An Internal Polypyrimidine-Tract-Binding Protein-Binding Site in the Hepatitis C Virus RNA Attenuates Translation, Which Is Relieved by the 3′-Untranslated Sequence

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Hepatitis C virus (HCV) RNA binds to several cellular proteins, which may regulate translation or replication of viral RNA. One of these is polypyrimidine tract-binding protein (PTB), which binds to the 5′-untranslated region (UTR) and the 3′-end 98 nucleotides (nt) (X region) of HCV RNA. Both of these PTB-binding sites regulate HCV translation. In this study, we further investigated the nature of PTB binding on HCV RNA. UV cross-linking studies using HeLa cell extracts and a recombinant PTB showed that the PTB-5′-UTR binding was much weaker than the PTB-3′-UTR binding. Unexpectedly, we found an even stronger PTB-binding site in the core-protein-coding region of HCV RNA. The binding domain was mapped to the 3′-end of this region, which contains a pyrimidine-rich sequence highly conserved among HCV isolates. Using a set of synthetic HCV RNAs with or without this sequence in in vitro translation studies, we showed that the PTB-binding sequence in the core-coding region strongly inhibited translation of HCV RNA. This inhibition was relieved by the presence of the X region at the 3′-end. Furthermore, the previously reported translational enhancement by the HCV 3′-UTR was more pronounced when this PTB-binding site was present in the RNA. These results suggest that PTB binding to an internal site of HCV RNA provides another mechanism for regulation of HCV translation.

INTRODUCTION

Many RNA viruses utilize host cellular factors to regulate their translation, replication, and/or transcription (see Lai (1998) for a review). Hepatitis C virus (HCV) provides such an example. It contains a 9.6-kb single-stranded, positive-sense RNA genome, which encodes a large polyprotein of about 3010–3033 amino acids (Choo et al., 1989; Kato et al., 1990). While the overall sequence of HCV RNA shows considerable diversity among various isolates (Bukh et al., 1995), the 5′-untranslated region (UTR) and the 3′-end 98 nucleotides (nt) (X region) of the HCV RNA are highly conserved (Bukh et al., 1992; Kolykhalov et al., 1996; Tanaka et al., 1996), suggesting their functional importance, probably in viral replication and/or translation.

The 3′-end X region has been reported to bind a cellular protein polypyrimidine-tract-binding protein (PTB) (Ito and Lai, 1997; Tsuchihara et al., 1997), which is a pre-mRNA splicing factor (Patton et al., 1991) and binds to several other viral RNAs as well (Lai, 1998). The X region enhances HCV translation from an internal ribosome entry site (IRES), and the sequence and structure of the PTB-binding site are important for this enhancement effect (Ito et al., 1998). PTB has also been reported to bind to the IRES in the 5′-UTR of HCV RNA and regulate HCV translation (Ali and Siddiqui, 1995), although this finding has been disputed (Kaminski et al., 1995; Reynolds et al., 1995). In the 5′-UTR of HCV RNA, several other cellular factors, including La antigen and hnRNP L, have been identified to bind and regulate its translation (Ali and Siddiqui, 1997; Fukushi et al., 1997; Hahm et al., 1998). These observations strongly suggest that host cellular factors binding to both ends of the HCV RNA are involved in the regulation of translation.

In this study, we further investigated the nature of PTB binding to both the HCV 5′-UTR and the 3′-UTR. We found that PTB binding to the 5′-UTR is significantly weaker than that to the X region. Unexpectedly, another PTB-binding site was discovered in the core-protein-coding region of HCV RNA. The binding domain was mapped to the 3′-end of this region, which contains a highly conserved pyrimidine-rich sequence. Furthermore, the PTB-binding sequence at the core-protein-coding region strongly attenuated HCV translation, which could be relieved by the X region at the 3′-end of the RNA. The presence of this sequence accounted for a significant part of the translational enhancement effects caused by the 3′-UTR of HCV. This property provides an additional mechanism for regulation of translation of HCV RNA.
RESULTS

