REPORT

A pseudoknot in the 3' non-core region of the *glmS* ribozyme enhances self-cleavage activity

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ABSTRACT

The recently described *glmS* ribozyme is a self-cleaving RNA sequence found in the 5' noncoding region of the transcript of the gene for glucosamine-6-phosphate (GlcN6P) synthase in many Gram-positive bacteria. This ribozyme is associated with the GlcN6P riboswitch, and ribozyme activity in response to binding of the metabolite, GlcN6P, is proposed to effect levels of gene expression. The previously defined core sequence of the GlcN6P-dependent ribozyme contained fewer than 80 nt of contiguous sequence, but a sequence containing conserved secondary structural features and encompassing the core was twice as long. Structural elements outside of the ribozyme core could contribute to ribozyme activity or participate in gene regulation as part of the expression platform or both. Here, a 174-nt transcript containing the *Bacillus anthracis glmS* ribozyme was used to examine the contribution of part of the non-core sequence to in vitro cleavage activity. The loop portion of hairpin loop 3, located just 3' of the ribozyme core, can potentially pair with a sequence ~80 nt downstream to form a pseudoknot tertiary interaction. Disruptive and compensatory mutations in the two duplex regions of the pseudoknot had effects on in vitro cleavage rates that support a role for the pseudoknot in enhanced ribozyme activity. Cleavage activity became less sensitive to disruptive mutations in the pseudoknot as MgCl₂ concentrations were raised from 2.5 to 10 mM, suggesting that one role of the pseudoknot could be to help stabilize the core structure.

Keywords: riboswitch; ribozyme; catalytic RNA; pseudoknot; glucosamine-6-phosphate; *Bacillus anthracis*

INTRODUCTION

The recent discovery and characterization of riboswitches has revealed a new mode of regulation of gene expression and has provided insight into how the riboswitch mechanism works (Barrick et al. 2004; Nudler and Mironov 2004; Tucker and Breaker 2005). Riboswitches are naturally occurring RNA sequences that have been found within the 5' noncoding regions of transcripts. They contain a structured metabolite-binding region, called the aptamer domain, that is functionally linked to an expression platform (Winkler et al. 2004; Tucker and Breaker 2005). Binding of a specific cellular metabolite to the aptamer domain causes changes in the expression platform that alter the level of protein expression by effecting transcription or translation of the message associated with the riboswitch (Tucker and Breaker 2005). This general configuration has been found

for a variety of naturally occurring riboswitches that link genetic control to a binding event with a specific metabolite. Riboswitches have been found that respond to lysine (Grundy et al. 2003; Rodionov et al. 2003; Sudarsan et al. 2003), adenine (Mandal and Breaker 2004a; Serganov et al. 2004; Noeske et al. 2005), coenzyme B12 (Nahvi et al. 2004), and other metabolites (Mandal and Breaker 2004b).

The self-cleavage activity of the *glmS* ribozyme could be involved in the regulation of gene expression, thereby giving ribozymes a place in a riboswitch mechanism (Barrick et al. 2004; Winkler et al. 2004). The *glmS* gene encodes the enzyme glucosamine-6-phosphate synthase, which catalyzes the conversion of fructose-6-phosphate and glutamine to glucosamine-6-phosphate (GlcN6P) and glutamate (Milewski 2002). GlcN6P is a precursor in the production of UDP-GlcNAc, a key component in the pathways of cell wall synthesis, lipopolysaccharide biosynthesis, and protein glycosylation. In a novel variation of riboswitch control, the *glmS* ribozyme, which is located in the 5' untranslated region of the *glmS* transcript, couples metabolite (GlcN6P) binding with RNA cleavage and gene expression (Barrick et al. 2004; Winkler et al. 2004). A 246-nt sequence in the 5' untranslated region of the *glmS* gene in *Bacillus*

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subtilis cleaved at a specific site in the RNA (Barrick et al. 2004; Winkler et al. 2004). This reaction required Mg^{2+} and, importantly, cleavage was stimulated by GlcN6P (Barrick et al. 2004; Winkler et al. 2004).

