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Analysis of *In Vivo* Interaction of HCV NS3 Protein and Specific RNA Aptamer with Yeast Three-Hybrid System

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Abstract We have previously isolated specific RNA aptamers with high affinity against the helicase domain of hepatitis C virus (HCV) nonstructural protein 3 (NS3). The RNA aptamers competitively and efficiently inhibited the helicase activity, partially impeding HCV replicon replication in human hepatocarcinoma cells. In this study, the RNA aptamers were tested for binding to the HCV NS3 proteins in eukaryotic cells, using a yeast three-hybrid system. The aptamers were then recognized by the HCV NS3 proteins when expressed in the cells, while the antisense sequences of the aptamers were not. These results suggest that the *in vitro* selected RNA aptamers can also specifically bind to the target proteins *in vivo*. Consequently, they could be potentially utilized as anti-HCV lead compounds.

Key words: HCV, NS3, RNA aptamer, yeast three-hybrid system

Hepatitis C virus (HCV) is the main agent that causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide [13]. Since currently available treatments for HCV infection are inefficient to many patients and frequently provoke side effects [5], more improved therapeutic approaches are needed.

Nonstructural protein 3 (NS3) encoded by HCV is multifunctional, with three known enzymatic activities that are separated into two distinct domains; the serine protease activity in the one-third amino-terminal domain and the nucleoside triphosphatase and helicase activities in the remaining carboxy-terminal domain [14]. Since NS3 is considered crucial for HCV replication, many therapeutic agents and protocols have been proposed to target the NS3 protease or helicase [7]. Recently, short RNA ligands, termed RNA aptamers, have been isolated 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 systematic evolution of ligands by exponential enrichment (SELEX) [4, 17]. These RNA ligands have been suggested as potential lead compounds against diverse human diseases because of their specificity and avidity as well as ease to be intracellularly overexpressed or chemically synthesized or modified [16].

Our laboratory has recently identified specific and high-affinity RNA aptamers against the HCV NS3 helicase domain, using the SELEX protocol [8]. These aptamers can act as potent decoys to competitively and efficiently inhibit helicase activity *in vitro*. Since helicase activity is reported to be crucial for RNA unwinding, presumably during the viral genome replication, and thus essential for the viral proliferation [12], the aptamers could be anti-HCV agents if the RNAs could also bind to the target protein in cells. Although the aptamers were shown to partially suppress RNA synthesis of the HCV subgenomic replicon in Huh-7 hepatoma cells lines, *in vivo* binding of the RNA aptamers to the HCV NS3 proteins should be confirmed in order to develop the RNAs as therapeutic leads. Specifically, since HCV NS3 is mainly functional as a multidomain form, *in vivo* interaction of the aptamers with full-length NS3 rather than the helicase domain alone should be analyzed.

In the present study, to explore the ability of the *in vitro* selected RNA aptamer to bind its protein target, HCV NS3, in the eukaryotic cells, a yeast three-hybrid system [15] was developed (Fig. 1). The system has been used to genetically screen RNA-binding proteins by using a hybrid RNA molecule as bait and also as a tool to dissect RNA-protein interactions [9], providing an ideal means for determining RNA aptamer-target protein in cells [3].

The sequence and predicted secondary structure of the RNA aptamer against the HCV NS3 helicase domain are presented in Fig. 1A [8]. This RNA specifically bound to the HCV helicase with high affinity of apparent dissociation constant (K_d) of about 990 pM. Deletion analysis showed

