RNA aptamer-mediated interference of HCV replication by targeting the CRE-5BSL3.2 domain

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SUMMARY

The RNA genome of hepatitis C virus (HCV) contains multiple conserved structural RNA domains that play key roles in essential viral processes. A conserved structural component within the 3' end of the region coding for viral RNA-dependent RNA polymerase (NS5B) has been characterized as a functional *cis*-acting replication element (CRE). This paper reports the ability of two RNA aptamers, P-58 and P-78, to interfere with HCV replication by targeting the essential 5BSL3.2 domain within this CRE. Structure probing assays showed the binding of the aptamers to the CRE to result in a structural reorganization of the apical portion of the 5BSL3.2 stem-loop domain. This interfered with the binding of the NS5B protein to the CRE and induced a significant reduction in HCV replication ($\approx 50\%$) in an autonomous subgenomic HCV replication system. These results highlight the potential of this CRE as a target for the development of anti-HCV therapies, and underscore the potential of antiviral agents based on RNA aptamer molecules.

Key words: HCV genome, CRE, RNA structure-function, RNA Aptamers.

INTRODUCTION

Hepatitis C virus (HCV) is the major cause of liver cirrhosis and hepatocellular carcinoma worldwide (1). Current therapeutic strategies based on the combination of interferon and ribavirin have enjoyed only limited success; the identification of new targets and the design of alternative therapies are therefore major goals in HCV research. The targeting of conserved viral genome regions is of particular interest in the quest for such therapies.

The HCV genome is a 9600 nt-long, positive single-stranded RNA molecule that encodes a unique open reading frame flanked by conserved untranslatable regions (5' and 3'UTRs) (Fig.1) (2-5). The genomic RNA acts as messenger RNA during early infection to generate viral proteins required for viral replication and infection (reviewed in 6-8). The regulation of these events involves functional RNA domains located throughout the entire genome. The initiation of HCV translation is directed by an internal ribosome entry site (IRES), most of which is located in the 5'UTR (Fig.1) (9,10). Viral RNA synthesis initiates at the 3'UTR and is strongly dependent on the presence of a *cis*-acting replication element (CRE) at the 3' end of the coding sequence. Identified as a region of conserved structure and sequence (11,12), this CRE folds into a cruciform structure defined by three stem-loops with different functional identities: 5BSL3.1, 5BSL3.2 and 5BSL3.3 (Fig.1) (12). The essential 5BSL3.2 domain consists of two GC-rich helices connected by an eight-base bulge and a 12-base apical loop (11,12). Disruptions in either the sequence or its folding lead to HCV genomes incompetent in terms of replication (13). The 5BSL3.2 domain interacts with both viral polymerase NS5B (14) and distant RNA domains of the viral genome (Fig.1). Indeed, a kissing complex is established between 5BSL3.2 and 3'SLII in the 3' UTR that is important for viral RNA synthesis (13). The internal loop of

5BSL3.2 is also involved in the formation of apical loop-internal loop (ALIL) interactions with structural elements located upstream in the NS5B coding sequence (15) and in the conserved apical loop of domain III_d in the IRES (16). This suggests the importance of long range RNA-RNA interactions in the modulation of multiple steps within the viral cycle. Together, these properties make the CRE region, particularly domain 5BSL3.2, a potentially good target for novel antiviral compounds.

RNA aptamers - short oligonucleotides that specifically bind their ligands with high affinity - are good candidates for targeting viral RNAs (17-20). These molecules access their targets by recognizing their three-dimensional folding; they therefore have an advantage over other inhibitory RNAs such as antisense oligonucleotides, ribozymes and siRNAs, which face greater challenges in terms of target sequence accessibility. The specificity of aptamers is also greater since it is determined by both the primary sequence and tertiary structure of the target (21). Extensive work has been performed on the isolation of RNA aptamers that efficiently bind to essential structural domains in viral RNA genomes (22-28).

