Antigenomic Hepatitis delta virus ribozymes self-cleave in 18 M formamide

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ABSTRACT

The ribozymes derived from Hepatitis delta virus (HDV) RNA appear unique in their sequence requirements for self-cleavage. While truncating the 1679 nucleotide antigenomic HDV RNA, we have characterized the cleavage requirements of a number of ribozymes of intermediate length. Two of these, containing 186 and 106 HDV nucleotides respectively, cleaved to completion in the presence of 18 M formamide. The 186 nucleotide ribozyme also cleaved to completion in 10 M urea. Removal of an additional 10 nts from the 3′ terminus of the 106 nt ribozyme resulted in a loss of the ability to cleave in high concentrations of the denaturants. The interaction of nucleotides near the cleavage site with a sequence within this 10 base region may confer unusual stability on these ribozymes.

INTRODUCTION

Hepatitis delta virus is a serious human pathogen with a structure unique among mammalian viruses. The viral genome is a single-stranded circular RNA, which appears to replicate through a rolling circle (1). We demonstrated that both genomic and antigenomic strands were capable of self-cleavage at 50°C in the presence of 10 mM magnesium chloride (2, 3). Since the sequences flanking the cleavage site resemble none of the other known ribozymes (3), the Delta ribozymes are likely to represent a new type of self-cleaving RNA.

We are truncating the 1679 nucleotide (nt) antigenomic Delta ribozyme to define a minimal molecule capable of self-cleavage activity. Intermediate antigenomic RNAs have been analyzed for cleavage activity under a variety of conditions of temperature and denaturants. A 186 nt ribozyme was capable of efficient cleavage in extremely high concentrations of formamide or urea. We have identified a sequence within the molecule that may contribute to the unusual stability of the ribozymes, both at high temperatures and high concentrations of denaturants.

MATERIALS AND METHODS

Subcloning the Hepatitis delta ribozyme fragments

A Sal I-Xba I fragment (antigenomic HDV nucleotides 962 to 781) from pD1 (a gift of Dr. J. Taylor) was subcloned in the polylinker of pGEM4 (Promega), forming pG-180. Linearizing the plasmid with Xba I produced a precursor RNA with 25 nts of vector 5′ sequence and 186 nts of Delta RNA, while linearization with Dde I truncated the Delta RNA by 40 nts at the 3′ end (Fig. 1). The truncation of the 5′ end to produce Ag136 and Ag96 was accomplished through digestion of the cDNA from pG-180 with a combination of Exonuclease III and mung bean nuclease (Stratagene protocol), resulting in pJB17.

The ten terminal nucleotides were reintroduced into Ag96 by using DNA oligonucleotides to amplify a fragment of the HDV molecule from nt 917 to nt 811 using the polymerase chain reaction (PCR, Perkin-Elmer Cetus protocol). The oligonucleotide at the distal end from the T7 polymerase promoter contained the additional ten nucleotides, as well as a Bam HI site for cloning. The amplified DNA was gel-purified and cloned into the Hind III-Bam HI sites in pJB17, replacing the original fragment. All mutations were confirmed by sequence analysis.

Transcription of the ribozymes *in vitro*

Transcription reactions were carried out as previously described (2) and the RNA purified by electrophoresis on an 8% polyacrylamide/7M urea gel and eluted (2). The recovered precursor RNA was then suspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA buffer.

Self-cleavage analysis

The precursor RNA was heated to 65°C in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA and cooled on ice in the absence of magnesium, then pre-incubated at the desired temperature for 5 min, and $MgCl_2$ was added to 9 mM to initiate the reaction. At the end of the indicated time, a 5 μl aliquot was added to 5 μl stop buffer containing 100 mM EDTA in 20 M formamide, heated to 65°C for 3 min, then immediately analyzed by fractionation on an 8% polyacrylamide/7 M urea gel. The excess of EDTA in the stop buffer is essential to prevent further cleavage before the sample is loaded on the gel. While heat and denaturation normally inhibit ribozyme activity, in the case of the Delta ribozymes, they increase the self-cleavage (4–6). Radiolabeled RNA markers of known size were used to calibrate the size of the reaction products. The percentage of uncleaved precursor and products was determined by densitometry of the gel autoradiograph.

