

Short Communication

Poly(A)-binding protein binds to the non-polyadenylated 3' untranslated region of dengue virus and modulates translation efficiency

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Poly(A)-binding protein (PABP) is a key player in mRNA circularization and translation initiation of polyadenylated mRNAs. It simultaneously binds the 3' poly(A) tail of an mRNA and eukaryotic initiation factor 4G (eIF4G), which forms part of the translation initiation complex assembling at the 5' end, thus circularizing the RNA molecule and enhancing translation initiation. Here, we report the binding of PABP to the non-polyadenylated 3' end of dengue virus (DENV) RNA. PABP binds the DENV 3' untranslated region (3'UTR) internally, upstream of the conserved 3' stem-loop near the two dumb-bell structures, and can be displaced by poly(A) RNA. The PABP-specific translation inhibitor PABP-interacting protein 2 (Paip2) interferes with the DENV 3'UTR–PABP interaction, and *in vitro* translation of DENV reporter RNAs in baby hamster kidney cell extracts is inhibited by Paip2 in a dose-dependent manner. Our findings show an expanded translation mechanism for PABP, binding to a viral RNA lacking a terminal poly(A) tail.

Dengue virus (DENV), a mosquito-borne member of the family *Flaviviridae*, is a major public health problem in tropical and subtropical areas worldwide, causing ~50 million cases of dengue fever and ~500 000 cases of the potentially fatal dengue haemorrhagic fever/dengue shock syndrome annually (Gibbons & Vaughn, 2002). The four DENV serotypes (DENV1–4) contain a 10.7 kb positive-stranded RNA genome encoding a single polyprotein flanked by a m⁷G-capped, ~100 nt 5' untranslated region (UTR) and a longer (~450 nt) 3'UTR (Clyde *et al.*, 2006). Unlike the majority of cellular mRNAs, DENV RNA lacks a poly(A) tail. Instead, the 3'UTR contains a terminal 3' stem-loop (3'SL), which is conserved across all flaviviruses (Brinton *et al.*, 1986; Hahn *et al.*, 1987; Mohan & Padmanabhan, 1991), and two dumb-bell (DB) structures containing the conserved sequences CS2 and RCS2 (Hahn *et al.*, 1987; Olsthoorn & Bol, 2001). The DENV 3'SL binds several cellular proteins such as La autoantigen, polypyrimidine-tract-binding protein (De Nova-Ocampo *et al.*, 2002), eukaryotic elongation factor 1A (Davis *et al.*, 2007) and Y-box-binding protein-1 (Paranjape & Harris, 2007). The 3'UTR plays an important role at the nucleotide level by harbouring two replication elements, the CS and the UAR (Alvarez *et al.*, 2005b; Hahn *et al.*, 1987; You & Padmanabhan, 1999), which promote 5' end–3' end RNA–RNA interaction. This 5' end–3' end RNA–RNA interaction is critical for viral RNA synthesis but appears not to be

necessary for DENV translation (Alvarez *et al.*, 2005a; Edgil & Harris, 2006).

The majority of eukaryotic translation is regulated by the termini of cellular mRNAs via RNA–protein association rather than RNA–RNA interactions. The 5' m⁷G-cap and the 3' poly(A) tail enhance translation initiation and have a synergistic effect when present on the same molecule (Gallie, 1991). The cap initiates assembly of eukaryotic initiation factors (eIFs) by binding eIF4E, which, by recruiting eIF4G, triggers the translational cascade. eIF4G further binds the eIF4A helicase and eIF3, which recruits the 43S ribosomal complex to the 5' end of the mRNA (Pestova *et al.*, 2007). eIF4G also binds poly(A)-binding protein (PABP), which simultaneously interacts with the 3' poly(A) tail, causing circularization of the mRNA and enhancement of its translation efficiency (Imataka *et al.*, 1998; Kahvejian *et al.*, 2005; Tarun & Sachs, 1996).

