

Construction of HBV-specific ribozyme and its recombinant with HDV and their cleavage activity *in vitro*

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Abstract

AIM To construct the recombinant of HDV cDNA and HBV-specific ribozyme gene by recombinant PCR in order to use HDV as a transporting vector carrying HBV-specific ribozyme into liver cells for inhibiting the replication of HBV.

METHODS We separately cloned the ribozyme (RZ) gene and recombinant DVRZ (comprising HDV cDNA and HBV-specific ribozyme gene) into the downstream of T7 promoter of pTAdv-T vector and studied the *in vitro* cleavage activity of their transcripts (rRZ, rDVRZ) on target RNA (rBVCF) from *in vitro* transcription of HBV C gene fragment (BVCF).

RESULTS Both the simple (rRZ) and the recombinant ribozyme rDVRZ could efficiently catalyze the cleavage of target RNA (rBVCF) under different temperatures (37°C, 42°C and 55°C) and Mg²⁺ concentrations (10 mmol/L, 15 mmol/L and 20 mmol/L) and their catalytic activity tended to increase as the temperature was rising. But the activity of rRZ was evidently higher than that of rDVRZ.

CONCLUSION The recombinant of HDV cDNA and ribozyme gene had the potential of being further explored and used in gene therapy of HBV infection.

INTRODUCTION

Hepatitis B virus (HBV) can cause acute and chronic B-type hepatitis in man. The conventional ways available for curing this disease have not been very efficient. This promotes people to explore novel genetic therapeutical ways. Hammerhead ribozyme is a kind of antisense RNA which can specifically cleave the target RNA^[1,2]. In the light of this, people have developed many effective genetic vectors containing ribozyme genes, the transcripts of which showed catalytic activity *in vitro* and *in vivo*^[3-5]. But how to improve the stability and efficiency of ribozyme and specifically carry ribozyme gene into only target cells or tissues has been a tackling problem. HDV, a human hepatitis agent, is a defective RNA virus, the replication cycle of which relies on the infection of HBV^[6]. So HDV can be developed as a specific transporting and replicating vector *in vivo* for ribozyme to reach liver^[7]. In this study, we constructed HBV-specific hammerhead ribozyme gene (RZ) and the recombinant (DVRZ) of HDV cDNA (DV) and ribozyme gene (RZ) and made a careful investigation of their *in vitro* catalytic activity under various conditions. The positive results encouraged us to further explore the feasibility of using HDV as a vector carrying ribozyme for inhibiting the replication of HBV *in vivo*.

MATERIALS AND METHODS

Plasmids

The plasmid pSVC-D3 (containing HDV cDNA) as one of two templates of recombinant PCR was kindly offered by Prof. Taylor of American. pTAdv-T vector used for cloning was purchased from Clontech Corporation.

Major reagents

RiboMax transcription kit, acrylamide, bisactylamide, dNTP, rNTP and Taq polymerase were purchased from Promega. [α -³²P]UTP from Beijing Yuhui Corporation. Advantage-TM PCR pure kit (gel purification kit) from Clontech. X-film from Kodak. DNA polymerase I from Biolabs. T7/Sp6 sequencing kit from Pharmacia.

PCR primers

Primers P1 and P2 covered the whole sequence of designed HBV-specific hammerhead ribozyme gene (RZ) and were used to amplify ribozyme gene (53bp) because of 9nt base-pairing of their 3' ends.

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The sequence of HBV-specific hammerhead ribozyme was designed according to the requirement of domains of ribozyme^[1] and the sequence of HBV C gene fragment.

The ribozyme gene (RZ: 53bp) was also used as one of two templates for recombinant PCR to construct the recombinant (DVRZ) of HDV cDNA and ribozyme gene (mentioned below).

P3, P4 were both recombinant primers, each of which was composed of partial sequences of ribozyme and HDV in order to replace the sequence (17-67) of near 5'-end of HDV with ribozyme by recombinant PCR. P5 was the 3'-end sequence of HDV cDNA.