The chemistry of the cleavage reaction has been defined, and linkage of ribozyme activity to gene expression has been demonstrated (Winkler et al. 2004). The mechanism of cleavage for this new class of ribozymes is similar to that of the small *cis*-acting ribozymes associated with RNA replicons, in that cleavage results in products with a 2',3'-cyclic phosphate group and a 5'-hydroxyl group. These end groups suggest that the reaction proceeds by nucleophilic attack of the 2' oxygen on the phosphorus of the adjacent scissile phosphate group. Ribozyme self-cleavage was linked to the regulation of gene expression by demonstrating that mutations of the *glmS* ribozyme that abolished cleavage activity in vitro resulted in derepression of gene expression in vivo (Winkler et al. 2004). However, the details of how ribozyme activity in the 5' untranslated region would regulate gene expression remain unclear.

The 5' noncoding sequences of the *glmS* transcripts from several Gram-positives share a conserved RNA secondary structure (Barrick et al. 2004; Winkler et al. 2004). The conserved structure extends beyond the GlcN6P-responsive 76-nt self-cleaving ribozyme core in *B. subtilis* defined by Winkler et al. (2004) (Fig. 1A). Given the proposed role of the longer sequence for regulation, the possibility that there could be a ribozyme domain and a regulatory domain (expression platform) that interact is an attractive and testable model. Specifically, it should be possible to test, in vitro, whether or not structural elements outside of the core ribozyme domain contribute significantly to ribozyme activity.

Within the non-core region, structural features immediately 3' to the ribozyme core could form a pseudoknot by pairing part of loop 3 with a single-stranded region just 3' of P4 (Fig. 1A). P3.1 was not a feature of the structures proposed in the original papers describing the *B. subtilis* *glmS* ribozyme (Barrick et al. 2004; Winkler et al. 2004), but was included in a secondary structure of the *Bacillus cereus* *glmS* ribozyme in a recent review by Tucker and Breaker (2005). The P3–P3.1 pseudoknot could form in all 18 sequences (Barrick et al. 2004; Winkler et al. 2004) that contain the conserved structural elements of the *glmS* riboswitch (Fig. 1B). Given that the pseudoknot structure is conserved, but resides outside the minimal domain defined for in vitro cleavage activity, it was not apparent whether the pseudoknot would contribute to ribozyme activity or, perhaps, would function in the yet-to-be-defined mechanism that links ribozyme activity to gene regulation. Here, we have measured the effect of mutations in P3 and P3.1 on *Bacillus anthracis* *glmS* ribozyme activity. The data suggest that the pseudoknot is required for high rates of ribozyme self-cleavage and this effect is most apparent at lower Mg^{2+} concentrations. The latter finding suggests that the pseudo-

knot could be important to ribozyme structural stability or folding under physiological conditions.

RESULTS AND DISCUSSION

The pseudoknot formed by P3 and P3.1 is a conserved structure in the *glmS* element

Breaker and coworkers (Barrick et al. 2004; Winkler et al. 2004) defined a secondary structure of the *B. subtilis* *glmS* ribozyme (Fig. 1A) and a consensus structural motif for this sequence in several Gram-positives. In addition to the duplex regions defined in that model (P1, P2, P2a, P3, P4, P4a), an additional pairing of sequence just 3' of P4 with L3 was possible (P3.1) (Fig. 1A). All 18 Gram-positive sequences containing a structure similar to the *glmS* riboswitch (Barrick et al. 2004) appeared to be capable of forming the P3.1 pairing (Fig. 1B). This conserved element contains 4–8 bp, depending on the species. In six instances, there are ambiguous pairing possibilities where 1 or 2 nt on the 3' side of P3 could alternatively form the 5' side of P3.1 (Fig. 1B). Although the presence of this secondary structural element was conserved, it was unclear as to whether the pseudoknot should be considered part of the ribozyme or, perhaps, part of a structure associated with an expression platform. Therefore, the contribution of the P3 and P3.1 structural elements to cleavage activity of the *glmS* ribozyme was examined.

