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6 **RNA aptamer-mediated interference of HCV replication by**  
7 **targeting the CRE-5BSL3.2 domain**  
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32 Running title: Inhibition of HCV-CRE function by RNA aptamers  
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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

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3 5BSL3.2 is also involved in the formation of apical loop-internal loop (ALIL)  
4 interactions with structural elements located upstream in the NS5B coding sequence  
5 (15) and in the conserved apical loop of domain IIIId in the IRES (16). This suggest  
6 the importance of long range RNA-RNA interactions in the modulation of multiple  
7 steps within the viral cycle. Together, these properties make the CRE region,  
8 particularly domain 5BSL3.2, a potentially good target for novel antiviral compounds.  
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17 RNA aptamers - short oligonucleotides that specifically bind their ligands with high  
18 affinity - are good candidates for targeting viral RNAs (17-20). These molecules  
19 access their targets by recognizing their three-dimensional folding; they therefore  
20 have an advantage over other inhibitory RNAs such as antisense oligonucleotides,  
21 ribozymes and siRNAs, which face greater challenges in terms of target sequence  
22 accessibility. The specificity of aptamers is also greater since it is determined by both  
23 the primary sequence and tertiary structure of the target (21). Extensive work has  
24 been performed on the isolation of RNA aptamers that efficiently bind to essential  
25 structural domains in viral RNA genomes (22-28).  
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38 The present work examines the effect on HCV replication of two RNA aptamers  
39 selected against the genomic HCV-CRE region. These molecules target the essential  
40 apical loop of the 5BSL3.2 domain, promoting structural reorganization that interferes  
41 with the proper recruitment of the viral polymerase. This was found to inhibit viral  
42 RNA synthesis in a stable subgenomic replicon system. These findings show that the  
43 CRE region is a potential target for interference with HCV replication, and confirm the  
44 efficacy of RNA aptamers as antiviral tools.  
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## MATERIALS AND METHODS

### *DNA templates and RNA synthesis*

HCV-CRE<sub>194</sub> 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 $\Delta$ 21 used for the production of recombinant NS5B $\Delta$ 21 protein was obtained as follows. NS5B $\Delta$ 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)*

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3 Aptamers were selected as previously described (29). Briefly, a theoretical starting  
4 RNA population of  $1 \times 10^{18}$  variants was obtained by the *in vitro* transcription of a  
5 synthetic DNA template containing a 30 nt-long random sequence region. Selection  
6 was achieved by challenging the binding of RNA variants to biotinylated HCV-CRE<sub>194</sub>  
7 viral RNA fragments fixed to a sepharose-streptavidin column. The elution and  
8 amplification of active molecules were carried out as described in the mentioned  
9 protocol, as was the selection procedure, which was repeated for nine rounds under  
10 the outlined experimental conditions.  
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#### 20 21 22 23 24 *Binding assays*

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27 Binding assays were performed by incubation of 20 fmol of <sup>32</sup>P 5' end-labeled  
28 aptamer (P-58 or P-78) with increasing concentrations (10 nM to 1250 nM) of  
29 unlabeled HCV-CRE<sub>194</sub> in the presence of binding buffer (2 mM MgCl<sub>2</sub>, 50 mM  
30 sodium cacodylate, 300 mM KCl). RNA molecules were independently denatured for  
31 3 min at 95°C in binding buffer and transferred to ice for 15 min. Reactions were  
32 initiated by mixing both RNA molecules at 37°C, and stopped on ice after 30 min. The  
33 resulting complexes were resolved in 5% native polyacrylamide gels in TB buffer  
34 (100 mM Tris-HCl pH 8.3, 86 mM boric acid) at room temperature. Gels were dried,  
35 scanned using a Storm 820 Phosphorimager (GE Healthcare, Little  
36 Chalfont, Buckinghamshire, United Kingdom) and analyzed using Image Quant v.5.2  
37 software (26). Dissociation constant ( $K_d$ ) values were calculated using Sigma Plot  
38 v.8.02 software and adjusted with the equation  $y = (B_{max}x)/(K_d + x)$  as previously  
39 described (33), where  $y$  is the percentage of complexed aptamer,  $B_{max}$  is the  
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3 amplitude of the reaction, and  $x$  is the concentration of the substrate HCV-CRE<sub>194</sub>  
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5 RNA.

#### 10 11 *RNA-RNA interaction probing assays*

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14 Probing assays of the aptamer:HCV-CRE<sub>194</sub> complex with RNase T1 and lead  
15 acetate were essentially performed as previously described (26). Briefly, binding  
16 reactions involved the use of trace amounts of the <sup>32</sup>P 5' end-labeled aptamer or 3'  
17 end-labeled HCV-CRE<sub>194</sub> plus 10 pmol of the unlabeled partner (HCV-CRE<sub>194</sub> for  
18 aptamer probing or the inhibitor RNA for the substrate assay). Aptamer RNA probing  
19 assays were performed in the presence of 0.01 U of RNase T1 (Ambion, Austin,  
20 Texas, USA) or 15 mM Pb<sup>2+</sup> acetate, and incubated for 2 min or 4 min at 4°C  
21 respectively in the presence of 2.8 µg of tRNA. Analysis of the HCV-CRE<sub>194</sub> RNA  
22 was performed under the same conditions but incubation was allowed to proceed for  
23 10 min or 20 min at 4°C respectively in the presence of 2.8 µg of tRNA. Digestion  
24 reactions were stopped by the addition of 100 mM EDTA and subsequent phenol  
25 extraction. RNA products were resolved by high resolution 7 M urea 6%  
26 polyacrylamide denaturing gel electrophoresis. Gels were dried, scanned and  
27 analyzed as above.  
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#### 45 46 47 48 49 *Aptamer competition with NS5BΔ21 binding to the HCV 3' genome end*

