Catalytic RNase P RNA from *Synechocystis sp*. cleaves the hepatitis C virus RNA near the AUG start codon

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Abstract Previously, we described two RNA structural motifs in the hepatitis C viral (HCV) genome that can be processed in vitro by human ribonuclease P (RNase P) enzyme [J. Biol. Chem. 277 (2002) 30606]. One of these structures is located in the internal ribosome entry site and is conserved in the related animal pestiviruses [J. Biol. Chem. 278 (2003) 26844]. Here, we tested two prokaryotic RNase P ribozymes (P RNA) against this conserved structural motif. In vitro experiments indicated that P RNA from *Synechocystis* sp. can specifically process the viral transcript preparations in a position close to the human RNase P cleavage site. This provides additional support for the presence of an RNA structure similar to tRNA near the AUG start codon and suggests that *Synechocystis* P RNA may be an active agent for HCV antigenomic interventions.

Keywords: Hepatitis C virus; tRNA-like; Ribozymes; Ribonuclease P

1. Introduction

Hepatitis C virus (HCV) is an enveloped virus classified in the *Flaviviridae* family. The genome consists of a single, 9600-nt non-segmented strand of RNA with a plus polarity. The genome comprises a 5′ untranslated region (5′ NCR), which functions as an internal ribosome entry site (IRES), a long open reading frame that encodes a polyprotein precursor of about 3010 amino acids, and a 3′ untranslated region [3].

It has been estimated that more than 1% of the world population is infected with HCV. The virus establishes a chronic infection in up to 85% of cases. HCV infection has become the most common cause of hepatocellular carcinoma and is the primary reason for liver transplantation among adults in Western countries [4]. Currently, there is no vaccine for HCV infection, and the only therapy for chronic hepatitis C is not completely effective, very expensive and has important adverse effects. Improved treatment for chronic HCV is clearly needed.

The use of RNA molecules as therapeutic agents is receiving increasing attention and among these are the ribozymes. The capacity of ribozymes to specifically inactivate other RNAs makes them potentially valuable tools for gene therapy against HCV. Up to now, hammerhead and hairpin ribozymes have been used, almost always against the 5′ NCR of HCV (the most highly conserved among the different isolates) in a sequence-specific manner (reviewed in[5]). Any approach directed to improve HCV therapy must deal with the high sequence variability of the viral genome that allows the virus to escape treatment attempts.

We have focused on developing an antigenomic strategy directed against RNA viral structures instead of viral sequences. The basis for this approach resides in the concept that although the viral genome undergoes mutations, its functional structures are theoretically more conserved [6] and, therefore, remain susceptible to treatment.

Ribonuclease P (RNase P) is a ubiquitous enzyme present in the three domains of life: Archaea, Bacteria and Eukarya [7]. RNase P recognizes and cleaves the 5′ end of all the various species of pre-tRNA present in cells [8]. But this is not the only substrate of RNase P; several other natural and artificial substrates have been identified. In *Escherichia coli*, RNase P can process RNAs, such as the precursors to 4.5 S RNA [9], tmRNA [10], a polycistronic pre-mRNA [11] and a small phage RNA [12]. Thus, this enzyme has evolved to recognize and process various substrates. This enzymatic plasticity has proved to be useful for identifying tRNA-like structures in divergent viral genomes [2,13].

In bacteria, the RNase P enzyme is composed of an RNA subunit (P RNA) and a protein subunit. The RNA subunit is the catalytic component; it can cleave substrates in the absence of the protein [14].

In a previous study, we described two tRNA-like structures in the first one-third of the viral genome that can be specifically cleaved by human RNase P [1]. One of these structures, located in the 5′ region of the viral genome, was found to be conserved in the genomes of viral isolates from different patients (Piron et al., unpublished results), and even in related animal pestiviruses [2]. Therefore, it can be considered a highly conserved structural element.

