INTRODUCTION

Japanese encephalitis virus (JEV) is the single most important causative agent of epidemic viral encephalitis in South-East Asia, leading to around 50 000 clinical cases annually with up to 10 000 deaths. JEV is a mosquito-borne virus belonging to the animal virus family Flaviviridae. The JEV genome consists of a single-stranded, positive-sense RNA with non-coding regions (NCRs) of 95 and 585 bases at its 5′ and 3′ ends, respectively. These may bind to viral or host proteins important for viral replication. It has been shown previously that three proteins of 32, 35 and 50 kDa bind the 3′ stem–loop (SL) structure of the JEV 3′ NCR, and one of these was identified as 36 kDa Mov34 protein. Using electrophoretic mobility-shift and UV cross-linking assays, as well as a yeast three-hybrid system, it was shown here that La protein binds to the 3′ SL of JEV. The binding was stable under high-salt conditions (300 mM KCl) and the affinity of the RNA–protein interaction was high; the dissociation constant (K_D) for binding of La protein to the 3′ SL was 12 nM, indicating that this RNA–protein interaction is physiologically plausible. Only the N-terminal half of La protein containing RNA recognition motifs 1 and 2 interacted with JEV RNA. An RNA toe-printing assay followed by deletion mutagenesis showed that La protein bound to predicted loop structures in the 3′ SL RNA. Furthermore, it was shown that small interfering RNA-mediated downregulation of La protein resulted in repression of JEV replication in cultured cells.

La protein binds the predicted loop structures in the 3′ non-coding region of Japanese encephalitis virus genome: role in virus replication

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Japanese encephalitis virus (JEV) genome is a single-stranded, positive-sense RNA with non-coding regions (NCRs) of 95 and 585 bases at its 5′ and 3′ ends, respectively. These may bind to viral or host proteins important for viral replication. It has been shown previously that three proteins of 32, 35 and 50 kDa bind the 3′ stem–loop (SL) structure of the JEV 3′ NCR, and one of these was identified as 36 kDa Mov34 protein. Using electrophoretic mobility-shift and UV cross-linking assays, as well as a yeast three-hybrid system, it was shown here that La protein binds to the 3′ SL of JEV. The binding was stable under high-salt conditions (300 mM KCl) and the affinity of the RNA–protein interaction was high; the dissociation constant (K_D) for binding of La protein to the 3′ SL was 12 nM, indicating that this RNA–protein interaction is physiologically plausible. Only the N-terminal half of La protein containing RNA recognition motifs 1 and 2 interacted with JEV RNA. An RNA toe-printing assay followed by deletion mutagenesis showed that La protein bound to predicted loop structures in the 3′ SL RNA. Furthermore, it was shown that small interfering RNA-mediated downregulation of La protein resulted in repression of JEV replication in cultured cells.

The positive-sense genomic RNA is converted to a replication-intermediate negative-sense RNA during the course of flavivirus replication, as with other positive-sense RNA viruses. The negative-sense RNA acts as template for the synthesis of positive-sense genomic RNA. The 3′ NCR nucleotide sequence and its structural elements such as the 3′ SL structure are predicted to contain cis-acting signals for flavivirus RNA synthesis initiation, and these may thus interact with viral and cellular proteins involved in viral RNA replication (Chambers et al., 1990; Chen et al., 1997; You & Padmanabhan, 1999). Indeed, a number of cellular proteins have been shown to interact specifically with the 3′ NCR of flavivirus and other positive-sense RNA viruses. For example, elongation factor-1α (EF-1α) interacts with the 3′ SL structure of dengue 4 virus (DEN4) and West Nile virus (WNV) (Blackwell & Brinton, 1997; Nova-Ocampo et al., 2002) and with the aminoacylated 3′ end of turnip yellow mosaic virus RNA (Joshi et al., 1986). Another protein, calreticulin, binds the 3′ NCR of the DEN4 and rubella virus genomes (Silva et al., 2007; Yocupicio-Monroy et al., 2003). The polypyrimidine tract-binding (PTB) protein has been shown to interact with the 3′ NCR of hepatitis C virus (HCV) (Ito and Lai, 1997; Tsuchihara et al., 1997) and Norwalk virus (Gutiérrez-Escalon et al., 2000, 2003). This protein also binds to the 3′ NCR of JEV (Kim & Jeong, 2006) and DEN4 (Nova-Ocampo et al., 2002). Yet another protein, La, has been

