Activity of HDV ribozymes to trans-cleave HCV RNA

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INTRODUCTION
Ribozymes are sorts of small RNAs which have catalytic activity, and some of them can bind specifically through Watson-Crick base pairs with and trans-cleave substrate RNA in appropriate target sites[1-4]. Hammerhead ribozymes and hairpin ribozymes have been tried widely to cleave many target RNAs, such as pathogenic microbes RNA[5-10], oncogene mRNA[11-15] and other kinds of pathogenicity-associated functional mRNA[16-18]. A great deal of experiments have demonstrated that these ribozymes, if well-designed, could be applied as antiviral or antitumor gene therapeutic drugs. Yet there are still few researches showing whether HDV ribozymes, owning a kind of pseudoknot-like secondary structure, have the ability to kill pathogenic virus or not. HDV ribozymes include genomic ribozymes (g.Rz) and antigenomic ribozymes (ag.Rz), of them the latter is the duplicate intermediates of HDV[19,20]. This study was to evaluate the ability of g.Rz to trans-cleave HCV RNA at molecular levels.

MATERIALS AND METHODS
Reagents
All the cDNA of ribozymes were synthesized in a DNA synthesizer and purified with 160 g·L⁻¹ denatured (7 mol·L⁻¹ urea) polyacrylamide gel electrophoresis in Shanghai Sangon Bioengineering Company. Plasmid pHCV-neo was kindly provided by Dr. Wang working in our institute. Polymerase chain reaction (PCR) reagents and T7 transcription kit (Ribomax ™) were purchased from Sino-American Bioengineering Company. Agarose Gel DNA Purification Kit, calf intestinal alkaline phosphatase (CIP) and T4 polynucleotide kinase were bought from Boehringer Mannheim Co. γ⁻³²P-ATP was the product of Beijing Yahui Co.and KODAK X-ray film was chosen to do autoradiography.

Preparation of template for preparation of substrate in vitro
pHCV-neo contains full length of HCV RNA 5'-NCR (341nt), translation-initiating codon AUG and 5'-fragment of C region (90nt). Sequence of HCV RNA 5'-NCR-C has been proved to be identical with that of HCV strains isolated from Chinese people reported by Bi et al. pHCV-neo has T7 phage promoter sequence ahead of HCV cDNA. Transcription template of HCV RNA 5'-NCR-C was prepared by the PCR method. The upper primer was T7 phage promoter sequence: 5'-TAA TAC GAC TCA TAG-3', and the reversed primer identified with the sequence from 413th to 383th nucleotide (nt) of HCV genome: 5' -GCGGATCCCCGGAATTGCTCCTG-3' (the italic letter was cleavage site of BamH I designed for future cloning). Route PCR process was adopted. PCR products were purified by Agarose Gel DNA Purification Kit, and the unpaired A at 3' end was cut off with Klenow enzyme. All these procedures were made according to the kit guidebook. After that, PCR products were regainged by phenol (pH8.0)/chloroform/isooamyl alcohol (volume ratio 25:24:1) extraction, precipitated with ethanol, and redisolved with RNAase-free water.

Design of ribozymes and preparation of their transcription templates
Based on the structure of our formerly reported g.Rz55, we designed here three kinds of HDV RNA-targetting g.Rzs namely Rx2C1, Rx2C2 and Rx2C3 respectively. The target sequences and their location in HCV RNA 5'-NCR-C are shown in Table 1. Routine PCR process was adopted to prepare the transcription templates of the ribozymes. PCR templates were formerly synthesized cDNA of these ribozymes. The upper primer contained T7 phage promoter

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sequence (Italic letters): 5’-TAATACGACTCACTATAG TCTAGAGTCCAGCTCTCTGCGGC-3’, and the reversed primer was 5’-CTGGGATCCGTCAGCTAGGCGATTCCAAG AAGATTGTGCCGCCC-3’. Purification of PCR products and treatment of their 3’-end were the same as that mentioned above.

