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Translation Elongation Factor-1α, La, and PTB Interact with the 3′ Untranslated Region of Dengue 4 Virus RNA

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The 384-nt long 3′ untranslated region (3′UTR) of dengue 4 virus (DEN4) is not polyadenylated, but contains the adjacent thermodynamically stable conserved short and long stem-loop structures (L-SL) and the conserved sequences CS1 and CS2. The latter are duplicated (CS2A and CS2B) in DEN4. Dengue virus replication, like that of other RNA viruses, might involve the *cis*-elements located within the 3′UTR and the *trans*-acting factors that could interact with the viral replicase to function as a replicase complex. The identification and characterization of viral and cellular proteins involved in the interaction with the 3′UTR of dengue virus will help us to understand the cellular requirements for viral replication. To determine these requirements, mobility shift and cross-linking assays were performed with uninfected and DEN4-infected C6/36 cell extracts as well as the different segments of the 3′UTR. Our results revealed that RNA–protein complexes were formed with the RNAs which involved the domains CS2A, CS2B, CS1, and L-SL. The minimum RNA sequence that was able to form specific and stable complexes with cellular proteins was the CS1–L-SL region. Using UV-induced cross-linking we identified eight proteins with molecular weights of 34, 39, 51, 52, 56, 62, 72, and 84 kDa that bound to the complete 3′UTR. The translation elongation factor-1α (EF-1α) bound to the complete 3′UTR and to the CS1–L-SL region. In addition, the recombinant GST–human La autoantigen bound to the 3′UTR and to the CS1–L-SL region as demonstrated by mobility shift and cross-linking assays. Although different antibodies against PTB were unable to react with any of the cellular proteins from C6/36, the recombinant His-PTB protein did bind to the complete 3′UTR and to the CS1–L-SL region. The specific binding of La and PTB to the sequences considered essential for viral RNA replication may suggest that these proteins could function as RNA chaperones to maintain RNA structure in a conformation that favors viral replication, while EF-1α may function as an RNA helicase.
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INTRODUCTION

Dengue viruses, the mosquito-borne members of the *Flaviviridae* family, are the causative agents of dengue fever (DF) and its associated complications dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Both DHF and DSS are endemic in tropical and subtropical areas of the world.

The dengue virus genome is composed of an 11-kb single-stranded RNA of positive polarity containing a unique open reading frame (ORF) that encodes a polyprotein arranged in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. Structural proteins are processed from the precursor polyprotein by a host signal peptidase associated with the endoplasmic reticulum (Markoff, 1989; Nowak *et al.,* 1989), while the processing of nonstructural proteins is catalyzed by the viral serine protease NS2B/NS3 during and after translation (Chambers *et al.,* 1990, 1991; Clum *et al.,* 1997; Falgout *et al.,* 1991). The ORF is flanked by two untranslated regions (UTRs) that contain a type I cap structure at the 5′-end and lack the poly(A) tract at the 3′-end. The 5′ and 3′UTRs from Dengue 4 virus (DEN4) are 101 and 384 nt long, respectively. Both regions must include the stem-loop (SL) structures that act as promoter elements involved in full-length RNA synthesis, as has been described for other RNA viruses (Brinton and Dispoto, 1986; Lai, 1998; Zeng *et al.,* 1998). The SL structures are highly conserved among flaviviruses, but those located in the 3′UTR seem to be more stable. Positive-polarity RNA viruses use the genomic 3′UTR as promoter for negative-strand RNA synthesis. Within the DEN4 3′UTR some important sequences involved in viral replication have been identified, including a long and short SL structure (L-SL) located in the last 98 nt and conserved sequences called CS1, CS2A, and CS2B (see Fig. 1) (Men *et al.,* 1996). Partial or total deletion of the conserved sequences CS2A or CS2B reduces viral replication without loss of viability. In contrast, deletion of some nucleotides in the conserved sequence CS1 or the L-SL produces a nonviable virus, suggesting that these regions are essential in viral replication (Men *et al.,* 1996). A similar

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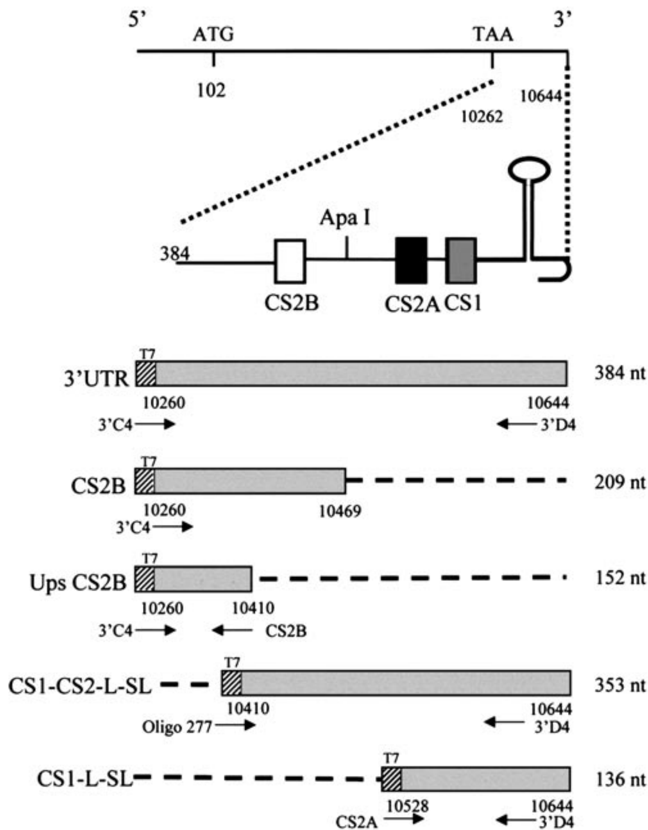

FIG. 1. Schematic representation of the 3′UTR from DEN4 and the different fragments used to produce truncated RNAs. The complete 3′UTR consisting of nt 10,260 to 10,644, CS2B consisting of nt 10,260 to 10,469, ups-CS2B consisting of nt 10,260 to 10,410, the CS1–CS2–L-SL region consisting of nt 10,410 to 10,644, and CS1–L-SL consisting of nt 10,528 to 10,644 RNAs were obtained after *in vitro* transcription of each PCR product. Oligonucleotides and length of each RNA are indicated. Dotted line indicates deleted sequences.

effect has also been observed with several deletions in the 3′UTR of other flaviviruses, such as tick-borne encephalitis and Kunjin viruses (Khromykh and Westaway, 1997; Mandl *et al.,* 1998). When the deletions were superimposed onto the models of the secondary structure for the 3′UTR for each virus, it was found that the overall structural integrity of the flavivirus 3′UTR was essential for optimal performance of its promoter function (Proutski *et al.,* 1999). The most 3′-terminal structures and sequences seem to be critical for the initiation of minus-strand RNA synthesis, while the more proximal structures possibly function as enhancers for viral RNA replication.

