Cellular Proteins Bind to the Poly(U) Tract of the 3’ Untranslated Region of Hepatitis C Virus RNA Genome

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Received November 17, 1998; returned to author for revision December 18, 1998; accepted February 3, 1999

UV cross-linking analyses were performed in an attempt to determine cellular protein–viral RNA interactions with the 3’ untranslated region (3’ UTR) of the hepatitis C virus RNA genome. Two cellular proteins, with estimated molecular masses of 58 kDa (p58) and 35 kDa (p35), respectively, were found to specifically bind to the 3’ UTR. The p58 protein was determined to be the polypyrimidine tract-binding protein. In addition to binding to the conserved 98 nucleotides (nt) of the 3’ UTR, p58 also binds to the poly(U) tract of the 3’ UTR. The p35 protein was found to interact only with the poly(U) tract of the 3’ UTR. These conclusions are supported by the following findings: (1) p58, and not p35, binds to the 3’ end conserved 98 nt, (2) both p58 and p35 bind to a 3’ UTR RNA with a deletion of the conserved 98 nt, (3) the 98-nt deletion mutant 3’ UTR competed out both p58 and p35 binding, (4) a poly(U) homopolymer competed out both p58 and p35 binding, (5) a 3’ UTR RNA with deletion of the poly(U) tract competed out only p58 binding but not p35 binding, and (6) an RNA containing the variable region of the 3’ UTR with a deletion of both poly(U) tract and 98 nt failed to compete for binding of either p58 or p35. Interaction of these cellular proteins with the HCV 3’ UTR is probably involved in regulation of translation and/or replication of the HCV RNA genome.

INTRODUCTION

Hepatitis C virus (HCV) is a positive-stranded RNA virus with a 9.5-kb RNA genome (Choo et al., 1989; Kato et al., 1990). The viral RNA is composed of a long 5’ untranslated region (5’ UTR) of 341 nucleotides, a single long open reading frame (ORF), and a 3’ untranslated region (3’ UTR) (Brown et al., 1992; Bukh et al., 1992; Han and Houghton, 1992; Kolykhalov et al., 1996; Major and Feinestone, 1997; Matsuura and Miyamura, 1993; Rice, 1996; Tanaka et al., 1995, 1996; Yamada et al., 1996). The ORF encodes a polyprotein of 3010–3040 amino acids, which is cotranslationally or posttranslationally processed by cellular and viral proteases into at least 10 structural and nonstructural proteins in the following order: C, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A, NS5B, (Choo et al., 1989; Kato et al., 1990; Matsuura and Miyamura, 1993; Miyamura and Matsuura, 1993; Rice, 1996). Based on the similarity of the genome organization and the amino acid sequences of HCV to those of pestiviruses and flaviviruses, it was classified as the third genus of the Flaviviridae family (Rice, 1996).

In the past several years, a great deal of progress has been made toward understanding the genomic organization of HCV, proteolytic processing of the viral polyprotein, and biochemical characterization of the individual viral proteins (Bartenschlager, 1997; Hijiikata et al., 1991; Major and Feinestone, 1997; Miyamura and Matsuura, 1993; Rice, 1996). Recent studies using recombinant systems indicated that the C, E1, and E2 proteins are sufficient for the assembly of viral particles (Baumert et al., 1998; Lagging et al., 1998). The nonstructural proteins NS2, NS3, and NS4A are viral proteases or cofactor required to process the viral polyprotein (Faiella et al., 1994; Kim et al., 1996; Love et al., 1996; Tomei et al., 1993). NS3 has also been found to possess ATPase (Jin and Peterson, 1995) and helicase (Kim et al., 1998; Yao et al., 1997) activity. Also, in vitro biochemical analyses have recently demonstrated that NS5B is an RNA-dependent RNA polymerase (Behrens et al., 1996; Lohmann et al., 1997). However, functions of the viral proteins in the context of the virus life cycle are not amenable for examination, due largely to the lack of an efficient tissue culture system for propagating virus (Bartenschlager, 1997; Major and Feinestone, 1997; Rice, 1996). Recent advances in the recovery of infectious HCV from cDNA-derived RNA genome in chimpanzee may provide an alternative approach toward genetic analysis and manipulation of the viral genome and viral proteins (Kolykhalov et al., 1997; Yanagi et al., 1997, 1998).

