Conformational heterogeneity and the determinants of tertiary stabilization in the hammerhead ribozyme from *Dolichopoda* cave crickets

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Repetitive DNA elements in *Dolichopoda* cave cricket genomes contain extended hammerhead ribozymes that are functional in adult crickets, but that exhibit very low self-cleavage activity in vitro relative to other extended hammerhead ribozymes. We find that the parental ribozyme tends to misfold into alternate secondary structures in vitro, complicating analysis of contributions by specific nucleotides to activity under biologically relevant magnesium concentrations. However, minor sequence alterations that stabilize the active secondary structure, without altering candidate tertiary interacting nucleotides, boosted observed rates more than 50-fold ($4.4 \pm 1.7 \text{ min}^{-1}$) and doubled the cleavage extent (>60%) in submillimolar magnesium. Productive alterations included flipping two base pairs in stem I, lengthening stem I and opening stem III to generate a trans-cleaving ribozyme. Specific peripheral nucleotides involved in tertiary stabilization were then identified through kinetic analysis for a series of sequence variants and by correlating plateau cleavage values with band intensity in native gel electrophoresis. These results demonstrate that conformational heterogeneity governs self-cleavage by the wild-type *Dolichopoda* hammerhead ribozyme in vitro, and they suggest a strategy for improving activity and enhancing the suitability of HHRz for intracellular and biotechnology applications.

Introduction

The hammerhead ribozyme (HHRz) is the smallest of the naturally occurring RNA endonucleases, with a secondary structure composed of three stems surrounding an 11 nt core.^{1,2} Self-cleaving HHRz fold into three distinct topologies (type 1, type 2 and type 3), depending on which of the three helices bears the 5' and 3' ends of the hammerhead motif (Fig. 1, inset). The essential HHRz sequence and structural elements are conserved across highly divergent evolutionary taxa. Much of this conservation is likely due to independent evolutionary origins, since self-cleaving HHRz can arise from within random libraries.^{3,4} However, the identification of HHRz motifs—at least some of which are active when transcribed in vitro—within multiple metazoan repetitive DNA elements⁵ and within highly conserved peripheral elements in intronic HHRz⁶ suggests that several HHRz subgroups share ancestry and have diverged following organismal speciation or retrotransposition. The biochemical and biological functions of HHRz from animals remain relatively unexplored.

Early structural and mechanistic studies of HHRz emphasized minimal constructs (<50 nts) that cleave site-specifically and to high degrees of completion in vitro in high concentrations of Mg^{2+} (>10 mM).⁷⁻¹¹ Much slower rates were observed in vitro at lower Mg^{2+} concentrations that better mimic intracellular conditions, because these minimal constructs lacked peripheral elements that are present in the native sequences. Loops or bulges in the native stems I and II provide stabilizing tertiary contacts that dramatically reduce the Mg^{2+} concentration required for efficient cleavage.¹²⁻¹⁴ These elements affect conformational dynamics by increasing the frequency with which productive conformation(s) are sampled,¹⁵ a model which has also been proposed¹⁶ for the P4 helix of kinase ribozyme Kin.46. For tertiary-stabilized HHRz, X-ray crystallography has also shown that both G8 and G12 are near the scissile bond, with G12 in position to act as general base,^{17,18} consistent with earlier predictions based on pH rate profiles for site-specifically substituted ribozymes.^{19,20} The potential role of G12 as general base is further supported by labeling of its N1 via nucleophilic attack on 2'-bromoacetamide.²¹

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Figure 1. Variations of *Dolichopoda baccettii* HHRz sequences. Paired regions I, II and III are indicated, as are Loop L2, Internal Loops IL1a and IL1b and cleavage site (arrow). Nucleotides that are changed during redesign of the hammerhead construct Dbp2 into Dbp2-m and trans-version (Dbp2-m, tDbp2-m) are highlighted. Inset shows three topologies for self-cleaving ribozymes.

Nucleobase-metal ion interactions also appear to contribute to shifting nucleobase pKa's within the active sites of some extended ribozymes,^{22,23} and a suitably positioned Mn^{2+} ion has been observed in HHRz crystals.²⁴

Both the type 1 HHRz from schistosomes¹⁷ and the type 2 HHRz from the satellite RNA of tobacco ringspot virus (sTRSV)¹⁸ utilize cross-strand base stacking, stabilizing metal ions and an assortment of hydrogen bonding across the major groove of the bulge or loop associated with stem I to achieve tertiary stabilization, although the precise details of these interactions are highly divergent between these two structures. In a study that included NMR structures of the isolated helical elements of chrysanthemum chlorotic mottle viroid (CCHMVd) ribozyme, structural modeling of the loop-loop interaction, comparative sequence analysis and comparison with structures of the schistosome and sTRSV hammerheads, Dufour et al. proposed that two loosely-defined motifs— UN_mYN in loop 1 and RN_nA in loop 2—form the required tertiary contacts in diverse naturally-occurring HHRz.²⁵ While the functional importance of these motifs is well demonstrated in the CCHMVd ribozyme, the corresponding sequences and their structural contexts are highly divergent across other taxa. Non-natural tertiary stabilizing elements can also be effective. For example, our lab and others have described trans-acting variants of natural HHRz with improved bioactivity,^{26–28} some of which exhibit cleavage rates as high as $3,000 \text{ min}^{-1}$ in 1 mM divalent metal ion.²² Similarly, an artificial interaction domain was engineered using GAAA tetraloop-receptor motifs that conferred efficient cleavage of target RNA at submillimolar Mg^{2+} concentrations in vitro and in cultured

