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Specific Interaction of Polypyrimidine Tract-Binding Protein with the Extreme 3′-Terminal Structure of the Hepatitis C Virus Genome, the 3′X

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We previously identified a highly conserved 98-nucleotide (nt) sequence, the 3′X, as the extreme 3′-terminal structure of the hepatitis C virus (HCV) genome (T. Tanaka, N. Kato, M.-J. Cho, and K. Shimotohno, Biochem. Biophys. Res. Commun. 215:744–749, 1995). Since the 3′ end of positive-strand viral RNA is the initiation site of RNA replication, the 3′X should contribute to HCV negative-strand RNA synthesis. Cellular factors may also be involved in this replication mechanism, since several cellular proteins have been shown to interact with the 3′-end regions of other viral genomes. In this study, we found that both 38- and 57-kDa proteins in the human hepatocyte line PH5CH bound specifically to the 3′-end structure of HCV positive-strand RNA by a UV-induced cross-linking assay. The 57-kDa protein (p57), which had higher affinities to RNA probes, recognized a 26-nt sequence including the 5′-terminal 19 nt of the 3′X and 7 flanking nt, designated the transitional region. This sequence contains pyrimidine-rich motifs and shows similarity to the consensus binding sequence of the polypyrimidine tract-binding protein (PTB), which has been implicated in alternative pre-mRNA splicing and cap-independent translation. We found that this 3′X-binding p57 is identical to PTB. The 3′X-binding p57 was immunoprecipitated by anti-PTB antibody, and recombinant PTB bound to the 3′X RNA. In addition, p57 bound solely to the 3′-end region of positive-strand RNA, not to this region of negative-strand RNA. We suggest that 3′X-PTB interaction is involved in the specific initiation of HCV genome replication.

In a positive-stranded RNA virus, the 3′ end of the viral genome is the initiation site for the synthesis of negative-strand RNA, an intermediate product of RNA replication. The 3′-end region of genomic RNA often forms stem-loop structures and interacts with viral and cellular proteins in RNA replication processes (3, 15, 20). Hepatitis C virus (HCV) (7, 17, 35), a member of the *Flaviviridae* family of positive-stranded RNA viruses, possesses a unique structure at the 3′-end region of the genome. The 3′ untranslated region (UTR) of HCV consists of three elements, a 30- to 40-nucleotide (nt) region of conventional 3′ UTR after the terminal codon, followed by a 20- to 200-nt poly(U) stretch and a specific 98-nt sequence, the 3′X, downstream of the poly(U) stretch which we recently identified (36, 37). The 3′X sequence is highly conserved among HCV genotypes, in contrast to the conventional 3′ UTR sequence, which is less conserved (13, 19, 37, 42). The 3′X RNA probably forms stable secondary structures and may therefore interact with viral proteins such as RNA polymerase (19, 36, 37, 42). The 3′X may act as a *cis* element, restricting the initiation point of RNA replication. RNA polymerase may require other host cellular proteins which interact with the viral genome, especially with the 3′X, and take part in the initiation of RNA replication. Although a number of host cellular proteins have been shown to interact with the 3′-end regions of genomic RNAs of several viruses, little is known about the features and functions of these proteins (3, 8, 15, 20, 21, 24–26, 32, 39, 43, 44). No interactions between the 3′-terminal structure and cellular proteins in HCV have previously been reported. We have identified a cellular protein that binds to the 3′X of the HCV genome; it is likely to be polypyrimidine tract-binding protein (PTB).

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MATERIALS AND METHODS

PCR. To make specific DNA fragments, PCR was performed with Pfu DNA polymerase for high-fidelity amplification. The reaction mixture (50 μl) contained 5 μl of 10-fold-concentrated Pfu buffer (Stratagene), forward and reverse primers (0.8 μM [each]), 0.2 mM (each) deoxynucleoside triphosphates, and 1 ng of template DNA. After the samples were denatured at 98°C for 2 min, 1.25 U of native Pfu DNA polymerase (Stratagene) was added at 82°C and 30 cycles of PCR (40 s at 93°C, 45 s at 55°C, and 60 s at 72°C) were performed.

Construction of plasmids. Plasmid pUC 9106-3′X carries the 3′-end sequences derived from the HCV-JS (34) genome, including nt 9106 to 9407, the poly(U) stretch, and the 3′X tail. The insert fragment of pUC 9106-3′X was made by PCR with primers ET7 9106 and SXRP2. The PCR template was the 3′-end clone of HCV-JS isolated from HCV-infected MT-2C cells (34). Forward primer ET7 9106 (5′-CCCCCGAATTCTG**TAATACGACTCACTATA**GGGCCAGAGG TGTCCGCGCGCT-3′) corresponds to nt 9106 to 9125 of the HCV-JS sequence with an additional sequence containing an *Eco*RI site (underlined) and T7 promoter sequence (boldface). Reverse primer SXRP2 (5′-AATACGTCGACT CTAGACATGATCTGCAGAGAGGCC-3′) is complementary to nt 79′ to 98′ of the 3′X sequence with a tag sequence containing a *Sal*I site (underlined). PCR was done as described above. The PCR product was digested with *Eco*RI and *Sal*I and ligated into the *Eco*RI-*Sal*I site of pUC18. The resulting plasmid was pUC 9106-3′X.