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For cleavage in formamide or urea, the radiolabelled precursor RNAs were preincubated as above in a reaction buffer containing the denaturants for 5 min, then $MgCl_2$ was added to 9 mM to initiate the reactions. After 5 min at 37°C, the reactions were terminated as described and the products analyzed. Controls to which no magnesium was added showed no cleavage.

RESULTS

Derivation of the antigenomic HDV ribozymes

Depending on the restriction enzyme used for linearizing plasmid pG-180, a precursor RNA containing either 186 nts (Ag180) or 146 nts of Delta sequence (Ag 140) was produced (Fig. 1). The self-cleavage of the two molecules at 22.5°C was extremely rapid, but only a proportion of the molecules cleaved (Fig. 2 a), with 5% of Ag180 RNA cleaving after 10 min, while 50% cleavage of Ag140 was seen. Since removing 40 nts from the 3′ terminus of Ag180 to form Ag140 increased the amount of RNA cleaved at each time point, sequences inhibitory to the cleavage might be located within these 40 nts. Alternatively, the longer molecule might form a greater number of RNA conformers, some of which were inactive at this temperature.

The extent of cleavage of Ag140 varied with the temperature (Fig. 2 b). Thus, at 0°C, 5% of Ag140 was cleaved in two minutes, while at 37°C, 50% was cleaved in two minutes, and no further cleavage occurred during incubation times of up to one hour (Fig. 2 b and data not shown). At 56°C, Ag140 cleavage reached 80% in 2 min, and increased another 10% upon further incubation (Fig. 2 b). Since the most likely effect of temperature would be to alter the structure of the RNA, these data implied even more strongly that the step limiting the extent of cleavage might be a conformational change.

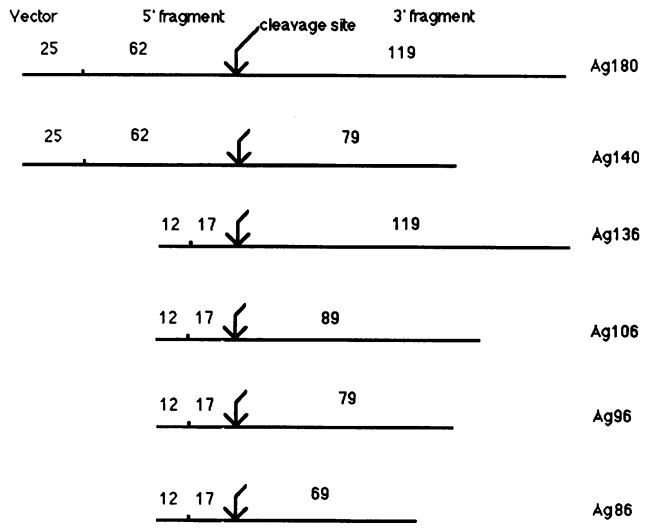
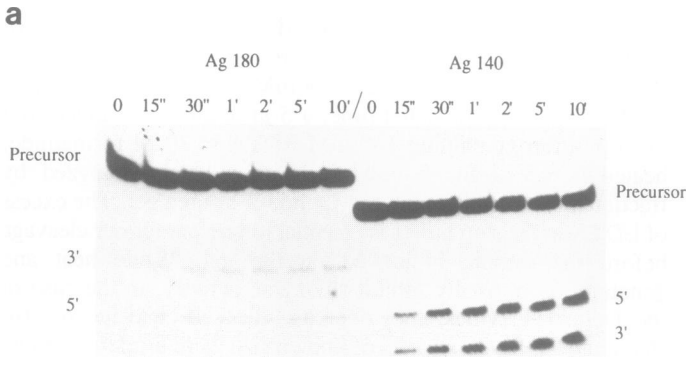


Figure 1. The antigenomic Delta ribozymes derived from *in vitro* transcription with T7 polymerase of a subcloned Sal I-Xba I fragment (antigenomic HDV nucleotides 962 to 781) from pD1 (a gift of Dr. J. Taylor) in the polylinker of pGEM4 (Promega). The original plasmid pG-180, was further deleted to form pJB17, from which the 5′ truncated RNAs were transcribed.

