Isolation of specific and high-affinity RNA aptamers against NS3 helicase domain of hepatitis C virus

BYOUNGHOON HWANG,¹ JUNG SUN CHO,¹ HYEON JU YEO,¹ JUNG-HYE KIM,¹ KYUNG MIN CHUNG,² KYUNGSOOK HAN,³ SUNG KEY JANG,² and SEONG-WOOK LEE¹

¹Department of Molecular Biology, Institute of Nanosensor and Biotechnology, Dankook University, Seoul 140-714, Korea

²Department of Life Science, Pohang University of Science and Technology, Kyungbuk 790-784, Korea

³Department of Computer Science and Engineering, Inha University, Incheon 402-751, Korea

ABSTRACT

Hepatitis C virus (HCV)-encoded nonstructural protein 3 (NS3) possesses protease, NTPase, and helicase activities, which are considered essential for viral proliferation. Thus, HCV NS3 is a good putative therapeutic target protein for the development of anti-HCV agents. In this study, we isolated specific RNA aptamers to the helicase domain of HCV NS3 from a combinatorial RNA library with 40-nucleotide random sequences using *in vitro* selection techniques. The isolated RNAs were observed to very avidly bind the HCV helicase with an apparent K_d of 990 pM in contrast to original pool RNAs with a K_d of >1 μ M. These RNA ligands appear to impede binding of substrate RNA to the HCV helicase and can act as potent decoys to competitively inhibit helicase activity with high efficiency compared with poly(U) or tRNA. The minimal binding domain of the ligands was determined to evaluate the structural features of the isolated RNA molecules. Interestingly, part of binding motif of the RNA aptamers consists of similar secondary structure to the 3'-end of HCV negative-strand RNA. Moreover, intracellular NS3 protein can be specifically detected *in situ* with the RNA aptamers, indicating that the selected RNAs are very specific to the HCV NS3 helicase. Furthermore, the RNA aptamers partially inhibited RNA synthesis of HCV subgenomic replicon in Huh-7 hepatoma cell lines. These results suggest that the RNA aptamers selected *in vitro* could be useful not only as therapeutic and diagnostic agents of HCV infection but also as a powerful tool for the study of HCV helicase mechanism.

Keywords: HCV; NS3 helicase; SELEX; RNA aptamer; intracellular protein detection; HCV replicon

INTRODUCTION

Hepatitis C virus (HCV) is the main causative agent of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Lauer and Walker 2001). Although the HCV infection causes worldwide health problems, efficient and specific antiviral therapy has not yet been developed.

HCV contains a single, positive-stranded RNA genome about 9600 nucleotides in length encoding a single polyprotein of about 3010 amino acids (Choo et al. 1991; Takamizawa et al. 1991). This polyprotein precursor is co- or posttranslationally processed by cellular and viral proteases to yield functional structural and nonstructural proteins (Bartenschlager et al. 1993; Grakoui et al. 1993; Hijikata et al. 1993; Tomei et al. 1993; Lin et al. 1994; Manabe et al. 1994; Mizushima et al. 1994). The structural proteins in-

clude the core protein, C, and the envelope glycoproteins E1 and E2. The nonstructural proteins are composed of NS2 protease, NS3 serine protease plus helicase, NS4A serine protease cofactor, NS4B and NS5A proteins, and NS5B RNA-dependent RNA polymerase, which are considered as components of a complex responsible for HCV replication (Moradpour et al. 2002).

HCV NS3 is multifunctional with three known enzymatic activities separated into two distinct domains. The serine protease activity is present in the one-third amino-terminal region of the protein and the nucleoside triphosphatase (NTPase) and helicase activities in the remaining carboxy-terminal domain (Kim et al. 1995; Satoh et al. 1995; De Francesco and Steinkühler 2000; Kwong et al. 2000). HCV NS3 has been demonstrated to be internally processed in cells within the RNA helicase sequence motif that is highly conserved in the Flaviviridae family (Shoji et al. 1999; Yang et al. 2000). Helicase activity is reported crucial for RNA unwinding presumably during the viral genome replication, and hence believed essential for the viral proliferation (Wardell et al. 1999; Kwong et al. 2000). Therefore, the

Reprint requests to: Seong-Wook Lee, Department of Molecular Biology, Institute of Nanosensor and Biotechnology, Dankook University, San8 Hannam-Dong, Yongsan-Gu, Seoul 140-714, Korea; e-mail: SWL0208@dankook.ac.kr; fax: (82-2) 798-4733.

Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.7100904>.

HCV NS3 helicase is an attractive target for anti-HCV drug discovery. The crystal or solution structure of the NS3 helicase along with or without oligonucleotide has been recently elucidated (Yao et al. 1997; Cho et al. 1998; Kim et al. 1998; Liu et al. 2001), providing more opportunities for rational design of NS3 inhibitors.

Characteristics of RNAs, which can adopt complex structures to bind target proteins with high affinities and encode amplifiable genetic information, make RNA a potentially very useful diagnostic and/or therapeutic lead compound (Gold et al. 1993; Burgstaller et al. 2002). Such short RNA ligands, termed RNA aptamers, have been identified from a random RNA library to bind several proteins including non-RNA-binding proteins with high affinity and specificity using *in vitro* selection techniques, called systemic evolution of ligands by exponential enrichment (SELEX; Ellington and Szostak 1990; Tuerk and Gold 1990). Several RNA aptamers have been successfully evaluated even in animal disease models (Sullenger and Gilboa 2002; Hwang et al. 2003).

Isolation and characterization of RNA aptamers specific for the HCV NS3 protease domain has been described (Kumar et al. 1997; Urvil et al. 1997; Fukuda et al. 2000). Intracellular expression of tandem repeats of the RNA aptamers against NS3 protease was demonstrated to inhibit the HCV protease activity in HeLa cells (Nishikawa et al. 2003). Moreover, high-affinity RNA aptamers to HCV NS5B RNA-dependent RNA polymerase were recently isolated that could inhibit the enzymatic activity *in vitro* (Biroccio et al. 2002; Vo et al. 2003). Although RNA ligands isolated from full-length HCV NS3 moderately inhibited HCV helicase activity (Kumar et al. 1997), no studies have been described for the isolation of RNA aptamers directly against HCV NS3 helicase domain. In the present study, we employed an RNA combinatorial library and isolated RNA aptamers for the HCV NS3 helicase domain essential for the HCV multiplication. This RNA was shown to bind the HCV helicase with high specificity and affinity. In addition, the RNA aptamer efficiently and competitively prevent RNA unwinding activity of the helicase with no effect on NTPase activity of the protein. Binding region of the RNA aptamer was enzymatically characterized. Moreover, only the cells that express HCV NS3 can be specifically recognized by the RNA aptamers, but not with control RNAs, indicating very specific interaction of the RNA with the HCV helicase. Importantly, replication of HCV subgenomic replicon (Lohmann et al. 1999) can be partially inhibited by the RNA aptamers in human liver cells.

RESULTS

In vitro selection of HCV helicase-specific RNAs with high affinity

An RNA library of approximately 10^{14} different molecules was generated with each molecule containing a 40-nt-long

sequence derived from a random sequence flanked by defined regions. To isolate high-affinity RNA ligands that bound the HCV NS3 helicase domain, we employed an *in vitro* selection procedure using the RNA library as described under Materials and Methods. After 21 cycles of selection, the bound RNAs were amplified by RT-PCR. The resulting cDNAs were then cloned, and 13 different clones sequenced.

