Biochemical and Biophysical Research Communications **290,** 105–112 (2002)
doi:10.1006/bbrc.2001.6167, available online at http://www.idealibrary.com on IDEAL®

Domains I and II in the 5′ Nontranslated Region of the HCV Genome Are Required for RNA Replication

Yoon Ki Kim, Chon Saeng Kim, Song Hee Lee, and Sung Key Jang[1]
Department of Life Science, Division of Molecular and Life Sciences, Pohang University of Science and Technology, San31, Hyoja-Dong, Pohang, Kyungbuk 790-784, Korea

Received November 26, 2001

Hepatitis C virus (HCV), a hepacivirus member of the *Flaviviridae* family, has a positive-stranded RNA genome, which consists of a single open reading frame (ORF) and nontranslated regions (NTRs) at the 5′ and 3′ ends. The 5′NTR was found to contain an internal ribosomal entry site (IRES), which is required for the translation of HCV mRNA. Moreover, the 5′NTR is likely to play a key role in the replication of viral RNA. To identify the *cis*-acting element required for viral RNA replication, chimeric subgenomic replicons of HCV were generated. Dissection of the replication element from the translation element was accomplished by inserting the polioviral IRES between the serially deleted 5′NTR of HCV and ORF encoding neomycin phosphotransferase. The deletions of the 5′NTR of HCV were performed according to the secondary structure of HCV. Replicons containing domains I and II supported RNA replication and further deletion toward the 5′ end abolished replication. The addition of domain III and the pseudoknot structure of the 5′NTR to domains I and II augmented the colony-forming efficiency of replicons by 100-fold. This indicates that domains I and II are necessary and sufficient for replication of RNA and that almost all of the 5′NTR is required for efficient RNA replication.

Key Words: **HCV; *cis*-acting element; replication; IRES.**

Hepatitis C virus (HCV) has been classified within the *hepacivirus* genus of the *Flaviviridae,* a group of enveloped viruses, to which the *flaviviruses* and the animal-pathogenic *pestiviruses* belong (1). These viruses have a single-stranded RNA genome of positive polarity that carries a single long open reading frame (ORF) that is flanked by nontranslated regions (NTRs) at its 5′ and 3′ ends. More specifically, the HCV genome has a length of about 9600 nucleotides, containing a single long open reading frame (ORF). Translation of the polyprotein is directed by an internal ribosome entry site (IRES) spanning most of the 5′NTR and a portion of the core-coding region which augments the IRES-dependent translation (2–9). The first 45 nucleotides of the 5′NTR are dispensable for IRES activity. The stop codon of the polyprotein coding sequence is followed by the 3′NTR, which is composed of a variable region, a poly(U/UC) tract of variable length, and 98 highly conserved 3′ terminal nucleotides, named X tail (10–13). Recent studies have demonstrated that the variable region is dispensable, but that the poly(U/UC) tract and the X tail are essential for viral proliferation (14, 15).

Investigation of the *cis*-acting elements in the HCV genome, which are required for viral RNA replication, has been hampered by the lack of a convenient animal model or an efficient *in vitro* virus cultivation system (16). This difficulty was partially solved by a HCV replicon, which replicates autonomously to very high levels upon transfection into the human hepatoma cell line Huh-7 (17–19). This replicon lacks the complete structural protein coding region (from C up to p7) and NS2. Instead, it contains the gene encoding neomycin phosphotransferase (*neo*) downstream of the HCV IRES. Translation of the HCV nonstructural proteins (from NS3 to NS5B) is directed by the IRES of *Encephalomyocarditis virus* (EMCV).

The 5′ and 3′ terminal sequences of *Flaviviridae* play keys roles in viral RNA replication, translation, and/or packaging (10, 11, 20–24). The 5′ terminal sequence of HCV is highly conserved among the its different strains (20), even though it is not required for IRES function (6, 25). This suggests that the 5′ end portion of HCV plays an important role in viral RNA replication, encapsidation, or other essential aspects of virus proliferation.

In this study, we undertook to identify the RNA sequences required for HCV RNA replication at the 5′NTR by using HCV replicons. Dissection of the replication element from the translation element was ac-

[1] To whom correspondence and reprint requests should be addressed. Fax: 82-54-279-2199. E-mail: sungkey@postech.ac.kr.

0006-291X/02 $35.00

complished by inserting polioviral IRES between the serially deleted 5′NTR of HCV and the ORF encoding neomycin phosphotransferase. In this configuration, the translation of *neo* gene is directed by the polioviral IRES. Analyses of the colony-forming efficiency of the replicons and viral protein production in the replicon-containing cells indicated that domains I and II are necessary and sufficient for RNA replication and that almost all of the 5′NTR is required for efficient RNA replication.

