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Review

Ribozymes of the hepatitis delta virus: Recent findings on their structure, mechanism of catalysis and possible applications★✪

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Although the delta ribozymes have been studied for more than ten years the most important information concerning their structure and mechanism of catalysis were only obtained very recently. The crystal structure of the genomic delta ribozyme turns out to be an excellent example of the extraordinary properties of RNA molecules to fold into uniquely compact structures. Details of the X-ray structure have greatly stimulated further studies on the folding of the ribozymes into functionally active molecules as well as on the mechanism of RNA catalysis. The ability of the delta ribozymes to carry out general acid-base catalysis by nucleotide side chains has been assumed in two proposed mechanisms of self-cleavage. Recently, considerable progress has been also made in characterizing the catalytic properties of *trans*-acting ribozyme variants that are potentially attractive tools in the strategy of directed RNA degradation.

Hepatitis delta virus (HDV) is a satellite virus that requires hepatitis B virus (HBV) for its life cycle. The genome of HDV is a single-stranded circular RNA, about 1700 nucleotides in length, and, similarly to plant viroids, it replicates *via* the general double rolling circle mechanism (reviewed in: Branch *et al.*, 1991; Taylor, 1991; 1992; 1993; 1996; 1999a, 1999b; Symons, 1992; Robertson, 1992; Govindarayan *et al.*, 1993; Lai, 1995). In the genomic RNA as well as in the antigenomic strand, which is generated dur-

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Abbreviations: HBV, hepatitis B virus; HDV, hepatitis delta virus.

ing vi rus rep li ca tion, there are two highly con-served sequences with ribozyme activities — the delta ribozymes (Fig. 1). These ribozymes are required for the self-cleavage of linear, multimeric RNA transcripts into monomeric RNAs that are sub se quently li gated into cir cu-lar forms. The important role of the delta ribozymes in functioning of the virus is well documented, although re cently ob tained data (Modahl & Lai, 1998; 2000; Filipovska & Konarska, 2000) somewhat complicate the simple double rolling circle mechanism pro-posed ear lier for rep li ca tion of the vi ral RNA.

RIBOZYME STRUCTURE

Re cently, the crys tal struc ture of the 3′ prod-uct of a genomic ribozyme has been de ter-mined by X-ray anal y sis (Ferre-D'Amare *et al.*, 1998; Ferre-D'Amare & Doudna, 2000). The structure confirms the ribozyme secondary structure model of the pseudoknot type (Fig. 1A) which has been proposed earlier