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52 Recombinant NS5BΔ21 protein was purified as previously described (34) from  
53 *Escherichia coli* BL21 transformed with the construct pET-NS5BΔ21. To generate  
54 3'HCV9181:NS5B complexes, the <sup>32</sup>P-labeled HCV 3' end RNA was denatured by  
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3 heating 1 min at 95°C and then cooled for 15 min at 4°C. 0.013 pmol of HCV RNA  
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5 were then mixed with 5 nM of recombinant NS5B $\Delta$ 21 protein in binding buffer (5 mM  
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7 HEPES pH 7.9, 2 mM MgCl<sub>2</sub>, 25 mM KCl). This interaction was subjected to  
8  
9 competition with increasing concentrations of the selected aptamers (15 to 2500 nM)  
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11 or the unrelated RNA<sub>80</sub> (26). Binding reactions lasted for 30 min at 37°C before being  
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13 transferred to ice for 5 min. The mixtures were then diluted to 100  $\mu$ l in the same  
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15 buffer and applied to pre-soaked 0.45 nm nitrocellulose membranes (GE Healthcare).  
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17 These membranes were then washed twice with binding buffer, scanned using a  
18  
19 Storm 820 Phosphorimager (GE Healthcare), and quantification performed as  
20  
21 previously described (26). The IC<sub>50</sub> values were calculated using Sigma Plot v.8.02  
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23 software and employing the equation  $y=y_{max}/(1 + 10^{(\text{Log}IC_{50} - X)})$ , where  $y_{max}$  is the  
24  
25 maximum percentage of relative binding of 3'HCV9181 RNA to the NS5B protein,  
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27 IC<sub>50</sub> the aptamer concentration that produces 50% of the maximum observed effect,  
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29 and X the aptamer concentration.  
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#### 38 *Cell culture and transfection assays*

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40 Intracellular activity of the aptamer RNAs was evaluated in a human  
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42 hepatocarcinoma cell line harboring an HCV subgenomic replicon system (Huh-7  
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44 NS3-3') (31,32), as previously reported (27,35). Cell monolayers were maintained in  
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46 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-  
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48 inactivated fetal bovine serum (FBS) (Invitrogen) and 0.5 mg ml<sup>-1</sup> G-418 at 37°C in a  
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50 5% CO<sub>2</sub> atmosphere. Twenty hours before transfection ~80,000 cells were seeded  
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52 onto a 24 well plate in DMEM supplemented with 10% FBS. Cells were transfected  
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54 using TransFectin reagent (Bio-Rad, Berkeley, California, USA) with 3  $\mu$ g of the  
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3 aptamer or the unrelated RNA molecule, RNA<sub>80</sub>, which was used as the internal  
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5 standard of the assay. Cells were harvested 20 h post-transfection and used in  
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7 subsequent analyses.  
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#### 10 11 12 13 *Quantification of the HCV RNA replicon* 14

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

#### 48 49 50 *Isolation of aptamers targeting the CRE-5BSL3.2 domain* 51

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53 To identify RNA molecules that specifically bind and possibly interfere with the  
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55 activity of the CRE located at the 3' end of the NS5B coding region (11), a SELEX  
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57 procedure (37) was performed in which the target was a 194 nt-long RNA fragment  
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3 (HCV-CRE<sub>194</sub>) of the HCV-1b Con1 genome (29). The target fragment spanned from  
4 nucleotide 9181 to 9371 and included the essential domains 5BSL3.1, 5BSL3.2 and  
5 5BSL3.3 that make up the CRE element (Fig. 2). Selection was performed as  
6 previously described (29). Sequence analysis of selected molecules allowed the  
7 identification of RNA aptamers P-58 and P-78 that contained the sequence motif  
8 GGYUGUG complementary to the 5BSL3.2 apical loop (Fig. 2) (29), suggesting this  
9 viral genomic domain to be their theoretical target site. Given the functional  
10 importance of the 5BSL3.2 domain for viral replication, RNA aptamers P-58 and P-78  
11 were characterized.

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14 Binding assays involving P-58, P-78 and their target HCV-CRE<sub>194</sub> RNA were  
15 undertaken to determine the specificity of the aptamers. For this, 20 fmol of <sup>32</sup>P  
16 internally labeled aptamers were incubated with increasing concentrations of  
17 unlabeled HCV-CRE<sub>194</sub> under the experimental conditions described in Materials and  
18 Methods. The complexes formed in the binding assays were resolved in 5%  
19 polyacrylamide gels (recognized by their gel mobility shift). Both P-58 and P-78  
20 bound to their target with K<sub>d</sub> values of 0.57±0.09 and 0.47±0.12 μM respectively  
21 (values are the mean of three independent experiments). No binding was observed  
22 for the starting pool P0 (data not shown), confirming the proper functioning of the  
23 selection procedure.

