III. MATERIALS AND METHODS

1. Virus and cell lines

Dengue serotype 2-virus strain 16681 isolated from a patient with dengue hemorrhagic fever in Thailand in 1964 was provided by Drs. Bruce Innis and Ananda Nisalak, Department of Virology, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Virus was grown at 29°C in C6/36 mosquito cell line (Igarashi, 1978), which was maintained in Leibovitz's L15 medium (GIBCO BRL, Carlsbad, California, USA) supplemented with 1.5% fetal bovine serum (FBS), 1X (0.26 gm %) tryptose phosphate broth (GIBCO BRL), 1X glutamine-penicillin-streptomycin solution (GIBCO BRL). Virus stock was stored in 20% FBS at –70°C. PS, a fibroblast porcine kidney cell line (Westaway, 1966) was maintained in L15 medium containing 10% FBS, 1X tryptose phosphate broth and 1X glutamine-penicillin-streptomycin solution at 37°C.

2. Antibodies

Monoclonal antibodies specific to flavivirus E protein (4G2) and dengue serotype 2 (3H5) E protein were used in the form of ascitic fluid (Henchal et al., 1982).

3. Plasmids and competent cell

Three recombinant plasmids, which were constructed based on pBluescript II KS (Stratagene, California, USA) and contained different regions of dengue cDNA sequence, were used for the construction of mutant, full-length complementary DNA clone (Figure 1). The subclone plasmid [pBK(S1SP6-1535)Δ402Pst I], contained nucleotides 1-1547 with mutated Pst I site at the base position 402, the 5' half-genome [pBK(S1SP6-4497)Δ402Pst I], contained nucleotide 1-4497 with mutated Pst I site at the base position 402, and the 3' half-genome [pBK(4166-10723)], contained nucleotide 4166-10723 (Sriburi et al., 2001). These plasmids were propagated in *E. coli* strain DH5αF' (GIBCO BRL) using Luria broth medium (Sigma, St. Louis, Missouri, USA) containing 25μg/ml ampicillin (GIBCO BRL) at 25°C and purified by

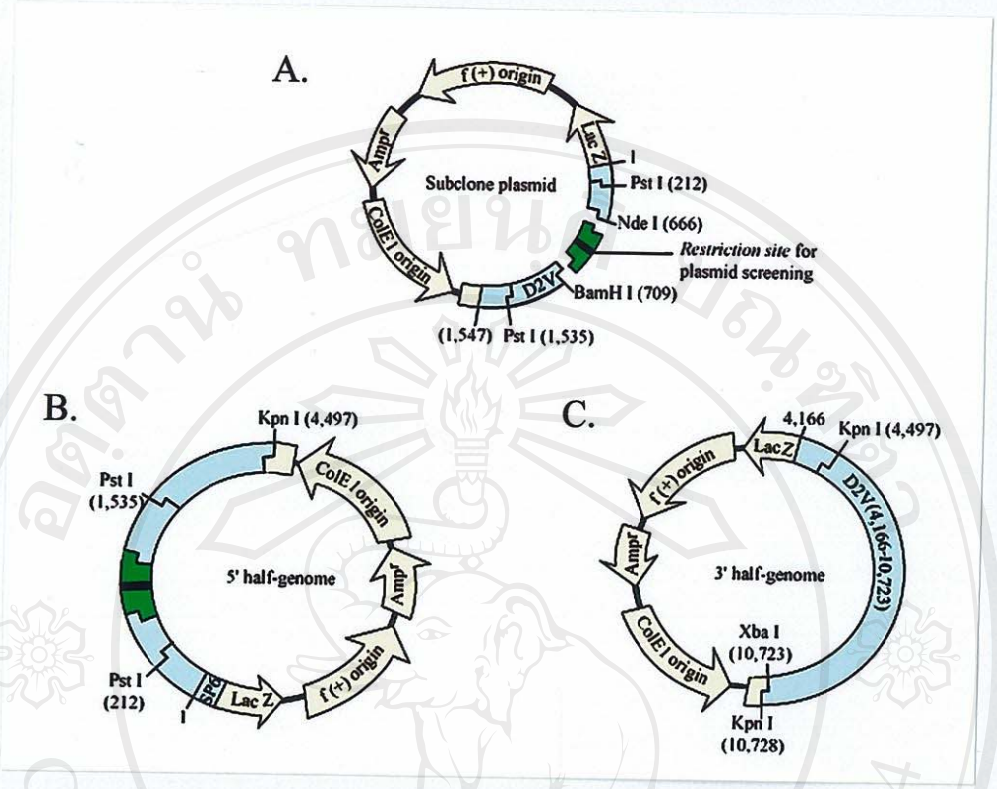

Figure 1. Three recombinant plasmids for the generation of prM mutant dengue viruses. Designated pBluescript II KS-based plasmids contained the following dengue cDNA sequences: A, the subclone plasmid [pBK(S1SP6-1535)Δ402Pst I], nucleotide 1-1547 with mutated Pst I site at the base position 402; B, the 5' half-genome [pBK (S1SP6-4497)Δ402Pst I], nucleotide 1-4497 with mutated Pst I site at the base position 402; and C, the 3' half-genome [pBK(4166-10723)], nucleotide 4166-10723 (Sriburi et al., 2001).

using QIAGEN plasmid mini kit or midi kit (QIAGEN, Hilden, Germany). In the preparation of competent cell for transformation, DH5αF' was expanded in 100 ml Luria broth medium by shaking at 200 rpm for 2-3 hours at 37°C and harvested by centrifugation at 5,000 rpm at 4°C for 10 minutes. The bacterial cells were resuspended gently with ice-cold 0.1 M $CaCl_2$ (20 ml), incubated on ice for 30 minutes, pelleted by centrifugation, and resuspended in 4 ml of 0.1 M $CaCl_2$. For

storage, $CaCl_2$-treated cells were adjusted to 20% glycerol and 400 μl aliquots were frozen in dry ice-ethanol bath and stored at -70°C. The transformation efficiency of the frozen competent cells, which was obtained by testing with pUC19 plasmid (Promega, Madison, Wisconsin, USA), was about 3×10^5 colonies forming unit per μg of plasmid DNA.

