

# La Protein Binding at the GCAC Site Near the Initiator AUG Facilitates the Ribosomal Assembly on the Hepatitis C Virus RNA to Influence Internal Ribosome Entry Site-mediated Translation\*

Received for publication, March 29, 2004, and in revised form, May 10, 2004  
Published, JBC Papers in Press, May 10, 2004, DOI 10.1074/jbc.M403417200

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Human La autoantigen has been shown to influence internal initiation of translation of hepatitis C virus (HCV) RNA. Previously, we have demonstrated that, among the three RRM of La protein, the RRM2 interacts with HCV internal ribosome entry site (IRES) around the GCAC motif near the initiator AUG present in the stem region of stem-loop IV (SL IV) (Pudi, R., Abhiman, S., Srinivasan, N., and Das S. (2003) *J. Biol. Chem.* 278, 12231–12240). Here, we have demonstrated that the mutations in the GCAC motif, which altered the binding to RRM2, had drastic effect on HCV IRES-mediated translation, both *in vitro* and *in vivo*. The results indicated that the primary sequence of the stem region of SL IV plays an important role in mediating internal initiation. Furthermore, we have shown that the mutations also altered the ability to bind to ribosomal protein S5 (p25), through which 40 S ribosomal subunit is known to contact the HCV IRES RNA. Interestingly, binding of La protein to SL IV region induced significant changes in the circular dichroism spectra of the HCV RNA indicating conformational alterations that might assist correct positioning of the initiation complex. Finally, the ribosome assembly analysis using sucrose gradient centrifugation implied that the mutations within SL IV of HCV IRES impair the formation of functional ribosomal complexes. These observations strongly support the hypothesis that La protein binding near the initiator AUG facilitates the interactions with ribosomal protein S5 and 48 S ribosomal assembly and influences the formation of functional initiation complex on the HCV IRES RNA to mediate efficient internal initiation of translation.

Translation of hepatitis C virus (HCV)<sup>1</sup> is mediated by an internal ribosome entry site (IRES) located mostly within the 5'-untranslated region (UTR) and extending a few nucleotides

\* This work was supported in part by grants from the Department of Science and Technology and Department of Biotechnology, India (to S. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a pre-doctoral fellowship from the Council of Scientific and Industrial Research, India.

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<sup>1</sup> The abbreviations used are: HCV, hepatitis C virus; UTR, untranslated region; IRES, internal ribosome entry site; La, human La antigen; RRM, RNA recognition motif; nt, nucleotide(s); eIF, eukaryotic initiation factor; PTB, polypyrimidine tract-binding protein; PCBP, poly-r(C)-binding protein; SL, stem-loop; RRL, rabbit reticulocyte lysate; RLuc, *Renilla* luciferase; FLuc, firefly luciferase; CD, circular dichroism; rpS5, ribosomal protein S5; DTT, dithiothreitol; MEM, minimal essential medium; I-RNA, inhibitor RNA.

into the open reading frame (1–5). HCV 5'-UTR is highly conserved and folds into a complex secondary structure comprising four major structural domains (I–IV) and a pseudoknot in the vicinity of the initiator AUG codon (6, 7). The *cis*-elements promote assembly of initiation complex independent of the 5'-end and thus mediate internal initiation of translation in a cap-independent manner (7). Domain I is not a part of IRES and most likely is involved in RNA replication, whereas domains II and III are complex and consist of multiple stem-loops and bulge-loops. Even minor mutations in domains II and III substantially reduce IRES activity, but this could in most cases be regained by compensatory second site mutations that restored secondary structure. Highly conserved residues are often unpaired and may thus be able to interact with the components of the translation apparatus. Domain IV consists of a stem-loop that contains initiator AUG codon and has been shown to play a key role in regulating the initiation of translation of the HCV RNA (5, 8–10). It appears from earlier reports that both the sequence and stability of the domain IV stem might control efficiency of HCV IRES translation (11, 12). These observations have led to a model for IRES function in which the structural elements in the IRES act as a scaffold that orients the potential binding sites in such a way that their interactions with initiation factors and ribosomes lead to assembly of functional ribosomal initiation complexes (13).

HCV IRES binds to the 40 S ribosomal subunit specifically and stably even in the absence of any initiation factors. Addition of eIF2/GTP/Met-tRNA<sub>i</sub> is sufficient for 40 S subunit to lock onto initiator AUG (13). eIF3, though not essential for the formation of 48 S complex formation, it has been shown to bind to the apical half of domain III and is likely to be a constituent of the 48 S-IRES complex *in vivo* (14, 15). 48 S complex formation on HCV IRES has no requirement for eIF4A, 4B, 4E, 4G, or for ATP hydrolysis (14–16). Because the viral 5'-UTR forms a binary complex with the 40 S ribosomal subunit in the absence of any canonical or non-canonical initiation factors, it is likely that the additional factors may stimulate internal initiation of translation following the assembly of RNA-40 S complex. Recently, binding of a 25-kDa cellular protein (p25) to HCV IRES has been shown to be important for the efficient translation initiation. p25 was originally suggested to be ribosomal protein S9 but later identified as rpS5 (14, 17, 18). In fact, HCV IRES has been suggested to have a prokaryotic-like mode of interaction with the 40 S ribosomal subunit, where the 40 S ribosomal subunit is thought to interact with the HCV-IRES through p25 (14). However, eukaryotic mRNAs and picornaviral IRESs have not been reported to require S5 protein for the ribosome assembly. Recently we have shown that a small RNA corresponding to the stem-loop III e+*f* domain of the HCV IRES, when introduced in *trans*, can antagonize cel-

lular protein binding to the viral IRES and inhibits HCV IRES-mediated translation. The RNA molecule showed strong interaction with the ribosomal S5 protein and prevented the recruitment of the 40 S ribosomal subunit by the HCV IRES (19). Therefore, it appears that any event that might facilitate S5 interaction with HCV IRES could be crucial for efficient ribosome assembly at the initiation site. It is believed that HCV IRES contains one set of determinants that is required for initial ribosome recruitment and a second set that promotes accurate placement of the initiation codon in the ribosomal P site (13). Several cellular *trans*-acting factors that are known to bind to HCV IRES and influence the internal initiation might play a role in the formation of functional initiation complex after the initial binding of 40 S ribosomes to the IRES. Most notable among these are the polypyrimidine tract-binding protein (PTB), (20) the La autoantigen (21), and poly(rC) binding protein 2 (PCBP 2) (22) etc. In case of picornaviruses, a model has been proposed in which the IRES *trans*-acting factors such as PTB help the RNA to attain or maintain an active conformation in which it is able to bind essential factors and the 43 S ribosome complex in a productive manner (23). In hepatitis A virus, glyceraldehyde-3-phosphate dehydrogenase was found to destabilize the folded structures of the RNA stem-loops and influence the IRES activity (24). Human La autoantigen was found to enhance the accuracy of initiation codon selection in poliovirus IRES (25).

The human La protein is an RNA-binding protein, belonging to the RNA recognition motif (RRM) superfamily (26, 27). La protein is ubiquitously expressed in many eukaryotic organisms, including *Saccharomyces cerevisiae*, *Xenopus laevis*, and *Drosophila melanogaster* (28). La protein has been shown to interact with a wide variety of cellular and viral RNAs and has been implicated in various cellular processes, which include RNA polymerase III transcription termination (27), telomere homeostasis (29), internal initiation of translation of Bip mRNA, poliovirus, coxsackievirus B3, encephalomyocarditis virus, and hepatitis C virus (30, 31). Also, La protein has been shown to be capable of unwinding DNA-RNA hybrids and double-stranded RNA in an ATP-dependent manner (32). La protein specifically interacts with both the 5'- and 3'-UTR of HCV RNA (33). La protein plays a functional role in internal initiation of translation where addition of purified La to RRL in the *in vitro* translation assays using HCV IRES resulted in stimulation of translation activity (21). Inhibition of HCV IRES activity caused by sequestration of La protein can be rescued by exogenous addition of purified La protein (34, 35).

