Supplementary Materials

Bacterial Riboswitches and Ribozymes Potently Activate the Human Innate Immune Sensor PKR

Chelsea M. Hull[1], Ananya Anmangandla[1] and Philip C. Bevilacqua[1,2,*]

[1]*Department of Chemistry, Center for RNA Molecular Biology, The Pennsylvania State University, University Park, PA 16802, USA.*

[2]*Department of Biochemistry and Molecular Biology, Center for RNA Molecular Biology, The Pennsylvania State University, University Park, PA 16802, USA.*

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**Corresponding author*: Email: pcb5@psu.edu; Phone: (814) 863-3812; Fax: (814) 865-2927

Postal address: **104 Chemistry Building, University Park, PA 16802.**

Supplementary Methods

Design of RNAs

The control RNA, dsRNA-79, was prepared as previously described.[1] This is a perfect dsRNA of 79 bp that is a classical activator of PKR. The Vc2FL and Vc2 constructs were prepared by transcription off a PCR product from a plasmid (gift from Scott Strobel's lab). The *glmS* ribozyme was also prepared by transcription from a PCR product from a plasmid (gift from Michael Been's lab). The ribozyme cleaved nearly to completion during transcription. RNA products were purified by denaturing PAGE. The twister ribozyme constructs were made by transcription from a hemi-duplex template. The +5, +10 and +15 twister constructs designed have the natural nucleotides present in the *C. bolteae* genome. Twister RNAs were also purified by denaturing PAGE and found to cleave during transcription. All sequences are provided in the Supplementary Methods.

PCR primers, hemiduplex templates and RNA sequences

All Vc2 and Vc2FL transcripts were made by amplifying the region of interest by PCR and fusing a T7 promoter at the 5'-end. PCR primers for the Vc2 and Vc2FL transcripts are listed below, followed by the complete RNA sequences. G83 is colored red, T7 promoters are in bold, and an additional guanosine inserted to promote transcription is shown by a lowercase 'g'. In Vc2FL the italicized region corresponds to the Vc2 RNA sequence.

Vc2 TS primer: 5'**TAATACGACTCACTATA**GGAAAAATGTCACG
Vc2 BS primer: 5'GTATGCATTTTGCCATCGGTAACC

Vc2 RNA:
5'*GGAAAAAUGUCACGCACAGGGCAAACCAUUCGAAAGAGUGGGACGCAAAGCCUCCGGCCUAA ACCAGAAGACAUGGUAGGUAGCGGGGUUACCGAUGGCAAAAUGCAUAC*

Vc2FL TS primer: 5'**TAATACGACTCACTATA**gGAAAGCTTGTC

Vc2FL BS primer: 5'TTTTAATACTGGTTTATCCATGCTGTTAGTCTC

Vc2 FL RNA:
5'gGAAAGCUUGUCACAUAAAUAUGACAAAACAUAUGUAGUGCGAGUAUUAUUAGUAGGGCUCA
UUCUCACAUUUGAAAUAUCAGUAUGUGAGAAUGACCCAAGAAUGAUUAAUUUUUAUCA*GGAAAA*
AUGUCACGCACAGGGCAAACCAUUCGAAAGAGUGGGACGCAAAGCCUCCGGCCUAAACCAGAAG
*ACAUGGUAGGUAGCGGGGUUACCGAUGGCAAAAUGCAUAC*ACUUUGUUGACUCAUCAUUGACAC
UAUGAAUGCAUGCUUUUGCUAAUUUUCUCGGACCUGACUUGGUGGCGUAUGUAACAAUACUCCG
AGACUAACAGCAUGGAUAAACCAGUAUUAAAA

Vc2 and Vc2FL G83C sequences have the G highlighted above changed to a C using QuikChange (Agilent) and the following PCR primers,

G83C TS: 5'CCAGAAGACATGGTAGGTACCGGGGTTACCGATGGC
G83C BS: 5'GCCATCGGTAACCCCGGTACCTACCATGTCTTCTGG.

All sequences were confirmed by standard Sanger sequencing.