Efficiency of cleavage in denaturing conditions varied with the ribozyme studied

If such a conformational change resulted in increased cleavage, then reagents that affect nucleic acid structure might be expected to allow the transition at a lower temperature. Zaug *et al.* (7) found that adding formamide or urea enhanced cleavage specificity in a ribozyme-substrate complex by destabilizing mismatched nucleotides, but the cleavage reaction was inhibited above 2 M urea or 5 M formamide. Recently Rosenstein and Been (4) reported that mild denaturing conditions increased the self-cleavage of a 135 nt genomic ribozyme from HDV, and this observation was broadened to include other genomic HDV ribozymes by Wu and Lai (5). Similarly, we reported that cleavage of a 114 nt genomic fragment at 37°C reached 90% in 5 min in the presence of 12.5 M formamide, while without formamide, the ribozyme was only 50% cleaved under the same conditions of time and temperature (6). In concentrations of formamide above 12.5 M, however, the cleavage was attenuated.

We characterized the cleavage of Ag140 and Ag180 at 37°C in the presence of urea or formamide. Ag140 showed an increase in cleavage up to 2 M urea or 7.5 M formamide (Figs. 3 a, c, d), then cleavage was inhibited. We interpreted this to mean there is an initial dissociation of interfering sequences from a 'core' sequence, or a conformational shift at the lower concentrations of denaturant to induce active core formation, leading to optimal cleavage. At higher concentrations of denaturant, the self-cleaving core dissociated, leading to inhibition of cleavage.



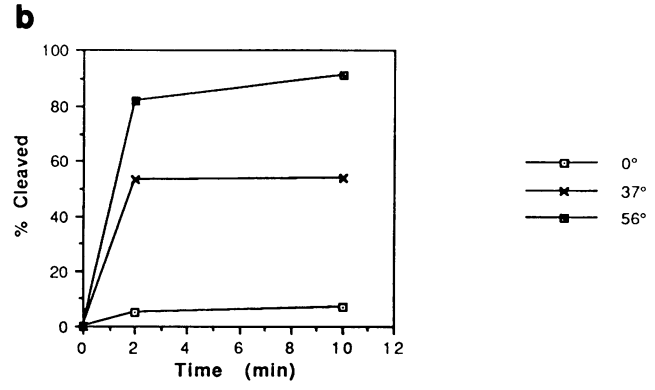


Figure 2 a. Self-cleavage at 22.5°C of Ag180 and Ag140. The two ribozymes show dramatically different efficiencies of cleavage at low temperatures. **Figure 2 b.** Increasing the temperature of the cleavage reaction increased the amount of cleavage, as seen with Ag140. Presumably a conformational change in a portion of the RNA molecules was necessary for the active cleavage conformation to form.

The Ag180 ribozyme, on the other hand, showed a strikingly different profile, both in urea and formamide. The longer ribozyme cleaved completely in up to 18 M formamide, or 9 M urea (Figs. 3 b, d). At 20 M formamide or 11 M urea, a partial inhibition of the self-cleavage reaction was seen (Figs. 3 c, d). Thus a sequence element located in the terminal 40 nts appeared to confer unusual stability on Ag180, allowing the self-cleavage reaction to proceed under highly denaturing conditions.

Truncating the 5′ end of the ribozyme did not affect the stability in formamide or urea

Reducing the length of the 5′ portion of the molecule to 12 vector nucleotides and only 17 HDV nucleotides while leaving the long 3′ end (Ag136, Fig. 1) produced a ribozyme that retained the capacity to cleave completely in 18 M formamide (data not shown). When both 5′ and 3′ deletions were combined, however, producing a precursor RNA with 17 Delta nts upstream and 79 nts downstream of the cleavage site, Ag96 (Fig. 1), the stability in both formamide and urea was intermediate between that of Ag140 and Ag180 (Fig. 3 c, d). Removal of an additional 10 nts from the 3′ terminus of Ag96 to create Ag86 (Fig. 1) destroyed the self-cleavage activity of the RNA (Fig. 4). Further experiments are in progress to determine whether this is an end point for required sequences or a 'window' of inactivity.

Reinsertion of the terminal 10 nucleotides restored the stability in formamide

To determine if unusually stable structures might be identified through computer analysis, we used the RNAFOLD program (8, a gift of M. Zuker) to analyze possible differences in the Ag180, Ag140 and Ag96 molecules at different temperatures. One folding revealed a possible stem-loop structure that might have been destroyed in truncating the 40 nts at the 3′ end to create Ag140 and Ag96 (Fig. 5 a).

