

Two independently selected capping ribozymes share similar substrate requirements

HANI S. ZAHER, R. AMMON WATKINS, and PETER J. UNRAU

Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada

ABSTRACT

We report the isolation and characterization of a second capping ribozyme, called 6.17. This ribozyme has substrate requirements that are very similar to a previously isolated capping ribozyme called Iso6. Both ribozymes promote capping and cap exchange reactions with a broad range of nucleotide substrates. The ribozymes mediate a reaction where the terminal phosphate of the nucleotide substrate attacks the α -phosphate found at the ribozyme's 5' terminus. This reaction involves the release of pyrophosphate during capping or a nucleotide during cap exchange. The second-order rate constants for 6.17 and Iso6 depend strongly on the length of the phosphate group found on the nucleotide substrate. Nucleoside diphosphates or triphosphates are efficiently utilized, while monophosphates are used ~ 20 -fold less efficiently by both ribozymes. These ribozymes also have rates that increase as pH is decreased. Despite these similarities, the ribozymes are not identical and 6.17 performs optimally when incubated with divalent magnesium ions, while Iso6 displays a preference for calcium ions. Further, the ribozymes have globally different secondary structures; 6.17 has a complicated pseudoknot structure consisting of five helical elements, while Iso6 likely consists of four helical elements. We hypothesize that capping proceeds via an invariant phosphate dependent mechanism that imposes a nearly identical "catalytic fingerprint" on these two distinct ribozymes.

Keywords: RNA capping; kinetics; mechanistic invariant; ribozyme; secondary structure

INTRODUCTION

Enzymes are noteworthy in that they efficiently perform specific chemistry. But how did these catalysts evolve and what were the fundamental constraints that governed their early emergence? These questions are difficult to address with protein enzymes, as billions of years of evolution have often erased the information required to understand the evolutionary progression from an emergent to a fully competent catalyst (Bartel and Unrau 1999). The emergent catalytic properties of RNA are much easier to study than that of protein, as RNA catalysts (ribozymes) can be selected artificially from pools of random sequence in the laboratory (Wilson and Szostak 1999; Joyce 2002; Fiammengo and Jaschke 2005). The emergent properties of these RNAs may be of particular importance, as catalytic RNAs are critical to modern metabolism (Ban et al. 2000; Winkler and Breaker 2005) and were likely to have played a dom-

inant role early in evolution prior to the emergence of protein catalysis (Gilbert 1986; Joyce 2002).

Artificially selected ribozymes, like protein enzymes, have generally been shown to exhibit good substrate discrimination over a range of chemistries (Cech et al. 1981; Unrau and Bartel 1998; Seelig and Jaschke 1999; Wilson and Szostak 1999; Lau et al. 2004; Tsukiji et al. 2004; Fiammengo and Jaschke 2005). In some cases the basis for substrate discrimination may be obvious; such is the case with self-cleaving ribozymes, where their base-pairing properties make possible highly specific phosphodiester bond cleavage reactions (Doherty and Doudna 2000). In other cases the mechanism behind substrate discrimination and its importance to catalysis is harder to resolve. For example, purine and pyrimidine nucleotide synthase ribozymes have been found to exhibit good substrate discrimination between small substrates having molecular weights < 170 Da (Unrau and Bartel 1998; Lau et al. 2004). This substrate specificity is achieved even though these ribozymes exploit a range of catalytic strategies—some exhibit good substrate binding affinity, while others have high intrinsic chemical rates but poor substrate affinity (Lau et al. 2004). This indicates that, at least for nucleotide synthesis ribozymes, a range of catalytic strategies is

Reprint requests to: Peter J. Unrau, Department of Molecular Biology and Biochemistry, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia V5A 1S6, Canada; e-mail: punrau@sfu.ca; fax: (604) 291-5583.

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available. A detailed investigation of the catalytic strategies utilized by other artificially selected ribozymes performing chemistries with a range of small molecule substrates will help to further delimit the catalytic properties of newly emergent ribozymes, similar to those possibly encountered early in evolution.

The chemistry of capping provides a unique window into the mechanism of substrate discrimination and RNA catalysis. Capping involves the exchange of nearly equivalent phosphate groups so as to form a nucleotide 5'-5' cap on an RNA strand using a small nucleotide substrate. Protein enzymes that catalyze the formation of capped nucleic acid, which include GTP-dependent RNA guanylyl transferase, ATP- and NADH-dependent DNA ligases, and ATP-dependent RNA ligases, all utilize a two-step mechanism that makes use of a lysine residue to form an enzyme phosphoramidate intermediate (Shuman and Lima 2004). In the second step, the terminal phosphate of the acceptor nucleic acid attacks the α -phosphate of the covalently linked nucleotide displacing the lysine residue and forming the 5'-5' cap structure. During the course of enzyme-mediated capping, the phosphate moieties of the nucleotide are required for chemistry, while the distal base and sugar of this nucleotide are utilized to provide substrate recognition and discrimination. As nucleotide substrates are, in principle, easily recognized by RNA, it might be expected that capping ribozymes, like their protein counterparts, would also distinguish between nucleotide substrates. An exploration of the catalytic and substrate utilization of capping ribozymes should therefore provide insight into how the requirements imposed by chemistry regulate RNA-mediated catalysis.

Previously, Huang and Yarus (1997a) isolated a general RNA capping ribozyme called Iso6. Iso6 accelerates the formation of a variety of 5'-5' RNA cap structures and releases pyrophosphate in the presence of Ca^{2+} ions. Notably, Iso6 is very promiscuous in its substrate requirements and only requires a terminal unblocked phosphate-containing nucleophile to attack the α -phosphate of the ribozyme's 5'-triphosphate (Equation 1, Huang and Yarus 1997c). Additionally, the ribozyme possesses pyrophosphatase, decapping, and exchange activities (Huang and Yarus 1997b). The ribozyme displays a similar $\sim 10 \mu\text{M}$ affinity for either guanosine triphosphate or diphosphate, but exhibits a substrate affinity toward guanosine monophosphate that is ~ 15 -fold lower (Huang and Yarus 1998). In this study, we report the isolation of a second capping ribozyme, called 6.17, from an independent selection and using a different selective approach. Intriguingly, this ribozyme has kinetic and substrate recognition properties similar to Iso6—even though both ribozymes have distinct metal ion preferences and are likely to adopt different secondary structures. This suggests that, in contrast to the majority of small molecule reactions mediated by RNA, the chemistry of capping is incompatible with facile substrate

discrimination, an interesting finding given the proven ability of RNA to distinguish between different nucleobases (Noeske et al. 2005).