The cleaved *glmS* ribozyme was generated using the same PCR method described above. PCR primers and final RNA product are provided below. The top strand primer is ~100 nt upstream (site not shown) of the T7 promoter found in the plasmid, and the bottom strand primer abuts the very end of the *glmS* sequence. The asterisk indicates the cleavage site of the ribozyme.

glmS TS primer: 5'-TCACTCATTAGGCACCCCAG
glmS BS primer: 5'-TCTCTCATCACACTTTCACCTTTG

glmS RNA:
5'ggGAAUUCAUUGUAAAUUAUAGAA*GCGCCAGAACUACAAGUAGUGUAGUUGACGAGGUGGG
GUUUAUCGAGAUUUCGGCGGAUGACUCCCGGUUGUUCAUCACAACCGCAAGCUUUUACUUAAAU
CAUUAAGGUGACUUAGUGGACAAAGGUGAAAGUGUGAUGAGAGA

Twister ribozyme transcripts were made using hemiduplex templates as shown below. Templates are listed 5'- to 3' in which the product is the reverse complement to the template starting after the boldface T7 binding site. Bottom strand (BS) templates were annealed with a top-strand T7 promoter. The asterisk indicates the cleavage site of the ribozyme. Colors indicate flanking nucleotides that were added in from the *C. bolteae* gene in order to extend the constructs and correspond to those in Figure 5. Those nucleotides added upstream of the cleavage site were not

present in any of the experiments because we worked the self-cleaved form that populated during transcription.

Twister (T) BS template:
5'TTCCCACTCTGCATTGATCAGGGCTTGTGACCTGCACCGGCTATAGGCCGGTGGCTGCATT*
AGGAAGGTATAGTGAGTCGTATTAATTTC

T+5 BS template:
5'CTCCTTTCCCACTCTGCATTGATCAGGGCTTGTGACCTGCA
CCGGCTATAGGCCGGTGGCTGCATT*AGGAAGGGGGCGGTATAGTGAGTCGTATTAATTTC

T+10 BS template:
5'ATTTCCTCCTTTCCCACTCTGCATTGATCAGGGCTTGTGACCTGCACCGGCTATAGGCCGGT
GGCTGCATT*AGGAAGGGGCAGCTGGTATAGTGAGTCGTATTAATTTC

T+15 BS template:
5'CATGGATTTCCTCCTTTCCCACTCTGCATTGATCAGGGCTTGTGACCTGCACCGGCTATAGG
CCGGTGGCTGCATT*AGGAAGGGGCAGCTGCCCCTGGTATAGTGAGTCGTATTAATTTC

RNA products were in the cleaved form lacking the 5'-RNA tail and are listed below.

T RNA:
5'AAUGCAGCCACCGGCCUAUAGCCGGUGCAGGUCACAAGCCCUGAUCAAUGCAGAGUGGGAA

T+5 RNA:
5'AAUGCAGCCACCGGCCUAUAGCCGGUGCAGGUCACAAGCCCUGAUCAAUGCAGAGUGGGAAA
GGAG

T+10 RNA:
5'AAUGCAGCCACCGGCCUAUAGCCGGUGCAGGUCACAAGCCCUGAUCAAUGCAGAGUGGGAAA
GGAGGAAAU

T+15 RNA:
5'AAUGCAGCCACCGGCCUAUAGCCGGUGCAGGUCACAAGCCCUGAUCAAUGCAGAGUGGGAAA
GGAGGAAAUCCAUG

RNA preparation and purification

In vitro T7 transcription reactions of 0.5–1 mL were carried out on the templates described above. Reactions were submerged in a water bath at 37˚C for 2–4 h, quenched with equal volume of 2X formamide loading buffer (95% v/v formamide, 20 mM EDTA), and fractionated on a

10% denaturing PAGE gel. RNA bands were detected via UV-shadowing, cut out with a razor blade, and eluted overnight into 1x TEN_{250} [10 mM Tris (pH 7.5), 1 mM EDTA, 250 mM NaCl]. RNA was then ethanol precipitated and dissolved in 1x TE [10 mM Tris (pH 7.5), 1 mM EDTA], quantified by NanoDrop, and stored at –20˚C.

For PKR activation assays, the highest concentration of RNA tested was prepared in 1xTEN_{100} and renatured at 90˚C for 2 min followed by room temperature (~22˚C) for 10 min. When binding cdiGMP to these riboswitches, a 2-fold excess cyclic di-GMP (Axxora) was incubated with the riboswitch in the desired concentration of Mg^{2+} in folding buffer [10 mM sodium cacodylate (pH 6.8), 10 mM KCl, 4 mM (or 0.5 mM) $MgCl_2$] at 70˚C for 5 min followed by room temperature for 30 min. Twister and *glmS* ribozymes were renatured at 90˚C for 2 min followed by room temperature for 10 min before each experiment.