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shown to interact with the 3’ NCR of DEN4 (Garcia-Montalvo et al., 2004; Nova-Ocampo et al., 2002) and Norwalk virus (Gutiérrez-Escolano et al., 2003).

We have shown previously that the 3’ SL structure of the JEV genome interacts with at least three cellular proteins with apparent molecular masses of 32, 35 and 50 kDa. One of these proteins was identified as the 36 kDa Mov34 protein (Ta & Vrati, 2000). In this study, we show that the ~50 kDa La protein binds to JEV 3’ SL RNA with high affinity and that small interfering (si)RNA-mediated downregulation of La protein results in repression of JEV replication in cultured cells.

METHODS

Preparation of RNA transcripts. Construction of pJE3SL, containing cDNA for JEV 3’ SL RNA, has been described previously (Ta & Vrati, 2000). The plasmid was linearized with XbaI and transcribed in vitro using T7 DNA polymerase by using the Riboprobe system (Promega) to produce 32P-labelled JEV 3’ SL RNA (Ta & Vrati, 2000).

Synthesis of recombinant La protein and its truncated forms. *Escherichia coli* plasmids expressing human La protein or its truncated forms under the control of the bacteriophage T7 promoter were kindly provided by Dr S. Das, The Indian Institute of Science, Bangalore, India (Pudi et al., 2003). The recombinant protein was expressed in *E. coli* BL21(DE3) cells and purified using Ni-NTA agarose (Qiagen).

Electrophoretic mobility shift assay (EMSA). Recombinant La protein was incubated in binding buffer [14 mM HEPES (pH 7.5), 6 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 60 mM KCl] with 500 ng poly(adenylic-cytidylic-uridylic) acid (polyACU), 1 μg yeast tRNA and 1 U RNasin in a 20 μl reaction for 10 min at 30 °C. After the addition of 1 ng 32P-labelled RNA, the reaction was continued for 20 min at 30 °C. RNA–protein complexes were separated at 4 °C by non-denaturing 5% PAGE (acylamide: bis-acylamide 50:1) containing 2.5% glycerol in 0.5× Tris/borate/EDTA buffer. The gel was then dried and autoradiographed.

UV-induced cross-linking of RNA and protein. The RNA–protein binding reaction set up as described above was placed on ice and irradiated for 30 min with a short-wavelength (254 nm) UV lamp (C). RNA toe-printing assay. An RNA toe-printing or primer-extension reaction, followed by incubation at 37 °C for 30 min. The UV-cross-linked products were boiled in Laemmli sample buffer and separated by 12% SDS-PAGE (acylamide:bisacylamide 29:1). The gel was fixed in 7% acetic acid, dried and autoradiographed.

La protein interaction with JEV RNA in vitro. The interaction of La protein with JEV RNA in vitro was studied by immunoprecipitation of La protein from JEV-injected cells followed by RT-PCR to detect La-protein–RNA complexes. Briefly, HEK293A cells were infected with JEV, and 18 h later, lysate was prepared followed by immunoprecipitation with anti-La antibody and protein A-Sepharose beads (Kaur et al., 2002). The beads loaded with La protein were washed with ice-cold wash buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM MgCl2, 0.05% NP-40, 0.5% SDS]. RNA was extracted using TriPure reagent (Roche) and used as template for RT-PCR using JEV-specific primers.