P3, P4, P5 were used to construct the recombinant (DVRZ) of HDV cDNA (DV) and HBV-specific ribozyme gene (RZ) by one-tube recombinant PCR^[8].

P6, P7 were used to amplify HBV C gene fragment (BVCF), which was the transcription template of target RNA (rBVCF). T7 was partial sequence of T7 promoter region of pTAdV-T vector and was used for sequencing (with Sp6) and identifying (with P2, P5, P7) whether 5'-end of foreign fragment forwardly inserted the downstream of T7 promoter.

The sequencing of all gene fragments was performed on ABI391 automatic sequencer (Pharmacia). The sequences and positions of these primers are listed in Table 1.

Table 1 The sequences and positions of primers

Primer name	Sequences	Positions
P1	5'-AACATTGACATAGCTCTGATGAGTCCGTGAG-3'	RZ:1-31
P2	5'-TCCAGGGAATTAGTACTTTTGCTCCTCACGGAC-3'	RZ:53-23
P3	5'-AGCAAGCTTGAGCCAAAACATTGACATAGCTCT-3'	HDV:1-16, RZ:53-37
P4	5'-CTCCGACGTTCCAATGCTCCAGGGAATTAGTACT-3'	HDV:84-68, RZ:53-37
P5	5'-GTCGAATTCGGGCTCGGGCGCGATCCAGCAGTC-3'	HDV:1680-1647
P6	5'-GATAAGCTTTTACATAGAGGACTCTTGG-3'	HBV:1650-1677
P7	5'-CTGGAATTCGGCGAGGGAGTTCTTCTTAG-3'	HBV:2480-2450

Construction and cloning of HBV-specific ribozyme gene(RZ)

Because of 9nt base-pairing between 3'ends of P1 and P2, direct PCR could produce complete 53bp ribozyme gene (Figure 1). After gel-purification (according to Advantage-TM PCR pure kit, Clontech Corporation), the PCR product (RZ) was directly cloned into the downstream of T7 promoter of pTAdV-T vector and so the resulting recombinant plasmid pTA-RZ was obtained. PCR with T7/P2 as primers and pTA-RZ as template could produce about 100 bp DNA fragment if 5'-end of ribozyme gene was forwardly inserted in to the downstream of T7 promoter of pTAdV-T vector and so could be used to identify the recombinant plasmid pTA-RZ of forward insert. The preparation of competent DH5 α cells and transformation of plasmids were performed according to the reference^[9]. The cloned ribozyme gene was finally verified by sequencing with T7/Sp6 primers.

Construction, cloning and sequencing of the recombinant (DVRZ) of HDV cDNA and HBV-specific ribozyme gene

Thirty μ L PCR reaction system was established^[8]: 20 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2 mmol/L MgCl₂, each dNTP 200 μ mol/L; Primers P3, P4, P5 were separately 0.05 μ mol/L, 0.005 μ mol/L, 0.05 μ mol/L. The two templates were recombinant plasmids pTA-RZ (containing ribozyme gene) and pSVC-D3 (containing HDV cDNA), each 10 ng. Taq polymerase 2 μ L, denaturation at 92°C-50s; annealing at 53°C-50s; elongation at 70°C-120s; 33cycles; the final elongation at 70°C lasted 5 min. PCR product (DVRZ, 1.7kb) was identified by agarose-gel (1.5%) electrophoresis and then purified with Advantage-TM PCR pure kit. The obtained DNA fragment (DVRZ) was directly cloned into the downstream of T7 promoter of pTAdV-T vector. The resulting recombinant plasmid pTA-DVRZ (positive clones on Ampr & white-blue plate) was verified by PCR with primers T7/P5 and sequencing with Sp6/T7 primers. PCR with primers T7/P5 could produce about 1.7kb-1.8kb DNA fragment and was used to identify the forward insertion of 5'-end of DVRZ. Sequencing was performed on ABI 391 automatic sequencer (Pharmacia).