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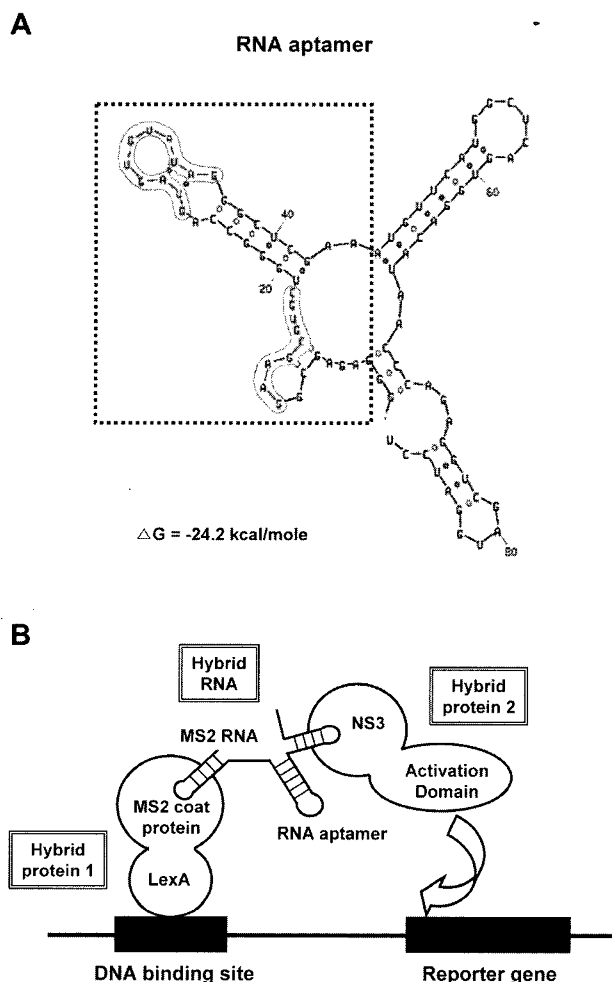

Fig. 1. Yeast three-hybrid system.
(A) Sequence and secondary structure of the RNA aptamer against the HCV NS3 helicase domain [8]. The minimal binding sequence of the aptamer is in the dotted box. Nucleotides 25 to 63 represent the sequences selected *in vitro*. Protected regions of the RNA from RNases in the presence of the target protein are marked as shaded areas. (B) Scheme of yeast three-hybrid analysis. Yeast strain L40-coat harbors chromosomal copies of reporter constructs such as *LacZ* or *HIS3* as well as the gene encoding hybrid protein 1, a LexA/Ms2 coat fusion protein. The LexA domain of the hybrid protein 1 binds to the LexA operator upstream of the reporter gene, while the MS2 coat protein part binds to the MS2 RNA sequence in hybrid RNA. The RNA aptamer sequence against HCV NS3 helicase comprises a second domain of the hybrid RNA. Hybrid protein 2 consists of the yeast GAL4 transcriptional activation domain fused to the HCV NS3 protein. Two vectors encoding the hybrid RNA and the hybrid protein 2, respectively, will be introduced to be expressed in the yeast. *In vivo* binding of the RNA aptamer to the HCV NS3 will cause the expression of the reporter genes.

that residues +1 to +45 of the RNA aptamer encompassed the minimal binding domain to the HCV helicase. Moreover, structure probing and footprinting analyses demonstrated that single-stranded loop regions in the left half (nucleotides 10 to 18 and 26 to 36) of the RNA aptamer are residues directly bound by the HCV helicase. This RNA was also shown to specifically bind to full-length HCV NS3 protein [8]. The yeast hybrid system contains two hybrid proteins and one hybrid RNA to interconnect the hybrid proteins to induce reporter gene activity in yeast, as a result of the interaction between the RNA aptamer and the HCV NS3 protein (Fig. 1B).

To generate an expression vector encoding the hybrid RNA molecule (RNA aptamer-MS2), the RNA aptamer sequence was amplified using PCR with two primers, 5'-AAACCCGGGAGAGCGGAAGCGTGCTG-3' and 5'-AAACCCGGGGGGATGTCCACTGAGCCATGAAC-3', digested with *Sma*I, and cloned into pIIIA/MS2-2 [15] (kindly supplied by S.-K. Jang, POSTECH University, Korea), upstream of two tandem MS2 sequences. The correct conformation of the hybrid RNA to bind the target protein was predicted by using the MULFOLD program [20] (data not shown). In addition, *in vitro* binding analysis of the *in vitro* transcribed hybrid RNA with the HCV helicase was conducted as described [8] (Fig. 2). The previously selected RNA aptamer exhibited high affinity with apparent dissociation constant (K_d) of about less than 1 nM. The hybrid RNA, RNA aptamer-MS2, was also shown to efficiently bind the HCV helicase, albeit less than the RNA aptamer alone with about 10 nM K_d. This result ensures that the hybrid RNA would adopt the correct conformation for binding to the target protein. As negative controls, no aptamer containing RNA (MS2) and antisense sequence of

