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Structural Requirements for Initiation of Translation by Internal Ribosome Entry within Genome-Length Hepatitis C Virus RNA

MASAO HONDA,* LI-HUA PING,* RENE C. A. RIJNBRAND,† ELIZABETH AMPHLETT,‡ BERWYN CLARKE,‡ DAVID ROWLANDS,‡ and STANLEY M. LEMON*,[1]

*Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7030; †Department of Virology, Institute of Medical Microbiology, Leiden University, Leiden 2300AH, The Netherlands; and ‡Department of Molecular Sciences, Glaxo-Wellcome, Beckenham, Kent, BR3-3BS, United Kingdom

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Cap-independent translation of hepatitis C virus (HCV) RNA is mediated by an internal ribosomal entry segment (IRES) located within the 5′ nontranslated RNA (5′NTR), but previous studies provide conflicting views of the viral sequences which are required for translation initiation. These discrepancies could have resulted from the inclusion of less than full-length 5′NTR in constructs studied for translation or destabilization of RNA secondary structure due to fusion of the 5′NTR to heterologous reporter sequences. In an effort to resolve this confusion, we constructed a series of mutations within the 5′NTR of a nearly full-length 9.5-kb HCV cDNA clone and examined the impact of these mutations on HCV translation *in vitro* in rabbit reticulocyte lysates and in transfected Huh-T7 cells. The inclusion of the entire open reading frame in HCV transcripts did not lead to an increase in IRES-directed translation of the capsid and E1 proteins, suggesting that the nonstructural proteins of HCV do not include a translational transactivator. However, in reticulocyte lysates programmed with full-length transcripts, there were multiple aberrent translation initiation sites resembling those identified in some picornaviruses. The deletion of nucleotides (nt) 28–69 of the 5′NTR (stem-loop IIa) sharply reduced capsid translation both *in vitro* and *in vivo.* A small deletion mutation involving nt 328–334, immediately upstream of the initiator AUG at nt 342, also resulted in a nearly complete inhibition of translation, as did the deletion of multiple intervening structural elements. An in-frame 12-nt insertion placed within the capsid-coding region 9 nt downstream of the initiator AUG strongly inhibited translation both *in vitro* and *in vivo,* while multiple silent mutations within the first 42 nt of the open reading frame also reduced translation in reticulocyte lysates. Thus, domains II and III of the 5′NTR are both essential to activity of the IRES, while conservation of sequence downstream of the initiator AUG is required for optimal IRES-directed translation.

Hepatitis C viruses (HCV) are positive-strand, enveloped RNA viruses which are classified within a genus of the family Flaviviridae (Houghton *et al.,* 1991; Matsuura and Miyamura, 1993). These viruses have a strong tendency to establish persistent infections in humans and are responsible for considerable morbidity and mortality related to chronic liver disease and hepatocellular carcinoma (Alter *et al.,* 1992; Kiyosawa and Furuta, 1994). There is extensive genetic, and possibly antigenic, heterogeneity among different HCV strains (Bukh *et al.,* 1995), and the rapid evolution of distinct HCV quasispecies in infected individuals may contribute to virus evasion of host immune responses and the persistence of infection (Weiner *et al.,* 1992; Honda *et al.,* 1994). Differences between the nucleotide sequences of various HCV stains are greatest in the envelope protein-coding region and the 3′ nontranslated region (3′NTR) of the genome, and least in the 5′NTR (Bukh *et al.,* 1995). The relatively low sequence heterogeneity observed within the 341-nucleotide (nt) 5′NTR reflects the presence of highly conserved secondary and tertiary RNA structures which are presumably essential for virus replication (Brown *et al.,* 1992; Smith *et al.,* 1995). This conservation of secondary and tertiary RNA structure extends to the 5′NTRs of the distantly related pestiviruses as well as the recently recognized primate hepatitis virus, GBV-B (M. Honda *et al.,* submitted for publication). Evidence from multiple laboratories indicates that these conserved RNA structures contribute to an internal ribosomal entry segment (IRES) within the 5′NTRs of these viruses which directs the initiation of virus translation in a cap-independent fashion similar to picornaviruses (Tsukiyama-Kohara *et al.,* 1992; Wang *et al.,* 1993; Fukushi *et al.,* 1994; Rijnbrand *et al.,* 1995; Reynolds *et al.,* 1995).

Internal initiation of translation appears to be a feature common to HCV and pestiviruses (Poole *et al.,* 1995) and distinguishes these viruses from other members of the Flaviviridae (such as yellow fever virus) which possess a much shorter 5′NTR and undergo 5′ cap-dependent translation similar to that of eucaryotic mRNAs. Although an understanding of the mechanism by which translation is initiated on HCV genomic RNA might be helpful in designing novel antiviral treatment strategies, this process is poorly understood. Definition of the RNA struc-

[1] To whom correspondence and reprint requests should be addressed. Fax: (919) 966-6714.

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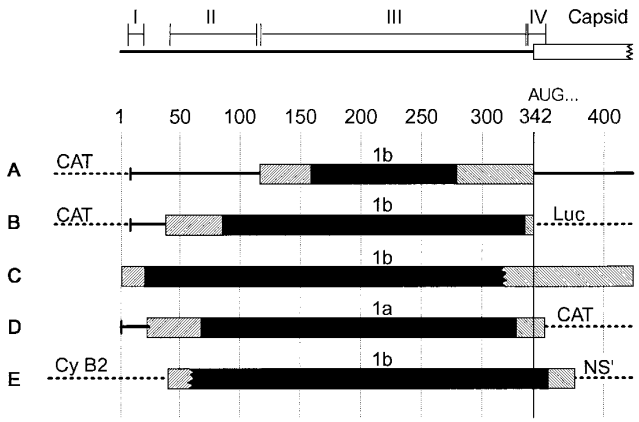

FIG. 1. Schematic summary of previous efforts to define the 5′ and 3′ limits of the HCV IRES. At the top is depicted the 5′ end of the HCV-N genome, with untranslated RNA shown as a solid line, and the polyprotein coding region commencing with the AUG at nt 342 as an open box. Locations of specific structural domains (I, II, and III) within the 5′NTR are indicated by brackets. Below are shown specific monocistronic or bicistronic transcripts analyzed in five previous *in vitro* or *in vivo* studies of the HCV IRES: (A) Tsukiyama-Kohara *et al.* (1992); (B) Wang *et al.* (1993); (C) Fukushi *et al.* (1994); (D) Rijnbrand *et al.* (1995); and (E) Reynolds *et al.* (1995). For each of the transcripts shown, heterologous upstream or downstream reporter sequences are shown as a dotted line with the specific reporter protein indicated above, while the HCV sequence appears as a solid line. The regions of the HCV sequence defined in each study as essential for IRES activity are indicated by the solid boxes: the possible ranges of the 5′ and 3′ limits of the IRES are shown as the shaded boxes. The genotype of the HCV sequence studied is indicated above the IRES. CAT, bacterial chloramphenicol acetyl transferase; Luc, firefly luciferase; β-gal, bacterial β-galactosidase; cyclin B2, *Xenopus laevis* cyclin B2; NS′, truncated influenza virus nonstructural protein.

tures which comprise the active IRES represents an important first step toward understanding this translational control mechanism, yet previous efforts to map the 5′ and 3′ limits of the IRES (Tsukiyama-Kohara *et al.,* 1992; Wang *et al.,* 1993; Fukushi *et al.,* 1994; Rijnbrand *et al.,* 1995; Reynolds *et al.,* 1995) have resulted in surprisingly different conclusions and offer no clear consensus concerning the structural requirements of this critical viral replication element (Fig. 1).