Plasmid pSP318-1R carries a sequence complementary to nt 1 to 318 of HCV-JT (38) under the control of an SP6 promoter sequence and was used to synthesize the 3′-end region of negative-strand HCV RNA. The insert sequence was obtained by PCR with primers HCV1EH (5′-TCAAGCTT**ATG**

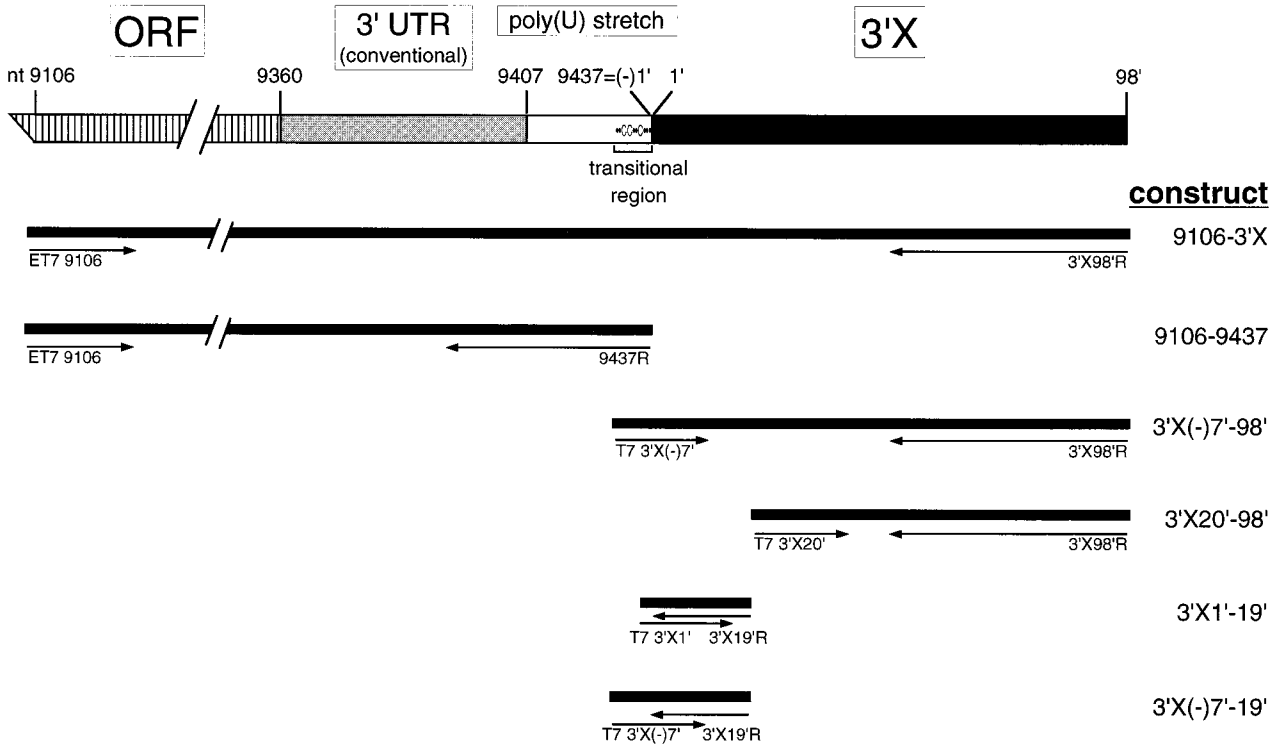

FIG. 1. Maps of RNA constructs representing the 3′-end region of the HCV genome. The structure of the 3′-end region from HCV-JS is shown at the top. The PCR primers used to make each corresponding DNA fragment (see Materials and Methods) are also shown. Nucleotides in the 3′X are numbered 1′ to 98′ to distinguish them from those in conventional HCV sequences. Nucleotides at the end of the poly(U) stretch (transitional region) are shown as negative numbers with primers in relation to nucleotides of the 3′X(−1 = nt 9437). ORF, open reading frame.

CATGCCAGCCCCCGATTGGGGG-3′) and HCV299RX (5′-TACTCTAGA GGGGCACTCGCAAGCACCCT-3′) on a template HCV-JT sequence. Forward primer HCV1EH has nt 1 to 20 of the HCV-JT sequence with a tag containing *Hin*dIII (underlined) and *Eco*T22I (boldface) sites, and reverse primer HCV299RX is complementary to nt 299 to 318 of the HCV-JT sequence with an *Xba*I tag sequence (underlined). The *Hin*dIII-*Xba*I-digested PCR product was inserted into the *Xba*I-*Hin*dIII site of pSP65 (Promega). The resulting plasmid, pSP318-1R, has nt 1 to 318 of the HCV-JT sequence with reverse direction under the control of an SP6 promoter sequence.