RESULTS

The selection was initially designed to isolate possible RNA polymerase ribozymes based on their ability to incorporate ^{45}S UTP onto their 3' ends when annealed to a poly(A) template and incubated with 1–2 mM concentrations of ^{45}S UTP. Such candidates were enriched based on their ability to be supershifted on a gel containing N-acryloyl-aminophenyl-mercuric acid (APM) (Igloi 1988). Due to the large incubation volumes of Rounds 1 and 2, crude ^{45}S UTP containing significant amounts of ADP and ATP was used, while pure ^{45}S UTP was used in Rounds 3–6. By Round 6, 2.5% of the pool RNA was retained on an APM gel after 4 h of incubation with no sign that activity increased in further rounds of selection. Twenty-five isolates were therefore cloned from Round 6 and screened for activity. Clone 6.17 showed the fastest kinetics, with $\sim 25\%$ reacting after 4 h, and was selected for further study. Analysis of the reaction mediated by this ribozyme indicated that this sequence did not perform a polymerase type activity at the 3' terminus but rather formed a nucleotide (nt) cap at its 5' terminus. This 272-nt-long ribozyme was truncated from its 3' end as well as internally, resulting in a fully active 94-nt *cis* construct referred to hereafter as c6.17. Nevertheless, the 94-nt size of the ribozyme and the intrinsic resolution of sequencing gels made it difficult to study capping with nucleotides lacking thiol tags. To facilitate analysis, a two-component *trans* ribozyme (called t6.17, shown in Fig. 1A) was designed by cutting c6.17 in the loop closing helix I. A short 33-nt-long sequence called module B, possessing a 17-nt-long tail that was not important for activity but that increased transcriptional yield, became capped when incubated with a 73-nt-long module A (residues 150–222 of the initial ribozyme). The t6.17 construct retained nearly all of the activity of the *cis* construct and had only a slightly lower rate constant and substrate binding affinity than its c6.17 parent under single turnover conditions.

Ribozyme 6.17 synthesizes 5'-5' caps

Trans-mediated capping of module B was confirmed by the following set of experiments. (1) Removing the triphosphate from the module B transcript and replacing it with a 5' hydroxyl or 5' monophosphate inactivated the ribozyme. (2) Unlabeled ribozyme incubated with [α - ^{32}P]-UTP became radiolabeled and did not lose this ^{32}P label upon treatment with calf intestinal phosphatase (CIP), suggesting that the α -phosphate of the incorporated nucleotide was not exposed. Furthermore, the location of this modification was mapped by alkaline hydrolysis to the very 5' end of

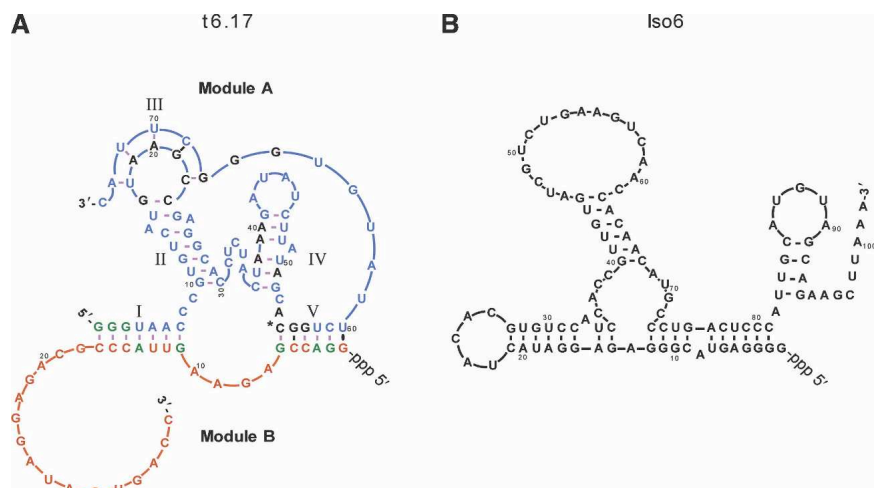
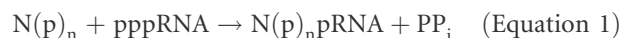


FIGURE 1. Proposed secondary structures of capping ribozymes 6.17 and Iso6. (A) The secondary structure of t6.17; module A is in blue, module B is in light red. RNA substrate recognition (module B) is accomplished by helices I and V. In addition, module A has two hairpins, II and IV. The loop of hairpin II forms a pseudoknot with the 3' terminus of module A, forming helix III. Black residues are protected from T1 nuclease and DEPC modification in the absence of the other module. Green residues are protected only in the presence of the other module. Pink bars represent proposed base-pairing interactions, while black bars indicate base pairing confirmed by manual covariation experiments. The black oval indicates the importance of residue U_{A60} in forming a wobble base pair with G_{B1} of module B. The asterisk indicates where the ⁴⁵S capped product quantitatively cross-links when exposed to UV light. (B) The secondary structure of Iso6 proposed by Huang and Yarus. Adapted with permission from Huang and Yarus (1997b) © 1997 American Chemical Society.

module B. (3) When module B was labeled with [γ -³²P]-GTP by transcription and then incubated in the presence of module A, radioactive pyrophosphate was released. (4) Unlabeled ribozymes became radiolabeled with no appreciable accumulation of radiolabeled phosphate or pyrophosphate when capped with [γ -³²P]-ATP as judged by gel electrophoresis containing phosphate and pyrophosphate standards. The simplest interpretation of these four independent pieces of data is that the ribozyme mediates a reaction where the α -phosphate of the 5' triphosphate found on module B is joined to the terminal phosphate found on the nucleotide substrate (Equation 1):



Secondary structure of the ribozyme

The bimolecular t6.17 capping ribozyme was particularly amenable to structural probing. Diethyl pyrocarbonate (DEPC) probing of the N7 position of adenosine residues and T1 nuclease partial digestion at unstructured G residues were particularly informative. Module A contained many residues that were enzymatically or chemically protected even in the absence of module B. Guanosine residues G_{A17}, G_{A21}, G_{A56}, G_{A57}, G_{A66}, G_{A67}, and G_{A68} were found to be protected from T1 in native conditions (Fig. 2A, left panel). Residues A_{A19}, A_{A20}, A_{A38}, A_{A39}, A_{A40}, A_{A51}, and A_{A54} were protected from DEPC modifi-

cation and were also independent of the presence or absence of module B (Fig. 2A, right panel). The protection of A_{A19}, A_{A20}, G_{A21}, and G_{A68} residues suggested the possibility of a pseudoknot interaction between residues U_{A18}-C_{A22} and G_{A68}-A_{A72} resulting in helix III. Point mutants designed to destabilize this helix were constructed and a complete covariation of the base pair G_{A21}:C_{A69} to U_{A21}:A_{A69} was found not to affect catalytic rate, while the individual point mutants had a significant impact on the ribozyme's ability to promote capping (Fig. 2B).