The conflicting results which are summarized in Fig. 1 may reflect the use of HCV sequences lacking the 5′ terminal sequence in some studies (Tsukiyama-Kohara *et al.,* 1992; Wang *et al.,* 1993; Reynolds *et al.,* 1995), or fusion of the 5′NTR to heterologous reporter sequences in monocistronic or discistronic transcripts which have been examined for translational activity (Wang *et al.,* 1993; Rijnbrand *et al.,* 1995; Reynolds *et al.,* 1995). It is difficult to predict the influence of these reporter sequences on the folding of the fused HCV RNA segment, as well as the impact of potentially altered secondary or tertiary structure on the activity of the HCV IRES. Another potential explanation for the conflicting results obtained in previous efforts to map the HCV IRES may be the use of different cell-free and *in vivo* systems to evaluate IRES activity. However, differences in the translation environment seem less likely to account for variation in the results because, in several studies, no differences were noted between *in vivo* and *in vitro* translation systems (Wang *et al.,* 1993; Rijnbrand *et al.,* 1995; Reynolds *et al.,* 1995). Most previous studies have been carried out with RNA from genotype 1b strains of HCV, and thus differences in the genotypes of viruses cannot explain the conflicting results shown in Fig. 1.

In an effort to clarify the location of the IRES within the 5′NTR of HCV, we carried out a mutational analysis of a 9.5-kb HCV cDNA clone, evaluating the impact of specific 5′NTR deletion mutations on translation of nearly genome-length HCV RNA transcripts both *in vitro* and *in vivo* under conditions designed to mimic those existing during replication of the virus. The results of these studies indicate that the IRES of HCV occupies most of the 5′NTR and suggest that the 5′ terminal hairpin (stem-loop I) (Rijnbrand *et al.,* 1995; Yoo *et al.,* 1992) acts to suppress translation. In addition, we show that mutations within the highly conserved capsid-coding region of the virus strongly influence the efficiency of IRES-directed translation both *in vitro* and *in vivo.*

MATERIALS AND METHODS

Plasmids

Plasmid pMN2-1G contains cDNA representing nt 1–9454 of the HCV genome under transcriptional control of the T7 promoter and fused at its 3′ end to a hepatitis δ ribozyme (Fig. 2). Thus this clone contains a nearly complete copy of the HCV genome, lacking only the ninety-four 3′ terminal nt identified recently by Tanaka *et al.* (1995). It was constructed by subcloning the *Hin*dIII–partial *Bam*HI fragment of HCV-N (genotype 1b) (Hayashi *et al.,* 1993) into a modified pBluescript II SK vector (Stratagene) in which the T7 promoter sequences between the *Kpn*I and *Bss*HII site had been deleted. The 5′ end of the cloned cDNA was modified by replacing the *Xho*I–*Nru*I fragment (nt 1–271 of HCV) with a PCR fragment amplified using the 5′ primer, 5′-CCGCTCGAGTAATACGACTCACTATAGCCAGCCC-3′, which contains an *Xho*I site and a T7 promoter sequence fused directly to the 5′ end of the HCV sequences (underlined), and a 3′ primer positioned just downstream of the 5′NTR to create p5′MN-2. This construction results in the T7 RNA polymerase initiating transcription at the first nucleotide of HCV which is a guanine. The 3′ cDNA insertion site was modified by subcloning the *Xba*I–*Eco*RI fragment (nt 7112–9454) into pBluescript II KS following removal of the *Cla*I site from this vector. The *Cla*I–*Eco*RI fragment (nt 9383–9454) of the resulting construct was replaced with a PCR-amplified fragment obtained using a 5′ primer upstream of the *Cla*I site and a 3′ primer which contained the HCV-N poly(U) sequence fused to downstream *Sfi*I/*Eco*RI sites. A synthetic oligonucleotide representing the hepatitis δ virus ribozyme with ends compatible with *Sfi*I and *Eco*RI was inserted to make p3′MN2. Finally, the

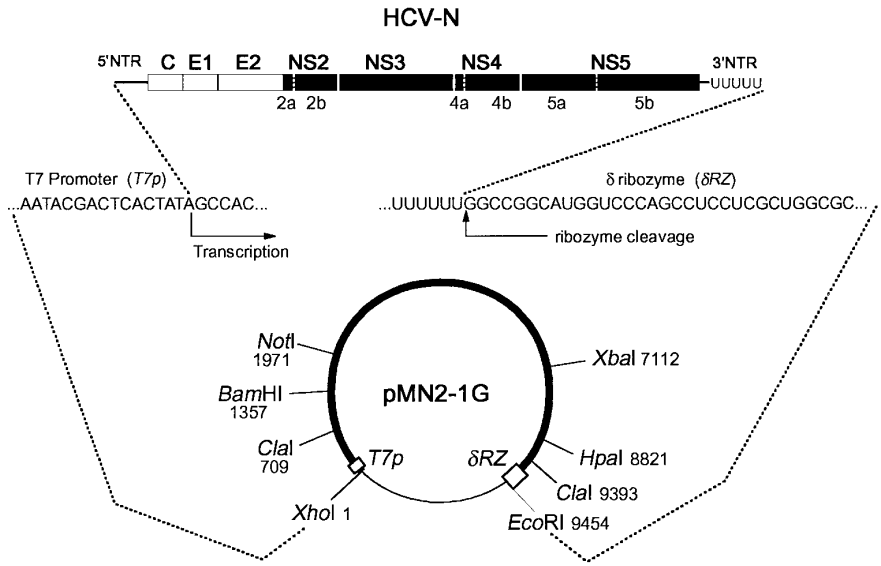

FIG. 2. Organization of the T7 transcriptional plasmid pMN2-1G. The HCV genome is indicated at the top, with the position of major proteins indicated within the open reading frame. The HCV cDNA is directly fused at its 5′ end to a minimal T7 RNA polymerase promoter sequence (T7p) and at its 3′ end to the hepatitis δ virus ribozyme sequence (δRZ).

large *Xmn*I/*Afl* III (nt 1–7815) fragment of p5′MN2 was ligated to the large *Afl* III/*Xmn*I fragment of p3′MN2 to create the genome-length pMN2-1G (Fig. 2). pN-/Fs contains a frameshift mutation within the E2 coding region of pMN2-1G. It was constructed by digestion of pMN2-1G with *Not*I, followed by a reaction with Klenow fragment and religation. The *Xmn*I/*Not*I (nt 1–1971) fragment of pMN2-1G was cloned into pBluescript II SK to create pN1-1971.

In previous work, a series of deletion mutations had been created within the 5′NTR of the Hutchinson (H) strain of HCV (Rijnbrand *et al.,* 1995). A series of chimeric, genome-length clones containing the wild-type and mutant H strain 5′NTRs were constructed as follows. Shuttle vectors were created by subcloning the small *Xho*I/*Not*I fragment of pMN2-1G (nt 1–1971) into pGEM11Z(+) and the *Eco*RI/*Hin*dIII fragment of the resulting construct (including the *Xho*I/*Not*I fragment) into pM13mp18 to make pM13/N1-1971. The mutated 5′NTR sequences (Rijnbrand *et al.,* 1995) were amplified by PCR using the 5′ primer, 5′-CGCTCGAGTAATACGACTCACTATAGCCAGCCCCCTGATGGG-3′, which contains an *Xho*I site and T7 promoter sequence fused directly to the 5′ end of the HCV sequence (underlined), and the 3′ primer, 5′-ATGGTGCACGGTCTACGAG-3′, which contains the *Apa*LI site at nt 335. For pΔ5–20, pΔ5–104, and pΔ1–130 (see Results), the 5′ primer sequences were 5′-CGCTCGAGTAATACGACTCACTATAGCCAGACACTCCACCATGAA-3′, 5′-CGCTCGAGTAATACGACTCACTATAGCCAGTGCAGCCTCCAGGAC-3′, and 5′-CGCTCGAGTAATACGACTCACTATAGGGAGAGCCATAGTGGTCTG-3′, respectively. For pΔ328–334, the 3′ PCR primer was 5′-ATGGTGCACGAGACCTCCCG-3′. Following digestion with appropriate restriction enzymes, each of the resulting PCR products was inserted into the *Xho*I/*Apa*L1 sites (nt 1–335) of pM13/N1-1971. *Xho*I/*Not*I fragments of the resulting series of constructs were subsequently inserted into pN1-1971, and *Xmn*I/*Not*I (nt 1–1971) fragments of these constructs were ligated with the large *Not*I/*Xmn*I fragment of pMN2-1G (nt 1972–9454) to create a series of genome-length cDNAs.

