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Structural determinant of human La protein critical for internal initiation of translation of hepatitis C virus RNA

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ABSTRACT:

Human La protein has been implicated to facilitate internal ribosome entry site (IRES) mediated translation of hepatitis C virus (HCV). Earlier, we have demonstrated that RNA recognition motif (RRM) encompassing 112-184 residues of La protein interacts with HCV-IRES near initiator AUG. A synthetic peptide LaR2C (24mer), derived from La-RRM (112-184), retained RNA binding ability, competes with the La protein binding to HCV IRES and inhibits translation. The peptide interferes with the assembly of 48S complexes resulting in the accumulation of pre-initiation complexes that are incompetent for the 60S ribosomal subunit joining. Here, NMR spectroscopy of the HCV-IRES bound peptide complex revealed putative contact points, mutations at which showed reduced RNA binding and translation inhibitory activity. The residues responsible for RNA recognition were found to form a turn in the RRM (112-184) structure. A 7-mer peptide comprising this turn showed significant translation inhibitory activity. The bound structure of the peptide inferred from transferred NOE experiments suggests it to be a β-turn. This conformation is significantly different from that observed in free RRM (112-184) NMR structure suggesting paths towards a better stabilized mimetic. Interestingly, addition of hexa-arginine tag enabled the peptide to enter Huh7 cells and showed inhibition HCV-IRES function. More importantly, the peptide significantly inhibited replication of HCV monocistronic replicon. Elucidation of the structural determinant of the peptide provides basis for developing small peptidomimetic as potent anti-HCV therapeutics.

INTRODUCTION:

The mechanism of internal initiation of translation of HCV RNA is unique and fundamentally different from the cap-dependent translation of host cell mRNAs and thus HCV-IRES offers a potential target for developing novel antiviral therapeutics (7).

The ribosome assembly at the Hepatitis C virus IRES has been shown to be 'prokaryotic like' and requires minimal number of initiation factors (28). HCV IRES has been shown to be capable of binding directly to 40S ribosomal subunit with the help of the ribosomal protein S5 (12, 21, and 32). Although, HCV IRES binds to the 40S ribosomal subunit specifically and stably even in the absence of any initiation factors, efficient translation requires some of the canonical initiation factors and non-canonical *trans*-acting factors, possibly to facilitate ribosome binding and to ensure proper positioning of the initiator AUG in the P site (15). Several cellular *trans*-acting factors have been reported to be critically required for HCV IRES mediated translation, which includes human La autoantigen (2, 7, 15 and 21).

Human La protein was originally identified in the sera from patients with systemic lupus erythematosus (SLE) and Sjögren syndrome (34). Earlier it was reported in the literature that La protein contains three RRMs, which were putatively located in between 1-100 (RRM1), 101-208 (RRM2) and between 209-300 (RRM3) [14, 29]. However, according to the recent nomenclature, the first structured domain in human La is termed as the 'La motif (16-75)', followed by two RRMs, RRM1 (112-184) and RRM2 (230-300) (27, 35). Although, based on the structure determinations (from NMR and PX data) the precise boundary of the structured cores were found to be slightly different [La-motif (7-92), RRM1 (110-194), RRM2 (229-327)] but they largely encompasses the above regions (1, 20).

La gets associated with the 3' termini of many newly synthesized small RNAs made by RNA polymerase III as well as certain small RNAs synthesized by other RNA polymerases (24, 38). The N terminal 80aa residues termed as La motif and the adjacent RRM part has been shown to be required for high affinity binding with pol III transcripts, where the La motif helps in specific recognition for poly uridylate sequence (UUU_{OH}) at the 3` end of the RNA. The C terminal RRM (230-300) has been shown to have a beta sheet comprising 5 strands and a long C terminal helix that binds to the putative RNA binding site (20). The central RRM (112-184) has been shown to possess a classical RRM-type fold containing four stranded beta sheet backed by two alpha helices. It also has an additional beta-strand inserted between alpha 2 and beta 4. The helix alpha 3 of RRM (112-184) is predominantly hydrophilic and protrudes away from the body of the domain. The beta sheet surface of the central RRM (112-184) contains 5 basic residues and no acidic residues, which makes it more basic and suitable for RNA interactions (1).

La protein has been shown to bind several viral and cellular IRES elements and influences their function. In fact it has been implicated as a molecular chaperone to facilitate IRES structure and translational competence (6). La protein has been shown to enhance poliovirus IRES function and corrects its aberrant translation in reticulocyte lysate (25).

Earlier we have demonstrated that La protein plays important role in mediating HCV IRES mediated translation. It interacts at GCAC motif near initiator AUG of HCV IRES, which might trigger some conformation alterations that facilitates formation of functional initiation complex and stimulate internal initiation of translation (29). It also seems to assists the binding of ribosomal protein S5 and probably plays a role in recruitment of 40S ribosomal subunit to HCV IRES (31). Recently, we have demonstrated that a 24-mer peptide (LaR2C) derived from the C-terminus of the RRM (230-300) of La protein competes with the cellular La protein binding to

90 the HCV IRES and interferes with the functional initiation complex formation (30). It appears
91 that LaR2C interferes with 48S ribosome complexes, rendering it incompetent for 60S joining
92 during internal initiation of translation of HCV RNA (30).

93 Here we report, characterization of the structural and functional domain of the LaR2C
94 peptide. Using NMR spectroscopy of the RNA bound peptide coupled with mutational analysis
95 we have delineated the minimal sequences within the peptide required for binding and inhibition
96 of HCV RNA translation. We have shown the presence of a unique β-turn at the N terminus of
97 the peptide which is more or less sufficient for its function. More importantly, we have
98 demonstrated direct delivery of the arginine-tagged peptide inside the Huh 7 cells. The study
99 constitutes the first report of a small anti-HCV peptide (7-mer) targeting HCV-IRES and
100 provides the 'proof of concept' that a peptide comprising of minimal RNA binding domain can
101 successfully inhibit HCV RNA translation and replication, which can be exploited as a target to
102 design efficient and more potent antiviral therapeutics against HCV infection in near future.

MATERIALS AND METHODS:

NMR spectroscopy:

Both peptide and RNA were dissolved in 20 mM potassium phosphate buffer, pH 7 containing 0.1 M KCl and 10% D2O. All spectra of LaR2C were recorded in a 500 MHz DRX-500 (Bruker Biospin, Switzerland) at 27ºC, unless stated otherwise. TOCSY (mixing time 80ms) and NOESY (mixing time 400ms) spectra were recorded with solvent suppression achieved using WATERGATE and the spinlock in TOCSY experiment was attained by MLEV sequence.

Determination of RNA-bound structure of the 7-mer peptide:

Mixing time for NOESY was 400 ms and the same for TOCSY (with MLEV spinlock) experiment was 60 ms for transferred NOE experiments. The distance between two protons was determined under transferred NOE conditions. NOESY experiments were done at a final concentration of 450μM 7mer peptide with 15 μM p383 RNA at 283K in 20mM deuterated Tris-HCl buffer, pH 7 containing 100mM NaCl. The one-dimensional spectrum under these conditions is significantly broadened compared to free peptide without showing any new peaks indicating fast exchange conditions. The distance between two adjacent protons in the aromatic ring of a tyrosine residue, i.e., 2.48 Å was considered as the standard. It was used to calculate all other distances by comparing their peak volumes, respectively. However, the distance constraints were given in terms of strong (<3.5 Å), medium (3.5–4.5 Å), and weak (>4.5 Å) and may be considered somewhat of an overestimate due to transferred NOE conditions used. The maximum limit kept was 5.0 Å and the minimum 2.8 Å. The angle constraints were derived by direct measurements of scalar coupling constants from one-dimensional spectrum under conditions mentioned above and corresponding dihedral angles were derived from Karplus relationships. An upper and lower limit of ± 20^{o} was used for angle constraints. The J-values were also cross-

checked with those obtained from DQF-COSY and procedure of Kim and Prestegard (22). Total of 30 distance constraints and 6 φ angle constraints (Table 1) were used for structure determination.

The angle and distance constraints, along with the complete protein sequence were fed into the software program DYANA to calculate the structures. Those with target function values were 40.00 or lower (i.e. deviation from the inputs) were combined and energy was minimized by DISCOVER 3.0 in molecular modeler INSIGHT II version 2005 (Biosym/MSI) using the simple minimization algorithm. Then the structures were validated by RCSB validation server and the statistics were given in Table 2.

