Biol. Chem., Vol. 386, pp. 183–190, February 2005 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2005.023

Interfering with hepatitis C virus IRES activity using RNA molecules identified by a novel *in vitro* selection method

Cristina Romero-López, Alicia Barroso-delJesus, Elena Puerta-Fernández and Alfredo Berzal-Herranz*

Instituto de Parasitología y Biomedicina 'López-Neyra', CSIC, Parque Tecnológico de Ciencias de la Salud, Avda. del Conocimiento s/n, Armilla, E-18100 Granada, Spain

* Corresponding author
e-mail: aberzalh@ipb.csic.es

Abstract

Hepatitis C virus (HCV) infection is one of the world's major health problems, and the identification of efficient HCV inhibitors is a major goal. Here we report the isolation of efficient anti-HCV internal ribosome entry site (IRES) RNA molecules identified by a new *in vitro* selection method. The newly developed procedure consists of two sequential steps that use distinct criteria for selection: selection for binding and selection for cleaving. The selection protocol was applied to a population of more than 10^{15} variants of an anti-hepatitis C virus ribozyme covalently linked to an aptamer motif. The ribozyme was directed against positions 357 to 369 of the HCV IRES, and the cleavage substrate was a 691-nucleotide-long RNA fragment that comprises the entire HCV IRES domain. After six selection cycles, seven groups of RNA variants were identified. A representative of each group was tested for its capacity to inhibit IRES activity using *in vitro* translation assays. All selected RNAs promoted significant inhibition, some by as much as 95%.

Keywords: anti-hepatitis C virus internal ribosome entry site (HCV IRES) RNAs; catalytic RNAs; hepatitis C virus internal ribosome entry site (HCV IRES); inhibitor RNAs; *in vitro* selection; RNA aptamers.

Introduction

The hepatitis C virus (HCV) is the major cause of transfusion-associated non-A, non-B hepatitis worldwide. Great efforts have been made to combat HCV infection, but the results obtained to date have not been satisfactory (reviewed in Dev et al., 2004). There is a real need to develop new therapeutic agents. Nucleic acids in general, and RNA molecules in particular, are firm candidates. On the other hand, HCV is an attractive target candidate for RNA-based therapeutic strategies since the viral genome is exclusively present as RNA: a ca. 9600-nucleotide-long plus polarity single-stranded RNA molecule (Choo et al., 1989) that codes for a single polyprotein. The initiation of translation occurs via an internal ribosome entry site (IRES). Most of the essential IRES domain is contained in the 5′-untranslated region (5′-UTR) and it spans as many as 30 nucleotides within the translatable domain (Reynolds et al., 1995; Wang et al., 2000). It folds into a complex, highly conserved secondary and tertiary structure essential for its activity (Honda et al., 1996). The HCV genome is highly variable, with the 5′-UTR showing the highest rates of conservation (Bukh et al., 1992).

Several attempts to develop RNA therapeutic agents (mainly ribozymes) against HCV infection have been reported in the literature in recent years, with the 5′-UTR and the coding region of the nucleocapsid the targets that have attracted most attention (Wakita et al., 1999; Puerta-Fernández et al., 2003b and references therein). Ribozymes interact with the substrate RNA molecule (e.g., viral RNA) and catalyze site-specific cleavage, but the efficiency of cleavage depends on the ribozyme binding to its target site, and the compact structure of the 5′-UTR region renders many potentially cleavable sites inaccessible. We have recently shown that the stable TAR-HIV-1 stem-loop motif can be used for anchoring hairpin and hammerhead ribozymes through a TAR antisense domain covalently linked to their 3′-end. This yields a hybrid molecule known as a catalytic antisense RNA. The interaction of TAR-antiTAR domains enhances ribozyme efficiency in *in vitro* HIV-1 RNA processing (Puerta-Fernández et al., 2003a). The present work examines the possibility of taking advantage of the conserved structural motifs of the HCV 5′-UTR to anchor anti-HCV ribozymes. Among other applications, *in vitro* selection strategies can be used to rapidly isolate nucleic acid motifs that efficiently bind specific ligands (reviewed in Breaker, 1997; Wilson and Szostak, 1999). These strategies have been applied with differing success for the identification of high-affinity aptamers that bind to specific domains of the HCV IRES (Aldaz-Carroll et al., 2002; Kikuchi et al., 2003; Tallet-Lopez et al., 2003).

The aim of the present work was to identify RNA molecules that specifically inhibit HCV IRES activity. To this end, a new *in vitro* selection method for the isolation of hybrid catalytic RNAs was developed. This method is composed of two sequential selection steps: selection for binding and secondly selection for cleavage. The RNA molecules identified carried an aptamer domain that encourages efficient binding to the HCV IRES covalently linked to a hammerhead ribozyme. In *in vitro* translation assays, the selected RNAs efficiently inhibited IRES function.

Results and discussion

In vitro selection of anti-HCV RNAs

An innovative *in vitro* selection method was designed in an attempt to isolate anti-HCV RNAs interfering with HCV

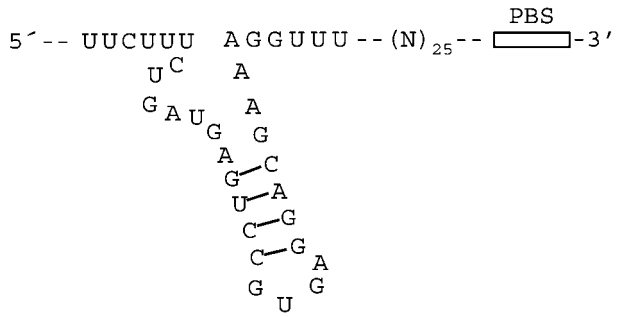

Figure 1 RNA library.
Representation of the catalytic RNAs used in the *in vitro* selection procedure. The nucleotide sequence corresponding to the hammerhead ribozyme that cleaves the HCV genome at nucleotide position 363 (HH363) is shown. N_{25} represents the 25-nucleotide-long randomized region. PBS, constant sequence used as primer binding site.