cells.²⁹ Among viroid HHRz's, tertiary stabilization has variable effects on the cleavage and ligation rates and the Hill coefficient for Mg^{2+} dependence (n_{Mg}), with values of k_2 (cleavage rate constant) varying by 750-fold, values of k_{-2} (ligation rate constant) varying by 100-fold, and values of n_{Mg} varying between 1 and 2,³⁰ highlighting the variable impact of these elements on HHRz activity. An intriguing possibility is that variation in the tertiary contacts translates to different rates or yields of RNA self-processing as a means of fine-tuning the biological role of the ribozyme elements, and that this feature can be exploited for intracellular application of engineered ribozymes.

The biological role of HHRz's is well established in pathogenic viroids, where they autocatalyze the cleavage of linear concatenated satellite RNAs into monomers during rolling circle replication—often in the absence of helper protein—for subsequent ligation into circular monomers.^{31,32} In contrast, the role(s) of HHRz's in eukaryotic biology is largely unclear. Early work identified HHRz's in crickets,³³ newts,³⁴ schistosomes,³⁵ *Arabidopsis*³⁶ and mice.³⁷ Total RNA from each of these organisms accumulates both cleaved and uncleaved RNA, indicating that these ribozymes are active in vivo. The mouse ribozyme lies between the translation termination and poly-adenylation signals within the 3' untranslated regions of genes for several CLEC2 proteins, which are C-type lectins that serve important roles in bone remodeling and immune responses.³⁷ Additional unverified motifs were later identified computationally in *Drosophila melanogaster*, *Caenorhabditis elegans* and *Homo sapiens*.³⁸ Within the past year, several groups have reported large numbers of HHRz motifs in phylogenetically diverse organisms that include methanogens, bacteria, algae, plants, metazoans and others, and a small handful of these elements were shown to be active when transcribed in vitro.^{5,6,39} Delineating their biological roles represents an important frontier in RNA biology.

In the case of the cricket ribozyme, Rojas et al. showed that HHRz sequences are present in the repetitive satellite pDo500 DNA family from several species of *D. baccetti* and *D. schivia-vazzii* cave crickets, and that transcripts from the crickets show evidence of self-cleavage in vivo. A recent study identified 135 additional HHRz sequences in repetitive DNA from 10 of 12 Dolichopoda species. Importantly, this study also presented evidence that the ribozyme nucleotides are more conserved than the flanking sequences and that they are under positive selection pressure to retain nucleolytic function,⁴⁰ again supporting a biological role for HHRz activity. When transcribed in vitro, the HHRz from *D. baccettii* displayed a higher rate of self-cleavage in vitro (0.01 min^{-1} in 5 mM Mg^{2+} at 25°C) than did HHRz from other Dolichopoda species,³³ but these rates were 100- to 10,000-fold lower than what has been observed for other extended HHRz.

This low activity has limited development of biotechnology applications based on the Dolichopoda ribozyme, and has complicated the interpretation of the potential biological significance of these ribozymes. Furthermore, they raise the possibility that tertiary stabilization by the extended portion of the Dolichopoda ribozyme may be weaker or less effective than the stabilization afforded in other ribozymes.

We therefore sought to determine whether stabilizing tertiary interactions analogous to those present in other extended HHRz are functional in the cricket ribozyme, and whether additional cis-acting elements might contribute to its poor kinetic behavior. Our results identify specific nucleotides critical for tertiary stabilization, and they indicate that efficient cleavage by the cricket HHRz can be restored through relatively minor alterations to secondary structure, ruling out serious defects in the tertiary interacting elements as being responsible for poor activity in vitro. Our data also establish that while cotranscriptional cleavage is efficient, in vitro cleavage upon refolding the natural form of the ribozyme is primarily limited by misfolding into alternate conformations.

Results

Peripheral elements are critical in the Dolichopoda hammerhead ribozyme for cleavage activity in submillimolar Mg^{2+} . Stem I of the *D. baccettii* HHRz (clone “Pst3”³³) has two helical elements, stems Ia and Ib, that flank an asymmetric 7:4 internal loop (IL₁) with the sequence 5'-UCU CCC U:UA UU-3' (Fig. 1). IL₁ is positioned to interact with a hexanucleotide element, 5'-GGG GGA-3', within Loop II (L₂). *D. baccettii* and *D. schiavazzii* are closely related phylogenetically,⁴¹ and their ribozyme sequences are nearly identical,⁴⁰ including the corresponding putative interacting elements, which in *D. schiavazzii* are 5'-UCC CUC U:UA UA-3' in IL₁ and 5'-AGGGAA-3' in L₂ (differences underlined). To test the significance of this juxtaposition, two ribozyme variants with different lengths of stem I were designed, denoted DbP2 (76 nt) (Dolichopoda baccettii Pst3 clone with 2 helical segments in stem I) and DbP1 (51 nt) (Fig. 1). Cotranscriptional cleavage was observed for both species at 37°C (30 mM free Mg^{2+} concentration, after adjusting for chelation by NTPs), with approximately 7-fold greater net cleavage observed for DbP2 than for DbP1 (Fig. 2A). Intact (uncleaved) RNA corresponding to each molecule was then gel-purified and allowed to react in various concentrations of Mg^{2+} (Table 1 and Fig. 2B). Increasing the Mg^{2+} concentration from 0.5 mM to 2 mM improved the initial rate of DbP2 by 8-fold, with no further increase at still higher Mg^{2+} concentrations. Gel-purified

