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Inhibition of the Replication of Hepatitis C Virus Replicon with Nuclease-Resistant RNA Aptamers

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Abstract Hepatitis C virus (HCV)-encoded nonstructural protein 5B (NS5B) possesses RNA-dependent RNA polymerase activity, which is considered essential for viral proliferation. Thus, HCV NS5B is a good therapeutic target protein for the development of anti-HCV agents. In this study, we isolated two different kinds of nuclease-resistant RNA aptamers with 2'-fluoro pyrimidines against the HCV NS5B from a combinatorial RNA library with 40 nucleotide random sequences, using SELEX technology. The isolated RNA aptamers were observed to specifically and avidly bind the HCV NS5B with an apparent K_d of 5 nM and 18 nM, respectively, in contrast with the original RNA library that hardly bound the target protein. Moreover, these aptamers could partially inhibit RNA synthesis of the HCV subgenomic replicon when transfected into Huh-7 hepatoma cell lines. These results suggest that the RNA aptamers selected *in vitro* could be useful not only as therapeutic agents of HCV infection but also as a powerful tool for the study of the HCV RNA-dependent RNA polymerase mechanism.

Key words: HCV, HCV replicon, NS5B RNA replicase, nuclease-resistant RNA aptamer, SELEX

Hepatitis C virus (HCV) is the main pathogen causing chronic hepatitis, liver cirrhosis, and in some instances, hepatocellular carcinoma [14]. Although HCV affects more than 3% of the world population, specific and efficient anti-HCV therapy has not yet been developed.

HCV contains a single positive-stranded RNA genome of about 9,600 nucleotides in length encoding a polyprotein of about 3,010 amino acids [6]. This polyprotein precursor is co- or post-translationally processed into at least 10 mature structural and nonstructural proteins (C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) by cellular and viral proteases [6, 23].

HCV NS5B harbors RNA-dependent RNA polymerase activity [2], which is considered crucial for the synthesis of negative-stranded and genomic viral RNA during the HCV genome replication. Therefore, HCV NS5B is believed to be essential for the viral proliferation, and hence, is a primary target for the development of antiviral drugs [18].

The characteristics of RNAs that can adopt complex but stable structures to specifically and avidly bind target proteins and can be chemically synthesized with ease render RNA a potentially useful diagnostic and/or therapeutic lead compound [4, 8]. Such short RNA ligands, termed RNA aptamers, have been identified from a random RNA library to bind a wide variety of proteins with high affinity and specificity using *in vitro* iterative selection techniques, called systemic evolution of ligands by exponential enrichment (SELEX) [7, 28]. Several aptamers have been successfully evaluated in animal disease models [9, 24, 26], and some of them are now in the therapeutically clinical development stage [27]. Of note, the US FDA recently approved an RNA aptamer against antivascular endothelial growth factor (VEGF), called pegaptanib sodium (Macugen), for the treatment of all types of neovascular age-related macular degeneration [19], which represents the marked therapeutic potential of RNA aptamers.

Isolation and characterization of high-affinity RNA aptamers specific for the HCV NS5B have recently been described [3, 29]. Although the isolated aptamers were shown to inhibit the enzymatic activity of RNA-dependent RNA polymerase *in vitro*, no studies have been described for the inhibition of intracellular HCV replication with RNA aptamers against the HCV NS5B. In the present study, we identified and characterized nuclease-resistant RNA aptamers for the HCV NS5B RNA-dependent RNA

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polymerase. Importantly, we assessed the replication process of HCV with the isolated aptamers in human hepatoma cell lines.

A random pool of RNA oligonucleotides of ~10^{14} different molecules was generated, with every pyrimidine modified at its 2' position by a fluoro group by the *in vitro* transcription of synthetic DNA templates with 2'-deoxy-2'-fluoro CTP and UTP (Epicentre Technologies) and normal GTP, ATP, and T7 RNA polymerase as previously described [25]. This modification of the 2' position of RNA increased its stability in human serum more than 10,000-fold, compared with unmodified 2'-hydroxyl RNA [15, 16, 25]. Moreover, RNAs with a 2' fluoro group have a high affinity, since the RNAs form substantially strong intramolecular helices, leading to thermodynamically stable and rigid tertiary structures [21]. The sequence of the resulting RNA library was 5'-GGGAUACCAGCUUAUUCAAUUN$_{40}$AGAUA-GUAAGUGCAAUCU-3', where N_{40} represents 40 nucleotides (nts) with the equimolar incorporation of A, G, C, and U at each position. Thus, each molecule in the library contained a 40-nt-long region derived from a randomized sequence flanked by defined sequences. A recombinant fragment of HCV NS5B RNA-dependent RNA polymerase was cloned into a pET21 expression vector (Novagen), which expresses recombinant proteins tagged with a hexahistidine at the C-terminus. Proteins were overexpressed in the *E. coli* BL21 (DE3) strain and purified with nickel-chelate resin (Ni-NTA agarose) as described elsewhere [22].