The present work examines the effect on HCV replication of two RNA aptamers selected against the genomic HCV-CRE region. These molecules target the essential apical loop of the 5BSL3.2 domain, promoting structural reorganization that interferes with the proper recruitment of the viral polymerase. This was found to inhibit viral RNA synthesis in a stable subgenomic replicon system. These findings show that the CRE region is a potential target for interference with HCV replication, and confirm the efficacy of RNA aptamers as antiviral tools.

MATERIALS AND METHODS

DNA templates and RNA synthesis

HCV-CRE₁₉₄ RNA was obtained by *in vitro* transcription of the *Sall* linearized plasmid construct pUC18-T7HCV9181-9371, as previously described (29). The 3'HCV9181 RNA used in *in vitro* NS5B binding assays was obtained as described elsewhere (16). P-58 and P-78 aptamer RNAs were synthesized by *in vitro* transcription of *Sall*-digested pUC19-derived plasmids containing the respective aptamer coding sequence in-between the *EcoRI* and *Sall* restriction sites. *In vitro* transcription reactions and RNA purification were performed as previously described (30).

The plasmid pET-NS5B Δ 21 used for the production of recombinant NS5B Δ 21 protein was obtained as follows. NS5B Δ 21 DNA was produced by RT-PCR of total RNA from Huh-7 cells harboring a HCV subgenomic replicon (Huh-7 NS3-3') (31,32). A total of 500,000 cells were lysed with Trizol reagent (Invitrogen, Carlsbad, California, USA) to extract the required RNA, as recommended by the manufacturer. 100 ng of this total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA) and a fraction of the resulting cDNA used for amplification with primers HCV-7999/*NheI* 5'-CATATGGCTAGCTCGATGTCCTACACATGGACA-3' and asHCV-9308_*XhoI* 5'-GTGGTGCTCGAGGCGGGGTCGGGCACGAGACAGG CT-3' (restriction sites are shown in italics). The DNA product was then cloned in the *NheI* and *XhoI* sites of the pET-24a plasmid (Novagen, Darmstadt, Germany).

Aptamer selection (SELEX)

Aptamers were selected as previously described (29). Briefly, a theoretical starting RNA population of 1×10^{18} variants was obtained by the *in vitro* transcription of a synthetic DNA template containing a 30 nt-long random sequence region. Selection was achieved by challenging the binding of RNA variants to biotinylated HCV-CRE₁₉₄ viral RNA fragments fixed to a sepharose-streptavidin column. The elution and amplification of active molecules were carried out as described in the mentioned protocol, as was the selection procedure, which was repeated for nine rounds under the outlined experimental conditions.

Binding assays

Binding assays were performed by incubation of 20 fmol of ^{32}P 5' end-labeled aptamer (P-58 or P-78) with increasing concentrations (10 nM to 1250 nM) of unlabeled HCV-CRE₁₉₄ in the presence of binding buffer (2 mM MgCl_2 , 50 mM sodium cacodylate, 300 mM KCl). RNA molecules were independently denatured for 3 min at 95°C in binding buffer and transferred to ice for 15 min. Reactions were initiated by mixing both RNA molecules at 37°C, and stopped on ice after 30 min. The resulting complexes were resolved in 5% native polyacrylamide gels in TB buffer (100 mM Tris-HCl pH 8.3, 86 mM boric acid) at room temperature. Gels were dried, scanned using a Storm 820 Phosphorimager (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and analyzed using Image Quant v.5.2 software (26). Dissociation constant (K_d) values were calculated using Sigma Plot v.8.02 software and adjusted with the equation $y = (B_{\text{max}}x)/(K_d + x)$ as previously described (33), where y is the percentage of complexed aptamer, B_{max} is the

amplitude of the reaction, and x is the concentration of the substrate HCV-CRE₁₉₄ RNA.