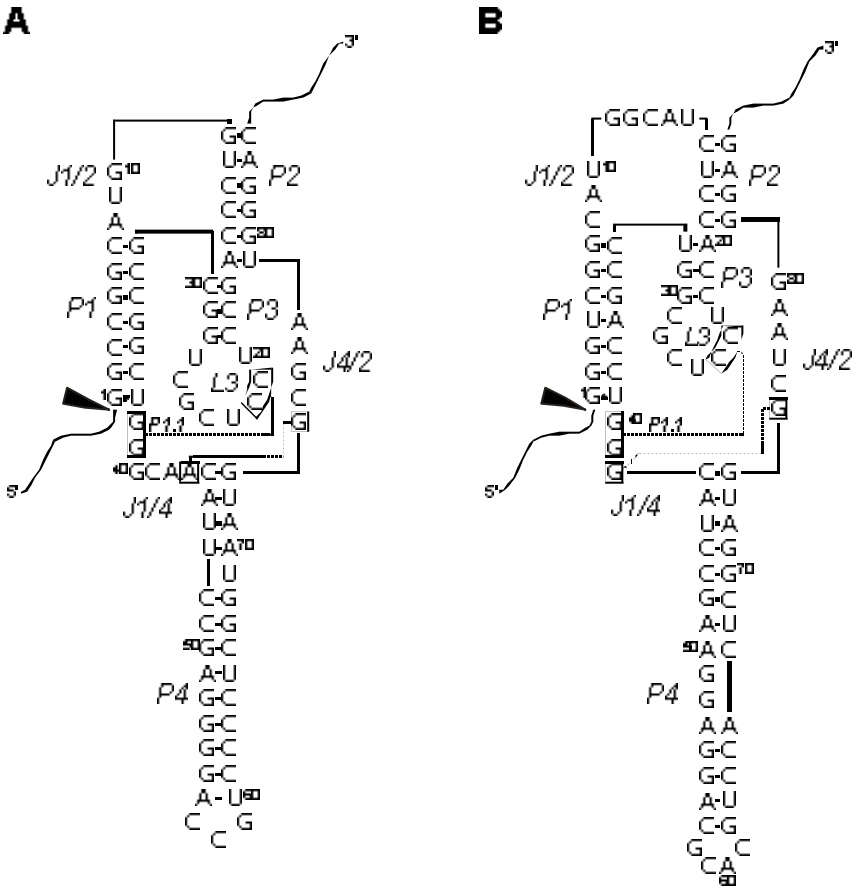

Fig ure 1. Sec ond ary struc ture mod els of the genomic (A) and antigenomic (B) delta ribo-zymes.

The se quences shown in the Fig-ure cor re spond to the 3′-cleavage prod ucts of the min i mal ribozyme se quences. Base-paired seg ments are de noted P1 to P4 and sin gle-stranded regions as J1/2, J1/4 and J4/2. Boxed nu cleo tides con-nected with dot ted lines in di cate the two- base-pair helices P1.1 and non-standard A–G or G–G in-teractions found in the crystal structure of the genomic ribo-zyme or suggested in the anti-genomic vari ant.

The delta ribozymes have been studied for more than ten years in several laboratories (for selected review articles see: Been, 1994; Lazinski & Taylor, 1995; Tan ner, 1995; Been & Wick ham, 1997). How ever, the most im por-tant information concerning their structure and mechanism of ca tal y sis were ob tained in based on the results of biochemical studies (Perrotta & Been, 1991; Been & Wickham, 1997). In addition, a new two-base-pair helix, P1.1, was found which introduces a second pseudoknot into the structure. This helix is crucial to the formation of the ribozyme spa-tial fold since it brings together two single-

stranded RNA stretches: L3 and J4/2. Nucleotides of these regions are important for catalysis (Been & Wickham, 1997) and in the tertiary structure they are located very close to the cleavage site. One of these nucleotides, C75, might be directly involved in the reaction mechanism (Ferre-D'Amare *et al.*, 1998; Ferre-D'Amare & Doudna, 2000, and discussion later in the text). The determination of the crystal structure of the genomic delta ribozyme is, undoubtedly, one of the most important achievements of 'ribozymology' in recent years. The structure is an excellent example of the extraordinary properties of RNA molecules to form uniquely compact folds stabilized by a variety of interactions. It has greatly stimulated further studies on delta ribozymes and, in particular, on the mechanism of RNA catalysis.

The antigenomic ribozyme has a secondary and, most likely, also tertiary structure very similar to its genomic counterpart (Perrotta & Been, 1991; Rosenstein & Been, 1991; Been & Wickham, 1997). Recently, a more direct evidence supporting this notion has been obtained from an experiment based on the information derived from the crystal structure of the genomic variant. The presence of the second pseudoknot was confirmed showing that variants with point mutations in the P1.1 helix were active if they had the potential to make standard Watson–Crick base pairs (Wadkins *et al.*, 1999). Similarly, nucleotides of the P1.1 helix were randomized in the genomic ribozyme (all possible variants were present simultaneously in the analyzed library) and the results of an *in vitro* selection experiment confirmed the crucial role of helix P1.1 in the active ribozyme structure (Nishikawa & Nishikawa, 2000).

The folding of the delta ribozymes into functionally active structures has recently attracted considerable interest. On the one hand, it touches on the very timely 'RNA folding problem'. On the other hand, there is an increasing number of examples suggesting that intermediates in an RNA's folding pathway can modulate its biological activity. It has been suggested that during replication of the viral HDV RNA the delta ribozymes may form sequentially from the growing RNA chain as soon as the corresponding RNA region has been transcribed (Lai, 1995). Alternatively, the multimeric RNA transcripts may be frozen in inactive conformations which subsequently interact with protein factors, facilitating ribozyme folding and RNA self-cleavage (Lazinski & Taylor, 1995). In order to get more information on the folding process of the genomic ribozyme we synthesized four RNA oligomers: 1–43, 1–73, 1–78 and 1–84 which can be considered as progressively elongated transcription intermediates of the ribozyme region (Matysiak *et al.*, 1999; see also Fig. 1A). We analyzed their structures by means of the Pb^{2+}-induced cleavage method, partial digestion with specific nucleases as well as by chemical probing. It turned out that events taking place during the folding of this ribozyme only partially reflect a hierarchical RNA folding pathway: RNA secondary structure elements fold first and then the tertiary structure is formed. In the case of the genomic delta ribozyme some secondary structure elements assume their final forms as soon as the corresponding RNA stretches have been synthesized. However, the region corresponding to the central hairpin, P3/L3, forms initially another stable hairpin that upon further elongation of the RNA chain is re-folded and the ribozyme adopts the final pseudoknotted structure. We suggested that this region might contribute to a mechanism responsible for controlling ribozyme cleavage activity by stabilizing the intermediates in its folding pathway (Matysiak *et al.*, 1999). RNA folding pathways of the genomic ribozyme were also modeled by a computer approach and the results supported the conclusion that sequential folding kinetics of the ribozyme might directly regulate its *in vivo* function (Isambert & Siggia, 2000). A correlation between the folding and catalytic behavior of delta ribozymes was demonstrated (Ananvo-