In several RNA viruses, viral replication requires the interaction of some structural elements located in the 3′UTR with cellular and viral proteins or with elements present in the 5′UTR (Barton *et al.,* 2001; Lai, 1998; You and Padmanabhan, 1999; You *et al.,* 2001). It has been suggested that the ribonucleoprotein complexes formed in the 3′UTRs are necessary for template-specific RNA-dependent RNA synthesis (Lai, 1998).

Interaction of some viral proteins with the 3′UTR(+) of some flaviviruses has been reported. For instance, NS5 and NS3 have been proposed as components of the RNA replicase for several flaviviruses (Kapoor *et al.,* 1995; Chen *et al.,* 1997) and have been found to be associated with the 3′UTR of Japanese encephalitis (JE) virus (Chen *et al.,* 1997). Thus, the 3′-SL sequence of JE virus competes with the full-length JE virus RNA for binding to the viral RNA-dependent RNA polymerase NS5. In addition, the recombinant NS3 protein from dengue 1 virus can form a complex with the 3′-SL structure of the 3′UTR, although other sites may also participate in binding.

On the other hand, the 3′UTR can also interact with cellular factors, as in the case of RNA transcripts of West Nile virus containing the 3′-SL, that bind the elongation factor-1α (EF-1α) and two as yet uncharacterized proteins of 84 and 105 kDa (Blackwell and Brinton, 1995). Translation factor EF-1α has also been found interacting with the 3′UTR of other RNA viruses such as vesicular stomatitis virus (Das *et al.,* 1998), poliovirus (Harris *et al.,* 1994) and turnip yellow mosaic virus (Joshi *et al.,* 1986).

RNA replication of dengue virus might depend on both *cis-* and *trans*-acting elements. The *cis*-elements, located within the 3′UTR, may act as promoters and enhancers during RNA synthesis (Men *et al.,* 1996; Proutski *et al.,* 1999), while *trans*-acting factors may interact with the viral replicase to function as the replicase complex. It is very likely that host factors are active participants in the process of RNA-dependent RNA synthesis. Thus, the identification and characterization of viral and cellular proteins involved in dengue virus replication will elaborate on the pathogenesis, virulence, and tropism of this virus. To determine the ability of cellular proteins to bind to the 3′UTR, mobility shift assays with uninfected and infected C6/36 cell extracts were performed.

Our results indicate that RNA–protein complexes were formed with the RNAs which contain the domains CS2A and CS1 and the L-SL element. The minimum RNA sequence that was able to form specific and stable complexes with cellular proteins was the CS1–L-SL region. Using UV-induced cross-linking we identified eight proteins from C6/36 cells with molecular weights of 34, 39, 51, 52, 56, 62, 72, and 84 kDa that bound to the complete 3′UTR. One of the proteins, identified as the translation factor EF-1α by supermobility shift assay, was able to bind to the complete 3′UTR and to the CS1–L-SL. In addition, the recombinant GST–human La autoantigen was also able to bind to both regions as demonstrated by mobility shift and cross-linking assays. Finally, although different antibodies against PTB were unable to react with any of the cellular proteins from C6/36, the recombinant His-PTB protein did bind to the complete 3′UTR and to the CS1–L-SL regions.

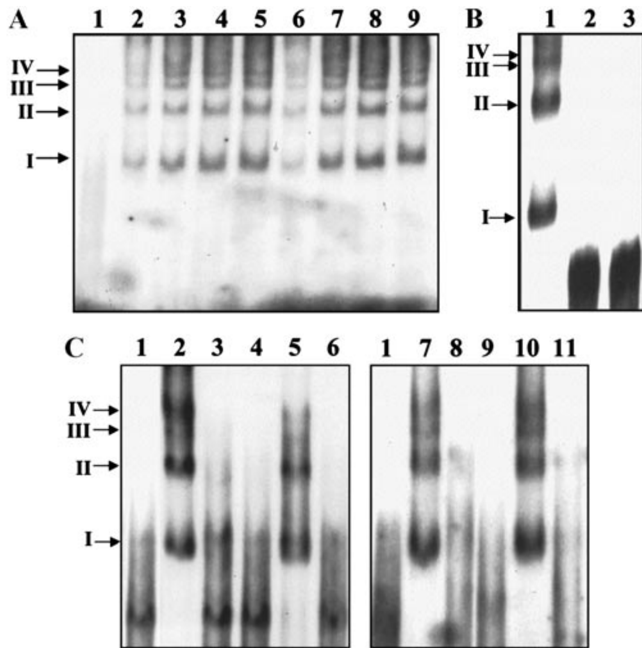

FIG. 2. Mobility shift assays of the 3′UTR RNA from DEN4 and S10 extract from C6/36 cells. (A) Labeled RNA of the complete 3′UTR (nt 10,260 to 10,644) incubated without (lane 1) or with 5 μg of S10 extracts from uninfected (lanes 2–5) or infected C6/36 cells (lanes 6–9) in the presence of 600 (lanes 3 and 7), 900 (lanes 4 and 8), and 1200 (lanes 5 and 9) mM KCl. (B) Labeled RNA of nt 10,260 to 10,644 of DEN4 was incubated with 5 μg of S10 extract from uninfected C6/36 cells (lanes 1–3) and in the absence (lane 1) or in the presence of 2 μg/ml of proteinase K (lanes 2 and 3). Treatment with proteinase K was performed for 15 min at 37°C before (lane 2) or after (lane 3) the addition of labeled RNA. (C) Labeled RNA of nt 10,260 to 10,644 of DEN4 was incubated without extract (lane 1) or with 5 μg of S10 extract from uninfected (lanes 2–6) or infected C6/36 cells (lanes 7–11) in the absence of competitor (lanes 2 and 7) or in the presence of a 20-fold molar excess of unlabeled homologous competitor (RNA from nt 10,260 to 10,644) (lanes 3 and 8), CS2B RNA consisting of nt 10,260 to 10,469 (lanes 4 and 9), ups-CS2B RNA consisting of nt 10,260 to 10,410 (lanes 5 and 10), and CS1–L-SL regions consisting of nt 10,528 to 10,644 (lanes 6 and 11). Complex formation was assayed by electrophoresis on native polyacrylamide gels and detected by autoradiography. Mobility of complexes is indicated on the left side.

RESULTS

C6/36 cellular proteins interact with the DEN4 virus 3′UTR

To determine if the 3′UTR from DEN4-bound cellular proteins is present in mosquito cells and/or viral proteins, mobility shift assays were performed using a ^{32}P-labeled RNA transcript from nt 10,260 to 10,644 (including the complete 3′UTR) and S10 extracts from uninfected and DEN4-infected C6/36 cells. Four similar RNA–protein complexes were formed with uninfected and infected C6/36 cell extracts (Fig. 2A, lanes 2 and 6, respectively). Complexes I and II revealed stronger RNA labeling than complexes III and IV.

