Riboswitches in Eubacteria Sense the Second Messenger Cyclic Di-GMP

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Cyclic di-guanosine monophosphate (di-GMP) is a circular RNA dinucleotide that functions as a second messenger in diverse species of bacteria to trigger wide-ranging physiological changes, including cell differentiation, conversion between motile and biofilm lifestyles, and virulence gene expression. However, the mechanisms by which cyclic di-GMP regulates gene expression have remained a mystery. We found that cyclic di-GMP in many bacterial species is sensed by a riboswitch class in messenger RNA that controls the expression of genes involved in numerous fundamental cellular processes. A variety of cyclic di-GMP regulons are revealed, including some riboswitches associated with virulence gene expression, pilus formation, and flagellum biosynthesis. In addition, sequences matching the consensus for cyclic di-GMP riboswitches are present in the genome of a bacteriophage.

The second messenger cyclic di-GMP (1–4) (fig. S1) is formed from two guanosine-5’-triphosphate molecules by diguanylate cyclase (DGC) enzymes. Once formed, the compound is degraded selectively by phosphodiesterase (PDE) enzymes that contain either EAL or HD-GYP amino acid domains (5). The activities of these synthesis and degradation enzymes are triggered by various stimuli and modulate cellular cyclic di-GMP concentrations (4) to signal physiological changes. Cyclic di-GMP can be bound by some DGC proteins to allosterically repress its own synthesis (6, 7). The only other protein targets known are Gluconacetobacter xylinus cellulose synthase (8, 9), Pseudomonas aeruginosa PelD protein (10), and PilZ domain proteins (11). However, cyclic di-GMP binding presumably affects only the pathways in which these proteins participate, and therefore these interactions cannot fully explain its global cellular effects (3, 12).

It has been hypothesized (3) that the existence of cyclic di-GMP riboswitches could explain how...
A cyclic di-GMP aptamer from *V. cholerae*. (A) Sequence and structure of the Vc2 RNA from *V. cholerae* chromosome 2, and its proximity to the ORF of VC1722. Nucleotides shown correspond to the 110 Vc2 RNA construct. Bold numbers identify regions of ligand-mediated structure modulation as observed in (B). Brackets identify the minimal 5’ or 3’ terminus (when the opposing terminus for 110 Vc2 RNA is retained) that exhibits structural modulation when tested with 10 nM cyclic di-GMP. Nucleotides in shaded boxes were mutated for studies depicted in Fig. 2A. Predicted G and U wobble pairs are identified with dots. (B) Polyacrylamide gel electrophoresis of RNA products generated by in-line probing of 5′-32P-labeled 110 Vc2 RNA. NR, no reaction; T1, partial digest with RNase T1; –OH, partial digest with alkali. RNA was incubated in the absence (−) or presence (+) of 100 μM cyclic di-GMP. (C) Plot of the normalized fraction of 110 Vc2 aptamer cleaved versus cyclic di-GMP concentration. Sites of structural modulation are as depicted in (B). (D) Comparison of *K*D values exhibited by 110 Vc2 aptamer for cyclic di-GMP (Fig. S5) and various analogs. G, guanosine; pG, pGpG, and pGPa, 5′ phosphorylated mono- and dinucleotides; GpGpG, trinucleotide; AMP and GMP, adenosine and guanosine monophosphate, respectively.

Fig. 2. Representative cyclic di-GMP aptamers are components of gene control elements. (A) Reporter fusion constructs carry wild-type (WT) or mutant (M1 through M3) riboswitches from *V. cholerae* (Vc2) in *E. coli*, or carry the equivalent WT and M3 riboswitches from *B. cereus* (Bc1 and Bc2) or *C. difficile* (Cd1) (fig. S6) in *B. subtilis*. (B) β-Galactosidase reporter gene assays for reporter fusion constructs described in (A). Maximum Miller units measured for the four aptamer representatives were 436, 47, 5, and 51, respectively. (C) *B. subtilis* cells carrying a β-galactosidase reporter construct fused to a wild-type Cd1 riboswitch and transformed with a plasmid lacking or carrying a normal or mutant (E170A) *V. cholerae* vieA gene encoding an EAL PDE. Plate diameter, 85 mm.

This second messenger controls the transcription and translation of many genes. Riboswitches are mRNA domains that control gene expression in response to changing concentrations of their target ligand (13, 14). We have discovered a highly conserved RNA domain called GEMM (15) residing upstream of the open reading frames (ORFs) for DGC and PDE proteins in some organisms and residing upstream of some genes that are controlled by cyclic di-GMP. The high conservation and genomic distributions of GEMM RNAs are characteristic of riboswitches.

Most GEMM RNAs conform to one of two similar architectures termed type 1 and type 2 (fig. S2) that are distinguished by the presence of specific tetraloop and tetraloop receptor sequences (5). Both types carry two base-paired regions (P1 and P2) that exhibit extensive covariation in the 503 representatives identified (15). The genome of the pathogenic bacterium *Vibrio cholerae* carries two sequences for type 1 GEMM RNAs (fig. S3). One (Vc1) resides upstream of the gbpA gene, and a second (Vc2) resides upstream of a gene (*VC1722*) homologous to tf06x of *V. cholerae* (15, 16).

