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Review of the substrate specificity for *delta* ribozyme cleavage

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ABSTRACT

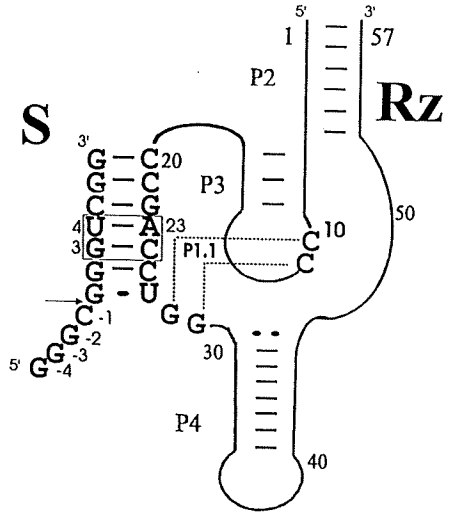
Recently, we reported the design and production of appropriate *delta* ribozymes cleaving in *trans* a mRNA. This work shown the potential of this ribozyme as a therapeutical tool. However, most of the designed ribozymes were inefficient for the cleavage of the targetted mRNA, therefore suggesting that the known substrate specificity might not be accurate. Consequently, we studied the contribution of the 5' sequence of the cleavage site to the substrate specificity. Using a collection of several small model substrates, we show that positions -4 to -1 contributed to the substrate specificity. We will also present preliminary results from a *delta* ribozyme combinatorial approach that aims to identify most potential site to target within a mRNA.

INTRODUCTION

The ability of ribozymes to specifically recognize a substrate, and subsequently catalyze its cleavage, makes them attractive therapeutical tools for the inactivation of both viral RNA and mRNA associated with human diseases. We engineered a ribozyme from the antigenomic HDV (hepatitis *delta* virus) RNA genome possessing several modified features in order to obtain a catalytic RNA of minimal length (57 nucleotides, see Fig. 1). This *delta* ribozyme was thoroughly characterized under both single- and multiple-turnover conditions (1, 2).

DELTA RIBOZYME CAN CLEAVE A mRNA IN *TRANS*, HOWEVER...

Delta ribozyme is unique in having the natural ability to function in the presence of human proteins; therefore, it can be considered as a suitable ribozyme for gene therapy development. According to the nested double-pseudoknot model (3), the secondary structure of *delta* ribozyme consists of five stems (P1, P1.1, P2, P3 and P4), two internal loops (L3 and L4) and two single-stranded junctions referred to as the linker stems (J1/4 and J4/2) (Fig. 1).



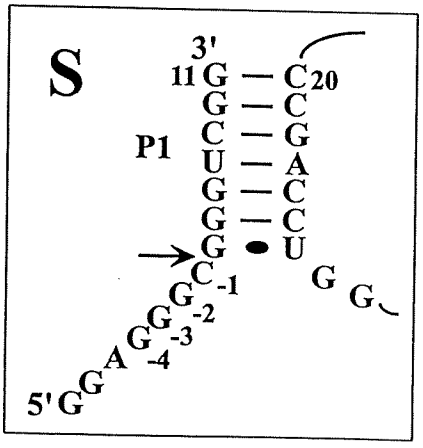


Figure 1. Delta ribozyme cleaving small model substrate

Substrate recognition (i.e. specificity) is based on the formation of the P1 stem which includes one G-U wobble basepair followed by six non-specific Watson-Crick basepairs (4). Since it has been shown that a pyrimidine (Y) immediately adjacent to the cleavage site is preferable (4), the logical design of a *delta* ribozyme against any RNA substrate is one containing the strech YGN_6 as its recognition sequence.

Recently, we have investigated the potential of the *delta* ribozyme in the *trans* cleavage of a mRNA. We reported the design and production of appropriate *delta* ribozymes cleaving the unique mRNA (~ 0.7 kb) produced by the HDV RNA genome (5). This work clearly illustrates the potential of this ribozyme as a therapeutical tool. However, most of the designed ribozymes were inefficient for cleavage of the mRNA, therefore hinting that determined specificity might not be accurate. For example, selected sites that includes several pyrimidines in the position upstream to the cleavage site, but at close proximity, were either poorly or not cleaved. Furthermore, this study clearly showed the importance of the single-stranded regions on the mRNA.

Here, we address two questions: what are the optimal sequence to target, and how to select the most potential sites to target in a mRNA.

SPECIFICITY OF *DELTA* RIBOZYME

The P1 stem: Concurrently with the demonstration of the potential to cleave in *trans* a mRNA, we studied the sequence specificity in the P1 stem using a small model substrate of 11 nucleotide in lenght (2, 6). We demonstrated that the middle basepairs (position Rz23-24 and S3-4 of the ribozyme and substrate, respectively) can not tolerate mismatch. These positions are essential for the binding, but more importantly, for the subsequent cleavage activity. While positions Rz24 and S3 can include any basepaired nucleotides, positions Rz23 and S4 have some restrictions. Although C_{23} and G_4 form a basepair, they result in a complete lack of cleavage activity.