Synthesis of nonlabeled and labeled 3′-end RNAs of HCV. Figure 1 shows maps of the 3′-end RNA constructs of HCV (positive strand). All RNAs were made by using an in vitro transcription kit (MEGAscript; Ambion, Inc.) with bacteriophage T7 RNA polymerase according to the manufacturer's protocol. To synthesize specific-sized RNAs, PCR-made DNA was employed as a template for in vitro transcription (Table 1). All forward primers for the first PCR contained the 3′-terminal 10 nt of the T7 promoter. The first PCR was done with 1 ng of pUC 9106-3′X as the template. Nonspecific larger bands which could be produced by misannealing of forward primer to upstream T7 sequence in pUC 9106-3′X were not detected. The second PCR used 3 μl of the first-PCR products as the template. The forward primer of the second PCR, T7pro (Table 1), contained an intact T7 promoter sequence; therefore, resulting DNA fragments were driven under the T7 promoter. The products were purified by agarose gel electrophoresis, extracted with a GENECLEAN fragment extraction kit (5 PRIME→3 PRIME, Inc.), and subjected to in vitro transcription. To make labeled RNA, 50 μCi of [α-^{32}P]UTP (800 Ci/mmol; Du Pont/NEN Research Products) was added to the reaction mixture where the cold UTP concentration was reduced to 0.375 mM (1/20 of the standard concentration).

To make 1-318R RNA, which represents the 3′-end region of the negative-strand HCV RNA genome, in vitro transcription was performed with *Eco*T22I-cut pSP318-1R DNA as the template and an SP6 RNA polymerase version of the MEGAscript kit. For labeling, 50 μCi of [α-^{32}P]UTP was added to the reaction mixture where the cold UTP concentration was reduced to 0.25 mM (1/20 of the standard concentration).

A DNA fragment representing 26 nt of PTB consensus binding sequence (33) was produced as follows. Partially complementary oligonucleotides PTB-F (5′-CTCACTATAGGCAGCCTGGTGCCTCCCT-3′) and PTB-R (5′-GGGCC AAGAGGGAGGCAC-3′) (2.0 μM [each]) were annealed, and end filling was performed by PCR without additional oligonucleotides. PTB-F contained the 3′-terminal 10 nt of the T7 promoter. The second PCR was done with 3 μl of the first-PCR products by using primers T7pro and PTB-R. The products were purified and subjected to in vitro transcription.

Preparation of cell extracts. S10 extracts from the nonneoplastic hepatocyte line PH5CH were prepared as described previously with some modifications (20, 21, 43). Briefly, PH5CH cells (10^7) were incubated with 0.5 mM diisopropylphosphofluoridate at 37°C for 5 min, washed with ice-cold phosphate-buffered saline twice, and collected as a cell pellet. Cells were frozen at −80°C, thawed, suspended in 100 μl of hypotonic buffer (5 mM Tris-HCl [pH 7.5], 25 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 5% glycerol, and 1 μg (each) of *Escherichia coli* tRNA and heparin sodium salt (Sigma) to reduce nonspecific RNA-protein interactions. The reaction was started by the addition of ^{32}P-labeled RNA probes and continued at 30°C for 30 min. For competition assays, competitor RNAs were added to the reaction mixture 10 min prior to the addition of radiolabeled probes. The resulting RNA-protein complexes were irradiated with a 254-nm-wavelength UV lamp (Bio-Rad), held 10 cm from the light source, on ice for 15 min. The samples were then incubated with RNase A (1.0 μg) and RNase T_1 (1.0 U) for 15 min at 37°C to digest the unbound region of the probe RNA. After RNase treatment, the samples were electrophoresed on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel.

UV-induced cross-linking assay. RNA-protein binding was detected by UV-induced cross-linking assay as described previously (22) with some modifications. The reaction mixture (10 μl) contained 50,000 cpm of ^{32}P-labeled RNA probe, PH5CH S10 extract (5 μg of protein), 5 mM Tris-HCl (pH 7.5), 25 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 5% glycerol, and 1 μg (each) of *Escherichia coli* tRNA and heparin sodium salt (Sigma) to reduce nonspecific RNA-protein interactions. The reaction was started by the addition of ^{32}P-labeled RNA probes and continued at 30°C for 30 min. For competition assays, competitor RNAs were added to the reaction mixture 10 min prior to the addition of radiolabeled probes. The resulting RNA-protein complexes were irradiated with a 254-nm-wavelength UV lamp (Bio-Rad), held 10 cm from the light source, on ice for 15 min. The samples were then incubated with RNase A (1.0 μg) and RNase T_1 (1.0 U) for 15 min at 37°C to digest the unbound region of the probe RNA. After RNase treatment, the samples were electrophoresed on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel.

Immunoprecipitation of the PTB–HCV 3′-end RNA complex. Rabbit anti-PTB antiserum was prepared as described previously (40). Briefly, 22-mer peptides corresponding to amino acids 267 to 288 of human PTB-1 (11) were synthesized, conjugated to keyhole limpet hemocyanin, and used to inoculate Japan White rabbits.