MATERIALS AND METHODS

Plasmid construction. Dissection of the replication element from the translation element of HCV was accomplished by inserting polioviral IRES between 5′NTR of HCV and the ORF encoding neomycin phosphotransferase in NK5.1 (19). For this purpose, plasmid NK/H(1–389)/P was constructed by ligating an *Asc*I fragment of NK5.1 (kindly provided by Dr. R. Bartenschlager) to an *Asc*I fragment of the PCR-amplified polioviral IRES element. PCR was carried out with pMPS1-ECAT (26) and the oligonucleotides H53, 5′-TTGGCGCGCCCCATTATGATACAATTGTCTGA-3′ and H57, 5′-TTGGCGCGCCCTCTAGACTCCGGTATTGCGGTACC-3′ as template and primers, respectively. H57 oligonucleotides contain the recognition sequence for *Xba*I, which contains an in-frame stop codon. To construct the serially deleted HCV 5′NTR, *Xba*I/*Hin*dIII fragments from plasmid NK/H(1–389)/P, spanning the polioviral IRES, the ORF encoding neomycin phosphotransferase, and a part of EMCV IRES were transferred into pSK plasmids to obtain plasmids pSK/PN. *Sac*II/*Xba*I fragments of the PCR-amplified and serially deleted HCV IRES were then ligated to *Sac*II/*Xba*I fragments of plasmids pSK/PN to generate plasmids pSK/H(1–377)/PN, pSK/H(1–354)/PN, pSK/H(1–341)/PN, pSK/H(1–330)/PN, pSK/H(1–119)/PN, pSK/H(1–20)/PN, pSK/H(21–377)/PN, and pSK/H(44–377)/PN. PCRs were carried out using NK 5.1 as a template and the following oligonucleotides: H69, 5′-TCCCCGCGGATTCATTCTGCTAACCAG-3′ and H80, 5′-TGCTCTAGAGTTTGGTTTTTCTTTGAGG-3′ for HCV 1-377, H69 and H70, 5′-TGCTCTAGAGATTCG TGCTCATGGTGCAC-3′ for HCV 1-354, H69 and H71, 5′-TGCTCTAGAGGTGCACGGTCTACGAGACC-3′ for HCV 1-341, H69 and H72, 5′-TGCTCTAGATACGAGACCTCCCGGGGCAC-3′ for HCV 1-330, H69 and H73, 5′-TGCTCTAGACCTGGAGGCTGCACGACAC-3′ for HCV 1-119, H69 and H74, 5′-TGCTCTAGAGCCCCCAATCGGGGGCTGGC-3′ for HCV 1-20, H76, 5′-TCCCCGCGGAAGCTTCGTAATACGACTCACTATAGACACTCCACCATAGAT CAC-3′ and H80 for HCV 21-377, and H77, 5′-TCCCCGCGGAAGCTTCGTAATACGACTCACTATAGCCTGTGAGGAACTACTGTC-3′ and H80 for HCV 44-377. Plasmid pSK/HΔIII/PN was constructed by self-ligation of the *Xma*I fragment of pSK/H(1–377)/PN. Plasmid pSK/HΔII/PN was generated by ligating a *Sac*II/*Xba*I fragment of plasmid pSK/PN, a *Sac*II/*Eco*RI fragment of PCR-amplified domain I, and a *Xba*I/*Eco*RI fragment of PCR-amplified domains III – IV. The PCRs were carried out with the NK 5.1 as template and the oligonucleotides H69 and H29, 5′-CCGGAATTCGGAGTGATCTATGGTGGAG-3′ for a fragment containing domain I, and H94, 5′-CCGGAATTCACCCCCCCTCCCGGGAGAG-3′ and H80 for a fragment containing domains III–IV as primers. And then, *Hin*dIII fragments of these plasmids, spanning the serially deleted HCV IRES, polioviral IRES, ORF encoding neomycin phosphotransferase, and a part of EMCV IRES were substituted back into NK5.1 on *Hin*dIII fragments to generate plasmids NK/H(1–377)/P, NK/H(1–354)/P, NK/H(1–341)/P, NK/H(1–330)/P, NK/H(1–119)/P, NK/H(1–20)/P, NK/H(21–377)/P, NK/H(44–377)/P, pSK/HΔIII/PN, and pSK/HΔII/PN. Plasmids NK/H(1–271)/P were constructed by self-ligation of the *Xba*I/Klenow-filled-*Nru*I fragment of NK/H(1–389)/P.

In vitro transcription and translation. To generate run-off transcripts of HCV replicons, plasmids were linearized with *Sca*I (Boehringer Mannheim). The linearized DNAs were then treated with phenol–chloroform and ethanol-precipitated. Transcription reactions were performed with T7 RNA polymerase (Boehringer Mannheim) as described by the manufacturer. After 2 h at 37°C, an additional T7 RNA polymerase was added, and the reaction was incubated for a further 2 h. The RNA transcripts were then treated with phenol–chloroform and ethanol-precipitated. After dissolving in RNase-free water, 3 U of RNase-free DNase (Promega) and 10 U of *Pst*I per μg of plasmid DNA were added and incubated for 1 h at 37°C. The reaction medium was then extracted twice with phenol–chloroform and with chloroform. After precipitation, the concentration of the RNA transcripts was determined using a UV-spectrophotometer. The integrity of the RNA was checked by agarose gel electrophoresis.

Translation reactions of the *in vitro* transcribed replicons in the HeLa cytoplasmic extracts were performed in 25 μl of a translation mixture containing 2 μg of mRNA. The reactions were carried out for 2 h at 30°C in the presence of [^{35}S]methionine (NEN), and the translation products were analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The intensity of the autoradiographic images obtained was enhanced by fluorography using salicylic acid. The gel was dried and exposed to Kodak XAR-5 or Agfa Curix RP1 for 12 to 18 h.

