Human La Antigen is Required for the Hepatitis C Virus Internal Ribosome Entry Site (IRES)-mediated Translation

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The abbreviation used are: HCV, hepatitis C virus; IRES, internal ribosome entry site (segment); SELEX, systematic evolution of ligands by exponential enrichment; 5‘NCR, 5’ non-coding (untranslated) region.

Running title: HCV IRES requires La antigen
The 5' noncoding region (5'NCR) of the hepatitis C virus (HCV) RNA genome serves as an internal ribosome entry site (IRES) and mediates translation initiation in a cap-independent manner. Previously, we reported the interaction between La antigen and the HCV IRES which appeared to occur in the context of initiator AUG. It was further shown that HCV IRES-mediated translation was stimulated in the presence of human La antigen. In this study, we have defined the cis- and trans-acting elements responsible for La-5'NCR interactions and established the dependency of the HCV IRES efficiency on cellular La antigen. During the La-IRES interaction, initiator AUG but not the neighboring codons was found to be the direct target of La binding. The C-terminus effector domain-dependent modulation of La binding to the HCV IRES is demonstrated by deletion and substitution mutagenesis of the protein. An RNA SELEX generated against La protein that selectively binds La in HeLa lysates and competes for the protein binding to the 5'NCR, was used to demonstrate the requirement of La for the HCV IRES function in the context of mono- and dicistronic mRNAs. Sequestration of La antigen by the RNA SELEX in HeLa translation lysates blocked the HCV and poliovirus IRES-mediated translation in vitro. The functional requirement of La protein for the HCV IRES activity was further established in a liver-derived cell line and in an add-back experiment in which the inhibited IRES was rescued by recombinant human La. These results strongly argue for the novel role of La protein during selection of the initiator AUG and its participation during internal initiation of translation of the HCV RNA genome.
The eukaryotic mRNAs contain short, unstructured 5’ noncoding region (5’NCR) and are usually translated by cap-dependent mechanism (see reviews, 1-3). In this scheme, the translation is initiated through binding of initiation factor-4F (eIF-4F) to the cap structure via its eIF-4E subunit. This event is followed by joining of 43S ribosomal complex that consists of 40S subunit, eIF-3, Met-tRNA\textsubscript{i} and GTP bound eIF-2. In contrast, a few cellular and viral mRNAs are translated by a distinct mechanism (4-7) because of their highly structured and relatively longer 5’NCR. These structural cis-elements constitute internal ribosome entry site (IRES) that promote assembly of initiation complex independent of the 5’ end and thus, mediate internal initiation in a cap-independent manner. A clinically important positive strand RNA virus, hepatitis C virus (HCV) that causes a variety of liver diseases in humans including hepatocellular carcinoma (8-10), has also been shown to translate by cap-independent mechanism through its unique IRES structure (11-17). Almost the entire 5’NCR (341 nt) of the HCV RNA except 40 nt at the extreme 5’ end has been shown to function as an IRES element (14, 16). The HCV IRES also extends to a short nt stretch (nt 12-30) downstream of the initiator AUG codon (18, 19). A pseudoknot structure (Fig. 1) preceding the translation start-site is another unique feature of the HCV IRES that is highly conserved among HCV genotypes and pestiviruses (17, 20). The HCV IRES, like other viral and cellular IRES elements, serves as a substrate for binding of multiple cellular factors. Among cellular proteins that participate during cap-independent translation initiation, two non-canonical translation initiation factors, polypyrimidine-tract binding protein (PTB) and La antigen (p52 or SS-B) are recognized for their ability to bind several viral 5’NCRs (see reviews, 2, 6). The functional importance of both the proteins during internal initiation of translation has been reviewed in detail (21).

La antigen is a multifunctional phosphoprotein that was originally identified as an autoantigen in patients with autoimmune disorders (see review, 22). Several studies have provided evidence for helicase activity of La antigen (23-25). The human La
antigen contains three putative RNA recognition motifs (RRM) and a basic region followed by a stretch of acidic region at the C-terminus (26). The C-terminus also contains a homodimerization domain that is required for the function of La in enhancing translation of poliovirus RNA (27). While a fraction of La antigen is found in the cytoplasm, majority of the protein is localized in the nucleus. However, cellular stress such as that resulting from a viral infection causes redistribution of the nuclear La to the cytoplasm (28). In the nucleus, La antigen transiently associates with the 3’ oligo (U) terminus of the RNA polymerase III transcripts and facilitates transcription termination and recycling of transcription complexes for reinitiation process (29). Phosphorylation of Ser 366 residue of La antigen by casein kinase II modulates its role in transcription but does not affect its RNA binding ability (30). In the cytoplasm, La antigen is associated with a subset of small ribosomal subunit possibly by direct association with 18S rRNA (31). La protein has been shown to interact with the 5’NCR or 5’ stem-loop structures of poliovirus (28), rubella virus (32), influenza virus (33), sindbis virus (34), the human immunodeficiency virus (HIV) TAR element (35), rhabdoviruses VSV (36) and rabies virus (37). For the most part, these La interactions with viral RNA elements do not appear to be mediated by the 3’ terminal oligouridylate sequences that are characteristics of La protein’s prototypical interactions with pre-processed polIII transcripts. RNA selection experiments with La protein have revealed a novel CACAA motif that may account for some non-protypical RNA interactions (38, DJK and JDK, manuscript in preparation). One RNA species, pre-tRNA_{met}-elongator (termed C5 RNA by Harada et al., [39]) was found to have a unique relationship to La protein, as determined by its ability to bind deletion mutant La 22-408 that was unable to bind any polIII transcripts in HeLa cells extracts (38). The C5 RNA contains both 3’ oligouridylates as well as the CACAA motif. The presence of a double binding motif likely accounts for the increased affinity of C5 RNA relative to other polIII transcripts. Many of the viral RNAs that interact with La protein also contain close or exact matches
to the CACAA motif within their 5’NCR sequences, suggesting that this element may play a role in translational regulation by La protein.

The aberrant translation initiation of poliovirus IRES-mediated RNA is corrected in the presence of La antigen that is also accompanied by a modest stimulation of translation (28, 40). La binding to the HIV TAR structure alleviates the translational repression exerted by TAR on the downstream reporter gene (41). We have recently shown that La antigen interacts with the HCV IRES which leads to significant level of stimulation of the HCV IRES-dependent translation (42). One of the interesting and novel aspects of this interaction is that it occurred in the context of the initiator AUG.