The RNA-protein binding reaction was performed with ^{32}P-labeled 3′X(−)7′-98′ RNA and PH5CH S10 as described above. After UV-induced cross-linking and RNase treatment, the samples were diluted with 250 μl of extraction buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM DTT, 100 mM NaCl, and 0.05% Nonidet P-40. The mixture was incubated with 10 μl of anti-PTB antiserum at 4°C for 1 h on a rotary shaker. The immunocomplexes were bound to protein G-Sepharose beads (Pharmacia) and washed four times with extrac-

TABLE 1. Oligonucleotides used to synthesize template DNA for in vitro transcription[a]

Construct	1st-PCR primer	Sequence	2nd-PCR primer	Sequence
9106-3′X	ET7 9106	CCCCCGAATTCTGTAATACGACTCACTATAGGGCCAGAGGTGTCCGCGCGCT	T7pro	GGATCCTAATACGACTCACTATAG
	3′X98′R	ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTCTGCAGTCATGCGGCT	3′X98′R	ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTCTGCAGTCATGCGGCT
9106-9437	ET7 9106	CCCCCGAATTCTGTAATACGACTCACTATAGGGCCAGAGGTGTCCGCGCGCT	T7pro	GGATCCTAATACGACTCACTATAG
	9437R	AAAGAAGAAAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGAATGGCCTATTGGC	9437R	AAAGAAGAAAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGAATGGCCTATTGGC
3′X(−)7′-98′	T7 3′X(−)7′	CTCACTATAGGCTTCTTTGGTGGCTCCATC	T7pro	GGATCCTAATACGACTCACTATAG
	3′X98′R	ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTCTGCAGTCATGCGGCT	3′X98′R	ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTCTGCAGTCATGCGGCT
3′X20′-98′	T7 3′X20′	CTCACTATAGGTAGTCACGGCTAGCTGTGA	T7pro	GGATCCTAATACGACTCACTATAG
	3′X98′	ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTCTGCAGTCATGCGGCT	3′X98′R	ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTCTGCAGTCATGCGGCT
3′X1′-19′	T7 3′X1′	CTCACTATAGGTGGCTCCATCTTAG	T7pro	GGATCCTAATACGACTCACTATAG
	3′X19′R	GGGCTAAGATGGAGCCACC	3′X19′R	GGGCTAAGATGGAGCCACC
3′X(−)7′-19′	T7 3′X(−)7′	CTCACTATAGGCTTCTTTGGTGGCTCCATC	T7pro	GGATCCTAATACGACTCACTATAG
	3′X19′R	GGGCTAAGATGGAGCCACC	3′X19′R	GGGCTAAGATGGAGCCACC
PTB consensus	PTB-F	CTCACTATAGGCAGCCTGGTGCCTCCCT	T7pro	GGATCCTAATACGACTCACTATAG
	PTB-R	GGGCCAAGAGGGAGGCAC	PTB-R	GGGCCAAGAGGGAGGCAC

[a] DNA fragments corresponding to each RNA construct were obtained by nested PCR with the indicated primer sets. PCR products were used as templates for in vitro transcription as described in Materials and Methods.

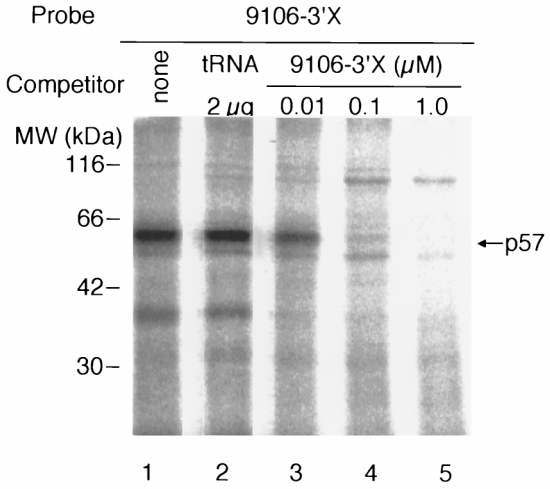

FIG. 2. Detection of cellular proteins which specifically bind to the 3′ end of HCV RNA by UV-induced cross-linking assay. ^{32}P-labeled 9106-3′X RNA probe was cross-linked with PH5CH S10 extract in the presence or absence (none) of competitors and electrophoresed on an SDS–10% polyacrylamide gel as described in Materials and Methods. Lane 1, no competitor; lane 2, 2.0 μg of tRNA as a nonspecific competitor; lanes 3 to 5, indicated concentrations of specific competitor 9106-3′X. The equivalent of 1.0 μM 9106-3′X RNA is 2.0 μg.

tion buffer. The materials bound to protein G-resin were electrophoresed on an SDS–10% polyacrylamide gel.

Expression of recombinant PTB. Plasmid pMalc-PTB1, which encodes the maltose-binding protein (MBP)–PTB-1 fusion protein, was constructed by inserting the full-length coding sequence of PTB-1 cDNA (11) except the first ATG codon into the *Stu*I and *Sal*I sites of plasmid pMAL-c (New England Biolabs, Inc.). The expression of MBP–PTB-1 in *E. coli* JM109 cells was induced by 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 2 h. The MBP–PTB-1 fusion protein was affinity purified on amylose-resin beads (New England Biolabs, Inc.) according to the manufacturer's protocol. A nonfusion form of MBP was also expressed by using plasmid pMAL-c in JM109 and purified on amylose-resin.

Nucleotide sequence accession number. The nucleotide sequence of pUC 9106-3′X has been deposited in the DDBJ, EMBL, and GenBank databases under accession no. AB001040.

