Isolation and characterization of RNA aptamers specific for the HCV minus-IRES domain I

Keisuke Konno, Syusuke Fujita, Mana Iizuka, Satoshi Nishikawa, Tsunemi Hasagawa and Kotaro Fukuda

1Department of Material and Biological Chemistry, Faculty of Science, Yamagata University, Yamagata 990-8560, Japan, and 2Age Dimension Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan

ABSTRACT
The minus-IRES (-IRES), corresponding to the 3'-terminal end of the negative strand of hepatitis C virus (HCV) RNA, is well conserved among HCV subtypes. The higher order structure of (-IRES) is essential for HCV replication, because the viral RNA dependent RNA polymerase, NS5B, recognizes it as the initiation site for plus-strand synthesis of the HCV genome. To inhibit the "de novo" synthesis of plus-strand RNA molecules, we performed an in vitro selection procedure for RNA aptamers that are specific for (-IRES) domain I. Among the selected aptamers, one RNA aptamer had two binding sites for the (-IRES) domain I. We found that this aptamer inhibited plus-strand synthesis by about 50%, suggesting that both binding sites are important for binding to its target within the (-IRES) domain I.

INTRODUCTION
The hepatitis C virus (HCV) is a member of the Flaviviridae family and is a major etiological agent of post-transfusion non-A, non-B hepatitis. HCV infection leads to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. One hundred and seventy million people worldwide are currently estimated to be infected with HCV. The combination therapy comprising polyethylene glycol-conjugated interferon and ribavirin represents the current standard treatment for chronic HCV infection, but leads to a sustained virological response in only about 50% of patients and has side effects. More effective antiviral drugs and therapies are therefore desirable.

HCV is composed of a positive single-stranded RNA genome of ~9.6 kb that encodes a large polyprotein of 3,010 amino acids. The 5'UTR of the RNA has a unique structure called the internal ribosomal entry site (IRES), which is essential for translation of the HCV polyprotein precursor. The non-structural protein NS5B has RNA dependent RNA polymerase activity and is the core enzyme that ensures the replication of the RNA viral genome. HCV RNA replication occurs in two steps. In the first step, the viral replicase containing NS5B synthesizes a minus-strand RNA that serves as a template for the synthesis of new plus-strand RNA molecules. Several lines of evidence show that the 3'-terminal nucleotide region of the HCV minus-strand RNA (the IRES sequence) is important for the initiation of plus-strand RNA synthesis. In this study, we performed an in vitro selection procedure for RNA aptamers that are specific for the IRES sequence within the minus-strand RNA. We found that one RNA aptamer inhibited plus-strand RNA synthesis by about 50%.

RESULTS AND DISCUSSION
In vitro selection of aptamers — Preparation of RNA and in vitro selection were carried out as previously described. We used a random-sequence RNA pool, whose constituents had a 40-nucleotide randomized core region, for the selection. After nine cycles, we obtained 42 RNA clones that could be grouped into three major families.
Family-I, -II, and -III. Family-I (25 clones) had the consensus sequence, 5'-UGGAUC-3', which is complementary to the apical loop of SL-E1. Family-II (13 clones) had the consensus sequence, 5'-GAGUAC-3', which is complementary to the apical loop of SL-D1. Family-III (2 clones) had both consensus sequences. The secondary structures of the RNA aptamers showed that most of them had a consensus sequence in their apical loop regions, indicating that these RNA aptamers bind to their targets through a loop-loop interaction.

Inhibition assay — The inhibitory activities of the forty RNA aptamers on RNA synthesis were evaluated using (-)IRES, which was the target used for the in vitro selection of the RNA aptamers. The RNA templates and one of the RNA aptamers were first pre-incubated for 5 min at room temperature in 10 µl Rdrp reaction buffer containing 40 mM NaCl, 20 mM Tris/HCl pH 7.5, 5 mM MgCl₂. Then 10 µl of the complete reaction mixture containing 40 mM NaCl, 20 mM Tris/HCl pH 7.5, 5 mM MgCl₂, 2 mM DTT, 1 mM NTP (ATP, GTP, UTP), 0.4 µM CTP, 1.72 pmol of RNA template, 0.8 pmol of NS5B and 2 µCi 32P-labeled α-CTP was added. We found that AP30 mostly inhibited plus-strand synthesis. As AP30 belonged to Family-III and had two consensus sequences, we decided to study it further. The predicted secondary structure of AP30 is indicated in Fig. 2.

Gel mobility shift assay — To determine the binding affinity of AP30 for (-)IRES and to determine whether the two consensus sequences are important for binding to the target, we performed gel mobility shift assays with AP30 and its mutants AP30mL1, AP30mL2 and AP30dm, which had a mutation in the consensus sequence 5'-GAGUAC-3', 5'-UGGAUC-3' and both sequences, respectively. 32P-labeled AP30 or one of the mutants (300 fmol) was incubated in 20 µl Rdrp reaction buffer with different concentrations of (-)IRES for 15 min at 24°C. Samples were loaded onto a nondenaturing 5% polyacrylamide gel and run at 100V. The gel was autoradiographed and scanned using BAS 2000 (Fig. 3). Kd values were calculated by using Prism. A Kd value of 36 nM for AP30 was obtained. Similarly, a Kd value of 109 nM was obtained for AP30mL1. A Kd value was obtained for AP30mL2 or AP30dm. In the case of AP30mL2, we confirmed its binding to (-)IRES at high concentrations of (-)IRES. This indicates that AP30 binds to (-)IRES mainly through its consensus sequence 5'-UGGAUC-3' and that the second consensus sequence 5'-GAGUAC-3' only assists in AP30 aptamer binding to (-)IRES.

Inhibition of plus-strand synthesis by AP30 — To determine whether consensus sequences are needed or not for the inhibition of plus-strand synthesis, the inhibition assay was performed as described above but with different concentrations of AP30 or its mutants. The results are shown in Fig. 4. AP30 inhibited plus-strand synthesis by ~50%. Interestingly, AP30mL1 also inhibited RNA synthesis but by ~40%. In contrast, AP30mL2 showed little or no inhibition. This suggests that the consensus sequence 5'-UGGAUC-3' is important for inhibition and that the consensus sequence 5'-GAGUAC-3' contributes to both inhibition and binding.

![Fig. 2](https://example.com/fig2.png) Predicted secondary structure of AP30, using mfold developed by Zuker. AP30 has both consensus sequences 5'-UGGAUC-3' and 5'-GAGUAC-3', which are circled. The loops which contain 5'-GAGUAC-3' and 5'-UGGAUC-3' are named L1 and L2, respectively.

![Fig. 3](https://example.com/fig3.png) The percentage of AP30 bound is plotted against the concentration of (-)IRES. The amounts of AP30(-)IRES complex (white squares), AP30mL1(-)IRES (black squares), AP30mL2(-)IRES (black triangles) and AP30dm(-)IRES (black circles) were determined by a BAS 2000 image analyzer. Values present results from three independent experiments.

![Fig. 4](https://example.com/fig4.png) Inhibition of RNA synthesis by AP30. Inhibition assays were performed with 86 nM of (-)IRES in the presence of 340 nM (black bar), 680 nM (white bar) or 1.36 µM (hatched bar) of AP30, it mutants or tRNA. The amount of RNA synthesized was determined by measuring the radioactivity of the products in a scintillation counter; 100% corresponds to the radioactivity of the products in the absence of an aptamer or tRNA. Values present results from three independent experiments.

REFERENCES


*Corresponding author.
E-mail: kotaro.f@sci.kj.yamagata-u.ac.jp