Specific Sequence of a Beta-turn in Human La Protein May Contribute to Species Specificity of Hepatitis C Virus

Anuj Kumar\textsuperscript{1†}, Asit Kumar Manna\textsuperscript{2†}, Upasana Ray\textsuperscript{1}, Ranajoy Mullick\textsuperscript{1}, Gautam Basu\textsuperscript{3}, Saumitra Das\textsuperscript{1*}, and Siddhartha Roy\textsuperscript{2*}

\textsuperscript{1}Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore - 560012, India

\textsuperscript{2}Division of Structural Biology and Bioinformatics, CSIR-Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata-700 032, India

\textsuperscript{3}Department of Biophysics, Bose Institute, P-1/12, C.I.T. Scheme VII M, Kolkata-700 054, India

Running title: A turn in human La protein is crucial for HCV tropism

# Address correspondence to Siddhartha Roy, sidroykolkata@gmail.com; Saumitra Das: sdas@mcl.iisc.ernet.in

*Corresponding Authors

†Contributed Equally

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ABSTRACT

Human La protein has been known to be an essential host factor for translation and replication of hepatitis C virus (HCV) RNA. Previously, we have demonstrated that residues responsible for interaction of human La protein with HCV internal ribosomal entry site (IRES) around the initiator AUG within the stem-loop IV form a β-turn in the RNA recognition motif (RRM) structure. In this study, sequence alignment and mutagenesis suggest that HCV RNA interacting β-turn is conserved only in the human and the chimpanzee, the species primarily known to be infected by the HCV. A 7-mer peptide corresponding to HCV RNA interacting region of human La inhibits HCV translation whereas another peptide corresponding to the mouse La sequence was unable to do so. Further, IRES mediated translation was found to be significantly high in the presence of recombinant human La protein in vitro in rabbit reticulocyte lysate. We observed an enhanced replication with HCV subgenomic and full length replicons upon overexpression of either human La protein or a chimeric mouse La protein harboring human La β-turn sequence in mouse cells. Taken together, our results raise a possibility of creating an immunocompetent HCV mouse model using human specific cell entry factors and a humanized form of La protein.

Importance: Hepatitis C virus is known to infect only humans and chimpanzees under natural conditions. This has prevented development of a small animal model, which is important for development of new antiviral drugs. Although a number of human specific proteins is responsible for this species selectivity and some of these proteins---mostly entry factors---have been identified, full multiplication of the virus in mouse cells is still not possible. In this study, we show that a turn in the human La protein that is responsible for the interaction with the viral RNA, is highly specific for the human sequence. Replacement of the corresponding mouse sequence with the human sequence, allows the mouse La to behave like the human counterpart.
and support viral growth in the mouse cell efficiently. This observation, in combination with previously identified cell entry factors, should open up the possibility of creating a mouse model of hepatitis C.
INTRODUCTION

Hepatitis C virus (HCV), a blood borne pathogen, is an enveloped positive sense RNA virus that causes post-transfusion and sporadic non-A, non-B hepatitis (NANBH). HCV belongs to the Hepacivirus genus of the Flaviviridae family (1, 2). HCV genome is approximately 9.6kb genome long containing an open reading frame (ORF) encoding a polyprotein precursor of ~3000 amino acid residues flanked at both the ends by untranslated regions (UTRs) (1-4). Unlike cap dependent translation of host cell mRNAs, HCV translation is mediated by an internal ribosomal entry site (IRES) located within 5'UTR and extending to 30 to 40 nucleotides downstream of the initiator AUG (iAUG) codon (5). Several reports have demonstrated that some host proteins interact with the HCV 5'UTR and form ribonucleoprotein complexes. These host proteins include human La protein (6), polypyrimidine tract binding protein (PTB) (7), and poly(rC)-binding protein 2 (PCBP2) (8). These interactions of host trans-acting factors with the 5'UTR of the viral genome may be important for HCV translation or/and replication.