To test the significance of the ten nucleotides necessary to form the putative stem-loop structure at the 3′ end terminal, we reinserted them. Analysis of the cleavage of the resulting ribozyme (Ag106, Fig. 1), revealed that the stability in formamide had been restored (Fig. 3 c).

DISCUSSION

In studying HDV antigenomic ribozymes of close to minimal length, we have found that some possess an unusual stability to denaturation in formamide or urea, which results in a very

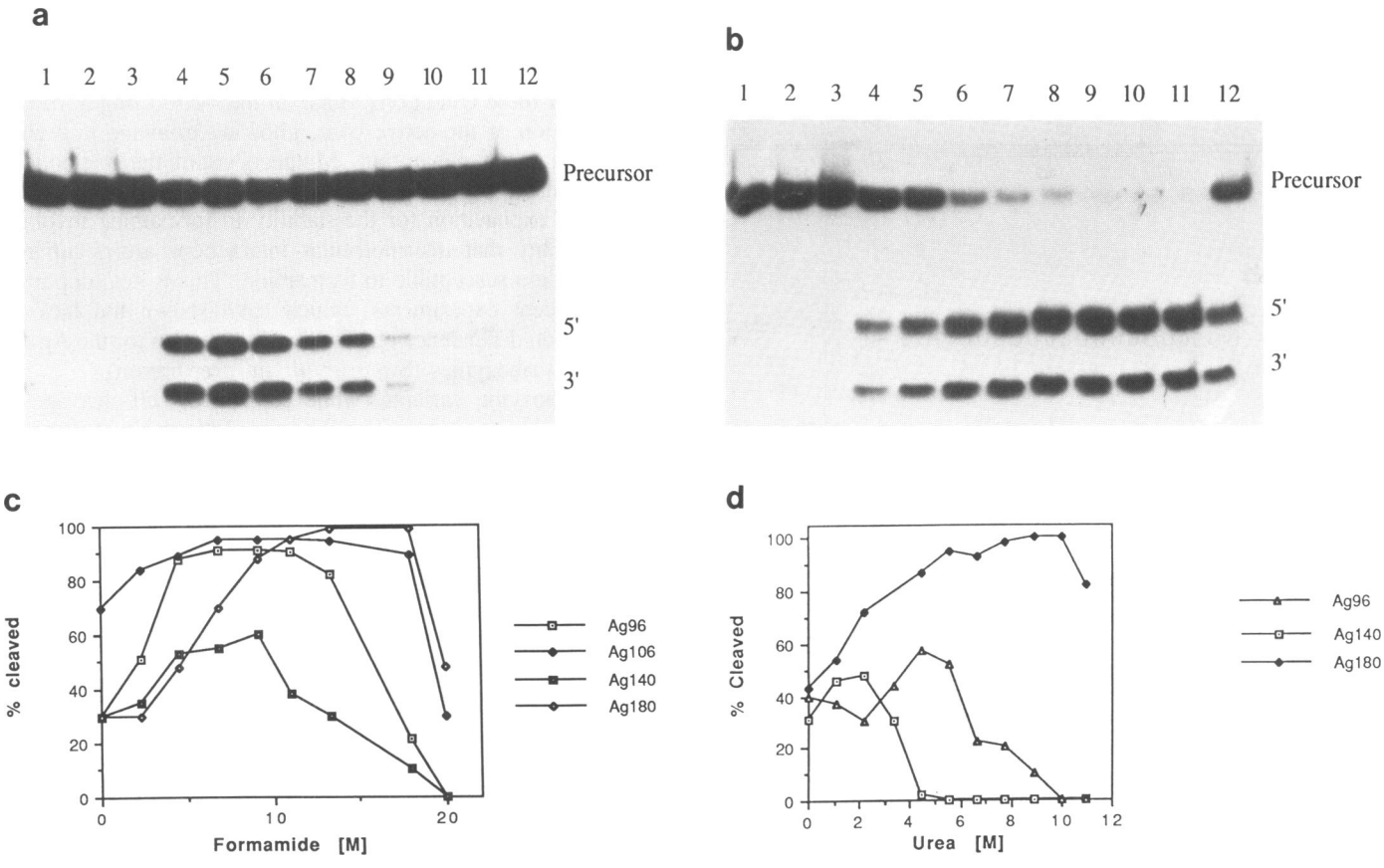


Figure 3. The four ribozymes show distinctly different cleavage patterns with increasing concentrations of formamide and urea. (a) Cleavage of the Ag140 ribozyme in formamide at 37°C. Lanes 1−3; No magnesium was added, but increasing concentration of formamide: 2.3 M, 6.8 M and 20 M. Lanes 4−12; the magnesium concentration was 9 mM, and the formamide concentrations were: (4) no formamide (5) 2.3 M (6) 4.5 M (7) 6.8 M (8) 9.1 M (9) 11.1 M (10) 13.4 M (11) 18 M (12) 20 M. (b) The Ag180 cleavage in