Structural Analysis of Ribozymes

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in
Encyclopedia of Analytical Chemistry
R.A. Meyers (Ed.)
pp. 5273–5285
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We use delta *ribozymes as a model in illustrating the various steps involved in the structural analysis of ribozymes.* Delta *ribozymes were originally identified as a self-cleaving motif located on the single-stranded RNA genome of hepatitis* delta *virus (HDV). The methodologies are presented in the order of their requirement for determination of the structure/function relationship of a ribozyme, beginning with the preparation of the RNA molecules required for the studies. Primary structural information is needed in order to identify potential ribozymes, then secondary and tertiary structural information is required for ribozyme characterization. The manuscript was written to guide an investigator from the initial observation of RNA catalytic activity (a ribozyme discovery) to the deduction of a structural model of the ribozyme.*

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1 INTRODUCTION

Ribozymes are a family of RNA molecules which possess various catalytic capabilities.[1–3] The best known ribozymes are the endoribonucleases which are capable of cleaving RNA molecules at specific sequences. This group of ribozymes has tremendous potential for the development of a novel approach for the selective inactivation of specific RNA molecules, including both those derived from pathogenic viruses and those associated with inherited diseases. The underlying theory of this inactivation is that the mRNA encoding a harmful protein would be intercepted and destroyed by the ribozymes before that mRNA is translated. In order to be able to fully exploit the potential of a ribozyme, it is crucial to have a complete understanding of the molecular mechanism of the ribozyme reaction. It has long been known that modification of RNA (ribozyme) structure often results in the alteration of its catalytic properties.

The various methodologies described in this chapter are presented in the order of their requirement for determination of the structure/function relationship of a ribozyme, beginning with the preparation of the RNA molecules required for the studies. Primary structural information is needed in order to identify potential ribozymes, then secondary and tertiary structural information are required for ribozyme characterization. The procedures described below are written so as to guide an investigator from the initial observation of RNA catalytic activity (a ribozyme discovery) to the deduction of a structural model of the ribozyme.

We use *delta* ribozymes as a model in illustrating the various steps involved in the structural analysis of ribozymes. *Delta* ribozymes were originally identified as a self-cleaving motif located on the single-stranded RNA genome of HDV.[4] The HDV genome is circular and replicates through a rolling circle mechanism involving only RNA intermediates.[5] Replication is initiated by the binding of the host RNA polymerase II to the parental RNA and results in the synthesis of a complementary multimeric copy of the HDV genome (Figure 1). The monomeric HDV genome is then released from this multimer by the catalytic activity of self-cleaving motif. This intramolecular cleavage is an RNA autocatalytic reaction. The RNA sequence responsible for the cleavage is known as the *delta*-cleaving RNA motif, or as the *cis*-acting ribozyme (Figure 1b). Based on subsequent

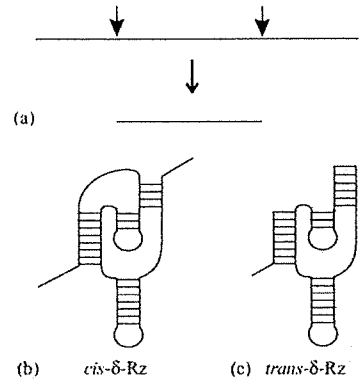


Figure 1 The discovery and development of *delta* ribozyme from HDV. (a) The single-stranded RNA genome of HDV is produced in a multimeric unit. Its self-cleaving motif is responsible for the release of multiple monomeric copies of the HDV genome, which are then re-circularized. (b) The *delta*-cleaving motif was subsequently identified. (c) From secondary structural analysis, a *trans*-acting *delta* ribozyme was derived by separating the junction between the P1 and P2 stems.

secondary structural information obtained by various investigators,[6–9] this motif has been modified into an intermolecular system (Figure 1c). This so called a *trans*-acting ribozyme system contains both a substrate molecule and an enzyme molecule.

2 HISTORY

RNA catalysis was first identified in the RNA components of both the group I intron by Cech[10] and RNaseP by Altman[11] in the early 1980s. These discoveries marked the first examples of enzymatic catalysis in the absence of any protein. These catalytically active ribonucleic acids were named ribozymes (RNA enzymes). During the past two decades several ribozymes have been identified that possess various catalytic capabilities which enable them to modify the phosphodiester bonds of their substrates. With the development of in vitro selection procedures, RNA molecules which catalyze a spectrum of reactions, for example phosphorylation and ligation, have been identified.[1–3] Without doubt in the decades to come, additional capabilities of ribozymes will be discovered.

3 SYNTHESIS OF RNA FOR RIBOZYME STUDIES

Ribozymes and their substrates can be produced either by in vitro transcription, or by chemical synthesis. The basic requirements and protocols for these two methods are described below.

3.1 In Vitro Transcription

This protocol makes use of enzymatic reactions catalyzed by purified bacteriophage T7 RNA polymerase, which uses DNA as a template. It has been demonstrated that the sequence immediately downstream of the T7 RNA promoter can affect the yield of transcript, and that the initiating nucleotides CCC or CCU give the best yield.[12] Large RNAs are routinely generated by this method. The model substrate of *delta* ribozyme, an oligomer 11 nt long, is also produced by this method (Figure 2a). Due to the propensity of T7 RNA polymerase to add one or two uncoded nucleotides to the 3′-end of the resultant transcripts,[1] the subsequent purification and verification of both the length and the sequence of the transcripts is required (section 5.2). The main limitation of this method is that it cannot be used when specific modifications are required.