HCV represents a serious health burden and remains a major medical problem. About 80-90% of the infected individuals fail to clear the virus and become chronic carriers, which may lead to severe liver complications. Current treatment options are suboptimal, and there is no vaccine available yet (1). Other natural hosts for HCV, besides humans, are chimpanzees and less studied tree shrews (9, 10). This highly restricted tropism has hampered the development of small animal models to facilitate drug and vaccine discovery and to better understand host-virus interactions. Murine cells are non-permissive to HCV entry and show inefficient viral replication in vitro and in vivo (11-13). Although different xenograft models, based on transplantation of severely immuno-compromised mice with human hepatocytes, have shown some promise (14, 15), these models pose many important challenges such as intra- and inter-experimental variability, low
throughput, donor-to-donor variability, and high costs (16). Based on the studies in Chinese hamster ovary (CHO) and mouse fibroblast (NIH3T3) cells, CD81 and occludin (OCLN) were found to be the minimal human factors required to render mouse cells permissive to HCV entry (17). Building on this observation, a genetically humanized immuno-competent mouse model has been developed in the recent past (13). However, even in this transgenic mouse model, only transient burst in viral replication was observed, suggesting a possible requirement of additional human specific factor(s) at various stages of viral translation and replication.

Human La autoantigen (hLa) is an important cellular factor required for HCV translation and replication (18). Earlier, we have demonstrated that a 24-mer peptide (LaR2C) derived from the C terminus of RNA recognition motif (RRM) (residues 112 to 184) of La protein competes with the cellular La protein binding to the HCV IRES and interferes with the formation of a functional translation initiation complex (19). Recently, we have demonstrated that a 7-mer peptide (hLa174-180) LaR2C-N7 derived from the 24-mer LaR2C, having an unique beta turn structure in HCV RNA bound condition, is sufficient to inhibit HCV IRES mediated translation (20). In this article, using mutational analysis, we have delineated the amino acid sequence requirement of this peptide for inhibition of HCV RNA translation. We have also shown that amino acids required for efficient inhibition are only conserved in La proteins of HCV natural hosts, human and chimpanzee. Furthermore, human La protein (hLa) and a chimeric mouse La protein bearing the human sequence in the heptameric β-turn region (mLahN7), but not the wild-type mouse La protein, was shown to stimulate HCV translation in vitro in the rabbit reticulocyte lysate. Moreover, we provide the evidence that overexpression of both hLa and mLahN7 can promote increased HCV RNA replication and viral protein production in mouse cells. Taken together, our
observations provide an important insight into one of the post-entry factors that need to be considered in generating a mouse model to study HCV infection.

**MATERIALS AND METHODS**

**Peptide synthesis and purification.** The wt LaR2C-N7 peptide (KYKETDL), alanine point mutants of LaR2C-N7 and the corresponding mouse La peptide, LaR2C-N7-mouse (KYKDTNL) were synthesized as described previously (21).

**Plasmids.** The HCV sub-genomic replicons SGR-JFH1/Luc (22, 23) and SGR-JFH1/Luc-GND (22, 23), and the full length JFH1 construct (24) were generous gifts from Takaji Wakita.

**Cell culture.** NIH3T3 mouse fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100U of penicillin/ml, 100 µg of streptomycin sulphate/ml, and 10% fetal bovine serum (FBS). H6 mouse hepatoma cells (25) were cultured in RPMI 1640 medium containing 25mM HEPES (Sigma), 5µM β-mercaptoethanol (Sigma), penicillin-streptomycin, and 2mM Glutamine. Cells were maintained at 37°C in the presence of 5% CO2 in an incubator (Sanyo).

**In vitro transcription and translation.** mRNAs were transcribed *in vitro* from linearized plasmid constructs under T7 promoters in run-off transcription reactions as per manufacturer’s instructions (Promega). *In vitro* translation reactions of *in vitro* transcribed mRNAs were carried out in micrococcal nuclease treated rabbit reticulocyte lysates (RRL) as per manufacturer’s instructions (Promega). Reaction mixtures were assayed for the luciferase activities according to Promega protocol using the dual luciferase reporter assay system as described previously (26).