Here, we have further characterized cis- and trans-elements involved in the La-5’NCR interactions. The functional importance of these interactions during transactivation of the HCV IRES is investigated using mutants derived from the HCV 5’NCR as well as those in the La protein. An RNA SELEX ligand that specifically interacts with La protein inhibited the HCV IRES-mediated translation in a liver derived cell line and HeLa lysates supporting the in vivo functional relevance of that interaction. The data presented here further reinforces our previous observations that initiator AUG codon of the HCV RNA is an essential recognition motif of La binding. This interaction appears to be responsible for the La-dependent translation initiation of the HCV RNA.

MATERIALS AND METHODS

Construction of plasmid DNA- The construction of recombinant plasmids were carried out by standard protocols. The plasmid pNCR-C(AUG) contains full length HCV 5’NCR (HCV-1) followed by 15 nt of the core region (42). The plasmid NCR-C(ΔAUG) is similar to the NCR-C(AUG) except that it lacks initiator AUG at nt 342. The AUG deletion in this plasmid was carried out by QuickChange Site-Directed Mutagenesis method (Stratagen). Using similar strategy, the plasmids pNCR-C(ΔACC), pNCR-
C(ΔC2), pNCR-C(ΔC3) and pNCR-C(ΔC4) were constructed which contain deletions of ACC (nt 339-341) and codons 2, 3, 4 of the core respectively. pNCR-C(Δ1-83) was derived from the wild type plasmid pNCR-C(AUG) in which nt 1-83 at the extreme 5' end of the HCV 5’NCR was enzymatically deleted using SalI and Nco1. PCR-based strategy was employed to construct plasmids pNCR-C(Δ1-120) and NCR-C(Δ1-135). Both the plasmids represent HCV 5’NCR sequence of NCR-C(AUG) except that they lack 120 and 135 nt from the 5' end of the NCR respectively. The plasmid NCR-C(AUG) was mutated at nt positions 126-129 (CUCC to AGAA) and 327-329 (CGU to GCA) of the HCV 5’NCR to construct pNCR-C(SI Mut) and pNCR-C(SII Mut) respectively. The plasmid NCR-C(Py-III Mut) was constructed by substitution of UUUCU to AAAGA at nt 194-198 in the pNCR-C(AUG). The nt sequence in these plasmids were confirmed by dideoxyribonucleotide sequencing method. The construction of His-tagged La encoding cDNA is described in detail by Goodier et al. (26). Selection of high affinity C5 RNA ligand against La antigen by SELEX method and construction of plasmid pC5-SP64 that expresses C5 RNA is described previously (38).

*In vitro transcription*-RNA transcripts were synthesized in vitro from linearized plasmid DNA that was purified by elution of the desired fragments from the agarose gels after digestion with an appropriate restriction endonuclease. The pNCR-C(AUG)] and all its derivatives were linearized with EcoRI and transcribed with T7 RNA polymerase using standard protocols. The plasmids pT7C1-341, (16) and pPB305 were linearized by HpaI and transcribed by T7 RNA polymerase to produce HCV and Thieler's murine encephalitis virus (TMEV) 5’NCR containing monocistronic luciferase RNA respectively. The plasmid T7P5NCLuc was linearized with Nhe1 and transcribed with T7 RNA polymerase to generate monocistronic RNA containing poliovirus 5’NCR followed by luciferase sequence. These monocistronic RNAs will thus contain respective IRES elements fused with the luciferase open reading frame followed by poly(A) tail. Plasmid pC5-SP64 and pGEM-4 were linearized with Dra1 and EcoR1 respectively for the
transcription with T7 RNA polymerase. For the preparation of a mutant C5 SELEX, pC5-SP64 was digested with Bsp1286I and transcribed similarly. The capped RNAs were synthesized in the presence of m7G(5')ppp(5')G cap analogue using Ampliscribe T7 transcription kit (Epicenter Technologies). The plasmid pRL-1b which encodes upstream renilla luciferase followed by the HCV IRES (nt 1-357 of the HCV genotype 1b) linked to the second reporter firefly luciferase gene (56) was linearized with HindIII and transcribed in the presence of cap analogue as described above. The capped T7DC1-341 RNA (16) was prepared similarly. The quality, integrity and quantity of RNA preparations were ensured by spectrophotometrically as well as agarose gel electrophoresis. The radioactive RNA probes were synthesized under similar reaction conditions with 4-thio-UTP and [α-32P]CTP.

Purification of La antigen and GST-PTB-A cDNA encoding the human La antigen without a tag in the pET-8c vector was used for the expression of recombinant La antigen. The expression of La antigen was induced by 1 mM IPTG in Escherichia coli (BL21(DE3)) cells and purified by a two-step chromatography to homogeneity as described earlier (42). The expression of full length human La (La 1-408) and its truncated versions that contain six-His tag at the C-terminus is described by Goodier et al. (26). The purification of these proteins were carried out with Ni-NTA agarose under non-denaturing conditions and eluted with imidazole according to manufacturer’s protocol (Qiagen). The La preparations were dialyzed against buffer D (5 mM HEPES [pH 7.6], 25 mM KCl, 1 mM EDTA, 1 mM DTT, 10% [v/v] glycerol, 0.2 mM PMSF, 1uM Leupeptin) and stored in aliquote at -80 C. The glutathione-S-transferase (GST) and GST-PTB fusion protein were expressed in bacteria using plasmids GEX-KT and GST-2TK/PTB respectively, and purified as described earlier (43).

UV cross-linking of proteins with RNA-4-Thio-UDP (Sigma) was phosphorylated with nucleoside 5'-diphosphate kinase to prepare 4-thio-UDP (44). RNA probes synthesized in the presence of 4-thio-UTP and [α-32P]CTP were incubated with the purified proteins
or lysates at 30 C for 30 min in RNA binding buffer (buffer D plus 2 mM MgCl2) as described previously (42). For all the competition assays, competitor RNAs were added along with the components of the reaction mixture prior to UV cross-linking. The UV cross-linking was carried out in Stratalinker (Stratagen) for 30 min at 4 C. The ribonucleoprotein complexes were treated with RNase A (10-20 U) (USB) and analyzed by sodium dodecyl sulfate-polyacrylamide (12%) gel electrophoresis (SDS-PAGE) followed by autoradiography. The intensity of the bands were quantitated by PhosphorImager.