Guanosine residues G_{B2}, G_{B6}, and G_{B11} of module B were enzymatically protected only in the presence of module A (Fig. 2C, left panel). DEPC modification of module B also showed the specific protection of adenosine residues A_{B3} and A_{B14} in the presence of module A (Fig. 2C, right panel). Based on the T1 and DEPC protection patterns for module B, we propose that modules A and B recognize each other through two base-paired regions: helix

I, consisting of nucleotides G_{A1}-G_{A7} and G_{B11}-C_{B17}, and helix V, consisting of nucleotides C_{A55}-U_{A60} and G_{B1}-G_{B6}. Point mutants that disrupted a proposed base pair in helix V (G_{A56}:C_{B5}) completely inhibited activity. Consistent with the existence of this helix, changing the G_{A56}:C_{B5} pair to a U_{A56}:A_{B5} pair restored capping activity (Fig. 2D).

A model of the t6.17 ribozyme, consisting of five helical elements, is shown in Figure 1A. Our ribozyme structure is consistent with DEPC and T1 probing as well as covariation data obtained for two of these helical elements. Module B recognition is achieved via helices I and V. In addition, module A has two helical elements, II and IV, with the pentaloop protruding from helix II interacting with the 3' tail of the ribozyme to form a pseudoknot, labeled helix III. Interestingly, this helix can be extended to form a 7-bp helix, and the exact interplay between helices II and III will require further study to resolve. The bulged helices II and IV are predicted to exist based on thermodynamic folding (mfold V3.1.2; Zuker et al. 1999). Consistent with this model, the two bulged adenosines in helix II were sensitive to DEPC treatment in native conditions. Helix IV was supported by the existence of protected residues A_{A38}, A_{A39}, A_{A40}, and A_{A51}. This relatively complex RNA motif consists of 31 bp and ~22 nt of interhelical sequence.

The t6.17 ribozyme appears likely to adopt a secondary structure different from Iso6. Using thermodynamic folding, S1 nuclease protection and lead cleavage mapping, Huang and Yarus (1997b) predicted that the secondary

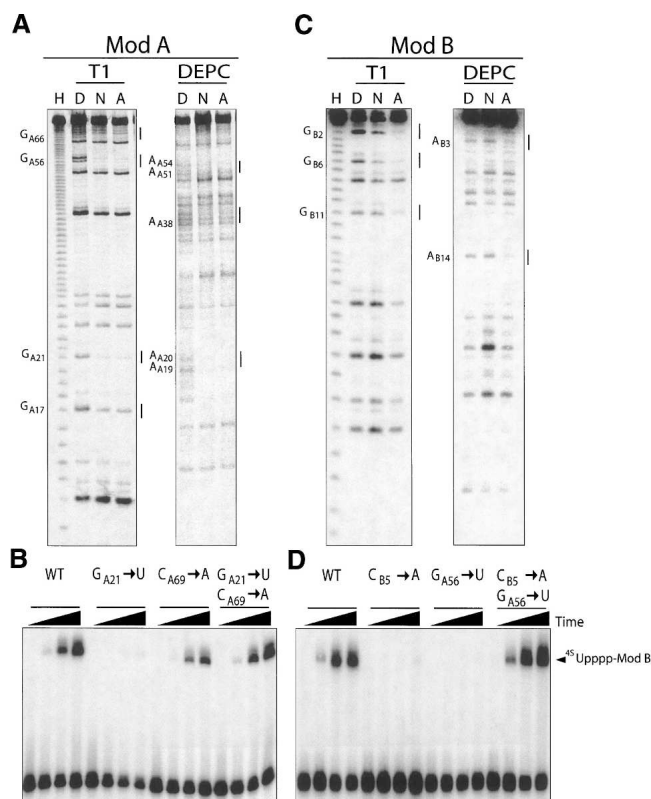


FIGURE 2. Secondary structure probing and manual co-variation experiments. (A) T1 partial hydrolysis reactions (*left panel*) and DEPC reactions (*right panel*) of ^{33}P -5'-end labeled module A. T1 hydrolysis showed the protection of module A guanine residues G_{A17} , G_{A21} , G_{A56} , G_{A57} , G_{A66} , G_{A67} , and G_{A68} in native conditions that were independent of the presence of module B. DEPC treatment indicated that adenosine residues A_{A19} , A_{A20} , A_{A38} , A_{A39} , A_{A40} , A_{A51} , and A_{A54} of module A were involved in secondary structure formation and were again independent of module B. In all cases H indicates hydrolysis ladder, D indicates denatured complex, N indicates native conditions in the absence of the other module, while A indicates probing of the fully assembled construct under native conditions. (B) A predicted pseudoknot in module A was confirmed by constructing point mutants in G_{A21} and C_{A69} . While mutating G_{A21} to U completely inhibited activity, altering C_{A69} to an A residue lowered the capping rate to one-third that of the wild type. When the double mutation was assayed, capping rate was restored to wild-type levels. (C) T1 partial hydrolysis reactions (*left panel*) and DEPC reactions (*right panel*) of ^{32}P -3'-end labeled module B. Residues G_{B2} , G_{B6} , and G_{B11} of module B were protected from T1 only in the presence of module A. DEPC treatment of module B showed the protection of adenosine residues A_{B3} and A_{B14} only in the presence of module A. (D) Kinetics of point mutants designed to test the interaction between modules A and B. Residues G_{A56} and C_{B5} were mutated so as to disrupt a predicted base pair. Only the double mutant that changed the base pair from a $C_{B5}:G_{A56}$ to an A:U was as functional as the wild-type sequence supporting the predicted secondary structure.

structure for Iso6 consists of four helical elements (Fig. 1B). Iso6 may potentially form a pseudoknot-like structure by the pairing of residues A_{54} – U_{57} with residues G_{96} – U_{99} . However, the resulting structures would remain topologically distinct from that of 6.17; in particular, helix IV of 6.17 is located 3' to the reactive 5'-terminal α -phosphate of

the ribozyme, while the corresponding 3'-end hairpin of Iso6 would be 5' to the reaction center.

Cross-linking of capped products

^{45}S UTP is an efficient photo-induced cross-linking reagent when excited by ~ 310 nm light. We found that when module B was capped with either ^{45}S UTP or ^{45}S UMP, a quantitative cross-link formed with module A. When these cross-linked RNA species were gel purified and subjected to base hydrolysis and T1 digestion, the cross-links were mapped in each case to residue C_{A55} , even though the length of the phosphodiester linkage varied from two to four phosphates (Fig. 3A). As expected, labeling module B at its 3' end with ^{32}P -pCp produced a cross-link with identical mobility that mapped to the extreme 5' end of module B.