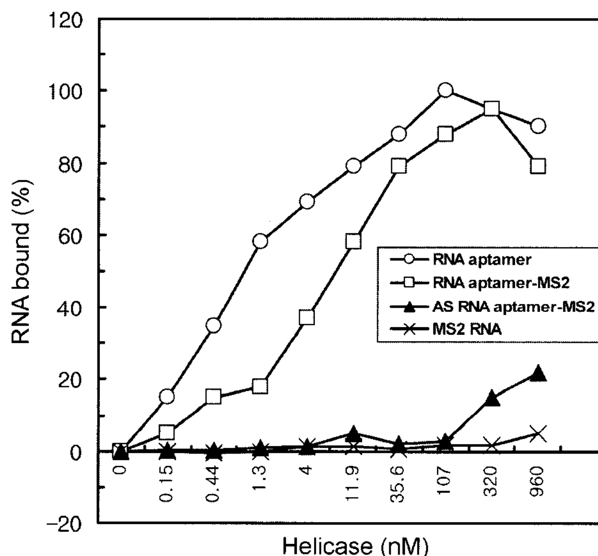

Fig. 2. Binding analysis of *in vitro* transcribed RNAs to the HCV helicase.
Internally radiolabeled RNA (50 pM) was incubated with increasing amount of the HCV helicase (0–960 nM) tagged with a hexahistidine at its C-terminus. RNA-HCV helicase complexes were precipitated with Ni-NTA beads, and the bound RNAs were extracted and counted. The percentage of RNA to HCV helicase was calculated by determining the fraction of radioactivity present in the RNA-HCV helicase complexes. A maximum percentage was seen on 107 nM helicase with the RNA aptamer. The plotted numbers have been normalized to that number.

Table 1. DNA construct sets for yeast three-hybrid analysis.

Strain	Hybrid RNA	Hybrid protein 2
I	MS2	AD-NS3
II	AS RNA aptamer-MS2	AD-NS3
III	RNA aptamer-MS2	AD-NS3
IV	IRE-MS2	AD-IRP
-	RNA aptamer-MS2	AD

MS2, two MS2 coat protein-binding RNA sequences; RNA aptamer, aptamer selected *in vitro* against HCV NS3 helicase [8]; AS RNA aptamer, anti-sense RNA sequence to the RNA aptamer; IRE, iron response element; AD, activation domain of Gal4 transcription of *S. cerevisiae*; NS3, HCV NS3 protein; IRP, iron regulatory protein.

the aptamer-harboring hybrid RNA (AS RNA aptamer-MS2) were used. As shown in Fig. 2, these RNAs bound poorly to the HCV helicase *in vitro*. IRE-MS2 positive control hybrid RNA carries the iron response element (IRE) RNA sequence upstream of two tandem MS2 sequences. AD-NS3 hybrid protein 2 expression plasmid encodes HCV full-length NS3 protein downstream of the yeast GAL4 transcriptional activation domain sequence in the yeast shuttle vector pGAD424 (Clontech, CA, U.S.A.). Expression vector for AD-IRP hybrid protein 2 encodes the rabbit iron regulatory protein (IRP) downstream of the GAL4 activation domain.