Peptide synthesis and purification:

The peptides LaR2C (KYKETDLLILFKDDYFAKKNEERK) corresponding to residue 174-197 of the La protein, the mutant LaR2C (KYKATDLLILFKDDYFAKKNEERK) peptide and the non-specific La peptide (aa71-98, Ref 19) termed as La-Nsp (ALSKSKAELMEISEDKTK**)** were custom synthesized at Sigma Genosys, with 90% purity. The peptides were dissolved in nuclease/protease free water, and integrity was checked by gel-electrophoresis followed by silver stain analysis.

All other smaller peptides (with or without 6XArg tag), were synthesized on a 0.1-mmol scale by solid-phase peptide synthesis strategy using Fmoc chemistry and Rink amide resin. Cleavage of the peptide from Rink amide resin and removal of all side chain-protecting groups were achieved in 95% trifluoroacetic acid solution. The crude peptides were purified by reversed-phase high-performance liquid chromatography (RP–HPLC, Waters Associates) on a Waters C-18 column (μBondapak) with linear gradients of water/acetonitrile containing 0.1% trifluoroacetic acid.

Peptide masses and purity (>95%) were checked by positive ion mode ESI-MS (WATERS Inc., USA).

Plasmids and cells:

The construction of the plasmids pRSET-A La, pHCV383Luc monocistronic construct, and the bicistronic construct containing HCV IRES have been described earlier (29). The HCV monocistronic replicon construct pFKi383hygubiNS3-3'5.1 used in the study was a generous gift from Dr. R. Bartenschlager (26). The mutant La clones were generated by megapriming PCR method and cloned into pRSET-A expression vector. The mutants carried substitutions only at the respective aminoacid positions (P2 or P4 or P15 or P21/22), all other positions remained unaltered. Thus the changes were only on RRM (112-184), not in other RRMs. The PV 5'UTR or HCV IRES (18-383nt) were cloned upstream of luciferase reporter gene in pCDNA3.1 to generate pCDPVLuc or pCDHCV Luc monocistronic construct respectively. Additionally, the HCV IRES (18-383) or HAV 5'UTR was cloned in between RLuc and FLuc reporter genes in pCDNA3.1 vector to generate the respective bicistronic constructs.

Huh7 monolayer cells were grown in DMEM supplemented with 10% FBS and for the Huh7 cells harboring HCV replicon were grown in DMEM with 10% FBS, 25µg /ml Hygromycin B (Calbiochem).

Purification of wild-type and mutant La proteins:

The wild-type and the mutant La proteins were over expressed in *E. coli* BL21 (DE3) cells and purified using nickel NTA agarose column as described earlier (29). Briefly, the cultures were induced with 0.6mM IPTG for 4 hours at 37^0C. The crude extracts were mixed with Ni-NTA

agarose slurry (Qiagen) and kept for rocking at 4°C for 4 hours. The lysate was loaded onto a column, washed with 50 ml of wash buffer (50mM NaH_2PO_4, 300mM NaCl, 40mM imidazole) and the bound proteins were eluted with 500µl of elution buffer containing 500mM imidazole. The eluted proteins were dialyzed at 4°C for 6h in dialysis buffer (50mM Tris/pH7.4, 100mM KCl, 7mM β-ME, 20% glycerol), aliquoted and stored in -70°C freezer.

in vitro transcription and translation

Radiolabeled mRNAs were transcribed *in vitro* using T7 RNA polymerase (Promega) and [α^{32}P] UTP. The HCV-IRES (18-383nt) cloned in pcDNA3 vector was linearized with EcoRI, gel eluted and transcribed *in vitro* in presence of [α^{32}P] UTP to generate the labeled HCV-IRES RNA as described previously(30). The HCV-IRES containing moncistronic construct (HCV luciferase) was linearized with XhoI to prepare HCV Luc RNA and the HCV bicistronic construct was linearized with PmeI to generate capped bicistronic RNA (Rluc-HCV IRES-Fluc in order) to use in the *in vitro* translation studies. The moncistronic construct, pCD Luc (containing luciferase gene in pCDNA3 backbone), was linearized downstream of luciferase gene with XhoI and the template was used to generate capped luciferase RNA by in vitro transcription in presence of cap analog using T7RNA polymerase. HAV bicistronic construct was linearized downstream of firefly luciferase and used as template for RNA synthesis as described earlier (30). The poly-linker sequence of pGEM-T-easy vector was linearized with SacI to generate the non-specific (Nsp) RNA.

in vitro translation of the capped Luciferase mRNA, capped bicistronic RNAs or uncapped HCV Luciferase RNA was carried out in micrococcal nuclease treated rabbit reticulocyte lysate (RRL, Promega Corporation, WI, and USA) as described previously (30).

UV cross-linking experiment:

The purified proteins or the synthetic peptides were incubated with ~75 fmoles of either [^{32}P]labeled HCV IRES RNA or the Nsp RNA at 37^{0}C for 15 minutes containing RNA binding buffer (5mM HEPES pH 7.6, 25mM KCl, 2mM $MgCl_2$, 3.8% Glycerol, 2mM DTT, 0.1mM EDTA and 5µg tRNA) in a reaction volume 15µl; the complex was then subjected for UV induced cross-linking as described earlier (29).The entire reaction mixture were then denatured followed by SDS-10% PAGE (for protein) or Tris-Tricine 17%PAGE (for peptide) and analyzed by phosphorimaging.

Filter binding assay:

The [α^{32}P] labeled HCV IRES RNA, or the Nsp RNA was incubated with the wild-type La or the mutant La proteins or the peptides at 30°C for 15 minutes in RNA binding buffer (containing 5mM HEPES pH 7.6, 25mM KCl, 2mM $MgCl_2$, 3.8% Glycerol, 2mM DTT and 0.1mM EDTA). The reaction mixtures were loaded onto nitrocellulose filters equilibrated with the 2ml RNA binding buffer. The filters were then washed four times with 1ml of binding buffer and air-dried. The counts retained were measured in liquid scintillation counter. The graph was plotted with protein or peptide concentration (µM) on x-axis and the percentage of bound RNA as the percentage of counts retained, on the y-axis.

Real time RT-PCR – RNA isolated from the from peptide treated and untreated replicon cells were reverse transcribed with HCV 5'primer using AMV RT (Promega) for the amplification of negative strand of HCV RNA . For real–time PCR analysis, the cDNA was used for PCR amplification using a Real-time assay mix (Finnzymes) as per manufacturer's instruction and the

219 data was analyzed by ABI-Prism's Real time PCR machine. Actin was used as an internal
220 control for the above reactions.
221
222 **Fluorescence microscopy**- Huh7 monolayer cells were grown on coverslip upto 70%
223 confluency. Before addition of the peptide, cells were washed with PBS twice and then
224 incubated with 1μM of Fluorescein labeled peptide (dissolve in DMEM) for 3 hours. After
225 extensive wash with PBS, cells were fixed with 3.7% formaldehyde for 30min at room
226 temperature, followed by washing with PBS for two times. Cells were observed under
227 Fluorescence microscope.
228

RESULTS:

NMR spectroscopy of the HCV IRES RNA bound peptide complex:

Identification of amino acid residues important for recognition of RNA can be performed by NMR spectroscopy. In this case, since the RNA is relatively large (18-383 nt of HCV IRES), obtaining the full structure of the RNA-peptide complex (1:1) would have been extremely difficult. Alternatively, NMR spectrum of the 24-mer peptide was studied, which was relatively simple, in the absence and presence of sub-stoichiometric amount of RNA. It was assumed that under fast exchange condition, the chemical shifts of peptide protons in the absence of RNA will be average of free and bound species (intensity of RNA protons will be insignificant due to sub-stoichiometric presence and broader line-width). Thus, comparison of peptide chemical shifts in the presence of RNA with that of the free peptide is expected to shed light on the residues that may be involved in recognition.

'Total Correlation Spectroscopy' (TOCSY) provides connectivity between all adjacent protons (three-bond connectivity) within an amino acid unit and hence a fingerprint for the type of amino acid. Figure 1A shows the TOCSY spectrum of the region that connects NH (approximately 7.5 to 9.5 ppm) with αH and other side-chain protons. Out of the expected 23 NH protons, 18-19 could be resolved. When sub-stoichiometric amount of RNA was added, significant shift of many protons (but not all), were observed. This perhaps indicated either an extensive peptide-RNA interface or a folding of the peptide coupled to RNA binding. Determination of the bound conformation of the peptide and the whole interface is beyond the scope of this work and we have focused on identifying at least one region that may be involved in recognition. This will allow us to mutate that residue in the whole protein and validate that the peptide is a good model for studying protein-RNA interaction.

