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hnRNP C and polypyrimidine tract-binding protein specifically interact with the pyrimidine-rich region within the 3′NTR of the HCV RNA genome

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ABSTRACT

Like other members of the *Flaviviridae* family, the 3′ non-translated region (NTR) of the hepatitis C virus (HCV) is believed to function in the initiation and regulation of viral RNA replication by interacting with components of the viral replicase complex. To investigate the possibility that host components may also participate in this process, we used UV cross-linking assays to determine if any cellular proteins could bind specifically to the 3′NTR RNA. We demonstrate the specific interaction of two host proteins with the extensive pyrimidine-rich region within the HCV 3′NTR. One host protein migrates as a doublet with a molecular weight of 57 kDa and is immunoreactive with antisera specific for polypyrimidine tract-binding protein (PTB), and the other protein (35 kDa) is recognized by a monoclonal antibody specific for heterogeneous nuclear ribonucleoprotein C (hnRNP C). These results suggest that recognition of the large pyrimidine-rich region by PTB and hnRNP C may play a role in the initiation and/or regulation of HCV RNA replication.

INTRODUCTION

Like the other members of the *Flaviviridae*, hepatitis C virus (HCV) is an enveloped virus containing a single strand RNA molecule of positive polarity. The HCV genome is ~9.6 kb with a long, highly conserved, non-capped 5′ non-translated region (NTR) of ~340 bases which functions as an internal ribosome entry site (IRES) (1). This element is followed by a single long open reading frame (ORF) encoding a polypeptide of 3011 amino acids. This large polypeptide is subsequently processed into the individual structural and non-structural viral proteins by a combination of host and virally-encoded proteinases (2). Following the termination codon of the long ORF, there is a 3′NTR which consists of three regions: an ~40 base region which is poorly conserved among various genotypes, a variable length poly(U)/polypyrimidine tract, and a highly conserved 98 base element (3–6). The 5′ and 3′NTRs form stable secondary structures (7–9) which are believed to function in the initiation and regulation of viral RNA replication. Since a robust, cell based virus replication system is not available for HCV, little is known about the mechanism of viral RNA replication and its regulation. Little more is understood about the mechanisms of RNA replication for the flaviviruses and pestiviruses (reviewed in 10,11), although what is known is similar to that of other positive stranded RNA viruses such as the *Picornaviridae*, in particular, the well studied poliovirus (reviewed in 12). Based upon the RNA replication of poliovirus, a model of the mechanism of HCV RNA replication has been proposed which is believed to begin with the synthesis of a full-length minus strand copy of the infecting plus strand viral RNA. This minus strand then serves as a template for the disproportionate synthesis of new full-length plus strand, which can be translated to synthesize more viral proteins or be packaged into progeny virus particles. Based upon this model, one of the functions of the 3′NTR would be to serve as the initiation site of the replicase complex for the synthesis of the minus strand template. In poliovirus RNA replication, host proteins appear to play some role in authentic viral RNA replication *in vitro* (reviewed in 12,13). Thus, it may be possible that host proteins play a similar role in the replication of HCV RNA. Although there is no *in vitro* RNA replication system currently available for HCV to explore this model directly, we sought to address this question by using RNA–protein UV cross-linking assays to determine if there were any cellular factors which could bind specifically to the 3′NTR RNA. These studies have identified two host proteins which specifically interact with the extensive pyrimidine-rich region within the HCV 3′NTR. One protein is the polypyrimidine tract-binding protein (PTB), which was previously reported to interact with the highly conserved 98 base element (9,14). Additionally, a novel protein which is recognized by a monoclonal antibody specific for heterogeneous nuclear ribonucleoprotein C (hnRNP C) was also found to bind to this pyrimidine-rich region, suggesting that recognition of the large pyrimidine-rich region by PTB and hnRNP C may play a role in the initiation and/or regulation of HCV RNA replication.