In order to determine the stability of the RNA–protein complexes, the samples were incubated with various concentrations of KCl. The four complexes formed with both uninfected and infected C6/36 S10 extracts were not altered in the range 600–1200 mM KCl (Fig. 2A, lanes 3–5 and 7–9, respectively); however, the intensity of the four complexes increased in the presence of KCl.

The four complexes observed with both uninfected and infected C6/36 cell extracts were ribonucleoproteins. This observation was corroborated by the susceptibility of the complexes to proteinase K. Proteinase K treatment after (Fig. 2B, lane 3) or before addition of labeled RNA (Fig. 2B, lane 2) inhibited the formation of the four RNA–protein complexes when compared to a similar reaction without proteinase K treatment (Fig. 2B, lane 1).

The specificity of the interactions between the complete DEN4 3′UTR and uninfected and infected C6/36 extracts was analyzed in competition experiments using a 20-fold molar excess of four different unlabeled RNA fragments as competitors. The four RNA–protein complexes formed with both uninfected (Fig. 2C, lanes 2–6) and infected (Fig. 2C, lanes 7–11) C6/36 extracts showed a significant reduction when samples were incubated in the presence of unlabeled RNAs containing the complete 3′UTR (Fig. 2C, lanes 3 and 8), CS2B (Fig. 2C, lanes 4 and 9), and the CS1–L-SL regions (Fig. 2C, lanes 6 and 11) but not with the unlabeled ups-CS2B RNA (Fig. 2C, lanes 5 and 10). These data strongly suggest that proteins from C6/36 cells bound specifically to the 3′UTR RNA from DEN4. Even though the region ups-CS2B is included in the 3′UTR it was noted to be an inefficient competitor, suggesting that the interaction took place only between nt 10,410 and 10,644.

To corroborate whether this region is indeed the major protein binding site, a mobility shift assay was performed using labeled RNA from nt 10,410 to 10,644 (CS1–CS2–L-SL). Three RNA–protein complexes were formed with 5 μg of uninfected (Fig. 3A, lane 2) and infected C6/36 cell extracts (Fig. 3A, lane 3). Complexes I and II revealed stronger RNA labeling than complex III. Infected cell extracts showed a stronger signal for complex I than uninfected C6/36 cell extracts.

Among the RNAs used in competition assays, the CS1–L-SL RNA containing the conserved stem-loop structure critical for dengue virus viability was the best competitor. To analyze if the double stem-loop structure was a target sequence for the protein interactions, ^{32}P-labeled CS1–L-SL RNA (nt 10,528 to 10,644) was incubated with S10 extract from uninfected and infected C6/36 cells. Despite the absence of CS2A and CS2B domains, our results indicate that the CS1 and L-SL domains alone were sufficient for the formation of three RNA–protein complexes with both uninfected and infected C6/36 cell extracts (Fig. 3B, lanes 2 and 5, respectively). Complexes I and II revealed stronger RNA labeling than complex III.

The stability of the CS1–L-SL RNA–protein complexes

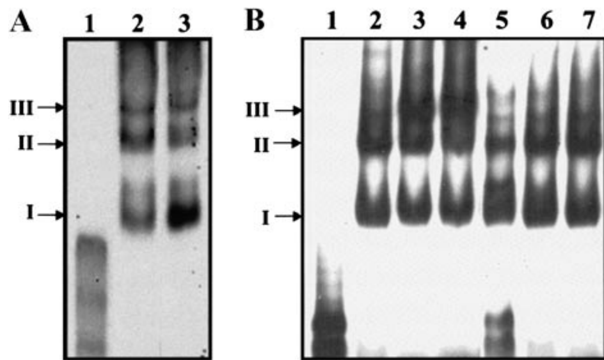

FIG. 3. Mobility shift assays of the CS1–CS2–L-SL and CS1–L-SL regions of RNA from DEN4 and S10 extract from C6/36 cells. (A) Labeled RNA consisting of nt 10,410 to 10,644 (CS1–CS2–L-SL region) was incubated without extract (lane 1) or with 5 μg of S10 extract from uninfected (lane 2) or DEN4-infected C6/36 cell extract (lane 3). (B) Labeled RNA consisting of nt 10,528 to 10,644 was incubated without extract (lane 1) or with 5 μg of S10 extract from uninfected (lanes 2–4) or DEN4-infected C6/36 cells (lanes 5–7), in the presence of 600 (lanes 3 and 6) and 1200 mM KCl (lanes 4 and 7). Complex formation was assayed by electrophoresis on native polyacrylamide gels and detected by autoradiography. Mobility of complexes is indicated on the left side.

was analyzed by their resistance to various KCl concentrations. The three complexes were not altered in the range 0–1200 mM KCl (Fig. 3B, lanes 2–4 and 5–7), although an increased intensity of complex III formed with uninfected C6/36 cell extracts was observed in the presence of the salt.

Identification of cellular proteins present in the RNA–protein complexes

To identify the cellular proteins present in the RNA–protein complexes, labeled DEN4 3′UTR (nt 10,260 to 10,644) was incubated with uninfected and infected C6/36 extracts, UV cross-linked, and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Eight proteins with apparent molecular weights of 34, 39, 51, 52, 56, 62, 72, and 84 kDa were detected in both uninfected and infected C6/36 cell extracts (Fig. 4A, lanes 1 and 2, respectively). However, the relative intensity of individual proteins was stronger in uninfected cell extracts (Fig. 4A, lane 1).

To identify specific regions within the 3′UTR of DEN4 responsible for the RNA–protein interactions, RNA probes of nt 10,410 to 10,644 and nt 10,528 to 10,644 (double stem-loop) were used in the UV cross-linking assays. The RNA probe from nt 10,410 to 10,644 bound to similar proteins as the complete 3′UTR; however, the relative intensity of individual proteins was different. The 34-, 39-, 51-, and 52-kDa proteins showed relative intensities similar to those cross-linked with the complete 3′UTR, while the 56-, 62-, 72-, and 84-kDa proteins showed lower relative intensities (Fig. 4B, lanes 1 and 2), suggesting that most of the RNA–protein interactions occur within nt 10,410 to 10,644 of DEN4. Additionally, the relative intensity of individual proteins was stronger in infected cell extracts than in uninfected cell extracts. This result correlates with the fact that infected cell extracts showed a stronger signal in mobility shift assays than those observed with uninfected C6/36 cell extract.

When a shorter probe was used (nt 10,528–10,644), the relative intensities of the 34-, 39-, 51-, and 52-kDa proteins from both uninfected and infected C6/36 cells were significantly stronger than the 56-, 62-, 72-, and 84-kDa proteins (Fig. 4C, lanes 1 and 2).