ranich & Perreault, 2000) us ing hy brid iza tion of com ple men tary oligodeoxy nucleotides and RNase H hydrolysis. An interesting suggestion was for mu lated that the P1.1 he lix forms only in the presence of Mg^{2+} ions.

Sev eral ear lier stud ies have shown an im portant role of vi ral RNA se quences sur round ing the delta ribozymes in modulating their catalytic activity. Recently, a more detailed picture of cer tain ef fects of this kind has be gun to emerge. For example, in the antigenomic RNA the for ma tion of a short du plex, P2a, in which the four-nucleotide sequence 10-UGGC-13 of J1/2 pairs with a sequence just outside the 3′-bound ary of the ribozyme, is pos sible. It was shown that P2a could both inhibit and enhance ribozyme activity depending on cleavage conditions (Perrotta & Been, 1998). This opens the pos si bil ity that P2a is part of a mechanism responsible for controlling ribozyme cleav age ac tiv ity. The im por tance of the conserved A14 which separates P2a and P2 forming a nucleotide bulge in the P2-P2a duplex has been stud ied in de tail (Perrotta *et al.*, 1999a). The data showed that the bulged A14 did not pro vide an es sen tial kink or hinge be tween P2 and P2a re quired for the cleav age ac tiv ity but, rather, fa vored the fold ing of the ribozyme into the pro-active structure by increas ing the rate of refolding from in ac tive to active conformations. In another report (Chadalavada *et al.*, 2000), a genomic variant containing 30 nucleotides immediately upstream of the cleav age site was found to have attenuated self-cleavage while adding more up stream nu cleo tides re stored cleav age in an *in vi tro* sys tem. A model was pro posed for the attenuation of ribozyme activity by alternative pair ing of the very 3′-end of the ribozyme with up stream flank ing nu cleo tides.

The delta ribozymes are now among the best-characterized small RNAs like yeast $tRNA^{Phe}$, the 'ham mer head' ribozyme, or the P4-P6 domain of the *Tetrahymena* group I intron. All of these RNAs are used as model molecules in studies requiring precise in formation on RNA ter tiary fold ing. In par tic u lar, the genomic delta ribozyme was used to gether with other RNAs for which the sec ond ary and ter tiary struc tures have pre vi ously been modeled to evaluate the inherent chemical instability of phosphodiester linkages to the spontaneous cleavage *via* intramolecular transesterification reactions (Soukup & Breaker, 1999). In the stud ies car ried out in our lab o ratory (Wrzesinski *et al.*, 2000), the genomic and antigenomic ribozyme 3′-cleavage products as well as their trun cates turned out to be useful for a better evaluation of how RNA structure influences the binding of complementary oligodeoxyribonucleotides. To map sites accessible to hybridization we applied semi-random oligonucleotide libraries and RNase H hydrolysis. We proposed an approach that correlates the RNase H cleavage sites and the most likely positions of DNA 6-mers hybridizing to the RNA targets. The data obtained exemplified the crucial role of tar get RNA struc tural fea tures in the bind ing of com ple men tary oligonucleotides and cor re lated well with the results obtained by other au thors (Mir & South ern, 1999) of an anal y sis of the hybridization of yeast $tRNA^{Phe}$ to an immobilized library of complementary oligonucleotides.

MECHANISM OF CATALYSIS

The ribozyme cleavage products containing a 5′-hydroxyl group and a 2′,3′-cyclic phosphate indicate that the reaction oc curs *via* transesterification rather then hydrolysis. In a mechanistic model the 2′-hydroxyl (or oxygen anion) is the nucleophile attacking the phosphorus atom of the phosphodiester linkage. Sub se quently, the break age of the chain occurs with the developing of a negative charge on the 5′-oxygen atom of the leaving group followed by its protonation.