#### 24 *Identification of the interacting sites between the aptamers and the HCV-CRE<sub>194</sub> RNA*

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26 RNA probing assays were performed to identify the residues involved in complex  
27 formation between the aptamers and HCV-CRE<sub>194</sub>. The secondary structure of the  
28 aptamer molecules was analyzed as follows. <sup>32</sup>P 5' end-labeled RNA aptamers were  
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3 subjected to partial digestion with RNase T1 or  $Pb^{2+}$ , which preferentially recognize  
4 nucleotides in single stranded RNA regions (G residues for RNase T1 degradation  
5 and any nucleotide for lead). The information obtained from the degradation patterns  
6 produced by T1 was then used for secondary structure prediction using Mfold  
7 software (38) (Fig. 2B). The results predicted the minimum free energy structure of  
8 both aptamers to be a Y shape (Fig. 2B), in which the sequence motif  
9 complementary to the 5BSL3.2 domain ( $G_{43}GCUGUG_{49}$  in P-58 and  $G_{40}GUUGUG_{46}$   
10 in P-78) is mainly located in an apical loop (Figs. 2 and 3). These residues had  
11 clearly become resistant to cleavage by RNase T1 and lead in the presence of a  
12 molar excess of the unlabeled HCV-CRE<sub>194</sub> molecule (Fig. 3), thus confirming their  
13 involvement in the interaction with the target HCV RNA.  
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28 Reciprocal probing assays were subsequently performed to identify the target  
29 regions in the substrate molecule. For this,  $^{32}P$  3' end-labeled RNA was partially  
30 digested with T1 and  $Pb^{2+}$  as described above. In the absence of inhibitor RNAs, the  
31 degradation pattern of the HCV-CRE<sub>194</sub> RNA resembled that previously described  
32 (12), with the proposed unpaired nucleotides in loops and single-stranded regions  
33 sensitive to cleavage (Figs. 2A and 4). In the presence of either aptamer, significant  
34 and reproducible changes were seen in the degradation pattern of the apical region  
35 of the 5BSL3.2 RNA domain, extending from the internal to the apical loop. The  
36 presence of P-58 rendered nucleotides  $C_{9282}$  to  $G_{9286}$  within the apical loop partially  
37 resistant to degradation, whereas the nucleotides of the upper portion of the stem,  
38 from  $G_{9271}$  to  $U_{9281}$ , and from  $U_{9291}$  to  $U_{9296}$ , showed greater susceptibility to RNase  
39 T1 and  $Pb^{2+}$  degradation (Fig. 4A). Similarly, the presence of P-78 led to the clear  
40 protection of nucleotides  $C_{9282}$  to  $C_{9288}$  within the apical loop to  $Pb^{2+}$  degradation, but  
41 with greater accessibility from  $U_{9277}$  to  $U_{9281}$  (Fig. 4B). This conformational  
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3 rearrangement rendered nucleotides G<sub>9297</sub> to C<sub>9300</sub> within the 5BSL3.2 internal loop  
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5 partially resistant to Pb<sup>2+</sup>-dependent hydrolysis in the presence of either RNA  
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7 aptamer (Fig. 4).  
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#### 10 11 12 *Competition for RNA-dependent RNA polymerase binding at the HCV genome 3' end*

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15 It has been reported that the 5BSL3.2 domain interacts with NS5B RNA-dependent  
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17 RNA polymerase (14). This raised the question of whether the binding and/or the  
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19 structural modification of the 5BSL3.2 domain induced by aptamers P-58 and P-78  
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21 might interfere with NS5B recruitment by the whole 3' end of the HCV genome. To  
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23 examine this, *in vitro* binding assays were performed involving the recombinant  
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25 protein NS5B $\Delta$ 21 and an RNA fragment that included 425 nt of the most 3' end of  
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27 HCV genomic RNA (containing the CRE region plus the 3'UTR [3'HCV9181]) (16,35).  
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29 Increasing concentrations of the aptamers P-58 and P-78 were used as binding  
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31 inhibitors, and their IC<sub>50</sub> values determined (Fig. 5). The results showed the effective  
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33 inhibition of the NS5B $\Delta$ 21 protein binding to its target RNA by the aptamer molecules  
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35 in a concentration-dependent manner, with IC<sub>50</sub> values in the nM range (185 $\pm$ 46 nM  
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37 for P-58 and 321 $\pm$ 89 nM for P-78). No inhibition of binding was observed when the  
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39 assay was performed in the presence of the unrelated 80 nt-long RNA control  
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41 molecule (RNA<sub>80</sub>; data not shown) used to normalize the binding inhibition data  
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43 obtained for the RNA aptamers. These data suggest that the aptamers' interaction  
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45 with the 5BSL3.2 domain, and the subsequent structural change induced, efficiently  
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47 impedes the binding of the viral RNA polymerase to the 3' end of the HCV genome.  
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#### 56 57 *Effect of aptamers in HCV replication*

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3 To assess the effect of the aptamers on HCV replication, Huh-7 cells supporting the  
4 autonomous replication of the subgenomic HCV 1b-Con1 derived replicon, were  
5 transfected with the P-58 and P-78 aptamers independently. Total RNA was  
6 extracted 20 h post-transfection and the (+) HCV RNA strand quantified using real  
7 time RT-PCR, as described in Materials and Methods. Transfection with the  
8 unrelated RNA<sub>80</sub> was used as an internal control. Figure 6 records the significant  
9 reduction seen in the HCV RNA levels when P-58 and P-78 were present (close to  
10 50% compared to the control). These results confirm the potential of aptamers  
11 targeting HCV-CRE to interfere with HCV replication.  
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## 27 DISCUSSION