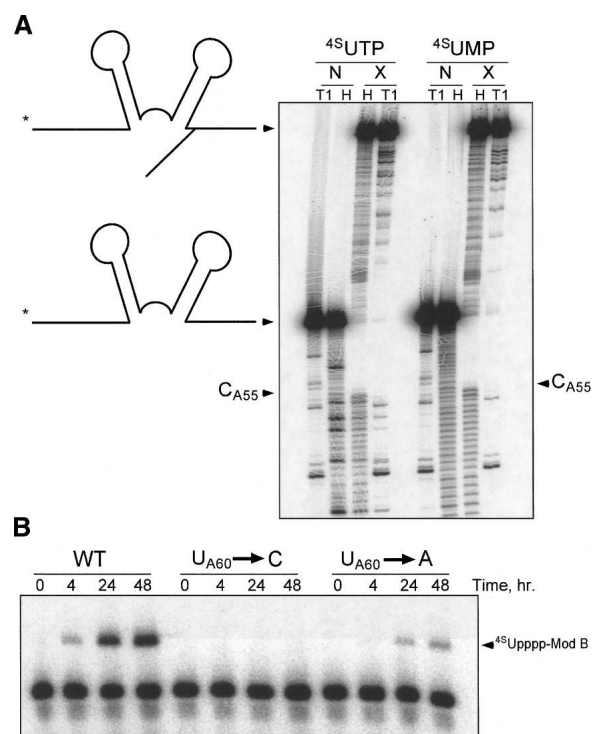


FIGURE 3. Cross-linking of capping products and flexibility of helix V. (A) Base hydrolysis (H) and T1 partial digestion (T1) of ^{45}S UTP-capped (*left lanes*) or ^{45}S UMP-capped (*right lanes*) module B cross-linked to a ^{32}P -5'-end labeled module A (X). The cross-link was formed by exposing a 24-h time point of a capping reaction to UV light with wavelength >300 nm for 30 min; nearly 100% of the ^{45}S capped product formed a single cross-linked species. Unreacted ribozyme that did not show a mobility shift in an APM gel did not cross-link when exposed to UV light (N) and was also subjected to base hydrolysis and partial T1 digestion. For both cap types, the cross-link was assigned to C_{A55} . (B) The importance of the $U_{A60}:G_{B1}$ wobble base pair was assessed by constructing point mutants, the first of which altered U_{A60} to C stabilizing the base pair. This change abolished capping. Destabilizing the end of the helix by mutating U_{A60} to an A residue was tolerated, but capping activity was lowered six- to sevenfold relative to the wild type.

The location of the cross-link, C_{A55} , is 6 bp away from the triphosphate required for capping activity (Fig. 1A, asterisk). We hypothesized that if helix V were flexible near the site of capping, it could allow the observed cross-link. This idea was tested by constructing point mutants that either stabilized or destabilized the triphosphate end of helix V. Replacing the terminal $U_{A60}:G_{B1}$ wobble pair with $C_{A60}:G_{B1}$ destroyed capping activity, while replacing the wobble pair with $A_{A60}:G_{B1}$ lowered capping efficiency by six- to sevenfold (Fig. 3B). These data indicate that helix V needs flexibility near the cap site, and that the wobble $U_{A60}:G_{B1}$ base pair is required for optimal activity.

Metal ion dependence and magnesium binding of 6.17 ribozyme

To test the metal ion requirements of 6.17, t6.17 was incubated in the presence of Mg^{2+} , Ca^{2+} , Mn^{2+} , $Co^{3+}(NH_3)_6$, Zn^{2+} , and Ni^{2+} . Capping took place only in the presence of Mg^{2+} , Ca^{2+} , and Mn^{2+} ; however, the capping rate slowed approximately twofold when Mn^{2+} was substituted for Mg^{2+} and by a factor of approximately sevenfold when only Ca^{2+} was present (Fig. 4A). Supplementing a reaction containing 20 mM Mg^{2+} with 5 mM Ca^{2+} or 5 mM Mn^{2+} had no effect on the capping rate, suggesting that Mg^{2+} was the preferred metal ion for catalysis and that Ca^{2+} and Mn^{2+} do not effectively compete for binding. A Hill analysis indicated that several ion binding sites were important for catalysis or folding and that magnesium was bound loosely by the ribozyme (see Fig. 4B). In contrast, the capping ribozyme Iso6 isolated by Huang and Yarus (1997a) exhibited a strong preference for Ca^{2+} and showed no activity even when incubated with 100 mM Mg^{2+} . In fact, both magnesium and strontium were found to be competitive inhibitors for the calcium-dependent capping activity of the Iso6 ribozyme (Huang and Yarus 1997b).

Capping is dependent on nucleotide phosphate chain length but not on base or nucleotide sugar composition

Substrates containing 5' phosphate groups were required for catalysis, although the length of this phosphate group could be varied. The general substrate requirements exhibited by 6.17 are similar to those displayed by Iso6, which also reacts with a range of substrates that contain at least one exposed phosphate group (Huang and Yarus 1997c). The first panel of Figure 5 shows t6.17 reacting with ^{45}S UTP, ^{45}S UDP, and ^{45}S UMP but not ^{45}S U. Capping reactions were observed with a broad range of ribose, deoxyribose, and dideoxyribose nucleotide triphosphates and nucleotide monophosphates, indicating that neither the sugar nor the base composition was essential for activity (Fig. 5, panels 2–5). Dinucleotides containing only internal phosphates were found not to react and implicate the importance of a

5' nucleotide phosphate of variable length in the ribozyme reaction (Fig. 5, panel 6).

Decreasing the phosphate chain length of the nucleotide substrate affected substrate binding affinity but left the ribozyme reaction rate invariant. Nucleotide titrations were performed and initial rates extracted for the c6.17 ribozyme. Fitting these rates to the Michaelis–Menten equation resulted in $k_{cat(app)}$ values of $0.079 \pm 0.002 \text{ h}^{-1}$, $0.080 \pm 0.002 \text{ h}^{-1}$, and $0.078 \pm 0.005 \text{ h}^{-1}$ for the nucleotides ^{45}S UTP, ^{45}S UDP, and ^{45}S UMP, respectively. The K_m values for the same series were $24 \pm 3 \mu\text{M}$, $13 \pm 2 \mu\text{M}$, and $535 \pm 100 \mu\text{M}$ (Fig. 6). Table 1 summarizes these parameters and the relative second-order rate constants for 6.17 and Iso6. Both ribozymes exhibit large decreases in $k_{cat(app)}/K_m$ when reacted with a nucleotide monophosphate. Notably, the $k_{cat(app)}$ for both ribozymes is nearly completely independent of substrate choice, averaging 0.079 h^{-1} for 6.17 and 0.083 min^{-1} for Iso6. This suggests that the intrinsic chemistry mediated by both ribozymes is independent of phosphate chain length, but that NMPs in contrast to NDPs or NTPs are poorly recognized.