PABP is a ~70 kDa protein with four RNA recognition motifs (RRMs) at the N terminus, the first two of which overlap with the eIF4G-binding site (Adam *et al.*, 1986; Burd *et al.*, 1991; Sachs *et al.*, 1986). The PABP–poly(A) RNA interaction is abrogated by PABP-interacting protein 2 (Paip2), resulting in translation inhibition (Khaleghpour *et al.*, 2001b). PABP has two Paip2-binding sites, one in the central RRM region and one at the proline-rich C-terminal end, but only the interaction in the RRM region abrogates the PABP–poly(A) interaction (Khaleghpour *et al.*, 2001a). Paip2 also represses the translation-enhancing effect of PABP by disrupting the PABP–eIF4G interaction (Karim *et al.*, 2006).

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Cellular translation initiation is a highly controlled process. Viruses lacking the cap-structure or a poly(A) tail have developed alternative ways to compete with cellular mRNAs for translation factors and to redirect cellular machinery in their favour. For example, among the members of the family *Flaviviridae*, the hepacivirus hepatitis C virus contains an internal ribosome entry site (IRES) that recruits the 40S ribosomal subunit and eIF3 directly, omitting the need for eIF4E and eIF4G (Pestova *et al.*, 1998). The enterovirus poliovirus (PV) also contains an IRES (Pelletier & Sonenberg, 1988), and its 2A protease cleaves eIF4G, shutting off host-cell translation. PV then uses the resulting C-terminal eIF4G fragment to promote cap-independent viral translation (Etchison *et al.*, 1982; Krausslich *et al.*, 1987; Lloyd *et al.*, 1988). In addition, the 3' pentanucleotide consensus sequence of non-polyadenylated rotavirus (*Reoviridae*) binds the viral NSP3 protein, which binds eIF4G with high affinity and displaces PABP from the eIF4F complex, thus inhibiting host-cell translation and promoting viral translation (Piron *et al.*, 1998; Vende *et al.*, 2000).

DENV neither shuts down host-cell protein synthesis nor harbours an IRES, but is able to translate well under eIF4E-limited conditions, suggesting a modified requirement for canonical eIFs (Edgil & Harris, 2006; Edgil *et al.*, 2006). We and others have shown that the non-polyadenylated DENV 3'UTR enhances viral translation efficiency (Chiu *et al.*, 2005; Holden & Harris, 2004; Holden *et al.*, 2006). To investigate the possibility of PABP interacting directly with the DENV 3'UTR, we first examined *in vitro* binding by electrophoresis mobility shift assay (EMSA). The complete DENV2 3'UTR (451 nt) was cloned downstream of the T7 polymerase promoter and labelled with [α - 32 P]ATP by *in vitro* transcription. The RNA probe was separated on a denaturing polyacrylamide gel and purified by elution, followed by phenol/chloroform extraction and precipitation with 2-propanol. The purified probe was combined with increasing amounts of purified, His-tagged PABP [a kind gift from R. Andino, UC San Francisco, USA (Herold & Andino, 2001)] in binding buffer [5 mM HEPES, 25 mM KCl, 2 mM MgCl₂, 3.8% glycerol (v/v), 20 mM DTT and 0.2 U SUPERaseIn (Ambion) μ l⁻¹], supplemented with 1 μ g tRNA μ l⁻¹ to reduce non-specific binding. Samples were incubated for 10 min at room temperature and then separated on a 4.5% native polyacrylamide gel at 8 °C (Fig. 1a, left panel). A shift was readily detected when the 3'UTR was incubated with PABP, whereas incubation with the non-specific protein BSA generated no shift (data not shown). The DENV 3'UTR–PABP interaction was further characterized by incubating increasing amounts of PABP with a fixed amount of labelled DENV 3'UTR under the conditions described above. The complexes were analysed for binding efficiency by slotblot and visualized by phosphorimaging (Typhoon2400, ImageQuant; GE Healthcare), generating a K_d of 0.8 μ M (Fig. 1b) using one-site binding (hyperbola) curvefit (Binding = $B_{max} \times [PABP] / (K_d + [PABP])$) in GraphPad Prism v.5.0.

