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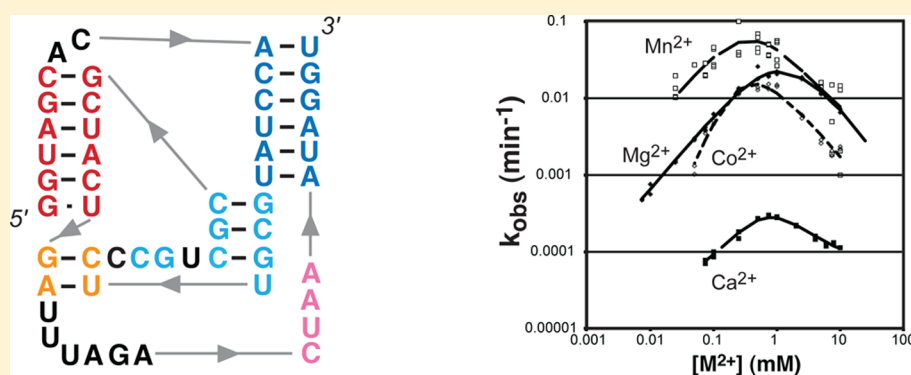
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S Supporting Information



ABSTRACT: HDV-like self-cleaving ribozymes have been found in a wide variety of organisms, implicated in diverse biological processes, and their activity typically shows a strong divalent metal dependence, but little metal specificity. Recent studies suggested that very short variants of these ribozymes exist in nature, but their distribution and biochemical properties have not been established. To map out the distribution of small HDV-like ribozymes, the drz-Spur-3 sequence was minimized to yield a core construct for structure-based bioinformatic searches. These searches revealed several microbial ribozymes, particularly in the human microbiome. Kinetic profile of the smallest ribozyme revealed two distinct metal binding sites, only one of which promotes fast catalysis. Furthermore, this ribozyme showed markedly reduced activity in Ca^{2+} , even in the presence of physiological Mg^{2+} concentrations. Our study substantially expands the number of microbial HDV-like ribozymes and provides an example of cleavage regulation by divalent metals.

The HDV ribozymes are among several RNAs that fold into structures promoting scission of their own backbone.¹ This activity occurs through 2'OH attack on the phosphate group of the adjacent nucleotide, producing a 2'-3' cyclic phosphate and 5' hydroxyl. Despite this common mechanism of self-scission, the secondary structures for the various classes of ribozymes are diverse.

The structure of the HDV ribozymes consists of five helical domains (P1, P2, P3, P1.1, and P4) arranged into a nested double-pseudoknot, with P1, P1.1, and P4 forming one coaxially stacked helix, and P2 and P3 forming another. Short single-stranded regions link the base-paired domains. The active site of the ribozyme is built around the L3, P1.1, and J4/2 regions, with the J4/2 containing the catalytic cytosine residue that acts as the proton donor in the cleavage reaction.^{2–8} The genomic and anti-genomic HDV ribozymes require a divalent metal ion for full catalytic activity, and the activity-pH profile is flat over a wide pH range, suggestive of two active-site titratable groups, one of which likely corresponds to the catalytic cytosine nucleobase.^{5,6,9–11}

Several other nucleotides in the ribozyme core form crucial interactions that promote catalysis. In the genomic HDV ribozyme, the U20 and G25 residues at the base of the P3 helix form a reverse G•U wobble pair that positions the N7 and O6 atoms of the guanine base to bind the catalytic metal.⁵ The G25 stacks on the conserved C24 residue, which in turn forms a hydrogen bond with the pro- S_p phosphate of C22 and creates a niche centered on the active site.^{2,4,5} In the J4/2 region, a conserved adenosine residue forms an A-minor interaction with the C18-G29 base-pair of the P3 helix and helps position the catalytic C75 residue into the cleavage site.² Finally, the pro- R_p oxygen of the scissile phosphate contacts the catalytic metal, and in one genomic ribozyme crystal structure, it forms a sharp U-turn kink that promotes release of the upstream product following scission.^{5,12,13} The identity of this nucleotide has a

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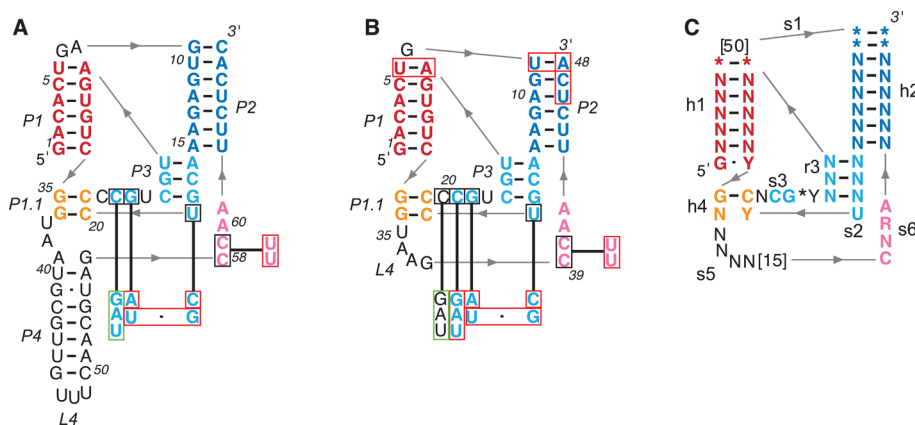


Figure 1. Minimization of HDV-like ribozymes. (A) drz-Spur-3 ribozyme. Black boxes indicate sites of mutagenesis, with connected boxes indicating variants. Green and red boxes indicate permissive and inhibitory substitutions, respectively. (B) Minimized drz-Spur-3 ribozyme (drz-Spur-3-min). Color scheme for substitutions is the same as in (A). Nucleotides in red boxes indicate deletions that abolished self-scission. (C) Structure-based descriptor used in genomic searches for minimal HDV-like ribozymes. Asterisks indicate nonessential insertions. Bracketed numbers correspond to variable-length regions with maximum lengths indicated in the brackets.

cleavage bands seen in the displayed in-line reaction in the L4 loop region as the same bands that appeared when the samples were run next to phosphorothioate sequencing ladders.

RESULTS

Minimization of drz-Spur-3 and Structure-Based Searches.