Previously, La protein has been shown to bind to HCV IRES in the context of initiator AUG (21). Recently, we showed that, of the three RRM2 present in La, RRM2 binds HCV RNA around the region encompassing the GCAC motif located in the stem region near the initiator AUG (36). However, the precise mechanism with which the La protein influences the stimulation of HCV IRES activity is yet to be elucidated. Here we report for the first time a possible functional role of La protein interaction near the initiator AUG of the HCV-IRES RNA to mediate efficient internal initiation of translation. We have demonstrated that mutations in the GCAC motif near initiator AUG within the SL IV, which alter the primary sequence while retaining the overall secondary structure affect the binding of La-RRM2 to HCV IRES. Also, these mutations have drastic effect on the HCV IRES-mediated translation both *in vitro* and *in vivo*, indicating that the sequence GCAC might play an important role in maintaining the IRES function. Further, we have observed that the mutations also alter the binding of certain cellular proteins to HCV IRES, especially p25 (S5), whose binding may have been influenced by the binding of La

protein to HCV IRES in the SL IV region. Interestingly, addition of increasing concentration of La protein helped in the binding of S5 protein suggesting that La protein might play a role in recruitment of the 40 S ribosomal subunit to the HCV IRES RNA. Additionally, the circular dichroism spectra of HCV stem-loop IV RNA showed a dose-dependent increase in the presence of increasing concentrations of La protein indicating that HCV IRES may undergo some conformational alterations upon binding to La protein, which assist in the formation of functional initiation complex. Finally, sucrose gradient centrifugation analysis of ribosome assembly implied that the mutations within HCV IRES lead to reduced efficiency in the formation of functional ribosomal complex. Taken together, the results strongly suggest that La protein interaction near the initiator AUG might be involved in conformational alteration to facilitate better contact with the 40 S ribosomal subunit required for efficient internal initiation of translation.

#### EXPERIMENTAL PROCEDURES

**Plasmids**—HCV 1b-encoding plasmid, pCV, was generously gifted by Dr. Akio Nomoto and Dr. Tsukiyama-Kohara, University of Tokyo, Japan. HCV 5'-UTR along with 42-nt coding sequence (18–383 nt) was cloned into the mammalian expression vector pcDNA3 (Invitrogen) to generate pcDHCV-383 as described earlier (36). pcD-SL IV containing nt 293–383 of HCV IRES was obtained by amplifying HCV SL IV using the primers, 5'-ATAGAAGCTTGCCTGATAGGTCTGCCGA-3' and 5'-CGCAATTCGTTACGTTTGGTTTT-3', from the template plasmid pcDHCV-383 and cloned between HindIII and EcoRI sites of the pcDNA3. The PCRs were carried out with 30 cycles, each cycle consisting of denaturation (95 °C for 40 s), annealing (55 °C for 1 min), and extension (68 °C for 1 min) using *Pfx* DNA polymerase (Invitrogen). The cDNA clone encoding human La autoantigen, pET-La was obtained from Dr. Jack Keene, Duke University. La coding sequence and La 101–208 (RRM2) were subcloned into pRSET-A vector (Invitrogen) (36). Ribosomal protein S5 containing plasmid pQE-RS5 was obtained from Dr. Shuetsu Fukushi, Biomedical Laboratories, Japan. S5 coding sequence was obtained from the vector by digesting with HindIII and BamHI and subcloned into pRSET-A vector.

All the mutants described in the study were generated by using a megaprimer PCR method as described previously (37). The method utilizes three oligonucleotide primers to perform two rounds of PCR. The product of the first PCR is used as one of the primers (a "megaprimer") for the second polymerase chain reaction. The first round of PCR was carried out using the 3' primer corresponding to 383 nt of HCV RNA, 5'-CGCAATTCGTTACGTTTGGTTTT-3', and the following 5' primers carrying the specific mutations, 5'-CCGTGCATC-AAGAGCACAAAT-3', 5'-TCGTAGACCTAATCATGATAGTAAATCCTAA-3', and 5'-TCGTAGACCCGGTATCATGAACCGAAATCTAA-3'. The PCRs were carried out for 35 cycles, each cycle consisting of denaturation (95 °C for 40 s), annealing (55 °C for 1 min), and extension (72 °C for 10 s) using *Taq* DNA polymerase (Bangalore Genie) or *Pfx* polymerase. The amplification products were subjected to 9% native PAGE and visualized by staining with ethidium bromide. The bands were cut and incubated in 400  $\mu$ l of elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 0.1% SDS) overnight at 37 °C. The supernatant was subjected to phenol-chloroform extraction, and the DNAs were alcohol-precipitated. The megaprimers thus obtained were then used as 3' primers in the second round of PCR along with the HCV 5' primer, 5'-TATAAGCTTGGATCCCCGGCGACA-3'. The PCRs were carried out for 35 cycles, each cycle consisting of denaturation (95 °C for 40 s), annealing (60 °C for 1 min), and extension (72 °C for 30 s) using *Taq* DNA polymerase or *Pfx* polymerase. The PCR products were digested with HindIII and EcoRI and cloned into pcDNA3 vector. pcD-SL IV M4mut was generated using the primers 5'-ATAGAAGCTTGCCTGATAGGGTGTGCGA-3', 5'-CGCAATTCGTTACGTTTGGTTTT-3', using the plasmid M4 as template and cloned between HindIII and EcoRI sites of the pcDNA3.

For the construction of bicistronic constructs, the two reporter genes, *Renilla* luciferase (RLuc) and Firefly luciferase (FLuc) coding sequences were PCR-amplified using *Pfx* polymerase using the following primers, 5'-GATGCTAGCACCATGACTTCGAAA-3', 5'-GGCCAAAGCTTACCA-TGATTCGAAA-3', 5'-GCATCTCGAGGAAAGACGCCAAAAAC-3', and 5'-ATTAGGGCCCTTACACGGCGATCTT-3'. The 5' primer of FLuc lacks the initiator AUG codon, because the HCV IRES has an initiator AUG located within. The amplified RLuc was cloned into NheI and XhoI

sites of pcDNA3.1 (+) vector (Invitrogen), and FLuc was cloned into XhoI and ApaI sites. The wild-type and mutant HCV IRES were cloned into HindIII and EcoRI sites between the two reporter genes.

**Preparation of HeLa S10 Cell Extract**—HeLa cells used for preparing cell extract were grown in T75 flasks in minimal essential medium (pH 7.0) supplemented with 10% fetal calf serum. Monolayer of HeLa cells were harvested, pelleted down, and washed thrice with cold isotonic buffer (35 mM HEPES, pH 7.4, 146 mM NaCl, 11 mM glucose), resuspended in 1.5× packed cell volume of hypotonic buffer (10 mM HEPES, pH 7.4, 15 mM KCl, 1.5 mM magnesium acetate, and 6 mM  $\beta$ -mercaptoethanol) and then incubated on ice for 10 min. Cells were then transferred to a Down's homogenizer and disrupted by 50 strokes on ice (lysis was checked under microscope). The lysate was incubated in 1× incubation buffer for 10 min. Cytoplasmic extract (S10 supernatant) was isolated by centrifuging the lysate at 10,000 × *g* for 30 min at 4 °C. The supernatant was dialyzed for 4 h against 100 volumes of dialysis buffer and aliquoted into pre-chilled tubes. The aliquots of S10 extract were stored at -70 °C.

**Purification of rpS5, La Full-length, and RRM2 Proteins Using a Nickel-Nitrilotriacetic Acid-Agarose Column**—*Escherichia coli* BL21(DE3) cells were transformed with pRSET-A vectors containing either the full-length or the deletion mutant of La or the full-length rpS5. Transformed colonies were inoculated into 100 ml of LB broth containing 75  $\mu$ g/ml ampicillin and grown at 37 °C in an incubator shaker at 200 rpm speed until the OD<sub>660</sub> reached 0.6. The cultures were induced with 0.6 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside and grown for further 4 h. The cells were pelleted and resuspended in 5 ml of lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole). The extract was made by sonication. The above crude extracts were mixed with 500  $\mu$ l of nickel-nitrilotriacetic acid-agarose slurry (Qiagen) and kept for rocking at 4 °C for 4 h. The lysate was loaded onto a column, and the flow-through was discarded. The column was washed with 50 ml of wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 40 mM imidazole). The bound protein was eluted with 500  $\mu$ l of elution buffer containing 500 mM imidazole. The eluted proteins were dialyzed at 4 °C for 4–6 h in 500 ml of dialysis buffer (50 mM Tris (pH 7.4), 100 mM KCl, 7 mM  $\beta$ -mercaptoethanol, 20% glycerol), aliquoted, and stored in -70 °C freezer.

