A Peptide Derived from RNA Recognition Motif 2 of Human La Protein Binds to Hepatitis C Virus Internal Ribosome Entry Site, Prevents Ribosomal Assembly, and Inhibits Internal Initiation of Translation

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Received 1 December 2004/Accepted 11 April 2005

Human La protein is known to interact with hepatitis C virus (HCV) internal ribosome entry site (IRES) and stimulate translation. Previously, we demonstrated that mutations within HCV SL IV lead to reduced binding to La-RNA recognition motif 2 (RRM2) and drastically affect HCV IRES-mediated translation. Also, the binding of La protein to SL IV of HCV IRES was shown to impart conformational alterations within the RNA so as to facilitate the formation of functional initiation complex. Here, we report that a synthetic peptide, LaR2C, derived from the C terminus of La-RRM2 competes with the binding of cellular La protein to the HCV IRES and acts as a dominant negative inhibitor of internal initiation of translation of HCV RNA. The peptide binds to the HCV IRES and inhibits the functional initiation complex formation. An Huh7 cell line constitutively expressing a bicistronic RNA in which both cap-dependent and HCV IRES-mediated translation can be easily assayed has been developed. The addition of purified TAT-LaR2C recombinant polypeptide that allows direct delivery of the peptide into the cells showed reduced expression of HCV IRES activity in this cell line. The study reveals valuable insights into the role of La protein in ribosome assembly at the HCV IRES and also provides the basis for targeting ribosome-HCV IRES interaction to design potent antiviral therapy.

Hepatitis C virus (HCV), a member of the Flaviviridae family, is an enveloped positive-sense, single-stranded RNA virus (10). The 9.6-kb-long genome encodes a single polyprotein of about 3,000 amino acids. The polyprotein is processed by host cell and viral proteases into three major structural proteins and several nonstructural proteins necessary for viral replication (4, 18). HCV causes a variety of liver diseases in humans, including liver cirrhosis and hepatocellular carcinoma (20, 32). It is estimated that about 3% of the world population is infected with HCV, and about 85% of infected individuals develop chronic infection. Current options for treating HCV involve alpha interferon alone or in combination with ribavirin (6, 13). However, these treatments fail to achieve sustained virological response in the majority of patients, thus emphasizing the need for novel therapeutic approaches to combat HCV infection.

Translation initiation of HCV occurs in a cap-independent manner wherein the ribosomes are recruited onto an internal ribosome entry site (IRES) located mostly within the 5′ untranslated region (UTR) and extending a few nucleotides into the coding region (31, 37, 39). HCV IRES has been shown to form three complex stem-loops and a pseudoknot, which encircles the initiator AUG codon (7). Although the HCV IRES binds to the 40S ribosomal subunit specifically and stably even in absence of any initiation factors, efficient translation requires canonical initiation factors like eIF2 and eIF3 (21, 27, 33) and other noncanonical trans-acting cellular proteins including polypyrimidine tract-binding protein (1), La autoantigen (2), poly(rC) binding protein (15), and heterogeneous nuclear ribonucleoprotein L (p68) (19). Recently, binding of a 25-kDa cellular protein (p25) to HCV IRES has been shown to be important for efficient translation initiation. p25 was originally suggested to be ribosomal protein S9 but was later identified as rpS5 (14, 16, 27).

Human La protein is known to interact with HCV IRES and stimulate translation initiation both in vitro and in vivo (2, 3). La protein has been shown to specifically interact with both the 5′ and 3′ UTRs of hepatitis C virus RNA (34). Sequestration of La in rabbit reticulocyte lysate (RRL) inhibits HCV IRES-mediated translation, which can be rescued by exogenous addition of purified La protein (3). However, it has been noted that HCV IRES has a lower requirement of La protein than poliovirus (PV) IRES (22). Due to the critical role played by the La protein in HCV IRES translation, the disruption of its interaction with HCV IRES is an attractive target for inhibiting HCV IRES activity. A 60-nucleotide inhibitor RNA (I-RNA) from the yeast S. cerevisiae that selectively blocked HCV and poliovirus IRES-mediated translation appeared to inhibit the translation by virtue of its ability to bind La protein (12). Recently, it has been shown that a synthetic peptide corresponding to the N-terminal “La motif” of human La autoantigen inhibits HCV IRES-mediated translation possibly by binding to other essential cellular proteins (23).

La protein has been shown to have three putative RNA recognition motifs (RRMs) and a basic region followed by a stretch of acidic region at the C terminus (17). We previously demonstrated that both N- and C-terminal halves of La protein are able to interact with HCV IRES and were able to stimulate HCV IRES-mediated translation, unlike the case in poliovirus...
IRES, suggesting that the deletion of the C terminus does not abrogate the ability of La protein to enhance the translation of hepatitis C virus. Among the three RRMs, RRM2 showed the highest affinity for binding to HCV IRES RNA. Additionally, using comparative sequence and structural analysis as well as primer extension inhibition analysis, we demonstrated previously that the RRM2 within the N-terminal domain of La protein binds HCV RNA at the GCAC motif near the initiator AUG (28). Recent reports from our laboratory also highlighted the fact that mutations within HCV SL IV lead to reduced binding to La-RRM2 and drastically affect HCV IRES-mediated translation. Also, the binding of La protein to SL IV of HCV IRES was shown to impart conformational alterations within the RNA so as to facilitate the formation of a functional initiation complex (29). Similarly, another recent report suggests direct involvement of La protein in the initiation of formation of 48S ribosome complexes on HCV IRES RNA (11).