RESULTS

PH5CH cell proteins that bind HCV 3′-end RNA. Hepatocytes are natural targets for HCV infection. Recently, we found that the nonneoplastic hepatocyte line PH5CH supported the replication of HCV in vitro (18) and may therefore be used to isolate specific cellular proteins which interact with the 3′-end region of HCV RNA and function in genomic replication. A UV-induced cross-linking assay was performed with S10 fractions of PH5CH cells to identify specific RNA-binding proteins. An HCV 3′-end RNA construct (9106-3′X) was designed to contain the 3′-end 269 nt of the open reading frame, the full-length conventional 3′ UTR, a 26-nt poly(U) stretch that includes the transitional region at the 3′ terminus where several cytosine residues appear (36, 37), and the full-length (98 nt) 3′X sequence (Fig. 1). The ^{32}P-labeled 9106-3′X RNA probe was incubated with PH5CH S10 extract and cross-linked by UV irradiation. Unbound probe RNA was digested by RNase A and RNase T_1, and the resulting RNA-protein complex was analyzed by SDS–10% polyacrylamide electrophoresis (PAGE). Five bands, whose molecular masses were estimated to be 110, 95, 57, 50, and 38 kDa, were detected by autoradiography (Fig. 2, lane 1). These bands disappeared after the reaction mixture was digested with proteinase K (data not shown), indicating that the bands were protein-RNA cross-linked complexes. The 57-kDa protein appeared most abundantly in PH5CH S10 extract, as reflected in the strength of the

signal on the radiogram (Fig. 2, lane 1). A shortly exposed radiogram showed the 57-kDa protein as a doublet band (data not shown).

A competition assay was used to confirm the specificities of these RNA-protein interactions. An additional 2.0 μg of *E. coli* tRNA (total, 3.0 μg) was tested as a nonspecific competitor. The intensities of the major RNA-binding protein bands were not reduced by additional tRNA (Fig. 2, lane 2). On the other hand, the 57- and 38-kDa signals were reduced by specific competitor 9106-3′X in a dose-dependent manner, but enhancement of the 110-, 95-, and 50-kDa bands was observed under these conditions (Fig. 2, lanes 3 to 5). Therefore, only the 57- and 38-kDa proteins bound specifically to the 9106-3′X RNA. We performed the same UV-induced cross-linking assay with S10 extracts of rabbit liver tissues and COS-1 cells, which are a nonhepatic monkey cell line. We found that the 57-kDa protein which specifically interacted with the 3′-end region of HCV RNA was present in these cell extracts (data not shown), suggesting that this protein is not specific to the human liver.

Mapping of protein binding sites at the 3′ end of HCV RNA. Our attention was focused on the most efficient RNA-binding 57-kDa protein (p57). The site of p57 binding to 9106-3′X RNA was examined by using various fragments of 3′-end HCV RNA as competitors in the UV-induced cross-linking assay. Maps of the 3′-end RNA constructs are shown in Fig. 1. 9106-9437 RNA lacks the 3′X sequence. 3′X(−)7′-98′ RNA contains the transitional region (7 nt) and full-length 3′X sequence.

The binding of 9106-3′X RNA to p57 was competed by an excess amount (0.1 μM) of cold 9106-3′X RNA (Fig. 3A, lane 2). Similarly, 0.1 μM RNA competitor 3′X(−)7′-98′ blocked RNA-p57 interaction and RNA competitor 9106-9437 slightly blocked RNA-protein binding (Fig. 3A, lanes 3 and 4, respectively). These results suggest that the p57 binding site is located across the transitional region and the 3′X.

By focusing further on the p57 binding site within the 3′X, the computer-predicted secondary structure suggested that the 3′X forms three stem-loop structures (19, 36, 42) which are involved in the interaction with p57. These structures were named (in order from 5′ to 3′) stem-loop I (nt 1′ to 20′), stem-loop II (nt 21′ to 52′), and stem-loop III (nt 53′ to 98′). The competitive activities of 3′X(−)7′-19′, corresponding to the transitional region and stem-loop I, and 3′X20′-98′, corresponding to stem-loop II and stem-loop III, were tested against 3′X(−)7′-98′–p57 binding. As shown in Fig. 3B, 3′X(−)7′-19′ RNA completely blocked the binding between p57 and RNA probe 3′X(−)7′-98′, as was seen with cold 3′X(−)7′-98′ RNA (lanes 2 and 3), but 3′X20′-98′ RNA was far less competitive for this RNA-protein binding (lane 4). These results show that p57 interacts most efficiently with a sequence that includes the transitional region and stem-loop I of the 3′X.

To confirm this observation, we made ^{32}P-labeled RNA probe 3′X(−)7′-19′ and tested its p57 binding ability. 3′X(−)7′-19′ RNA bound specifically to p57 (Fig. 3C, lanes 1 and 2). Furthermore, RNA probe 3′X1′-19′, which contains only stem-loop I, also bound to p57, but the strength of the signal was weaker than that of RNA probe 3′X(−)7′-19′ (Fig. 3C, lanes 3 and 4).