**In Vitro Transcription**—mRNAs were transcribed *in vitro* from different linearized plasmid constructs under T7 promoters in run-off transcription reactions. The HCV bicistronic constructs were linearized with PmeI downstream of firefly luciferase and used as templates for RNA synthesis. The linear DNA were electrophoresed on agarose gels and extracted by using Qiagen gel elution kit and capped bicistronic RNA were synthesized using Ribomax Large scale RNA production system-T7 (Promega) under standard conditions with addition of 5' cap analog (Promega).

Radiolabeled mRNAs were transcribed *in vitro* using T7 RNA polymerase (Promega) and [<sup>32</sup>P]uridine triphosphate (PerkinElmer Life Sciences). The pcDNA3 vectors containing wild-type or mutant HCV IRES were linearized with EcoRI, gel-eluted, and transcribed *in vitro* to generate the <sup>32</sup>P-labeled RNA. The transcription reaction was carried out under standard conditions (Promega protocol) using 2.5  $\mu$ g of linear template DNA at 37 °C for 1.5 h. After alcohol precipitation, the RNA was resuspended in 25  $\mu$ l of nuclease-free water. 1  $\mu$ l of the radiolabeled RNA sample was spotted onto DE81 filter paper, washed with phosphate buffer, and dried, and the incorporated radioactivity was measured using liquid scintillation counter.

**Filter Binding Assay**—The <sup>32</sup>P-labeled wild-type or mutant HCV IRES RNAs were incubated with the La RRM2 or RRM3 proteins at 30 °C for 15 min in RNA binding buffer (containing 5 mM HEPES, pH 7.6, 25 mM KCl, 2 mM MgCl<sub>2</sub>, 3.8% glycerol, 2 mM DTT, and 0.1 mM EDTA) and loaded onto nitrocellulose filters equilibrated with the 2-ml RNA binding buffer. The filters were then washed four times with 1 ml of binding buffer and air-dried. The counts retained were measured in liquid scintillation counter. The graph was plotted with protein concentration (micromolar) on the x-axis, and the percentage of bound RNA was plotted as the percentage of counts retained, on the y-axis.

**UV Cross-linking Experiment**—The <sup>32</sup>P-labeled wild-type or mutant HCV IRES RNAs were incubated with the purified proteins or HeLa S10 extract at 30 °C for 15 min in RNA binding buffer (containing 5 mM HEPES, pH 7.6, 25 mM KCl, 2 mM MgCl<sub>2</sub>, 3.8% glycerol, 2 mM DTT, and 0.1 mM EDTA) and then irradiated with a hand-held UV lamp for 10 min. The mixture was treated with 30  $\mu$ g of RNase A (Sigma) at 37 °C for 30 min. The protein-nucleotidyl complexes were electrophoresed on SDS-10% PAGE analyzed by phosphorimaging analysis. In the experiments performed in the presence of La protein, the RNA was incubated with the purified proteins at 30 °C for 10 min prior to binding with HeLa S10 extracts.

**In Vitro Translation**—*In vitro* translation of the capped bicistronic mRNAs were carried out in micrococcal nuclease treated rabbit reticulocyte lysates (RRL, Promega Corp.). Briefly, 12.5- $\mu$ l reaction mixtures contained 8.75  $\mu$ l of RRL containing 0.25  $\mu$ l each of minus methionine mixture, 5  $\mu$ Ci of [<sup>35</sup>S]methionine (PerkinElmer Life Sciences) and 10 units of RNasin (Promega) were incubated at 30 °C for 1.5 h. 1  $\mu$ l of the reaction mixtures was assayed for both the *Renilla* and firefly luciferase activity according to Promega protocol using Dual-Luciferase reporter assay system. 7  $\mu$ l of the reaction mixtures was electrophoresed on SDS-10% polyacrylamide gel, dried, and analyzed by phosphorimaging.

**Transfection of HeLa/Huh7 Monolayer Cells**—HeLa/Huh7 cells grown in 30-mm dishes at 60–70% confluence were transfected with 2  $\mu$ g of the bicistronic constructs using Tfx 20 transfection reagent (Promega) according to manufacturer's protocol. Briefly, the DNA was mixed with 5  $\mu$ l of Tfx 20 reagent and diluted to 1 ml using the MEM (Invitrogen) and incubated at room temperature for 15 min. The cells were washed with medium and overlaid with 1 ml of MEM containing the above DNAs. After 1 h of incubation at 37 °C, 0.8 ml of MEM, and 0.2 ml of fetal bovine serum (Invitrogen) was added to the dishes. After 36 h, the cells were washed with phosphate-buffered saline and lysed using 1× passive lysis buffer (Promega) and assayed for RLuc and FLuc according to Promega protocol using the Dual-Luciferase reporter assay system.

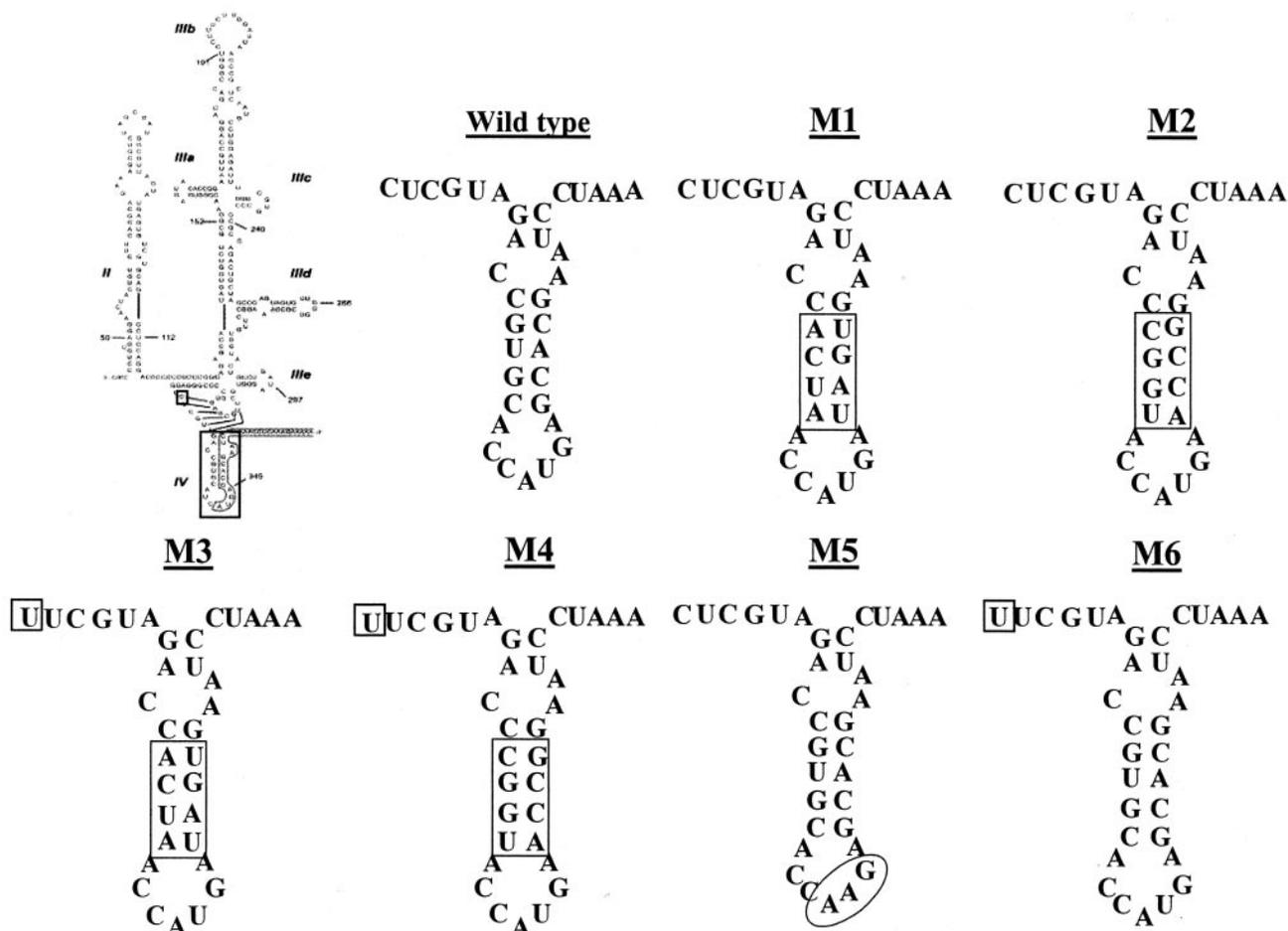
**CD Spectroscopy**—Measurements of CD spectra were performed with a Jasco J-715 spectropolarimeter as described earlier (38). Spectra were obtained in 0.5 ml of RNA binding buffer (5 mM HEPES, pH 7.6, 25 mM KCl, 2 mM MgCl<sub>2</sub>, 3.8% glycerol, 2 mM DTT, 0.1 mM EDTA). CD spectra were obtained in the 240- to 320-nm range at 20 °C with HCV stem-loop IV WT or stem-loop IV M4 RNA (250 or 300 nM) and increasing concentration of purified recombinant La protein (0.5–2  $\mu$ M). The molar ellipticity values were normalized for the contribution of the La protein at each concentration.