On the other hand, little is known about the molecular mechanism of HCV replication. Comparative sequence analyses have revealed that the 5’ UTR is one of the most conserved RNA sequence among different genotypes of HCV (Brown et al., 1992; Bukh et al., 1992; Matsuura and Miyamura, 1993), suggesting that it may act as cis signal or signals for translation and replication of the viral RNA ge-
nomic (Brown et al., 1992; Bukh et al., 1992; Matsuura and Miyamura, 1993; Rice, 1996). A number of studies have demonstrated that the 5’ UTR functions as an internal ribosomal entry site for cap-independent initiation of translation (Matsuura and Miyamura, 1993). Whether the 5’ UTR also plays a role in viral RNA replication remains to be determined. In the case of poliovirus, however, the 5’ UTR is involved in both translation and replication of the viral RNA genome (Andino et al., 1993; Rohli et al., 1994; Gamarik and Andino, 1998). The 3’ UTR of HCV consists of a variable region (type-specific region), a polyuridine tract of variable length, and a highly conserved 3’-terminal 98 nucleotides (nt) (Kolykhalov et al., 1996; Tanaka et al., 1995, 1996; Yamada et al., 1996). The 3’-terminal 98 nucleotide sequence was predicted to form an RNA structure with three stem-loops and may be involved in viral RNA replication or translation (Kolykhalov et al., 1996; Tanaka et al., 1996; Yamada et al., 1996). The stem-loop structures of the 98-nt RNA have been experimentally confirmed by ribonuclease digestion (Blight and Rice, 1997; Ito and Lai, 1997). In addition, a cellular factor, polyuridine tract-binding protein (PTB), was found to specifically bind to these stem-loop structures (Ito and Lai, 1997; Tsuchihara et al., 1997). The function or functions of the conserved 98 nt and its interaction with PTB largely remain speculative (Ito and Lai, 1997; Kolykhalov et al., 1996; Tanaka et al., 1996; Tsuchihara et al., 1997; Yamada et al., 1996). However, it has recently been described that the conserved 3’ end 98 nt (X region) enhanced translation by a factor of threefold to fivefold in an internal ribosome entry site (IRES)-dependent manner, and it did not affect the cap-dependent translation (Ito et al., 1998). Furthermore, it has also been shown that deletion of either the 3’-terminal 98 nt or the poly(U) tract of the HCV 3’ UTR failed to generate virus infection in a chimpanzee model. This experiment demonstrated that both the conserved 98 nt and the poly(U) tract are essential for recovery of infectious virus, indicating their important roles in HCV replication (Yanagi et al., 1998a; Dr. Robert Purcell, personal communication).

In an attempt to further understand the function or functions of the 3’ UTR of the HCV RNA genome, the interaction of the 3’ UTR with cellular proteins was analyzed by UV cross-linking. The results described in this study herein reveal that two cellular proteins, p58 and p35, bind to the 3’ UTR of the HCV RNA genome. In agreement with previous reports (Ito and Lai, 1997; Tsuchihara et al., 1997), p58 protein binds to the 5’ terminal 28 nt of the conserved 98 nt of the 3’ UTR. In addition, p58 was also found to interact with the poly(U) tract of the 3’ UTR. The other protein, p35, specifically binds to the poly(U) tract of the 3’ UTR. Binding of p58 (PTB) to the conserved 98 nt of the 3’ UTR was recently reported to be partially responsible for stimulation of the IRES-dependent translation (Ito et al., 1998). Sequence analyses has revealed that the poly(U) tract of the HCV 3’ UTR is highly variable in length among different isolates and often contains interspersed cytidine substitutions (Kolykhalov et al., 1996; Major and Feinstone, 1997; Rice, 1996; Tanaka et al., 1995, 1996; Yamada et al., 1996). One possibility is that the binding of p58 and p35 to the poly(U) tract may also play a regulatory role in the translation and/or replication of the HCV RNA genome.

RESULTS

Identification of cellular proteins that bind to the 3’ UTR

It has been recently reported by two independent research groups that a cellular factor, PTB (p58), specifically binds to the conserved 98 nt of the HCV 3’ UTR (Ito and Lai, 1997; Tsuchihara et al., 1997). We are also interested in the interaction of the HCV 3’ UTR with cellular proteins because a number of cellular factors have been identified that bind to the 3’ end of the RNA genome of several other RNA viruses (Blackwell and Brinton, 1995, 1997; Kusov et al., 1996; Leopardi et al., 1993; Paradigon and Strauss, 1992; Shi et al., 1996; Todd et al., 1995; Yu and Leibowitz, 1995). Because the HCV 3’ UTR is composed of three distinct regions, the variable region, a poly(U) tract, and a conserved 98-nt sequence at the 3’ end (Fig. 1), an attempt was made to identify cellular proteins that bind to any of these regions. A uniformly [α-32P]UTP-labeled 3’ UTR/full RNA (Fig. 1) was incubated with Huh 7 cell extract that was preincubated with excess yeast tRNA to limit potential nonspecific RNA–protein interactions. The RNA–protein complexes were then UV cross-linked, extensively treated with RNase A and T1, and analyzed by electrophoresis on SDS-PAGE. This analysis identified two major protein bands that bind to the 3’ UTR, with estimated molecular masses of 58 kDa (p58) and 35 kDa (p35). The intensity of both protein bands increases with increasing amounts of cell extracts (Fig. 2A). The p58 protein was detected at a ~25-fold lower protein concentration than p35. Binding of p58 to 3’ UTR starts to appear at a protein concentration of 72 ng (more obvious in a longer exposure of Fig. 2A) and reaches a peak at 9 μg of cellular protein. However, p35 protein was not detected until 1.8 μg of cell extract was used in the UV cross-linking assay (Fig. 2A).

Binding of p35 protein to the 3’ UTR also increases with increasing amount of cell extract, and saturates at ~45 μg of cellular protein (Fig. 2A, and data not shown). Interestingly, it appears that binding of p35 protein to the 3’ UTR reduced binding of p58 (Fig. 2A, lane 6), suggesting that binding of p35 may interfere or compete with the binding of p58 to the 3’ UTR (also see below).