Among other shifted residues in the TOCSY spectra, one residue at 8.27 ppm shows significant shift upon complex formation (Figure 1; indicated by an arrow). Chemical shifts and connectivity patterns indicate that this residue is a glutamic acid (no glutamine is present in the peptide). There is only one threonine (position 5) in the peptide. Threonine α, β and methyl protons have very characteristic chemical shifts and can be identified easily. Figure 1B shows the position of T178. T178 is connected to the shifted glutamic acid (E177) by NOE (Figure 1C) indicating that the glutamic acid at 8.27 ppm is E177. Thus, at least E177 is likely to be involved in recognition of HCV IRES RNA. Additionally, significant shifts were observed corresponding to Y175 (Tyr) at the N terminus and also Y188 (Tyr) and K192-N193-E194 positions at the C terminus of the LaR2C peptide.

Effect of point mutation within the LaR2C region of La protein on HCV IRES binding:

As mentioned above, the chemical shift perturbation can be due to direct interaction or indirect coupled folding events. In order to investigate whether the above amino acid residues of La protein are actually involved in recognition of HCV IRES RNA, we have generated corresponding point mutations in the full-length protein using site-directed mutagenesis (Fig 2A). The RNA binding activities of the mutant La proteins were tested and compared with that of Wt-La protein by UV-crosslinking assay using [^{32}P] labeled HCV IRES RNA. The results showed that mutations at the La175$_{Y\text{-}A}$ and La177$_{E\text{-}A}$ (corresponding to N-terminus P2 and P4 positions of LaR2C peptide) mostly affected the HCV IRES binding. Whereas, mutations at La188 $_{Y\text{-}A}$ and La 194 $_{E\text{-}A}$-195 $_{E\text{-}A}$ (corresponding to C terminus, positions P15 and P21/22) didn't significantly alter the RNA binding ability of La protein (Fig 2B).

Additionally, filter binding assay was performed using [^{32}P] labeled HCV IRES RNA and increasing concentration of purified recombinant proteins (Wt-La or the mutants). Considering the mid point of transition, it appeared that the mutation at the P4 residue significantly affected the RNA binding ability of La protein. Mutation at P2 residue also showed decrease in RNA binding ability, but to some extent only. However mutation at P15 or P21/22 didn't alter the binding ability of La protein (Fig 2C). As expected, a non specific RNA probe didn't show considerable binding with the wt-La protein in the same filter-binding assay.

Earlier, we have shown that LaR2C peptide can effectively compete with full-length La protein for binding near the iAUG within HCV IRES RNA (30). Here, in the competition UV-crosslinking experiment we have observed that the full-length Wt-La protein can also compete out binding of LaR2C with the HCV IRES RNA (Fig 2D). However, the mutant P4-La protein failed to compete the binding of LaR2C peptide significantly with the HCV IRES RNA (Fig 2D). The result suggests, that the domain of La protein encompassing the amino acid P4 ($La177_{E-A}$) might be involved in making contact with the HCV IRES RNA near initiator AUG where LaR2C peptide also binds. However, binding of La protein to other sites within HCV IRES RNA might not be affected as much with the above mutation.

Effect of mutation in the LaR2C peptide on RNA binding and translation

To further investigate the role of N terminal amino acids in the peptide activity we have generated a mutant LaR2C peptide where E_{177} was changed from Glutamic acid to Alanine (Fig 3A). The RNA binding ability of the wild type and mutant peptide were tested by UV-cross-linking assay. For this purpose [α ^{32}P] labeled HCV IRES RNA was UV cross-linked with increasing concentration of either wt LaR2C or the mutant LaR2C or a non-specific La peptide. Result showed that the mutation at P4 ($La177_{E-A}$) did affect the RNA binding ability of the

mutant peptide (Fig 3A). The non specific peptide (Nsp) didn't show any RNA binding activity (Fig 3A). To further confirm the RNA binding ability of the peptides, filter binding assay was performed using increasing concentration of wild-type and mutant peptide and $\alpha\ ^{32}P$ labeled HCV IRES RNA. The results showed reduced level of saturation for the mutant peptide-RNA complex compared to the wt LaR2C peptide, suggesting critical role of the P4 residue in the activity of the LaR2C peptide (data not shown).

Interestingly, results from similar filter binding assay suggest that the deletion of N-terminal amino acids almost abrogated the RNA binding activity of the peptide (ΔLaR2C-C14), whereas retention of only 14 amino acids in the N terminus (ΔLaR2C-N14) appeared to be sufficient for significant RNA binding activity (Fig 3B).

Additionally, the effect of mutation in the peptide was tested in the *in vitro* translation assays in Rabbit Reticulocyte Lysate (RRL) using uncapped monocistronic RNA containing HCV IRES upstream of Firefly luciferase gene. Results showed significant decrease in HCV IRES mediated translation of HCV Luc RNA in presence increasing concentration (30μM -50%, and 60μM-70% inhibition) of Wt LaR2C peptide. However similar concentration of mutant peptide failed to inhibit the HCV IRES function significantly (Fig 3C). Also, it appears that the C terminal truncated peptide ΔLaR2C-N14 retained the translation inhibitory activity as compared to wild-type peptide control. However, deletion of N terminal amino acids (ΔLaR2C-C14) resulted in abrogation of the translation inhibitory activity (Fig 3C).

Characterization of conformation of the N-terminal seven residue peptide:

The N-terminal part of the LaR2C (174-196aa) has been shown to constitute β4-sheet and β4' strand of RRM (101-200) (1). Interestingly, the residues of the peptide responsible for RNA

recognition were found to map to a turn in the context of RRM (112-184) NMR structure. Based on earlier NMR structure information (PDB ID 1S79), when the LaR2C peptide was modeled, these critical N-terminal amino acids were found to be located in a similar turn that appears to be exposed for RNA binding (Fig 4A).

Since, the RRM (112-184) structural model suggests that the N-terminal seven residues completely cover the turn that sticks out of the globular structure of the domain (Fig 4A) we were interested to investigate the structure and function of the 7 residue peptide.

The HCV IRES RNA bound structure of LaR2C-N7 structure was determined by NMR spectroscopy. Under bound conditions, the peptide gave several new NOEs and change of value of J for several amide protons in addition to significant line broadening (data not shown). This indicates that conformational parameters derived from this experiment indeed reflect the bound form. The sequence KETD forms a β-turn as distance between Cα atoms of K and D is less than 7Å (Fig 4B). However, the Ramachandran angles do not fall within any defined turn category. The KETD sequence in the RRM (112-184) structure is also a β-turn but the conformational parameters do not fall strictly into any well-defined category (Fig 4C). Although, the structures in two contexts are similar, there are noticeable differences in Ramachandran angles. Thus, there could be significant remodeling of the structure upon binding of La to the RNA (Fig 4D).

The smaller peptide (LaR2C-N7) inhibits HCV IRES mediated translation *in vitro*

After determining the preference for beta turn conformation in the N-terminal 7-residues we were interested to investigate whether the 7mer peptide comprising of these amino acids will also inhibit HCV IRES mediated translation. For this purpose we have used monocistronic RNA containing HCV IRES upstream of reporter luciferase gene in the *in vitro* translation assays in

presence or absence of increasing concentration of LaR2C-N7 peptide. Interestingly, the smaller peptide (LaR2C-N7) also showed translation inhibitory activity which is only slightly weaker than the 24-mer peptide (Fig 5A). However, mutation at the P4 position of the LaR2C-N7 completely abrogated its translation inhibitory activity (Fig 5B). Interestingly, the peptide LaR2C-N7 didn't show significant inhibition of capped-Luciferase RNA (representing cap-dependent translation) suggesting the specificity of the inhibition (Fig 5C). Also, LaR2C-N7 showed selective inhibition of HCV IRES mediated translation in the context of HCV bicistronic RNA (Fig 5D). Further, the LaR2C-N7 peptide failed to inhibit IRES mediated translation of hepatitis A virus, but showed significant inhibition of Polio virus IRES function at higher concentration (Fig 5E).

More importantly, addition of increasing concentration purified wt-La protein showed significant rescue of the suppressive effect of LaR2C-N7 (Fig 5F). However similar concentration of BSA protein was not able to rescue the inhibition. Addition of increasing concentration of recombinant La protein (25ng, and 50ng) in the reaction (in absence of peptide) showed dose dependent stimulation in HCV IRES mediated translation (data not shown) as observed earlier (29). The result reinforces the idea that the LaR2C-N7 peptide mediated inhibition of translation could be due to competition with the endogenous La protein. Taken together the results suggest that the turn at the N terminus of the LaR2C peptide could be critical for its RNA binding as well as translation inhibitory activity.