**Sucrose Gradient Centrifugation Analysis of Ribosomal Assembly on HCV IRES**—The <sup>32</sup>P-radiolabeled RNAs (~2 × 10<sup>5</sup> cpm) were added to 50  $\mu$ l of ribosome assembly reactions containing 35  $\mu$ l of RRL, 0.5  $\mu$ l each of minus methionine and minus leucine amino acid mixtures and 10 units of RNasin (Promega) and incubated at 30 °C for 15 min. The reactions were diluted to 150  $\mu$ l with gradient buffer (20 mM Tris (pH 7.5), 100 mM KCl, 3 mM MgCl<sub>2</sub>, 1 mM DTT) and overlaid on a 5–30% (w/v) linear sucrose gradient. The ribosomal complexes were sedimented by ultracentrifugation for 3 h at 4 °C and 30,000 rpm using a Beckman SW41 swing bucket rotor. Fractions (5 drops) were manually collected from the bottom of the tube and counted in a Rackbeta liquid scintillation counter.

## RESULTS

**Mutations at the GCAC Motif Near Initiator AUG in Stem-loop IV of HCV IRES Affect Binding to RRM2 of La Protein**—We have demonstrated earlier that the RRM2 of human La protein binds to hepatitis C virus IRES in the region encompassing GCAC motif near the initiator AUG located in stem-loop IV. Interestingly, we have also noticed that this GCAC sequence motif is relatively conserved across the genotypes of HCV (data not shown). Thus it was interesting to investigate whether the binding of RRM2 of La protein to HCV RNA in this region influences the IRES-mediated translation. Several mutations in SL IV were designed in such a way as to alter the primary nucleotide sequence of the GCAC motif, whereas the compensatory mutations in the other strand would allow the secondary structure to be retained (Fig. 1A). Two mutants, M1 and M2, were generated in the GCAC region, either altering the GC content (M1) or retaining it (M2) and confirmed by sequencing. Interestingly, during the sequencing of the above clones we also found two more mutant clones that harbored an additional mutation, C325U (at the beginning of the pseudoknot) along with the designed mutations in SL IV region. Consequently these additional mutants were named as M3 and M4 (Fig. 1A). To investigate the contribution of this additional mutation at the 325 position, we have generated one more mutant with substitution at this position, C325U (named as M6). Earlier studies from other laboratories have indicated the importance of La binding at the initiator AUG, hence we

A



B

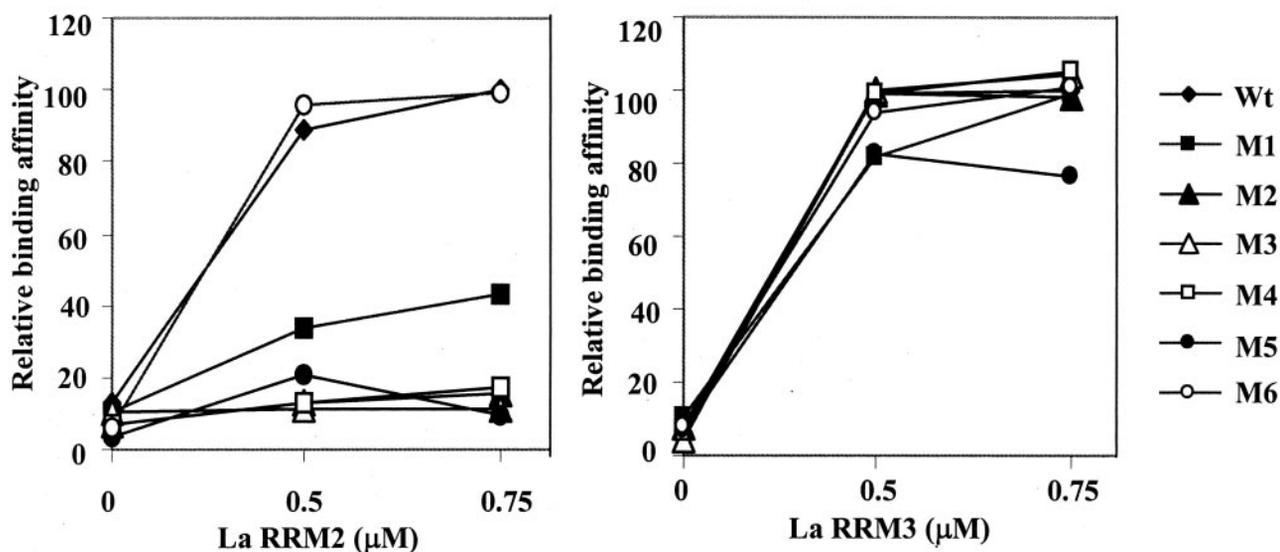


FIG. 1. Effect of mutations in SL IV on binding to La-RRM2 and La-RRM3 proteins. A, schematic representation of the predicted secondary structure of HCV IRES indicating the stem-loops (adapted from Brown *et al.* (6)). The positions of the mutations are highlighted in the magnified SL IV region. B, filter binding assay to study the binding of HCV wild-type and mutant IRES with La-RRM2 and RRM3. The  $^{32}\text{P}$ -labeled wild-type or mutant HCV IRES RNAs, M1–M6 (as indicated by the symbols to the right of the panel), were bound to increasing concentrations of either La-RRM2 or La-RRM3 proteins. The amount of bound RNA was determined by binding to the nitrocellulose filters. The percentage of bound RNA was plotted against the protein concentration (micromolar).

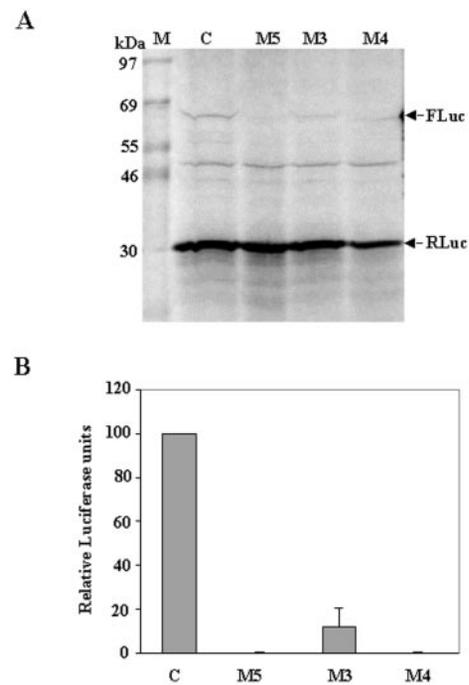
also generated another mutant M5 where AUG was substituted with AAG, to use as a control in our studies.