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MATERIALS AND METHODS

Cells, protein extracts and protein purification

Hep G2, a human hepatoblastoma cell line (ATCC HB-8065) was obtained from the American Type Culture Collection (Rockville, MD) and was propagated at 37°C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and penicillin/streptomycin (5000 U/ml). To prepare cellular extracts, subconfluent monolayers were trypsinized, pooled, and washed three times with ice-cold phosphate buffered saline without calcium or magnesium. A packed cell pellet (2×10^8 cells) was resuspended in five times packed cell volume (~8 ml) of hypotonic lysis buffer (10 mM N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid [HEPES], pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol), and incubated on ice for 10 min. Cells were pelleted and resuspended in one packed cell volume, transferred to a Dounce homogenizer and lysed by 10 strokes with a B type pestle. The lysate was spun for 10 min at 4°C at 2000 r.p.m. in a Beckman GS-6R centrifuge, and the extract was aliquoted and stored at –80°C until use. A plasmid encoding the entire PTB ORF as a hexahistidine-tagged fusion protein was a generous gift from Jim Patton (Vanderbilt University). PTB was expressed and purified from *Escherichia coli* as described in Perez *et al*. (15). All protein concentrations were determined using a BioRad protein assay as per the manufacturer's instructions.

Plasmid constructs

The 5′ and 3′NTRs of HCV were constructed from four overlapping oligonucleotides covering the HCV 3′ or 5′NTR. The sequences used to generate the 5′ and 3′NTRs were derived from the published sequence for the infectious HCV H77 strain (GenBank accession no. AF009606), and the sequence used for the 3′NTR was derived from the sequence of the Hutchinson strain derived from patient serum H77 as previously reported (4). The sequence of synthesized region was confirmed by automated DNA sequencing. The end of the 3′NTR was designed so that the terminal bases (GUU) from HCV were used to create a *Hpa*I restriction site followed by an *Age*I site (**GTT**AACCGGT); thus, cleavage with *Hpa*I followed by *in vitro* run off transcription would generate an authentic HCV 3′ end (GUU) (6). The *Age*I–*Age*I fragment containing the 3′NTR was then subcloned into a modified Bluescript II KS (+) (Stratagene, La Jolla, CA) which had an *Age*I site added via linker at the *Sac*I site generating pKSHCV3′NTR. The HCV 3′ X-tail with an upstream T7 promoter was amplified by PCR using the following primers: (+), 5′-CGGAATTCTGTAATACGACTCACTATAGGTGGCTCCATCTTAGCC-3′; (–), 5′-GGGGTACCAACATGATCTGCAGAGAG-3′.

The PCR product was digested with *Eco*RI and *Kpn*I and cloned into the *Eco*RI/*Kpn*I sites of pUC18 to produce pUC-3Xtail. The HCV 5′NTR was designed with an *Xba*I site at the 5′ end followed by the T7 promoter (indicated by underlining) directly preceding the HCV 5′NTR (indicated in bold) (5′-TCTAGATAATACGACTCACTATA**GCCAGCC.....**) so that transcription with T7 RNA polymerase would initiate on the first base of HCV genomic RNA. The 3′ end of 5′NTR ended at a naturally occurring *Kpn*I site within the core protein region (amino acid 81 in the core protein of Hutchinson strain of HCV). The HCV 5′NTR region was cloned into pUC19 between the *Xba*I and *Kpn*I sites generating pHCV5′NTR. pECM1RNA, containing the *E.coli* RNase P (M1) RNA sequence downstream of a T7 promoter, was a generous gift from Lisa Hegg (SB Pharmaceuticals). Plasmid pBVDV3′NTR contains the 3′NTR region of BVDV (NADL strain) and was constructed by cloning the *Eag*I–*Sac*II fragment of pVVNADL (16) downstream of the T7 promoter in pBluescript II SK(+) (Stratagene). All plasmids were constructed, sequenced, propagated and prepared using standard techniques (17).

Synthesis of labeled RNA

Radiolabeled RNAs were synthesized by *in vitro* transcription of pKS-HCV3′NTR linearized with *Hpa*I using T7 RNA polymerase and either [α-^{32}P]UTP or [α-^{32}P]CTP. Non-specific control RNA was synthesized by using pECM1RNA linearized with *Kpn*I. RNA corresponding to the HCV 5′NTR was synthesized by using pHCV5′NTR linearized with *Kpn*I. RNA corresponding to the HCV 3′ X-tail was synthesized using pUC-3Xtail plasmid linearized by *Acc65I* to produce an RNA transcript of 100 nt. Unlabeled RNAs were synthesized using MEGAshortscript kit (Ambion Inc., Austin, TX). RNAs corresponding to HCV 3′-NTR nt 178–197, 168–197 and 138–177 were chemically synthesized by Cybersyn, Inc. Random 30mer RNA was a generous gift from Hu Li (SB Pharmaceuticals).