3.1.1 Oligonucleotide Templates

A pair of synthetic DNA oligonucleotide templates can be designed so that one contains the complementary sequences of the T7 RNA promoter and the sequence coding for ribozyme or substrate, while the other contains the sequence of the T7 RNA promoter. Prior to the preparation of an in vitro transcription reaction mixture, the two oligonucleotides (500 pmol each) are mixed in diethylene pyrocarbonate (DEPC)-treated water (20 µL) containing 10 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl pH 7.5, 10 mM $MgCl_2$ and 50 mM KCl, heated at 95 °C for 5 min and allowed to cool slowly to 37 °C. The partial duplexes formed then serve as templates for RNA synthesis by T7 RNA polymerase (Figure 2a).

3.1.2 Cloned Templates

The DNA template of *delta* ribozyme was cloned into the plasmid pUC19 using recombinant DNA techniques (section 4.2). The resultant recombinant plasmid containing the ribozyme sequence is then digested so as to either linearize or release the *delta* ribozyme insert. T7 RNA polymerase will use the resultant DNA duplex as a template and produce transcripts extending until the end of the duplex, so called run-off transcription reactions[13] (Figure 2b).

3.1.2.1 Materials and Methods Mix, in a final volume of 100 µL:

DNA template either as a partial duplex (500 pmol) or as a digested cloned template (5 µg)	20 µL

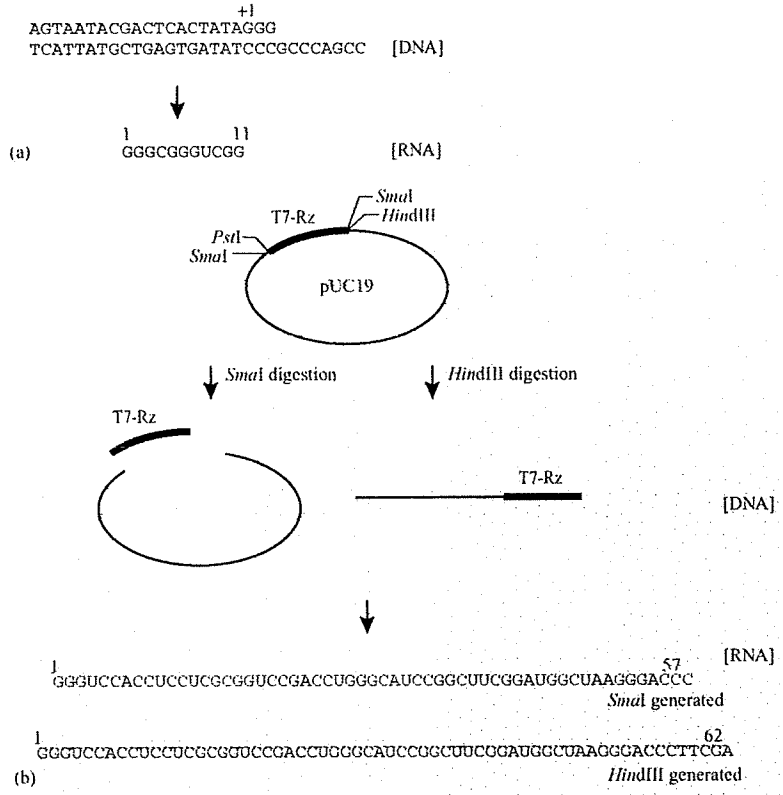
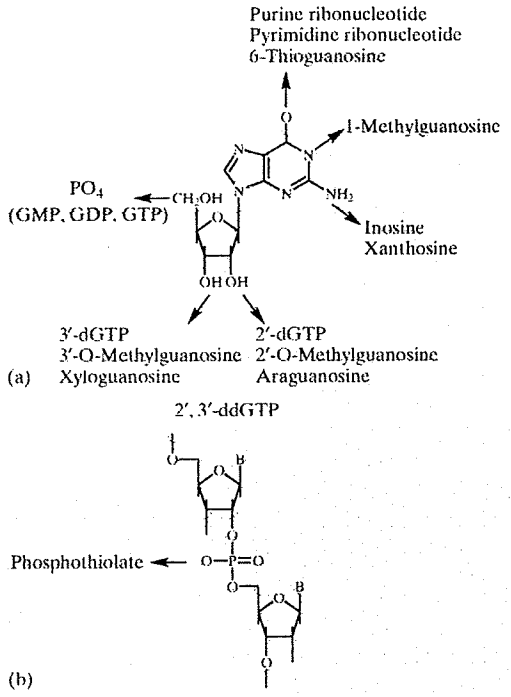


Figure 2 DNA templates for in vitro transcription reactions catalyzed by bateriophage T7 RNA polymerase. (a) The partial duplex formed by two oligonucleotides. The substrate (11 nt) of *delta* ribozyme is illustrated. (b) The double-stranded DNA. The plasmid pUC19 harboring the sequence of *trans* acting *delta* ribozyme is digested with either *Sma*I or *Hind*III and the resulting linear DNA is used as a template. RNA and DNA molecules are identified in brackets.