To study the effect of mutations in the SL IV region of the

HCV IRES RNA on binding to La-RRM2 protein, filter binding assays were performed. The  $^{32}\text{P}$ -labeled wild-type or mutant HCV IRES RNAs were incubated with the increasing concen-

tration of purified recombinant La-RRM2 protein (0.5 and 0.75  $\mu\text{M}$ ). The amount of bound RNA was determined by binding to nitrocellulose filters followed by plotting to obtain the saturation curve. RRM2 showed efficient binding with the wild-type HCV IRES RNA. All the four mutants with mutations in GCAC sequence (M1, M2, M3, and M4) showed a significant reduction in their ability to bind to RRM2 to different extents (between 70 and 90%). However, the mutants M2, M3, and M4 showed much more drastic effect on the binding ability compared with the mutant M1. Interestingly, the single mutant M6 (C325U) did not show significant reduction in binding to RRM2 indicating that this mutation alone may not contribute to the reduction in binding ability, but along with the mutations at GCAC motif, may result in pronounced effect. As expected, the M5 mutant, where initiator AUG was mutated, showed a drastic decrease in binding to La-RRM2. As a control, when the same experiment was performed with La RRM3 protein, it was observed that the binding of the HCV IRES was not affected due to the mutations in stem-loop IV. The result was consistent with our earlier observation that RRM2 indeed binds at the GCAC near the initiator AUG and RRM3 binds to HCV IRES at regions other than this RRM2 binding site. When RNA toe-printing assay was performed using the mutant M4 RNA and La protein, no toe-prints were detected corresponding to the GCAC, as obtained with the wild-type HCV IRES RNA (data not shown). The result further confirmed the observations made from the filter-binding experiments.

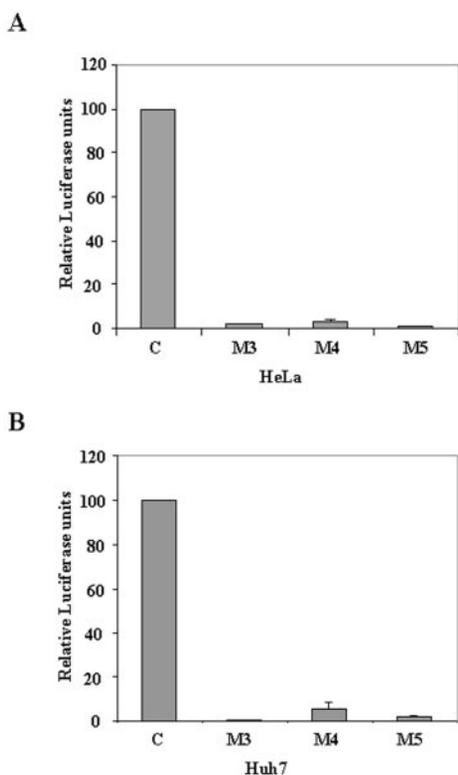
**Mutations in Stem Loop IV Region That Affect the Binding to La-RRM2 Results in Reduced IRES-mediated Translation *in Vitro***—Human La protein interaction with the HCV IRES has been shown to be critical for mediating internal initiation of translation. Previously we have shown that La protein interacts with the HCV IRES near the initiator AUG through RRM2. To study the effect of mutations in HCV IRES that abrogate RRM2 interaction, an *in vitro* translation experiment has been performed. Because the mutants M3 and M4 showed almost total abrogation of the ability to interact with RRM2, these mutants were used for further studies. Additionally as a negative control AUG mutant, M5 was included in the experiment. To compare the translation efficiencies of mutants to that of wild-type, *in vitro* transcribed capped bicistronic RNAs containing two reporter genes, *Renilla* luciferase (RLuc) and firefly luciferase (FLuc) flanked by either wild-type or mutant HCV IRES were assayed for translation activity in rabbit reticulocyte lysate (RRL). In the bicistronic RNAs, translation of first cistron, RLuc occurs in cap-dependent manner while FLuc translation is mediated by the HCV IRES. To avoid alternative initiation site, the initiator AUG was deleted from the second cistron, FLuc, such that initiation takes place at the initiator AUG located within the SL IV of the HCV RNAs. Half of the reaction mixtures were electrophoresed on SDS-10% PAGE and analyzed by phosphorimaging. Lane C represents the translation from HCV wild-type bicistronic RNA, showing the efficient translation of both the reporter genes, RLuc and FLuc. Lane M5 represents AUG mutant bicistronic RNA, used as a negative control, where the translation of second cistron FLuc was totally abrogated. Lanes M3 and M4 represent the mutant bicistronic RNA where the translation of FLuc was found to be highly impaired (Fig. 2A). Alternatively, to quantitate the translation products, the luciferase activities were measured from the reaction mixtures using dual luciferase assay system (Promega). The ratio of FLuc to RLuc activities was calculated in case of each bicistronic RNA for normalization. The translation activities of mutant RNAs were plotted as a percentage to that of wild-type RNA. Both the mutants, M3 and M4, showed pronounced effect on the HCV IRES-mediated translation (Fig. 2B). However, mutant M4 showed



**FIG. 2. Effect of mutations in SL IV in HCV IRES mediated translation *in vitro*.** A, 1  $\mu\text{g}$  of wild-type and mutant capped HCV bicistronic constructs were translated in rabbit reticulocyte lysate (RRL), and the products were analyzed on SDS-10% polyacrylamide gel followed by phosphorimaging. *Renilla* luciferase (RLuc) and firefly luciferase (FLuc) products are indicated by arrows. Lane M represents the mobility of the  $^{14}\text{C}$  protein molecular weight marker. The corresponding molecular masses are indicated to the left of the panel. Wild-type (C) and mutant (M3–M5) bicistronic RNAs used in the translation reactions are indicated at the top of the lanes. B, both the reporter genes, *Renilla* luciferase (RLuc) and firefly luciferase (FLuc) activities were measured by Dual-Luciferase assay (Promega). The ratio of FLuc to RLuc activity representing the efficiency of wild-type bicistronic RNA (C) and the mutants (M3–M5) were plotted. The relative FLuc activities of the mutants were represented as a percentage of the control reaction (expressed as 100%). Results represent an average of three independent experiments.

marginally more inhibition as compared with M3. The control AUG mutant did not show any FLuc translation as expected. Additionally, when the *in vitro* translation experiments were repeated with mutant RNAs M1 and M2, significant translation inhibition was observed (data not shown). The results suggest that the binding of La protein through the RRM2 in the SL IV region of HCV IRES might play a critical role in internal initiation of translation.

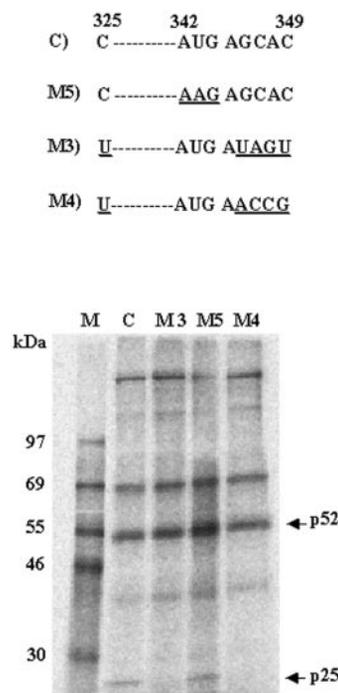
**Mutations in the HCV Stem-loop IV Had a Drastic Effect in Mediating Internal Initiation of Translation *in Vivo***—The results of *in vitro* translation in RRL indicated that the mutations in SL IV reduced the HCV IRES activity. To verify the results of the *in vivo* conditions, the bicistronic construct DNAs were transiently transfected into mammalian monolayer cells. Because it was earlier shown that HeLa and Huh 7 (liver-derived) cell lines support efficient translation by HCV IRES, both these cell lines were used for comparing the translation efficiency of the mutants with the wild-type HCV IRES. 60–70% confluent monolayer cells were transfected with an equal amount of wild-type or mutant bicistronic constructs using Tfx 20 transfection reagent (Promega). 36 h post-transfection, the cells were lysed, and the extracts were used to measure luciferase activities using the Dual-Luciferase assay system (Promega). The transfection efficiency was normalized by taking the FLuc/RLuc ratio for each bicistronic construct. The AUG mutant bicistronic construct, M5, did not show any FLuc activity due to the lack of functional initiation codon for the