To examine the specificity of the interaction between the HCV 3’ UTR and cellular proteins, RNA binding competition experiments were performed with two different RNA competitors, unlabeled 3’ UTR/full and 5’ UTR RNAs. In these experiments, two different protein con-
centrations (1.8 μg and 45 μg) of cell extract were used because binding of p58 and p35 reaches their respective peak at different protein concentrations (Fig. 1A). Cell extract was incubated with an excess of yeast tRNA at 30°C for 10 min before [α-32P]UTP-labeled 3’ UTR/full RNA was added together with an increasing amount of unlabeled competitor RNA and incubated for an additional 30 min at 30°C. When unlabeled specific 3’ UTR/full RNA was used, the binding of both p58 and p35 to labeled RNA was competed out in proportion to the increasing amount of unlabeled 3’ UTR/full RNA (Figs. 2, B–D). At a protein concentration of 45 μg, ~50 times more competitor RNA is required to reduce binding of p58 by 50% compared with that required to reduce binding of p35 by 50% (Fig. 2D). However, when cell extract was reduced to 1.8 μg, competition of p58 binding by 3’ UTR/full RNA is comparable with that of p35 (Fig. 2D). The binding of p58 and p35 to the 3’ UTR was not affected by the addition of increasing amounts of 5’ UTR RNA when 45 μg of cell extract was used. When 1.8 μg of cell extract was used in this assay, however, 20% of p58 binding was competed out by 5’ UTR at 1000 ng, but p35 binding was still not reduced at this concentration (Fig. 3). These experiments clearly demonstrate that p58 preferentially and p35 specifically bind to the 3’ UTR of HCV RNA genome.

FIG. 1. (A) Sequences of the HCV 3’ UTR used in this study. The 3’ UTR consists of three distinct regions: the 5’ end variable region (42 nt, italic), a poly(U) tract (49 nt, underlined), and the 3’ end conserved 98 nt (bold). ■ and ▼ indicate sites where 39- (D39) and 70- (D70) nt deletion mutants were produced. (B) Schematic diagram of the full-length 3’ UTR and deletion mutants. Generation of these RNA transcripts is described in the text. Hatched box represents the variable region of the 42 nt immediate downstream of the ORF; □, poly(U) tract of 49 nt; ■, 3’ end conserved 98 nt. The T7 promoter is indicated at the front of each transcript.
Determination of p58 and p35 binding sites

To further delineate the binding region for p58 and p35 in the 3’ UTR RNA, mutant 3’ UTR RNAs were transcribed and examined in the RNA–protein UV cross-linking analysis (Fig. 1). As shown in Fig. 4, a truncated 3’ UTR with a 39-nt deletion from the 3’ end retains full activity for both p58 and p35 binding (lane 3). Extended analysis
further revealed that deletion of the last 70 nt from the 3′ end did not reduce the ability of the 3′ UTR to interact with both p58 and p35 (lane 4). This indicates that the binding sites for p58 and p35 are located in the variable region, poly(U) tract and/or the 5′ end 28 nt of the conserved 98 nt. To further determine the binding sites for p58 and p35, deletion mutant RNAs consisting of either only the conserved 98 nt or only the variable region and the poly(U) tract sequence were then examined by UV cross-linking analysis for their ability to bind to p35 and p58. When the conserved 98 nt RNA was uniformly labeled and tested, only p58 was able to be cross-linked (Fig. 4, lane 2). This experiment demonstrated that p35 binds to the sequence outside the conserved 98 nt. However, the intensity of p58 binding to the 98 nt RNA is 3.5 times less compared with its binding to a 3′ UTR/full RNA (Fig. 4, lane 1), suggesting that the variable region and poly(U) tract may be also involved in p58 binding. To directly determine the role of the variable region and poly(U) tract in p58 and p35 binding, 3′ UTR/D98, a 3′ UTR RNA with deletion of the 98 nt, was examined in a competition experiment. The results are shown in Fig. 5. The 3′ UTR/D98 RNA potently competed out p35 binding (Fig. 5). Binding of p35 to labeled 3′ UTR was dramatically reduced in proportion to increases in the amount of unlabeled 3′ UTR/D98 RNA. Its potency in competing p35 binding is close to that of 3′ UTR/full RNA (Fig. 2D). Like 3′ UTR/full RNA, 3′ UTR/D98 RNA also competed out p58 binding to a lesser extent when 45 μg of cell extract was used (Fig. 5A and 5B). From these results, it is apparent that two separate binding sites exist within the 3′ UTR. One binding site resides in the 5′ 28 nt of the conserved 98 nt to which p58 binds, whereas the other is within the nonconserved upstream sequences to which p35 and also possibly p58 bind.
Cross-linking competed by 3' only in the absence of cell extract. (B) Comparison of protein–RNA interaction with the amounts of competitor RNA in log scale. The percentage of control (%) was calculated from the data using protein–RNA complex in the absence of competitor RNA as 100%. The percentage of control (%) was derived from a competition experiment using a [32P]UTP-labeled 3' UTR/D98 RNA directly used in the UV cross-linking assay. Interestingly, both p58 and p35 were found to bind to 3' UTR/D98 RNA (Fig. 6A). Binding of p58 and p35 to 3' UTR/D98 is also proportional to the amount of cell extract used. To further verify this finding, an unlabeled 3' UTR/D98 RNA was used in a competition experiment. Because p58 binding is more sensitive to competitor RNA at a lower protein concentration, 1.8 µg of cell extract was used instead of 45 µg of cell extract, as described in Fig. 5A. Results derived from this competition experiment clearly show that 3' UTR/D98 RNA competed out p58 binding to an equivalent extent as it competed for p35 binding (Figs. 5B and 6). These findings confirmed that p58 also interacts with a sequence besides the 3' end 98 nt. To determine whether p58 is a PTB, an immunoprecipitation experiment was performed using a monoclonal antibody specific for PTB. After the UV cross-linking of cellular protein–3' UTR RNA and RNase treatment, protein–RNA complexes were incubated with anti-PTB antibody and precipitated by protein A-Sepharose. As shown in Fig. 6C, an anti-PTB antibody specifically precipitated p58 but not p35. This experiment verified that p58 is the PTB.