Two negative strains (I and II DNA set), the test strain (III DNA set), a positive control strain (IV DNA set), and a negative control strain (-DNA set) were constructed by transformation of the host yeast strain L-40 coat, which carries a gene encoding hybrid protein 1 (LexA-MS2 coat fusion protein) as well as chromosomal copies of LexA-dependent *LacZ* and *HIS3* reporter constructs (kindly gifted by S.-K. Jang), using lithium acetate-mediated transformation methods [15] (Table 1). The positive control strain harbors a naturally occurring RNA-protein interaction: IRP binds IRE present at the 5' untranslated region of ferritin mRNA to inhibit translation of the mRNA encoding the iron storage protein [10]. The negative control strain will indicate any induction of reporter gene activity by nonspecific interaction between the RNA aptamer-MS2 hybrid RNA and AD.

In vivo binding of the RNA aptamer to the HCV NS3 has been validated first by using *LacZ* color assays as described [1] (Fig. 3A). Two negative strains showed no *LacZ* expression in the colony color assay, confirming no or very weak nonspecific interaction between HCV NS3 and RNA (MS2 or AS RNA aptamer-MS2 RNA) in yeast cells. By sharp contrast, only the positive control (set IV of Table 1) and the test strain (set III of Table 1) displayed *LacZ* expression in the colony. These results indicate an *in vivo* interaction between the *in vitro* selected specific RNA aptamer and HCV NS protein. As shown in Fig. 2, less color illumination in the strain III than strain IV could be due to the 10-fold less affinity between the hybrid RNA, RNA

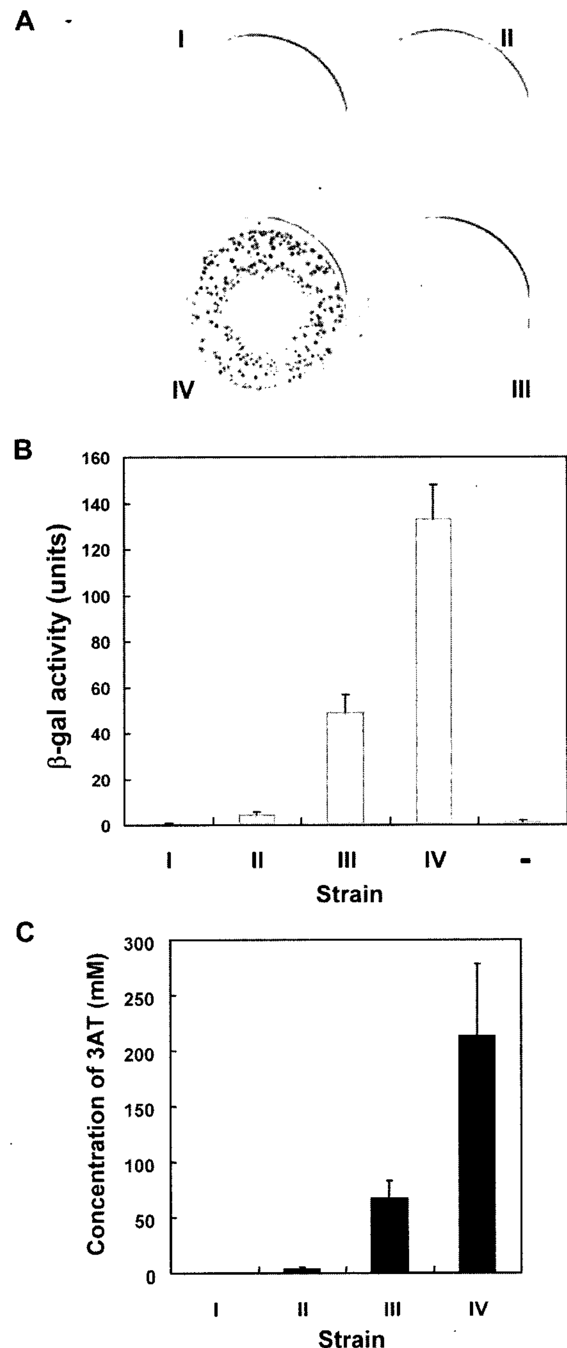

Fig. 3. RNA aptamer to HCV NS3 helicase binds to the NS3 protein *in vivo*.