Protein expression and purification

Full-length PKR with an N-terminal $(His)_6$ tag was cloned into pET-28a and transformed into BL21 (DE3) Rosetta cells (Novagen).[2,3] Sonicated cells were passed over a Ni^{2+} -NTA agarose column (Qiagen) then eluted with imidazole gradient in the background of 750 mM NaCl on a Bio-Rad FPLC. Protein was dialyzed into storage buffer [10 mM Tris (pH 7.6), 50 mM KCl, 2 mM $Mg(OAc)_2$, 10% glycerol and 7 mM β-mercaptoethanol]. Concentration was determined by spectroscopy and the protein was aliquotted, flash-frozen, and stored at –80˚C.

PKR Activation Assays

RNAs were assessed for activation of unphosphorylated PKR. Upon purification from *E. coli*, PKR is phosphorylated. We dephosphorylated it by treatment with lambda protein phosphatase

at 30˚C for 1 h followed by inactivation of the phosphatase with freshly made 2 mM sodium orthovanadate.[3,4] Immediately after dephosphorylation, PKR, at a final concentration of 0.8 μM, was incubated with the RNA or RNA-ligand complex of interest at 30˚C for 10 min (normal conditions) or 20 min (for Mg^{2+} dependence). RNAs were diluted in $1xTEN_{100}$ at 4-fold dilutions. Reactions were performed in PKR activation buffer [20 mM HEPES (pH 7.5), 50 mM NaCl] and 1.5 mM DTT, 100 μM ATP (Ambion) plus 15 μCi [γ-^{32}P]-ATP. Magnesium chloride was added at either 4 or 0.5 mM Mg^{2+} and RNAs were incubated for 5 min at 30˚C to aid folding. Reactions were stopped with addition of SDS loading buffer and fractionated on a 10% Bis-Tris (Novex) SDS PAGE pre-cast gel, dried and exposed to a storage PhosphorImager screen (Molecular Dynamics). Phosphorylated PKR bands were detected with a PhosphorImager (Molecular Dynamics) and quantified using ImageQuant software. Each activation assay gel contains a no-RNA negative control, as well as a 0.1 μM dsRNA-79 positive control used for normalization.

