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A Conserved Helical Element Is Essential for Internal Initiation of Translation of Hepatitis C Virus RNA

CHANGYU WANG, PETER SARNOW, AND ALEEM SIDDIQUI*

Department of Microbiology, Department of Biochemistry, Biophysics and Genetics, and Program in Molecular Biology, University of Colorado Medical School, Denver, Colorado 80262

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Translation of hepatitis C virus (HCV) RNA is initiated by cap-independent internal ribosome binding to the 5′ noncoding region (NCR). To identify the sequences and structural elements within the 5′ NCR of HCV RNA that contribute to the initiation of translation, a series of point mutations was introduced within this sequence. Since the pyrimidine-rich tract is considered a characteristic feature of picornavirus internal ribosome entry site (IRES) elements, our mutational analysis focused on two putative pyrimidine tracts (Py-I and Py-II) within the HCV 5′ NCR. Translational efficiency of these mutant RNAs was examined by in vitro translation and after RNA transfection into liver-derived cells. Mutational analysis of Py-I (nucleotides 120 to 130), supported by compensatory mutants, demonstrates that the primary sequence of this motif is not important but that a helical structural element associated with this region is critical for HCV IRES function. Mutations in Py-II (nucleotides 191 to 199) show that this motif is dispensable for IRES function as well. Thus, the pyrimidine-rich tract motif, which is considered as an essential element of the picornavirus IRES elements, does not appear to be a functional component of the HCV IRES. Further, the insertional mutagenesis study suggests a requirement for proper spacing between the initiator AUG and the upstream structures of the HCV IRES element for internal initiation of translation.

Human hepatitis C virus (HCV) infects hepatocytes and causes acute and chronic hepatitis (6, 15). HCV infection accounts for about 80% of posttransfusion non-A, non-B hepatitis. A strong association between the HCV infection and hepatocellular carcinoma has been observed (15). The viral genome has been cloned and shown to be a positive-stranded RNA of about 9.5 kb (7, 20, 36, 38, 45). The structure and organization of the HCV genome (Fig. 1A) are similar to those of pestiviruses and flaviviruses. However, unlike the flavivirus RNAs, the HCV and pestivirus RNAs have a relatively long 5′ noncoding region (NCR) preceding the single large open reading frame encoding the viral polyprotein. The 5′ NCR of HCV is highly conserved among different HCV isolates and represents the most conserved region of the genome (5). It contains about 341 nucleotides (nt) with multiple AUG triplets. A complex secondary structure has been proposed for the HCV 5′ NCR from phylogenetic comparative sequence analysis and substantiated by enzymatic probing of the RNA (4).

The majority of eukaryotic mRNAs are translated by a mechanism known as ribosome scanning (22). However, picornavirus RNAs and a few cellular mRNAs are translated by an alternate mechanism involving internal entry of ribosome into the 5′ nontranslated region (2, 13, 17, 23, 31, 37, 39). This mechanism involves binding of ribosomes to an RNA sequence that folds into a complex secondary structure known as the internal ribosome entry site (IRES) (17) or ribosome landing pad (39). Although HCV is not a member of the family *Picornaviridae*, recent studies have demonstrated that translation of the HCV genomic RNA is initiated by a cap-independent internal ribosome binding mechanism (21, 46, 47). Deletion analysis of the HCV 5′ NCR indicates that most of this sequence, which folds into a complex secondary structure, constitutes the IRES (47).

* Corresponding author. Phone: (303) 270-7016. Fax: (303) 270-8330.

A common feature of the picornavirus IRES elements is the presence of a pyrimidine-rich tract followed by an AUG triplet located about 20 nt downstream (18, 23, 35, 42), designated Yn-Xm-AUG motif (Y, pyrimidine; X, any base) (48). This AUG is not used as the initiation site of protein synthesis for enteroviruses and rhinoviruses but serves as an initiator AUG for the cardioviruses and aphthoviruses. The Yn-Xm-AUG motif and/or spacing requirement has not been shown to exist for the other RNAs that are capable of directing internal ribosome binding, such as the cellular immunoglobulin heavy-chain-binding protein mRNA (31), the *Antennapedia* mRNA of *Drosophila melanogaster* (37), and the RNA 3 of infectious bronchitis virus (29). A comparison of the RNA secondary structures among these cellular and viral IRES elements reveals no common structural features. Thus, it appears difficult to precisely define the structural and sequence components of the IRES element. Several studies have identified cellular proteins that interact with the picornavirus IRES elements (10, 12, 18, 30, 33). A 57-kDa protein (or polypyrimide tract-binding protein) (14) and a 52-kDa protein (or La autoantigen) (34) have been implicated in picornavirus IRES-mediated internal ribosome binding. However, the exact role of these proteins in IRES function remains unclear.