The presence of these structural motifs in the HCV genome opens the door to new antiviral strategies with RNA ribozymes that recognize tRNA-like molecules, e.g., bacterial RNase P ribozymes. In this study, we tested the ribozymes from *E. coli* and *Synechocystis* sp.

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Our results indicate that cyanobacterial P RNA specifically cuts the HCV genome in the IRES of the virus in vitro and it does so in a position very close to where cleavage of the human enzyme maps.

2. Materials and methods

2.1. Preparation of ribozymes and RNA substrates

The RNA transcripts used as substrates in the cleavage assays were derived from the following: plasmids pN (1–4728) Bluescript, containing nt 1 to 4728 of hepatitis C virus under the T7 promoter; a pUC18, containing a PCR product which includes the T7 promoter + the HCV sequence from nucleotides 1 to 418 followed by an exogenous *Eco*RI DNA cleavage site; a pUC19 TyrT, containing the sequence of the naturally occurring precursor to $tRNA^{Tyr}$; and a plasmid containing a 400-nt unrelated RNA corresponding to hepatitis B virus (HBV) surface antigen mRNA. Uniformly labeled RNA substrates were obtained by in vitro transcription of 1–2-μg DNA templates (1 h at 37 °C) in the presence of [α-^{32}P]GTP or [α-^{32}P]ATP followed by treatment with RNase-free DNase I for 5-min at 37 °C. Cellulose CF11 chromatography was used to eliminate DNA fragments and non-incorporated nucleotides. Transcripts were then purified by gel electrophoresis under denaturing conditions on 4% polyacrylamide gels containing 7 M urea. Bands were visualized by autoradiography, excised from the gel and eluted in T1 buffer (100 mM Tris–HCl, pH 7.5, and 10 mM EDTA, pH 7.5). The concentration of radioactive transcripts was determined by calculating the amount of incorporated [α-^{32}P]NTP based on scintillation counting. In order to obtain 3′ end labeled transcripts, uniformly labeled and unlabeled RNAs were both transcribed, run in parallel and bands were eluted from acrylamide gel. The "cold" transcript was subsequently labeled with T4 RNA ligase and [^{32}P]pCp 5′ triphosphate under conditions similar to those described by the manufacturer. The reaction was carried out in 30 μL of 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 0.01% BSA, and 10% DMSO. The reaction mixture was incubated overnight (12–15 h) at 4 °C. The labeled RNA was purified again using the electrophoretic procedure described above. For the 5′ end labeled transcripts preparations, "cold" transcripts prepared as before were labeled with [α-^{32}P]GTP and the enzyme guanylyl transferase.

pFL117 and pT76803 were used as DNA templates for the T7-dependent runoff transcription of *E. coli* P RNA and *Synechocystis* sp. PCC6803 RNase P RNA, respectively. An unlabeled and a uniformly labeled transcription were done in parallel and gel purified. The amount of isotopically labeled RNA enzyme was measured in a scintillation counter and it was estimated that the yield of recovery of unlabeled RNA enzyme was equivalent. Unlabeled ribozymes were used in the cleavage assays. Different purification of the ribozymes gave identical results.

2.2. Cleavage assays

For the M1 cleavage assays, re-natured ribozyme (1.8 nM) was incubated with substrate transcripts (1.8 nM) in a volume of 10 μL for 60 min at 37 °C in 50 mM Tris–HCl, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$ and 4% (wt/vol) polyethylene glycol. For the cyanobacterial P RNA assays, varying amounts of the ribozyme were incubated in assay buffer (50 mM Tris–HCl, pH 7.5, 100 mM $MgCl_2$, and 1 M KCl) for 15 min at 37 °C before addition of the substrate (1.8 nM). Ionic conditions have been obtained from previous work of Pascual and Vioque in [17]. In the present study, titration of Mg^{2+} concentrations from 10 to 100 nM revealed that cleavage of tRNA precursor and HCV RNA starts at 50 nM and increases at 100 nM (data not shown), in agreement with previously mentioned authors.