The mutant pN-CapSi contains multiple silent mutations between nt 350 and nt 383 and was constructed by PCR-based mutagenesis using the 5′ primer, 5′-AGACCGTGCACCATGAGCACcAAcCCgAAACCaCAgcGtAAgACaAAgaGgAAtACCAACCGCC-3′ (nucleotide substitutions from HCV-N in lower case), containing the *Apa*LI site at nt 335 and a 3′ primer, 5′-AGGCCCTGGCAGCGCCCCCTAG-3′ downstream of the *Avr*II site at nt 770. Amplified PCR fragments were digested with *Apa*LI and *Avr*II and cloned into pM13/N1-1971. A genome-length plasmid was constructed as above. A similar strategy was used for construction of p351-12, which contains a 12-nt insertion at nt 351 (5′-primer, 5′-AGACCGTGCACCATGAGCACGggcggcagcacgAATCCTAAACCTC-3′), p342-AUU (5′-AGACCGTGCACCATtAGCACGAATCC-3′), and p342-CUU, (5′-AGACCGTGCACCcTtAGCACGAATCC-3′).

PCRs were carried out using *Pfu* (*Pyrococcus furiosus*) DNA polymerase with 35 cycles of 95° for 1 min, 42° for 1.5 min, and 75° for 3 min. The plasmid DNAs were purified by two cycles of CsCl gradient centrifugation. The sequence of the manipulated regions and presence of the expected mutations were confirmed by DNA sequence analysis.

Cells

Huh-T7 cells (Schultz *et al.,* 1996) are derived from Huh7 human hepatocellular carcinoma cells. They are stably transformed and constitutively express bacterio-

phage T7 RNA polymerase. Cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and 400 μg (active compound) of geneticin (Gibco/BRL) per milliliter.

Antibodies

Murine monoclonal antibody to the HCV capsid protein, 27D5G5, was generously provided by G. Inchauspé of INSERM Unite 271, Lyon, France. Polyclonal guinea pig antibodies to NS3, NS4b, and NS5 were raised against a fusion protein, a synthetic peptide, and a baculovirus expression product, respectively.

In vitro transcription and translation

Plasmids were linearized by digestion with *Eco*RI (for pMN2-1G and related deletion mutants) or *Not*I (pN1-1971). RNA was transcribed by T7 RNA polymerase using Riboprobe System II reagents as recommended by the manufacturer (Promega). Transcription products were treated with RQ1 DNase for 15 min at 37°, extracted with phenol–chloroform and precipitated with ethanol/7.5 *M* ammonium acetate. The concentration of RNA was estimated by spectrophotometry. *In vitro* translation was carried out in micrococcal nuclease-treated rabbit reticulocyte lysates, supplemented with canine pancreatic microsomal membranes (Promega). Translation reactions (25 μl) were programmed with 2.5 μg (for full-length clones) or 0.5 μg (for pN1-1971) of RNA, and carried out at 30° for 1 hr. Total translation products were separated by 11 or 12% SDS–polyacrylamide–6 *M* urea gel electrophoresis, followed by autoradiography.

Expression of HCV capsid protein in transfected Huh-T7 cells

Huh-T7 cells were seeded into 4-well tissue culture chamber slides 24 hr prior to transfection. Cells (90% confluent) were infected with recombinant vaccinia virus vTF7-3 (expressing T7 RNA polymerase) (Fuerst *et al.,* 1986) in 100 μl of Opti-MEM (Gibco-BRL) at an m.o.i. of 10. Following a 1-hr incubation at 37°, the virus inoculum was removed and replaced with a mixture containing 1 μg of plasmid DNA and 3 μl of lipofectin (Gibco-BRL) in 40 μl of Opti-MEM, followed by 200 μl of Opti-MEM. After a 24-hr incubation at 37°, the cells were washed once with PBS and fixed in acetone:methanol (1:1) for 10 min at room temperature. Following removal of the fixative and washing in PBS for 10 min, the cells were incubated for 1 hr with a monoclonal antibody to the capsid protein, 27D5G5, diluted 1:2000 in PBS containing 3% bovine serum albumin (BSA) in PBS. Following additional washes with PBS, the cells were incubated with FITC-conjugated rabbit antibody to mouse immunoglobulin (DAKO), diluted 1:40 in PBS containing 3% BSA for 30 min. Cells were washed three times in PBS for 10 min, mounted in Vectashield fluid (Vector), and examined with a fluorescence microscope. Alternatively, the intensity of fluorescence displayed by 100 cells in each transfected culture was quantified by an automated fluorescence-based image cytometer (Lockett *et al.,* 1992).

Ribonuclease protection assay for HCV RNA

A ribonuclease protection assay for quantitation of RNA transcripts containing the HCV capsid sequence was developed using reagents and protocols from the Lysate Ribonuclease Protection Assay Kit (United States Biochemical Co.). pC342-709, which contains HCV capsid sequence from the initiator AUG to the *Cla*I site (nt 709) inserted into *Hin*dIII/*Cla*I sites of pSP73 (Promega), was linearized by digestion with *Aat*II (nt 401). A 0.33-kb ^{32}P-labeled antisense RNA probe was transcribed from this DNA with SP6 RNA polymerase, and a 0.14-kb probe to human glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) was transcribed from a GAPDH control template (United States Biochemical Co.) with T7 RNA polymerase. These antisense probes produce protected fragments of 0.31 and 0.10 kb, respectively. vTF7-3-infected Huh-T7 cells in 6-well culture plates were transfected with 2.5 μg plasmid DNA in 15 μl lipofectin as described above. Twenty-four hours later, the cells were washed once with PBS and lysed by the addition of 45 μl of 4 *M* guanidine thiocyanate, 25 m*M* sodium citrate, 0.5% sarcosyl. The resulting cell lysate was hybridized with a mixture of ^{32}P-labeled antisense RNA probes for 16 hr at 37°C, then diluted and extensively digested with ribonucleases and proteinase K (United States Biochemical Co.). Protected RNA fragments were precipitated with isopropanol and electrophoresed on a 6% denaturing polyacrylamide gel. The radioactivity present in protected RNA fragments was determined quantitatively by PhosphorImager analysis (Molecular Dynamics).