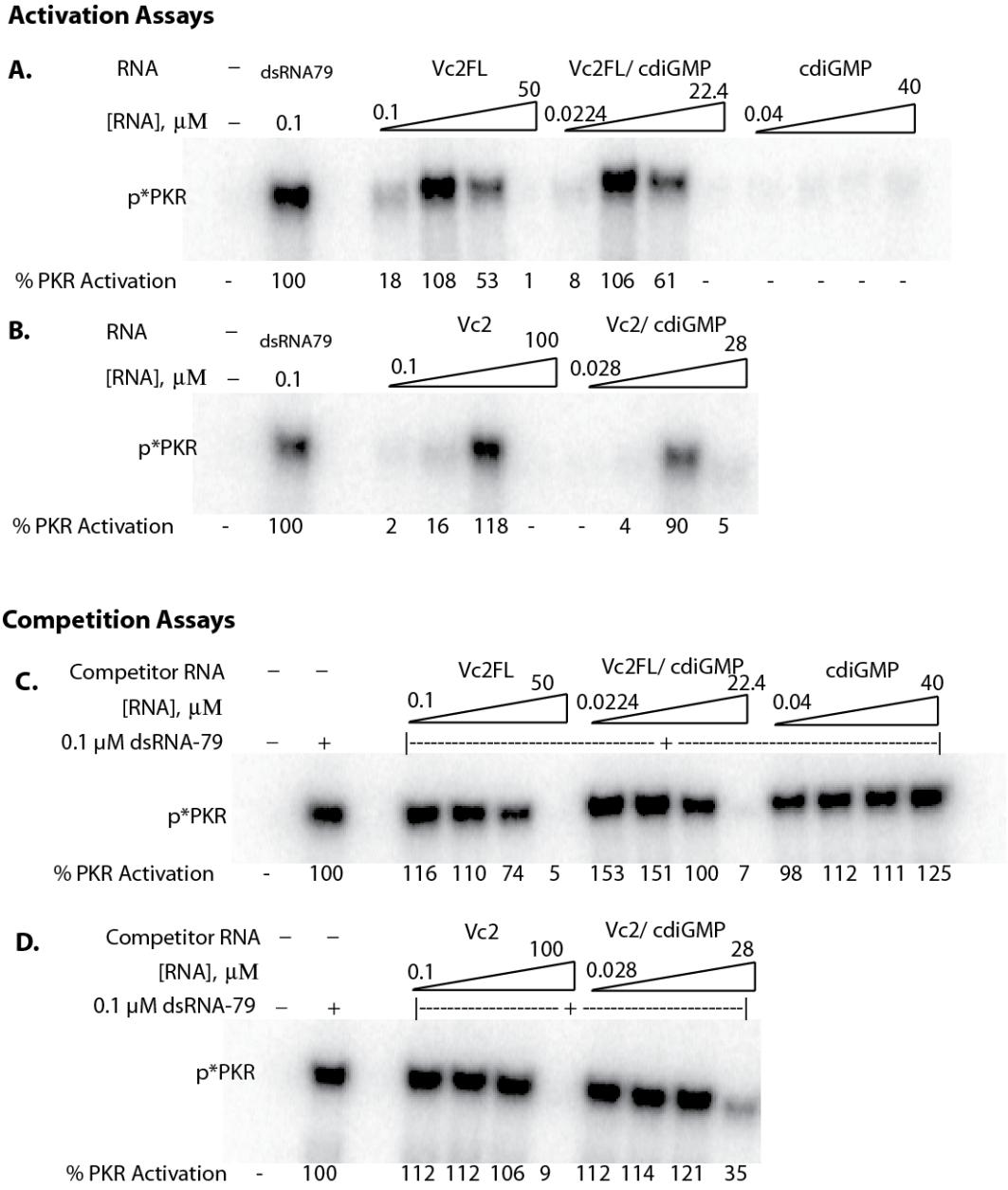
Structure Mapping and PKR Footprinting

RNAs were 5-end labeled with [γ-^{32}P]-ATP using T4 PNK (NEB) and purified by denaturing PAGE. Labeled RNAs were renatured in $1xTEN_{100}$ at 90˚C for 2 min and room temperature for 10 min and if applicable, pre-incubated in binding buffer with 8 μM cdiGMP ligand at 70˚C for 5 min followed by room temperature for 30 min. To minimize background, nonspecific RNA ($tRNA^{phe}$) and protein (BSA) were added to the reactions at final concentrations of 130 ng and 1.5 μM respectively. RNA was subjected to limited digestion by single-stranded specific nucleases (RNase T1 or RNase A) or a double-stranded specific nuclease (RNase V1) under native conditions in structure buffer [20 mM HEPES (pH 7), 100 mM NaCl, and 4 mM $MgCl_2$].