Cloning and isolation of target gene fragment (HBV C gene fragment-BVCF)

PCR with primers P6, P7 was performed to amplify HBV C gene fragment from serum of HBV. Extraction of sample DNA and PCR reaction were performed according to the reference^[10]. PCR product (BVCF) was directly cloned into the downstream of T7 promoter of pTAdV-T vector and the resulting recombinant plasmid pTA-BVCF (positive clones on Ampr & blue-white plate) was verified by PCR with T7/P7 and bidirectional sequencing with T7/Sp6 primers. PCR with primers T7/P7 was utilized to identify the forward-direction insertion of 5'-end of BVCF.

Linearization of recombinant plasmids pTA-RZ, pTA-DVRZ and pTA-BVCF

These plasmids were separately digested with BamHI and then filled with Klenow fragment for *in vitro* transcription of inserted gene fragments (RZ, DVRZ, BVCF).

In vitro transcription of linearized plasmids pTA-RZ and pTA-DVRZ

³²P-UTP-labeled transcription of the two plasmids was first carried out in order to test the effect of transcription. But the plasmids' transcripts (rRZ, rDVRZ) used for catalyzing the cleavage of target RNA (rBVCF-transcript of plasmid pTA-BVCF containing HBV C gene fragment-BVCF) were not ³²P-labeled. In vitro transcription was performed with T7 RNA polymerase according to RiboMax Transcription kit and the reference^[11]. The

transcripts (rRZ, rDVRZ) would additionally contain 100nt partial sequence of pTAdV-T vector at their both ends. rRZ and rDVRZ were extracted by phenol/chloroform/iso-propyl alcohol (25:24:1), precipitated with ethanol and resuspended with H₂O (RNase-free). The latter was the recombinant of HDV and ribozyme.

Preparation of ³²P-UTP-labeled target RNA (rBVCF)

Linearized plasmid pTA-BVCF (containing HBV C gene fragment) was *in vitro* transcribed and the target RNA (rBVCF) was produced. 20 μ L transcription system was so established: ATP, CTP, GTP each 2.5 mmol/L and UTP 0.1 μ mol/L, 0.5 μ Ci/ μ L [α -³²P]UTP, 2 μ g linearized plasmid pTA-BVCF, T7 RNA polymerase 20U, RNasin 10U, 37°C-60 min. The product was loaded for 5% PAGE-7M urea autoradiographed electrophoresis. Gel-band of 1cm length and 0.5 cm breadth was cutted off from the position of target RNA and soaked overnight with NES buffer (0.5 mol/L NH₄Ac, 1 mmol/L EDTA, 0.1% SDS) and extracted with phenol/chloroform/iso-propyl alcohol (25:24:1). The supernate was precipitated with ethanol and then the pellet was harvested and resuspended with H₂O (RNase-free). rBVCF(831+100nt) contained also 100nt partial sequence of pTAdV-T vector at its two ends.

Cleavage reaction

Ten μ L reaction system was established: 0.1 mol/L Tris-HCl (pH 8.0), 20 mmol/L MgCl₂, rRZ or rDVRZ and their target RNA (rBVCF) each 2 μ L, mixed and incubated under different temperatures (37°C, 42°C, 55°C) for 1h. Negative control with ³²P-labeled rBVCF incubated at 55°C without rRZ & rDVRZ was performed. In the meantime, rRZ and rDVRZ were separately incubated with target RNA (rBVCF) at 37°C under different Mg²⁺ concentrations (10mmol/L, 15mmol/L). In the end, 10 μ L ion-free formamide and 1 μ L loading buffer (50% glycerol, 1mmol/L EDTA, 0.04% bromophenol blue) were added to terminate the reaction. Then after incubated at 65°C for 10 min, 5 μ L reaction sample was loaded for 5% PAGE-7M urea electrophoresis.