IRES function. An RNA library of putative inhibitor RNA molecules was constructed and subjected to selection. It consisted of a hammerhead ribozyme that cleaves the HCV IRES region between nucleotide positions 363 and 364 (HH363) (Lieber et al., 1996), carrying a variety of 25-nucleotide-long RNA molecules covalently attached to its 3′-end (Figure 1). During chemical synthesis of the template, the sequence coding for the 25-nucleotide-long RNA domain was completely randomized to generate a population theoretically consisting of more than 10^{15} variants. The initial population was cloned and its complexity analyzed by sequencing (data not shown). The aim was to select aptamer motifs with high affinity for the IRES region without depleting the catalytic activity of the hammerhead motif. This new procedure, outlined in Figure 2, consists of two selection steps, and allows the identification of molecules that bind and cleave the HCV IRES. The first step involves the use of 5′HCV-356 substrate RNA, which contains only the first 356 nucleotides

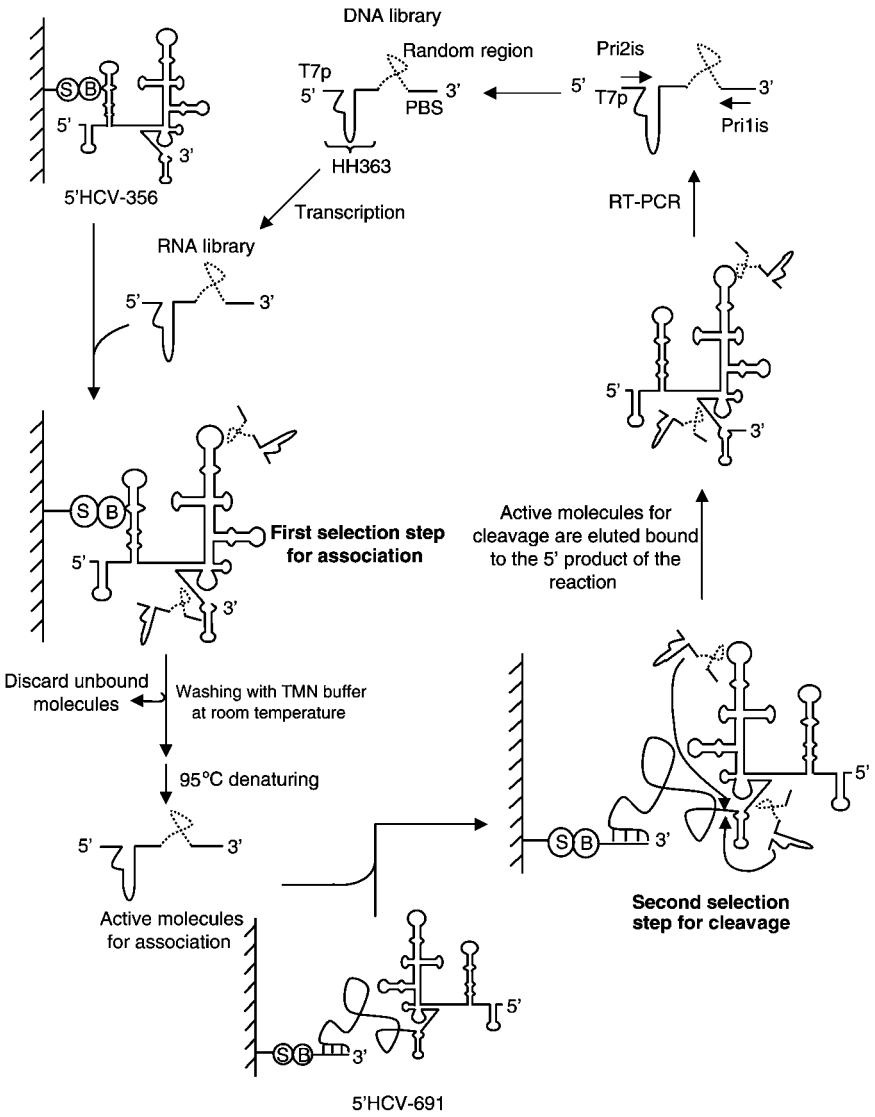

Figure 2 *In vitro* selection of anti HCV IRES inhibitor RNAs.
For methodological details see the materials and methods section. S, streptavidin; B, biotin ligand. Pri1is and Pri2is are primers for cDNA synthesis and PCR amplification; T7p, T7 promoter; PBS, as described in the legend of Figure 1.