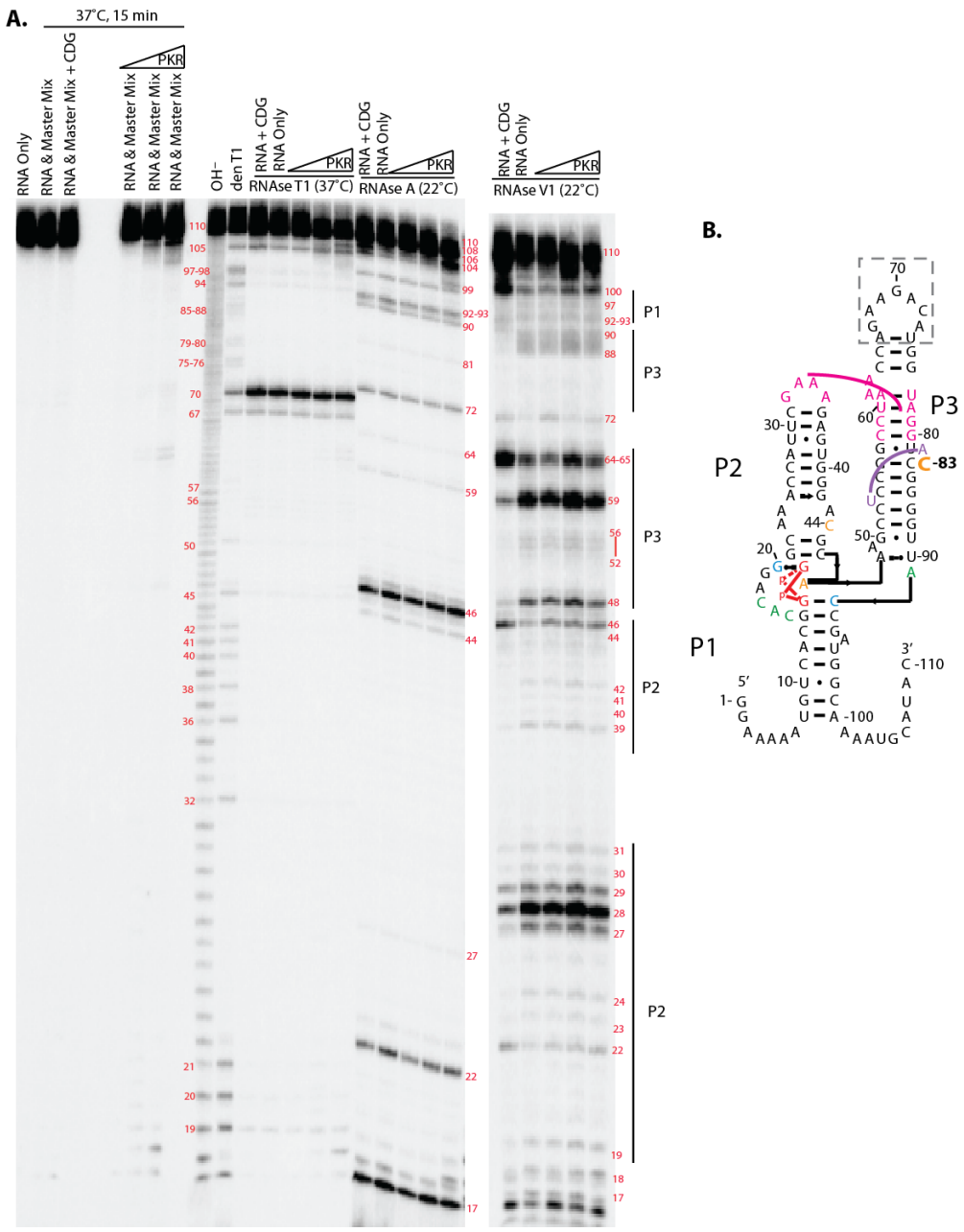
Conditions that gave single-hit digestion were 0.001 U μL^{-1} RNase T1 (Ambion), 37˚C, 15 min; 0.005 ng μL^{-1} RNase A (Ambion), room temperature, 15 min; and 0.001 U μL^{-1} RNase V1 (Ambion), room temperature, 15 min.

To generate a hydrolysis ladder, labeled RNA was incubated in 100 mM $Na_2CO_3/NaHCO_3$ and 2 mM EDTA for 8 min at 90˚C. To generate a T1 ladder (=all Gs), labeled RNA was incubated under denaturing conditions in 0.1 U μL^{-1} RNase T1, 18 mM Na-citrate (pH 3.5), 0.9 mM EDTA, and 6 M urea for 20 min at 50˚C. For PKR footprinting, the RNA and master mix [1x TEN_{100}, 130 ng $tRNA^{phe}$, 1.5 μM BSA, 4 mM Mg^{2+}] was incubated with increasing concentrations of PKR (0.44, 1.75, and 7 μM) for 30 min at room temperature prior to nuclease digestions. Controls were performed on structure mapping and footprinting experiments by incubating the RNA with each component of the reaction in the absence of nuclease at 37˚C for 15 min. All samples were quenched with 2x EDTA/formamide/0.2% SDS loading buffer (pH 11) and loaded onto a 12% polyacrylamide, 8.3 M urea sequencing gel, dried and exposed to a storage PhosphorImager screen overnight.