Figure 2. (A) Time course of co-transcriptional cleavage in the presence of 4 mM each NTPs & 30 mM excess Mg^{2+} ; DbP1 (diamonds); DbP2 (squares); DbP3 (triangles). (B) Self-cleavage by gel-purified, refolded RNAs at 20°C for ribozymes DbP1, DbP2 and DbP3 at the Mg^{2+} concentrations indicated. Curve fitting was done with single-exponential equation for kinetics in 0.5 mM Mg^{2+} and the rest of the kinetics were fit to bi-exponential equation; uncertainties of the fits are reported in Table 1.

Table 1. Self-cleavage rate constants measured at 20°C¹

Mg^{2+} (mM)	DbP2, k_{obs} (min ⁻¹)	DbP3, k_{obs} (min ⁻¹)
0.5	$k_1 = 0.037 \pm 0.003$	$k_1 = 0.075 \pm 0.008$
2	$k_1 = 0.32 \pm 0.05$ $k_2 = 0.008 \pm 0.008$	$k_1 = 0.36 \pm 0.07$ $k_2 = 0.012 \pm 0.01$
10	$k_1 = 0.47 \pm 0.13$ $k_2 = 0.027 \pm 0.015$	$k_1 = 0.41 \pm 0.08$ $k_2 = 0.027 \pm 0.015$

¹See Figure 2 legends for details on calculations.

DbP1 did not show any detectable self-cleavage activity in 0.5 mM or 2 mM Mg^{2+} after 2 h at 20°C. In 10 mM Mg^{2+} the self-cleavage rate is 25-fold slower for DbP1 (0.016 ± 0.003 min⁻¹) than for DbP2. Similar poor cleavage was observed for ribozyme DbP4, in which IL₁ of DbP2 was replaced with Watson-Crick base pairs (5'-AAU A/UAU U-3') (data not shown). Appending additional bulge and helical elements from *D. baccettii* and *D. schiavazzii* yielded ribozyme “DbP3.” However, the additional 10 base pairs in stem Ic of DbP3 had only marginal effects on activity (Fig. 2); therefore, subsequent analysis focused on ribozyme DbP2.

Point mutants of DbP2 interacting elements show complex, multiphasic kinetic behavior in self-cleavage reactions. Although DbP2 is clearly stabilized for reactivity relative to DbP1, it is still one to three orders of magnitude less active than other extended HHRz. To evaluate the contributions of specific nucleotides to tertiary stabilization of the Dolichopoda HHRz, point mutations

Figure 3. Cleavage kinetics of DbP2 construct with IL₁ and L₂ mutants. (A) Cleavage plots of six IL1 mutants in 0.5 mM Mg²⁺ at 20°C. IL₁C2A, IL₁U5A and IL₁U7A showed no activity and are not plotted. (B) Cleavage plots of four IL₁ and six L₂ mutants in 0.5 mM Mg²⁺ at 20°C. All kinetic profiles were fit to single-exponential equation except IL₁C6A in (A), which was fit to bi-exponential equation. (C) Cleavage plots of the same IL₁ mutants as in (A), but in 10 mM instead of 0.5 mM Mg²⁺.

were introduced into IL₁ and L₂ of DbP2. Mutations are denoted first by the structural feature into which they have been introduced, numbered in the 5' to 3' direction within each structural element, followed by the substitution. Thus, mutation IL₁C2A designates a C-to-A mutation at position 2 within internal loop IL₁. For this analysis, all nucleotides in L₂ were changed to uridine; all nucleotides in IL₁ were replaced by adenosine, except nucleotide IL₁A9 which was replaced with uridine.

Nearly all mutant ribozymes showed reduced rates of self-cleavage relative to the wild-type DbP2 in 0.5 mM Mg²⁺. In most cases the initial activity was too weak to quantify accurately, and some of the mutants showed a small burst of cleavage in the first minute followed by slower cleavage activity (Fig. 3A and B and Table 2). The exception was mutant IL₁C6A, which showed biphasic kinetics and a higher rate and extent of self-cleavage than DbP2. Rescue experiments at 10 mM Mg²⁺ improved the extent of cleavage after 2 h for some of the mutants, but these conditions result in multiphasic kinetic profiles in the early time-points (Fig. 3C). In most mutants there was no improvement in observed activity (Table 2). Addition of 150 mM K⁺ had only a slight effect on the extent of DbP2 self-cleavage in 0.5 mM Mg²⁺, and no effect in 10 mM Mg²⁺ even after 22 h of reaction (data not shown).

Electrophoretic evidence for conformational heterogeneity. Failure to regain well-behaved kinetics at high Mg²⁺ suggests that the loss in activity at low Mg²⁺ may arise from overall misfolding in vitro, rather than solely from loss of tertiary interactions. This interpretation is supported by the observation that the extent of co-transcriptional cleavage for DbP2 is greater than the extent of self-cleavage using gel-purified, refolded RNA (compare Fig. 2A and B). For most of the mutants, similar discrepancies were observed upon comparing the extent of self-cleavage during transcription with the extent of cleavage observed for gel-purified RNA (data not shown), again supporting the “misfolding” model.