Cell culture and transfection. Cell monolayers of the human hepatoma cell line Huh-7 were routinely grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL), 10% fetal bovine serum (FBS; Hyclone), penicillin, and streptomycin. Cells were transfected with various amounts of the transcribed RNAs using the electroporation procedure. Cells were immediately transferred to 9 ml of DMEM containing 10% FCS, and 5 ml was seeded in a 10 cm-diameter cell culture dish. 24 h after electroporation, the medium was replaced with DMEM supplemented with 10% FBS and G418 (600 μg/ml; Calbiochem). The medium was changed every 3 days, and 2 to 3 weeks after electroporation, colonies were stained with Crystal violet (Sigma; 0.5% in 70% methanol). It should be noted that given G418 concentrations have not been corrected for the amounts of active substance as given by the manufacturer.

RESULTS

Identification of the cis-Acting Element Required for Replication at the 5′NTR of HCV

To determine the *cis*-acting element required for viral RNA replication, chimeric subgenomic replicons of HCV were generated. Since the 5′NTR of HCV was thought to contain conserved *cis*-acting elements important for replication and translation, to identify the replication element it was necessary to separate the replication and translation elements. This was accomplished by inserting polioviral IRES between the serially deleted 5′NTR of HCV and the ORF encoding neomycin phosphotransferase. The 5′NTR deletions were conducted according to the secondary structure of HCV (Fig. 1). Several constructs NK/H(1–389)/P, NK/H(1–377)/P and NK/H(1–354)/P, include the N-terminal part of the core, which augments HCV IRES-dependent translation. Plasmid NK/H(1–341)/P contains the complete 5′NTR without initiator AUG. Plasmids NK/H(1–330)/P, NK/H(1–271)/P, NK/H(1–119)/P and NK/H(1–20)/P contain the domains I–III, I–II, and a part of domain III, I–II, and I, respectively.