In order to determine the minimal sequence required for an HDV-like ribozyme activity, a series of deletions were designed based on the drz-Spur-3 ribozyme. This ribozyme was chosen because it exhibited robust cleavage activity even though it contained a shortened six base-pair P1 helix (Figure 1A).¹⁶ Furthermore, the drz-Spur-3 ribozyme maintains the sequence requirements important for self-scission in the HDV-like fold: mutations of the active site cytosine (C58) abolish activity and self-scission is abrogated when the U19•G24 reverse wobble pair at the base of the P3 helix is either swapped or individually mutated to form traditional Watson–Crick base-pairs (U19C or G24A).^{2,4,5,23,24} When we mutated the G-U pair in drz-Spur-3, self-scission was abolished, supporting an interaction similar to the genomic HDV ribozyme.⁵ Interestingly, mutations to the C23 residue in drz-Spur-3 showed minimal effect on self-scission. This nucleotide is highly conserved in the HDV ribozyme, and it extends the P3 helix by stacking on the reverse G•U wobble in HDV ribozyme crystal structures.^{2,5,21,23} Overall, these data support a model of the drz-Spur-3 ribozyme core highly similar to the HDV ribozyme, while the reduced P1 helix made it an ideal candidate for minimization studies.

We started minimization of the drz-Spur-3 ribozyme by investigating the P4 helix. Previous reports indicated that this helix is not essential, and can be replaced with an eight-nucleotide loop without abolishing catalysis.²¹ To test whether this reduction has the same effect on the drz-Spur-3 sequence, we sequentially deleted nucleotides in this region until only four nucleotides joined the P1.1 helix to the active site cytosine. The composition of these nucleotides appears unimportant, as both the wild-type UAAG sequence as well as a GAAA mutant supported self-scission. Shortening this strand further to a three-nucleotide joining region abolished self-cleavage.

We next sought to examine the nucleotide requirements in the P1, J1/2, and P2 regions of the ribozyme. Several HDV-like ribozymes contain only a single nucleotide in the J1/2 region,

and indeed this length was tolerated in the drz-Spur-3 sequence with no compositional preference, despite the presence of the shorter P1 helix.¹⁸ The P2 helix was less susceptible to deletions, as removal of more than a single base-pair abolished activity. Deletions to the 3' side of this helix also proved deleterious to self-scission. Finally, further shortening of the P1 helix failed to yield active constructs, even in conjunction with additional reductions to the P2 helix on the expectation that similar helical length for the P1 and P2 regions would maintain the same register along the two helical stacks. Combined, these deletions yielded a 48-nt minimal motif, drz-Spur-3-min, which possessed residual cleavage activity ($3 \times 10^{-3} \pm 1 \times 10^{-3} \text{ min}^{-1}$, 37 °C and 10 mM Mg²⁺) (Figure 1B).

To verify that the minimized ribozyme, drz-Spur-3-min, was undergoing HDV-like self-scission, we again mutated residues predicted to be essential to catalysis. A C39U/C40U double mutation abolished self-scission, as did reversal of the predicted G•U wobble pair at the base of the P3 helix. Unlike the drz-Spur-3 parent sequence, mutation of the C21 residue that stacks on the G•U wobble was detrimental to self-scission. However, mutations to C20, which is predicted to act only as a spacer nucleotide, did not affect catalysis. These results indicate that the drz-Spur-3-min ribozyme, despite being only 48 nucleotides long, maintains the HDV-like ribozyme core.

Using the drz-Spur-3-min sequence as the template, we designed secondary-structure descriptors to use in searches for minimal genomic HDV-like ribozymes (Figure 1C).²⁵ Because the minimal HDV-like motif, as defined by drz-Spur-3-min, contained several features that were excluded when HDV-like ribozymes were first identified through structure-based searches, we allowed the P1 helix to be six or seven base pairs long, and the J1/2 and P4/L4 regions to be up to 50 and 19 nucleotides in length, respectively. We searched through the available genomic sequences of viruses, bacteria, and eukaryotes, as well as a several microbial metagenomic data sets for sequences capable of forming the minimal HDV-like secondary structure. The result was a relatively permissive structure-descriptor that uncovered numerous putative HDV-like ribozymes that possessed some or all of the newly defined minimal features. Of particular interest were a family of sequences found in the lancelet *Branchiostoma floridae*, where canonical HDV-like ribozymes were previously found, a

