6. Summary

- DEN NTRs fold into stable stem loop structures as evaluated by RNA secondary structure prediction softwares. The ΔG values range from -17 to -27 kcal/mol for shorter 5'(+) and 3'(-) NTRs and -123 to -148 kcal/mol for longer 3'(+) and 5'(-) NTRs.

- cDNAs corresponding to DEN NTRs were cloned in pBS/KS+ vector flanked by T3 and T7 promoters on either sides. This facilitated the generation of (+) and (-) NTR transcripts from the same clone. PCR amplified products that carried either T3 or T7 promoters were used as templates for in vitro synthesis of radiolabelled (α-[32P]-ATP) and photoreactive (Br-UTP) transcripts.

- Host cell proteins that interact with DEN NTRs were analyzed by using the in vitro generated transcripts as probes in EMSA and UV-CL assays. These assays revealed that DEN NTRs formed several stable and specific complexes with BHK cell lysate proteins. Several proteins of the molecular mass between 30-60 kDa interacted with the DEN NTRs, however the N-terminal amino acid sequences of these proteins could not be determined as the proteins were either N-terminally blocked or present in very low amounts.

- Host cell proteins that interact with DEN NTRs were identified by screening mouse macrophage cDNA expression library. Of about a million plaques screened, 16 plaques were rescued and amplified for sequencing. The five different clones that were sequenced corresponded to four different mouse proteins that showed >85% homology with the corresponding human proteins and are implicated in various cellular functions.

- Clone #1 corresponded to Glutamyl-Prolyl-tRNA synthetase (EPRS) gene, encoding a 165 kDa protein, which catalyzes the aminoacylation of glutamic acid and proline tRNA species during translation of cellular proteins. The sequence alignment of the full length EPRS gene with the identified clone revealed that the cDNA insert carried a partial gene (1381-2890 bp) coding for 461-964 amino acids out of 1021 amino acid residues.
Clone #5 corresponded to Chromatin modifying protein 2A (CHMP2A) gene, encoding a 25 kDa protein, which is a component of the mammalian Endosomal sorting complex required for transport (ESCRT-III) complex, required for multivesicular bodies (MVBs) formation and sorting of endosomal cargo proteins into MVBs. This protein is also reported to be involved in chromosome condensation and is suggested to play a role in stable cell cycle progression and in gene silencing. The sequence alignment of the full length CHMP2A gene with the identified clone revealed that the cDNA insert carried its full-length gene.

Clone #6 corresponded to the gene encoding the beta subunit of an actin filament capping protein, Cap-Zβ, which is a 25 kDa protein that regulates growth of the actin filament by capping the barbed end of growing actin filaments. The sequence alignment of the full length Cap-Zβ gene with the identified clone revealed that the cDNA insert carried C-terminal part of the gene (783-820 bp) coding for 262-272 amino acids.

Clone #4 and #16 corresponded to the gene encoding a 25 kDa RuvBL-2 protein, which is a mammalian DNA helicase showing structural similarity with the bacterial recombination factor RuvB. The sequence alignment of the full length Cap-Zβ gene with the identified clone revealed that the cDNA insert carried a partial gene (243-1390 bp) coding for 82-463 amino acids.

Full-length genes of RuvBL-2 and Cap-Zβ were isolated from the cDNA library through PCR amplification and cloned separately in prokaryotic expression vector pQE-60 and were induced with IPTG. Expression levels of both the proteins were low as neither protein could be visualized in induced cell lysates. However both were visible by inclusion bodies preparation. Neither of these proteins could bind to the Ni-NTA column. It is speculated that these proteins could have strongly aggregated upon formation of inclusion bodies, and as a result the 6x His-tag of the recombinant proteins is not accessible to bind to the column.

We characterized CHMP2A protein as the full-length gene was present in the cDNA insert sequenced. CHMP2A ORF was cloned in the expression vector pQE-31 and the recombinant His-tagged protein was purified using Ni-NTA affinity column
Summary

chromatography. Polyclonal antibodies against rCHMP2A were raised in mice and were used for immunological studies.

- CHMP2A appears to be ubiquitously present in several insect and mammalian cell lines and mammalian tissues including brain, liver and spleen. Immunofluorescence analysis demonstrates that the CHMP2A protein is predominantly a nuclear protein with low levels of diffuse cytoplasmic staining.

- In vitro binding assays between rCHMP2A and DEN NTRs were carried out in two different formats. In one, we demonstrated that the specific binding of the radiolabelled probe to rCHMP2A immobilized on Ni-NTA beads and in the other we used a UV-CL approach. Both these assays demonstrated that the recombinant murine CHMP2A protein shows significant interaction with the terminal regions of the minus sense/replication intermediate DEN RNA but not with the plus sense/genomic NTRs.

- Further, yeast-based confirmatory assay (in vivo yeast three hybrid assay) was employed to validate the in vitro binding of DEN NTRs with rCHMP2A protein. DEN-2 NTRs were separately used as the bait RNA and the rCHMP2A protein as the prey and in vivo interaction of the bait with prey in the yeast host was monitored by His prototrophy and reconstitution of lacZ expression. Consistent with the in vitro observations above, we found that the DEN-2 virus (-) NTRs but not the complementary (+) NTRs interacted in vivo with rCHMP2A protein.

- This study has revealed for the first time that DEN virus, through its NTRs, may interact with three or more additional host proteins like CHMP2A, Cap-Zβ and RuvBL-2. It is known that dengue virus exclusively replicates in vesicular bodies and we propose that CHMP2A (present in vesicular bodies) may keep the minus strand in proper topology through its interaction with both the NTRs of minus sense/replication intermediate RNA, so that it could be copied into plus genomic RNA by viral RdRp. It is further speculated that the dengue virus may recruit Cap-Zβ for intracellular trafficking and RuvBL-2 may be involved in RNA metabolism of DEN virus. Further investigations are needed to explore the significance of interaction of these proteins with DEN NTRs.