since the mosquito control programs are difficult to implement and maintain, the development of new antiviral drugs and a safe vaccine become imperative (Solomon and Mallewa, 2001; CDC, USA, 2005).

1.2 Genome of Dengue virus

Dengue virus possesses a positive-sense, single-stranded RNA wrapped in a nucleocapsid protein within an envelope. The whole genome is approximately 10.8kb in length and contains a single open reading frame. A single polyprotein translated from the viral RNA is cleaved co- and post-translationally by the host and viral proteases into three structural proteins — capsid, membrane protein (M, which is expressed as prM, the precursor to M), and envelope (E) — that constitute the virus particle and seven nonstructural proteins (NS) that are essential for viral replication encoded by the remainder of the genome. The reported gene order is 5'-C-prM/M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' (Gubler and Kuno, 1997; Chao *et al*, 2005; Mukhopadhyay *et al*, 2005).

1.3 Properties of viral proteins

The major function of NS proteins is the replication of RNA. Flavivirus NS3 is a multi-functional protein possessing protease, helicase, and RNA triphosphatase activities. The dengue virus NS3 protease shares with other flavivirus NS3 in possessing a trypsin-like character with a classic serine protease catalytic domain (Leung *et al.*, 2001). Unlike trypsin, it has a marked preference for dibasic residues

requiring a co-factor provided by the nonstructural protein NS2B (Rohana *et al.*, 2000). NS2B is an essential cofactor for the efficient catalysis of the proteolytic cleavage of the dengue virus type 2 polyprotein (Clum *et al.*, 1997). The NS2B-NS3 conjugate may cleave the precursor polyprotein *in vivo* at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions as well as at internal sites within C, NS2A, NS3, and NS4A, whereas the host proteases, signalase and furin, act on the remaining cleavage sites (Fig. 1.2; Brinkworth *et al.*, 1999).

NS5 protein contains seven motifs. RNA-dependent RNA polymerase situates in the C-terminal two-thirds of the protein, while the other two methyltransferase motifs situate in the N-terminal part of the protein (Liu *et al.*, 2002). NS5 proteins of dengue virus, West Nile virus, and Kunjin virus have been shown to possess non-specific RNA-dependent RNA polymerase activity (Brook *et al.*, 2002).

1.4 Four small non-structural proteins of Dengue virus

NS2A, 2B, 4A and 4B are four small non-structural proteins encoded by flavivirus genome (Fig.1.3). All four proteins are poorly conserved in sequence but exhibit conserved hydrophobicity profiles among flavivirus, which suggest that they may be membrane-associated (Gublur and Kuno, 1997). The crude membrane fractions of West Nile virus-infected cells contain a set of viral encoding proteins as major constituents after weak or stringent salt washes. The NS2A, NS2B and NS4B are membrane or integral membrane proteins that are separated from the membranes after stringent salt washes. In addition, expression in human A549 cells (Giard *et al.*, 1973) of the dengue virus nonstructural proteins NS2A, NS4A, or NS4B enhances replication of an IFN-sensitive virus. Moreover, expression of NS4B and, to a lesser extent, of NS2A and NS4A proteins results in

down-regulation of IFN-β-stimulated gene expression (Muñoz-Jordán *et al.*, 2003). This phenomenon is also observed in West Nile virus and Kunjin virus (Liu *et al.*, 2005).

1.5 Properties of NS2A

NS2A of dengue virus is a small hydrophobic protein of about 24-kDa that migrates anomalously by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It is about 20-kDa in Kunjin virus (Mackenzie *et al.*, 1998) and 22-kDa in yellow fever virus (Lindenbach and Rice, 1999). Two forms of NS2A are found in yellow fever virus-infected cells. Full-length NS2A (224 amino acids) is the product of cleavage at the NS1/2A and NS2A/2B sites. NS2Aα, a C-terminally truncated form of 190 amino acids, results from partial cleavage by the viral NS2B-NS3 serine protease at the sequence QK↓T within NS2A. Changing lysine to serine at this site (QKT→QST) blocks the production of both NS2Aα and infectious virus (Kümmerer and Rice, 2002). In addition, studies in RNA replication with Kunjin virus have shown that NS2A co-localizes with double-strand RNA in discrete cytoplasmic foci and interacts with the 3' untranslated region of Kunjin viral RNA, as well the proposed replicase components NS3 and NS5 in cell lysates. The result indicates that the flavivirus replication complex includes NS2A (Mackenzie *et al.*, 1998). NS2A of Kunjin virus inhibits IFN-ß promoter-driven transcription. An identified single-amino-acid mutation (Ala30-to-Pro) in NS2A dramatically reduces this inhibitory activity. The findings determine a new function for NS2A in virus-host interactions and identify NS2A as a new target for attenuation in the development of live flavivirus vaccines (Liu *et al.*, 2004).

1.6 Properties of NS2B

Dengue virus possesses a protease complex made up of the non-structural proteins NS2B and NS3. This protease complex catalyzes autocleavage (*cis*) at the junction between NS2A and NS2B as well as between NS2B and NS3. It also catalyzes the *trans* cleavage at the junctions between NS3 and NS4A as well as NS4B and NS5. The *cis* cleavage at the NS2B–NS3 junction has been demonstrated in *Escherichia coli* by linking a 40-residue hydrophilic segment of NS2B to a NS3 N-terminal protease domain carrying the NS2B–NS3 cleavage site (Clum *et al.*, 1997). Hydrophobic regions of NS2B whose structural integrity may not be essential for proteolytic processing may have additional functions during viral replication (Chambers *et al.*, 1993). Modeling of the putative interactions between the dengue virus 2 (DV2) NS3 protease and its cofactor, NS2B, suggests that a 12 amino acid hydrophobic region within this sequence (^{70}GSSPILSITISE^{81}) may associate directly with NS3 (Fig. 1.4). Modeling also suggests that the substrate binds in an extended conformation to the solvent-exposed surface of the protease, with a P1-binding site that is significantly different from its HCV counterpart. The model described (Fig. 1.5) not only reveals unique features of the flavivirus protease but also provides a structural basis for both cofactor and substrate binding that should prove to be useful in the early design and the development of inhibitors (Brinkworth *et al.*, 1999).

A series of 46 charged-to-alanine mutations in the yellow fever virus NS2B-NS3 protease, previously characterized in cell-free and transient cellular expression systems, have been tested for their effects on virus recovery. Four distinct plaque phenotypes are observed in cell culture: parental plaque-size (13 mutants), reduced plaque-size (17 mutants), small plaque-size (8 mutants) and no plaque-formation (8 mutants). These data indicate that certain mutations which

reduce NS2B-NS3 protease cleavage activity cause growth restriction of yellow fever virus in cell culture. The existence of reversion mutations primarily in NS2B rather than NS3, suggests that the protease domain is less tolerant of structural perturbation compared with the NS2B protein (Chambers *et al.*, 2005). Approximately 17.5 and 51.5 % of Vero cells expressing DV2 NS3 serine protease and NS2B-$NS3_{185}$ serine protease precursor protein [NS2B-$NS3_{185}$(pro)], respectively, are apoptotic. Site-directed mutagenesis which replaced His^{51} with Ala within the protease catalytic triad significantly reduces the ability of the expressed NS3 and NS2B-$NS3_{185}$(pro) to induce apoptosis. The results show that DV2-encoded NS3 serine protease induced apoptosis, which is enhanced in cells expressing its precursor, NS2B-$NS3_{185}$(pro). These findings suggest the importance of NS2B as a cofactor to NS3 protease-induced apoptosis (Shafee and AbuBakar, 2003).

1.7 Properties of NS4A

The small hydrophobic protein NS4A has been positively identified by N terminal sequencing only in Kunjin virus-infected cells. Its size is 16-kDa and migrates in SDS-PAGE in the region between C and NS2B (Speight and Westaway, 1989). A precursor protein comprising NS3 and NS4A has been reported in dengue virus and many other flaviviruses either in wild-type virus infected cells or after expression from vectors (Cahour *et al.*, 1992). In a previous study on the nonstructural proteins of Kunjin virus using immunoelectronic microscopy, NS4A is found localized within a majority of the virus-induced membranes, including vesicle packets, convoluted membranes and distinct paracrystalline. This study also showed that NS4A bound strongly to most of the other nonstructural proteins, such as NS3 and NS5, and was included in replication

complex (Mackenzie *et al.*, 1998).

1.8 Properties of NS4B

NS4B is the largest of the four small hydrophobic nonstructural proteins and comprises 248 amino acid residues. In DV2-infected cells, NS4B is first produced as a peptide of apparent size of 30 kDa; NS4B is then post-translationally modified, in an unknown way, to produce a polypeptide of apparent size 28 of kDa. The modification of NS4B is found to be cell-dependent and most likely mediated by a cellular enzyme (Preugschat and Strauss, 1991). By using immunofluorescence and immunoelectronic microscopy, the subcellular localization of NS4B is analyzed in Vero cells infected by Kunjin virus. NS4B translocated from cytoplasm to the nucleus independently of other viral proteins and enter the nucleus late during the latent period (Westaway *et al.*, 1997). The nonstructural protein NS4B of DV2 partially blocks activation of nuclear signal transducer and activator of transcription 1 (STAT1) and interferon-stimulated response element (ISRE) promoters in cells stimulated with interferon (IFN) (Muñoz-Jordán *et al.*, 2003). This function of NS4B is conserved in West Nile virus and yellow fever virus. Deletion analysis shows that the first 125 amino acids of dengue virus NS4B are sufficient for inhibition of alpha/beta IFN (IFN-α/ß) signaling. Co-expression of dengue virus NS4A and NS4B together results in enhanced inhibition of ISRE promoter activation in response to IFN-α/ß, but proper viral polyprotein processing is required for anti-interferon function (Muñoz-Jordán *et al.*, 2005). However, the actual role of NS4B in dengue replication cycle has never been studied.