The MFOLD algorithm^{42,43} predicts multiple conformations for DbP2, each of which is poorly defined by p-num⁴⁴ analysis (not shown). Native gel electrophoretic mobility of DbP2 was therefore monitored to establish whether multiple conformations exist in solution. To prevent self-cleavage during native gel electrophoresis, the adenosine nucleotide 5' to the cleavage site was replaced by deoxy-adenosine to generate construct dA-DbP2 (Fig. 4A). Native gel mobility of dA-DbP2 shows three dominant bands and at least three weaker bands (Fig. 4B), indicating at least six conformations of the molecule. It is not possible to identify from these data which band corresponds to the active hammerhead structure, but the multitude of bands implies a conformational heterogeneity that can explain the low extent of reaction observed during self-cleavage assays.

Stem modifications improve self-cleavage activity. The DbP2 sequence is GC-rich, consistent with its origin from within a eukaryotic repetitive DNA element. The native stem Ia contains four consecutive G-C pairs that could potentially pair with the pyrimidine-rich IL₁ or the G-rich L₂ to yield misfolded, inactive structures. To generate a stable and unique secondary structure, changes were introduced into the base-pairing stems

without altering the loops. To generate ribozyme DbP2-m, two base pairs in stem Ia were inverted from G-C to C-G to discourage alternate base pair formation (Fig. 1). In addition, two cytosines were appended at the 3' end of the transcript to pair with the 5' terminal GG overhang, further stabilizing stem I against potential alternate secondary structures. MFOLD and p-num analysis predict a well-defined stem I for ribozyme DbP2-m.

Ribozyme DbP2-m self-cleaves more rapidly and to a greater extent than DbP2, both during transcription and in assays using gel-purified RNA. During co-transcriptional cleavage, nearly 90% of DbP2-m is cleaved in the first 2 h, compared with ~65% for DbP2 (Fig. 5A). For gel-purified, refolded RNA, the rate of self-cleavage by DbP2-m in 0.5 mM Mg²⁺ at 20°C (0.16 ± 0.01 min⁻¹) is improved by 5-fold relative to DbP2. However, refolded DbP2-m reached a plateau at ~30% cleavage within 30 min (Fig. 5B), and the extent of cleavage did not improve even at 10 mM Mg²⁺ (data not shown). Native gel electrophoretic mobility of refolded DbP2-m still showed the same three major bands as for DbP2, albeit without the additional minor bands (Fig. 4B). Thus, although higher yields are obtained both during co-transcriptional cleavage and upon refolding for DbP2-m relative to DbP2, alternative folding still limits net reactivity.

Trans-cleaving ribozymes are further improved in yield and rate. DbP2-m was next converted into a trans-acting, two-piece molecule (tDbP2-m) by opening loop III (Fig. 1). The observed rate increased more than 10-fold to 2.0 ± 0.7 min⁻¹ for single-turnover cleavage in trans (1:1 stoichiometry of the two strands; 100 nM each). In addition, the cleavage extent at long times increased from about 30% for the cis-acting DbP2-m ribozyme to about 50% for the trans-acting tDbP2-m ribozyme. Native gel assays show a decrease in the number of alternate conformers for tDbP2-m relative to DbP2-m (Fig. 4B and lanes 4–8). Increasing the ribozyme concentration to 500 nM (5-fold excess over substrate), did not alter the rate significantly (2.4 ± 0.5 min⁻¹) but did increase the cleavage extent to 65% (Fig. 5C). Any further increase in ribozyme concentration decreased the cleavage extent marginally, with a concomitant increase in the intensity of the low-mobility alternative band on native gels (Fig. 4B, 1R). Thus, the low-mobility band likely corresponds to a less-compact, inactive structure. The highest initial rates were observed at ribozyme concentrations of 2 μM (20-fold excess over the substrate, 4.39 ± 1.72 min⁻¹); hence, this stoichiometry was chosen for further studies.

Magnesium and temperature dependence of the trans reaction with tDbP2-m. To evaluate the Mg²⁺-dependence of the reaction with these modified HHRz, trans-cleavage rates for tDbP2-m were measured at 15°C, which is a typical temperature for the caves in which Dolichopoda crickets are naturally found. Well-behaved cleavage kinetics were observed at all concentrations assayed, and initial rates steadily increased from 0.26 ± 0.05 at 0.1 mM Mg²⁺ to 5.8 ± 0.7 min⁻¹ in 10 mM Mg²⁺ (Fig. 6A). A plot of log(rate) as a function of log(Mg²⁺ concentration) is roughly linear over this range, with a slope indicating that n_{Mg} ≈ 0.51. Mg²⁺-dependence of tDbP2-m mirrored observations with cis-cleaving version of the Dolichopoda ribozyme. Increasing