Four different RNAs were selected that specifically bind to the HCV helicase (Fig. 1). As shown in Figure 1A, all the RNAs were 1 nt shorter than the original 92-nt-long library RNAs. This change might result from deletion of part of the random sequence during RT-PCR amplification. In addition, the four RNAs contained very similar sequences. To evaluate binding specificity of the selected RNA, precipitation experiments were performed with internally radiola-

FIGURE 1. SE RNA sequences and their specific binding to the HCV NS3 helicase domain. (A) After 21 rounds of *in vitro* selection, the sequence of 13 selected RNAs was determined. Only four different but very similar RNA sequences were found in these clones with each being present multiple times (numbers in parentheses). The line drawn for sequences #2, #3, and #4 indicates that nucleotides found at these positions are identical to those shown for sequence #1. (B) SE RNA #1 specifically binds the HCV helicase. Internally radiolabeled library RNA (lanes 1–3, 350 pM) or SE RNA #1 (lanes 4–6, 350 pM) was incubated with (lanes 3, and 6–8, 160 nM) or without HCV helicase (lanes 2 and 5) and the RNA–protein complexes were precipitated with Ni-NTA beads (lanes 2, 3, and 5–8). Bound RNAs were extracted and analyzed on a 6% polyacrylamide gel with urea. Unlabeled library RNA (lane 7, 1.7 μ M) or unlabeled SE RNA #1 (lane 8, 1.7 μ M) were added in a 5000-fold excess amount to the binding reaction to determine if either could inhibit the formation of SE RNA–HCV helicase complexes. Lanes 1 and 4 contain 10% of the input-labeled library RNA or SE RNA #1, respectively. (C) Internally radiolabeled SE RNA #1 (95 pM) was incubated with (lanes 3–7, 80 nM) or without HCV helicase (lane 2) and binding activity of RNA to the HCV helicase was analyzed as Figure 1B. Poly U (lane 4, 655 nM), tRNA (lane 5, 655 nM), SE RNA #1 (lane 6, 655 nM) or no RNA competitor (lane 3) were added in a 6700-fold excess amount to the binding reaction to determine if they could inhibit the formation of SE RNA–HCV helicase complexes. In addition, 670-fold excess amount of unlabeled SE RNA #1 (65.5 nM) were added (lane 7). Lane 1 contains 10% of the input-labeled SE RNA #1.

beled RNAs (Fig. 1B,C). Selected RNA (SE RNA) #1 was shown to bind strongly and specifically to HCV helicase, but not to the agarose beads (Fig. 1B, lanes 4–6). In contrast, original-library RNA hardly bound to the protein (Fig. 1B, lanes 1–3). Other selected RNA clones also showed avid and specific binding to the HCV helicase, but not to the beads (data not shown). The SE RNAs can bind to GST-tagged full-length HCV NS3, but not to the his-tagged other HCV protein such as NS5B protein, thus excluding possible nonspecific binding of the RNA to the histidine moieties tagged at the C-terminal end of the helicase (data not shown). SE RNA #1 was chosen for further characterization because it appeared the most frequently in the selected RNA clones. Formation of helicase-SE RNA #1 complex was shown to be competitively blocked by addition of excess amount of unlabeled SE RNA #1 in a dose dependent manner (Fig. 1B, lane 8, and Fig. 1C, lanes 6,7). However, a nonspecific competitor, such as the original RNA library or tRNA, could not inhibit the binding of SE RNA to the HCV helicase (Fig. 1B, lane 7, or Fig. 1C, lane 5, respectively). Moreover, 90-nt-long poly(U) sequence that the HCV helicase has been shown to preferentially bind most likely at its RNA binding domain (Kanai et al. 1995) also showed little competition of helicase-binding activity of SE RNA #1 even in the excess amount (Fig. 1C, lane 4). These results suggest that the SE RNA specifically and efficiently binds the HCV helicase.

To estimate the affinity of the SE RNA-HCV helicase interaction, a precipitation experiment was used with trace amounts of radiolabeled RNAs and increasing amounts of the helicase (Fig. 2). The original-library RNA containing 40-nt-long random sequences was shown to have little af-

finity to the HCV helicase even at the highest concentration of the protein. By contrast, SE RNA exhibited high affinity with apparent dissociation constant (K_d) of about 990 pM, demonstrating that SE RNA binds tightly to the HCV helicase.

Effects of SE RNA on RNA binding, ATPase and RNA helicase activities of the HCV helicase

Given the observation that SE RNA selectively and avidly binds the HCV helicase, we next determined if the binding of the selected RNA affected the biochemical activities of the HCV helicase. First, effect on RNA template binding capacity of the helicase by SE RNA was measured in a gel retardation assay which was performed in the absence of ATP with a internally radiolabeled RNA substrate used as a longer template for the assay of RNA helicase activity (Fig. 3). Poly(U) sequence efficiently protected the helicase from RNA binding as shown previously (Fig. 3, lane 5; Gwack et al. 1996). The selected RNA was also effective at inhibiting the substrate RNA binding to the HCV helicase in a concentration-dependent manner (Fig. 3, lanes 3,4), whereas the original pool RNA showed much less inhibition even at the highest concentration tested in this study (Fig. 3, lane 6). Furthermore, SE RNA efficiently impeded the helicase from binding to the poly(U) sequence (data not shown). This result, together with the previous observations in Figures 1 and 2, indicates that SE RNA appears to specifically and avidly bind to the HCV helicase at the substrate RNA binding site or distinct region that could affect substrate RNA binding capacity of the protein.

FIGURE 2. High binding affinity of the SE RNA to the HCV helicase. (A,B) Internally radiolabeled library RNA (A, 50 pM) or SE RNA #1 (B, 50 pM) was incubated with increasing amount of the HCV helicase (0–960 nM). RNA-HCV helicase complexes were precipitated with Ni-NTA beads. Bound RNAs were extracted and electrophoresed on a 6% polyacrylamide gel with urea. “Input” lane contains 10% of labeled RNAs used in this study. (C) The percentage of RNA to HCV helicase was calculated by determining the fraction of radioactivity present in the RNA-HCV helicase complexes. A maximum binding percentage of RNA to HCV helicase was seen on 320 nM concentration of HCV helicase. The plotted numbers have been normalized to that amount and values shown represent means of three separate measurements.

FIGURE 3. Inhibition of substrate RNA binding to the HCV helicase by SE RNA. Radiolabeled substrate RNA (520 pM) was incubated with (lanes 2–6, 20 nM) or without HCV helicase (lane 1). The protein–RNA complexes were separated from unbound RNAs in a 4% non-denaturing polyacrylamide gel. In lanes 2–6, no competitor RNA (lane 2), or excess amount of unlabeled SE RNA (lane 3, 4.2 nM; lane 4, 42 nM), poly U (lane 5, 42 nM), or library RNA (lane 6, 42 nM) was added to the binding reaction to determine if they could compete with radiolabeled substrate RNA for binding to the HCV helicase. Binding activity of the substrate RNA to the helicase in each sample is given below each lane as a percentage relative to the activity in the absence of any competitor RNAs.

ATPase activity of the HCV helicase has been previously reported to be highly stimulated by oligomeric nucleic acids, with poly(U) having the greatest effect (Suzich 1993; Preugschat et al. 1996; Wardell et al. 1999). Therefore, we determined next whether SE RNA could stimulate ATPase activity of the helicase (Fig. 4). ATPase activity was monitored by measuring the amount of hydrolyzed ATP using thin-layer chromatography. Consistent with the previous findings, poly(U) demonstrated a dose-dependent stimulation of ATPase activity up to an approximately 6-fold increase over activity in the absence of any RNA (Fig. 4, lanes 2–9). On the contrary, neither original library RNA nor SE RNA could affect the ATPase activity even at the highest amount tested here, when compared with the activity without RNA (Fig. 4, lanes 10–19).

Once we had determined that the selected RNA specific to the HCV helicase with high-affinity interfered with the substrate RNA binding activity of the protein without effect on the ATPase activity, we wanted to investigate if SE RNA could inhibit RNA helicase activity of the protein (Fig. 5). RNA unwinding activity of the HCV helicase was measured

with the partially dsRNA substrate generated as described in Materials and Methods. Since the HCV helicase unwinds RNA/RNA duplexes in a 3' to 5' direction (Gwack et al. 1996; Tai et al. 1996), the RNA substrates contained single-stranded RNA regions at their 3' ends. Previous reports demonstrated that poly(U) RNA efficiently inhibited RNA helicase activity of the HCV helicase probably through competitive binding to the protein with RNA substrates (Tai 1996). In accordance with this finding, here, poly(U) sequence showed impediment to the unwinding of RNA duplexes mediated by the HCV helicase in a concentration-dependent manner by up to 44% (Fig. 5A, lanes 10–14). Noticeably, more efficient inhibition of RNA helicase activity was observed in the case of SE RNA by up to 90%, whereas control RNA, such as tRNA, showed very little inhibition (Fig. 5A, lanes 5–9 and 15–19). In addition, SE RNA-engendered inhibition of HCV RNA helicase was dose dependent with an IC_{50} of approximately 12.5 nM (Fig. 5B). Therefore, RNA selected in this study prohibited RNA helicase activity of the HCV NS3 helicase much more efficiently than poly(U) RNA by more than 10-fold [IC_{50} of poly(U) RNA $\gg$ 121.5 nM, see Fig. 5B). The mechanism of helicase inhibition by the SE RNA was determined with respect to the decreasing amounts of the substrate RNAs (Fig. 5C). K_m was affected by the presence of the SE RNA, whereas V_{max} was not, which indicates that the RNA aptamer inhibited the helicase activity of HCV NS3 by competitively interfering with the substrate binding to the protein.