RESULTS

IRES-directed translation of the complete HCV polyprotein

Previous work confirms that translation of HCV RNA is initiated by a 5′ cap-independent mechanism involving binding of the 40S ribosome subunit at a position located internally on the RNA, but provides conflicting views of the structural requirements of the IRES (Fig. 1). These differences in conclusions concerning the location of the IRES could have resulted from the use of less than full-length 5′NTR sequences in some studies or from the presence of heterologous reporter protein-coding sequences fused to 5′NTR segments under study. We thus sought to map the 5′ and 3′ boundaries of the HCV IRES within the context of genome-length RNA using expression of the HCV polyprotein as a measure of the efficiency of HCV translation. We constructed a plasmid (pMN2-1G, Fig. 2) in which the nearly full-length cDNA sequence (nt 1–9454) of HCV-N, a Japanese genotype

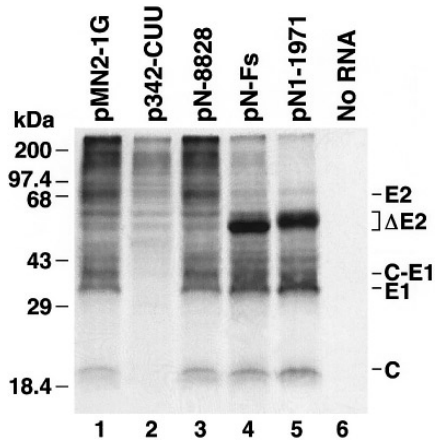

FIG. 3. SDS–PAGE analysis of the products of *in vitro* translation of genome-length and 3′ truncated RNAs. Lanes contain products of reticulocyte lysates programmed with RNAs prepared from: (1) pMN2-1G, (2) p342-CUU in which the initiator AUG codon is changed to CUU, (3) pMN2-1G linearized by digestion with *Hpa*I (nt 8828), (4) pN-Fs containing a frameshift mutation within the E2 coding region, (5) pN1-1971 in which the sequence downstream of the *Not*I site at nt 1971 has been removed, or (6) no RNA control. The size and position of marker proteins are indicated at the left, while the positions of the capsid (C), E1, nonprocessed capsid-E1 (C-E1), E2 and truncated E2 (ΔE2) proteins are shown at the right. Reticulocyte lysates were supplemented with microsomal membranes.

1b virus (Hayashi *et al.,* 1993), is flanked upstream by the bacteriophage T7 RNA polymerase promoter and downstream by a hepatitis δ virus ribozyme sequence (Fig. 2). T7 polymerase-directed transcription from this plasmid initiates at nt 1 of the HCV genome (a guanine base). Although the presence of only a single guanine at the T7 initiation site can be expected to result in less efficient transcription than when two or more guanines are present (Milligan *et al.,* 1987), *in vitro* T7-directed transcription from this vector was only modestly less efficient than transcription from a similar plasmid containing an additional guanine (data not shown). The δ ribozyme directs cleavage of transcribed RNA immediately 3′ of the HCV 3′ terminal poly(U) sequence.

To assess translation from the construct, genome-length RNA was transcribed *in vitro* by T7 polymerase and used to program *in vitro* translation reactions in lysates of rabbit reticulocytes which were supplemented with microsomal membranes. Under these conditions, the ~21-kDa capsid protein and glycosylated ~34-kDa E1 and ~68-kDa E2 proteins were readily identified within the products of translation (Fig. 3, lane 1). These proteins were present when runoff RNA transcripts truncated at nt 2705 were translated *in vitro* and could be precipitated with multiple sera from HCV-infected patients (data not shown). Other HCV proteins were present among a number of high-molecular-weight products when the full-length RNA was used to program translation (Fig. 3, lane 1), but no attempt was made to specifically identify these proteins. The capsid and E1 proteins were not expressed from a modified genome-length construct, p342-CUU, in which the initiator AUG was substituted with CUU (Fig. 3, lane 2). However, multiple high-molecular-weight proteins were translated from this modified RNA which were not produced in control reactions containing no RNA (Fig. 3, compare lanes 2 and 6). Since these large proteins are translated from a mutant RNA lacking the authentic initiation codon, they are likely to have resulted from the inappropriate initiation of translation at alternative start codons within the 9.0-kb HCV open reading frame in this *in vitro* system.

To further characterize the multiple high-molecular-weight proteins evident in Fig. 3, we modified pMN2-1G by creating a frameshift mutation at nt 1975 which leads to termination of translation within the E2 protein (plasmid pN-Fs). In addition, we created another mutant in which sequence downstream of the *Not*I site at nt 1971 was completely removed (plasmid pN1-1971). The amounts of capsid, E1, and (truncated) E2 products from pN-Fs and pN1-1971 were increased from that produced from the genome-length pMN2-1G transcripts (Fig. 3, compare lanes 4 and 5 with lane 1). However, several high-molecular-weight translation products (>100 kDa) were present in lysates programmed with the frameshift mutant, pN2-Fs, but not pN1-1971 in which sequence downstream of E2 was deleted (Fig. 3, compare lanes 4 and 5). The largest protein in the products from pN1-1971 was a faint band which migrated with an apparent mass of ~68 kDa (close to that of glycosylated E2) (Fig. 3, lane 5), which correlates with the expected size of nonprocessed C-E1-ΔE2. These findings thus indicate that there are multiple sites of aberrant translation initiation within the open reading frame of HCV when genome-length HCV RNA is used to program translation in rabbit reticulocyte lysates. This aberrant initiation of translation resembles that observed with genome-length poliovirus and hepatitis A virus RNAs in reticulocyte lysates (Brown and Ehrenfeld, 1979; Jia *et al.,* 1991; Meerovitch *et al.,* 1993). Thus, in subsequent *in vitro* translation assays, the amount of capsid and E1 proteins produced was taken as a measure of IRES activity.

Compared with lysates programmed with full-length pMN2-1G RNA, qualitatively and quantiatively similar translation products were obtained in lysates programmed with RNA transcripts with a 3′ truncation at nt 8828 (Fig. 3, compare lanes 1 and 3). This suggests that the presence of the 3′ poly(U) sequence in the full-length transcript has little if any influence on the efficiency of IRES-directed translation *in vitro.*

Expression of HCV proteins in transfected Huh-T7 cells

Intact pMN2-1G plasmid DNA was transfected into Huh-T7 cells which are derived from human hepatocellular carcinoma cells and constitutively express cytoplasmic T7 RNA polymerase (Schultz *et al.,* 1996). To boost the level of T7 polymerase expression, cells were

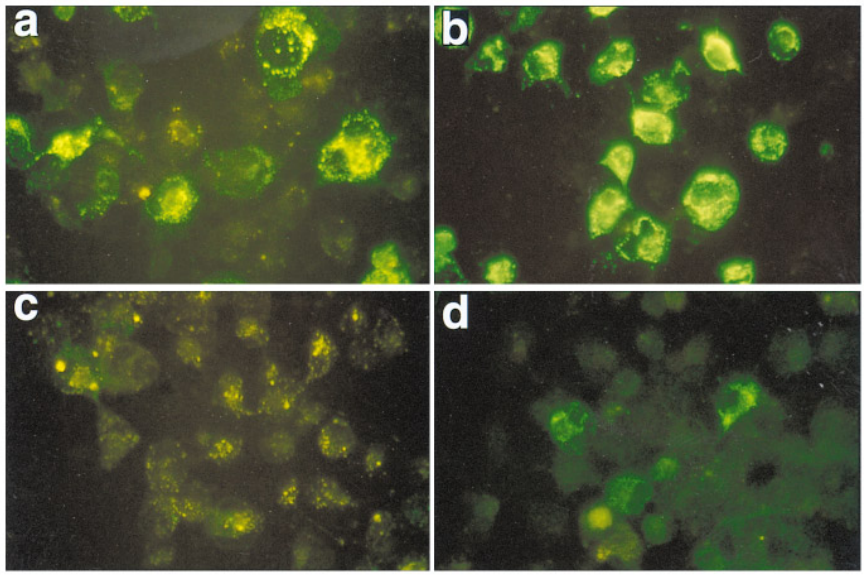

FIG. 4. Indirect immunofluorescence demonstrating HCV proteins expressed from genome-length transcripts in vTF7-3-infected Huh-T7 cells transfected with plasmid DNA. Cells were stained with (a–c) monoclonal antibody to capsid protein or (d) polyclonal anti-NS5A peptide antibody. Transfections were carried out by a liposome-mediated method with plasmids (a and d) pMN2-1G, (b) pΔ5–20, or (c) p342-CUU.

infected with recombinant vaccinia virus vTF7-3 (Fuerst *et al.*, 1986) immediately prior to DNA transfection. HCV capsid protein was readily identified in up to 50% of cells by indirect immunofluorescence staining using a specific monoclonal antibody (Fig. 4a). In contrast, transfection of p342-CUU (in which the initiator AUG has been changed to CUU) resulted in no detectable expression of the capsid protein (Fig. 4c). This confirmed the specificity of the indirect immunofluorescence reaction and provided evidence that expression of the capsid antigen in cells transfected with pMN2-1G was due to translation initiated at the AUG codon at nt 342. Cells transfected with pMN2-1G (containing the intact initiator codon) were also stained with polyclonal guinea pig antibodies specific for NS3, NS4 (data not shown), and NS5 (Fig. 4d) proteins. Each of these nonstructural proteins was expressed in transfected cells. However, the immunofluorescence signal was significantly weaker than that observed with monoclonal antibody staining of the capsid protein and present in a smaller proportion of cells. This is likely to reflect lower affinity antibody–antigen interactions, but premature termination of translation or degradation of these HCV translation products cannot be excluded. Nonetheless, identification of NS5 antigen in cells transfected with pMN2-1G (Fig. 4d) served to confirm the integrity of the HCV open reading frame in these transcripts.