1.9 The overview of experimental designs

Since the functions of these four small nonstructural proteins regarding to

dengue viral replication have not been well-studied, it is necessary to establish the tool and assay system for future studies. As the first step, it was decided to obtain expression clones of individual genes for protein production, functional study, subunit vaccine development, and, when possible, for construction of DNA vaccines.

Here, I started with constructing the expression clones of these four nonstructural proteins from Dengue virus type 2 PL046 strain with the influenza hemagglutinin protein and six histidines as tags. Then, I expressed the four nonstructural proteins in two different expression systems, *E. coli* and mammalian cells. Finally, I have initiated the functional study by plaque assay.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Virus

Dengue virus type 2 PL046 strain (Taiwan local strain)

2.1.2 Cell lines

BHK-21 (baby hamster kidney cell)

293T (human embryo kidney epithelial cell)

2.1.3 Bacterial strains

Escherichia coli DH5α strain: for general cloning (Invitrogen)

Escherichia coli NovaBlue (*DE3*) strain: for protein expression (Novagen)

2.1.4 Plasmids

Plasmid	Description	Source
pcDNA3	T7 promoter and CMV promoter. (Selection marker: Ampicillin)	Invitrogen
pcDNA3(pro)	modified pcDNA3 (3~8 bases upstream of *Bam*HI site modified as SD sequence)	Yang laboratory collection
pcDNA3(pro)-D34B-HAHis	DV3 NS4B with C-terminal HA and His tag in pcDNA3(pro)	Yang laboratory collection
pBluescript II SK (+)	T7 promoter and *lac* promoter. (Selection marker: Ampicillin)	Stratagene
pET-30a (+)	T7 promoter, HIS-tag, and S-tag. (Selection marker: Kanamycin)	Novagen

pET△5T	pET-30a (+) w/o N-terminal His-tag and S-tag	This study
pNS2A-HAHis pNS2B-HAHis pNS4A-HAHis pNS4B-HAHis	*Bam*HI-*Xba*I fragment containing 2A, 2B, 4A, or 4B* with C-terminal HA and His tag in pcDNA3 Note: 4B*, abnormality in cloned fragment	徐婕琳, 2003 交大碩士論文; Yang laboratory collection
pcDNA3-D24B-HAHis	Recloned *Bam*HI-*Xba*I fragment containing 4B with C-terminal HA and His tag in pcDNA3	This study
pcDNA3-D24A	*Bam*HI-*Xho*I fragment containing DV2 NS4A in pcDNA3	This study
pcDNA3(pro)-D22A-HAHis pcDNA3(pro)-D22B-HAHis pcDNA3(pro)-D24A-HAHis pcDNA3(pro)-D24B-HAHis	*Bam*HI-*Xba*I fragment containing 2A, 2B, 4A, or 4B with C-terminal HA and His tag in pcDNA3 (pro)	This study
pET△5T-D22A-HAHis pET△5T-D22B-HAHis pET△5T-D24A-HAHis pET△5T-D24B-HAHis	*Bam*HI-*Xba*I fragment containing 2A, 2B, 4A, or 4B with C-terminal HA and His tag in pET△5T	This study
pKRY	Truncated DV2 E protein (nt 1 ~ nt 1205) with C-terminal HA and His tag in pcDNA3	Yang laboratory collection
pET-30b-HeptB	pET-30b containing HeptB ORF	Gift from Dr. H-L Peng

2.1.5 Primers

Gene	Position in Dengue genome*	Primer	Primer sequence (5' to 3')
NS4B	6826-7569	D26825F	CGG GAT CCA TGA ACG AGA TGG GTT TCC TGG AA
		D27570R	TTG TGT TGG TTG TGC TCT TCC GAG CTC GCC

*According to the sequence of Dengue type 2, New Guinea C strain (Accession No. M29095).

2.1.6 Chemicals, enzymes, and reagents

Chemical	Source	Catalog number	Application
1kb DNA ladder	SibEnzyme	SEM11C001	DNA electrophoresis
2-propanol	Sigma	I 9516	RNA extraction
Acetic acid	Fluka	33209	PAGE (Destain buffer)
Acryl/Bis 37.5:1 solution	AMRESCO	0254	PAGE
Agarose	VEGONIA	9201-05	DNA/RNA electrophoresis
Ampicillin	Applichem	A0839	Bacterial culture
Anti-DIG-AP	Roche	1 093 274	Northern Blot
APS	Bio-Rad	161-0700	PAGE
β-mercaptoethanol	MERCK	1.1543.0100	Reducing reagent
Blocking reagent	Roche	1 096 176	Northern Blot

CDP-Star	PerkinElmer	NEL602001KT	Northern Blot
Chloroform	Riedel-de Haën	32211	RNA extraction
Coomassie Brilliant Blue R-250	J.T.Baker	F792-01	Protein staining
Crystal Violet	Sigma	C-3886	Cell staining
DEPC	Sigma	D 5758	RNase inactivation for Northern Blot
DIG DNA Labeling mix	Roche	1 277 065	Northern Blot
DNA Polymerase I, Large (Klenow) Fragment	BioLabs	M0210S	Klenow fill in
EDTA	AMRESCO	0105	TE buffer
EtBr	Sigma	E-7637	DNA/RNA staining
Ex Tag polymerase	TaKaRa	RR001B	PCR reaction
Fetal Bovine Serum	Biological industries	04-001-1A	Cell culture
Formaldehyde	Riedel-de Haën	33220	Cell fixation Northern Blot
Formamide	Riedel-de Haën	33272	Northern Blot
Glutaraldehyde	Fluka	49630	Cell fixation
Hexanucleotide mix	Roche	1 277 081	Northern Blot (Probe labeling)
Isopropyl-b-D-thiogalacto pyranoside (IPTG)	MDBio,Inc.	105039	Induction of *lacZ* promoter
$K_3Fe(CN)_6$	Sigma	P-3667	X-gal staining
$K_4Fe(CN)_6 \cdot 3H_2O$	Sigma	P-3289	X-gal staining

Kanamycin	Sigma	K4000	Antibiotics
Klenow enzyme	Roche	1 008 404	Northern Blot (Probe labeling)
LB agar	Alpha Biosciences	L12-111	Bacterial medium
LB broth	Scharlau	02-385	Bacterial medium
Lipofectamine 2000	Invitrogen	11668-019	Transfection
Maleic acid	Fluka	63190	Northern Blot (Maleic acid buffer)
MEM	GIBCO	41500-034	Cell culture medium
Methanol	Mallinckrodt	3016-08	Western Blot
Methylcellulose	Sigma	M 0512	Plaque assay
MOPS	MERCK	1.06126.0025	Northern Blot
NaCl	AMRESCO	0241	Buffer
$NaHCO_3$	Sigma	S-5761	Additional ingredient to cell culture medium
NaOH	Riedel-de Haën	30620	Buffer
Nitrocellulose Transfer Membrane	Schleicher & Schuell	10401396	Western Blot membrane
Nonfat powdered milk	New Zealand Milk Brands Ltd	EMB 53084-D	Western Blot (Blocking)
Nytran SuPerCharge	Schleicher & Schuell	10416296	Northern Blot
PMSF	Fluka	78830	Protein inhibitor

Prestain protein marker	TBB	0901	SDS-PAGE
Restriction enzyme	TakaRa, BioLabs	—	Plasmid construction
SDS	Riedel-de Haën	62862	Northern Blot Western Blot
SuperSignal® West Femto Maximum Sensitivity Subatrate	PIERCR	34095	Western Blot
SuperSignal® West Pico Chemiluminescent Subatrate	PIERCR	34079	Western Blot
T4 DNA ligase	Fermentas	1812	Plasmid construction
TEMED	Sigma	T-9281	Western Blot
Tris (base)	AMRESCO	0826	Northern Blot Western Blot
Trypsin	GIBCO	12605-010	Cell culture
Tween-20	Sigma	P-1379	Northern Blot Western Blot
Urea	Fluka	SK-2644U	Denature reagent
X-ray film	Midwest Scientific	LA7111	Northern Blot Western Blot
X-gal	MDBio,Inc.	613049	X-gal staining

2.1.7 Antibodies

Antibody	Source	Catalog number
HA-probe (F-7) HRP (from mouse)	Santa Cruz	SC-7392
His-probe (H-3) HRP (from mouse)	Santa Cruz	SC-8036 HRP
6xHis Monoclonal Antibody (from mouse)	BD Bioscience	8916-1
Goat anti-mouse HRP	PIERCE	31430

2.1.8 Kits

Kit	Source	Catalog number	Application
DIG DNA Labeling Kit	Roche	1 175 033	Northern Blot
TRI reagent	Molecular Research Center, Inc.	TR-118	RNA extraction
ExcelPure™ Plasmid Miniprep Purification Kit	Premier	N-PM050	Plasmid extraction
PCR Clean-up/Gel Extraction Kit	Premier	N-DCE050	DNA purification