Table 2. Activities of DbP2 single-point mutants

Mutant	k _{obs} (min ⁻¹) 0.5 mM Mg ²⁺	F _{max} 0.5 mM Mg ²⁺	F _{2h} 10 mM Mg ²⁺
DbP2	0.036 ± 0.002	0.24 ± 0.01	~0.27
IL ₁ U1A	0.018 ± 0.003	0.19 ± 0.01	~0.30
IL ₁ C2A	-	-	~0.45
IL ₁ C3A	≤0.018	0.05 ± 0.003	~0.05
IL ₁ C4A	≤0.018	0.05 ± 0.003	~0.10
IL ₁ U5A	-	-	~0.55
IL ₁ C6A	0.083 ± 0.024 0.008 ± 0.005	0.55 ± 0.12	~0.55
IL ₁ U7A	-	-	~0.55
IL ₁ U8A	-	-	0.09
IL ₁ A9U	-	-	0.09
IL ₁ U10A	N.C.	0.05*	0.05
IL ₁ U11A	N.C.	0.06*	0.13
L ₂ G1U	N.C.	0.12*	0.21
L ₂ G2U	N.C.	0.06*	0.20
L ₂ G3U	-	-	0.14
L ₂ G4U	N.C.	0.04*	0.20
L ₂ G5U	N.C.	0.03*	0.04
L ₂ A6U	-	-	0.02

Self-cleavage activities were measured at 20°C in 50 mM Tris.HCl, pH 7.5. The values of k_{obs} and F_{max} (net self-cleavage fraction at equilibrium) were obtained from curve-fitting. Biphasic kinetic behavior was observed for IL₁C6A, yielding the two reported rate constants. “-,” inactivating mutations that showed no appreciable self-cleavage. N.C., not calculated; values for k_{obs} could not be reliably calculated for these mutants due to complex kinetics and weak overall activity in the early time points (Fig. 2). Asterisks (*) indicate data sets that could not be fit to simple kinetic equations due to their complex kinetic nature and values quoted are actual cleavage extents after 2 h. The experimental cleavage extent after 2 h (F_{2h}) in 10 mM Mg²⁺; except DbP2, all plots could not be fit to any equation.

the Mg²⁺ concentration to >10 mM only marginally improved the rates and extents of cleavage for ribozyme DbP2 (data not shown), consistent with previous studies in which optimal activity for the 558 nt full length Pst3 transcript was observed at 5 mM Mg²⁺ over the range of 35°C to 50°C.³³

The temperature dependence of tDbP2-m showed a hill-shaped curve in initial rates with peak activity (4.15 ± 0.16 min⁻¹) at 25°C in 0.5 mM Mg²⁺ (Fig. 6B). This is in contrast to the optimal temperature of 55°C (0.25 ± 0.05 min⁻¹) observed previously for the 558 nt, full-length Pst3 transcript at 5 mM Mg²⁺.³³ The differences in the rates, Mg²⁺ requirements and temperature profiles of tDbP2-m and Pst3 may indicate that the rate-limiting step in the catalysis of the same HHRz depends on the RNA scaffold in which it is embedded and the Mg²⁺ concentration at which it is assayed. For example, the lower rate and higher required temperature of the ribozyme within the longer transcript may reflect a high-energy barrier associated with a required conformational change.

Relative contributions of tertiary stabilization for cis- and trans-cleaving ribozymes. A trans-cleaving version of DbP1

Figure 4. (A) Schematic of reactions used to assemble cis-DbP2 with an internal dA and sequence of assembled ligation complex. Monophosphates added prior to ligation are shown explicitly. (B) Native polyacrylamide gel showing the electrophoretic mobilities of several variants of the Dolichopoda HHRz. The two cis-constructs with deoxyadenosine at the cleavage site, dA-DbP2 ("2wt") and dA-DbP2-m ("2m"), show several prominent bands, indicated by arrows 1L to 6L on the left. Note that for DbP2, band 2L resolves into two bands at lower exposure. For lanes 3 to 8, the trans version of DbP2-m is composed of two strands, where the ribozyme strand (Rz) and radiolabeled substrate strand (sub) have been mixed in different stoichiometries as indicated at the top of the gel; the substrate concentration was kept constant at 100 nM. The trans construct shows two predominant bands. Band 2R is the major ribozyme-substrate complex in solution, and band 1R is a lower mobility complex that intensifies with increased ribozyme strand concentration, and which may be an alternate conformer, a dimer or a higher-order aggregate. The substrate band (3R) decreases in intensity at higher concentration of ribozyme.

(denoted tDbP1-m) was generated based on the stem design of tDbP2-m. Ribozyme tDbP1-m lacks IL₁, but it carries the base-flip in stem Ia, the two extra base-pairs at the end of stem Ia, and the opened loop L₃ that characterize tDbP2-m. These mutations improved substrate cleavage by tDbP1-m relative to tDbP1 (Fig. 6C), even though this ribozyme lacks tertiary stabilization. However, for the modified, trans-cleaving Dolichopoda ribozyme, catalysis in sub-millimolar Mg²⁺ is even more strongly dependent on tertiary stabilization for cleavage in trans than it is for cleavage in cis. Specifically, the initial rate of trans-cleavage by ribozyme tDbP1-m (0.001 min⁻¹) is 4,400-fold lower than tDbP2-m in 0.5 mM Mg²⁺ at 20°C [k_{obs} (tDbP2-m)/ k_{obs} (tDbP1-m) = 4,400]. In contrast, cis-cleavage by DbP1 is only 25-fold lower than DbP2 [k_{obs} (DbP2)/ k_{obs} (DbP1) ≈ 25].

Loop nucleotide requirements for catalysis by tDbP2-m. To determine the nucleotides responsible for tertiary stabilization, point mutations were introduced into tDbP2-m (Fig. 7A), and the ribozyme variants were tested for trans-cleavage activity under single-turnover conditions in 0.5 mM Mg²⁺ at 20°C.