SELEX was performed to isolate nuclease-resistant RNA aptamers specific to the HCV NS5B, essentially as described previously [1, 10, 25] with a few modifications. 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 $MgCl_2$, 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 any RNAs that nonspecifically bound to agarose beads. The precleared supernatant was transferred to a new tube and further incubated with 2 μg of his-tagged HCV NS5B for 30 min at room temperature. The NS5B-RNA complexes were precipitated with beads, and the pellets washed five times with 0.5 ml of the binding buffer. The RNAs were recovered, amplified with RT-PCR and *in vitro* transcription, and used for 7 more rounds of selection. After 8 rounds of selection, the amplified DNA was cloned and 14 clones were sequenced. Interestingly, 2'-fluoro-selected RNA aptamers (2'-F SE RNAs) were classified only into 2 major groups (Fig. 1A). Both aptamer groups contained totally different selected sequences. As a working model, the most stable secondary structure of the SE RNAs, as shown in Fig. 1B, was predicted using MULFOLD program [30]. The predicted structures also showed that the two aptamer groups have different configurations. SE RNA #9 is comprised of one

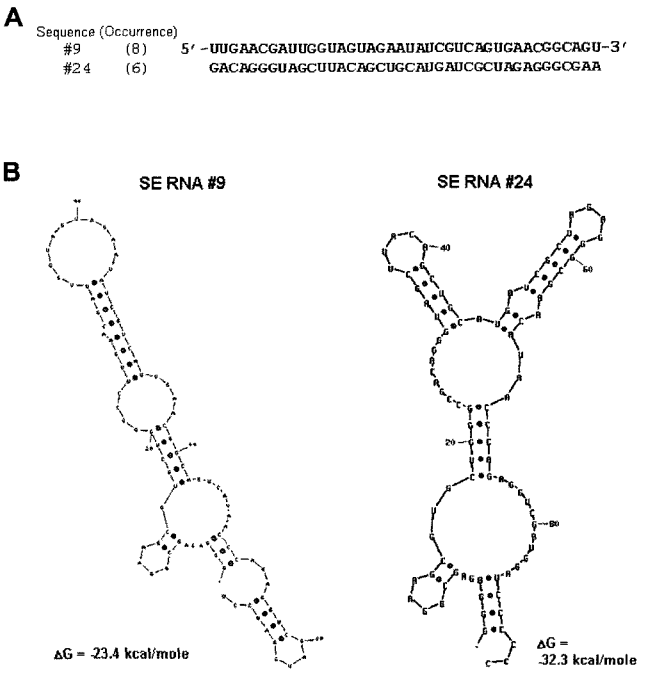

Fig. 1. Sequences and predicted structures of selected 2'-fluoro RNA aptamers (2'-F SE RNA).

A. Selected sequences of 2'-F SE RNAs. After 8 rounds of *in vitro* selection, the sequences of 14 selected RNAs were determined. Two different RNA sequences were found in these clones and each was present in multiple times (number in parentheses). C and U in this figure correspond to 2'-fluoro C and 2'-fluoro U, respectively. **B.** Predicted model of secondary structure of 2'-F SE RNA #9 and #24. The most stable secondary RNA structure was determined by using the MULFOLD program [30]. Nucleotides 25 to 64 represented the sequences selected from a randomized region of the RNA library.

apical stem-loop, in contrast with SE RNA #24 with its two apical stem-loop structures. Sequences of both aptamers selected from a random region of the RNA library are present in these apical stem loop part(s), suggesting the possibility that the apical stem-loop configuration would be involved in the direct binding to the NS5B.