Since cellular mRNAs bind PABP at their terminal poly(A) tail, we investigated whether DENV mimics this terminal

3'-end binding. The final 102 nt of the DENV genome, comprising the 3'SL, were cloned as above and labelled by *in vitro* transcription with [α - 32 P]ATP. The probe was incubated with increasing amounts of PABP, and the interaction was visualized by EMSA. Interestingly, no interaction could be detected (Fig. 1a, right panel), even at the highest concentrations that generated a clear shift with the complete 3'UTR (Fig. 1a, left panel). This implies that PABP binds DENV internally upstream of the 3'SL. To confirm this, we examined the 3'UTR–PABP interaction by competing with unlabelled 3'UTR, 3'SL or 3'UTR lacking the 3'SL (3' Δ SL) (Fig. 1c). The 3'UTR–PABP binding was unaffected even by a 25-fold molar excess of 3'SL, but as expected, the interaction was disrupted with equal efficiency by the 3' Δ SL and the control 3'UTR (Fig. 1d, upper panel). The negative control for non-specific binding, tRNA, had no effect on the 3'UTR–PABP complex (data not shown). To narrow down further the PABP-binding region, competition EMSA experiments were performed with 3'UTR constructs containing a deletion of DB1, DB2 or both, as well as with a construct containing both DB regions and the A-rich stretches on either side (Fig. 1c). All three DB deletion mutants still contained the A-rich sequences flanking the DB region. The four constructs competed effectively with the labelled WT-3'UTR probe for PABP binding (Fig. 1d, bottom panel). The fact that all three DB deletion mutants as well as the DB1+2 construct competed with the WT-3'UTR probe for PABP binding and that the only common sequences found in these four constructs were the A-rich regions flanking the DBs suggests that these A-rich regions are likely to be involved in PABP binding.

To validate the specificity of the DENV 3'UTR–PABP interaction, the canonical RNA target of PABP, poly(A) RNA, was also used as a competitor (Baer & Kornberg, 1983). Addition of poly(A) RNA (average length 423 nt; GE Healthcare) to the DENV 3'UTR RNA–PABP EMSA mixture disrupted the DENV–PABP interaction in a dose-dependent manner, confirming the specificity of the RNA–protein interaction (Fig. 2a). The dissociation of the 3'UTR–PABP complex caused by small increases in the amount of poly(A) RNA is likely due to the multiple PABP-binding sites present on the poly(A) RNA.

To characterize further the nature of the 3'UTR–PABP interaction, two PABP-specific antibodies were used to supershift the complex observed by EMSA: anti-PABP N terminus (α -PABP-N; Cell Signaling Technology) and anti-PABP C terminus (α -PABP-C; Abcam PLC) antibodies. DENV 3'UTR was incubated in the presence of PABP (see above) with two different concentrations of either antibody. A supershift was observed with the anti-PABP-C but not with the anti-PABP-N antibody, the latter of which may even have slightly inhibited the PABP–3'UTR interaction (Fig. 2b). It is possible that the DENV 3'UTR and the anti-PABP-N antibody compete for the same site on PABP. Overall, the supershift observed with anti-PABP-C antibody confirms the 3'UTR–PABP interaction.