Positions upstream to the cleavage site: In order to undertake a systematic study of the 5' sequence of the cleavage site, we designed a novel substrate of 14 nucleotides long yielding two distinct products of 7 nucleotides (Fig. 1, inset). This 5'-extended substrate allows substitution of nucleotides from positions -4 to -1 without modifying the *in vitro* transcription efficiency since the 5'-end sequence remains intact (i.e. 5'-GGA). A collection of several substrates including all single mutants for positions -4 to -1 compared to the original substrate, as well as multiple mutations were synthesized. The cleavage reactions of these mutants were characterized under pre-steady-state conditions (i.e. [Rz] >> [S]). We observed that the base requirement varies for each position. Not only the position -1 appeared of primary importance, but also position -2. Furthermore, positions -3 and -4 contributes significantly to provide good substrates. In addition, the presence of secondary structures in this region was evaluated. Clearly, these results shed light on new features that contribute to the substrate specificity requirement of the *delta* ribozyme cleavage. The specificity appears to be defined by at least 9 consecutive nucleotides including single- and double-stranded positions.

SPECIFICITY AND STRUCTURE

Ribozyme target sequences located in single-stranded regions of a mRNA have a higher potential as target sites because they should be more accessible to ribozyme attack than those found in double-stranded regions. In order to verify whether the specificity defined above was accurate when targetted sites are located in a full-length RNA molecules, and to take into account the intrinsic secondary structure of a mRNA, we undertook the development of a strategy that is based on the use of a combinatorial library of the ribozyme. The rationals of this approach are: i. target site acessibility; and, ii. the ability to form an active ribozyme-substrate complex. It is clear that these two factors are inter-independent and that their relationship is complex. Such an unpredictable interdependence can best be approached by using combinatorial methods. This strategy was used for other ribozymes including the designed of hairpin ribozymes for targetting the highly structured Sindbis RNA virus (7). Theoretically this approach includes three steps:

1) The synthesis of a combinatorial library of *delta* ribozymes encompassing all possible

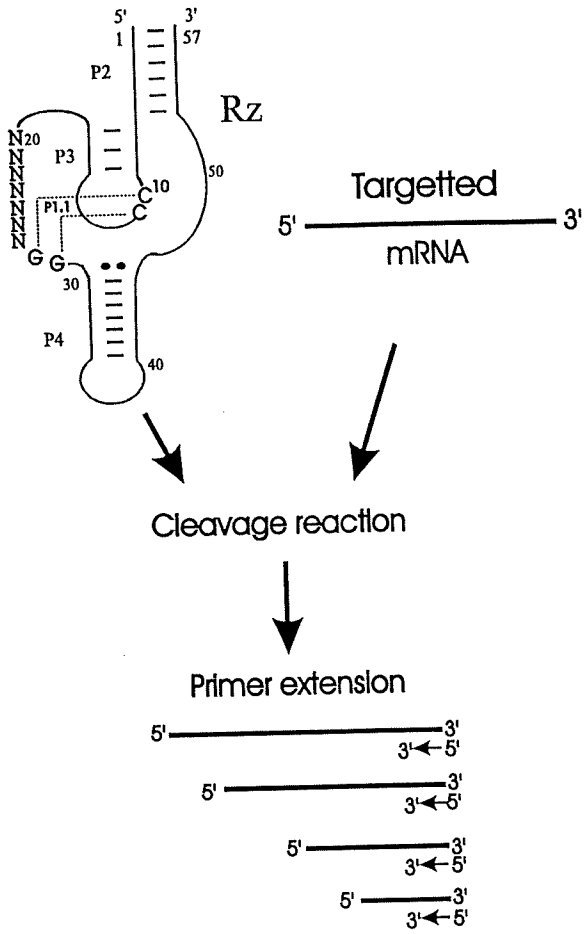


Figure 2. Combinatorial approach
(N indicates U,C,A or G)

sequence specificities to identify the most readily cleavable sites within targeted RNA molecules (see Fig. 2).

2) The incubation of the mRNA substrate with the pool of ribozymes under conditions permissive for cleavage to occur.

3) The identification of the mRNA cleaved site by primer extension assays. Knowning the sequence of the substrate, the sequence at a cleavage site is determined, and therefore, the sequence of the ribozyme P1 domain that acts to this site. Such an experiment using a bacterial mRNA is currently in progress and results will be presented.

CONCLUSION

The demonstration that more than 9 nucleotides contribute to define the ability and the specificity of a substrate to be cleaved by *delta* ribozyme make it as good as that of any known ribozyme. General rules of substrate specificity will be suggested, which aims to facilitate the design of efficient *delta* ribozymes as therapeutical tools.

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