In contrast to the complex kinetic behavior noted above for the single-point mutations within the cis-acting DbP2 (Fig. 3), the kinetic profiles for most of the tDbP2-m mutants were well behaved (Fig. 7B) and yielded interpretable kinetic parameters (Fig. 7C). Initial cleavage rates were unaltered, within error, for variants IL₁U1C, L₂G1A and L₂G5A relative to tDbP2-m, although they gave lower cleavage plateaus. All the other mutations yielded 4-fold or greater decrease in activity. No cleavage was observed for mutants L₂G3A and L₂A6G.

Electrophoretic mobilities of each of these mutant RNAs were monitored on a native gel using a non-cleavable substrate to determine the effect of each mutation on overall folding (Fig. 8A). For mutants IL₁U1C, IL₁U5C, IL₁U7C, L₂G1A and L₂G5A, the native gel shows two predominant bands that migrate with (or in the case of L₂G5A, just ahead of) the two major bands of ribozyme tDbP2-m. Each of these mutations affects the fraction of RNA in the faster-moving band (F₂) and the observed cleavage fraction plateaus (F_{max}) to approximately the same degree, producing a marked correlation between the high-mobility F₂

Figure 5. (A) Co-transcriptional self cleavage by DbP2 and DbP2-m in the absence of blocking oligo. (B) Self-cleavage plots of gel-purified DbP2 and DbP2-m in 0.5 mM Mg^{2+} at 20°C. (C) Kinetic profiles of tDbP2-m in 0.5 mM Mg^{2+} at 20°C with different stoichiometries of the two strands. Substrate concentration was held constant at 100 nM, and ribozyme concentration was varied from 100 nM (1:1) to 6 μ M (60:1), as indicated. Cleavage data were fit to a bi-exponential equation. Inset shows same data at longer times.

Figure 6. Mg^{2+} and temperature dependence of trans cleavage by tDbP2-m (Rz:sub = 20:1). (A) Mg^{2+} concentration was varied from 0.1 to 10 mM, and k_{obs} values were obtained from curve-fitting with single-exponential equation; errors indicate uncertainty of the curve fit. All reactions were carried out at 15°C. Trend line gives an apparent Hill coefficient of 0.51, indicating only modest cooperativity in binding to Mg^{2+} . (B) Dependence of single-turnover cleavage activity of tDbP2-m on temperature in 0.5 mM Mg^{2+} , 50 mM Tris.HCl, pH 7.5, in the range of 10 to 70°C. Uncertainties of the observed rate constants were calculated from the curve-fit. (C) k_{obs} values of Dolichopoda variants were measured at 20°C in 0.1 mM Mg^{2+} (dark gray), 0.5 mM Mg^{2+} (black) or 10 mM Mg^{2+} (diagonal lines). Dotted line indicates lower limit of detection under the conditions of the assay (background).

Figure 7. Unpaired nucleotides implicated in function of the Dolichopoda HHRz. (A) Nucleotides in L₂ and IL₁ targeted for mutagenesis within the context of tDbP2-m. Nucleotides for which transition mutations have strong effects on cleavage rate are circled. Nucleotides that were least sensitive to mutation are in italics. (B) Cleavage reactions tDbP2-m (filled squares) and single-point mutants in loop L₂ and bulge IL₁ as indicated. Reactions were carried out for 30–60 min in 0.5 mM Mg²⁺, pH 7.5, at 20°C. (C) Initial observed cleavage rates (k_{obs} or $k_{\text{obs},1}$) values obtained from fitting the data in (B); tDbP2-m, IL₁U1C, L₂G1A and L₂G5A plots are fit to bi-exponential equation; the other data sets are fit to single exponential equation. Error bars reflect uncertainties of the fit. Note that mutants IL₁U1C, L₂G1A and L₂G5A show similar initial rate constants (k_{obs}) as tDbP2-m, even though their net cleavage rates are reduced because of their lower plateau values.

species and the kinetic parameter F_{max} (Fig. 8B). This correlation identifies the faster-migrating species as the RNA that is folded into the catalytically competent structure. Three of the remaining four constructs showed three bands of almost equal intensity, while L₂G4A was less well resolved. In each of these four RNAs, the mobility of the fastest-migrating species was less than that of the fastest-moving species of ribozyme tDbP2-m, suggesting that their folded structures are less compact. Nevertheless, the amount of RNA in the fastest-migrating fractions in L₂G2A and L₂G4A again falls near the diagonal in the plot of F_{max} vs. F_2 . Therefore, most of these mutations appear to alter the partitioning among alternative conformations, the most compact of which is most strongly implicated in catalysis.

Discussion

The pDo500 satellite DNA is a repetitive element comprising roughly 5% of the Dolichopoda cave cricket genome.⁴⁵ The HHRz within this element exhibits self-cleavage in adult crickets³³ and appears to be under positive selection to retain activity (non-random retention of base pairing potential and fewer inactivating mutations than expected by chance);⁴⁰ thus, it may be important for the normal biology of these insects. The fact that it is active in vivo also suggests that it could potentially be exploited as a tool for biotechnology and for artificial gene regulation. Consistent with previous observations on extended HHRz, we find that tertiary stabilizing motifs are critical for obtaining efficient catalysis by the Dolichopoda ribozyme at sub-millimolar concentrations of Mg²⁺, and that 10 mM or higher Mg²⁺ is required when these peripheral sequences are removed (DbP1 and tDbP1-m). Native gel shift analysis establishes that the natural cricket HHRz sequence misfolds into alternate conformations that can account for the weak activity observed here and previously.³³ This heterogeneity also confounded initial assessment of the functional roles of individual nucleotides in the tertiary interaction domain. Introducing minor changes in the DbP2 primary sequence and converting it into a trans-cleaving species increased the initial rate of cleavage in submillimolar Mg²⁺ by more than 50-fold, doubled the final extent of product formation to >60% and decreased heterogeneity on native gels. The resulting tDbP2-m ribozyme is therefore suitable for further exploration as a tool for biotechnology applications.