In two very recently proposed mechanisms of self-cleavage the ability of the delta ribozymes to carry out a gen eral acid-base catalysis has been assumed (Perrotta *et al.*,

1999; Nakano *et al.*, 2000; see also Fig. 2). Both papers postulate a crucial role of one of the cytosine residues of the J4/2 region, C75 in the genomic ribozyme and C76 at the corresponding position of the antigenomic variant, in the cleavage mechanism. The authors of the first mechanistic proposition (Perrotta *et al.*, ring nitrogen N3 of C75 is shifted upwards to about 7, most likely as a consequence of interaction with the phosphate residue of C22 and the presence of a tight metal ion binding site in the vicinity. In the proposed mechanisms an ionized metal ion hydrate acts as a general base (Nakano *et al.*, 2000).

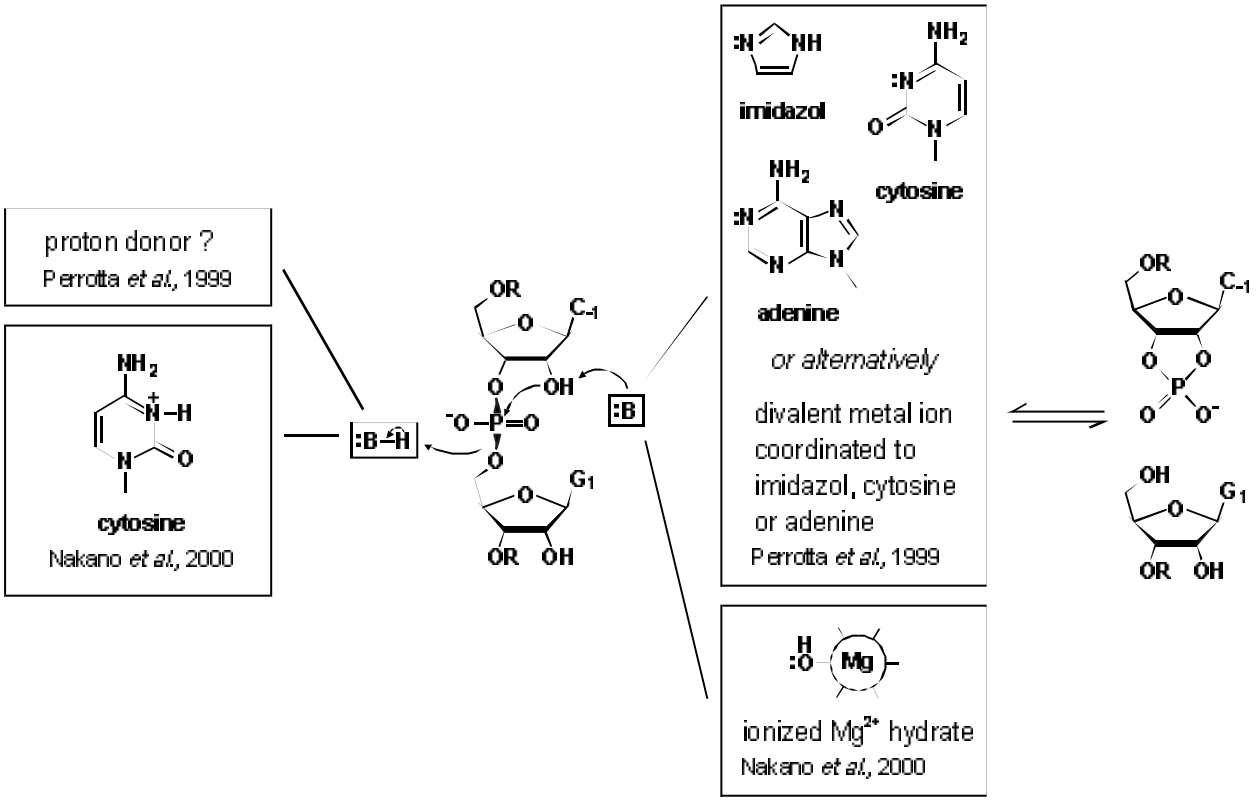

Figure 2. Proposed acid-base catalysis of RNA cleavage in the delta ribozymes (Perrotta *et al.*, 1999; Nakano *et al.*, 2000).