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29 The identification and characterization of functional RNA domains in viral genomes  
30 opens up the possibility of designing novel antiviral strategies aimed at interfering  
31 with their activity (17,20). Conserved RNA sequences have been extensively tested  
32 as potential targets for inhibitory RNA molecules. In particular, SELEX procedures  
33 have been successfully used to isolate RNA aptamers capable of binding to viral  
34 structural RNA domains with essential functions, such as the trans-activation  
35 response (TAR) element of human immunodeficiency virus (HIV), the HCV-IRES,  
36 and the HCV 3'X tail, among others (22-28,39). The present work characterizes the  
37 efficiency of two aptamer RNAs - P-58 and P-78 - isolated by a SELEX procedure  
38 against the CRE region of the HCV 1b genome. Both molecules share a common  
39 sequence motif complementary to the apical loop of 5BSL3.2 in the HCV-CRE. RNA  
40 probing assays indicate that this common motif is involved in both aptamers'  
41 interaction with viral RNA (Fig. 3). Interestingly, MFold software analysis, plus the  
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3 structural restrictions derived from RNA probing assays with RNase T1, predict both  
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5 aptamer molecules to have a Y-shaped structure (Fig. 2C), a conformation described  
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7 for RNA aptamers identified as efficient inhibitors of other viral genomic structural  
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9 domains (22,40). The present results indicate that the interaction of these aptamers  
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11 and the HCV-CRE target RNA lead to a refolding of the apical portion of the 5BSL3.2  
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13 (Fig. 4), involving nucleotides in the internal and apical loops as well as the helical  
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15 region between them. Thus, the overall structural rearrangement of the 5BSL3.2  
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17 domain may be directly related to the inhibitory effect of the aptamers on HCV  
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19 replication (Fig. 6).  
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24 5BSL3.2 is known to bind to HCV NS5B (14) at the time that the apical loop is  
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26 involved in an RNA-RNA interaction with the distantly located apical loop of the 3'  
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28 SLII structural domain within the 3'UTR sequences of the genomic HCV-RNA (13)  
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30 (Fig. 1). Via its internal loop, 5BSL3.2 is involved in two additional functional  
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32 interactions, one with a domain located within the NS5B coding region centered on  
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34 nucleotide 9110 (15), the other with domain IIIId within the IRES region (16) (Fig. 1).  
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36 This network of interactions plays an important role in various essential steps of the  
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38 viral cycle, such as viral replication and translation, suggesting it may be a key  
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40 element in the modulation of the switch between them. The interference with any of  
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42 these interactions may challenge the viral cycle, therefore these RNA aptamers have  
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44 a great potential as base for the development of anti-HCV agents.  
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49 It has been shown that viral NS5B RNA polymerase binding to the 5BSL3.2 domain  
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51 can be interfered with by 5BSL3.2 RNA decoys, but not with similar decoys lacking  
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53 three nucleotides in the internal loop (14). In addition to its binding to the 5BSL3.2  
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55 domain, the polymerase binds to the poly (U) and to the SLII domain, as well as to  
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57 the linker between SLII and SLI within the 3' X-tail region (41,42). Very recently it has  
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3 also been shown that targeting of the SLIII domain within the 3' X-tail region with  
4 antisense peptide nucleic acids (PNAs) interferes with the binding of the NS5B  
5 protein to the 3' X-tail, though there is no evidence of a direct SLIII RNA-NS5B  
6 polymerase interaction (43). An explanation for the incomplete inhibition (80%) of  
7 polymerase binding to the CRE region in the presence of saturating concentrations of  
8 either P-58 and P-78, might be the interaction of the polymerase with any of the other  
9 identified binding sites in the 3' end of the HCV RNA genome (43). It is also likely that  
10 the structural change induced by the P-58 and P-78 in the internal loop of 5BSL3.2 is  
11 responsible for the partial interference in the binding of the polymerase to the 3' end.  
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24 RNA aptamers P-58 and P-78 efficiently interfered with HCV replication in Huh-7  
25 cells (Fig. 6). It has been shown in this system that the apical loop of 5BSL3.2  
26 interacts with a complementary sequence in the SLII loop of the 3' X-tail, forming a  
27 kissing complex essential for replication (13). Therefore, one cannot rule out the  
28 possibility that the inhibitory effect on HCV replication induced by the aptamers might  
29 also be due to the interference with this or some other long distance RNA-RNA  
30 interaction, as mentioned above (15) (Fig. 1). Neither can it be ruled out that  
31 aptamers might interfere with the proper formation of the efficient replication  
32 complex, as previously described (43). The authors of the latter work also report that  
33 the treatment of HCV subgenomic replicons with antisense PNAs targeting the SLI,  
34 SLII or SLIII within the 3'X-tail RNA leads to the inhibition of HCV replication in the  
35 same range as described in this work (between 40 to 50%) (43). They also show that  
36 the PNA targeting SLIII inhibits the formation of a replication-efficient RNA-NS5B  
37 dimer complex, suggesting it to be the cause of replication inhibition.  
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56 The lack of efficient treatments against HCV infection necessitates new therapeutic  
57 strategies be sought, and interfering with functional genomic RNA elements is an  
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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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44 laboratory is partially supported by FEDER funds from the EU.  
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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-CRE<sub>194</sub> used in this work is shaded in the diagram.

**Figure 2. Secondary structure of HCV-CRE<sub>194</sub> and RNA aptamers.** (A) Sequence and secondary structure of the HCV-CRE<sub>194</sub> 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-CRE<sub>194</sub> target RNA are indicated in italics.