4. Mutagenesis of dengue pr-M junction cDNA clones

4.1. Mutagenesis of the subclone plasmid

To study the effect of changes of charged amino acid content of the prM cleavage junction on the replication of dengue virus, mutations were introduced into the pr-M junction sequence within the subclone plasmid. Previous results from the study of JEVpr/16681 chimeric virus, which contained seven positive charges and lacked the negative charge within the 13-amino acid region proximal to the pr-M junction, revealed that these changes in the charge content resulted in the reduction of virus replication as compared with the parent dengue virus, which contained only four positive charges together with two negative charges in the same region. It was not yet known whether the reduction of virus replication was due solely to the increase of positively charged amino acids, or the absence of the negatively charged amino acids, or both. To determine the effect of each of the first two possible changes of the 13-amino acid region proximal to the prM cleavage junction of JEVpr/16681 on viral replication, we designed the two mutagenic oligonucleotides, 16681pr(+7,-2), 16681pr(+4,-0) for the mutagenesis of pr-M junction (Table 2). The 16681pr(+7,-2) oligonucleotide resulted in an increase of three positive charges whereas the 16681pr (+4,-0) oligonucleotide caused the absence of the two negative charges. Additional oligonucleotide, 16681pr(+7,-0), was designed to determine the effect of non-charged residues of the 13-amino acid region proximal to the pr-M junction of JEVpr/16681 on virus replication. Also, the oligonucleotide, 16681pr(+9,-0), was designed to evaluate the effect of further increasing the positive charges (to 9 positive charges) of the pr-M junction on virus replication. The oligonucleotides were designed 5' and 3' compatible end with Nde I and BamH I site and synthesized by the Bio-Service Unit, National Center for Genetic Engineering and Biotechnology, Bangkok.

Table 2. Oligonucleotides for site-directed mutagenesis and mutant plasmid construction.

Amino acid Position / Cleavage Position / Designation		14	13	12	195 11	10	9	8	200 7	6	5	4	3	2	205↓ 1	-1	
		Nucleotide sequence															
16681pr(+7, -2)	amino acid residues	Tyr	Gly	Arg	Cys	Thr	Arg	Met	Arg	Glu	His	Arg	Arg	Glu	Lys	Arg	Ser	
	Coding 5'	TAT	GGG	**AGG**	TGT	ACC	**CGC**	ATG	**CGA**	GAG	CAT	**AGA**	**AGA**	GAA	**AAA**	**A**		
	Non-coding 3'		A	CCC	**TCC**	ACA	TGG	**GCG**	TAC	**GCT**	CTC	GTA	**TCT**	**TCT**	CTT	**TTT**	**TCT**	AG 5'
16681pr(+4, -0)	amino acid residues	Tyr	Gly	Thr	Cys	Thr	Thr	Met	Gly	His	His	Arg	Arg	Ser	Lys	Arg	Ser	
	Coding 5'	TAT	GGG	ACG	TGT	ACC	ACA	ATG	GGA	**CAT**	CAT	**AGA**	**CGT**	**TCG**	**AAA**	**A**		3'
	Non-coding 3'		A	CCC	TGC	ACA	TGG	TGT	TAC	CCT	**GTA**	GTA	**TCT**	**GCA**	**AGC**	**TTT**	**TCT**	AG 5'
16681pr(+7, -0)	amino acid residues	Tyr	Gly	Arg	Cys	Thr	Arg	Met	Arg	His	His	Arg	Arg	Ser	Lys	Arg	Ser	
	Coding 5'	TAT	GGG	**AGA**	TGT	ACA	**CGA**	ATG	**CGA**	**CAT**	CAT	**AGA**	**AGA**	TCA	**AAA**	**A**		3'
	Non-coding 3'		A	CCC	**TCT**	ACA	TGT	**GCT**	TAC	**GCT**	**GTA**	GTA	**TCT**	**TCT**	AGT	**TTT**	**TCT**	AG 5'
16681pr(+9, -0)	amino acid residues	Tyr	Gly	Arg	Cys	Arg	Arg	Ser	Arg	Arg	Ser	Arg	Arg	Ser	Lys	Arg	Ser	
	Coding 5'	TAT	GGG	**AGA**	TGT	**AGA**	**CGC**	TCA	**CGA**	**CGC**	TCG	**AGA**	**AGA**	**TCA**	**AAA**	**A**		3'
	Non-coding 3'		A	CCC	**TCT**	ACA	**TCT**	**GCG**	AGT	**GCT**	**GCG**	AGC	**TCT**	**TCT**	**AGT**	**TTT**	**TCT**	AG 5'

Note. Bolded letters encoded amino acid residues of designed prM oligonucleotides indicate specific mutations introduced into the pr-M junction of dengue virus genome. Underlined sequences indicate engineered restriction enzyme sites of Sph I for 16681pr(+7, -2), Sfu I for 16681pr(+4, -0), BsrG I for 16681pr(+7, -0) and Xho I for 16681pr(+9, -0), respectively. ↓, furin cleavage site.

