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Role of La autoantigen and polypyrimidine tract-binding protein in HCV replication

Angela M. Domitrovich[a,1], Kevin W. Diebel[a,1], Naushad Ali[b,1], Shameema Sarker[a], Aleem Siddiqui[a,*]

[a]*Program in Molecular Biology, Department of Microbiology, B172, University of Colorado Health Sciences Center, 4200 E. 9th Avenue, Denver, CO 80262, USA*

[b]*Avidity LLC, Eleanor Roosevelt Institute, 1899 Gaylord Street, Denver, CO 80206, USA*

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Abstract

To determine if the cellular factors La autoantigen (La) and polypyrimidine tract-binding protein (PTB) are required for hepatitis C virus (HCV) replication, we used siRNAs to silence these factors and then monitored their effect on HCV replication using quantitative RT-PCR. In addition, we determined the influence of PTB on the activity of the 3′ noncoding region (NCR) of HCV and investigated its interaction with the components of the HCV replicase complex. We found that La is essential for efficient HCV replication while PTB appears to partially repress replication. PTB does, however, block the binding of HCV RNA-dependent RNA polymerase (RdRp, NS5B) to the 3′NCR. Indirect immunofluorescence microscopy showed co-localization of cytoplasmic PTB with the HCV RdRp in hepatoma cells (Huh-7) expressing HCV proteins, while in vitro translation of viral proteins from the HCV replicon revealed the interaction of PTB isoforms with NS5B polymerase and NS3.

Keywords: HCV; La; PTB; HCV replication; HCV translation; NS5B; NS3

Introduction

Hepatitis C virus (HCV) infection is a major world health problem with a worldwide carrier rate estimated at 300 million people (Manns et al., 2001). HCV infection can often lead to chronic hepatitis in up to 60–80% of infected adults (Giannini and Brechot, 2003) and can then progress to liver cirrhosis and hepatocellular carcinoma (Di Biscegli, 1997). HCV is composed of a 9.6-kb positive-sense single-stranded RNA genome (Bartenschlager and Lohmann, 2000) and encodes a polyprotein precursor of approximately 3000 amino acids. The HCV polyprotein is synthesized on ribosomes in the rough endoplasmic reticulum (ER) and is cleaved co- and post-translationally by viral and host proteases to yield three structural proteins (core, E1, and E2) and six nonstructural proteins (NS2 to NS5A/B) (Bartenschlager and Lohmann, 2000; Dubuisson et al., 2002). Many of the nonstructural proteins have been found to be essential for productive viral replication (Kolykhalov et al., 2000). The 5′ noncoding region (5′-NCR) and the 3′-NCR of HCV are highly conserved sequences that contain elements that are directly involved in RNA replication (Luo et al., 2003). The HCV 5′-NCR has been shown to function as an internal ribosome entry site (IRES) permitting cap-independent translation (Tsukiyama-Kohara et al., 1992; Wang et al., 1993). The 3′-NCR has a unique non-poly (A) tail and consists of a short variable region, a U/(UC) motif, and a terminal 3′X tail composed of highly conserved stem-loop structures (Kolykhalov et al., 1996).

During the initiation of HCV replication, an RNP complex is formed at the HCV 3′-NCR of the viral genome.

* Corresponding author. Fax: +1 303 315 8330.
E-mail address: aleem.siddiqui@uchsc.edu (A. Siddiqui).

[1] These authors contributed equally to this work.

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All of the nonstructural proteins, except NS2, are required for efficient HCV replication (Blight et al., 2000; Lohmann et al., 1999). Within the replication initiation complex there is an RNA-dependent RNA polymerase (NS5B) that serves as a catalytic subunit that synthesizes minus-strand RNA. The newly synthesized negative-strand RNA then serves as a template for the production of the plus-strand viral RNA.

Changes in intracellular events resulting from HCV replication are poorly understood. Studying the HCV replication processes in the context of a full-length genome has been difficult since there are no current tissue culture or practical animal model systems available for HCV infection. However, the development of a selectable and efficiently replicating HCV subgenomic replicon in a human hepatoma cell line, Huh-7, has provided a suitable model for investigating the mechanisms of viral persistence and pathogenesis in the context of HCV replication (Lohmann et al., 1999). These replicons are bicistronic constructs composed of the HCV IRES (nucleotides 1–377 of the 5′-NCR) directing the translation of the neomycin phosphotransferase (neo) gene; the encephalomyocarditis IRES, which mediates the translation of HCV nonstructural proteins NS3 through NS5; and the 3′NCR.

The entire HCV replication cycle appears to occur in the ER (Dubuisson et al., 2002). However, Shi et al. reported that lipid-rafts derived from intracellular membranes could serve as the site of HCV replication (Shi et al., 2003). This notion is supported by the fact that the majority of the HCV NS proteins localize to the ER and other membranous structures (Bartenschlager and Lohmann, 2000; Dimitrova et al., 2003; Shi et al., 2003). The ER localization of these proteins causes severe morphological changes in the intracellular membranes and induction of ER stress, a factor responsible for activation of transcription factors that alter cellular metabolism (Dubuisson et al., 2002; Gong et al., 2001; Tardif et al., 2002, 2005; Waris et al., 2002). How these metabolic changes affect the process of HCV gene expression, persistent infection, or viral maturation have been the subjects of intense investigations. A significant number of studies in the recent past have focused on the cellular and viral proteins that directly form ribonucleoprotein (RNP) complexes with the 5′ and 3′NCRs (Ali and Siddiqui, 1995, 1997; Ali et al., 2000; Anwar et al., 2000; Banerjee and Dasgupta, 2001; Buratti et al., 1998; Fukushi et al., 1997; Hahm et al., 1998; Ito and Lai, 1999; Kolupaeva et al., 2000). Indeed, a whole host of the cellular proteins have been shown to interact with the viral cis-elements (Ali and Siddiqui, 1995, 1997; Buratti et al., 1998). Chief among them are the polypyrimidine tract-binding protein (PTB, p57 or hnRNP1) and the La antigen, each of which have been shown to be involved in the HCV IRES-mediated translation initiation process (Ali and Siddiqui, 1995, 1997; Ali et al., 2000; Anwar et al., 2000; Gosert et al., 2000).

PTB belongs to an hnRNPI family of RNA binding proteins (Ghetti et al., 1992). It is mainly found not only in the nucleus of cells but also shuttles to the cytoplasm. The major function of PTB in the nucleus is to regulate the splicing of precursor mRNAs. The protein has been shown to facilitate translation directed by the picornavirus IRES elements in the cytoplasm (Gosert et al., 2000). We have previously shown that PTB interacts independently within three separate domains of the HCV 5′-NCR, each of which has a sequence motif that closely resembles the core of the consensus pyrimidine tract (CYYYYCYYYYGG; Y, pyrimidine) (Ali and Siddiqui, 1995), with the optimal binding site for PTB being UCUU within a pyrimidine rich context (Perez et al., 1997). Also, by using a SELEX RNA approach, the binding of PTB to the 5′-NCR was shown to be required for HCV IRES function in vivo (Anwar et al., 2000). In addition to binding to the 5′-NCR, PTB has also been shown to bind to the 3′NCR at both the U/(UC) motif region as well as the 3′ X region (Ito et al., 1998). The functional importance of these binding sites remains unknown.

The La autoantigen is a highly conserved RNA-binding phosphoprotein that is mainly associated with newly synthesized RNA polymerase III transcripts via a binding interaction with their UUU-OH 3′ tails within the nucleus of normal, healthy human cells (Maraia and Intine, 2001). Up to 80% of all La protein within a cell is located in the nucleus, but the location can vary based on different environmental conditions within the cell permitting higher concentrations of La to reside within the cytoplasm of the cell as is the case during HCV transient transfections in tissue culture cells (Izumi et al., 2004; Maraia, 2001). It has been shown previously that La can interact with the HCV genome at the 5′ and 3′NCR regions near the initiator AUG and within the poly U/UC region, respectively (Ali and Siddiqui, 1997; Spangberg et al., 1999). The interaction of La with the 5′NCR has been determined to be important for efficient HCV translation (Ali and Siddiqui, 1997). In addition to its role in HCV translation, La is also associated with other IRES structures and has been found to be critical for their function as well (Maraia and Intine, 2001). Although the translational requirements associated with La and its interaction with the HCV IRES have already been characterized, the functional role of this protein in the context of HCV replication has not been investigated.