**FIG. 3. Translational abilities of SL IV mutants in transient transfection analysis.** *A* and *B*, 2  $\mu$ g of plasmid DNAs containing wild-type or mutant HCV bicistronic constructs were transiently transfected into HeLa (*Panel A*) or Huh 7 (*Panel B*) monolayer cells. 36 h post-transfection, the expression of FLuc and RLuc from the constructs was assayed by Dual-Luciferase assay (Promega). The ratio of FLuc to RLuc activity representing the efficiency of wild-type bicistronic construct (*C*) and the mutants (*M3–M5*) were plotted. The relative FLuc activities of the mutants were represented as a percentage of the control reaction (expressed as 100%). The results represent an average of three independent experiments.

second cistron. Consistent with the *in vitro* results, in the case of mutants M3 and M4, translation efficiency was reduced up to 90% as compared with the wild-type IRES (Fig. 3). Similar results were obtained in the case of both the cell types, HeLa (Fig. 3*A*) and Huh 7 (Fig. 3*B*), ruling out any cell-type-specific effect in the IRES activity. The results suggest that the mutations at the GCAC motif in the SL IV reduced the efficiency of the HCV IRES-mediated translation *in vivo*.

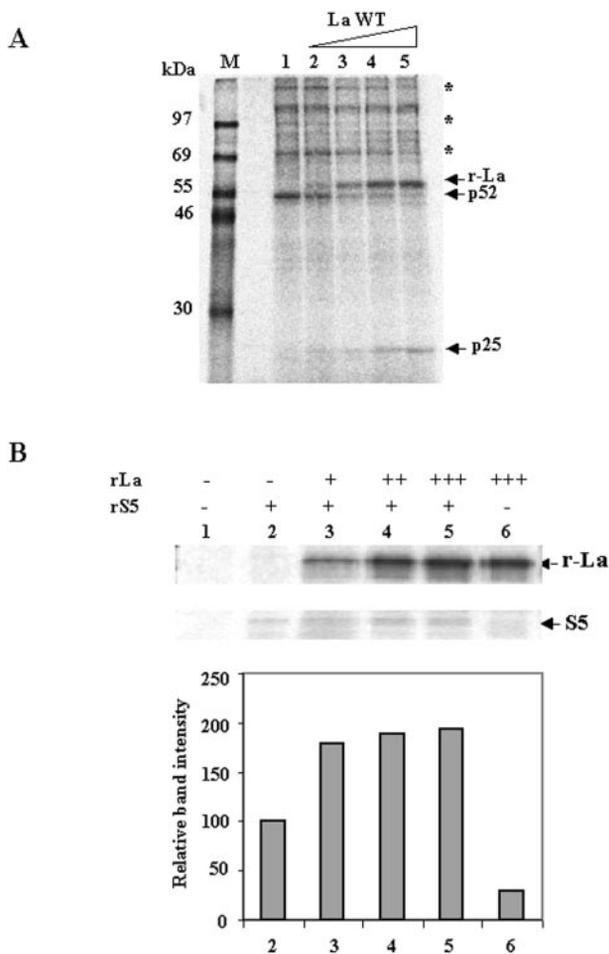
**Mutations in the GCAC Motif in Stem-loop IV Affect Cellular Protein Binding to HCV IRES**—HCV IRES is known to bind several cellular proteins, including canonical initiation factors and non-canonical *trans*-acting factors. These include polypyrimidine tract-binding protein (PTB), human La autoantigen (La), poly(rC)-binding protein (PCBP), heterogenous nuclear ribonucleoprotein L, ribosomal protein factors, S5, and others. To investigate if cellular protein binding is affected due to the mutations in SL IV, UV cross-linking experiments were performed with HeLa S10 cytoplasmic extract using wild-type and mutant HCV IRES RNAs. The  $^{32}$ P-labeled RNAs were incubated with HeLa S10 extract in binding buffer and cross-linked by UV irradiation. After digesting the unbound RNA with RNase A, the reactions were electrophoresed on SDS-polyacrylamide gels and analyzed by phosphorimaging. Although the overall protein binding profiles between wild-type and mutant HCV IRES RNA were very similar, the most notable difference was noticed in the binding of a 25-kDa protein (p25). Mutations in SL IV totally abrogated the binding of p25 to mutants M3 and M4 (Fig. 4). Similarly, when mutants M1 and M2 were



**FIG. 4. Effect of mutations in HCV SL IV on cellular protein binding.** The  $^{32}$ P-labeled wild-type and mutant HCV IRES RNAs (as described above the panel) were UV cross-linked to HeLa S10 cytoplasmic extract, digested with RNase A, and resolved by SDS-10% PAGE followed by phosphorimaging. Positions of p25 and p52 are indicated. Lane *M* represents  $^{14}$ C protein molecular weight marker. The corresponding molecular masses are indicated to the left of the panel.

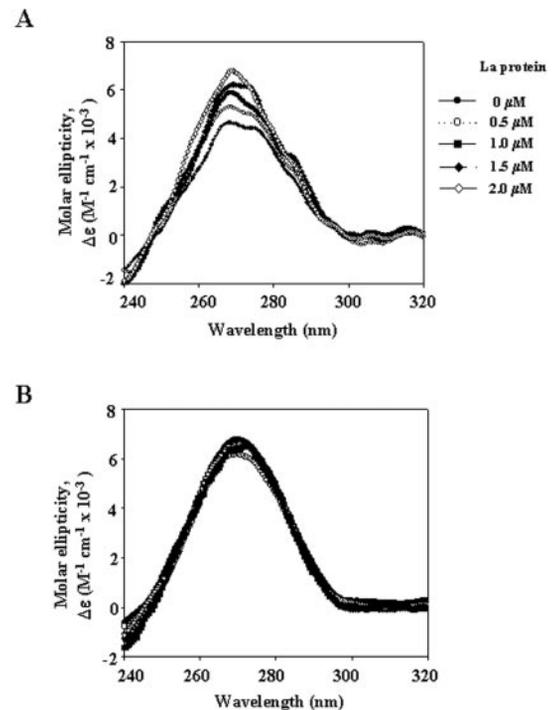
used, binding to p25 was affected, whereas mutant M6 (C325U) did not affect the p25 binding (data not shown).

**La Protein Facilitates the Binding of Ribosomal Protein S5 to HCV IRES RNA**—Because we have observed that binding of cellular protein p25 was altered with the mutant RNAs, it was interesting to investigate whether La protein binding to HCV IRES RNA somehow facilitates interaction of p25 protein. To address this, the wild-type HCV RNA was first incubated with increasing amounts of purified recombinant La protein (50, 100, 200, and 300 ng) followed by incubation with HeLa S10 extract. Interestingly, it was observed that, with the addition of recombinant exogenous La protein, there was dose-dependent increase in the binding of a 25-kDa protein (p25). Recombinant La protein also competed with the endogenous La (p52) for the binding as apparent from the dose-dependent decrease in p52 band intensity and increase in the band corresponding to the recombinant La protein. Some other protein binding also seemed to be affected along with the p52 binding suggesting that La protein might influence interaction of other cellular proteins as well (Fig. 5*A*). Earlier, the 25-kDa protein (p25) that interacts with HCV IRES has been identified as ribosomal protein S5 (rpS5). The S5 protein binding to HCV IRES has been shown to be crucial for the 40 S interaction. To investigate whether La protein assists the binding of S5 to HCV IRES, recombinant purified S5 protein was bound to HCV IRES in the absence or presence of increasing concentrations of recombinant La protein (100, 200, and 300 ng, respectively). Exogenous addition of recombinant La protein was found to influence the binding of S5 to HCV IRES significantly as apparent from the dose-dependent increase in band intensity of recombinant S5 (Fig. 5*B*). Thus the observations strongly suggest that the binding of La protein is important for the binding of S5 protein, which in turn might influence the ribosome assembly for efficient translation initiation activity of the HCV IRES RNA.