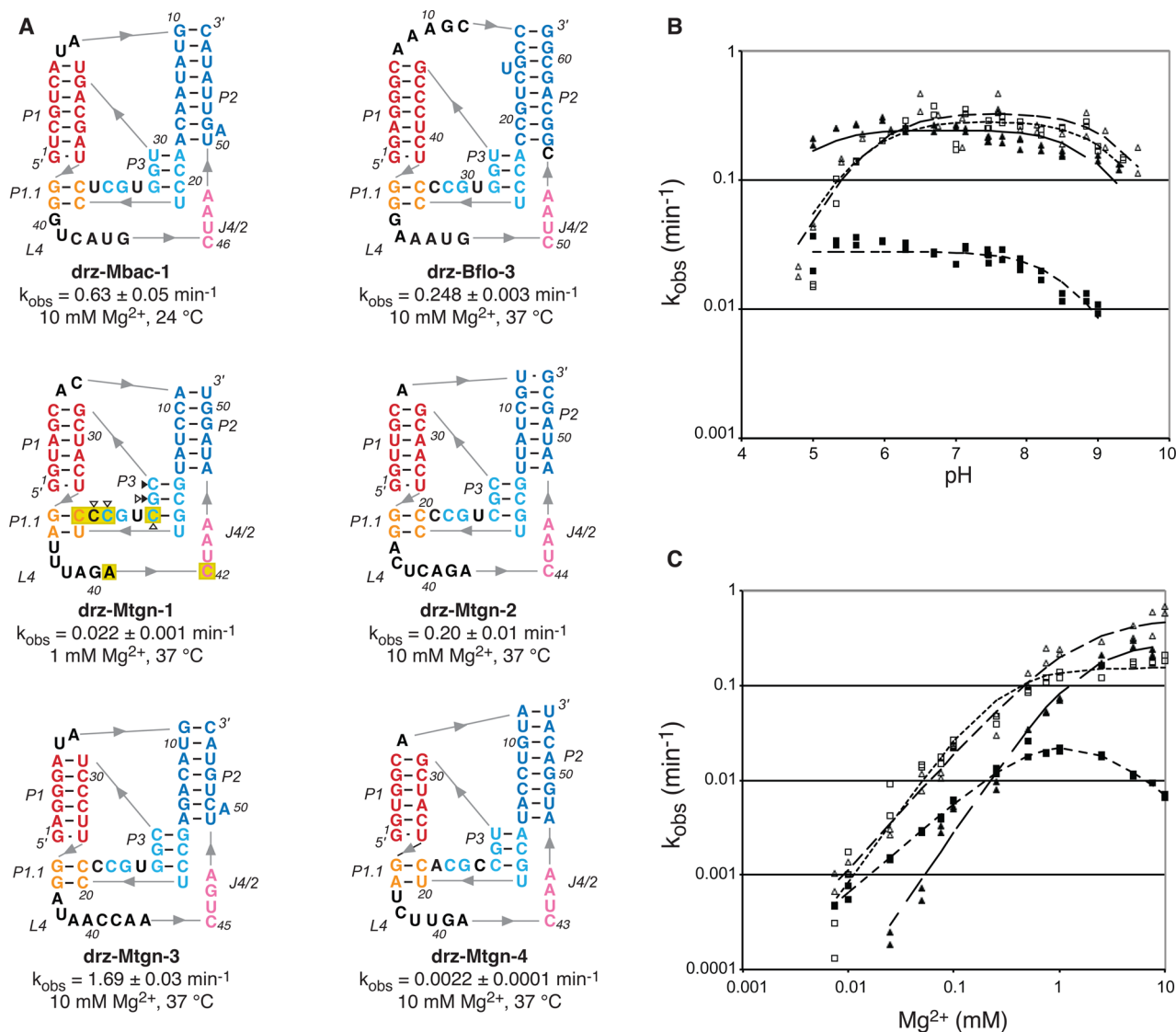


Figure 2. Minimal HDV-like ribozymes. (A) Secondary structures and activity of the genomic minimal HDV-like ribozymes, drz-Mbac-1, drz-Bflo-3, drz-Mtgn-1, 2, 3, and 4, respectively. Nucleotides boxed in yellow in the drz-Mtgn-1 ribozyme correspond to positions subject to phosphorothioate interference shown in Figure 4, closed arrows correspond to positions subject to in-line degradation at low Mg^{2+} , and open arrows correspond to positions showing in-line degradation at high Mg^{2+} concentrations. (B) pH profiles of the drz-Mbac-1 (open triangles), drz-Bflo-3 (filled triangles), drz-Mtgn-1 (open squares), and drz-Mtgn-2 (filled squares) ribozymes. The estimated pK_a s for each ribozyme are 5.7 and 9.4, 4.7 and 9.0, undefined and 8.6, and 5.6 and 9.2, respectively. (C) Mg^{2+} -dependence of the ribozymes. Symbols are the same as in (B). The calculated Mg^{2+} $K_{1/2}$ s are 1.5 mM, 1.7 mM, 1.0 mM, and 0.28 mM, respectively.

ribozyme from *Mollicutes bacterium* D7 (also known as *Coprobacillus* sp. D7), and a number of sequences identified in a metagenomic data set derived from the human microbiome (Figure 2).^{16,22} The metagenomic ribozymes represent the smallest naturally occurring HDV-like ribozymes.

Characterization of Minimal HDV-Like Ribozymes. In the lancelet *B. floridae*, three minimal HDV-like ribozymes were found in close proximity to each other. This organism was previously shown to harbor two families of canonical HDV-like ribozymes (drz-Bflo-1 and -2).¹⁶ The new drz-Bflo-3 family is characterized by a seven base-pair P1 helix and a five nucleotide J1/2 region. Unlike the extended J1/2 domains found in the drz-Agam-2 ribozymes and related retrotransposon-associated HDV-like ribozymes, no secondary structure is predicted for the J1/2 region of the drz-Bflo-3 sequences.^{16,26–28} The drz-Bflo-3 family also lacks the entire P4 helix, instead employing just six nucleotides to join the P1.1 helix to the active-site

cytosine. Mutation of the active site cytosine to a uridine abrogated self-scission, confirming an HDV-like catalytic core.

The three drz-Bflo-3 ribozymes identified through structure-based searches reside on the same strand within a 4000-nucleotide region of a single genomic scaffold. No annotated transcripts are present in this locus, although several ESTs are found within 20 kb of the ribozymes. Sequence-based searches of the *B. floridae* genome revealed three additional drz-Bflo-3-like ribozymes. Two of these sequences contain four point mutations, none of which are predicted to lower the ribozyme activity; however, the third instance lacks a fully base-paired P2 helix. Notably, the drz-Bflo-3–5 sequence resides within a large intronic region of EST 869853, which is predicted to code for the catalytic core domain of a cysteinyl tRNA synthetase, but the ribozyme’s function within this intron is unknown.

Microbial Ribozymes. Two strains of the *M. bacterium* were found to harbor a single copy of a small HDV-like