Mapping the binding domain of SE RNA

To evaluate the structural features of the 91-nt-long SE RNA that are critical for helicase binding, deletion analysis was performed to determine the minimal binding domain of the RNA (Fig. 6A,B). This analysis demonstrated that the last 25 nt of the RNA (MD1, MD2, and MD3) could be removed without severe effect on binding, whereas deletion of only the first 19 nt (MD6) seriously affected binding. Deletion of the last 46 nt (MD4) reduced, but did not abolish, binding. However, larger deletions from the 3' end of the selected RNA (MD5) showed nearly complete abolishment of binding. In addition, RNA truncated at both ends of SE RNA (the first 19 nt plus the last 46 nt) also did not retain the ability to bind the helicase (data not shown). Therefore, residues +1 to +45 of SE RNA are the most important for the recognition of the HCV helicase, while residues +46 to +66 probably stabilize the structure of SE RNA that is required for binding to the HCV helicase.

To characterize how SE RNA interacts with the HCV helicase, structure probing and footprinting analyses were performed with RNase T1, S1, and V1 (Fig. 6C,D). As a working model, the most stable secondary structure of the SE RNA, as shown in Figure 6E, was predicted using

FIGURE 4. No effect of SE RNA on the ATPase activity of the HCV helicase. 1 μ Ci of (α - 32 P) ATP was incubated with (lanes 2–19, 20 nM) or without HCV helicase (lane 1). Hydrolyzed (α - 32 P) ATP was spotted onto polyethyleneimine cellulose sheets and separated from nonhydrolyzed ATP by ascending chromatography. In lanes 3–19, increasing amount of poly U, library RNA, or SE RNA #1 (lanes 3,10,15: 16 nM; lanes 4,11,16: 80 nM; lanes 5,12,17: 400 nM; lanes 6,13,18: 2 μ M; lanes 7,14,19: 5 μ M; lane 8: 10 μ M; lane 9: 20 μ M) was added to the reaction to determine if the RNAs could affect the ATPase activity of HCV helicase. Conversion percentage was calculated by determining the amount of hydrolyzed ATP.

MULFOLD program (Jaeger et al. 1989). The general secondary structure predicted by computer program is comprised of four stem-loops separated by internal loops and appeared to be similar to the results determined by structure probing. However, apical loop-stem-bulge structure predicted in the left upper corner (nt 26–36) of the SE RNA is very sensitive to RNase T1 and S1 (Fig. 6C, lanes 2,3), suggesting that this part would rather form a large loop configuration. To determine which regions are affected when HCV helicase binds to the SE RNA, footprinting studies were performed with three different concentrations of the protein (Fig. 6D). In accordance with the deletion mapping studies, single-stranded loop regions in the left half (nt 10–18 and 26–36) of the SE RNA are well protected by the HCV helicase (Fig. 6D, lanes 3–10). Moreover, such helicase-mediated protection of the RNA aptamer against RNases is dose dependent. In contrast, the sequences at nt positions 19–24 and 37–42 with a strong stem configuration, which are beneath the apical large single-stranded region, does not interact directly with the protein. However, interestingly, such a strong stem structure with high GC content in the selected RNA aptamers is very similar to that in the 3'-terminal 127 nt regions of HCV negative-strand RNA (Fig. 6E) that were recently described to be involved in the specific interaction with HCV NS3 helicase (Banerjee and Dasgupta 2001). The stem sequences at nt positions 23–25 and 41–42 are rather more sensitive to RNase V1 in the presence of the protein (Fig. 6D, lanes 11–14). These results suggest that conformational change of the SE RNA can be induced in the presence of HCV helicase such that stem structure between the protein-bound single-stranded regions would become more stacked. Other selected RNA clones as described in Figure 1A showed the same pattern of secondary structure and binding motif to the HCV helicase as clone #1 (data not shown).

Taken together with the results of deletion mapping and

structural probing analyses, the essential and minimal binding domain of the SE RNA to the HCV helicase was denoted shaded in a dotted box in Figure 6E. This scheme indicates that the HCV helicase binds predominantly to the single-stranded sequences at nt positions 10–18 and 26–36 (5'-GAAGCGUGC-3' and 5'-GUAPuUGUPuUAG-3', Pu represents purine nucleotide) in loop regions of left half of the SE RNA, which alters the conformation of the stem portion between the bound regions.

Application of SE RNA for in situ detection of intracellular HCV NS3

To determine whether the SE RNA specifically interact with the HCV helicase, but not with the other cellular proteins, and, if so, can the RNA be applied as a diagnostic agent for the intracellular HCV NS3, we set out to do experiments to specifically identify NS3-expressing cells using the SE RNA (Fig. 7). For those studies, we first developed stable Saos-2 cell lines that express HCV NS3 in the absence of tetracycline, but not in the presence of tetracycline, using the plasmid encoding NS3 under the Tet-off control system. The expression of the HCV NS3 proteins in such cells was confirmed by immunofluorescence study and western analysis with NS3 antibodies (data not shown). Cells expressing HCV NS3 proteins were identified using alkaline phosphatase-induced color reaction generated from the biotin-labeled SE RNA binding to the fixed cell constituents as described in Materials and Methods. No cells were stained with control RNA in either presence or absence of tetracycline, indicating no nonspecific interaction occurred between RNA and cellular components in our setting condition (Fig. 7A[b] and 7A[c]). Moreover, incubation with the SE RNA with fixed cells did not also create any color reaction in the presence of tetracycline (Fig. 7A[d]), suggesting the SE RNA cannot interact with any cellular components. By sharp contrast, distinct color reactions with the SE RNA were shown in the NS3-expressing Saos-2 cells (Fig. 7A[e]). To determine whether the SE RNA can specifically bind to the intracellular HCV NS3 protein, NS3-expressing cells were reacted with the biotin-labeled SE RNA preincubated with excess amount of NS3 protein (Fig. 7A[f]) or control cellular helicase protein (Fig. 7A[g]). While preincubation of the RNA with control protein did not affect any color reaction with the NS3-expressing cells, no staining occurred when incubated with the SE RNA along with the cells after preincubation of the RNA with the NS3 protein. Thus, preincubation of the SE RNA with the NS3 protein, but not with other cellular helicase, can prevent the SE RNA from interaction with NS3-expressing cells, which indicates that

FIGURE 5. Strong and competitive inhibition of SE RNA against RNA unwinding activity of the HCV helicase. (A) Internally radiolabeled longer substrates were hybridized with unlabeled shorter substrates. 0.25 nM α - 32 P-labeled duplex RNAs were incubated with (lanes 3–19, 1 nM) or without HCV helicase (lane 2) in the absence (lanes 2,3) or presence of ATP (lanes 4–19, 5 mM). The unwound RNAs were separated from duplex RNAs in a 6% nondenaturing polyacrylamide gel. The completely unwound RNA was shown by heating (lane 1). In lanes 5–19, increasing amount (lanes 5,10,15: 1.5 nM; lanes 6,11,16: 4.5 nM; lanes 7,12,17: 13.5 nM; lanes 8,13,18: 40.5 nM; lanes 9,14,19: 121.5 nM) of unlabeled SE RNA #1, poly U, or tRNA was added to the reaction to determine if they could inhibit RNA unwinding activity of HCV helicase. (B) The unwinding percentage by HCV helicase in the presence of increasing amount of competitor RNAs was calculated by determining the ratio of the unwound RNA to total radiolabeled substrate RNA. The plotted numbers have been normalized to the amount of unwound RNA generated by the helicase without any RNA competitors (lane 4 of Fig. 5A) and values shown are averages of measurements performed in triplicate. (C) Inhibition mechanism by SE RNA. Helicase reactions were performed as described above with the serially diluted substrate RNAs along with tRNA or SE RNA. The substrate was varied with twofold decreases between 1 μ M and 15.6 nM. Twenty nanomolars of the SE RNA shows competitive inhibition, with a calculated K_i of 9.1 nM.

the SE RNA specifically binds to the intracellular HCV NS3 protein.