RNA-RNA interaction probing assays

Probing assays of the aptamer:HCV-CRE₁₉₄ complex with RNase T1 and lead acetate were essentially performed as previously described (26). Briefly, binding reactions involved the use of trace amounts of the ³²P 5' end-labeled aptamer or 3' end-labeled HCV-CRE₁₉₄ plus 10 pmol of the unlabeled partner (HCV-CRE₁₉₄ for aptamer probing or the inhibitor RNA for the substrate assay). Aptamer RNA probing assays were performed in the presence of 0.01 U of RNase T1 (Ambion, Austin, Texas, USA) or 15 mM Pb²⁺ acetate, and incubated for 2 min or 4 min at 4°C respectively in the presence of 2.8 µg of tRNA. Analysis of the HCV-CRE₁₉₄ RNA was performed under the same conditions but incubation was allowed to proceed for 10 min or 20 min at 4°C respectively in the presence of 2.8 µg of tRNA. Digestion reactions were stopped by the addition of 100 mM EDTA and subsequent phenol extraction. RNA products were resolved by high resolution 7 M urea 6% polyacrylamide denaturing gel electrophoresis. Gels were dried, scanned and analyzed as above.

Aptamer competition with NS5BΔ21 binding to the HCV 3' genome end

Recombinant NS5BΔ21 protein was purified as previously described (34) from *Escherichia coli* BL21 transformed with the construct pET-NS5BΔ21. To generate 3'HCV9181:NS5B complexes, the ³²P-labeled HCV 3' end RNA was denatured by

heating 1 min at 95°C and then cooled for 15 min at 4°C. 0.013 pmol of HCV RNA were then mixed with 5 nM of recombinant NS5B Δ 21 protein in binding buffer (5 mM HEPES pH 7.9, 2 mM MgCl₂, 25 mM KCl). This interaction was subjected to competition with increasing concentrations of the selected aptamers (15 to 2500 nM) or the unrelated RNA₈₀ (26). Binding reactions lasted for 30 min at 37°C before being transferred to ice for 5 min. The mixtures were then diluted to 100 μ l in the same buffer and applied to pre-soaked 0.45 nm nitrocellulose membranes (GE Healthcare). These membranes were then washed twice with binding buffer, scanned using a Storm 820 Phosphorimager (GE Healthcare), and quantification performed as previously described (26). The IC₅₀ values were calculated using Sigma Plot v.8.02 software and employing the equation $y = y_{\max} / (1 + 10^{(\text{LogIC}_{50} - X)})$, where $y_{\max}$ is the maximum percentage of relative binding of 3'HCV9181 RNA to the NS5B protein, IC₅₀ the aptamer concentration that produces 50% of the maximum observed effect, and X the aptamer concentration.

Cell culture and transfection assays

Intracellular activity of the aptamer RNAs was evaluated in a human hepatocarcinoma cell line harboring an HCV subgenomic replicon system (Huh-7 NS3-3') (31,32), as previously reported (27,35). Cell monolayers were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 0.5 mg ml⁻¹ G-418 at 37°C in a 5% CO₂ atmosphere. Twenty hours before transfection ~80,000 cells were seeded onto a 24 well plate in DMEM supplemented with 10% FBS. Cells were transfected using TransFectin reagent (Bio-Rad, Berkeley, California, USA) with 3 μ g of the

aptamer or the unrelated RNA molecule, RNA₈₀, which was used as the internal standard of the assay. Cells were harvested 20 h post-transfection and used in subsequent analyses.

Quantification of the HCV RNA replicon

Intracellular HCV RNA was quantified as previously described (27). Briefly, total RNA was extracted with Trizol, following the manufacturer's instructions. 20 ng of the extracted RNA were then used for reverse transcription with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). A fraction of the cDNA was diluted with Taqman Gene Expression Master Mix (Applied Biosystems) and amplified by PCR over 40 cycles with specific oligonucleotides targeting the IRES region (C-149 and C-342) (36). The fluorogenic Taqman probe (FT-275) was added to the PCR mixture to a final concentration of 150 nM (36). Quantification of the mRNA GAPDH was performed with the human GAPD (GAPDH) Endogenous Control kit (Applied Biosystems). Reactions were run in an ABI PRISM 7000 Sequence Detector System and the collected data analyzed using ABI PRISM 7000 SDS v.1.1 software (both from Applied Biosystems).