The arginine-tagged LaR2C-N7 peptide inhibits HCV IRES function *in vivo*

To investigate whether the LaR2C-N7 peptide would be equally effective in inhibiting HCV IRES mediated translation *in vivo* in Huh7 cells; we have explored delivery of the peptides

inside the cells with the help of hexa-arginine fusion tag. Arginine-tagged peptide has the property to internalize into mammalian cells when supplied exogenously into the medium (13). The RNA binding ability of the arginine tagged LaR2C-N7 peptide was demonstrated by UV cross-linking experiment using [^{32}P] labeled HCV IRES RNA. The mutant mLaR2CN7-4 (arginine-tagged) peptide didn't show appreciable RNA binding activity. A non-specific RNA probe was also used as negative control in the experiment (Fig 6A).

To investigate the internalization of the arginine–tagged peptides, we have used fluorescein labeled hexa arginine-tagged peptides and found that both the peptides are internalized inside Huh7 cells (Fig 6B). To investigate the effect of these peptides on HCV IRES function inside the cells, we have used these arginine-tagged peptides in Huh7 cells. Huh7 monolayer cells were first transfected with the pcDNA3-HCV bicistronic construct and incubated for 3 hours, washed and layered with medium containing 2μM of Arg-LaR2C-N7 peptide and incubated further for either 4 hours or 6 hours. Similarly, as a negative control, another set of dishes was layered with mutant peptides (Arg-mLaR2C-N7-4). After incubation with the peptide, the cells were washed and then lysed and the luciferase activities (Fluc and Rluc) were measured. The relative luciferase activities were represented where Rluc represents cap dependent translation and Fluc represents HCV IRES mediated translation. The results showed significant decrease (inhibition up to 70%) in the HCV IRES mediated translation over the control, when cells were incubated with Arg-LaR2C-N7 peptide. However, the mutant (Arg-LaR2C-N7-4) did not show appreciable inhibitory effect (Figure 6C and 6D). The absolute levels of RLuc and FLuc activities of a representative experiment are presented in the table (Fig 6E).Taken together, these results indicated that LaR2C-N7 does compete with the interaction of

391 cellular La protein to HCV IRES RNA and exert a dominant negative effect to inhibit HCV
392 IRES mediated translation *in vivo*.

393 Additionally, to test whether this peptide would inhibit HCV replication as well, we have
394 treated Huh7 cells harboring HCV monocistronic replicon (26) with either Wt (Arg-LaR2CN7)
395 or mutant (Arg-mLaR2C-N7-4) peptide for 24 hours. The peptides (4μM) were added twice with
396 12 hours intervals of time. As the measure of negative strand synthesis indicates replication of
397 HCV RNA (4), we have quantitated it by real time RT-PCR. The results showed almost 50%
398 decrease in levels of HCV negative strand RNAs compared to the untreated cells when 4μM of
399 ArgLaR2C-N7 peptide was used (Fig 6F). However, the mutant peptide (Arg-mLaR2C-N7-4)
400 didn't show appreciable decrease in negative strand synthesis (Fig 6F). At lower concentration of
401 the peptide (2μM) the inhibition was around 30% and at higher concentration (10μM),
402 considerable increase in the inhibitory activity was observed (data not shown).

403 Taken together, the results provide the 'proof of concept' that the peptide LaR2C-N7
404 might be effective against HCV IRES function and consequently inhibit replication of HCV
405 RNA in Huh7 cells.

406

407 **DISCUSSION:**

408 Hepatitis C is a major public health problem with limited established therapeutic options.
409 Most common established therapy is interferon α, which is not effective in majority of the cases.
410 Newer experimental therapies include nucleoside analogs, protease inhibitors and polymerase
411 inhibitors, but there is a growing need for agents that are directed against new targets (35).

412 Human La protein plays an important role in HCV infection. Earlier studies indicated that
413 for HCV IRES RNA, which lacks 3'$UUU_{OH,}$ several sub-domains of La protein contributes to

RNA recognition. The central RRM (112-184) binds strongly to HCV IRES in the context of iAUG, whereas the C-terminal RRM (230-300) and the hydrophobic domain have also been shown to contribute to HCV IRES binding perhaps at other sites (2, 6, 29, 31). Earlier, La motif was shown to indirectly influence the RNA binding ability of the La protein via other RRMs (19). However, we have demonstrated that RRM (112-184) of La protein directly binds to HCV IRES RNA at the GCAC near iAUG (29-31). We have also demonstrated that LaR2C peptide (corresponding to 174-196 aa) interacts with the GCAC near iAUG in the SLIV region and act in a dominant negative manner by competing with the endogenous La protein binding at this site (30, 31). Thus, La-HCV IRES interaction could be a potential target for drug design. In fact, earlier Izumi et al also reported a short peptide LAP (aa11-28) derived from La motif, which could selectively inhibit IRES mediated translation of hepatitis C and poliovirus (19).

Our results are consistent with the earlier study by Izumi *et al* (19) that a single point mutation in the La-motif of the full-length La protein can completely abrogate the RNA binding activity. Both the studies indicate that perhaps the secondary structure and the tertiary interactions between the domains influence La protein folding which might contribute to RNA binding. Thus point mutation at any of the hot spots (La motif or RRM) could have drastic consequence in the La protein structure and its RNA binding ability.

The inhibitory activity of the peptides may not be attributed to their RNA-binding ability alone. In fact in our earlier publication (30) we have mentioned that, in addition to HCV RNA binding, the LaR2C peptide might also interfere with the binding of some other cellular protein factors to HCV IRES RNA and thus the inhibitory effect of the peptide could be the combination of both effects. It is possible that the mutation at P4 in the LaR2C peptide might have affected both the possibilities and hence have drastically reduced the inhibitory activity. It

437 is worth mentioning that the LAP peptide (as reported earlier) didn't show HCV IRES RNA
438 binding, but still could inhibit the translation effectively (19).

439 La protein have been shown to interact with the HCV 3'UTR and influence the viral
440 RNA replication (8). It would be interesting to investigate whether LaR2C peptide or its smaller
441 derivative (LaR2C-N7) dislodges La binding to HCV 3'UTR as well. In that case the inhibitory
442 effect of the peptide on HCV RNA replication should have been more pronounced. However, at
443 this point we can't comment on whether the 50% inhibition of HCV RNA replication achieved
444 by LaR2C-N7 peptide (4μM) is mere consequence of translation inhibition. Future experiments
445 would be directed to address this issue using higher concentration of the peptide and also test the
446 efficacy of this peptide in protecting cells from HCV infection in cell culture model.

447 Although, a large peptide has been used as a drug in other viruses, e.g. against HIV (T-
448 20), in general the potential of drug development increases sharply as the molecular weight of
449 the peptide decreases. In this study we have demonstrated that a small 7-mer peptide (hexa-
450 arginine tagged) corresponding to an exposed turn can significantly inhibit HCV IRES mediated
451 translation in cell culture at even significantly lower concentration (2μM) than required for
452 inhibition of translation *in vitro*. The hexa-arginine tagged 7-mer peptide is also capable of
453 inhibiting HCV RNA replication. The fact that the effect of the peptide (at 4μM concentration)
454 on HCV replication is only 50% could be due to its relative instability during long incubation.
455 But designing peptidomimetics through mimicking of this turn might improve both the affinity
456 and *in vivo* stability (Bioavailability).

457 The bound structure of the peptide inferred from transferred NOE experiments suggests it
458 to be a β-turn, but falling into no defined category (37). The NMR structure of the un-liganded
459 domain indicates this conformation to be a β-turn as well. However, the Ramachandran angles of

460 i+1 and i+2 residues are different from the prescribed values of any type of β-turn (Hutchinson
461 and Thornton 1988). Even though the domain structure and the bound peptide structure are both
462 β-turns and falling into any defined category, they are themselves different. This suggests
463 conformational change of the turn upon binding to target RNA. Thus, stabilization of the bound
464 β-turn conformation in a peptidomimetic by suitable residues may enhance binding.

465 Specificity is a crucial issue in designing peptidomimetics as well as other therapeutic
466 entities. Lack of inhibition of IRES function with E4 mutations (mLaR2C-N7-4) strongly
467 suggests highly specific mode of inhibition. In addition, the fact that the LaR2C-N7 peptide did
468 not inhibit HAV IRES suggests the specificity of its inhibitory activity. It is possible that La
469 protein is not as critical for HAV-IRES function. On the other hand, La protein has been shown
470 to interact and enhance IRES mediated translation of Poliovirus RNA. However, LaR2C-N7
471 peptide was not as effective against Polio virus IRES mediated translation again indicating high
472 degree of selectivity. Domains other than RRM (101-200) have been attributed for this
473 stimulatory function in polio virus (6). Also, the LaR2C-N7 peptide can selectively inhibit HCV
474 IRES mediated translation *in vivo* at 2μM concentration without affecting cap-dependent
475 translation, suggesting specificity of the approach.

