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# Cellular proteins bind to the 3' and 5' untranslated regions of dengue 2 virus genome

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***Abstract***

***In vitro*** generated cloned full length dengue 2 virus untranslated regions (UTRs) were used in RNA gel mobility shift assays to examine cellular factors binding to the virus genomes. Cellular factors in lysates of Vero (monkey) and C6136 (mosquito) cells bound specifically and non-specifically to the dengue 2 virus 3' UTR. Non-specific interaction with the 5' UTR, resulting in formation of at least 4 band shift complexes was noted with lysate of the C6136 cells only. Pre-treating the cell lysates with proteinase K affected binding of cellular factors to the dengue 2 virus UTRs, suggesting that the cellular factors were proteins. These findings suggest that cellular proteins could interact with specific sites on the dengue virus genomes.

***Key words:*** C6/36, Dengue, RNA, UTR, Vero, Virology

## INTRODUCTION

Dengue virus infection is still a major public health problem in many South East Asian countries.[1] The positive polarity single-stranded RNA virus, belonging to the family *Flaviviridae*,[2] is propagated in the laboratory using a variety of mosquito, mammalian and human cells.[3] The efficiency of dengue virus replication in these cells varies however, suggesting that there are probably cellular factors which are important in regulation of the viral genome replication. These factors could also play important roles in determining tissue tropism of the dengue virus ***in vivo.*** A possible mechanism of regulation could involve interaction of specific cellular factors with the viral genome. The sites on the genome to which these factors could bind may include the 3' and 5' UTRs which are predicted to fold into stem-loop structures, similar to most other flaviviruses.[4, 5] Although the RNA sequences which give rise to these structures are not conserved among the flaviviruses, the size and shape of these structures are strikingly similar suggesting that they may be functionally important. In the present study, possible cellular factor interaction with the dengue 2 virus UTRs was investigated.

## MATERIALS AND METHODS

### *Cells and cell lysate preparation*

The C6136 and Vero cells used in the present study were purchased from the American Type Culture Collection (ATCC), USA. Cell lysates were prepared by resuspending cells in lysis solution consisting of 25 mM Tris-HCl [pH 7.5], 40 mM KCl, 100 µM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK, Sigma, USA), 10 µg/ml aprotinin (Boehringer Mannheim, Germany) and 0.5% NP-40 (Boehringer Mannheim, Germany) on ice. The nuclei and membranous debris were removed by centrifugation (11000 x g, 4°C, 5 min) and protein concentration was determined using the micro bicinchoninic acid (BCA) assay kit (Pierce, USA). Samples were kept at -70°C after glycerol was added to a final concentration of 10%.

### *Amplification and cloning of dengue 2 virus UTRs and E.coli trxA genes*

The 5' and 3' UTRs of dengue 2 NGC strain were amplified following cDNA synthesis by the polymerase chain reaction (PCR) using the 5S/5AS and 3S/3AS oligonucleotide pairs, respectively (Table 1). The *trxA* gene of ***Escherichia coli (E. coli;*** K-12 strain), on the other hand, was amplified from boiled supernatant of the bacterial suspension in the presence of STRX/ASTRX oligonucleotide pair (Table 1). The temperature cycling parameters of each amplification reaction are as shown in Table 2.

Following amplification the 5' UTR, 3' UTR and *trxA* amplicons were ligated directly into the pGEM-T Cloning Vector Systems™ (Promega, USA) and transformed into E. ***coli*** (JM109 strain) following the manufacturer's protocols. The resulting recombinant plasmids were purified and

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***TABLE 1:*** **Oligonucleotides used as primers in the polymerase chain reaction amplification of DNA sequences.**

| Primer | Oligonucleotide sequence (5' to 3' direction) |
|---|---|
| 5S | AGTTGTTAGTCTACGTGGACCGACAAAGAC |
| 5AS | CAGAGATCTGCTCTCTAATTAAAAAACTGTTAG |
| 3S | TAGAAGGCAAAACTAACATGAAACAAGGC |
| 3AS | AGAACCTGTTGATTCAACAGCACC |
| STRX | AGATCTATGAGCGATAAAATTATTCACCTGACTGACG |
| ASTRX | GGATCCCGCCAGGTTAGCGTCGAGGAACTC |
| T7 | TAATACGACTCACTATAGGG |
| T75S | TACGTAATACGACTCACTATAGGGAGTTGTTAGTCTACGTGGACCGAC |
| T73S | TACGTAATACGACTCACTATAGGGTAGAAGGCAAAACTAACATGAAACAAGG |

Notes: (i) Restriction enzyme recognition sequences added to the oligonucleotides are in bold. (ii) The T7 RNA polymerase recognition sequence is underlined. (iii) The oligonucleotides were designed based on published nucleic acid sequences of DEN-2 NGC and *trxA*.[11,12,13]

their inserts were verified by DNA sequencing (T7 Sequenase Quick-Denature plasmid DNA sequencing kit™; Amersham, UK).