2.1. 9 Buffers

- 0.25% Coomassive blue stain solution

  2.5g Coomassive brilliant blue，50% methanol，10% acetic acid added dd H_2O to 1000 ml

- 0.5% glutaraldehyde

  25% glutaraldehyde stock diluted in 1X PBS

- 10X MOPS Electrophoresis buffer

  0.22 M MOPS (pH 7.0)，20 mM sodium acetate，10 mM EDTA (pH 8.0)

- 10X SDS-PAGE running buffer

  0.25 M Tris base，1.92 M Glycine，1% SDS

- 10X transfer buffer

  39 mM Glycine，48 mM Tris base，10% SDS，20% methanol

- 1X PBS (pH 7.4)

  137 mM NaCl，10 mM Na_2HPO_4，2.7 mM KCl，1.8 mM KH_2PO_4

- 20X SSC buffer (pH 7.0)

  3 M NaCl，300 mM sodium citrate

- 2X SDS-PAGE loading buffer

  0.5% bromphenol blue，0.5M Tris-HCl (pH 6.8) ，10% SDS ，100% glycerol

- 5% Blocking buffer

  2.5g nonfat powdered milk dissolved in 50ml 1X TBS buffer

- 50X TAE buffer

  48.4 g Tris base，0.5 M EDTA (pH 8.0) 20 ml，11.42 ml acetic acid added dd H_2O to 200 ml

- Blocking solution

  1 % (w/v) blocking reagent dissolved in maleic acid buffer

- Cell lysis buffer（RIPA buffer）

  0.1% SDS，1% Triton X-100，1% NP-40，10 mM Tris-HCl (pH7.4)，1 mM $MgCl_2$，1 mM PMSF

- Detection buffer (pH 9.5)

  0.1 M Tris-Cl，0.1 M NaCl
- Maleic acid buffer (pH 7.5)

  0.1 M Maleic acid，0.15 M NaCl
- Prehybridization/Hybridization solution (pH 7.0)

  0.5 M sodium phosphate (pH 7.2)，7% (w/v) SDS，1 mM EDTA
- TBS buffer (Tris-buffered saline)

  10mM Tris(pH 8.0)，150 mM NaCl
- TBST buffer

  10mM Tris(pH 8.0)，150 mM NaCl，0.05% Tween 20
- Washing buffer (pH 7.5) (for Northern Blot)

  0.1 M Maleic acid，0.15 M NaCl，0.3% (v/v) Tween 20
- X-gal staining buffer

  5 mM $K_4Fe(CN)_6 \cdot 3H_2O$，5 mM $K_3Fe(CN)_6$，1 mM $MgSO_4 \cdot 7H_2O$，1mg/ml X-gal

2.1.10 Media

- LB (Luria-Bertani) broth

  1% tryptone，0.5% yeast extract，1% NaCl
- LB (Luria-Bertani)/Ampicillin broth

  1% tryptone，0.5% yeast extract，1% NaCl，1.5% agar，50 μg/ml ampicillin
- LB (Luria-Bertani)/Kanamycin broth

  1% tryptone，0.5% yeast extract，1% NaCl，1.5% agar，50 μg/ml kanamycin
- LB (Luria-Bertani)/ Ampicillin agar

  1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, 50μg/ml ampicillin
- LB (Luria-Bertani)/Kanamycin agar

  1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, 50μg/ml kanamycin

2.1.11 Equipments

- -20℃ low temperature refrigerator (WHITE-WESTINGHOUSE)
- -80℃ low temperature refrigerator 925/926 (FIRSTEK SCIENTIFIC)
- Auto dry box DX100 (Taiwan Dry Tech Co. Ltd)
- Bench top orbital shaker S101 (FIRSTEK)
- Centrifuge model 5100 (Kubota Corporation)
- Digital scale PB153-S (METTLER TOLEDO)
- GeneQuant pro DNA/RNA calculator (AMERSHAM PHARMACIA BIOTECH)
- Gene Cycler^{TM} (BIO-RAD)
- Image system GEL DOC 2000 (BIO-RAD)
- Microcentrifuge MICRO 240A (DENVILLE SCIENTIFIC INC.)
- Mini-Protein electrophoresis cell 3 (BIO-RAD)
- Mini Trans-Blot Electrophoretic Transfer Cell (BIO-RAD)
- Orbital shaker IKA-VIBRAX-VXR
- pH meter Φ360 (BACKMAN)
- 4℃ refrigerator KS-101MS (MINI KINGCON)
- SHORTER MINI Horizontal Gel Electrophoresis Apparatus MJ-105 (MEDCLUB)
- Thermal Cycler PTC-100^{RT} (MJ RESEARCH INC.)
- Vertical acrylamide electrophoresis unit (BIO-RAD)
- VORTEX-GENIE2 G560 (SCIENTIFIC INDUSTRICS)
- Water bath B206-T1 (FIRSTEK SCIENTIFIC)

2.2 Methods

2.2.1 Transformation of *E. coli*

2.2.1.1 Preparation of competent cells (for chemical method)

A single colony of *E. coli* was inoculated in 5ml of LB broth and grew overnight at 37°C with vigorous shaking (~165rpm). 2ml of the overnight culture was transferred into 100ml LB broth (containing 5% glucose and 2mM $MgCl_2$) and was then incubated at 37°C with shaking (~165rpm) until the OD_{600} is between 0.4 and 0.7. The cultures were stored on ice for 20 minutes. The cells were recovered by centrifugation at 3000rpm for 10 minutes and then resuspended in 50ml ice-cold 0.1M $CaCl_2$. The cells were let stand on ice for 30 minutes and were pelleted by centrifugation at 2000rpm for 10 minutes at 4°C. The pellet was resuspended in 10ml ice-cold 0.1M $CaCl_2$. The cells were incubated at 4°C for 18 hours and were then recovered by centrifugation at 2000rpm for 10 minutes. The pellet was resuspended in 10ml ice-cold 0.05M $CaCl_2$ (containing 15% glycerol). The cells were dispensed as 100µl per eppendorf tube and then were stored at -80°C.

2.2.1.2 Transformation

Stored competent cells were thawed on ice. 0.1~1µg of plasmid DNA was mixed with 50µl competent cells and was then stored on ice for 25 minutes. The mixture was incubated in a preheated 42°C circulation water bath for 1 minute. Then 500µl of LB broth was added to the cells. The culture was incubated at 37°C with shaking (~165rpm) for 1 hour. 100µl of the culture was plated on LB agar plate with 50µg/ml ampicillin or kanamycin. The plate was stored at room temperature until the liquid had been absorbed. The plate was inverted and was then incubated at 37°C for 12~18 hour.

2.2.2 Plasmid DNA extraction

Plasmid DNA in *E. coli* was extracted with ExcelPure™ Plasmid Miniprep Purification Kit (Premier). The procedure is as following:

A single colony of *E. coli* was inoculated in 5ml of LB broth (with antibiotics) and grew overnight at 37°C with vigorous shaking (~165rpm). The cells were recovered by centrifugation at 2500rpm for 12 minutes and then resuspended in 200μl Solution I buffer (Premier, Inc.; San Diego, USA). The *E. coli* solution was transferred to an eppendorf. 200μl of Solution II buffer (Premier, Inc.; San Diego, USA) was added and mixed gently. 200μl of Solution III buffer (Premier, Inc.; San Diego, USA) was added to the mixture and mixed gently again. Cells were spun at 13000 rpm for 5 minutes at room temperature (RT), the supernatant was transferred to Mini-M™ Column. The solution was centrifuged at 13000rpm for 1 minute, and the filtrate in the collection tube was discarded. 700μl of Washing solution (Premier, Inc.; San Diego, USA) was added in and spin for 1 minute. This step was repeated once again. After the filtrate discarded, the column was centrifuged at 13000rpm for 3 minutes to remove residual ethanol. Mini-M™ Column was transferred to a new eppendorf and incubated at 60°C for 5 minutes to evaporate the ethanol. Finally, DNA was eluted by 30-50μl Elution Solution (Premier, Inc.; San Diego, USA) and centrifuged at 13000rpm for 1 minute. Plasmid DNA was stored at -20°C.

2.2.3 Restriction enzyme digestion

0.5~1μg of DNA was dissolved in appropriate volume of water and was digested with restriction enzyme (following the commercial protocol). Generally, 1μg DNA was digested with 1 unit of restriction enzyme in a 10μl reaction at

37°C for 1 hour or longer. The reaction was stopped by heat inactivation (65°C for 10 minutes in general).

2.2.4 Klenow enzyme fill-in and blunt-ended ligation

Klenow fill-in reaction was performed with DNA polymerase I, large (Klenow) fragment (BioLabs). 0.5μg DNA was dissolved in 1X EcoPol buffer containing 33μM dNTPs. 0.5 unit of Klenow enzyme was added to the reaction (total volume = 50μl) and incubated at 25°C for 15 minutes. The reaction was stopped by 10mM EDTA (pH8.0) and 75°C heating for 20 minutes. And blunt end ligation was achieved with T4 DNA ligase (Fermentas). 50-400ng DNA was dissolved in 1X T4 DNA ligation buffer containing 5% PEG 4000. 5 units of T4 DNA ligase was added to the reaction (total volume = 50μl) and incubated at 22°C for 1 hour. The reaction was stopped by 65°C heating for 10 minutes. Half volume of the reaction was then transformed into *E. coli.*

2.2.5 Cell culture

BHK-21 cells were grown in MEM (Minimum Essential Medium; Gibco) supplemented with 5% fetal bovine serum and 0.22% $NaHCO_3$. 293T cells were grown in MEM supplemented with 10% fetal bovine serum and 0.22% $NaHCO_3$. Cells were incubated in tissue culture incubator with 5% CO_2.