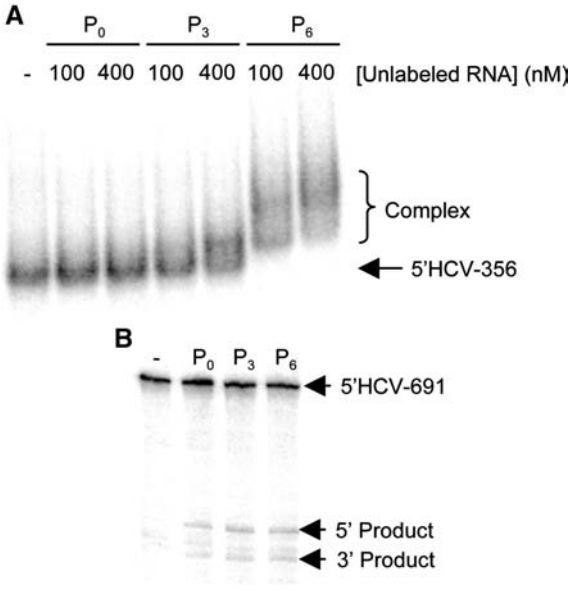

Figure 3 Binding and cleavage activities during the course of selection.
(A) An autoradiogram of *in vitro* binding assays is shown. The internally labeled substrate 5′-HCV-356 RNA was incubated with the pool of RNA variants corresponding to the selection cycle shown at the top of each lane. Complexes showed a reduced electrophoretic mobility and at least two conformers can be identified. The concentration of each RNA pool is indicated. Reactions proceeded as described in the materials and methods section. (B) Autoradiogram of *in vitro* cleavage assays. The internally labeled substrate 5′-HCV-691 RNA was challenged with the RNA pool corresponding to the selection cycle shown at the top of each lane. P_0, initial population; P_3, P_6, pool of RNA variants correspond to the third and sixth rounds of selection, respectively. Extra bands correspond to non-specific degradation that occurs independently of the catalytic RNAs. '-' indicates reaction in the absence of the RNA pool.

of the viral RNA genome, and is therefore not cleavable by HH363. The 5′HCV-356 substrate was internally biotinylated and trapped in a Sepharose-streptavidin column. The initial population of RNA variants (P_0) was loaded onto the column and left to bind to the substrate. Those molecules that associated with the HCV IRES (P_{1-1}) were recovered by denaturing the RNA-RNA interactions, as described in the Materials and methods section. The catalytic activity of the pool of selected variants (P_{1-1}) was then challenged in a second step involving a cleavable HCV substrate molecule, 5′HCV-691, containing the first 691 nucleotides of the HCV genome. This was immobilized to a sepharose-streptavidin column by its 3′-end through a 5′ biotinylated oligonucleotide complementary to positions 671–691 in the substrate molecule. The P_{1-1} population was then loaded onto the column and incubated with the 5′HCV-691 substrate in conditions favoring RNA cleavage. After cleavage, active molecules (P_{1-2}) (no longer attached to the column) were eluted together with the 5′ cleavage product by washing with cleavage buffer under non-denaturing conditions. The non-active molecules remained bound to the column-immobilized substrate. The mix containing the selected population of inhibitors (P_{1-2}) was concentrated and amplified by RT-PCR using primers Pri1is and Pri2is. A fraction of the amplified DNA was used for cloning and sequence analysis; the remainder was *in vitro* transcribed and went through a new round of the selection cycle. The initial step allows the recovery of active variants that bind to the HCV-IRES region and the second allows removal of variants that impede the catalytic activity among them. Splitting the selection strategy into two steps that use substrates differing in length allows the selection of binding domains different to the simple extension of the substrate recognition domain (helix III) of the hammerhead ribozyme.

The cycle was repeated six times, with the stringency of the selection increased for the fourth generation by raising the temperature of the association step (to 37°C) and reducing the incubation time for both selection steps (from 30 to 15 and 60 to 30 min, respectively). The efficiency of the selection procedure was then monitored by gel mobility shift assays as described in the materials and methods section. No mobility shift was observed for the 5′HCV-356 substrate when incubated with the initial RNA pool (P_0). On the contrary, almost no free target molecules were detected in the presence of the pool recovered from the sixth cycle (P_6; Figure 3A). Cleavage experiments showed that the catalytic activity was not challenged during the selection procedure (Figure 3B). These results prompted us to analyze the P_6 population.

It is worth noting that, in contrast to previous *in vitro* processes for selecting aptamers against specific domains of the HCV IRES, the present selection process covers the entire HCV IRES RNA molecule as target (Aldaz-Carroll et al., 2002; Kikuchi et al., 2003). This novel feature allows the recovery of binders specific to tertiary structural domains.

Analysis of selected sequences

After six rounds of selection we sequenced 62 clones, half of which were different variants carrying single or multiple base substitutions (Figure 4). These were classified into seven groups defined by common sequence motifs. Some variants were classifiable in several groups since they had more than one consensus domain. Group 1, composed of 55% of the selected variants, was more frequently represented. No sequence bias was observed among the variants of each group for any of the nucleotides flanking the consensus domain. Furthermore, the consensus sequences were distributed along the 25-nucleotide domain (Figure 4). Sequence analysis of the HCV IRES region revealed that each of the selected consensus sequences showed complementarity with a specific domain of the HCV IRES region (Figure 5). Interestingly, the consensus sequence that defines group 2 corresponds exactly to that selected by other groups who exclusively used the IRES domain II as a target (Kikuchi et al., 2003; Da Rocha Gomes et al., 2004). Similarly, Toulmé and co-workers defined a consensus sequence against domain IV that is included in the present consensus 5 (Aldaz-Carroll et al., 2002). These authors determined that their consensus sequences were involved in an interaction with the complementary sequence in the HCV genome. It is therefore very plausible that the consensus sequences of each of the groups selected in the present work are responsible for