Yeast three-hybrid system. An RNA–protein Hybrid Hunter kit (Invitrogen) was used for studying RNA–protein interactions. Briefly, 87 nt encoding JEV 3’ SL RNA (nt 10890–10976) was fused to the 3’ end of the MS2 RNA-coding sequence in plasmid pR3H3’ between the Agd and XmnI sites to generate the RNA-hybrid vector. To make the protein-hybrid vector, cDNA encoding La protein was fused at the 3’ end of the B42 domain-encoding sequence in the prey plasmid pYESTrp2 (Invitrogen) between the BamHI and EcoRI sites. Yeast strain L40uraMS2 was co-transformed with the protein-hybrid and RNA-hybrid plasmids by using a lithium acetate method (Gietz & Woods, 2002). The transformed yeast was grown in selective medium overnight at 30 °C and assayed for β-galactosidase activity.

RNA toe-printing assay. An RNA toe-printing or primer-extension inhibition assay was performed as described previously (Pestova et al., 1998). Briefly, 60 fmol in vitro-transcribed RNA was incubated with 0.5 pmol 32P-end-labelled oligonucleotide (complementary to the 3’ end of RNA) in primer extension buffer [50 mM Tris/HCl (pH 8.3), 50 mM KCl, 10 mM MgCl2, 10 mM dNTPs, 0.5 mM spermidine] at 58 °C for 20 min followed by cooling at room temperature. Increasing amounts of protein (0–100 ng) were then added to the reaction, followed by incubation for 30 min at 30 °C. The primer was then extended by using 1 U avian myeloblastosis virus reverse transcriptase at 42 °C for 90 min. The DNA synthesized was precipitated and electrophoresed on a 6% polyacrylamide/7 M urea denaturing gel along with a nucleotide sequence ladder of the corresponding cDNA generated using the same end-labelled primer.

Filter-binding assay. Increasing amounts of protein (1 nM–10 μM) were incubated in a 20 μl reaction with 10 fmol 32P-labelled RNA in binding buffer [14 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 60 mM KCl] containing 500 ng yeast tRNA at 30 °C for 15 min. The reaction products were loaded onto nitrocellulose filters (HAWP 02500; Millipore), pre-equilibrated with 2 ml binding buffer and filtered under vacuum. The filters were then washed twice with 2 ml binding buffer and dried, and the counts retained were measured in a liquid scintillation counter. The percentage of bound RNA was calculated from the counts and a saturation binding curve was plotted. The binding affinity of the RNA–protein complex, denoted as the dissociation constant (Kd) of the binding reaction, was calculated as the protein concentration at which 50% of the RNA was bound. This was carried out by fitting the data into the binding curve (Langmuir) equation using GraphPad Prism software.

siRNA-mediated downregulation of La and JEV replication. The small hairpin (sh)RNA plasmid encoding La siRNA was constructed using appropriate oligonucleotides cloned in the pSIREN-RetroQZsGreen plasmid (Clontech). The La RNA sequence between nt 85 and 103 of the ORF was targeted. This siRNA has previously been used successfully to knock down La protein levels by >50% in HeLa cells (Costa-Mattioli et al., 2004). Monolayers of HEK293A cells grown overnight were transfected with the shRNA plasmid using Effectene reagent (Qiagen). Cells were harvested 2 days later and lysates were prepared. These were subjected to a Western blot with anti-La antibody to measure La expression. For virus replication studies, transfected cells were infected with JEV at 48 h post-transfection at an m.o.i. of 0.1. JEV titres in culture supernatants collected 24 h later were measured by plaque assay (Vrati et al., 1999). All experiments were carried out in triplicate. Untransfected cells or cells transfected with shRNA plasmid encoding siRNA to luciferase (Clontech) were used as negative controls.