The translational activities of pN-Fs (containing the frameshift mutation within the E2 coding region) and pN1-1971 (lacking HCV sequence downstream of nt 1971) were evaluated in vTF7-3 infected Huh-T7 cells. With transfection of equivalent molar quantities of these plasmids, there was no consistent difference in the expression of capsid protein determined by indirect immunofluorescence (data not shown). These data thus provide no support for the presence of a significant transactivator of translation within the NS2-NS5 region of the polyprotein, such as the poliovirus IRES transactivating activity that may be associated with the nonstructural poliovirus 2A protein (Hambidge and Sarnow, 1992; Macadam *et al.,* 1994). However, these data do not exclude such an activity within the structural proteins of HCV.

Required structural elements of the HCV IRES in rabbit reticulocyte lysates

Previously, we evaluated the translational activity of RNA transcripts containing the 5′NTR of the H strain of HCV (genotype 1a) (Rijnbrand *et al.,* 1995) fused to RNA encoding the reporter protein CAT. For these studies, an extensive series of deletion mutations was created within the H strain 5′NTR which specifically removed putative 5′NTR stem-loop structures. To assess the impact of these mutations on internal initiation of translation from full-length HCV RNA, we constructed a series of genome-length chimeric cDNAs in which the 5′NTR of the N2 strain present in pMN2-1G was replaced by the wild-type or mutated H strain 5′NTRs (Fig. 5A). Genome-length RNAs were transcribed from the resulting chimeric constructs and used to program translation in rabbit reticulocyte lysates supplemented with canine pancreatic microsomal membranes (Fig. 5B). These results suggested that the 5′NTR of the H strain genotype 1a virus was moderately more efficient in directing internal initiation of translation (pMN2/H, Fig. 5B, lane 2) than the genotype 1b N2 5′NTR (pMN2-1G, Fig. 5B, lane 1). The difference in the quantities of capsid and E1 proteins in the translation products from pMN2-1G and pMN2/H RNAs shown in Fig. 5B was reproducibly present in multiple experiments.

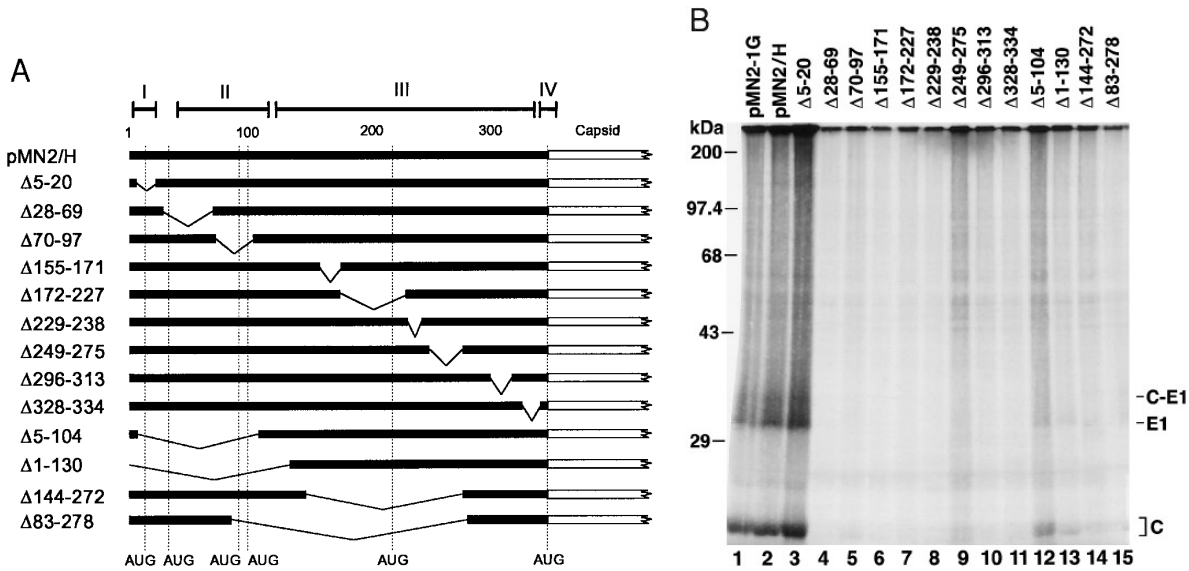

FIG. 5. (A) Schematic depicting the location of 5′NTR mutations within chimeric genome-length cDNA transcripts. Solid bars represent nontranslated RNA, while the open bars represent the 5′ region of the capsid encoding RNA. Nucleotide numbering and structural domains of the 5′NTR (Brown *et al.,* 1992) (M. Honda *et al.,* submitted for publication) are indicated at the top. Vertical lines indicate position of AUG codons. (B) SDS–PAGE analysis of translation products from rabbit reticulocyte lysates programmed with RNA transcripts prepared from (lane 1) the pMN2-1G plasmid, (2) the chimeric pMN2/H plasmid containing the 5′NTR of H strain HCV, and (3–15) various 5′NTR deletion mutants prepared in the background of pMN2/H, as shown in (A). SDS–PAGE was carried out in the absence of urea, resulting in reduced resolution of the high-molecular-weight translation products.

Most of the deletion mutations created within the H strain 5′NTR eliminated or caused a very substantial reduction in the translation efficiency of the RNA in rabbit reticulocyte lysates. Thus, the deletion of nt 28–69 completely abrogated translation *in vitro* (Fig. 5B, lane 4), indicating that internal initiation of translation on full-length HCV RNA is dependent upon retention of the RNA sequence in this region, which contains the 5′ nucleotides of a complex stem-loop comprising domain II in a recently refined model of the 5′NTR secondary structure (see Fig. 6) (Brown *et al.,* 1992) (M. Honda *et al.,* submitted for publication). Similarly, removal of the 28 nt comprising the apex of this structure (stem-loop IIb, Δ70–97) also completely inactivated the IRES (Fig. 5B, lane 5). These data thus refute the conclusion reached by Tsukiyama-Kohara *et al.* (1992) that the 5′ limit of the IRES lies between nt 111 and nt 138 and are consistent with the results of Wang *et al.* (1993) and Rijnbrand *et al.* (1995) (Fig. 1). As expected, deletions which are predicted to destabilize the base of the complex domain III structure (mutants Δ249–275 and Δ296–313) (Fig. 5B, lanes 9 and 10) (Fig. 6) (Brown *et al.,* 1992) eliminated IRES activity. However, the absence of translational activity in other mutants, Δ155–171, Δ172–227, and Δ229–238 (Fig. 5B, lanes 6–8, respectively) provided compelling data that the apical extensions of this structure, stem-loops IIIa, IIIb, and IIIc (Brown *et al.,* 1992), are also individually critical elements of the IRES (Fig. 6). These deletion mutations result in the precise removal of these predicted stem-loops and should have minimal effect on the secondary structure of the remaining sequence. Finally, a small deletion (Δ328–334) which destabilizes the pseudoknot involving stem-loop IIIf immediately upstream of the initiator AUG (Wang *et al.,* 1995) also resulted in a near total inhibition of translation (Fig. 5B, lane 11).