UV cross-linking assays

In vitro binding reactions were carried out using 200 ng of total HepG2 cellular protein (or 250 ng of purified PTB) in a reaction volume of 30 µl containing 50 mM Tris–HCl pH 7.6, 2 mM $MgCl_2$ and 100 mM KCl. Reaction mixtures containing 10 fmol of radiolabeled RNA (in the presence or absence of the indicated unlabeled competitor RNAs) were incubated at 30°C for 20 min, then transferred to Parafilm on ice and exposed to UV light (254 nm) at a distance of 4 cm for 10 min. Following treatment with 1 mg of RNase A/ml at 37°C for 15 min, the samples were resolved by SDS–PAGE on 10% gels. To control for equivalent amounts of protein loaded in each lane, gels were stained with Coomassie Blue prior to autoradiography (data not shown). For UV cross-linking and immunoprecipitation assays, 5 µl of anti-PTB antibody mAb 7G12 or 3 µl of anti-hnRNP C mAb 4F4 (a generous gift from Gideon Dreyfuss, University of Pennsylvania, PA) were added to the RNase-digested cross-linking reactions as described above, along with 200 µl of NET-2 buffer (50 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.05% NP-40 and 0.5 mM DTT). After 1 h at 4°C, 50 µl of protein G sepharose (Pharmacia) was added and allowed to bind for 1 h. After four washes with NET-2 buffer, bound proteins were removed from the beads by adding 40 µl of SDS–PAGE sample buffer and heating to 75°C for 5 min; proteins were then resolved on a 10% gel.

RESULTS

HepG2 cytoplasmic proteins bind specifically to the HCV 3′NTR RNA

To examine the possibility of cellular factors interacting with the HCV 3′NTR, radiolabeled RNA corresponding to the full-length 3′NTR sequence (Fig. 1) was incubated with cytoplasmic extracts from HepG2 cells in the absence or presence of unlabeled competitor RNAs. The reaction mixtures were then exposed to UV light to create covalent cross-links between the protein and the radiolabeled RNA, digested with RNase A to degrade any unbound RNA, then analyzed by SDS–PAGE (Fig. 2). In the

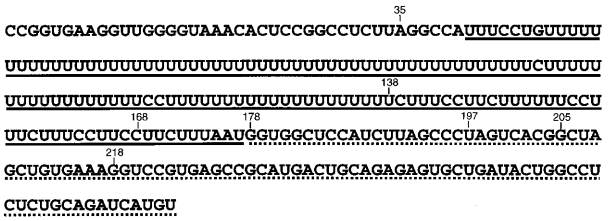

Figure 1. Sequence of the HCV 3′NTR region. The sequence of the HCV 3′NTR, H77 strain (4) used in this study is shown. The highly variable poly-pyrimidine tract is indicated with a solid underline, and the highly conserved 3′ X-tail is indicated with a dotted underline.

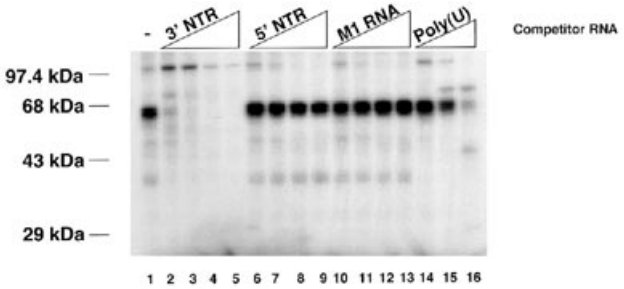

Figure 2. HepG2 cytoplasmic proteins interact specifically with the HCV 3′NTR. Radiolabeled HCV 3′NTR RNA was incubated in HepG2 cytoplasmic extracts with or without competitor RNAs. Binding reactions were incubated in the presence of UV light to form covalent cross-links between the labeled RNA and proteins, treated with RNase A and fractionated by SDS–PAGE on a 10% gel. Competitions were performed using 25-, 125-, 500- and 1000-fold molar excess of unlabeled RNAs [lanes 2–5, HCV 3′NTR; lanes 6–9, HCV 5′NTR; lanes 10–13, *E.coli* M1 RNA; lanes 14–16, poly(U)]. The positions of the molecular weight markers are indicated to the left.