### *Incorporation of T7 promoter into cDNAs*

To enable ***in vitro*** transcription to be performed, T7 promoter was incorporated into the cDNAs generated above by the polymerase chain reaction. The cDNA inserts of pGEM-5' UTR and pGEM-3' UTR were excised from the plasmid vector and then subjected to the PCR in the presence of T75S/5AS, T73S/3AS and T7/ STRX oligonucleotide pairs, respectively (Table 1). The resulting T7 promoter incorporated amplicons were purified and subsequently used as templates for ***in vitro*** transcriptions.

### *Generation of in vitro transcripts*

***In vitro*** transcription was performed at 37°C for 2 hr in 80 µl volume comprising of 1X transcription buffer (Amersham, UK), 10 mM DTT, 80 U RNasin™ (Promega, USA), 1 mM of each of the four ribonucleoside-5'-triphosphates (rNTPs; Promega, USA), 8 pmol of T7' promoter incorporated amplicon and 80 U T7 RNA polymerase (NEB, USA). On the other hand, radiolabelled transcripts (probes) were generated by incubating a 20 p1 reaction volume comprising of 1X transcription optimised buffer (Promega, USA), 10 mM DTT, 20 U RNasin™, 0.5 mM of each of the three rNTPs (ATP, CTP and GTP), 15 µM UTP, 50 µCi [α-$^{33}$P]UTP (2000 Ci/mmol; New England Nuclear, USA), 1.5 pmol of T7 promoter incorporated amplicon and 40 U T7 RNA polymerase (NEB, USA) at 37°C for 1 hr. Template DNA was then removed by digesting with RNase-free DNase (Promega, USA) at 37°C for 45 min. The 3' UTR and a*trxA* (antisense *trxA*) transcripts were purified through a DEPC-water

TABLE 2: Temperature cycling conditions used in the PCR

| Oligo pair | | Denature | | Anneal | | Extend | | Cycles | Final extend | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fwd | Rev | Temp (°C) | Time (s) | Temp (°C) | Time (s) | Temp (°C) | Time (s) | Number | Temp ("C) | Time (min) |
| 5S | 5AS | 94 | 60 | 55 | 60 | 72 | 30 | 30 | 72 | 6 |
| 3S | 3AS | 94 | 60 | 55 | 60 | 72 | 60 | 30 | 72 | 12 |
| STRX | ASTRX | 94 | 60 | 60 | 60 | 72 | 90 | 30 | 72 | 7 |
| T75S | 5AS | 94 | 45 | 60 | 45 | 72 | 15 | 30 | 72 | 1 |
| T73S | 3AS | 94 | 45 | 60 | 45 | 72 | 35 | 30 | 72 | 2 |
| T7 | STRX | 94 | 45 | 55 | 45 | 72 | 30 | 30 | 72 | 2 |

Notes: (i) The final extension was carried out for 1 cycle only. (ii) The expected sizes of the amplicons of each oligonucleotide pair in the order of the table above are 96,454,339,120,478 and 408 bp, respectively. (iii) 'Fwd' and 'Rev' refer to the forward and reverse oligonucleotides, respectively. (iv) The T7-UTR amplicons will generate full length UTR transcripts corresponding to the positive strand of the dengue virus genome whereas the T7-*trxA* amplicon will generate antisense *trxA* transcript during *in vitro* transcription with T7 RNA polymerase.

ChromaSpin™100 column (Clontech, USA) whereas a ChromaSpin™30 column was used for the 5' UTR transcript. Radioactivity of the probe was determined using a scintillation counter (Tri-Carb 2500 TR; Packard, USA) and the theoretical maximum amount of transcript generated was calculated based on the assumption of 100% incorporation of the limiting UTP.