The 3′X-binding 57-kDa protein was PTB. The sequence of 3′X(−)7′-19′ RNA is aligned with the consensus binding sequence of PTB in Fig. 4A (33). The transitional region is a pyrimidine cluster, and the 3′-terminal 19 nt of the 3′X are also pyrimidine rich, suggesting that p57 recognizes pyrimidine clusters. It is known that several eukaryotic RNA-binding proteins preferentially interact with pyrimidine-rich sequences (33). PTB, a tentative pre-mRNA splicing repressor, is one

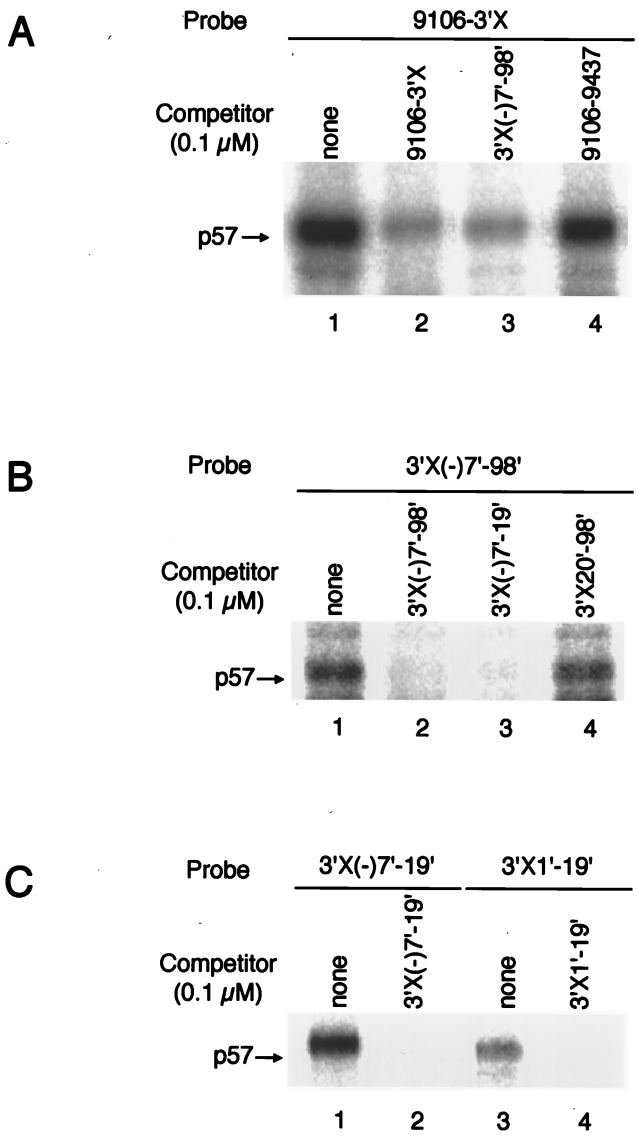

FIG. 3. Mapping of the p57 binding site within 9106-3′X RNA. (A) Competition assay for 9106-3′X RNA–p57 binding was performed with no competitor (none; lane 1) or with the indicated competitors (0.1 μM [each]; lanes 2 to 4). (B) Competition assay with the p57 binding site sequence. Probe 3′X(−)7′-98′ was cross-linked with PH5CH S10 in the absence (none; lane 1) or presence of 0.1 μM (each) specific competitor (lanes 2 to 4). (C) Determination of the binding site. The indicated RNA probes were cross-linked with PH5CH S10 in the absence (none; lanes 1 and 3) or presence of 0.1 μM (each) specific competitor (lanes 2 and 4).

such protein and has a molecular mass of 57 kDa (5, 16). As shown in Fig. 4A, the p57 binding site [3′X(−)7′-19′] sequence resembles the PTB consensus binding sequence (69.2% [18 of 26 residues identical]).

To determine whether p57 is PTB, we first tested the PTB consensus binding sequence as a competitor in our p57-RNA binding assay. A 26-nt fragment of PTB consensus sequence RNA (GCAGCCUGGUGCCUCCCUCUUGGCCC) was synthesized by in vitro transcription and used as a competitor for binding between 3′X(−)7′-98′ and p57. RNA-p57 complex formation was blocked by PTB consensus RNA as efficiently as it was by 3′X(−)7′-98′ competitor (data not shown).

Immunoprecipitation of cross-linked RNA-p57 complexes

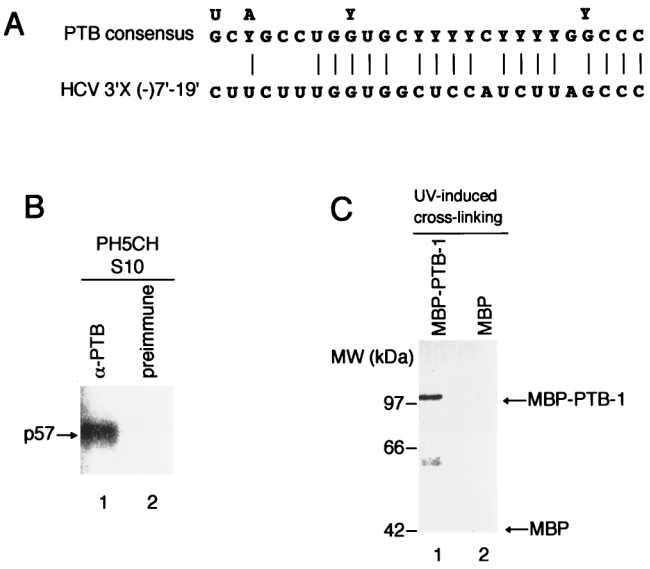

FIG. 4. Confirmation of p57 identity with PTB. (A) Alignment of PTB consensus binding and p57 binding sequences. Vertical lines indicate identical residues. (B) Immunoprecipitation of p57 with anti-PTB antibody. PH5CH proteins cross-linked with probe 3′X(−)7′-98′ were immunoprecipitated with anti-PTB antiserum (α-PTB; lane 1) or preimmune serum (lane 2). (C) Interaction between recombinant PTB and 3′X RNA. UV cross-linking assays were performed with probe 3′X(−)7′-98′ and affinity-purified MBP–PTB-1 (lane 1) or MBP (lane 2).