2.2.6 Transfection of mammalian cell

Cells were transfected with different expression plasmid by use of Lipofectamine™ 2000 (Invitrogen) and the appropriate amount of plasmid according to the manufacturer's instructions. 35mm culture dish as example, 4μg DNA was diluted in 250μl serum-free medium and mixed gently. 10μl Lipofectamine™ 2000 was gently mixed with 250μl serum-free medium and

incubated for 5 minutes at RT. The diluted DNA was combined with diluted Lipofectamine™ 2000 and incubated for 20 minutes at RT. The mixture was added to 90-95% confluent cells and incubated at 37°C in CO_2 incubator for 4-6 hours. The medium was replaced with fresh serum-free medium. Cells were incubated at 37°C in CO_2 incubator for 48 hours prior to the following assay.

2.2.7 Expression of four nonstructural proteins in mammalian cell, BHK-21

Forty-eight hours after transfection, transfected BHK-21 cells were lysed with 100~200μl RIPA buffer (containing 1mM PMSF) at RT. The lysates were sedimented by centrifugation (12000×g for 5 min at 4°C). The supernatant and pellet were respectively mixed with 1 volume 2× SDS-PAGE loading dye (containing 8M urea, 200mM DTT, and 200mM β-mercaptoethanol) and analyzed by Western blotting.

2.2.8 Plaque assay

Forty-eight hours posttransfection, MEM without FBS was mixed with virions of DV2 PL046 strain in the amount of 100-200 PFU/well. The mixtures were mixed gently and added onto the transfected BHK-21 cells in 35mm dishes and then incubated at 37 °C with 5% CO_2 for 1 h. MEM containing 5% FBS and 1.1% methylcellulose were added to the well and incubated at 37 °C with 5% CO_2 for 5-7 days. The medium was discarded before the cells were fixed with 3.7% formaldehyde. After 15 minutes, the solution was removed and the cells were stained with 0.5% crystal violet in 3.7% formaldehyde. The plates were washed with water before the plaque numbers were scored.

2.2.9 Statistical analysis

All determinations were conducted three times at least. Analysis of variance (ANOVA) of the data was evaluated by the Statistical Analysis System (SAS ver. 9.0). Duncan's multiple-range test was employed to determine the statistical significance of the differences between the means ($P < 0.05$).

2.2.10 Expression of four nonstructural proteins in *E. coli*

E. coli NovaBlue (*DE3*) cells were transformed with different expression plasmid and a single colony was inoculated into 5ml LB broth supplemented 50 μg/ml ampicillin/kanamycin and cultured overnight. Overnight culture was diluted by 100X to 5-10ml LB broth and also supplemented with 100 μg/ml ampicillin and incubated at 37°C until the OD_{600} reached 0.4-0.6, after which the culture was added with 1mM isopropyl-β-D-thiogalactoside for 4 hours to induce the expression of NS genes. Then bacterial cells were collected by centrifugation and stored at -80°C until used. Cells were lysed by freeze and thaw. The lysate was resuspended in 250μl 1M Tris-Cl (pH 8.0)-1mM PMSF and sedimented by centrifugation (12000× g for 5 minutes at 4°C). The supernatant was collected and the pellet was resuspended in 250μl 1M Tris-Cl (pH 8.0) with 8M urea and 1mM PMSF. The urea-dissolved pellet was then subjected to centrifugation (12000× g for 5 minutes at 4°C) and the supernatant were used for protein gel analysis. Bacterial protein samples from supernatant and pellet were mixed with 1 volume 2× SDS-PAGE loading dye (containing 8M urea, 200mM DTT, and 200mM *β*-mercaptoethanol) and analyzed by Western blotting.

2.2.11 Western blot analysis

E. coli or mammalian cells expressed proteins were separated by SDS-PAGE

(14% gel) using a Mini-Protein electrophoresis cell 3 (Bio-Rad) and electrophoretically transferred to Nitrocellulose membrane (Schleicher & Schuell) using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 0.09A for 37 minutes. The membrane was blocked for 1hr at room temperature with 5% nonfat powdered milk (New Zealand Milk Brands Ltd) in TBS buffer. Then, the membrane was incubated with 6xHis monoclonal antibody (BD Bioscience) or HA-probe (F-7) HRP (Santa Cruz Biotechnology) for 1 hour at RT and washed in PBST 3 times for 5 minutes. The membrane incubated with 6xHis monoclonal antibody was then incubated with Goat anti-mouse HRP (PIERCE) for 1 hour at RT and washed in PBST 3 times for 5 minutes. For visualization of the fusion protein, the blots were developed by SuperSignal® West Pico Chemiluminescent Substrate (for *E. coli*) or SuperSignal® West Femto Maximum Sensitivity Substrate (for cells) and then exposed to an X-ray film.

2.2.12 RNA extraction

RNA was isolated from *E. coli* or mammalian cells using TRI reagent (Molecular Research Center, Inc.). The procedure is as following:

E. coli NovaBlue (*DE3*) cells were transformed with different expression plasmids A single colony of the transformants was inoculated into 5ml LB broth supplemented 50 μg/ml ampicillin/kanamycin and culture overnight. Overnight culture was diluted by 100X to 5-10ml LB broth and also supplemented with 100 μg/ml ampicillin and incubated at 37°C until the OD_{600} reached 0.4-0.6, after which the culture was added with 1mM isopropyl-β-D-thiogalactoside for 4 hours to induce the expression of NS genes. Then bacterial cells were collected by centrifugation and were resuspended

with 1ml TRI reagent. The solution was incubated at 65°C for 10 minutes. 200µl chloroform was added to the reaction and vortexed. The mixture was stored at RT for 15 minutes and centrifuged at 12000rpm for 15 minutes at 4°C. The aqueous phase was transferred to a new eppendorf. 40µl of sodium acetate (pH 5.2) and 600µl of isopropanol were added and stored at RT for 8 minutes to precipitate RNA. RNA was recovered by centrifugation at 12000rpm for 8 minutes at 4°C. 1ml of 75% ethanol was added to wash the precipitated RNA and centrifuged at 7500rpm for 5 minutes at 4°C. Ethanol was removed, and the RNA pellet was air-dried for 10 minutes. RNA was dissolved in 40-60µl of DEPC-treated ddH_2O and stored at -20°C.

2.2.13 Northern blot analysis

Totally 9µg of RNA was used for each lane for Northern blot analysis. The RNA was loaded on to 1.2% formaldehyde agarose gel. The RNA was blotted onto Nytran SuPerCharge (Schleicher&Schuell), and the membrane was UV crosslinked (254nm; 120 joules) twice. NS2A gene fragment was obtained using *Bam*HI-*Xho*I digestion followed by gel extraction. The DNA probe was labeled by DIG DNA labeling kit (Roche) as instructed by the manufacturer. DNA to be labeled was dissolved in ddH_2O to a final volume of 15µl. Diluted DNA was denatured by 95°C heating for 10 minutes and chilled quickly on ice. 2µl of Hexanucleotide Mix, 2µl of DIG DNA labeling mix, and 1µl of Klenow enzyme were added to the reaction and incubated at 37°C for 20 hours. The reaction was stopped by adding 2µl of 0.2M EDTA and heating at 65°C for 10 minutes. Hybridization overnight at 45°C was followed by washes (20 minutes at room temperature in 2× SSC, 0.1% SDS and then 20 minutes at 60°C in 0.5× SSC, 0.1% SDS). The blocking of the blot was preformed with Blocking reagent

(Roche) after washing (20 minutes at RT in 1× SSC, 0.1% SDS) twice. 10000× diluted anti-DIG-AP (Roche) was added to the blot. After shaking at RT for 30 minutes, the blot was washed (20 minutes at RT in 1× SSC, 0.1% SDS) twice. Then the blot was shaded and incubated with 250μl CDP-Star (PerkinElmer) at 37℃ for 20 minutes. The result was achieved after exposure to the X-ray film.

Chapter 3. Results

3.1 Construction of pcDNA3-D24B-HAHis expression plasmid

Full-length cDNA fragment of NS4B (744bp) was obtained by polymerase chain reaction (PCR) amplification with primers D26825F and D27570R on dengue virus type 2 (DV2) PL046 strain full-length cDNA (賴建孛, 2006, 交大碩士論文) as the template. The contents of the PCR reaction mixture are listed in Table 3.1 and the condition of the PCR reaction was performed with the program described in Table 3.2. The PCR products were digested with restriction enzyme *Bam*HI at the 5'-end and *Xho*I at the 3'-end. The restricted fragment was then ligated with the large *Bam*HI-*Xho*I fragment of pNS4A-HAHis (pcDNA3 based). Thus the NS4B gene was cloned into pcDNA3 carrying C-terminal HA and His tag, named pcDNA3-D24B-HAHis (Fig. 3.1 A). This construct was assessed by *Afl*III and *Ava*I digestions. pcDNA3-D24B-HAHis was digested into 3.5kb, 2kb, and 872bp fragments (indicated by A, B, and C in Fig. 3.1 B) by *Afl*III and 4.3kb, 1.4kb, and 678bp fragments (indicated by D, E, and F in Fig. 3.1 B) by *Ava*I.