In the HCV 5′ NCR, the pyrimidine tract Py-I is located at nt 120 to 130 and is not followed by an AUG codon in the approximate vicinity on a primary sequence level. However, in the proposed RNA folding model (4), a helical structure is formed by base-pairing interaction between sequences at nt 125 to 133 and a distant sequence located at nt 315 to 323. This long-range interaction brings the initiator AUG in closer proximity (about 20 nt) to the pyrimidine tract. This structural arrangement would then resemble the characteristic Yn-Xm-AUG motif. A second pyrimidine tract, Py-II, is located at nt 191 to 199 and is followed by a noninitiator AUG triplet about 16 nt downstream of it. This sequence combination appears to contain the characteristic feature of the picornavirus IRES elements. In this study, a series of point mutations was

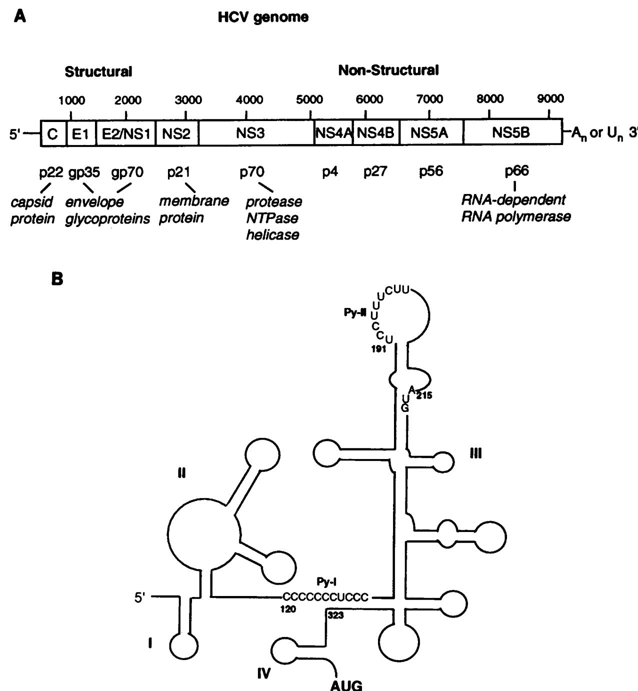

FIG. 1. (A) Structure and organization of the HCV genome. NTPase, nucleoside triphosphatase. (B) Schematic map of the computer-predicted RNA secondary structure of the 5′ NCR of HCV RNA (the structure is adapted from Brown et al. [4]). The numbering of nucleotides in the structure refers to the HCV-1 strain. The sequences of the two pyrimidine tracts are shown.

introduced in the HCV 5′ NCR with the aim of understanding the functional significance of the two putative pyrimidine tracts. The results of our mutagenesis studies argue against the requirement of a specific sequence in the pyrimidine tracts for IRES function. Instead, the maintenance of a secondary and/or tertiary structure seems to play a key role in internal initiation of translation of the HCV RNA genome.

MATERIALS AND METHODS

Plasmid constructions and site-directed mutagenesis. Standard methods were used for the manipulation, growth, and purification of plasmid DNAs (43). The T7-based luciferase construct (T7C1-341), which contains the full-length 5′ NCR of HCV (HCV-1 strain) preceding the coding region of the reporter gene (luciferase) in plasmid pGEM4, has been described previously (47). Plasmid T7C1-341P was made by inserting a 32-nt sequence of the polylinker region of pGEM4 (Promega) between the HCV 5′ NCR and the reporter gene. Plasmid T7CΔ152-278 was generated by deleting an *Age*I-*Stu*I fragment (nt 152 to 278) within the HCV 5′ NCR from plasmid T7C1-341. For site-directed mutagenesis, the Altered Sites in vitro mutagenesis system (Promega) was used as instructed by the manufacturer. The basic plasmid for the mutagenesis experiments was pHC5NC. This plasmid was produced by subcloning the *Hin*dIII-*Hpa*I fragment of the T7C1-341 plasmid DNA that contains the HCV 5′ NCR (nt 1 to 341) and the coding region of the luciferase gene into the *Hin*dIII-*Sma*I site of the phagemid vector pAlter-1. In pHC5NC and its derivative plasmids, the luciferase-encoding gene is under control of the bacteriophage SP6 promoter. Mutagenic oligonucleotides were purchased from Oligo Etc. Mutations in the HCV 5′ NCR were subsequently confirmed by double-stranded DNA sequencing by the dideoxynucleotide method. Mutants are named according to the first nucleotide position of each mutated sequence. For example, in plasmid pHC5NC/126, the mutated sequence starts at nt 126. The mutated sequences and their locations are detailed in the figures in the context of the secondary structure.

In vitro transcription and translation. Plasmid DNAs were purified by CsCl density gradient centrifugation. Plasmid DNAs of T7C1-341, T7C1-341P, and T7CΔ152-278 were linearized with *Hpa*I and transcribed in vitro with T7 RNA polymerase (New England Biolabs) as instructed by the manufacturer. Plasmid pHC5NC and its derivatives were linearized with *Bam*HI and transcribed in vitro with SP6 RNA polymerase (Promega). In vitro translation experiments were carried out in rabbit reticulocyte lysates (Promega). Proteins were labeled with Trans^{35}S-label (ICN Biomedicals) and subsequently fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

RNA transfection. HepG2, a human hepatoblastoma cell line, was used in this study for the direct RNA transfection experiments. Cells were maintained in Dulbecco's modified Eagle medium with 8% fetal calf serum. In vitro-synthesized RNAs were introduced into cultured cells with Lipofectin (GIBCO/BRL). Nearly confluent cell monolayers (>80%) in 60-mm-diameter dishes were transfected with 5 to 10 μg of RNA mixed with 30 μg of Lipofectin in 3 ml of Opti-MEM I (GIBCO/BRL) as described previously (1). Five to six hours after transfection, cell lysates were prepared and assayed for luciferase activity as described by de Wet et al. (9) with a luminometer (Mode Opticomp I; MGM Instruments, Inc.).