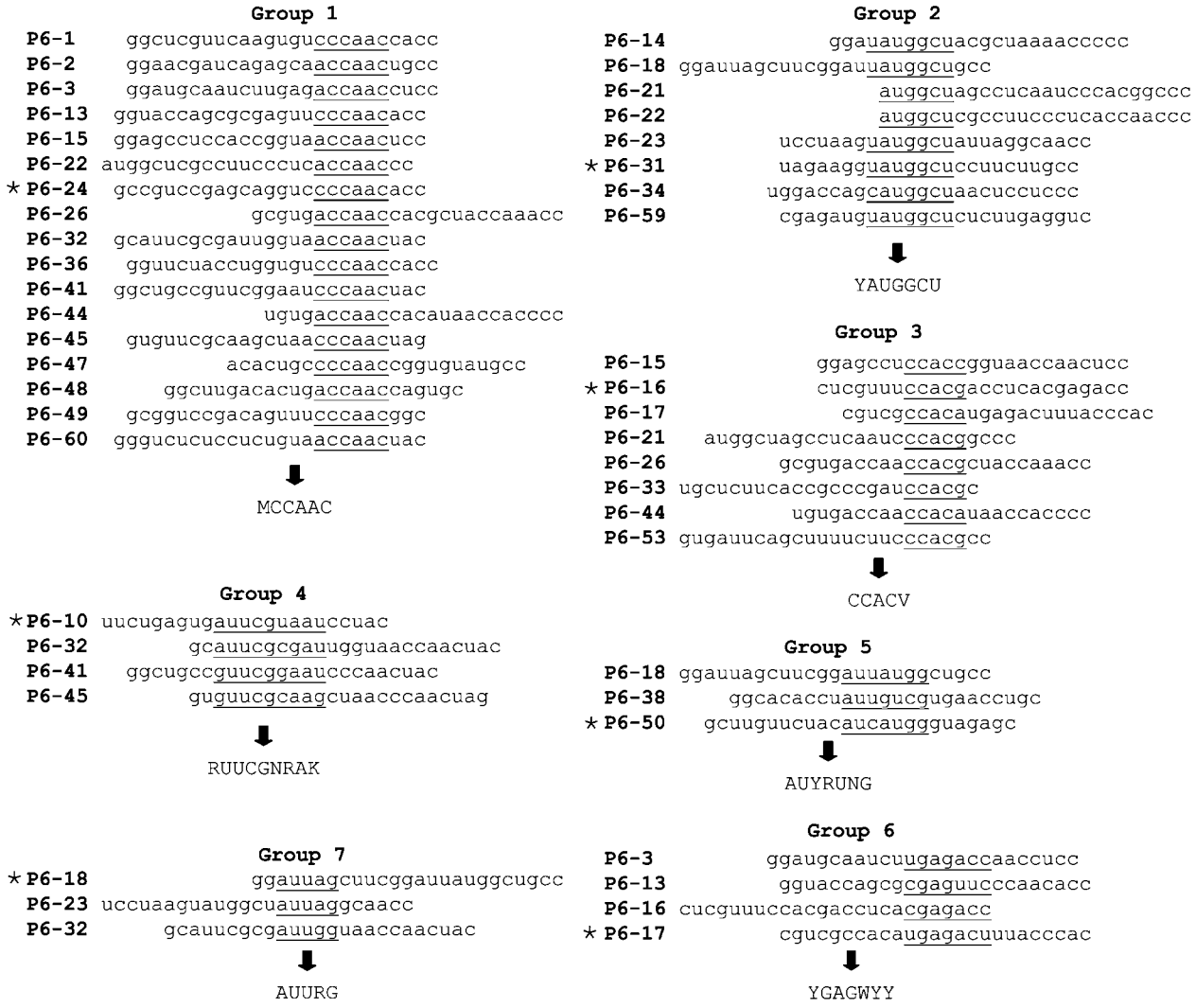

Figure 4 Selected RNA aptamers against HCV IRES.
Sequence of the 25-nucleotide-long RNA motif after six rounds of selection-amplification, classified in groups according to common consensus sequences (underlined). The consensus sequence is shown below each group. Y: C or U; R: G or A; M: A or C; K: G or U; W: A or U; V: A, C or G; and N: any nucleotide. The representative variant of each group used in further analysis is indicated with an asterisk.

the efficient interaction with the substrate observed. This possibility is being investigated in our laboratory.

Inhibition of HCV IRES function by selected RNAs

To test the inhibitory activity of the selected RNA molecules, *in vitro* coupled transcription-translation assays using rabbit reticulocyte extracts were carried out. A plasmid DNA containing a bicistronic cassette was used as a template. The first unit in the transcript corresponded to the *CAT* gene (chloramphenicol acetyl transferase), translation of which was cap-dependent and served as an internal control of the efficiency of the translation reaction. The second unit was the *Fluc* gene (firefly luciferase), translation of which is dependent on HCV IRES function.