### *RNA gel mobility shift assay*

RNA gel mobility shift assay was performed as described previously[6] with slight modifications. An 18 µl binding mix (25 mM Tris-HCl [pH 7.5], 40 mM KCI, 0.5% NP-40, 100 µM TPCK, 10 µg/ml aprotinin, 2 mM $MgCl_2$, 0.5 mM DTT, 300 µg/ml E. *coli* tRNA, 5 µg cell lysate) was incubated at 22°C for 20 rnin before 1 µl of probe (corresponding to 85000 and 350000 CPM for 5' and. 3' UTR probes, respectively) was added. After a further incubation of 15 rnin, 1 µl of heparin (corresponding to 1.5 and 4.5 U for 5' and 3' UTR probes, respectively) was added, followed by a 12 rnin incubation. Subsequently, 4 p1 of 6X loading buffer was added and samples were electrophoresed on a native polyacrylamide gel (acrylamide/bisacrylamide ratio of 30:0.8; 4% and 5% for 3' and 5' UTR probes, respectively) in 0.5 X TBE at 10 V/cm. The gel was vacuum dried and exposed to Hyperfilm MP™ (Amersham, UK) with intensifying screens at -70°C for approximately 15 hr.

For competition experiments, unlabeled transcripts and non-specific competitors were included into the binding reaction mix prior to the initial incubation. In experiments involving proteinase K, cell lysates were pre-treated with the protease at 37°C for 20 rnin before they were added to the binding mix. For control samples, the protease was heat inactivated at 95°C for 30 rnin prior to use.

## RESULTS AND DISCUSSION

The presence of factors in Vero and C6136 cell lysates that bind to the 3' UTR of dengue 2 virus was assessed by examining the ability of the 3' UTR probe (radiolabelled 3' UTR transcript) to form mobility retarded RNA complexes in the presence or absence of unlabelled competitors. A broad mobility retarded RNA complex with no distinctive band was noted when Vero cell lysate was added to the labelled probe in the absence of any competitor (Fig. 1, BV). In contrast, a single distinctive mobility retarded RNA complex was detected with the C6136 cell lysate (BC). Addition of excess unlabelled 3' UTR transcript as competitor, reduced in a dose-dependent manner the intensity of the BV and BC complexes. This suggests that formation of the complexes repre-

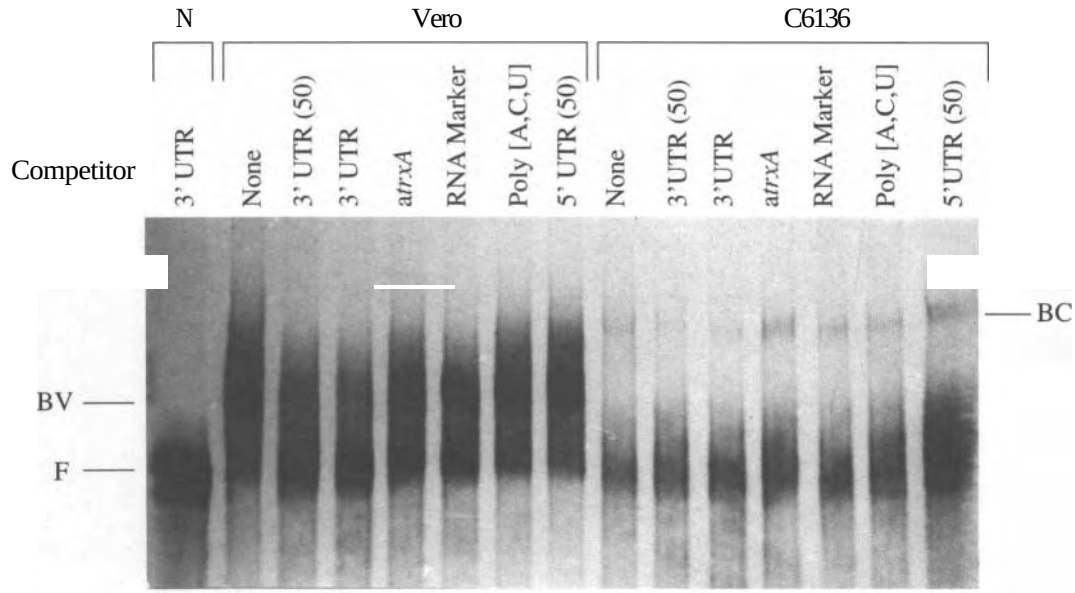


FIG. 1: Gel mobility shift assay to determine cellular factor binding to dengue 2 virus 3' UTR. Specificity of the binding was assessed by competition using unlabelled competitors. Each competitor (75-fold excess unless indicated otherwise as 50-fold excess in round brackets) was pre-incubated with 5 μg of cell lysate (Vero or C6/36) before the addition of 350,000 CPM of $^{33}$P-labeled 3' UTR transcript (478 nt probe) and 4.5 U heparin. The competitors used are indicated at the top of each lane. The free probes (F) and band shift complexes (BV and BC for Vero and C6136 cell lysates, respectively) are also indicated. Sample without cell lysate is marked 'N' whereas, those containing either Vero or C6/36 cell lysate are marked accordingly.