In order to determine the effects that both La and PTB have on HCV replication, we set out to silence both genes and then monitor their roles in HCV replication using quantitative RT-PCR. In this study using the subgenomic replicon expression system we have found that La is required for efficient HCV replication, while PTB may act as a repressor of replication. In agreement with previous studies our data show that both La and PTB are needed for HCV translation (Ali et al., 2000; Anwar et al., 2000; Gosert et al., 2000; Zhang et al., 2004). In addition, we demonstrate direct interactions between PTB and HCV NS3 and NS5B proteins, both of which are required for HCV replication. These interactions may regulate the functions of the viral

RNP complexes during translation/replication activities of the HCV RNA genome.

Results

La and PTB mRNA expression levels are diminished in the presence of their corresponding siRNAs

To determine if La and PTB are necessary for efficient HCV replication, we synthesized siRNA specific to La and PTB proteins. Huh-7 cells were transiently transfected with the HCV replicon (BM4–5) and/or siRNA against La or PTB. Total RNA was then extracted from various cells and subjected to quantitative RT-PCR analysis using specific primers and probes for both La and PTB to determine the overall mRNA expression levels of La and PTB. Using this approach we observed a 62% reduction of La mRNA levels at 24 h and a 72% reduction at 48 h post-transfection (Fig. 1A). For PTB there was about a 50% reduction of PTB mRNA levels at 24 h and a 71% reduction at 48 h post-transfection (Fig. 1B). Huh-7 cells that were not transfected with either the HCV replicon RNA, La siRNA, or PTB siRNA did not show any decrease in either La or PTB RNA levels (Figs. 1A and B). In addition, Western blots were done to determine the protein levels of La and PTB during silencing. Huh-7 cells were transfected as above and cellular lysates were prepared as described in Materials and methods. We observed modest reductions in both La and PTB protein

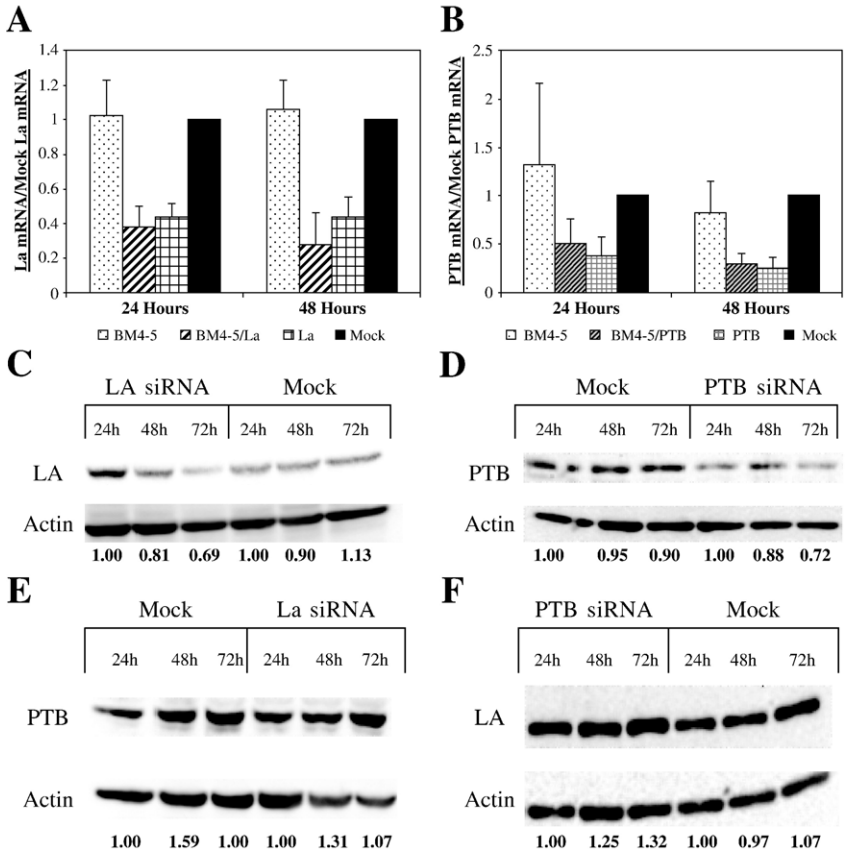

Fig. 1. Effects of silencing on La and PTB mRNAs. (A) Quantitative RT-PCR analysis of La mRNA. Huh-7 cells were transiently transfected with HCV subgenomic replicon RNA (BM4–5) only or along with La siRNA (BM4–5/La) or La siRNA only (La) or neither (mock) as described in Materials and methods. RNA was harvested at 24 or 48 h post-transfection. The amount of La mRNA was determined as described in Materials and methods. The columns and bars represent the averages and standard deviations of three sets of duplicate reactions. *P* values were determined to be the following: BM4–5 24 h 0.89, BM4–5/La 24 h 0.02, La 24 h 0.01, BM4–5 48 h 0.88, BM4–5/La 48 h 0.03, and La 48 h 0.02. Significant *P* values are values less than 0.05. (B) Quantitative RT-PCR analysis of PTB mRNA. Reactions were carried out the same as above except using PTB siRNA during the transfection. The amount of PTB mRNA was determined as described in Materials and methods. The columns and bars represent the averages and standard deviations of three sets of duplicate reactions. *P* values were determine to be the following: BM4–5 24 h 0.57, BM4–5/PTB 24 h 0.08, PTB 24 h 0.03, BM4–5 48 h 0.44, BM4–5/PTB 48 h 0.01, and PTB 48 h 0.01. (C) Western blot analysis of La protein with La siRNA. Huh-7 cells were transfected as above. Total protein was extracted at 24, 48, and 72 h post-transfection and subjected to 10% SDS–PAGE and immunoblotted as described in Materials and methods. The blot was probed with anti-La antibody. The protein bands were then quantified by densitometry analysis from three sets of transfection experiments each normalized to the levels of actin. The remaining amount of protein relative to the 24-h time point is listed. "Mock" samples had no La siRNA. (D) Western blot analysis of PTB protein in the presence of PTB siRNA. (E) Western blot analysis of PTB protein in the presence of La siRNA. (F) Western blot analysis of La protein in the presence of PTB siRNA. All were done as stated above. The average amount La or PTB protein remaining from three data sets relative to the 24-h time points is listed below each gel. Bottom panel in each figure represents the protein loading control in which lysates were immunoblotted with anti-actin antibody.

levels in the presence of corresponding siRNAs (Figs. 1C and D). This modest reduction of La and PTB proteins probably reflects the abundance of these proteins in Huh-7 cells. We next determined that the effects of siRNAs on these proteins were specific. As shown in Figs. 1E and F, in the presence of La siRNA, PTB protein levels were not diminished and, similarly, La protein levels were not reduced in the presence of PTB siRNA.

HCV IRES translation decreases when La or PTB is silenced

Using RNA SELEX technique, we have previously shown that La and PTB are required for HCV IRES function (Anwar et al., 2000). In a recent study, results showing that La is necessary for HCV IRES function were reported using La siRNA (Costa-Mattioli et al., 2004). Here, we have undertaken an siRNA approach to establish the role of La and PTB on HCV translation and replication using the subgenomic replicon expression system. Huh-7 cells were transfected with the HCV subgenomic replicon (BM4–5) and/or La siRNA or PTB siRNA. Cellular lysates were prepared as described in Materials and methods. Western blot analysis was used to monitor neomycin protein levels, which are under the translational control of the HCV IRES. La siRNA caused a 46% decrease in neomycin protein levels at 24 h and a 58% reduction at 48 h (Fig. 2A). For PTB, we found that there was a 50% reduction of neomycin protein levels at 24 h and a 69% and 86% reduction at 48 h and 72 h, respectively (Fig. 2B). As expected, we did not see neomycin protein expression in the Huh-7 cells that were only transfected with La siRNA/PTB siRNA or in the Huh-7 cells that were not transfected with La siRNA/PTB siRNA and BM4–5 RNA since these cells do not contain the neomycin gene. In a previous study by Zhang et al., siRNA against PTB and La in the context of a bicistronic HCV replicon similar to the one used in this paper had no affect on EMCV IRES translation, indicating that the changes in neomycin levels that we detected are a result of decreased HCV IRES activity uncoupled from EMCV IRES translation (Zhang et al., 2004).

La is required for HCV subgenomic replication

Next, we determined the effects of La siRNA on HCV replication. As shown above, the La siRNA is efficient at reducing the amount of La mRNA being produced by the cell. Here we show that in the presence of La siRNA there is a reduction in the HCV subgenomic RNA replication. By using quantitative RT-PCR, we observed a significant reduction of HCV replication activity. Huh-7 cells cotransfected with replicon RNA and La siRNA displayed a 63% and 67% reduction at 24 and 48 h post-transfection, respectively (Fig. 3A). These results indicate that La is required for efficient HCV subgenomic replication.