Table 1 Target sequences and their location in HCV RNA 5’-NCR-C

| Target sequence | Location in HCV RNA 5’ NCR-C | Ribozyme |
|-----------------|-------------------------------|----------|
| 5’-CGU | GCCAGCU-3’ | 107-113 nt | RzC1 |
| 5’-GGU | GGGUGCC-3’ | 268-274 nt | RzC2 |
| 5’-CAU | GAGACG-3’ | 345-351 nt | RzC3 |

Transcription in vitro

Each transcription reaction was done under 37 ℃ for 4 h, with a total volume of 20 µL containing template DNA 1.5 µg, α-32P-nITP (preservation concentration 25 mmol·L⁻¹) 6 µL, 5× transcription buffer 4 µL, T7 RNA polymerase 2 µL (preservation concentration 1 µg·µL⁻¹). Digestion of DNA templates, purification and quantification of transcription products were executed according to the kit guidebook.

Radiolabel of substrate RNA at 5’-end

Phosphate at the 5’-end of substrate RNA was deleted with CIP, then the substrate RNA was radiolabelled at the 5’-end with T4 polynucleotide kinase and γ-32P-ATP. After that, substrate RNA was purified by routine phenol (pH4.5)/chloroform/isoamyl alcohol (volume ratio 25:24:1) extraction, precipitated with ethanol, and redissolved with RNAase-free water and then quantified, and stored at -20 ℃. All operations were made according to the guidelines.

Trans-cleavage reaction and measurement of effects-time relationship

Trans-cleavage reaction was done under conditions with or without deionized formamide having a final concentration of 2.5 mol·L⁻¹. Each reaction system contained: radiolabelled substrate RNA 50 nmol·L⁻¹, ribozymes 5 µmol·L⁻¹, Tris·Cl (pH7.5) 50 mmol·L⁻¹, with a total volume of 10 µL. The standard reaction protocol was as follows: heat the tube containing the reaction mixtures to 95 ℃ for 3 min → place on ice for 10 min → dip into 37 ℃ water for another 10 min → add prewarmed MgCl₂; to the final concentration of 20 mmol·L⁻¹ → keep the reaction temperature at 37 ℃ for 2 h → separate reaction products by electrophoresis on a 80 g·L⁻¹ polyacrylamide gel containing 7 mol·L⁻¹ urea → display the products by autoradiography at -70 ℃ (the X-ray film was exposed for 0.5 µs beforehand) for about 24 h → measure A value of the images with Gel Documentation-Analyzing Systems (Gel Doc™ 2000, Bio-Rad) and then calculate the percentage of substrate cleaved by the ribozymes.

Under the optimized cleavage conditions, following reactions were made in four tubes containing 15 µL mixtures each, of which three reactions were designed for each ribozyme to trans-cleave the target RNA, separately, and the fourth reaction for all the three ribozymes to trans-cleave the substrate RNA simultaneously and cooperatively. The reaction procedures were the same as that mentioned above, except that after MgCl₂ was added to the mixtures, the reactions were terminated at 10, 30 and 90 min time points. Five µL solution was removed from each tube at different time points for investigating the effects-time relationship.

Percentage of cleavage (%) = (A value of cleaved substrate RNA / (A value of cleaved substrate RNA + A value of uncleaved substrate RNA)) × 100%

RESULTS

Effects of the ribozymes to trans-cleave substrate RNA under different conditions

Transcription templates of RzC1, RzC2, RzC3 and HCV RNA 5’-NCR-C were proven to be obtained successfully by 20 g·L⁻¹ agarose gel electrophoresis. The ribozymes and substrate RNA were successfully synthesized by transcription in vitro known from 80 g·L⁻¹ denatured (7 mol·L⁻¹ urea) polyacrylamide gel electrophoresis.

According to the design, HCV RNA 5’-NCR-C should be trans-cleaved into 106nt (5’ product) and 324nt (3’ product) fragments by RzC1, 267nt and 163nt fragments by RzC2, and 344nt and 86nt fragments by RzC3. As HCV RNA 5’-NCR-C was radiolabelled at its 5’-end, only the 5’-products were displayed in X-ray films after autoradiography. The results showed that RzC1 and RzC2 are able to trans-cleave HCV RNA 5’-NCR-C under the selected reaction conditions, and the length of their cleavage products was set in accordance with the design. Percentage of substrate RNA cleaved by RzC1 and RzC2 under the conditions without deionized formamide at 2 h time point was 4.2 % and 3.5 %, respectively. With the addition of deionized formamide to the trans-cleavage reaction systems to a final concentration of 2.5 mol·L⁻¹, percentage of cleaved substrate RNA reached up to 26 % and 21.8 %, respectively at the same time point (Figure 1). It was surprising that under both conditions with or without deionized formamide, RzC3 almost had no trans-cleavage activity to HCV RNA 5’-NCR-C. Based on the whole experiment, we think that the reaction conditions with deionized formamide was better than that without it, thus being adopted in the future investigations.