RESULTS

La protein binds to the 3’ SL RNA of JEV genome in vitro

La protein has been shown to bind to the NCRs of many RNA viruses, including the flavivirus DEN4. La protein is
La protein binds JEV RNA in a specific manner, as the binding reaction contained an excess of yeast tRNA and poly(ACU). In addition, no RNA–protein complexes were seen in the binding reaction when in vitro-synthesized, radiolabeled tobacco etch virus (TEV) RNA was incubated with large amounts of La protein (Fig. 1b). La protein sometimes forms dimers at higher concentrations (Craig et al., 1997) and this could have led to the formation of the minor RNA–protein complex of lower mobility in these experiments.

Fig. 1(c) shows that the rhLa interaction with JEV 3′ SL RNA was stable in the presence of high salt. Thus, the presence of 300 mM KCl, large amounts of non-specific nucleic acids such as 1 μg yeast tRNA, poly(ACU) or plasmid DNA, or an unrelated protein (1 μg BSA) in the binding reaction did not affect the interaction of La with JEV 3′ SL RNA.

The binding of JEV RNA to rhLa was also studied by a UV cross-linking assay. Fig. 1(d) shows that a radiolabeled protein band of ~50 kDa was detected in the reaction containing rhLa protein, whereas no band was seen in the control reaction where BSA was used in place of rhLa. The binding of rhLa protein with JEV 3′ SL RNA was also confirmed by Northwestern blotting (data not shown). These experiments thus demonstrated that JEV 3′ SL RNA interacts with rhLa in vitro in a strong and specific manner.

La protein binds JEV RNA in vivo

A yeast three-hybrid system was employed to study the interaction of La protein with JEV 3′ SL RNA under in vivo conditions. Human La cDNA was cloned into plasmid pYESTrp2 fused in-frame with the B42 activation domain to produce the ‘prey’ protein, whereas 3′ SL RNA was cloned in plasmid pRH3′ fused to MS2 RNA to produce the ‘bait’ RNA. The L40uraMS2 strain of yeast, constitutively expressing the LexA–MS2 hybrid coat protein, was transformed with these two plasmids. This strain of yeast has two reporter genes (lacZ and HIS3) whose expression is regulated by LexA operator sequences. Interaction of ‘bait’ RNA and the ‘prey’ protein in the nucleus will bring the B42 activation domain together with the LexA DNA-binding domain, resulting in transcriptional activation of

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Fig. 1. La protein interaction with the JEV 3′ SL RNA. (a) La protein binding to the JEV 3′ SL RNA structure in the presence of anti-La antibody. An RNA–protein binding reaction was carried out using 1 ng 32P-labelled JEV 3′ SL RNA and 2 ng rhLa protein in the presence or absence of mouse anti-La mAb, followed by EMSA. Lanes: 1, JEV 3′ SL RNA (free probe); 2, RNA–protein complex in the absence of antibody; 3–5, RNA–protein complexes in the presence of anti-E protein, anti-hsp60 or anti-La antibody, respectively. (b) Specificity of JEV RNA binding to La protein. An RNA–protein binding reaction was carried out using 1 ng 32P-labelled RNA and different amounts of rhLa, followed by EMSA. Lanes: 1, JEV 3′ SL RNA (free probe); 2 and 3, JEV 3′ SL RNA binding to 10 and 20 ng rhLa, respectively; 4, TEV RNA (free probe); 5 and 6, TEV RNA binding to 10 and 20 ng rhLa, respectively. (c) Stability of JEV RNA binding with La protein. An RNA–protein binding reaction was carried out using 1 ng 32P-labelled JEV 3′ SL RNA and 10 ng rhLa protein in the presence of increasing amounts of salt. Lanes: 1, JEV 3′ SL RNA (free probe); 2–6, binding in the presence of 60, 120, 160, 240 and 300 mM KCl, respectively. (d) UV-induced cross-linking of JEV 3′ SL RNA with rhLa. An RNA–protein binding reaction was carried out using 50 ng rhLa or BSA and 1 ng 32P-labelled JEV 3′ SL RNA, followed by UV cross-linking, RNase A treatment and PAGE. Lanes: 1, JEV 3′ SL RNA binding to BSA; 2, JEV 3′ SL RNA binding to rhLa. Numbers indicate the positions of molecular mass markers (kDa).
the two reporter genes. Thus, a positive interaction of the RNA with the protein will allow the yeast to grow on plates lacking histidine and will also result in the synthesis of β-galactosidase. Our experiments showed that yeast transformed with the ‘prey’ La protein and 3′ SL ‘bait’ RNA grew on histidine-lacking medium and synthesized a large amount of β-galactosidase (Fig. 2). Similarly, in a positive-control experiment, yeast transformed with ‘bait’ containing the iron-responsive element (IRE) RNA sequence and the ‘prey’ containing the iron-regulatory protein (IRP) grew on histidine-lacking medium and synthesized a significant amount of β-galactosidase. In both cases, the transformed yeast grew in the presence of 10 mM 3-aminotrizole, indicating a specific and strong RNA–protein interaction. In these experiments, yeast transformed with plasmids for 3′ SL RNA plus IRP, and IRE RNA plus La protein, produced only small amounts of β-galactosidase. However, these interactions were weak and non-specific, as these yeasts did not grow in the presence of a lower concentration of 5 mM 3-aminotrizole. These results demonstrated a strong and specific interaction of the JEV 3′ SL RNA structure with La protein in an in vivo environment.