RESULTS

Construction and cloning of ribozyme gene

53bp DNA fragment was obtained by PCR with primers P1/P2 (Figure 1). This was in accordance with the size of anticipated ribozyme gene. PCR using T7/P2 as primers and plasmid pTA-RZ as the template amplified a DNA fragment of about 100 bp, which identified forward-direction insertion of ribozyme gene into the downstream of T7 promoter of pTAdV-T vector. Sequencing verified the HBV-specific ribozyme gene. The sequence of ribozyme gene was as follows: 5'-AACATTGACATAGCTCTGATGAGTCCG-TGAGGACAACTACTAATTCCTGGA-3'

Construction, cloning and sequencing of the recombinant (DVRZ) of HDV cDNA(DV) and HBV-specific ribozyme gene(RZ)

About 1.7kb DNA was amplified by recombinant PCR, which indicated the anticipated recombinant DNA molecule DVRZ (Figure 1). After DVRZ's cloning into pTAdV-T vector and transforming into DH5 α on Ampr & white-blue plate, PCR with primers T7/P5 produced about 1.8 kb-DNA fragment and identified the positive recombinant plasmid pTA-DVRZ of correct DVRZ's insertion direction. Sequencing of DVRZ with primers T7/Sp6 (pTA-DVRZ as template) confirmed the construct DVRZ.

Cloning and isolation of target gene fragment (HBV C gene fragment-BVCF)

Anticipated 831bp target DNA fragment was amplified by PCR from the serum of HBV (Figure 1). After its cloning into pTAdV-T vector and then transformed into DH5 α , PCR with primers T7/P7 produced an about 0.9 kb DNA fragment and so identified the positive recombinant plasmid pTA-BVCF of BVCF's correct insertion direction. Finally, sequencing with primers T7/Sp6 confirmed the inserted BVCF.

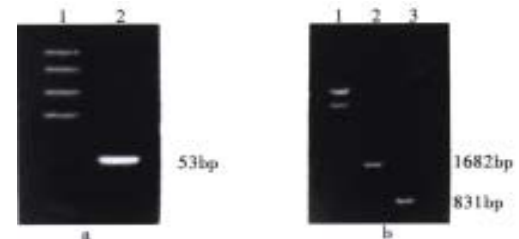


Figure 1 Construction of HBV-specific ribozyme gene (RZ), its recombinant with HDV cDNA (DVRZ) and target DNA (BVCF) by PCR. a: Lane 1-PCR marker, Lane 2-ribozyme gene RZ (53bp). b: Lane 1- λ DNA/-Hind III+*Eco* RI marker, Lane 2-recombinant DVRZ (1682bp) Lane 3-target gene BVCF (831bp).

In vitro transcription of linearized recombinant plasmids pTA-BVCF, pTA-RZ and pTA-DVRZ

Target RNA (rBVCF) of 931nt was transcribed from pTA-BVCF. HBV-specific ribozyme (rRZ) of 153nt was transcribed from pTA-RZ.

HBV-specific recombinant ribozyme (rDVRZ, containing HDV) of 1782nt was transcribed from pTA-DVRZ (Figure 2). All these transcripts (rBVCF, rRZ, rDVRZ) contained the same partial sequence of pTAdV-T vector at their both ends after transcription with T7 RNA polymerase.

Cleavage reaction

Ribozymes rRZ and rDVRZ without ³²P-label were separately incubated with target RNA (³²P-labeled rBVCF) under different temperatures (37°C, 42°C, 55°C) and Mg²⁺ concentrations. The results from autoradiographed electrophoresis showed that under these temperatures and Mg²⁺ concentrations, both

rRZ and rDVRZ could catalyze the cleavage of target rBVCF into two RNA fragments (721nt, 210nt) and their catalytic activity tended to increase with the rising of temperature. Comparatively, the catalytic activity of rRZ was higher than that of rDVRZ. But it seemed that Mg^{2+} from 10 mmol/L to 20 mmol/L had no obvious effect on their cleavage activity (Figure 3).