RESULTS

Isolation of aptamers targeting the CRE-5BSL3.2 domain

To identify RNA molecules that specifically bind and possibly interfere with the activity of the CRE located at the 3' end of the NS5B coding region (11), a SELEX procedure (37) was performed in which the target was a 194 nt-long RNA fragment

(HCV-CRE₁₉₄) of the HCV-1b Con1 genome (29). The target fragment spanned from nucleotide 9181 to 9371 and included the essential domains 5BSL3.1, 5BSL3.2 and 5BSL3.3 that make up the CRE element (Fig. 2). Selection was performed as previously described (29). Sequence analysis of selected molecules allowed the identification of RNA aptamers P-58 and P-78 that contained the sequence motif GGYUGUG complementary to the 5BSL3.2 apical loop (Fig. 2) (29), suggesting this viral genomic domain to be their theoretical target site. Given the functional importance of the 5BSL3.2 domain for viral replication, RNA aptamers P-58 and P-78 were characterized.

Binding assays involving P-58, P-78 and their target HCV-CRE₁₉₄ RNA were undertaken to determine the specificity of the aptamers. For this, 20 fmol of ³²P internally labeled aptamers were incubated with increasing concentrations of unlabeled HCV-CRE₁₉₄ under the experimental conditions described in Materials and Methods. The complexes formed in the binding assays were resolved in 5% polyacrylamide gels (recognized by their gel mobility shift). Both P-58 and P-78 bound to their target with K_d values of 0.57±0.09 and 0.47±0.12 μM respectively (values are the mean of three independent experiments). No binding was observed for the starting pool P0 (data not shown), confirming the proper functioning of the selection procedure.

Identification of the interacting sites between the aptamers and the HCV-CRE₁₉₄ RNA

RNA probing assays were performed to identify the residues involved in complex formation between the aptamers and HCV-CRE₁₉₄. The secondary structure of the aptamer molecules was analyzed as follows. ³²P 5' end-labeled RNA aptamers were

subjected to partial digestion with RNase T1 or Pb^{2+} , which preferentially recognize nucleotides in single stranded RNA regions (G residues for RNase T1 degradation and any nucleotide for lead). The information obtained from the degradation patterns produced by T1 was then used for secondary structure prediction using Mfold software (38) (Fig. 2B). The results predicted the minimum free energy structure of both aptamers to be a Y shape (Fig. 2B), in which the sequence motif complementary to the 5BSL3.2 domain ($G_{43}GCUGUG_{49}$ in P-58 and $G_{40}GUUGUG_{46}$ in P-78) is mainly located in an apical loop (Figs. 2 and 3). These residues had clearly become resistant to cleavage by RNase T1 and lead in the presence of a molar excess of the unlabeled HCV-CRE₁₉₄ molecule (Fig. 3), thus confirming their involvement in the interaction with the target HCV RNA.

Reciprocal probing assays were subsequently performed to identify the target regions in the substrate molecule. For this, ^{32}P 3' end-labeled RNA was partially digested with T1 and Pb^{2+} as described above. In the absence of inhibitor RNAs, the degradation pattern of the HCV-CRE₁₉₄ RNA resembled that previously described (12), with the proposed unpaired nucleotides in loops and single-stranded regions sensitive to cleavage (Figs. 2A and 4). In the presence of either aptamer, significant and reproducible changes were seen in the degradation pattern of the apical region of the 5BSL3.2 RNA domain, extending from the internal to the apical loop. The presence of P-58 rendered nucleotides C_{9282} to G_{9286} within the apical loop partially resistant to degradation, whereas the nucleotides of the upper portion of the stem, from G_{9271} to U_{9281} , and from U_{9291} to U_{9296} , showed greater susceptibility to RNase T1 and Pb^{2+} degradation (Fig. 4A). Similarly, the presence of P-78 led to the clear protection of nucleotides C_{9282} to C_{9288} within the apical loop to Pb^{2+} degradation, but with greater accessibility from U_{9277} to U_{9281} (Fig. 4B). This conformational

rearrangement rendered nucleotides G₉₂₉₇ to C₉₃₀₀ within the 5BSL3.2 internal loop partially resistant to Pb²⁺-dependent hydrolysis in the presence of either RNA aptamer (Fig. 4).