To aid in the insertion of mutagenic oligonucleotides into the subclone plasmid, the plasmid pBK(S1SP6-1547)Δ402Pst I was previously modified by introducing two restriction enzyme recognition sites for Nde I and BamH I at the base positions 666 and 709, respectively (Figure 2) (Sriburi et al., 2001; Keelapang et al., 2003). The Nde I- and BamH I-added subclone plasmid was prepared for ligation with oligonucleotides by digesting to completion with BamH I and Nde I, and separating from a small BamH I-Nde I fragment by agarose gel electrophoresis. The large fragment was extracted from agarose gel using the gel extraction kit (QIAGEN).

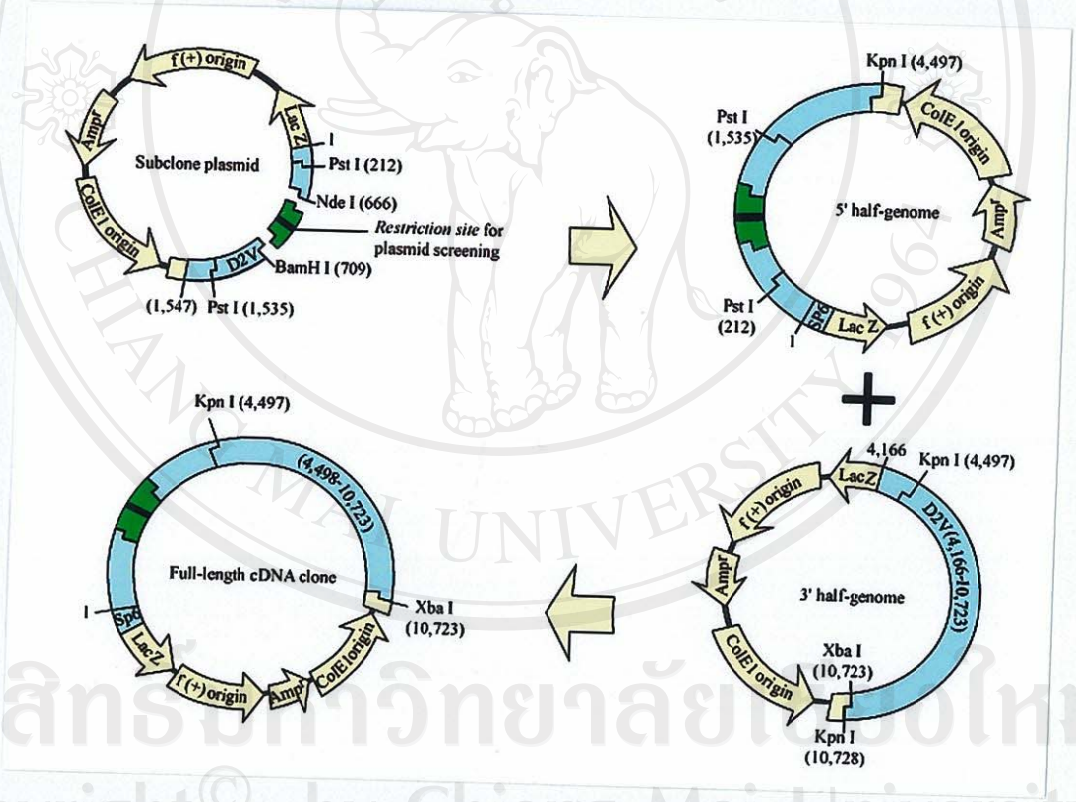

Figure 2. Steps involved in the construction of dengue full-length cDNA plasmids with introduced mutations within the 13-amino acid region proximal to the pr-M junction.

Prior to ligation, the oligonucleotides were phosphorylated at the 5' end by using T4 polynucleotide kinase (GIBCO BRL) in a reaction buffer containing 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 100 mM ATP, and 100 pmol of each oligonucleotide. Following 30 minutes of incubation at 37°C, the reaction was stopped by heat inactivation at 65°C for 2 minutes. The corresponding pair of oligonucleotides was then annealed by mixing the phosphorylated oligonucleotides together and incubating at 37°C for 30 minutes. For the ligation of the annealed oligonucleotide to the Nde I- and BamH I-digested subclone plasmid, the molar ratio of 3:1 oligonucleotide to the subclone plasmid was used. The ligation mixture consisted of 250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothreitol, 25% (w/v) polyethylene glycol 8000, 0.03 pmol (97.6 ng) of Nde I- and BamH I-digested plasmid vector, 0.1 pmol of annealed double-stranded oligonucleotide and 2 units of T4 DNA ligase in the total volume of 10 μl. The mixture was incubated at 14°C overnight and then used to transform into DH5αF' frozen competent cells. One hundred μl of $CaCl_2$-treated competent cell were gently added with 10 μl of ligation mixture and incubated on ice for 30 minutes prior to swirling at 42°C in a water bath for 60 seconds. Then, 900 μl of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) were added into the transformation mixture. The transformation mixture was shaken at 220 rpm for 90 minutes in a 37°C shaking water bath to allow the transformant bacteria to express the ampicillin resistance gene, which was encoded by the plasmid. The transformation mixture was spread onto Luria agar plate containing 25 μg/ml ampicillin and was then incubated at 37°C overnight. Isolated bacterial transformant colonies were picked for an expansion in 5 ml of Luria broth containing 25 μg/ml ampicillin. The subclone plasmid was separated from this bacterial transformant by using plasmid mini/midi kit and then the plasmid was screened the oligonucleotide insertion by digesting with restriction endonuclease, the specific recognition site of which was introduced within the oligonucleotide before expanding the large scale for preparation of the intended mutation of subclone plasmid.