Biochemical and genetic analyses were carried out with both *V. cholerae* GEMM RNAs to determine whether they function as aptamers for cyclic di-GMP. A 110-nucleotide Vc2 RNA construct (110 Vc2) (Fig. 1A) was subjected to in-line probing (17) in the absence or presence of 100 μM cyclic di-GMP (Fig. 1B). The changing patterns of spontaneous cleavage at the base of stems P1 and P2 suggest that the conserved nucleotides near the regions undergoing structural modulation (labeled 1 through 3) are important for ligand binding. Similar results were obtained for a construct encompassing the Vc1 RNA and for representative cyclic di-GMP aptamers from *Bacillus cereus* and *Clostridium difficile*. Despite the constrained structure of the second messenger, cyclic di-GMP exhibited a half-life for spontaneous degradation of no poorer than ~150 days under in-line probing conditions (fig. S4). Therefore, the biologically relevant ligand for this aptamer class most likely is cyclic di-GMP and not one of the breakdown products of this second messenger.

The 110 Vc2 RNA appeared to form a one- to-one saturable complex with a dissociation constant (*K*D) of ~1 nM for cyclic di-GMP (Fig. 1C and fig. S5). This interaction was nearly three orders of magnitude tighter than the affinity measured for cyclic di-GMP binding by HD-GYP PDEs was not bound by the
aptamer even at a concentration five orders of magnitude higher than the $K_D$ for cyclic di-GMP.

Bacterial riboswitches typically carry a conserved aptamer located immediately upstream of a less well-conserved expression platform. Most expression platforms in bacteria form structures that control transcription termination or translation initiation (18). Similar architectures are associated with many GEMM RNA representatives (5), which suggests that cyclic di-GMP aptamers are likely to be components of riboswitches. Four $5'$ untranslated regions from *V. cholerae*, B. cereus, and *C. difficile* were examined for riboswitch function in cells. DNAs encompassing *Vc2*, its predicted native promoter, and the adjoining expression platform were used to prepare translational fusions with the *E. coli lacZ* reporter gene. Transformed *E. coli* cells carrying the aptamer-reporter fusion constructs were grown in liquid medium and assayed for $\beta$-galactosidase activity.

The reporter construct carrying the wild-type *Vc2* aptamer (Figs. 1A and 2A) exhibited a high level of gene expression (Fig. 2B). In contrast, constructs M1 and M3 that carried mutations disrupting P1 or altering an otherwise strictly conserved nucleotide in the P2 bulge expressed the reporter gene at less than 10% of the wild-type level. Furthermore, an aptamer that carried four compensatory mutations (M2) that restore the structure of P1 exhibited near–wild-type gene expression. These results parallel the ligand-binding activities of these RNAs and suggest that the riboswitch integrating the *Vc2* aptamer functions as an “on” switch. Similarly, transcriptional fusions were prepared for cyclic di-GMP riboswitches from *B. cereus* (Bc1 and Bc2) and *C. difficile* (Cd1) (fig. S6) and cloned into *B. subtilis*. The expression patterns for wild-type and equivalent M3 variants were consistent with “on” switch function for Bc1 and “off” switch function for Bc2 and Cd1 (Fig. 2B).

Cyclic di-GMP riboswitch modulation of gene expression was established by examining “off” switch action of the Cd1 RNA. The cellular concentration of the second messenger was manipulated by expression of *V. cholerae* VieA, an EAL PDE that is expected to lower the cellular concentration of cyclic di-GMP (19). This is consistent with our data indicating that the *Vc2* riboswitch associated with the *FC1722* mRNA is a genetic “on” switch that yields higher gene expression when cyclic di-GMP concentrations are elevated (Fig. 2C).

The remaining *V. cholerae* cyclic di-GMP riboswitches (*Vc1*; fig. S3) is associated with the *gpbA* gene, which codes for a sugar-binding protein reported to be the key determinant permitting the bacterium to colonize mammalian intestines, leading to cholera disease (20). It has been shown that *V. cholerae* lowers its cyclic di-GMP levels when colonizing mammalian host intestines (19). The *V. cholerae* vieA gene used in our study to demonstrate Cd1 riboswitch response to changing cyclic di-GMP levels (Fig. 2C) is known to be essential for bacterial infection. Thus, it is possible that a reduction in cyclic di-GMP levels brought about by the action of VieA is sensed by the *Vc1* riboswitch to facilitate expression of GbpA and *V. cholerae* infection.

Some organisms have a strikingly high number of cyclic di-GMP riboswitch representatives (fig. S3) (5). *Geobacter uranireducens*, with 30 representatives identified, has the largest number of cyclic di-GMP aptamer RNAs among bacterial species whose genomes have been sequenced. These are distributed upstream of 25 different transcriptional units, with five RNAs carrying tandem cyclic di-GMP aptamers. Also intriguing is the identification of cyclic di-GMP riboswitch representatives residing within *PhicD119* bacteriophage DNA that is integrated within the *C. difficile* genome. The riboswitch sequence, located within the lysis module of the bacteriophage genome, is also evident in DNA packaged into bacteriophage particles (23). Although more than 20 metabolite-sensing riboswitch classes have been reported, and thousands of representatives of these classes have been identified, the cyclic di-GMP–binding RNAs are the only examples of bacteriophage-associated riboswitches found after exhaustive bioinformatics searches. Perhaps viruses have little need for sensing fundamental metabolic products, but might gain an evolutionary advantage by monitoring the physiologic transformations of bacterial cells brought about by changing concentrations of the second messenger cyclic di-GMP.

References and Notes

5. See supporting material on Science Online.
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