476 One notable point here is that in the *in vitro* translation assays approximately 60μM
477 concentration of 7mer peptide was necessary to achieve around 70% inhibition. However, the
478 hexa-arginine tagged 7mer peptide was found to be more effective and similar levels of
479 inhibition were achieved at much lower concentration (5-10μM) (data not shown). Even in the *in*
480 *vivo* assay 2-4μM hexa-arginine tagged peptide was sufficient to achieve 50% inhibition. It is
481 possible that the hexa-arginine residues might have played some unintended positive roles by
482 contributing in the net positive charge of the peptide and thus enhanced its RNA binding ability

thereby increasing the inhibitory activity as well. Also, in the *in vivo* situation, in the context of properly folded HCV IRES RNA in presence of other *trans* acting factors, the inhibitor could be more effective.

Field of therapeutic peptide analogs is at its infancy. Many of the problems associated with use of therapeutic peptides are gradually being solved. In fact a number of peptidomimetics are currently being tried as antiviral agents (9, 17). One of the best approaches in design of effective peptidomimetic is to replace naturally occurring amino acids with unnatural amino acids that stabilize the interacting conformation guided by structure of the peptide. This not only reduces the entropy cost of binding to the receptor (disorder to order), but also stabilize the small peptides from proteolysis and degradation (3). Design of peptidomimetic inhibitor such as BILIN–2061 against HCV protease was possible on the basis of structural relationship studies (SAR), availability of crystal structure of the protease, replacement of the natural amino acids etc (33, 39). Availability of the structure of this small peptide will be helpful in developing more stable peptidomimetic with higher affinity for HCV IRES so that it could effectively inhibit IRES dependent translation at much lesser concentration while increasing the bioavailability and solving the stability issues.

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507

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FIGURE LEGENDS:

Figure 1: NMR analysis of HCV IRES RNA bound peptide:

Panel A: Overlay of two TOCSY spectra, pink colored one is for the LaR2C peptide without RNA and the green colored one is for the La derived peptide with HCV-IRES RNA. The arrow indicates the shifting of the E177 peaks after addition of HCV-IRES RNA. All the spectra in this figure were recorded in a Bruker DRX-500 NMR spectrometer. The experimental details are given in the Materials and Methods section.

Panel B: TOCSY spectrum of the LaR2C with spin system identification of two amino acid residues, labeled with their corresponding one-letter symbols. The subscripts indicate the amino acid position in the peptide.

Panel C: Overlay of TOCSY (red) and NOESY (blue) spectra of the LaR2C and demonstration of TOCSY-NOESY connectivity between T178 and E177. The boxes identify the location of the NH-αH TOCSY cross peaks for the residues E177 (down field) and T178 (up field).

Figure 2: Effect of point mutation in La protein on HCV IRES binding

Panel A: Schematic representation of the domain organization of human La protein. The residues mutated in full-length La protein (between 174-197aa) with their corresponding positions with in 24-mer LaR2C peptide is indicated.

Panel B: UV cross-linking analysis. [α^{32} P] UTP labeled HCV IRES RNA (~75fmole) was UV cross-linked with increasing concentration (150, 300ng) of either wt La protein or the mutants (as indicated on top of the lanes). The protein-nucleotide complex was resolved in SDS-10% PAGE followed by phosphor imaging analysis. The position of La protein (p52) is indicated. The band intensities corresponding to La were quantified by densitometry. The numbers below the lanes 3, 5, 7 and 9 represents the relative intensities taking lane 1 (150ng protein) as control,

642 whereas the numbers (in bold) below lane 4, 6, 8 and10 represents the relative intensities taking
643 lane 2 (300ng protein) as control.
644 Panel C: Filter-binding assay: [α^{32}P] labeled HCV IRES RNA was bound to increasing
645 concentrations of either wild-type La, or the mutant La proteins (as indicated). Additionally,
646 [α^{32}P] labeled nonspecific RNA was also used along with the wild-type La protein. The amount
647 of bound RNA was determined by binding to the nitrocellulose filters. The percentage of bound
648 RNA was plotted against the protein concentration (μM).
649 Panel D: Competition UV-cross linking: [α^{32}P] UTP labeled HCV IRES RNA was pre-incubated
650 with LaR2C peptide followed by addition of either wt-La protein (lanes 2-3) or mutant La
651 protein (P4, lanes 4-5) in the reaction mixture for competition. The UV cross-linked complex
652 was treated with RNase and resolved by SDS 15% Tris-Tricine gel. The relative position of the
653 band corresponding to LaR2C peptide is indicated with an arrow. The numbers below the lanes
654 represent the relative band intensities taking lane 1 as control.
655
656 **Figure 3: Effect of P4 point mutation in LaR2C peptide activity**
657 Panel A: Schematic representation of the peptide used in the UV cross-linking analysis. The
658 residue mutated in mutant LaR2C peptide is indicated in italics. [α^{32}P] UTP labeled HCV IRES
659 RNA (~75fmole) was UV cross-linked with increasing concentration (30μM, 60μM) of LaR2C,
660 mLaR2C and La-NSP. The peptide-nucleotide complex was resolved in 15% Tris-tricine PAGE
661 followed by phosphor imaging analysis. The band intensities were quantified by densitometry.
662 The numbers below the lanes represent the intensities taking lane 1 (no peptide) as control.
663

664 Panel B: Filter-binding assay: [^{32}P] labeled HCV IRES RNA was bound to increasing
665 concentrations of either wild-type LaR2C peptide or mutant peptides as indicated. The amount
666 of bound RNA was determined by binding to the nitrocellulose filters. The percentage of bound
667 RNA was graphically represented against the peptide concentration (μM).
668 Panel C: Effect on HCV IRES mediated translation *in vitro*: One microgram of uncapped HCV-
669 IRES-Luc RNA was translated in RRL in absence (lane1) or in presence of increasing
670 concentration (30 and 60 μM) of either Wt LaR2C or mutant peptides (as indicated). The
671 relative FLuc activities were represented as a percentage of the control reaction (expressed as
672 100%).
673
674 **Figure 4: Structural characterization of the LaR2C-N7 peptide**
675
676 Panel A: Structural model of LaR2C in the context of structural model of RRM (101-200) of La
677 protein (PDB ID 1S79). The helix regions are colored pink, β-sheets are colored yellow and turns
678 and random coils are colored grey. The wtLaR2C-N7 sequence lies in the grey region (174 to
679 180 AA residues). The glutamic acid (177) is colored blue, threonine (178) is colored green and
680 aspartic acid (179) is colored black.
681 Panel B*:* Superimposition of best 16 structures (backbone) of 7-mer peptide (residuo174-180)
682 under RNA bound conditions simulated using DYANA (Version 1.5. Peter Guentert & Kurt
683 Wuthrich, Zurich, Switzerland). The amino acid residues are represented by three letter code and
684 numbered corresponding to their position in RRM (101-200).
685 Panel C: Backbone structure of RNA bound LaR2C-N7.

Panel D: 16 superimposed structures from the same region of RRM (101-200) structure for comparison. The amino acid residues are represented by three letter code and numbered from amino terminus starting from 174.

Figure 5: Effect of LaR2C-N7 on HCV IRES-mediated translation *in vitro*

Panel A and B: Above the panels, schematic representation of the wild-type 7mer peptide (LaR2C-N7) or the mutant 7mer peptide along with the 24-mer wild-type LaR2C peptide. The residue mutated is indicated in italics. One microgram of HCV IRES-Luc monocistronic RNA was translated in rabbit reticulocyte lysate (RRL) in absence or presence of increasing concentration (15, 30 and 60μM) of either the wtLaR2C-N7 or the mutant peptide mLaR2C-N7-4. Respective luciferase activities were measured and plotted against different peptide concentration. The relative FLuc activities were represented as a percentage of the control reaction (expressed as 100%). Results represent an average of three independent experiments.

Panel C: Similarly, one microgram of capped Luciferase RNA was translated in RRL in absence or presence of increasing concentration (15, 30, 60μM) of LaR2C–N7 peptide and luciferase activities were plotted against different concentration of the wtLaR2C-N7 peptide. The relative FLuc activities were represented as a percentage of the control reaction (expressed as 100%). Results represent an average of three independent experiments.

Panel D: One microgram of capped bicistronic RNA was translated in RRL in absence or presence of increasing concentration (15, 30, 60μM) of LaR2C–N7 peptide and luciferase activities were plotted against different concentration of the wtLaR2C-N7 peptide. The relative RLuc and FLuc activities were represented as a percentage of the respective control reactions (expressed as 100%). Results represent an average of three independent experiments.