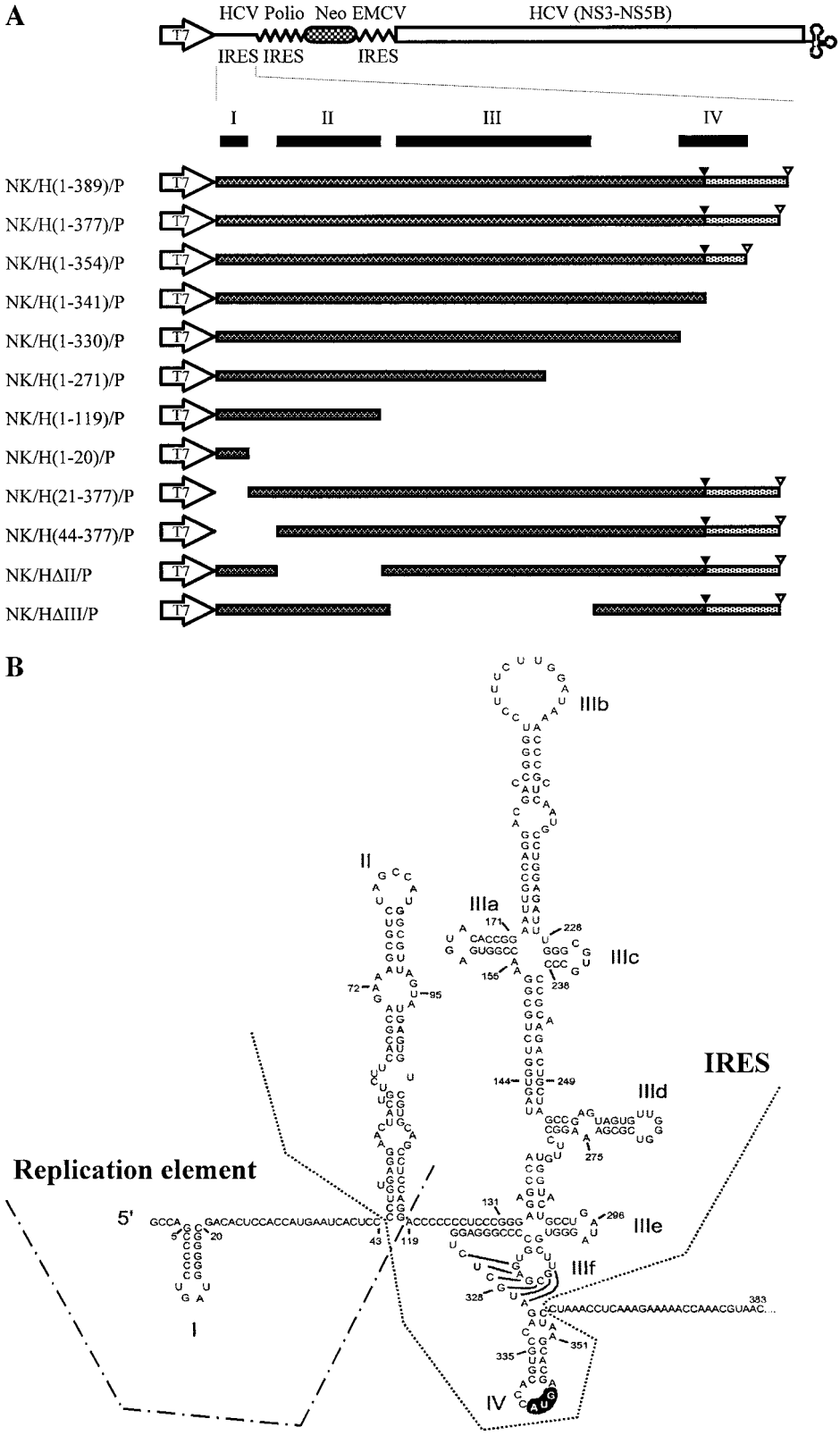

FIG. 1. Schematic representation of the chimeric subgenomic replicon of HCV. [A] HCV replicons contain different portions of HCV 5′NTR, the polioviral IRES, the *neo* gene, the EMCV IRES, and the HCV sequence from NS3 to the authentic 3′ end. The positions of the translational start and stop codons are indicated by solid and open arrowheads, respectively. The 5′ variable parts of HCV are shown on the bottom panel. Domains I, II, III, and IV, which comprise the stem-and-loop structure are indicated by the solid bars. [B] Schematic diagrams of the secondary structure of HCV 5′NTR and immediately downstream open reading frame is shown as determined by Honda *et al.* (38).

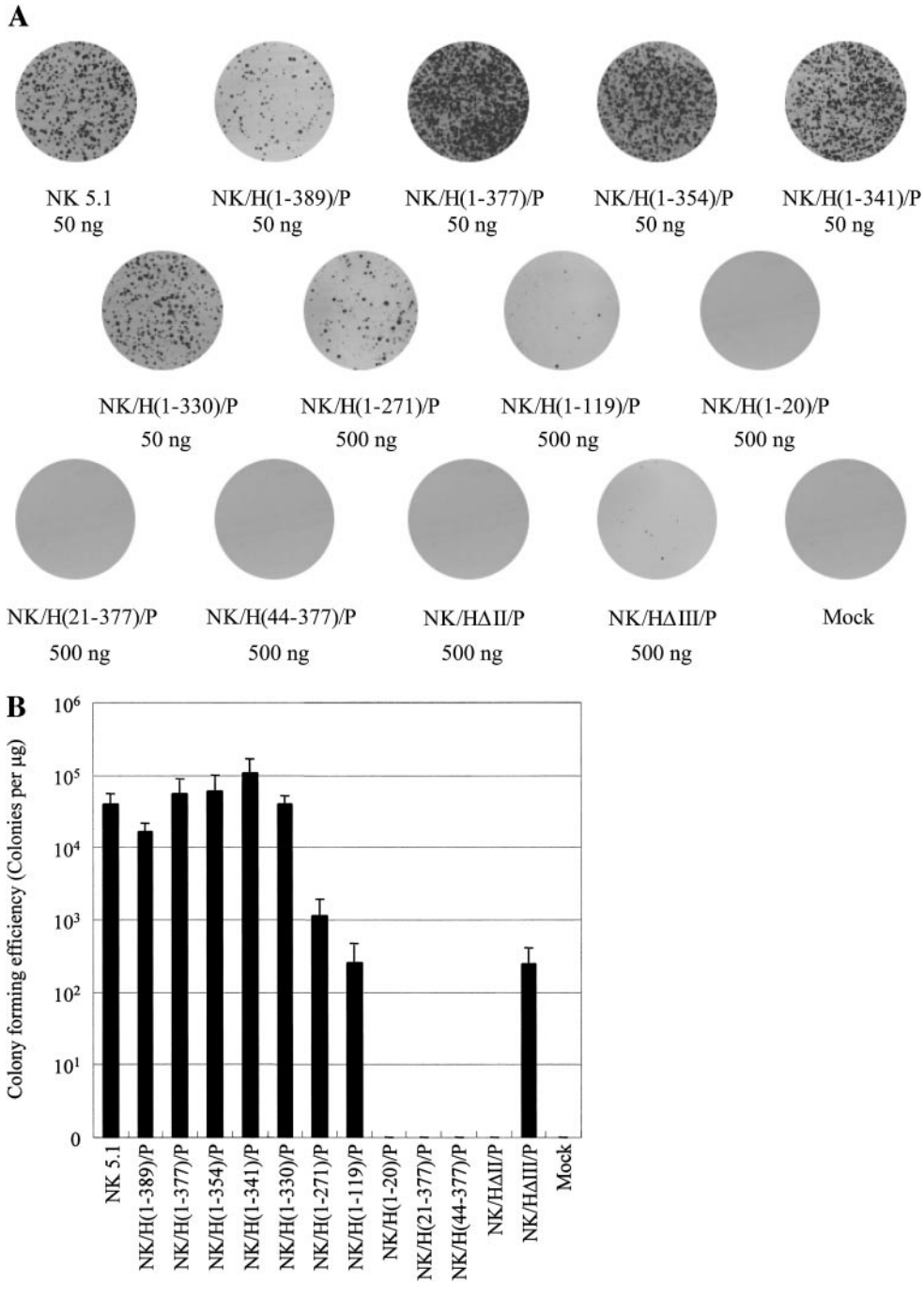

FIG. 2. Determination of the *cis*-acting element required for replication. [A] Huh-7 cell colonies are shown by Crystal violet staining after G418 selection of cells transfected with replicon RNAs. [B] Graphical representation of the colony-forming efficiency of each replicon. Numbers refer to the number of colonies per microgram of *in vitro* transcribed replicon RNA. The columns and bars represent the means and standard deviations of five independent transfections.

Plasmids NK/H(21–377)/P and NK/H(44–377)/P lack domain I, and plasmids NK/HΔII/P and NK/HΔIII/P lack domains II and III, respectively.