PTB binding at the 5′-end of HCV RNA requires the core-protein-coding sequence

Previously it has been shown that PTB binds to both the 5′- and the 3′-UTRs of HCV RNA (Ali and Siddiqui, 1995; Ito and Lai, 1997; Tsuchihara et al., 1997). To compare its relative binding strength to either end of the RNA, we carried out a UV cross-linking experiment using 32P-labeled RNAs of HCV 3′-X, the 5′-UTR alone, the 5′-UTR plus the 5′-end of the core-coding sequence (nt 342–512 from the 5′-end), and the 5′-UTR plus the entire core-coding sequence (nt 342–914). When HeLa cell extracts were used, a 58-kDa protein, which was previously identified as PTB (Ito and Lai, 1997), was found to cross-link to the 3′-UTR (Fig. 1A, lane 1). However, PTB was not cross-linked to the 5′-UTR (lane 2), in contrast to the previous report (Ali and Siddiqui, 1995). Instead, several smaller proteins were detected to bind to the 5′-UTR; the nature of these proteins has not been identified. The lack of PTB binding to the 5′-UTR of HCV RNA was confirmed by the competition assay, in which a 500 times molar excess of the unlabeled 5′-UTR RNA could not abolish the PTB–3′-X binding (Fig. 1B), whereas only a 5-fold molar excess of the 3′-X region RNA almost completely abolished the PTB–3′-X binding (Ito and Lai, 1997) (data not shown). Unexpectedly, when the 5′-UTR RNA plus the entire core-coding sequence [5′-UTR+core(342-914)] was used, a very strong PTB binding was observed (Fig. 1A, lane 4). A smaller RNA containing the 5′-UTR plus the 5′-end of the core-protein-coding sequence did not bind this protein (Fig. 1A, lane 3). These results suggest that the 3′-half of the core-protein-coding sequence contains a previously undetected PTB-binding site. The binding affinity of PTB to this region was at least as strong as that to the 3′-UTR. Immunoprecipitation of this UV cross-linked protein by a PTB-specific antibody, but not by the antibody against an unrelated protein (TATA-binding protein), confirmed that the 5′-UTR plus the entire core-coding RNA bound PTB (Fig. 1C).

When a recombinant glutathione S-transferase (GST–PTB) was used for UV cross-linking, a weak binding between PTB and the 5′-UTR RNA was observed (Fig. 1A, lane 6), but it was considerably weaker than its binding to the 3′-UTR (lane 5). When the 5′-end of the core-coding sequence [5′-UTR+core(342-512)] was joined to the 5′-UTR, the amount of PTB binding was enhanced (lane 7). Significantly, when the entire core-protein-coding region was included, the PTB binding was even stronger than that to the 3′-UTR (lane 8). These results combined indicate that the core-protein-coding region contains a strong PTB-binding site and that the previously reported PTB binding to the 5′-UTR region (Ali and Siddiqui, 1995) is very weak at best.

Mapping of the PTB-binding site in the core-coding region of HCV RNA

To map the PTB-binding site in the core-coding region, we performed the UV cross-linking experiment using the

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**FIG. 1.** UV cross-linking analysis of HCV RNA with HeLa cell extracts and a recombinant GST–PTB. (A) UV cross-linking of proteins from cytoplasmic extracts of HeLa cells (lanes 1–4) and GST–PTB (lanes 5–8) to the various RNAs. 20 μg of HeLa extracts or 50 ng of GST–PTB was mixed with 32P-labeled RNA, UV-irradiated, digested with RNase A, and separated by SDS–PAGE on 10% polyacrylamide gels. Arrows indicate the GST–PTB (86 kDa) and the endogenous PTB (58 kDa) in HeLa cells. Molecular mass markers are indicated on the left. (B) Competition analysis of UV cross-linked PTB. A fixed amount of the 32P-labeled 3′-UTR–X RNA (2 pmol of RNA) was UV cross-linked with a fixed amount of HeLa cytoplasmic extracts (20 μg of RNA) and separated on SDS–PAGE on 10% polyacrylamide gels. Arrows indicate the GST–PTB (86 kDa) and the endogenous PTB (58 kDa) in HeLa cells. Molecular mass markers are indicated on the left. (C) Competition analysis of UV cross-linked PTB. A fixed amount of the 32P-labeled 3′-UTR–X RNA (2 pmol of RNA) was UV cross-linked with a fixed amount of HeLa cytoplasmic extracts (20 μg of RNA). Increasing amounts of the unlabeled HCV 5′-UTR RNAs were used as a competitor. Lanes 2 to 5, 1-, 5-, 50-, and 500-fold molar excesses of unlabeled HCV-5′-UTR over the 32P-labeled 3′-UTR–X, respectively. Lanes 1 and 4, UV cross-linked proteins without immunoprecipitation. Lanes 2 and 3, UV cross-linked proteins with anti-PTB antibody. Lanes 5 and 6, UV cross-linked proteins with anti-TBP antibody.
entire core-coding RNA [core(342-914)] and its 3′-end truncation mutants [core(342-854), core(342-800), and core(342-686)]. Figure 2A shows that only the core(342-914) RNA bound PTB from the HeLa cell lysates, suggesting that the PTB-binding site is located at the 3′-end of the core-coding region (nt 854–914). The competition assay using the recombinant GST–PTB binding to the 32P-labeled core(342-914) RNA showed that only the core(342-914) RNA, which contains the entire core-coding region, but not core(342-800) RNA, inhibited the PTB binding (Fig. 2B), confirming that the PTB-binding site is at the 3′-end of the core-coding region. As expected, the 32P-labeled core(342-800) RNA did not bind GST–PTB (lanes 1–4).