formamide continues far beyond the concentration that inhibited the Ag140. The lane demarcations are the same as in Fig. 3 a. (c) The pattern of enhanced cleavage and cleavage inhibition in formamide. The Ag180 displays the remarkable ability to cleave in up to 20 M formamide, and Ag96 cleaves to 70% in 13 M formamide. On the other hand, Ag106 has regained the ability to cleave completely in 18 M formamide. (d) Delta ribozyme cleavage in the presence of urea. Above 4.5 M urea the Ag140 cleavage was completely inhibited, while only a partial inhibition was seen above 10 M urea with Ag180. Ag96 shows inhibition of cleavage above 6 M urea, but is still capable of some cleavage at 9 M urea concentration.

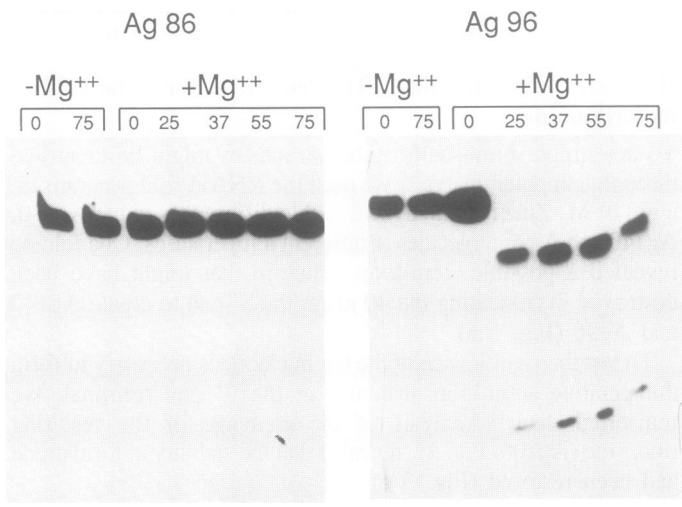


Figure 4. Deletion of an additional ten nucleotides from the 3′ terminus of Ag 96 to form Ag86 abolished autocatalytic cleavage, even at elevated temperatures. (Left) Ag86 shows no cleavage with increasing temperature. (Right) Ag96 cleavage activity continues even at 75°C.