4.2. Mini- and midi-preparations of plasmid from cultured *E. coli*

Isolated transformant colonies were picked and expanded in Luria broth (20/100 ml) containing 25 μg/ml ampicillin by shaking at 220 rpm, 37°C overnight. Bacterial cells were harvested by centrifugation at 3,000 rpm for 10 minutes at 4°C. Bacterial pellet was resuspended in 0.3/3 ml of suspension buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 μg/ml of RNAse A]. One volume of lysis buffer [200 mM NaOH, 1% SDS], was added and mixed by inverting gently 4-6 times at room temperature. Following a 5-minute of incubation, one volume of ice-cold neutralizing buffer [3.0 M potassium acetate, (pH 5.0)], was added. Bacterial lysate was mixed again by inversion and further incubated on ice for 10/20 minutes before centrifugation at 14,000 rpm, 4°C for 30 minutes. The supernatant containing plasmid DNA was then applied onto the QIAGEN-tip 20/100 column, which was equilibrated with 750 mM NaCl, 50 mM MOPS (pH 7.0), 15% isopropanol, 0.15% Triton X-100. The mini/midi column was allowed to empty by gravity flow and then was washed with 4/20 ml of washing buffer [1.0 M NaCl, 50 mM MOPS (pH 7.0), 15% isopropanol]. The plasmid DNA was eluted from column with 0.8/5 ml of elution buffer [1.25 M NaCl, 50mM Tris-HCl (pH 8.5), and 15% isopropanol] and precipitated by adding 0.7 volume of isopropanol and spinning at 14,000 rpm for 30 minutes at 4°C. Following one wash with 500 μl of 70% ethanol, plasmid DNA pellet was dried at room temperature or under vacuum and dissolved with 10 mM Tris-HCl (pH 8.0). The purified plasmid DNA was analyzed by agarose gel electrophoresis and quantitated by using a UV/VIS spectrophotometer at the wavelength 260 nm.

4.3. Screening of the subclone plasmid

To aid in the screening of the subclone plasmid for the insertion of oligonucleotide, restriction endonuclease recognition site was introduced in each of the mutagenic oligonucleotides without affecting the intended amino acid sequence. Based on these introduced restriction sites, the four subclone plasmids, 16681pr (+7,-2), 16681pr(+4,-0), 16681pr(+7,-0) and 16681pr(+9,-0), were screened for the presence of intended oligonucleotide by digesting with Sph I, Sfu I, BsrG I and Xho I, respectively. Following the analysis of the digested product by 0.7% agarose gel electrophoresis, a 4.5 kb DNA band was expected for 16681pr(+4,-0), 16681pr(+7,-0)

and 16681pr(+9,-0) mutant plasmids, and two bands (0.6 and 3.9 kb in size) were expected for the 16681pr(+7,-2) subclone plasmid.

4.4. Preparation of 1.3 kb Pst I fragment for the construction of mutant 5' half-genome cDNA clones

To prepare the 1.3 kb Pst I fragment (nt 212-1535) containing the inserted mutagenic oligonucleotide from the subclone plasmid for subsequent construction of the mutant 5' half-genome (Figure 2), the subclone plasmid with confirmed oligonucleotide insertion was digested with Pst I. Following complete Pst I digestion, the digested product was electrophoresed in 0.7% agarose gel and the 1.3 kb Pst I fragment was excised from the agarose gel and weighed. For extracting the DNA fragment from agarose gel, three volumes of solubilization and binding buffer mixture were added to 1 volume of gel slice (100 mg ~100 μl) and the tube was incubated at 50°C for 10 minutes or until the gel had completely dissolved. Solubilized gel mixture was applied into QIAquick spin column and centrifuged at 10,000 rpm for 1 minute to allow DNA to bind onto the silica membrane inside the column. The spin column was washed with 750 μl of PE washing buffer and centrifuged for 1 minute at room temperature. To eliminate the remaining PE buffer, the column was centrifuged again at maximum speed for 1 minute. To elute the 1.3 kb DNA fragment, 30 μl of 1 mM Tris-HCl (pH 8.5) were dropped onto the center of membrane for 2 minutes before centrifugation at maximum speed for 1 minute. The eluted DNA band was visualized by agarose gel electrophoresis and quantitated by UV absorption at 260 nm.

4.5. Construction and characterization of mutant 5' half-genome plasmids

To construct the 5' half-genome mutant plasmid containing the purified mutagenic Pst I fragment derived from the subclone plasmid, a 2:1 molar ratio of the purified 1.3 kb Pst I fragment (24.59 ng) and the 6.1 kb dephosphorylated Pst I-digested 5' half-genome (100 ng) was employed in the ligation reaction using T4 DNA ligase. The dephosphorylated Pst I-digested 5' half-genome vector was prepared by digesting with Pst I to completion and by dephosphorylating the 5' terminus using calf intestine alkaline phosphatase (Promega) at 37°C for 1 hour. The large fragment of dephosphorylated Pst I-digested 5' half-genome was separated by 0.7% agarose gel

electrophoresis and then was extracted from the gel by using gel extraction kit. The purified Pst I-digested 5' half-genome vector was visualized by agarose gel electrophoresis and quantitated by UV absorption at 260 nm. Following ligation and transformation, bacterial transformant colonies were selected with 25 μg/ml ampicillin.