We also performed a gel-mobility shift assay using 32P-labeled core(342-914) RNA to further assess the specificity of the PTB–RNA binding. Figure 2C shows that an RNA–protein complex of slower electrophoretic mobility was formed between the recombinant PTB and the entire core-coding RNA (lane 3). The formation of this RNA–protein complex was inhibited by an excess of core(342-914) RNA but not core(342-800) RNA (lanes 4 and 5). Interestingly, HCV core protein, which has been shown to have general RNA-binding activities (Hwang et al., 1995; Santolini et al., 1994), did not form an RNA–protein complex with the core-coding RNA under these conditions (lane 2). These results further confirm that the 3′-end of the core-coding region of HCV RNA has a strong and specific PTB-binding site (Fig. 3A). This region contains a stretch of pyrimidine-rich sequence, which is highly conserved among various HCV isolates (Fig. 3B). This sequence conservation suggests that PTB can bind to this region in almost all HCV isolates. GST–PTB also appeared to bind to other regions of the core-coding sequence weakly; the specificity of these bindings was not further evaluated.

The PTB-binding sequence in the core-coding region inhibits the translation of HCV RNA

To investigate the biological significance of PTB binding at the core-coding region, we studied whether this region affected the translation of HCV RNA, since the binding of PTB to both ends of HCV RNA (Ali and Siddiqui, 1995; Ito and Lai, 1997; Tsuchihara et al., 1997) has been shown to enhance IRES-dependent translation (Ito et al., 1998). We used a reporter RNA (HCV-5CL), which contains the 5′-UTR and the entire core-coding region of HCV preceding the luciferase (LUC) gene, and a corresponding RNA, HCV-5CLD752-914, which has the PTB-binding site deleted, for in vitro translation in rabbit reticulocyte lysates (Fig. 4A). Figures 4B and 4C show that HCV-5CL RNA was translated very poorly (lane 1), whereas the corresponding deletion mutant RNA was translated three- to fourfold more efficiently (lane 2), indicating that the PTB-binding sequence at the 3′-end of the core-coding region strongly inhibited its translation.

FIG. 2. Mapping of the PTB-binding sites in the core-coding region of HCV RNA. (A) UV cross-linking using cytoplasmic extracts of HeLa cells and various 32P-labeled RNAs. (B) Competition analysis of UV cross-linked GST–PTB. A fixed amount of the 32P-labeled HCV core(342-800) (lanes 1–4) or core(342-914) (lanes 5–11) RNA (2 pmol of RNA) was UV cross-linked with a fixed amount of GST–PTB (50 ng). Increasing amounts of the unlabeled HCV core(342-800) (lanes 1–4) or core(342-914) (lanes 5–11) RNAs were used as a competitor. (–) Lane, no competitor. Lanes 2–4, 6–8, and 9–11, 1, 10, and 50-fold molar excesses over the radiolabeled probe, respectively. (C) Gel-mobility shift assay of RNA–protein complexes. 32P-labeled core(342-914) RNA was mixed with GST–core protein (lane 2) or GST–PTB that had been predigested with thrombin (lanes 3–5) and various unlabeled competitor RNAs. The RNA–protein complexes were separated in 3% polyacrylamide gels under nondenaturing conditions.