To confirm that intracellular HCV NS3 specifically interact with the SE RNA, NS3-expressing cells were incubated with the biotin-labeled SE RNA along with the increasing excess amounts of unlabeled SE RNA or control RNA that is aptamer to myasthenia gravis autoantibody (Fig. 7B). No color reaction was changed in the cells even in the highest amounts of the unlabeled control RNA (Fig. 7B[f–h]). In contrast, color reaction was effectively diminished in the presence of unlabeled SE RNA in a dose-dependent manner (Fig. 7B[b–d]). Thus, unlabeled SE RNA, but not control RNA, can compete with the biotin-labeled SE RNA for binding to the NS3-expressing cells, indicating that the HCV NS3 protein in mammalian cells specifically binds the SE RNA.

Intracellular detection experiments using the SE RNA strongly suggest that the SE RNA can specifically interact with the intracellular HCV NS3 protein and the protocols developed in this study could be applied for the specific identification of human cells expressing HCV proteins with RNA aptamers.

Effect of SE RNA on the intracellular RNA synthesis of HCV replicon

Once we had observed that the selected RNA in this study efficiently inhibited the helicase activity of HCV NS3 by competitively interfering with the substrate RNA binding to the HCV helicase, we next evaluated RNA aptamer activity to inhibit HCV replication in human liver cells using the recently developed HCV subgenomic replicon systems (Fig. 8; Lohmann et al. 1999; Krieger et al. 2001). To determine

FIGURE 6. Minimal domain and structural determinations of SE RNA that binds to the HCV helicase. (A) The 91-nt-long SE RNA (MD1) was truncated at its 3' end (MD 2–5) or 5' end (MD 6). The sequences (+25 nt to + 63 nt) selected from the randomized region in the library RNA are presented as shaded box. (B) Three hundred and fifty picomolars of radiolabeled pool RNA or MD1–6 RNAs were incubated without (lane *b*) or with (lane *c*, 200 nM) HCV helicase and the RNA–protein complexes were precipitated with Ni–NTA beads. Bound RNAs were extracted and analyzed on a 6% polyacrylamide gel with 7 M urea. Lane *a* contains 10% of the input-labeled RNAs. Binding % denotes the amount of each bound RNA relative to the input RNA. (C) Enzymatic mapping of the RNA secondary structure of the SE RNA. RNA labeled at the 5' end (2.7 nM) was enzymatically digested with RNases T1 (lane 2, 1U), nucleases S1 (lane 3, 2 U), and RNases V1 (lane 4, 0.0001 U). The partially digested products were then separated on a 12% polyacrylamide gel with urea along with partial alkaline hydrolysis ladder (lane 1, AH). (D) Enzymatic footprinting of HCV helicase–SE RNA complex. The 5'-end-labeled SE RNA was incubated in the absence of (–; lanes 3,7,11) or presence of increasing amounts of HCV helicase (0.105 μ M in lanes 4,8,12; 1.05 μ M in lanes 5,9,13; 10.5 μ M in lanes 6,10,14). Protein–RNA mixtures were digested with RNases T1 (lanes 3–6), nucleases S1 (lanes 7–10), and RNases V1 (lanes 11–14). The cleaved RNA fragments were then resolved on a 12% polyacrylamide gel with urea together with RNA size markers, T1 lad (lane 1, partially digested SE RNAs with RNases T1) and AH (lane 2, partially alkaline hydrolyzed SE RNAs). The protected sites from each nuclease by the protein are indicated by bars on the *left* side. The asterisks on the *right* side of the autoradiogram indicate the sequences whose accessibility to RNase V1 increased in the presence of the HCV helicase. (E) The computer-predicted model of the secondary structure of SE RNA #1 and the first 127 nucleotide of 3' UTR of the HCV negative strand, HCV(–)3'UTR. The minimal binding domain of the SE RNA was shown in dotted box. Nucleotides 25–63 represented the sequences selected from randomized region of RNA library. Digestion patterns of the RNA to various nucleases are mapped. Squares, triangles, and circles indicate RNases T1, S1 nucleases, and RNases V1 cleavage sites, respectively. The size of each symbol denotes the susceptible intensities on the SE RNA to each nuclease. Protected regions of the RNA in the presence of the target protein are marked as shaded areas. Asterisks indicate the sites at which cleavage by RNases V1 was enhanced following binding of the HCV helicase.

FIGURE 7. SE RNA as a diagnostic agent of HCV NS3-expressing cells. (A) Tet-off Saos-2 cells were cultured in the presence (b,d; -NS3) or in the absence of tetracycline (a,c,e-g; +NS3) for 48 h. Cells were fixed and incubated without RNA (a), with biotin-labeled control RNA (b,c), or with biotin-labeled SE RNA #1 (d-g) after preincubation of SE RNA without any proteins (d,e), or with NS3 protein (f) or control protein (g) to determine if only SE RNA could specifically bind to intracellular HCV NS3 protein. (B) Saos-2 cells were cultured in the presence (e) or in the absence of tetracycline (a-d,f-h), fixed and incubated with biotin-labeled SE RNA #1. As competitors, excess amount of unlabeled SE RNA (b-d, 30-, 300-, 3000-fold, respectively) or control RNA (f-h, 30-, 300-, 3000-fold, respectively) was added to the cells to determine if they could compete with biotin-labeled SE RNA for binding to the HCV NS3-expressing cells.

whether the SE RNA inhibit the intracellular HCV replication, we quantified the level of HCV negative (–) strand RNA in Huh-7 hepatocarcinoma cells by RT-PCR 72 h after cotransfection with the HCV replicon RNA and the various RNA competitors, and compared the amount of HCV RNA in each cell with cells transfected with the HCV replicon alone. As expected, tRNA that showed very low affinity to the HCV helicase (Fig. 1) and no effect on the helicase activity (Fig. 5) hardly affected the HCV subgenomic RNA synthesis. By contrast, RNA aptamer to the HCV helicase (SE RNA) showed inhibition of RNA synthesis of the HCV replicon by up to 48%. Another RNA aptamer, which was selected against myasthenia gravis autoantibody (MG RNA aptamer: Lee and Sullenger 1997), could not efficiently protect cells from the replication of HCV replicon RNA, which strongly suggests that the inhibition of HCV replication by SE RNA identified in this study is mainly due to the

specific interaction of the SE RNA with the HCV helicase expressed by the HCV replicon in cells.

DISCUSSION

Since the helicase domain of HCV NS3 is probably crucial for the proliferation of HCV, identification of inhibitors for the helicase has been employed mainly through random screening of small molecules (Borowski et al. 2003) or phage-display antibody screening (Artsaenko et al. 2003). However, potential limitations of those molecules would stem from nonspecificity or low affinity to the target protein. Therefore, development of more specific and effective agents is necessary.

In vitro selected RNA aptamers are specific antagonists against a wide variety of proteins with high affinities. Moreover, aptamers can be chemically synthesized in large quan-

FIGURE 8. Inhibition of replication of HCV replicon by SE RNA. (A) RT-PCR analysis of HCV replicon RNA products in cells. Huh-7 cells were mock transfected (lane 2), or transfected with HCV subgenomic replicon RNA without any competitor RNAs (lane 3), or along with tRNA (lane 4), MG aptamer RNA (lane 5), or SE RNA (lane 6). HCV (-) subgenomic RNA strand was amplified by RT-PCR, yielding a DNA fragment of 210 bp. An amplified β -actin cDNA with 800 bp size was loaded as an internal control. (B) Relative RNA amount of HCV replicon in cells. HCV RNA values were first normalized to β -actin RNA amounts. HCV RNA level was then expressed relative to the level in cells transfected with HCV replicon RNA alone. Averages of measurements performed with five separate times are shown with bars indicating standard errors.

ties, easily modified for *in vivo* utility, and readily regulatable with oligonucleotides antisense to the aptamers (Rusconi et al. 2002), which renders them strong candidates for small-molecule lead compounds.