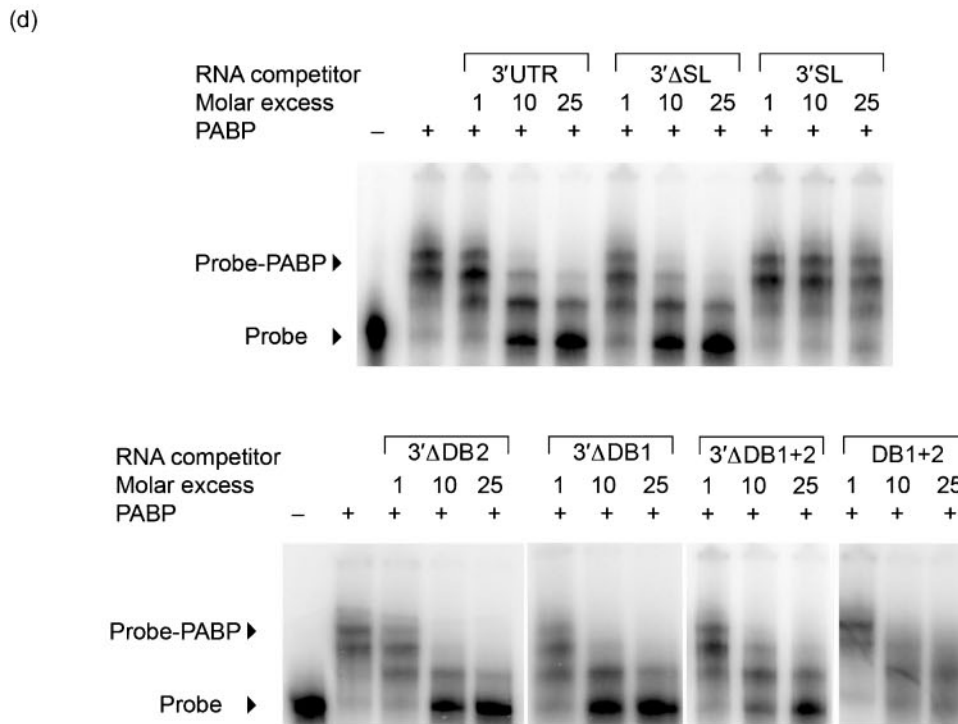
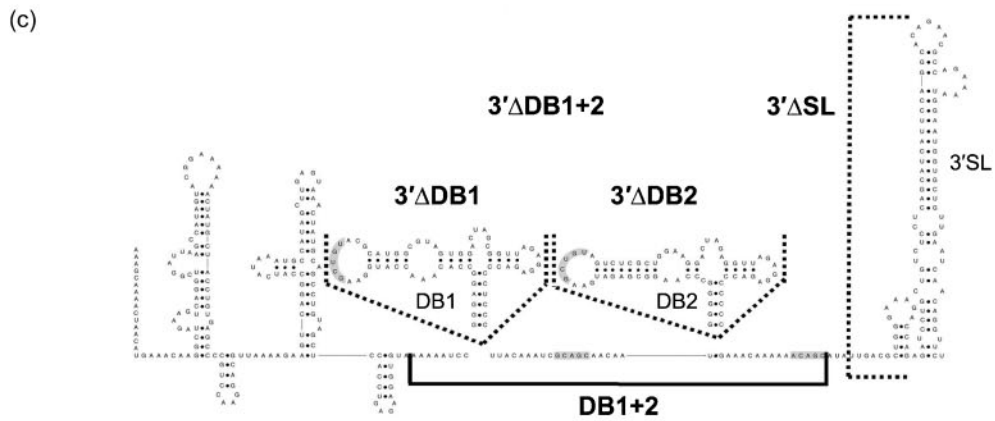
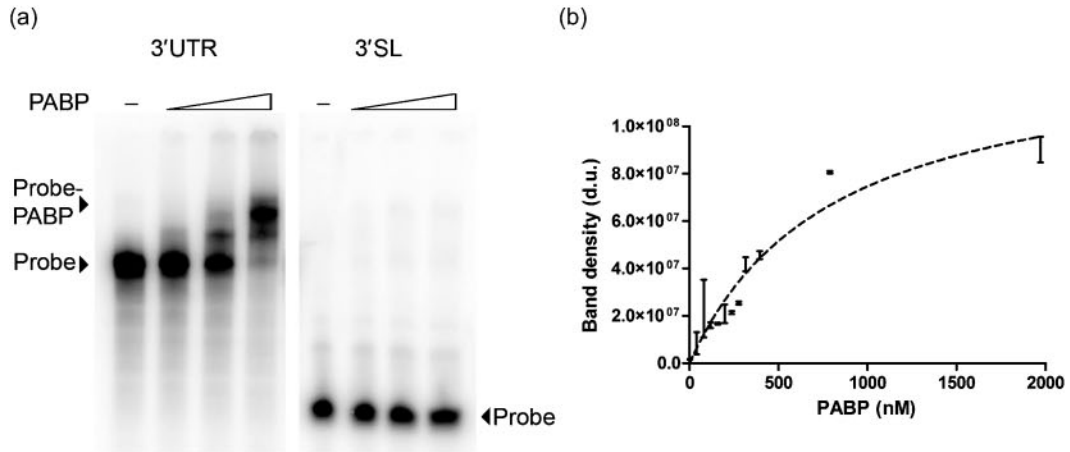