RNAGuard® RNase inhibitor (Pharmacia)	27 units
rNTP (Pharmacia) 10 mM each,	5 µL
Transcription buffer (5×)	20 µL
(400 mM *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (HEPES)-KOH pH 7.5, 120 mM $MgCl_2$, 10 mM spermidine) dithiothreitol (DTT) (100 mM)	2 µL
Pyrophosphatase (1 unit μL^{-1}, Boehringer Mannheim)	1 µL
Purified T7 RNA polymerase (2 µg μL^{-1})	2 µL

Incubate at 37 °C for 2–4 h. Add 5 µL RQ1 RNase-free DNase (1 unit μL^{-1}, Promega) to eliminate the DNA template, then extract twice with buffered phenol. Add 0.1 volume of 3 M sodium acetate pH 5.2 and 2 volume of ethanol to the aqueous phase. Chill for 15 min at −80 °C and centrifuge at 4 °C for 15 min in a microfuge. Discard the supernatant then add an equal volume of 70% ethanol and repeat the centrifugation step. Dissolve the pellet in 20 µL of DEPC-treated water and add 10 µL of 5× gel loading buffer (95% formamide, 10 mM ethylenediaminetetraactetic acid (EDTA), 0.05% bromophenol blue and 0.05% xylene cyanol). The mixtures are fractionated by denaturing 20% polyacrylamide gel electrophoresis (PAGE, 19:1 ratio of acrylamide to bisacrylamide) containing 45 mM Tris-borate pH 7.5, 7 M urea and 1 mM EDTA. The reaction products are visualized by ultraviolet (UV) shadowing, and the bands corresponding to the correct sizes of the ribozymes and substrates cut out, and the transcripts eluted overnight at 4 °C in a solution (200 µL) containing 0.1% SDS and 0.5 M ammonium acetate. The transcripts are then precipitated by the addition of 0.1 volume of 3 M sodium acetate pH 5.2 and 2.2 volume of ethanol. Transcript yield is determined by spectrophotometry at 260 nm. The sequence and size of the RNA products are then verified by RNase digestion (section 5.2).



Scheme 1 Specific modifications of an RNA molecule.[32] (a) Some modifications on the mononucleotide can be on the sugar or the base residue. (b) The phosphodiester backbone can be modified to a phosphothiolate group.

3.2 Chemical Synthesis

RNA molecules can be chemically synthesized in the same fashion as DNA molecules from 3′ to 5′ by taking the advantage of the high chemical reactivity of the 5′-hydroxyl group. It has been reported that the enzymatic activity of the resultant RNA is equivalent to that of RNA derived by transcription methods.[14] Most importantly, chemical synthesis allows the introduction of single-atom substitutions at specific positions in the RNA molecules. There are three major targets for modification in RNAs: the exocyclic base, the sugar, and the internucleotide phosphodiester linkage (Scheme 1). Presently the cost of production is still prohibitive, especially when large RNA molecules are required. An improved method using the solid-phase chemistry is capable of production in the 200 μmol range; however the chemical synthesis of RNAs longer than 45 nt is not advised when 2′-*O*-*t*-butyldimethylsilyl-5′-*O*-DMT-ribonucleosides (tBDMS-amidites) are used, and of not more than 90 nt when 2′-*O*-triisopropylsilyl-oxymethyl-ribonucleosides (TOM-amidites) are used.[16] In some cases where longer RNA molecules are required, the investigator might combine T7 transcription and chemical synthesis (section 3.3) in order to produce the desired RNA.

An automated DNA/RNA synthesizer (for example, ABI Model 392 or 394 from Applied Biosystem, Foster City, CA) and high-performance liquid chromatography (HPLC) system are required for the synthesis of RNA oligonucleotides using tBDMS-amidites phosphoramidites or TOM-amidites, in addition to nucleoside-functionalized CPG or polystyrene supports (A, G, C, U). Due to the special amidites and deprotection reagents currently available, various modifications can be incorporated into the synthesized RNA. For example, inosine can be introduced at selective bases. 2′-deoxy or 2′-O-methyl RNA can be substituted for the 2′-OH of the ribose residue. The internucleotide phosphodiester bonds can be replaced by phosphothioate or phosphonate linkages. Moreover, other modifications, such as biotinylated RNA and halogenated deoxy and ribonucleotide incorporation, aimed at facilitating the downstream use of the resulting RNAs can also be incorporated.

Due to the high cost of equipment and materials in general, investigators are advised to order custom-made RNA oligonucleotides from facilities such as Keck Oligonucleotide Synthesis Facility (Yale University, CT), and Xeragon AG (Zurich, Switzerland). Following their synthesis RNA oligonucleotides are subjected to deprotection and desalting steps analogous to those for synthetic DNA oligonucleotides. The deprotection removes protecting groups from 2′-hydroxyl of the ribose residue using either TBAF or $Et_3N(HF)_3$ according to the manufacturer's instruction;[15] the subsequent desalting step removes inorganic salts, trace organic compounds, low-molecular-weight impurities and short failure sequences. The latter step can be performed using size-exclusion chromatographic columns such as G-25 Sephadex or reverse phase HPLC.[16] However, gel electrophoresis is the method of choice for removal of failure sequences. The sequence and size of the RNA products are verified by RNase digestion (section 5.2).

3.3 Combination Method

Both enzymatic and chemical techniques can be combined in order to obtain target RNA molecules with site-specific modifications at affordable prices. For example, target RNA molecules with a specific phosphothiolate linkage isomer can be generated by enzymatic ligation of two individual RNAs, one of which is a chemically synthesized RNA containing a phosphothiolate linkage at the desired position. The resultant Sp and Rp isomers are then separated by HPLC. The desired RNA isomer is then joined to the other RNA by T4 RNA ligase.[17] For more examples and details on using a combination of

chemical and enzymatic methods to generate the RNAs of interest, see Vinyak et al.[14]

4 PRIMARY STRUCTURAL INFORMATION

The observation of self-cleaving RNA molecules naturally lead to experiments aimed at identifying the responsible element whether its sequence or surroundings causes the cleavage. In the case of *delta* ribozymes, a linear dimer of the HDV genome was reported to be initially processed into a monomeric RNA when the responsible cDNA was transfected into a monkey kidney cell line.[4] These findings suggested that either HDV RNA has an unusual secondary structure that allows a specific attack by a cellular RNase, or that the HDV RNA has a specific self-cleaving activity. The self-cleaving motif of HDV was mapped on both strands of the HDV genome in order to locate the cleavage position.[6] The steps required for this identification are described below.