**Purification of La Protein.** *Escherichia coli* BL21 (DE3) cells were transformed with a bacterial expression vector encoding hLa, mLa or mLahN7. Transformed colonies were
inoculated into LB medium containing 80µg/ml ampicillin (HiMedia) and grown at 37°C in an incubator shaker until A_600 reached 0.6. The culture was induced with 0.6mM isopropyl-β-D thiogalactopyranoside (Sigma) for four hours at 37°C. The crude extracts were mixed with Ni-NTA agarose slurry (Qiagen) and allowed to rock for 2hr at 4°C. The lysate was loaded onto a column, washed with 50 ml of wash buffer (50mM Tris pH 7.4, 300mM NaCl, and 40mM imidazole), and the bound proteins were eluted with elution buffer containing 500mM imidazole. The eluted proteins were dialyzed in dialysis buffer (50mM Tris pH 7.4, 100mM KCl, 7mM β-mercaptoethanol, and 20% glycerol), aliquoted and stored at -80°C.

**Transient transfection.** Mouse fibroblasts or hepatoma cells were transiently cotransfected with in vitro transcribed subgenomic or full length JFH1 RNA and plasmid construct encoding hLa, mLahN7 or mLa using Lipofectamine 2000 reagent (Invitrogen) in an antibiotic free medium. Cells were harvested at different time points and total RNA was isolated using TRIzol reagent (Sigma) followed by reverse transcriptase PCR (RT-PCR). Cell lysates were prepared by using reporter lysis buffer (Promega) and luciferase activities were measured using luciferase assay kit (Promega).

**Quantitative RT-PCR.** For RT-PCR, 2 µg of total RNA was used for first strand cDNA synthesis using RevertAid™ Moloney murine leukemia virus reverse transcriptase (M-MuLV RT, Thermo Scientific). cDNA (1:10 diluted) was used for PCR amplification using SYBR Green real time assay mixture (Thermo Scientific). The data were obtained by using an ABI-Prism’s real time PCR machine and analyzed using the comparative ΔΔC_T method (27). Amplification of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization. Primers used for PCR are listed below:

HCV 5’ primer (Forward): 5’-TGCGGAACCGGTGAGTACA -3’
HCV 3’ primer (Reverse): 5’-CTTAAGGTTAGGATCCTGCTCAT-3’

GAPDH 5’ primer (Forward): 5’-CATGAGAAGTATGACAACAGCCT-3’

GAPDH 3’ primer (Reverse): 5’-AGTCCTTCCACGATACCAAAGT-3’

**Strand Specific tagged cDNA RT-PCR.** Total RNA was reverse transcribed with Avian myeloblastosis virus reverse transcriptase (AMV-RT, Promega), using a tagged HCV 5’ primer (forward). Two rounds of PCR were performed by using Hot Start Taq DNA polymerase (Genei). The first round of PCR was done on cDNA using the tag-only primer (forward) and the HCV 3’ primer (reverse). The resulting PCR product was diluted 1:100 and used for the second round of PCR using two internal primers. The final PCR product was analyzed by agarose gel electrophoresis. Amplification of GAPDH was used as an internal control.

**Preparation of cell lysates and Western blot analysis.** Cells were washed with PBS, scrapped off the culture dish and collected by centrifugation. Lysis of cell pellets was done in RIPA buffer constituting 50 mM Tris pH 7.4 containing 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 1 mM PMSF, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 12 µl/ml protease inhibitor cocktail for 30 min at 4°C followed by centrifugation at 12,000×g for 10min. Cellular debris was removed and supernatant was used for Western Blotting. Protein concentrations in supernatant were determined by Bradford’s method of protein quantification. Equal protein amounts were separated by SDS-10% PAGE and transferred onto nitrocellulose membrane (PALL Life Sciences) by semidy Western blot apparatus (Bio-Rad).

Non-specific binding was blocked with 5% non-fat dry milk powder in TBST (20mM Tris-HCl pH-7.4, 137mM NaCl, and 0.1% Tween-20) for one hour. The blots were incubated with primary antibody (anti-La) followed by a secondary antibody (horseradish peroxidase conjugated anti-
rabbit IgG; Sigma). Immunoblots were developed using ECL system (Amersham Pharmacia Biotech).

**Two cycle-RNase protection assay.** A two cycle RNase protection assay was performed as previously elsewhere (28). Briefly, total cellular RNA was self-hybridized overnight at 60°C in the absence of radiolabeled probe. This was followed by RNase treatment for 1hr in digestion mix (10 mM Tris-HCl buffer, pH 7.5 containing 500mM NaCl, 5mM EDTA, 4.5µg of RNase A/ml, and 350U of RNase T1/ml). Samples were treated with proteinase K followed by phenol-chloroform extraction and ethanol precipitation with tRNA (5µg). Precipitated RNA samples were then suspended in hybridization buffer containing radiolabeled probe (75 fmol) and subjected to a standard RNase protection procedure.