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5 **Figure 3: Structure probing assays of the RNA aptamers.** Structure probing  
6 assays for P-58 (A) and P-78 (B) complexed with the HCV-CRE<sub>194</sub>. Trace amounts of  
7 <sup>32</sup>P 5' end-labeled aptamers were partially digested with RNase T1 or Pb<sup>+2</sup> in either  
8 the presence (+) or absence (-) of HCV-CRE<sub>194</sub> RNA. Cleavage products were  
9 resolved in 10% high resolution polyacrylamide denaturing gels. T1L = T1 cleavage  
10 ladder; OH = alkaline ladder; C = the corresponding aptamer incubated in binding  
11 buffer. Nucleotides involved in the interaction are shown on the right of each gel. Size  
12 fragments are indicated on the left.

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25 **Figure 4: Structure probing assays of the HCV-CRE<sub>194</sub> RNA.** Trace amounts of  
26 the <sup>32</sup>P 3' end-labeled HCV-CRE<sub>194</sub> RNA were partially digested with RNase T1 or  
27 Pb<sup>+2</sup> in the presence (+) or absence (-) of P-58 (A) or P-78 (B). Specific cleavage  
28 products were resolved in 6% high resolution polyacrylamide gels. The RNA region  
29 where changes in the degradation pattern were observed in the presence of the RNA  
30 aptamers is indicated by a bracket labeled with an asterisk at the right of the gel.  
31 Structural domains are highlighted at the right of the gel. Specific nucleotide positions  
32 are indicated on the left of the gel. T1L = T1 cleavage ladder; OH = alkaline ladder; C  
33 = the corresponding aptamer incubated in binding buffer.