Interaction of EF-1α with the 3′UTR of DEN4 virus RNA

Cellular proteins of 57/60, 52, 50, and 39 kDa have been reported to bind viral RNAs of picornavirus, hepa-

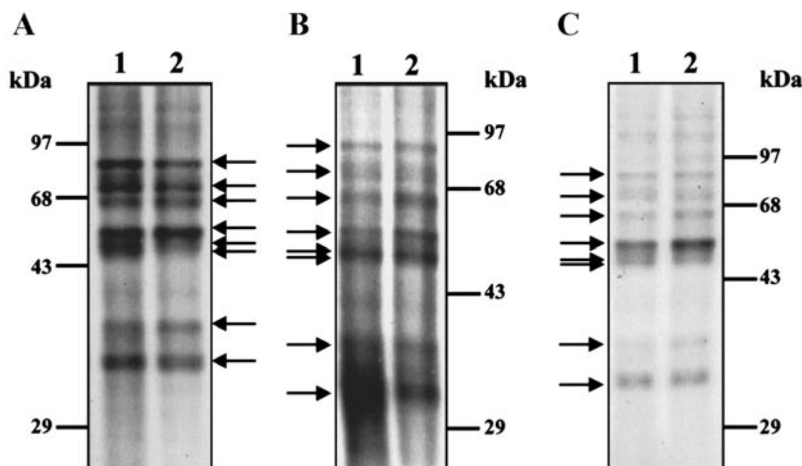

FIG. 4. UV-induced cross-linking of C6/36 S10 extracts to labeled RNAs. Labeled RNAs of nt 10,260 to 10,644 (A) or nt 10,410 to 10,644 (B) or nt 10,528 to 10,644 of DEN4 (C) were incubated with 60 μg of S10 extract from uninfected (lanes 1) or DEN4-infected C6/36 cells (lanes 2). Proteins were separated by 10% SDS–PAGE and detected by autoradiography. Arrows indicate cross-linked proteins with the molecular masses in kilodaltons.

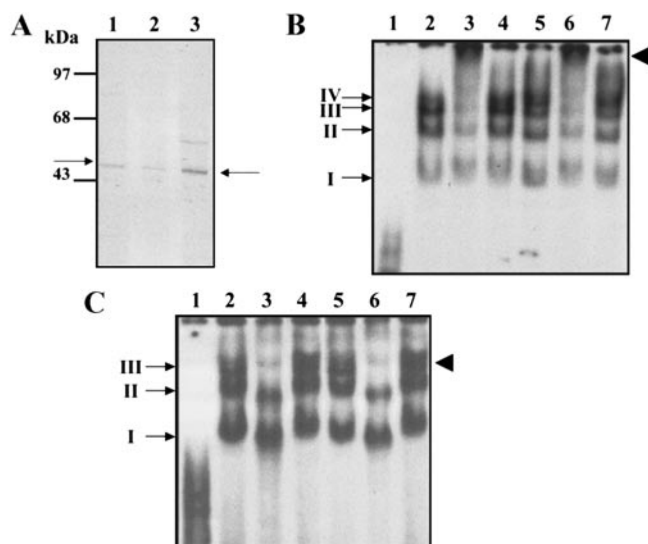

FIG. 5. Mobility supershift assay with an anti-EF-1α antibody. (A) Western blot assay with polyclonal antibodies against EF-1α protein. Thirty micrograms of uninfected and infected C6/36 (lanes 1 and 2, respectively) or HeLa cell S10 extracts (lane 3) were run on SDS–PAGE, transferred to nitrocellulose membrane, and incubated with polyclonal antibodies against EF-1α protein, diluted 1:100. Color was developed with BCIP and NBT. Labeled RNAs of nt 10,260 to 10,644 (B) or nt 10,528 to 10,644 of DEN4 (C) were incubated without (lanes 1) or with 5 μg of S10 extract from uninfected (lanes 2–4) or infected C6/36 cells (lanes 5–7) in the absence (lanes 2 and 5) or in the presence of anti-EF-1α (lanes 3 and 6) or anti-actin (lanes 4 and 7) antibodies. Complex formation was assayed by electrophoresis on native polyacrylamide gels and detected by autoradiography. Mobility of complexes is indicated on the left.

titis C, and other flaviviruses. These proteins, identified as PTB, La, EF-1α, and PCBP2, have essential roles in viral translation and replication (Blackwell and Brinton, 1997; Blyn *et al.,* 1997; Hahm *et al.,* 1998; Hellen *et al.,* 1993; Ito and Lai, 1999; Kaminski *et al.,* 1995; Luz and Beck, 1991; Meerovitch *et al.,* 1993; Svitkin *et al.,* 1994). Because of their similarities to the molecular weights of proteins detected in this study, the presence of EF-1α, La, and PTB in the C6/36 cell extracts was investigated. To determine whether EF-1α binds to the DEN4 3′UTR RNA, a supermobility shift assay was performed using polyclonal anti-EF-1α antibodies (kindly provided by W. Merrick). Initially, the ability of the anti-EF-1α antibody to react with the translation factor present in the mosquito cells was analyzed by Western blot. The anti-EF-1α antibody reacted with a 50-kDa protein present in uninfected and infected C6/36 cell extracts (Fig. 5A, lanes 1 and 2, respectively). The anti-EF-1α antibody also reacted with a molecule of approximately the same molecular weight (50 kDa) in the HeLa cell extract (Fig. 5A, lane 3) and to a lesser extent with a 60-kDa protein. Since EF-1α has a molecular weight of 50–52 kDa, the detection of the 50-kDa protein in our extracts indicated that this factor was present in the mosquito extracts and was detected using the polyclonal antibody. Incubation of anti-EF-1α antibodies with uninfected (Fig. 5B, lanes 2–4) and infected cell extracts (Fig. 5B, lanes 5–7) before the addition of labeled 3′UTR RNA resulted in the formation of a fifth complex with less mobility (indicated with an arrowhead) and in the reduction of the radioactive signal present in complexes II, III, and IV (Fig. 5B, lanes 3 and 6). These effects were not observed in the absence of antibodies (Fig. 5B, lanes 2 and 5) or in the presence of an anti-actin control antibody (Fig. 5B, lanes 4 and 7).

To further investigate if EF-1α cross-linked to the CS1–L-SL region, a supermobility shift assay was performed with labeled RNA from nt 10,528 to 10,644. Incubation of anti-EF-1α antibody with uninfected (Fig. 5C, lanes 2–4) and infected C6/36 extracts (Fig. 5C, lanes 5–7) before the addition of labeled RNA resulted in a significant reduction of complex III formation (Fig. 5C, lanes 3 and 6), compared to the complexes formed in the absence of antibodies (Fig. 5C, lanes 2 and 5) or in the presence of an anti-actin antibody (Fig. 5C, lanes 4 and 7). These results indicate the presence of EF-1α in the CS1–L-SL region, within the complete 3′UTR.