**Statistical Analysis.** The data in graphs represent mean ± S.D. Student’s t-test was performed to determine the levels of statistical significance for comparison between different samples. The criterion for defining statistical significance was $P < 0.05$ (* represents $P < 0.05$, ** represents $P < 0.005$, *** represents $P < 0.0005$).

**RESULTS**

**Role of different amino acid residues of 7-mer LaR2C-N7 in inhibiting HCV translation**

Human La protein is known to interact with HCV RNA via a $\beta$-turn (176-180) present in the RRM (112-184) (26). Previously, it was reported that a hepta-peptide LaR2C-N7 (KYKETDL), encompassing this $\beta$-turn inhibits HCV RNA translation (20, 26). However, it was unknown which amino acid residues are crucial for translation inhibitory activity. Thus, to understand the role of each amino acid in HCV translation, peptides with single alanine substitution at each position were synthesized. The inhibitory activities of all seven alanine mutant peptides (Table 1)
were checked in an *in vitro* translation assay system. A considerable reduction of translation inhibitory activity was observed in case of LaR2C-N7-2A, N7-3A, N7-4A, N7-5A and N7-6A, but not for N7-1A and N7-7A, when compared to the wild-type peptide (Fig. 1). Thus, the five internal residues (Y, K, E, T, D), comprising the β-turn in the RNA bound conformation of the peptide, appears to be important for the HCV RNA recognition process.

**Conservation of LaR2C-N7 sequence determines efficiency of HCV translation**

We further investigated the conservation of the LaR2C-N7 heptapeptide sequences in different species. Sequence alignment of several orthologs of La protein (Figs. 2A and 2B) suggests that the exact sequence is only preserved in human and chimpanzee (and some other primates of unknown host status). We observed that rabbit and mouse sequences differ from the human sequence in this region by one and two amino acids, respectively. As deduced from amino acid substitution experiments, these amino acids are important for interacting with HCV RNA. Moreover, we found that LaR2C-N7 peptide (KYKETDL) could more efficiently inhibit HCV translation as compared to the corresponding mouse La peptide (KYKDTNL) (Fig. 2C), further validating the importance of conservation of amino acid sequence of β-turn in determining translation efficiency of HCV RNA.

**Exogenous supplementation of mouse La carrying human N7 sequence (mLahN7) enhances HCV translation**

As human La protein (hLa) is crucial for supporting the HCV IRES mediated translation, supplementation of human La protein in the mouse or the rabbit translation system should enhance viral translation. As rabbit reticulocyte lysate (RRL) supports translation of hepatitis C
virus RNA inefficiently, we next investigated the effect of exogenous addition of human La protein on translation efficiency of the HCV RNA in RRL. We clearly observed a dose dependent stimulation of protein synthesis (Fig. 3A). In contrast, P4La (a mutant human La; E177A) (26), which interacts with HCV RNA inefficiently, failed to show similar stimulation (data not shown), suggesting that the β-turn mediated HCV RNA interaction plays an important role in host-specific IRES mediated translation. In order to investigate if the β-turn residues (KYKETDL) are solely responsible for higher efficiency of IRES mediated translation of HCV, we supplemented rabbit reticulocyte lysate with a chimeric mouse La protein carrying the human heptamer sequence (KYKETDL), mLahN7. It was observed that the chimeric protein but not the wildtype mouse La (mLa), could efficiently stimulate viral RNA translation like the human La protein (Fig. 3A).

Next, we investigated if the results hold true within a mouse cell. For this, the non-replicative, SGR-JFH1/GND RNA carrying a mutation in NS5B was used to exclude the differences due to RNA replication. Mouse fibroblast cells, NIH3T3, were cotransfected with SGR-Luc/JFH1-GND RNA and the construct encoding hLa, mLahN7 or mLa. However, a significant increase in luciferase activity, representing HCV IRES mediated translation, was observed upon overexpression of hLa or mLahN7 while no change was found when mLa was overexpressed (Figs. 3B and 3C). Taken together, our results suggest that the β-turn sequence corresponding to human La plays an important role in determining host specific translation of HCV.