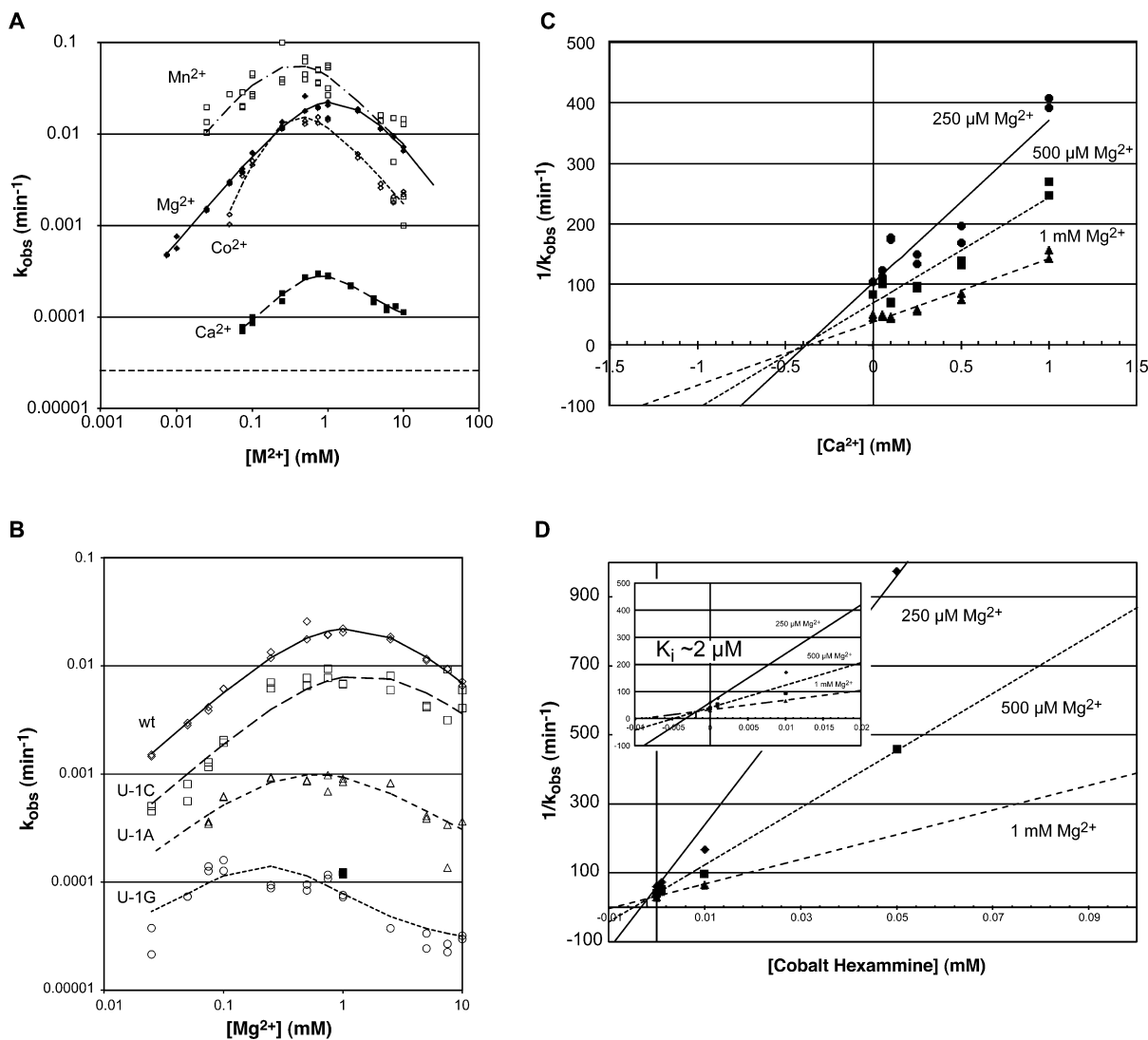


Figure 3. *In vitro* activity of the drz-Mtgn-1 ribozyme. (A) Divalent metal ion profile of the drz-Mtgn-1 ribozyme. The calculated $K_{1/2}$ s for Mg²⁺ (filled diamonds), Mn²⁺ (open squares), Co²⁺ (open diamonds), and Ca²⁺ (filled squares) are 0.96 and 1.3, 0.23 and 0.51, 0.26 (Hill coefficient \sim 1.9) and 0.53, and 0.58 and 2.0, respectively, at 37 °C, in 140 mM KCl and 10 mM NaCl. The dashed line corresponds to the observed rate of cleavage in the absence of divalent metal ions. (B) Mg²⁺ response of the WT (diamonds) and U-1 variant ribozymes with U-1A (triangles), U-1C (squares), and U-1G (circles) substitutions. The bell-shaped response suggests that the -1 nucleotide does not affect binding of additional, rate-reducing metals (see Discussion). The activity for the U-1G mutant in calcium (filled squares) suggests that the low activity for the WT sequence in Ca²⁺ is due to unfavorable interactions Ca²⁺ forms with the upstream nucleobase. (C) Ca²⁺ inhibition of the Mg²⁺-dependent activity. The intersection point for linear models for 1/ k_{obs} at 250 μ M (circles), 500 μ M (squares), and 1 mM (triangles) Mg²⁺ over a range of Ca²⁺ concentrations indicate noncompetitive (allosteric) inhibition with a Ca²⁺ K_i of \sim 400 μ M. (D) Cobalt hexammine inhibition of the Mg²⁺-dependent activity. The intersection point for linear models for 1/ k_{obs} at 250 μ M (circles), 500 μ M (squares), and 1 mM (triangles) Mg²⁺ over a range of cobalt hexammine concentrations indicates competitive inhibition with a K_i of \sim 2 μ M. Displayed values are the average of two independent trials.

ribozyme that contains a two-nucleotide J1/2 region, a seven base-pair P1 helix, and a six-nucleotide L4 loop. The ribozyme self-cleaved efficiently at ambient temperature in physiological-like metal ion concentrations ($0.23 \pm 0.01 \text{ min}^{-1}$; 24 °C and 1 mM Mg²⁺). Drz-Mbac-1 resides \sim 700 nts downstream of a predicted PolY gene (EHM88375) and \sim 300 nt upstream of several other predicted genes of unknown function (EHM88373 and EHM88374) as well as tRNA^{Met}. This sequence represents the second confirmed bacterial HDV-like ribozyme, suggesting that HDV-like ribozymes may be more common in bacteria than originally found.¹⁶

Several small HDV-like ribozymes were identified in metagenomic data sets, which were derived from deep sequencing of human sewage and previously yielded 14 new

hammerhead ribozymes.^{22,29–31} Four of these (drz-Mtgn-1 to 4) were active *in vitro*, showed no self-scission when the active site cytosine is mutated to a uridine, and exhibited remarkably small HDV-like structures. In particular, drz-Mtgn-1 consists of the minimal P1 and P2 helices of six base-pairs seen in the engineered minimal ribozymes, along with only two nucleotides in the J1/2 region and six nucleotides joining the P1.1 to the active site cytosine, and self-cleaves with a rate constant of $(0.021 \pm 1) \times 10^{-3} \text{ min}^{-1}$ in 1 mM Mg²⁺ at 37 °C.

