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Design of ribozymes to selectively cleave the human hepatitis *delta* virus mRNA

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ABSTRACT

At least 15 million people worldwide are carriers of the human hepatitis *delta* virus (HDV). Thus, HDV infection could be an important public health problem. HDV contains two RNA catalytic domains known as *delta* motif derived from its genomic or antigenomic RNA. It has been proposed that these catalytic motifs are involved in the self-cleavage reaction of RNA multimeric copies produced during its rolling circle replication. Our goal is to develop a ribozyme-mediated gene inactivation system which selectively inhibits the HDV replication. For this purpose, the unique mRNA encoding HDV antigen (HDAg) is targeted. To identify the potential cleavage sites on the HDAg mRNA, we used various procedures to assess its secondary structure, namely computer-aided secondary structure prediction and RNAse susceptibility assays. The accessibility of the target sites was further characterized by gel retardation and RNAse H assays. Putative target sequences were then used in the construction of *delta* ribozymes. The cleavage activity of engineered *delta* ribozymes were assayed *in vitro* using various substrates. The most efficient ribozymes will be chosen for further studies *in vivo*.

INTRODUCTION

HDV is a subviral particle that propagates only in the presence of hepatitis B virus (see 1, 2 and 3 for review). HDV is unique among animal viruses because it contains a single-stranded circular RNA genome of 1.7 kb, which codes for the two isoforms of *delta* antigen (HDAg). These proteins are crucial for the HDV life cycle, especially for its replication and its packaging. HDV shares several characteristics with plant viroids, which are small (246-463 nt), circular RNAs devoided of coding sequence that infect plants (4). Like viroids, HDV replicates through a DNA-independent rolling circle mechanism. HDV contains both the genomic and the antigenomic self-cleaving motifs (*i.e.* ~85 nucleotides) involved in the release of monomers (3,5). It has been possible to separate the self-cleaving sequence in two molecules to develop a *trans* system where one molecule, identified as a *delta* ribozyme, possesses the catalytic properties required to catalyse successive cleavage of several molecules of substrate (i.e. turnover) (6).

We aim to develop a gene inactivation system which selectively inhibits the HDV replication by inactivation of the HDAg mRNA. This gene inactivation system is developed based on a specific cleavage activity of ribozymes. In principle, ribozymes recognize their substrates by base-pairing to form an enzyme-substrate complex and then cleave their substrates at a specific site. We chose the *delta* ribozyme for our studies because it is the only known ribozyme that naturally occurs in human cell and is active under physiological concentration of magnesium and calcium (3, 6). In addition, the *delta* ribozyme presents an advantage over other ribozymes due to the fact that it requires only a relatively short span of recognizable nucleotides on the substrate molecule to form an enzyme-substrate complex prior to cleavage (figure 1A). These sequences can be described as a stretch composed of a pyrimidine, a guanosine, and six other nucleotides (PyGN6) (7). Since the recognizable sequence is not long and restricted, one can theoretically design a *delta* ribozyme to cleave any substrates containing the span of PyGN6. However, one might encounter difficulties in defining accessible cleavage sites on the RNA substrate molecules. In the present report, we describe the identification and characterization of putative cleavage sites of *delta* ribozymes on the HDAg mRNA. Using the acquired data, we have