The poly(U) tract of 3' UTR is the binding site for both p58 and p35

The nonconserved region of the 3' UTR consists of a variable region of ~40 nt immediately downstream of the ORF and a poly(U) tract of variable length that may contain interspersed cytidine substitutions [poly(U/C)] in some isolates (Fig. 1). To further delineate the specific sequences to which p58 and p35 bind, competition experiments were performed with RNA homopolymers [poly(U), poly(C), poly(A), or poly(G)] as competitors. The results are shown in Fig. 7. Both p58 and p35 bindings were competed out by a poly(U) homopolymer in a similar way to competition by 3' UTR/full and 3' UTR/D98 RNAs. It reduced p35 labeling by >50% at 1 ng (Fig. 7A, lane 4), whereas ≥10 ng nearly competed out all the binding of p35 to 3' UTR (Fig. 7A). Although it competed out p58 binding only at 1000 ng (Fig. 7A), its competition for p58 binding is similar to that of p58 binding by either 3' UTR/full or 3' UTR/D98 RNA at a protein concentration of 45 µg (Figs. 2B and 5A). When cell extract used in the assay was reduced from 45 to 1.8 µg, p58 binding was more sensitive as p35 binding to competition by a poly(U) homopolymer (data not shown). However, a poly(A) homopolymer also competed out both p58 and p35 binding, whereas a poly(C) homopolymer did not significantly compete out either p58 or p35 binding (Fig. 7A). The poly(A) homopolymer reduced p35 binding by a factor of 18%, 37%, 59%, and 90% and p58 binding by a factor of

Interaction of p58 with another sequence besides the 3' end 98 nt

The p58 protein was previously reported to specifically bind to the 3' end conserved 98 nt and was determined to be a PTB (Luo and Lai, 1997; Tsuchihara et al., 1997). The finding that p58 binds to the 3' end 98 nt was also identified in this study as described above. In addition, results derived from a competition experiment using a mutant 3' UTR RNA with deletion of the 3' end 98 nt (3' UTR/D98) suggest that it may also bind to a region outside the 98 nt (Fig. 5A). To further explore this possibility, an [α-32P]UTP-labeled 3' UTR/D98 RNA was directly used in the UV cross-linking assay. Interestingly, both p58 and p35 were found to bind to 3' UTR/D98 RNA (Fig. 6A). Binding of p58 and p35 to 3' UTR/D98 is also proportional to the amount of cell extract used. To further verify this finding, an unlabeled 3' UTR/D98 RNA was used in a competition experiment. Because p58 binding is more sensitive to competitor RNA at a lower protein concentration, 1.8 µg of cell extract was used instead of 45 µg of cell extract, as described in Fig. 5A. Results derived from this competition experiment clearly show that 3' UTR/D98 RNA competed out p58 binding to an equivalent extent as it competed for p35 binding (Figs. 5B and 6). These findings confirmed that p58 also interacts with a sequence besides the 3' end 98 nt. To determine whether p58 is a PTB, an immunoprecipitation experiment was performed using a monoclonal antibody specific for PTB. After the UV cross-linking of cellular protein–3' UTR RNA and RNase treatment, protein–RNA complexes were incubated with anti-PTB antibody and precipitated by protein A-Sepharose. As shown in Fig. 6C, an anti-PTB antibody specifically precipitated p58 but not p35. This experiment verified that p58 is the PTB.

The poly(U) tract of 3' UTR is the binding site for both p58 and p35

The nonconserved region of the 3' UTR consists of a variable region of ~40 nt immediately downstream of the ORF and a poly(U) tract of variable length that may contain interspersed cytidine substitutions [poly(U/C)] in some isolates (Fig. 1). To further delineate the specific sequences to which p58 and p35 bind, competition experiments were performed with RNA homopolymers [poly(U), poly(C), poly(A), or poly(G)] as competitors. The results are shown in Fig. 7. Both p58 and p35 bindings were competed out by a poly(U) homopolymer in a similar way to competition by 3' UTR/full and 3' UTR/D98 RNAs. It reduced p35 labeling by >50% at 1 ng (Fig. 7A, lane 4), whereas ≥10 ng nearly competed out all the binding of p35 to 3' UTR (Fig. 7A). Although it competed out p58 binding only at 1000 ng (Fig. 7A), its competition for p58 binding is similar to that of p58 binding by either 3' UTR/full or 3' UTR/D98 RNA at a protein concentration of 45 µg (Figs. 2B and 5A). When cell extract used in the assay was reduced from 45 to 1.8 µg, p58 binding was more sensitive as p35 binding to competition by a poly(U) homopolymer (data not shown). However, a poly(A) homopolymer also competed out both p58 and p35 binding, whereas a poly(C) homopolymer did not significantly compete out either p58 or p35 binding (Fig. 7A). The poly(A) homopolymer reduced p35 binding by a factor of 18%, 37%, 59%, and 90% and p58 binding by a factor of
10%, 22%, 64%, and 86% at 1, 10, 100, and 1000 ng, respectively (Fig. 7B). It seems that affinity of poly(A) binding to p35 is 100-fold less compared with poly(U). A poly(G) homopolymer was unable to compete out p35 cross-linking to 3′ UTR RNA until 1000 ng was used and then was able to reduce p35 binding by only 15%. However, poly(G) did cause a 20–30% reduction of p58 binding at 100 and 1000 ng, respectively (Fig. 6B). Taken together, these results revealed that the affinity of p58 and p35 binding to ribonucleotide homopolymers is in the order of poly(U), poly(A), poly(G), and poly(C). These findings also suggest that both p58 and p35 may specifically interact with the poly(U) tract of the 3′ UTR.