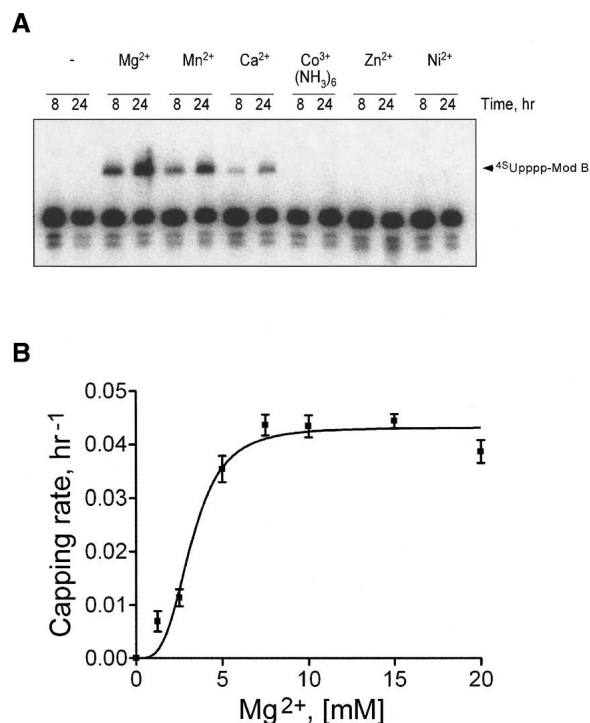


FIGURE 4. Metal ion requirements and Mg^{2+} dependence. (A) Standard ^{45}S UTP capping reactions (pH 7.6) were performed in the presence of 5 mM of the indicated metal ions. Samples were taken at the indicated times and run on a 10% APM gel. Mn^{2+} supported capping with a rate half that observed in the presence of only Mg^{2+} . Capping also tolerated Ca^{2+} , but with a rate one-seventh of that observed with Mg^{2+} . (B) A cooperative binding analysis using a fit to three independent magnesium titration experiments. ^{45}S UTP capping rates were determined and plotted against Mg^{2+} concentration. A Hill coefficient of 3.5 ± 0.6 , with a binding constant of $3.2 \pm 2 \text{ mM}$, was measured (assuming equivalent binding sites).

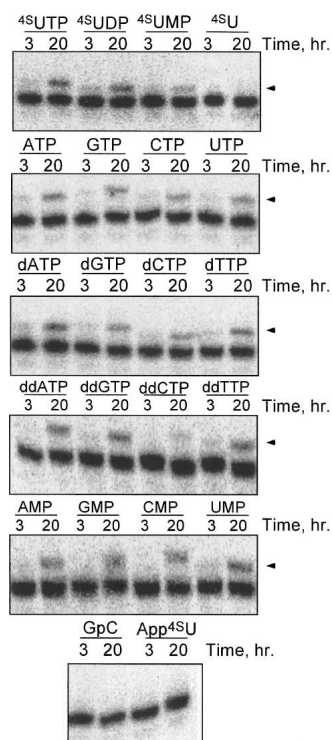


FIGURE 5. General substrate utilization by the capping ribozyme. Capping takes place with nucleotides having at least one 5'-phosphate group, while substrates having internal phosphates (GpC and App⁴⁵U) do not support capping. Module B was [³²P]-pCp labeled and annealed to a 10-fold excess of module A to form a reactive ribozyme complex. Reactions were started by the addition of 1 mM of the indicated substrate to the ribozyme incubation. Time points were taken at 3 and 20 h and resolved using 15% PAGE.

Decapping and exchange reactions

In addition to being able to react when activated with a triphosphate, 6.17 was also able to perform cap exchange and decapping. t6.17 was first incubated in the presence of 1 mM UTP and the resulting uridine tetraphosphate cap product (Upppp-Mod B) was carefully gel purified away from unreacted or hydrolyzed module B RNA. The Upppp-Mod B construct was then reannealed to module A and incubated with varying concentrations (31–500 μ M) of

⁴⁵UTP (Fig. 7). Capping by exchange of UTP with ⁴⁵UTP had a saturated rate of capping of $0.070 \pm 0.006 \text{ h}^{-1}$ —a value very similar to that observed when the ribozyme was initially activated with a triphosphate (Fig. 6A). These observations indicate that capped RNA is a good substrate for cap exchange and implies that neither the pyrophosphate leaving group of the triphosphate activated ribozyme nor the nucleotide released during exchange strongly influence catalytic rate. Again this property is shared with Iso6; using a guanine triphosphate cap, Huang and Yarus (1997b) demonstrated that Gppp-Iso6, when incubated with Gppppp, efficiently performed cap exchange to form Gppppp-Iso6.

pH profile

t6.17 showed an increase in reactivity with decreasing pH. The effect of pH on the capping rate was measured from 5.7 to 9.5 using three replicates (Fig. 8). The pH-rate profile is consistent with two apparent pK_a's, the first below 6 and the second at ~ 8 and will require a more detailed investigation to fully understand. Generally, the pH dependence of 6.17 is similar to that observed for Iso6, where maximal activity is also observed at low pH (Huang and Yarus 1997a). Both capping ribozymes are in striking contrast to ribozymes that perform phosphodiester chemistry, where chemical rate increases with pH (i.e., Bergman et al. 2000).

DISCUSSION

Why do two capping ribozymes selected from two different laboratories both show a near complete lack of nucleotide substrate discrimination? The procedure used in the early rounds of selection to isolate Iso6 involved the circularization of RNAs that were able to generate a 5'-terminal monophosphate by liberating a pyrophosphate from their 5' termini. Only in later rounds of selection were ribozymes enriched based on their ability to produce cap structures when incubated with UTP-agarose (Huang and Yarus 1997a). The selection resulting in the isolation of Iso6

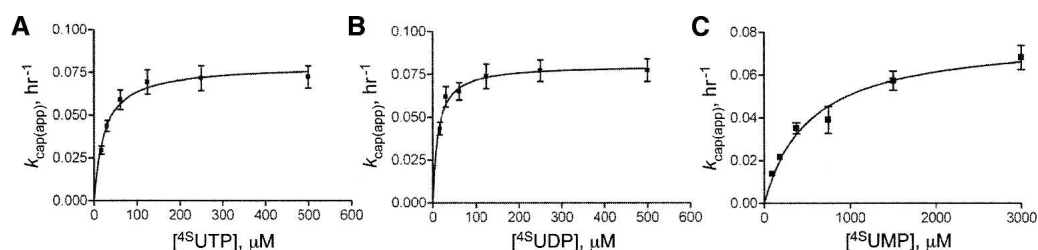


FIGURE 6. Determining the kinetic parameters of capping dependence on 5'-nucleotide phosphate chain length. Internally labeled c6.17 was incubated with (A) ⁴⁵UTP, (B) ⁴⁵UDP, and (C) ⁴⁵UMP at the indicated concentrations. Time points were taken at 1, 2, 4, 8, and 24 h and resolved on an 8% APM gel. Initial rates were then extracted from each time course, plotted against the nucleotide concentration, and fit to the Michaelis-Menten equation.