To evaluate the binding specificity of the 2'-F SE RNA, precipitation experiments were performed with internally radiolabeled RNAs (Fig. 2). Labeled and purified RNAs were incubated with proteins as described above. NS5B-RNA complexes were then precipitated with Ni-NTA agarose beads, and bound RNAs extracted from the pellets using 15 μl of 0.1 M EDTA and phenol. Bound RNAs were analyzed on a 6% polyacrylamide gel with urea. The original library RNA with 2-fluoro pyrimidines bound to neither the Ni-NTA beads nor target HCV NS5B protein (Fig. 2, lanes 1–3). In contrast, both SE RNA #9 and #24 were shown to bind to HCV NS5B, but not to the agarose beads (Fig. 2, lanes 4–9). Notably, SE RNA #24 bound to the target protein more strongly than SE RNA #9. This may be because the SE RNA #24 binds the HCV NS5B with more high affinity. In addition, the selected RNA

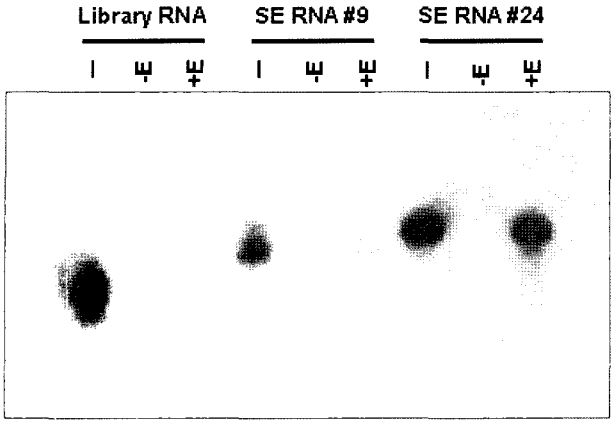

Fig. 2. Binding of 2'-F SE RNA to HCV NS5B.
One nM of internally radiolabeled original library RNA, SE RNA #9, or SE RNA #24 was incubated with (+E) or without (-E) NS5B (100 nM), and RNA-protein complexes were precipitated with Ni-NTA beads. Bound RNAs were extracted and analyzed on a 6% polyacrylamide gel with urea. Lane I contains 10% of each input-labeled RNA.

aptamers could not bind to other his-tagged HCV proteins such as NS3 helicase (data not shown), thus excluding possible nonspecific binding to the histidine moieties of the NS5B protein, and moreover, indicating the specific interaction of the aptamers with the target HCV NS5B.

To estimate the affinity of the SE RNA-HCV NS5B interaction, a gel retardation experiment was used with trace amounts of radiolabeled RNAs and increasing amounts of the RNA-dependent RNA polymerase (Fig. 3). For a gel shift analysis, the radiolabeled SE RNA was incubated with target proteins at room temperature for 30 min. Thereafter, the complexes were analyzed on a 4% nondenaturing polyacrylamide gel. The original library RNA containing 40-nt-long random sequences contained little affinity to the HCV NS5B even at the highest concentration of the protein. By contrast, both 2'-F SE RNAs efficiently formed a shifted nucleoprotein complex with the HCV NS5B in a dose-dependent manner and exhibited high affinity with apparent dissociation constant (K_d) of about 18 nM or 5 nM (SE RNA #9 or #24, respectively), demonstrating that SE RNAs bound tightly to the HCV NS5B. Notably, SE RNA #24 could bind to the target protein 4-fold better than #9, indicating a more efficient binding activity of the SE RNA #24, as shown in Fig. 2.

Once we had observed that the RNA aptamers selected in this study specifically and avidly bound to the HCV