Reactions were stopped by addition of 2 volumes of gel loading buffer. Cleavage products were separated on denaturing polyacrylamide gels and visualized by autoradiography.

2.3. Partial and total T1 nuclease digestion of RNA

For partial RNase T1 reaction, the digestion mixture (10 μL) contained about 2×10^4 Cerenkov cpm of the 3′ end labeled transcript and 2.5 μg tRNA of carrier in T1 buffer. The cleavage reaction was initiated by addition of 0.001 μg nuclease T1, incubated at 37 °C for 20 min. The reaction was stopped by addition of 2 volumes of gel loading buffer and products were resolved on 8% denaturing polyacrylamide gel. For total RNase T1 digestion, similar conditions were employed, but the amount of nuclease in the reaction was 1μg/μl final concentration, and the products were run in 20% denaturing polyacrylamide gel. Gels were visualized by autoradiography.

2.4. Nucleotide sequence accession number of type 1b and HCV variants

The nucleotide sequence for the "type 1b" HCV genome used here is available in the GenBank™ data base under Accession No. S62220. The nucleotide sequence for HCV variants, var1, var 2, and var 3, presented in this article can be accessed through EMBL data base under EMBL data base Accession Nos. AY576553, AY576574, and AY576577, respectively.

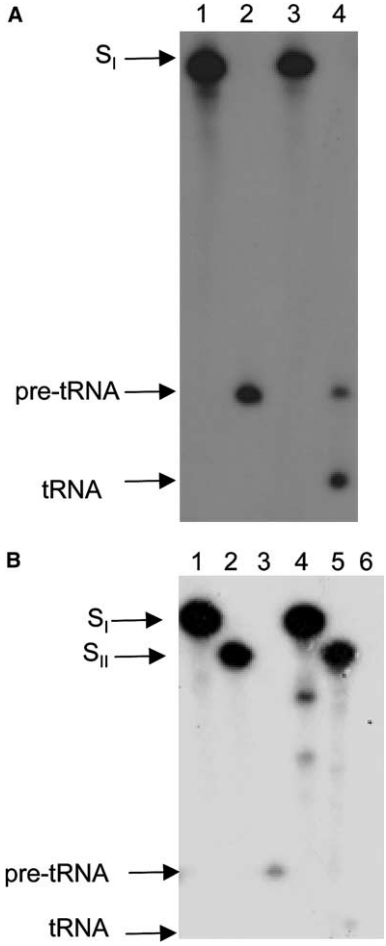

Fig. 1. Activity of P RNA from *E. coli* and *Synechocystis* in a cleavage reaction over an HCV transcript. S_I is an HCV *Blp*I 570 transcript containing the 5′ NCR and the beginning of the core region of the HCV genome. S_{II} is a 400-nt unrelated RNA corresponding to hepatitis B virus (HBV) surface antigen mRNA. As a positive control, pre-tRNA was used. (A) Autoradiogram of the *E. coli* P RNA assay of cleavage of S_I and pre-tRNA substrates (1.8 nM). Lanes 1 and 2 represent the control reaction in the absence of ribozyme. Lanes 3 and 4 represent the reaction in the presence of ribozyme (1.8nM). (B) Autoradiogram of cyanobacterial P RNA cleavage of S_I, S_{II} and pre-tRNA substrates (1.8 nM). Lanes 1, 2 and 3 represent the control reaction in the absence of ribozyme. Lanes 4, 5 and 6 represent the reaction in the presence of ribozyme (67.5 nM).

