## VI. SUMMARY

Dengue virus, a causative agent of dengue fever (DF) and dengue hemorrhagic fever (DHF), belongs to the genus *Flavivirus* in the family *Flaviviridae*. It is a spherical, enveloped RNA virus with a diameter of about 40-60 nm. The single-stranded RNA viral genome, about 10.7 kb in size, contains the $m^7$GpppAmp cap at the 5' terminus and serves as a messenger RNA for viral protein production. During dengue virus replication, a long polyprotein is cleaved by several proteinase enzymes within the cytoplasm of infected cells, resulting in three structural proteins capsid (C), premembrane (prM) and envelope (E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5). The viral RNA genome and structural proteins assemble with lipid bilayer of ER to generate immature viral particles, which bud into the ER lumen. The surface of immature virions consists of 180 molecules each of the two glycoproteins E and prM, which associate by non-covalent interactions at the molecular ratio of 1:1 (prM-E heterodimer). Viral particles are exported by the secretory pathway from ER, Golgi apparatus, *trans*-Golgi network and secretory vesicles to the extracellular compartment. During the transport, the prM protein on immature virions is cleaved into soluble pr protein and the envelope-bound M protein by subtilisin-like proprotein convertases, especially the furin protease, resulting in the dissociation of the prM-E heterodimer, the rearrangement of E into E homodimer, and the acquisition of mature virion morphology with high infectivity. Cleavage of the prM protein by furin occurs at the consensus sequence (Arg-Xaa-Lys/Arg-Arg or Arg-Xaa-Xaa-Arg, where Xaa is any amino acid), which is located at the pr-M junction within the prM protein of dengue and other flaviviruses. A flavivirus with specific mutation of an amino acid within the consensus sequence of the pr-M junction is not infectious, possibly due to the failure of prM cleavage. For dengue virus, however, not all of the prM protein is cleaved and up to 40-60 molecules of prM remains uncleaved in the mature virions released from infected mosquito cell despite the presence of the consensus furin cleavage sequence at the pr-M junction. Failure to cleave all dengue prM proteins does not appear to affect the infectivity of dengue virions.

Failure to completely cleave dengue prM protein in the presence of intact consensus sequence for furin suggests that certain amino acids surrounding the consensus sequence may affect the efficiency of prM cleavage. In a recent study, the 13-amino acid region just proximal to dengue pr-M junction was replaced with the homologous region derived from Japanese encephalitis virus. Analysis of the resultant JEVpr/16681 chimeric virus revealed that the cleavage of prM in JEVpr/16681 was clearly enhanced as compared with the parent dengue virus strain 16681. The 13-amino acid region proximal to the pr-M junction of JEVpr/16681 shares with the parent strain 16681 the furin consensus sequence, but differs by the presence of three additional positively charged amino acids [at the amino acid positions 193 (P13), 196 (P10) and 198 (P8)], resulting in an increase from a total of four positive charges to seven positive charges and an absence of two negatively charged amino acids [at the amino acid positions 199 (P7) and 203 (P3)]. In addition, the side chains of two uncharged amino acids at the amino acid positions 197 (P9) and 200 (P6) of JEVpr/16681 prM also differ from the parent virus. These results suggested that the cleavage of prM protein in dengue virus, while requires the presence of furin consensus sequence, can be affected by other amino acids at the pr-M junction proximal region. Unexpectedly, while the prM protein of JEVpr/16681 is cleaved to the greater extent than the parent strain, the replication of this chimeric virus is substantially reduced from the parent virus. The titer of JEVpr/16681 following an expansion in the C6/36 cell line was $1.54 \times 10^6$ FFU/ml, which was 100-fold lower than the parent virus ($1.40 \times 10^8$ FFU/ml). The focus size of JEVpr/16681 as determined in PS cells of $17.68 \pm 9.59$ cells/focus was much smaller than that of the 16681 parent ($222.20 \pm 77.36$ cells/focus). Also, the kinetics of virus replication following an infection of PS cells, and to the lesser extent in C6/36 cells, was markedly reduced as compared with the parent virus. It is not yet clear that the reduction of JEVpr/16681 replication is caused by each or the combination of the three types of changes of the amino acid sequence at the13-amino acid region proximal to the pr-M junction in JEVpr/16681: 1) the different side chains of two uncharged amino acid residues at the amino acid positions 197 (P9) and 200 (P6), 2) the absence of the two negatively charges at the amino acid positions 199 (P7) and

203 (P3), and 3) the presence of three additional positively charges at the amino acid positions 193 (P13), 196 (P10) and 198 (P8).