A representative variant of each group of selected inhibitors (Figure 4) was *in vitro* transcribed and added to extracts. The capacity to inhibit IRES-dependent translation was determined. Figure 6 shows very significant inhibition of IRES activity for all the molecules tested: three variants (HH363-17, HH363-18 and HH363-50) exhibit up to 95% inhibition, which suggests very potent anti-HCV activity. No significant variation in the CAT levels was observed upon addition of the RNAs tested. To further investigate the contribution of each domain to the total inhibition of HCV IRES activity, the inhibition exerted by selected RNAs was then compared to that of the catalytic domain HH363, as well as of the aptamers. RNA molecules consisting only of either one (HH363 or each aptamer independently) were synthesized and their inhibitory activity tested (Figure 6). With the exception of HH363-10 and 16, all the selected chimeric inhibitors showed significantly greater inhibition than the hammerhead ribozyme alone ($p<0.05$). Furthermore, the independent RNA aptamers also significantly inhibit HCV IRES activity. Among them, aptamer 18 was the most potent, inducing a reduction in translation activity close to 90%. These aptamers may compete for translation factors in order to bind to their respective targets, or induce conformational changes in the IRES that result in less efficient translation initiation. Although in four cases

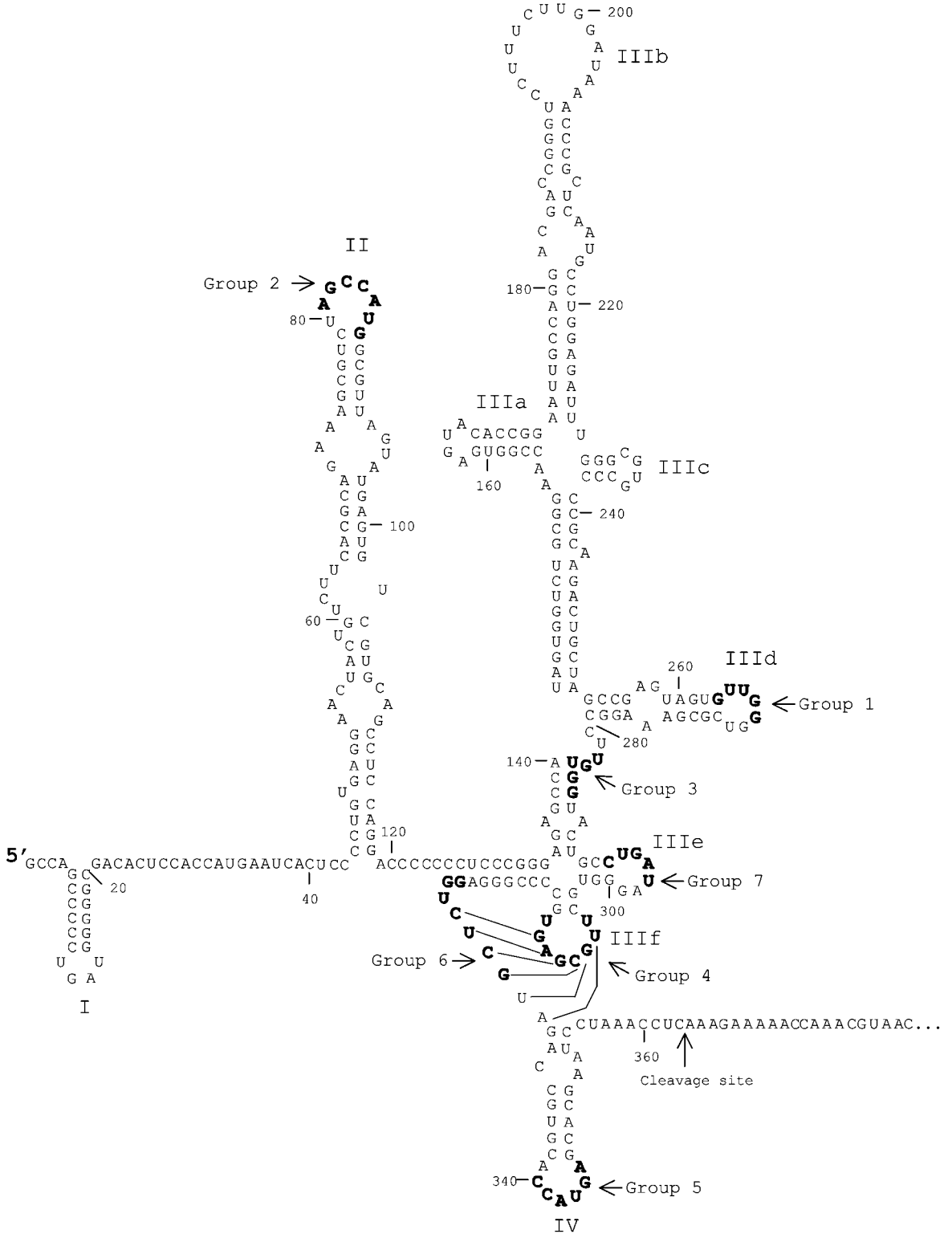

Figure 5 Secondary structure of the 5′-UTR HCV containing the IRES region.
Complementary domains to selected consensus sequences are shown in bold font. The Figure was adapted from Honda et al. (1999).

the inhibition exerted by the aptamers was comparable to that shown by the chimeric molecules, aptamers 18, 17 and 50 had significantly less influence on IRES-dependent translation. Combining the latter aptamer domains with the HH363 catalytic motif in a single molecule led to an enhanced inhibitory effect, resulting in the most efficient inhibitors ($p<0.07$; Figure 6), indicating the advantage of combining two domains with different inhibitory activities as previously described (Puerta-Fernández et al., 2003a). *In vitro* cleavage and binding assays indicated that the enhanced inhibitory effect might respond to more efficient binding of the inhibitor to the IRES, rather than an increase in the cleavage efficiency (Table 1). Inhibition effects obtained with the aptamers indicate that the efficiency of the first selection step in the identification of inhibitors of IRES activity is in good