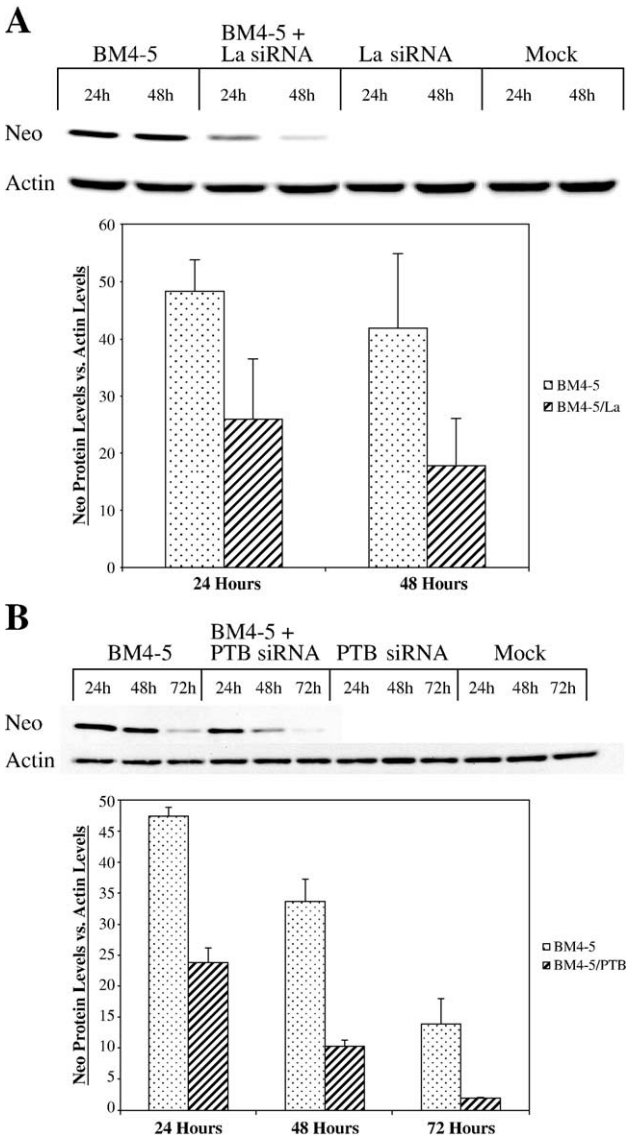

Fig. 2. Effects of La and PTB silencing on HCV IRES translation (A) Western blot analysis of neomycin with La siRNA. Huh-7 cells were transfected. Total protein was extracted at 24 or 48 h post-transfection and then run on a 12% SDS–PAGE gel and immunoblotted. The blot was probed with an anti-neomycin phosphotransferase antibody followed by a monoclonal anti-actin antibody. The columns and bars of the graph represent the averages and standard deviations of the densitometry analysis from three sets of transfection experiments each normalized to the levels of actin. (B) Western blot analysis of neomycin with PTB siRNA at 24, 48, and 72 h post-transfection. The blots and graph were done as stated above and also represent data from three sets of transfection experiments.

PTB effect on HCV replication

Again by using quantitative RT-PCR, we analyzed the effect of PTB siRNA on HCV replication. We found that even though PTB mRNA expression levels were decreased to 50% and 71% at 24 and 48 h, respectively (Fig. 1B), we observed a 45% reduction of HCV replicon RNA at 24 h and only a modest 6% reduction at 48 h (Fig. 3B). We then further investigated the role of PTB using subgenomic

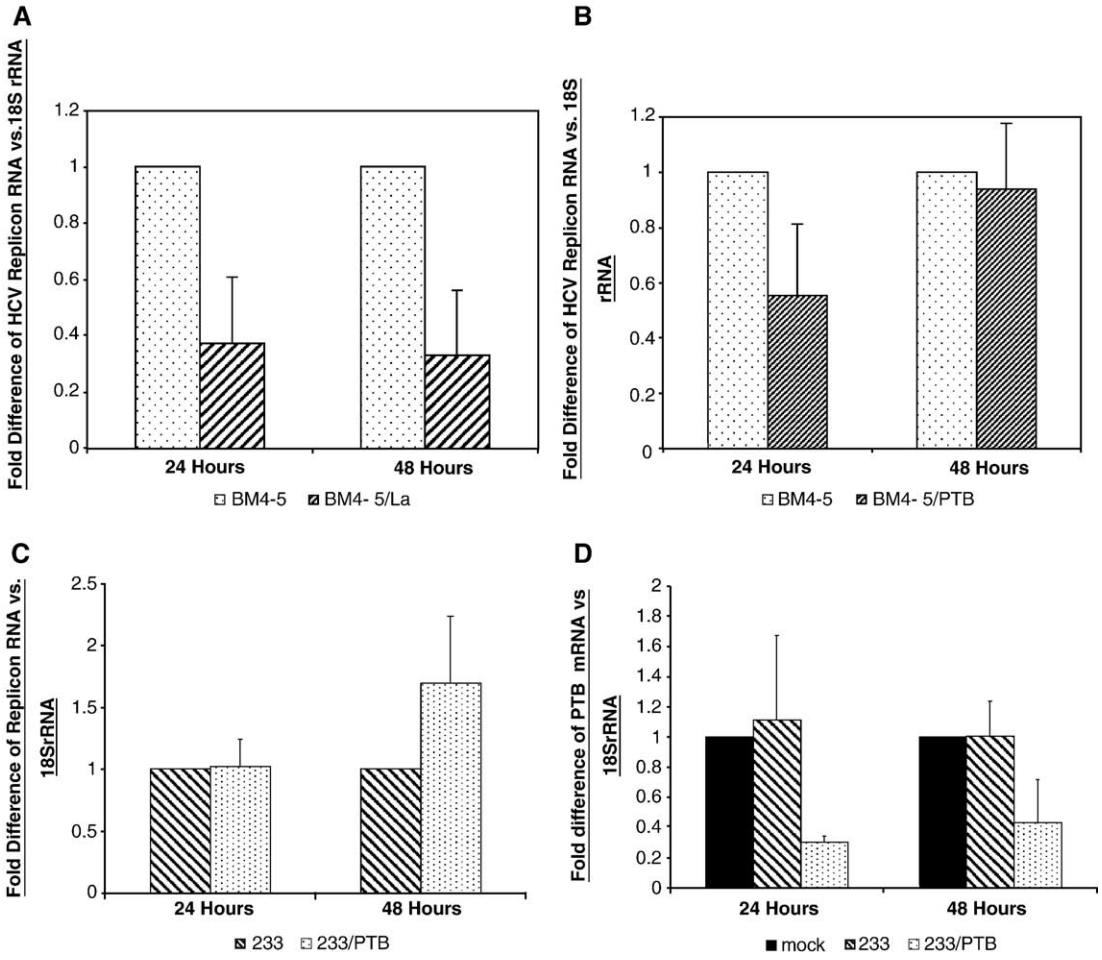

Fig. 3. Effect of La siRNA on BM4–5 replication. (A) Quantitative RT-PCR analysis of subgenomic replicon RNA (BM4–5) in the presence of La siRNA. The reactions were carried out as stated in Fig. 1 except the levels of BM4–5 mRNA and 18S rRNA were detected and the mRNA levels of BM4–5/La were normalized to the levels of BM4–5 mRNA. *P* values were determined to be the following: BM4–5/La 24 h 0.04 and BM45/La 48 h 0.04. (B) Quantitative RT-PCR analysis of subgenomic replicon RNA (BM4–5) in the presence of PTB siRNA. Same as above except using PTB siRNA. *P* values were determined to be the following: BM4–5/PTB 24 h 0.05 and BM4–5/PTB 48 h 0.69. (C) Quantitative RT-PCR analysis of subgenomic replicon RNA (BM4–5) in the presence of PTB siRNA using RNA from the translation defective mutant, 233. Same as above expect RNA from the 233 mutant was used in place of the BM4–5 RNA and the levels of 233/PTB were normalized to the levels of 233 mRNA. *P* values were determined to be the following: 233/PTB 24 h 0.8228 and 233/PTB 48 h 0.0474. (D) Quantitative RT-PCR analysis of PTB mRNA in the presence and absence of 233 RNA. Reactions were carried out as described in Fig. 1. *P* values were determined to be the following: 233 24 h 0.6857, 233 48 h 0.9709, 233/PTB 24 h 0.0001, and 233/PTB 48 h 0.0112.

replicon RNA in which the HCV IRES has been inactivated by mutagenesis. Mutant 233 has mutations in the stem-loop IIIc of the HCV IRES starting at nucleotide 233 and is shown to inhibit the ability for the HCV IRES to perform its role in translation initiation (Fig. 4). When this mutant was transfected into Huh-7 cells we observed an increase of HCV replicon RNA at 48 h in the presence of PTB-specific siRNA (Figs. 3C and D). Together, these results suggest that PTB may not be required for HCV replication.