**FIG. 5. La protein binding helps in the interaction of ribosomal protein S5 to HCV IRES RNA.** *A*,  $^{32}$ P-labeled HCV IRES RNA was UV cross-linked to HeLa S10 extract in the absence (*lane 1*) or presence of increasing concentrations of recombinant purified La full-length protein (*lanes 2–5*). The reactions were treated with RNase A, resolved on SDS-10% PAGE, and analyzed by phosphorimaging. *Lane M* shows  $^{14}$ C protein molecular weight marker. The corresponding molecular masses are indicated to the left of the panel. Positions of p25, p52, and recombinant-La (*r-La*) are indicated to the right. Other protein bands, which showed altered intensities upon addition of La protein, are indicated with asterisks. *B*, wild-type  $^{32}$ P-labeled HCV IRES RNA was UV cross-linked to recombinant purified S5 protein in the absence (*lane 2*) or presence of increasing concentrations of recombinant purified La protein (*lanes 3–5*). *Lane 6* shows binding of highest concentration of La protein to HCV IRES in absence of S5. *Lane 1* is no protein control. The bands corresponding to La and S5 protein obtained from the same gel are represented separately for clarity. The mean band intensity of the S5 band corresponding to each lane (as indicated) was quantitated by phosphorimaging analysis and graphically represented below the panel.

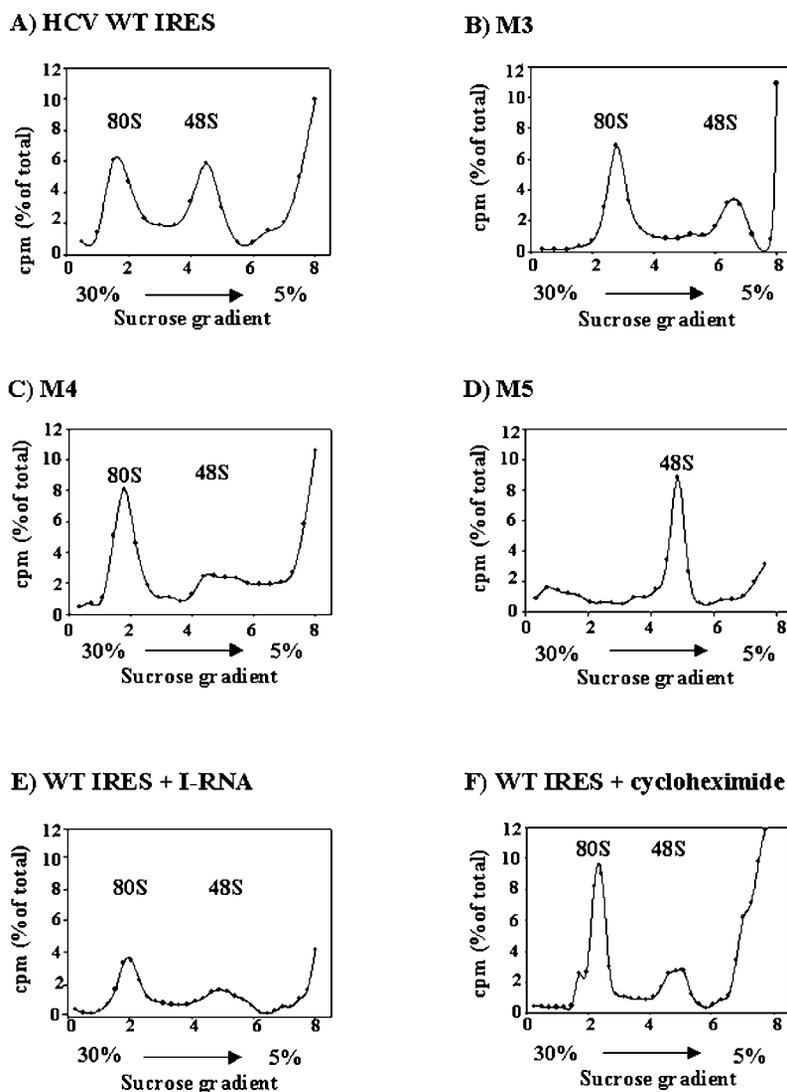
**Binding of La Protein to Stem-loop IV of HCV IRES Leads to Conformational Alterations within the RNA**—Our results suggested that perhaps interaction of La protein through RRM2 at the GCAC motif near initiator AUG facilitates 40 S ribosomal binding through S5. Thus, to further investigate if interaction of La protein at this region brings about any conformational alterations within the HCV IRES, which would favor the 48 S ribosome assembly, the CD spectra of HCV RNA was analyzed in the absence and presence of purified recombinant La protein. CD spectra were obtained in the 240- to 320-nm range at 20 °C with stem-loop IV of HCV wild-type RNA (250 nM) in 0.5 ml of RNA-binding buffer. The CD spectrum of the SL IV RNA was characterized by a signature band centered at 268 nm that increases in magnitude upon incubation with increasing concentration of purified recombinant La protein (0.5–2  $\mu$ M). The



**FIG. 6. Analysis of CD spectra of wild-type and mutant HCV SL IV RNA in absence and presence of increasing concentration of La protein.** *A* and *B*, CD spectra were obtained in 0.5 ml of RNA-binding buffer between 240- and 320-nm wavelength range at 20 °C with stem-loop IV of HCVWT (*panel A*) or stem-loop IV of mutant RNA (corresponding to M4) (*panel B*), in the absence or presence of increasing concentration of purified recombinant La protein (0.5–2  $\mu$ M). The molar ellipticity values were normalized for the contribution of the La protein at each concentration. Symbols in each curve corresponding to the concentration of La protein used are indicated to the right of the panels.

molar ellipticity values were minimal for the La protein at wavelengths above 240 nm, however, the values were still normalized for the contribution of the La protein at each concentration. The molar ellipticity value of SL IV RNA increased significantly (Fig. 6*A*) upon incubation with La protein indicating that the protein binding was inducing certain conformational alterations within the RNA. When a similar experiment was performed using mutant RNA, corresponding to SL IV of M4, no significant change was observed in the CD spectrum of the RNA upon incubation with the La protein (Fig. 6*B*). However, UV cross-linking studies revealed that both wild-type SL IV and mutant SL IV do bind to La protein (data not shown), thus ruling out the possibility that the altered molar ellipticity values were simply due to RNA-protein interaction. The results indicated that the effect of La protein was specific for the wild-type HCV SL IV RNA and not for the mutant SL IV RNA.

**Mutations in the Stem-loop IV Affect the Ribosomal Assembly on HCV IRES RNA**—UV cross-linking experiments showed that the mutations at the GCAC in HCV IRES RNAs affected the interaction with S5 ribosomal protein. Also, from our study, it appears that La binding near the GCAC at the SL IV region results in conformational alterations in the RNA, which might facilitate the ribosome assembly at the initiation site. Thus it was interesting to investigate if the lack of interactions of La-RRM2 and S5 proteins at this region could affect the functional ribosome complex formation near the initiator AUG of HCV IRES RNA. For the purpose, sucrose gradient centrifugation experiments were performed, followed by the analysis of 48 S and 80 S ribosomal peaks. The  $^{32}$ P-labeled wild-type and mutant HCV IRES RNAs were incubated in translation reactions containing RRL and amino acid mixture and loaded onto 5–30% sucrose gradient and ultracentrifuged for 3 h at 30,000



**FIG. 7. Sucrose gradient centrifugation analysis of the ribosomal assembly on the wild-type and mutant HCV IRES RNAs.** Sucrose gradient sedimentation profiles of  $^{32}\text{P}$ -labeled wild-type (panel A) or mutant (panels B–D) HCV IRES RNAs after incubated in RRL and separated on 5–30% sucrose gradient. The fractions (5 drops) were manually collected from the bottom of the tube, and scintillation counts were measured. The counts per minute of each fraction, shown as percentage of the total counts added to the reaction ( $\sim 2 \times 10^5$ cpm) and were plotted against the volume of the gradient solution (0–8 ml). The ribosomal peaks corresponding to 48 S and 80 S are indicated. Panel E represents sedimentation profile of wild-type HCV IRES RNA incubated in RRL in the presence of 750-fold excess of yeast inhibitor RNA (I-RNA), and panel F represents the profile in the presence of cycloheximide (500  $\mu\text{g/ml}$ ).