3.2 Sequence analysis of NS4B gene

The cDNA of Taiwan local strain, DV2 PL046 was used as the template for the construction of NS4B gene. The cDNA sequence of NS4B was sequenced and compared to the prototype virus, dengue virus type 2 New Guinea C strain (NGC strain) (Table 3.3). There were 22 nucleotides of NS4B in PL046 strain different from that of NGC strain (Fig. 3.2). All except two, F112L and T198I, were silent mutations (Table 3.3). The percentage of identity of nucleotides is 97% and that of amino acids is 99.2% in NS4B (Table 3.3).

3.3 Construction of pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)-D22B-HAHis, pcDNA3(pro)-D24A-HAHis, and pcDNA3(pro)-D24B-HAHis expression plasmids

Fragments of NS2A, 2B, 4A, and 4B were obtained by restriction enzyme digestions with *Bam*HI and *Xba*I from pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis (徐婕琳, 2003, 交大碩士論文), and pcDNA3-D24B-HAHis and followed by gel purification. pcDNA3(pro)-D34B-HAHis (Appendix 1) was also treated with the same restriction enzyme digestion and gel purified. The small *Bam*HI-*Xba*I fragments of NS2A, 2B, 4A, and 4B were ligated to the large *Bam*HI-*Xba*I fragment of pcDNA3(pro)-D34B-HAHis. Thus those four nonstructural genes mentioned above were cloned into pcDNA3(pro) carrying C-terminal HA and His tag. The plasmids were named pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)-D22B-HAHis, pcDNA3(pro)-D24A-HAHis, and pcDNA3(pro)-D24B-HAHis (Fig. 3.3). pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)-D22B-HAHis, and pcDNA3(pro)- D24B-HAHis were confirmed by restriction digestions (Fig. 3.4) while pcDNA3(pro)-D24A-HAHis was assessed by sequencing (Fig. 3.5). pcDNA3(pro)-D22A-HAHis was digested into 4.3kb, 1.5kb, and 581bp fragments (indicated by A, B, and C in Fig. 3.4) by *Ava*I; 5.7kb and 768bp fragments (indicated by D and E in Fig. 3.4) by *Spe*I digestion. pcDNA3(pro)-D22B-HAHis was digested into 4.7kb and 1.5kb fragments (indicated by F and G in Fig. 3.4) by *Ava*I; 5.4kb and 759bp fragments (indicated by H and I in Fig. 3.4) by *Spe*I digestion. For pcDNA3(pro)-D24B-HAHis, it was digested into 3.6kb, 2kb, and 872bp fragments (indicated by J, K, and L in Fig. 3.4) by *Afl*III; 5.3kb and 1.3kb fragments (indicated by N and O in Fig. 3.4) were obtained by *Nde*I digestion. The extra band obtained from *Nde*I digestion of pcDNA3(pro)-D24B-HAHis (indicated by M in Fig. 3.4) was caused by

incompletely digestion. Sequence analysis of pcDNA3(pro)-D24A-HAHis was shown in Fig. 3.5.

3.4 Construction of pETΔ5T-D22A-HAHis, pETΔ5T-D22B-HAHis, pETΔ5T-D24A-HAHis, and pETΔ5T-D24B-HAHis expression plasmids

3.4.1 Construction of pETΔ5T expression vector

pETΔ5T (Fig. 3.6) was constructed by removing N-terminal His-tag and S-tag from pET-30a(+) (Appendix 2). pET-30a(+) was digested with *Nde*I and *Nco*I. The truncated linear pET-30a(+) was then blunt-end ligated after a Klenow fill-in reaction. pETΔ5T was confirmed by *Mlu*I and *Xho*I double digestion (Fig. 3.7), and the blunt-end ligated region was verified by sequencing (Fig. 3.8). pETΔ5T was digested into 4.4kb and 888bp fragments (indicated by A and B in Fig. 3.7) by *Mlu*I and *Xho*I while pET-30a(+) was digested into 4.4kb and 1018bp fragments (indicated by C and D in Fig. 3.7) by contrast. The sequence of blunt-end ligated region is showed in Fig. 3.6. The upstream CATA was from *Nde*I digestion, and the downstream ATGG was from *Nco*I digestion.

3.4.2 Construction of pETΔ5T-D22A-HAHis, pETΔ5T-D22B-HAHis, pETΔ5T-D24A-HAHis, and pETΔ5T-D24B-HAHis expression plasmids

Fragments of NS2A, 2B, 4A, and 4B were obtained by restriction enzyme digestions with *Hind*III and *Xba*I on pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pcDNA3-D24B-HAHis, followed by gel purification. The small *Hind*III-*Xba*I fragments containing the coding region and HA-His of NS2A, 2B, 4A, and 4B were first ligated to the *Hind*III and *Xba*I digested cloning vector, pBluescript II SK(+) (Appendix 3). Then the small *Bam*HI-*Not*I fragments of the resulting plasmids were ligated to the large *Bam*HI-*Not*I

fragment of pETΔ5T. Thus four nonstructural genes were cloned into pETΔ5T carrying C-terminal HA and His tag, named pETΔ5T-D22A-HAHis, pETΔ5T-D22B-HAHis, pETΔ5T-D24A-HAHis, and pETΔ5T-D24B-HAHis (Fig. 3.9 A to D). These constructs were confirmed by restriction digestions (Fig. 3.9 E). pETΔ5T-D22A-HAHis was digested into 3.1kb, 2.1kb, and 1kb fragments (indicated by A, B, and C in Fig. 3.9 E) by *Afl*III; 5.6kb and 639bp fragments (indicated by D and E in Fig. 3.9 E) by *Nde*I and *Spe*I digestion. pETΔ5T-D22B-HAHis was digested into 5.6kb and 384bp fragments (indicated by F and G in Fig. 3.9 E) by *Nde*I and *Spe*I; 5.7kb and 212bp fragments (indicated by H and I in Fig. 3.9 E) by *Eco*RV digestion. For pETΔ5T-D24A-HAHis, it was digested into 3.4kb, 1.4kb, 875bp, and 301bp fragments (indicated by J, K, L, and M in Fig. 3.9 E) by *Bsp*HI; 5.9kb and 136bp fragments (indicated by N and O in Fig. 3.9 E) were obtained by *Nco*I digestion. As for pETΔ5T-D24B-HAHis, 4.1kb, 1.2kb, 678bp, and 305bp fragments (indicated by P, Q, R and S in Fig. 3.9 E) were obtained after *Ava*I digestion, and 3.2kb, 2.1kb, and 1kb (indicated by T, U, and V in Fig. 3.9 E) after *Afl*III digestion.

3.5 Expression of four nonstructural genes in mammalian cells

3.5.1 Expression of pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pcDNA3-D24B-HAHis in BHK-21

Four nonstructural protein constructs, pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pcDNA3-D24B-HAHis, were expressed in BHK-21 cells. The plasmids were based on pcDNA3 having a CMV immediate-early gene transcription control region. BHK-21 cells transfected with these four plasmids

separately were harvested 48 hours posttransfection and were lysed by RIPA buffer (formula on page 16). The cell lysates were analyzed by SDS-PAGE with Coomassie blue staining and Western blot with antibody against the C-terminal HA tag (Fig. 3.10). The predicted molecular weights of NS2A-HAHis, NS2B-HAHis, NS4A-HAHis, and NS4B-HAHis are 29, 19.4, 22, and 31.6 kDa respectively. Compared with cell transfected with vector only, there was no extra band on the Coomassie blue stained gel (Fig. 3.10 A and B). For cells transfected with pNS2B-HAHis, a single band between 24kDa and 17kDa was detected both in the supernatant and the pellet of the cell lysate by Western blot (Lane 3, Fig. 3.10 C and D). For cells transfected with the pNS4A-HAHis construct, protein was detected both in the supernatant and the pellet of the cell lysate at about 22 kDa, which is as expected (Lane 4, Fig. 3.10 C and D). As for NS4B, there was a single band below the 33kDa marker as shown in Lane 5, Fig. 3.10 (C) and (D). pNS2A-HAHis was also transfected into BHK-21 cells but expression was not detected (Lane 2, Fig. 3.10 C and D).

3.5.2 Expression of pNS2A-HAHis in 293T

293T cells transfected with pNS2A-HAHis were harvested 48 hours after transfection and were lysed by RIPA buffer. The cell lysates were analyzed by SDS-PAGE with Coomassie blue staining and Western blot with antibody against the C-terminal HA and His tags (Fig. 3.11). The predicted molecular weight of NS2A-HAHis is 29kDa. Compared with cell transfected with vector only, there was no extra band on the Coomassie blue stained gel either in the supernatant or in the pellet (Fig. 3.11 A and B). As for Western blot analysis, a single band between 33kDa and 24kDa was detected both in the supernatant and the pellet of the cell lysate (Lane 2 of Fig. 3.11 C and D).