RESULTS

Mutational analyses of pyrimidine tracts Py-I and Py-II. A conserved feature of the 5′ NCR of all picornavirus RNAs, which conduct translation by the mechanism of internal ribosome entry, is the presence of a pyrimidine-rich stretch of nucleotides (16). The functional significance of this motif in translation initiation has been amply demonstrated by extensive mutational analyses by several investigators (18, 23, 32, 42). In the present study, we have focused on two pyrimidine tracts located in the HCV 5′ NCR (Fig. 1B). Py-I spans from nt 120 to 130 and is located immediately upstream of the domain III stem-loop structure (Fig. 1B). From the primary sequence arrangement, Py-I is not followed by an AUG triplet, which is an important feature of the Yn-Xm-AUG motif found in picornavirus IRES elements (48). However, in the predicted secondary structure model of the 5′ NCR (4), the initiator AUG located at nt 342 is brought into close structural proximity by base pairing between nt 125 to 133 and 315 to 323 (Fig. 1B). Part of this pyrimidine tract sequence also participates in a base-pairing interaction. The second putative pyrimidine tract is located within the apical loop of the domain III structure (Fig. 1B), and a noninitiator AUG triplet is located 16 nt downstream. A series of mutations was generated to investigate the functional relevance of these two pyrimidine tracts in the HCV IRES element. Base substitutions were introduced into an HCV 5′ NCR cDNA which was cloned in front of the reporter gene luciferase. Translation of in vitro-synthesized RNAs was analyzed for luciferase activity in rabbit reticulocyte lysates and in transfected liver-derived HepG2 cells.

The first series of base substitutions was made in Py-I located at nt 120. Plasmid pHC5NC/126 contains four nucle-

A

pHC5NC pHC5NC/126 pHC5NC/319 pHC5NC/126/319

pHC5NC/121/126/319 pHC5NC/196 pHC5NC/217

B

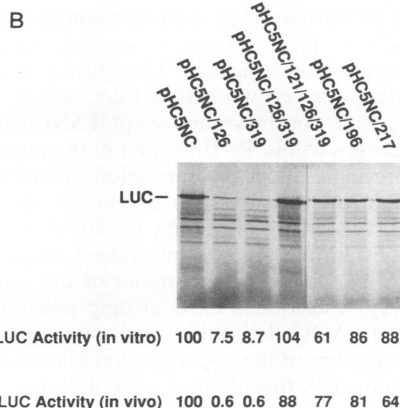

FIG. 2. (A) Mutagenesis of the 5′ NCR of HCV RNA. Nucleotide sequences of the mutated regions are presented in the context of the predicted secondary structure. The altered nucleotides are boxed and shown in lowercase. (B) Translation of the RNAs in vitro (rabbit reticulocyte lysates) and in vivo (transfected HepG2 cells). SDS-PAGE patterns of the [^{35}S]methionine/cysteine-labeled translation products of the RNAs from rabbit reticulocyte lysates are shown. The position of luciferase protein (LUC) is marked. The lower panel indicates the percentage of luciferase activity from reticulocyte lysates (in vitro) and transfected HepG2 cellular extracts (in vivo). Luciferase activity is normalized against the value obtained with pHC5NC RNA containing full-length wild-type 5′ NCR, arbitrarily set at 100.

otide changes between nt 126 and 129 (CUCC to AGAA) within the pyrimidine sequences (Fig. 2A). Results from in vitro and in vivo translation studies of the mutant pHC5NC/126 RNA are shown in Fig. 2B. Mutations in these nucleotides caused a dramatic reduction in translation initiation by the HCV 5′ NCR, as determined by expression of the luciferase reporter gene. Interestingly, a much greater inhibitory effect on translation of the mutant RNA was observed in vivo than in vitro, indicating that the HCV IRES element is more sensitive to this mutation in the cellular environment. These results from both in vitro and in vivo experiments suggest that the sequence within Py-I might be important for HCV IRES function. However, a closer examination of the secondary structure of the 5′ NCR in this region predicts a likely disruption of a helical structure by the mutations in the pHC5NC/126 RNA. The apparent perturbance of the helical structure might have caused the observed decrease in transla-

tional efficiency of the mutated RNA. Therefore, a second mutation was introduced in the region which was complementary to the mutated pyrimidine sequences of pHC5NC/126. Plasmid pHC5NC/319 contains nucleotide alterations between nt 319 and 322 (GGAG to UUCU) in the 5′ NCR (Fig. 2A). These base substitutions should also cause disruption of the base-pairing interaction similar to those found for mutant pHC5NC/126. As expected, the translational efficiency of pHC5NC/319 RNA was dramatically reduced both in vitro and in vivo (Fig. 2B). It must be noted that in this mutant, the primary sequence in Py-I remained intact and only the complementary sequences at nt 319 to 322 were altered. Failure of this mutant HCV 5′ NCR to initiate translation efficiently strongly suggests the importance of the relevant secondary structure. To provide conclusive evidence in support of this notion, a compensatory mutant, pHC5NC/126/319, was generated by combining the nucleotide alterations in pHC5NC/126 and pHC5NC/319 (Fig. 2A). This compensatory mutant maintains the nucleotide changes of pHC5NC/126 that interrupt the pyrimidine tract sequence but restores the predicted helical structure with the introduction of complementary sequences. As shown in Fig. 2B, the translational efficiency of this mutant RNA was fully recovered, reflecting the restoration of the secondary structure. Further mutations in plasmid pHC5NC/126/319 in the upstream pyrimidine sequences at nt 121 to 122 (CC to AA) (pHC5NC/121/126/319; Fig. 2A) resulted in only a modest inhibitory effect on translation of the mutant RNA (Fig. 2B). It should be emphasized that in plasmid pHC5NC/121/126/319, majority of the pyrimidine bases have been altered with apparently no appreciable effect on translation. Together, these mutational studies demonstrate a functional importance of the secondary structure which includes the pyrimidine-rich region and preclude any specific role for the pyrimidine sequences per se.