The sequencing tracks containing drz-Mtgn-1 and -4 were obtained from a human diarrheal sample and exhibited partial overlap, indicating that these ribozymes occur in a single organism. Drz-Mtgn-4 also lacks a P4 helix, but has a predicted 8-bp P2 helix, self-cleaves with a rate constant of (2.0×10^{-3})

$\pm (1 \times 10^{-4}) \text{ min}^{-1}$ in 10 mM Mg^{2+} at 37 °C, and appears in the metagenomic contig In-A_000423 (GenBank BABBO1000423.1). The sequencing track from which drz-Mtgn-1 was isolated shows similarity to a locus of the *Bacteroides cellulosilyticus* gene coding for a 3-deoxy-7-phosphoheptulonate synthase, with the ribozyme located approximately 400 nts upstream of the protein-coding domain.³² This mapping suggests that the drz-Mtgn-1 and -4 ribozymes originated from a bacterial genome.

The drz-Mtgn-2 and -3 ribozymes shared similar structural features with drz-Mtgn-1 and -4; however, they possess 7-bp P2 helices and slightly longer joining strands between the P1.1 helix and the active-site cytosine. These sequences self-cleave faster than the drz-Mtgn-1 and 4 ribozymes ($k_{\text{obs}} = 0.20 \pm 0.01 \text{ min}^{-1}$ for drz-Mtgn-2; $1.69 \pm 0.03 \text{ min}^{-1}$ for drz-Mtgn-3 in 10 mM Mg^{2+} at 37 °C), on par with many larger HDV-like ribozymes.¹⁶ The drz-Mtgn-2 ribozyme, like the drz-Mtgn-1 and -4 sequences, was identified in a human diarrheal sample, whereas drz-Mtgn-3 was obtained from deep sequencing of human sewage.

Kinetic pH Profiles. To probe the effects of the minimal HDV-like structures on the catalytic mechanism, we examined pH profiles for the most active constructs (Figure 2B). This analysis was conducted in physiological monovalent ion concentrations (140 mM KCl, 10 mM NaCl), which was previously shown to reduce the apparent pK_a of the catalytic cytosine in the HDV ribozymes.³³ All of the tested small HDV-like ribozymes, along with the full-length drz-Spur-3 sequence, exhibited flat pH profiles between pH 6 and pH 8, consistent with a mechanism in which two titratable groups affect the rate-limiting step of the reaction. However, neither the drz-Bflo-3 family nor the drz-Mtgn-1 ribozyme showed a decline in activity at lower pH, suggesting a pK_a for the active-site cytosine comparable to the unperturbed value of ~ 4.2 , and likely resulting from the presence of 150 mM monovalent salts.³⁴

In addition to the activity–pH relationship, we examined the divalent metal ion dependence of the minimal ribozymes, along with the drz-Spur-3 sequence (Figure 2C). The drz-Spur-3, drz-Bflo-3, drz-Mbac-1, and drz-Mtgn-2 ribozymes all showed a strong metal dependence up to 10 mM Mg^{2+} . The Hill coefficients for these sequences varied between 1 and 1.6, indicating some binding cooperativity, as was previously suggested for the HDV and *CPEB3* ribozymes.^{17,33} Again, the moderately high physiological-like levels of monovalent ions included in the buffer solution likely partially stabilized the active structure, perhaps resulting in slightly lower Hill coefficient than would be observed in a monovalent ion-free solution. In all cases the self-scission followed a monoexponential decay. Biexponential behavior, typical of larger HDV-like ribozymes and indicative of misfolded ribozyme subpopulations, was not observed for any of the constructs.

Metal Dependence and Role of the Upstream Nucleobase on drz-Mtgn-1 Scission. Surprisingly, the drz-Mtgn-1 ribozyme did not show an increase in its catalytic rate constant at higher Mg^{2+} concentrations. This ribozyme exhibited a positive activity–metal correlation up to ~ 1 mM Mg^{2+} , but at higher concentrations, cleavage rate constants decreased, resulting in bell-shaped activity–metal curves. A parsimonious explanation for this pattern is a model with two metal-binding modes, one of which promotes fast catalysis and a second that either inhibits catalysis or causes it to proceed at a much slower rate. A similar model was previously used to explain hammerhead ribozyme cleavage in the presence of

lanthanides, and a related metal ion response has been observed for both genomic and anti-genomic HDV ribozymes with distinct combination of metal ions and the nucleotide identity at the -1 position, though not with Mg^{2+} as the catalytic metal.^{9,15,35}

To further study the bell-shaped metal dependence of the drz-Mtgn-1 ribozyme, its self-cleavage activity was analyzed with a panel of divalent metal ions. The activity of the drz-Mtgn-1 ribozyme in the tested metal ions was variable, but did not follow any physiochemical trends, such as pK_a or the ionic radius. Unlike HDV ribozymes, drz-Mtgn-1 exhibited bell-shaped activity–metal curves for all divalent ions tested, suggesting that the second metal-binding mode for this ribozyme is inherent to the fold and not a product of a particular metal's unique interaction with the ribozyme's structure (Figure 3A). These results agree with a two-channel model for self-cleavage, wherein at low divalent metal concentrations only the catalytic metal-binding site is occupied and self-scission proceeds efficiently, but as the concentration of divalent ions increases, the metal ion binds an additional site that promotes a suboptimal conformation, causing the observed cleavage rate to decrease.^{8,15} We also observed that the self-scission in Ca^{2+} proceeded approximately 100-times slower than in other divalent metals. Such a pronounced preference against a particular divalent metal has not been seen in other HDV-like ribozymes, including the full-length drz-Spur-3 sequence. Calcium dependence retained a bell-shaped metal-response curve, indicating that it could bind in both rate-activating and rate-reducing modes; however, the absence of efficient catalysis suggests that Ca^{2+} interacts with the ribozyme in a profoundly different manner than the other tested divalent metals.

We next sought to examine the Ca^{2+} dependence further by constructing variants with U-1A/C/G mutations, because in HDV ribozymes the identity of the nucleotide immediately upstream of the cleavage site was previously shown to influence the metal ion dependence and results in a bell-shaped k_{obs} plot.¹⁵ These constructs exhibited highly variable kinetics in Mg^{2+} , with the U-1C construct cleaving at approximately the same rate as the wild-type sequence, and the U-1A and U-1G mutants cleaving ~ 100 -times more slowly (Figure 3B). These data agree with previous analysis of the -1 position's effect on self-scission in HDV ribozymes.^{12,14} However, all drz-Mtgn-1 U-1 constructs continued to exhibit a bell-shaped metal dependence in Mg^{2+} , although for the U-1G construct, it was less pronounced. The slow self-scission of the U-1G mutant, coupled with the low metal response, correlates with previous models of the genomic HDV ribozyme active site, where the larger guanosine base is unable to assume the proposed U-turn kink that leads to efficient metal binding and is necessary for fast self-scission.^{12,13} As the U-1A construct also self-cleaved slowly, it is likely that a purine base at this site in general impairs metal binding, leading to slow catalysis. Because substitutions at the -1 nucleotide did not alter the overall shape of the activity–metal curves, the proposed second metal-binding mode appears independent of the upstream nucleotide; instead, this secondary binding mode seems to influence the core of the ribozyme.