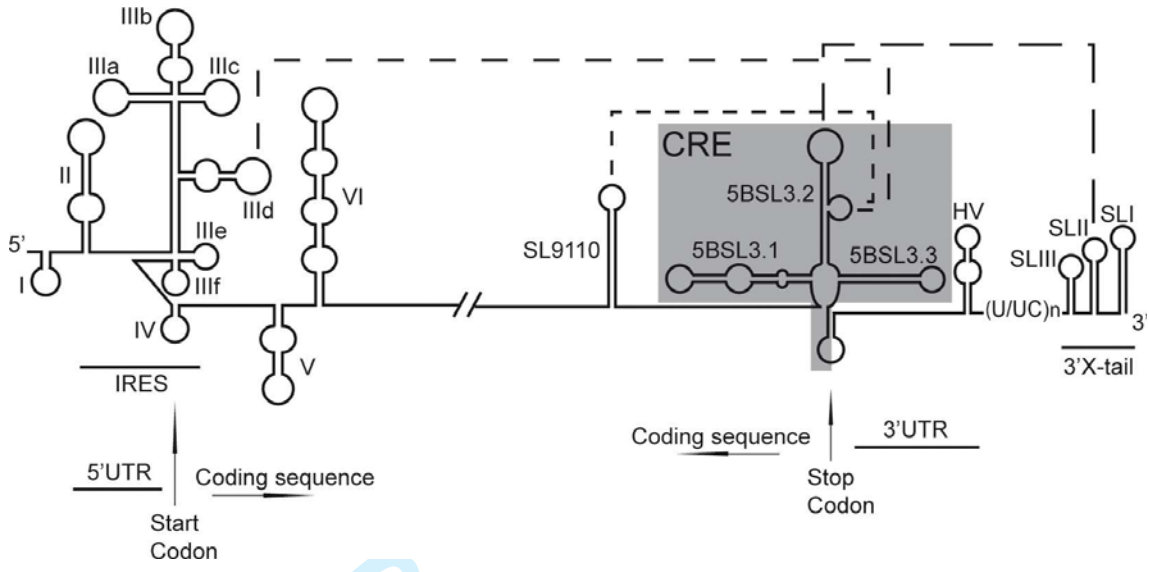
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47 **Figure 5: NS5B $\Delta$ 21 binding to the HCV-3' end fragment: aptamer competition**  
48 **assays.** <sup>32</sup>P-3' end-labeled HCV-3' was incubated in the presence of increasing  
49 concentrations of P-58 (□) or P-78 (•). An 80 nt-long unrelated transcript of the  
50 pBSSK plasmid (RNA<sub>80</sub>) (6) was used as a control of the competition reaction. The  
51 data show the percentage of the HCV 3' end bound to the protein in the presence of  
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3 the aptamers compared to those recorded in the presence of the experimental  
4 control (RNA<sub>80</sub>). Values are the mean ( $\pm$  SD) of at least three independent assays.  
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10 **Figure 6: Aptamer inhibition of HCV replication in Huh-7 cells expressing a**  
11 **HCV subgenomic replicon.** Cells were transfected with 3  $\mu$ g of P-58 or P-78. At 20  
12 h post-transfection, total RNA was isolated with Trizol reagent and RT-PCR was  
13 performed using specific primers plus the Taqman probe for HCV 5' UTR or GAPDH  
14 mRNA. The results were analyzed using ABI PRISM SDS v.1.1 software (Applied  
15 Biosystems). HCV RNA levels were normalized to those obtained for GAPDH mRNA  
16 and referred to the control assay in the presence of 3  $\mu$ g of the RNA<sub>80</sub>. Significant  
17 differences ( $P < 0.02$ ) between the relative HCV RNA amounts in the presence of  
18 aptamers (P-78 or P-58) with respect to the control RNA were observed. Values are  
19 the mean of three independent experiments  $\pm$  standard deviation.  
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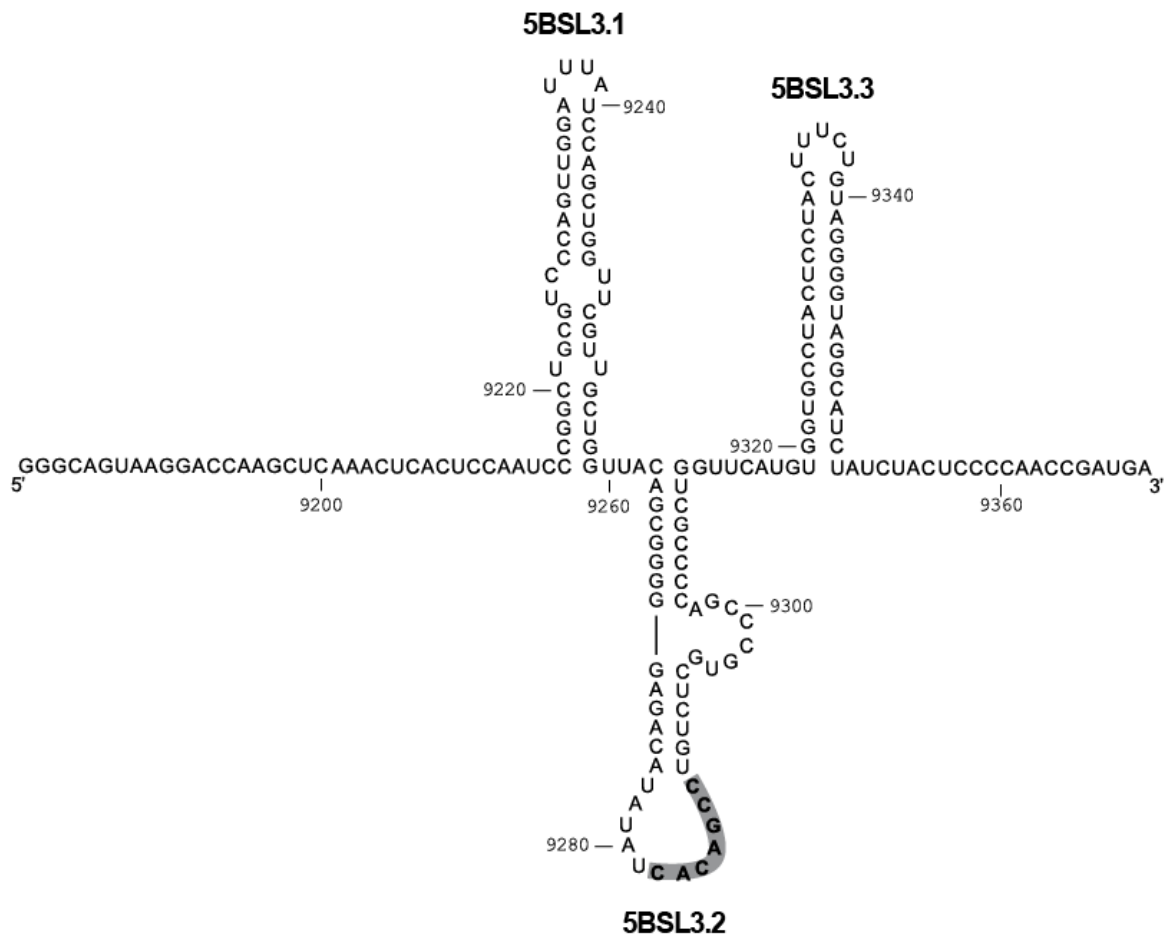
Figure 1



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Figure 2

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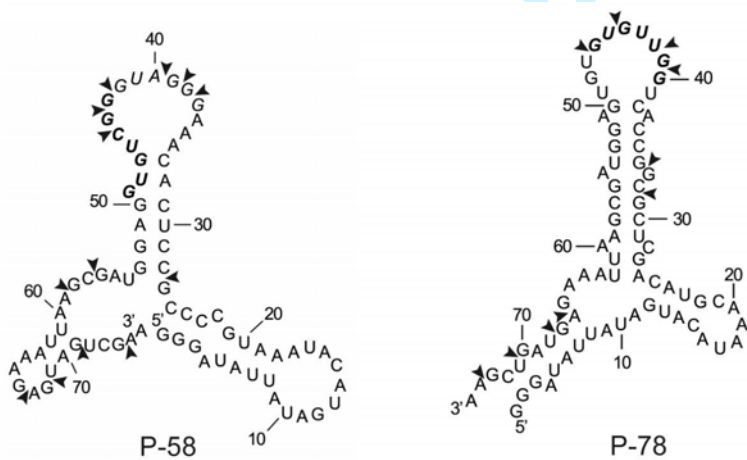