**Immunoprecipitation of cellular La antigen-HCV 5’NCR complexes**- HeLa S10 cytoplasmic fractions from suspension cultures of HeLa S3 were prepared essentially as described by Barton et al. (45). These lysates were active for the HCV IRES-mediated translation of reporter RNAs. Huh7 cells were cultured on petri dishes and the S10 lysates were prepared using the same method. S10 fraction (50 ug protein) maintained in RNA binding buffer was mixed with full length NCR-C(AUG) RNA probe in a total volume of 50 ul. After UV cross-linking and ribonuclease treatment, the samples were diluted to 500 ul with NETS buffer (50 mM Tris-HCl, [pH 7.4], 5mM EDTA, 1 mM DTT, 100 mM NaCl, 0.05% Nonidet P-40), mixed with affinity purified human anti-La autoantibodies (V3) and incubated at 4 C for 1 hr. The immunocomplexes were immobilized on ProteinA-Sepharose 4B beads for 2 hr at 4 C. The unbound materials were washed six times with the same buffer. The bound protein(s) were analyzed by SDS-PAGE followed by autoradiography. A parallel reaction mixture was performed with normal human IgG (NH IgG) purified by ProteinA-Sepharose chromatography and served as a control.

**Translation**- HeLa S10 lysates and initiation factor (IF) lysates (45) were used during translation-inhibition assay. Twenty five ul of the translation mixture that included 5 ul of the S10 lysates, 3 ul IF, 3 ul 10XCPK (300 mM creatine phosphate, 4 mg/ml phosphocreatine kinase, 600 mM KCH3CO2 and 155 mM Hepes-KOH, pH 7.4), 1 ul 35S-
methionine and 20U RNasine, were programmed with IRES containing luciferase RNA (each 100 ng) in the absence or presence of varying amounts of C5 SELEX RNA. The reaction was carried out for 1 hr at 30 C and luciferase activity was assayed from a 2 ul aliquote as described by de Wet (46). Inhibition of translation by C5 RNA was plotted against the ligand concentrations. Rabbit reticulocyte lysates (RRL, Promega) were used for translation-stimulation studies in the presence of His-La (1-408) or its truncated versions as described earlier (42). Translation of the capped T7DC1-341 was carried out using 10 ul RRL, 2 ul HeLa S100, 1 ul 35S-methionine, 1 ul amino acid mixture without methionine and 20U RNasine in a total vol. of 25 ul for one hour at 30 C. Two ul of translation reactions were subjected to SDS-PAGE (12%) electrophoresis. The gels were treated with Fluoro-Hance (RPI), dried and used for PhosphorImage analysis (BioRad).

A liver-derived cell line (Huh7) was used for the co-transfection assay. The cells were transfected with in vitro transcribed uncapped T7C1-341 or capped dicistronic dual luciferase RNA using lipofectin transfection protocol (Gibco BRL). To demonstrate the inhibitory activity of C5 SELEX or its mutant on the HCV IRES-controlled translation, the cells were directly co-transfected with the reporter RNAs along with C5 SELEX. An RNA derived from pGEM-4 multiple cloning site served as a control during a similar co-transfection experiment. Each transfection was carried out in triplicate. The luciferase activity was assayed as described above.

RESULTS

The C-terminus of La protein modulates its interaction with the HCV IRES-La antigen contains three putative RNA recognition motifs (RRMs). RRM1 and RRM2 are located at the N-terminal half of La protein and RRM3 is located at the beginning of the C-terminal portion (Fig. 2A). Each of these RRMs play important role during direct recognition of the RNA motifs and increase the affinity of La during RNA binding by the other RRM (26). The C-terminus contains an adjacent basic and acidic region, phosphorylation sites
and a homodimerization domain that regulate its nuclear and cytoplasmic functions (26, 27, 30). Using various La antigen mutants shown in Fig. 2A, we investigated if these structural elements play significant role during recognition of the HCV 5’NCR. The bacterially expressed His-tagged La and those containing deletion mutations were purified by affinity chromatography using Ni-NTA-resins (26). A silver-stained SDS-PAGE pattern of the purified proteins is presented in Fig. 2B. The affinity purified proteins were UV cross-linked with the wild type [³²P]-labeled HCV 5’NCR probe and subjected to SDS-PAGE as shown in Fig. 2C. La 1-408, which represents full length wild type La antigen binds 5’NCR efficiently. RRM1 represented by La 1-103 alone did not interact with La antigen. However, when part of the RRM2 was included to the RRM1 in La 1-160, binding with the 5’NCR probe was observed but with lesser extent compared to the wild type (La 1-408). Although La 1-235 contains both the RRMs, it exhibited poor binding. Two additional mutants, La 188-408 and La 229-408, in which aa 187 and 228 were deleted from the N-terminus respectively, efficiently cross-linked with the 5’NCR. These binding data suggest that affinity of La for the 5’NCR interaction is directed more towards the RRM3 than the other two RRMs that are located at the N-terminal half of La. Contrary to these observations, the binding of C-terminal half of La protein to hY4 RNA was not detected in an mobility shift analysis (26). Using different preparations of La mutants, we further confirmed the unusual binding ability of La 188-408 and La 229-408 mutants. Because of the fact that the RNA targets of La antigen significantly differ from each another in their sequence and structural organization, the requirement of cis-elements within La antigen that regulate its RNA binding ability may also differ. It is also possible that these diverse group of RNAs present distinct structural elements for La binding. This promiscuous binding ability of La may also be an important element for its distinct nuclear and cytoplasmic functions. As suggested by competition studies described previously (42, see below), La antigen appears to make multiple contacts with the HCV 5’NCR. It is highly likely that this novel RNA binding
domain (RRM3) is able to make direct contact(s) with the 5'NCR to form RNP complex that was easily detected by the UV cross-linking assay.