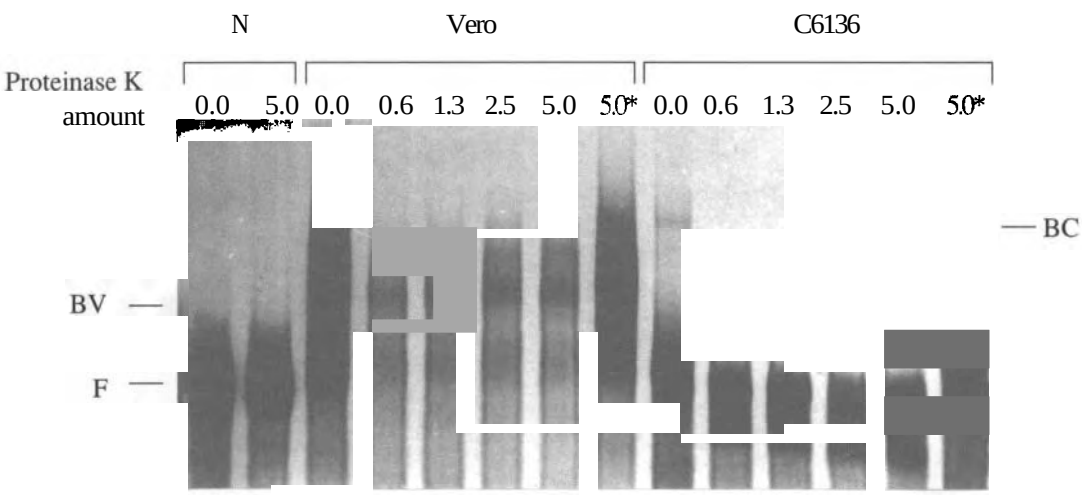


**FIG.** 2: Gel mobility shift assay to determine the effects of proteinase K pre-treatment of the Vero and C6/36 cell lysates on the formation of dengue 2 virus 3' UTR mobility retarded RNA complexes. Vero and C6/36 cell lysates (5 μg) were pre-incubated with selected concentrations of proteinase K (indicated by the units at the top of each lane) prior to addition of 350.000 CPM of 3' UTR probe and 4.5 U heparin. Samples without cell lysate are marked 'N' whereas those containing Vero or C6/36 cell lysates are marked accordingly. The free probes (F) and mobility retarded RNA complexes (BV and BC for Vero and C6/36 cell lysates, respectively) are also indicated. Asterisk shows that the proteinase K is heat-inactivated prior to use.

sents specific interaction of cellular factors with the dengue 3' UTR probe. Furthermore, addition of non-specific competitors; *atrx*A transcripts, RNA marker, poly [A,C,U] and dengue virus 5' UTR to the reaction mixes did not affect the presence and the intensity of the mobility shifted complexes. However, in the Vero cell lysate-treated samples distinctive bands representing the BV complex became prominent. It is suggested that non-specific cellular factors which interacted with the dengue 3' UTR probe, forming the broad RNA band shift complex, bind to the unlabelled non-specific competitors also, thus allowing detection of the specific dengue virus 3' UTR -Vero cell factor mobility retarded RNA complex.

The nature of these cellular factors were examined by performing gel mobility shift assays using proteinase K (a broad spectrum serine protease)-treated cell lysates. Addition of 5 U of proteinase K alone to the radiolabelled probes did not result in degradation of the RNAs or formation of any mobility retarded band shift complex (Fig. 2). This suggests that the proteinase K preparation contained no RNase activities and did not bind to the radiolabelled probe. However, substantial reduction in the intensity of the BV complex was noted when Vero cell lysate was pre-treated with selected concentrations of the proteinase K. A distinctive mobility retarded RNA complex akin to that observed when non-specific competitors were added became apparent in the proteinase K treated samples. Addition of heat inactivated proteinase K to the reaction mix, however, restored the broad BV band shift complex suggesting that proteinase K sensitive cellular factors bind specifically and non-specifically to the dengue virus 3' UTR probes. Pre-treatment of the C6/36 cell lysate with proteinase K, on the other hand, resulted in no de-