The rate of self-scission for the U-1G construct was similar to the rate of self-cleavage of drz-Mtgn-1 in the presence of Ca^{2+} , suggesting that relatively slow cleavage in the presence of Ca^{2+} could be due to the metal's interaction with the -1 nucleotide. To test this model, we measured the self-cleavage for the U-1G

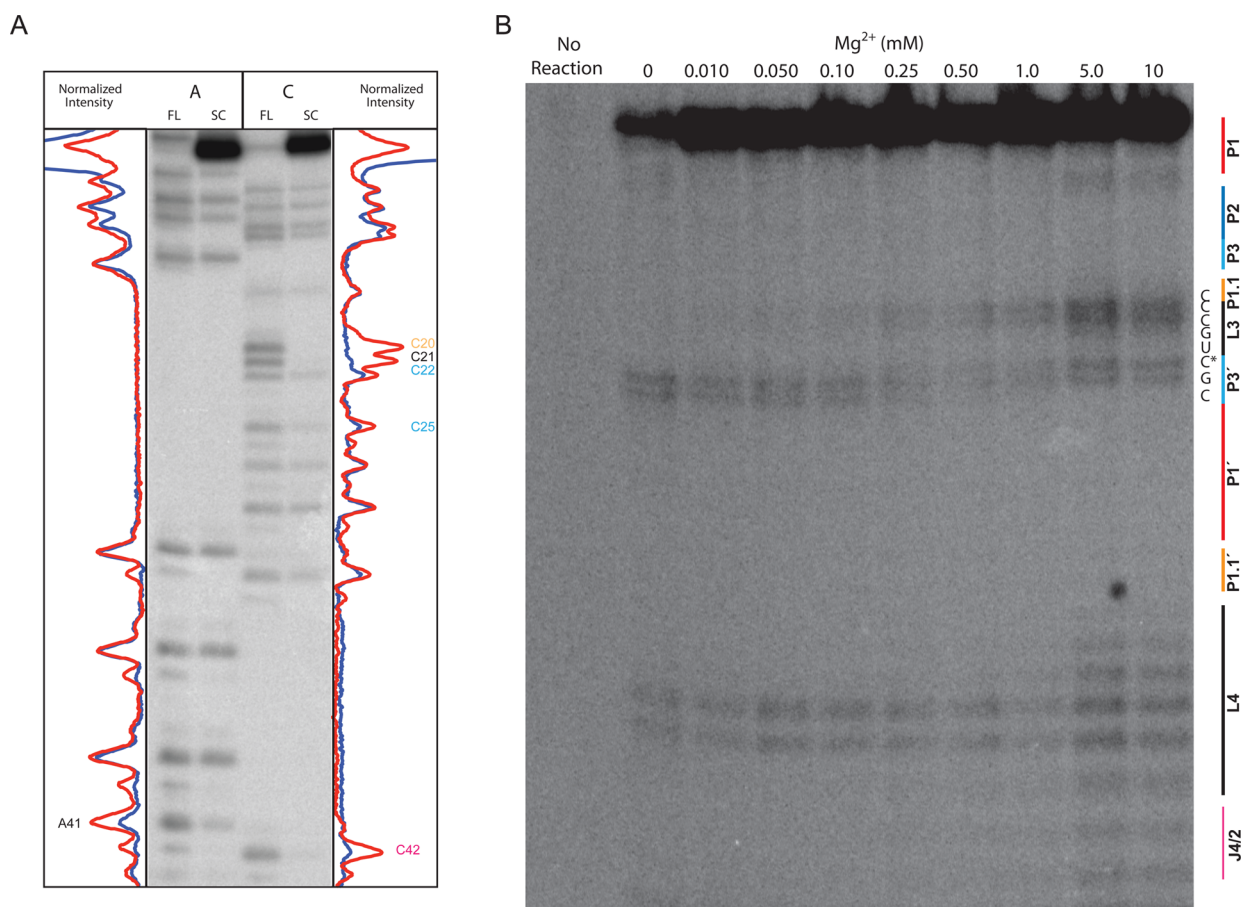


Figure 4. Structural analysis of drz-Mtgn-1. (A) Phosphorothioate interference mapping of A and C positions in the drz-Mtgn-1 ribozyme under cotranscriptional conditions. FL corresponds to full-length ribozyme and SC indicates the self-cleaved fraction. The normalized intensity for the A and C nucleotides are displayed to the left and right, respectively, with the red traces corresponding to the FL lanes, and the blue traces corresponding to the SC lanes. Positions demonstrating enrichment in the FL fraction are indicated and colored as in Figure 2. (B) In-line probing of the drz-Mtgn-1 sequence under increasing magnesium concentrations. No reaction corresponds to drz-Mtgn-1 RNA that was not subjected to in-line degradation and contains a full length, precursor form of the ribozyme (not shown). The various domains comprising the ribozyme's structure are indicated and colored as in Figure 2. The asterisk indicates the position where the degradation pattern shifts by a single nucleotide between low and high Mg^{2+} regimes. The sequence for the displayed PAGE is correlated with the appearance of the strong cleavage points in the L4 region in independent sequencing experiments (see Figure S1).

mutant in the presence of Ca^{2+} and compared it to the rate obtained for this construct in the presence of Mg^{2+} (Figure 3B). Unlike in the wild-type drz-Mtgn-1 ribozyme, where cleavage occurs roughly 100-times slower when calcium is present instead of magnesium, there is almost no difference in scission between these two metals in the U-1G construct. If the slow self-scission for the wild type ribozyme in calcium was due to the metal's interaction with a region other than the nucleotide upstream of the cleavage site, the U-1G mutant would be expected to also cleave more slowly in calcium. That is, the effect of the metal ion and U-1 mutation on the cleavage rate would be additive. Because the U-1G ribozyme rate constants in Mg^{2+} and Ca^{2+} are almost identical, the slow self-scission of the wild type ribozyme in Ca^{2+} is likely due to formation of a nonproductive conformation around the -1 nucleotide when calcium is the only divalent metal present.