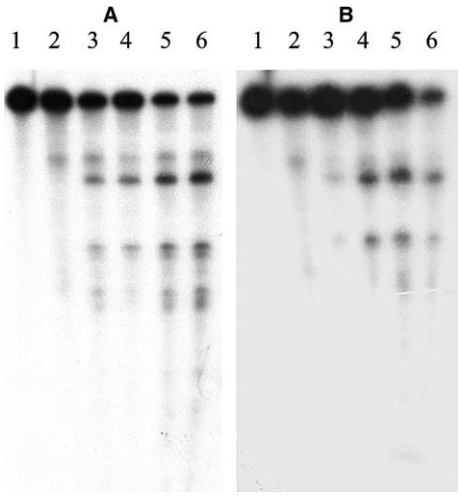

Fig. 2. Kinetics of HCV IRES cleavage by cyanobacterial P RNA. The HCV *Blp*I transcript was incubated with increasing amounts of enzyme (A, lanes 3–5) or at different incubation periods (B, lanes 3–5). Lane 1 shows the transcript alone, lane 2, the control reaction in the absence of ribozyme. (A) The HCV *Blp*I transcript was incubated with P RNA for 120 min with 8.4 nM (lane 3), 16.9 nM (lane 4), 33.75 nM (lane 5), and 67.5 nM (lane 6) of ribozyme. (B) The HCV *Blp*I transcript was incubated with 67.5 nM of cyanobacterial P RNA during 60 min (lane 3), 120 min (lane 4), 180 min (lane 5) and 240 min (lane 6).

3. Results

3.1. *E. coli* P RNA does not cleave HCV RNA

We found that the HCV transcript (1–570) was not cleaved, under optimal in vitro conditions, by the *E. coli* ribozyme, as shown in Fig. 1A. Even when we tried with an excess of ribozyme we obtained identical negative results (data not shown). Furthermore, the transcript was resistant to cleavage by *E. coli* holoenzyme (data not shown).

3.2. *Synechocystis P RNA specifically cleaves an HCV transcript in vitro*

In contrast to the *E. coli* ribozyme, the *Synechocystis* ribozyme does not require a CCA sequence at the 3′ end of the pre-tRNA substrate for efficient activity; this sequence motif is not present in the viral genome [17]. As shown in Fig. 1B, *Synechocystis* P RNA cleaved the 570-nt HCV transcript although with lower efficiency than cleavage of tRNA precursor.

However, a similar amount of an unrelated 400-nt RNA that is not cleaved by the human enzyme was not affected by the *Synechocystis* ribozyme, indicating the specific nature of the reaction on the HCV transcript. The ribozyme cleaves the viral transcript in a single position and yields two products, around 2/3 (larger) and 1/3 (shorter) in length. Fig. 2A shows the direct