3.5.3 Expression of pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)-D22B-HAHis, pcDNA3(pro)-D24A-HAHis, and pcDNA3(pro)-D24B-HAHis in BHK-21

BHK-21 cells transfected with pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)-D22B-HAHis, pcDNA3(pro)-D24A-HAHis, and pcDNA3(pro)-D24B-HAHis separately were harvested 48 hours posttransfection and were lysed by RIPA buffer. The cell lysates were analyzed by SDS-PAGE with Coomassie blue staining and Western blot with antibody against the C-terminal HA and His tags (Fig. 3.12). The predicted molecular weights of NS2A-HAHis, NS2B-HAHis, NS4A-HAHis, and NS4B-HAHis are 29, 19.4, 22, and 31.6 kDa respectively. Compared with cell transfected with vector only, there was no extra band on the Coomassie blue stained gel (Fig. 3.12 A). For cells transfected with the pcDNA3(pro)-D24A-HAHis construct, there was a single band below the 24kDa marker in both the supernatant and the pellet as shown in Lane 4 and 9 of Fig. 3.12 (B). pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)-D22B-HAHis, and pcDNA3(pro)-D24B-HAHis were also transfected into BHK-21 cells but expression was not detected (Lane 2, 3, 5, 7, 8, 10, Fig. 3.12 B).

3.6 Plaque assay of pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pcDNA3-D24B-HAHis in BHK-21

BHK-21 cells transfected with pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pcDNA3-D24B-HAHis separately were infected with DV2 virus PL046 strain. Seven days post-infection, the cells were fixed and stained with crystal violet. The numbers and sizes of plaques were recorded in Table 3.4 and analyzed by Duncan's test. The differences of plaque numbers between negative control and each treatment were not significant. The pictures of assay plates were

shown in Fig. 3.13. However, when the plaque size of cells transfected with different plasmids was compared to cells transfected with pcDNA3, it showed that cells transfected with pNS2B-HAHis exhibited reduced plaque size (0.35 ± 0.072 mm) relatively to pcDNA3 (0.52 ± 0.123 mm). Plaque diameter of cells transfected with pNS4A-HAHis (0.7 ± 0.14 mm) was significantly larger than that of cells transfected with pcDNA3. As for cells transfected with pNS2A-HAHis (0.52 ± 0.099 mm) or pcDNA3-D24B-HAHis (0.51 ± 0.124 mm), the plaque diameters were not different from that of cells transfected with pcDNA3.

3.7 Expression of four nonstructural genes in *E. coli*

3.7.1 Expression of pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pcDNA3-D24B-HAHis in *E. coli*

E. coli NovaBlue (*DE3*) transformed with pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pcDNA3-D24B-HAHis separately were cultured and induced using 1mM IPTG at 37℃ for 4 hours. Then the cells were lysed by the method of freeze-and-thaw. The cell lysates were analyzed by SDS-PAGE with Coomassie blue staining and Western blot with antibodies against the C-terminal HA and His tag (Fig. 3.14). The predicted molecular weights of NS2A-HAHis, NS2B-HAHis, NS4A-HAHis, and NS4B-HAHis are 29, 19.4, 22, and 31.6 kDa, respectively. Compared with cells transformed with vector only, there was no extra band on the Coomassie blue stained gel (Fig. 3.14 A and B). Protein expression of the four nonstructural proteins was not detected by Western blot, either (Fig. 3.14 C to F).

3.7.2 Expression of pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)-D22B-HAHis, pcDNA3(pro)-D24A-HAHis, and pcDNA3(pro)-D24B-HAHis in *E. coli*

E. coli NovaBlue (*DE3*) transformed with pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)-D22B-HAHis, pcDNA3(pro)-D24A-HAHis, and pcDNA3(pro)-D24B-HAHis separately were cultured and induced using 1mM IPTG at 37℃ for 4 hours. Then the cells were lysed by the method of freeze-and-thaw. The cell lysates were analyzed by SDS-PAGE with Coomassie blue staining and Western blot with antibodies against the C-terminal HA and His tags (Fig. 3.15). The predicted molecular weights of NS2A-HAHis, NS2B-HAHis, NS4A-HAHis, and NS4B-HAHis are 29, 19.4, 22, and 31.6 kDa, respectively. Compared with cells transformed with vector only, there was no extra band on the Coomassie blue stained gel (Fig. 3.15 A). For cells transformed with the pcDNA3(pro)-D24A-HAHis construct, proteins were only detected by anti-HA antibody in the supernatant of the cell lysate at about 22 kDa, which is as expected (Lane 10, Fig. 3.15 B). pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)- D22B-HAHis, and pcDNA3(pro)-D24B-HAHis were also transformed into NovaBlue (*DE3*) but expression was not detected (Lane 3 to 9, 11, Fig. 3.15 B and C).

3.7.3 Expression of pETΔ5T-D22A-HAHis, pETΔ5T-D22B-HAHis, pETΔ5T-D24A-HAHis, and pETΔ5T-D24B-HAHis in *E. coli*

E. coli NovaBlue (*DE3*) transformed with pETΔ5T-D22A-HAHis, pETΔ5T-D22B-HAHis, pETΔ5T-D24A-HAHis, and pETΔ5T-D24B-HAHis separately were cultured and induced using 1mM IPTG at 37℃ for 4 hours. Then the cells were lysed by the method of freeze-and-thaw. The cell lysates were analyzed by SDS-PAGE with Coomassie blue staining and Western blot with antibodies against the C-terminal HA and His tag (Fig. 3.16). The predicted

molecular weights of NS2A-HAHis, NS2B-HAHis, NS4A-HAHis, and NS4B-HAHis are 29, 19.4, 22, and 31.6 kDa, respectively. Compared with cell transformed with vector only, there was no extra band on the Coomassie blue stained gel (Fig. 3.16, A and B). For cells transfected with pETΔ5T-D22B-HAHis, a single band between 24kDa and 17kDa were detected in the pellet of the cell lysate by both the anti-HA and the anti-His antibody (Lane 3, Fig. 3.16 D and F). For cells transformed with the pETΔ5T-D24A-HAHis construct, proteins were detected in the pellet of the cell lysate by both the anti-HA and the anti-His antibody at about 22 kDa, which is as expected (Lane 4, Fig. 3.16 D and F). As for NS4B, there was a single band below the 33kDa marker in both the supernatant and the pellet detected by anti-HA (Lane 5, Fig. 3.16 C and D). However, this protein was detected only in the pellet by anti-His antibody (Lane 5, Fig. 3.16 F). pETΔ5T-D22A-HAHis was also transformed into NovaBlue (*DE3*) but its expression was not detected (Lane 2, Fig. 3.16 C to F).

3.7.4 Detection of NS2A mRNA in *E.coli*

E. coli NovaBlue (*DE3*) transformed with pETΔ5T-D22A-HAHis was cultured and induced using 1mM IPTG at 37℃ for 4 hours. The bacterial RNA was extracted with TRI reagent (Molecular Research Center, Inc.) and then analyzed by Northern blot. The length of the NS2A-HAHis open reading frame is 792bp. There were two bands below 16S rRNA (about 1.5kb) as shown in Fig. 3.17 (B).

Chapter 4. Discussions

4.1 Construction of pcDNA3-D24B-HAHis expression plasmid and sequence analysis

Previously, a researcher in our laboratory has cloned four nonstructural genes of DV2 PL046 strain into the expression vector pcDNA3 along with C-terminal HA and His tags (徐婕琳, 2003, 交大碩士論文). They were named pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pNS4B-HAHis (Appendix 4). However, restriction digestion of pNS4B-HAHis did not match the predicted pattern of the DNA fragments (Fig. 4.1), suggesting there were some abnormality with this construct. Thus, I reconstructed this plasmid by amplifying the NS4B gene from full-length cDNA of dengue virus type 2 PL046 strain and then replaced the sequence of NS4A on pNS4A-HAHis with this newly amplified NS4B fragment to create a new expression plasmid, named pcDNA3-D24B-HAHis. This construct was assessed by restriction digestions (Fig. 3.1).

Alignment and comparison of the nucleotide and deduced amino acid sequences of NS4B gene in PL046 revealed that nucleotide sequence (97%) and amino acids (99.2%) are similar to that of NGC strain (Table 3.3). Between the two mutations, F112L and T198I, phenylalanine (F), leucine (L), and isoleucine (I) are nonpolar amino acids, while threonine (T) is polar uncharged amino acid. Thus, T198I may cause some conformational and functional changes to NS4B. Besides, phenylalanine has bulky side chain. Therefore, its replacement of leucine may also cause conformational differences.

4.2 Expression of four nonstructural proteins in mammalian cells

4.2.1 Expression of pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pcDNA3-D24B-HAHis in BHK-21

The predicted molecular weights of NS2A-HAHis, NS2B-HAHis, NS4A-HAHis, and NS4B-HAHis are 29, 19.4, 22, and 31.6 kDa, respectively. All the nonstructural proteins except NS2A, can be detected both in the supernatants and the pellets of the cell lysates with the expected size by Western blot (Fig. 3.10). As for NS2A, perhaps its expression level was too low to be detected. It is possible that NS2A possesses certain toxicity to BHK-21.