In the present study, we isolated RNA aptamers against the HCV NS3 helicase domain with SELEX technology. These aptamers bind specifically (Fig. 1) and very avidly to the helicase with subnanomolar binding constant (Fig. 2). The aptamers are very effective inhibitors of RNA unwinding activity, but not NTPase, of the helicase with $IC_{50} \sim 12.5$ nM by competitively interfering with the substrate RNA binding (Figs. 3–5). Moreover, interaction between the RNA aptamers and the HCV helicase is shown to be very specific and selective from intracellular protein detection experiments with the RNA aptamers (Fig. 7). In addition,

intracellular RNA synthesis of HCV replicon can be partially suppressed by the RNA aptamers (Fig. 8).

The binding site of the selected RNA aptamers on the HCV helicase is not defined in this study. However, from the observation that the RNA aptamers efficiently inhibit the binding of RNA substrate with the helicase in a dose-dependent way (Fig. 3) and competitively impeded the helicase activity of NS3 with respect to the substrate (Fig. 5), we could speculate that the aptamers interact with the helicase at a substrate binding domain. The structure of HCV helicase consists of three nearly equal-sized subdomains, which form a Y-shaped molecule (Yao et al. 1997). Two structural models for nucleic acid binding mode and coupling process of NTP hydrolysis and RNA unwinding of the HCV helicase have been suggested. One is that a deoxyuridine octamer oligonucleotide lies in a groove between the first two and the third in monomer helicase and ATP hydrolysis could facilitate the conformational change of interdomain cleft, resulting in the movement of the helicase along the substrates with unwinding them (Kim et al. 1998). The other is that a single-stranded RNA would be positioned in the channel that was formed in the middle of dimer through the interaction of two helicase monomers. ATP hydrolysis would cause the dissociation of the substrate RNA from one molecule of the helicase and then the substrate RNA can be bound by the other molecule through rotation of dimer. In such a way, the helicase unwinds double-stranded RNA by passing one strand of the RNA through the channel (Cho et al. 1998). Recent data support the latter dimer model for the HCV helicase mechanism (Khu et al. 2001). Considering those structures of the HCV helicase, there are at least two RNA binding sites in the helicase, one at the catalytic pocket probably in the interdomain cleft (Kim et al. 1998; Kim et al. 2003) and the other at the substrate holding site near the Arg-rich motif (460QRRGRTGR467) in domain 2 (Kim et al. 1997). In this study, the RNA aptamer efficiently prohibited the binding of substrate RNA to the helicase in a competitive manner (Figs. 3, 5). Therefore, single-stranded regions at the left half of the RNA aptamer, which were identified as primary binding domain to the HCV helicase (Fig. 6), are likely to bind to one of either or both above substrate binding sites of the helicase. Strikingly, the RNA aptamer contains stem configuration with high GC content in the minimal binding domain that is also found in the 3'-terminal 127 nt regions of HCV-negative-strand RNA (Fig. 6). Sequences and stem-loop structures in the 3'-end of HCV-negative RNA was recently described to specifically interact with the HCV NS3 helicase (Banerjee and Dasgupta 2001), suggesting that the RNA aptamers selected in this study could competitively sequester the HCV helicase from binding to the HCV-negative RNA. If so, the RNA aptamers could efficiently inhibit HCV genome replication since the viral RNA synthesis is likely to be initiated at the 3' terminus of positive and negative RNA strand. Taken

together, it is conceivable that the selected RNA aptamers would fit into the substrate binding regions with or without the nearby motif, resulting in the fixation of the helicase conformation in an inactive state such that they prevent binding of the RNA substrate and movement of the helicase, and hence inhibit RNA unwinding activity. More detailed molecular and structural analysis of binding region of the RNA aptamers on the HCV helicase, which is now on progress, will provide us more hints for the confined function and mechanism of the HCV helicase.

The observation that the RNA aptamers bind very specifically to the HCV helicase, but not any other cellular proteins including cellular helicase (Fig. 7) is surprising in a way because HCV RNA helicase contains the motifs highly conserved for RNA helicase activity and is classified as a DEXH protein of the SFII superfamily of helicase. However, it is not also unexpected because RNA aptamers can distinguish even the very similar domains of individual proteins in the same classes (Bae et al. 2002). The RNA aptamers would thus bind to distinct regions that could not be found in other cellular proteins. To our knowledge, this is the first report on the selection of RNA aptamers against a specific viral RNA helicase.

In addition, we presented our efforts at developing those RNA aptamer-based in situ diagnostic methods of HCV NS3 proteins. In the circumstances we have conditioned, only NS3-expressing cells can be stained with the RNA aptamers but not with control RNAs. Specific staining with the RNAs were confirmed from the experiments of competitive binding assay and with the RNAs preincubated with NS3 helicase (Fig. 7). These suggested that RNA aptamers selected in vitro be very useful for in situ diagnosis of the target proteins. Further developments will facilitate RNA-based diagnostic methods for HCV infection, which could replace conventional time-consuming and expensive methods based on antibodies.

In this study, we suggest that an RNA aptamer against the HCV helicase could provide new insights into the development of small agents modulating HCV replication. The RNA aptamers would function as decoys against the helicase involved in HCV regulatory circuits and thus limit HCV replication in human liver cells. Indeed, transient transfection of the RNA aptamers into the liver cells was observed to suppress RNA replication of HCV replicon system, although not completely (Fig. 8). This partial inhibition may be due to insufficient amount and/or improper localization of the RNA aptamers in cells. Since HCV NS3 is mainly localized in the cytoplasmic side on the ER membrane (Penin et al., 2004), overexpression of such short RNA aptamers in cytoplasm using a pol III promoter system such as 7SL promoter (Paul et al. 2003) could further improve the bioactivity of the RNA aptamers to interfere with HCV replication. In addition to this therapeutic potential, the RNA aptamers would be useful to genetically

elucidate the intracellular role of the HCV helicase during HCV replication.

MATERIALS AND METHODS

Expression and purification of the his-tagged HCV NS3 helicase domain

A recombinant fragment of HCV NS3 helicase domain encompassing the C-terminal two-thirds of the HCV type 1b NS3 protein (amino acids 1193–1658) was cloned into pET21 expression vector (Novagen), which expresses recombinant proteins tagged with a hexahistidine at C-terminus. Proteins were overexpressed in *Escherichia coli* BL21 (DE3) strain and purified with nickel-chelate resin (Ni-NTA agarose, Qiagen) and a poly(U)-Sephacrose column (Amersham Pharmacia) as described (Cho et al. 1998).

Selection procedure

In vitro selection was carried out essentially as described previously (Lee and Sullenger 1996, 1997; Bae et al. 2002), with a few modifications. A random pool of RNA oligonucleotides was produced by in vitro transcription of synthetic DNA templates with NTPs and T7 RNA polymerase. The sequence of the generated RNA is 5'-GGGAGAGCGGAAGCGUGCUGGGCCN₄₀CAUAA CCCAGAGGUCGAUGGAUCCCCC-3', where N₄₀ represents 40 nt with equimolar incorporation of A, G, C, and U at each position. First, 10 µg of the RNA library was preincubated with 20 µl of Ni-NTA agarose beads in 100 µl binding buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM dithiothreitol, and 1% BSA) for 30 min at room temperature with shaking. The RNA-bead complexes were then precipitated and discarded to remove RNAs with nonspecific binding activity to agarose beads. The precleared supernatant was transferred to a new tube and further incubated with 2 µg of his-tagged HCV NS3 helicase domain for 30 min at room temperature. Helicase-RNA complexes were precipitated with beads, and pellets washed five times with 0.5 ml of the binding buffer. RNAs were recovered, amplified with RT-PCR and in vitro transcription, and used for next rounds of selection. Eight subsequent rounds of selection were performed in the same manner. In contrast, from round nine, a more stringent condition was employed by reducing helicase concentration by 5–10 fold at every fourth or fifth round: 0.2 µg (rounds 9–13), 0.04 µg (rounds 14–17), or 0.008 µg (rounds 18–21). After 21 rounds of selection, the amplified DNA was cloned and several clones were sequenced.