Competition for RNA-dependent RNA polymerase binding at the HCV genome 3' end

It has been reported that the 5BSL3.2 domain interacts with NS5B RNA-dependent RNA polymerase (14). This raised the question of whether the binding and/or the structural modification of the 5BSL3.2 domain induced by aptamers P-58 and P-78 might interfere with NS5B recruitment by the whole 3' end of the HCV genome. To examine this, *in vitro* binding assays were performed involving the recombinant protein NS5B Δ 21 and an RNA fragment that included 425 nt of the most 3' end of HCV genomic RNA (containing the CRE region plus the 3'UTR [3'HCV9181]) (16,35). Increasing concentrations of the aptamers P-58 and P-78 were used as binding inhibitors, and their IC₅₀ values determined (Fig. 5). The results showed the effective inhibition of the NS5B Δ 21 protein binding to its target RNA by the aptamer molecules in a concentration-dependent manner, with IC₅₀ values in the nM range (185 $\pm$ 46 nM for P-58 and 321 $\pm$ 89 nM for P-78). No inhibition of binding was observed when the assay was performed in the presence of the unrelated 80 nt-long RNA control molecule (RNA₈₀; data not shown) used to normalize the binding inhibition data obtained for the RNA aptamers. These data suggest that the aptamers' interaction with the 5BSL3.2 domain, and the subsequent structural change induced, efficiently impedes the binding of the viral RNA polymerase to the 3' end of the HCV genome.

Effect of aptamers in HCV replication

To assess the effect of the aptamers on HCV replication, Huh-7 cells supporting the autonomous replication of the subgenomic HCV 1b-Con1 derived replicon, were transfected with the P-58 and P-78 aptamers independently. Total RNA was extracted 20 h post-transfection and the (+) HCV RNA strand quantified using real time RT-PCR, as described in Materials and Methods. Transfection with the unrelated RNA₈₀ was used as an internal control. Figure 6 records the significant reduction seen in the HCV RNA levels when P-58 and P-78 were present (close to 50% compared to the control). These results confirm the potential of aptamers targeting HCV-CRE to interfere with HCV replication.

DISCUSSION

The identification and characterization of functional RNA domains in viral genomes opens up the possibility of designing novel antiviral strategies aimed at interfering with their activity (17,20). Conserved RNA sequences have been extensively tested as potential targets for inhibitory RNA molecules. In particular, SELEX procedures have been successfully used to isolate RNA aptamers capable of binding to viral structural RNA domains with essential functions, such as the trans-activation response (TAR) element of human immunodeficiency virus (HIV), the HCV-IRES, and the HCV 3'X tail, among others (22-28,39). The present work characterizes the efficiency of two aptamer RNAs - P-58 and P-78 - isolated by a SELEX procedure against the CRE region of the HCV 1b genome. Both molecules share a common sequence motif complementary to the apical loop of 5BSL3.2 in the HCV-CRE. RNA probing assays indicate that this common motif is involved in both aptamers' interaction with viral RNA (Fig. 3). Interestingly, MFold software analysis, plus the

structural restrictions derived from RNA probing assays with RNase T1, predict both aptamer molecules to have a Y-shaped structure (Fig. 2C), a conformation described for RNA aptamers identified as efficient inhibitors of other viral genomic structural domains (22,40). The present results indicate that the interaction of these aptamers and the HCV-CRE target RNA lead to a refolding of the apical portion of the 5BSL3.2 (Fig. 4), involving nucleotides in the internal and apical loops as well as the helical region between them. Thus, the overall structural rearrangement of the 5BSL3.2 domain may be directly related to the inhibitory effect of the aptamers on HCV replication (Fig. 6).

5BSL3.2 is known to bind to HCV NS5B (14) at the time that the apical loop is involved in an RNA-RNA interaction with the distantly located apical loop of the 3' SLII structural domain within the 3'UTR sequences of the genomic HCV-RNA (13) (Fig. 1). Via its internal loop, 5BSL3.2 is involved in two additional functional interactions, one with a domain located within the NS5B coding region centered on nucleotide 9110 (15), the other with domain IIIId within the IRES region (16) (Fig. 1). This network of interactions plays an important role in various essential steps of the viral cycle, such as viral replication and translation, suggesting it may be a key element in the modulation of the switch between them. The interference with any of these interactions may challenge the viral cycle, therefore these RNA aptamers have a great potential as base for the development of anti-HCV agents.