The role of acidic and basic regions of La during 5'NCR interaction was ascertained by three La mutants containing substitutions or substitutions plus deletions at the carboxy terminal end. In GXK>DE, glycine and lysine-rich region (aa 328-341) was replaced with alternating aspartate (D) and glutamate (E) residues while La K>DE contains amino acids D, E, and D in place of lysine residues at positions 339, 341, and 344 respectively. The mutant, La ΔDXE>GK contains deletion of residues 337 to 367 and residues 368 to 376 within the acidic region were replaced with alternating glycines and lysines and juxtaposed to residue 336. The binding of La K>DE and La ΔDXE>GK was comparable to the wild type La antigen, whereas GXK>DE showed considerably reduced binding to the 5'NCR. These results identify the basic region as an important element of La protein-HCV 5'NCR recognition.

Because of the fact that La 229-408 contains regulatory cis-elements essential for translational and transcriptional activities of La protein (26, 27, 30), and that it binds the 5'NCR with affinity similar to the wild type La (Fig. 2C), we investigated if this mutant represents functional elements sufficient for stimulation of the HCV IRES activity. An in vitro synthesized uncapped monocistronic T7C1-341 RNA in which the HCV IRES drives translation of luciferase, was used as a template during translation stimulation studies in rabbit reticulocyte lysates (RRL). The RRL contains sub-optimal levels of La protein and has been previously used as a suitable system for similar studies (27, 40, 42). The translation of the HCV IRES-driven luciferase RNA was increased modestly, by 2.5-fold in the presence of La 229-408 (Fig. 3, lanes, 3-5) compared to the basal translation without the exogenously added La (lane 2). However, the full length La stimulated >6-fold at similar concentration of the protein (lanes 6-8). The La-stimulated HCV IRES activity was completely blocked by monoclonal La antibody (SW5) but not by a non-specific control (data not shown). These data support the stimulatory role of La binding
during the HCV IRES-driven translation initiation (42). Further, these data also demonstrate that the N-terminal half of La antigen is important for translational stimulatory activity of La. While the C-terminal half binds the 5’NCR efficiently, it is unable to stimulate translation at wild type levels.

*The La antigen targets initiator AUG but not the flanking nucleotides for its binding to the HCV RNA-* We have previously shown La antigen interaction with the HCV 5’NCR in the context of the initiator AUG (2). To further characterize this unique pattern of interaction, several additional 5’NCR mutants were used during competition assay. A mutant RNA representing a full length wild type HCV 5’NCR that lacked the initiator AUG codon [NCR-C(ΔAUG)] was used as a competitor for La binding to the thio-U containing [32P]-labeled HCV IRES [NCR-C(AUG)] probe (Fig. 4A). The intensities of binding signal due to cross-linking of La with the wild type probe progressively decreased at increasing concentrations of the unlabeled homologous competitor RNA (Fig. 4A, lanes 2-4). However, AUG deletion containing mutant RNA showed considerably low level of competition (lanes 5-7). Consistent with our previous findings, the results with these additional mutants show that initiator AUG at this position is required for La interaction. Interestingly, a series of mutant RNAs which retained the initiator AUG but had three nt deletion in its vicinity showed La binding similar to the wild type 5’NCR (Fig. 4B). Additional mapping analysis further suggested that nt 1-120 from the 5’ end of the NCR does not contribute to the La binding. The disruption of the helical structure within the stem 1 of the pseudoknot marginally affected La-5’NCR interaction. Similarly, several substitutions from pyrimidines to purines within the apical loop of domain also showed no affect on La binding (data not shown). These results clearly suggest that the La binding is focused on initiator AUG and the structural elements located within the domains III and IV.

*Multiple forms of cellular La antigen bind the HCV 5’NCR*-Since the functional significance of La antigen interaction to the 5’NCR was investigated in both the liver
(Huh7) and non-liver (HeLa) derived translation systems (see below), we first
determined the presence of La protein in the cytoplasmic fractions (S10) of these cell
lines. The S10 lysates were UV cross-linked with thio-U containing $^{32}$P-labeled wild type
HCV 5'NCR [NCR-C(AUG)] and immunoprecipitated using affinity purified human La
antibodies (V3) (Fig. 5). Two closely migrating UV cross-linked 5'NCR-La complexes of
the HeLa S10 at 50/52 kDa were observed following immunoprecipitation with the
human La antibodies (lane 4) but not with the normal human IgG (NHIgG) (lane 3).
When Huh7 S10 lysates were used during this assay, a 43 kDa and a 50 kDa UV cross-
linked proteins were immunoprecipitated (lane 2). These results are consistent with our
previous observations of a predominant 43 kDa La protein from Huh7 cell line bound
the HCV 5'NCR. Interestingly, this La-5'NCR complex was not immunoprecipitated
from the HeLa S10 preparation after UV cross-linking with the 5'NCR. However, one of
the two 50/52 kDa HeLa S10 derived La antigen that was bound to the probe, was also
detected in Huh7 S10 (compare lanes 2 and 4). Using immunoblot assay in earlier
studies, Pruijn (55) also demonstrated the existence of only 52 kDa La species in HeLa
cell lysate whereas several other cell lines were shown to contain both 50/52 kDa as well
as 43 kDa La in their lysates. Thus, the 52 kDa La form which was used for binding
assay appears to be expressed in both the cell lines. The diversity and the origin of these
La forms are not clearly understood. However, results from several laboratories
suggest that different La proteins may result from alternative splicing, phosphorylation
or cleavage at the PEST sites within the C-terminus (30, 47, 55).

La antigen is required for the HCV IRES-mediated translation-As shown above, La
antigen directly interacts with the critical components of the HCV IRES and stimulates its
activity to a considerable extent. Thus, it is conceivable that La protein might be
required for the HCV IRES activity. To initiate these studies, we first used full length
5'NCR as a competitor during translation of T7C1-341 (Fig. 6) in RRL. The basal level of
the HCV IRES activity (lane 2) as determined by the translation of luciferase was
completely inhibited by the 5’NCR competitor (lane 3). This inhibition was rescued by the addition of recombinant human La antigen (lane 4), but not by an unrelated protein or BSA (data not shown). This La add-back experiment clearly indicates that La antigen has functional requirement for the HCV IRES function.