In order to examine whether La protein interacted with JEV RNA during the course of virus infection, a co-immunoprecipitation experiment was carried out. HEK293A cells were infected with JEV and a cell lysate was prepared 24 h later. La protein was immunoprecipitated from this lysate using an anti-La mAb. A control immunoprecipitation reaction was carried out using anti-hsp60 mAb. The RNA extracted from the immunoprecipitated pellet was subjected to RT-PCR using primers specific to the JEV genome sequence. A product of 390 bp was expected in cases where the pellet contained JEV RNA. Fig. 3 shows that a distinct band of the expected size was seen only when anti-La antibody was used for immunoprecipitation. These data demonstrated that, during JEV infection, endogenous La protein associates with JEV RNA.

**The N-terminal half of La protein is involved in binding with JEV RNA**

The La protein has been shown to have three putative RNA recognition motifs (RRMs) and a basic region followed by a stretch of acidic amino acids at the C terminus (Wolin & Cedervall, 2002). The N-terminal (aa 1–208) and C-terminal (aa 209–408) halves of La protein were over-expressed in *E. coli* (Fig. 4a). The purified full-length protein and the N-terminal and C-terminal halves of La protein were used in an EMSA to study their interaction with JEV RNA. Whilst 50 ng of the N terminus of La protein showed clear binding to 3′ SL RNA, its C terminus showed no binding using this amount (Fig. 4b). As the protein amounts increased in the RNA–protein binding reaction, multimers of RNA–protein complexes were seen for the full-length and N-terminal half of the protein. The C-terminal half of La protein showed no binding with JEV RNA, even when very large amounts of protein were used (400 ng).

Interaction of La protein with 3′ SL RNA was further studied by UV cross-linking of the RNA–protein complexes followed by RNase A treatment. Fig. 4(c) shows that a distinct band of ~50 kDa was seen with La protein. Similarly, the N-terminal half of La protein produced a band of ~23 kDa with 3′ SL RNA. However, only a very faint band was seen with the C-terminal half of La protein. These experiments thus demonstrated that the 3′ SL RNA of JEV interacts strongly with La protein and that it is the N-terminal half of the protein that is involved in binding with RNA.
La protein binds the loop structures in JEV 3' SL RNA

To map La protein binding points on JEV 3' SL RNA, toe-printing assays were performed. Fig. 5 shows the specific reverse transcriptase pauses (toe prints) at A-10929, C-10930 and A-10931 residues in loop I, and at A-10940, A-10941 and G-10942 residues in loop II of the predicted secondary structure of the 3' SL RNA.