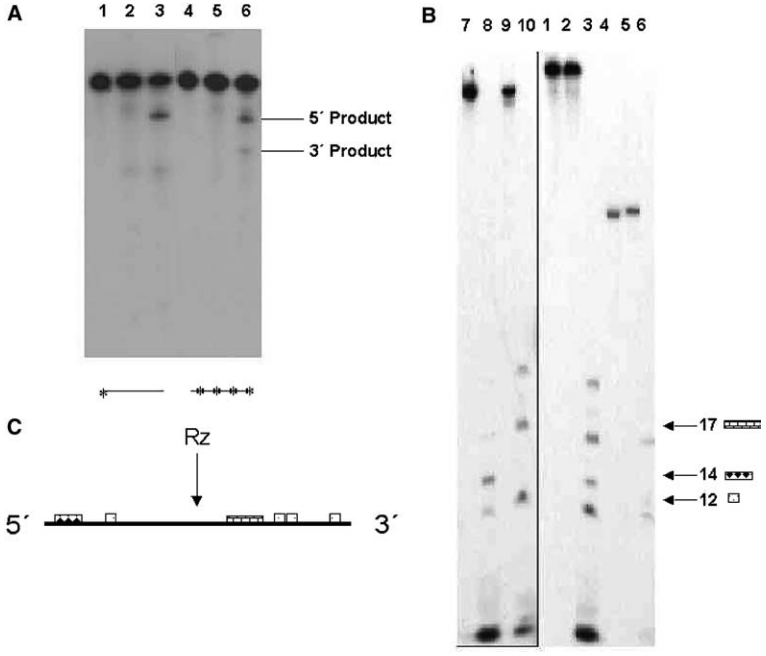

Fig. 3. Mapping the cleavage site interval in the HCV *Blp*I RNA transcript (1–570). (A) Determination of the cleavage polarity. Cleavage of an asymmetrically, 5′ end labeled transcript (lanes 1–3), in parallel to another uniformly labeled transcript (lanes 4–6). Lanes 1 and 4 show the transcript alone, lanes 2 and 5, the control reaction in the absence of ribozyme, and lanes 3 and 6, the reaction in the presence of ribozyme (64.5 nM). The HCV *Blp*I transcript (1.8 nM) was incubated with the ribozyme for 120 min. (B) RNase T1 mapping the 5′ and 3′ fragments products of *Blp*I RNA in a 20% acrylamide-urea electrophoresis. The largest T1 oligonucleotides of *Blp*I RNA (12–17 bases long) in the uncut RNA or its 5′ and 3′ fragment products were identified by comparison to the band mobility of an internal HCV RNA transcript "tracer", containing bases (A330 to C389) of known RNase T1 pattern (Piron unpublished). Lanes 1 and 2, uniformly labeled *Blp*I transcript alone and incubated in RNase T1 buffer, respectively. Lane 3, RNase T1 complete digestion of the uncut substrate. This pattern contains all the RNase T1-resistant oligonucleotide bands of the *Blp*I RNA. Lanes 4 and 5 are the "tracer" HCV RNA transcript alone and incubated with RNase T1 buffer, respectively. Lane 6 is the total RNase T1 digestion of "tracer" RNA. It contains the 17-base resistant product $_{351}$AAUCCUAAACCUCAAAG$_{367}$. Lanes 7 and 8 correspond to the ribozyme 5′*Blp*I RNA cleavage product, untreated and completely digested with RNase T1, respectively. Lane 8 lacks the 17-base band. Lanes 9 and 10 correspond to the ribozyme 3′*Blp*I RNA cleavage product untreated or completely digested with RNase T1, respectively. In contrast to lane 8, lane 10 contains the 17-base band. Other 14- and 12-base length resistant oligonucleotides could be traced similarly to the 17-base length, as is indicated in the (B) and (C) with filled boxes. (C) HCV *Blp*I transcript is represented with a black line. The localization of the largest oligonucleotides (boxes) generated by T1 digestion is shown. The localization of the cleavage produced by the ribozyme (Rz) is indicated.

relationship between the amount of ribozyme and the percentage of cleavage. Fig. 2B shows the kinetics of *Synechocystis* P RNA cleavage of the HCV transcript (1–570), showing an increasing proportion of cleavage over a 240-min time course. We notice that different preparations of RNase P RNA of *Synechocystis* have been used, ones directly after RNA transcription and purification and that folding of the ribozyme before each experiment is required. These two factors may explain, at least in part, variations of cleavage efficiency between different experiments.

To determine whether the cleavage is maintained in a larger RNA transcript and to know the polarity of the cleavage, we assayed three transcripts that were identical at the 5′ end (position 1 of the HCV genome) and progressively longer at the 3′ end (positions *Aat*II: 402, *Blp*I: 570 and *Bam*HI: 1360 of the HCV genome, respectively). In the presence of the ribozyme, the three transcripts rendered one product of identical length which coincides with the ''largest'' fragment of the 570-base substrate cleavage reaction and another of a different length in each reaction (data not shown). We can infer that the ''largest'' product of identical size in the three reactions must localize at the 5′ end of the transcripts. A parallel cleavage reaction of the 570-base transcript uniformly labeled or labeled at the 5′ end rendered a common 2/3 length fragment product, supporting the proposed polarity of the cleavage (Fig. 3A).