PTB competes with HCV NS5B on the 3′NCR

We set out to further investigate the role of PTB in HCV replication by focusing on the interactions of PTB with HCV nonstructural proteins. The interaction of NS5B with the 3′NCR is considered a critical step during the replication initiation process. Interestingly, PTB binds cis-elements within the 3′NCR that are located in the vicinity of the NS5B-binding site (Ito et al., 1998; Tsuchihara et al., 1997). Therefore, it is likely that binding of PTB to these regulatory elements may affect the replicase function during the initiation of replication. To address this issue, recombinant NS5B that is capable of replicating full-length HCV RNA in vitro (Oh et al., 1999) was UV cross-linked with a radiolabeled 3′NCR probe and subjected to SDS–PAGE followed by autoradiography. The appearance of a radio-labeled protein band indicates binding of NS5B to the 3′NCR (Fig. 5A, lane 2). In similar reactions, increasing amounts of GST-PTB (lanes 3–6) or GST (lane 7) were added during the UV cross-linking assay. The binding of NS5B to the 3′NCR gradually decreased with increasing amounts of PTB. However, a 10- to 20-fold molar excess of NS5B exhibited a low level of interaction with the 3′NCR

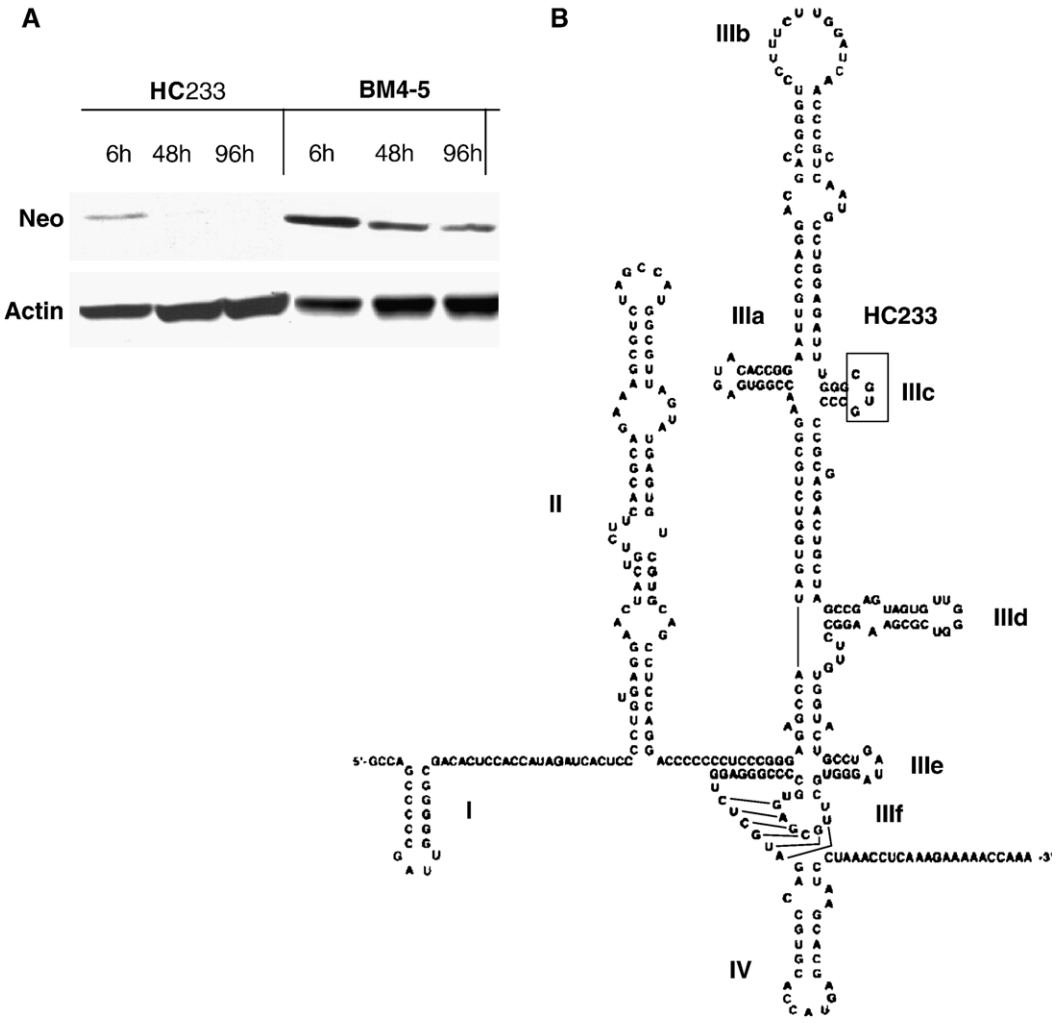

Fig. 4. Effect of 233 mutant on HCV IRES translation. (A) Western blot analysis of neomycin with 233 RNA. Huh-7 cells were transiently transfected via electroporation with the HCV IRES translation defective BM4–5 subgenomic replicon mutant, 233, as described in Materials and methods. Total protein was extracted at 6, 48, and 96 h post-transfection and then subjected to 12% SDS–PAGE gel and immunoblotted with anti-neomycin phosphotransferase antibody followed by a monoclonal anti-actin antibody. (B) HCV IRES with 233 mutation indicated in the boxed area (HC233).

under these conditions (lanes 4–5). The NS5B–3′NCR interaction was modestly affected by the GST control which is not uncommon in GST pull-down assays and does not impact our overall results (lane 7). These results demonstrate that GST-PTB but not GST, interferes with the interaction of NS5B polymerase with the 3′NCR.

In addition to the 3′NCR, HCV polymerase also interacts with the 3′ terminal region of the negative-strand RNA intermediate for plus strand synthesis. This region is complementary to the 5′NCR and is represented here as (−)5′NCR RNA. Using a (−)5′NCR probe in a UV cross-linking assay as described above, we further investigated whether PTB affects the binding of NS5B to this sequence (Fig. 5B). Neither GST-PTB (lanes 3–6) nor GST alone (lane 7) affected the interaction between NS5B and (−)5′NCR. Based on these results, we conclude that the interaction between PTB and 3′NCR may influence the ability of NS5B to bind this region in the replication initiation step. It is also possible that the observed inhibition may result from direct protein–protein interactions between PTB and NS5B as described below.

HCV RNA polymerase co-localizes with cytoplasmic PTB

Huh-7 cells were transiently transfected with a pCMV/729-3010 vector that expresses all the HCV NS proteins (NS2 through NS5B) under the control of the CMV promoter (Hijikata et al., 1993). The transfected cells were subjected to indirect immunofluorescence microscopy using polyclonal anti-PTB and monoclonal anti-NS5B antibodies (Fig. 6A). The transfected cells expressing the NS proteins showed cytoplasmic punctate staining of NS5B (marked as ‘a’) as previously reported (Shi et al., 2003). Although PTB is predominantly found in the nucleus (Back et al., 2002; Perez et al., 1997) (cells indicated as ‘b’, also see PTB staining in Fig. 6B, panel b), the transfected cells (panel a, indicated with an arrow) exhibited cytoplasmic localization of PTB. The expression pattern was similar to that of NS5B

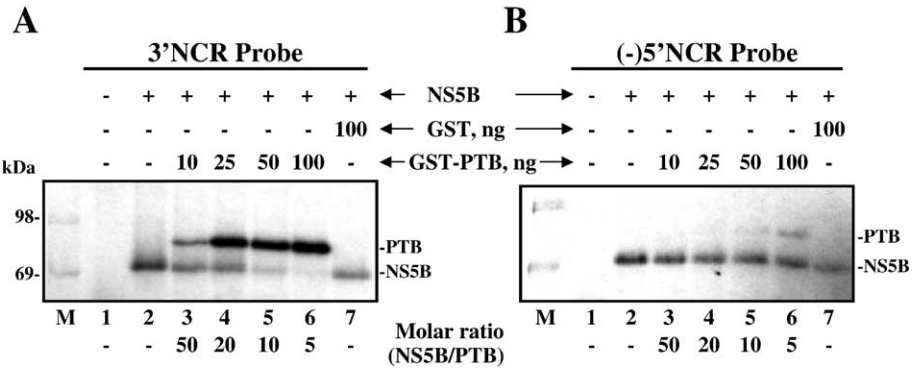

Fig. 5. Competition of PTB with NS5B for the HCV promoters. (A) Competition for 3′NCR. The in vitro synthesized ^{32}P-labeled 3′NCR was UV cross-linked with the bacterially expressed NS5B (lane 2). In similar reactions, indicated amounts of GST-PTB (lanes 3–6) or GST (lane 7) were added. The UV cross-linked products were analyzed by SDS–PAGE and autoradiography. The experiment was repeated three times and a representative result is shown here. The individual PTB and NS5B bands in the mixtures were identified and characterized in separate experiments (not shown). Lane 1, control without protein; lane M, ^{14}C-labeled marker proteins. The molar concentration of PTB and NS5B was calculated in each sample and presented as a molar ratio of NS5B to PTB (on bottom of the figure). (B) Competition for the (−)5′NCR. UV cross-linking was carried out with a (−)5′NCR probe and analyzed as described above.