4.2.2 Expression of pNS2A-HAHis in 293T

The possible reason why no protein was detected in BHK-21 may be because the protein expression level was too low to be detected. To improve the expression level of NS2A, I chose to change the expression host. The human embryonic kidney cell line 293 transfected with the SV40 T antigen (293T) is particularly suited for transient expression assays because it is readily transfected at high efficiency and the SV40 T antigen replicates plasmids containing the SV40 origin (DuBridge *et al.*, 1987; Morskoy and Rich, 2005), which leads to increased copy number of the transfected plasmid. As it turned out, NS2A-HAHis was detected both in the supernatant and the pellet of the 293T cell lysate with the predicted size, 29kDa, by Western blot (Fig. 3.11). Thus, the protein expression level was improved in 293T cells so that NS2A could be detected.

4.3 Plaque assay of pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pcDNA3-D24B-HAHis in BHK-21

The number of plaques in an assay plate indicates the numbers of successful virion infection events (Chiu and Yang, 2003). Thus, reducing number of plaques implies the decreased infectivity. Although cells transfected with pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, or pcDNA3-D24B-HAHis exhibited slightly reducing numbers of plaques compared with cells transfected with pcDNA3 (Table 3.4). However, the differences were not significant. On the other hand, cells expressing NS2B-HAHis formed smaller plaques than cells transfected with vector only (Fig. 3.13). In cells expressing NS4A-HAHis, larger plaques were formed under dengue type 2 virus infection (Fig. 3.13). It has been reported that viruses showed a decrease in replication kinetics in cells forming small plaques and an increase in cells forming large plaques (Henley *et al.*, 2003). In other words, plaque size can reflect replication efficiency. Therefore, this result suggests that the expression of NS2B expressed in BHK-21 cells may decrease replication of dengue virus type 2 while NS4A may increase the replication.

4.4 Expression of the four nonstructural genes in *E. coli*

Under economical consideration, large amount of protein expression is usually proceeded in *E. coli*. So I transformed the four nonstructural genes into NovaBlue (*DE3*), trying to produce the four nonstructural proteins in *E. coli*.

4.4.1 Expression of pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pcDNA3-D24B-HAHis in *E. coli*

The four plasmids, pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pcDNA3-D24B-HAHis, were based on pcDNA3 having a CMV and T7 promoter upstream to the multiple cloning sites. Not ideal for expression of most

soluable proteins in *E. coli*, it can be very useful when low level of functional expression of hydrophobic or toxic proteins is the main purpose (Lewin *et al.*, 2005). These four plasmids were transformed into NovaBlue (*DE3*) separately for expression of the four hydrophobic proteins. But in this study, protein expression was not detected by either Coomassie blue staining or Western blot. The reason may be due to the sequence contents in noncoding region and/or open reading frames of those four nonstructural genes were not suitable for protein expression under the experimental setup (Folley LS and Yarus M, 1989; Gustafsson *et al.*, 2004; Laursen *et al.*, 2005).

Thus, I re-cloned the four nonstructural genes into another vector, pcDNA3(pro), described in the next section, which contains the Shine-Dalgarno (SD) prokaryotic ribosome binding site upstream to the multiple cloning sites.

4.4.2 Construction of pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)-D22B-HAHis, pcDNA3(pro)-D24A-HAHis, and pcDNA3(pro)-D24B-HAHis expression plasmids

Previously, researchers in the laboratory has introduced the prokaryotic ribosome binding site, Shine-Dalgarno sequence (AGGAGG) into 9-14 bases upstream to the translation start codon of a pcDNA3-based plasmid containing a DV E gene fragment with C-terminal HA and His tags (陳逸修, Yang lab unpublished data). This construct is known as the pcDNA3(pro) backbone. The E gene fragment was then replaced by the sequence of DV3 NS4B and named pcDNA3-D34B-HAHis (陳欣悟, Yang lab unpublished data) (Appendix 1). I replaced the DV3 NS4B gene with DV2 NS2A, NS2B, NS4A, and NS4B respectively and obtained the expression clones of the four nonstructural genes

on the pcDNA3(pro) backbone.

4.4.3 Expression of pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)-D22B-HAHis, pcDNA3(pro)-D24A-HAHis, and pcDNA3(pro)-D24B-HAHis in *E.coli* and BHK-21

I tried to express pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)-D22B-HAHis, pcDNA3(pro)-D24A-HAHis, and pcDNA3(pro)-D24B-HAHis in *E.coli* NovaBlue (*DE3*) strain. But only NS4A-HAHis could be detected in the pellet of the cell lysate by Western blot. Thus, the introduction of SD sequence upstream to the start codon seemed to improve the expression of NS4A-HAHis. However, the expressions of the other three nonstructural proteins were not significantly improved.

These four clones were also expressed in BHK-21 cells. Only NS4A-HAHis was detected both in the supernatant and the pellet of the cell lysate by Western blot (Fig. 3.12). The possible reason might be the sequence changes upstream to the start codon affect the translation initiation region, thus resulting in the low expression levels of NS2A, NS2B, and NS4B (Gustafsson *et al.*, 2004).

4.4.4 Construction of pETΔ5T-D22A-HAHis, pETΔ5T-D22B-HAHis, pETΔ5T-D24A-HAHis, and pETΔ5T-D24B-HAHis expression plasmids

Since the expression of the four clones on pcDNA3 was not dramatically improved by the introduction of the SD ribosome-binding site, I decided to change the expression vector again. I chose pET-30a(+) (Appendix 2) as the new expression vector, which contains a His-tag and an S-tag upstream to the

multiple cloning sites. I removed the N-terminal His-tag and S-tag by blunt-ended ligation after *Nde*I-*Nco*I double digestion and Klenow fill-in reaction to obtain a truncated pET-30a(+) vector, named pETΔ5T. But there are no proper restriction sites for direct insertion of the four nonstructural genes along with the C-terminal HA and His tag in the desired direction. Therefore, I cloned the sequences of the NS genes into the *Hind*III and *Xba*I sites on pBluescript II SK(+) (Appendix 3), and than transferred the *Bam*HI and *Not*I fragment from the pBluescript II SK(+)-based plasmid into pETΔ5T (Fig. 3.6).

4.4.5 Expression of pETΔ5T-D22A-HAHis, pETΔ5T-D22B-HAHis, pETΔ5T -D24A-HAHis, and pETΔ5T-D24B-HAHis in *E. coli*

Recombinant proteins of NS2B, NS4A, and NS4B were expressed from pETΔ5T in *E. coli* NovaBlue (*DE3*) strain and confirmed by Western blot (Fig. 3.16). The molecular weights of NS2B, NS4A, and NS4B were in the range of the expected sizes. As for NS2A, it was not detected by Western blot, thus I tried to detect the mRNA of NS2A in NovaBlue(*DE3*) by Northern blot (Fig. 3.17). In addition to the target band (792bp), there was an extra band detected. If the extra band was resulted from degraded mRNA, the target band would be the larger one. Otherwise, if the extra band was a read through product, then the target band would be the smaller one. This datum meant that NS2A was transcribed into mRNA eventhough the protein expression was not detected. This result suggested that NS2A might be degraded soon after being produced or NS2A might be toxic to *E. coli*. Alternatively, since the construct was only assessed by restriction mapping, mutations introduced during the construction of the plasmid may also be a possibility.

Chapter 5. Conclusion

The four small nonstructural proteins of dengue virus type 2 were expressed as HA-His (influenza hemagglutinin-polyhistidine protein) fusion proteins in *E. coli* and mammalian cells. For expression of these proteins in *E. coli* system, the coding regions of NS2A, 2B, 4A and 4B were successfully cloned into a truncated pET-30a(+) vector, pETΔ5T. These constructs were confirmed by restriction digestions and then used for protein expression.

The observation of expressions of NS2B, 4A and 4B in *E. coli* were established by Western blotting analysis. As for NS2A, the expression was not detected by Western blot analysis perhaps due to its toxicity to *E. coli*. However, the transcripts of NS2A were detected by Northern blot analysis. Expressions of NS2B, 4A, and 4B in mammalian cells, BHK-21, were also confirmed by Western technique. Whereas, NS2A was detected in 293T cells but not in BHK-21 cells.

Functional assay of these proteins was attempted with the use of plaque assay. The results suggested that cells expressing NS2B formed smaller plaques than negative control while cells expressing NS4A formed larger plaques.

Reference

1. Beeck, A.O.D., Y. Rouillé, M. Caron, S. Duvet, and J. Dubuisson. 2004. The transmembrane domains of the prM and E proteins of yellow fever virus are endoplasmic reticulum localization signals. J. Virol. 78: 12591-12602.
2. Brinkworth, R.I., D.P. Fairlie, D. Leung, and P.R. Young. 1999. Homology model of the dengue 2 virus NS3 protease: putative interactions with both substrate and NS2B cofactor. J. Gen. Virol. 80: 1167-1177.
3. Brooks, A.J., M. Johansson, A.V. John, Y. Xu, D.A. Jans, and S.G. Vasudevan. 2002. The interdomain region of dengue NS5 protein that binds to the viral helicase NS3 contains independently functional importin beta 1 and importin alpha/beta-recognized nuclear localization signals. J. Biol. Chem. 277 (39): 36399-36407.
4. Cahour, A., B. Falgout, and C.J. Lai. 1992. Cleavage of the dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junctions is mediated by viral protease NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. J. Virol. 66 (3): 1535–1542.
5. Centers for Disease Control and Prevention (CDC, USA). 2005. Website of CDC, USA <http://www.cdc.gov>
6. Chambers, T.J, A. Nestorowicz, S.M. Amberg, and C.M. Rice. 1993. Mutagenesis of the yellow fever virus NS2B protein: effects on proteolytic processing, NS2B-NS3 complex formation, and viral replication. J. Virol. 67 (11): 6797-6807.
7. Chambers, T.J., D.A. Droll, Y. Tang, Y. Liang, V.K. Ganesh, K.H.M. Murthy, and M. Nickells. 2005. Yellow fever virus NS2B–NS3 protease: characterization of charged-to-alanine mutant and revertant viruses and analysis of polyprotein-cleavage activities. J. Gen. Virol. 86: 1403-1413.