A second putative pyrimidine-rich sequence, Py-II, is located within the apical loop of the domain III structure and contains a stretch of 9 nt from nt 191 to 199. This pyrimidine tract is particularly interesting because a noninitiator AUG triplet is located 16 nt downstream. By analogy to the picornavirus IRES elements, it is very likely that Py-II may participate in functioning as a Yn-Xm-AUG motif for the HCV IRES element. To address the functional role of this suspected motif, two mutants were made. Plasmid pHC5NC/196 contains three nucleotide substitutions between nt 196 and 198 (UCU to AGA) within the pyrimidine tract (Fig. 2A), and plasmid pHC5NC/217 contains a one-base substitution which converts the AUG triplet at nt 215 to AUC (Fig. 2A). As shown in Fig. 2B, the mutation in Py-II caused only a slight decrease in translation of luciferase both in vitro and in vivo. Similarly, mutation of the noninitiator AUG triplet caused only a slight decrease in translation of the pHC5NC/217 RNA. These results demonstrate that the second pyrimidine tract and the AUG triplet following it are not a part of the functional IRES element. Thus, from these mutational analyses, it appears that neither of the putative pyrimidine tracts is critical for HCV IRES function. This feature of the HCV IRES element, therefore, is distinct from that of picornavirus IRES elements.

Insertion of additional nucleotides upstream of the initiator AUG. It has been shown that picornavirus IRES elements require a spacing of about 20 nt between an AUG triplet and a pyrimidine tract (18, 23, 32, 42). Studies described above demonstrate that a pyrimidine tract-related helical structure is critical for its function. According to the predicted secondary structure model, the helical structure is located about 20 nt upstream of the initiator AUG codon (Fig. 1B). To determine whether spacing between the initiator AUG and the upstream structural elements is crucial for IRES function, a random sequence of 32 nt was inserted immediately upstream of the initiator AUG (T7C1-341P). As shown in Fig. 3, this insertion significantly impaired the ability of the HCV 5′ NCR in directing translation initiation of the mutant RNA both in vitro and in vivo. In a previous report, we showed that a 9-nt deletion at the 3′ border of the HCV 5′ NCR abolished translation (47). In light of this result, it is likely that a proper spacing between a functional motif of the HCV 5′ NCR (i.e., the helical structure associated with Py-I) and the initiator AUG is required for efficient translation. Alternatively, this insertion may have disrupted the nearby structures which are crucial for IRES function. These results also explain the failure by Yoo et al. (49) to detect IRES function in the HCV 5′ NCR. The constructs used by Yoo et al. (49) contained an additional 30 nt upstream of the initiator AUG of the reporter gene which could have destroyed the IRES function, an effect similar to that observed here with the T7C1-341P construct.

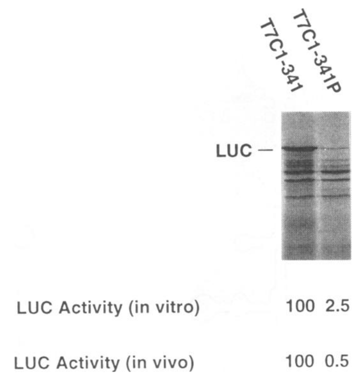

FIG. 3. Inhibitory effect on translation initiation caused by an insertion mutation. The ^{35}S-labeled protein synthesis in reticulocyte lysates (in vitro) is illustrated by the SDS-PAGE pattern. The percentage of luciferase (LUC) activity of the mutant RNA normalized against the wild-type 5′ NCR-containing T7C1-341 RNA from translation in reticulocyte lysates (in vitro) and transfected HepG2 cellular extracts (in vivo) is shown in the lower panel.

Mutational analysis in the stem-loop structure domain III. Domain III of the HCV 5′ NCR represents a stable, large structure with multiple stem-loops (Fig. 1B). The apical loop of this domain harbors the second pyrimidine tract, which is followed by an AUG codon. As shown above (pHC5NC/196; Fig. 2B), nucleotide changes within Py-II located in the apical loop exhibited no appreciable effect on translation initiation. This finding suggests that the primary sequence in the apical loop of domain III structure is not important for HCV IRES function. To further substantiate this, we introduced another mutation in the region immediately downstream of the loop (pHC5NC/206; Fig. 4A). Nucleotides CCC at map positions 206 to 208 were altered to AGG. This mutation is expected to significantly affect the structure of the upper portion of domain III (Fig. 4A). Surprisingly, data from both in vitro and in vivo studies show that this structural alteration did not result in any significant decrease in translation initiation (Fig. 4B). The translational efficiency of the mutant pHC5NC/206 RNA was reduced only by 40% compared with the wild-type pHC5NC RNA. This modest inhibitory effect could be due to the structural perturbance caused by the mutation. Next, a 127-nt deletion was made in domain III (T7CΔ152-278; Fig. 4A). This deletion mutation essentially eliminates the upper portion of domain III, including the apical loop containing Py-II motif.