as evidenced by the co localization of both proteins (panel c). We further explored the possibility of the cytoplasmic localization of PTB in cells that were transiently transfected with the in vitro transcribed HCV subgenomic BB7 and BB7 Pol$^-$ replicon RNAs (Blight et al., 2000). Both RNAs express NS proteins in the cells (Fig. 6B, panel a, indicated with arrow). However, in contrast to BB7, which efficiently replicates in Huh-7 cells, the BB7 Pol$^-$ is replication-defective due to a mutation in the GDD motif of NS5B. In both cases, PTB and NS5B were co-localized in the cytoplasm (panel c). Together, these results demonstrate an interaction between PTB and the NS5B polymerase, and indicate that the catalytic activity of NS5B polymerase may not be required for this interaction.

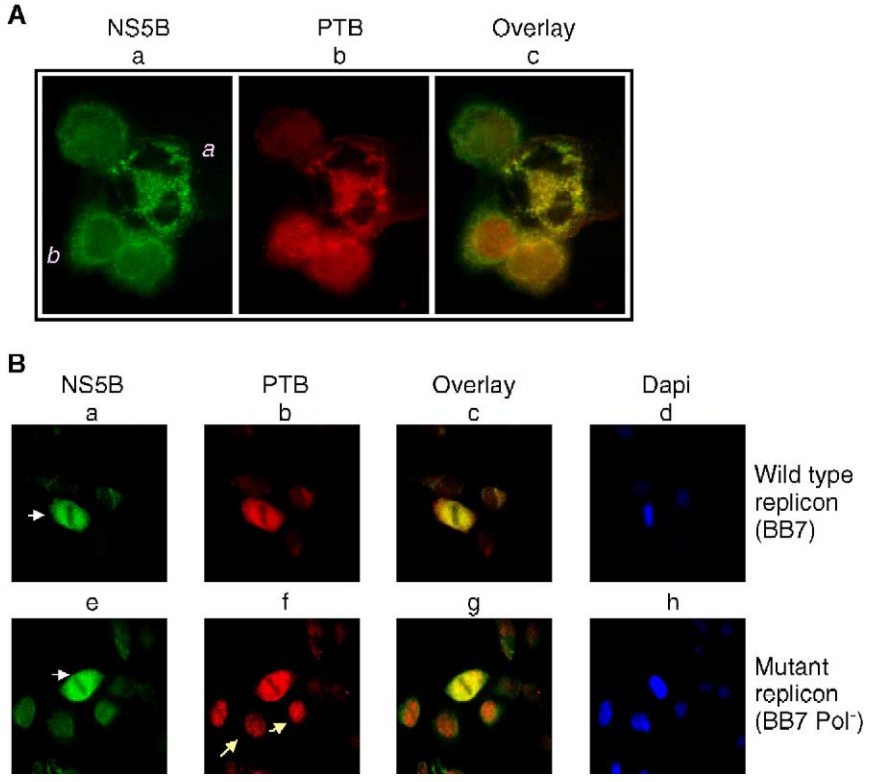

Fig. 6. Indirect immunofluorescence microscopy for the localization of NS5B and PTB in Huh-7 cells expressing HCV proteins. (A) Huh-7 cells transfected with the plasmid pCMV/720–3010 for 48 h and double-stained with monoclonal anti-NS5B antibody and polyclonal anti-PTB antibodies. (a) Cells expressing NS5B, (b) example of untransfected Huh-7 cells. (B) Huh-7 cells were transfected with 5 μg each of in vitro transcribed BB7 (panels a–d) or BB7 Pol$^-$ (panels e–h) RNAs and the cells were stained as above. The immunofluorescence of PTB and NS5B was detected using appropriate antibodies. Arrows (panel a) indicate the Huh-7 cell expressing NS5B. Arrows in panel b indicate staining of PTB in the nucleus of untransfected cells. Dapi, nuclear staining with bis-benzimide.

PTB isoforms interact with the HCV NS5B and NS3 in vitro

To further investigate the interaction between PTB and HCV NS proteins, the HCV NS5B gene was transcribed in vitro from pEMCV-5B and translated in rabbit reticulocyte lysates. The translation of NS5B protein was confirmed by radiolabeling with ^{35}S-methionine and analyzed by SDS–PAGE. The radiolabeled NS5B was allowed to interact with GST, GST-PTB, and GST-La proteins that were immobilized on glutathione-Sepharose beads (Fig. 7A). To one of the reaction mixtures, RNase A was added to show that the template RNA present in the translation lysate did not mediate these interactions. The binding of NS5B polymerase to GST-PTB in the RNase treated (lane 4) or untreated (lane 3) lysates was detected, whereas the GST control did not show any binding (lane 1). The GST-La protein also failed to exhibit any appreciable interaction with NS5B under similar conditions (lane 2). These results indicate that NS5B may directly interact with PTB via protein–protein interaction. This novel interaction was further investigated in the presence of other nonstructural (NS) proteins that form complexes with NS5B in vivo. The in vitro transcribed BB7 subgenomic replicon was translated in a HeLa S10 translation system (Barton et al., 1996). The polyprotein processing and translation products were verified by ^{35}S-methionine labeling of the proteins followed by SDS–PAGE and autoradiography (data not shown). In parallel reactions, the unlabeled viral NS proteins were translated and subjected to immunoprecipitation with antibodies against NS5B, NS3, and human La protein in the presence of excess amount of RNase A. PTB binding was subsequently detected by Western blot analysis of the gel using a monoclonal anti-PTB antibody (Fig. 7B). Both NS5B (lane 2) and NS3 (lane 3) were found to interact with PTB present in HeLa cytoplasmic lysates. Interestingly, the mobility of the PTB bands in both lanes was slightly different. PTB has been shown to exist in three alternatively spliced isoforms (PTB-1, PTB-2, and PTB-4). PTB-2 and PTB-4 isoforms differ from PTB-1 by an insertion of 19 and 26 amino acids in the middle region of the protein respectively (Wagner and Garcia-Blanco, 2001; Wollerton et al., 2001). Because of this property, two closely migrating PTB bands are usually detected in HeLa lysates (Ali and Siddiqui, 1995). In the present analysis, we observed a slight shift in the mobility of PTB, reflecting the possibility that different isoforms of PTB maybe causing the difference in mobility.

Interaction of PTB with replication proteins in the cell-free replication system

We have recently developed a cell-free HCV replication system that utilizes cytoplasmic lysates prepared from Huh-7 cells harboring the HCV subgenomic replicon (designated as FCA4) (Ali et al., 2002). The FCA4 lysates prepared by gentle lysis from Huh-7 cells which stably express the HCV replicon (Guo et al., 2001) were shown to contain preformed ribonucleoprotein complexes that actively replicate the endogenous replicon RNA templates (Ali et al., 2002). We utilized the FCA4 cell-free replication lysates to investigate PTB interaction with the replicase complex. The integrity of the complex was determined by the presence of intact NS5A and NS5B proteins in the FCA4 lysate preparations using a Western blot assay (Fig. 8A). The cell-free lysates derived from the parent Huh-7 cells showed no cross-reactivity with the antisera used in the Western blot analysis. Both FCA4 and Huh-7 lysates were treated with a large excess of RNase A and subjected to immunoprecipitation using antisera against NS3 and NS5B proteins. The liquid immunoprecipitates were fractionated by SDS–PAGE and immunoblotted with an anti-PTB antibody. The interactions of both NS3 as well as NS5B with PTB were detected in FCA4 lysates (Fig. 8B, lanes 1 and 3) but not in control samples (Fig. 8B, lanes 2 and 4). These results confirm that PTB physically interacts with the HCV proteins in vivo in the context of the RNP complex. However, we cannot rule out the possibility