Overexpression of mLahN7 in mouse cells supports HCV replication

Since mLahN7 enhances HCV translation in mouse cells, we were interested in investigating the effect of overexpression of mLahN7 on HCV replication in mouse cells. NIH3T3 cells were
cotransfected with SGR-Luc/JFH1 RNA and the construct expressing either hLa or mLahN7 or mLa, and replication was detected by quantifying HCV RNA levels (Figs. 4A and 4B) as well as luciferase expression (Fig. 4C) at longer time points (24, 48 and 72hr) after transfection. As expected, increased replication was observed in the presence of hLa and mLahN7 (but not mLa). In contrast, the level of SGR-JFH1/GND RNA rapidly declined even in presence of hLa or mLa-hN7, indicating the requirement of continuous replication for the maintenance of HCV RNA in the transfected mouse cells. Overexpression of La orthologs was confirmed by Western blot analysis (Fig. 4D).

Although NIH3T3 mouse fibroblasts have been shown to support HCV replication in principle, the efficiency is not likely to be very high due to lack of miR-122 (11, 29). Thus, similar experiments (as described above) were performed with mouse hepatoma cells transiently transfected with full length JFH1 RNA. Here also we observed an enhanced HCV RNA levels upon hLa or mLahN7 overexpression. In contrast, cells treated with a polymerase inhibitor, hemin (30, 31) showed degradation of transfected RNA in the absence of replication (Figs. 5A-5D). Similar observation was observed in protein levels also (Fig. 5E). Taken together, our results suggest that mouse cells are unable to maintain HCV replication but overexpression of hLa or mLahN7 significantly improves HCV replication and sustain it for relatively longer time point.

Increased synthesis of HCV negative strands in the presence of mLahN7

Replication of HCV positive strand genome is preceded by the production of a negative strand intermediate which is then used as template for production of new positive strands. Thus, detection of negative strand RNA is a true indicator of viral replication (28, 32). Considering the
fact that the presence of a large number of HCV positive strands might interfere with hybridization based methods to quantify negative strands levels, more sensitive strand-specific RT-PCR using tagged primers (32) (Figs. 6A-6C) and two cycle RNase protection assay (Figs. 6D and 6E) were performed and HCV negative strands were detected. We found that overexpression of hLa or mLahN7 (not mLa) enhances HCV negative strand synthesis, which further strengthens our observation that the $\beta$-turn corresponding to human La is important for HCV RNA replication in mouse cells.

**DISCUSSION**

HCV infection is a major cause of mortality and morbidity, with approximately 3 percent of the worldwide population is infected with it. Chronic HCV infections can lead to cirrhosis of liver, liver failure, and hepatocellular carcinoma (1). Elucidation of the HCV life cycle and the development of different protective and therapeutic strategies have been hampered due to the lack of a suitable small animal model (33). Host-virus interactions that determine host tropism significantly influence pathogenesis of viral infection. Due to the narrow host specificity of the HCV, the full-blown infection could not been established in mice models, except in humanized immunosuppressed mouse. Host factors determining liver tropism of HCV are only partly understood. Restriction of HCV replication in murine cells can be attributed to the presence or absence of some essential human host factors that affect virus attachment, entry, replication, packaging, and assembly (17, 34-37). Previous reports have investigated different determinants for HCV entry into rodent cells. Although expression of human CD81 and OCLN were shown to overcome the receptor entry block of HCV particles, no infectious virions are released from rodent cells expressing both of the host factors (17, 36). A trans-complementation based system
demonstrated apolipoprotein E (apoE) as the limiting factor for assembly and release of HCV in mouse hepatoma cells (36).

Following uptake into murine cells, the intracellular viral RNA is translated but the level is not sustained for long, suggesting that HCV RNA replication is impaired in these cells (13). It is likely that other human specific orthologs are needed for efficient replication of viral RNA in mouse cells. Several reports have demonstrated that host antiviral defenses limit efficient HCV RNA propagation (37, 38). For example, mouse embryo fibroblasts (MEFs) with deficiencies of protein kinase R (PKR) or interferon regulatory factor 3 (IRF 3) are more supportive for HCV replication (34, 37). Exogenous introduction of liver-specific miR-122 into mouse fibroblasts increases viral RNA abundance (37). However, other factors may also contribute.