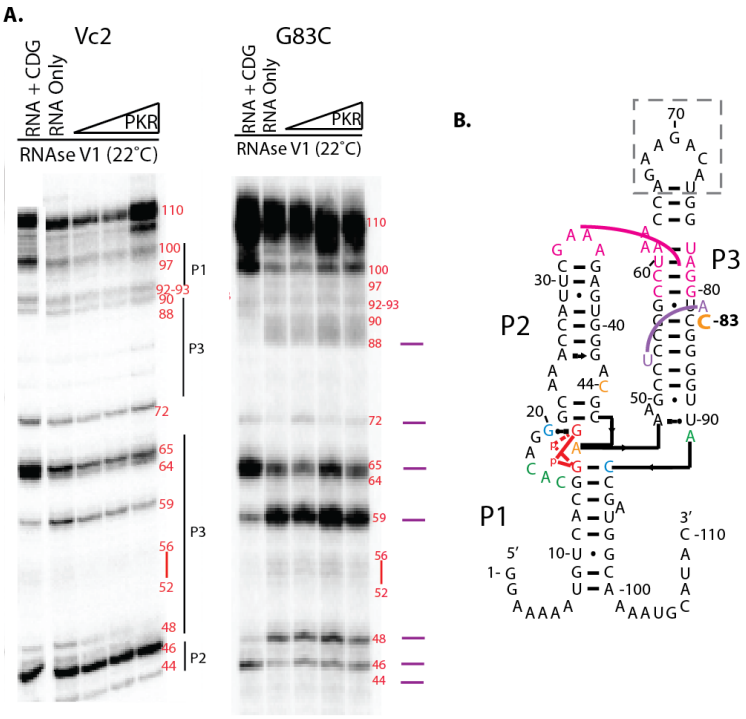
Supplementary Table 1: Excel file of activation assay data available online.



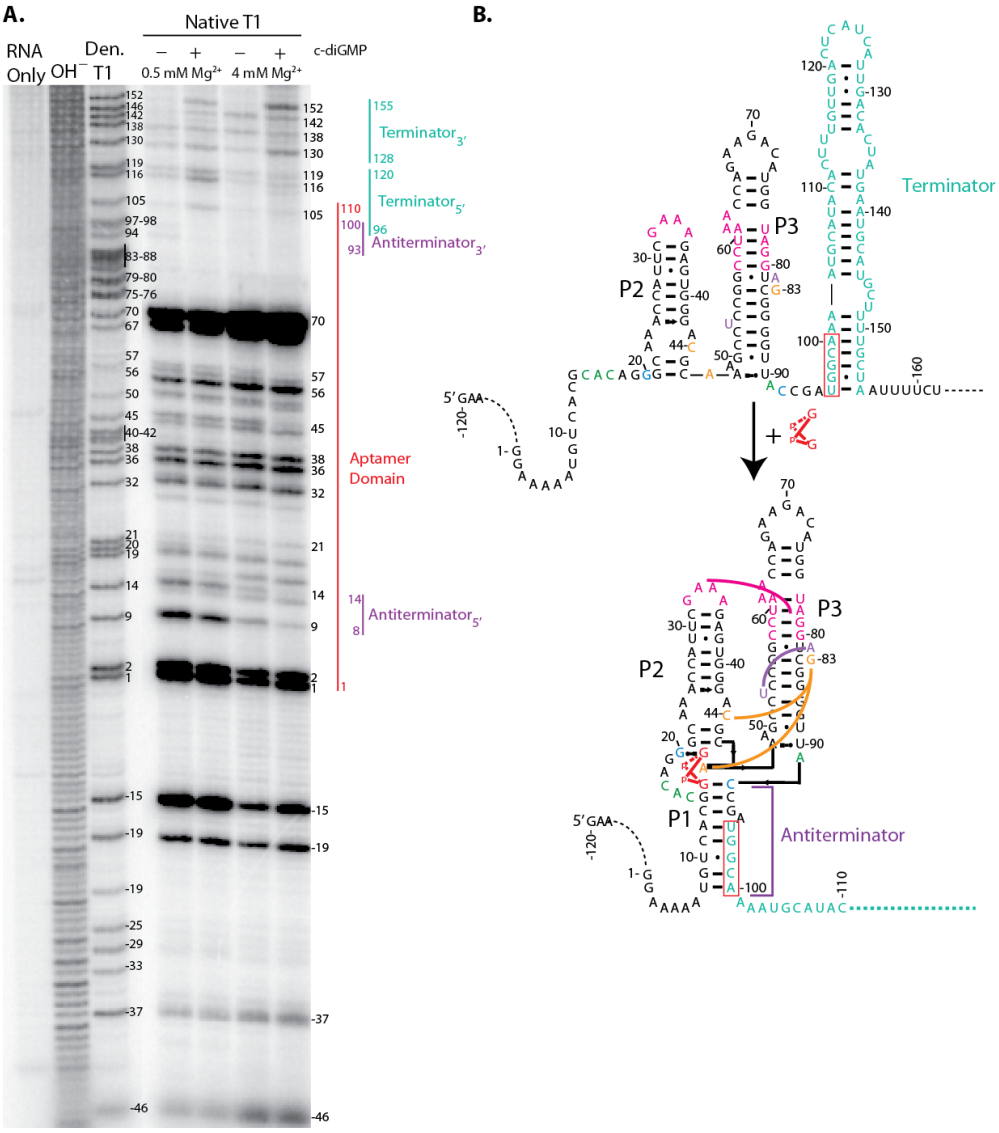
Supplemental Figure 1 Additional activation and competition assays for Vc2 and Vc2FL. a) Activation of PKR by Vc2FL in the absence and presence of cdiGMP, as well as cdiGMP alone. b) Activation of PKR by Vc2 in the absence and presence of cdiGMP. c) Competition for dsRNA-79-mediated activation of PKR by Vc2FL in the absence and presence of cdiGMP, as well as cdiGMP alone. d) Competition for dsRNA-79-mediated activation of PKR by Vc2 in the absence and presence of cdiGMP. Bands indicate phosphorylated PKR, 'p*PKR'. Percent PKR activation was normalized to that of 0.1 μM dsRNA-79. Shown are SDS-PAGE gels and all experiments were in 4 mM Mg^{2+}.