It has been shown that viral NS5B RNA polymerase binding to the 5BSL3.2 domain can be interfered with by 5BSL3.2 RNA decoys, but not with similar decoys lacking three nucleotides in the internal loop (14). In addition to its binding to the 5BSL3.2 domain, the polymerase binds to the poly (U) and to the SLII domain, as well as to the linker between SLII and SLI within the 3' X-tail region (41,42). Very recently it has

also been shown that targeting of the SLIII domain within the 3' X-tail region with antisense peptide nucleic acids (PNAs) interferes with the binding of the NS5B protein to the 3' X-tail, though there is no evidence of a direct SLIII RNA-NS5B polymerase interaction (43). An explanation for the incomplete inhibition (80%) of polymerase binding to the CRE region in the presence of saturating concentrations of either P-58 and P-78, might be the interaction of the polymerase with any of the other identified binding sites in the 3' end of the HCV RNA genome (43). It is also likely that the structural change induced by the P-58 and P-78 in the internal loop of 5BSL3.2 is responsible for the partial interference in the binding of the polymerase to the 3' end.

RNA aptamers P-58 and P-78 efficiently interfered with HCV replication in Huh-7 cells (Fig. 6). It has been shown in this system that the apical loop of 5BSL3.2 interacts with a complementary sequence in the SLII loop of the 3' X-tail, forming a kissing complex essential for replication (13). Therefore, one cannot rule out the possibility that the inhibitory effect on HCV replication induced by the aptamers might also be due to the interference with this or some other long distance RNA-RNA interaction, as mentioned above (15) (Fig. 1). Neither can it be ruled out that aptamers might interfere with the proper formation of the efficient replication complex, as previously described (43). The authors of the latter work also report that the treatment of HCV subgenomic replicons with antisense PNAs targeting the SLI, SLII or SLIII within the 3'X-tail RNA leads to the inhibition of HCV replication in the same range as described in this work (between 40 to 50%) (43). They also show that the PNA targeting SLIII inhibits the formation of a replication-efficient RNA-NS5B dimer complex, suggesting it to be the cause of replication inhibition.

The lack of efficient treatments against HCV infection necessitates new therapeutic strategies be sought, and interfering with functional genomic RNA elements is an

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2
3 interesting option. These domains retain a conserved sequence and structure that
4 imposes considerable constraints on genome plasticity, reducing the risk of
5 appearance of escape variants, and therefore making them potential targets (44).
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7 This fact would contrast with other anti-HCV strategies aimed to inactivate viral
8 proteins, like the recently approved HCV-NS3 protease inhibitors which main
9 limitation is the rapid appearance of resistant variants (45). The present work reports
10 the inhibition of replication of subgenomic HCV replicons by two independent
11 aptamers targeting the CRE domain. The combined used of both aptamers or in
12 combination with other aptamers targeting different structural genomic RNA domains
13 might result in a strong inhibition of the HCV cycle. Work to test this possibility is
14 currently underway at our laboratory. In addition to their potential use as novel HCV
15 inhibitors, these molecules could also be used to further identify novel RNA-RNA
16 interactions involving the CRE and to study their role in the viral cycle.
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FIGURE LEGENDS

Figure 1. Diagram showing the secondary structure of the 5' and 3' ends of the HCV genome. The diagram shows the 5' UTR plus domains V and VI located in the core coding sequence, all at the 5' end of the genome. The main structural domains (I-VI) and subdomains (IIIa-IIIc) are identified. The translation start codon is indicated by an arrow. The representation of the 3' end includes: the 3' UTR containing the hypervariable region (HV), the polyU/UC stretch and the 3' X-tail including the SLI, SLII and SLIII domains; in addition the stem-loop domain that contains the translation stop codon (indicated by an arrow), the CRE region that includes the 5BSL3.1, 5BSL3.2 and 5BSL3.3 domains, and the SL9110 stem-loop domain are also included in the 3' end representation. Long distance RNA-RNA interactions are shown by dashed lines. The genomic fragment HCV-CRE₁₉₄ used in this work is shaded in the diagram.