3.3. Mapping the cyanobacterial RNase P RNA cleavage site in the HCV IRES

In a first attempt to localize more precisely the RNase P cleavage site, an internally labeled 570-base was digested with the ribozyme. The uncut substrate as well as the 5′ ''largest'' and 3′ ''shortest'' products were eluted from the gel and subjected to nuclease RNase T1 total digestion. The products of this reaction were run in 20% polyacrylamide gel in parallel with a known pattern of RNase T1 products. This pattern was originated by a total RNase T1 digestion of a transcript corresponding to bases 330 to 389 of HCV genome, which covers the relevant sequence analyzed here (Piron unpublished). We observed that a ''marker'' 17-base RNase T1-resistant oligonucleotide (bases $_{351}$AAUCCUAAACCUCAAAG$_{367}$) was absent from the large 5′ fragment but present in the shorter 3′ product (Figs. 3 and 4B). This result implies that cleavage is upstream of position 351 of HCV genome (summarized in Fig. 3C).

To allow determination of the cleavage site, a 402 nt transcript was labeled at the 3′ end and incubated with nuclease T1 to give partial digestion. In parallel, this substrate was incubated with the cyanobacterial ribozyme (Fig. 4A). In Fig. 4A, lane 1, partial digestion of this substrate with nuclease T1 rendered a ladder of oligonucleotides starting at HCV nt number 402 and ending at every ''G'' of increasing length. Two large gaps without RNase T1 sensitive sites could be clearly

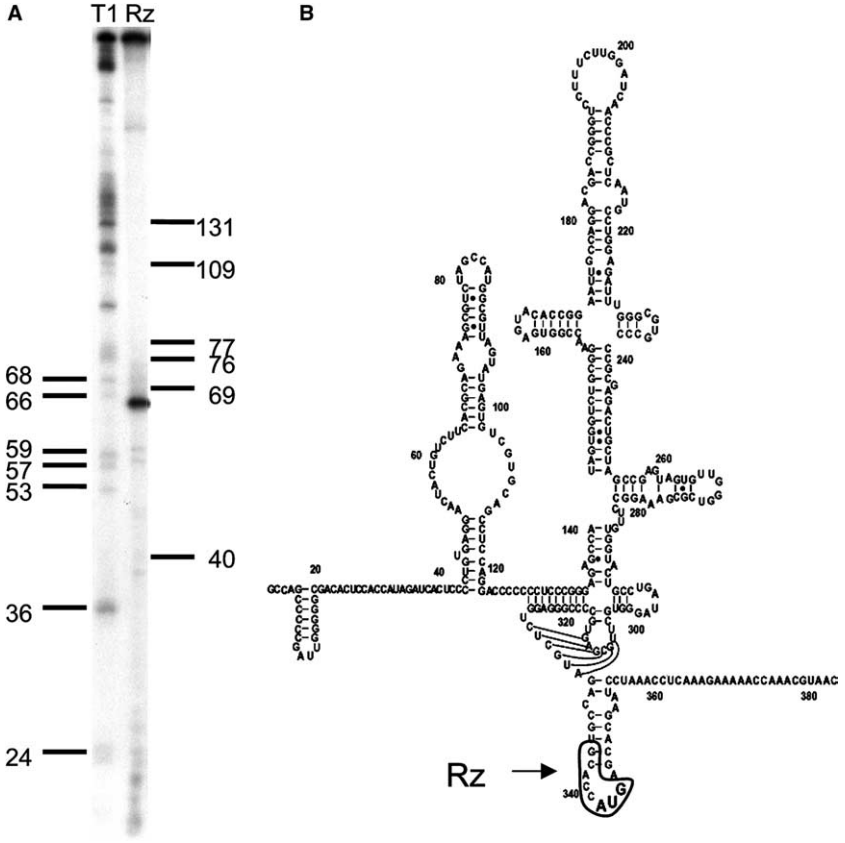

Fig. 4. Determination of the cyanobacterial P RNA cleavage site. The reaction was performed with the HCV *Aat*II transcript (1–402 nts) labeled at the 3′ end (1.8 nM). (A) T1 represents the nuclease partially hydrolyzed size marker. Primary cleavage sites are indicated at the left; the numbers refer to nucleotide positions in the HCV transcript sequence. Rz represents the cleavage reaction with cyanobacterial P RNA (67.5 nM). The numbers at the right refer to the electrophoretic position of transcripts of known length. (B) Positioning of the cleavage site on the predicted secondary structure of the HCV IRES.