Fig. 1. PABP interacts with the DENV 3'UTR RNA. (a) Increasing amounts of purified PABP (0.4, 1 and 2 mM) were incubated with ^{32}P -labelled DENV 3'UTR (left) or 3'SL (right) and separated on a native 4.5% polyacrylamide gel by EMSA. 'Probe-PABP', DENV 3'UTR-PABP complex. (b) Interaction of ^{32}P -labelled DENV 3'UTR with increasing amounts of PABP was measured by filter binding. Dashed line illustrates the fitted binding isotherm; error bars represent SEM ($n=2-4$ for each PABP concentration). (c) Schematic diagram [adapted from Proutski *et al.* (1997) and Olsthoorn & Bol (2001)] of the DENV 3'UTR depicting conserved regions (3'SL, DB1 and DB2) targeted for deletion in the mutant constructs (dashed lines) and the DB1 + 2 construct (solid line). (d) The specificity of the PABP-DENV 3'UTR interaction was determined by adding increasing amounts (1-, 10- and 25-fold molar excess) of RNA competitors 3'UTR (positive control), 3' Δ SL or 3'SL (upper panel), and 3' Δ DB2, 3' Δ DB1, 3' Δ DB1 + 2 or DB1 + 2 (lower panel). Complexes were separated on a 4.5% polyacrylamide gel. 'Probe-PABP', DENV 3'UTR-PABP complexes; 'Probe', free probe. All data shown in Figs 1-3 are representative of at least two experiments. d.u., Density units.

PABP has been directly implicated in enhancing translation of viral mRNAs. Bradrick *et al.* (2007) showed that viruses with different IRES exhibit differential requirements for PABP. The more dependent translation from polyadenylated IRES-containing constructs was on the presence of the poly(A) tail and PABP binding, the more sensitive it was to suppression of translation by Paip2. We therefore investigated the effect of Paip2 by using purified Paip2-

glutathione S-transferase (GST) to interrupt the DENV 3'UTR-PABP interaction. The Paip2-GST expression plasmid was kindly provided by S. Bradrick (Duke University, USA) and Paip2-GST was purified as described previously (Bradrick *et al.*, 2007). Increasing amounts of Paip2 were added to a constant amount of DENV 3'UTR probe and PABP. Low amounts of Paip2 did not interfere with the PABP-3'UTR interaction (Fig. 3a, lanes 3-6), but at higher concentrations (>69 nM) Paip2 disrupted the PABP-3'UTR complex and the 3'UTR-PAPB shift disappeared (Fig. 3a, lanes 7-8), which was likely replaced by the PABP-Paip2 interaction. We speculate that the weak, higher-mobility shift shown in lanes 7 and 8 could represent a DENV 3'UTR-PABP-Paip2 complex, where Paip2 is bound to the C-terminal end of PABP, which is still bound to the DENV RNA via the N-terminal RRM site. Paip2 does not interact with DENV 3'UTR alone, as no shift was detected in the absence of PABP even with the highest Paip2 concentration used (Fig. 3a, lane 9).