TABLE 1. Kinetic parameters of Iso6 and c6.17 and their relative efficiencies

Iso6 ^a				c6.17 ^b			
Nucleotide	$k_{\text{cat(app)}} \text{ (min}^{-1}\text{)}$	$K_m \text{ (}\mu\text{M)}$	Rel. $k_{\text{cat(app)}}/K_m^c$	Nucleotide	$k_{\text{cat(app)}} \text{ (h}^{-1}\text{)}$	$K_m \text{ (}\mu\text{M)}$	Rel. $k_{\text{cat(app)}}/K_m^c$
GTP	0.084 ± 0.004	13 ± 2	1.0	⁴⁵ UTP	0.079 ± 0.002	24 ± 3	1.0
GDP	0.089 ± 0.005	11 ± 2	1.3	⁴⁵ UDP	0.080 ± 0.002	13 ± 2	1.9
GMP	0.077 ± 0.004	200 ± 40	0.060	⁴⁵ UMP	0.078 ± 0.005	535 ± 100	0.044

^aTaken from Huang and Yarus (1998). Reaction conditions: 20 mM Mes (pH 5.5), 20 mM Ca²⁺, 37°C.

^bReaction conditions: 50 mM Tris (pH 7.6), 20 mM Mg²⁺, 150 mM K⁺, 22°C.

^cRel. $k_{\text{cat(app)}}/K_m$ is given as the ratio of the $k_{\text{cat(app)}}/K_m$ of the appropriate nucleotide to that of the triphosphate one.

therefore favored ribozymes with the general ability to activate the 5'-terminal α -phosphate irrespective of substrate. A completely different approach was utilized to isolate 6.17. Ribozymes able to cap themselves with ⁴⁵UTP were selected in early rounds (using thiol-sensitive APM gels) after having been incubated with a solution containing significant levels of ATP and ADP in addition to ⁴⁵UTP. As a consequence a mild selective pressure in favor of ⁴⁵UTP-dependent capping should have existed during our selection. Even without this pressure, ribozymes with good levels of substrate discrimination are typically selected by incubation of RNA pools with pure small molecule substrates (Unrau and Bartel 1998; Seelig and Jaschke 1999; Wilson and Szostak 1999; Lau et al. 2004; Tsukiji et al. 2004; Fiammengo and Jaschke 2005). It therefore appears that selection conditions were not directly responsible for the inability of 6.17 to differentiate between nucleotide substrates and that the chemistry of capping may have in some way prevented the isolation of ribozymes able to utilize a specific substrate.

The primary sequences of 6.17 and Iso6 bear no obvious sequence similarities when compared using a dot plot or manual alignment. Furthermore, the two ribozymes are likely to adopt globally different secondary structures (Fig. 1) as indicated by structural probing and covariation experiments (Fig. 2). However, the possibility of the two ribozymes adopting similar tertiary structures cannot be completely excluded. Intriguingly, 6.17 and Iso6 are both predicted to have helices that terminate at the site of capping. This helix in 6.17 (labeled helix V in Fig. 1A) must be quite distorted, as the capped product quantitatively cross-links to C_{A55} at the distal end of this helix (Fig. 3). Consistent with this interpretation, stabilizing helix V by converting the wobble base pair found at its terminus to a Watson-Crick pair inhibited activity (Fig. 3B). A similar situation may exist for Iso6, as the nucleotide immediately proximal to the site of capping is predicted to lack a base-pairing partner (Huang and Yarus 1997b). The helical element, which at least for 6.17 appears likely to be in a distorted geometry, may play an important role in stabilizing the phosphate chemistry that takes place at the reactive terminal α -phosphate of both ribozymes.

Iso6 and 6.17 display an unusual pH dependence, with capping rates increasing as the pH is decreased (Fig. 8; Huang and Yarus 1997a). Ribozymes that mediate phosphodiester cleavage and ligation reactions exhibit reaction rates with opposite behavior (Dahm et al. 1993; Pyle and Green 1994; Bergman et al. 2000). The pH profile of the class I ligase, for example, is explained by the fact that the attacking 3' hydroxyl group must be deprotonated to facilitate its attack onto the α -phosphate of the triphosphate on the incoming RNA strand so as to form a 5'-3' phosphodiester linkage (Bergman et al. 2000). The pH dependence of capping can be explained by assuming that protonation of the triphosphate chain is required to facilitate catalysis. In support of this argument, the hydrolysis of ATP, where water attacks the α - or β -phosphate to yield AMP or ADP, respectively, is increased at lower pH (Ramirez et al. 1980). In this instance, the neutralization of the γ - or β -phosphate oxyanions increases the electrophilicity of the reaction center, stabilizing a proposed oxyphosphorane intermediate. This reaction is accelerated by a factor of 10–50 in the presence of divalent metal ions such as Mg²⁺ or Ca²⁺ at pH values >3 (Ramirez et al. 1980), suggesting that both 6.17 and Iso6 utilize a similar divalent metal-ion-dependent process during capping. Consistent with this hypothesis, both ribozymes are unreactive in the

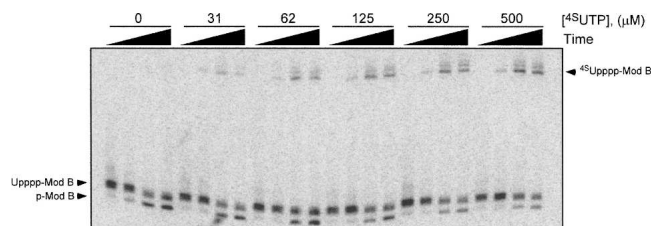


FIGURE 7. Decapping and exchange activity. A [³²P]-3'-end labeled module B was capped with UTP (Upppp-Mod B), carefully gel purified, and annealed to module A. ⁴⁵UTP was titrated from 0 to 500 μM . Time points were taken at 0, 2, 24, and 48 h and run on a 15% APM gel. In the absence of ⁴⁵UTP, Upppp-Mod B is hydrolyzed to a monophosphate-module B (p-Mod. B). As the ⁴⁵UTP concentration is elevated, a competing exchange activity, where ⁴⁵UTP attacks the α -phosphate releasing UTP and forming a ⁴⁵UTP cap (⁴⁵Upppp-Mod B), is observed.

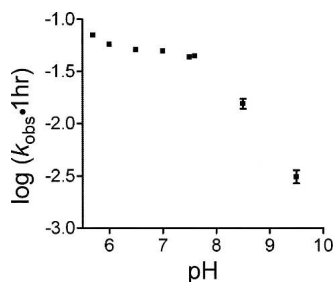


FIGURE 8. pH profile of the ⁴⁵S-UTP capping reaction. ⁴⁵S-UTP capping reactions were carried out with pH varying from 5.7 to 9.5, in the presence of 20 mM Mg²⁺. Log capping rates from three different experiments were averaged and plotted against pH.

presence of cobalt hexamine. Like other magnesium-dependent ribozymes, 6.17 tolerates Mn²⁺ and Ca²⁺ (Fig. 4A; Fedor 2002); however Iso6 prefers Ca²⁺ and only reacts slowly with Mn²⁺ (Huang and Yarus 1997b). Ribozyme 6.17 has a relatively loose affinity for Mg²⁺ and may as a consequence tolerate ions with slightly larger diameters (the ionic radii of Mg²⁺, Mn²⁺, and Ca²⁺ are 0.72 Å, 0.83 Å, and 1.00 Å, respectively). Iso6 in contrast has a fairly high affinity for Ca²⁺, possibly explaining its ability to tolerate Mn²⁺ but not Mg²⁺ (Huang and Yarus 1998).