The addition of the X sequence to the 3′-end of 5CL RNA (HCV-5CL-X) enhanced the translation to almost the same level as the HCV-5CLΔ752-914 (lane 3), indicating
that the X region, which binds PTB, can relieve the inhibitory effects of the PTB-binding sequence in the core-protein-coding region of HCV RNA. The effects of the 3′-end X region on both RNAs with or without the PTB-binding site in the core-protein-coding region were further assessed. Comparison of lanes 1 and 3 indicated that the X region enhanced the translation on the RNA that contains this internal PTB-binding site by three- to fivefold (Fig. 4B and 4C), consistent with the previous observation (Ito et al., 1998). However, on the RNAs that do not have this internal PTB-binding site, the translation enhancement by the X sequence was less than twofold. Similar results were obtained when the LUC activities of the various translation products were measured (Fig. 4C). These results combined suggest that the PTB-binding sequence in the core-coding region serves as a translational repressor and that the 3′-end X sequence, which also binds PTB, relieves this translational inhibition.

To investigate whether the translational inhibition by the PTB-binding site was due to destabilization of mRNAs, we monitored the stability of these RNAs in reticulocyte lysates during in vitro translation, by a primer extension study using a primer complementary to the 5′-end of the HCV RNA. HCV genotypes are described on the left.

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**FIG. 3.** (A) Diagram of the structure of HCV RNA constructs and their PTB binding. The relative extents of PTB binding to the various RNAs are expressed in semiquantitative terms. (B) Nucleotide sequences of the potential PTB-binding site in the HCV core-coding region among individual HCV isolates (Bukh et al., 1992). Numbers on the top represent the nucleotide numbers from the 5′-end of the HCV RNA. HCV genotypes are described on the left.

DISCUSSION

Our previous works have shown that stem loops 2 and 3 of the X region of the 3′-UTR of HCV RNA bind PTB (Ito and Lai, 1997) and that this X region enhanced HCV translation from an IRES; mutations at the PTB-binding site reduced the level of the translational enhancement (Ito et al., 1998). From these results, we speculated that the binding of PTB to the X region enhanced the translation of HCV RNA by a mechanism similar to that of the poly(A) tail in eukaryotic mRNAs. The poly(A) tail binds a poly(A)-binding protein (Pab1p), which has been shown to interact with the 5′-end of mRNAs through additional cellular factors (Craig et al., 1998; Tarun and Sachs, 1996), resulting in the circularization of mRNA (Wells et al., 1998). The communication between the 5′-end and the 3′-poly(A) tail of eukaryotic mRNA enhanced translation efficiency (Craig et al., 1998; Munroe and Jacobson, 1990; Tarun et al., 1997). PTB has also...
been reported to interact with the IRES of HCV, raising the possibility that PTB has a bridging role for both ends of the HCV RNA (Ali and Siddiqui, 1995), similar to that of Pab1p for eukaryotic mRNAs.

Our studies presented here showed that PTB–5'-UTR binding was much weaker than PTB–3'-X binding. Using HeLa cell extracts, the PTB–5'-UTR binding could not be demonstrated (Fig. 1A); only when a purified recombinant GST–PTB was used did a weak binding become detectable. Furthermore, unlabeled 5'-UTR RNA could not inhibit the binding between the 3'-X and PTB even at a 500-fold molar excess (Fig. 1C), whereas the homologous RNA could abolish this binding at a 5-fold molar excess (Ito and Lai, 1997), suggesting that PTB–5'-UTR binding is at least 100-fold weaker than the corresponding PTB–3'-UTR binding. This finding is consistent with an earlier report that PTB failed to bind to an RNA consisting of the 5'-UTR plus 11 nt of the core-coding region (Kaminski et al., 1995). Kaminski et al. also concluded that PTB itself did not affect HCV translation, although the RNA used in that study did not contain the X region at the 3'-end (Kaminski et al., 1995). These results suggest that the HCV 3'-end is unlikely to interact with the 5'-end through PTB alone. Blight and Rice (1997) also showed that the 3'-X sequence could not interact with the 5'-UTR directly. All of these findings suggest that other cellular factors, in addition to PTB, are required for the interaction between the 5'- and the 3'-ends of HCV RNA to account for the 3'-UTR-mediated enhancement of HCV translation (Ito et al., 1998).