Cleavage of the *Bacillus anthracis* *glmS* ribozyme was stimulated by GlcN6P

B. anthracis, *Bacillus thuringiensis*, and some strains of *B. cereus* share an identical 5' *glmS* sequence that is slightly shorter than the *B. subtilis* sequence. This sequence was selected for study. A plasmid construct (pANX1) for transcribing the *B. anthracis* *glmS* ribozyme (Fig. 2A) was generated by annealing synthetic oligos containing the wild-type sequence and ligating the sequence into a plasmid vector downstream of a T7 RNA polymerase promoter. Precursor ANX1 ribozyme RNA was transcribed from the linearized plasmids with T7 RNA polymerase, and the transcript was screened for ribozyme cleavage activity, with and without GlcN6P, during transcription. Precursor and 3' cleavage products were separated by electrophoresis on a denaturing polyacrylamide gel after a 15-min transcription at 37°C, and the extent of cleavage was quantified (Fig. 2B). Under these conditions, the ANX1 transcript cleaved to 82% when 1 mM GlcN6P was included in the transcription reaction, and there was 12% cleavage in the reactions without GlcN6P (Fig. 2B). McCarthy et al. (in press) have recently demonstrated that TRIS buffer can substitute for GlcN6P in *glmS* ribozyme cleavage reactions. In the absence of GlcN6P, TRIS buffer supported cleavage activity, but it has a lower affinity than

FIGURE 1. (A) Secondary structure of the *glmS* ribozyme of *B. subtilis*, as defined by Winkler et al. (2004). The ribozyme core was defined from A-1 to C75 (enclosed region) (Winkler et al. 2004). Sequences with potential to form P3.1 are indicated. (B) Compilation of P3-P3.1 pseudoknots. Partial structures from 18 Gram-positive bacteria are shown. For sequences of high similarity, base changes for a second bacterial species are denoted in parentheses. G-U and potential either-or base pairs are shown with black dots instead of dashes. Abbreviations: Ban, *Bacillus anthracis*; Bce, *B. cereus*; Bha, *B. halodurans*; Cac, *Clostridium acetobutylicum*; Cpe, *C. perfringens*; Cte, *C. tetani*; Dha, *Desulfotobacterium hafniense*; Efa, *Enterococcus faecalis*; Gst, *Geobacillus stearothermophilus*; Lpl, *Lactobacillus plantarum*; Lin, *Listeria innocua*; Lmo, *L. monocytogenes*; Oih, *Oceanobacillus iheyensis*; Sau, *Staphylococcus aureus*; Sep, *S. epidermidis*; Tte, *Thermoanaerobacter tengcongensis*; Fnu, *Fusobacterium nucleatum*.

GlcN6P. TRIS-dependent cleavage could account for the 12% cleavage seen in our transcription reactions in the absence of GlcN6P. When precursor RNA was purified and tested, the ribozyme cleaved at a rate of $\sim 1 \text{ min}^{-1}$ in the presence of 1 mM GlcN6P and 2.5 or 5 mM MgCl_2 (Table 1). A 5'-end-labeled ribozyme generated a product, under self-cleavage conditions, that migrated in a sequencing gel with a mobility consistent with cleavage 5' of G1 (Fig. 2A; data not shown). Thus, the *B. anthracis* form of

the *glmS* ribozyme appears to share the essential features of self-cleavage activity and response to GlcN6P with the *B. subtilis* sequence (Winkler et al. 2004).

P3.1 duplex contributes to ribozyme activity

Base changes that would disrupt base pairing in P3.1 were introduced into 5' and 3' sides of that structural element. Activity of these mutant ribozymes (P3.1-5' and P3.1-3')

FIGURE 2. (A) Proposed secondary structure of the *glmS* ribozyme from *B. anthracis* with P3.1. Lowercase letters indicate sequence derived from the cloning vector (pTZ18U). This structure was modeled after the *B. subtilis* structure. The P2 and P3 alignments differ slightly from those proposed by Barrick et al. (2004) and Tucker and Breaker (2005). (B) Cleavage of ANX1 and P3 and P3.1 mutant ribozymes during transcription in the presence or absence of 1 mM GlcN6P. Products were separated on a denaturing polyacrylamide gel and the positions of the precursor and 3' product bands are indicated on the left. The 5' product is not visible. The percent cleaved in the presence and absence of GlcN6P is given below each lane. (C) Mutations in the P3-P3.1 pseudoknot. Mutated regions are bracketed and the modified sequence is shown to the right. Lowercase designates mutant bases.