Interaction of La protein with the 3′UTR of DEN4 virus RNA

The mosquito homologue of the La autoantigen obtained from C6/36 cells (Pardigon and Strauss, 1996) has a molecular weight similar to that of the 50-kDa protein cross-linked to the complete 3′UTR and CS1–L-SL region. Therefore, as a first step, polyclonal antibodies directed against human La protein were used to detect this molecule in S10 extracts from uninfected and infected C6/36 cells by Western blot, using S10 extract from Vero cells as positive control. Human anti-La antibody detected a 52-kDa protein in Vero cell extract (Fig. 6A, lane 1) while a 49- to 50-kDa protein was detected in uninfected and infected C6/36 cell extracts (Fig. 6A, lanes 2 and 3, respectively). An additional band of 40 kDa was also detected in mosquito cells. Although anti-La antibodies directed against human La protein were able to detect the La protein from mosquito tissues, they were inefficient at supershifting the ribonucleoprotein complexes formed with mosquito cell extracts (data not shown). Then we decided to analyze the ability of La protein to bind to DEN4 3′UTR RNA, using mobility shift and cross-linking assays with the recombinant GST–human La protein. The addition of 1 and 5 μg of GST–La protein to the complete 3′UTR (Fig. 6B) or to the CS1–L-SL RNAs (Fig. 6C) in mobility shift assays induced the formation of an RNA–protein complex (Figs. 6B and 6C, lanes 4 and 5), while a similar assay with the same concentration of GST protein alone (Figs. 6B and 6C, lanes 2 and 3) did not, suggesting that the 3′UTR contains elements that interact with La protein.

To corroborate the interaction of La protein with the 3′UTR and the CS1–L-SL region, UV cross-linking assays

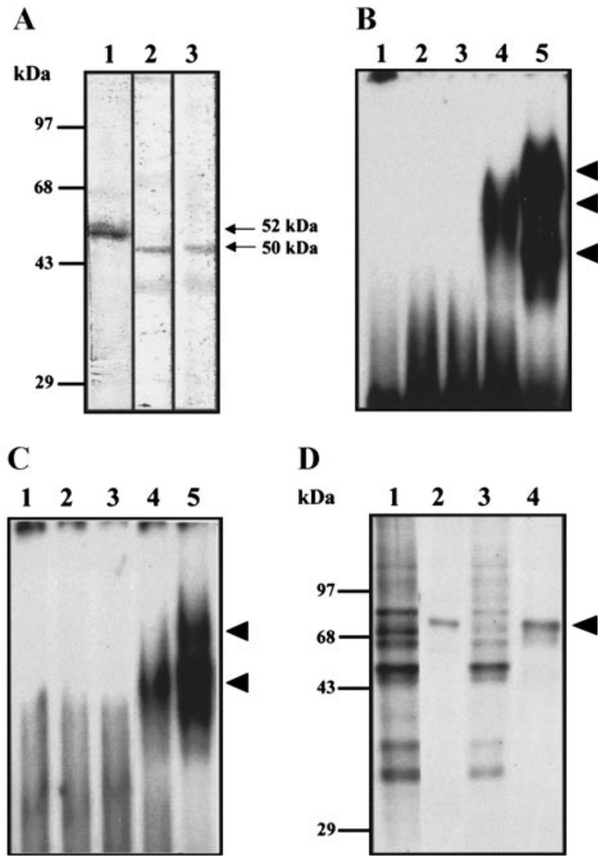

FIG. 6. Mobility shift and UV-induced cross-linking with the recombinant La protein. (A) Western blot assay with polyclonal antibodies against La protein. Thirty micrograms of Vero cell (lane 1) and uninfected and infected C6/36 cell (lanes 2 and 3, respectively) S10 extracts were run on SDS–PAGE, transferred to nitrocellulose membrane, and incubated with polyclonal antibodies against La protein, diluted 1:1000. Color was developed with BCIP and NBT. Interaction between recombinant GST–La protein and RNA. Labeled RNAs consisting of nt 10,260 to 10,644 (B) or 10,528 to 10,644 (C) were incubated without extract (lanes 1), with 1 and 5 μg of GST protein (lanes 2 and 3, respectively), or with 1 and 5 μg of GST–La protein (lanes 4 and 5, respectively). (D) Labeled RNAs of nt 10,260 to 10,644 (lanes 1 and 2) or nt 10,528 to 10,644 of DEN4 (lanes 3 and 4) were incubated with 60 μg of S10 extract from infected C6/36 cells (lanes 1 and 3) or with 5 μg of GST–La protein (lanes 2 and 4). Proteins were separated by 10% SDS–PAGE and detected by autoradiography. Arrows indicate the complex formed with GST–La protein and the recombinant protein cross-linked to viral RNA.

with GST–La protein were performed. The GST–La protein cross-linked with both the complete 3′UTR (Fig. 6D, lane 2) and the CS1–L-SL labeled RNAs (Fig. 6D, lane 4). As one would expect due to the GST moiety, the molecular weight of the cross-linked GST–La protein was 76 kDa and not 51 kDa. The proteins that cross-linked to the complete 3′UTR (Fig. 6D, lane 1) and to the CS1–L-SL region (Fig. 6D, lane 3) were also shown.

Interaction of PTB with the 3′UTR of DEN4 virus RNA

The 56-kDa protein from C6/36 cells that cross-linked to the DEN4 3′UTR resembles the molecular weight of PTB protein that binds the UTR from several other RNA viruses (Gutiérrez-Escolano *et al.,* 1997; Hahm *et al.,* 1998; Hellen *et al.,* 1993, 1994; Jang and Wimmer, 1990; Kaminski *et al.,* 1995; Luz and Beck, 1991; Rojas-Eisenring and del Angel, 1995). In addition, one of the target binding sites for PTB, UCUAA, is present within the DEN 3′UTR, specifically in the L-SL region. To investigate the presence of PTB in C6/36 cells, a Western blot assay was performed using monoclonal antibodies against the amino- and carboxy-terminal regions of human PTB. These antibodies were unable to react with any of the protein in C6/36 cell extracts (Fig. 7A, lane 3) while the doublet of 57/60 kDa present in HeLa cell extracts and the 62-kDa recombinant His-PTB protein were clearly detected (Fig. 7A, lanes 2 and 1, respectively). The presence of a histidine tract caused an increase in the molecular weight of the His-PTB recombinant protein compared with the native PTB isoforms present in HeLa cells. Several polyclonal antibodies against PTB were also used to identify PTB in C6/36 cells without success (data not shown). However, due to the importance of the PTB factor in the translation and replication of several RNA viruses and the presence of a putative binding site for PTB within the DEN4 3′UTR RNA, we decided to further analyze if the DEN4 3′UTR was able to interact with PTB, using a UV cross-linking assay with the His-PTB recombinant protein. The complete 3′UTR and the CS1–L-SL region were able to cross-link with the 62-kDa recombinant protein (Fig. 7B, lanes 2 and 4, respectively), indi-