rpm. The fractions were collected from the bottom of the tube, and scintillation counts were measured. The percentage of counts in each fraction was plotted against the fraction volume of the gradient. When wild-type HCV IRES RNA was used, two peaks were observed in equilibrium, corresponding to 80 S and 48 S ribosomes associated with the labeled HCV RNA (Fig. 7A). When AUG mutant RNA (M5) was used, the peaks corresponding to 80 S ribosomes were not observed while the 48 S ribosomes were found to be associated with the RNA indicating that functional initiation AUG codon is essential for association of 60 S ribosomal subunit in the 80 S complex (Fig. 7D). Addition of cycloheximide resulted in the increase in 80 S peak due to the trapping of 80 S ribosomal complexes on the RNA (Fig. 7F). When the experiments were performed with either M3 or M4 RNAs, we observed a marginal increase in 80 S peak, whereas the 48 S ribosome peak was significantly reduced (Fig. 7, B and C). To verify if this effect was due to altered binding of La protein to SL IV, we have taken help of an inhibitor RNA (I-RNA) that was earlier shown to sequester La protein from translation reactions and inhibit HCV IRES-mediated translation. When an excess of I-RNA (750-fold) was added to the translation reactions using the wild-type HCV RNA, both the 48 S and 80 S peaks were reduced significantly (Fig. 7E).

Taken together, the results strongly suggest that the mutations at the SL IV region affect the binding of La-RRM2 at the GCAC near the initiator AUG, as a consequence of which formation of functional ribosomal initiation complex is se-

verely affected and thus resulting in reduced HCV IRES-mediated translation.

#### DISCUSSION

The ribosome assembly at the Hepatitis C virus IRES is known to require minimal number of eukaryotic initiation factors. HCV IRES has been shown to be capable of binding directly to purified small ribosomal subunit (40 S) with the help of the ribosomal protein S5, followed by correct positioning on to the initiator AUG (14, 18). It is possible that in the absence of any canonical initiation factors, some additional cellular *trans*-acting factors might also be involved in the formation of functional initiation complex on the HCV IRES RNA.

Although, it has been known for a while that human La protein binds to HCV IRES and helps in stimulation of HCV IRES-mediated translation, the exact mechanism is yet to be elucidated. Earlier we have demonstrated that La protein might bind to the GCAC sequence motif present near the initiator AUG through its RRM2. It has been speculated that La protein might mediate the unwinding of SL IV region in a way that would facilitate the interaction of the 40 S ribosomal subunit with the HCV IRES RNA (7). In this study the results strongly support the hypothesis and demonstrate that indeed La protein binding near the initiator AUG facilitates the interactions with p25 (S5) and the 48 S ribosome assembly and influence the functional initiation complex formation on the HCV IRES RNA.

We have observed a direct correlation between the reduced interaction of mutant RNAs with the La-RRM2 and their ability to mediate efficient internal initiation. The fact that mutant M5, where AUG has been mutated, failed to bind RRM2 is consistent with the earlier observation that the presence of initiator AUG is important for La protein binding at the SL IV region. Substantial decrease in binding of mutants M1 and M2 to La-RRM2 suggest that the GCAC sequences at SL IV stem region might be important even in the presence of initiator AUG. We ensured that the GCAC mutations are associated with compensatory mutations in the other strand to maintain the stem-structure within the SL IV region. Interestingly, mutations upstream of this region such as C325U (M6 RNA) did not affect the RRM2 interaction, although it might affect the translation because of the disruption of the pseudoknot structure. In fact, it has been demonstrated that the maintenance of this structural element is critical for internal initiation of translation (39). La-RRM3 interaction was not affected with any of these mutants and supports our earlier observation that La-RRM3 is likely to bind regions other than GCAC at the SL IV of the HCV IRES RNA.

We have observed that the mutations also alter the binding of certain cellular proteins to HCV IRES, whose binding may have been influenced by the binding of La protein to SL IV region. The effect on the cellular La protein interaction with mutant RNAs was not apparent in the UV cross-linking experiment; this could be due to the fact that the other La binding sites were not altered. In fact, filter binding experiments demonstrated that the mutations in SL IV resulted in a drastic decrease in the binding of La-RRM2 to the HCV IRES, whereas the La-RRM3 binding was unaffected. La protein might assist the binding of other essential cellular factors to HCV RNA either directly through protein-protein interactions or by mediating structural alterations of the RNA, which would facilitate the interaction with other *trans*-acting factors. One of the proteins whose binding was drastically affected was found to be p25 (S5). Interestingly, earlier it has been demonstrated that the CAC triplet within the stem of domain IV region is protected in foot-printing experiments with HCV IRES and 40 S complexes (40), which overlaps with the La-RRM2 binding site as shown in this study. Hence, it is likely that La protein binding to SL IV may have a role in positioning the S5 protein onto HCV IRES. This is further confirmed by the observations that mutations in SL IV, which reduced the binding to La-RRM2 failed to alter the conformations of the IRES RNA, which affected the interactions with S5 and thereby the formation of 48 S ribosome complexes. The possibility that La protein might help in S5 interaction with HCV IRES RNA was further supported by the observation that addition of increasing concentration of recombinant La protein showed dose-dependent increase of p25/S5 protein interaction with HCV IRES. However, the effect was more pronounced in the presence of other cellular proteins in HeLa S10. So, we do not rule out the possibility that the other cellular proteins along with La protein might also be involved in facilitating the S5 interaction with IRES.

La protein has been shown to have double-stranded RNA unwinding activity (32). It is possible that La might help in unwinding the stem structure that immediately follows the initiator AUG and thus facilitate the advancement of initiation complex. Earlier it was demonstrated that glyceraldehyde-3-phosphate dehydrogenase, an IRES *trans*-acting factor, mediates conformational alterations of hepatitis A virus IRES RNA (24). Significant increase in the CD spectra of HCV stem-loop IV RNA in the presence of La protein was observed suggesting that RNA is undergoing some conformational alterations upon binding to La

protein. However, it is not clear at this stage if the alterations in the conformation are due to the secondary or tertiary level of interactions. It is also possible that RNA unwinding of stem-loop IV facilitates the movement of initiation complex. In fact, it has been shown earlier that La protein interacts with DDX15/hPrp43, which is a putative DEAH-box RNA helicase (41). Furthermore, HCV RNA toe-printing analysis in the presence of increasing concentrations of La protein revealed a decrease in certain toe-prints corresponding to the RNA structure, which suggests that La protein might help in melting some secondary structures and facilitate the extension of the primer by the reverse transcriptase (Ref. 36 and data not shown).

Analysis of ribosome assembly on the mutant RNAs (M3 and M4), indicates that the 40 S interaction is affected. This could be due to reduced binding of the RNA with p25 as a consequence of reduced La-RRM2 interaction at this site. The 80 S peak was found to be marginally increased in the case of mutant M3 and M4 RNAs. It appears that the initiation complex was trapped, similar to what was observed in the presence of cycloheximide. In contrast, addition of I-RNA resulted in much more drastic effect in both the 48 S and the 80 S peaks. I-RNA is known to sequester La protein and inhibit interaction with all the binding sites on the HCV IRES, whereas M3 and M4 mutations affect only La-RRM2 binding to the SL IV RNA but the interaction with La-RRM3 remained unaltered.

Taken together, the study reveals the possible role of La protein in unwinding of HCV IRES RNA structure to facilitate the binding of 40 S ribosomal subunit through S5 ribosomal protein and help in the formation of the functional initiation complex to mediate efficient internal initiation of translation. Future studies in an *in vitro* reconstituted system using isolated components of the initiation complex will unfold the precise role of La protein in HCV IRES-mediated translation.

*Acknowledgments*—We thank Dr. Akio Nomoto and Dr. Tsukiyama-Kohara for the HCV 1b encoding plasmid pCV, and Dr. Jack Keene for the pET-La construct. We also thank Dr. Shuetsu Fukushima for the pQE30-S5 plasmid. Help from members of our laboratory is highly appreciated.

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