To further characterize La antigen requirement for the HCV IRES function, we explored the possibility whether SELEX RNA molecules could serve as a specific reagent that can sequester La protein in the translation lysates, thereby render its function during the HCV IRES-controlled translation initiation process. The systematic evolution of ligands by exponential enrichment (SELEX) is an in vitro selection procedure which leads to isolation of oligonucleotides with selective affinity for a given target. In this study, we have used a C5 SELEX RNA generated against La antigen that binds the protein with high affinity (DJK and JDK, manuscript in preparation). The C5 SELEX contains dual recognition motifs for La antigen; motif I contains CACAA target whereas motif II is located at the 3’ end of the RNA ending with terminal UAAUUU residues. The overall structure of C5 SELEX resembles with a typical t-RNA. The SELEX binds only La protein in the HeLa translation lysates. This conclusion was derived from direct UV cross-linking of C5 SELEX probe with HeLa S10 fraction and subsequent immunoprecipitation by La antibodies (Fig. 7A). The binding specificity of the C5 RNA was further determined in a competition assay. La antigen binding to the wild type HCV 5’NCR probe was successfully inhibited by the C5 RNA (Fig 7B, compare lanes 2-4 with the homologous competitor shown in lanes 5-7). Since PTB was previously shown to bind the 5’NCR at multiple sites (43), we used a similar competition assay as a control. The PTB interaction to the probe was not affected by C5 RNA even at 200 molar excess (Fig. 7C, lanes 2-4). However, under similar conditions, the homologous competitor showed higher degree of competitive inhibition (lanes 5-7). These results clearly demonstrate the specificity of C5 RNA to bind La antigen with high affinity and further indicate that the C5 ligand may not perturb the structural integrity of the 5’NCR, as it
permits PTB interaction. Because of these specific features, we used C5 RNA as a specific probe to determine the requirement of La during HCV IRES-controlled translation.

The C5 SELEX RNA was introduced into HeLa S10 lysates during translation of the HCV (T7C1-341), poliovirus (T7P5NCLuc) and TMEV (PB305) IRES-controlled luciferase reporter RNA. In this experiment, poliovirus IRES-Luc was used as a positive control because of its well-characterized interaction with La antigen. The translation of TMEV IRES-controlled luciferase occurred efficiently in the presence of C5 SELEX RNA (Fig 7D). However, the translation of HCV and poliovirus IRES-containing luciferase RNA was diminished with increasing concentrations of the C5 SELEX ligand under similar translation conditions. Thus, addition of C5 RNA to the lysates specifically sequestered La antigen that was required for the poliovirus and the HCV IRES function. Interestingly, general translational machinery was not affected by the SELEX RNA as evidenced by the continued translation of the TMEV IRES-controlled reporter cistron. A similar inhibitory pattern of the HCV IRES by C5 RNA was also detected in RRL (data not shown).

The ability of C5 SELEX to inhibit the HCV IRES activity was also tested in the context of a capped dicistronic mRNA (T7DC1-341). The upstream cistron (CAT) of this mRNA is translated by cap-dependent mechanism whereas the translation of downstream luciferase (LUC) coding region is dictated by the HCV IRES (16). The translation of luciferase of the capped T7DC1-341 mRNA was found to be efficient in the HeLa translation system that was completely abolished by the C5 SELEX. However, cap-dependent translation of CAT was inefficient under these conditions. This phenomenon was also observed when capped Brome Mosaic Virus mRNAs were used as templates for translation in HeLa lysates (data not shown). We, therefore, utilized reticulocyte translation system supplemented with the HeLa S100 as a source of cellular factors including La antigen. The translation of T7DC1-341 was then investigated in the presence of either wild type C5 SELEX or a mutant C5 RNA (Fig. 7E). The C5 mutant
lacks La recognition motif II which is located at the 3' end of the RNA. Since motif I (the internal site for La recognition) remains intact in this mutant, its overall structure and stability remains similar to that of wild type SELEX molecule except that this mutagenesis resulted in a moderate lose of affinity for La antigen. The wild type C5 SELEX dramatically inhibited translation of the luciferase but not the CAT cistron (lanes 3, 4). The C5 mutant, however, was less effective in inhibiting the HCV IRES-dependent translation compared to that of wild type C5 (compare lanes 5, 6 with lanes 4, 5). The mutant SELEX also failed to inhibit cap-dependent translation of CAT cistron (lanes 5, 6). These observations further reiterate the specific inhibition of the HCV IRES function by C5 SELEX by sequestering La antigen without inhibiting general translation process.

Since HCV targets human hepatocytes for completing its life-cycle, we further investigated if La antigen is required for the HCV IRES activity in vivo. To address this question, a human liver-derived cell line (Huh7) was co-transfected with T7C1-341 (HCV IRES-Luciferase) and C5 SELEX or a control RNA of similar length derived from pGEM-4 vector. The cell lysates were assayed for the HCV IRES-dependent luciferase expression. The HCV IRES activity was specifically inhibited by the C5 RNA but not by the control RNA (unrelated) (Fig. 8A). The TMEV IRES which served as a negative control in our in vitro assay (Fig. 7D), was found to be inactive in Huh7 cells after repeated transfection studies. However, other controls such as poliovirus IRES was efficiently inhibited by the C5 SELEX (data not shown). A dual luciferase assay system was employed to further evaluate the specificity of C5 SELEX inhibitory activity. In vitro transcribed capped RL-1b contains upstream renilla luciferase (RL) that is translated by cap-dependent mechanism whereas the translation of downstream firefly luciferase (FL) is controlled by the HCV IRES (Fig 8B, upper panel). During transfection studies as described above, a dramatic inhibition of the HCV IRES in the context of the dual reporter RNA was observed by wild type (WT) C5 RNA (Fig. 8B) whereas the mutant (Mut) C5 exhibited moderate inhibitory activity similar to that seen during our in vitro analysis (Fig. 7E).
Interestingly, the moderate inhibition of cap-dependent translation was also observed by both WT as well as Mut C5 SELEX. A possible explanation of such inhibition could be that La antigen targets initiator AUG codon embedded in the context of Kozak consensus sequence (ACCAUGG). The recognition of AUG by La antigen is thought to be involved for the start-site selection during cap-dependent translation (48). Therefore, it could be possible that C5 SELEX may have interfered with La antigen binding to the initiator AUG during cap-dependent translation. These results which are consistent with the in vitro inhibition data presented in Fig. 7D, provide an in vivo evidence that La protein is an essential feature of the HCV IRES-mediated translation. Taken together, La antigen requirement for the HCV IRES was established in the context of several reporter constructs both in vitro as well as in living cells.