Ca^{2+} Inhibition of drz-Mtgn-1 Activity. Because drz-Mtgn-1 cleaved slowly in Ca^{2+} , we sought to test whether Ca^{2+} affects the self-cleavage reaction in the presence of Mg^{2+} . We measured the ribozyme scission at various concentrations of Ca^{2+} and Mg^{2+} , and the inverses of the observed rates were plotted against the concentration of Ca^{2+} , yielding the Dixon

plot for inhibition analysis. A fundamental assumption of the Dixon analysis is that the rate of catalysis increases as the nontitrated reactant is increased. This method of inhibition analysis was only valid for Mg^{2+} concentrations below 1 mM, because above 1 mM Mg^{2+} , additional magnesium reduced the observed rate (Figure 3A). At low Mg^{2+} concentrations, cleavage is reduced in the presence of Ca^{2+} and the inhibition appears to be noncompetitive with a K_i of approximately 400 μM , whereby the Ca^{2+} ion binds at a site other than the active site and prevents fast catalysis (Figure 3C). Whether this effect is a result of calcium's presumed interaction with the nucleotide upstream of the cleavage site, or calcium interfering with other, noncatalytic magnesium binding sites, cannot be distinguished. In a control experiment, a titration of cobalt hexammine, an exchange-inert isostere of hydrated Mg^{2+} , produced a Dixon plot suggesting competitive inhibition over the same concentrations of Mg^{2+} , as has previously been reported for the HDV ribozyme (Figure 3D).³⁶ This result indicates that, at low concentrations, Mg^{2+} forms the same interactions within the active site of drz-Mtgn-1 as in the HDV ribozymes.

The Dixon analysis cannot be used over Mg^{2+} concentrations that yield decreasing cleavage rates in the absence of calcium;

however, information regarding metal binding can still be inferred. When Ca^{2+} is present at concentrations greater than 1 mM in reactions over this range of Mg^{2+} concentrations, rate constants are found to increase with additional Mg^{2+} , contrary to what occurs in the absence of Ca^{2+} . Below this calcium threshold, rate constants again decrease with rise of Mg^{2+} concentration. The calcium concentration at which this inversion occurs can be observed on a Dixon plot at the intersection point of the linear fits for calcium inhibition in higher Mg^{2+} regimes. In 1, 5, and 10 mM Mg^{2+} , linear fits cross at $\sim 300 \mu\text{M}$, which agrees well with the estimated K_i from rate-enhancing Mg^{2+} regimes. This result suggests that the primary cause of reduced catalysis at elevated Ca^{2+} and Mg^{2+} concentrations is likely due to a large population of the ribozyme forming an inactive fold (e.g., a misfolded U-turn) that is rescued when increasing amounts of Mg^{2+} displace calcium binding. At the lower Ca^{2+} concentrations, the inactive population arising from Ca^{2+} interactions is smaller, and the primary factor contributing to reduced catalysis is autoinhibition due to increasing Mg^{2+} levels occupying a second binding site, leading to inefficient catalysis. In combination, the calcium inhibition patterns in various magnesium regimes support a model in which calcium promotes formation of a nonproductive fold near the scissile phosphate, precluding efficient Mg^{2+} -catalyzed scission, and both metals retain dual binding modes that result in autoinhibition at elevated metal ion concentrations.

Structural Mapping of drz-Mtgn-1. In order to further define the bell-shaped metal profiles of drz-Mtgn-1, we sought to probe its metal-binding sites. To begin, we performed phosphorothioate interference mapping, in which the pro- R_p oxygen was substituted with a sulfur to define regions of the backbone where oxygen atoms are required for catalysis, either by binding a metal or by forming interactions with other regions of the ribozyme to help stabilize the active conformation.³⁷ Phosphorothioate substitution revealed several positions in the phosphate backbone essential for catalysis during cotranscriptional self-cleavage in the presence of Mg^{2+} (Figure 4A). Bands corresponding to thiophosphate-substituted nucleotides around the active site, as well as cytosines in the P1.1 and L3 core, were enriched in the inactive species of ribozyme (see Figure 2A for mapping of the interference sites onto the secondary structure). These regions correspond to known sites of Mg^{2+} binding in the HDV ribozymes and overlap with sites of phosphorothioate interference in the genomic HDV and human *CPEB3* ribozymes, suggesting that drz-Mtgn-1 ribozyme forms the conserved HDV ribozyme active site, despite the reduction in secondary structural elements.^{5,17,38} While this interference pattern supports a model wherein a single or multiple Mg^{2+} ions are forming contacts to the backbone around the active site prior to efficient catalysis, it failed to differentiate between the conformational changes that lead to subtle rate differences in active species under changing metal concentrations.

Ribozyme activity depends on the ability of the sequence to assume the catalytically competent conformation. Such folds are highly structured, and consequently, the phosphate backbone exhibits defined susceptibility to in-line degradation.^{39,40} The varied activity under changing Mg^{2+} concentrations seen for the drz-Mtgn-1 sequence indicates that the ribozyme is capable of forming multiple folding states. These states likely correspond to different backbone segments forming stable structures, and thus, analysis of the backbone for its

propensity to in-line attack under changing metal levels reveals sites of metal-dependent reorganization of local structure.

When the drz-Mtgn-1 ribozyme was incubated at various concentrations of Mg^{2+} , several regions exhibited changing degradation patterns (Figure 4B). At low magnesium levels, backbone scission is observed in the 3' side of the P3 helix as well as the L4 loop region. With increasing Mg^{2+} concentration yielding the fastest self-cleavage rates, the bands corresponding to the loosely structured L4 loop remained susceptible to in-line attack; however, the bands corresponding to the P3 domain diminished, indicating the formation of a stable helix. When Mg^{2+} was present at levels where autoinhibition was observed, extensive degradation is seen throughout the J4/2 and L4 regions. Significantly, a single-nucleotide shift occurs in the 5' direction from the degradation pattern observed for the P3 helix at low Mg^{2+} concentrations, corresponding to a stabilized cytosine at the top of the helix and a labile cytosine at the base of P3 adjacent to L3 (Figures 2A, 4B, and S1). Furthermore, new bands mapping to the two cytosines in the L3 domain appear at high Mg^{2+} . Combined, these changes in cleavage patterns correlate well with the observed kinetic data: breakdown in P3 motif decreases from low Mg^{2+} levels to the concentrations yielding the fastest self-scission, corresponding to the formation of a stable helix, whereas above these Mg^{2+} levels, degradation products appear in the L3 and P3 domains indicating a rearrangement of the catalytic core at higher metal concentrations.