(A) Expression of *LacZ* reporter gene in three-hybrid strains. The yeast strain L40-coat was transformed with plasmid sets I-IV, indicated in Table 1. Transformants were analyzed for the reporter gene expression by a colony color assay. (B) Analysis of β-gal enzymatic activity of the yeast strain L40-coat which was transformed with plasmid sets I-IV or –, as indicated in Table 1. Averages of assays from three to six transformants for each sample are shown with bars indicating standard errors. (C) Expression of *HIS3* reporter gene by three-hybrid growth analysis. The yeast strain was transformed with the plasmid sets as in (A) and plated on medium lacking uracil, leucine, and histidine, but supplemented with increasing amount of 3-AT (0–300 mM). Levels of reporter gene expression were assessed by the highest 3-AT concentration allowing growth of the yeast.

aptamer-MS2, and the HCV NS3 helicase than that of the RNA aptamer and the target protein. In addition, the *LacZ* reporter gene assays of the transformants were employed by direct measurements of liquid β-galactosidase (β-gal) activity using chlorophenol red β-D-galactopyranoside as a substrate (Fig. 3B). As shown by *LacZ* color assay, β-gal production was largely stimulated in the tester strain III with only 3-fold less activity than the positive strain, thus providing evidence of an *in vivo* interaction between the RNA aptamer and its HCV NS3 target protein. In contrast, replacement of the RNA aptamer with the antisense aptamer sequence (strain II) and elimination of the RNA aptamer (strain I) or NS3 (- strain) dramatically reduced the reporter gene activation, indicating that the β-gal stimulation in the strain III was not due to nonspecific interaction, not only between nonspecific RNA and HCV NS3, but also between the RNA aptamer and the Gal4 activation domain. Next, a *HIS3* growth assay was conducted to confirm this *in vivo* binding (Fig. 3C). To quantify the *HIS3* expression level and to determine the affinity between hybrid RNA and hybrid protein 2 in each transformant, 4 μl drops of the yeast transformants grown to 0.1 OD_{600} were applied to plates with YBN media lacking uracil, leucine, and histidine but supplemented with increasing amounts (0–300 mM) of the *HIS3* gene product inhibitor 3-AT. After 3 days of incubation, the growth of each L-40-coat cell expressing the hybrid constructs was monitored. The level of *HIS3* expression is defined as the highest concentration of 3-AT that still allows the transformed strain to grow. Two negative strains, I and II, showed negligible resistance to 3-AT, implying no expression of *HIS3* gene in these strains due to neither nonspecific binding of the HCV NS3 to MS2 nor AS RNA aptamer-MS2 RNA. However, as expected, positive strain IV efficiently grew on plates containing up to 220 mM 3-AT. In addition, our test strain III clearly exhibited 3-AT-resistant growth on plates with up to 70 mM 3-AT, indicating the specific interaction between the selected RNA aptamer and the HCV NS3 protein in cells.

In this study, with a yeast three-hybrid system, we observed that *in vitro* selected RNA aptamers against HCV NS3 helicase could also be recognized by their target proteins in eukaryotic cells. Although the HCV NS3 proteins are localized in the cytoplasmic side on the ER membrane [14] and the yeast hybrid system functions in the nucleus, the present results implied the possibility of specific binding of the RNA aptamer to its target protein in eukaryotic cell milieu. In addition to our laboratory, others recently isolated RNA aptamers against different HCV regulatory proteins including NS3 protease [6, 11, 18] and NS5B RNA replicase [2, 19]. However, to the best of our knowledge, this study provides the first evidence that RNA aptamers against HCV regulatory proteins can interact with their targets in cells. These aptamers could function as decoys in HCV-infected cells, explaining the mechanism underlying inhibition phenomena of HCV replicon replication observed in cells by the specific RNA aptamers against HCV NS3 helicase [8] and providing the rationale to develop the specific RNA aptamer-based anti-HCV modalities. In addition, the yeast three-hybrid system could provide an efficient experimental means to define the *in vivo* binding site on the HCV helicase as well as the selected RNA aptamers for interaction with each other.

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