Here, we studied the role of La protein in assisting the assembly of a translation initiation complex by using a dominant negative synthetic peptide, LaR2C, derived from the C terminus of La-RRM2. The peptide retained the RNA binding ability and successfully competed with cellular La protein for binding to SL IV region of the HCV IRES RNA. More importantly, it appears that the LaR2C peptide interferes with the assembly of 48S complexes, resulting in the accumulation of preinitiation complexes that are incompetent for the 60S ribosomal subunit joining. The results reinforce the role of La protein in the formation of the functional initiation complex on the HCV IRES RNA (11, 29). Finally, the peptide has been successfully exploited as a dominant negative inhibitor of HCV IRES-mediated translation. The LaR2C sequences expressed as a fusion to HIV-TAT peptide, when introduced into the HuH7 cell line constitutively expressing the HCV bicistronic construct, significantly reduced the HCV IRES-mediated translation. The study put forward the idea of targeting ribosome-IRES interaction to design potent antiviral therapeutic agents against HCV infection.

**MATERIALS AND METHODS**

**Plasmids, peptides, and cells.** The plasmids pRSET A-La, pRSET A-La101-208 (RRM2), pCD HCV-383 bicistronic construct, pCD HCV-383 WT and pCD HCV-383 M2, and pHCV-383 GFP have been described previously (28, 29). The PV 5′ UTR was cloned in between HindIII and EcoRI sites replacing HCV IRES in the bicistronic plasmid (30), and the pFluc-HAV-BF20 bicistronic construct was cloned as described previously (30). pcDGFP was obtained by inserting green fluorescent protein (GFP) into ECoRI/Xhol of the pcDNA3 vector. La101-180 and La208-208 coding sequences were PCR amplified using the primers 5′-GGCCGGATCCGAGTGACTTTGGAA-3′, 5′-GGCTGAAATTCCCGCTTCCTTCGTTT-3′, 5′-GCATGGATCCACTGATGCAACTCTT-3′, and 5′-CTGAAATCTCAGGTCATCTTCGC-3′ and pRSET A-La as template DNA. The amplified products were cloned into BamHI and EcoRI sites of the vector pRSET A to obtain pRSET A-La101-180 and pRSET A-La208-208. pTAT and pTAT-HA vectors were obtained from S. Vijaya of our department. La101-180 and La120-208 coding sequences were PCR amplified using comparative sequence and structural analysis as well as primer extension inhibition analysis, we demonstrated previously that the RRM2 within the N-terminal domain of La protein binds HCV RNA at the GCAC motif near the initiator AUG (28). Recent reports from our laboratory also highlighted the fact that mutations within HCV SL IV lead to reduced binding to La-RRM2 and drastically affect HCV IRES-mediated translation. Also, the binding of La protein to SL IV of HCV IRES was shown to impart conformational alterations within the RNA so as to facilitate the formation of a functional initiation complex (29). Similarly, another recent report suggests direct involvement of La protein in the initiation of formation of 48S ribosome complexes on HCV IRES RNA (11).

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**Preparation of HeLa Si10 cell extract.** HeLa Si10 cell extract was prepared as described previously (29). Briefly, cells were harvested, pelleted down, and washed three times with cold isocetic buffer (35 mM HEPES, pH 7.4, 1 mM MgCl2, 1 mM EGTA, 100 mM NaCl, 11 mM glucose), resuspended in 1.5× packed cell volume of hypotonic buffer (10 mM HEPES, pH 7.4, 15 mM KCl, 1.5 mM Mg-acetate, and 6 mM β-mercaptoethanol), and then incubated on ice for 10 min. Cells were then transferred to a Dow's homogenizer and disrupted by 50 strokes on ice (lysis was checked under a microscope). The lysate was incubated in 1× incubation buffer for 10 min. Si10 cytoplasmic extract was isolated by centrifuging the lysis at 10,000 × g for 30 min at 4°C. The supernatant was diluted for 4 h against 100 volumes of dialysis buffer and aliquoted into prechilled tubes. The aliquots of Si10 extract were stored at −70°C.

In vitro transcription mRNAs were transcribed in vitro from different linearized plasmid constructs under T7 promoters in runoff transcription reactions. The monocistronic construct pCD GFP was linearized with XhoI downstream of GFP to be used as templates for RNA synthesis. The linear DNAs were electrophoresed on agarose gels and extracted by using a QIAGEN gel elution kit, and capped bicistronic RNAs were synthesized using a Ribomax large-scale RNA production system-T7 (Promega) under standard conditions with the addition of 5′ cap analog (Promega). For the synthesis of uncapped HCV-GFP RNA, pCD HCV-383 GFP linearized with Xhol was used, and for the synthesis of HCV wild-type and mutant IRES RNAs, pCD HCV-383 WT or pCD HCV-383 M2 (29) linearized with EcoRI downstream of HCV IRES was used. The transcription was performed using Ribomax large-scale RNA production system-T7 (Promega) according to the manufacturer's protocol.

Radiolabeled mRNAs were transcribed in vitro using T7 RNA polymerase (Promega) and [α-32P]UTP (NEB). pCD HCV-383 was linearized with EcoRI, gel eluted, and transcribed in vitro to generate the 32P-labeled RNA. The trans-
To this reaction, 32P-end-labeled primer complementary to 25 nucleotides of the HCV IRES RNA, 17.5 μl of RRL containing 0.5 μl of minus methionine, 20 μCi [32P]methionine, and 10 units of RNasin (Promega). The reaction mixtures were incubated at 30°C for 1 h 30 min. The expression from capped GFP or uncapped HCV-GFP RNAs in monocistronic constructs was resolved on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and dried before analysis using a PhosphorImager.

**Filter-binding assay.** The 32P-labeled HCV 383 RNA was incubated with increasing concentrations of purified recombinant proteins or peptides at 30°C for 15 min in RNA binding buffer (containing 5 mM HEPES, pH 7.6, 25 mM KC1, 2 mM MgCl2, 3.8% glycerol, 2 mM dithiothreitol, 0.1 mM EDTA), and loaded onto nitrocellulose filters equilibrated with the 2 μl RNA binding buffer. The filters were then washed twice with 2 μl of binding buffer and dried, and the counts retained were measured with a liquid scintillation counter. The graph was plotted with protein concentration (nanomolars) on the x axis and the percentage of bound RNA as the percentage of counts retained on the y axis. Each experiment was repeated three times, and the representative experiments are shown in the figures.