Figure 1 Effects of RzC1, RzC2 and RzC3 to trans-cleave HCV RNA 5’-NCR-C under different conditions (results came from image analysis).

Roles of the three ribozymes to trans-cleave substrate RNA at different time points

Under the optimized reaction conditions, fragments of HCV RNA 5’-NCR-C trans-cleaved by RzC1 and RzC2 were produced at the fixed time points (Figures 2 and 3). When the three ribozymes were added to one tube to trans-cleave HCV RNA 5’-NCR-C simultaneously, images of 106nt and 267nt fragments corresponding to the cleavage products of RzC1 and RzC2 respectively were observed in the same X-ray film (Figure 4), yet no marked image of 344nt fragment meaning the substrate RNA cleaved by RzC3 turned up at the same time.

Effects-time relationship of the ribozymes to trans-cleave substrate RNA

Percentage of cleaved substrate RNA by RzC1 and RzC2 was
6.1% and 4.6% at 10 min time point, 14.0% and 11.7% at 30 min time point, and 24.9% and 20.3% at 90 min time point respectively. These results showed that the percentage of RzC1 and RzC2 to trans-cleave HCV RNA 5′-NCR-C increased with time. At the same time, nearly no effects of RzC3 to trans-cleave HCV RNA 5′-NCR-C was observed. The percentage of these ribozymes to trans-cleave HCV RNA 5′-NCR-C in one tube turned out to be 8.4% at 10 min, 19.5% at 30 min and 37.3% at 90 min time point respectively (Figure 5). These results demonstrated that the combination of ribozymes aiming at different target sites could be applied to cleave substrate RNA more efficiently than using only one of them.

![Figure 2](image_url) Results of trans-cleavage by RzC1. A: HCV RNA 5′-NCR-C in the system without ribozymes; B,C,D: Results of HCV RNA 5′-NCR-C trans-cleaved by RzC1 10, 30 and 90 min time points respectively.

![Figure 3](image_url) Results of trans-cleavage by RzC2. A: HCV RNA 5′-NCR-C in the system without ribozymes; B,C,D: Results of HCV RNA 5′-NCR-C trans-cleaved by RzC2 at 10, 30 and 90 min time points respectively.

![Figure 4](image_url) Results of trans-cleavage by combination of RzC1, RzC2 and RzC3. A: HCV RNA 5′-NCR-C in the system without ribozymes; B,C,D: Results of HCV RNA 5′-NCR-C trans-cleaved by the ribozymes at 10, 30 and 90 min time points respectively.

![Figure 5](image_url) Effects-time relationship of RzC1, RzC2 and RzC3 to trans-cleave HCV RNA 5′-NCR-C.

**DISCUSSION**

HDV ribozymes were first reported for their self-cleavage activity, also named cis-cleavage, which meant splicing reaction occurring within the catalytic RNA molecule itself, i.e., intramolecular cleavage[25-29]. Several years later, it was found that a self-cleavage HDV ribozyme could also be divided into two parts, one part ("ribozyme" component) still maintained the catalytic activity and the other part (homologous "substrate" component) could be cleaved by the former intermolecularly when they were taken together again. This kind of intermolecular cleavage was called trans-cleavage, and the "ribozyme" components having trans-cleavage activity were usually described as trans-HDV ribozymes[30-36]. Our previous studies have shown that trans-HDV genomic ribozyme g.Rz55 was able to trans-cleave its homologous substrate S87 with a percentage of about 69% under conditions Tris-Cl 50 mmol·L⁻¹ (pH7.5) and MgCl₂ 20 mmol.L⁻¹[22]. In recent years, some researches have found that the substrate-binding region of trans-HDV ribozymes might be changeable to extent[37]. These findings are the theoretical and experimental bases for reconstruction and application of HDV ribozymes to trans-cleave HCV RNA 5′-NCR-C.