To further verify that p35 specifically binds to the poly(U) tract of the 3′ UTR, a specific poly(U) deletion mutant of 3′ UTR, 3′ UTR/dU, was generated and used in a competition analysis (Fig. 1). As shown in Fig. 8, the unlabeled 3′ UTR/dU RNA specifically competed out p58 binding but not p35 binding. It caused a reduction of p58 binding by 32% and 74% at 100 and 1000 ng, respectively (Fig. 8B). However, competition of p58 binding by 3′ UTR/dU RNA was not significantly increased even when cell extract was reduced to 1.8 μg (data not shown). In contrast to 3′ UTR/full and 3′ UTR/D98 RNAs, 3′ UTR/dU RNA was unable to entirely compete out p58 binding. The exact reason is not yet known. A possible explanation is that p58 may have different binding sites for 98 nt and poly(U) tract or that the affinity of p58 binding to poly(U) may be better than its binding to 98 nt. Regardless, these results demonstrate that the poly(U) tract of the 3′ UTR is specifically recognized by p35.

The p58 protein was found to bind to both the 3′ end 98 nt (3′ UTR/98 RNA, Fig. 4) and the nonconserved sequences consisting of the variable region and a poly(U) tract (3′ UTR/D98; Figs. 5 and 6). Because 3′ UTR/D98 RNA also contains the variable region in addition to a poly(U) tract, the variable region may also interact with p58. To rule out this possibility, an RNA containing the variable region sequence (3′ UTR/VRm) (Fig. 1) was generated and tested in a competition experiment. As shown in Fig. 9, 3′ UTR/VRm has no effect on either p58 or p35 binding at amounts up to 5000 ng, regardless of the amount of cell extract used. This experiment clearly showed that the variable region has no role in p58 and p35 binding. Therefore, the poly(U) tract of the 3′ UTR was determined to be the binding site for both p58 and p35.

Evaluation of p58 and p35 in different cell types

UV cross-linking was used to determine whether p58 and p35 are present in a number of different cell types. These include human liver cell lines Huh7 and HepG2; human nonliver cell lines HeLa, Hep2, and 293; and
animal cell lines BHK (hamster), COS-1 (monkey), and MDCK (canine). As shown in Fig. 10, both p58 and p35 proteins are ubiquitously present in all different cell types tested here.

**DISCUSSION**

In vitro UV cross-linking analysis has been broadly used to study RNA–protein interactions, leading to an understanding of the in vivo functions of a protein and its specific interaction with RNA (Belsham and Sonenberg, 1996; Frankel et al., 1991; Nagai, 1993). In this study, this type of analysis was used to show that two cellular proteins specifically interact with the 3' UTR of the HCV RNA genome. Based on their mobility in SDS–PAGE, it was estimated that the molecular masses of these two proteins are ~58 and ~35 kDa, respectively. Competition experiments showed that unlabeled 3' UTR RNA specifically competed out cross-linking to both p58 and p35 (Fig. 2). However, unlabeled 5' UTR RNA failed to compete out cross-linking to p35 and reduced cross-linking to p58 by only 20%, even at a >1000 times higher concentration compared with 3' UTR/full RNA (Fig. 2D). These findings demonstrated that p35 specifically and p58 preferentially bind to the 3' UTR of the HCV RNA genome. The p58 protein was confirmed to be the PTB, which was previously found to interact with the HCV 5'
which is consistent with previous studies (Ito and Lai, 1997; Tsuchihara et al., 1997). In addition to binding to the 3’ end conserved 98 nt, p58 was identified here to also interact with the poly(U) tract of the 3’ UTR. This conclusion was based on substantial evidence from several experiments. First, p58 was directly UV cross-linked with an [α-32P]UTP-labeled 3’ UTR RNA with deletion of the 3’ end 98 nt (3’ UTR/D98). Cross-linking of p58 to 3’ UTR/D98 is similar to that seen with 3’ UTR/full RNA, as shown in Fig. 6A. When unlabeled 3’ UTR/D98 RNA was used in a competition analysis, it competed out the binding of p58 to a similar extent as that by the intact 3’ UTR/full (Figs. 2D and 5B). In addition, the intensity of cross-linking of p58 to the 3’ end 98 nt was substantially decreased (3.5 times) compared with its cross-linking to 3’ UTR/full (Fig. 4), suggesting that p58 may also bind to a sequence besides the 3’ end 98 nt. In addition, a poly(U) homopolymer could compete for p58 binding like 3’ UTR/full and 3’ UTR/D98 RNAs (Fig. 7A and data not shown). In contrast to 3’ UTR/full and 3’ UTR/D98 RNAs, however, a mutant 3’ UTR with a specific poly(U) tract deletion, 3’ UTR/du, was unable to entirely compete out p58 binding (Fig. 8). Although the binding affinity of p58 to poly(U) and the 3’ end 98 nt was not determined, it appears that the binding of p58 to poly(U) is much stronger that that to the 3’ end 98 nt (Figs. 2D, 5B, 6, 7A, and 8, and data not shown). Furthermore, an RNA consisting of the variable region of the 3’ UTR and 6 nt from the 5’ end of the 98 nt (3’ UTR/VRm), which does not have either a poly(U) tract or the conserved 98 nt, failed to compete out p58 binding. This finding further delineates the binding site of p58 from within the nonconserved region to a poly(U) tract. The p58 protein was previously determined to be the PTB (Ito and Lai, 1997; Tsuchihara et al., 1997). All the characteristics of p58 found in this study suggest that it is the PTB, which was confirmed by a monoclonal antibody specific for PTB (Fig. 6C). Taken together, findings described in this study demonstrated that PTB binds not only to the 3’ end 98 nt but also to the poly(U) tract of the HCV 3’ UTR.

