Nucleic Acids Symposium Series No. 51 393–394
doi:10.1093/nass/nrm197

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

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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 that is specific for the (-)IRES domain I. After confirming the binding convergence in the ninth RNA pool, 42 RNA clones were sequenced and analyzed. Of these, 25 clones (Family-I) had the consensus sequence, 5'-UGGAUC-3', which is complementary to the apical loop of SL-E1, an important region for NS5B recognition. Another 13 clones (Family-II) had the consensus sequence, 5'-GAGUAC-3', which is complementary to the apical loop of SL-D1. Biochemical analyses are in progress to evaluate whether these RNA aptamers have the ability to inhibit HCV replication.

INTRODUCTION

The hepatitis C virus (HCV) is a member of *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 about 50% of patients and has side effects. More effective antiviral drugs and therapies are therefore required.

HCV has a positive single-stranded RNA genome of ~9.6 kb that encodes a large polyprotein consisting 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 protein 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 called (-)IRES, which is complimentary to the IRES sequence, is important for the synthesis of plus-strand RNA (1, 2). The (-)IRES domain I, which is composed of 228 nt from the 3' terminus, has a stable secondary structure as shown in Fig. 1 (3, 4).

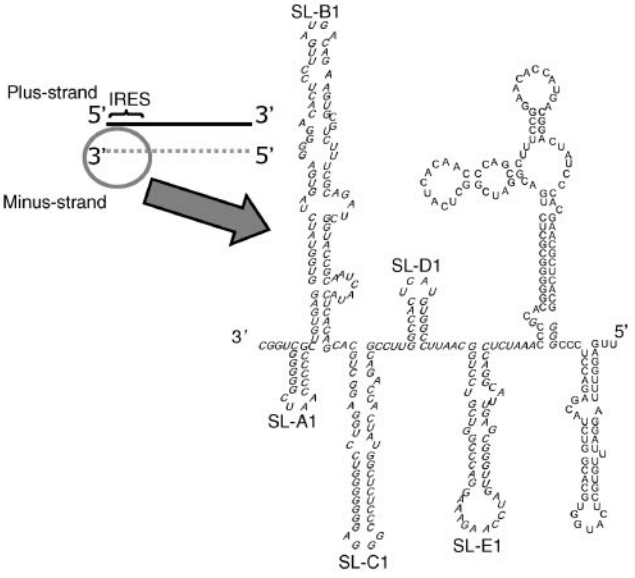

Fig. 1 The predicted secondary structure of HCV (-)IRES. Domain I is drawn in *italics*. It is composed of five stem-loops which are named SL-A1, Sl-B1, SL-C1, SL-D1 and SL-E1 (3, 4).

As RNA synthesis is a critical step in the viral replication cycle, the search for compounds which could interfere specifically with HCV RNA replication is expected to lead to the development of inhibitors of HCV proliferation. *In vitro* selection is a useful tool to obtain ligands that bind to a target with high affinity and specificity. In our previous studies, an RNA aptamer that binds to HCV IRES was selected and shown to inhibit polyprotein translation (5). To obtain RNA inhibitors that could block the synthesis of the new plus-strand of the HCV genome, we performed an *in vitro* selection procedure specific for the (-)IRES domain I.

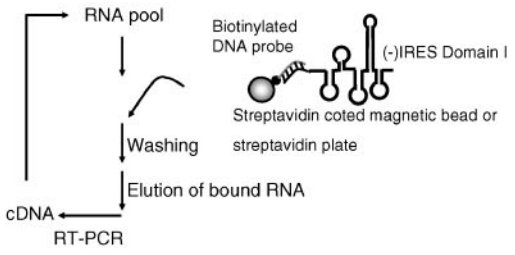

Fig. 2 Schematic of the *in vitro* selection procedure to isolate RNAs that bind to HCV (-)IRES.

RESULTS AND DISCUSSION

Preparation of RNA pool and target RNA — The random-sequenced RNA pool, which has a 40-nucleotide randomized core region, was generated by *in vitro* transcription of template DNA with T7 RNA polymerase and purified on an 8% PAGE containing 7 M urea. HCV (-)IRES domain I (1-228 nt), additional sequences for the hybridization tag and three G bases to improve translation efficiency were generated by *in vitro* transcription with T7 RNA polymerase and a PCR fragment from an (-)IRES-encoding vector. Transcribed RNA was then purified as described above.