After linearizing the plasmids by *Sca*I, run-off transcripts were generated using T7 RNA polymerase, and the purified RNAs were electroporated into Huh-7 cells. Cells were then selected with G418 after incubating for 24 h, in order to isolate cells supporting the continuous replication of HCV RNAs. The selected colonies were either expanded for further analyses or fixed on the cell culture dish, stained with crystal violet, and counted.

Transfection of Huh-7 cells with NK/H(1–389)/P, which contains the *neo* gene, the expression of which is

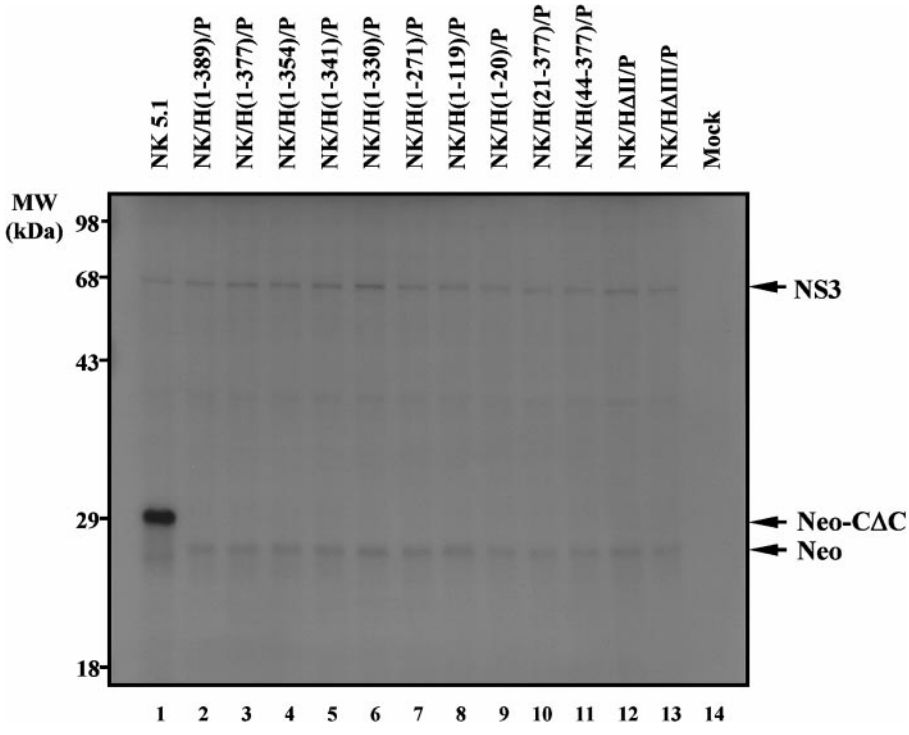

FIG. 3. *In vitro* translation of chimeric subgenomic replicon RNA. *In vitro* translations were performed in HeLa cell cytoplasmic extracts using the same replicon RNAs as Fig. 2. The positions of HCV NS3 and the neomycin phosphotransferase (*neo*) are indicated. Note that the translation product of the *neo* gene (*neo*-core) of NK 5.1, which is fused with the N-terminal of HCV core protein, is larger than that of other RNAs (*neo*).

directed by polioviral IRES, produced approximately 1.7×10^4 colonies per μg of RNA. This was similar to that of NK 5.1, in which *neo* gene is expressed by HCV IRES [Fig. 2, compare NK/H(1–389)/P and NK 5.1]. Further deletions up to nucleotides 330 of HCV 5′NTR had little influence on the efficiency of colony formation [Fig. 2, NK/H(1–377)/P, NK/H(1–354)/P. NK/H(1–341)/P, and NK/H(1–330)/P]. All of these clones contain domains I-III and the pseudoknot structure of HCV 5′NTR. The overall number of colonies were drastically reduced (about 50-fold) when the pseudoknot structure was deleted [Fig. 2, NK/H(1–271)/P]. Additional deletions of domain III further reduced colony forming efficiency by about 3-fold [Fig. 2, NK/H(1–119)/P]. NK/HΔIII/P, which had domain III only deleted, showed the same colony forming efficiency as NK/H(1–119)/P. However, deletion up to domain II [NK/H(1–20)] completely abolished the colony forming activity of the replicon, and the deletion of domain II alone (NK/HΔII/P) also abolished the replication of HCV RNA (lane NK/HΔII/P in Fig. 2). The deletion of domain I also abolished the replication of HCV RNA [lanes NK/H(21–377)/P and NK/H(44–377)/P in Fig. 3]. These results indicate that domains I and II are essential for replication and that the domain III and the pseudoknot structure augment the replication of HCV RNA.