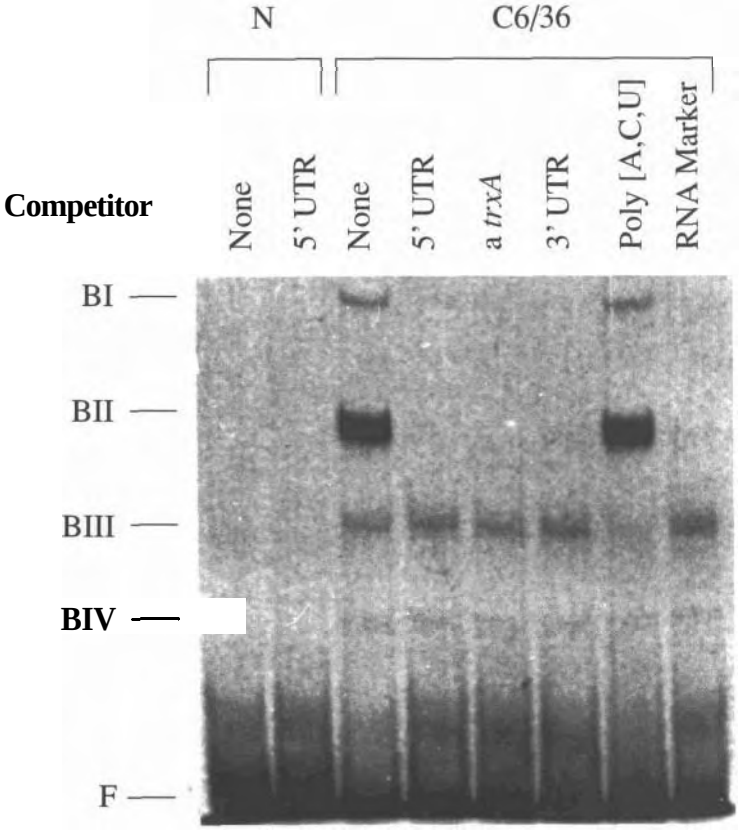


**FIG. 3: Gel mobility shift assay to determine cellular factor binding to dengue 2 virus 5' UTR. Specificity of the binding is assessed by competition using unlabeled competitors. Each competitor (present at 50-fold excess) was pre-incubated with 5 μg of C6/36 cell lysate before the addition of 85,000 CPM of $^{33}$P-labeled 5' UTR transcript (120 nt probe) and 1.5 U heparin. The competitors used are indicated at the top of each lane. The free probes (F) and mobility retarded RNA complexes (BI to BIV) are also indicated. Samples without cell lysate are marked 'N' whereas those containing C6/36 cell lysate are marked accordingly.**

tectable RNA-protein complex formation. The effects were specific since heat-inactivated enzyme restored the formation of the RNA-protein complexes. These findings suggest that there are specific cellular proteins in both Vero and C6/36 cell lysates which bind to the dengue virus 3' UTR with the former having substantially more non-specific interaction too.

Possible binding of cellular proteins to dengue 2 virus 5' UTR was investigated by performing similar competitive binding experiments and gel mobility shift assays as above. No band shift RNA complexes were detected when the 5' UTR probe was added to the Vero cell lysate (data not shown). On the other hand. four mobility retarded RNA-cellular factor complexes were present when C6/36 cell lysate was used (Fig. 3). The BII RNA complex was the most predominant in comparison to other complexes. To determine the specificity of the binding, unlabelled *atrx*A transcripts, RNA marker, poly [A,C,U] and dengue virus 3' and 5' UTR transcripts were added as competitors. Except for poly [A,C,U], addition of all other competitors abrogated the formation of the BI and BII RNA complexes. The presence of the BIII and BIV RNA complexes, however, was not affected by the competitors except poly [A,C,U]. These findings suggest that cellular factors which bind to the dengue 2 virus 5' UTR bind non-specifically to other RNAs also. The failure of poly [A,C,U] to compete for binding in formation of the BI and BII RNA complexes could be due to the absence of the *G* ribonucleotide which is probably needed to form a stable stem loop structure. On the other hand, the failure of the non-specific competitors to abrogate the formation of the BIII and BIV RNA complexes could be due to abundant presence of these non-specific RNA binding cellular factors. Addition of proteinase K-digested C6136 cell lysate to the radiolabelled probes resulted in no mobility shifted RNA complex formation (Fig.