Unexpectedly we found that PTB bound to the 3'-end of the core-coding region, instead of the 5'-UTR, in HCV RNA (Fig. 2). This binding was at least as strong as its binding to the 3'-X region. This PTB-binding site contains a pyrimidine-rich sequence, which is conserved among all HCV isolates (Bukh et al., 1995), suggesting the functional importance of this region (Fig. 3B). Interestingly, deletion of this PTB-binding site increased the level of translation of the HCV RNA, which does not have the X region at the 3'-end, suggesting that this internal PTB-binding site serves as a translational attenuator. This translational inhibition was not due to RNA destabiliza-

FIG. 4. Functional analysis of the PTB-binding site in the core-coding region by in vitro translation. (A) Schematic diagrams of the RNAs used in this study. pHCV-5CL-X contains T7 promoter (large open arrow), 5'-UTR (single line), core-encoding region (hatched box) of the HCV 1b strain fused to LUC genes (open box), and the HCV 3'-X region in the pGL vector (Promega). pHCV-5CL-Δ752–914 has a deletion of the nt 752–914 region in pHCV-5CL-X. (B) In vitro translation products of various RNAs. In vitro translation was carried out in rabbit reticulocyte lysates at 120 mM KCl, and the translation products were separated by SDS–PAGE on 10–20% polyacrylamide gradient gels. The autoradiogram was scanned by NIH image 1.62, and relative amounts of the core-LUC fusion protein were determined. The translation product of HCV-5CL RNA is artificially set at 100%. (C) Relative LUC activity of the in vitro translation products of various RNAs. The LUC activity of HCV-5CL RNA is artificially set at 100%. The columns and bars represent the means and standard deviations of two sets of triplicate studies. (D) RNA stability of HCV-5CL (left) and -5CL-Δ752–914 (right) RNA. 2 μg of each RNA was mixed with rabbit reticulocyte lysates. At 0, 30, 60, and 90 min after in vitro translation, RNAs were harvested and used as a template for primer extension experiments using a 5'-UTR primer. Arrow indicates the primer-extended products (265 nt in length), which correspond to the intact 5'-end of HCV RNA.
tion by this PTB-binding site, since the kinetics of RNA degradation was similar between HCV-5CL and -5CL-XΔ752-914 RNAs (Fig. 4C). Surprisingly, most of the translational attenuation caused by this internal PTB-binding site was relieved by the presence of the X region at the 3'-end, again suggesting a long-distance interaction between the 3'- and 5'-ends or between the 3'-end and the internal PTB-binding site. Previously we reported that the 3'-end X region enhanced translation of HCV-5CL RNA by three- to fivefold (Ito et al., 1998); this finding was confirmed in this study (Fig. 4B). However, when the internal PTB-binding sequence was deleted, the 3'-X-mediated translation enhancement was not more than twofold (Figs. 4B and 4C, compare HCV-5CLΔ752-914 and HCV-5CL-XΔ752-914). Thus, the 3'-X-mediated translation enhancement of HCV RNA (Ito et al., 1998) is partly due to the relief of the translational suppression by the internal PTB-binding site in the core-protein-coding region. Since this sequence is present in the full-length HCV RNA, both the positive regulation and the negative regulation of translation by the PTB-binding sequences are likely to be biologically relevant in HCV infection. This finding also shows that the effects of translational enhancement by the 3'-UTR of HCV were best demonstrated using RNAs that contain the complete core-protein-coding region following the 5'-UTR of HCV RNA (Ito et al., 1998). In any case, the inclusion of more HCV sequences would more likely reflect the mechanism of the translational regulation in the natural HCV life cycle.