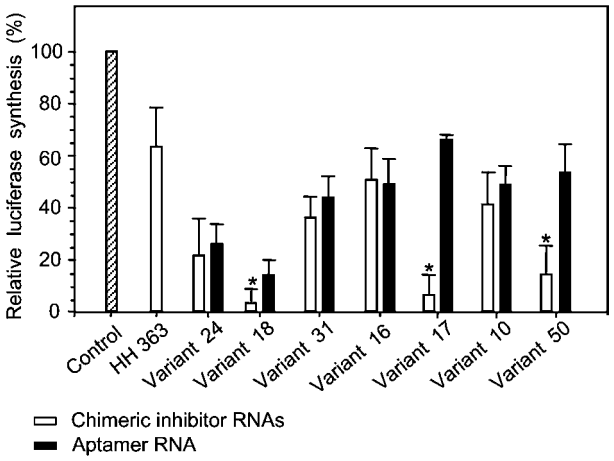

Figure 6 Inhibition of IRES-dependent *in vitro* translation. *In vitro* translation assays of the firefly luciferase gene under control of the HCV IRES were carried out using rabbit reticulocyte extracts. The bar chart shows the amount of luciferase with respect to the control CAT protein. A concentration of 5 μM of either a chimeric inhibitor RNA or an aptamer were added to the extracts and their ability of inhibit IRES-dependent translation measured as relative luciferase gene translation with respect to *CAT* gene translation. Values were normalized to that obtained in the absence of any inhibitor RNA. Values are the mean of four independent experiments. The asterisk indicates significant differences ($p<0.07$) between inhibition observed with the chimeric RNA and the aptamer corresponding to a selected variant.

agreement with previous data (Aldaz-Carroll et al., 2002; Kikuchi et al., 2003). The addition of the second selection step for a different inhibitory activity resulted in identification of the most efficient anti-HCV RNAs. Further analysis is required to clarify the mechanism of action of these new inhibitory RNAs. Since the selection of inhibitor RNAs was achieved from a pool of variants by binding (and cleavage) to the nude HCV IRES using an *in vitro* selection strategy, it is unlikely that the inhibition observed responds to a non-specific translation inhibition by binding to non-HCV IRES RNA sequences or protein factors (Breaker, 1997; Wilson and Szostak, 1999). Similarly, it is difficult to consider that the inhibition observed is exerted at the transcription level by specific binding to the DNA. Nevertheless, we cannot completely rule out that the inhibition observed with some of the variants could be due to an effect on transcription by binding to the nascent RNA of the *Fluc* cistron.

Taken together, these results validate and give support to the power of the *in vitro* selection method described, and suggest it could be used as an experimental tool for obtaining new antiviral RNAs (either as catalytic antisense or as independent aptamer molecules). The selection conditions of the strategy and the number of cycles used could be modified according to the goal in mind. In this first attempt, several inhibitor RNAs were identified that were able to almost completely block the activity of the HCV IRES. The quasi-species structure of the HCV genome population must be taken into account when considering the potential application of these inhibitor RNAs as anti-HCV agents. The combination of various RNAs with different specificity may represent a strategy to overcome this fact.

Table 1 Cleavage and binding of HCV IRES by selected RNAs.

Variant	k_{obs} (min^{-1})	K_d (nM)
HH363-16	0.012±0.031	228.26±68.42
HH363-17	0.009±0.004	114.44±16.29
HH363-18	0.013±0.003	14.49±1.93
HH363-50	0.039±0.005	5.47±1.07

Values are the mean of four independent experiments.

Materials and methods

Construction of the RNA library

An RNA library was produced by *in vitro* transcription of a synthetic DNA template as previously described (Barroso-delJesus et al., 1999). The DNA template was constructed by annealing and extension of oligodeoxynucleotides 5′HH363is (5′-TAT GAA TTC TAA TAC GAC TCA CTA TAG GGT TCT TTC TGA TGA GTC CGT gag gac gaa agg ttt-3′) and 3′HH363is (5′-GCT GAA AGC TTG GAT CCG CTC AN_{25}a aac ctt tcg tcc tc-3′; lowercase letters indicate the complementary sequences; N is any nucleotide). Samples of 6 nmol of each deoxyoligonucleotide were hybridized in ddH_2O by heating at 95°C for 2 min, followed by slow cooling to room temperature. Double-stranded DNA was generated by an extension reaction for 1 h at 37°C with 2.5 U of Klenow and 50 μM of each dNTP (Amersham Biosciences GmbH, Uppsala, Sweden). The DNA pool was then amplified by PCR with the primers Pri1is (5′-GCT GAA AGC TTG GAT CCG CTC A-3′) and Pri2is (5′-TAT GAA TTC TAA TAC GAC TCA CTA TAG GGT TCT TTC TGA TGA GTC CGT-3′; the T7 promoter sequence is underlined), using 3 U of *Taq* DNA polymerase (Biotools B&M Labs, Madrid, Spain). The resulting DNA template coded for the hammerhead 363 ribozyme and a 25-nucleotide-long random sequence linked to its 3′-end. The T7 promoter at the 5′-end, and a primer-binding site sequence at the 3′-end flanked the coding sequence.