1999a) show that imidazole buffer rescues the activity of a mutant antigenomic ribozyme with the cytosine-76 to uracil substitution. These data are consistent with imidazole-enhanced cleavage by a general base mechanism in which imidazole accepts a proton from the 2′-hydroxyl group. This means that in the wild-type antigenomic ribozyme C76 could act as a general base. Another possibility is that imidazole could coordinate a catalytic metal ion, presumably replacing a lost ligand with the mutation of C76 (Perrotta *et al.*, 1999). In the other mechanism (Nakano *et al.*, 2000), C75 of the genomic ribozyme acts as a general acid. As shown experimentally, pK_a of the

Most evidence has consistently supported a requirement for divalent metal ions in HDV catalysis while other ribozymes such as the 'hammerhead', 'hairpin' and 'VS' versions are also active at very high concentration of monovalent ions (Murray *et al.*, 1998). However, in the crystal structure of the 3′-cleavage product of the genomic ribozyme no tightly bound divalent ions were found in the vicinity of the cleavage site. Thus, the crystallographic data did not reveal how metal ions might participate in catalysis. In solution, the presence of 'general' metal ion binding sites in the delta ribozymes has been suggested based on the results of metal ion-induced cleavage experi-

ments (for a review on the method, see Ciesio³ka, 1999). Specific cleavages were induced in the J4/2 region with Pb^{2+} (Rogers *et al.*, 1996), and, as shown in our laboratory also with Ca^{2+}, Mn^{2+} and Mg^{2+} (Matysiak *et al.*, 1999). In the *trans*-acting antigenomic ribozyme, a specific Mg^{2+}-induced cleavage occurs at the bottom of the P2 stem (Lafontaine *et al.*, 1999). On the other hand, although the 3′,5′-phosphodiester linkage at the functional cleavage site is cleaved slightly faster in the presence of Ca^{2+} than Mg^{2+}, the 2′,5′-linkage is cleaved in the presence of Mg^{2+} (or Mn^{2+}) but not Ca^{2+} (Shih & Been, 1999). This dramatic difference is strongly suggestive of a crucial metal-ion interaction at the ribozyme active site. The role of divalent metal ions in ribozyme folding and catalysis remains, however, still a puzzle despite years of experimental efforts in several laboratories. Recently, we have compared the catalytic activity of four structural variants of the antigenomic delta ribozyme in the presence of various divalent metal ions that effectively support catalysis. The ribozyme variants differed in their catalytic activities but, strikingly, with a given variant we observed relatively small differences in the reactions induced by several metal ions although the ions differed substantially in the ability of their hydrates to ionize. Thus the availability of an ionized metal ion hydrate which has been proposed to act as the general base in the reaction mechanism (Nakano *et al.*, 2000) does not seem to be the rate-limiting step in the ribozyme self-cleavage (unpublished results of our laboratory).

A general acid-base catalysis that has been, for the first time, proposed to operate in the delta ribozymes seemed to be unique among the earlier known catalytic RNAs. A similar strategy has been, however, suggested for a newly discovered ribozyme – the ribosome and peptide bond formation (Ban *et al.*, 2000; Muth *et al.*, 2000; Nissen *et al.*, 2000). An adenine residue of the large ribosomal RNA acts as the general base in the peptidyl transferase center. General acid-base catalysis by nucleotide side chains increases the catalytic repertoire of RNA and suggests that similar strategies could operate in other RNA-catalyzed reactions. Very recently, differences among mechanisms of ribozyme-catalyzed reactions as well as the roles of metal ions in ribozyme functions have been discussed in excellent review articles (Warashina *et al.*, 2000; Hanna & Doudna, 2000). Recent findings have demonstrated the diversity of RNA catalysis mechanisms and challenged the traditional paradigms of the relationships between metal ions and ribozymes.

POSSIBLE APPLICATIONS OF DELTA RIBOZYMES

The delta ribozymes are potentially attractive tools in the strategy of directed RNA degradation. These ribozymes are naturally active in human cells and at physiological Mg^{2+} ion concentration they show the highest cleavage rates among all known ribozymes. Several *trans*-acting ribozymes have been constructed showing the variants cleaved in the J1/2 region to be the most promising in practical applications (reviewed in: Been & Wickham, 1997). There are, however, certain limitations to designing ribozymes with a desirable specificity. The substrate-ribozyme recognition element (helix P1 in the wild type, *cis*-acting ribozyme, see Fig. 1) is directly involved in the formation of the catalytic core and changes in its base-pair composition influence ribozyme activity. Unlike, for instance, the 'hammerhead' ribozyme in which the recognition elements and catalytic core are separated and the desired ribozyme specificity can be readily achieved. Moreover, the P1 helix is relatively short which suggests low selectivity of *trans*-acting variants unless there are additional discriminating factors of substrate-ribozyme interactions.