To examine the role of these nucleotides in La protein binding with JEV RNA, deletion mutants were produced. 3' SLΔ1 had all of the La-binding nucleotides (nt 10929–10942) deleted. This led to deletion of the first loop, whilst making the second loop smaller. The second mutant, 3' SLΔ2, had both loops deleted (Fig. 5). The binding affinity of these RNAs with rhLa protein was determined as the K_d of the binding reaction using filter-binding assays (Fig. 5). Thus, the mean K_d for 3' SL RNA binding with La protein was 12.2±1.7 nM, whereas it was 9.4±1.0 and 511.3±192.0 nM for the 3' SLΔ1 and 3' SLΔ2 RNAs, respectively. These results showed that La protein binds JEV 3' SL RNA with high affinity. Deletion of nt 10929–10942 from 3' SL RNA did not affect La protein binding significantly. However, when both loops were removed, La protein binding with JEV RNA was drastically reduced, indicating that La protein binds to the loop structures in JEV 3' SL RNA.

La protein regulates JEV replication

In order to examine the role of La protein in JEV replication, La protein levels were downregulated in HEK293A cells using siRNA, and the JEV titre was obtained in these cells at 24 h post-infection. Fig. 6 shows that JEV titres were significantly reduced (P<0.05) in a dose-dependent manner when cells were transfected with plasmid encoding La siRNA. Thus, the JEV titre was not affected when 200 ng siRNA encoding-plasmid was used, whereas it was reduced by 75 and 97% when 400 and 800 ng plasmid were used, respectively. A control plasmid encoding a siRNA to luciferase failed to affect JEV titres significantly. The viability of the cell monolayer, determined by microscopy and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Western blotting, was not affected by transfection with siRNAs.

DISCUSSION

RNA-dependent RNA transcription, a process involved in flavivirus replication, is not well understood. However, extrapolating from our understanding of DNA transcription, which requires several transcription factors besides the enzyme DNA polymerase, it is likely that RNA-dependent RNA transcription involves proteins other than the RNA-dependent RNA polymerase (RdRp). As RNA viruses have a limited coding capacity, it is conceivable that these viruses make use of the host-cell proteins for RdRp-mediated transcription and replication. Indeed, host-cell proteins have been shown to be part of the RNA replicase complex of phage Qβ (Blumenthal & Carmichael, 1979), brome mosaic virus (Quadt et al., 1993), cucumber mosaic virus (Hayes & Buck, 1990), tobacco mosaic virus (Osman & Buck, 1997), vesicular stomatitis virus (VSV) (Behrens et al., 1998), measles virus (Moyer et al., 1990), influenza virus (O’Neill & Palese, 1994) and poliovirus (Harris et al., 1994; Hayes & Buck, 1990). In addition, a number of host-cell proteins have been shown to interact specifically with
the 3' ends of RNA viral genomes of different genera (see Introduction). In the case of flaviviruses, EF-1α has been shown to bind to the 3' SL RNA of WNV (Blackwell & Brinton, 1997), whilst the PTB, La and EF-1α proteins bind the 3' NCR of DEN4 RNA in vitro (Nova-Ocampo et al., 2002). EF-1α interacted with WNV RNA with high affinity and bound the stem of the SL structure of the 3' NCR. Biochemical characterization and fine mapping of host-protein binding with DEN4 RNA, however, was not undertaken. We have shown previously that at least three proteins with apparent molecular masses of 32, 35 and 50 kDa from mouse brain cells bind with the 3' SL RNA of JEV genome. One of these proteins was identified as the 36 kDa Mov34 protein, belonging to a family of proteins whose members are involved in RNA transcription and translation. In the current study, we have shown that the ~50 kDa La protein binds to the 3' SL of JEV RNA genome with high affinity and we identified its binding sites to the top loops of its predicted secondary structure.