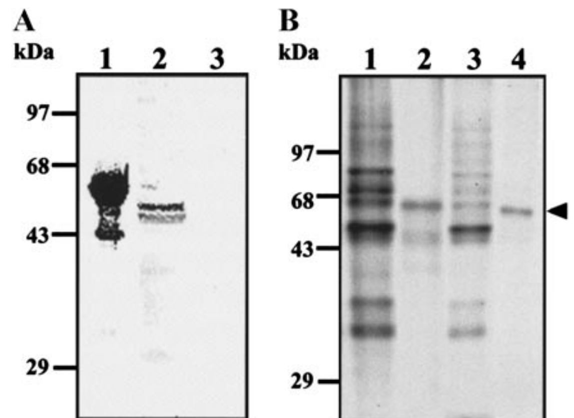

FIG. 7. Interaction between recombinant His-PTB protein with RNA of DEN4. (A) Western blot assay with polyclonal antibodies against human PTB protein. Ten micrograms of the recombinant protein His-PTB (lane 1) and 50 μg of HeLa cell (lane 2) and 50 μg of C6/36 cell (lane 3) S10 extracts were run on SDS–PAGE, transferred to nitrocellulose membrane, and incubated with polyclonal antibodies against human PTB protein, diluted 1:100. Color was developed with BCIP and NBT. (B) Labeled RNAs of nt 10,260 to 10,644 (lanes 1 and 2) or nt 10,528 to 10,644 of DEN4 (lanes 3 and 4) were incubated with 60 μg of S10 extract from infected C6/36 cells (lanes 1 and 3) or with 500 ng of His-PTB protein (lanes 2 and 4). Proteins were separated by 10% SDS–PAGE and detected by autoradiography. Arrows indicate the recombinant protein cross-linked to viral RNA.

cating that a PTB binding site is present within both regions.

DISCUSSION

Replication in several RNA viruses requires *cis*- and *trans*-acting elements. Some of the *cis*-elements are located within the 3′UTR and other are also present within the 5′UTR and the coding regions (You and Padmanabhan, 1999). The integrity of the structures present in the 3′UTR of flavivirus family members is essential for optimal performance of its promoter function (Zeng *et al.,* 1998). The most 3′-terminal structures and sequences, which include the L-SL and CS1 domains, have been considered critical for the initiation of minus-strand RNA synthesis, while the more proximal structural elements including the CS2A and CS2B domains may function as enhancers for viral RNA replication. Additionally, *in vitro* RNA synthesis from DEN requires both 5′ and 3′UTR to be present in the same or in different template molecules (You and Padmanabhan, 1999; You *et al.,* 2001).

The *trans*-acting elements involved in the replication of RNA viruses include some viral and cellular proteins. In flavivirus, NS5 and NS3 have been proposed as components of the RNA replicase complex (Kapoor *et al.,* 1995; Chen *et al.,* 1997); however, there is no evidence of specific host factors involved in this process.

In other RNA viruses, host factors and viral proteins interact with the viral RNA and participate in viral RNA replication (Lai, 1998). To initially characterize components that could be involved in DEN replication, we decided to first identify some cellular proteins that interact with the 3′UTR of DEN4 RNA. Analyses were performed using mobility shift and cross-linking assays with uninfected and infected C6/36 cell extracts and different segments of the 3′UTR from DEN4.

The complete 3′UTR from DEN4 was able to form stable and specific RNA–protein complexes with factors from uninfected and infected C6/36 S10 extracts. We were unable to differentiate between the complexes formed with these extracts, since the cross-linked proteins showed similar molecular weights. These results agree with those obtained with West Nile virus, another member of the flavivirus family, where no differences were found with the RNA–protein complexes or the cross-linked protein patterns formed with the 3′UTR by uninfected or infected cell extracts (Blackwell and Brinton, 1995). There are two possible explanations: first, the viral RNA might interact with cellular factors to form ribonucleoprotein complexes that in turn interact with the viral replicase in the presence of other structures or molecules, such as membranes, membrane-associated viral proteins, and/or the 5′UTR (You and Padmanabhan, 1999; You *et al.,* 2001). The second possibility is that there is no direct binding of RNA to viral proteins. The 3′UTR of JE virus interacts with different proteins from uninfected and infected HK cell extracts; NS3 and NS5 are two of the viral proteins detected in infected cell extracts (Chen *et al.,* 1997). The inability to detect viral proteins in our assays could be due to the use of S10 extracts that do not contain the membrane-associated proteins that are present in total cell extracts, such as those used with JE virus. Alternatively, our experiments were performed with C6/36 proteins obtained 4 days postinfection at a m.o.i. of 0.1, while the infected extract from BHK cells used in JE virus experiments was obtained 24 h after infection at a m.o.i. of 5. It is possible that after 4 days, a smaller amount of NS3 and NS5 is present in an S10 extract.

The cellular proteins bound specifically to the 3′UTR, since the efficiency of the interaction was affected when competed with similar 3′UTR sequence. We also found that the CS2B or CS1–L-SL regions acted as efficient competitors. The ability of both RNAs to compete for all the proteins that bound to the 3′UTR could be explained if the cellular proteins which bound to the CS2B and CS1–L-SL region could be available for the binding of additional proteins. This reaction would hinder the interaction with the remaining proteins and affect the RNA–protein complex formation.

The RNA containing the ups-CS2B domain was unable to compete with the 3′UTR, suggesting that this region may not be a target for protein binding. Competition assays suggested that the RNA–protein interaction is located within CS2A, CS2B, and CS1–L-SL sequences. This observation was confirmed by mobility shift and cross-linking assays, where the labeled RNA sequence containing the CS1, CS2A, CS2B, and L-SL regions (CS1–CS2–L-SL) bound to the same proteins that bound with the complete 3′UTR. The formation of ribonucleoprotein complexes with CS1, CS2A, and CS2B as well as the L-SL seems to confirm their critical roles in DEN4 replication (Men *et al.,* 1996). The ability of CS1–L-SL to cross-link efficiently with the eight proteins when compared with the complete 3′UTR suggests that the target domains for protein binding are contained in this region. However, the higher intensity of signals when some proteins interacted with RNA containing the CS2A and CS2B domains suggests that some of the proteins that bound to the CS1–L-SL region must have bound to the CS2B and CS2A sequences. This could explain the requirement of both sequences for efficient replication.