**In vitro translation.** In vitro translation of the capped or uncapped monocistronic mRNAs was carried out in micrococcal nuclease-treated RRL (Promega Corporation, WI). Briefly, 25-μl reaction mixtures contained 1 μg template RNA, 17.5 μl of RRL containing 0.5 μl of minus methionine, 20 μCi [32P]methionine, and 10 units of RNasin (Promega). The reaction mixtures were incubated at 30°C for 1 h 30 min. The expression from capped GFP or uncapped HCV-GFP RNAs in monocistronic constructs was resolved on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and dried before analysis using a PhosphorImager.

**Primer extension inhibition analysis.** Primer extension inhibition (toe-printing) assay was performed as described previously (21). Briefly, increasing concentrations of the peptides LaR2C and NSP were incubated with 5 pmol of in vitro-transcribed RNA corresponding to the HCV IRES (residues 18 to 383), and binding reaction was performed in a final volume of 20 μl at 30°C for 20 min. To this reaction, 32P-end-labeled primer complementary to 25 nucleotides of the 3’ end of the HCV-383 was added and allowed to extend using 3 units of AMV-Reverse Transcriptase (Promega) at 30°C for 1 h. The cDNAs were alcohol precipitated, resuspended, and compared with the dideoxynucleotide sequence ladders by electrophoresis on a 6% polyacrylamide–7 M urea denaturing gel.

**UV cross-linking assay.** Radiolabeled RNA corresponding to the HCV IRES was allowed to form a complex with the purified recombinant La protein, cell extract, or synthetic peptides in binding buffer (5 mM HEPES [pH 7.6], 25 mM KC1, 2 mM MgCl2, 3.8% glycerol, 2 mM dithiothreitol, 0.1 mM EDTA) at 30°C for 15 min. These RNA-protein complexes were then irradiated with short-wavelength UV light for 15 min. The excess and unbound RNA was digested with 30 μg of RNase A (Sigma) for 30 min at 37°C. The proteins were then denatured in 1× SDS buffer and resolved on an SDS-polyacrylamide gel, which was dried before it was exposed to the PhosphorImager. For competition experiments, the labeled RNA was preincubated with the peptides for 10 min before adding the LaR2C peptide.

**Sucrose gradient centrifugation analysis of ribosomal assembly on HCV IRES.** The ribosome assembly analysis experiment was performed as described previously (29). Briefly, 32P radiolabeled HCV IRES RNA (~2 × 10⁸ cpm) was added to 25 μl of ribosome assembly reaction mixtures in the presence or absence of the La peptide (LaR2C) or 10 mM 5’-guanylyl-3’-imidophosphate (GMP-PNP; Sigma-Aldrich). The reaction mixtures were diluted to 150 μl with gradient buffer (20 mM Tris [pH 7.5], 100 mM KC1, 3 mM MgCl2, 1 mM dithiothreitol) and overlaid on a 5 to 30% (wt/vol) linear sucrose gradient. The ribosomal complexes were sedimented by ultracentrifugation for 3 hours at 4°C and 30,000 rpm using a Beckman SW41 swing bucket rotor. Fractions (200 μl) were manually collected from the bottom of the tube, and the radioactivity was counted with a Rackbeta liquid scintillation counter. Each experiment was repeated three times, and the representative experiments are shown in the figures.

**RESULTS**

**RRM2 of La protein binds to HCV IRES through its C-terminal residues.** Previously, we have shown that RRM2 of La protein binds to HCV IRES with high affinity. To precisely identify the region that is important for the binding, two deletion constructs of LaRRM2 (La101-180 and La120-208) with deletions of 20 amino acids from the N terminus and 28 amino acids from the C-terminal region were generated (Fig. 1A). The overexpressed and purified proteins were analyzed by gel electrophoresis followed by silver staining (Fig. 1B) and used to study their ability to bind HCV IRES RNA using the filter-binding assay. 32P-labeled HCV IRES RNA was incubated with increasing concentrations of La-RRM2 (La101-208), La101-180, or La120-208 proteins in RNA-binding buffer. The RNA-protein complexes were bound to nitrocellulose filters and washed with binding buffer to remove unbound RNA. The counts retained were plotted against the protein concentration to obtain a saturation curve. The results showed that La120-208 retained the ability to bind HCV IRES RNA with an affinity of 0.16 μM, which was comparable to the affinity of La-RRM2 (0.14 μM), while the RNA binding ability was drastically affected in La101-180, where the C-terminal 28 amino acids were deleted (Fig. 1C). The results indicated that the C-terminal amino acids of La-RRM2 contribute to the binding with HCV IRES RNA.