These results indicate that domains II and III, which

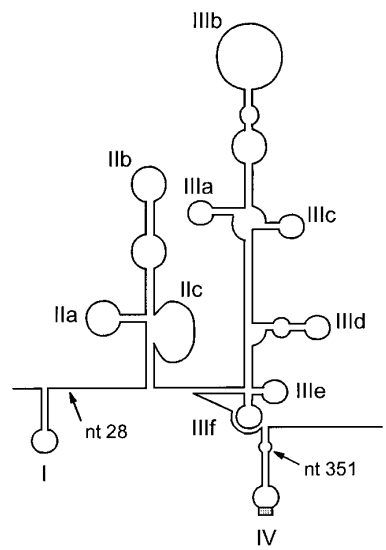

FIG. 6. Schematic of a model depicting predicted RNA secondary and tertiary structure within the 5′NTR and the immediately adjacent capsid-coding sequence of HCV. The model contains recent refinements of an earlier proposed structure (Brown *et al.,* 1992) and is supported by phylogenetic comparisons with GBV-B virus (Honda *et al.,* submitted for publication). Individual stem-loops are labeled according to the major domain, while the stippled box indicates the location of the initiator AUG within the loop of stem-loop IV. The 5′ end of the most 5′ deletion eliminating IRES function (nt 28, Δ28–69) and the site of the insertional mutation in p351_12 (nt 351) are indicated.

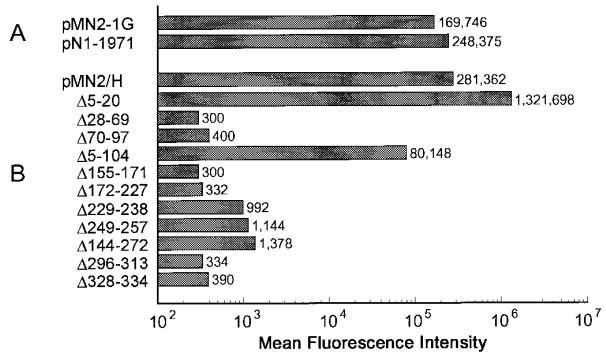

FIG. 7. Quantitative microfluorometry of vTF7-3 infected Huh-T7 cells which were transfected with the indicated plasmid DNAs and stained for capsid protein expression at 24 hr by indirect immunofluorescence with specific monoclonal antibody. The mean fluorescence intensity was derived from data collected on 100 individual cells analyzed in each transfected culture; the background fluorescence in mock-infected cells was set arbitrarily to 0 units.

together comprise much of the 5′NTR (Brown *et al.,* 1992) (Fig. 6), are essential for IRES activity. The small amounts of capsid and E1 proteins produced from mutants with deletions of nt 5–104 or to a lesser extent nt 1–130 (Fig. 5B, lanes 12 and 13) may represent translation initiated by ribosomes scanning in a 3′ direction from the 5′ end of these monocistronic RNAs (see below and Discussion).

In contrast to the striking decreases in translation observed with deletions in domains II and III, removal of the most 5′ stem-loop (domain I, Fig. 6) in the mutant Δ5–20 resulted in a demonstrable and reproducible enhancement of HCV translation (Fig. 5B, lane 3). This finding supports earlier observations made by Yoo *et al.* (1992), who found increased translation from transcripts with a 5′ deletion mutation, and Rijnbrand *et al.* (1995), who found that deletion of nt 5–20 resulted in enhanced expression of CAT when fused as a reporter protein to the 5′NTR of the H strain virus. Thus, in rabbit reticulocyte lysates programmed with these nearly full-length RNAs, the efficiency of internal initiation of translation is determined by RNA sequences located within every region of the 5′NTR.

Structural requirements of the HCV IRES *in vivo*

To assess the impact of the mutations described above on translation from genome-length viral RNA *in vivo,* plasmid DNAs were transfected into vTF7-3-infected Huh-T7 cells. Translation was assessed by indirect immunofluorescence using monoclonal antibody to the capsid protein, as shown for the parent plasmid in Fig. 4a. To quantify the expression of capsid protein by each of the mutants, the mean fluorescence intensity displayed by 100 cells was determined with an automated, fluorescence image-based cytometer (Lockett *et al.,* 1992). In general, these results (Fig. 7) mirrored the results of the *in vitro* translation of these RNAs described above. The H strain 5′NTR (pMN2/H) was approximately 1.7-fold more efficient than the N2 strain 5′NTR (pMN2-1G) in directing translation of the capsid protein (Fig. 7), confirming the higher efficiency of the H strain IRES found in reticulocyte lysates (Fig. 5B). Consistent with the enhanced translational activity of the Δ5–20 mutant in reticulocyte lysates (Fig. 5B, lane 3), expression of the capsid protein from this plasmid was enhanced approximately 5-fold over expression from the parent pMN2/H in Huh-T7 cells (Figs. 4b and 7). In contrast, removal of nt 28–69 eliminated (>99% reduction) expression of the capsid protein from the full-length RNA *in vivo,* as did most of the other deletion mutations made within the 5′NTR of the chimeric genome-length constructs (Fig. 7).

However, two deletion mutants (Δ5–104 and Δ1–130) resulted in substantial levels of capsid expression *in vivo.* Although reduced, capsid expression from Δ5–104 was still approximately one-third that of pMN2/H (Fig. 7). This was consistent with a low but detectable level of translation from this RNA *in vitro* (Fig. 5B, lane 12). In contrast, deletion of nt 1–130 (Δ1–130) resulted in a fourfold enhancement of capsid expression in transfected Huh-T7 cells (data not shown), while this transcript demonstrated little if any translational activity in reticulocyte lysates (Fig. 5B, lane 13). With both of these mutants, it is likely that the translation of capsid protein observed *in vivo* resulted from ribosomes scanning from the 5′ end of the transcripts (see Discussion). In addition, the deletion in Δ1–130 places three guanine bases at the T7 transcriptional start site (rather than the single guanine present in pMN2-1G). As T7 transcription is optimal when three guanine bases are present at the beginning of the transcript, the fourfold enhanced expression of capsid antigen from this construct is likely to reflect enhanced transcriptional activity *in vivo* (Milligan *et al.,* 1987).

To determine whether differences in capsid protein expression observed with various mutants in transfected Huh-T7 cells reflect varied efficiencies of transcription from the T7 promoter, we quantified the relative abundance of selected HCV transcripts in these cells using a ribonuclease protection assay. These results indicated that the abundance of HCV RNA was comparable in cells transfected with pMN2-1G, pMN2/H, and Δ5–104, but substantially increased in cells transfected with Δ1–130 (>12-fold increase by PhosphorImager analysis) (data not shown). Thus, the enhanced translational activity of pMN2/H compared with that of pMN2-1G cannot be attributed to enhanced transcription or stability of the RNA transcripts. However, increased transcription of Δ1–130 was at least partially responsible for the very efficient expression of capsid protein from this construct in transfected Huh-T7 cells and likely reflects the presence of multiple guanines at the T7 transcriptional start site of this plasmid. The abundance of Δ5–20 transcripts in transfected cells was moderately increased over levels of the parent pMN2/H transcripts detected by RNase protection assay (approximately 2-fold increase by PhosphoImager analysis) (data not shown), suggesting that