In vitro selection of aptamers —The in vitro selection method for isolating aptamers specific for (-)IRES is shown in Fig. 2. A biotinyated DNA probe was hybridized to domain I of (-)IRES and then mixed with streptavidin magnetic beads in 100 µl of buffer E (20 mM HEPES-KOH, pH 7.6, 200 mM KCl and 5 mM $MgCl_2$). After washing the beads with the same buffer, three nanomoles of the RNA pool was pretreated three times for 5 min with the streptavidin bead-immobilized DNA probe to remove non-specific binders. The free RNA recovered was then mixed with the streptavidin bead-immobilized probe-(-)IRES. The reaction mixture (300 µl) was incubated for 10 min in buffer E at room temperature.

The (-)IRES RNA-aptamer complexes were magnetically separated and washed with buffer E. To minimize contamination of non-specific binders, we used a streptavidin plate instead of streptavidin beads after the sixth generation. Probe-(-)IRES was immobilized to a streptavidin plate and washed with buffer E. The reaction mixture (100 µl) was then added to the plate and incubated for 10 min in buffer E at room temperature. The plate was washed with buffer E. To increase the stringency of the selection conditions, the amount of RNA was decreased from 3 nmol in the first generation to 150 pmol in the ninth generation (Table 1). The (-)IRES-bound RNAs were eluted at 98℃ with 7 M urea and recovered by ethanol precipitation. The RNA pool was reversed-transcribed at 42℃ for 70 min. The dsDNA product was amplified by PCR, transcribed *in vitro* by T7 RNA polymerase, and purified on an 8% PAGE containing 7 M urea. The RNAs obtained were used for the next cycle of selection.

Analysis of RNA aptamer sequences — After confirming the binding convergence in the eighth RNA pool, the PCR product at the eighth cycle was introduced into the pGEM-T easy vector and cloned into *Escherichia coli* JM109 strain. Plasmid DNA was isolated from individual clones and sequenced. The secondary structure models of selected aptamaers were drawn with the MulFold program based on the Zuker algorithm.

Table.1 Selection conditions. SP means use of a Streptavidin Plate.

cycle	RNA pool (pmol)	Target (pmol)	Wash (µl×times)
1	3000	50	300×1
2	600	40	300×1
3	600	40	1000×1
4	500	40	500×2
5	400	40	800×2
6(SP)	250	40	300×1
7(SP)	150	40	300×1
8(SP)	150	40	300×2
9(SP)	150	40	300×3

The 42 clones obtained could be grouped into two major families, Family-I and -II. Family-I (25 clones) had the consensus sequence, 5'-UGGAUC-3', which is complementary to the apical loop of SL-E1. On the other hand, Family-II (13 clones) had the consensus sequence, 5'-GAGUAC-3', which is complementary to the apical loop of SL-D1. The secondary structures of the RNA aptamers showed that most of them had a consensus sequence in each apical loop, indicating that RNA aptamers bind to the target through a loop-loop interaction.

It is notable that SL-E1 has a sequence similar to the SL-II observed in the 3'UTR of the HCV plus-strand, which is important for synthesis of the minus-strand. This indicates that SL-E1 may be recognized by the NS5B replicase (4). Furthermore, in agreement with the above report (1), it has been shown that demolishing of SL-E1 decreases the transcription of (-)IRES. The isolated RNA aptamers, especially Family-I containing the sequence complementary to SL-E1, are expected to inhibit NS5B binding to (-)IRES and transcription of the HCV minus-strand .

REFERENCES

1. Astier-Gin, T. *et al.* (2005) *FEBS Journal*, **272**, 3872-3886.
2. Reigadas, S. *et al.* (2001) *Eur. J.Biochem.*, **268**, 5857-5867.
3. Smith, R.M. *et al.* (2002) *J.Virol.*, **76**, 9563-9574.
4. Schuster, C. *et al.* (2002) *J.Virol.*, 76, 8058-8068.
5. Kikuchi, K. *et al.* (2005) *Nucleic Acids Res.*, **33**, 683-692.

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