(Fig. 2C) was evaluated in transcription reactions identical to those used to evaluate the wild-type ribozyme. Changes were introduced at four positions in either side of P3.1 to decrease the stability of the interaction. These mutations reduced the amount of cleavage in the presence of GlcN6P to 13% for the P3.1-5' ribozyme and to 30% for the P3.1-3' ribozyme (Fig. 2B). These data indicate that ribozyme cleavage activity was reduced with mutations in the sequences that are predicted to disrupt P3.1. To test whether the loss

of ribozyme activity was due to disruption of P3.1, the compensatory mutation was made and tested. Combining the 5' and 3' mutations restored the potential to form a base-paired P3.1 (P3.1-5'/3') with the identity of base pairs at the mutated positions reversed relative to the wild-type P3.1 (Fig. 2C). In the transcription-cleavage reaction, the ribozyme with the compensatory mutation in P3.1 cleaved to the same extent in GlcN6P (82%) as the wild-type ribozyme, ANX1. These data provide support for a model

TABLE 1. Rate constants for self-cleavage of the ANX1 and mutant ribozymes in varying $MgCl_2$ concentrations

Ribozyme	2.5 mM $MgCl_2$		5 mM $MgCl_2$		10 mM $MgCl_2$	
	k_{obs} (min^{-1})	F	k_{obs} (min^{-1})	F	k_{obs} (min^{-1})	$F(F_1/F_2)$
ANX1 (wt)	0.82 ± 0.1	0.84	0.99 ± 0.2	0.84	15 ± 3	0.28
P3.1-5'	0.0026 ± 0.0002	(1)	0.073 ± 0.002	0.70	1.1 ± 0.3	0.59
P3.1-3'	0.0088 ± 0.0005	0.76	0.172 ± 0.01	0.78	0.23 ± 0.04	0.70
P3.1-5'/3'	0.92 ± 0.05	0.78	1.6 ± 0.4	0.75	0.59 ± 0.03	0.75
					14 ± 3	0.33
					1.0 ± 0.1	0.49
P3-5'	0.00053 ± 0.0001	(1)	0.0052 ± 0.0001	(1)	0.02 ± 0.0045	0.79
P3-3'	0.00039 ± 0.0001	(1)	0.0032 ± 0.0008	0.73	0.0089 ± 0.0008	0.62
P3-5'/3'	0.42 ± 0.08	0.83	1.2 ± 0.4	0.76	16 ± 3	0.22
					0.82 ± 0.06	0.62

For reactions that followed a simple first-order rate equation, rate constants were determined by nonlinear curve fitting to the exponential form. For biphasic kinetics in 10 mM $MgCl_2$, rate constants were determined by fitting the data to an equation for the sum of two exponentials. F is the fraction of the total that cleaved with the given rate. For very slow reactions in which the endpoint was not defined, rates were determined using data points from the first 10% of the reaction fit to a single exponential assuming 100% cleavage ($F = 1$). Values given are the average of at least three independent determinations with the standard deviation from the mean. For the data shown, all reactions were carried out at 37°C with 1 mM GlcN6P. All of the ribozymes constructs cleaved slower in the absence of GlcN6P.

in which the two mutated regions physically interact and suggested that P3.1 was part of the extended ribozyme structure that enhances activity of the core region.