The faster moving protein, p35, has not been identified previously. In contrast to p58, which binds to the 3’ end conserved 98 nt as well as the poly(U) tract, p35 specifically interacts with the poly(U) tract of the 3’ UTR. Deletion analysis of the 3’ UTR mapped the binding site of p35 to the sequences consisting of the variable region and a poly(U) tract because it does not bind to the 3’ end 98 nt (Fig. 4). Like p58, it binds to a mutant 3’ UTR with a deletion of the 3’ end 98 nt (3’ UTR/D98) (Fig. 6A). Unlabeled 3’ UTR/D98 RNA efficiently competed out p35 cross-linking. When a poly(U) homopolymer was used, it also potently competed out p35 radiolabeling (Fig. 7A). In addition, a poly(U)-deficient 3’ UTR RNA specifically competed for p58 binding without any effect on the p35 binding (Fig. 8). Furthermore, an RNA containing the variable region had no effect on the p35 binding (Fig. 9).
These results clearly demonstrate that the poly(U) tract of the 3′ UTR is also the binding site for p35.

The possible functions of p58 and p35 in the HCV infectious cycle remain to be determined. It is known that RNA–protein interactions are required for numerous biological processes, including pre-mRNA splicing, RNA transportation, RNA modification, protein translation, and regulation (Ali and Siddiqui, 1997; Belsham and Sonenberg, 1996; Frankel et al., 1991; Miranda et al., 1997; Nagai, 1993; Von Hippel et al., 1995). For RNA viruses, cellular proteins are found to interact with the untranslated region of the viral RNA genome. In the case of poliovirus, cellular proteins are associated with viral proteins and the 5′ UTR of the viral RNA genome, which are involved in both internal initiation of translation and RNA replication (Ali and Siddiqui, 1997; Andino et al., 1993; Belsham and Sonenberg, 1996; Blackwell and Brinton, 1997; Gamarnik and Andino, 1998; Hellen et al., 1993; Liu et al., 1996). A number of cellular proteins have been identified that specifically interact with the untranslated region of the viral RNA genome. In the case of poliovirus, cellular proteins are associated with viral proteins and the 5′ UTR of the viral RNA genome, which are involved in both internal initiation of translation and RNA replication (Ali and Siddiqui, 1997; Andino et al., 1993; Belsham and Sonenberg, 1996; Hellen et al., 1993). Two cellular proteins, La antigen and PTB, were identified and extensively characterized (Ali and Siddiqui, 1997; Hellen et al., 1993). Both of them interact with the IRES elements and enhance protein translation (Ali and Siddiqui, 1997; Belsham and Sonenberg, 1996; Hellen et al., 1993). In addition, RNA–protein interactions have been demonstrated to play an important role in Qβ phage RNA replication (Miranda et al., 1997). Furthermore, a number of cellular proteins have been identified that bind to either the 5′ UTR or 3′ UTR of the viral RNA genome or antigenome of other RNA viruses; these include human rhinovirus (Todd et al., 1995), hepatitis A virus (Kusov et al., 1996), rubella virus (Liu et al., 1996; Singh et al., 1994), mouse hepatitis virus (Furuya and Lai, 1993; Yu and Leibowitz, 1995), measles virus (Leopardi et al., 1993), Sindbis virus (Pardigon and Strauss, 1992), and West Nile virus (Blackwell and Brinton, 1995, 1997; Shi et al., 1996). Although functions of most of these RNA–protein interactions must be estab-
lished, identification of the RNA–protein interactions usually represents the first step toward further understanding of their functions in the virus life cycle. Recently, PTB was identified to specifically bind to the 3’ end 98 nt of the HCV RNA genome and was speculated to play a role in translation and replication of the viral RNA genome (Ito and Lai, 1997; Tsuchihara et al., 1997). In fact, the 3’ end 98 nt of the HCV 3’ UTR was recently found to stimulate translation in an IRES-dependent manner. The binding of PTB to the 3’ end 98 nt was believed to be partially responsible for stimulation of translation (Ito et al., 1998). In this study, it was shown that the PTB also interacts with the poly(U) tract of the 3’ UTR (Figs. 4–6, 8, and 9). It seems that the binding of p58 to poly(U) is stronger than that to the 3’ end 98 nt (Figs. 6, 7A, and 8, and data not shown). Furthermore, an additional cellular protein, p35, was identified that specifically binds to the poly(U) tract of the HCV 3’ UTR. As shown in a chimpanzee model of infection, both the poly(U) tract sequences and the conserved 98 nt of the 3’ UTR are essential for recovery of infectious virus. However, the variable region immediately following the ORF was dispensable as suggested by Kolykhalov et al., 1997; Dr. Robert Purcell, personal communication). Therefore, interactions of p58 and p35 with the poly(U) tract and the binding of p58 to the 3’ end 98 nt of the 3’ UTR may play important roles in translation and replication of the HCV RNA genome. The poly(U) tract varies in length in clinical isolates, and some isolates contain interspersed cytidine substitutions (Kolykhalov et al., 1996; Major and Feinstein, 1997; Rice, 1996; Tanaka et al., 1995; Yamada et al., 1996). A possible function of these protein–RNA interactions is to enhance translation of the viral RNA genome, as reported recently (Ito et al., 1998). In addition, the cellular proteins may inhibit replication of the viral RNA genome through binding to the 3’ UTR early in infection because the binding of cellular proteins to the 3’ UTR blocks that of NS5B (data not shown). However, the accumulation of viral proteins may begin to favor the interaction of 3’ UTR with the replication complex rather than cellular proteins. As reported previously, NS3 protein binds preferentially to poly(U) sequences (Kanai et al., 1998), suggesting that it can be involved in the regulation process of switching from translation to replication. The RNA sequences controlling the translation and replication of the viral RNA genome have been recently reported for poliovirus (Gamarnik and Andino, 1998). In this case, an RNA structure at the 5’ end of the poliovirus genome, next to the IRES, controls viral translation and replication. When cellular protein PCBP binds to this RNA structure, it up-regulates viral translation, whereas the binding of the viral protein 3CD represses translation and promotes negative-strand RNA synthesis. It is possible that the interactions of cellular proteins with the conserved 98 nt and the poly(U) tract of HCV 3’ UTR might regulate viral translation and replication. It is also possible that the length of the poly(U) tract with interspersed substitution of cytidines may determine the level of virulence of HCV in vivo. Obviously, further investigations are needed to define the functions of the 3’ UTR and its interaction with cellular proteins.