Construction of HCV targets

Viral RNA was obtained as described by Perez-Ruiz et al. (1997) and used as a template for cDNA synthesis with primer 24 (Inchauspe et al., 1991). Templates for the HCV RNAs were obtained by PCR amplification of the viral cDNA product with primers ET7 (5′-GAG AAT TCT AAT ACG ACT CAC TAT A-3′) and 691-*Bam*H1 (5′-TTG GAT CCA CCC AAA TTG CGC GAC CTA C-3′; the restriction sites for *Eco*R1 and *Bam*H1 are underlined). The amplification reaction was carried out in 50 μl with 1.5 U of *Taq* DNA polymerase (Biotools B&M Labs). Amplified DNA containing nucleotides +1 to 691 of the HCV genome was cloned into the *Eco*R1 and *Bam*H1 restriction sites of the pUC19 vector to render the pU5′HCV-691 plasmid. Similarly, the pU5′HCV-356 plasmid was constructed by cloning the DNA fragment encoding the first 356 nucleotides of the HCV genome into pUC19. This fragment was amplified from plasmid pU5′HCV-691 with primers ET7 and Pri356 (5′-ATA AAG CTT AGG ATT CGT GCT CAT-3′; the restriction site is underlined).

RNA targets 5′HCV-356 and 5′HCV-691 were *in vitro* transcribed from plasmid pU5′HCV-356 and pU5′HCV-691 linearized with *Hin*dIII and *Bam*HI, respectively. RNAs were synthesized and gel-purified as described by Barroso-delJesus et al. (1999). The 5′HCV-691 was immobilized in a streptavidin column (HiTrap Streptavidin HP Columns, Amersham Biosciences GmbH) using 5 nmol of a 5′ biotinylated DNA probe (5′-biotin-ACC CAA ATT GCG CGA CCT ACG-3′). For synthesis of the internally bio-

tinylated 5′HCV-356 substrate, 42 μM biotin-16-uridine-5′-triphosphate (biotin-16-UTP; Roche Diagnostics, Molecular Biochemicals, Mannheim, Germany) were added to the transcription reaction. The amount of biotin-modified nucleotide was calculated to give approximately one biotin residue per molecule (Theissen et al., 1989).

RNA-RNA binding

To select molecules that bound to the HCV IRES, a sepharose-streptavidin column (HiTrap Streptavidin HP Column, Amersham Biosciences GmbH) loaded with 300 nmol of internally biotinylated 5′HCV-356 substrate and equilibrated with TMN buffer (10 mM Tris-acetate pH 7.5, 10 mM magnesium acetate, 100 mM sodium chloride), was loaded with 4 nmol of the RNA library. The binding reaction was allowed to proceed for 30 min at room temperature, unless otherwise indicated. Unbound molecules were discarded by repeated washing with TMN buffer at room temperature. IRES-binding variants were recovered by washing with 10 volumes of TMN at 90°C. Samples were then concentrated with Centricon YM-10 (Millipore, Bedford, USA).

To evaluate the ability of the selected populations to bind with the HCV 5′UTR region, 2 nM internally labeled 5′HCV-356 substrate and increasing concentrations of selected pools were incubated separately for 7 min at 65°C. After a further 10 min at 37°C, they were mixed and allowed to react for 30 min in TMN buffer. Samples were resolved on a 5% native polyacrylamide gel at 4°C with 50 mM Tris-acetate (pH 7.5) and 5 mM $MgCl_2$.

RNA cleavage assays

For the second selection step, a sepharose-streptavidin column was used, previously loaded with 300 nmol of target 5′HCV-691 and equilibrated with cleavage buffer (50 mM Tris-HCl, 10 mM magnesium chloride). The pool of RNA variants resulting from the first selection step was loaded onto the column in the same buffer. The reaction proceeded for 1 h at 37°C, unless otherwise indicated. Catalytically active RNA molecules were then eluted by washing the column with cleavage buffer at room temperature, concentrated as described above and then precipitated with ethanol.

In order to compare the catalytic activity of the selected molecules, 2 nM internally labeled 5′HCV-691 substrate and a five-fold excess of different RNA pools were subjected to a denaturing step, as described previously, in cleavage buffer. The reaction was initiated by mixing the substrate and the catalytic RNAs, followed by incubation for 60 min at 37°C. The reaction products were resolved on a 4% denaturing polyacrylamide gel.

Amplification and analysis of selected RNA molecules

Molecules that bound and cleaved IRES RNA were amplified using *Tth* DNA polymerase (Promega, Madison, USA) and the primers Pri1is and Pri2is. The amplified DNA was cloned in pGEMT vector (Promega) for sequence analysis and transcription where indicated.

In vitro translation assays

In vitro translation assays were carried out with reticulocyte rabbit lysates using the T7 quick-coupled transcription/translation system (Promega) according to the manufacturer's instructions. Reactions were carried out in 5 μl containing 4 μl of extracts, 50 ng of plasmid pCMVCatIREcluc (a kind gift from Dr. P. Fortes) used as template, 1 μCi [^{35}S]-methionine (Redivue Pro-mix L-[^{35}S] *in vitro* cell labeling mix; Amersham Biosciences) and the indicated concentration of inhibitory RNA. Reactions were stopped on ice and were resolved on 12.5% (w/v) sodium dodecyl sulfate polyacrylamide gels. Dried gels were quantified using a Storm 820 instrument (Amersham Biosciences).