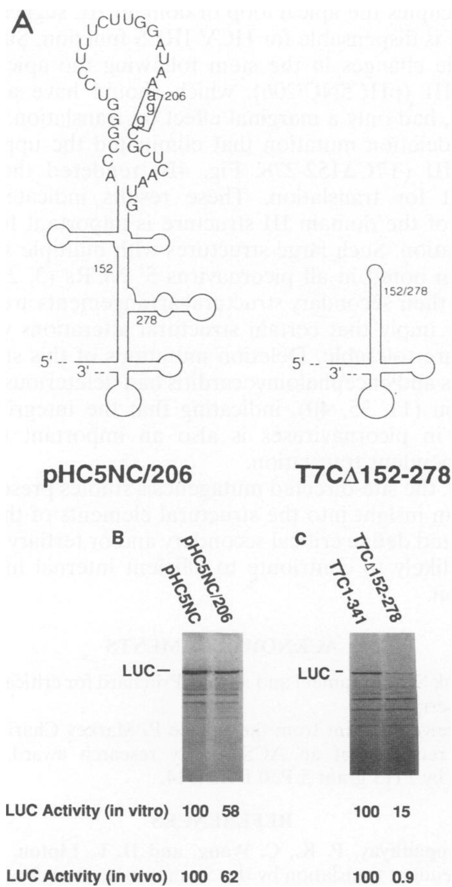

FIG. 4. (A) Mutational analysis of domain III structure of the HCV 5′ NCR. The altered sequences from point mutations are boxed and shown in lowercase. (B and C) SDS-PAGE patterns of the in vitro translation products of the RNAs. The lower panel indicates the percentage of luciferase (LUC) activity of the mutant RNAs normalized against the wild-type RNAs in reticulocyte lysates (in vitro) and transfected HepG2 cellular extracts (in vivo).

Translation of T7CΔ152-278 RNA in transfected cells was dramatically reduced (Fig. 4C), whereas in vitro the effect of mutation was 85% less efficient. This finding indicates that the integrity of the domain III structure is important for translation initiation, while structural and sequence alterations in the apical part are tolerable.

DISCUSSION

Although HCV has been tentatively classified as a member of the family *Flaviviridae*, the translational strategy utilized by this virus is similar to that of picornaviruses. Translation of the picornavirus RNA genomes occurs in a cap-independent manner by the internal entry of ribosome into the 5′ NCR. Using the dicistronic expression vector scheme, we and others have previously demonstrated that HCV contains an IRES element within the 5′ NCR (21, 46, 47). The HCV IRES element can initiate cap-independent translation efficiently in poliovirus-infected cells (47) and in coxsackievirus-infected cell extracts (46) in which cap-dependent translation has been suppressed (28). While the mechanism of how the IRES elements function in picornaviruses is not clearly understood, several lines of

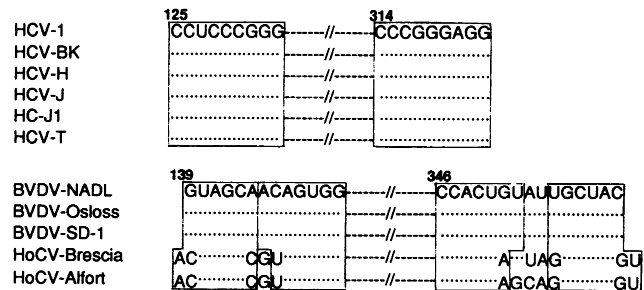

FIG. 5. Sequence comparison among different HCV and pestivirus isolates in the predicted helical structural element of the 5′ NCR. Sequences were obtained from GenBank. The nucleotide positions in HCVs are according to the HCV-1 isolate sequence (GenBank); BVDV-NADL, bovine viral diarrhea virus; HoCV, hog cholera virus.

evidence suggest a functional role for a Yn-Xm-AUG motif (48).