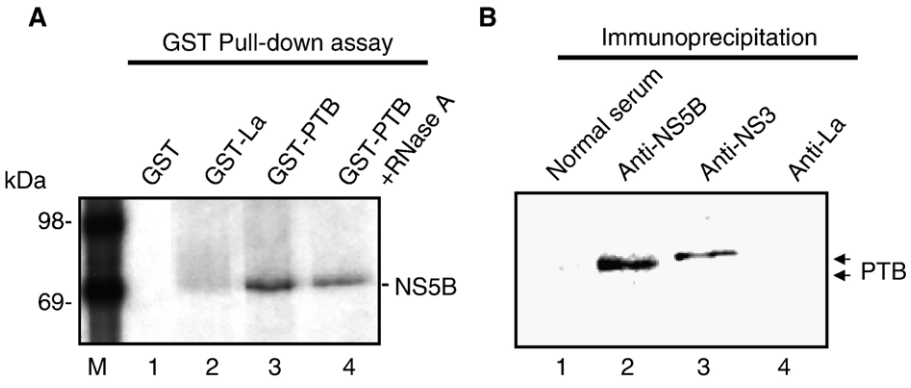

Fig. 7. Interaction of PTB with NS5B and NS3 in vitro. (A) Interaction of in vitro labeled NS5B with PTB. An EMCV-NS5B template was used to translate NS5B in the presence of ^{35}S-methionine and subjected to a GST-pull down assay as described in Materials and methods. Lane 4 is identical to lane 3 except that the lysate was treated with RNase A (20 μg) after translation was completed. M, molecular weight marker. (B) The BB7 replicon RNA was translated in HeLa cell-free lysates and immunoprecipitated with antibodies against NS3 (lane 3), NS5B (lane 2), normal human serum (lane 1), and anti-La antigen (lane 4). The immunocomplexes were immobilized on Protein G-Sepharose beads and subjected to Western blot analysis. The blot was probed for PTB.

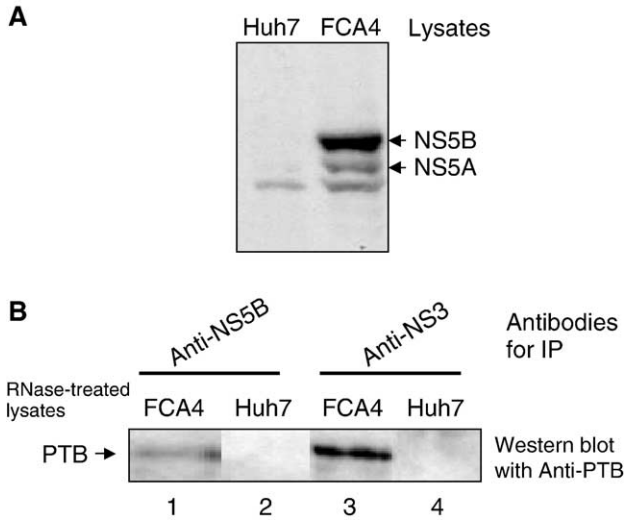

Fig. 8. Interaction of cellular PTB with the HCV NS proteins. (A) Western blot for the detection of viral proteins in FCA4 lysates. The cell-free HCV replication lysate (FCA4) was subjected to direct Western blot analysis to confirm the presence of intact NS5B proteins. Similar Huh-7 lysates served as a control. The blot was probed with antibodies that identify NS5A and NS5B proteins. (B) Binding of cellular PTB with the HCV NS3 and NS5B. Immunoprecipitation (IP) was performed using RNase A-treated FCA4 replication lysates (lanes 1 and 3) or Huh-7 cells (lanes 2 and 4) with polyclonal anti-NS5B and monoclonal anti-NS3 antibodies as indicated. The samples were fractionated on 10% SDS–PAGE and electrophoretically transferred to nitrocellulose membranes. The blot was probed with a monoclonal antibody against PTB.

of interactions outside of RNP complexes in the reaction mixtures.

Discussion

In order for both HCV translation and replication to occur, one of these processes must be blocked to allow the other process to take place since they occur in opposite directions. Cellular factors that have previously been shown to bind to the 5′ and 3′NCRs of HCV have been proposed to play pivotal roles in translation and replication. In this study, we have used transient transfections of Huh-7 cells with siRNA oligonucleotides specific to two such factors PTB and La to determine if silencing of these factors affects both translation and replication. We found that in the case of La, both translation and replication levels were decreased. However, with PTB, translation levels were reduced while replication levels were moderately enhanced in the presence of siRNA. In addition, we provide evidence for interactions between PTB and two viral NS proteins (NS3 and NS5B) that are required for HCV replication (Bartenschlager and Lohmann, 2000; Ikeda et al., 2002; Pause et al., 2003; Pietschmann et al., 2002). Further, we also showed that PTB competes with the NS5B polymerase for binding to the 3′NCR.

La, a 52-kDa autoantigen, was one of the first cellular proteins identified to interact with IRES elements and to stimulate HCV IRES-mediated translation (Dasgupta et al., 2004; Maraia and Intine, 2001). In this study, we have found that in addition to interacting with the HCV IRES to initiate translation, La also serves as a required cellular factor necessary for efficient HCV replication. It has previously been shown that the La protein interacts with the HCV IRES within the stem-loop IV of the HCV 5′NCR in the context of the initiator AUG codon (Ali and Siddiqui, 1997). In addition to this interaction, La protein can also interact and change the conformation of the large ribosomal subunit protein S5 (Pudi et al., 2004). In light of these findings, it is possible that the La protein could be required to assist in the unwinding of the RNA within stem-loop IV of the HCV 5′NCR to allow the initiator AUG accessible for processing by the mature ribosomal complex in order to start translation of the HCV polyprotein. By helping to bring together the mature ribosomal complex to engage in translation, we hypothesize that La could guide the movement of the ribosomal complex in the 5′NCR and beyond. Previous work has shown that stem-loops I and II are required for HCV replication and that stem-loops II through IV are required for translation (Pudi et al., 2003; Qi et al., 2003). These data suggest a dual role for the sequences located within stem-loop II of the HCV 5′NCR. This dual role could play a part in the molecular switch between viral translation and replication by selectively binding to different host and/or viral factors during different phases of the viral life cycle. It is also known that during translation stem-loop II makes extensive contacts with the 40S ribosomal subunit (Spahn et al., 2001).

PTB has been shown to interact with multiple pyrimidine tracts in the HCV genome that are found in the 5′-NCR, the core protein coding region, and within the 3′-NCR including the 3′X region (Ali and Siddiqui, 1995; Anwar et al., 2000; Ito and Lai, 1999; Ito et al., 1998; Tardif et al., 2002). In many instances these interactions have been found to be required for the regulation of HCV IRES function. In this study, we have provided evidence that PTB does play an active role in HCV IRES translation acting as an enhancer. However, PTB appears to impart negative constraints on HCV replication. This, most probably, is medicated via its interaction with the 3′NCR and nonstructural proteins (NS3 and NS5B).

When we transiently cotransfected Huh-7 cells with both HCV subgenomic RNA and PTB siRNA, we saw an initial lag in RNA replication (Fig. 3B, 24 h) followed by an increase in RNA replication levels close to the levels of cells that were only transfected with HCV subgenomic RNA at 48 h post-transfection (Fig. 3B, 48 h). In addition, when a translationally defective mutant 233 was used, there was a 1.5- to 2-fold increase in HCV RNA replication at 48 h post-transfection in the absence of PTB (Fig. 3C). It has previously been determined that PTB binds to stem-loops I and II of 3′X in the 3′NCR (Chung and Kaplan, 1999). It has further been shown that deletions of either stem-loops I or II within the 3′X region are detrimental for HCV replication (Frieb and Bartenschlager, 2002).

The binding of PTB to both 5′ and 3′ cis-elements within the NCRs may help to block the initiation of replication

from the 3′NCR by exerting a positive influence on the IRES while preventing NS5B from binding to the 3′NCR needed for the assembly of the replication initiation complex. It seems possible that once a threshold level of viral proteins is synthesized, NS5B and NS3 proteins may physically engage PTB. This engagement will diminish PTB's ability to enhance the HCV IRES function. By the same token, such interactions may also relieve the inhibitory effect of PTB on NS5B–3′NCR interactions, resulting in the overall regulation of both translation and initiation of replication processes. This hypothesis is partially supported by the quantitative evaluation of the competition data presented in Fig. 5A. It seems possible that an excess of NS5B may alleviate the PTB inhibition. For example, a 10- to 20-fold molar excess of NS5B showed a significant amount of 3′NCR binding in the presence of PTB. Recent studies suggest that oligomerization of NS5B is required for efficient HCV replication (Qin et al., 2002). In this respect, the oligomerization of NS5B may increase the local concentration of NS5B in the vicinity of the 3′NCR, thus overcoming the PTB inhibitory effect on the ability of the polymerase to bind to the 3′NCR. In fact, poliovirus RdRp, which is similar to HCV NS5B (RdRp) in many respects, has been shown to form sheet and tube-like structures resulting from oligomerization and these structures are also known to be critical for poliovirus replication (Lyle et al., 2002).