The disruption of DENV 3'UTR-PABP interaction by Paip2 indicates that PABP may regulate DENV translation and cellular translation in a similar manner. We previously developed an *in vitro* translation assay using baby hamster kidney (BHK) cell extract [cells lysed in 100 mM HEPES pH 7.5, 120 mM KOAc, 2.5 mM MgOAc, 100 mM sucrose, 1 mM DTT and 11% glycerol (v/v), supplemented with 1 mM PMSF, 10 μM leupeptin and 0.2 μM aprotinin], where translation from DENV luciferase reporter RNAs can be assessed (Edgil *et al.*, 2003). Here, we used a m⁷G-capped DENV reporter RNA containing the DENV2

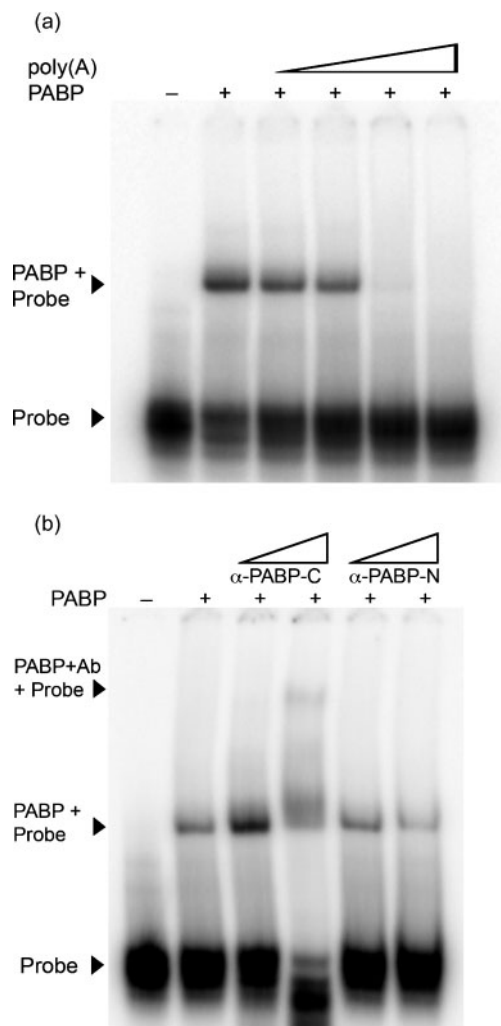


Fig. 2. The DENV 3'UTR-PAPB interaction is disrupted by poly(A) RNA and is specifically recognized by anti-PABP-C terminus antibodies. (a) The DENV 3'UTR-PAPB complex is disrupted by increasing amounts of poly(A) RNA (0.1-, 0.2-, 0.5- and 1-fold molar excess over the DENV 3'UTR) in EMSA. 'PABP+Probe', DENV 3'UTR-PAPB complex; 'Probe', free probe. (b) The DENV 3'UTR-PAPB complex can be supershifted in EMSA with antibody against the PABP-C terminus ('PABP+Ab+Probe') but not the PABP-N terminus. Anti-PABP-C antibody was assayed at 25 and 125 $\mu\text{g ml}^{-1}$ and anti-PABP-N antibody at 12.5 and 62.5 $\mu\text{g ml}^{-1}$. Despite the addition of an RNase inhibitor, minor probe degradation is nevertheless noticeable upon addition of higher amounts of anti-PABP-C antibody.