In this study, we have identified human La protein as a crucial component that determines efficient translation and hence replication of HCV RNA in mouse cells. Our mutational analyses demonstrate the importance of the primary sequence of the β-turn within the La-RRM (112-184) in determining species specificity of HCV infection. Our observations reflect that even a point mutation, for example at the fourth position (E177D), may prevent HCV translation or render HCV non-permissive to many species. These results are consistent with one of the earlier studies that showed a single point-mutation in the La motif of the full-length La protein could completely abrogate the RNA binding activity (39). Since many secondary and tertiary interactions between the different domains of La influence La protein folding, a mutation at any of such hot spots could have drastic consequences for the La protein structure and its RNA binding ability.
Development of a selectable and efficiently replicating HCV sub-genomic replicon in the human hepatoma cell line, Huh-7, has allowed to study replication in cell culture. Additionally, the ability to select for replication enhancing mutations eventually led to the establishment of HCV replication in other hepatic (HepG2 and IMY-N9) and non-hepatic (HeLa and HEK293) cell lines (40, 41). Consistent with earlier observations (11), we also found that JFH1 genotype 2a sub-genomic and full length replicons could replicate in both mouse hepatocytes and embryonic fibroblasts without any requirement of adaptive mutations. Interestingly, HCV replication was significantly higher and maintained for longer time scale in the presence of a chimeric mouse La with humanized β-turn.

The regulatory functions of different cis-acting elements of HCV RNA and picornaviruses are mediated through their interactions with many cellular and viral proteins. Recently, we demonstrated that human La protein interaction with a highly conserved GCAC-tetrancleotide sequence within IRES enhances HCV RNA replication by promoting linkage between 5’ and 3’ UTRs (42). It is possible that mouse La might not be able to perform similar function. Further studies should focus on investigating whether mouse La interacts with different viral proteins (such as NS5B, NS3 etc.) that participate in replication as a part of functional replication complex.

Recently, a transgenic mouse model stably expressing human CD81, SR-B1, CLDN1 and /or OCLN has been established (43). Although this mouse is permissive to HCV entry, RNA replication is not efficiently maintained. We speculate that transgenic mice having two additional mutations in mouse La β-turn sequence (D177E and N199D) may boost translation and replication of HCV RNA, thus opening up possibilities of establishing a better mouse model of hepatitis C by offering improvements over existing in vivo HCV systems.
ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

FOOTNOTES

The abbreviations used are: HCV, hepatitis C virus; IRES, internal ribosome entry site; UTR, untranslated region; RRM, RNA recognition motif; SL, stem loop; PTB, polypyrimidine tract binding protein; PCBP, poly (rC)-binding protein; DTT, dithiothreitol; RRL, rabbit reticulocyte lysate; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction; OCLN, occludin; SR-B1, scavanger receptor class B type 1; CLDN1, claudin-1.

REFERENCES


TABLE 1. Sequence of synthetic peptides and their nomenclatures. Mutation is marked in bold.

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<td>N7-wt (Human N7)</td>
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FIGURE LEGENDS

FIGURE 1: Effect of alanine substitution of each amino acid of the heptapeptide on HCV IRES mediated translation. In vitro translation inhibition by wild-type (wt) and alanine mutated LaR2C-N7 peptides. 1 µg of capped luciferase HCV-IRES RNA was translated in RRL in the absence and presence of 60 µM of wt or mutated LaR2C-N7 peptides. The bars represent average of two independent measurements. The rectangles on top of the bars represent high and the low values.

FIGURE 2: Conservation of La 174-180 amino acid residues in different species. (A) Multiple sequence alignment of La sequences from several mammalian species. La174-180 amino acid sequence is represented in bold. Differences in this sequence are marked in red. (B)
Sequence Logo plots (residues 174-180 shown) of 90 sequences obtained from a BLAST search of human La protein (sequence from pdb: 1S79). (C) Inhibitory effect of wt LaR2C-N7-human (sequence from human La-protein) or wt LaR2C-N7-mouse (sequence from mouse La-protein) peptides on HCV IRES-mediated translation in vitro. 1 µg of HCV bicistronic RNA (RLuc-HCV IRES-FLuc) was translated in RRL in the absence or in the presence of increasing concentrations of either wt LaR2C-N7-human or wt LaR2C-N7-mouse peptides (as indicated). In both cases, the relative FLuc activities were represented as percentages of the control reaction (expressed as 100%). Values represent an average of three independent experiments.