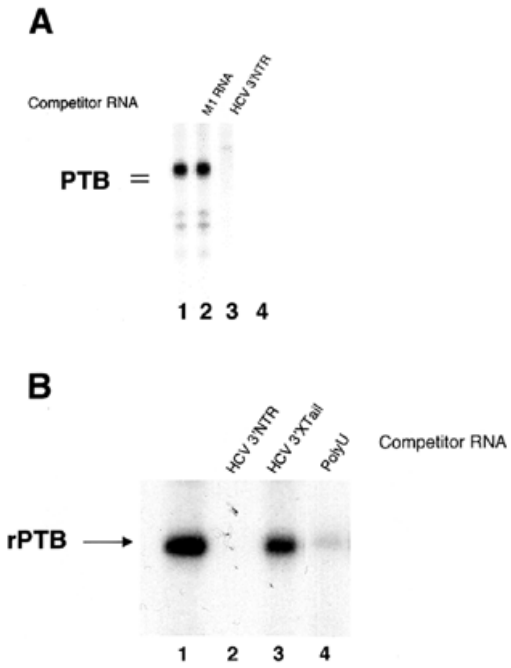

Figure 3. Specific binding of PTB to the HCV 3′NTR. (**A**) UV cross-linking and immunoprecipitation reactions using anti-PTB antisera were performed with labeled HCV 3′NTR RNA alone (lane 1), or with 125-fold molar excess of the unlabeled HCV 3′NTR (lane 3) or *E.coli* M1 (lane 2) competitor RNA. Lane 4 represents a mock immuno-precipitation without antibody. (**B**) Recombinant PTB interacts specifically with the HCV 3′NTR. UV cross-linking reactions were using hexahistidine-tagged PTB and no competitor RNA (lane 1) or the indicated competitor RNAs (lanes 2–4).

absence of any unlabeled competitor RNA, one major protein doublet species was detected bound to the radiolabeled 3′NTR with an apparent molecular size of 57 kDa; two other minor species were also detected (35 and 100 kDa, respectively) (Fig. 2, lane 1). To examine the specificity of the interactions of these proteins with the labeled RNA, additional reactions were carried out using 25-, 125-, 500- and 1000-fold molar excess of unlabeled HCV 3′NTR (Fig. 2, lanes 2–5), HCV 5′NTR (Fig. 2, lanes 6–9), or another non-specific RNA, *E.coli* M1 RNA (Fig. 2, lanes 10–13). When radiolabeled 3′NTR was incubated in the presence of unlabeled 3′NTR RNA, binding of the 57 and 35 kDa proteins was almost completely abolished (Fig. 2, lanes 2–5). In contrast, when either 5′NTR or M1 RNA was used as the unlabeled competitor, binding of the 57 and 35 kDa proteins remained unchanged. Because the HCV 3′NTR RNA contains an extensive U/C-rich tract, poly(U) was also included as a competitor (Fig. 2, lanes 14–16). This showed that poly(U) efficiently competes for binding of the 57 and 35 kDa proteins to the labeled RNA. From these results, it can be concluded that the 57 and 35 kDa protein species from the HepG2 cytoplasmic extracts bind specifically to the HCV 3′NTR; that poly(U) is such an effective competitor suggests that the pyrimidine-rich tract within the 3′NTR might be an important binding determinant.

PTB interacts specifically with the HCV 3′NTR

PTB, also known as hnRNP I (18), is a 57 kDa cellular protein that has been shown to cross-link efficiently to pyrimidine-rich RNAs (18–22). Because the size of the protein doublet observed cross-linking to the 3′NTR is consistent with the size of PTB, a cross-linking/immunoprecipitation experiment was performed using a monoclonal antibody against PTB. Figure 3A shows that this antibody immunoprecipitated a doublet of 57 kDa when labeled 3′NTR was used as the binding substrate in the absence of competitor (Fig. 3A, lane 1). Competition experiments revealed that unlabeled 3′NTR RNA (Fig. 3A, lane 3) was able to compete efficiently for binding of PTB, while unlabeled M1 RNA (Fig. 3A, lane 2) was not able to compete (Fig. 3A, compare lane 3 with lane 2). These results confirm the identity of PTB as the protein doublet which interacts specifically with the HCV 3′NTR.