8. Chao, D.Y., C.C. Kingi, W.K. Wang, W.J. Chen. 2005. Strategically examining the full-genome of dengue virus type 3 in clinical isolates reveals its mutation spectra. Virol. J. 2: 72-81.
9. Chiu, M.W. and Y.L. Yang. 2003. Blocking the dengue virus 2 infections on BHK-21 cells with purified recombinant dengue virus 2 E protein expressed in *Escherichia coli*. Biochem. Biophys. Res. Commun. 309 (3): 672-678.
10. Clum, S., K.E. Ebner, and R. Padmanabhan. 1997. Cotranslational membrane insertion of the serine proteinase precursor NS2B-NS3(Pro) of dengue virus type 2 is required for efficient *in Vitro* processing and is mediated through the hydrophobic regions of NS2B. J. Biol. Chem. 272 (49): 30715-30723.
11. DuBridge, R.B., P. Tang, H.C. Hsia, P.M. Leong, J.H. Miller, and M.P. Calos. 1987. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol. Cell Biol. 7 (1): 379-387.
12. Folley, L.S. and M. Yarus. 1989. Codon contexts from weakly expressed genes reduce expression *in vivo*. J. Mol. Biol. 209 (3): 359-378.
13. Gubler, D.J. 2001. Human arbovirus infections worldwide. Ann. NY Acad. Sci. 951: 13-24.
14. Gubler, D.J. and G. Kuno. 1997. Dengue and Dengue Hemorrhagic Fever. CAB International, New York, New York.
15. Guha-Sapir, D. and B. Schimmer. 2005. Dengue fever: new paradigms for a changing epidemiology. Emerg. Themes Epidemiol. 2: 1-10
16. Giard, D.J., S.A. Aaronson, G.J. Todaro, P. Arnstein, J.H. Kersey, H. Dosik, and W.P. Parks. 1973. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J. Natl. Cancer Inst. 51 (5): 1417-1423.

17. Gustafsson, C., S. Govindarajan, and J. Minshull. 2004. Codon bias and heterologous protein expression. Trends Biotechnol. 22 (7): 346-353.
18. Henley, K.A., L.R. Manlucu, L.E. Gilmore, J.E. Blaney, Jr., C.T. Hanson, B.R. Murphy, and S.S. Whitehead. 2003. A trade-off replication in mosquito versus mammalian systems conferred by a point mutation in the NS4B protein of dengue virus type 4. Virology 312: 222-232.
19. Kümmerer, B.M. and C.M. Rice. 2002. Mutations in Yellow Fever Virus nonstructural protein NS2A selectively block production of infectious particles. J. Virol. 76 (10): 4773-4784.
20. Laage, R. and D. Langosch. 2001. Strategies for prokaryotic expression of eukaryotic membrane proteins. Traffic 2: 99-104.
21. Laursen, B.S., H.P. Sorensen, K.K. Mortensen, and H.U. Sperling-Petersen. 2005. Initiation of protein synthesis in bacteria. Microbiol. Mol. Biol. Rev. 69 (1): 101-123.
22. Leung, D., K. Schroder, H. White, N.X. Fang, M.J. Stoermer, G. Abbenante, J.L. Martin, P.R. Young, and D.P. Fairlie. 2001. Activity of recombinant dengue 2 virus NS3 protease in the presence of a truncated NS2B co-factor, small peptide substrates, and inhibitors. J. Biol. Chem. 276 (49): 45762–45771.
23. Lewin, A., M. Mayer, J. Chusainow, D. Jacob, and B. Appel. 2005. Viral promoters can initiate expression of toxin genes introduced into *Escherichia coli*. BMC Biotech. 5:19.
24. Lindenbach, B.D. and C.M. Rice. 1999. Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. J. Virol. 73: 4611-4621.
25. Lithwick, G. and H. Margalit. 2003. Hierarchy of sequence-dependent

features associated with prokaryotic translation. Genome Res. 13 (12): 2665-2673.

26. Liu, W.J., P.L. Sedlak, N. Kondratieva, and A.A. Khromykh. 2002. Complementation analysis of the Flavivirus Kunjin NS3 and NS5 proteins defines the minimal regions essential for formation of a replication complex and shows a requirement of NS3 in *cis* for virus assembly. J. Virol. 76 (21): 10766-10775.

27. Liu, W.J., H.B. Chen, X.J. Wang, H. Huang, and A.A. Khromykh**.** 2004. Analysis of adaptive mutations in Kunjin Virus replicon RNA reveals a novel role for the Flavivirus nonstructural protein NS2A in inhibition of beta interferon promoter-driven transcription. J. Virol. 78 (22): 12225-12235.

28. Liu, W.J., X.J. Wang, V.V. Mokhonov, P.Y. Shi, R. Randall, and A.A. Khromykh. 2005. Inhibition of interferon signaling by the New York 99 strain and Kunjin subtype of West Nile Virus involves blockage of STAT1 and STAT2 activation by nonstructural proteins. J. Virol. 79 (3): 1934-1942.

29. Mackenzie, J.M., A.A. Khromykh, M.K. Jones, and E.G. Westaway. 1998. Subcellular localization and some biochemical properties of the Flavivirus Kunjin nonstructural proteins NS2A and NS4A. Virology 245: 203–215.

30. Morskoy, S. S. and B. E. Rich. 2005. Bioactive IL7-diphtheria fusion toxin secreted by mammalian cells. Protein Eng. Des. Sel. 18(1): 25-31.

31. Mukhopadhyay, S., R. J. Kuhn and M. G. Rossmann. 2005. A structural perspective of the flavivirus life cycle. Nat. Rev. Microbiol. 3: 13-22.

32. Muñoz-Jordán, J.L., G.G. Sánchez-Burgos, M. Laurent-Rolle, and A. García-Sastre. 2003. Inhibition of interferon signaling by dengue virus. Proc. Natl. Acad. Sci. 100 (24): 14333-14338.

33. Muñoz-Jordán, J.L., M. Laurent-Rolle, J. Ashour, L. Martínez-Sobrido, M.

Ashok, W.I. Lipkin, and A. García-Sastre. 2005. Inhibition of alpha/beta interferon signaling by the NS4B protein of Flaviviruses. J. Virol. 79 (13): 8004-8013.

34. Niimura, Y., M. Terabei, T. Gojobori, and K. Miura. 2003. Comparative analysis of the base biases at the gene terminal portions in seven eukaryote genomes. Nucleic Acids Res. 31 (17): 5195-5201.

35. Preugschat, F. and J.H. Strauss. 1991. Processing of nonstructural proteins NS4A and NS4B of dengue 2 virus *in vitro* and *in vivo*. Virology 185: 689–697.

36. SAS. 2000. SAS User's Guide: Statistic Version 8.1. SAS Institute, Inc. Cary, NC, USA.

37. Schumann, W. and L. C. S. Ferreira. 2004. Production of recombinant proteins in *Escherichia coli*. Genet. Mol. Biol. 27 (3): 442-453.

38. Shafee, N. and S. AbuBakar. 2003. Dengue virus type 2 NS3 protease and NS2B-NS3 protease precursor induce apoptosis. J. Gen. Virol. 84: 2191-2195.

39. Solomon, T. and M. Mallewa. 2001. Dengue and Other Emerging Flaviviruses. J. Infect. 42: 104–115.

40. Spehr, V., D. Frahm, and T. F. Meyer. 2000. Improvement of the T7 expression system by the use of T7 lysozyme. Gene 257 (2): 259-267.

41. Speight, G. and E.G. Westaway. 1989. Positive identification of NS4A, the last of the last of the hypothetic nonstructural proteins of Flavivirus. Virology 170: 299–310.

42. Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods in Enzymology 185: 60-89.

43. Westaway, E.G., A.A. Khromykh, M.T. Kenney, J.M. Mackenzie, and M.K.

Jones. 1997. Proteins C and NS4B of the Flavivirus Kunjin translocate independently into the nucleus. Virology 234: 31-41.

44. World Health Organization. 2000. Website of World Health Organization <http://www.who.org>.

45. Yusof, R., S. Clum, M. Wetzel, H.M.K. Murthy, and R. Padmanabhan. 2000. Purified NS2B/NS3 serine protease of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids *in Vitro*. J. Biol. Chem. 275 (14): 9963–9969.

46. 徐婕琳. 2003. 表現登革熱病毒2型PL046非結構性蛋白：NS2A、NS2B、NS4A及NS4B在大腸桿菌、哺育類細胞及真菌*Pichia pastoris*表現系統. 交大碩士論文.

47. 賴建孝. 2006. 探討登革熱二型病毒PL046質體建構及報導基因的選擇. 交大碩士論文.