4.1 Sequence Mapping and Self-cleaving Activity

In general, cDNA coding for the RNA molecules of interest is generated and cloned into a plasmid for identification using standard recombinant DNA techniques. Exonuclease III, a 3′ to 5′ exonuclease specific for double-stranded DNA carrying either a blunt end, a 5′-overhang, or nick, is commonly used for the construction of unidirectional nested deletion sets from the plasmids. The nested deletion clones can be generated from either end using an appropriate restriction enodonuclease that leaves either a blunt end or 5′-overhang to linearize the DNA, followed by exonuclease III treatment. The sequence of each of the resulting deletions is determined by dideoxynucleotide chain-termination sequencing.[13]

RNA transcripts are then produced by in vitro transcription reactions in the presence of 0.05 mCi [α-^{32}P]GTP, and are purified and subjected to various buffered conditions so as to identify the responsible element. To prove that the self-cleaving activity is solely the result of the RNA transcripts, and not due to RNase or proteins present in the in vitro transcription reaction or cell extracts, the purified primary transcripts are incubated in 50 mM Tris-HCl, pH 8.0 and 10 mM $MgCl_2$ (standard conditions for many ribozyme cleavage assays, including *delta* ribozymes).[6,18] The newly formed products are resolved on denaturing polyacrylamide gels, and are visualized by exposure of the gels to either X-ray films or phosphorImaging™ screens.

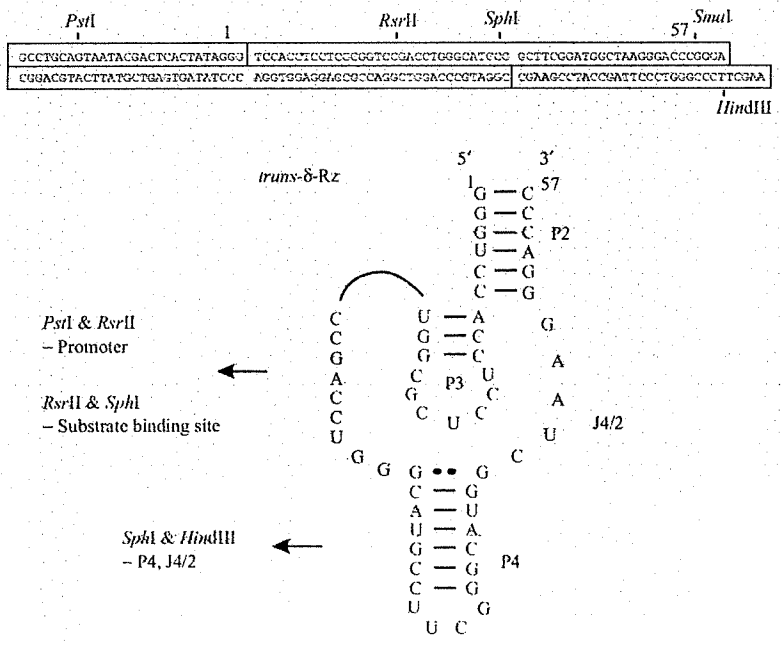


Figure 3 Cloning of the *trans*-acting *delta* ribozyme. Four overlapping oligonucleotides were designed so that they encode the entire sequence of *trans*-acting *delta* ribozyme. Following annealing of these four oligonucleotides, the resulting fragment was cloned into *PstI*/*Hin*dIII-digested pUC19. The resultant clone was verified by dideoxynucleotide sequencing. The restriction endonuclease sites are identified on the double-stranded DNA.

To characterize the biochemical properties of the self-cleaving motif, the cleavage efficiency is determined in various buffer systems (pH 5.0–9.0) containing either monovalent (Na^+, K^+ or NH^{4+}) or divalent ions (Mg^{2+}, Ca^{2+} or Mn^{2+}). The *delta* ribozyme self-cleaving motif located on the HDV genome was found to be able to use either Mg^{2+} or Ca^{2+} as a cofactor for efficient cleavage in Tris-HCl pH 5.0–8.0.[6] The cleavage products were identified as a 3′ fragment with 5′-hydroxyl end, and a 5′ fragment with 2′,3′-cyclic phosphate terminus similar to the products of other ribozyme cleavage reactions.

4.2 Construction of Ribozymes and Their Variants

In order to explore enzymatic properties of a ribozyme, cDNA clones coding for the ribozymes are constructed using recombinant DNA techniques. From the primary structural information (i.e. the sequence and the cleavage site), deoxyoligonucleotides can be designed and synthesized for cloning purposes. The *delta* ribozymes used in our studies were initially constructed using four overlapping oligonucleotides (Figure 3). Several restriction sites were included in order to facilitate the creation of variant ribozymes. For example, ribozyme mutants carrying a single mutation in the P1 stem can be produced by digestion of the plasmid carrying the ribozyme with the restriction endonucleases, *Rsr*II and *Sph*I. Subsequent ligation of this predigested plasmid to new oligonucleotides having the altered sequence flanked by *Rsr*II and *Sph*I sites led to the production of the ribozyme variant.[19]

5 SECONDARY STRUCTURE ANALYSIS

Secondary structural information of RNA molecules is generally considered to be a simplification of what is in fact a three-dimensional complex. RNA secondary structure is predicted using either computer-aided alignment, or experimental data. Both approaches suggest putative base pairs in the three-dimensional structure of the RNA molecule.