To both confirm that PTB binds to the HCV 3′NTR and to further localize the RNA-binding site, recombinant PTB was used in UV cross-linking experiments. Figure 3B shows that the recombinant PTB binds to the full-length HCV 3′NTR substrate RNA (lane 1); this binding is competed by a 125-fold excess of unlabeled HCV 3′NTR (lane 2) and poly(U) (lane 4), but not by a 125-fold excess of unlabeled RNA corresponding to the terminal 98 nt of the NTR (the ‘3′ X-tail’ element, lane 3). This experiment suggests that nucleotides within the full-length NTR

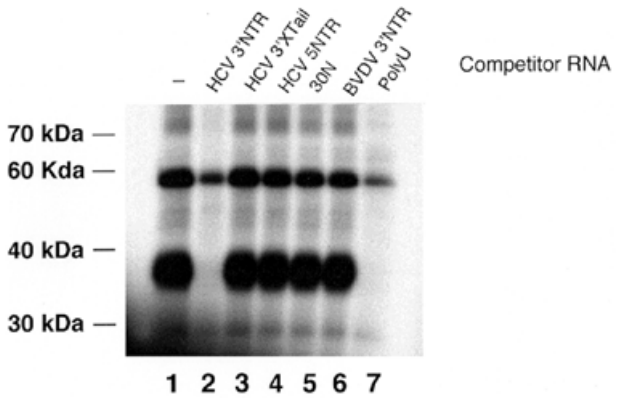

Figure 4. Labeling of the HCV 3′NTR with [α-^{32}P]UTP results in more efficient cross-linking of PTB and a 35 kDa protein species. UV cross-linking reactions were performed using labeled HCV 3′NTR RNA as substrate either alone (lane 1), or in the presence of 25-fold molar excess of unlabeled HCV 3′NTR (lane 2), HCV 3′ X-tail (lane 3), HCV 5′NTR (lane 4), random 30mer RNA (lane 5), BVDV 3′NTR (lane 6) or 100 ng of unlabeled poly(U) RNA (lane 7). The positions of the molecular weight markers are indicated to the left.

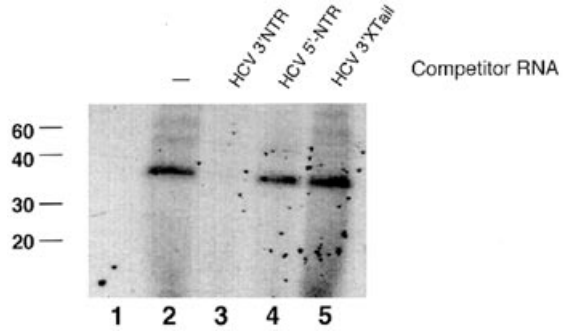

Figure 5. Specific binding of hnRNP C to the HCV 3′NTR. UV cross-linking and immunoprecipitation reactions using anti-hnRNP C antisera were performed with labeled HCV 3′NTR RNA alone (lane 2), or with 25-fold molar excess of the unlabeled HCV 3′NTR (lane 3), HCV 5′NTR (lane 4) or HCV 3′ X-tail competitor RNA (lane 5). Lane 1 represents a mock immuno-precipitation without antibody.

upstream of the 3′ X-tail element are required for PTB-binding, and further suggests the importance of the large pyrimidine tract.

hnRNPC binds specifically to the HCV 3′NTR

The experiment shown in Figure 2 revealed that in addition to the 57 kDa protein (PTB), another protein migrating at 35 kDa also interacted specifically with the HCV 3′NTR RNA. Another UV cross-linking experiment was performed, this time using HCV 3′NTR RNA labeled with [α-^{32}P]UTP instead of CTP. Labeling of the RNA with UTP resulted in a much stronger signal for both PTB and the 35 kDa species (Fig. 4, lane 1). Inclusion of a 25-fold molar excess of unlabeled competitor RNAs revealed that only the HCV 3′NTR RNA (Fig. 4, lane 2) and poly(U) (Fig. 4, lane 7) effectively competed for both cross-linked species, while the other RNAs did not (Fig. 4, lanes 3–6). Increased labeling of the cross-linked proteins by the [α-^{32}P]UTP-labeled RNA also further suggests that the extensive pyrimidine-tract within the 3′NTR is important for protein binding.