**Peptide LaR2C, derived from the C terminus of La-RRM2, is capable of binding to HCV IRES RNA.** Based on the filter-binding assay results with La101-180 and La120-208 and the information obtained from the computational modeling (28), a synthetic peptide (LaR2C) of 24 amino acids (La174-197), corresponding to the C terminus of La-RRM2, was custom synthesized and tested for its ability to bind to HCV IRES using a filter-binding assay. A nonspecific peptide of similar length was used as a negative control. Additionally, another peptide (NSP-La), corresponding to the sequences closer to the N-terminal region of La protein (amino acids 71 to 88), which has been used previously as a nonspecific control in a similar study (23), was used as a negative control. The integrities of the La peptide (LaR2C), NSP-La, and NSP were checked by gel electrophoresis followed by silver staining (Fig. 2A). The filter-binding results showed that LaR2C retained the ability to bind to HCV IRES with an affinity of 75 μM, although the affinity was much lower than that of La protein (0.12 μM). The nonspecific peptides NSP and NSP-La did not show any binding to the RNA (Fig. 2B). Since both of the nonspecific peptides failed to bind HCV IRES RNA, we decided to carry out the rest of the experiments with the NSP as a negative control, which is of similar length to that of LaR2C peptide. To further confirm the LaR2C binding with HCV IRES RNA, a UV cross-linking assay was used where increasing concentrations of LaR2C or NSP were used to bind to 32P-labeled HCV IRES RNA. After the removal of unbound RNA by RNase A treatment, the protein-nucleotidyl complexes were subjected to SDS–15% polyacrylamide gel electrophoresis (PAGE) followed by phosphorimaging analysis. LaR2C showed efficient binding to HCV IRES RNA in a dosage-dependent manner (Fig. 2C, lanes 2 to 4), while the NSP of similar length did not show any binding (Fig. 2C, lanes 5 to 7). To study the specificity of the interaction further, a
competition UV cross-linking assay was performed where the binding of LaR2C with 32P-labeled HCV IRES was competed with 100- and 200-fold molar excesses of unlabeled HCV IRES RNA (self-RNA). The results showed that the unlabeled HCV wild-type IRES RNA efficiently competed out the binding (Fig. 2D, lanes 3 to 4), confirming the specificity of the interaction of LaR2C with HCV IRES RNA.

Previously, we have shown that La-RRM2 binds to HCV IRES around GCAC near the initiator AUG located in the stem-loop IV region, and the substitution mutations in SL IV affect the binding of La-RRM2 to HCV IRES drastically (29). Since LaR2C encompasses the RNA binding domain of RRM2 and has the ability to bind to HCV IRES, we wanted to verify if LaR2C binds to HCV IRES at the same region where RRM2 binds. For this purpose, unlabeled HCV mutant IRES RNA M2, where GCAC residues in SL IV have been substituted with ACCG (29), was used in the competition UV cross-linking assay. Even a 100- to 200-fold excess of M2 RNA failed to compete significantly with the 32P-labeled HCV IRES RNA for binding to LaR2C (Fig. 2D, lanes 5 to 6). The results clearly indicated that LaR2C specifically binds to HCV IRES RNA and perhaps at the same site within the SL IV region where La-RRM2 has been shown to interact.

LaR2C binds to HCV IRES in the SL IV region near iAUG. To further confirm the region on HCV IRES RNA where LaR2C binds, we performed a primer extension-inhibition assay (toe printing) using HCV IRES RNA in the absence or presence of increasing concentrations of the peptide LaR2C. Increasing concentrations of LaR2C or the NSP were incubated with 5 pmol of in vitro-transcribed HCV IRES RNA. To this complex, 32P-end-labeled primer complementary to the 3’ end of the HCV 3’-end was added and extended using AMV Reverse Transcriptase. The resulting extended products were analyzed on a 6% polyacrylamide–7 M urea denaturing gel. For precise mapping of the contact points, a DNA sequencing reaction corresponding to the HCV 383 RNA and using the same end-labeled primer was electrophoresed alongside. The results demonstrated specific reverse transcriptase pauses (toe prints) with the addition of increasing concentrations of LaR2C (Fig. 3), indicating possible binding sites. Two specific toe prints corresponding to C334 and A342 were observed around the initiator AUG (iAUG) in the SL IV region, which showed an increase in band intensity with the addition of increasing concentrations of LaR2C (Fig. 3, compare lane 5 with lanes 6 and 7). The relative band intensities were quan-
titated by densitometry and analyzed for the severalfold increase with respect to the no-protein control (data not shown).

However, no significant increase in toe prints was observed when a similar concentration of NSP was used (Fig. 3, compare lane 9 with lane 8). Similar toe prints were observed around this region when purified full-length La or RRM2 was added in the reaction (reference 28 and data not shown). The results indicate that the LaR2C peptide might interact with the SL IV region of HCV IRES RNA around the iAUG.

LaR2C competes with binding of full-length La protein to the HCV IRES RNA. We have shown previously that RRM2 of human La protein binds to the HCV IRES RNA near the initiator AUG (29). Since we observed that LaR2C also binds to the SL IV region of the HCV IRES RNA near the initiator AUG in the present study, we were interested to investigate whether LaR2C can compete with the full-length protein for binding to the HCV IRES RNA and, if so, what could be the possible consequences. To address this, a competition UV cross-linking experiment was performed with purified recombinant La protein and 32P-labeled HCV IRES RNA in the absence or presence of increasing concentrations of LaR2C peptide or NSP. The results showed that the binding of La protein to HCV IRES RNA was partially competed out (up to 40%) with the addition of 20 μM and 40 μM of LaR2C peptide.