La is a multifunctional RNA-binding protein interacting with a variety of RNAs. In the nucleus, La protein associates transiently with the 3' oligo(U) terminus of RNA polymerase III transcripts to facilitate transcription termination (Maraia et al., 1994). Most La protein is localized in the nucleus, although some is found in the cytoplasm, especially under conditions of cellular stress such as viral infection (Meerovitch et al., 1993). In addition to binding to the NCRs of many RNA viruses, La protein has been shown to bind small viral RNAs such as Epstein–Barr virus-encoded RNAs (Rosa et al., 1981), VA RNAs of adenovirus (Francoeur & Mathews, 1982) and the leader

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**Fig. 5.** La protein binding to JEV RNA. RNA–protein binding reactions were carried out by using *in vitro*–synthesized 86 nt JEV 3' SL RNA (nt 10891–10976 of JEV genomic RNA) together with a 30 nt non-viral sequence from the plasmid at its 3' end, and increasing amounts of full-length rhLa protein. A γ-32P-labelled synthetic primer, complementary to the vector sequence, was then annealed to the RNA and extended by reverse transcriptase. The primer-extension products (RNA toe prints) were analysed by 7M urea/12% PAGE. Top left: autoradiograph of the primer-extension reaction products obtained in the presence of 0, 10, 20, 50 and 100 ng rhLa protein (right-hand lanes, left to right, respectively). The lanes on the left show the RNA sequencing reaction products. The arrows indicate La protein-binding sites. Top right: predicted secondary structures of JEV 3' SL RNA. The nucleotides interacting with La protein have been circled. Also shown are the predicted secondary structures of 3' SLΔ1 and 3' SLΔ2 RNA. The lower panels show the results of filter-binding assays using rhLa and different RNAs. The RNA–protein binding affinity measured as $K_d$ is indicated.
La protein binding to Japanese encephalitis virus RNA

sequence of a unique RNA produced during replication of VSV (Wilusz et al., 1983). Several putative roles have been ascribed to La protein in various processes related to virus replication such as transcription termination (Gottlieb & Steitz 1989a; Maraia et al., 1997; Horke et al., 1994; Meerovitch et al., 1994), translation enhancement (Chang et al., 1994; Meerovitch et al., 1993; Svitkin et al., 1994) and viral RNA stabilization (Spangberg et al., 2001).

In the present study, La protein was shown to bind JEV RNA with high affinity; the apparent $K_d$ for La protein binding with 3' SL RNA was 12 nM. These values are comparable to the affinity with which La protein binds to its known natural ligands in the normal cell. For example, pre-t-RNA Val , hY1 and hY4 RNAs interact with La protein with $K_d$ values of 7.3, 6 and 5 nM, respectively (Goodier et al., 1997; Horke et al., 2004). The $K_d$ values for La protein interaction with JEV RNA were similar to those obtained for La protein binding to Sindbis virus negative-strand RNA (15.4 nM) (Pardigon & Strauss 1996), human immunodeficiency virus trans-activation response element RNA (17 nM) (Chang et al., 1994), rubella virus RNA (2 nM) (Duncan & Nakhasi 1997) and poliovirus 5' NCR RNA (4 nM) (Meerovitch et al., 1993).

La is a highly abundant protein; about 20 million copies of the protein are estimated to be present in a mammalian cell (Gottlieb & Steitz 1989b). The concentration of La protein in the S100 extract of HeLa cells is calculated to be 50 nM (Fan et al., 1998). It would appear that binding of La protein to JEV RNA is physiologically plausible, and using a co-immunoprecipitation experiment, we demonstrated that the JEV genome did indeed interact with endogenous La in JEV-infected HeLa cells. In addition, La protein was shown to bind JEV 3' SL RNA under in vivo conditions using a yeast three-hybrid system.

La protein contains three RRMs: RRM1 and RRM2 are located in the N-terminal half of the protein, whilst RRM3 is located in the C-terminal half. The N-terminal domain of La protein has been shown to interact with the poliovirus 5' NCR (Izumi et al., 2004; Svitkin et al., 1994), whilst the C-terminal domain interacts with the HCV 5' NCR in vitro (Ali et al., 2000); however, both N- and C-terminal halves are able to interact with the viral RNA independently in vivo (Pudi et al., 2003). We have shown that La protein interaction with the JEV 3' NCR involves its N-terminal half only.