hnRNP C is another cellular RNA-binding protein with known affinity for pyrimidine stretches (23). To investigate the possibility that the 35 kDa cross-linked species was hnRNP C, another cross-linking/immunoprecipitation was performed, this time using a monoclonal antibody specific for hnRNP C. Figure 5 shows that that this antibody immunoprecipitated a protein of 35 kDa when labeled 3′NTR was used as the binding substrate in the absence of competitor (Fig. 5, lane 2). Competition experiments revealed that unlabeled 3′NTR RNA (Fig. 5, lane 3) was able to compete efficiently for binding of hnRNP C, while unlabeled HCV 5′NTR (Fig. 5, lane 4) and HCV 3′ X-tail RNA (Fig. 5, lane 5) were not able to compete. These results confirm that the 35 kDa species that cross-links specifically to the HCV 3′NTR is hnRNP C, and also further suggests that hnRNP C recognizes nucleotides upstream of the 3′ X-tail.

In an effort to more precisely localize the binding sites of PTB and hnRNP C, a UV cross-linking experiment was performed using HepG2 cell extracts and labeled full-length HCV 3′NTR RNA substrate and additional competitor RNAs (Fig. 6A). In the absence of any competitor RNAs, bands corresponding to PTB and hnRNP C were detected (Fig. 6A, lane 1). Unlabeled competitor RNA corresponding to HCV 3′NTR nt 1–35 did not compete for binding of these two proteins (Fig. 6A, lane 2), but RNA corresponding to nt 1–205 and 1–218 (Fig. 6A, lanes 3 and 4, respectively) did compete. To further localize the sequence requirements for protein binding, additional competitor RNAs spanning 3′NTR nt 178–197, 138–177 and 169–197 were included in the binding reactions (Fig. 6A, lanes 5, 6 and 7). From these results it can be clearly concluded that the region upstream of the 3′ X-tail element contains critical determinants for recognition by hnRNP C and PTB, and that the U/C-rich elements are important for binding. To examine if recombinant PTB exhibited the same binding specificity as HepG2 cell-derived PTB, a cross-linking experiment employing the same unlabeled competitor RNAs was performed (Fig. 6B). The results of this experiment strongly suggest that purified, recombinant PTB clearly exhibits specific binding to the pyrimidine-rich region of the HCV 3′NTR (nt 138–177; see Fig. 7 for a summary of competition studies); we further conclude that both hnRNP C and PTB do not bind to the 3′ X-tail sequence.

DISCUSSION

The results presented here demonstrate that two cellular protein species of 35 and 57 kDa derived from HepG2 cytoplasmic extracts specifically interact with and could be covalently crosslinked using UV light to RNA sequences comprising the 3′NTR of HCV. These two proteins were identified as hnRNP C and PTB based on immunoprecipitation analysis and studies with purified recombinant PTB. We further localized PTB and hnRNP C binding within the HCV 3′NTR to the polypyrimidine tract stretch located between the non-conserved region and the 3′ X-tail. Interestingly, we were unable to show that the 5′NTR was able to efficiently compete for PTB binding to the 3′NTR despite a previous report that PTB interacts with the HCV 5′NTR (24). This apparent difference may be explained by the fact that the 3′NTR contains a long polypyrimidine tract (137 bases) and may therefore contain more potential binding sites for PTB; thus it may be a much more efficient competitor for PTB binding than