identified. These gaps could match the 12-base resistant sequence between positions A369 and G380 followed by the "marker" 17-base resistant sequence A351 to G368. These two gaps permit to follow the HCV RNase T1 pattern in this region. In comparison with the RNase T1 pattern, in Fig. 4A, lane 1, the 3′ product of the ribozyme reaction in lane 2 migrated to a position between the fragment ending at G344 and the next ending at C338. This allows to map the cleavage site at the interval between 59 and 66 bases away from the 3′ end of the 402 bases fragment (Fig. 4A).

In a secondary structure model of the HCV IRES, cleavage is localized in domain IV of the HCV IRES and 5′ to the AUG codon (Fig. 4B).

3.4. *Synechocystis RNase P RNA cleaves HCV variants*

To define the cleavage by RNase P RNA as a general property of HCV RNA, three viral sequences obtained from different patients (Fig. 5A), together with the sequence used in this study (referred to as type 1b here), were compared for RNase P RNA accessibility (Fig. 5B). We used transcripts from cloned HCV PCR fragments corresponding to HCV genome positions 1–418, carrying mutations in the vicinity of the AUG start codon. Mutations reside within the minimal RNA fragment which is readily recognized by the human RNase P enzyme and proposed to be the tRNA-like motif (Piron, unpublished). Cleavage was consistently observed in all sequences tested with similar efficiencies. The appearance of two bands probably implies a new secondary cleavage which is not yet characterized.

4. Discussion

This study demonstrates that the RNase P ribozyme from the cyanobacterium *Synechocystis* sp. was able to specifically cleave the IRES of hepatitis C viral RNA in vitro, whereas the *E. coli* enzyme, M1 RNA, was unable to perform this reaction. M1 catalytic activity is highly dependent on the presence of a 3′ CCA terminus on its natural tRNA substrates [15,16], a sequence motif that is not present in the tRNA-like motif of the HCV IRES. In contrast, the cyanobacterium ribozyme does not present this dependence [17] and can effectively cleave