Pestova et al. (1998) reported that the constitution of the eukaryotic initiation factors at the IRES of HCV RNA was different from that of other IRES-containing viral RNAs. They showed that only eIF3 bound to HCV IRES, similar to prokaryotic mRNAs (Pestova et al., 1998; Sizova et al., 1998). Thus, it is unlikely that the HCV 3'-UTR interacts with the 5'-end through the same set of translation initiation factors as eukaryotic mRNA does. Although the role of PTB in the translation of HCV RNA has not been directly demonstrated, the fact that all of the PTB-binding sites on the HCV RNA can regulate HCV translation suggests that PTB likely

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**FIG. 4—Continued**

B

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C

D

HCV-5CL

HCV-5CLΔ752-914

(min) 0 30 60 90

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265 nt
plays an important role in translation. It is possible that PTB may bind to eIF-3 to effect 5'- and 3'-end interactions. Furthermore, other cellular and/or viral factors may bind to HCV RNA to play a bridging role between the 5'- and the 3'-ends. Several such factors have been reported to bind the 5'-UTR of HCV RNA (Ali and Siddiqui, 1997; Fukushi et al., 1997; Hahm et al., 1998); some of these may interact with PTB.

Based on these findings, we propose a working model for the translational regulation of HCV RNA (Fig. 5). At the IRES of HCV RNA, an unknown factor (Y) interacts with both viral RNA and PTB. When the factor Y interacts with the PTB at the core-coding region, HCV translation is inhibited because of the steric hindrance of RNA structure (Fig. 5A). When the 3'-end X region is present, the factor Y will interact with the PTB at the 3'-X region instead, preventing its binding with the PTB at the core-coding region and thus allowing elongation of translation. The interaction between the 5'- and the 3'-ends also enables HCV RNA to be circularized to enhance translational initiation.

Finally, what is the biological significance of translational suppression exerted by an internal PTB-binding RNA sequence? One possibility is that this regulatory mechanism ensures that only the full-length HCV RNA can be efficiently translated. When HCV RNA is degraded from the 3'-end and loses the X region, translation of the degraded RNA will be strongly inhibited because of the internal PTB-binding sequence. Another possibility is that PTB binding to an internal site of viral RNA may be fortuitous, due to the random occurrence of the pyrimidine-rich sequence. This binding will inhibit translation, which must be relieved by some mechanism, such as PTB binding at the 3'-UTR (Fig. 5A). Thus, the interaction between PTB and the HCV RNA genome at the 3'-UTR and an internal region may provide both positive and negative regulation of translation.

MATERIALS AND METHODS

Construction of cDNA clones

To generate in vitro transcripts of the various regions of HCV RNA, plasmid HCV-X(+) (Ito and Lai, 1997), which contains the core-coding region of HCV-T cDNA [core(342–914)] (Chen et al., 1992) and its deletion mutants [core(342–854), core(342–800) and core(342–686)], and the plasmid containing the entire HCV cDNA of HCV-1 isolate (Choo et al., 1989) were used as templates. These plasmids contain the T7 promoter upstream of the HCV sequence.

The construction of the plasmid pGEX-PTB has been described previously (Ito and Lai, 1997). The pGEX-HCV core, which encodes the GST-HCV core fusion protein, was constructed from the HCV cDNA of HCV RH isolate (Lo et al., 1995).

Plasmid pHCV-5CL-X was constructed as described previously (Ito et al., 1998). To construct pHCV-5CL-XΔ752-914, which is a deletion mutant of pHCV-5CL-X, the cDNA fragment containing the T7 promoter, the 5'-UTR, and a truncated core-coding region (752–914 nt) of HCV RNA was subcloned to pGL-basic vector (Promega), and the HCV-X sequence was inserted following the LUC gene.

In vitro RNA transcription

To synthesize RNA in vitro, various DNAs were linearized with the appropriate restriction enzymes and subjected to in vitro transcription using T7 RNA polymerase (Promega). In vitro transcription was carried out according to the manufacturer’s recommended procedure (Promega). 32P-labeled RNAs were obtained by transcription in the presence of 50 μCi of [α-32P]UTP [3000 Ci/mmol (ICN Biomedicals)], 12.5 μM UTP, and 500 μM each of ATP, GTP, and CTP. Unlabeled RNAs were transcribed in the presence of 500 μM each of ATP, GTP, CTP, and UTP. After incubation at 37°C for 2 h, 1 U of RQ DNase I