To determine the effect of each type of changes in the pr-M junction proximal region of JEVpr/16681, the three types of changes were introduced into the full-length cDNA clone of dengue virus strain 16681 separately and each mutant viruses were then compared with JEVpr/16681 and the parent strain. In the first set of experiment, the effects of the change of two uncharged residues were studied. The 16681pr(+7, -0) dengue mutant was constructed by changing threonine at the amino acid position 197 (P9) and serine at the amino acid position 200 (P6) of JEVpr/16681 into methionine and histidine, respectively, as occurred in strain 16681. The mutant virus 16681pr(+7, -0) was viable after transfection into C6/36 cells with the virus titer of $7.32\times10^6$ FFU/ml after one round of amplification. Determinations of the focus size in PS cell (17.80 ± 8.61 cells/focus) and the kinetic of infection both C6/36 and PS cells revealed the reduced focus size and decreased replication indistinguishable from JEVpr/16681. This result indicated that the two uncharged amino acids at the amino acid positions 197 and 200 was not involved in reducing the replication of JEVpr/16681 in these cell lines. In the second set of experiment, the effects of an absence of two glutamic acid residues at the amino acid positions 199 (P7) and 203 (P3) in JEVpr/16681 was examined by constructing the mutant virus 16681pr(+4, -0) in which the two negatively charged amino acid residues of strain 16681 were changed into histidine and serine, respectively, as in JEVpr/16681. The mutant virus 16681pr(+4, -0) replicated in C6/36 cells to the titer of $4.18\times10^7$ FFU/ml, which was similar to the parent strain 16681. In contrast, its focus size in PS cell, at 42.30 ± 21.16 cells/focus, was markedly reduced from 16681 but was approximately 2-fold larger than JEVpr/16681. Similarly, the kinetic of infection of 16681pr(+4, -0) in PS cell was lower than the parent virus, but higher than JEVpr/16681. In C6/36 cells the replication of 16681pr(+4, -0) was similar to the parent virus. The result indicated that an absence of the two negatively charged residues at the position 199 and 203 cause some reduction of dengue virus replication, but not to the extent observed in JEVpr/16681, and is only detected in PS cells. The phenotype of 16681pr(+4, -0) also suggested that the marked reduction of JEVpr/16681 replication is unlikely to be due solely to the lack of two negatively charged amino acids in the prM cleavage proximal

region. In the third set of experiment, the effects of increasing three positively charged amino acids at the amino acid positions 193 (P13), 196 (P10) and 198 (P8) were determined. The mutant virus 16681pr(+7, -2) was constructed by changing the three uncharged amino acids of strain 16681 to arginine as occurred in JEVpr/16681. The results revealed efficient replication of 16681pr(+7, -2) in C6/36 cells resulting in the virus titer of $1.27\times10^7$ FFU/ml, which was lower than strain 16681. In contrast, the focus size of 16681pr(+7, -2) in PS cell, at 22.64 ± 12.61 cells/focus, was greatly reduced when compared with 16681 parent and was indistinguishable from JEVpr/16681. The replication kinetics of 16681pr(+7, -2) following infection of both PS and C6/36 cells was lower than the parent virus, but higher than JEVpr/16681. The data indicated that the addition of three positively charged amino acid residues at the amino acid positions 193 (P13), 196 (P10) and 198 (P8) also caused some reduction of dengue virus replication, and that the marked reduction of virus replication as observed in JEVpr/16681 was likely due to the combination of an absence of the two negative charges at the amino acid positions 199 (P7) and 203 (P3) and an increase of the three positive charges at the amino acid positions 193 (P13), 196 (P10) and 198 (P8).

If an increase of the total positive charge of the 13-amino acid region proximal to the prM cleavage junction from +2 in strain 16681 to +7 in JEVpr/16681 caused a marked reduction of virus replication, it may be possible that further increase of the positive charges would affect virus replication more profoundly. To test this hypothesis, the mutant virus 16681pr(+9, -0) was constructed by changing the uncharged amino acids at the position 193 (P13), 195 (P11), 196 (P10), 198 (P8) and 199 (P7) of strain 16681 to arginine and also changing a negative charge at the amino acid position 203 (P3) to serine, resulting in the total of nine positive charges. Following transfection of the full-length mutant *in vitro* transcripts into C6/36 cells, the mutant virus 16681pr(+9, -0) was viable and replicated to the titer of $1.71\times10^5$ FFU/ml, which was about 10-fold lower than JEVpr/16681. The focus size of 16681pr(+9, -0) as determined in PS cell, at 1.48 ± 1.15 cells/focus, was the smallest among the four mutant viruses. The kinetics of virus replication following infection of 16681pr(+9, -0) in both C6/36 and PS cells was clearly lower than JEVpr/16681 and other mutant viruses. Therefore, an increase in the content of positively charged

amino acids in the 13-amino acid region proximal to the pr-M junction to nine positive charges further reduced dengue replication in both PS and C6/36 cell lines.

In conclusion, this study revealed that an alteration of two uncharged amino acids at the amino acid positions 197 (P9) and 200 (P6) of the 13-amino acid region proximal to the prM cleavage did not affect the replication of JEVpr/16681. An absence of the two negative charges at the amino acid positions 199 (P7) and 203 (P3) of dengue pr-M junction region had some affect in reducing of dengue replication as well as an increase of three positive charges at the amino acid positions 193 (P13), 196 (P10) and 198 (P8). Taken together, the results indicated that both of the increase of three positive charges and the absence of two negative charges within the 13-amino acid region proximal to the pr-M junction contributed to the marked reduction of virus replication of JEVpr/16681. In addition, further reduction of dengue virus replication occurred when the net positive charge of the 13-amino acid region proximal to the pr-M junction was increased by an in vitro mutagenesis to the level of nine positive charges.