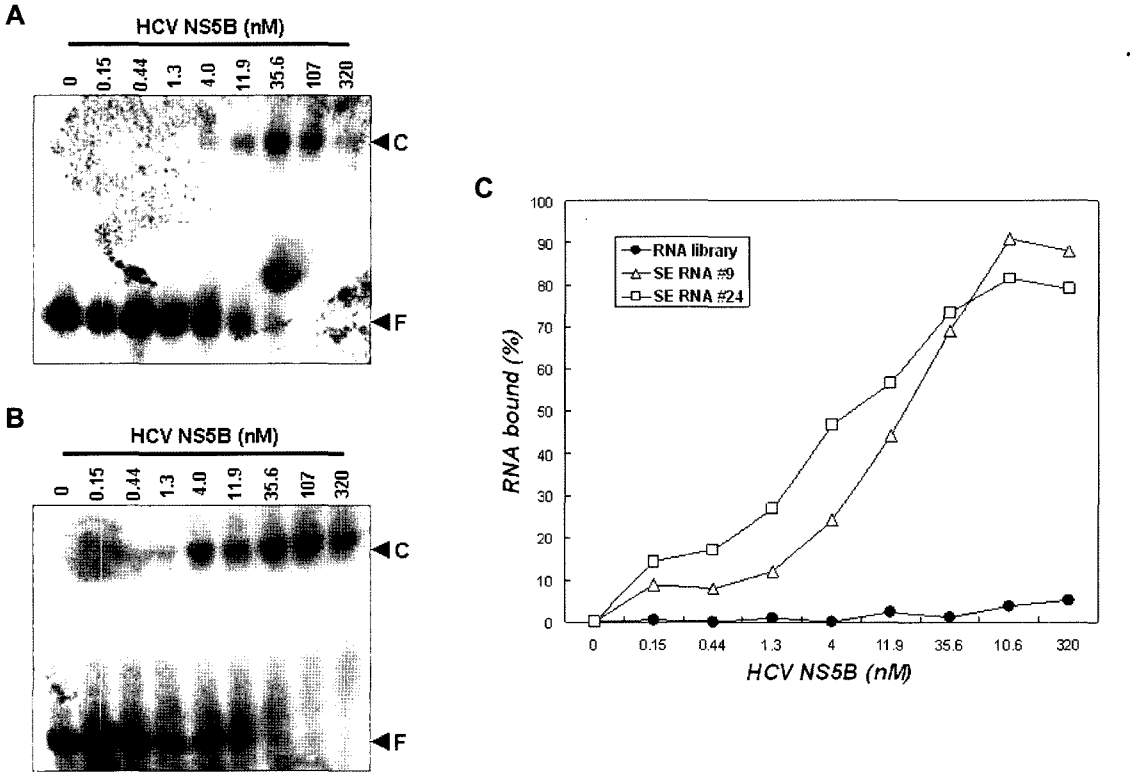

Fig. 3. High binding affinity of the SE RNA to the HCV NS5B replicase.
A, B. Internally radiolabeled SE RNA #9 (A, 50 pM) or SE RNA #24 (B, 50 pM) was incubated with increasing amount of the HCV NS5B replicase (0–320 nM). The resulting NS5B-SE RNA complexes, C, were separated from the unbound free RNA, F, in a 4% nondenaturing acrylamide gel. **C.** The percentage of RNA bound to HCV NS5B was calculated by determining the fraction of radioactivity present in the RNA-HCV NS5B complexes. Values shown represent the mean of three separate measurements.

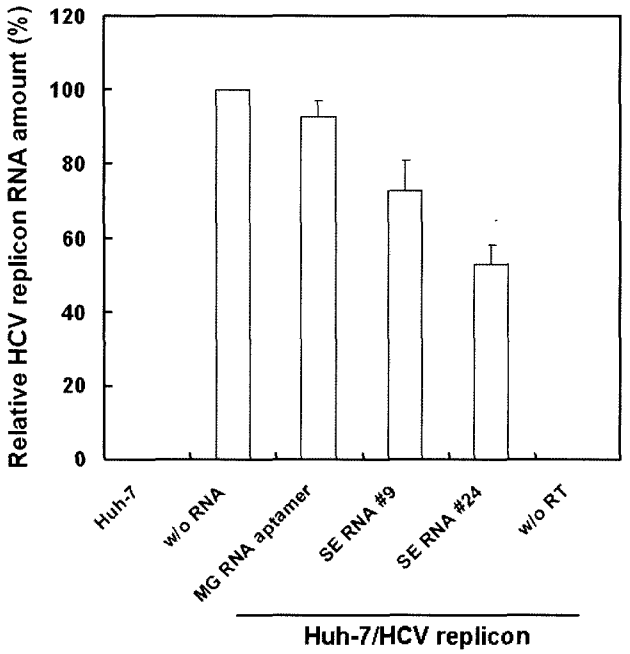

Fig. 4. Inhibition of replication of the HCV replicon by 2'-F SE RNA.

Huh-7 cells were mock transfected, or transfected with HCV subgenomic replicon RNA without any competitor RNAs (w/o RNA), or along with MG aptamer RNA, SE RNA #9, or SE RNA #24. HCV (-) subgenomic RNA strand was amplified by RT-PCR. No cDNA was amplified by PCR without RT (w/o RT) from cells transfected with SE RNA #24. An amplified β-actin cDNA was loaded as an internal control. HCV RNA values were first normalized to β-actin RNA amounts, and the HCV RNA level was then expressed relative to the level in cells transfected with HCV replicon RNA alone. Averages of measurements performed three separate times are shown.