Many studies have shown that HDV ribozymes owned a kind of special pseudoknot-like secondary structure which was different from that of hammerhead ribozymes and hairpin ribozymes[25-29], so did their requirements to the target sequences of substrate RNA[30-36]. Roy et al. reported that trans-ag.Rz was able to cleave 814nt HDAg mRNA efficiently at several target sites, and concluded that all the sequences having the characteristic of 5′-R.R.R.R.G.N₆ (N₆ = A/C/U), N₆₇, N₆₈, N₆₉, 3′ (R=A or G,Y=U or C) in HDAg mRNA might be the most possible sites to be cleaved by trans-ag.Rz[37]. The results demonstrated that the combination of ribozymes aiming at different target sites could be applied to cleave substrate RNA more efficiently than using only one of them.

In comparison of the self-cleavage and trans-cleavage
activity of HDV ribozymes at natural target sites in homologous substrate RNA and other target sites in HDV mRNA, the activity of RzC1 and RzC2 to trans-cleave HCV RNA 5'-NCR-C was a little lower, and RzC3 had almost no cleavage activity. By comparing the sequences and structure of RzC1, RzC2 and RzC3 to cis-HDV ribozymes and trans-HDV ribozymes reported by others, and the differences among the target RNAs, we suppose that the possible reasons may lie in several respects. Firstly, the target sites in HCV RNA 5'-NCR-C and the structure of trans-HDV ribozymes should be further optimized by more methods. For example, the calculation and application of the free energies required for forming pseudoknot and binding of ribozyme to substrate[38], or other strategies to map the accessible sites in substrate for ribozyme to bind[39,40]. Secondly, it is likely that the activity of trans-HDV ribozymes might be weakened if the A-U or U-A base pair just adjoined the G·U wobble pair when A-U or U-A and C-G or G-C base pairs co-existed in the binding regions of ribozymes and substrate RNA. Thirdly, the special secondary structure of HCV RNA 5'-NCR-C might impede the cleavage activity of trans-HDV ribozymes[31,41]. Fourthly, other sequences at both ends of trans-HDV ribozymes might diminish their cleavage activity[42]. Finally, different reaction conditions such as the kind of buffer might significantly influence the cleavage effects sometimes[43]. Certain denaturing agents such as deionized formamide in an appropriate concentration may reduce the formation of complex secondary structure of substrate RNA, thus improve the trans-cleavage activity to some extent[44,45]. A suitable pH of solution was very important for the trans-cleavage to occur too, and pH7.0-7.5 was commonly used during researches in vitro. Divalent cations such as Mg2+, Ca2+ and Mn2+ could bind and interact with the ribozymes’, phosphate-pentose skeleton full of negative charges[44,45], thus facilitating the ribozyme to fold into and maintain active structure, and/or take part in trans-cleavage reaction directly[46,47].

HCV is an important pathogenic factor of chronic hepatitis, and related to the formation of cirrhosis and occurrence of hepatocarcinoma or cholangiocarcinoma[48]. Finding new ways to control HCV infection is difficult but necessary. IRES and translation-initiating codon of HCV RNA are usually chosen as the trans-cleave target sequences not only because they are the important functional regions, but also they are very conservative in each HCV variant[49]. As a result, ribozymes aiming at the two regions will have a universal cleavage effects on all of the HCV variants. Effects of HDV ribozymes to trans-cleave HCV RNA 5'-NCR-C at extracellular molecular levels are not completely in accordance with that exhibited intracellularly, because intracellular conditions and factors that influence the ribozyme activity are far more complicated than the extracellular ones. On the other hand, HDV ribozymes are the only kind of viral ribozymes which exist in mammalian cells naturally, especially in human hepatocytes. Based on these opinions and facts, it is expected that the intracellular location and cleavage activity of HDV ribozymes might be more efficient than that of hammerhead ribozymes and hairpin ribozymes, thus it is necessary and valuable to assess the roles of HDV ribozymes in trans-cleaving HCV RNA intracellularly.

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