DISCUSSION

In this study, we have demonstrated novel features of the La-HCV IRES interaction and their functional consequences during the IRES-controlled translation. Although several cellular factors have been recently shown to interact with the HCV 5’NCR, the binding of La antigen appears to have fundamental impact on the IRES efficiency. These conclusions are based on the extensive mutational analysis and the strong inhibitory effect of SELEX RNA that sequesters La antigen with high affinity.

La antigen has been shown to be evolutionarily conserved from yeast to human and its presence in the nucleus as well as cytoplasm of a wide variety of cell types has been documented (46). Because, Huh7 and HeLa cells support HCV IRES-mediated translation (12, Fig. 7D), we investigated the possible existence of various forms of La that may bind the HCV 5’NCR in a translationally active cellular extracts. At least three forms of La antigen were found to interact with the HCV 5’NCR; two forms migrating at 50/52 kDa from HeLa lysates and a 43 kDa form that was found only in Huh7 lysates.
One of the 50/52 kDa form found in HeLa extract was also observed in the Huh7 lysates. Fan et al., (30) have previously demonstrated that HeLa cells contain phosphorylated and unphosphorylated forms of La antigen. Though both the states of La antigen differ in their transcriptional activity, these La forms were equally active for RNA binding. However, both the La forms were shown to have similar electrophoretic mobility on SDS-PAGE. Thus, it appears that the two 5’NCR bound La species from HeLa S10 lysates (Fig. 2) could be alternately spliced RNA encoded isoforms. The 43 kDa La is thought to be the cleavage product of the 50/52 kDa form. The functional importance of three La species described above in the context of their binding to the 5’NCR remains to be investigated in detail.

Several lines of evidence presented here demonstrate that initiator AUG codon is an essential component of La antigen's recognition motif within the HCV 5’NCR. Since complete inhibition of La interaction was not achieved when initiator AUG was deleted (Fig. 4A) or substituted to AAG/AUU (2), it is likely that additional cis-elements of the 5’NCR may be required for La binding to the 5’NCR. Several mutants containing deletions from 5’ end and substitutions in the pseudoknot structure of the 5’NCR were used to address this problem. The deletion of either oligopyrimidine-II tract (Py-II, Fig. 1) or substitutions that disrupt the base pairing in the stem I of the pseudoknot (17) only affected the La binding modestly. Using synthetic RNA molecules as a substrate, McBratney and Sarnow (48) noted that La antigen interacts with the AUG triplet and that this interaction was influenced by the Kozak sequence context. In this study, the deletion of triplets surrounding AUG codon failed to affect La interaction. The three 5’NCR mutants, NCR-C(ΔC2), NCR-C(ΔC3) and NCR-C(ΔC4) that showed efficient binding with La antigen had complete ACCAUGA Kozak sequence except that they lacked in specific codon (see Fig. 3B, upper panel). Interestingly, the deletion mutant NCR-C(ΔACC) in spite of having UGCAUGA in place of usual ACCAUGA, interacts efficiently with La antigen.
Svitkin et al., (40) have shown that N-terminal half of La antigen (amino acids 1-194) binds poliovirus 5’NCR but fails to correct or stimulate the translation initiation. In this study, we demonstrate that the mutant protein representing C-terminal half of La antigen (La 229-408) also binds the 5’NCR efficiently and modestly stimulates the HCV IRES-directed translation in rabbit reticulocyte lysates (RRL). The C-terminal half of La protein functions as an effector part of La antigen during poliovirus IRES-mediated translation and contains a dimerization domain that spans between aa 226-348 (27). In light of these observations, one can argue that if La binding to the IRES elements and its homodimerization via C-terminal domain are the only criteria for its translational stimulation activity, La 229-408 should achieve a translation stimulation similar to the wild type. However, we observed that stimulatory activity of the La 229-408 was comparatively lower. Thus, C-terminal effector domain is important as suggested by previous studies (27, 40) but in addition to this domain, N-terminal half of La antigen may also play an important role during IRES-mediated translation. This is also supported by the interaction between La1-160 mutant and the 5’NCR.

The functional importance of La binding to the HCV 5’NCR was demonstrated by the translation-stimulation studies in which several-fold increase in the HCV IRES activity was observed in the presence of recombinant human La protein in RRL (Fig. 3). Because of the fact that RRL contain comparatively little La protein than HeLa lysates, the effect of recombinant La was more pronounced in this system. Belsham et al. (49) attempted La-depletion strategy to demonstrate the functional importance of La protein during poliovirus IRES-mediated translation. In that assay, La antigen was immunodepleted from HeLa lysate. Interestingly, the La-depletion dramatically abolished the translation ability of the lysate which was not rescued by the addition of purified La protein. The explanation was that such an approach suffers from the removal of La-associated factors that may be essential element(s) of translation initiation. A La-depletion/add-back approach was also employed by Das et al., (50)
during their studies on the inhibition of the HCV IRES-mediated translation by yeast I-RNA that strongly binds human La antigen. In this experiment the translation lysates were passed through an I-RNA affinity column, and the flow through that was assayed for HCV IRES function failed to support translation. During an add-back experiment described here (Fig. 6), again the HCV IRES activity was rescued to the basal level that suggested the requirement of additional factor(s) for the stimulated translation. In light of the failure of some of these approaches, we sought an alternative strategy of employing SELEX RNA molecules with high affinity to La protein to establish the requirement of La antigen for the HCV IRES activity in vivo. We have utilized a high affinity RNA ligand, termed C5 SELEX RNA, that binds to the La antigen (Fig. 7A). This approach allowed us to provide two additional lines of evidence in support of the functional role of La protein in the HCV IRES-mediated translation. First, the sequesteration of La in the HeLa translation mixture was achieved by the addition of large amounts of SELEX RNA. This led to a dramatic decrease in the HCV and poliovirus IRES functions, whereas cardiovirus (TMEV) IRES activity was unaffected. We further demonstrate an in vivo inhibition of the HCV IRES activity by the C5 SELEX but not by an unrelated control RNA. These studies together provide unequivocal evidence for the requirement of La in vivo for efficient HCV IRES function.