by anti-PTB antibody was attempted to further confirm that p57 is homologous to PTB. The antibody against PTB-1 recognized endogenous PTB from HeLa (40) and PH5CH (data not shown) cell extracts by Western blot analysis. This antibody also specifically recognized recombinant PTB-1 by Western blot analysis (data not shown). The 57-kDa protein which was cross-linked with radiolabeled 3′X(−)7′-98′ RNA was immunoprecipitated from the reaction mixture by anti-PTB antiserum, but the RNA-p57 complex was not precipitated by preimmune serum (Fig. 4B).

A PTB fusion protein (MBP–PTB-1) which was expressed in *E. coli* and affinity purified with amylose-resin was used to examine PTB affinity for the 3′X RNA. The molecular mass of purified MBP–PTB-1 was estimated to be 97 kDa by SDS-PAGE (data not shown). The 97-kDa MBP–PTB-1 fusion efficiently cross-linked with probe 3′X(−)7′-98′ RNA, but no binding between the nonfusion form of MBP (42 kDa) and 3′X(−)7′-98′ RNA was observed (Fig. 4C). This complex of the 3′X and recombinant PTB was also immunoprecipitated with anti-PTB-1 antibody (data not shown). We conclude that the 3′X-binding 57-kDa protein in hepatocytes is PTB.

p57 binds specifically to the 3′ end of positive-strand HCV RNA but not to the negative strand. During initiation of HCV genomic replication, p57 may also potentially interact with the 3′ end of HCV negative-strand RNA. To examine this, a UV-induced cross-linking assay with RNA probe 318-1R, which corresponds to the 318 nt of the 3′ end of HCV negative-strand RNA, was performed. The assay results revealed that the 318-1R probe bound specifically to 120-, 70-, and 50-kDa proteins but not to a 57-kDa protein (Fig. 5, lanes 1 and 2). Nonlabeled 3′X(−)7′-98′ RNA did not compete for protein binding with negative-strand RNA (Fig. 5, lane 3). Furthermore, nonlabeled 318-1R RNA did not prevent interaction between 3′X RNA and p57 (Fig. 5, lanes 4 to 6). The p57 hepatocyte protein, which is probably PTB, binds specifically to the 3′ end of HCV positive-strand RNA but not to the negative strand.

DISCUSSION

Here, we have demonstrated that various hepatocyte cellular proteins may interact with the 3′-end region of HCV positive-strand RNA (Fig. 2). Of these proteins, a 57-kDa protein (p57) clearly showed specific binding to the 3′X, the extreme 3′-terminal structure of the HCV genome (Fig. 3A). Specifically, the p57 binding site was located at the 5′-terminal 19 nt of the 3′X and 7 flanking nt of the transitional region (Fig. 3B and C). The 5′-terminal nucleotide sequence of the 3′X is perfectly conserved among HCV genotypes and corresponds to the 5′ first stem-loop structure of the 3′X, suggesting an important role for this region (37). A common feature of the transitional region is the appearance of several C, CC, or CCC residues at intervals of two to five U residues, and the sequence of this region is considerably heterogeneous even within individuals (36, 37). The transitional region may act as pyrimidine-rich tract different from a pure poly(U) stretch.

It was important to determine whether p57 is a novel protein. Pyrimidine motifs were noted in the 26-nt sequence recognized by p57 (Fig. 4A). In higher eukaryotes, pyrimidine-rich sequences are often recognized by several RNA-binding proteins, including the essential splicing factor U2AF^{65}, the splicing regulator Sex-lethal, and PTB, which is also known as hnRNP1 and is thought to regulate alternative splicing by selectively repressing 3′ splice sites (9–12, 23, 27, 33). From several lines of experimental evidence, we have concluded that p57 is PTB. (i) p57 appears as doublet bands by SDS-PAGE. PTB is also known to be a 57-kDa protein with isoforms which closely migrate around 57 kDa (5, 16, 27). (ii) The sequence of the p57 binding site is similar to the consensus binding sequence of PTB (Fig. 4A). PTB consensus sequence RNA blocked 3′X-p57 binding (data not shown). (iii) p57 reacted with anti-PTB antibody (Fig. 4B). (iv) Recombinant PTB-1 showed specific binding to the 3′X sequence (Fig. 4C). PTB is known to be a nuclear protein, but cytosolic localization has also been shown by immunofluorescence (10). Although we did not determine the subcellular localization of p57, we confirmed by Western blotting that the PH5CH S10 fraction con-