In this report, the roles of two putative pyrimidine tract sequences, Py-I and Py-II, of the HCV 5′ NCR were investigated by site-directed mutagenesis. The results of this analysis indicate that both motifs were dispensable for translation initiation. This finding suggests that the Yn-Xm-AUG motif may not be an important component of the HCV IRES element. However, our studies strongly implicate the involvement of a helical secondary structure associated with Py-I for HCV IRES function. The secondary structure of this region involves base-pairing interactions between nt 125 to 133 and nt 315 to 323. The maintenance of such a structure appears to be the critical element of HCV IRES, as evidenced by the mutational analysis presented here. The key evidence is based on the recovery of translational efficiency by the compensatory mutants pHC5NC/126/319 and pHC5NC/121/126/319 (Fig. 2B). An important feature of this result is that one of the mutants (pHC5NC/121/126/319), which contained extensive changes in the pyrimidine sequences, was efficiently translated. This finding suggests that the primary sequences of the pyrimidine tract may not be important for IRES function, but a higher-order structure in this region regulates internal initiation of translation. This view is also supported by the recent computer-based structural analysis of the picornavirus 5′ NCR. In this analysis, conserved tertiary structural elements such as pseudoknots, which may play a key role in cap-independent translation, have been predicted (24, 25). It is striking that this helical structural element appears to exist in all HCV isolates and the related pestiviruses (4, 8). Sequences in the helical region are highly conserved among different variants of HCV or are substituted by coupled mutations in pestiviruses (Fig. 5). This high degree of conservation suggests that this secondary structure may be important for viral function. Further, this helical element is predicted to be a thermodynamically stable structure ($\Delta G = -20.5$ kcal [ca. -85.6 kJ] mol^{-1}). From the data presented in Fig. 2B, it is tempting to implicate this region as a functional component of the HCV IRES element. However, it should be emphasized that the maintenance of the helical structure alone is not sufficient for IRES function; other sequences and relevant structures of the 5′ NCR may play an equally important role in translation. For instance, the functional role of the domain III structure was demonstrated by the deletion mutation (T7CΔ152-278) which abolished translation (Fig. 4B). In this mutant, the helical structure should remain intact, while the major portion of domain III is deleted.

The mutational studies presented here provide the first

genetic evidence in support of the RNA folding model of the HCV 5′ NCR proposed by Brown et al. (4), which is also supported by the RNase probing analysis. Tsukiyama-Kohara et al. (46) proposed a different model in which the helical structure associated with Py-I is not included. This model, therefore, is not in agreement with our mutational data.

Pyrimidine-rich tracts have been suggested to be important elements of the picornavirus IRES. This notion is supported by previous mutational studies (18, 23, 32, 42). However, a recent study on the encephalomyocarditis virus IRES shows that mutation of all the pyrimidine residues to purines in the pyrimidine-rich tract caused only a marginal decrease in translation initiation (19). These data and the studies presented here on HCV suggest that primary sequences of the pyrimidine-rich tract may not be important for IRES functions.

On the basis of secondary structural models, it was predicted that the region surrounding the pyrimidine tract is unstructured in the picornavirus 5′ NCR (3, 27, 41, 44). However, it has been recently proposed that the pyrimidine tract could be involved in forming some tertiary structures such as pseudoknots (24, 25). Such high-order structures may be important components of the IRES element. Of interest in this respect is the mutational study of the poliovirus IRES by Pilipenko et al. (42), in which deletion of most of the sequences in the pyrimidine tract, while retaining only five nucleotides, had no major effect on translation. Of the five nucleotides, three uridine residues were predicted to be involved in base-pairing interactions that have been implicated in pseudoknot structure according to the recently revised RNA folding model of the poliovirus 5′ NCR (24). Thus, it is possible that the inhibitory effect of mutations in the pyrimidine tract of picornavirus 5′ NCR is due to structural perturbances caused by the mutations rather than changes in the primary sequence. These mutations in the pyrimidine tract could have disrupted the tertiary structure and may have led to a loss of IRES function. Interestingly, a relevant tertiary structure has also been found in the 5′ NCR of the infectious bronchitis virus RNA 3, which is capable of directing internal initiation of translation (26). Similarly, our preliminary data from additional mutations in the HCV 5′ NCR indicate the presence of a tertiary structure (a pseudoknot structure) in the vicinity of the initiator AUG. This superstructure includes the pyrimidine tract-related helical secondary structure (47a).

On the basis of several criteria, the picornavirus IRES elements are classified into two groups (16, 48). In group I IRES (enteroviruses and rhinoviruses), the ribosomes bind to the IRES element which includes a noninitiator AUG triplet and scan until a proper AUG triplet is encountered to initiate translation. In the case of group II IRES (cardioviruses and aphthoviruses), the ribosome binds to the IRES element that includes an initiator AUG triplet and translation is initiated without scanning. In both cases, a proper spacing between an AUG triplet and certain upstream motifs is essential for IRES function. Similarly, in the case of the HCV IRES, a spacing requirement between the initiator AUG and an upstream motif in the 5′ NCR appears to be essential, as suggested by the data for the insertion mutant T7C1-341P (Fig. 3). It is likely, although not yet proven, that the initiator AUG may be involved in HCV IRES function, similar to the group II IRES elements. The HCV IRES element shares yet another feature of the group II IRES elements in that both can direct internal initiation of translation efficiently in vitro in reticulocyte lysates.

Domain III of the 5′ NCR represents a relatively large secondary structure with multiple stem-loops and harbors the pyrimidine tract Py-II (Fig. 1B). Point mutations in Py-II, which occupies the apical loop of domain III, suggest that this sequence is dispensable for HCV IRES function. Surprisingly, nucleotide changes in the stem following the apical loop of domain III (pHC5NC/206), which should have altered the structure, had only a marginal effect on translation. However, a major deletion mutation that eliminated the upper half of domain III (T7CΔ152-278; Fig. 4B) rendered the 5′ NCR inefficient for translation. These results indicate that the integrity of the domain III structure is important for translation initiation. Such large structures with multiple stem-loops have been noted in all picornavirus 5′ NCRs (3, 27, 41, 44), although their secondary structural arrangements are different. This may imply that certain structural alterations within this domain are tolerable. Deletion mutations of this structure in poliovirus and encephalomyocarditis had deleterious effects on translation (11, 35, 40), indicating that the integrity of such domains in picornaviruses is also an important feature of cap-independent translation.