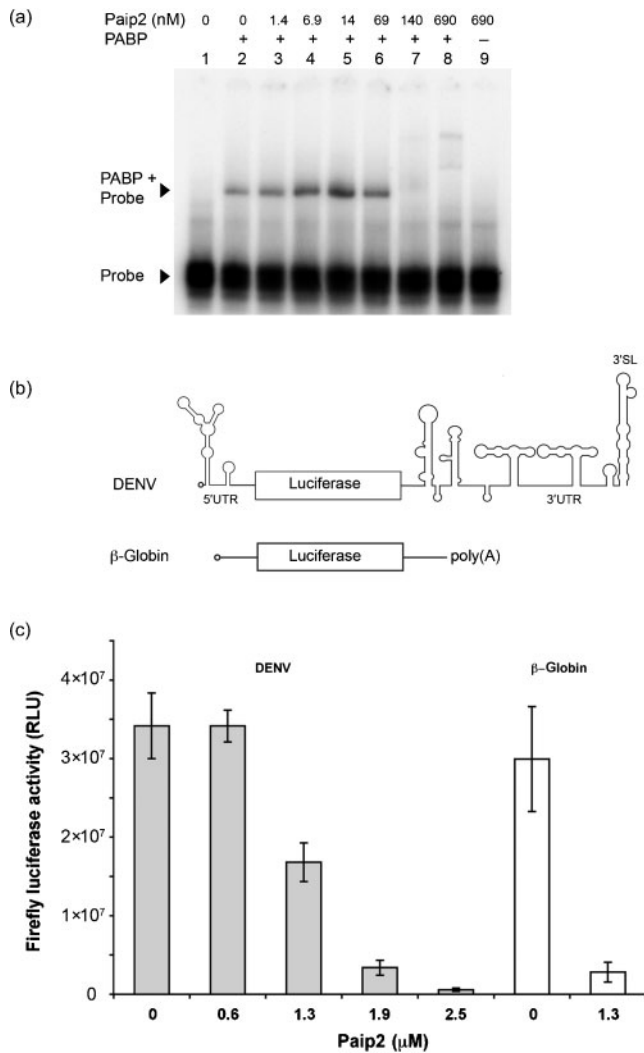


Fig. 3. Paip2 functionally interferes with the DENV 3'UTR-PABP interaction. (a) The effect of increasing amounts of Paip2 on the DENV 3'UTR-PABP interaction was assessed by EMSA in the presence or absence of 0.37 μ M PABP. Higher concentrations of Paip2 disrupt the 3'UTR-PABP interaction (lanes 7-8). Paip2 alone does not bind the 3'UTR directly (lane 9). (b) Schematic overviews of the DENV-luciferase reporter RNA (top) with the DENV 5'UTR, 3'UTR and 3'SL indicated flanking the firefly luciferase-coding region and the β -globin reporter (bottom), with the luciferase gene between the β -globin 5'UTR and poly(A) tail. Circles at the 5' end indicate the m⁷G-cap. (c) Paip2 interferes with DENV luciferase reporter RNA (grey bars) and β -globin reporter RNA (white bars) translation *in vitro*. Each reporter RNA was added to BHK extracts in the presence of increasing amounts of Paip2 and assayed for expression of firefly luciferase after 90 min of translation at 30 $^{\circ}$ C.

5'UTR followed by the first 72 nt of the viral capsid gene fused to the firefly luciferase gene and the DENV2 3'UTR to provide all the necessary elements for DENV translation (Fig. 3b, top). Increasing amounts of Paip2 added to the BHK extract inhibited translation of the DENV luciferase

reporter in a dose-dependent manner (Fig. 3c), confirming the importance of PABP in DENV translation. A β -globin reporter RNA containing the 5'UTR of β -globin, followed by the firefly luciferase gene and a poly(A) tail (Fig. 3b, bottom) was used as a positive control for translation inhibition by Paip2 (Fig. 3c).

Here, we have demonstrated the biochemical affinity of PABP for the DENV 3'UTR. The specific interaction occurs upstream of the 3'SL, likely involving the A-rich sequences that flank the two DB structures. To the best of our knowledge, no other viruses or mRNAs lacking a poly(A) tail have been reported to use PABP to enhance translation. Thus, this interaction is distinct from the 3'-terminal poly(A) interaction typical of cellular and some viral mRNAs, but can nonetheless be interrupted by poly(A) RNA. The affinity of PABP binding to the DENV 3'UTR and to a poly(A) tail was not directly compared in this study, but the latter has been determined previously and appears to have a higher affinity than that of the PABP-DENV 3'UTR interaction (Görlach *et al.*, 1994; Sladic *et al.*, 2004). Addition of the cellular translation inhibitor and PABP-binding partner Paip2 disrupted the PABP-DENV 3'UTR interaction as assessed by EMSA and also inhibited translation of DENV reporter RNAs as well as a β -globin reporter control RNA *in vitro*. Thus, PABP could serve as the bridging factor for circularization of DENV RNA and translation enhancement, as DENV appears to require eIF4G (C. Polacek & E. Harris, unpublished results). Overall, our data suggest that PABP is a universal translation factor that plays a role in DENV translation machinery despite the lack of viral poly(A) tail.

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