Translational Efficiency of Poliovirus and EMCV IRES in Replicon RNAs

In vitro translation experiments were performed using replicon RNA transcripts and HeLa cell cytoplasmic extract in order to compare the relative translational efficiencies of the *neo* gene directed by polioviral IRES among the replicon constructs (Fig. 3). Although the colony forming efficiencies varied enormously among the different replicons, translation levels of the NS proteins directed by EMCV IRES and of the *neo* gene directed by polioviral IRES were similar for all subgenomic replicons containing polioviral IRES (Fig. 3, lanes 2 to 13). NK5.1 yielded a slightly larger *neo* gene product, since it contains the N-terminal core sequences (16 amino acids) fused with the *neo* gene. The translation level of this protein, which is directed by HCV IRES, was higher than those of the *neo* genes in the other constructs, which are directed by polioviral IRES, under the translation conditions used (compare lane 1 with lanes 2–13 in Fig. 3). Here we used a HeLa extract optimized for HCV mRNA translation with higher potassium concentrations for *in vitro* translation. Polioviral IRES functions better than HCV IRES in certain *in vitro* translation conditions (data not shown). These results indicate that the differences in the colony forming efficiencies of the different replicons

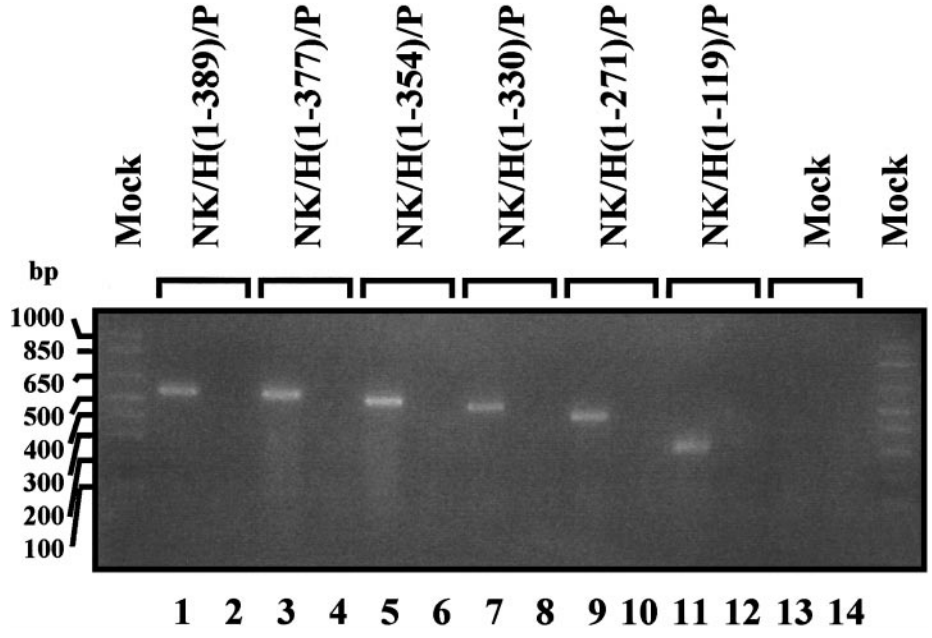

FIG. 4. RT-PCR and PCRs using total RNAs purified from cells containing chimeric subgenomic replicon RNA. RT-PCR and PCRs were performed using total cellular RNAs purified from the pools of G418-resistant colonies. RNAs were converted into cDNA using oligonucleotide H126 as a primer. The cDNAs were amplified for 30 cycles using oligonucleotides H127 and H128 as primers.

are not caused by the differences in the translational efficiencies of the genes in the replicons. Verification of Replicon RNAs and Viral Proteins in Colonies Containing Replicons

To verify the integrity of transfected RNAs in colonies containing HCV replicons, reverse transcription followed by the polymerase chain reaction (RT-PCR) or PCR without reverse transcription was performed using total RNAs from colonies selected by G418 after transfecting the replicon constructs (Fig. 4). Primer H126, 5′-CTCGAAGTACATAAGCGGATA-3′ corresponding to antisense nt 228–247 of polioviral IRES, was used for RT. Primer H127, 5′-CGACACTCCACCATAGATCAC-3′ corresponding to nt 20–40 of HCV IRES and primer H128, 5′-CGACACTCCACCATAGATCAC-3′ corresponding to antisense nt 207-227 of polioviral IRES were used for PCR. DNAs of the expected sizes were detected by RT-PCR from G418-resistant colonies (Fig. 4, lanes 1, 3, 5, 7, 9, and 11). On the other hand, no band was detected by PCR alone (Fig. 4, lanes 2, 4, 6, 8, 10 and 12). These results indicate that the input RNAs were replicated in the colonies without changes, such as insertions and deletions.

To investigate the expression of viral proteins encoded in the replicons, Western blot analyses were performed using the total cell extracts from replicon-containing cells (Fig. 5). The amounts of total proteins in the extracts were normalized against the actin levels in the extracts (Fig. 5, panel α-actin). Various amounts of viral proteins (NS3, NS4B, NS5A, and NS5B) were detected by their corresponding antibodies (Fig. 5, panels α-NS3, α-NS4B, α-NS5A, and α-NS5B). The levels of viral proteins correlated roughly with the colony forming efficiencies of the corresponding replicons. That is, NK 5.1, NK/H(1–389)/P, NK/H(1–377)/P, NK/H(1–354)/P, and NK/H(1–330)/P, which showed higher colony forming efficiency, expressed more viral protein than NK/H(1–271)/P and NK/H(1–119)/P, which showed lower colony forming efficiency. However, the protein level in each colony did not precisely match the colony forming efficiency of each replicon. For example, NK/H(1–377)/P produced slightly lower amounts of viral proteins than replicons that had higher colony forming efficiencies (Fig. 5). The reason for the lower protein yield of NK/H(1–377)/P is not known. Interestingly, the size of each colony after G418 selection of the replicon transfectant was related to its colony forming efficiency. Replicons showing lower colony forming efficiencies [NK/H(1–271)/P, NK/H(1–119)/P, and NK/HΔIII/P] formed smaller colonies than the higher yielding replicons (Fig. 2A).