Since the molecular weights of 50 and 51 kDa of mosquito cell proteins were similar to some factors that interacted with other viral RNAs, supermobility shift and cross-linking assays were performed in the presence of antibodies or recombinant proteins. The presence of EF-1α, a highly conserved translation factor of approximately 50 kDa involved in the interaction with the 3′UTR of other RNA viruses (revised in Lai, 1998), has also been demonstrated by mobility shift assay. Antibodies against this factor induced two different patterns in the RNA–protein complex formation: the appearance of an addi-

tional complex of lower mobility with the complete 3′UTR and competition of complex III with the CS1–L-SL region. Both effects indicate the presence of this translation factor in the RNA–protein complexes. These effects may be explained by different changes induced by antibodies in the conformation of ribonucleoprotein complexes. EF-1α, which has been described to be associated with the RNA-dependent RNA polymerase of poliovirus (Harris *et al.,* 1994) and vesicular stomatitis virus (Das *et al.,* 1998), paradoxically is not involved in the regulation of translation of RNA viruses, since it does not bind to the translation regulation sites of the RNA virus analyzed. This factor, like other translation factors, is involved in viral replication, and its ability to interact with the structurally conserved 3′-SL regions of the genomes of divergent flaviviruses such as West Nile and dengue 2 virus (Blackwell and Brinton, 1997) supports a role for this protein in flavivirus replication. Since EF-1α is highly conserved among different eukaryotic species it is possible that this protein could be used in flaviviral replication in vertebrate and invertebrate hosts. All these data support the role of EF-1α in flaviviral replication; however, its precise role remains to be determined.

The 52-kDa La protein from mammalian cells is also associated with several viral RNAs (Meerovitch *et al.,* 1993; Rojas-Eisenring and del Angel, 1995; Svitkin *et al.,* 1994). In mosquito cells, this protein has a molecular weight of 50 kDa and could bind to Sindbis virus RNA (Pardigon and Strauss, 1996). Polyclonal antibodies against human La protein were able to detect La protein in mosquito cell extracts by Western blot, although the efficiency of reacting with the mosquito La homologue was low compared to the La protein from Vero cells. To analyze the possible interaction of La protein with the DEN4 3′UTR, the human recombinant La protein was used for mobility shift and cross-linking assays. The recombinant GST–La protein was indeed able to bind to the 3′UTR within the CS1–L-SL region.

La is a nuclear protein involved in RNA polymerase III transcription termination (Gottlieb and Stelitz, 1989). This protein has also been involved in protein transport from nucleus to cytoplasm (Meerovitch *et al.,* 1993) and the relocalization of this cellular protein from the nucleus to the cytoplasm in poliovirus-infected cells has been demonstrated (Meerovitch *et al.,* 1993). Because DEN replication occurs in membrane vesicles located in the perinuclear zone, the presence of La in this cellular structure is possible. It has been demonstrated that La has an essential role in RNA translation, in poliovirus (Meerovitch *et al.,* 1993; Svitkin *et al.,* 1994), rhinovirus (Rojas-Eisenring and del Angel, 1995), etc. However, its role in replication has not been analyzed. Although a direct biochemical role of La protein in translation remains to be demonstrated, it is postulated that La may function as an RNA chaperone to maintain RNA structure in a conformation that favors translation. Moreover, binding of La protein to the hepatitis C virus 3′UTR protects the RNA from rapid degradation, since this viral RNA and the DEN RNA do not contain a poly(A) tail (Spandberg *et al.,* 2001).

PTB, a 57/60-kDa protein that plays a role in RNA splicing (Lin and Patton, 1995), binds to multiple sites in the UTRs of several RNA viruses (Hellen *et al.,* 1994; Jang and Wimmer, 1990; Lai, 1998). Since one of the cross-linked proteins has a molecular weight of 56 kDa, we decided to analyze the presence of PTB in the 3′UTR. We were unable to demonstrate the presence of this protein in mosquito S10 extract using four different antibodies against mammalian PTB. Even though these antibodies did not react with C6/36 cells, we cannot exclude the possibility that this protein is present in mosquito cells. As PTB is a ubiquitous protein, it is possible that its concentration is very low or could have been modified in this cell line.

Within the CS1–L-SL region, the UCUA sequence could be a putative PTB binding site, and the ability of recombinant PTB to interact with the complete 3′UTR and the CS1–L-SL regions was therefore analyzed. The recombinant protein bound efficiently to both regions, suggesting that PTB may be part of the ribonucleoprotein complex formed with the 3′UTR. Preliminary results from our laboratory indicate that the PTB protein is present in the RNA–protein complexes formed within the 3′UTR of DEN4 and Vero cell extracts. Further analysis using mosquito cell extracts would enable us to determine if this cell line contains a PTB-like protein.

PTB has been involved in viral RNA translation through its interaction with the 5′UTR and the 3′UTR. PTB, as well as La, may function as a chaperone to stabilize the correct RNA conformations that allow translation and may also be required for replication. Further studies with mammalian cell extracts would indicate the specific binding sites for PTB and its possible role in DEN replication.

The data presented in this paper indicate that EF-1α from C6/36 cells and mammalian La and PTB proteins bound to the 3′UTR from DEN4. Two putative binding sites for EF-1α and PTB have been located within the DEN4 3′UTR at nt 10,596 to 10,601 and nt 10,577 to 10,579, respectively. It is possible that these molecules facilitate the interaction with other cellular or viral proteins to initiate replication. This conclusion is supported by the fact that the CS1–CS2–L-SL region is important and the CS1–L-SL region is essential for DEN replication and both are target sites for cellular protein interaction. Additionally the binding of proteins such as La, EF-1α, and PTB to the RNA sequence involved in replication strongly suggests that they may function as helicases, chaperones, or *trans*-acting factors in viral replication. Further analyses, including functional studies, are necessary to demonstrate the role of each protein in DEN virus replication.

MATERIALS AND METHODS

Cells and virus

Monolayers of C6/36 cells (*Aedes albopictus* larvae) were grown at 34°C in minimal essential medium supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin. C6/36 cells were infected with dengue 4 virus strain H-241 (kindly provided by David Vaughn, Walter Reed Army Institute of Research, Washington, DC) and after 5 days of infection, cell extracts were prepared. Viruses were propagated in suckling mice and LLC-MK2 cells as described previously by Gould and Clegg (1991).

Preparation of S10 extracts

C6/36 cells, uninfected and infected with dengue 4 virus, were scraped in detached buffer (40 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl) and centrifuged at 3000 rpm at 4°C in a Sorvall GSA rotor. The cells were resuspended in 5 vol of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) and centrifuged again. Finally, cells were resuspended in 2 vol of buffer A and subjected to 20 strokes in a Dounce homogenizer. The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C in a Sorvall GSA rotor and the supernatant (S10 extract) was divided into aliquots. The concentration of protein was determined with the Bradford (1976) method (Pierce).