PTB predominantly resides in the nucleus but translocates to the cytoplasm under certain conditions (Back et al., 2002; Perez et al., 1997; Zang et al., 2001). Using an indirect immunofluorescence procedure, we show that PTB is localized in the cytoplasm of Huh-7 cells expressing HCV proteins and those harboring subgenomic replicon RNAs (Fig. 6). The subgenomic RNA that transiently transfected cells similarly displayed cytoplasmic distribution of PTB. It should be noted that replication defective subgenomic (BB7 Pol$^-$) RNA can only translate the viral proteins but is crippled in its ability to replicate due to GDD mutations in NS5B. Nonetheless, cytoplasmic staining of PTB was clearly evident in both cases. This implies that the viral protein expression or events such as formation/assembly of HCV ribonucleoprotein complexes may be sufficient to trigger similar redistribution of PTB in the cells as seen with wild-type HCV RNA.

The IRES elements of HCV and picornaviruses recruit translation initiation factors and nuclear proteins such as La antigen and PTB (Hellen and Sarnow, 2001; Sarnow, 2003). It has been hypothesized that 'cross-communication' between the 5′ and 3′ ends of mRNA plays a critical role during cap-dependent and picornavirus IRES-mediated translation. This communication is established by a protein-bridge formed between the translation factor eIF-4G and poly (A) binding protein (PABP) in an RNP supercomplex [i.e., Cap/IRES-(eIF-4G)-PABP-(polyA)] (Sachs et al., 1997). In the case of HCV, since it contains a unique 3′NCR, it is possible that both PTB and/or La protein may be engaged in 5′ and 3′ cross communication. At present, there is no evidence for any such interactions. Further investigations are needed to examine these interactions.

Materials and methods

Cell culture

Huh-7 human hepatoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin/ml, and 100 μg of streptomycin sulfate/ml. Cells were maintained at 5% CO_2 and 37 °C.

Plasmids and in vitro RNA transcription

The plasmid containing the HCV subgenomic replicon BM4–5 was obtained through a generous gift from C. Seeger (Fox Chase Cancer Center). The BM4–5 plasmid contains within it a bicistronic, subgenomic HCV replicon that can be transcribed in vitro from a T7 promoter using DNA linearized by *Sca*I. This replicon consists of the HCV-IRES (nucleotides 1–377 of the 5′ HCV NCR) directing the translation of the neomycin phosphotransferase (neo) gene and the encephalomyocarditis (EMCV) IRES directing the translation of the HCV nonstructural proteins NS3 through NS5B, followed by the HCV 3′NCR.

HCV replicon RNA was transcribed in vitro from linear BM4–5 DNA cut by the *Sca*I restriction enzyme. RNA was synthesized using the Ampliscribe T7 transcription kit (Eppicentre) according to the manufacturer's protocol. The quantity of the RNA was assessed by spectrophotometer and the quality was checked visually on a 1% TBE gel.

The plasmids pNCR-C(AUG) and p3′NCR encode the HCV IRES (nt 1–356 of HCV-1a) and the 3′NCR (HCV-1b) respectively under the control of T7 promoter. The plasmid pEMCV-5B encodes an RNA that contains the HCV NS5B gene between the upstream EMCV IRES and the downstream HCV 3′NCR. These HCV sequences were cloned into the vector pGEM-4 (Promega). The plasmid encoding the HCV replicon BB7 construct and a corresponding replication-deficient BB7 mutant (BB7 Pol$^-$) construct was a generous gift from Dr. C. M. Rice (Blight et al., 2000). The plasmid pCMV/720–3010 encodes HCV 720–3010 amino acids of the genotype 1b (Hijikata et al., 1993).

The plasmids pNCR-C(AUG) and p3′NCR were linearized with *Eco*RI whereas pEMCV-5B, pBB7, and pBB7 Pol$^-$ were linearized with *Sca*I. The linear DNAs were eluted from agarose gels and transcribed with T7 RNA polymerase using AmpliScribe T7 transcription kit (Epicenter Technologies) according to the manufacturer's instruction. To synthesize the complementary strand of the HCV IRES [(−)5′NCR], the NCR-C(AUG) plasmid was linearized with *Hin*dIII and transcribed with SP6 polymerase as described above.

Site-directed mutagenesis and transfection of mutant

The BM4–5 plasmid was used as the wild-type template that the site-directed mutation was constructed from (nucleotides 233–236 in HCV IRES region). Site-directed mutagenesis reactions were carried out using the PfuTurbo DNA Polymerase PCR system (Stratagene) according to the manufacturer. Following the PCR reaction, the PCR products were digested with *Dpn*I (Promega). The *Escherichia coli* strain DH5 alpha was then transformed by electroporation with the digested PCR product using standard procedures. Mutations were confirmed by DNA sequence analysis. 1 μg of in vitro transcribed RNA from the HCV 233 construct was electroporated into 4×10^6 Huh-7 cells as described by Lohmann et al. (2003).

La siRNA cotransfection with bicistronic HCV replicon

siRNA targeted against La autoantigen mRNA was created using the oligonucleotides below. The procedure for generating siRNA using a T7 primer system has been described previously (Donze and Picard, 2002). The sequences for generating the siRNAs are as follows: T7 primer, 5′-TAATACGACTCACTATAG-3′; La siRNA sense primer, 5′-ATGTCTCTACAAATTTCTTAGCTATAGTGAGTCGTATTA-3′; and the La siRNA antisense primer, 5′-CTGCTAAGAAATTTGTAGAGACTATAGTGAGTCGTATTA-3′. One day prior to cotransfection, Huh-7 cells were trypsinized and resuspended in complete DMEM and plated onto 6-cm plates at approximately 30% confluency. Cotransfection experiments were performed using Lipofectamine 2000 (Invitrogen). For each transfection two solutions were made, A and B. Solution A: 500 pmol of La siRNA, 10 μg of HCV replicon RNA, or both, were added to 100 μl of OPTIMEM media (Invitrogen). Solution B: 6 μl of Lipofectamine 2000 was added to 100 μl OPTIMEM media. Solutions A and B were allowed to incubate at room temperature for 15 min. After 15 min, solutions A and B were combined, gently mixed, and allowed to incubate another 20 min at room temperature. The combined solutions were then added to the cells in the 6-cm plates. The cells were then incubated for 4 h at 37 °C and 5% CO_2. After 4 h, the transfection solution was replaced with 3 mL of complete DMEM growth media.

PTB siRNA cotransfection

The day before the transfection, Huh-7 cells were trypsinized and resuspended in complete DMEM and then seeded at 9×10^4 cells in 24-well dishes. Cotransfection experiments were performed as above with the following changes: 50 pmol of PTB siRNA (Dharmacon, SMARTpool) and 0.5 μg of HCV replicon RNA were added to 50 μl of OPTIMEM media. In addition, solutions A and B were allowed to incubate for 5 min separately before being combined. The transfection solution was replaced with DMEM after 24 h of incubation.

Western blotting

Total protein was obtained at 24 and 48 h post-transfection (and 72 h for PTB). For the 233 mutant, protein samples were taken at 6, 48, and 96 h post-transfection. Growth media were removed from the transfected Huh-7 cells and the cells were washed once with PBS-EDTA. Cells were then allowed to incubate at room temperature in PBS-EDTA for 20 min. Cells were then removed from the plates and centrifuged at 4 °C. Total protein was harvested using the corresponding lysis buffers: for La and 233 proteins, 10 mM HEPES-KOH [pH 7.2], 140 mM KCl, 5 mM MgCl2, 1 mM EGTA, 1% NP-40, 1× protease inhibitor cocktail [Roche] and for PTB the lysis buffer was the same with the following changes: 5 mM KCl and 1 mM EDTA instead of EGTA. Cell pellets were resuspended in 50–100 μl of lysis buffer and were allowed to incubate on ice for 25 min. The resuspended cells were then centrifuged for 10 min at 4 °C. The supernatant was run on a 12% SDS–PAGE gel and transferred to a nitrocellulose membrane (BioRad, Trans-Blot Transfer Medium) at 100 V for 90 min at 4 °C. The membranes were probed and developed using SuperSignal West Dura Extended Duration Substrate (Pierce). Images were captured on the Kodak 440CF Imaging System. The band intensities from the Western Blots were measured with 1D Image Analysis Software from Kodak. La protein was probed using the monoclonal anti-La antibody (SW5). PTB protein was detected with a polyclonal anti-hnRNPI antibody (Santa Cruz Biotechnologies). The polyclonal anti-neomycin phosphotransferase antibody was obtained from Cortex Biochem. The monoclonal anti-Actin antibody was purchased from NeoMarkers (Fremont, CA.).