Finally, the site-directed mutagenesis studies presented here provide an insight into the structural elements of the 5′ NCR of HCV and define critical secondary and/or tertiary structures that are likely to contribute to efficient internal initiation of translation.

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REFERENCES

1. **Bandyopadhyay, P. K., C. Wang, and H. L. Lipton.** 1992. Cap-independent translation by the 5′ untranslated region of Theiler's murine encephalomyelitis virus. J. Virol. **66:**6249–6256.
2. **Borman, A., and R. J. Jackson.** 1992. Initiation of translation of human rhinovirus RNA: mapping the internal ribosome entry site. Virology **188:**685–696.
3. **Brown, E. A., S. P. Day, R. W. Jansen, and S. M. Lemon.** 1991. The 5′ nontranslated region of hepatitis A virus RNA: secondary structure and elements required for translation in vitro. J. Virol. **65:**5825–5838.
4. **Brown, E. A., H. Zhang, L.-H. Ping, and S. M. Lemon.** 1992. Secondary structure of the 5′ nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. Nucleic Acids Res. **20:**5041–5045.
5. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1992. Sequence analysis of the 5′ noncoding region of hepatitis C virus. Proc. Natl. Acad. Sci. USA **89:**4942–4946.
6. **Choo, Q.-L., G. Kuo, A. Weiner, K.-S. Wang, L. Overby, D. Bradley, and M. Houghton.** 1992. Identification of the major, parenteral non-A, non-B hepatitis agent (hepatitis C virus) using a recombinant cDNA approach. Semin. Liver Dis. **12:**279–288.
7. **Choo, Q.-L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, A. Medina-Selby, P. J. Barr, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:**2451–2455.
8. **Deng, R., and K. V. Brock.** 1993. 5′ and 3′ untranslated regions of pestivirus genome: primary and secondary structure analyses. Nucleic Acids Res. **21:**1949–1957.
9. **de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7:**725–737.
10. **Dildine, S. L., and B. L. Semler.** 1992. Conservation of RNA-protein interactions among picornaviruses. J. Virol. **66:**4364–4376.
11. **Duke, G. M., M. A. Hoffman, and A. C. Palmenberg.** 1992. Sequence and structural elements that contribute to efficient encephalomyocarditis virus RNA translation. J. Virol. **66:**1602–1609.