To characterize the mutant 5' half-genome plasmid contained the mutagenic oligonucleotide, the 5' half-genome plasmid was first doubly digested with Sph I and EcoR I and separated by agarose gel electrophoresis. Three expected DNA fragments of 0.5, 0.9 and 5.9 kb in size indicated the correct orientation of the 1.3 kb Pst I fragment whereas the other set of three fragments, 0.5, 1.9 and 4.9 kb in size, revealed an incorrect orientation. Subsequently, each of the mutant 5' half-genome was appropriately digested with Sph I, Sfu I, BsrG I and Xho I to again confirm the presence of intended mutations. Also, the 5' half-genome plasmid were digested with additional restriction enzymes, Hind III, Pst I, Kpn I, Nco I, and EcoRI, to make sure that these recognition sites were still present.

5. Construction of full-length cDNA clones containing mutations of the pr-M junction

Mutant, full-length cDNA clone was constructed by ligating the 6.2 kb Kpn I-digested fragment encoding the nucleotide sequences 4,497-10,723 (94.5 ng) from the 3' half-genome plasmid into the Kpn I site at the nucleotide position 4,497 of the mutant 5' half-genome (50 ng) using the 2:1 molar ratio. To minimize the possible deletion of viral sequence from plasmid during the culture of *E. coli*, transformed *E. coli* cells were incubated at 20-25°C. After three days of incubation at this low temperature, distinctively large and small bacterial colonies were observed. A number of transformant colonies were grown in 5-ml Luria broth in the presence of 25μg/ml ampicillin. Plasmid DNA was prepared and analyzed by Hind III and Xba I digestion and agarose gel electrophoresis. From previous findings, none of the large transformant colonies retained full-length cDNA sequence whereas most of the small colonies did (Sriburi et al., 2001). Therefore, only small transformant colonies were picked for expansion in 5 ml Luria broth containing 25μg/ml ampicillin at 20-25°C. The plasmid DNA was then isolated using QIAGEN plasmid mini kit (QIAGEN) and

the direction of inserted 3' half-genome fragment within the full-length cDNA clone was determined by Hind III and Xba I digestion and agarose gel electrophoresis. In addition, the presence of intended mutations in each of the other four mutant, full-length plasmid cDNA clones was determined by digesting with Sfu I, Sph I, BsrG I, and Xho I restriction enzymes followed by agarose gel electrophoresis. Two *E. coli* colonies containing the full-length cDNA clone with the intended mutation and the correct orientation of the Kpn I-digested 3' half-genome were then expanded in the larger scale (100 ml) in Luria broth containing 25μg/ml ampicillin at 20-25°C. Then, the full-length plasmid was again isolated and digested with additional restriction enzymes, Pst I, Kpn I, Nco I, and EcoRI, to make sure that these recognition sites were still present.

6. *In vitro* transcription and transfection of full-length RNA into C6/36 cells

To prepare plasmid DNA template for *in vitro* transcription, the full-length cDNA clone was digested at the 3' end with Xba I and purified with DNA mini kit (QIAGEN). *In vitro* transcription was performed in a 20 μl reaction containing 1 μg linearized cDNA, 80 mM Hepes-KOH (pH 7.5), 32 mM $MgCl_2$, 2 mM spermidine, 40 mM dithiothreitol, 5 mM each of ATP, CTP, and UTP, 1.5 mM GTP, 3.5 mM m^7G(5')ppp(5')G cap analog (New England Biolabs, Beverly, Massachusetts, USA) and 20-40 units SP6 RNA polymerase-pyrophosphatase mix (SP6 Ribomax kit, Promega). Following an incubation at 30°C for 4-6 hours, the reaction mixture was treated with RNAse-free DNAse and purified using the RNAeasy mini kit (QIAGEN) before transfection.

The yield and approximate size of the *in vitro* transcription products were analyzed by adding 8 μl of RNA sample buffer [62.5% deionized formamide (v/v), 1.14 M formaldehyde, 200 μg/ml bromophenol blue, 200 μg/ml xylene cyanol, 1.25X MOPS-EDTA-sodium acetate buffer (0.25 M MOPS, pH 7.0; 100 mM sodium acetate; 12.5 mM EDTA, pH 8.0)] to 2 μl of the *in vitro* transcript, and subject to heating (65°C) for 10 minutes and electrophoresis in 0.7% agarose gel containing 3.7% formaldehyde in 1X MOPS-EDTA-sodium acetate running buffer (0.2 M MOPS, pH 7.0; 80 mM sodium acetate; 10 mM EDTA, pH 8.0). The gel was stained

with 10 μg/ml ethidium bromide for 30 seconds, destained extensively with water, and visualized with an ultraviolet light source.