DISCUSSION

We have defined the minimal sequence needed for self-scission by an HDV-like motif to be just 48 nucleotides, and used this minimal motif to identify small HDV-like self-cleaving sequences throughout genomic space. The structure descriptors allowed for several variants of the minimal motif, uncovering numerous putative ribozymes. These included a sequence family in *B. floridae*, an organism that appears to be rich in HDV-like self-cleaving sequences, and several examples of microbial origin.

The drz-Bflo-3 family represents the third structurally unique HDV-like family of ribozymes found in the lancelet genome. This family differs significantly from the drz-Bflo-1 and 2 sequences in that they contain an extended J1/2 domain and no P4 region. The close proximity of the three drz-Bflo-3 ribozymes raises questions regarding their biological function, which is presently unknown. A similar arrangement of HDV-like ribozymes was found in the *S. purpuratus* drz-Spur-1 family;¹⁶ however, in this organism the ribozymes are found within a single intron or UTR, whereas we found no annotated genes near the drz-Bflo-3 family. Given that the drz-Bflo-3 family of ribozymes is found to be highly conserved throughout the *B. floridae* genome, as well as the broad distribution of the drz-Bflo-1 and 2 families within *B. floridae* genome, it is likely that this organism harbors repeat elements that employ the ribozymes in a manner analogous to that seen in many other ribozyme-terminated retrotransposons.^{26–28}

The majority of the minimal HDV-like ribozymes were discovered in microbial genomes, contrasting with previous studies which identified larger HDV-like ribozymes primarily in multicellular eukaryotes, with single examples identified in an insect virus, a human gut bacterium, and a marine unicellular diplomonid.^{16–18} Whereas many eukaryotic ribozymes are associated with retrotransposons,^{26–28} the microbial examples do not appear to have a uniform biological function informed

by their genomic loci. Further analysis of the effect that these elements have on the expression of surrounding genes will likely reveal the biological roles of these ubiquitous catalytic RNAs.

The HDV-like ribozymes found in human metagenomic samples are the smallest confirmed HDV-like self-cleaving sequences. Although sequence homology is only confirmed for the contig on which the drz-Mtgn-1 motif is found, it is likely that all of these ribozymes are of viral or bacterial origin. The ribozymes, along with the drz-Mbac-1 sequence, expand the number of microbial HDV-like ribozymes and indicate that this class of RNAs is widespread in bacteria, and suggest that the fold may be ancient in origin.

Metagenomic data sets have proven highly valuable in identifying self-cleaving ribozymes. Previously, a number of examples of hammerhead ribozymes were obtained from analysis metagenomic sequence tracks,³¹ and now several HDV-like ribozymes have been found in similar metagenomic samples. This finding indicates that the usefulness of these types of sequence repositories extends beyond the mapping of viral and bacterial populations, as they provide highly diverse data sets rife with functional RNAs.

The metagenomic ribozymes exhibited an array of cleavage rate constants, despite very similar sequence composition. As a whole, the rates of self-scission for these metagenomic ribozymes suggest that the optimal orientation for linking of a 6-bp P1 domain to the P2 helix in the context of the HDV-like fold occurs when the P2 helix is 7 bp long and the J1/2 strand consists of two nucleotides. Such an arrangement is observed in the drz-Spur-3 sequence, and the fast-acting drz-Mtgn-3 ribozyme described here.

Among the minimal ribozymes analyzed in this study, the drz-Mtgn-1 proved to be most unusual. The presence of two distinct titration points in the metal-activity relationship and the marked preference against Ca^{2+} as the catalytic metal was not expected for an HDV-like self-cleaving sequence, because these ribozymes typically show robust activity in any divalent metal.⁴¹ In-line probing showed that the smallest HDV-like self-cleaving sequence can sample several conformational states that are biased by the divalent metal ion concentrations and are absent in larger examples of the class. The mapping experiment suggested that these conformational states arise from direct perturbations to the ribozyme core, particularly around the P3 and L3 domains.

The drz-Mtgn-1 ribozyme also exhibits Ca^{2+} modulation of the Mg^{2+} -dependent self-scission reaction, resulting in a unique metal-activity profile for the combination of these two ions. The rate modulation caused by Ca^{2+} correlates well with the observed K_d s for each metal individually, as the inhibitory effect is greatest when the Mg^{2+} concentration is below its predicted K_d for the active site and the Ca^{2+} concentration is in the millimolar range. The noncompetitive nature of inhibition indicates that calcium does not interfere with magnesium binding in the catalytic pocket; instead, Ca^{2+} appears to bind another location, likely in the leader sequence just upstream of the cleavage site, in a manner that prevents efficient self-scission.

The biological roles of HDV-like ribozymes have not been characterized outside of retrotransposons and the hepatitis delta virus, but the unique metal-ion dependence of the drz-Mtgn-1 ribozyme may allow for modulation of its activity by calcium. Calcium levels are tightly regulated in cells and can increase dramatically during signaling events.⁴² Elevated

calcium levels inhibit self-scission of the drz-Mtgn-1 ribozyme, potentially affecting downstream events coupled to the ribozyme activity and linking calcium signaling with ribozyme activity.

Our results describe the broad distribution of tiny HDV-like ribozymes. This finding builds on the previously shown prevalence of this fold throughout nature and indicates that the HDV ribozyme motif is a common tool for self-scission of RNA. The results indicate that the reduced structure of minimal HDV-like ribozymes is perhaps more susceptible to folding into diverse conformations stabilized by different divalent metal ion concentrations and identities, leading to unique kinetic profiles that differ significantly from canonical, and presumably more structured, HDV-like ribozymes.

■ ASSOCIATED CONTENT

📄 Supporting Information

Figure S1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ABBREVIATIONS

HDV, Hepatitis Delta Virus; drz, delta-like ribozyme

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