DISCUSSION

The *cis*-acting elements required for the replication of bovine viral diarrhea virus (BVDV) that belongs to the family *Flaviviridae* along with HCV were identified using infectious cDNA clones or viral subgenomic replicons. When the IRES of BVDV was replaced with that of EMCV or HCV, only the first 3-4 nucleotides of the genomic RNA were found to be essential for viral replication (27). However, the stem-loop motif at the immediate 5′ terminus of BVDV, which spans up to nt 32,

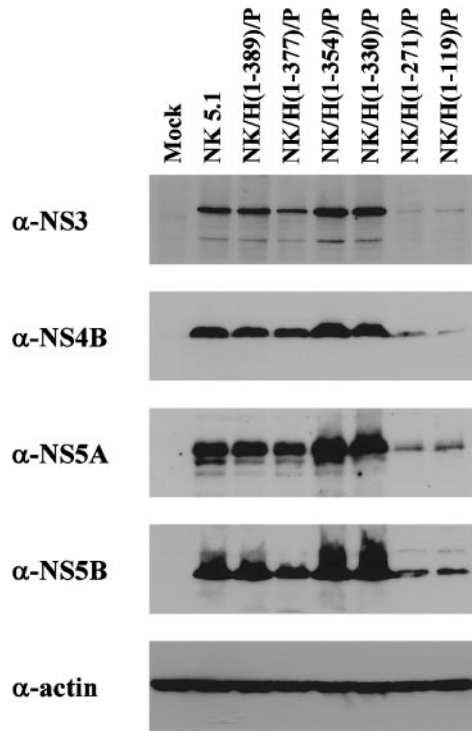

FIG. 5. Detection of HCV proteins in G418-resistant Huh-7 colonies. To monitor the expression levels of viral proteins in the Huh-7 cells containing the HCV subgenomic replicon, Western blot was performed using a monoclonal antibody against NS3, and polyclonal antibodies against NS4B, NS5A, and NS5B. A monoclonal antibody against human actin was used as a control.

plays a key role in both translation and replication of replicon RNA (28). Studies using other approaches to identify the *cis*-acting element at the 5′ end of the HCV genome have been prevented due to the lack of an efficient HCV cultivation system. We tried to identify the 5′ terminal region required for replication of HCV RNA by using chimeric subgenomic replicons, as reported by Lohmann *et al.* (17, 18). The polioviral IRES was inserted into the replicons in order to separate the *cis*-acting element of replication from the translation element. In these replicons, translation of *neo* gene and HCV nonstructural proteins were directed by polioviral IRES and EMCV IRES, respectively. The constructs containing domains I and II replicated in Huh-7 cells. Further deletions toward the 5′ end abolished replication. The addition of domain III and the pseudoknot structure in the 5′NTR to domains I and II enhanced colony forming efficiency by ca. 100-fold. This result indicated that domains I and II are essential for RNA replication and that almost all regions in 5′NTR, except the sequence adjacent to the initiation codon, are required for efficient RNA replication.

The first 40 nucleotides of the HCV RNA genome, including domain I, were suggested to be excluded from the IRES element (6, 25), but were shown to be involved in RNA replication (29). On the other hand, the dinucleotide at positions 34 and 35 was shown to contribute to the differential translation efficiencies of genotype 1a and 1b isolates (30). The remaining three stem-loop domains (II-IV) form the IRES.

Interestingly, the *cis*-acting element for RNA replication determined in this study partially overlaps the HCV IRES. That is, domain II is required for both replication and translation. Therefore, it is possible that domain II is involved in switching from translation to replication. In the case of poliovirus, it has been shown that RNA replication is inhibited while translation is in progress (31). The switch from translation to replication of poliovirus was suggested to be controlled by an RNA-binding protein poly(rC)-binding protein (PCBP) (31–33). Several proteins have been shown to interact with the 5′NTR of HCV (34–36). Among these, PCBP2 was shown to interact with domains I and II (34). The role of PCBP2 in either the translation or the replication of RNA remains to be elucidated.

The requirement for domains I and II in replication is possibly related to the characteristics of the RNA-dependent polymerase encoded in NS5B. The minimal sequence necessary for *de novo* synthesis of positive sense RNA from the 3′ end of negative sense RNA was investigated *in vitro* using purified full-length NS5B (37). The authors showed that 239 nt at the 3′ end of negative sense RNA served as a template for the *de novo* synthesis of positive sense RNA (37). Moreover, the 122 nt at the 3′ end of negative sense RNA also served as a template for the *de novo* synthesis of RNA, albeit with much weaker activity was detected (personal communication with Dr. J.-W. Oh). This result is in agreement with the findings of the present study. We found that domains I and II represent the minimal requirement for replication, and that the downstream sequence in the 5′NTR augments replication.

ACKNOWLEDGMENTS

We are indebted to Dr. Ralf Bartenschlager at the Johannes Gutenberg University Mainz, for kindly providing us with the HCV subgenomic replicon NK 5.1. The present study was supported in part by the NRL, G7 Program and the Molecular Medicine Research Group Program of MOST, and by KOESEF through PNRC.