Figure 3

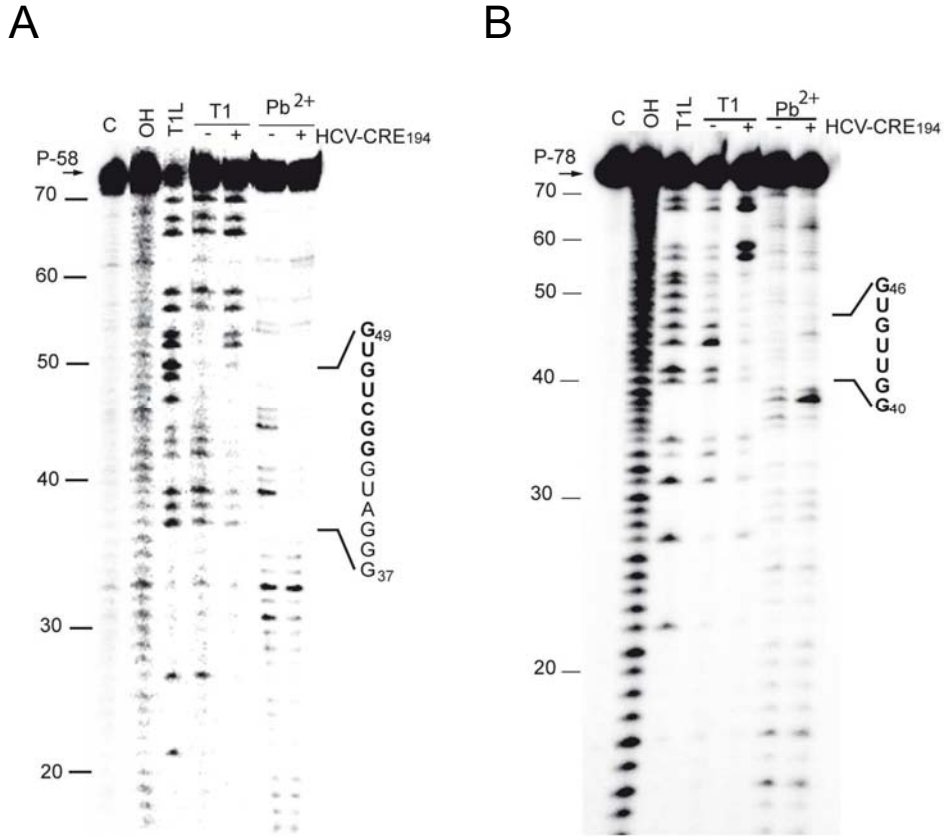


Figure 4

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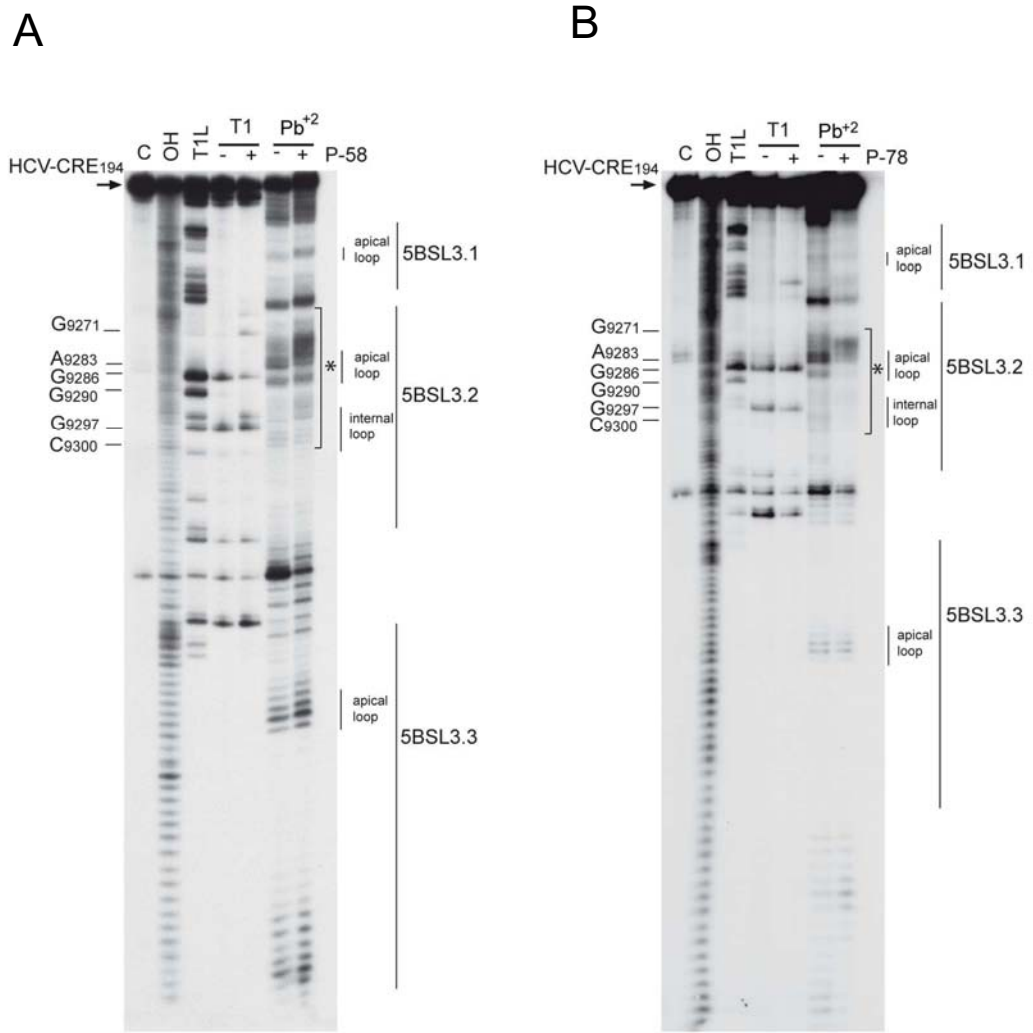