Comparing rate measurements and gel mobility shift assays among variants of the well-behaved, trans-cleaving tDbP2-m ribozyme allows a preliminary assessment of the requirements for specific nucleotides in establishing the tertiary stabilization of the native Dolichopoda ribozyme. The G-rich L₂ hexaloop at the end of stem 2 (L₂ = GGG GGA) tolerated G-to-A substitutions at positions 1 and 5. Similar substitutions at positions 2, 3 and 4 strongly reduced the observed rate, as did the A-to-G substitution at position 6. Each of these observations is consistent with the loosely-defined motif proposed previously (L₂ = RN_nA),²⁵ and with the presence of adenosines at both these positions in five other Dolichopoda species,⁴⁰ including *D. schiavazzii* (L₂ = AGG GAA). In the schistosome and sTRSV HHRz, the terminal A forms a reverse Hoogsteen pair with IL₁U1 or L₁U1,

respectively,¹⁷ and it has been modeled as making similar interactions in the CChMVd hammerhead.²⁵ However, we observe here that mutating IL₁U1 to cytosine in the Dolichopoda HHRz has only a minor effect (<2-fold) on the observed rate. We suggest that the cytosine at this position rotates slightly to provide an equivalent reverse Hoogsteen pairing.⁴⁶ Alternatively, this minor deviation from the Dufour motif may suggest that the tertiary interactions within the Dolichopoda ribozyme are distinct from those described previously, or that the loop-bulge interaction is dominated by the other interacting nucleotides. For four mutations (L₂G3A, L₂A6G, IL₁U5C and IL₁U7C), the net yield for catalysis is affected more severely than compaction into a high-mobility form, causing these species to fall well below the diagonal in **Figure 8B**. The values of $F_{\max}$ are less than half the corresponding F_2 values for these species, and their catalytic rates are reduced. Therefore, mutations at these positions may perturb both the tertiary contact (affecting the observed rate for the active fraction) and the global compaction that is probed by gel mobility.

Significant redesign was required to achieve efficient cleavage *in vitro* in these studies. In contrast, intracellular RNA assembly takes place co-transcriptionally in the presence of proteins, ions and small molecule ligands that can redirect folding to on- and off-pathway structures. The present work, along with that of others,⁴⁷ provides examples in which *in vitro* cation-induced folding differs markedly from co-transcriptional folding. For the Dolichopoda ribozyme, the effects of conformational heterogeneity are more pronounced in assays using gel-purified RNA than during transcription. Co-transcriptional cleavage proceeds to 60% completion in DbP2 and nearly 90% in DbP2-m in 1-h reactions (**Fig. 5A**), which is in contrast to the approximately 15 to 30% yield for both RNAs using gel-purified materials (**Fig. 5B**). Rapid and sequential folding of individual secondary structural elements during transcription likely reduces the opportunity for misfolding, while the gel-purified RNA is more subject to becoming trapped in alternate structures during refolding. Addition of divalent cations then induces further compaction and formation of higher order tertiary structures.^{48–51} Dividing the molecule into two parts for trans cleavage also reduces the propensity for forming these alternate structures. Several group I and group II introns require specific proteins to effect splicing *in vivo*,^{52–57} and the newt HHRz—which is also transcribed from repetitive satellite DNA—exists in ovaries as part of a ribonucleoprotein (RNP) complex⁵⁸ that may include the NORA RNA-binding protein.⁵⁹ In contrast, the 20- to 50-fold intracellular acceleration of self-splicing by the Tetrahymena Group I intron, relative to *in vitro* self-splicing rates, is observed in both Tetrahymena⁶⁰ and *E. coli*⁶¹ cells, making it more likely due to transcriptional context or non-specific factors than to specifically required proteins. Although the cricket ribozyme is active in adult crickets,³³ its catalytic activity is tremendously decreased *in vitro*. While conformational heterogeneity of the wild-type sequence is clearly a limiting factor for the purified RNA *in vitro*, a significant fraction is cleaved during transcription. It remains to be determined whether self-cleavage activity of the ribozyme within crickets is an intrinsic

Figure 8. (A) Electrophoretic gel mobility shift for mutants of loop L₂ and bulge IL₁ of tDbP2-m. Identities of the ribozyme strands are indicated above the lanes. Arrows indicate the two major high-mobility band shared by several of the ribozymes. For clarity, only the substrate strand was radiolabeled. (B) Correlation between the fractional cleavage of the substrate by each ribozyme, $F_{\max}$ (obtained from curve-fitting in **Fig. 7**) and the fraction of RNA in the highest-mobility band in comparison to all bands in that lane (F_2). Filled diamonds within the gray area are within 30% of the $x = y$ diagonal line. Open diamonds indicate ribozymes for which the fraction that is productively folded is affected more strongly than electrophoretic compaction.

or regulated property, and whether protein-assisted folding, co-transcriptional folding or other factors ultimately govern its biological activity.