DNA templates

Oligonucleotides were synthesized by Gibco BRL and used for PCR amplification using the full-length cDNA from DEN4 (kindly provided by Ching Juh Lai, NIAID NIH, Bethesda, MD) as template. The following primers were used: Oligo 3′C4, 5′TAATACGACTCACTATAGGGTAATTACCAACAACA3′; Oligo 3′D4, 5′AGAACCTGTTGGATCAAC3′; Oligo CS2B, 5′TATGCCACGCGTACAGC3′; Oligo CS2A, 5′TAATACGACTCACTATAGGGCAACACAAAA3′; Oligo 277, 5′TAATACGACTCACTATAGGGCCCCAGGGAGGCCATGCGC3′.

Oligonucleotides 3′C4 and 3′D4 were used to copy the complete 3′ end cDNA sequence of DEN4 (nt 10,260 to 10,644) by PCR. Regions amplified by other primers are represented in the 3′UTR map (Fig. 1). The first 16 nt of primers 3′C4, CS2A, and 277 correspond to the bacteriophage T7 promoter; three G residues were included in the dengue virus-specific sequence to increase the efficiency of transcription by T7 polymerase, as described previously (Rojas-Eisenring and del Angel, 1995). PCR was carried out in a Perkin–Elmer Cetus Model 480 DNA thermal cycler (1 cycle at 94°C for 2 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, and 75°C for 1 min). Amplification products were purified with a Qiaquick Gel Extraction kit (Qiagen) and used for *in vitro* transcription.

Transcription of PCR products

PCR products were used to synthesize ^{32}P-labeled RNA transcripts with T7 RNA polymerase following the conditions described by the manufacturer (Promega), in the presence of [α-^{32}P]UTP (DuPont). Transcription reactions were treated with DNase RQ1 (1 U) at 37°C for 30 min and precipitated with sodium acetate and ethanol. Purified RNAs were resuspended with DEPC-treated water (Sigma).

Mobility shift electrophoresis assay

Five micrograms of S10 extract from uninfected and DEN4-infected C6/36 cells was incubated with 4 μg of yeast tRNA (Sigma) in a buffer containing 50 mM HEPES, pH 7.4, 0.1 mM DTT, 40 mM $MgCl_2$, 0.5 mM EDTA, 20 mM spermidine, 1.5 mM ATP, 10 mM GTP, and 50% glycerol in a final volume of 10 μl at 4°C for 15 min. Labeled RNA (600,000 cpm) was added and the mixture was incubated for 15 min at 4°C to allow complex formation. Samples were incubated with RNases A (20 μg) and T1 (20 U) at room temperature for 30 min, loaded gently on a 6% polyacrylamide gel (acrylamide:bis-acrylamide, 80:1) prepared in 0.5× TBE buffer, and electrophoresed at 20 mA for 4 h. Gels were dried and autoradiographed.

For competition experiments, unlabeled RNAs were included in the preincubation reaction. For supermobility shift assays, polyclonal antibodies against EF-1α (kindly provided by William Merrick, Case Western Reserve University, Cleveland, OH), were preincubated for 15 min in the binding reaction before or after the addition of labeled RNA.

UV-induced cross-linking

UV-induced cross-linking of the RNA–protein complexes was performed as described (Gutiérrez-Escolano *et al.,* 1997) with 60 μg of S10 extract or 500 ng of recombinant GST–La and PTB proteins and 1×10^6 cpm of each ^{32}P-labeled RNA. Samples were analyzed by 10% SDS–PAGE. Gels were fixed, dried, and autoradiographed. The molecular weight of cross-linked proteins was determined by Kodak Digital Science version 2.02 ID Image Analysis Software.

Cloning, expression, and purification of recombinant His-PTB and GST-La proteins

The pGEX-La plasmid (kindly provided by Akio Nomoto, Institute of Medical Science, University of Tokyo, Tokyo, Japan) was used to express and purify the recombinant La protein. Briefly, BL21 bacteria transformed with the pGEX-La plasmid were grown at 37°C in 2XYT medium supplemented with 2% glucose and 100 μg ampicillin until an OD of 0.5 at 600 nm was reached. Protein expression was induced at room temperature for 20 h by addition of 0.5 mM IPTG. Bacteria were pelleted, soni-

cated at 4°C in a buffer containing 20 mM HEPES–KOH, pH 7.9, 200 mM NaCl, 1 mM EDTA, 20% glycerol, 10 mM β-mercaptoethanol, and protease inhibitors, and centrifuged for 20 min at 7000 rpm in a Sorvall GSA rotor. The recombinant La protein was purified from the clarified supernatant using a glutathione–Sepharose 4B column and treated with 10 mM reduced glutathione for 1 h at 4°C. GST–La protein was dialyzed against 50 mM NaCl, 1 mM EDTA, and 50 mM Tris–HCl, pH 7.5, and quantified by absorbance at 280 nm (Smith and Johnson, 1988).

The His-PTB recombinant protein was obtained after subcloning the PTB coding sequence (kindly provided by Stanley M. Lemon, The University of North Carolina at Chapel Hill, Chapel Hill, NC) into a *Hin*dIII site from the pProEX-HTb vector (Gibco BRL). DH5α bacteria were transformed with recombinant plasmid pProEX-HTb-PTB and grown in LB-ampicillin (100 μg/ml), at 37°C until an OD of 0.5 at 590 nm was reached. Protein expression was induced by addition of 0.6 mM IPTG for 3 h at 37°C. Bacteria were pelleted, resuspended in (4 v/w) lysis buffer (50 mM Tris–HCl, pH 8.5, 150 mM NaCl, and protease inhibitors), and sonicated on ice for 4 min. The His-PTB recombinant protein was purified from clarified lysate through chromatography on Ni-NTA resin (Qiagen). The His-PTB was eluted with 20 mM Tris–HCl, pH 8.5, 100 mM KCl, 5 mM β-mercaptoethanol, 10% glycerol, and 100 mM imidazole. The eluted protein was dialyzed against 50 mM HEPES, pH 7.5, 20% glycerol, 0.1 mM DTT, and 1 mM $MgCl_2$ (Gu *et al.,* 2000). Protein concentration was measured using the Bradford (1976) assay (Pierce).

The purification of both recombinant proteins was monitored by Western blot using a monoclonal anti-La antibody (kindly provided by N. Sonenberg, McGill University, Montreal, Quebec, Canada), with monoclonal anti amino-terminal PTB and anti-carboxy-terminal PTB antibodies (kindly provided by E. Wimmer, State University of New York at Stony Brook, Stony Brook, NY) and polyclonal anti-PTB antibody (kindly provided by D. L. Black, Howard Hughes Medical Institute, University of California, Los Angeles, Los Angeles, CA).

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