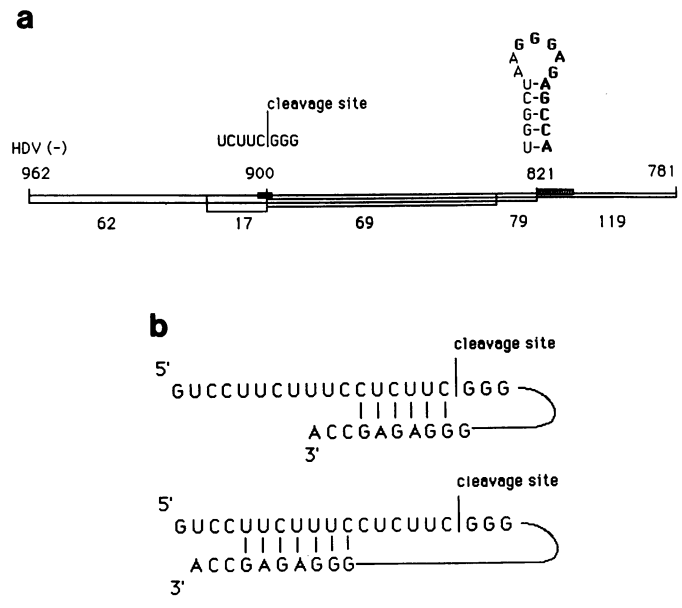


Figure 5 a. Regions involved in self-cleavage, cleavage inhibition and stabilization of the ribozyme have been identified in a 106 nt fragment of the antigenomic HDV ribozyme. Numbers above the figure locate the fragment within the antigenomic HDV molecule (14). The lengths of the proposed domains in the ribozyme are noted below the figure. Written in **bold** letters are the ten nucleotides inserted to form Ag106 from Ag96. **Figure 5 b.** Possible base-pairings between the ten nucleotides which confer stability in formamide and two regions near the cleavage site.

efficient self-cleavage under highly denaturing conditions. Thus, a 186 nt ribozyme cleaved to 100% in five min at 37°C in 18 M formamide. The cleavage time was measured after magnesium was added, and following five min preincubation in the denaturant, when presumably all but the most stable structures would have melted out. Truncation of the terminal 40 nts resulted in a loss of the stability, and maximal cleavage, which never reached 100%, was seen in 10 M formamide. An increase in both activity and stability was noted with further truncation of the 5′ end (compare Ag140 with Ag96, Fig. 3 c). Ag140 requires a higher concentration of formamide to achieve its maximal cleavage, which is still less than that of Ag96. Presumably, the deleted 5′ sequences could inhibit formation of the active core structure through formation of alternate conformers.

We propose a model for antigenomic Delta ribozyme cleavage and stability (Fig. 5 a). A maximum of 17 nts upstream and 79 nts downstream of the cleavage site are necessary for self-cleavage. Between 17 and 62 nts upstream and between 79 and 119 nts downstream are sequences which interfere with the cleavage, possibly by causing formation of RNA conformers. Eliminating the upstream and downstream sites, forming Ag96, allows the active core to form, with resultant cleavage. Cleavage is still enhanced by concentrations up to 15 M formamide, because the active core itself is resistant to formamide. Above 15 M formamide, the active core dissociates, and cleavage is inhibited. The region between antigenomic HDV nts 821 and 811 contains a sequence element that confers an exceptional stability on the Ag180 and Ag106 Delta RNAs, allowing cleavage in up to 18 M formamide and, in the case of Ag180, in 10 M urea. Sequence analysis of possible interactions between these ten nucleotides and the remainder of the ribozyme revealed two potential interactions. In one, six of the ten added nucleotides could base pair with the six nucleotides immediately 5′ of the cleavage site (Fig. 5 b, upper pairing). The second would involve seven of the terminal ten in a pairing with seven nucleotides (−7 to −13) upstream of the cleavage site (Fig. 5 b, lower pairing). The postulated stem-loop at the 3′ terminus (Fig. 5 a) would not form under these conditions. Such an interaction might lead to a stabilization of the active core, allowing cleavage in higher concentrations of denaturant. Mutagenesis of the nucleotides involved in the proposed interations is currently in progress.

Another explanation for the stability in formamide involves the possibility that intermolecular interactions are occurring, which are less susceptible to formamide. This is not supported by our recent experiments, which have shown the lack of concentration dependence on the cleavage reaction for the Ag180 and Ag140 ribozymes (Smith *et al.*, in preparation).

Delta ribozyme variants, while capable of self-cleavage at physiological temperatures (Fig. 2 b), are also capable of cleavage under biologically adverse conditions. The 186 nt antigenomic Delta cleaves at temperatures up to 75°C (data not shown), 25° above the highest temperatures seen with the hammerhead (9−11) or the hairpin (12) ribozymes. Ag96, in addition to cleaving in temperatures up to 75°C (Fig. 4), cleaves rapidly to completion at 37°C, with a first order rate constant of 1.4 min^{-1} (data not shown). Antigenomic Delta ribozymes thus combine efficiency of cleavage at physiological temperatures with an unusual degree of internal stability. These attributes should prove most useful in the design of a ribozyme for *in vivo* activity (13). Finally, the tolerance of Delta ribozymes to high temperature and denaturants indicates that some ribozymes are capable of activity in conditions inhospitable to current life or enzymatic activity.

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