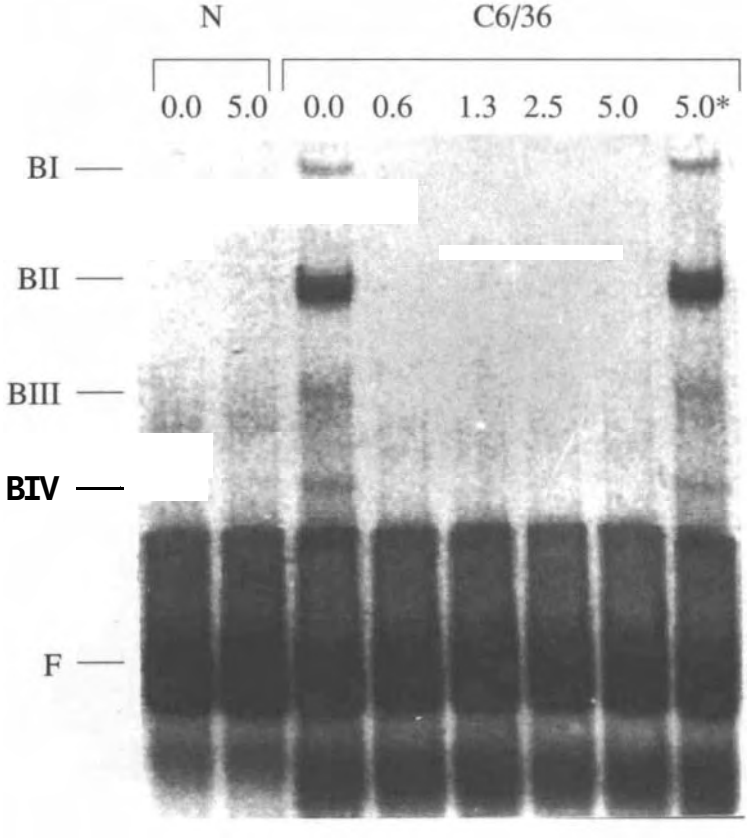


**FIG. 4: Gel mobility shift assay to determine the effects of proteinase K pretreatment of the C6/36 cell lysate on the formation of RNA band shift complexes with the 5' UTR of dengue 2 virus.** C6136 **cell lysate (5 µg) was pre-incubated with selected concentrations of proteinase K (indicated by the units at the top of each lane) prior to the addition of 85.000** CPM **of 5'** UTR **probe and 1.5 U** heparin. **Samples without cell lysate are marked 'N' whereas those containing C6/36 cell lysate are marked accordingly. The free probes (F) and RNA band shift complexes (BI to BIV) are also indicated. Asterisk shows that the proteinase K is heat inactivated prior to use.**

4). However,.addition of heat-inactivated proteinase K restored the ability of the cells' proteins to form the RNA band shift complex. Hence, these'findings suggest that the cellular factors which non-specifically interacted with the dengue 2 virus 5' UTR are also proteins.

Formation of virus 3' and 5' UTR -protein complexes in vitro have been reported for a number of viruses including the flaviviruses.[6,7,8,9] These suggest the possible importance of cellular factors in regulation of viral replication especially in determining host cell susceptibility and efficiency of virus replication. As indicated in the present study, binding of cellular factors from the Vero cell lysates differ from those of the C61 36 cells, suggesting that different cellular proteins are involved. Since the 3' UTR is thought to be important in initiation of flavivirus replication: the finding that different cellular proteins in Vero and C6/36 cell lysates bind to this viral genomic site suggests that efficient dengue virus replication probably depends on the availability of these specific cellular proteins.

The importance of cellular proteins binding to the dengue 2 virus 5' UTR is as yet still unknown. However, deletions of 5 to 6 nucleotides of dengue 4 virus 5' UTR was reported to differentially affect replication of the virus in LLCMK/ 2 cells (simian kidney derived) and C6/36 cells,[10] suggesting that this viral genomic site could also be important in conferring cell tropism. In the present study, it is noted that non-specific interactions resulting in formation of dengue 5' UTR RNA-protein complexes were detected only with the C6/36 cell lysates. This supports the notion that possibly different cellular proteins are involved in regulation of dengue virus replication in different hosts.

Results obtained from the present investigation and those from studies performed by others highlight the importance of pursuing further investigations on the interaction between the flavivirus UTRs and host proteins. Findings from these studies may lead to a better understanding of flavivirus replication strategies in general.

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