FIG. 2. Ability of the peptide LaR2C to bind to HCV IRES RNA. (A) The sequences of the peptides LaR2C, NSP, and NSP-La are indicated. The LaR2C, NSP, and NSP-La peptides were analyzed by resolving by SDS–12% Tris-Tricine gel electrophoresis followed by silver staining. (B) Filter-binding assay to study the binding of the peptide LaR2C to HCV IRES. 32P-labeled HCV IRES RNA was bound to increasing concentrations of the peptide LaR2C, NSP, or NSP-La (as indicated by the symbols within the panel). The amount of bound RNA was determined by binding to the nitrocellulose filters. The percentage of bound RNA was plotted against the peptide concentration (micromolars). (C) UV cross-linking of LaR2C and NSP to HCV IRES. 32P-labeled HCV IRES RNA was UV cross-linked with increasing concentrations (20, 40, and 60 μM) of either LaR2C or NSP (as indicated above the panel), digested with RNase A, and resolved by SDS–15% PAGE followed by phosphorimaging. Lane 1 represents the no-protein control. Lane M represents the 14C protein molecular weight marker. The corresponding molecular masses are indicated to the left of the panel. (D) Competition assay to determine specificity of the binding of LaR2C to HCV IRES RNA. LaR2C preincubated with 100- and 200-fold excesses of unlabeled HCV wild-type (wt) RNA or HCV M2 RNA (where the SL IV region was mutated), as indicated above the lanes, was bound to 32P-labeled HCV IRES RNA and UV cross-linked. The complexes were treated with RNase A and resolved by SDS–15% PAGE followed by phosphorimaging. The band corresponding to LaR2C is indicated to the right of the panels by arrows. Lane 1 represents the no-protein control. Lane M represents the 14C protein molecular weight marker. The corresponding molecular masses are indicated to the left of the panel.
in the binding reaction (Fig. 4, lanes 3 and 4). At a further high concentration of the LaR2C peptide (20 and 40 μM [lanes 6 and 7]) or 40 μM NSP (lane 9) was incubated with HCV IRES RNA (18 to 383 nucleotides) as described above and analyzed for the toe prints. Lanes 5 and 8 represent the no-protein control. Lanes 1 to 4 show the DNA sequencing ladder corresponding to the HCV RNA (18 to 383 nucleotides) obtained by using the same end-labeled primer. The nucleotide indicated on the right of the panel signifies the corresponding positions on the HCV IRES RNA. The nucleotide positions corresponding to GCAC and iAUG are indicated on the left.

FIG. 3. Primer extension inhibition (toe-printing) analysis. Increasing concentrations of the LaR2C peptide (20 and 40 μM [lanes 6 and 7]) or 40 μM NSP (lane 9) was incubated with HCV IRES RNA (18 to 383 nucleotides) as described above and analyzed for the toe prints. Lanes 5 and 8 represent the no-protein control. Lanes 1 to 4 show the DNA sequencing ladder corresponding to the HCV RNA (18 to 383 nucleotides) obtained by using the same end-labeled primer. The nucleotide indicated on the right of the panel signifies the corresponding positions on the HCV IRES RNA. The nucleotide positions corresponding to GCAC and iAUG are indicated on the left.

FIG. 4. Effect of LaR2C on the binding of recombinant La protein and other cellular proteins to HCV IRES RNA. (A) 32P-labeled HCV IRES RNA was preincubated with increasing concentrations (20, 40, and 60 μM) of LaR2C or NSP as indicated above the lanes and then bound to recombinant purified La protein. The UV cross-linked complexes were treated with RNase A and resolved by SDS–10% PAGE followed by phosphorimaging. The band corresponding to recombinant La (rLa) is indicated to the right of the panel. Lane M represents the 14C-protein molecular weight marker. The corresponding molecular masses are indicated to the left of the panel. The intensity of the band corresponding to La protein in each lane was quantitated using densitometry and represented in numbers below the lanes. (B) 32P-labeled HCV IRES RNA was preincubated with increasing concentrations (20, 40, and 60 μM) of LaR2C and then bound to HeLa cytoplasmic extract (2.5 μg). The UV cross-linked RNA-protein complexes were treated with RNase A and resolved by SDS–5 to 15% gradient PAGE followed by phosphorimaging. The band corresponding to p52 is indicated to the right of the panel. The protein bands whose intensities were reduced are indicated by asterisks. The intensity of the band corresponding to p52 in each lane was quantitated using densitometry and represented in numbers below the respective lanes. Lane M represents the 14C-protein molecular weight marker. The corresponding molecular masses are indicated to the left of the panel.

To further verify whether LaR2C peptide can compete with full-length La protein in the context of cytoplasmic extract, a UV cross-linking experiment was performed using 32P-labeled HCV IRES RNA and HeLa cell extract in the absence or presence of increasing concentrations of LaR2C. The results demonstrated that in the presence of increasing concentrations of LaR2C, the binding of a 52-kDa polypeptide to the 32P-labeled HCV IRES RNA was found to be drastically reduced (Fig. 4B, lanes 2 to 4). Immunoprecipitation of the UV cross-linked nucleoprotein complex using anti-La antibody showed a
52-kDa cross-linked band from HeLa cell extract corresponding in size to the 52-kDa protein band observed in the above-described UV cross-linking experiment (data not shown). Results suggest that the 52-kDa cross-linked band, which showed decreased binding with HCV IRES upon addition of the LaR2C peptide, is indeed the La protein. Interestingly, in the presence of LaR2C peptide, significant changes in band intensities were observed in the binding of some other cellular proteins to HCV IRES RNA (as indicated with asterisks in Fig. 4). The sizes of some of them were found to be similar to that of eIF3 subunits (p170 and p66) that were reported to interact with the HCV IRES RNA. From the above-mentioned observation, it appears that the LaR2C peptide can compete with the full-length La protein for binding to the HCV IRES RNA even in presence of other trans-acting factors in the HeLa cytoplasmic extract.