**MATERIALS AND METHODS**

**Construction of plasmid DNA**

A plasmid containing the cDNA of the 3’ UTR of HCV genotype 1b was kindly provided by Drs. Tanaka and Shimotoho (Tanaka et al., 1995, 1996). This 3’ UTR is composed of a variable region, a poly(U) tract, and the conserved 3’-terminal 98 nt (Fig. 1; Tanaka et al., 1995, 1996). The 3’ UTR was amplified by PCR using primers T7/3’ UTR (5’-GCCGGGTACCTTAATACGACTCAGTACCTATAGGGCTAAGCGTGAACGGGAGCTAACA-3’) and 3’ UTR/E (5’-GCCGGGTACCTTAATACGACTCAGTACCTATAGGGCTAAGCGTGAACGGGAGCTAACA-3’). The PCR DNA fragment was digested with KpnI and Sall and inserted into a puC19 plasmid that was also digested by both KpnI and Sall. This plasmid was designated pUC19/T7–3’ UTR. The pUCRT/3’ UTR/98 DNA, which contains only the 3’-terminal conserved 98 nt, was constructed by cloning only the conserved 98 nt from the 3’ UTR into vector puC19/RT (Luo et al., 1997). This sequence was amplified through PCR using oligonucleotide primers T7/HCV5 (5’-GCCGGGTACCTTAATACGACTCAGTACCTATAGGGCTAAGCGTGAACGGGAGCTAACA-3’) and 3’ UTR/Ear (5’-GCCGGGTACCTTAATACGACTCAGTACCTATAGGGCTAAGCGTGAACGGGAGCTAACA-3’). The amplified DNA fragment was digested with KpnI and inserted into puC19/RT, which was cut by Eagl, end filled, and then digested with KpnI. Plasmid puC19/T7–3’ UTR/D98, which contains only the variable region and poly(U) tract of the 3’ UTR, was constructed through PCR priming with oligonucleotides T7/3’ UTR and HCV3/D98 (5’-GCCGGGTACCTTAATACGACTCAGTACCTATAGGGCTAAGCGTGAACGGGAGCTAACA-3’). The amplified DNA was digested with KpnI and SalI and ligated into similarly digested puC19. To generate a specific poly(U) deletion mutant RNA of 3’ UTR, plasmid puC19/3’ UTR/dU was constructed. Two separate PCRs were performed. The first PCR was carried out using oligonucleotides 3’ UTR/dU (5’-GGTGCAAAGGGAGCTAACCATCCAGGCAATAGGGCAGCTCGGCTCCATCTTGAGCC-3’). The amplified DNA was digested with KpnI and SalI and inserted into puC19 vector as described above.

Plasmid puCRT/T7–5’ UTR, which contains the 5’ UTR of HCV genotype 1a, was constructed by PCR using oligonucleotides T7/HCV5 (5’-GCCGGGTACCTTAATACGACTCAGTACCTATAGGGCTAAGCGTGAACGGGAGCTAACA-3’) and HCV/Clai (5’-CCGCACTTTCATCTGGTTCCGCTATTCGATG-3’) as primers and pCRII/NS3 as template. PCRII/NS3 contains the
cDNA of the 5’ UTR of an HCV genotype 1a virus (Heidi Wang, unpublished) and is nearly identical in sequence to that described by others (Brown et al., 1992). The PCR product was digested with KpnI and inserted into the pUCRT vector (Luo et al., 1997), which was linearized by EagI digestion and end filled, followed by KpnI digestion. All plasmid constructs were confirmed by DNA sequencing.