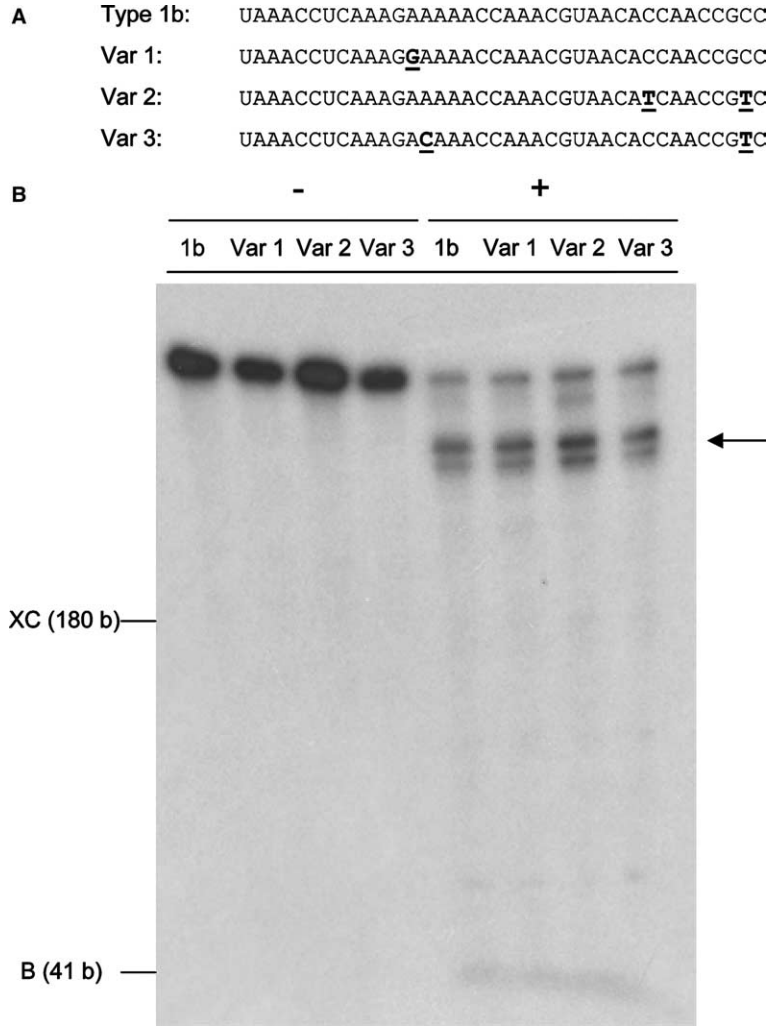

Fig. 5. RNase P RNA cleavage of HCV 1–418 RNA variants. (A) Sequence of the HCV variants with mutations between positions 356 and 393 is indicated in bold and underlined. (B) Autoradiogram shows the RNase P RNA cleavage of three different variants: var 1, var 2, var 3 and "type 1b" RNA. +, presence of the ribozyme in the reaction. The 5′ cleavage products are indicated by an arrow. XC and B indicate, respectively, the xylene-cyanol and bromophenol blue.

HCV RNA. The position of cleavage resides within the recently determined minimal RNA fragment recognized by human RNase P (Piron, unpublished results), indicating that the ribozyme recognizes the same structural RNA motif. Although this was not a surprising finding, the fact that the two enzymes are of very different nature–human RNase P is a ribonucleoprotein composed of RNA and at least nine proteins, and the *Synechocystis* RNA moiety is only a ribozyme–supports the concept that the HCV RNA structure near the AUG is similar to a tRNA molecule.

It is known that some forms of the catalytic RNA moiety from *E. coli* P RNA (either specifically modified or selected in vitro) can be introduced into mammalian cells for the purpose of carrying out targeted cleavage of mRNA molecules. In this sense, the *Synechocystis* ribozyme might be a candidate tool for inactivation of HCV RNA within the cell. This strategy is supported by the assumption that viral RNA is not a natural substrate for endogenous human RNase P activity. This assumption is, in turn, based on several observations that argue against an active role of endogenous RNase P cleavage in HCV biology: the presence of HCV RNA in the nucleus (where most RNase P is found) has never been demonstrated [18] and no evidence of subgenomic HCV RNAs has been reported [19].

In previous studies, the IRES portion of HCV RNA has been considered a target region for inactivating HCV with the use of nucleic acids, including complementary oligonucleotides, ribozymes and aptamers [5]. There are two reasons for this: (1) Cleavage within the viral IRES should, in theory, eliminate translation of the polyprotein; hence, no individual proteins should be produced. (2) The 5′ UTR sequence is the most conserved among different viral isolates. However, regarding this second argument, fine analysis of the sequence composition of the virus population within single viral isolates has demonstrated that variants carrying single and double mutations are frequent, and that these might be responsible for viral evasion of treatment when positive selection pressure is introduced with an antigenomic agent. Recognition of structures instead of sequences may represent a great advantage in the fight against the highly variable HCV. As an indicative of the requisite for conservation of this structural motif is the fact that it is conserved even in HCV-related pestiviruses, classic swine fever virus, bovine diarrhea virus and border disease virus [2] because it is implicated in HCV IRES function [20,21]. In fact, several sequences with point mutations in the HCV IRES tRNA-like motif obtained from infected patients show similar sensitivity to synechocystis sp. ribozyme.

Although the activity of the natural *Synechocystis* ribozyme is low, methods including in vitro selection of mutated variants or engineered molecules can be set up to increase its antiviral efficacy.

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