Panel E: 1µg of either PV–Luciferase monocistronic RNA or capped HAV-bicistronic RNA (containing Rluc-HAV-Fluc in order) translated in absence (lane 1) and presence of increasing concentrations (15, 30, 60µM) of LaR2C-N7 peptide. The translation of the firefly luciferase activities were measured and plotted against the peptide concentration for the respective construct as indicated. The relative FLuc activities were represented as a percentage of the control reaction (expressed as 100%). Results represent an average of three independent experiments.

Panel F: One microgram of HCV IRES-Luc monocistronic RNA was translated in rabbit reticulocyte lysate (RRL) in absence or presence of wtLaR2C-N7 (40µM). Increasing concentrations (25ng, 50ng) of purified wild-type La protein or BSA (50ng) was added to the reactions as indicated below the lanes. Respective luciferase activities were measured and plotted in the graph. The relative FLuc activities were represented as a percentage of the control reaction (expressed as 100%). Results represent an average of three independent experiments.

Figure 6: Effect of arginine-tagged LaR2C-N7 on HCV IRES function in Huh7 cells

Panel A: UV cross linking: Increasing concentration (2µM and 4µM) of hexa-arginine tagged peptides, Wt ArgLaR2C-N7 or the mutant Arg-mLaR2C-N7-4 was UV cross-linked with [α^{32}P] UTP labeled HCV IRES RNA or a non-specific RNA probe and analyzed in SDS-17% Tris-Tricine gel analysis followed by phosphorimaging.

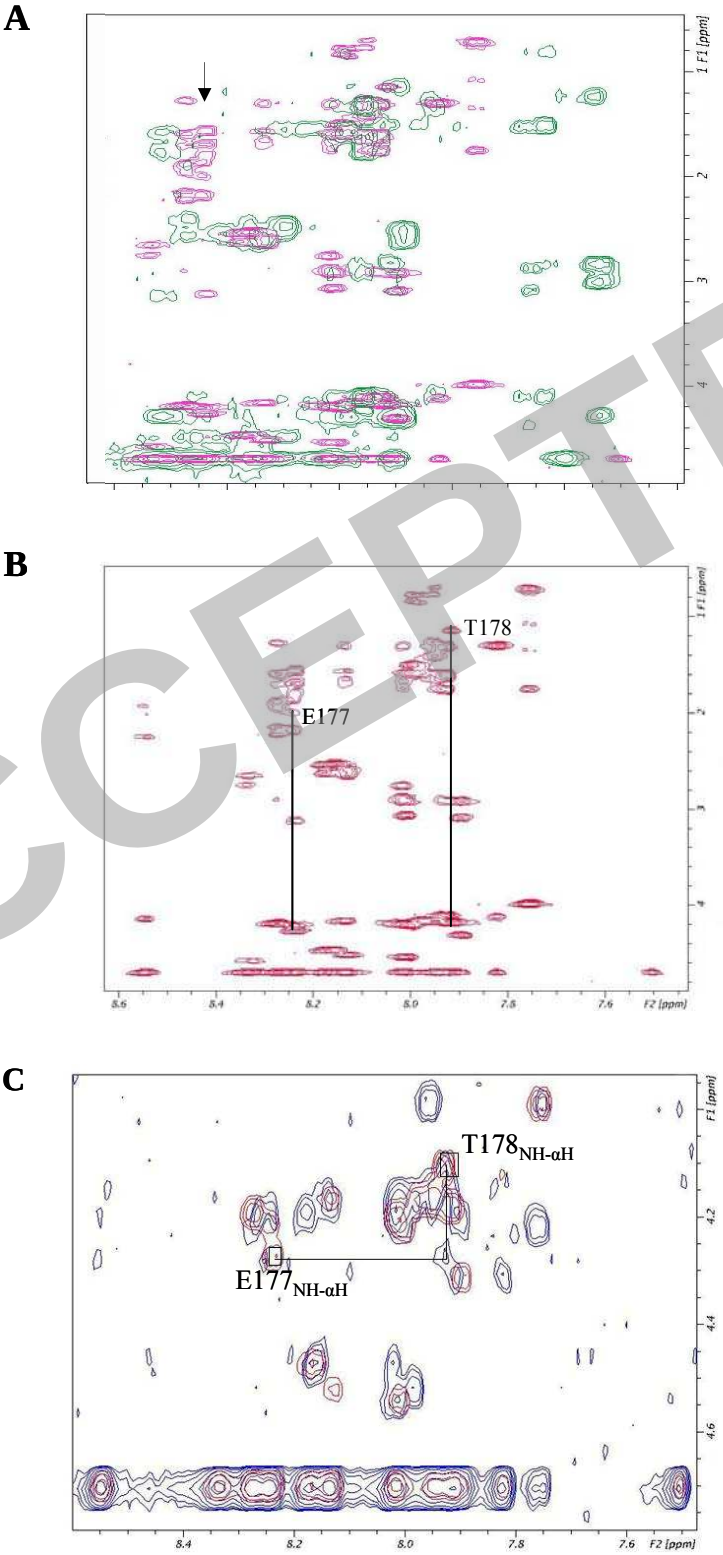
Panel B: Fluorescein tagged hexa-arginine peptides (both wild-type and the mutant) were incubated with the Huh7 cells for 3 hours, extensively washed with PBS, followed by observation under a fluorescence microscope. Left panel is for Wt ArgLaR2C-N7 and right panel is mutant Arg- mLaR2C-N7-4 peptide.

Panel C and D: Huh7 monolayer cells were transfected with 2μg of HCV bicistronic DNA (containing Rluc-HCV IRES-Fluc in order). After 3 hours of transfection, cells were overlaid with 2μM of either Wt or mutant Arg-LaR2C-N7 peptide. Cells were harvested at different time points (as indicated), lysed and luciferase activities were measured using dual luciferase assay system. The Rluc (grey bar) and Fluc (white bar) activities were plotted as fold increase or decrease in presence of the peptide with respect to the corresponding control (in the absence of peptide) taken as 100.

Panel E: Absolute values of RLuc and FLuc activities (in relative light units) of a representative experiment are presented in the table.

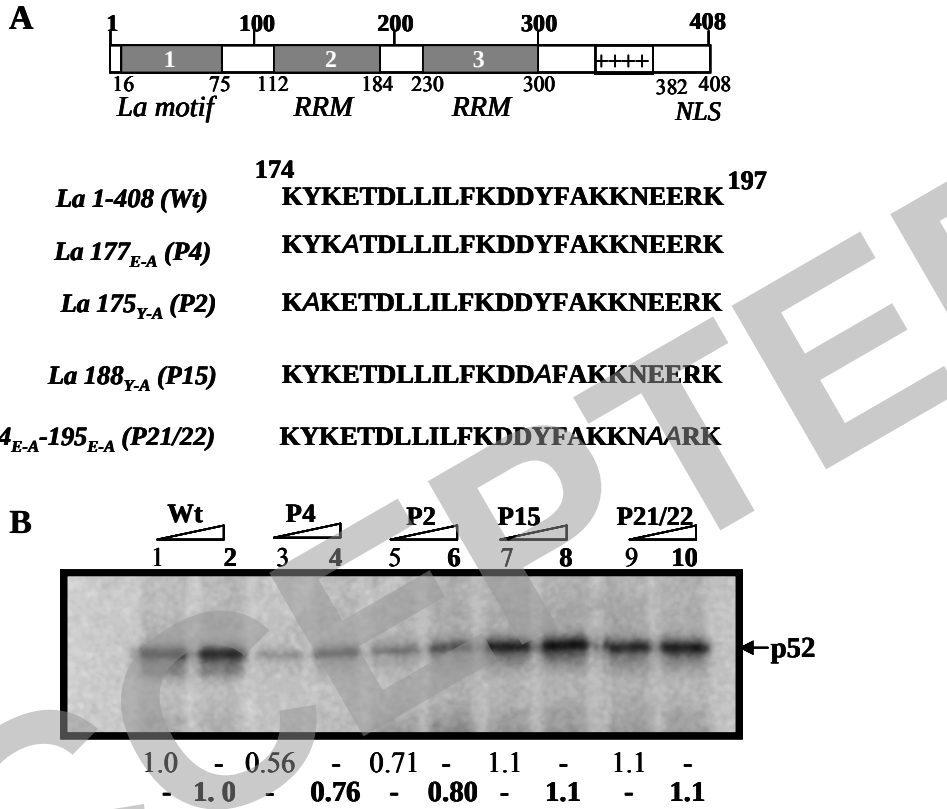
Panel F: Schematic representation of the HCV monocistronic replicon RNA adopted from Michael *et al,* 2003 (Ref 26). Monolayer Huh7 cell harboring above replicon was overlaid with either Wt 7-mer (ArgLaR2C-N7) or mutant7-mer (Arg-mLaR2C-N7-4) peptide (4μM each), added twice at 0 and 12^{th} hour. RNA was isolated at 24^{th} hour time point and subjected to cDNA synthesis. HCV negative strand was detected using real time PCR. Data were normalized with actin control and negative strand synthesis was expressed as fold change compared to control cells (in absence of peptide).