The HCV 5’NCR has been shown to form RNP complexes with several cellular factors including La antigen and PTB (42, 43). Recently, in vitro reconstitution studies by Pestova et al. (51) revealed a direct interaction of 40S ribosomal subunit via S9 protein that is located on the other end of the mRNA binding cleft from the eIF-3 binding site. In a similar study, 4 subunits of the eIF-3 (p170, p116, p66 and p47) were also shown to make direct contact with the apical half of the domain III of the 5’NCR (52). In our studies, we observed that La antigen binding to the 5’NCR was unperturbed when increasing concentrations of 40S ribosomal subunit were added to the assay mixture (unpublished data). These and other studies suggest that the interactions of cellular
proteins may be directed toward specific sites in the 5'NCR. Furthermore, eIF-3 and La antigen are known to associate with native 40S ribosomal subunit in the cytoplasm (1, 31). Since structural integrity of the 5'NCR is important for its IRES function, it is highly likely that the structural elements of the HCV IRES provide a "docking facility" for the ribosomes. The ribosomal docking must be mediated by these cellular factors to direct the complex to the designated initiation site. In this scenario, La antigen, may be functioning as a molecular chaperon during assembly of the ribosomal complex directly at the initiation site.

Based on the inherent feature of La antigen for its ability to unwind the double stranded RNA in a ATP-dependent manner (24), it is conceivable that the La binding to the translation initiation site provides additional advantage for the initiation complex in acquiring RNA helicase activity. Interestingly, the flanking sequences of the initiator AUG form a stable stem-loop structure (53, Fig. 1, Domain IV) and this structure must be melted away to facilitate translocation of the 80S complex to the second and subsequent codons. Indeed, Honda et al., (53) demonstrated that the mutations that destabilize this stem-loop structure promote the IRES efficiency. Thus, because of strategic binding and its dsRNA helicase activity, La antigen may play a pivotal role in accelerating translation efficiency of the HCV IRES. A more plausible role of La protein may be that it functions like an RNA chaperon in positioning the translation machinery at the start-site. This study further revealed a unique property of La antigen during RNA recognition through its putative RRM3 motif. Extensive mutagenesis of RRM3 and the C-terminal regulatory elements is needed to further delineate the functional importance of these motifs during HCV IRES-driven translation. That remains the goal of our future investigation.

In summary, the present communication and a previous report (2) provide several lines of evidence which suggest that initiator AUG is the primary target of the La protein, and that additional structural motifs of the IRES such as domains III and IV must
The C-terminus effector domain plays a pivotal role during La-5’NCR interaction. The requirement of La for the HCV IRES was established using the C5 SELEX RNA that sequesters La protein both in vitro and in vivo. The La antigen add-back experiment reiterated these observations.

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FIGURE LEGENDS

FIG. 1. Schematic representation of computer-generated RNA folding model of the HCV 5’NCR as proposed by Honda et al., (53) and Wang et al., (20, 54). The conserved AUGs, three putative polypyrimidine-tracts (Py) as well as stem I (SI) and stem II (SII) of the pseudoknot structure are shown.

FIG. 2. Interaction of La antigen mutants with the wild type HCV 5’NCR. A. His-La mutants used during direct UV cross-linking of the HCV 5’NCR probe. The three putative RNA recognition motifs (RRMs) and the adjacent acidic (+++) and the basic (- - -) regions at the C-terminal end are elaborated. B. SDS-PAGE pattern of the purified His-La proteins. The purified proteins (100 ng) were subjected to electrophoresis and stained with silver staining method to visualized the protein bands C. Direct UV cross-linking of the purified His-La proteins and its truncated mutants with the NCR-C(AUG) RNA probe. Hundred nanogram of each of full length His-La (La 1-408) and the truncated versions were UV cross-linked with the RNA probe and fractionated by SDS-PAGE after digestion with RNase. The dried gel was exposed for autoradiography.

FIG. 3. Translational trans-activation of the HCV IRES-controlled luciferase RNA in rabbit reticulocyte lysates (RRL) by full length and C-terminal half of La antigen. Translation of the HCV IRES-Luciferase RNA (T7C1-341) with increasing amounts of bacterially expressed purified full length His-La antigen (lanes 6-8) or its truncated version representing C-terminal half (lanes 3-5). Lanes; 1, no template RNA; 2, without added His-La antigen. The translation was carried out with 50 ng in vitro transcribed uncapped T7C1-341 RNA in RRL and the translation products were radiolabeled with [35S]methionine. The final volume (20 ul) of the reaction mixtures was adjusted with RNA binding buffer (see Materials and Methods). Ten microliter of translation mixtures
were fractionated by SDS-12%PAGE. The gels were treated with Fluoro-Hance (RPI Corp., USA) before autoradiography. Luciferase activity was measured with a 2 ul aliquot of the translation mixture according to de Wet et al. (10).

**FIG. 4.** La antigen binds the HCV 5’NCR in the context of the initiator AUG. A. Competition assay using mutant RNA lacking initiator AUG. Purified human recombinant La preparation expressed in bacteria was UV cross-linked with the wild type 5’NCR RNA probe and fractionated by SDS-12%PAGE after digestion with RNase. The NCR-C(AUG) probe contained 4-thio-U and was labeled with $^{32}$P]CTP. The binding reactions were carried out with 15 ng purified La. Lane 1, no competitor RNA, lanes 2-4, homologous competitor; lanes 5-6, mutant 5’NCR. Upper panel shows the nucleotide sequence at the 3’ end of the wild type NCR-C(AUG) and the AUG deletion (indicated with dash) containing mutant RNA [NCR-C(ΔAUG)]. The band intensities were calculated using PhosphorImager. The numbers in the upper panel indicate nt position in the HCV RNA. B. Effect of deletion of nt sequences surrounding initiator AUG on La protein binding with the wild type probe. The three nt deletions in each of the mutant 5’NCR are shown by dash line in upper panel. These RNAs were used as unlabeled competitors during the competition assay as described above. Lanes; 1, no competitor RNA; 2-4, increasing amounts of unlabeled homologous RNA; 5-16, mutant RNAs as competitors.