LaR2C inhibits HCV IRES-mediated translation in vitro. Human La autoantigen has been shown to enhance HCV IRES-mediated translation. Since the RNA-protein interaction studies revealed that the peptide LaR2C binds to HCV IRES near iAUG and competes with the binding of full-length La protein to HCV RNA, it was interesting to study whether LaR2C has any effect on HCV IRES-mediated translation. For this purpose, monocistronic RNA, HCV-GFP containing the reporter gene GFP downstream of HCV IRES, was translated in vitro in rabbit reticulocyte lysate using [35S]methionine in the absence or presence of increasing concentrations of LaR2C (20 μM, 40 μM, and 60 μM). The cap-independent initiation of translation occurring internally from HCV IRES resulted in the synthesis of GFP. The translated GFP was analyzed by electrophoresis followed by phosphorimaging analysis, and the band intensities were quantitated by densitometry. It was observed that the addition of the peptide LaR2C led to a significant decrease in the HCV IRES activity (80 to 95%) in a dosage-dependent manner, leading to reduced synthesis of GFP (Fig. 5A, lanes 2 to 4). However, the addition of similar concentrations of NSP to the translation reactions did not show any significant effect (Fig. 5A, lanes 5 to 7). Also, the peptide LaR2C didn’t affect the translation when capped GFP RNA was used (Fig. 5B, lanes 2 to 4), indicating the specificity of the peptide to selectively inhibit HCV IRES-mediated translation. Furthermore, the addition of similar concentrations of LaR2C peptide didn’t show inhibition of hepatitis A virus (HAV) IRES (Fig. 5C). However, the addition of increasing concentrations (20 μM, 40 μM, and 60 μM) of LaR2C peptide showed only up to 44 to 49% inhibition of the PV IRES-mediated translation of the reporter gene firefly luciferase. The results suggest that the LaR2C peptide might be more effective in inhibiting HCV IRES compared to other IRES function.

LaR2C inhibits HCV IRES-mediated translation in vivo. As we have observed that LaR2C showed a dominant negative effect on HCV IRES-mediated translation in vitro, we wanted to investigate whether the same peptide can inhibit the HCV IRES-mediated translation in vivo as well. As a first step, sequence coding for the peptide LaR2C was cloned into bac-
Fusion protein in His tag in the fusion protein enabled the overexpressed TAT into the medium (5) (Fig. 6A). Additionally, the presence of a internalize into mammalian cells when supplied exogenously tide as a fusion of HIV TAT peptide that has the property to material expression vector pTAT, which would produce the pep- 

![Diagram]

**FIG. 6.** Effect of TAT-LaR2C fusion protein on HCV IRES-mediated translation in vivo. (A) Schematic representation of the TAT-LaR2C fusion protein. The amino acid sequences of TAT and LaR2C are highlighted. (B) Huh7 monolayer cells expressing HCV bicistronic RNA were overlaid with 100 nM of either HA-TAT or TAT-LaR2C fusion protein for 10 min. The cells were then harvested after different time points (10 min, 6 h, and 12 h) and lysed, and the RLuc and FLuc levels were measured using a Dual Luciferase assay system. The relative ratio of FLuc to RLuc was plotted at each time point. Black bars represent control cells, white bars represent cells overlaid with HA-TAT, and gray bars represent cells treated with TAT-LaR2C fusion protein. The HCV bicistronic construct used in the cell line is indicated on top of panel B. Data from the transfection experiments are expressed as means ± standard deviations of three independent replicates. (C) Absolute levels of RLuc and FLuc activities (in relative light units) of a representative experiment are presented in the table.

terion, the cells were washed and incubated with fresh medium and then harvested at different time intervals, 10 min, 6 h, and 12 h. The cells were then lysed, and FLuc and RLuc reporter activities were measured. The relative luciferase activities were represented as a ratio of FLuc to RLuc for normalization. The results indicated that the cells layered with TAT-LaR2C showed a decrease in the HCV IRES-mediated translation over the control cells, while the TAT-HA did not have any effect on HCV IRES-mediated translation (Fig. 6B). At 6 h posttransfection, the inhibitory effect was pronounced (inhibition up to 60%), while the inhibitory effect was found to be gradually decreasing upon prolonged incubation (12-h time point), possibly due to degradation of TAT-LaR2C within the cells. The presence of the His-tagged TAT-LaR2C protein transduced into the cells was monitored by immunoblotting of the cell extracts using anti-His antibody. The results showed a dosage-dependent increase in the presence of TAT-LaR2C protein inside the cells upon addition of increasing concentrations of the fusion protein into the medium (data not shown). Taken together, these results indicated that LaR2C does compete with the interaction of cellular La protein with HCV IRES RNA and exerts a dominant negative effect to inhibit HCV IRES-mediated translation.

**LaR2C** peptide prevents the assembly of ribosomal complexes on the HCV IRES. Previously, we demonstrated that full-length La protein binding at the SL IV region triggers a conformational change that facilitates ribosome assembly at the HCV IRES RNA. Since the LaR2C peptide successfully competes with the full-length La protein for binding with the HCV IRES, we have investigated the effect of ribosome assembly upon the addition of the LaR2C peptide in the reaction. For this purpose, sucrose gradient centrifugation experiments were performed, followed by the analysis of 48S and 80S ribosomal peaks. 32P-labeled HCV IRES RNA was incubated in translation reactions containing RRL and an amino acid mixture and loaded onto a 5 to 30% sucrose gradient and ultracentrifuged for 3 h at 30,000 rpm. The fractions were collected from the bottom of the tube, and scintillation counts were measured. The percentage of counts in each fraction was plotted against the fraction volume of the gradient. When wild-type HCV IRES RNA was used, two peaks were observed in equilibrium, corresponding to 80S and 48S ribosomes associated with the labeled HCV RNA (Fig. 7A). Upon addition of 20 μM of the LaR2C peptide, the peaks corresponding to 80S and 48S ribosomes were found to be reduced to some extent (compare Fig. 7A and B). At a higher concentration (40 μM) of LaR2C, the 80S peak was found to be completely abolished, and even the 48S peak was further reduced compared to the control (compare Fig. 7C and A). However, the addition of a 40 μM concentration of the NSP didn’t show significant alterations in either of the 48S or 80S peaks in the ribosomal assembly experiment (Fig. 7D). Interestingly, upon addition of a further high concentration (60 μM) of LaR2C peptide, even the 48S peak was found to be drastically reduced along with the 80S peak (data not shown).