12. **Gebhard, J. R., and E. Ehrenfeld.** 1992. Specific interactions of HeLa cell proteins with proposed translation domains of the poliovirus 5′ noncoding region. J. Virol. **66:**3101–3109.
13. **Glass, J. M., X.-Y. Jia, and D. F. Summers.** 1993. Identification of the hepatitis A virus internal ribosome entry site: in vivo and in vitro analysis of bicistronic RNAs containing the HAV 5′ noncoding region. Virology **193:**842–852.
14. **Hellen, C. U. T., G. W. Witherell, M. Schmid, S. H. Shin, T. V. Pestova, A. Gil, and E. Wimmer.** 1993. A cytoplasmic 57 kDa protein (p57) that is required for translation of picornavirus RNA by internal ribosome entry is identical to the nuclear polypyrimidine tract-binding protein. Proc. Natl. Acad. Sci. USA **90:**7642–7646.
15. **Houghton, M., A. Weiner, J. Han, G. Kuo, and Q.-L. Choo.** 1991. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology **14:**381–388.
16. **Jackson, R. J., M. T. Howell, A. Kaminski.** 1990. The novel mechanism of initiation of picornavirus RNA translation. Trends Biochem. Sci. **15:**477–483.
17. **Jang, S. K., M. V. Davies, R. J. Kaufman, and E. Wimmer.** 1989. Initiation of protein synthesis by internal entry of ribosomes into the 5′ nontranslated region of encephalomyocarditis virus RNA in vivo. J. Virol. **63:**1651–1660.
18. **Jang, S. K., and E. Wimmer.** 1990. Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-KD RNA-binding protein. Genes Dev. **4:**1560–1572.
19. **Kaminski, A., G. J. Belsham, and R. J. Jackson.** 1994. Translation of encephalomyocarditis virus RNA: parameters influencing the selection of the internal initiation site. EMBO J. **13:**1673–1681.
20. **Kato, N., M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugimura, and K. Shimotohno.** 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. USA **87:**9524–9528.
21. **Kettinen, H., K. Grace, S. Grunert, B. Clarke, D. Rowlands, and R. Jackson.** Mapping of the internal ribosome entry site at the 5′ end of the hepatitis C virus genome. Proceedings of the International Symposium on Viral Hepatitis and Liver Disease, in press.
22. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108:**229–241.
23. **Kuhn, R., N. Luz, and E. Beck.** 1990. Functional analysis of the internal translation initiation site of foot-and-mouth disease virus. J. Virol. **64:**4625–4631.
24. **Le, S.-Y., J.-H. Chen, N. Sonenberg, and J. V. Maizel.** 1992. Conserved tertiary structure elements in the 5′ untranslated region of human enteroviruses and rhinoviruses. Virology **191:**858–866.
25. **Le, S.-Y., J.-H. Chen, N. Sonenberg, and J. V. Maizel, Jr.** 1993. Conserved teriary structural elements in the 5′ nontranslated region of cardiovirus, aphthovirus and hepatitis A virus RNAs. Nucleic Acids Res. **21:**2445–2451.
26. **Le, S.-Y., N. Sonenberg, and J. V. Maizel, Jr.** 1994. Distinct structural elements and internal entry of ribosomes in mRNA3 encoded by infectious bronchitis virus. Virology **198:**405–411.
27. **Le, S.-Y., and M. Zuker.** 1990. Common structures of the 5′ non-coding RNA in enteroviruses and rhinoviruses. J. Mol. Biol. **216:**729–741.
28. **Lee, K. A. W., and N. Sonenberg.** 1982. Inactivation of cap-binding proteins accompanies the shut-off of host protein synthesis by poliovirus. Proc. Natl. Acad. Sci. USA **79:**3447–3451.
29. **Liu, D. X., and S. C. Inglis.** 1992. Internal entry of ribosomes on a tricistronic mRNA encoded by infectious bronchitis virus. J. Virol. **66:**6143–6154.
30. **Luz, N., and E. Beck.** 1990. A cellular 57 kDa protein binds to two regions of the internal initiation site of foot-and-mouth disease virus. FEBS Lett. **269:**311–314.
31. **Macejak, D. G., and P. Sarnow.** 1991. Internal initiation of translation mediated by 5′ leader of a cellular mRNA. Nature (London) **353:**90–94.
32. **Meerovitch, K., R. Nicholson, and N. Sonenberg.** 1991. In vitro mutational analysis of *cis*-acting RNA translational elements within the poliovirus type 2 5′ untranslated region. J. Virol. **65:**5895–5901.
33. **Meerovitch, K., J. Pelletier, and N. Sonenberg.** 1989. A cellular protein that binds to the 5′-noncoding region of poliovirus RNA: implications for internal translation initiation. Genes Dev. **3:**1026–1033.
34. **Meerovitch, K., Y. U. Svitken, H. S. Lee, F. Lejbkowicz, D. J. Kenan, E. K. L. Chan, V. I. Agol, J. D. Keene, and N. Sonenberg.** 1993. La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. J. Virol. **67:**3798–3807.
35. **Nicholson, R., J. Pelletier, S.-Y. Le, and N. Sonenberg.** 1991. Structural and functional analysis of the ribosome landing pad of poliovirus type 2: in vivo translational studies. J. Virol. **65:**5886–5894.
36. **Ogata, N., H. J. Alter, R. H. Miller, and R. H. Purcell.** 1991. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:**3392–3396.
37. **Oh, S.-K., M. P. Scott, and P. Sarnow.** 1992. Homeotic gene *Antennapedia* mRNA contains 5′-noncoding sequences that confer translational initiation by internal ribosome binding. Gene Dev. **6:**1643–1653.
38. **Okamoto, H., S. Okada, Y. Sugiyama, K. Kurai, H. Iizuka, A. Machida, Y. Miyakawa, and M. Mayumi.** 1991. Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. J. Gen. Virol. **72:**2697–2704.
39. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature (London) **334:**320–325.
40. **Percy, N., G. J. Belsham, J. K. Brangwyn, M. Sullivan, D. M. Stone, and J. W. Almond.** 1992. Intracellular modifications induced by poliovirus reduce the requirement for structural motifs in the 5′ noncoding region of the genome involved in internal initiation of protein synthesis. J. Virol. **66:**1695–1701.
41. **Pilipenko, E. V., V. M. Blinov, L. I. Ramanova, A. N. Sinyakov, S. V. Maslova, and V. I. Agol.** 1989. Conserved structural domains in the 5′-untranslated region of picornaviral genomes: an analysis of the segment controlling translation and neurovirulence. Virology **168:**201–209.
42. **Pilipenko, E. V., A. P. Gmyl, S. V. Maslova, Y. V. Svitkin, A. N. Sinyakov, and V. I. Agol.** 1992. Prokaryotic-like cis elements in the cap-independent internal initiation of translation on picornavirus RNA. Cell **68:**119–131.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Skinner, M. A., V. R. Racaniello, G. Dunn, J. Cooper, P. D. Minor, and J. W. Almond.** 1989. New model for the secondary structure of the 5′ non-coding RNA of poliovirus is supported by biochemical and genetic data that also show that RNA secondary structure is important in neurovirulence. J. Mol. Biol. **207:**379–392.
45. **Takamizawa, A., C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, and H. Okayama.** 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. J. Virol. **65:**1105–1113.
46. **Tsukiyama-Kohara, K., N. Iizuka, M. Kohara, and A. Nomoto.** 1992. Internal ribosome entry site within hepatitis C virus RNA. J. Virol. **66:**1476–1483.
47. **Wang, C., P. Sarnow, and A. Siddiqui.** 1993. Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. J. Virol. **67:**3338–3344.

47a. **Wang, C., and A. Siddiqui.** Unpublished data.

48. **Wimmer, E., C. U. T. Hellen, and X. Cao.** 1993. Genetics of poliovirus. Annu. Rev. Genet. **27:**353–436.
49. **Yoo, B. J., R. R. Spaete, A. P. Geballe, M. Selby, M. Houghton, and J. H. Han.** 1992. 5′ end-dependent translation initiation of hepatitis C viral RNA and the presence of putative positive and negative translational control elements within the 5′ untranslated region. Virology **191:**889–899.