5.1 Computer-aided Prediction

RNA secondary structure elucidation is similar to an alignment of protein or nucleic acid sequences, except that the RNA sequence folds back on to itself rather than on to identical or similar bases.[20] The complementary bases, G–C and A–U, form stable base pairs through hydrogen bonds between donor and acceptor sites on the bases, and are known as Watson–Crick base pairs. In addition, the weaker G–U wobble pair can be formed in a skewed fashion. These three types of base pairs are called canonical base pairs. Other base pairs (i.e. G–G or C–C) are called noncanonical base pairs.[21] The prediction of secondary structure can be made either from a single RNA sequence by minimizing the free energy of folding, or from a common folding pattern observed for a family of aligned, homologous RNAs. To calculate the free energy of RNA folding, arbitrary energy profiles are assigned to each individual base pair type and motif.[22] For the past decade, the formulae defined by Turner and his co-investigators have been widely used to define the free energy of stacking of canonical base pairs, hairpin loops, and both interior and bulge loops.[20,22] In general, the secondary structural information obtained using aligned RNA sequences is very valuable because the RNA structure is considered to be conserved to a greater degree than the sequence is. Over a period of sequence drift, the structural similarity might remain essentially the same through the phenomenon known as compensatory base changes, which conserve the base pairs,[22] as is observed in the structures of tRNAs or 5S RNA.

5.2 Nuclease Mapping or Enzymatic RNA Sequencing

Enzymatic RNA sequencing is generally used in both the mapping of RNA secondary structures and in the determination of RNA length. This method takes advantage of the cleavage specificity of a variety of ribonucleases and nucleases (Table 1) that are incubated with the substrate RNA in separate reactions. The reaction mixtures are then fractionated by denaturing gel electrophoresis. The resulting bands are compared to an RNA ladder generated from the same RNA. To determine the length of the RNA, several nucleases can be used (Table 1).

Table 1 Ribonucleases and nucleases that are commercially available, and are commonly used, are listed with their optimal buffer[23]

Nucleases	Cleavage	Buffers (5×)
CV or V1	Prefers double-stranded RNA	125 mM Tris-HCl, pH 7.2
Phy M	Ap↓N and Up↓N	250 mM sodium citrate, pH 5.0
S1	Single-stranded nucleic acid	200 mM sodium acetate, pH 4.5, 1 M NaCl, 50 mM $ZnSO_4$
T1	Single-stranded RNA and Gp↓N	250 mM sodium citrate, pH 5.0
T2	Prefers single-stranded RNA and Ap↓N	250 mM sodium acetate, pH 5.0 250 mM Tris-HCl, pH 7.5
U2	Single-stranded RNA and Ap↓N	50 mM sodium citrate, pH 4.5

5.2.1 Materials and Methods

5.2.1.1 5'-Dephosphorylation of RNAs Mix, in total volume of 10 µL:

RNA purified from in vitro transcription reaction (10 pmol µL^{-1})	2 µL
Calf intestine alkaline phosphatase (Pharmacia)	0.2 unit
1 M Tris-HCl, pH 8.0	5 µL
RNAGuard® RNase inhibitor (Pharmacia)	10 units
DEPC-treated water	

Incubate at 37 °C for 30 min. Extract twice with buffered phenol. Collect the aqueous phase, add 0.1 volume of 3 M sodium acetate pH 5.2 and 2 volume of ethanol. Chill the mixture for 15 min at −80 °C. Centrifuge at 4 °C for 15 min in a microfuge, add an equal volume of 70% ethanol and repeat the centrifugation step. Dissolve the pellet in 20 µL of DEPC-treated water to obtain 1 pmol µL^{-1} of dephosphorylated RNA.

5.2.1.2 5'-End-labeling of Transcripts Mix, in total of 10 µL:

Dephosphorylated RNA (1 pmol µL^{-1})	2 µL
10 × T4 polynucleotide kinase buffer (USB)	1 µL
RNAGuard® RNase inhibitor (Pharmacia)	10 units
γ-^{32}P ATP (Amersham, 10 µCi µL^{-1})	3.2 pmol
T4 polynucleotide kinase (USB)	3 units
DEPC-treated water	

Incubate at 37 °C for 30 min. Add 5 µL of loading buffer (95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) and fractionate on a denaturing PAGE. The radioactively labeled RNA band is cut out following exposure of the gel to an X-ray film. The 5'-end-labeled RNA is eluted overnight at 4 °C in a solution (200 µL) containing 0.01% SDS and 0.5 M ammonium acetate.

5.2.1.3 Alkaline Hydrolysis Generation of an RNA Ladder Mix, in total of 5 µL, 5'-end-labeled RNA (50 000 cpm µL^{-1}) in solution containing 50 mM $NaHCO_3$ and 5 mM EDTA. Incubate at 95 °C for 5 min. Add 5 µL of loading buffer (95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) and fractionate on a denaturing PAGE gel.

5.2.1.4 Nuclease Assay The specific cleavage by nucleases at the ribose-phosphate backbone are carried out using various buffering conditions as listed in Table 1. Frequently $MgCl_2$ or EDTA is also added in order to obtain conditions corresponding to either native or partially denatured folding. Note that the addition of heavy metal ions should be omitted in assays using RNase T2. Moreover, the optimal time required for the mapping is determined depending on the efficiency of each nuclease.[23] The cleavage reaction mixtures are fractionated on denaturing PAGE gels along with the corresponding RNA ladder.

5.3 Chemical Interference

Chemical reagents interact with the heterocyclic bases, the phosphodiester bonds and the ribose moieties resulting in a modified structure (Table 2). The modified residues are detected by primer extension,[2] except when using Fe (II)-EDTA and imidazoles where primer extension is not required in order to resolve the reaction products. Fe (II)-EDTA generates hydroxyl radicals which nonspecifically interact with nucleic acids. Consequently Fe (II)-EDTA is often used for elucidation of the surface residues of an RNA molecule. These interactions occur at the heterocyclic bases and ribose residues, the latter of which results in strand breaks which are detected following gel fractionation. Imidazole and its conjugates rapidly cleave the phosphodiester bonds located in single-stranded regions whereas those located in double-stranded regions are cleaved much more slowly.[23] For *delta* ribozymes, several research groups have used RNase mapping, chemical interference, and UV-cross-linking procedures to determine *delta* ribozyme structures.[24–26] Three versions of the secondary structure are illustrated in Figure 4. The differences result from the different assay conditions used and from the analysis of the mixed population of *delta* ribozymes present in aqueous solution.