P3 and P3.1 form a pseudoknot required for ribozyme activity

To test the P3–P3.1 pseudoknot model, mutations were introduced into the 5′ and 3′ sides of P3 and the resulting ribozymes were characterized as previously described for the P3.1 mutants. Three nucleotide positions were changed in either side of P3 (Fig. 2C) to decrease the stability of the duplex. The extent of cleavage during transcription for the ribozyme containing the P3-5′ mutation was nearly undetectable ($\leq 2\%$) both in the presence and absence of GlcN6P. A small amount of cleavage (3.5%) was seen in GlcN6P with the ribozyme containing the P3-3′ mutation. To demonstrate that activity of the ribozyme was lost because of a disruption to P3, the compensatory mutation was constructed (P3-5′/3′; Fig. 2C) and tested for cleavage activity (Fig. 2B). The ribozyme with the P3-5′/3′ compensatory mutation again cleaved to 82% in GlcN6P. These findings are consistent with a P3 requirement for optimal ribozyme activity and, together with the data for P3.1, suggest that a pseudoknot that forms just 3′ to the ribozyme core can affect ribozyme activity.

The contribution of P3.1 to cleavage rates is most apparent at low Mg^{2+} concentration

To better characterize the contribution of the pseudoknot to cleavage activity, precursor RNA was isolated from transcription reactions without GlcN6P and used in kinetic studies to examine cleavage rates as a function of $[MgCl_2]$. Because the pseudoknot falls outside of the ribozyme core, it is unlikely to participate directly in catalysis or form part of the active site. It could, however, contribute to the structural stability of the ribozyme. If so, the sensitivity of ribozyme activity to the mutations in P3 and P3.1 may vary with Mg^{2+} concentration. At 37°C in 2.5 mM $MgCl_2$ and 1 mM GlcN6P, the ANX1 ribozyme reaction followed first-order kinetics (Fig. 3A), cleaving with a k_{obs} of 0.82 min^{-1} (Table 1). The rate constant for cleavage increased slightly ($k_{obs} = 0.99 \text{ min}^{-1}$) when $MgCl_2$ was raised to 5 mM. However, in 10 mM Mg^{2+} , biphasic kinetics (Fig. 3B) were observed. These data could be fit to the sum of two exponentials to generate a curve consistent with a fast-cleaving fraction ($\sim 28\%$, $k_{obs} = 15 \text{ min}^{-1}$) and a remaining, larger fraction ($\sim 59\%$), that cleaves at 1.1 min^{-1} (Table 1). The biphasic kinetics in high $[Mg^{2+}]$ suggested that cleavage chemistry can be fast, but taken together, the $[Mg^{2+}]$ data revealed that a step that occurs at about 1 min^{-1} was rate determining at the low to moderate Mg^{2+} concentrations.

The contribution of P3.1 to ribozyme activity, under some conditions, can be relatively large, but the effect was

FIGURE 3. Kinetics of wild-type and mutant ribozyme cleavage. (A) Cleavage of ribozymes ANX1 (squares), P3-5′/3′ (open diamonds), and P3.1-5′/3′ (closed diamonds) show first-order kinetics and generated similar rate constants, (0.82 , 0.42 , and 0.92 min^{-1} , respectively) in 1 mM GlcN6P and 2.5 mM $MgCl_2$. (B) Cleavage of ANX1 in 1 mM GlcN6P and 10 mM $MgCl_2$ (see Table 1). The data fit poorly to an equation for a first-order single exponential (dashed line) so it was fit to an equation for the sum of two exponentials (solid line). (C) Comparison of the fast-cleaving compensatory mutants, P3-5′/3′ (open diamonds) and P3.1-5′/3′ (closed diamonds) to the slower cleaving 5′ and 3′ mutants: P3-5′ (open circles), P3-3′ (open triangles), P3.1-5′ (closed circles), and P3.1-3′ (closed triangles) in 1 mM GlcN6P and 2.5 mM $MgCl_2$. The slopes give values within a factor of two of the rate constants reported in Table 1.

suppressed as the $MgCl_2$ concentration was raised. In 2.5 mM $MgCl_2$, the 5′ and 3′ mutations in P3.1 decreased the cleavage rate of the ribozyme 320- and 93-fold, respectively (Fig. 3C; Table 1). The ribozyme with the P3.1-5′/3′ compensatory mutation cleaved at a rate (0.92 min^{-1}) nearly identical to that of the wild-type ribozyme sequence (Fig. 3A; Table 1). This difference in rate constants was consis-

tent with the extents of cleavage seen in the transcriptions. In 5 mM MgCl₂, the magnitude of the effect of the 5' and 3' mutations in P3.1 was less than in 2.5 mM MgCl₂ and resulted in decreases in the rate constants by 14- and 6-fold, respectively. At 10 mM MgCl₂, the P3.1-5' and P3.1-3' mutant ribozymes cleaved at rates of 0.23 and 0.59 min⁻¹, respectively, which are only two- to fourfold slower than the 1.1 min⁻¹ rate of ANX1 (the slower phase) under these conditions. For the P3.1 compensatory mutant (P3.1-5'/3'), the biphasic kinetics of cleavage closely mimicked the kinetics of cleavage seen with the wild-type ribozyme in the higher [MgCl₂] reactions.