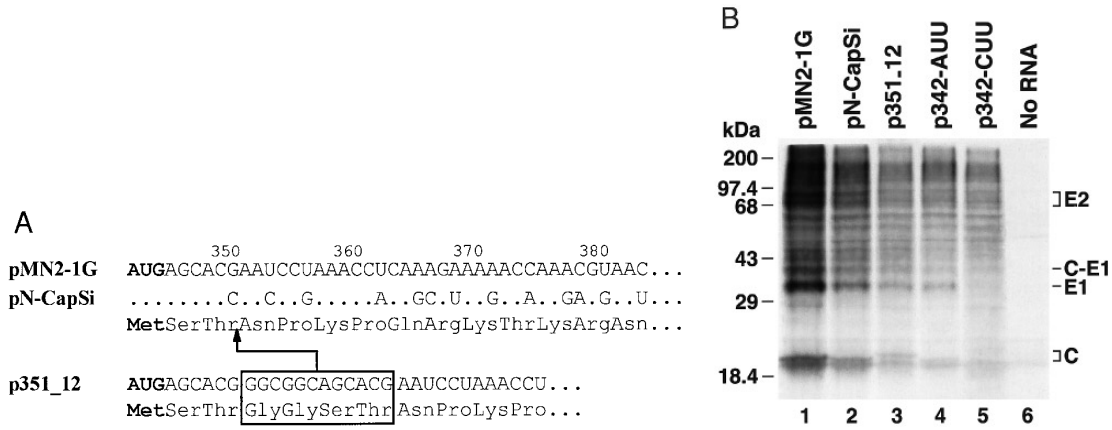

FIG. 8. Mutations downstream of the initiator AUG influence HCV IRES activity. (A) Top: Alignment of nucleotide sequences of pMN2-1G and pN-CapSi, which contains multiple substitutions in the second and third bases of codons which do not alter predicted amino acid sequence of the capsid protein. Bottom: Nucleotide sequence and predicted amino acid sequence of the capsid protein expressed from p351_12, which contains an in-frame 12-nt insertional mutation (boxed segment) at nt 351. The authentic initiator codon is shown in boldface type. (B) Products of *in vitro* translation reactions programmed with RNAs produced from (lane 1) pMN2-1G, (2) pN-CapSi, (3) p351_12, (4) p342-AUU, (5) p342-CUU, or (6) no RNA.

this stem-loop may interfere with T7 transcription. It is not clear whether this increased abundance of the Δ5–20 RNA transcripts is sufficient to fully account for the nearly 5-fold increase observed in capsid expression *in vivo* (Fig. 7). These results were confirmed in replicate transfection assays.

Mutations downstream of the initiator AUG influence the efficiency of translation initiated by the HCV IRES

Results published recently by Reynolds *et al.* (1995) suggest that the IRES of HCV might extend downstream to involve sequence within the highly conserved capsid-coding region of HCV (Bukh *et al.,* 1994). To test this hypothesis, we created two additional mutant constructs. pN-CapSi was identical to pMN2-1G, except for 13 silent nucleotide substitutions at second and third base positions of the codons between nt 350 and nt 383 (Fig. 8A). The capsid protein expressed from this construct should be identical in sequence to the native HCV-N protein. The second mutant construct, p351_12, contained a 12-nt insertional mutation placed in frame between the third and the fourth codon of the open reading frame. This mutation was designed to place a Gly-Gly dipeptide spacer upstream of reiterated amino acids 2 and 3 of the capsid protein (Fig. 8A). The capsid protein expressed by this construct is thus predicted to contain a Ser-Thr-Gly-Gly insertion immediately downstream of Met[1], followed by the native sequence starting at Ser[2]. The products of translation in reticulocyte lysates programmed with RNAs transcribed from these modified plasmids are shown in Fig. 8B. Compared with reticulocyte lysates programmed with the parent pMN2-1G RNA (Fig. 8B, lane 1), the translation of capsid and E1 proteins was reduced in lysates programmed with the pN-CapSi and, to a greater extent, p351_12 RNAs (Fig. 8B, compare lanes 2 and 3 with lane 1). The HCV proteins (C, E1) were translated from p351_12 transcripts with an efficiency similar to that of transcripts in which the AUG codon was changed to AUU (p342-AUU, Fig. 8B, lane 4), but with an efficiency significantly greater than that of p342-CUU (Fig. 8B, lane 5).

The products of these and other translation reactions included a protein migrating with an apparent mass of ~20 kDa, slightly more rapidly than the capsid protein in this gel system (Fig. 8B). However, the identity of the authentic capsid protein was readily apparent from the shift in its mobility that was evident in the products of p351_12, which expresses a mutant capsid protein containing four additional amino acid residues (Fig. 8B, lane 3). Although the identity of the 20-kDa protein is uncertain, it is an HCV-specific product which is generated by initiation of translation at the authentic initiation codon, since the quantity of this product was closely correlated with the quantity of the authentic capsid and E1 proteins produced from the various mutant RNAs that were translated in Fig. 8B. However, a faint, nonspecific protein band of similar size was also present in the control reaction programmed with no RNA in this experiment (Fig. 8B, lane 6).

In transfected Huh-T7 cells, the expression of capsid antigen following transfection with pN-CapSi was similar to that of the pMN2-1G parent (data not shown). However, the 12-nt insertion in p351_12 and modification of the initiator AUG in p342-AUU resulted in very low overall levels of capsid protein expression in transfected Huh-T7 cells. Because the p351_12 mutation alters the sequence of the capsid protein, the reduction in translation activity was also confirmed by staining transfected cells with antibody specific for NS3 (data not shown). These data thus confirm that efficient internal initiation of translation by the HCV IRES, within the context of nearly full-length viral RNA, is dependent upon sequence down-

stream of the initiator AUG and thus provide a possible explanation for the strongly conserved nature of this segment of the genome (Bukh *et al.,* 1994).

DISCUSSION

Since the 5′NTR of HCV is considerably shorter than that of any picornavirus, the IRES of HCV is likely to be a more compact structure and to occupy a greater proportion of the 5′NTR sequence than IRES elements present in these other viruses. Tsukiyama-Kohara *et al.* (1992) presented strong evidence that the 5′ border of the HCV IRES is located between nt 111 and nt 257 (Fig. 1). However, equally compelling data from Wang *et al.* (1993) and Rijnbrand *et al.* (1995) suggest that the 5′ limit of the IRES is between nt 29 and nt 69, while the results of Fukushi *et al.* (1994) suggest that IRES activity is positively influenced by even the most 5′ stem-loop within the 5′NTR, lying between nt 1 and nt 20 (Fig. 1). In an effort to clarify these conflicting results, we evaluated translation of the viral capsid and E1 proteins from HCV RNA transcripts which represented nearly the complete HCV genome (nt 1–9454) and which contained no foreign reporter protein-coding sequences. This strategy precluded the possibility that fortuitous basepair interactions between the viral RNA and fused heterologous reporter sequences might alter HCV RNA structure and lead to misinterpretation of the structural requirements of the IRES. Moreover, the parent construct for these experiments, pMN2-1G, contained the complete 5′NTR, while several previous investigations have studied truncated RNAs with missing 5′ terminal sequences (Tsukiyama-Kohara *et al.,* 1992; Wang *et al.,* 1993; Reynolds *et al.,* 1995).

We sought to evaluate RNA transcripts that were as close as possible to native viral RNAs and thus evaluated translation from genome-length constructs encoding all of the structural and nonstructural proteins of HCV. We inserted a ribozyme sequence to direct cleavage of these transcripts immediately 3′ of the terminal poly(U) sequence in HCV-N (Fig. 2). However, during the preparation of this paper, Tanaka *et al.* (1995) reported the presence of an additional 94 nt of HCV sequence located 3′ of the poly(U) tract. Although we found that the presence or absence of the 3′ poly(U) tract had little effect on HCV translation from these nearly genome-length RNAs in rabbit reticulocyte lysates (Fig. 3), additional experiments will be needed to determine whether the newly recognized 3′ terminal sequence has any influence on translational efficiency.