Table 2 Chemical interference[23]

Chemicals	Attack at functional groups
Dimethyl sulfate	Nucleophilic centers of heterocyclic bases
Ethyl nitrourea	Internucleotide phosphates and nucleophilic centers of heterocyclic bases
CMCT	Uridine (N3), guanosine (N1)
Ketoxal	Guanosine in single-stranded regions
DEPC	Purine (N7)
Fe (II)-EDTA	Heterocyclic bases and ribose
Imidazole and conjugates	Phosphodiester bonds

CMCT, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metro-*p*-toluene sulfonate.

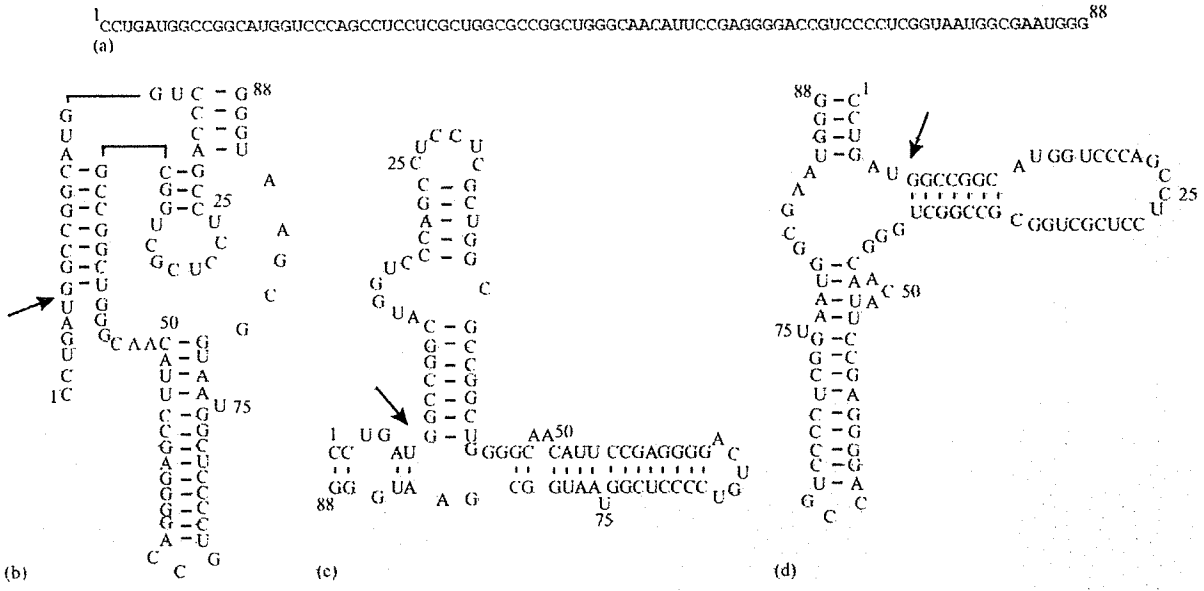


Figure 4 The primary and secondary structural information of genomic *delta* ribozymes. (a) The sequence of the genomic *cis*-acting *delta* ribozyme is 88 nt long.[33] (b–d) The secondary structures of *delta* ribozyme as determined by Perrotta and Been (b),[7] Wu et al. (c),[6] and Branch et al. (d).[8]

6 NATIVE OR TERTIARY STRUCTURE ANALYSIS

Using both intramolecular and intermolecular (i.e. with surrounding molecules) interactions, ribozymes adopt their native or tertiary structures and thereby gain their catalytic activity. The surrounding molecules used in the intermolecular interaction are metal ions, proteins and water. Over the last few years there has been considerable progress in our understanding of the kinetics of RNA folding and cleavage due to simultaneous advances in both experimental and theoretical methods.[27] Ribonuclease mapping, chemical interference and mutational analyses have been used to define the possible conformations of various RNA molecules. Most significantly, advanced techniques of crystallography, X-ray diffraction and NMR have determined precisely several RNA structures. Currently, the relationship between structure and function has been most thoroughly studied for the group I intron-derived ribozyme. Several of the approaches used in the study of this large ribozyme have also been applied to smaller ribozymes, the best studied of which are the hammerhead ribozymes. However, in some cases structural information from different methods resulted in discrepancies in the proposed structure–function relationship due to the heterogenous population of ribozyme present in aqueous solution. Kinetic characterization has been used widely in the survey of native structural analysis as it relates to enzymatic activity.

6.1 Cleavage Assay

A cleavage assay is the primary method of demonstrating that a ribozyme has adopted its native structure. Like protein enzymes, cleavage assays can be carried out in buffered solution in the presence of trace amounts of radioactive labeled substrate. The radioactive RNA molecules are either the *cis*- ribozyme (RNA carrying a self-cleaving motif), or the substrate for a *trans*-ribozyme. The cleavage reactions catalyzed by both *cis*- and *trans*-δ-Rz require metal ions as cofactors (i.e. $MgCl_2$, $CaCl_2$ and $MnCl_2$). Denaturing agents such as formamide and urea are sometimes included in the reaction mixture in order to disturb any misfolded molecules and thereby enhance the refolding of the ribozyme–substrate complexes.