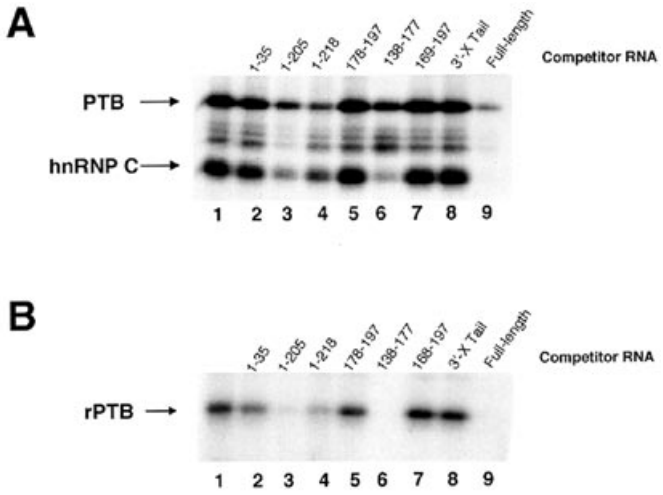

Figure 6. Localization of HCV 3′NTR sequences required for hnRNP C and PTB binding. (**A**) Radiolabeled HCV 3′NTR RNA was cross-linked with HepG2 cytoplasmic extracts with or without a 25-fold molar excess of the indicated RNAs. Bands corresponding to PTB and hnRNP C are indicated to the left. (**B**) Purified, recombinant PTB was cross-linked to radiolabeled HCV 3′NTR RNA with or without a 25-fold molar excess of the indicated competitor RNAs.

the 5′NTR under the experimental conditions used here. In fact, we did detect competition by the 5′NTR RNA when higher levels of RNA were used (data not shown). It is noted, however, that another study examining cellular proteins that bound to the HCV 5′NTR did not report PTB binding to the HCV 5′NTR (25). Rather, they reported that two cellular proteins (87 and 120 kDa) bound to the HCV 5′NTR.

Two other groups have recently reported binding of PTB to the HCV 3′NTR (9,14). In contrast to our findings, Tsuchihara *et al.* (14) concluded that PTB binds specifically to a 26 nt region at the 3′-end of the HCV RNA genome containing the 5′-terminal 19 nt of the 3′ X-tail and the 7 nt immediately upstream. From our own experimental data (Fig. 6), PTB (as well as hnRNP C) is clearly not competed by a 25-fold excess of unlabeled RNA corresponding to this region (nt 168–197). Furthermore, they state as data not shown that poly(U) was only a weak competitor of PTB binding to the 3′ X-tail, which is very unusual given that a strong interaction of PTB and poly(U) is well-documented by others (21,26), and that PTB is also routinely purified to homogeneity using a poly(U) matrix. Ito and Lai (9) also reported binding of PTB to the HCV 3′NTR, but they localize the binding site to sequences within the 3′ X-tail region (nt 178–197 by our numbering convention). Again, our experimental data do not reproduce this result, as unlabeled RNA corresponding to the exact same sequence identified by Ito and Lai (9) as containing the primary binding determinant for PTB clearly is unable to compete for PTB binding to the labeled full-length HCV 3′NTR sequence. Further, when we used labeled HCV 3′ X-tail as the RNA binding substrate, we were unable to detect binding of PTB (data not shown). Tsuchihara *et al.* (14) used cell extracts prepared from the non-neoplastic hepatocyte cell line PH5CH in their localization studies, and they state as data not shown that they observe similar binding in several different mammalian cell extracts. Although Ito and Lai (9) used HeLa cell extracts in their reported binding studies, they also show similar binding using extracts prepared from five different cell lines (including HepG2 cells) and from mouse and human primary hepatocytes. Because our studies also used cell extracts derived from HepG2 cells, a human hepatoblastoma cell line, a difference in cell type does not seem likely to account for the differences we observed in binding specificity. Using a large panel of unlabeled competitor RNAs, we also demonstrated unequivocally that purified PTB binds to the UC-rich region located upstream of the 3′ X-tail element. It is worth noting here that the sequence of the HCV 3′NTR upstream of the 3′ X-tail contains at least seven repeats of the sequence 'UCUU', which is the exact core consensus sequence element that was identified in a stringent *in vitro* selection procedure as a high-affinity binding site for PTB (15).

The identification of hnRNP C as another cellular protein that exhibits an affinity for the pyrimidine-rich region within the HCV 3′NTR is interesting, particularly in light of the fact that hnRNP C has been identified as having potent RNA annealing activity (27). It has been suggested that one function of hnRNP proteins may be to modulate the interactions between RNAs and trans-acting factors, possibly by altering RNA conformation; hnRNPs may also act as molecular 'matchmakers', in that they may recruit other protein factors to the RNA, possibly through protein–protein interactions (27). Interestingly, Hsieh *et al.* (28) recently reported that another hnRNP protein, hnRNP K, interacts with a hydrophilic region of the N-terminus of the HCV core