To purify the full-length *in vitro* transcripts, 20 μl of the *in vitro* transcripts were adjusted with RNAse- and DNAse-free water to 100 μl and then transferred into 350 μl of RLT buffer (components unknown) containing 1% (v/v) β-mercaptoethanol. Following thorough mixing, 250 μl of absolute ethanol were added and mixed gently by pipetting. The mixture was then applied into the spin column and the RNA transcripts were allowed to bind to the membrane by centrifuging at 8,000 rpm for 15 seconds. Non-specific and unbound contents were removed from the membrane by washing twice with 500 μl of RPE buffer (components unknown) containing 70% ethanol by centrifuging at 8,000 rpm for 15 seconds and 2 minutes. The column was then centrifuged at maximum speed (14,000 rpm) for 2 minutes to eliminate the remaining solution. The bound RNA was eluted by adding 30 μl of RNAse-free water to the center of the membrane, followed by incubation at room temperature for 5 minutes and spinning at 8,000 rpm for 1 minute. The yield and approximate size of the purified *in vitro* products were analyzed by electrophoresis as mentioned above. To quantitate the full-length *in vitro* transcripts, destained gel was photographed with the gel documentation system (BioRad) and the proportion of full-length RNA band against total RNA was determined digitally with the Molecular Analyst software (BioRad). The total RNA concentration of the purified in vitro transcripts was determined by UV absorption at the wavelength of 260 nm. From the total RNA concentration and the proportion of the full-length RNA transcript in the purified *in vitro* products, the quantity of the full-length RNA transcript was determined from: full-length RNA concentration = total RNA concentration × proportion of full-length RNA transcript in total *in vitro* transcript.

Transfection of C6/36 cells was carried out by mixing 1 μg full-length *in vitro* transcription product with 5 μl lipofectin (GIBCO BRL) in 1 ml L15 medium (without fetal bovine serum) before adding onto a twice-washed confluent cell monolayer in 35 mm^2 plastic dish. Following a 4-hour incubation at room temperature, RNA-lipofectin mixture was removed and 3 ml of the L15 maintenance media containing 1.5% fetal bovine serum were added. Transfected cell monolayer was returned to 29° C for the generation of progeny virus particles. To harvest the virus released from

transfected cells into the culture supernatant, 1.3 ml of the culture supernatant were collected on day 0, 1, 3, 5, 7, 9, 11 and 14 after transfection, spun briefly to pellet the cells, and adjusted to 20% fetal bovine serum. The virus suspension was stored in two aliquots of 500 μl /tube and three small aliquots (200 μl /tube) at -70°C. In addition, two aliquots of 140 μl/tube were stored without adding of fetal bovine serum for the preparation of viral genomic RNA. For further virus characterization, a large viral stock was prepared by expanding once in C6/36 cells using a T75 culture flask.

7. Detection of virus replication

7.1. Virus titration by focus immunoassay

Quantitation of virus in supernatant of transfected cells was performed by the 2-step focus immunoassay. Culture supernatant of transfected C6/36 cells was serially diluted (ten-fold dilution series) with L15 medium containing 3.0% fetal bovine serum. From each dilution, 50 μl were transferred, in duplicate, onto each well of the confluent PS cell monolayer in 96 well plate (with 25 μl media remaining in each well). Following a 2-hour incubation at 37°C, 125 μl of the overlayer L15 medium containing 3 % fetal bovine serum and 1.5% carboxymethylcellulose (Sigma) was added and then the plate content was mixed gently by tapping. The cell monolayer was returned to incubation at 37°C in moist chamber. After 3 days of incubation, the overlayer was removed from each well and the monolayer was washed gently 5 times with PBS (200 μl). The infected PS cell monolayer was then fixed with 3.7% formaldehyde in PBS (100 μl/well) for 10 minutes and washing with PBS (200 μl). Following the fixation of infected PS cells, the cells were permeabilized by adding of 2% Triton-X 100 in PBS (100 μl/well) for 10 minutes and washing with PBS (200 μl) for 5 times at room temperature. Fixed and permeabilized PS cell monolayer was reacted with an anti-flavivirus E protein (4G2) monoclonal antibody at the dilution of 1:750 in PBS (50 μl) at 37°C for 1 hour. Unreacted 4G2 was removed by washing 5 times with PBS (200 μl) and then cells were incubated with rabbit anti-mouse IgG conjugated with alkaline phosphatase enzyme at the dilution of 1:1,500 in PBS 0.05% Tween-20, 2% fetal bovine serum (PBSTF, 50 μl) at 37°C for 1 hour. After incubation, the cells were again washed with PBS (200 μl) to eliminate unbound

conjugated antibody and then reacted with NBT-BCIP substrate-chromogen mixture (0.335 mg/ml of NBT, 0.165 mg/ml of BCIP) in AP buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 0.005 M $MgCl_2$) in the dark at room temperature and the reaction was stopped by washing with distilled water. Clusters of infected cells (foci), with purple deposit in the cytoplasm, were directly counted under a light microscope. Depending on the concentration of virus, only a certain number of selected wells which displayed isolated clusters of infected cells were counted and the total counts were pooled for each viral sample. The absolute virus titer [in focus forming unit (FFU) per ml] was then calculated from: virus titer = (1/the first dilution of virus suspension that resulted in countable wells) × the total number of cluster counted in all countable wells/total volume of virus suspension inoculated in all countable wells (relative to the first countable wells).