Materials and Methods

Materials. DNA and RNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA), radiolabeled nucleotides from ICN (Costa Mesa, CA). Deoxynucleotide triphosphates were purchased as powders from Amersham Pharmacia (Piscataway, NJ). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) at molecular biology grade or higher.

Cleavage assays and kinetics. All RNA constructs for self-cleavage kinetic assays were synthesized through *in vitro* transcription at 37°C using T7 RNA polymerase, 3 mM each NTPs, 30 mM DTT, 30 mM MgCl₂ and 300 nM [α -³²P] UTP. Full-length transcripts were purified by gel electrophoresis in 6%

denaturing polyacrylamide. Refolding was accomplished by heating the RNA in water to 90°C for 2 min, followed by slow cooling to room temperature. Self-cleavage reactions were initiated by adding 5 μ L of MgCl₂ solution (at 15-times the desired final concentration) to 70 μ L of 100 nM refolded RNA at 20°C in 50 mM Tris.HCl and 100 μ M EDTA, pH 7.5. Aliquots (5 μ L) were withdrawn at each time point and quenched with 5 to 10 μ L of 90% formamide, 50 mM EDTA and 0.005% each of xylene cyanol and bromophenol blue tracking dyes. Kinetic experiments with trans-constructs were performed under single-turnover conditions in which the ribozyme strand (2 μ M) was in 20-fold excess over the substrate strand (100 nM), unless otherwise noted. The ribozyme strands were made through in vitro transcription, whereas the substrate strand was synthesized commercially. Substrate strand was 5' radiolabeled using [γ -³²P] ATP and polynucleotide kinase, and the two separate strands were annealed by heating and cooling as above. Cleavage data were fit to a single-exponential equation:

$$F_t = F_0 + (F_{\max} - F_0)\{1 - \exp(-k_{\text{obs}}t)\} \quad (1)$$

or to a bi-exponential equation,

$$F_t = F_0 + (F_{\max} - F_0)\{1 - \alpha \exp(-k_{\text{obs},1}t) - (1 - \alpha) \exp(-k_{\text{obs},2}t)\} \quad (2)$$

where F_t is fraction cleaved at time “t,” F_0 is the zero point correction, $F_{\max}$ is the estimated plateau value at infinite time, α is the fraction of the cleaved population which has a rate constant of $k_{\text{obs},1}$ and (1- α) fraction reaches equilibrium with a rate constant of $k_{\text{obs},2}$. The errors were calculated from the curve fit using KaleidaGraph 3.5 software. Co-transcriptional cleavage experiments were performed at 37°C using 4 mM each NTPs, 46 mM MgCl₂ (30 mM excess of total NTP) and 300 nM [α -³²P] UTP.

Electrophoretic gel mobility assays. The cis- and trans-constructs studied by native gel mobility shift were modified at the cleavage site from ribose to deoxyribose to prevent cleavage during these experiments. For trans-constructs, the ribozyme strand was synthesized through in vitro transcription and the dA-modified substrate strand was chemically synthesized by Integrated DNA Technologies. The substrate strand was 5' radiolabeled using [γ -³²P] ATP and polynucleotide kinase. To modify the cis-acting ribozymes so that they contain an internal

deoxyadenosine, full-length RNAs were prepared from shorter RNA oligos using T4 DNA ligase (Fig. 4A). In the case of cis-Dbp2, the construct was made from three oligos, a 48mer RNA (transcribed using T7 RNA polymerase), a 10mer synthetic RNA with a 3' terminal deoxy adenosine and a 15mer synthetic RNA. The two shorter oligos were 5' phosphorylated in separate reactions using ATP and polynucleotide kinase and then gel-purified. The 48mer RNA was annealed to a bridging DNA oligo in a 2:1 ratio of DNA:RNA by heating to 70°C for 5 min and slow cooling to 50°C. The two shorter oligos were then heated separately to 50°C and added to the RNA:DNA mixture at a concentration equal to that of the DNA oligo. After 5 min incubation at 50°C, the mixture was cooled to room temperature. Ligation was initiated by adding 500 μ M ATP and 0.3 U/ μ L of T4 DNA ligase at 16°C for ~20 h. Finally, polynucleotide kinase and [γ -³²P] ATP were added to the reaction mixture (T4 DNA ligase and PNK share same buffer) to radiolabel the 5' end of the ligated product (73mer oligo) at 37°C for 2 h. Full-length product was separated from unreacted oligos by gel purification.

Native gels used for electrophoretic gel shifts contained 15% polyacrylamide (purged under vacuum to remove bubbles and dissolved gasses), 0.5 mM MgCl₂, 200 mM Tris.HCl (pH 7.5) and 10 μ M EDTA. The high concentration of Tris.HCl was essential to maintain the pH of the gel and running buffer close to neutrality during electrophoresis. The 0.4 mm gels were run in the cold room (4°C) at 15W for >8 hrs to minimize heating. The RNA constructs in the study were refolded as in the cleavage reactions (see above) and incubated in 0.5 mM Mg²⁺ for 5 min before loading onto the gel. Gels were dried and exposed to activated phosphor screens, and the bands were imaged on a Molecular Dynamics Typhoon PhosphorImager.

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