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**FIG. 5.** A proposed model for translational regulation of HCV RNA. (A) In the absence of the X region at the 3'-end, an unknown factor (Y) binds to the IRES in the 5'-UTR and interacts with PTB at the 3'-end of the core-coding region. These interactions inhibit ribosome scanning on HCV RNA because of the steric hindrance of RNA structure. (B) In the presence of the X region at the 3'-end, the Y factor binds to the 3'-end of the HCV RNA through PTB, preventing the binding of the Y factor with the PTB at the core-coding region and thus allowing translational elongation. The interaction between the 5'- and the 3'-ends also enables HCV RNA to be circularized to enhance translational initiation.
Immunoprecipitation of UV cross-linked protein

exposed to X-ray film. containing 0.1% SDS. After being dried, the gel was

sodium dodecyl sulfate (SDS), 62.5 mM Tris–HCl (pH 8.0), 50% glycerol, 0.1 mM EDTA, 2 mM DTT. Following incubation at 30°C for 10 min, 32P-labeled RNA (2 pmol of RNA) was added, and the reaction mixture was incubated for an additional 10 min. The reaction mixture was then mixed with 1/5 vol of 5X loading buffer [50% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol] and separated by electrophoresis on a 3% polyacrylamide gel made in 0.5X TBE [45 mM Tris–borate, 1 mM EDTA]–5% glycol. The polyacrylamide gels had been preelectrophoresed at 200 V for 30 min prior to loading of the samples, and electrophoresis was performed at 200 V at room temperature for 2 h. After being dried, the gel was exposed to X-ray film.

Gel-mobility shift assay

The gel-mobility shift assay was carried out essentially as described previously (Furuya and Lai, 1993) with slight modification. Briefly, 500 ng of recombinant proteins was first mixed with 20 μg of yeast tRNA in a final volume of 20 μl of binding buffer [5 mM HEPES (pH 7.8), 25 mM KCl, 2 mM MgCl2, 3.8% glycerol, 0.1 mM EDTA, 2 mM DTT]. Following incubation at 30°C for 10 min, 32P-labeled RNA (2 pmol of RNA) was added, and the reaction mixture was incubated for an additional 10 min. The reaction mixture was then mixed with 1/5 vol of 5X loading buffer [50% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol] and separated by electrophoresis on a 3% polyacrylamide gel made in 0.5X TBE [45 mM Tris–borate, 1 mM EDTA]–5% glycol. The polyacrylamide gels had been preelectrophoresed at 200 V for 30 min prior to loading of the samples, and electrophoresis was performed at 200 V at room temperature for 2 h. After being dried, the gel was exposed to X-ray film.

In vitro translation

In vitro translation was carried out in micrococcal nuclease-treated rabbit reticulocyte lysates (Flexi; Promega). Translation reactions (25 μl) were programmed with 2 μg of RNA, 10 μl of lysates, 0.5 U of RNase inhibitor (RNasin; Promega), 2 mM dithiothreitol, 20 μM amino acid mixture minus methionine, and 120 mM KCl in the presence of 1 μl of [35S]methionine (10 mCi/ml; NEN) and carried out at 30°C for 90 min. At the end of the reactions, stop buffer [50 μg/ml RNase A, 10 mM EDTA (pH 7.5)] was added to the reaction mixture. Aliquots of the translation products were separated by SDS–PAGE. The dried gel was autoradiographed and the radioactivities of the specific bands were quantitated by National Institutes of Health image 1.62 (Brown et al., 1998).

Primer extension

Two-microgram quantities of various RNAs were incubated with rabbit reticulocyte lysates in the presence of 120 mM KCl under the conditions for in vitro translation. Total RNA was extracted from the lysates at 0, 30, 60, and 90 min after the reaction using Trizol reagent (Gibco BRL) and analyzed by primer extension using a 32P-end-labeled primer (5'-AACACTACTGGCTAGCAGT-3') complementary to the 5'-UTR of HCV RNA as previously described (Ito et al., 1998).

LUC assays

For LUC assay of the in vitro translation product, 5 μl of reaction mixture was mixed with 100 μl of Luciferase assay reagent (Promega), and the LUC activity was measured after 20 s by Luminometer (Berthold).
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