NS5B *in vitro*, we next evaluated RNA aptamer activity to suppress HCV replication in human liver cells using the recently developed HCV subgenomic replicon systems [12, 17] (Fig. 4). A subgenomic replicon construct, pFK-I_{389}neo/NS3-3'/5.1 [12], carrying two cell culture adaptive mutations in NS3 and one in NS5A, was obtained from Dr. Ralf Bartenschlager, University of Heidelberg, Germany. HCV replicon RNA was then constructed by *in vitro* transcription with the AseI and ScaI-digested replicon plasmid as described elsewhere [10]. To determine whether the SE RNA inhibits the intracellular HCV replication, we quantified the level of synthesized HCV-negative (-) strand RNA in hepatocarcinoma Huh-7 cells by RT-PCR, 72 h after cotransfection with the HCV replicon RNA and the various RNA competitors. We compared the amount of HCV RNA in each cell with cells transfected with the HCV replicon alone. Electroporation experiment was employed for RNA transfection in a condition of 950 μF and 250 V using a Gene Pulser system (BioRad) into 400 μl of a suspension of 4×10^6 Huh-7 cells with 500 ng of the HCV replicon RNA along with 5 μg of tRNA, or with RNA aptamer against unrelated target protein such as autoantibody causing myasthenia gravis (MG RNA aptamer) [25], or with 5 μg of SE RNA #9 or #24. Plasmid pcDNAluc encoding Renilla luciferase was also added to each sample to assess transfection efficiency. Similar transfection efficiency in each sample was confirmed by RT-PCR analysis of the luciferase gene (data not shown). After 72 h of transfection, total RNA was isolated and 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'-GGGAAGC-TTCTCGTCCTGCAGTTCAT) and a 3' primer specific for the HCV (-) strand cDNA. Values were normalized to that of β-actin, which was amplified with a 5' primer (5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG) and a 3'-primer (5'-CGTCATACTCCTGCTTGCTGATCCAC-ATCTGC). As shown, nonspecific RNA such as tRNA hardly affected the HCV subgenomic RNA synthesis. By contrast, RNA aptamers #9 and #24 to the HCV NS5B showed inhibition of RNA synthesis of the HCV replicon by up to 27% and 47%, respectively. Unrelated 2'-fluoro MG RNA aptamer could not protect liver cells from the replication of HCV replicon RNA, which strongly indicates that the inhibition of HCV replication by the SE RNAs identified in this study was mainly due to the specific interaction of the SE RNAs with the HCV NS5B expressed by the HCV replicon in cells. In accordance with the analysis of binding efficacy and affinity of the SE RNAs (Figs. 2 and 3), SE RNA #24 inhibited HCV replicon RNA replication more efficiently than SE RNA #9 (Fig. 4). This implies that the bioactivity of RNA aptamers to prohibit HCV replication could be improved with enhancement of the binding affinity of the aptamers to the target HCV proteins.

In the present study, we identified nuclease-resistant RNA aptamers against the HCV NS5B RNA-dependent RNA polymerase with SELEX technology. These aptamers bound specifically and very avidly to the target protein with nanomolar binding constant. Importantly, the RNA aptamers could partially suppress intracellular RNA synthesis of HCV replicon when introduced into human liver cells. The molecular mechanism of how the aptamers inhibit HCV replicon replication is now being investigated. Recently, besides NS5B RNA replicase, several studies have reported the isolation of RNA aptamers against another HCV regulatory proteins such as the NS3 helicase domain [10,11] or NS3 protease domain [13]. However, such aptamers contained a normal 2'-hydroxyl group, and thus the aptamers have to be expressed from their cDNA counterparts to inhibit the HCV replication [20], an approach will have large complications to apply to the development of antiviral agents. By contrast, the obvious advantage of the nuclease-resistant RNA aptamers developed in this study will be that we can directly transfer the aptamers into target cells like small chemicals. Further development such as minimization,

optimization, and modification of the aptamers will enhance therapeutic potential of the aptamers, facilitate their chemical synthesis, and increase their durability *in vivo* [5], which will be greatly useful to apply the selected aptamers as candidates for HCV therapeutics if combined with the development of a feasible intracellular delivery system of the RNA aptamers. In addition to therapeutic agent, the RNA aptamers could be used as diagnostic probes for HCV infection and as genetic tools to elucidate the intracellular role of the HCV NS5B during HCV multiplication.

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