6.1.1 Materials and Methods

Mix, in total of 20 μL, either radioactively labeled *cis*-acting ribozyme or radiactively labeled substrate of *trans*-acting ribozyme (ca. 50 000 cpm) in buffered solution containing 50 mM Tris-HCl, pH 7.5–8.0, and 5–50 mM $MgCl_2$. In the cases of *trans*-acting ribozyme, an approximate amount of ribozyme is added. Incubate the reaction for the time required (i.e. 10–30 min). Stop the reaction by adding 5 μL of loading buffer

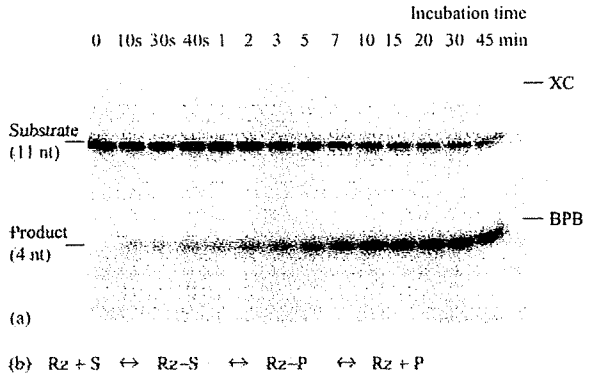


Figure 5 Kinetic analysis of *trans*-acting *delta* ribozyme. (a) An example of a time course experiment. Trace amounts of 5′-end-labeled substrate were incubated with 100 nM *delta* ribozyme in a solution containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$. (b) Simplified kinetic pathway of the cleavage reaction catalyzed by *delta* ribozyme.

(95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) and fractionate on a denaturing PAGE (Figure 5a).

6.2 Metal Ion Dependence

In general, ribozymes require the presence of metal ions for their folding and cleavage activity. These metal ions (Mg^{2+}, Ca^{2+}, Mn^{2+}, Sr^{2+} etc.) are added to the cleavage reactions in either the presence or absence of monovalent ions (Na^+, K^+ or NH_4^+) in order to determine the metal ion requirement.

6.3 Kinetic Determination

Time course experiments are performed at various substrate and ribozyme concentrations in order to determine kinetic parameters, such as a maximum rate of cleavage and substrate association constant.

6.3.1 Single Turnover Conditions

Various amounts of ribozyme are mixed with trace amounts of substrate (final concentration < 1 nM) in a 18-µL reaction mixture containing 50 mM Tris-HCl pH 7.5, and are then subjected to denaturation by heating at 95 °C for 2 min. The mixtures are quickly placed on ice for 2 min and equilibrated to 37 °C for 5 min prior to initiation of the reaction. Cleavage is initiated by the addition of $MgCl_2$ to 10 mM final concentration. The cleavage reactions are incubated at 37 °C, and followed for 3.5 h or until the end-point of cleavage is reached. The samples are quenced by the addition of 5 µL stop solution (95% formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), and are analyzed by 20% PAGE as described above. Both the substrate (11 nt) and the reaction product (4 nt) bands are detected using a molecular dynamic radioanalytic scanner after exposition of the gels to a phosphorImaging™ screen (Figure 4a).

6.3.1.1 Measurement of Pseudo-first-order Rate Constant (k_{cat}, K_M and k_{cat}/K_M) Kinetic analyses are performed under single turnover conditions as described by Hertel et al.[28] with some modifications for *delta* ribozyme. Trace amounts of end-labeled substrate (<1 nM) are cleaved by various ribozyme concentrations (5–500 nM), and the fraction cleaved is determined. The rate of cleavage (k_{obs}) is obtained from the fitting of the data to the equation $A_t = A_\infty(1 - e^{-kt})$, where A_t is the percentage of cleavage at time t, A_∞ is the maximum percent cleavage (or the end-point of cleavage), and k is the rate constant (k_{obs}). Each rate constant should be calculated from at least two measurements. The values of k_{obs} obtained are then plotted as a function of ribozyme concentration in order to determine the other kinetic parameters: k_{cat}, K_M and k_{cat}/K_M.

6.3.2 Multiple Turnover Conditions

Trace amounts of labeled substrate are mixed with unlabeled substrate in order to obtain various substrate final concentrations. Fixed amounts of ribozyme (50 nM) are then added to the substrate mixtures in an 18-µL reaction mixture containing 50 mM Tris-HCl pH 7.5, and then subjected to denaturation by heating at 95 °C for 2 min. Again the mixtures are quickly placed on ice for 2 min and equilibrated to 37 °C for 5 min prior to the initiation of the reaction. Cleavage is again initiated by the addition of $MgCl_2$ to 10 mM.

6.3.3 Inhibition Analysis

The inhibitory effects of the substrate and the product can be kinetically tested under both single- and multiple-turnover conditions.

6.3.3.1 Single Turnover Conditions Conditions similar to those described above can be used with various amounts of potential inhibitors (either substrate, product or oligonucleotide). The reactions are initiated by mixing inhibitors (0.5–20 µM) with substrate (1 nM) prior to addition of the ribozyme (50 nM) in 20 µL enzyme assay buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$). Aliquots (2 µl) are withdrawn at various intervals during the 40-min incubation period, and are quenched by the addition of ice-cold stop solution (6 µL). The samples are fractionated on 10% denaturing PAGE gels, and the reaction products are quantified following exposure to phosphorImaging™ screens. Control reactions are carried out in the absence

of inhibitor. To evaluate the effect of an inhibitor on the intrinsic rate of *delta* ribozyme cleavage, the data are analyzed as described by Clouet-d'Orval et al.[29] in order to determine the fraction of inhibition (I) at each inhibitor concentration. Equation (1) is used:

$$I = 1 - \left(\frac{k_{2_{\text{inhibitor}}}}{k_2}\right) \quad (1)$$

where $k_{2_{\text{inhibitor}}}$ and k_2 are the rates of cleavage in the presence and absence of the inhibitor, respectively. The values of k_2 are obtained from fitting the experimental data to the pseudo-first-order rate equation (Equation 2):

$$A_t = A_\infty(1 - e^{k2t}) \quad (2)$$

where A_t is the percentage of product formed at time t, and A_∞ is the maximum amount of product formed. The fraction of inhibition (I) is plotted versus inhibitor concentration, and the data fitted to a hyperbolic equation in order to obtain N_I, the inhibitor concentration needed to reduce the rate of cleavage by one-half.