7.2. Determination of focus size by 4-step focus immunoassay

Viruses generated from transfected C6/36 cells were expanded once in C6/36 cell monolayer ($T75cm^2$ flask) and stored in small aliquots at -70°C. To determine the number of infected cell in each focus (focus size), approximately fifty FFU per 50 μl of L15 medium containing 3% fetal bovine serum were added onto the PS monolayer in the 96-well culture plate and incubated at 37°C. Following 2 hours of incubation, 125 μl/well of 1.5% carboxymethylcellulose in L15 medium-3% fetal bovine serum were overlayered on the infected cell monolayer and the plate returned to incubation at 37°C for exactly 72 hours. The overlayer was removed and the monolayer was washed gently with PBS (200 μl/well) for at least 5 times or until the overlayer was completely removed. The infected cell monolayer was then fixed with 3.7% formaldehyde in PBS (100 μl/well) for 10 minutes and subsequently permeabilized with 2% Triton X-100 in PBS (100 μl/well) for 10 minutes before washing with PBS (200 μl/well) for 5 times at room temperature.

To visualize the infected cells, cell monolayer was first reacted with 4G2, an anti-flavivirus E protein monoclonal antibody, at the dilution of 1:750 in PBS (50 μl /well) at 37°C. After an hour of incubation, unreacted antibody was removed by washing with PBS (200 μl/well) for 5 times. In the second step, the cells were reacted with rabbit anti-mouse IgG (Cappel, Organon Teknika N.V., Turnhout, Belgium) at

the dilution of 1:500 in PBS, 0.05% Tween-20, 2% fetal bovine serum (PBSTF, 50 μ l/well) at 37°C for 1 hour. Unbound antibodies were again removed by washing with PBS for 5 times and the plate were then were incubated with 1:500 dilutions of goat anti-rabbit IgG (Cappel) in PBSTF (50 μl/well) at 37°C for 1 hour. The infected cells were washed with PBS for 5 times and the monolayer was reacted with peroxidase-rabbit anti-peroxidase complex (Dako Cytomation, Glostrup, Denmark) at the dilution of 1:500 in PBSTF (50 μl/well) at 37°C for 1 hour. Finally, the unbound peroxidase-rabbit anti-peroxidase complex was removed by 5 washes of PBS before adding the substrate-chromogen mixture [0.05 mg/ml of 3,3' diaminobenzidine (Sigma) and 0.06% (v/v) of 6% H_2O_2 in PBS] in the dark at room temperature for 15-30 minutes. Dengue-infected cells were readily observed by light microscope as cells with dark-brown deposit in the cytoplasm surrounding clear nuclear area. The number of infected cell in each focus (focus size) was assessed by photographing at least 23 foci under light microscope and followed by manual counting. To compare the change in the focus size of the prM mutant viruses against that of the parent 16681 virus, the reduction in focus size (in percent) was calculated from: [1- (the mean focus size of each prM mutant virus / the mean focus size of 16681)] $\times$ 100.

7.3. Extraction of viral genomic RNA and amplification of the prM gene by RT-PCR

Viral RNA extraction was performed by using the QIAamp viral RNA mini spin kits (QIAGEN). The viral sample (140 μl) was lysed with 4.0 M guanidine-isothiocyanate in AVL lysis buffer (560 μl) by pulse-vortexing and added with 560 μl of ethanol for 15 seconds and incubated at room temperature for 10 minutes to inactivate RNAse and to ensure the isolation of intact viral RNA. Carrier RNA was added to buffer AVL to improve the binding of viral RNA to the membrane and limits possible degradation of the viral RNA due to any residual RNAse activity. Viral RNA in AVL buffer was transferred to the column and allowed to adsorb onto the silica-gel membrane by two brief-centrifugation at 8,000 rpm for 1 minute. Viral genomic RNA bound to the membrane was washed with 500 μl of 70% ethanol washer buffer 1 and 2 (AW1 and AW2 buffer) during two centrifugation steps at 8,000 rpm for1 minute and 14,000 rpm for 3 minutes. To elute viral genomic RNA, 30 μl of RNAse-free

water containing 0.04% sodium azide were applied onto the center of membrane within the spin column and incubated at room temperature for 5 minutes. Following incubation, the viral RNA was eluted by centrifugation at 8,000 rpm for 1 minute. The elution was repeated two more times and the eluted materials were pooled, aliquoted and stored at -70°C. An aliquot of 10 μl of viral genomic RNA was used in the reverse transcription reaction at 56°C for 1 hour in the reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 10 mM dithiothrietol, 0.5 mM each of four dNTPs, 100 pmol of anti-sense primer (C2504) and 20 units of Superscript II RNAse H $^{(-)}$ reverse transcriptase enzyme (GIBCO BRL). The reverse transcription reaction was stopped by heating at 94°C for 10 minutes.

The complementary DNA from the reverse transcription reaction was amplified in a 100-μl volume of mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 0.2 mM each of four dNTPs, 100 pmol of sense primer (D2J134) and 2.5 units of Taq DNA polymerase (Promega). As the template of the PCR reaction, 5 μl of cDNA product of the reverse transcription reaction was employed. After overlaying the reaction mixture with 40 μl of mineral oil, DNA amplification was carried out in the thermal cycler (Perkin Elmer, Foster City, California, USA). For the primer pair, D_2J134 and C2504 (Table 3), the cycling conditions was: denaturation at 94°C for 1 minute, annealing at 65°C for 1 minute, and extension at 72°C for 10 minutes. The last extension step was extended to 20 minutes. Following 30 cycles of amplification, the size and relative quantity of the PCR product were determined by agarose gel electrophoresis. With this primer pair, a 2.3 kb DNA band was expected after staining the agarose gel with ethidium bromide and observing under the ultraviolet light source.