La protein has been shown to bind and stabilize unpolyadenylated RNAs, such as histone mRNAs (McLaren et al., 1997), cytoplasmic Y RNAs (Rutjes et al., 1999) and HCV genomic RNA (Spangberg et al., 2001). JEV genomic RNA lacks a poly(A) tail at its 3' end. We have shown that La protein binds 3' SL RNA with high affinity. It is thus possible that La protein binding protects the JEV genomic RNA from rapid exonucleolytic degradation in the cytoplasm of the infected cell.

The JEV 3' SL RNA has a stable stem of dsRNA, which must unwind for transcription to proceed from the 3' end of the genomic RNA for production of the negative-sense replication intermediate. Both recombinant and native La from different species have been shown to possess ATP-dependent dsRNA unwinding activity (Bachmann et al., 1990; Huhn et al., 1997; Xiao et al., 1994). La protein binding to JEV 3' SL RNA may have a role in unwinding the RNA involved in the stem formation, thus enabling the virus to replicate via synthesis of the replication intermediate.

RNA selection experiments with La protein have revealed a novel CACAA motif involved in RNA–protein interactions (Kenan, 1995). Many of the viral RNAs interacting with La protein also contain a close match or exact CACAA sequence (Ali et al., 2000). For example, La protein interacts with the HCV 5' NCR with a GCAC sequence (Pudi et al., 2003). The toe-printing experiments described here indicated that La protein interacted with sequence ACA (nt 10929–10931) in loop I of the JEV 3' SL RNA, whilst it bound to sequence AAG (nt 10940–10942) in loop IV.
II. The ACA sequence is a part of the GCACA sequence located between nt 10927 and 10931 of JEV RNA. Interestingly, deletion of nt 10929–10942 had only a small effect on La binding to the RNA. It is likely that La protein binds to the loop structure, as its removal in 3′ SL2 RNA drastically reduced the binding of La protein to the RNA.

Importantly, the ACA sequence is a part of the pentanucleotide sequence CACAG located between nt 10928 and 10932 in loop I of the JEV 3′ SL RNA. The top loop structures and the pentanucleotide sequence are well conserved in arthropod-borne flaviviruses, and the pentanucleotide sequence is absolutely required for replication of yellow fever virus (Silva et al., 2007) and WNV (Tilgner & Shi, 2004). Whilst this sequence was not necessary for translation, no RNA replication (transcription) took place in its absence. La protein has been shown to bind the NS3 and NS5 (RdRp) proteins and also the 5′ and 3′ ends of the genomic RNA in the case of DEN4. For JEV, NS3 and NS5 proteins have also been shown to bind the 3′ SL RNA (Chen et al., 1997). These data suggest that the replicase complex assembly at the 3′ end of the JEV genome, necessary for negative-strand synthesis, may involve La protein binding to JEV 3′ SL RNA.

La protein interaction with JEV 3′ SL RNA may thus have an important role in virus replication. Indeed, our studies showed that JEV titres were reduced by 97% when the levels of La protein were downregulated using siRNA. La protein has been shown to bind poliovirus and HCV NCRs. Downregulation of La using siRNA was shown to decrease poliovirus replication fivefold (Costa-Mattioli et al., 2004) and replication of the HCV subgenomic replicon by 50% (Domitrovich et al., 2005). In these studies, La protein was predicted to assist in unwinding of the RNA, leading to efficient translation and replication. Experiments are in progress to study the mechanistic role of La protein in JEV replication.

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REFERENCES


La protein binding to Japanese encephalitis virus RNA


Wilusz, J., Kurilla, M. G. & Keene, J. D. (1983). A host protein (La) binds to a unique species of minus-sense leader RNA during