The effect of the disruptive mutations in P3 (P3-5' and P3-3') on ribozyme activity was larger than what was seen in P3.1. In 2.5 mM MgCl₂, the P3 mutants cleaved approximately 1500- and 2100-fold slower, respectively, than the ANX1 ribozyme (Fig. 3C; Table 1). Even as the MgCl₂ concentration was raised, cleavage rates for these mutants were substantially slower relatively than the wild type, suggesting that the mutations that disrupted P3 introduced a more deleterious effect than the mutations in P3.1. The P3-5'/3' compensatory mutation restored cleavage rates to near wild-type levels, although in 2.5 mM MgCl₂ those rates are approximately half the rate of the ANX1 ribozyme (Table 1).

CONCLUSION

Enhancement of ribozyme activity by extra-core elements that stabilize the active core structure appears to be a frequent feature of ribozymes. The core of the HDV ribozyme is stabilized by P2 (Perrotta and Been 1991), the hairpin ribozyme by a four-way junction (Walter et al. 1998), and the hammerhead ribozyme by the interaction of hairpin loops 1 and 2 (Khvorova et al. 2003). The P5abc subdomain of the *Tetrahymena* Group I intron ribozyme, although considered a peripheral element in the Group I introns, contributes to stability and activity (Engelhardt et al. 2000). This work provides support for a pseudoknot in the *glmS* ribozyme that falls outside the ribozyme core but enhances ribozyme cleavage activity in the presence of GlcN6P and low Mg²⁺ concentrations. This pseudoknot consists of the previously described P3 and an additional paired region, P3.1, that we describe here. The data suggest that the mutations in P3.1 had a smaller effect on activity than the mutations in P3. This observation could be explained by the relative proximity of P3 and P3.1 to the ribozyme core. The secondary structure is consistent with the potential for P3 stacking on the end of P2 (Figs. 1A, 2A). That interaction would be expected to stabilize P2 and the *glmS* ribozyme core. P3.1, on the other hand, may form a coaxial helix with P3 but would be less intimately associated with the core and, as a result, its contribution could be less. Additional studies are necessary to test this hypothesis and further account for the differing effects of mutation in P3 and P3.1. We also note that while P3.1 did

contribute to ribozyme activity, an additional role in regulation, as part of an expression platform, is possible.

MATERIALS AND METHODS

Enzymes and reagents

T7 RNA polymerase was purified by M. Puttaraju from an over-expressing clone provided by W. Studier (Davanloo et al. 1984). Oligonucleotides were ordered from Integrated DNA Technologies. Other enzymes and chemical supplies were purchased from commercial sources.

Plasmid construction

A plasmid containing the wild-type *B. anthracis glmS* ribozyme sequence (pANX1) was prepared by inserting a synthetic double-stranded DNA containing 161 nt of the *glmS* ribozyme sequence into the pTZ18U plasmid. This DNA duplex was constructed using three complementary pairs of oligonucleotides. The oligonucleotide pairs were annealed and ligated, producing a double-stranded DNA fragment with 5' EcoRI and 3' BamHI sticky ends. pTZ18U was digested with BamHI and EcoRI. The dsDNA *glmS* ribozyme sequence was inserted into the EcoRI/BamHI sites of the plasmid, pTZ18U, such that transcription with T7 RNA polymerase yields a transcript containing the 161-nt ANX1 sequence with 8- and 5-nt vector-derived sequences at the 5' and 3' ends, respectively. The DNA was transformed into *Escherichia coli* (JM83), and DNA from several colonies was isolated using a boiling lysis miniprep protocol modified from Holmes and Quigley (1981). Miniprep DNA was sequenced by primer extension with modified T7 DNA polymerase and dideoxynucleotides (Sanger et al. 1977; Tabor and Richardson 1987; Perrotta and Been 1992) to verify the presence of proper sequence. The miniprep DNA was retransformed and plasmid DNA was prepared from overnight cultures and purified by CsCl equilibrium density ultracentrifugation with ethidium bromide (Maniatis et al. 1982). Mutagenesis of regions in the P3 or P3.1 duplexes was performed by oligonucleotide-directed mutagenesis of a uracil-containing single-stranded form of the pANX1 as a template (Kunkel et al. 1987; Vieira and Messing 1987; Perrotta and Been 1991). Sequences were verified, and plasmid DNA for each mutant was prepared as described above.