Acknowledgments

We thank Dr. P. Fortes for the generous gift of plasmid p-CMVCATIREcLuc, and for her help with the *in vitro* translation assays. V. Augustin is acknowledged for excellent technical assistance. C.R.-L. was the recipient of an I3P fellowship from the CSIC funded by the European Social Funds. A.B.-delJ. is the recipient of a CSIC-I3P program contract funded by the European Social Funds. This work was supported by Grant BMC2003-669 from the Spanish Ministerio de Ciencia y Tecnología.

References

Aldaz-Carroll, L., Tallet, B., Dausse, E., Yurchenko, L., and Toulmé, J.J. (2002). Apical loop-internal loop interactions: a new RNA-RNA recognition motif identified through in vitro selection against RNA hairpins of the hepatitis C virus mRNA. Biochemistry *41*, 5883–5893.

Barroso-delJesus, A., Tabler, M., and Berzal-Herranz, A. (1999). Comparative kinetic analysis of structural variants of the hairpin ribozyme reveals further potential to optimize its catalytic performance. Antisense Nucleic Acid Drug Dev. *9*, 433–440.

Breaker, R.R. (1997). *In vitro* selection of catalytic polynucleotides. Chem. Rev. *97*, 371–390.

Bukh, J., Purcell, R. H., and Miller, R. H. (1992). Sequence analysis of the 5′ noncoding region of hepatitis C virus. Proc. Natl. Acad. Sci. USA *89*, 4942–4946.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D.W., and Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science *244*, 359–362.

Da Rocha Gomes, S., Dausse, E. and Toulmé, J.-J. (2004). Determinants of apical loop-internal loop RNA-RNA interactions involving the HCV IRES. Biochem. Biophys. Res. Commun. *322*, 820–826.

Dev, A., Patel, K., and McHutchison, J.G. (2004). New therapies for chronic hepatitis C virus infection. Curr. Gastroenterol. Rep. *6*, 77–86.

Honda, M., Ping, L.H., Rijnbrand, R.C., Amphlett, E., Clarke, B., Rowlands, D., and Lemon, S.M. (1996). Structural requirements for initiation of translation by internal ribosome entry within genome-length hepatitis C virus RNA. Virology *222*, 31–42.

Honda, M., Beard, M.R., Ping, L.H., and Lemon, S.M. (1999). A phylogenetically conserved stem-loop structure at the 5′ border of the internal ribosome entry site of hepatitis C virus is required for cap-independent viral translation. J. Virol. *73*, 1165–1174.

Inchauspe, G., Abe, K., Zebedee, S., Nasoff, M., and Prince, A.M. (1991). Use of conserved sequences from hepatitis C virus for the detection of viral RNA in infected sera by polymerase chain reaction. Hepatology *14*, 595–600.

Kikuchi, K., Umehara, T., Fukuda, K., Hwang, J., Kuno, A., Hasegawa, T., and Nishikawa, S. (2003). RNA aptamers targeted to domain II of hepatitis C virus IRES that bind to its apical loop region. J. Biochem. (Tokyo) *133*, 263–270.

Lieber, A., He, C.Y., Polyak, S.J., Gretch, D.R., Barr, D., and Kay, M.A. (1996). Elimination of hepatitis C virus RNA in infected human hepatocytes by adenovirus-mediated expression of ribozymes. J. Virol. *70*, 8782–8791.

Pérez-Ruiz, M., Torres, C., García-López, P.A., Ruiz-Extremera, A., Salmerón, J., and Berzal-Herranz, A. (1997). Determination of HCV RNA concentration by direct quantitation of the

products from a single RT-PCR. J. Virol. Methods *69*, 113–124.

Puerta-Fernández, E., Barroso-delJesus, A., Romero-López, C., and Berzal-Herranz, A. (2003a). HIV-1 TAR as anchoring site for optimized catalytic RNAs. Biol. Chem. *384*, 343–350.

Puerta-Fernández, E., Romero-López, C., Barroso-delJesus, A., and Berzal-Herranz, A. (2003b). Ribozymes: recent advances in the development of RNA tools. FEMS Microbiol. Rev. *27*, 75–97.

Reynolds, J.E., Kaminski, A., Kettinen, H.J., Grace, K., Clarke, B.E., Carroll, A.R., Rowlands, D.J., and Jackson, R.J. (1995). Unique features of internal initiation of hepatitis C virus RNA translation. EMBO J. *14*, 6010–6020.

Tallet-López, B., Aldaz-Carroll, L., Chabas, S., Dausse, E., Staedel, C., and Toulmé, J.J. (2003). Antisense oligonucleotides targeted to the domain IIId of the hepatitis C virus IRES compete with 40S ribosomal subunit binding and prevent *in vitro* translation. Nucleic Acids Res. *31*, 734–742.

Theissen, G., Richter, A., and Lukacs, N. (1989). Degree of biotinylation in nucleic acids estimated by a gel retardation assay. Anal. Biochem. *179*, 98–105.

Wakita, T., Moradpour, D., Tokushihge, K., and Wands, J.R. (1999). Antiviral effects of antisense RNA on hepatitis C virus RNA translation and expression. J. Med. Virol. *57*, 217–222.

Wang, T.H., Rijnbrand, R.C., and Lemon, S.M. (2000). Core protein-coding sequence, but not core protein, modulates the efficiency of cap-independent translation directed by the internal ribosome entry site of hepatitis C virus. J. Virol. *74*, 11347–11358.

Wilson, D.S., and Szostak, J.W. (1999). *In vitro* selection of functional nucleic acids. Annu. Rev. Biochem. *68*, 611–647.

Received October 15, 2004; accepted December 9, 2004