It has been demonstrated that in the case of HCV IRES RNA, the 40S ribosomal subunit can form a stable binary complex which is distinct from the 48S preinitiation complex
FIG. 7. Effect of LaR2C peptide on ribosomal assembly on the HCV IRES RNA. Sucrose gradient sedimentation profiles of 32P-labeled HCV IRES RNA in the absence (A) or presence of increasing concentrations of the LaR2C peptide (B and C) or the NSP (D) after incubation in RRL and separated on a 5 to 30% sucrose gradient are shown. The fractions (200 µl) were manually collected from the bottom of the tube, and scintillation counts were measured. The counts per minute of each fraction were shown as the percentage of the total counts added to the reaction (~2 × 10^5 cpm) and were plotted against the volume of the gradient solution (0 to 8 ml). The ribosomal peaks corresponding to 48S and 80S are indicated. Panel E represents the sedimentation profile of HCV IRES RNA incubated in RRL in the presence of 10 mM GMP-PNP alone, and panel F is that obtained in the presence of both LaR2C (40 µM) and GMP-PNP (10 mM).
(26). However, this binary complex might not be separable from the true 48S peak in the sucrose gradient centrifugation. The actual 48S preinitiation complex would be competent for the 60S joining, whereas the binary complex would not lead to functional initiation complex formation. To further clarify whether the inhibition at the 80S complex formation by the LaR2C peptide was due to its effect at the preinitiation complex formation, we have performed the ribosome assembly reaction in the presence of 10 mM GMP-PNP. GMP-PNP, which is a nonhydrolyzable analog of GTP, inhibits translation initiation at the 48S stage by preventing the release of eIF2. As expected, the addition of GMP-PNP abolished the 80S peak and caused the accumulation of 48S ribosome preinitiation complexes in the control reaction (Fig. 7E). Interestingly, when LaR2C peptide (40 μM) was added to the reaction (in the presence of 10 mM GMP-PNP), in addition to a drastic decrease in the 80S peak, a significant decrease in the 48S peak was also observed (compare Fig. 7A, E, and F). The results suggest that LaR2C peptide might interfere with the transition step between the HCV-40S binary complex to the 48S complex, resulting in the formation of defective complex that is incompetent for 60S joining. Since the addition of LaR2C didn’t show accumulation of the 48S peak (as observed in the case of GMP-PNP), and rather decreases the 48S peak at higher concentrations, it seems that the complex might be dissociating eventually.

The result clearly reconfirms the role of La protein in the formation of a functional 48S ribosome complex on HCV IRES, as suggested by our earlier work (28, 29) and also a recent report (11).

Taken together, the results strongly suggest that the LaR2C peptide acts as a dominant negative and might compete for the binding of La protein to the SL IV region of the HCV IRES; as a consequence, the formation of a functional ribosomal initiation complex is severely affected, resulting in the inhibition of HCV IRES-mediated translation.

DISCUSSION

A recent report proposed a revised model depicting a sequential pathway of HCV IRES-mediated translation which is distinct from canonical initiation of cellular mRNAs. According to this model, HCV IRES begins translation as an IRES-40S complex that is followed by formation of a 48S intermediate prior to GTP hydrolysis and 60S joining to assemble the 80S complex. The model also suggests additional function during translation initiation, which requires maintenance of the HCV IRES structure (26). Although the 48S assembly with HCV IRES-40S is known to require only eIF3 and ternary complex eEF2-GTP-Met-tRNAi (21), trans-acting factors like La protein, polypyrimidine tract-binding protein, etc., which have been shown to stimulate IRES activity, might help in stabilizing the initiation complex. Previous reports from our laboratory showed that La binding to SL IV of HCV IRES RNA through RRM2 might induce conformational alterations within the RNA which facilitate internal initiation (29). We have shown that La protein binding (through RRM2) at the GCAC, near iAUG, apparently has a double effect, and first, it indirectly facilitates the S5 interaction with the HCV IRES and thus helps in 48S assembly. Second, the RRM2 binding at the SL IV region induces conformational alteration required for functional initiation complex formation. Thus, the mutant HCV IRES RNA, where GCAC sequences were altered, failed to bind La-RRM2 and thus prevented the S5 interaction as well as affected the conformational alteration leading to trapping of the ribosomes at the 80S complex, resulting in inhibition of IRES activity. However, LaR2C retained the ability to bind to HCV IRES, but it might not be capable of other essential functions performed by full-length La protein in stimulating HCV IRES function. This might explain the dominant negative activity shown by LaR2C on HCV IRES-mediated translation both in vitro and in vivo.

Interestingly, a recent report demonstrated that a dominant negative La mutant, where amino acids 226 to 348 (encompassing part of RRM3) had been deleted, inhibits the initiation of formation of the 48S ribosome complex and proposed a possible role for the La protein in the formation of functional initiation complex (11). Since the mutant La lacked the RRM3 but retained the RRM2, it might still be able to establish partial contact with the SL IV region near the iAUG. Our preliminary studies showed that RRM3 interacts with the SL III region, which largely encompasses the 40S contact points (reference 24 and data not shown). Thus, the La mutant lacking RRM3 is expected to have a drastic effect on 48S complex formation and the consequent decrease in 80S functional complex formation.

This study suggests that LaR2C peptide prevents full-length La protein binding to the HCV SL IV, thereby preventing the conformational alterations required for the functional initiation complex formation. In all the three approaches, the result reinforces the role of La protein in ribosome assembly during HCV IRES-mediated translation.