6.3.3.2 Multiple-turnover or Steady-state Conditions Various concentrations of substrate are mixed with trace amounts of end-labeled substrate (<1 nM) so that final concentration is between 75 and 500 nM in the reaction mixtures. The reaction mixtures contain substrate, ribozyme (50 nM), and inhibitor (0.5–20 μM), and are performed as described for single-turnover conditions. The cleavage rates (v_i, μM min^{-1}) are determined at various substrate and inhibitor concentrations. Lineweaver-Burk, or reciprocal plots of $1/v_i$ and $1/[S]$ at all inhibitor (I) concentrations, are plotted, and the slopes and intercepts calculated by weighted linear regression analyses.

6.4 Three-dimensional Structural Information

6.4.1 Binding Shift Assay

Nondenaturing electrophoresis is commonly used in resolving the isomers that result from the folding of ribozymes. Moreover, kinetic parameters, such as equilibrium dissociation constant (K_d), the association and dissociation of substrate and product (Figure 5b), can be determined by this assay.[19]

6.4.1.1 Materials and Methods The equilibrium dissociation constants can be determined as follows. Various ribozyme concentrations, ranging from 5 to 600 nM, are individually mixed with trace amounts of end-labeled substrate (<1 nM) in a 9-μL solution containing 50 mM Tris-HCl pH 7.5. This reaction is then heated at 95 °C for 2 min and cooled to 37 °C for 5 min prior to addition of $MgCl_2$ to a final concentration of 10 mM in a manner similar to that of a regular cleavage reaction. The reactions are incubated at 37 °C for 1.5 h, at which point 2 μL of sample loading solution (50% glycerol, 0.025% of each bromophenol blue and xylene cyanol) is added and the resulting mixture electrophoresed through a nondenaturing polyacrylamide gel (20% acrylamide with a 19 : 1 ratio of acrylamide to bisacrylamide, in 45 mM Tris-borate pH 7.5 and 10 mM $MgCl_2$ buffer system). The gels are pre-run at 20 W for 1 h prior to sample loading, while the actual electrophorsis is carried out at 15 W for 4.5 h at room temperature. Quantification of bound and free substrate molecules is performed following exposure of the gels to a PhosphorImaging™ screen.

6.4.2 In Vitro Evolution

An in vitro selection procedure can be used for the identification of important residues in the structure of *delta* ribozymes.[30] A pool of *trans*-acting ribozymes with sequences that have been randomized are produced from synthetic DNA templates using T7 RNA polymerase. In each selection cycle, the inactive ribozyme–substrate complexes can be separated from the active complexes using a biotinylated substrate against avidin. Using this procedure, the nucleotides essential for maintenance of the activity of *trans*-acting *delta* ribozymes were identified.[30]

6.5 Data Analysis

Delta ribozyme is able to catalyze the cleavage of an 11-mer RNA substrate under both single- and multiple-turnover conditions.[18–19,31] In both cases only small differences in the kinetic parameters are observed in the presence of either magnesium or calcium as cofactor. Under multiple-turnover conditions, the catalytic efficiency of the ribozyme (k_{cat}/K_M) is higher at 37 °C than at 56 °C.[17] The cleavage reaction seems to be limited by the product release step at 37 °C, and by the chemical cleavage step at 56 °C. Substrate inhibition is detected at high concentrations of the 11-mer substrate. The 2′-hydroxyl group adjacent to the scissile phosphate is involved in binding with the ribozyme, while the essential cytosine residue of the J4/2 junction has been shown to contribute to substrate association. The kinetic pathway of *delta* ribozyme is believed to involve a conformational transition step that is essential for the formation of the active ribozyme–substrate complex (Figure 5b).

7 CONCLUSION

In addition to fundamental methods for the ribozyme structural analysis, several advanced protocols are being

developed for determination of the structure–function relationship of ribozymes. For example, X-ray crystallography, nuclear magnetic resonance and in vitro selection of RNA molecules have provided the scientific community with considerable information. We believe that these topics should be reviewed by experts in those particular domains.

ACKNOWLEDGMENTS

The authors thank the Medical Research Council of Canada (MRC) and the Natural Sciences and Engineering Research Council of Canada (NSERC) for providing operational and supporting grants, respectively. S.A. was recipient of an NSERC postdoctoral fellowship, and J.P.P is an MRC scholar.

ABBREVIATIONS AND ACRONYMS

CMCT	1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimide metro-*p*-toluene sulfonate
DEPC	Diethylene Pyrocarbonate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraactetic Acid
HDV	Hepatitis *Delta* Virus
HEPES	*N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic Acid
HPLC	High-performance Liquid Chromatography
PAGE	Polyacrylamide Gel Electrophoresis
tBDMS-amidites	2′-*O*-*t*-butyldimethylsilyl-5′-*O*-DMT-ribonucleosides
TOM-amidites	2′-*O*-triisopropylsilyl-oxy-methyl-ribonucleosides
Tris	Tris(hydroxymethyl)amino-methane
UV	Ultraviolet

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