FIGURE 3: mLahN7 enhances HCV translation in vitro and in mouse fibroblasts. (A) 1 µg of HCV bicistronic RNA (RLuc-HCV IRES-FLuc) was translated in the absence or presence of increasing concentrations of purified hLa or mLahN7 or mLa protein (25, 50 ng). BSA (50 ng) was used as a negative control. Respective RLuc and Fluc activities were plotted against different concentrations of proteins (‘ns’ represents values that are not significant). (B) Enhancement of translation in mouse fibroblasts by overexpression of human La protein. NIH3T3 cells were cotransfected with in vitro transcribed SGR-JFH1/Luc-GND replicon RNA and increasing concentrations (0.5 and 1µg) of construct expressing hLa or mLahN7 or mLa. At 24h post-transfection, a luciferase assay was performed to measure HCV IRES mediated translation and results were represented graphically. (C) For the experiment performed in panel B, overexpression of hLa, mLahN7 and mLa was checked by Western blot analysis using a polyclonal anti-La antibody. ‘C’ denotes no transfection control.

FIGURE 4: Mouse fibroblasts support HCV replication upon mLahN7 overexpression. (A and B) NIH3T3 cells were cotransfected with (A) SGR-Luc/JFH1 or (B) SGR-Luc/JFH1-GND replicon RNA, and the plasmid construct expressing hLa, mLahN7, or mLa. At 24, 48, and 72h
post transfection, HCV-positive strand RNA levels were quantified by using qRT-PCR. (C) For the experiments performed in panels A and B, luciferase assay was performed to determine HCV IRES mediated translation. Results were represented graphically. The data plotted represent the mean ± SD of three independent experiments. (D) For the experiments performed in panels A to C, overexpression of hLa, mLahN7 and mLa was checked by Western blot analysis (at 72hr post transfection) using anti-La antibody. Actin was used as an internal control to ensure equal loading.

**FIGURE 5: mLahN7 overexpression facilitates HCV replication in mouse hepatoma cells.** (A-D) Mouse hepatoma cells were cotransfected with *in vitro* transcribed full length HCV JFH1 RNA and the plasmid construct expressing hLa, mLahN7, or mLa in the absence (black line) or presence (gray line) of 100µM hemin. At 24, 48, and 72h post transfection, HCV-positive strand RNA levels were quantified by using qRT-PCR. (E) Similar to the experiments performed in panels A to D, cells were harvested at 24h post transfection, and Western blotting for NS5B and La was performed. Equal loading was confirmed by using actin as an internal control. ‘C’ denotes no transfection control.

**FIGURE 6: mLahN7 enhances HCV negative strand synthesis.** (A and B) Mouse hepatoma cells were cotransfected with *in vitro* transcribed full length HCV JFH1 RNA along with a plasmid construct expressing hLa, mLahN7 or mLa. The cells were harvested at (A) 24h and (B) 48h post transfection, and HCV negative strand RNA levels were quantified by strand specific tagged cDNA RT-PCR. ‘C’ denotes no transfection control. (C) NIH3T3 cells were cotransfected with *in vitro* transcribed full length HCV JFH1 RNA along with a plasmid construct expressing hLa, mLahN7 or mLa. The cells were harvested at 48h post transfection, and HCV negative strand RNA levels were quantified by strand specific tagged cDNA RT-PCR.
(D) Mouse hepatoma cells were cotransfected with \textit{in vitro} transcribed HCV JFH1 RNA along with a plasmid construct expressing hLa, mLahN7 or mLa. At 24h post transfection, HCV-negative strand RNA was detected by using a two-cycle RNase protection assay. ‘C’ denotes no transfection control. Numbers below figure panels denote densitometry values. (E) NIH3T3 cells were cotransfected with \textit{in vitro} transcribed HCV JFH1 RNA along with a plasmid construct expressing hLa, mLahN7 or mLa. At 48h post transfection, HCV-negative strand RNA was detected by using a two-cycle RNase protection assay.