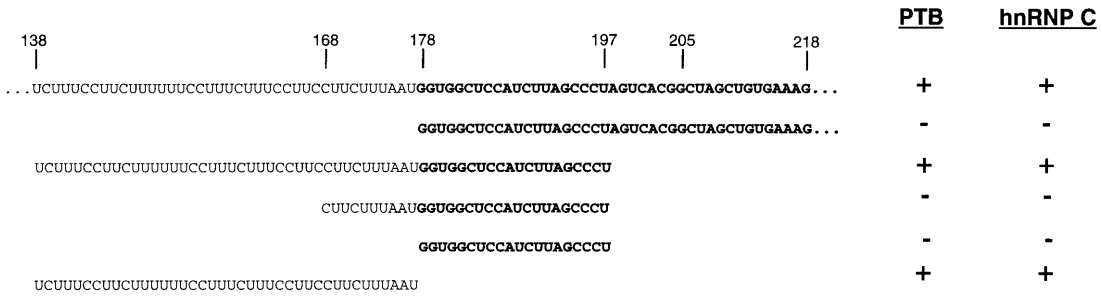

Figure 7. PTB and hnRNP C recognize the pyrimidine-rich region upstream of the HCV 3′ X-tail element. HCV 3′NTR sequences 138–218 are shown (3′ X-tail sequences are in bold). Binding data are qualitatively summarized from the results shown in Figures 3–6. +, competition observed at a 25-fold molar excess of unlabeled competitor RNA; –, no observed competition.

protein. Thus, one possible role for hnRNP protein binding to the pyrimidine-rich tract within the HCV 3′NTR may be to modulate the secondary and/or tertiary structure of the viral RNA to facilitate recognition by the viral replicase complex; that hnRNPs may also be involved in protein–protein interactions further suggests that they may also participate in the recruitment of such factors.

In the *Flaviviridae*, it has been proposed that the 3′NTR serves as the initiation site for the replicase complex for minus strand synthesis. Similar to findings obtained with other RNA viruses (29–34), several studies support the idea that this event involves the interaction of both viral and host factors with the sequences and structures in the 3′NTR (35–37). Deletions made within the 3′NTR of infectious cDNA clones for several members of the *Flaviviridae* family such as Dengue type 4 virus (38), Kunjin virus (39), tick-borne encephalitis virus (40), bovine viral diarrhea virus (41) and Dengue type 2 virus (42) had various effects ranging from either abolishing virus replication, causing growth restriction in cell culture, or reducing or preventing the development of viremia in infected animals. Although these 3′NTR elements are essential for virus growth in several cases, it is still unknown how they function at a molecular level. Although the interaction of PTB with IRESs in the 5′ non-coding regions of several members of the *Picornaviridae*, and its role in the initiation of translation has been previously described and well characterized (26,43–49), a role for this protein in viral RNA replication is still not clear; however, Kaminski *et al*. (49) recently reported that PTB-binding to the encephalomyocarditis virus (EMCV) IRES may help to maintain the appropriate higher-order structure of the RNA. One can envisage that it may play a similar role by interacting with the pyrimidine-rich region within the HCV 3′NTR RNA. The recent development of infectious cDNA clones of HCV (50–52) will allow the biological relevance of these elements to be experimentally addressed. Recently, it has been shown that deletion of the polypyrimidine tract from the HCV 3′NTR abolishes the infectivity of RNA transcripts from an infectious cDNA clone of HCV in chimpanzees, demonstrating that this region is essential for virus replication (53). Interestingly, in another study using HCV genomic RNA transcripts from infectious cDNAs, two different lengths of polypyrimidine tract (75 and 133 bases) were tested for infectivity in chimpanzees (50). Although both were capable of initiating an infection, the HCV RNA with the longer polyU/UC tract appeared to be preferred, suggesting that the longer polyU/UC tract may have conferred a selective advantage to the virus in the host. From this, it is tempting to speculate that the essentiality of the polypyrimidine tract is in part due to its interaction with hnRNP C and PTB. Studies with purified HCV NS5B RNA-dependent RNA polymerase (RdRp) indicate that, *in vitro*, this protein demonstrates no apparent specificity for viral RNA templates (54–58), although it binds well to poly(U) (56). It is possible that the interaction of hnRNP C and PTB with the polypyrimidine tract of the HCV genomic RNA may help regulate the initiation or elongation steps of viral RNA replication *in vivo* by direct interaction with components of the viral replicase complex such as NS5B.

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