Supplemental Figure 2 Structure mapping and PKR footprinting of the Vc2 G83C mutant riboswitch. a) 5'-end labeled RNA at 4 mM Mg^{2+} was incubated alone or in the presence of cdiGMP or PKR, subjected to limited nuclease digestion, and fractionated by 12% denaturing PAGE. Leftmost lanes are controls performed in the absence of nuclease; underlined lanes were incubated at 37˚C for 15 min to simulate the RNase activity (note some lanes were skipped due to defects in the gel). 'OH-' is a limited alkyline hydrolysis ladder and 'den T1' is RNAse T1 in denaturing conditions. The right side of the gel displays RNase digestion in native conditions for RNases T1, A and V1 for 15 min at the temperature noted. The following were probed: Vc2 G83C RNA bound to cdiGMP ('RNA+CDG'), RNA-only, and RNA with PKR titrated from 0.44 to 7 µM. b) Secondary structure of Vc2 with G83C labeled and tertiary interactions that involved this nucleotide removed.



Supplemental Figure 3 Vc2 G83C structure mapping comparison to WT Vc2. a) Excerpts from the RNase V1 lanes in Figure 6 in the main text (left side of the above gel) and Supplemental Figure 2 (right side of the above gel) for direct comparison of nucleotides that have changes in cleavage between WT Vc2 and G83C Vc2, marked to the right of the gel with purple lines. Comparison between RNA+CDG and RNA-only lanes in G83C at nucleotides C46, A48, C59, C64, and C65 support ligand binding to G83C. Positions A48 and C59, which are at the docked interface, have enhanced cleavages in G83C. Cleavage comparisons at these nucleotides (and others) reveal footprinting of PKR in WT but not G83C. This suggests that PKR has many weak binding sites on the undocked Vc2 G83C structure, allowing RNase V1 to cleave double-stranded regions. b) Secondary structure of Vc2 with G83C labeled and tertiary interactions that involved this nucleotide removed. (Same as Supplemental Figure 2B, provided here for convenience.)



Supplemental Figure 4 Structure mapping of the Vc2FL riboswitch and prediction of the terminator and antiterminator. a) RNA was 5'-end labeled, incubated alone or in the presence of cdiGMP, subjected to limited RNase T1 digestion, and fractionated by 12% denaturing PAGE. Lanes are as follows: RNA-only, hydrolysis ladder (OH^-), denaturing RNase T1 ladder ('Den T1'), and Native T1 mapping. The right side of the gel displays RNase T1 digestion under native conditions at 0.5 or 4 mM Mg^{2+} in the absence or presence of cdiGMP. Vc2FL is a 350 nt 5'-UTR containing the Vc2 aptamer and expression domains. The numbering scheme includes the aptamer as 1–110 and nucleotides in the UTR flanking this domain are labeled as numbers below or above this range. Cleavages of interest are labeled with the corresponding nucleotides and labels at the right of the gel denote the aptamer domain and predicted antiterminator and terminator stem-loops. b) Model for switching between the antiterminator and terminator forms. Formation of the antiterminator prevents formation of the overlapping terminator (red box), which gives transcription readthrough. This model is supported by gain of RNase T1 cleavage at G152 in the presence of c-diGMP (panel a) and is consistent with a model proposed for the cdiGMP riboswitch in *Clostridium difficile*.[5]

Supplementary References

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