748 **Figure 1**



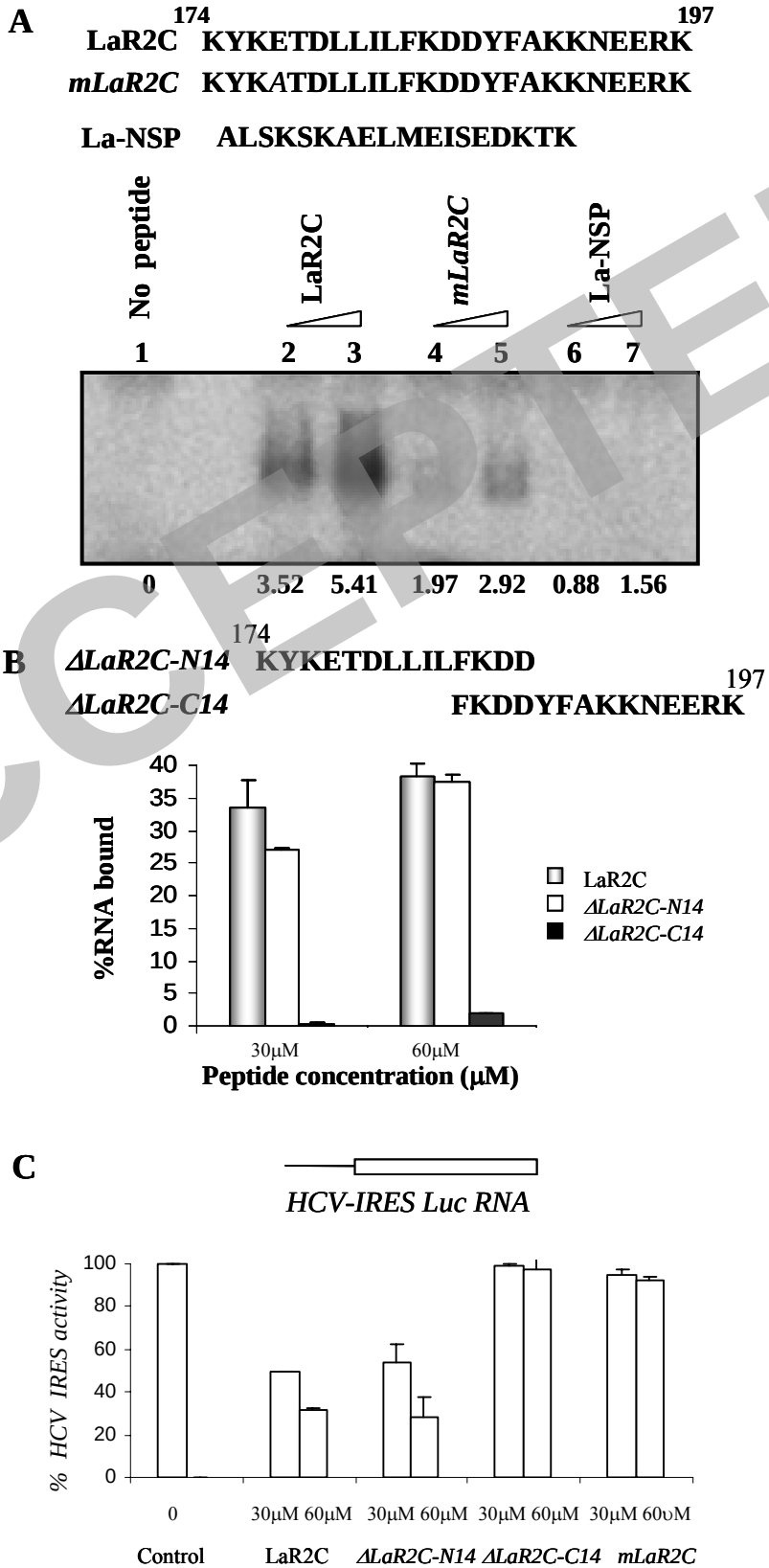
749

Figure 2



750

Figure 3



751

Figure 4

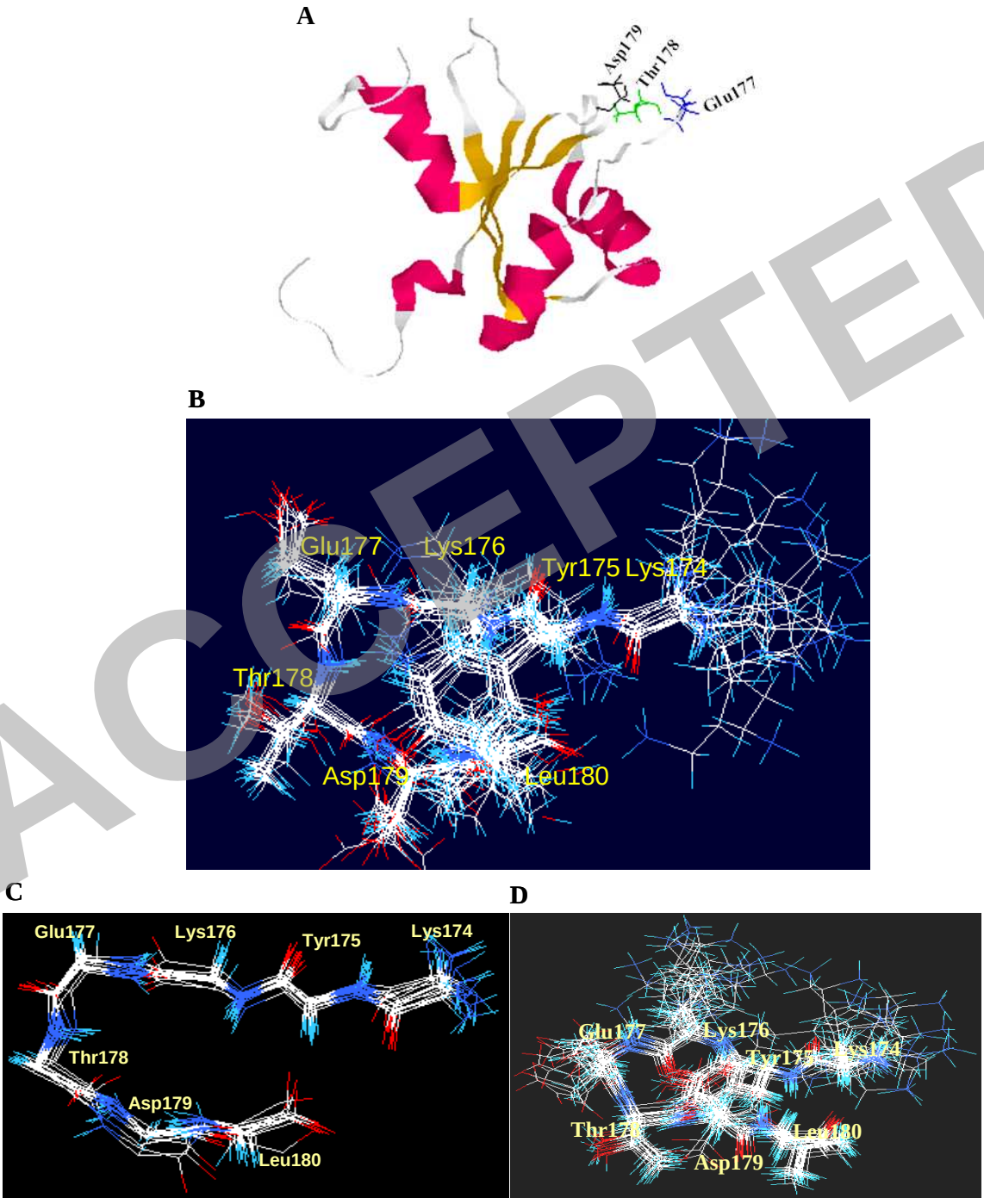


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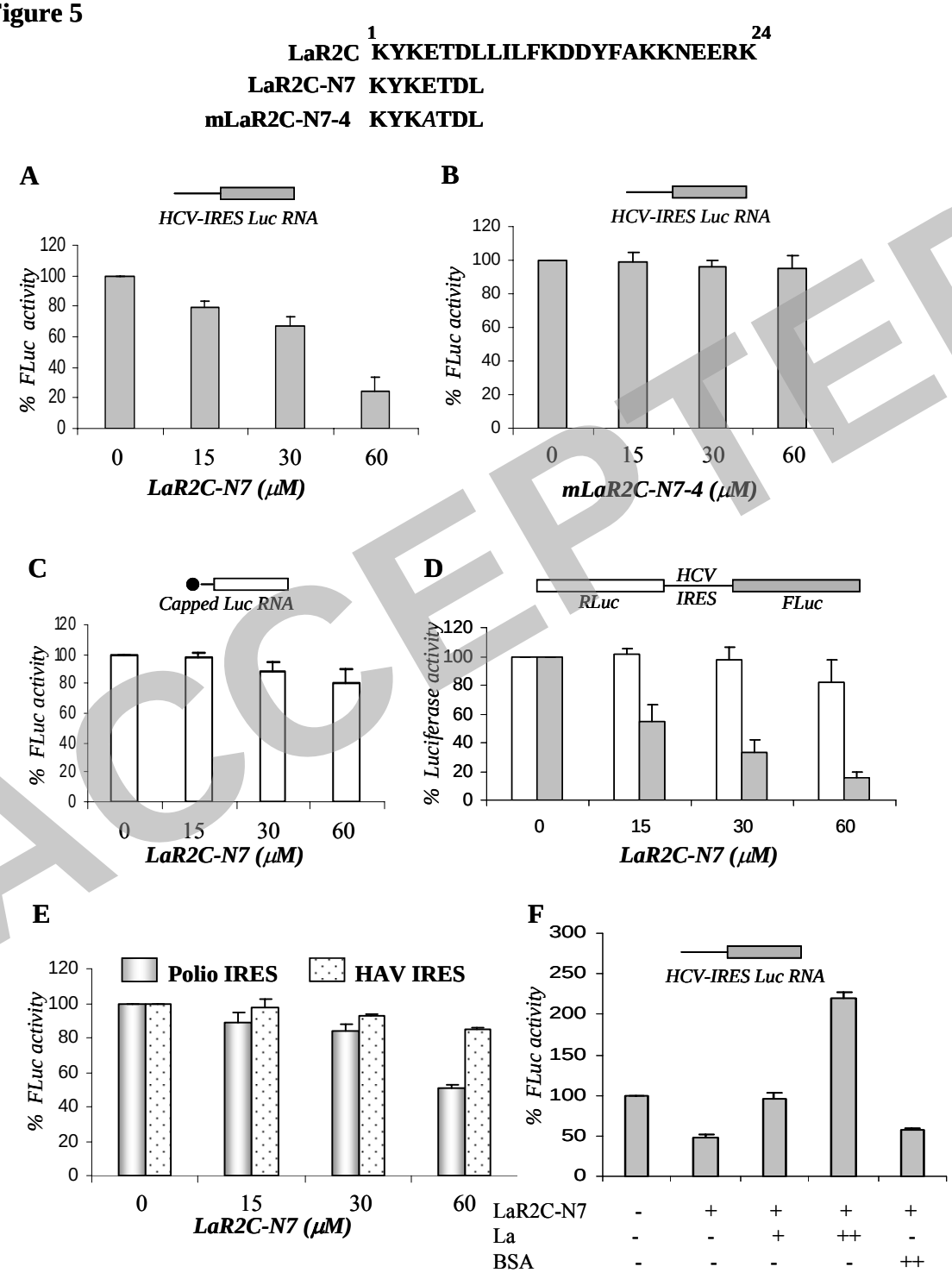
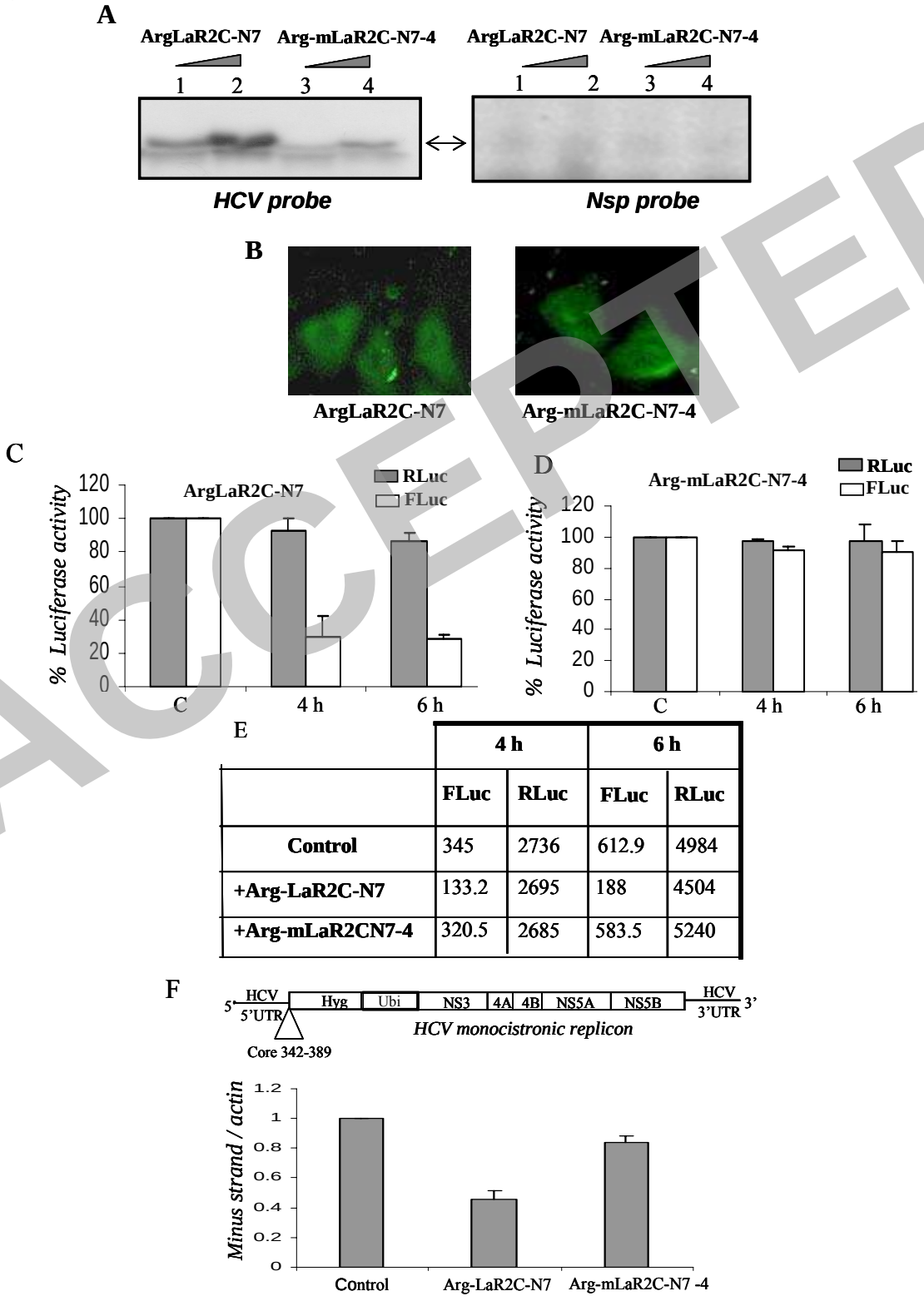
Figure 5



753

Figure 6



E

	4 h		6 h	
	FLuc	**RLuc**	**FLuc**	**RLuc**
Control	345	2736	612.9	4984
+Arg-LaR2C-N7	133.2	2695	188	4504
+Arg-mLaR2CN7-4	320.5	2685	583.5	5240

754

Table 1: Restraints for wtLaR2C-N7 (residue 174-180) peptide structure calculation

Restraints for wtLaR2C-N7 (residuo174-180) peptide structure calculation	
Restraints	no.
Total NOE distance restraints	30
Short-range(<3.5A0)	4
Medium-range(3.5-4.5A0)	16
Long-range(>4.5A0)	10
Dihedral angle restraints	6
Hydrogen bond restraints	0

Table 2: Structure Determination Statistics for wtLaR2C-N7

Ramachandran plot statistics (%)	
Number of non-glycine and non-proline residues	7
Number of end-residues (excluding Gly and Pro)	2
Number of glycine residues (shown as triangles)	0
Number of proline residues	0
Total number of residues	7
Residues in most favored regions	40.0%
Residues in additional allowed regions	60.0%
Residues in generously allowed regions	0.0%
Residues in disallowed regions	0.0%
Deviation from idealized geometry	
Bond lengths (Å)	0.015
Bond angles (°)	1.9
RMSD from experimental restraints	
NOE(Å)	0.154
Dihedral angle restraints (°)	13.6
Atomic RMSD	
Backbone (all residues)	0.33
Heavy atoms (all residues)	1.62