**FIG. 5.** Immunoprecipitation of La antigen from a liver cell-derived (Huh7) and a non-liver derived (HeLa) S10 fractions after UV cross-linking with the 4-thio-U containing $^{32}$P-labeled HCV 5’NCR probe. UV cross-linked Huh7 (lanes 1, 2) and HeLa (lanes 3, 4) lysates were subjected to immunoprecipitation with human anti-La antibodies (V3) (lanes 2, 4) or normal human IgG (lane 1, 3). The samples were fractionated by SDS-PAGE and autoradiographed. Normal human IgG (NHIgG) was isolated from a serum
of healthy individual by ProteinA-Sepharose chromatography. Anti-La antibodies were isolated from autoimmune sera by affinity chromatography using recombinant human La as a ligand.

FIG. 6. Rescue of the HCV IRES activity by recombinant human La antigen. The T7C1-341 RNA was translated in RRL as described in the legend to Fig. 3. The template RNA was translated in the absence (lane 2) or presence (lane 3) of full length wild type 5’NCR (competitor). Lane 4 is similar to lane 3 except that recombinant human La antigen (100 ng) was added to counteract the inhibition by the competitor 5’NCR. A similar concentration of bovine serum albumin (BSA) failed to counteract such inhibition (data not shown). Lane 1, translation without T7C1-341 RNA template as a control.

FIG. 7. Inhibition of the HCV IRES activity by sequestration of La antigen with the C5 RNA SELEX. A. C5 RNA SELEX binds only La antigen in the HeLa lysates. HeLa S10 lysates were UV cross-linked with thio-U and 32P-labeled C5 RNA (lane 1). A similar cross-linked sample was immunoprecipitated using normal human IgG (lane 2) or human anti-La antibodies (lane 3). Lane M, molecular weight markers. B. Competitive inhibition of La-5’NCR interaction by C5 RNA (lanes 2-4) during UV cross-linking of the wild 5’NCR probe with La antigen. Lanes 5-6 shows competition with the homologous RNA. C. Specificity of the C5 RNA binding. GST-PTB interaction with the 5’NCR (lane 1) is inhibited by the homologous competitor (lanes 5-7) but not with the C5 RNA (lanes 2-4). GST alone does not interact with the HCV 5’NCR (1). D. Selective inhibition of the La antigen-dependent IRES activity. In vitro transcribed uncapped viral IRES-containg luciferase RNAs (100 ng each) were translated in HeLa cell lysates in the presence of 1, 2 and 3 ug of C5 RNA. The luciferase activity in the C5 RNA containing samples was compared with sample without the ligand that was set at 100% activity. The template RNAs used in this experiment are indicated with the plot. The results presented here are
representative of several repeats. **E.** Inhibition of the HCV IRES by C5 SELEX RNA in the context of dicistronic mRNA template. In vitro transcribed capped T7DC1-341 RNA (1 ug) was translated in rabbit reticulocyte lysates supplemented with HeLa S100 (lane 2). The HCV IRES activity is represented by the translation of luciferase (LUC) while chloramphenicol acetyltransferase (CAT) is translated by cap-dependent mechanism. Lane 1, translation without RNA. The RNA template was translated in the presence of 2 ug (lane 3) and 4 ug (lane 4) wild type (WT) C5 SELEX RNA or similar concentrations of the mutant C5 RNA (lanes 5, 6) respectively. The translation of LUC and CAT were quantitated by autoradiography of the gel directly exposed to the PhosphorImager screen (BioRad). The translation of the control without inhibitors (lane 2) was considered as 100% and compared with lanes 3-6.

**FIG. 8.** In vivo inhibition of the HCV IRES by C5 SELEX. **A.** The Huh7 cells cultured on 60 mm petri-dishes were transfected with T7C1-341 (3 ug) alone (control) or co-transfected with C5 SELEX or pGEM-4 RNAs (15 ug each). Luciferase activity in the cytoplasmic lysates was assayed to determine the HCV IRES efficiency after 16 hr of transfection. The ‘unrelated’ control represents 75 nt RNA derived from the multiple cloning site of pGEM-4 vector. Each transfection experiment was carried out in triplicate and standard error was calculated. The standard error for C5 SELEX is calculated to be < 1% and is not visible on this scale. **B.** Three ug of capped dual luciferase reporter RNA (upper panel) was transfected into Huh7 cells either alone (control) or co-transfected with wild type (WT) or mutant (Mut) C5 SELEX RNA (4 ug) as described above. Each transfection was carried out in duplicate. The enzymatic activities of renilla (RL) and firefly (FL) luciferase were assayed from an equal amount of protein sample using Dual-Luciferase Reporter Assay System (Promega). The transfection experiments were repeated several times.
Fig. 1

Ali et al., 2000
Fig. 2A

Ali et al., 2000
La antigen mutants

Fig. 2B

Ali et al., 2000
Fig. 2C

Ali et al., 2000
Fig. 3

Ali et al., 2000
NCR-C(AUG):
UGCACCAGAGCACGAAUCCUGAAUU

NCR-C(ΔAUG):
UGCACC-----AGCACGAAUCCUGAAUU

Fig. 4A

Ali et al., 2000
Fig. 4B

Ali et al., 2000
Fig. 5

Ali et al. 2000
|                | 1 | 2 | 3 | 4 |
|----------------|---|---|---|---|
| **La antigen** | - | - | - | + |
| **5'NCR competitor** | - | - | + | + |
| **HCV IRES-LUC** | - | + | + | + |

LUC activity $\times 10^3$

7.3 0.2 14.3

Fig. 6

Ali et al., 2000
Fig. 7A

Ali et al., 2000
Figs. 7B & 7C

Ali et al., 2000
Fig. 7D

Ali et al. 2000
Fig. 7E

Ali et al., 2000
Fig. 8A

Ali et al., 2000

% Luciferase Activity

Control

CS SELEX

Unrelated
Fig. 8B. Ali et al., 2000

% Luciferase Activity

RL(Cap-dependent) FL (HCV IRES)

Cap

Renilla Luc HCV IRES Firefly Luc

(A)
Human La antigen is required for the hepatitis C virus internal ribosome entry site (IRES)-mediated translation

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