Binding analysis of selected RNAs

SE RNAs were internally radiolabeled and isolated as described (Lee and Sullenger 1996, 1997; Bae et al. 2002). Purified RNAs were incubated with proteins as described above. Helicase-RNA complexes were precipitated with Ni-NTA agarose beads, and bound RNAs extracted from the pellets using 15 µl of 0.1 M EDTA and phenol. RNAs were then analyzed on a 6% polyacrylamide gel with urea.

ATPase assay

Standard ATPase assays were conducted as described (Suzich et al. 1993) with a few modifications. ATPase activity was measured in

a final volume of 20 μ l containing 30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 2 mM DTT, 1% BSA, 40 U RNase inhibitor (Kosco), 1 mM ATP, 1 μ Ci [α -³²P]ATP (3000 Ci/mmol, Amersham), and 20 nM HCV helicase in the presence of RNA samples, if any. After a 45-min incubation of reaction mixtures at 37°, reactions were terminated by the addition of EDTA to a final concentration of 20 mM. Reaction products were analyzed by thin-layer chromatography. Two microliters of the reaction mixture were spotted onto a polyethyleneimine cellulose sheet (Baker) and then substrate and products were separated by ascending chromatography in 0.35 M potassium phosphate (pH 3.5). The chromatogram was air dried and autoradiographed and the hydrolyzed ATP was quantified by PhosphorImager analysis (FUJIS BAS1000).

Preparation of helicase substrates

The substrates are composed of two annealed complementary RNA strands, namely double-stranded RNA. The longer RNA strand was generated by in vitro transcription with T7 RNA polymerase from PvuII-digested pSK(-) Δ SacII-HincII, which was generated by deletion of SacII/HincII site from pBluescriptSK(-) (Stratagene), and radio-labeled with [α -³²P]UTP (3000 Ci/mmol, Amersham). The shorter strand was transcribed with T3 RNA polymerase (Kosco) from pBluescriptSK(-) that had been cleaved with BamHI. The transcripts were treated with DNase I (Promega), extracted with phenol-chloroform, purified with Sephadex G-25 column (Sigma), and then precipitated with ethanol. Both RNA strands were mixed at a molar ratio of longer to shorter strand of 1:3 in a hybridization buffer solution (20 mM HEPES, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS). The mixture was boiled for 5 min, transferred to 65° for 30 min, and then incubated at room temperature for 12 h. The generated partial duplex RNA substrates were electrophoresed on a 6% nondenaturing polyacrylamide gel, eluted with 400 μ l of elution buffer (0.6 M ammonium acetate, 1 mM EDTA, 0.2% SDS) from the gel, and then purified.

RNA binding assay of helicase

Radiolabeled 261 nt RNA substrate (520 pM) that was generated as the longer strand for helicase assay described above was incubated with 20 nM of HCV helicase, 80 U of RNase inhibitor, and 3 nM unlabeled substrate in binding buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM dithiothreitol, and 1% BSA) at room temperature for 30 min in the presence of competitor RNA, if any. The protein-RNA complexes were then analyzed with gel shift assay on a 4% nondenaturing polyacrylamide gel containing 20% glycerol.

Helicase assay

RNA helicase activity was performed as described (Kim et al. 1995) with modifications. Helicase activity was assayed in 20 μ l reaction mixture containing 25 mM MOPS-KOH (pH 6.5), 3 mM MgCl₂, 2 mM DTT, 0.01% of BSA, 40 U RNase inhibitor, 1 nM HCV helicase, and 0.25 nM ³²P-labeled partial duplex RNA substrate.

After preincubation for 15 min at room temperature, 5 mM ATP was added to start the helicase reaction. The reaction mixtures were further incubated for 30 min at 37°C, and then stopped by adding 5 μ l of 5X termination buffer (0.1 M Tris-HCl, pH 7.4, 20 mM EDTA, 0.5% SDS, 0.1% NP40, 0.1% xylene cyanol, 0.1% bromophenol blue, 50% glycerol). Effects of various RNAs on HCV helicase activity were studied by direct addition of the RNAs to the reaction mixture during preincubation. An aliquot (12.5 μ l) of each reaction mixture was electrophoresed on a 6% nondenaturing polyacrylamide gel. Strand separation was then visualized by autoradiography, and the efficiency of the helicase reaction was calculated by quantification of the radioactivity of the bands with PhosphorImager (FUJIS BAS1000).

Inhibition mechanism of SE RNA to the helicase activity was determined by conducting substrate titration experiments. For assessing reaction velocities, the products of helicase reactions were measured at different time intervals in the presence of increasing concentration of the substrate RNA. The slopes of linear plots of the measured values versus time, representing the initial velocities of the reaction, were replotted in Woolf-Augustinsson-Hofstee format with or without SE RNA. Aptamer K_i was determined using 20 nM aptamer in the presence of serially diluted substrate RNA and calculated by changes in K_m .

Enzymatic analysis of RNA secondary structure

The in vitro transcribed SE RNA was dephosphorylated with calf intestine alkaline phosphatase (Ambion) and 5' end-labeled with [γ -³²P]ATP (3000 Ci/mmol, Amersham) and T4 polynucleotide kinase (Ambion). The labeled RNA (54 fmole) was first heated at 65°C for 5 min and renatured at room temperature for 5 min. The RNA was then digested with RNase T1 (USB), nuclease S1 (Amersham Pharmacia), or RNase V1 (Ambion). RNase T1 digestion was carried out at 37°C for 10 min in a reaction mixture (20 μ l) containing 20 mM NaCitrate (pH 5.0), 7 M urea, 1 mM EDTA, 0.25 mg/ml tRNA, and 1 U of RNase T1. Nuclease S1 digestion was employed at 37°C for 10 min in a buffer (20 μ l) containing 2 μ l of 10 $\times$ S1 buffer and 2 U of nuclease S1. RNase V1 (0.0001 U) was treated at room temperature for 15 min in a 20 μ l solution that contained 2 μ l of 10 $\times$ V1 buffer. The nuclease-treated reactions were stopped by the addition of 10 μ l of RNA loading dye (10 ml formamide, 10 mM EDTA, 0.01% bromophenol blue, 0.01% xylene cyanol). To generate a sequencer ladder, alkaline hydrolysis was performed at 95°C for 15 min in a 20 μ l reaction volume that contained 50 mM NaHCO₃ (pH 9.0), 1 mM EDTA, 0.25 mg/ml tRNA, and 54 fmole of SE RNA. The cleaved RNA fragments were denatured at 80°C for 5 min and analyzed on a 12 to 15% polyacrylamide gel with 7 M urea.

Enzymatic footprinting assay for probing the HCV helicase-binding site on the SE RNA

The 5'-end labeled SE RNA (54 fmole) was incubated with the HCV helicase at room temperature for 30 min in a 20 μ l binding buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 2 mM DTT) and then digested and analyzed with RNase T1, nuclease S1, or RNase V1 as described for enzymatic analysis of RNA secondary structure.