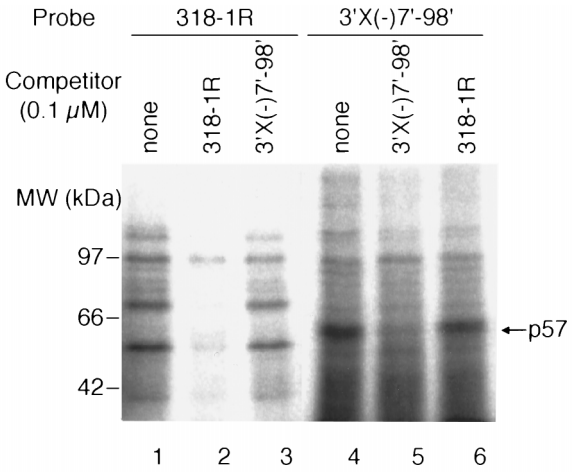

FIG. 5. p57 did not bind to the 3′-end region of HCV negative-strand RNA. UV-induced cross-linking assays of PH5CH S10 were performed with probe 318-1R (lanes 1 to 3) and probe 3′X(−)7′-98′ (lanes 4 to 6) in the presence of no competitor (none; lanes 1 and 4), 0.1 μM 3′X(−)7′-98′ (lanes 3 and 5), and 0.1 μM 318-1R (lanes 2 and 6).

tained PTB (data not shown). This also supports our conclusion. It is known that PTB has an affinity to poly(U). We observed that poly(U) weakly interfered with 3′X-p57 binding (data not shown) and that RNA competitor 9106-9437, which contained a poly(U) stretch without the 3′X, also weakly competed for 9106-3′X RNA–p57 binding (Fig. 3A, lane 4). These results suggest that the poly(U) stretch of the HCV genome also interacts with PTB but that the 5′ terminus of the 3′X binds to PTB with much greater specificity. The 7-nt sequence of the transitional region, which does not closely align with the PTB consensus sequence, was not essential for p57 binding but increased binding affinity (Fig. 3C).

Behrens et al. reported that HCV nonstructural protein 5B had RNA-dependent RNA polymerase (RdRp) activity (2). They used a conventional 3′ UTR sequence of HCV followed by a poly(U) stretch as a specific template for RdRp assay. However, a nonstructural protein 5B-mediated reaction was found not only with HCV 3′ UTR but also with unrelated input RNA, suggesting that the conventional 3′ UTR does not act as the specific recognition signal for viral RNA replication and that HCV RNA polymerase requires other factors to restrict the initiation of negative-strand synthesis from the extreme 3′ terminus of positive-strand RNA. The 3′X, which is the extreme 3′-end structure of positive-strand RNA, should be a *cis* element for negative-strand RNA synthesis; p57 may therefore act as a *trans* element to restrict the initiation of RNA synthesis.

Furthermore, we identified another feature of HCV RNA-p57 interaction; p57 bound specifically to the 3′ end of positive-strand RNA but not to that of negative-strand RNA (Fig. 5). Similar to p57, bacterial protein HF-I recognizes only the 3′ end of Qβ phage positive-strand RNA (31). HF-I is involved in the Qβ phage RdRp complex, which is a well-characterized component of viral RNA replication, and regulates the affinity of the RdRp to genomic RNA. Without HF-I, phage RdRp is incapable of initiating negative-strand RNA synthesis from the 3′ terminus of positive-strand RNA template, but positive-strand RNA is able to be synthesized. As a result of this differential efficiency of RNA synthesis, Qβ phage gets a larger amount of positive-strand RNA; such disproportional positive- and negative-strand phage RNA synthesis is advantageous for phage replication. It is possible that HCV RdRp regulates a similar process of disproportional RNA synthesis and that p57 regulates this mechanism by specific binding to positive-strand, not negative-strand, RNA.

PTB binds to the internal ribosomal entry site in several positive-strand RNA viruses, such as poliovirus (14, 28, 40), human rhinovirus (4, 30), encephalomyocarditis virus (5, 16, 41), hepatitis A virus (6), and HCV (1), and is involved in a cap-independent protein translation mechanism. Multiple PTB binding sites in the HCV internal ribosomal entry site have previously been reported (1), and we noticed that one of these sites, nt 110 to 136, shows similarity to the PTB binding sequence (19 of 26 nt match). PTB is a candidate enhancer of ribosome and viral RNA interactions because PTB associates with both ribosomes and RNAs (16, 27). Although there is no experimental evidence to indicate that ribosomes are involved in HCV RNA replication, it is not unusual for viral RNA replication to require host factors which are implicated in the translation mechanism. For example, brome mosaic virus RdRp requires the p41 subunit of wheat germ eukaryotic initiation factor 3, which is needed for 40S ribosome-mRNA binding (29), and Qβ phage RdRp requires ribosomal protein S1 for template recognition and initiation of RNA replication (31). It is possible that PTB connects ribosomes and HCV RNA and promotes RNA replication when the RNA replication mechanism of HCV requires ribosomal protein.

We have shown the first evidence that the 3′-end region of HCV RNA interacts with hepatocyte proteins, but the questions of whether 3′X-PTB interaction is necessary for HCV RNA replication in vivo and whether PTB is really a member of the replication machinery of HCV remain. Since an efficient virus replication system in vivo, as well as an in vitro system which mimics in vivo genomic replication initiated from the 3′X, remains to be established, the molecular mechanism of HCV replication is still unclear, but cellular factors such as PTB may play key roles in HCV replication.

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