To make sure that there was no DNA (plasmid or PCR product) contamination during the RNA extraction step, a PCR tube was set up with 10 μl of viral genomic RNA without the previous reverse transcriptase step. In separate tubes, a known concentration of DNA template was used as the PCR positive control, whereas the negative control of PCR contained only PCR reagent without added DNA template.

Table 3. Specific primers of reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequencing

Designation	Sequence (5' to 3')	Recognition Position
S D_2J-134[AB]	TCAATATGCTGAAACGAGAGAGAAACCG	134-162
C 2504[A]	GGGGATTCTGGTTGGAACTTATATTGTTCTGTCC	2504-2471
C 1518[B]	CATCTGCAGCAACACCATCTC	1539-1519
C 1204*	GGAGTGTTTGCAGACGAA	1204-1186
S 350*	GGAAAGAGATTGGAAGGATGA	350-371

A, primer pair for reverse transcriptase-polymerase chain reaction of Lewis et al. (1993). B, primer pair for semi-nested polymerase chain reaction. *, primer pair for nucleotide sequencing. C, reverse direction. S, forward direction.

When relatively low level of the RT-PCR produce was observed by agarose gel electrophoresis, the product was amplified again in the semi-nested PCR by using the primer pair D2J134 and C1518 (Table 3). The cycling parameter was: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 3 minutes. Following 30 cycles, 10 μl of the semi-nested PCR product were analyzed by agarose gel electrophoresis. A 1.3 kb DNA band was expected after ethidium bromide staining and visualization with an ultraviolet light source. The template of the negative control tube for the semi-nested PCR was the product of the RT-PCR in which the reverse transcriptase was omitted during the reverse transcription step.

The PCR product was purified by using the QIAquick purification kits (QIAGEN). One volume of the PCR product was mixed gently with 5 volumes of the binding buffer, applied onto the column, and centrifuged at 13,000 rpm for 30-60 seconds. The column was washed with 750 μl of 70% ethanol-washing buffer by centrifugation at 13,000 rpm for 1 minute. To elute the PCR product from the

column, 30 μl of the elution buffer (10 mM Tris-HCl, pH 8.5) were applied and followed by centrifugation at 13,000 rpm for 1 minute. Elution was repeated for two more times. The quantity of the DNA in the eluted materials was determined by spectrophotometer using the wavelength of 260 nm.

7.4. Nucleotide sequence analysis

The whole prM gene was analyzed by using two oligonucleotide primers, S350 and C1204 (Table 3), for the sense strand and anti-sense strand, respectively. Following the purification of RT-PCR product, 500-600 ng of DNA template was gently mixed with 4 μl of the Big Dye reaction mix (Applied Biosystem, Foster City, California, USA) and 3.2 pmol of each of the sequencing primers in the total volume of 20 μl. The mixture was subject to amplification in a DNA thermal cycler for 25 cycles with the following parameters: denaturing at 94°C for 30 seconds, annealing at 50°C for 15 seconds and extension at 60°C for 4 minutes. The sequencing product was added with 50 μl of 0.3 M sodium acetate in absolute ethanol, incubated on ice for 1 hour, and then centrifuged at 14,000-rpm for 30 minutes at 4°C to precipitate the sequencing products. The pellet was washed with 500 μl of 70% ethanol, dried in room air or under vacuum, and dissolved in 20 μl of template suspension reagent. The tube was shaken by pulse-vortexing for 5 minutes or more until the pellet was completely resuspended. The sequencing product was then analyzed by an automated DNA sequencer (ABI PRISM 310 genetic analyzer, Applied Biosystem).

8. Determination of virus replication kinetics

Two cell lines, C6/36 and PS, were used to study the kinetics of viral replication. The multiplicity of infection of 0.001 and 0.01 were used to infect C6/36 cell and PS cell, respectively. The two-day-old monolayers of C6/36 cells and PS cells grown in T 25cm^2 tissue culture flask were disaggregated and the single cell suspension was counted in a hemacytometer counting chamber to determine the total number of cells in the flask. From the number of cells in the T-25 flask and the virus titer of the virus stock, the amount of virus needed to infect cells at the multiplicity of infection of 0.001 and 0.01 was calculated and prepared using L15 containing 3% fetal bovine serum as the diluent. To infect the monolayer with dengue virus, the

culture medium in each T25cm^2 tissue culture flask was removed by pipetting and replaced with 1.5 ml of the diluted virus suspension. Viruses were allowed to adsorbed to C6/36 monolayer by gentle shaking at room temperature for 16-18 hours. Virus adsorption to PS cell monolayer was performed at 37°C for 2 hours. After infection, free viruses were removed by washing for five times with serum-free L15 medium before replacing with 5 ml of L15 medium containing 1.5% (C6/36 cells) or 3%(PS cells) fetal bovine serum, 1X (0.26 gm %) tryptose phosphate broth (GIBCO BRL), 1X glutamine-penicillin-streptomycin solution. Following adsorption, the infected C6/36 cells were returned to 29°C for further incubation whereas the PS infected cells were cultured at 37°C. To harvest the virus that liberated from infected cells into the culture supernatant, 800 μl of the culture supernatant were collected on day 0, 1, 2, 3, 4, 5, 7, 9, 11 and 14 after infection, spun briefly to pellet the cells, and adjusted to 20% fetal bovine serum. The virus suspension was stored in small aliquots (200 μl /tube) at -70°C. Virus titration was subsequently performed with the focus immunoassay for each viral sample.