Detection procedure of intracellular NS3 protein

Nonradioactively labeled SE RNA was prepared as described (Rosemeyer et al. 1995). Briefly, *in vitro* transcribed SE RNA (121 pmole) was labeled at its 3' end by incubation with 5 X reaction buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.25 mg/ml BSA, 0.5 mM dNTP), 0.5 mM biotin-dUTP (Boehringer Mannheim), 5 mM CoCl₂, 40 U RNase inhibitor, and 20 U terminal deoxynucleotide transferase (TdT, Boehringer Mannheim) in a final volume of 30 µl for 30 min at 37°C. Saos-2 cells that control the expression of the HCV NS3 protein under Tet-off transcriptional regulatory system was developed and cultured in the presence or in the absence of tetracycline for 48 h on 35 mm dish. Cells were then fixed with 70% ethanol, permeated with 1% Triton in PBS for 10–15 min at room temperature, and washed two times with 0.1% Triton in PBS. To block proteins that non-specifically bind RNAs, permeated cells were preincubated with binding buffer solution (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM dithiothreitol, and 1% BSA) containing 50 µg tRNA and 40 U RNase inhibitor for 30 min to 1 h and washed 3–5 times with the binding buffer without BSA. RNA binding procedure was employed by incubating cells with binding buffer comprising of 0.1 pmole of biotin-labeled RNA and 40 U RNase inhibitor for 30 min to 1 h at room temperature. After rinsing the cells 3–5 times with excess amount of binding buffer, cells were treated with 1:150 dilution of alkaline phosphatase-conjugated streptavidin in binding buffer for 30–60 min at room temperature and washed with binding buffer without BSA at least 10 times. Color reaction was then detected by reacting the cells with 1:50 dilution of NBT/BCIP in buffer solution containing 0.1 M Tris-HCl (pH 8.5), 0.05 M MgCl₂, and 0.1 M NaCl for 1 h at room temperature.

Electroporation of HCV replicon and bioassay of RNA aptamer

A subgenomic replicon construct, pFK-I₃₈₉neo/NS3–3'/5.1, carrying two cell-culture adaptive mutations in NS3 and one in NS5A, (Krieger et al. 2001) was obtained from Dr. Ralf Bartenschlager (Univ. of Heidelberg, Germany). HCV replicon RNA was constructed by *in vitro* transcription with the AseI and ScaI-digested replicon plasmid as described (Krieger et al. 2001). Electroporation of 500 ng of the HCV replicon RNA was performed in a condition of 950 µF and 250 V using a Gene pulser system (Bio-Rad) into 400 µl of a suspension of 4 × 10⁶ Huh-7 cells along with or without 5 µg of tRNA, MG aptamer RNA, or SE RNA. Plasmid pcDNA_{luc} encoding Renilla luciferase was also added to each sample to determine transfection efficiency. Cells were transferred to 3 ml complete DMEM and plated in 35-mm cell culture dishes. After 72 h, total RNA was isolated from the cells with guanidine isothiocyanate (Feramisco et al. 1982). RNA (5 µg) was reverse-transcribed with a 3' primer specific for the (–) strand of HCV cDNA (5'-GGGGAATTCCGTAACACCAACGGGCGC) or random primer for β-actin cDNA. The resulting cDNAs were amplified for 30 cycles with a 5' primer (5'-GGGAAGCTTCTCGTCCTGCAGTTCAT) and a 3' primer specific for the HCV (–) strand cDNA. Values were normalized to that of β-actin, which were amplified with a 5' primer (5'-ATCTGGCACCACACCTTCTCAATGAGCTGCG) and a 3' primer (5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC).

ACKNOWLEDGMENTS

We deeply thank Dr. R. Bartenschlager for supplying the HCV replicon construct, Drs. Y.J. Kim, Y.S. Seo, and I.-H. Kim for their support throughout this project, and Dr. B.A. Sullenger for his useful discussion. This work was supported by grants of the '98 Good Health R&D project (HMP-98-B-3–0020), Ministry of Health & Welfare, R.O.K., the international mobile telecommunications 2000 R&D project (01-PJ11-PG9–01BT00B-0012), Ministry of Information & Communication, R.O.K., and the Intelligent Microsystem Center project (MS-04–211), one of the 21st century's Frontier R&D Projects, Korea Ministry of Science & Technology.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

Received January 29, 2004; accepted May 12, 2004.

REFERENCES

- Artsaenko, O., Tessmann, K., Sack, M., Haussinger, D., and Heintges, T. 2003. Abrogation of hepatitis C virus NS3 helicase enzymatic activity by recombinant human antibodies. *J. Gen. Virol.* **84**: 2323–2332.
- Bae, S.-J., Oum, J.-H., Sharma, S., Park, J., and Lee, S.-W. 2002. *In vitro* selection of specific RNA inhibitors of NFATc. *Biochem. Biophys. Res. Comm.* **298**: 486–492.
- Banerjee, R. and Dasgupta, A. 2001. Specific interaction of hepatitis C virus protease/helicase NS3 with the 3'-terminal sequences of viral positive- and negative-strand RNA. *J. Virol.* **75**: 1708–1721.
- Bartenschlager, R., Ahlborn-Laake, L., Mous, J., and Jacobsen, H. 1993. Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. *J. Virol.* **67**: 3835–3844.
- Biroccio, A., Hamm, J., Incitti, I., De Francesco, R., and Tomei, L. 2002. Selection of RNA aptamers that are specific and high-affinity ligands of the hepatitis C virus RNA-dependent RNA polymerase. *J. Virol.* **76**: 3688–3696.
- Borowski, P., Deinert, J., Schalinski, S., Bretner, M., Ginalski, K., Kulikowski, T., and Shugar, D. 2003. Halogenated benzimidazoles and benzotriazoles as inhibitors of the NTPase/helicase activities of hepatitis C and related viruses. *Eur. J. Biochem.* **270**: 1645–1653.
- Burgstaller, P., Girod, A., and Blind, M. 2002. Aptamers as tools for target prioritization and lead identification. *Drug Discov. Today* **7**: 1221–1228.
- Cho, H.-S., Ha, N.-C., Kang, L.-W., Chung, K.M., Back, S.H., Jang, S.K., and Oh, B.-H. 1998. Crystal structure of RNA helicase from genotype 1b hepatitis C virus. *J. Biol. Chem.* **273**: 15045–15052.
- Choo, Q.L., Richman, K.H., Han, J.H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, R., Barr, P.J., et al. 1991. Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci.* **88**: 2451–2455.
- De Francesco, R. and Steinkühler, C. 2000. Structure and function of the hepatitis C virus NS3-NS4A serine protease. *Curr. Top. Microbiol. Immunol.* **242**: 149–169.
- Ellington, A.D. and Szostak, J.W. 1990. *In vitro* selection of RNA molecules that bind specific ligands. *Nature* **346**: 818–822.
- Feramisco, J.R., Smart, J.E., Burridge, K., Helfman, D.M., and Thomas, G.P. 1982. Co-existence of vinculin and a vinculin-like protein of higher molecular weight in smooth muscle. *J. Biol. Chem.* **257**: 11024–11031.
- Fukuda, K., Vishnuvardhan, D., Sekiya, S., Hwang, J., Kakiuchi, N.,