Studies from different laboratories suggest that the La protein binds to various RNAs using different combinations of RRMs. Although RRM1 (sometimes referred to as the “La motif”) does not bind RNA on its own, it contributes significantly to the recognition of UUU-OH-containing RNAs (25). Also, it has been demonstrated previously that deletion of the N-terminal residues of RRM1 decreased the affinity for binding to HIV leader RNA as well as HCV IRES RNA (9, 23). While the N-terminal region of the La protein has been shown to be an interacting domain with poliovirus RNA (35), in the case of HCV, both the N-terminal (containing RRM1 and RRM2) and C-terminal (containing RRM3 and the hydrophobic domain) regions of the La protein have been shown to be capable of binding independently to the HCV IRES and stimulate translation to some extent (3, 28). Interestingly, the RRM2-derived peptide LaR2C didn’t inhibit PV IRES-mediated translation as efficiently as observed in case of HCV IRES, suggesting that La-RRM2 may not be as essential in PV IRES function. Although there is no report on the requirement of La protein in HAV IRES-mediated translation, it is possible that even if it interacts with HAV IRES, it might not interact through RRM2. Thus, as expected, the LaR2C peptide didn’t show an inhibitory effect on HAV IRES-mediated translation. From our studies, it appears that the role of La-RRM2 in ribosome assembly could be more effective for the hepatitis C virus IRES-mediated translation.

Interestingly, it appeared from the competition UV cross-linking experiment that in addition to La protein, the peptide...
LaR2C might have altered the binding of some other cellular protein factors to the HCV IRES RNA. Since La protein also binds to the SL III region of HCV IRES (data not shown), where the majority of the eIF3 subunits have been reported to interact (8), it is tempting to speculate that the competition of full-length La protein binding by the LaR2C peptide might indirectly affect the interaction of eIF3 subunits. In fact, bands corresponding to some cellular protein binding showed more intensity upon addition of the LaR2C peptide, which could be due to the increasing availability of the binding sites on the HCV IRES RNA that were otherwise occupied by full-length La protein binding. Alternatively, the peptide might sequester some other protein factors giving better access to these cellular proteins to bind more strongly to the HCV IRES RNA. Thus, the effect of the peptide on HCV IRES-mediated translation could be a combination of such effects.

La protein binding through other domains (RRMs and the hydrophobic region) is not expected to be affected due to the RRM2-derived peptide binding at the HCV IRES. Thus, at lower concentrations of LaR2C peptide (20 and 40 μM), the competition UV cross-linking results demonstrated only a 40% decrease in La binding. However, at a higher concentration (60 μM), a significant reduction of La binding with the HCV IRES was observed. It is possible that at higher concentrations, the peptide LaR2C might sequester cellular La protein and also interfere with its binding to other binding sites within HCV IRES, resulting in a much more pronounced effect. In fact, a previous report did suggest that a peptide derived from RRM1 of the La protein might bind full-length La protein and sequester its binding with the HCV IRES RNA to inhibit internal initiation of translation (23).

The TAT-LaR2C fusion protein was capable of inhibiting, but not total abrogation of, HCV IRES-mediated translation of FLuc activity in vivo. This might be due to the fact that the TAT-LaR2C might not bind HCV IRES in vivo with similar affinity as the LaR2C peptide. Alternatively, the HCV IRES requires very low concentrations of La protein for its activity, and TAT-LaR2C protein was not able to compete out the binding of La protein totally in HuH7 cells. But considering the half-life of both luciferase activities, it appears that the 60% decrease in FLuc activity at the 6-h time point actually reflects the residual luciferase activity. Since the RLuc activity didn’t change during this time period, it is possible that the Tat-LaR2C protein might have selectively blocked the HCV IRES function, resulting in a drastic reduction in new synthesis of FLuc protein. However, the specificity and efficacy of the peptide to act as a dominant negative could possibly be further enhanced if the size of the peptide could be shortened after further characterizing and delineating the RNA-binding domain within LaR2C. Alternatively, the LaR2C peptide sequence could be delivered in vivo by using lentiviral vectors under an inducible promoter to test the selective inhibitory activity. This would then be useful to develop an effective inhibitor of HCV IRES translation.

Several strategies have been used to target essential RNA-protein interactions to block the HCV IRES-mediated translation. Antisense oligonucleotides targeted to domain IIId of HCV IRES have been used to compete with 40S ribosomal subunit binding and prevent in vitro translation (36). Reports from our laboratory have shown that a small RNA molecule (SLIIIe+f) corresponding to the subdomain e+f within stem-loop III of the HCV IRES RNA, when introduced in trans, can bind to the cellular proteins and antagonize their binding to the viral IRES, thereby inhibiting HCV IRES-mediated translation (30). It would be interesting to investigate whether SLIIIe+f RNA and LaR2C peptide could be used in combination to prevent the ribosome assembly to block HCV RNA translation more effectively. Recently, it has been shown that a peptide corresponding to 11 to 28 amino acids of La protein is capable of inhibiting both poliovirus and HCV IRES-mediated translation (23). Although the mode of action of this peptide is yet to be elucidated, it appeared that La peptide lacks the HCV RNA binding ability. In this study, we have identified the peptide LaR2C, which retains the RNA binding ability and also inhibits HCV IRES-mediated translation specifically without having a significant effect on cap-dependent translation. Due to its potential dominant negative effect, it would also be interesting to investigate the efficacy of the peptides in combination to develop an effective antiviral strategy against hepatitis C virus infection.

ACKNOWLEDGMENTS

We thank Akio Nomoto and Tsukiyama-Kohara for the HCV 1b encoding plasmid pCV, Jack Keene for the pET-La construct, and Asim Dasgupta for providing us the NSP-La peptide sequence. We also thank Steven F. Dowdy and S. Vijaya for the pTAT and pHATAT plasmids and R. Varadarajan for providing NSP. We gratefully acknowledge Sankar Bhattacharyya, Debojyoti Dhar, Partho Sarothi Ray, and other lab members for their help and critical comments on the manuscript.

This work was supported by a grant from the Department of Biotechnology (DBT), India, to S.D. and also partially supported by funds from the Department of Science and Technology (DST), India. R.P. was supported with a predoctoral fellowship from the Council of Scientific and Industrial Research, India. S.S.R. was supported from DBT-PDF.

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