In two variants of the *trans*-acting genomic ribozyme the interactions between nucleo-

tides around the cleavage site were analyzed by changing every possible base pair between the substrate and the ribozyme at positions –1 and +1 relative to the cleavage site (Nishikawa *et al.*, 1997). While the G38 in the ribozyme was indispensable to the cleavage, the –1 position in the substrate could accommodate any base, although the rate of cleavage differed among bases and the two studied ribozymes. In the ribozyme, G39 was favored for cleavage, and interestingly, the +1 base of the substrate affected the structure of the catalytic core in one of the studied variants, shifting the cleavage site (Nishikawa *et al.*, 1997). On the other hand, stem P1 extension from 7 to 8 or 9 base pairs caused a loss of activity indicating that the original 7-base-pair stem was the most favorable (Nishikawa *et al.*, 1999). The original GC-rich sequence was replaced with AU-rich sequences containing six AU or UA base pairs with the natural GU wobble base pair at the cleavage site. The ribozymes in *cis*-arrangement showed an activity similar to the wild type molecule while in *trans*-arrangement, due to stem P1 instability, cleavage efficiency depended strongly on the concentration of the complex and temperature, showing multiple turnover at 37°C (Nishikawa *et al.*, 1999).

The role of substrate–ribozyme interactions in efficient cleavage of *trans*-acting antigenomic ribozyme was also investigated with substrates that varied in either the length or the nucleotide sequence of their P1 stems (Ananvoranich & Perreault, 1998). A minimum of six base pairs was sufficient for the cleavage to occur although the observed cleavage rate decreased 10-fold compared to a 7-base-pair stem. Moreover, any mismatches within the helix strongly decreased the activity, with those introduced in the middle of the P1 stem resulting in a complete lack of cleavage. Further studies on the effect of alterations in the P1 stem suggested that a tertiary interaction involving base moieties in the middle of P1 is likely to play a role in defining the active site (Ananvoranich *et al.*, 1999). Moreover, a closer examination (Deschenes *et al.*, 2000) of the role of nucleotides from –1 to –4 in efficient cleavage with a collection of small substrates that possessed single and multiple mutations in this region showed the optimal sequence to be –1HRHY–4 (H = U, C, A; R = purine; Y = pyrimidine). Thus, although the –1 to –4 region is not a part of the recognition domain it is an external determinant of the ability of a substrate to be cleaved. This region extends from the seven-nucleotide recognition stretch 1-GNNHNNN-7 proposed earlier (Ananvoranich & Perreault, 1998) to 11 contiguous nucleotides that contribute to determining the ability of an RNA molecule to be cleaved by the delta ribozyme (Deschenes *et al.*, 2000).

The cleavage of an mRNA in *trans* by a delta ribozyme derived from the antigenomic strand has been demonstrated, for the first time, with mRNA encoding the only protein of HDV, the two isoforms of the delta antigen (Roy *et al.*, 1999). Ribozymes were synthesized to cleave selected potential cleavage sites within mRNA sequence (i.e. YGN_6). Of the nine ribozymes tested three specifically cleaved the target RNA molecule *in vitro*. Although the catalytic rate constant of the selected ribozyme for mRNA cleavage was 50-fold lower than that for the cleavage of the small substrate, multiple-turnovers were observed, an essential property for further applications of the delta ribozymes (Roy *et al.*, 1999). Kinetic schemes for intermolecular RNA cleavage by variants of the antigenomic delta ribozymes have been studied in detail (Mercure *et al.*, 1998; Shih & Been, 2000) and the results should be helpful in designing ribozymes with the desired high cleavage activity and multiple turnovers.

Summarizing, considerable progress has recently been made in characterizing the catalytic properties of *trans*-acting delta ribozymes. The factors influencing substrate-ribozyme interactions, multiple turnovers, differences in catalytic activity of various structural variants are now better recog-

nized and understood. Further studies are, however, needed toward successful applications of the delta ribozymes as therapeutic agents or useful biochemical tools.

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