Quantitative RT-PCR

Total RNA was harvested at 24 and 48 h post-transfection using RNA-STAT60 (Tel-Test), following the manufacturer's standard procedures. RNA was quantified using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Quantification runs were done in duplicate. Amplifications were conducted using the following primers and dual-labeled probes (Qiagen): La mRNA probe, 5′-6FAM-TAC GGC TAT CTT TAA GGT TCC GGC CCA-TAMRA-3′; La mRNA forward primer, 5′-CTG TGG CGC GGC TTC T-3′; La mRNA reverse primer, 5′-TCC AGG GCA GCC ATC TTT T-3′; PTB mRNA probe, 5′-6FAM-CAC CAG ATT TTC TCC AAG TTC GGC ACA-TAMRA-3′; PTB mRNA forward primer, 5′-ACC CTG TGA CCC TGG ATG TG-3′; PTB mRNA reverse primer, 5′-TGT TCT TGG TGA AGG TGA TGA TCT-3′; HCV Replicon probe, 5′-6FAM-CCT TCA TCT CCT TGA GCA CGT CCC G-TAMRA-3′; HCV replicon forward primer, 5′-CTT TGA CAG ACT GCA GGT CCT G-3′; HCV replicon

reverse primer, 5′-GCC TTA ACT GTG GAC GCC TTC-3′; 18S rRNA probe, 5′-6FAM-TGC TGG CAC CAG ACT TGC CCT C-TAMRA-3′; 18S rRNA forward primer, 5′-CGG CTA CCA CAT CCA AGG AA-3′; 18S rRNA reverse primer, 5′-GCT GGA ATT ACC GCG GCT-3′. The sequences for the primers and probes were designed on Primer Express software (Applied Biosystems). Amplification reactions were performed in a 25-μl reaction mix using the Quantitect RT PCR kit (Qiagen) containing a final concentration of each primer at 0.5 μM, and each probe at 0.2 μM. For the quantification reactions using the La mRNA primer/probe set, the PTB mRNA primer/probe set and the HCV replicon primer/probe set the total RNA input amount were 250 ng. For the quantification reaction using the 18S rRNA primer/probe set, the total RNA input amount was 250 pg. Reactions were performed under the following conditions: 30 min at 50 °C (reverse transcription reaction); 15 min at 95 °C (heat inactivation of the reverse transcriptase and activation of the Taq polymerase); 35–40 cycles of 15 s at 95 °C and 1 min at 60 °C (PCR amplification and data acquisition). The results from each run were calculated using the comparative ΔΔCt method as described in the ABI Prism 7000 handbook (appliedbiosystems.com, user bulletin #2: "Relative Quantification of Gene Expression," part #4303859B). All experimental results were normalized to the 18S rRNA endogenous control. Each normalized result was then used to compare differences within various experimental controls such as mock for the silencing experiments and BM4–5 when compared to BM4–5 cotransfected with siRNA. Graphed results are the averages of three separate duplicate runs analyzed in the manner described above. Two-tailed *P* values were determined by performing a paired *t* test calculated using the statistical program "GraphPad InStat" version 3.06, GraphPad Software, San Diego, CA, www.graphpad.com.

Expression and purification of recombinant proteins

The plasmid GST-2TK/PTB, which encodes the glutathione S-transferase (GST)-human PTB fusion protein, was a generous gift from Dr M. A. Garcia-Blanco. The expression of GST-PTB was induced with 1 mM IPTG in *E. coli* (JM101) and affinity-purified on glutathione-Sepharose beads (Pharmacia). The final preparation was dialyzed against 5 mM Hepes pH 7.6, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 10% (v/v) glycerol (Ali and Siddiqui, 1995). GST and GST-La proteins were also purified by a similar procedure. The $(His)_6$-tagged HCV NS5B was expressed in *E. coli* (*BL21*) and purified according to the procedure described by Oh et al. (1999).

UV cross-linking of proteins with RNA

4-Thio-UDP (Sigma) was phosphorylated with nucleoside 5′-diphosphate kinase to prepare 4-thio-UTP (Ali and Siddiqui, 1995). The 3′NCR and (−)5′NCR RNA probes were synthesized in the presence of 4-thio-UTP and [^{32}P]CTP and extracted according to standard procedures. The probes were mixed with purified HCV NS5B in RNA binding buffer (5 mM HEPES [pH 7.6], 25 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10% [v/v] glycerol) and incubated at 30 °C for 30 min as described previously (Ali and Siddiqui, 1995, 1997). For competition analysis, increasing amounts of GST-PTB or GST were added along with the components of the reaction mixture prior to UV cross-linking. The UV cross-linking was carried out in Stratalinker (Stratagen) for 30 min at 4 C. The ribonucleoprotein complexes were treated with RNase A (10–20 U) (USB) and analyzed by 12% SDS–PAGE followed by autoradiography. The identity of cross-linked ribonucleoprotein products (NS5B-RNA and PTB-RNA) was verified by immunoprecipitation and by comparison of their relative mobility in the same gel.

GST pull-down assay

The HCV NS5B was translated in rabbit reticulocyte lysates (RRL) using in vitro transcribed EMCV-5B RNA and the protein was radiolabeled with ^{35}S-Methionine according to the recommended protocol (Promega). The protein synthesis was confirmed by SDS–PAGE and autoradiography. GST, GST-PTB, or GST-La proteins were immobilized on glutathione-Sepharose beads (Amersham). The RRL containing labeled NS5B was diluted in 500 μl buffer B (25 mM HEPES, pH 7.9, 150 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, protease inhibitor cocktail) and added to the beads. One of the samples was treated with 10 μg of RNase A to digest the template RNA. The binding was carried out at 4 °C for 2 h. The unbound materials were washed six times and the samples were analyzed by SDS–PAGE followed by autoradiography.

Indirect immunofluorescence microscopy

Huh-7 cells were grown on glass cover slips and transfected with in vitro transcribed BB7 or BB7 Pol$^-$ RNA. The cells were washed twice with PBS and fixed with PBS containing 4% formaldehyde for 20 min at RT. After washing the cells 4 times, the cells were permeabilized with cold acetone for 7 min. The cover slips were washed and treated with blocking buffer (10% fetal bovine serum, 0.1% Triton X-100 in PBS buffer) for 1 h at RT. The polyclonal anti-PTB antibodies and monoclonal anti-NS5B antibodies were added to the cells and incubated for 1 h. The unbound antibodies were washed and FITC and TRITC conjugated secondary antibodies were added to detect NS5B and PTB, respectively. The cover slips were mounted on the glass slides in the presence of Slowfade Light Antifade (Molecular Probes). The immunofluorescence was detected using a Zeiss microscope (Axioplan2). In a similar experiment, the pCMV/720–3010 DNA was transfected into Huh-7 cells

and subjected to immunofluorescence staining 48 h post-transfection as described above.

Immunoprecipitation

HeLa S10 cytoplasmic fractions from suspension cultures of HeLa S3 were prepared as described by Barton et al. (1996). For immunoprecipitation and Western blot analysis, the HCV NS proteins (NS3 through NS5B) were synthesized in a reaction mixture containing 150 μl S10 lysates, 60 μl IF (initiation factors) lysates, 20 μl ATP generating system, 5 μl RNasin, and 16 μg BB7 RNA in a total volume of 300 μl. The translation was carried out at 30 °C for 2 h followed by treatment with RNase A/RnaseT1 mixture (Ambion) for 30 min. Two hundred microliters of NETS buffer (50 mM Tris–HCl, [pH 7.4], 5 mM EDTA, 1 mM DTT, 100 mM NaCl, 0.05% Nonidet P-40) was added to an aliquot of 50 μl translation mix and the immunoprecipitation was carried out with antibodies against NS3 and NS5B. Normal human IgG served as control. The immunocomplexes were immobilized on Protein G-Sepharose 4B beads for 2 h at 4 C. The unbound materials were washed six times with the NETS buffer. The bound proteins were fractionated by SDS–PAGE and subjected to a Western blot analysis using a monoclonal anti-PTB antibody (7G12). The blot was developed using ECL techniques (Amersham Pharmacia Biotech).

The cell-free replication lysates derived from the FCA4 cell line were prepared as described previously (Ali et al., 2002). This cell line is derived from Huh-7 cells selected in the presence of G418 after RNA transfection with subgenomic HCV replicon. These cells stably express the HCV replicon at a high efficiency and contain adaptive mutations (Guo et al., 2001). These lysates were treated with RNase A/T1 mix (20 μg) and subjected to immunoprecipitation and Western blot analysis as described above. The Huh-7 lysates that were prepared similarly were used as a control.

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