**In vitro RNA transcription**

The products of the in vitro T7 RNA transcription of full-length 3’ UTR and deletion mutants are schematically shown in Fig. 1. The full-length 3’ UTR was transcribed from pUC19/T7–3’ UTR DNA, which was linearized by EarI digestion. Deletion mutants 3’ UTR/D39 and 3’ UTR/D70 were transcribed from pUC19/T7–3’ UTR plasmid that was digested by PstI and blunt ended by Klenow fragment (New England Biolabs, Beverly, MA) and Nhel, respectively. The poly(U) deletion mutant was derived from EarI-linearized pUC19/3’ UTR/dU DNA. 3’ UTR/D98 was synthesized from pUC19/T7–3’ UTR/D98 after digestion with EarI. 3’ UTR/98 was synthesized from undigested plasmid pUCRT/3’ UTR/98 in which the T regulatory sequence terminates T7 RNA transcription and a ribozyme derived from HDV (Pattnaik et al., 1992) autocatalytically processes the RNA transcripts to produce precise 3’ UTR/98. 5’ UTR/full RNA was transcribed from EarI-linearized pUCRT/T7–5’ UTR and processed by HDV ribozyme at 3’ end. 3’ UTR/VRm RNA consisting of the variable region of the 3’ UTR and 6 nt (GGUGGC) derived from the 5’ end of the conserved 98 nt was generated from plasmid pUC19/3’ UTR/dU that was digested with NalI. All RNA transcripts were synthesized in vitro using a commercial T7 RNA transcription kit (Stratagene, La Jolla, CA). To generate [α-32P]UTP-labeled RNA, 10 μl of [α-32P]UTP (NEN, Boston, MA) and 1 μl of 0.4 mM cold UTP were added to a 40-μl reaction containing other ingredients as specified. All RNAs were purified by electrophoresis on a 6% polyacrylamide gel containing 7.7 M urea. RNA was eluted from the gel by incubation with an elution buffer (0.5 M ammonium acetate, 1 mM EDTA, and 0.1% SDS) at 37°C overnight and then collected by ethanol precipitation (Luo et al., 1992). RNA concentration was determined by measurement of spectrophotometer.

**Preparation of cell extracts**

Huh7, HepG2, HeLa, 293, Hep2, BHK, Cos-1, and MDCK cells were grown in DMEM supplemented with 10% FBS. Cell extracts were prepared according to the procedure of Andino et al. (1993) with slight modifications. Briefly, cells were washed twice with cold PBS, scraped, collected by centrifugation, and washed twice with cold PBS. The cell pellet was then resuspended in a 3× volume of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 1 mM DTT, 1% Nonidet P-40, and 0.1 mM phenylmethylsulfonyl fluoride). After incubation on ice for 20 min, cells were homogenized by 30 strokes in a Dounce homogenizer, and nuclei were removed by centrifugation. The supernatant was transferred to a new tube and centrifuged for 15 min at 10,000 rpm in an SS rotor (Sorvall). The resulting supernatant was removed and mixed with glycerol to a final concentration of 10% and stored at −80°C.

**UV cross-linking of RNA with cellular proteins**

Procedures for UV cross-linking of RNA with cellular proteins were modified from the published report (Andino et al., 1990). Cell extracts were first incubated with 20 μg of yeast RNA in binding buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM DTT, 0.5% Nonidet P-40, 5% glycerol) at 30°C for 10 min. Then 2 μl of [α-32P]UTP-labeled RNA (3 × 105 cpm) was added for an additional 30-min incubation in a final volume of 20 μl. For competition experiments, different amounts (as indicated) of cell extract was first mixed with 20 μg of yeast RNA at 30°C for 10 min, and different amounts of either un labeled RNA, poly(U), poly(G), poly(A), or poly(C) homopolymer (Pharmacia Biotech, Piscataway, NJ) were subsequently mixed together with [α-32P]UTP-labeled RNA. After 30-min further incubation at 30°C, the reaction was irradiated under UV light (8 W; Sylvania/GTE, Japan) on ice at a distance of 3 cm for 20 min. The reaction was then treated with 2 μg/ml RNase A and 100 units of RNase T1 (Gibco BRL, Gaithersburg, MD) at 30°C for 30 min. The reaction was mixed with an equal volume of Laemmli’s sample buffer (Bio-Rad, Hercules, CA) and boiled for 5 min before loading on an SDS–10% or 12% polyacrylamide gel. The gel was dried and autoradiographed. The UV cross-linked protein–RNA complex was quantified with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA) (Johnston et al., 1990).

**Immunoprecipitation of p58–RNA complex with an anti-PTB antibody**

UV cross-linking of the 3’ UTR RNA to cellular proteins were performed as described above. After UV cross-linking and RNase treatment, the reaction was diluted with 300 μl of RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA, and 1% Triton X-100) and incubated with 1:100 diluted monoclonal antibody against PTB (kindly provided by Dr. Eckard Wimmer, State University of New York at Stony Brook) (Hellen et al., 1993) at 4°C overnight. The protein–RNA complex was then precipitated with protein A-Sepharose (Pharmacia Biotech). The p58–RNA complex-bound Sepharose beads were washed four times with RIPA buffer and boiled for 5 min before 12% SDS–polyacrylamide gel electrophoresis. The gel was dried and autoradiographed.
ACKNOWLEDGMENTS

Sincere thanks are given to Drs. Torahiko Tanaka and Kunitada Shimotohno for kindly providing a plasmid containing the 3′ UTR cDNA of HCV genotype 1b, to Dr. Eckard Wimmer for kindly providing the anti-PTB antibody, and to Dr. Heidi Wang for a plasmid containing cDNA of HCV 5′ UTR. Thanks are also extended to Dr. Robert Hamatake for preparation of HuH7 cell extracts and NS5B protein used in the early experiments and Dr. David Standing for stimulating discussions. I am grateful to Dr. Robert Purcell (National Institutes of Health) for sharing the results before publication and Drs. Mark Krystal and Richard Colombo for critical reading of the manuscript and for their continued strong support.

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