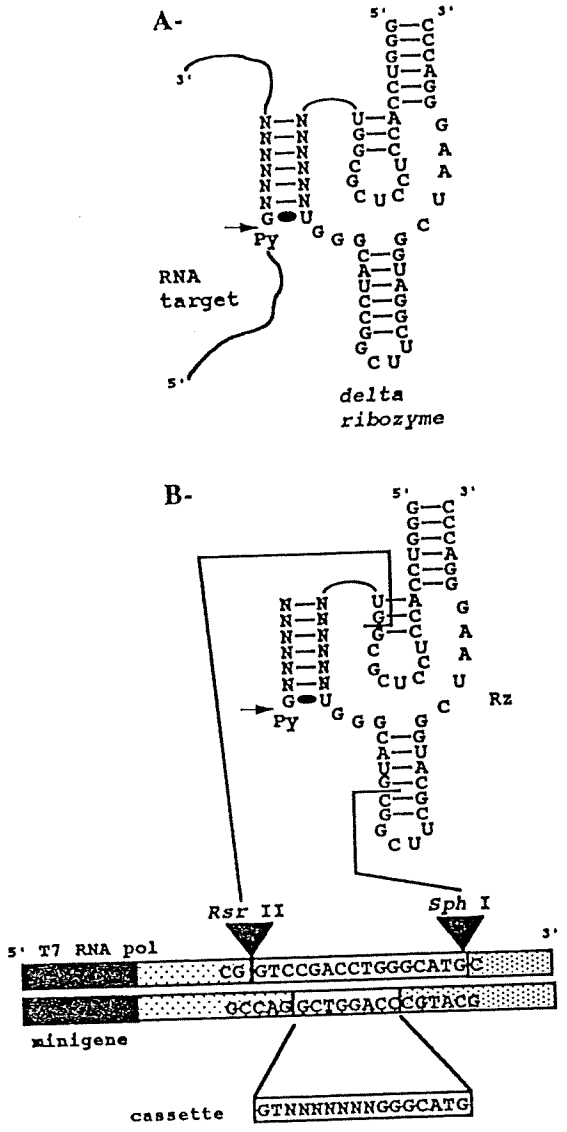

Figure 1: Schematic representation of the d ribozyme and the minigene including a casette for the modification of substrate recognition domain. A) Sequence and secondary structure of the antigenomic d ribozyme that cleaves any target RNA. B) Minigene with a cassette for the modification of the substrate recognition domain. pUC19 vector had been engineered to have a unique Rsr II and Sph I restriction site flanking the recognition sequence. Solid arrow show the cleavage site; Py indicate a pyrimidine while N is for any nucleotide (G, U, C, A).

designed several *delta* ribozymes to bear recognition sites corresponding to the sequence of the HDAg mRNA.

RESULTS AND DISCUSSION

Reported sequences of HDV were retreived from our public data bank (8). In order to identify possible cleavage sites of a *delta* ribozyme on the HDAg mRNA, we aligned all the open reading frames (ORF) of the 17 known variants of HDV. Sequence analysis was performed on the aligned ORFs to define conserved areas containing the consensus sequence (PyGN6) of the *delta* ribozyme recognition domain. Preliminarily, 12 sites located throughout the ORFs were chosen. We then used various methods to evaluate the potential of these sites of being a target site for the cleavage reaction by a *delta* ribozyme. The potential of these putative cleavage sites were scored based on the hypothesis that the sites located on the double-stranded region are poor substrates because ribozyme would have to compete with intramolecular base-pairing to recognize these sequences. In addition, the accessibility of the sites and binding specificity was also used as criteria in the evaluation.

Computer generated secondary structures of each ORFs were created using *mfold* version 2.3 (9). We found that eight possible sites were located mostly on the single-stranded region of the predicted structures. RNAse susceptibility assays were performed to assess the single- and double-stranded regions of the HDAg mRNA. HDAg mRNA was synthesized *in vitro* by T7 RNA polymerase, then 5' kinased in presence of [γ^{32}P]ATP and purified following a polyacrylamide gel electrophoresis. The synthesized HDAg mRNA was then subjected to RNAse T1, V1 and U2 partial digestions. The data suggest that five chosen sites were mostly located on single-stranded regions. In order to assay for the accessibility of the proposed target sites, oligonucleotides having complementary sequences to the target sites of the HDAg mRNA were synthesized and used in gel retardation analyses. Theoretically, the oligonucleotide which were able to hybridize the HDAg mRNA (e.g. forming a DNA-RNA duplex) will be retarded in a native gel electrophoresis. Half of the oligonucleotides could form such RNA-DNA complexes. The binding specificity of these oligonucleotides to the mRNA were then assayed by RNAse H digestion analysis. RNAse H which specifically cleaves transcripts at the site formed a RNA:DNA duplex was added into each mixture containing the randomly labelled HDAg mRNA and an individual oligonucleotides. The RNAse H cleavage products were then resolved on a denaturaing gel electrophoresis. Some of oligonucleotides exhibit a specific cleavage while others bind non-specifically.

Following analyses by computer-aided structure prediction, RNase susceptibility assays, gel retardation and RNase H assays, we further our investigation by choosing nine sites consisting of three sites giving positive results to all biochemical tests, five sites giving posivitive results to at least two tests, and one site giving negative results to all test. Nine oligonucleotides having sequences complementary to their corresponding sites of the HDAg mRNA were designed for the construction of modified *delta* ribozymes. The construction of *delta* ribozyme was carried out using a prepared pUC19 plasmid harboring antigenomic *delta* ribozyme currently used in our laboratory. In these constructions the substrate recognition domain of the ribozyme is flanked with the restriction sites of *Rsr* II and *Sph* I (figure 1B). The designed oligonucleotides were ligated to replace the region named as cassette of the *delta* ribozyme. The engineered *delta* ribozymes were prepared by *in vitro* transcription. Their cleavage activity was assayed using various synthetic substrates. Presently, we are investigating the cleavage activity of these engineered ribozymes *in vitro* to obtain their kinetic parameters. The engineered ribozymes with cleavage activity will be chosen for *in vivo* assays.

CONCLUSION

The gene inactivation by ribozymes has important therapeutic implications because they could be designed to harbor a specific recognition sequence and specifically cleave target cellular or viral RNAs (10). Ribozymes could thus be used as a modulator of gene expression. Here we have shown the experimental approach to obtain a better design of a *delta* ribozyme directed to cleave a target molecule. Severals studies have shown that the engineered ribozymes can inactivate target RNA like oncogenes (ras, c-fos, BCR-ABL) and viral RNA (in particular against HIV) (11). To our knowledge, no report to date shows the inhibition of HDV replication by using *delta* ribozyme.

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