Transcriptions

Plasmid DNA was linearized by cutting with BamHI endonuclease, and the DNA was purified by phenol/chloroform extraction and ethanol precipitation. Transcription reactions (20 µL, 15 min, 37°C) contained 40 mM TRIS-HCl (pH 7.5), 15 mM MgCl₂, 5 mM dithiothreitol, 2 mM spermidine, 1 mM each ATP, UTP, and GTP, 0.5 mM CTP, 2 µg DNA, 10 µCi [α -³²P]CTP, and 300 U T7 RNA polymerase. To test for GlcN6P-stimulated ribozyme activity in the transcriptions, GlcN6P was added at 1 mM. Reactions were terminated with an equal volume of formamide containing 50 mM EDTA, and reaction products were separated by electrophoresis on a 6% polyacrylamide gel containing 7 M urea. Extent of cleavage was quantified with a Phosphorimager (Molecular Dynamics). To isolate precursor RNA for kinetic studies, the transcription was

scaled up to 50 μ L. Following electrophoresis, precursor RNA was identified by autoradiography. RNA was eluted from a gel slice, recovered by ethanol precipitation, and stored in 10 mM TRIS-HCl (pH 7.5), 1 mM EDTA at -20°C .

Cleavage assays

Radiolabeled precursor RNA was heated at 95°C for 3 min in 10 mM TRIS-HCl (pH 7.5) and 1 mM EDTA and placed on ice. The RNA was then preincubated at 37°C for 1 min in the cleavage cocktail minus GlcN6P, and the cleavage reactions were started by addition of GlcN6P (37°C). For these reactions, GlcN6P was used at 1 mM. The K_d for GlcN6P with the *B. subtilis* *glmS* ribozyme is about 0.2 mM (Winkler et al. 2004), and therefore, 1 mM GlcN6P should be at or near saturation for these reactions. Final concentrations for the cleavage reactions were 50 mM TRIS-HCl (pH 7.5), 50 mM KCl, 1 mM GlcN6P, and MgCl_2 at 2.5, 5.0, or 10 mM, as specified. Radiolabeled RNA was used at trace levels without carrier RNA. Aliquots of the reactions were removed and stopped in two volumes of formamide containing 50 mM EDTA on ice. The samples were warmed to 95°C prior to electrophoresis on 6% polyacrylamide gels containing 7 M urea. The gel was dried and the fraction of precursor cleaved was quantified with a Phosphor-imager (Molecular Dynamics). In 2.5 and 5 mM Mg, the ribozymes cleaved with apparent first-order kinetics and data were fit to the equation $f_t = F \times (1 - e^{-kt})$, where f_t is the fraction cleaved at time t , and F is the fraction that cleaved with the first-order rate constant k . For slow reactions, rate constants were estimated from the slope of the initial 10%–20% of the reaction. In 10 mM MgCl_2 , the wild-type ribozyme and ribozymes with compensatory changes cleaved with biphasic kinetics. The rate constants were determined by fitting the data to the equation $f_t = F1 \times (1 - e^{-k_1t}) + F2 \times (1 - e^{-k_2t})$, where f_t is the fraction cleaved at time t , and $F1$ and $F2$ are fraction of the total that cleaved at rates of k_1 and k_2 , respectively.

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