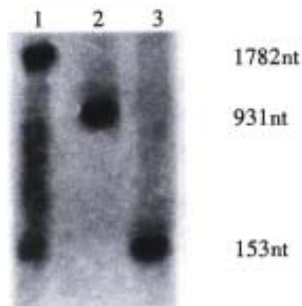


Figure 2 *In vitro* transcription of linearized pTA-RZ, pTA-DVRZ and pTA-BVCF (Lane 1-rDVRZ,1782nt. Lane 2-rBVCF,931nt. Lane 3-rRZ,153nt).

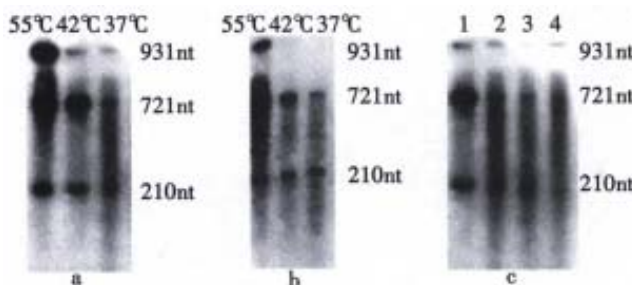


Figure 3 Cleavage of target RNA(rBVCF) catalyzed by rRZ and rDVRZ. a: Cleavage of target RNA (rBVCF) by rRZ under 37°C, 42°C and 55°C with 20 mmol/L Mg^{2+} . b: Cleavage of target RNA (rBVCF) by rDVRZ under 37°C, 42°C and 55°C with 20 mmol/L Mg^{2+} . c: Cleavage of target RNA (rBVCF) separately by rRZ and rDVRZ at 37°C under different Mg^{2+} concentrations (10 mmol/L, 15 mmol/L) (Lane 1,2-cleavage by rRZ separately under 10 and 15 mmol/L Mg^{2+} ; Lane 3,4-cleavage by rDVRZ separately under 10 and 15 mmol/L Mg^{2+})

DISCUSSION

Hammerhead ribozyme is a kind of antisense RNA with specific catalytic activity, which can catalyze the specific cleavage of target RNA^[1-3]. So far, many specific ribozyme constructs have demonstrated their catalytic activity both *in vitro* and *in vivo*^[12-15]. HDV, is a defective RNA virus, the replication cycle of which must be dependent on the infection of HBV^[6]. In view of the characteristics of hammerhead ribozyme and HDV, we constructed the recombinant(rDVRZ) of HDV and ribozyme by one-tube recombinant PCR with 3 primers and intended to have HBV-specific ribozyme carried into liver cells by using HDV as a transporting vector. As the first step, we studied the *in vitro* cleavage activity of HBV-specific ribozymes-rRZ and rDVRZ. The results showed that both rRZ and rDVRZ had the ability of catalyzing the specific cleavage of target RNA (rBVCF-*in vitro* transcript of HBV C gene fragment) into two RNA fragments (721nt, 210nt).

The activity of ribozyme rRZ (without containing HDV) was much higher than that of recombinant rDVRZ, which in part cleaved the target RNA. One possible reason is that rDVRZ has much longer two arms outside its base-pairing region and can easily form a complex secondary or tertiary structure, an obstacle to subsequent base-pairing with target RNA (rBVCF). Its three-dimensional structure simulated by computer (not presented here) supports this conclusion. However, we could conclude that the post-transcripted hammerhead ribozyme should not be too long though we are unsure how long ribozyme and its target RNA are optimal to their interaction.

We found that with the temperature rising, the ribozyme activity increased, possibly because higher temperature helped transit the complex tertiary structure into comparatively extended state and so partially delete the structural obstacle to base-pairing. We also found that the requirement of ribozyme for Mg^{2+} was not strict. It appeared that different Mg^{2+} concentrations from 10 mmol/L to 20 mmol/L could meet the ribozymes' activity. But overhigh Mg^{2+} seemed to cause the non-specific cleavage of target RNA (not presented). The reason for this was unclear. Presently, we are studying the activity of the recombinant ribozyme *in vivo* and its repressive effect on HBV replication.

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