REFERENCES

1. Miller, R. H., and Purcell, R. H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2057–2061.
2. Tsukiyama-Kohara, K., Iizuka, N., Kohara, M., and Nomoto, A. (1992) *J. Virol.* **66,** 1476–1483.
3. Zhao, W. D., Lahser, F. C., and Wimmer, E. (2000) *J. Virol.* **74,** 6223–6226.
4. Hwang, L. H., Hsieh, C. L., Yen, A., Chung, Y. L., and Chen, D. S. (1998) *Biochem. Biophys. Res. Commun.* **252,** 455–460.
5. Honda, M., Brown, E. A., and Lemon, S. M. (1996) *RNA* **2,** 955–968.
6. Honda, M., Ping, L. H., Rijnbrand, R. C., Amphlett, E., Clarke, B., Rowlands, D., and Lemon, S. M. (1996) *Virology* **222,** 31–42.

7. Lu, H. H., and Wimmer, E. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1412–1417.
8. Wang, C., Sarnow, P., and Siddiqui, A. (1994) *J. Virol.* **68,** 7301–7307.
9. Wang, T. H., Rijnbrand, R. C., and Lemon, S. M. (2000) *J. Virol.* **74,** 11347–11358.
10. Kolykhalov, A. A., Feinstone, S. M., and Rice, C. M. (1996) *J. Virol.* **70,** 3363–3371.
11. Tanaka, T., Kato, N., Cho, M. J., Sugiyama, K., and Shimotohno, K. (1996) *J. Virol.* **70,** 3307–3312.
12. Tanaka, T., Kato, N., Cho, M. J., and Shimotohno, K. (1995) *Biochem. Biophys. Res. Commun.* **215,** 744–749.
13. Yamada, N., Tanihara, K., Takada, A., Yorihuzi, T., Tsutsumi, M., Shimomura, H., Tsuji, T., and Date, T. (1996) *Virology* **223,** 255–261.
14. Kolykhalov, A. A., Mihalik, K., Feinstone, S. M., and Rice, C. M. (2000) *J. Virol.* **74,** 2046–2051.
15. Yanagi, M., St Claire, M., Emerson, S. U., Purcell, R. H., and Bukh, J. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 2291–2295.
16. Bartenschlager, R., and Lohmann, V. (2001) *Antiviral Res.* **52,** 1–17.
17. Lohmann, V., Korner, F., Dobierzewska, A., and Bartenschlager, R. (2001) *J. Virol.* **75,** 1437–1449.
18. Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. (1999) *Science* **285,** 110–113.
19. Krieger, N., Lohmann, V., and Bartenschlager, R. (2001) *J. Virol.* **75,** 4614–4624.
20. Bukh, J., Purcell, R. H., and Miller, R. H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4942–4946.
21. Deng, R., and Brock, K. V. (1993) *Nucleic Acids Res.* **21,** 1949–1957.
22. Cahour, A., Pletnev, A., Vazielle-Falcoz, M., Rosen, L., and Lai, C. J. (1995) *Virology* **207,** 68–76.
23. Men, R., Bray, M., Clark, D., Chanock, R. M., and Lai, C. J. (1996) *J. Virol.* **70,** 3930–3937.
24. Mandl, C. W., Holzmann, H., Meixner, T., Rauscher, S., Stadler, P. F., Allison, S. L., and Heinz, F. X. (1998) *J. Virol.* **72,** 2132–2140.
25. Rijnbrand, R., Bredenbeek, P., van der Straaten, T., Whetter, L., Inchauspe, G., Lemon, S., and Spaan, W. (1995) *FEBS Lett.* **365,** 115–119.
26. Jang, S. K., and Wimmer, E. (1990) *Genes Dev.* **4,** 1560–1572.
27. Frolov, I., McBride, M. S., and Rice, C. M. (1998) *RNA* **4,** 1418–1435.
28. Yu, H., Isken, O., Grassmann, C. W., and Behrens, S. E. (2000) *J. Virol.* **74,** 5825–5835.
29. Boyer, J. C., and Haenni, A. L. (1994) *Virology* **198,** 415–426.
30. Honda, M., Rijnbrand, R., Abell, G., Kim, D., and Lemon, S. M. (1999) *J. Virol.* **73,** 4941–4951.
31. Gamarnik, A. V., and Andino, R. (1998) *Genes Dev.* **12,** 2293–2304.
32. Gamarnik, A. V., and Andino, R. (2000) *J. Virol.* **74,** 2219–2226.
33. Silvera, D., Gamarnik, A. V., and Andino, R. (1999) *J. Biol. Chem.* **274,** 38163–38170.
34. Fukushi, S., Okada, M., Kageyama, T., Hoshino, F. B., Nagai, K., and Katayama, K. (2001) *Virus Res.* **73,** 67–79.
35. Fukushi, S., Okada, M., Kageyama, T., Hoshino, F. B., and Katayama, K. (1999) *Virus Genes* **19,** 153–161.
36. Fukushi, S., Kurihara, C., Ishiyama, N., Hoshino, F. B., Oya, A., and Katayama, K. (1997) *J. Virol.* **71,** 1662–1666.
37. Oh, J. W., Ito, T., and Lai, M. M. (1999) *J. Virol.* 73, 7694–7702.
38. Honda, M., Beard, M. R., Ping, L. H., and Lemon, S. M. (1999) *J. Virol.* **73,** 1165–1174.