Although 6.17 and Iso6 ribozymes fail to recognize either the sugar or base of their substrates, they both exhibit a high affinity for the phosphate moiety of the capping nucleotide. Both ribozymes have affinities ranging from 11 to 24 μM for nucleotide diphosphates and triphosphates. This affinity decreases by a factor of 15–22 when the ribozymes are given nucleotide monophosphates. Intriguingly, chemistry is performed in such a way that even when binding interactions are improved by providing a substrate with two or more phosphate groups, the maximum rate of capping remains invariant (Table 1). These data suggest that both capping ribozymes possess complicated active sites that can activate the α-phosphate toward chemistry independent of the attack of the ultimate substrate. Using thio-phosphate modifications to track the stereochemistry of the 6.17 capping reaction, we have recently shown that capping in fact proceeds via a two-step mechanism that utilizes a covalent intermediate (Zaher and Unrau 2006)—closely paralleling the reaction mechanism of protein enzymes. This mechanism implies that the preferential recognition afforded substrates containing at least two phosphate groups is intertwined with the displacement of the pyrophosphate-leaving group that initiated the process of cap formation.

By reasons of symmetry, the initial state of either ribozyme when activated with a triphosphate or a nucleotide cap looks chemically indistinguishable from the product of the ribozyme reaction—provided that the ribozymes do not interact with the sugar or base of the nucleotide at the distal end of the cap. Breaking this symmetry would allow the ribozyme to distinguish reactant from product and would

be expected to reduce the promiscuous nature of either ribozyme. The finding that Iso6 and 6.17 do not break this symmetry has two implications: First, as we have argued through the finding that 6.17 and Iso6 cannot distinguish between different nucleobases and sugars (Fig. 5), both ribozymes must focus their catalytic attention in the immediate vicinity of the phosphate groups involved in capping chemistry. Second, the amount of informational complexity required to specify the three-dimensional structure of the active site is sufficiently high to preclude the easy isolation of capping ribozymes that can recognize particular nucleotides. This is unusual, as in principle the recognition of a nucleobase via Watson–Crick hydrogen bond interactions is easily achieved by RNA, as was recently demonstrated by the solution structures of the adenine and guanine riboswitches (Noeske et al. 2005). We hope that the future evolution of these capping ribozymes using selection conditions where substrate discrimination is required will allow the isolation of RNA capping ribozymes that utilize a specific nucleotide substrate. Such a ribozyme would be predicted not only to utilize a substrate with a defined base, but (as a consequence of the formation of a nucleotide binding pocket) would also be expected to react only with a nucleotide of defined phosphate chain length. The additional sequence information required to specify this substrate discrimination would provide important clues as to the evolutionary potential of RNA and extend the ideas of Szostak concerning the information content of functional RNA sequences (Szostak 2003; Carothers et al. 2004).

MATERIALS AND METHODS

Pool construction and isolation of capping ribozyme 6.17

A high-diversity DNA pool having the sequence *TTCTAATAC-GACTTATA* GGACCGAGAAGTTACCC-N₍₇₆₎-CCTTGG-N₍₇₆₎-GGCACC-N₍₇₆₎-ACGCACATCGCAGCAAC (italics indicate the T7 promoter sequence, N indicates a random nucleotide position) was made from three synthetic single-stranded pools, as described previously (Unrau and Bartel 1998; Zaher and Unrau 2005). RNA transcripts were made by in vitro T7 transcription (40 mM Tris-HCl at pH 7.9, 2.5 mM spermidine, 26 mM MgCl₂, 0.01% Triton X-100, 10 mM DTT, 8 mM GTP, 4 mM ATP, 4 mM CTP, 2 mM UTP, 4 U/μL T7 RNA polymerase incubated for 1 h at 37°C) (Zaher and Unrau 2004). A total of ~1.5 × 10¹⁵ different RNA sequences were used with 5–6 copies of each sequence in the initial pool (a total of 15 nmol of RNA). Initially the selection was designed to isolate RNA polymerase ribozymes. This was carried out by incubating the RNA with an RNA template designed to hybridize to the 3' end of the pool which provided a template for ⁴⁵S-UTP incorporation using the following incubation conditions: 0.3–0.5 μM RNA, 1.0 μM template, 2 mM ⁴⁵S-UTP, 50 mM Tris-HCl (pH 7.6), 25 mM MgCl₂, 150 mM KCl at 22°C for 20 h. The crude ⁴⁵S-UTP used in Rounds 1 and 2 of the selection was synthesized by incubating 10 mM ⁴⁵S-UDP and 20 mM ATP with

50 mM Tris-HCl (pH 7.8), 2.5 mM MgCl₂, 0.1 mM EDTA, 5 mM DTT, and 1 mM spermidine together with 0.15 U/μL of nucleoside-5'-diphosphate kinase (Sigma) for 30 min at room temperature. This preparation was then crudely enriched and used in the pool incubation. The final composition of the synthesis was ~62% ⁴⁵UTP, 12% ADP, and 26% ATP as determined by analytical HPLC. Further rounds used ⁴⁵UTP from USB. After incubation with ⁴⁵UTP, the pool was dehybridized from the template by the addition of a competing oligonucleotide able to hybridize to the template and EDTA to chelate Mg²⁺. The pool was then gel purified. Reactive RNA able to incorporate a ⁴⁵U residue was then purified on a 5% PAGE gel containing APM and visualized using a Storm 820 PhosphorImager. Round 4 of this pool was found to contain both template-dependent and template-independent sequences (the former to be discussed elsewhere). A template-independent selection was carried out by excluding the template from future rounds in order to isolate primer-independent self-tagging ribozymes. In Rounds 5 and 6 of the selection the ⁴⁵UTP concentration was lowered to 1 mM. Incubation times during the selection for Rounds 1–6 were 24, 24, 24, 23, 1, and 1 h, respectively. By Round 6, the selection was stopped; the RNA was cloned and 25 isolates sequenced. After screening for activity, isolate 6.17 was studied in detail due to its faster kinetics. The truncated *cis* sequence c6.17 has been submitted to GenBank and has accession number DQ371299.