- Taira, K., Shimotohno, K., Kumar, P.K., and Nishikawa, S. 2000. Isolation and characterization of RNA aptamers specific for the hepatitis C virus nonstructural protein 3 protease. *Eur. J. Biochem.* **267**: 3685–3694.
- Gold, L., Allen, P., Binkley, J., Brown, D., Schneider, D., Eddy, S.R., Tuerk, C., Green, L., Macdougall, S., and Tasset, D. 1993. RNA: the shape of things to come. In *The RNA World* (eds. R.F. Gesteland and J.F. Atkins), pp. 497–510, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Grakoui, A., McCourt, D.W., Wychowski, C., Feinstone, S.M., and Rice, C.M. 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polypeptide cleavage sites. *J. Virol.* **67**: 2832–2843.
- Gwack, Y., Kim, D.W., Han, J.H., and Choe, J. 1996. Characterization of RNA binding activity and RNA helicase activity of the hepatitis C virus NS3 protein. *Biochem. Biophys. Res. Comm.* **225**: 654–659.
- Hijikata, M., Mizushima, H., Akagi, T., Mori, S., Kakiuchi, N., Kato, N., Tanaka, T., Kimura, K., and Shimotohno, K. 1993. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. *J. Virol.* **67**: 4665–4675.
- Hwang, B., Han, K., and Lee, S.-W. 2003. Prevention of passively transferred experimental autoimmune myasthenia gravis by an *in vitro* selected RNA aptamer. *FEBS Lett.* **548**: 85–89.
- Jaeger, J., Turner, D., and Zuker, M. 1989. Improved predictions of secondary structures for RNA. *Proc. Natl. Acad. Sci.* **86**: 7706–7710.
- Kanai, A., Tanabe, K., and Kohara, M. 1995. Poly(U) binding activity of hepatitis C virus NS3 protein, a putative RNA helicase. *FEBS Lett.* **376**: 221–224.
- Khu, Y.-L., Koh, E., Lim, S.P., Tan, Y.H., Brenner, S., Lim, S.G., Hong, W.J., and Goh, P.-Y. 2001. Mutations that affect dimer formation and helicase activity of the hepatitis C virus helicase. *J. Virol.* **75**: 205–214.
- Kim, D.W., Gwak, Y., Han, J.H., and Choe, J. 1995. C-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase activity. *Biochem. Biophys. Res. Comm.* **215**: 160–166.
- Kim, D.W., Kim, J., Gwak, Y., Han, J.H., and Choe, J. 1997. Mutational analysis of the hepatitis C virus RNA helicase. *J. Virol.* **71**: 9400–9409.
- Kim, J.L., Morgenstern, K.A., Griffith, J.P., Dwyer, M.D., Thomson, J.A., Murcko, M.A., Lin, C., and Caron, P.R. 1998. Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Structure* **6**: 89–100.
- Kim, J.W., Seo, M.Y., Shelat, A., Kim, C.S., Kwon, T.W., Lu, H.-h., Moustakas, D.T., Sun, J., and Han, J.H. 2003. Structurally conserved amino acid W5011 is required for RNA helicase activity but is not essential for DNA helicase activity of hepatitis C virus NS3 protein. *J. Virol.* **77**: 571–582.
- Krieger, N., Lohmann, V., and Bartenschlager, R. 2001. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. *J. Virol.* **75**: 4614–4624.
- Kumar, P.K., Machida, K., Urvil, P.T., Kakiuchi, N., Vishnuvardhan, D., Shimotohno, K., Taira, K., and Nishikawa, S. 1997. Isolation of RNA aptamers specific to the NS3 protein of hepatitis C virus from a pool of completely random RNA. *Virology* **237**: 270–282.
- Kwong, A.D., Kim, J.L., and Lin, C. 2000. Structure and function of hepatitis C virus NS3 helicase. *Curr. Top. Microbiol. Immunol.* **242**: 171–196.
- Lauer, G.M. and Walker, B.D. 2001. Hepatitis C virus infection. *New Engl. J. Med.* **345**: 41–52.
- Lee, S.-W. and Sullenger, B. 1996. Isolation of a nuclease resistant decoy RNA that selectively blocks autoantibody binding to insulin receptors on human lymphocytes. *J. Exp. Med.* **194**: 315–324.
- . 1997. Isolation of a nuclease-resistant decoy RNA that can protect human acetylcholine receptors from myasthenic antibodies. *Nature Biotechnol.* **15**: 41–45.
- Lin, C., Lindenbach, B.D., Pragai, B.M., McCourt, D.W., and Rice, C.M. 1994. Processing in the hepatitis C virus E2-NS2 region: Identification of p7 and two distinct E2-specific products with different C termini. *J. Virol.* **68**: 5063–5073.
- Liu, D., Wang, Y.S., Gesell, J.J., and Wyss, D.F. 2001. Solution structure and backbone dynamics of an engineered arginine-rich subdomain 2 of the hepatitis C virus NS3 RNA helicase. *J. Mol. Biol.* **314**: 543–561.
- Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**: 110–113.
- Manabe, S., Fuke, I., Tanishita, O., Kaji, C., Gomi, Y., Yoshida, S., Mori, C., Takamizawa, A., Yashida, I., and Okayama, H. 1994. Production of nonstructural proteins of hepatitis C virus requires a putative viral protease encoded by NS3. *Virology* **198**: 636–644.
- Mizushima, H., Hijikata, M., Tanji, Y., Kimura, K., and Shimotohno, K. 1994. Analysis of N-terminal processing of hepatitis C virus nonstructural protein 2. *J. Virol.* **68**: 2731–2734.
- Moradpour, D., Brass, V., Gosert, R., Wolk, B., and Blum, H. 2002. Hepatitis C: molecular virology and antiviral targets. *Trends Mol. Med.* **8**: 476–482.
- Nishikawa, F., Kakiuchi, N., Funaji, K., Fukuda, K., Sekiya, S., and Nishikawa, S. 2003. Inhibition of HCV NS3 protease by RNA aptamers in cells. *Nucleic Acids Res.* **31**: 1935–1943.
- Paul, C.P., Good, P.D., Li, S.X., Kleihauer, A., Rossi, J.J., and Engelke, D.R. 2003. Localized expression of small RNA inhibitors in human cells. *Mol. Ther.* **7**: 237–247.
- Penin, F., Dubuisson, J., Rey, F., Moradpour, D., and Pawlotsky, J.-M. 2004. Structural biology of hepatitis C virus. *Hepatology* **39**: 5–19.
- Preugschat, F., Averett, D.R., Clark, B.E., and Porter, D.J.T. 1996. A steady-state and pre-steady-state kinetic analysis of the NTPase activity associated with the hepatitis C virus NS3 helicase domain. *J. Biol. Chem.* **271**: 24449–24457.
- Rosemeyer, V., Laubrock, A., and Seibl, R. 1995. Nonradioactive 3'-end-labeling of RNA molecules of different lengths by terminal deoxynucleotidyltransferase. *Anal. Biochem.* **224**: 446–449.
- Rusconi, C.P., Scardino, E., Layzer, J., Pitoc, G.A., Ortel, T.L., Monroe, D., and Sullenger, B.A. 2002. RNA aptamers as reversible antagonists of coagulation factor IXa. *Nature* **419**: 90–94.
- Satoh, S., Tanji, Y., Hijikata, M., Kimura, K., and Shimotohno, K. 1995. The N-terminal region of hepatitis C virus nonstructural protein 3 (NS3) is essential for stable complex formation with NS4A. *J. Virol.* **69**: 4255–4260.
- Shoji, I., Suzuki, T., Sato, M., Aizaki, H., Chiba, T., Matsuura, Y., and Miyamura, T. 1999. Internal processing of hepatitis C virus NS3 protein. *Virology* **254**: 315–323.
- Sullenger, B.A. and Gilboa, E. 2002. Emerging clinical applications of RNA. *Nature* **418**: 252–258.
- Suzich, J.A., Tamura, J.K., Palmer-Hill, F., Warrenner, P., Grakoui, A., Rice, C.M., Feinstone, S.M., and Collett, M.S. 1993. Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with the related pestivirus and flavivirus enzymes. *J. Virol.* **67**: 6152–6158.
- Tai, C.-L., Chi, W.-K., Chen, D.-S., and Hwang, L.-H. 1996. The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). *J. Virol.* **70**: 8477–8484.
- Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I., and Okayama, H. 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. *J. Virol.* **65**: 1105–1113.
- Tomei, L., Failla, C., Santolini, E., DeFrancesco, R., and LaMonica, N. 1993. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J. Virol.* **67**: 4017–4026.
- Tuerk, C. and Gold, L. 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**: 505–510.
- Urvil, P.T., Kakiuchi, N., Zhou, D.-M., Shimotohno, K., Kumar, P.K.R., and Nishikawa, S. 1997. Selection of RNA aptamers that

- bind specifically to the NS3 protease of hepatitis C virus. *Eur. J. Biochem.* **248**: 130–138.
- Vo, N.V., Oh, J.W., and Lai, M.M. 2003. Identification of RNA ligands that bind hepatitis C virus polymerase selectively and inhibit its RNA synthesis from the natural viral RNA templates. *Virology* **307**: 301–316.
- Wardell, A.D., Errington, W., Ciaramella, G., Merson, J., and McGarvey, M.J. 1999. Characterization and mutational analysis of the helicase and NTPase activities of hepatitis C virus full-length NS3 protein. *J. Gen. Virol.* **80**: 701–709.
- Yang, S.H., Lee, C.G., Song, M.K., and Sung, Y.C. 2000. Internal cleavage of hepatitis C virus NS3 protein is dependent on the activity of NS34A protease. *Virology* **268**: 132–140.
- Yao, N., Hesson, T., Cable, M., Hong, Z., Kwong, A.D., Le, H.V., and Weber, P.C. 1997. Structure of the hepatitis C virus RNA helicase domain. *Nat. Struct. Biol.* **4**: 463–467.