Figure 5

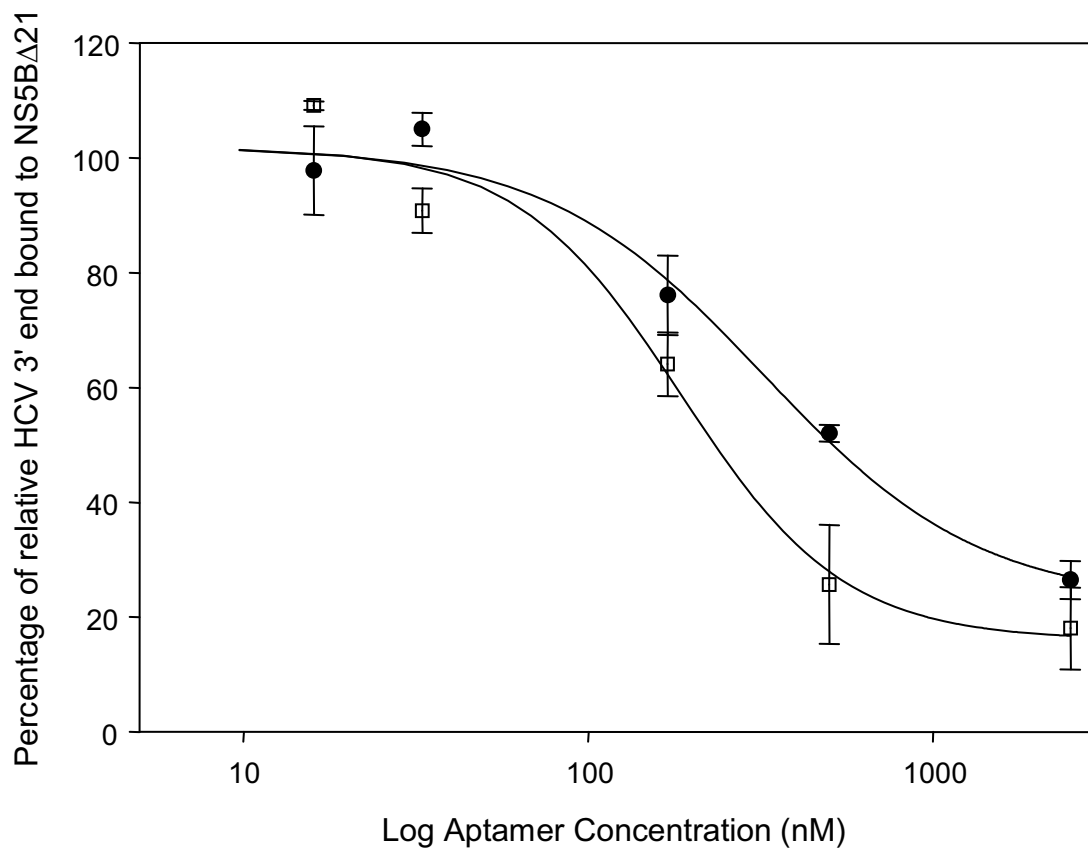
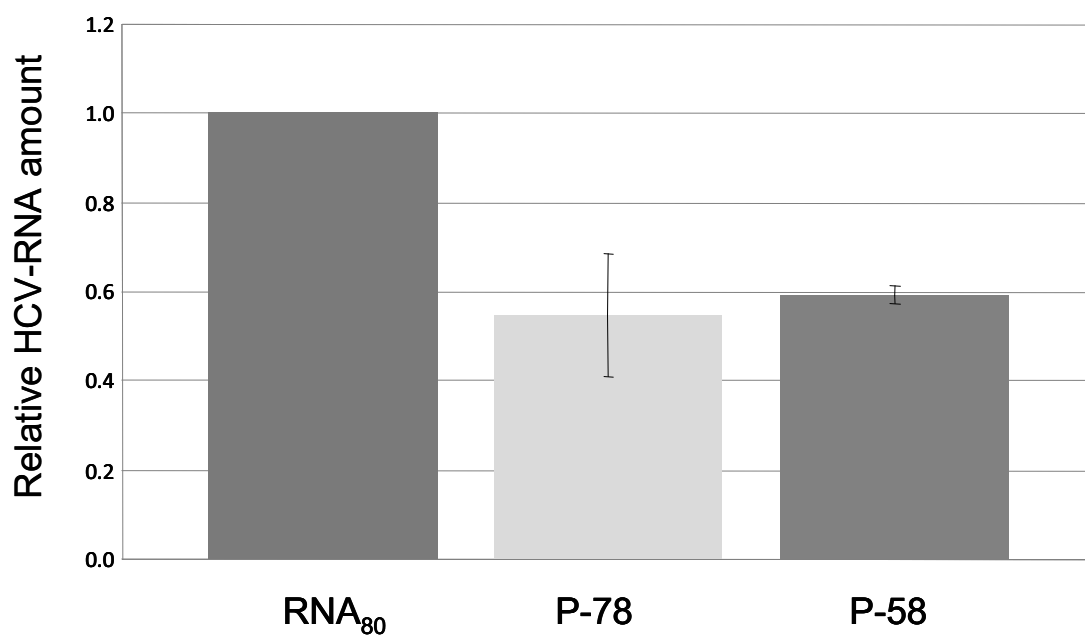


Figure 6



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