Characterization of *cis*-acting capping ribozyme 6.17

The site of nucleotide addition was mapped by incubating the ribozyme with [α -³²P]-UTP and subjecting the resulting radio-labeled RNA to partial alkaline hydrolysis (5 μM RNA in 50 mM NaHCO₃ at 90°C for 30 min in the presence of 0.5 μg/μL tRNA). The reaction was stopped by adjusting the pH to 7.0 using 1 M Tris-HCl. A G-ladder was produced by partial T1 digestion (5 μM RNA, 0.5 μg/μL tRNA, 6 M urea, 0.02 U/μL T1 [Fermentas] at 22°C for 2 min). The reaction was stopped by the addition of 2× formamide loading dye (95% formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue) and storing the sample on dry ice before loading onto a sequencing gel.

The phosphates of the incorporated nucleotide were shown to be protected from phosphatase by subjecting a ribozyme capped with [α -³²P]-UTP to CIP (New England Biolabs); 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT supplemented with 1 U/μL of enzyme and incubating for 1 h at 37°C.

Kinetic reactions were carried out under selection conditions using 0.5 μM ³²P-body-labeled RNA. To calculate the *K_m* of isolate c6.17, time courses with nucleotide concentrations ranging from 17 μM to 3 mM were performed. Initial rates were extracted from time-course data and plotted against ⁴⁵UTP, ⁴⁵UDP, and ⁴⁵UMP concentrations and fit to the Michaelis–Menten equation.

6.17 capping assay

Isolate 6.17 was truncated and separated into two modules called A and B to facilitate analysis. Module A consisted of residues 150–222 of the 6.17 isolate, while module B consisted of residues 1–33 and contains the reactive 5' triphosphate that is capped. Module B was 3'-end labeled by incubating 5 μM RNA with 2 μM [³²P]-pCp in 50 mM HEPES (pH 8.3), 10 mM DTT, 10 μg/mL BSA,

8.3% (v/v) glycerol, 1 mM ATP, 1 U/μL T4 RNA ligase for 2 h at 22°C (Wang and Unrau 2002). The labeled RNA was then gel purified by 12% PAGE. [³²P]-pCp was synthesized by incubating 30 μM Cp (Sigma) in polynucleotide kinase buffer (70 mM Tris-HCl, 10 mM MgCl₂, 5 mM DTT at pH 7.6) supplemented with 5 μCi/μL of [γ -³²P]-ATP (3000 Ci/mmol, NEN) and 0.5 U/μL of T4 polynucleotide kinase (Invitrogen) at 37°C for 30 min. The enzyme was then inactivated by heating at 65°C for 15 min.

Capping reactions were carried out, in single turnover conditions, by annealing module A (2 μM) and module B (0.2 μM) by heating at 80°C for 5 min and gradually cooling to 22°C before the addition of the selection buffer. Reactions were started by the addition of nucleotide at a concentration of 1 mM. Time points were taken at 3 h and 24 h, and samples were then resolved using 15% sequencing PAGE.

Decapping and exchange activity

Module B was first capped with UTP and carefully gel purified on a 12% gel. The resulting capped construct was then incubated with a fivefold excess of module A under varying concentrations of ⁴⁵UTP. Time points were taken and resolved using 15% APM gels in order to separate ⁴⁵UTP caps from the initial UTP capped substrate and hydrolyzed products.

Capping rate as a function of pH

[³²P]-pCp labeled module B was annealed to module A as before and incubated in 50 mM Mes (pH 5.7), Mes (pH 6.0), Mes (pH 6.5), Bes (pH 7.0), Bes (pH 7.5), Tris (pH 7.6), Epps (pH 8.5), and Ches (pH 9.5) buffers, in the presence of 20 mM MgCl₂, 150 mM KCl, and 1 mM ⁴⁵UTP. Aliquots were taken at 4, 8, 24, and 48 h and separated on a 10% PAGE containing 10 μM APM. The time courses from three independent experiments were fit to first-order kinetics, and apparent capping rates were then extracted and plotted against the pH values.

Metal requirement and Mg²⁺ titrations

[³²P]-pCp-labeled module B was annealed to module A as before and incubated in 50 mM Tris (pH 7.6), 150 mM KCl, and 1 mM ⁴⁵UTP in the presence of one of the following metal ions: Mg²⁺, Mn²⁺, Ca²⁺, Co³⁺(NH₃)₆, Zn²⁺, and Ni²⁺ at 5 mM. Time points were taken and resolved on an APM gel. Dependence of capping on Mg²⁺ was carried out by performing capping reactions in the presence of Mg²⁺ concentrations spanning 1.25–20 mM. Aliquots were taken at 4, 8, 24, and 48 h and separated on a 10% PAGE containing 10 μM APM. The apparent capping rates from three different experiments, resulting from a first-order fit, were plotted against the Mg²⁺ concentrations and fit to the Hill equation.

Secondary structure determination

The protection of A and G residues within either module A or B was probed using chemical and enzymatic methods under three different incubation conditions: denaturing, native, and assembled. Denaturing reactions were carried out in 25 mM sodium cacodylate (pH 7.5), 150 mM KCl, and 5 mM EDTA and at a temperature of 90°C, while the native reactions were performed in 25 mM sodium cacodylate (pH 7.5), 150 mM KCl, and 25 mM MgCl₂ at 22°C. Probing of hybridized modules A and B was

performed in native buffer. Modifications of the N7 position of adenosine residues were carried out by incubating 200 μ L of an RNA sample with 1 μ L DEPC (Sigma) at 90°C for 15 min (denaturing conditions) or 10 μ L DEPC at 22°C for 1 h (native and assembled conditions). The RNA was then ethanol precipitated twice. Cleavage was initiated by resuspending the pellet in 20 μ L of 1 M aniline-acetate (pH 4.5) at 60°C and incubating for 15 min in the dark. The aniline was removed by speed vacuuming for 2 h. The nucleic acid was resuspended in formamide loading dye. Partial enzymatic T1 digestion of G residues was carried out for the three incubation conditions. The cleavage products from the chemical modifications and T1 digestion were resolved by 15% PAGE alongside a base-hydrolysis ladder of the appropriate end-labeled module.

Photo-cross-linking of ^{45}U cap structures to the ribozyme

Module A was dephosphorylated using CIP and the resulting RNA 5'-labeled with [γ - ^{32}P]-ATP (3000 Ci/mmol, NEN) at 2 $\mu\text{Ci}/\mu\text{L}$ in the presence of T4 polynucleotide kinase at 0.5 U/ μL in polynucleotide kinase buffer for 30 min at 37°C. This was followed by gel purification on a 10% gel. A small amount of the resulting radio-labeled module A was annealed to an excess of module B and a capping reaction initiated by the addition of ^{45}UTP or ^{45}UMP at a concentration of 1 mM. After 24 h of incubation the sample was exposed to UV light through an ELISA plate (selecting >300 nm) from a UV transilluminator (Fotodyne, Bio/Can Scientific) for 30 min. The cross-linked RNA was gel purified using 10% PAGE. The site of the cross-link was mapped using T1 digestion in urea and base hydrolysis as described earlier, and the resulting fragments were resolved on a 12% sequencing gel. The same analysis was also performed for 3'-labeled module B (using [α - ^{32}P]-pCp).

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