Figure 2. Secondary structure of HCV-CRE₁₉₄ and RNA aptamers. (A) Sequence and secondary structure of the HCV-CRE₁₉₄ target RNA fragment. The 5BSL3.1, 5BSL3.2 and 5BSL3.3 structural elements are indicated. Numbers refer to the nucleotide positions of the HCV 1b Con1 isolate (32). The sequence region complementary to the selected aptamer consensus sequence is shaded. (B) Sequence and proposed minimum energy secondary structure of the P-58 and P-78 aptamers, as determined by Mfold software using the experimental constraints obtained in RNase T1 analysis. G nucleotides highly accessible to RNase T1 are indicated by solid arrow heads. Consensus-selected complementary nucleotides to the 5BSL3.2 apical loop are indicated in bold. Nucleotides involved in the interaction with the HCV-CRE₁₉₄ target RNA are indicated in italics.

Figure 3: Structure probing assays of the RNA aptamers. Structure probing assays for P-58 (A) and P-78 (B) complexed with the HCV-CRE₁₉₄. Trace amounts of ³²P 5' end-labeled aptamers were partially digested with RNase T1 or Pb²⁺ in either the presence (+) or absence (-) of HCV-CRE₁₉₄ RNA. Cleavage products were resolved in 10% high resolution polyacrylamide denaturing gels. T1L = T1 cleavage ladder; OH = alkaline ladder; C = the corresponding aptamer incubated in binding buffer. Nucleotides involved in the interaction are shown on the right of each gel. Size fragments are indicated on the left.

Figure 4: Structure probing assays of the HCV-CRE₁₉₄ RNA. Trace amounts of the ³²P 3' end-labeled HCV-CRE₁₉₄ RNA were partially digested with RNase T1 or Pb²⁺ in the presence (+) or absence (-) of P-58 (A) or P-78 (B). Specific cleavage products were resolved in 6% high resolution polyacrylamide gels. The RNA region where changes in the degradation pattern were observed in the presence of the RNA aptamers is indicated by a bracket labeled with an asterisk at the right of the gel. Structural domains are highlighted at the right of the gel. Specific nucleotide positions are indicated on the left of the gel. T1L = T1 cleavage ladder; OH = alkaline ladder; C = the corresponding aptamer incubated in binding buffer.

Figure 5: NS5B Δ 21 binding to the HCV-3' end fragment: aptamer competition assays. ³²P-3' end-labeled HCV-3' was incubated in the presence of increasing concentrations of P-58 ($\square$) or P-78 ($\bullet$). An 80 nt-long unrelated transcript of the pBSSK plasmid (RNA₈₀) (6) was used as a control of the competition reaction. The data show the percentage of the HCV 3' end bound to the protein in the presence of

the aptamers compared to those recorded in the presence of the experimental control (RNA₈₀). Values are the mean ($\pm$ SD) of at least three independent assays.

Figure 6: Aptamer inhibition of HCV replication in Huh-7 cells expressing a HCV subgenomic replicon. Cells were transfected with 3 μ g of P-58 or P-78. At 20 h post-transfection, total RNA was isolated with Trizol reagent and RT-PCR was performed using specific primers plus the Taqman probe for HCV 5' UTR or GAPDH mRNA. The results were analyzed using ABI PRISM SDS v.1.1 software (Applied Biosystems). HCV RNA levels were normalized to those obtained for GAPDH mRNA and referred to the control assay in the presence of 3 μ g of the RNA₈₀. Significant differences ($P < 0.02$) between the relative HCV RNA amounts in the presence of aptamers (P-78 or P-58) with respect to the control RNA were observed. Values are the mean of three independent experiments $\pm$ standard deviation.

Figure 1

Figure 2

A

B

Figure 3

Figure 4

Revised

Figure 5

Figure 6