We observed sharply reduced synthesis of the capsid and E1 proteins within *in vitro* translation reactions programmed with RNA containing a deletion between nt 28 and nt 69 (Fig. 5B, lane 4). This supports the conclusion reached by both Wang *et al.* (1993) and Rijnbrand *et al.* (1995) that the 5′ limit of the IRES lies within this region. Translation of this deletion mutant was also reduced over 100-fold compared with the parent sequence in vTF7-3 infected Huh-T7 cells (Fig. 7), providing further evidence for this conclusion. This segment of the 5′NTR contains nucleotides forming the 5′ end of a complex stem-loop structure comprising domain II (nt 44–118) in a refined model of the secondary structure of the 5′NTR (Fig. 6). The existence of this RNA structure is strongly supported by a combination of phylogenetic comparisons, thermodynamic modeling, and probing of synthetic RNAs by single- and double-strand specific RNases (Brown *et al.,* 1992) (M. Honda *et al.,* submitted for publication). The experimental data presented here indicate that this complex structure plays an essential role in HCV translation and that destabilization of its base with removal of stem-loop IIa (Δ28–69) or IIb (Δ70–97, Fig. 5B, lane 5, and Fig. 7) destroys IRES activity within the context of nearly genome-length RNA both *in vitro* and *in vivo.*

We also found, however, that removal of the most 5′ stem-loop I (nt 5–20) (Fig. 6) resulted in a reproducible increase in the translational efficiency of the IRES *in vitro* (Fig. 5B, lane 3). This was matched by a roughly fivefold increase in capsid protein expression *in vivo* (Fig. 4b and Fig. 7), although a ribonuclease protection assay suggested a twofold increased abundance of the Δ5–20 transcripts in transfected Huh-T7 cells. The results of the *in vitro* and *in vivo* experiments thus appear to confirm similar observations made with a series of reporter constructs by Rijnbrand *et al.* (1995) and the earlier observations of Yoo *et al.* (1992), who found increased translation of HCV transcripts with deletions involving the 5′ terminal 22–34 residues of the genome. However, these data conflict with results reported by Fukushi *et al.* (1994), who found that removal of nt 1–20 resulted in a marked decrease in translation of the capsid protein *in vitro.* The explanation underlying these discordant observations is not clear, but might relate to a role for nt 1–4 in preserving essential IRES structure or otherwise stabilizing HCV transcripts. Taken together, however, these results indicate that the efficiency of IRES-directed translation is dependent upon sequence involving almost the entire 5′NTR.

We found that minimal *in vitro* translational activity was retained by the Δ5–104 RNA and to a lesser extent by the Δ1–130 RNA (Fig. 5B, lanes 12 and 13, and Fig. 7). While these observations are consistent with the possibility that a minimal IRES element might be located within the 236 nt preceding the initiator AUG, as suggested by Tsukiyama-Kohara *et al.* (1992), they cannot be considered strongly supportive of this view. These RNAs were monocistronic and only contain a single AUG (at nt 215) upstream of the initiator AUG-342 (Fig. 5A). As AUG-215 is in an unfavorable context for initiating translation (Kozak, 1986; Grünert and Jackson, 1994), it seems likely that the translation of these RNAs may be due to initiation at the authentic AUG-342 by ribosomes which have bypassed AUG-215 while scanning from the 5′ end of the RNA. This interpretation is consistent with the relatively

increased translational activity of Δ5–104 and Δ1–130 transcripts in transfected Huh-T7 cells (Fig. 7) compared with reticulocyte lysates (Fig. 5B, lanes 12 and 13), as vaccinia virus induced capping of even a minor fraction of the T7 transcripts in the Huh-T7 cells (Fuerst and Moss, 1989) would be expected to greatly enhance translation initiated by a scanning mechanism.

Previous studies indicate that the IRES extends in a 3′ direction to involve sequence immediately upstream of the initiator AUG (Wang *et al.,* 1993, 1995; Rijnbrand *et al.,* 1995). Consistent with these earlier results, deletion of 7 nt immediately upstream of the AUG (Δ328–334, Fig. 5B) abrogated HCV translation from these full-length RNAs both *in vitro* (Fig. 5B, lane 11) and *in vivo* (Fig. 7). This deletion eliminates basepair interactions within the pseudoknot which is present at the base of domain III and thus confirms the importance of the integrity of this pseudoknot to activity of the HCV IRES (Wang *et al.,* 1995).

A recent report by Reynolds *et al.* (1995) also suggests that sequence extending from 12 to 30 nt 3′ of the initiator AUG is required for efficient IRES activity (Fig. 1). However, this observation conflicts with results obtained by Tsukiyama-Kohara *et al.* (1992) and Wang *et al.* (1993), who both noted efficient internal initiation of translation of reporter proteins (CAT or luciferase) fused directly to the initiator AUG of HCV. In support of Reynolds *et al.* (1995), we observed that an in-frame insertion of 12 nt at nt 351, 9 nt downstream of the AUG, resulted in a sharp reduction of capsid and NS3 protein synthesis both *in vitro* (Fig. 8B, lane 3) and *in vivo.* This insertional mutation results in a change in the amino terminal sequence of the expressed capsid protein. Thus, these data do not indicate whether the reduction in translational efficiency is due to an effect on RNA structure or an alteration in capsid protein sequence. However, we also found that the creation of multiple silent mutations within the RNA encoding the amino terminal sequence of the capsid protein resulted in a lesser but still substantial decrease in translational activity *in vitro* (Fig. 8B, lane 2). It was surprising that the translational activity of this mutant was not equally reduced in transfected Huh-T7 cells, but it remains possible that such a reduction might be observed *in vivo* under conditions of less efficient T7-directed transcription.

These data indicate that HCV IRES activity is dependent upon the 5′ sequence of the expressed open reading frame. This aspect of the HCV IRES distinguishes it from the IRES elements of picornaviruses which act independently of downstream sequence (Jackson *et al.,* 1994) and suggests a fundamental difference in the mechanism of translation initiation. Possibly, this requirement for sequence within the open reading frame might relate to involvement of a stem-loop structure overlapping the initiator AUG (Fig. 6) (Smith *et al.,* 1995). The existence of this stem-loop is supported by nuclease-mapping studies as well as recent phylogenetic comparisons with the newly discovered GBV-B virus (Honda *et al.,* submitted for publication). Both the p351_12 and pN-CapSi mutations alter this structure. Possible interactions between protein coding sequence and the IRES require further investigation, but are also consistent with recent studies of chimeric polioviruses in which HCV 5′NTR-capsid sequences replace the native picornaviral IRES (Lu and Wimmer, 1996).

Both *in vitro* and *in vivo,* chimeric HCV RNAs containing the H strain 5′NTR were translated with greater efficiency than the N strain RNA (Figs. 5B, lane 2, and Fig. 7). Although this effect was modest (approximately 1.7-fold in Huh-T7 cells), the cumulative effects of such interstrain variation in IRES activity could be important in explaining differences in levels of HCV replication in patients infected with various strains or genotypes of HCV. The nucleotide sequences of the H and N strains of HCV differ at four locations within the 5′NTR, all of which represent bases that are not involved in canonical Watson–Crick basepair interactions (Brown *et al.,* 1992) (M. Honda *et al.,* submitted for publication). These include the loop sequence of stem-loop I (UGAU vs GAUU, in H and N respectively), nt 34–35 (GA vs AG), nt 204 (A vs C) and nt 243 (A vs G). These differences in primary structure could directly influence interactions between the 5′NTR and *trans*-acting cellular translation initiation factors or, alternatively, they might stabilize or destabilize tertiary structure within the RNA. It is tempting to speculate that the most significant of these changes, the multiple substitutions in the loop segment of stem-loop I, might alter the translation-suppressing activity of this structure. Further studies will be required to determine whether this hypothesis is correct.

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