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Riboswitches as versatile gene control elements

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Riboswitches are structured elements typically found in the 5′ untranslated regions of mRNAs, where they regulate gene expression by binding to small metabolites. In all examples studied to date, these RNA control elements do not require the involvement of protein factors for metabolite binding. Riboswitches appear to be pervasive in eubacteria, suggesting that this form of regulation is an important mechanism by which metabolic genes are controlled. Recently discovered riboswitch classes have surprisingly complex mechanisms for regulating gene expression and new high-resolution structural models of these RNAs provide insight into the molecular details of metabolite recognition by natural RNA aptamers.

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Introduction

From microRNAs [1] to 'riboregulators' [2], one of the more salient concepts to have emerged from gene regulation research over the past several years is that RNA frequently plays a more direct and intimate role in controlling gene expression than previously assumed [3,4]. It has long been known that differential folding of RNA plays a major role in transcriptional attenuation [5]. Other RNA-based regulatory mechanisms have subsequently been discovered, including pathways involving antisense [6] and tRNA–mRNA interactions [7], control of translation by temperature-dependent modulation of RNA structure [8–11] and the involvement of microRNAs as *trans*-acting genetic factors [1]. The frequency at which these discoveries have been occurring suggests an even greater role for RNA in cellular control processes. This already appears to be true of bacteria, as recent descriptions of gene control by riboswitches are revealing a pervasive system of RNA-mediated gene control [12–16].

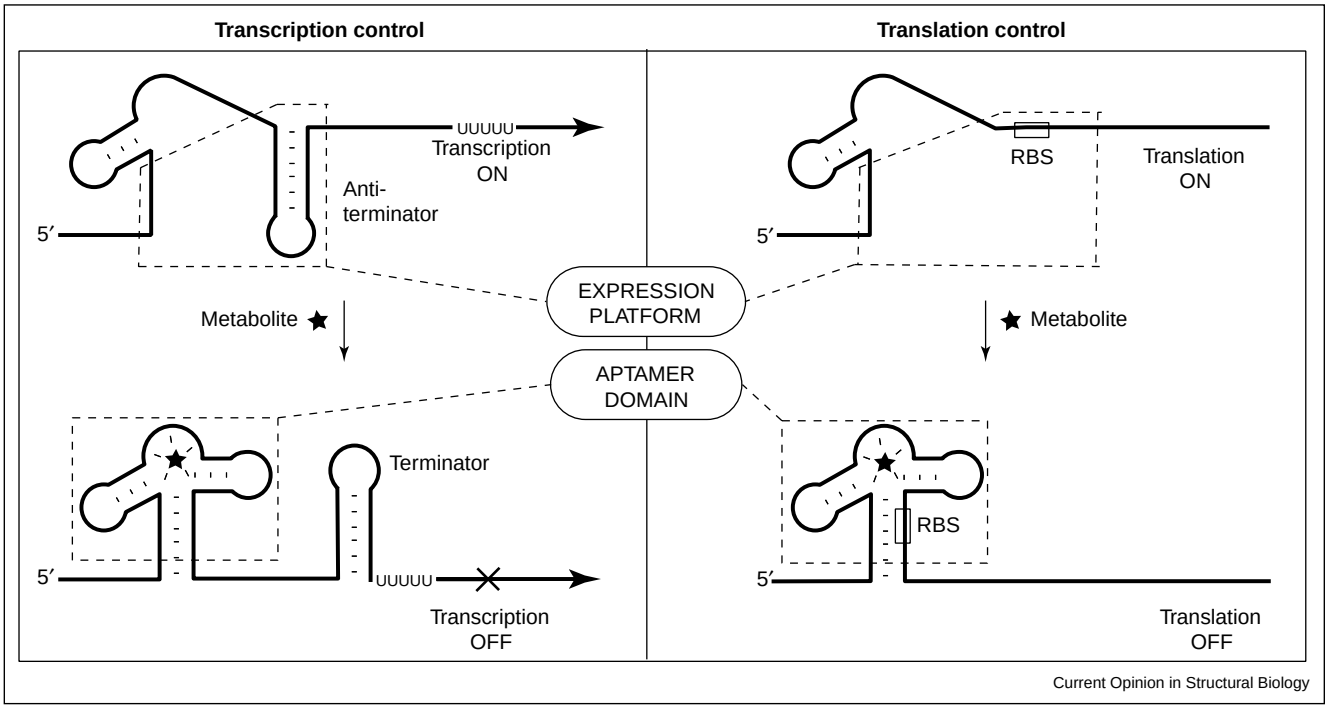
Riboswitches are widespread in bacteria, with nine classes already reported, some of which describe previously known conserved regulatory elements (e.g. [17–19]). The metabolites sensed by riboswitches are diverse, among them guanine [20], flavin mononucleotide (FMN) [21,22] and lysine [23•–25•]. One riboswitch class that binds thiamine pyrophosphate (TPP) [26] also has been found in plants and fungi [27•]. However, riboswitches that occur in eubacteria have been most extensively studied and this review will primarily focus on recent advances concerning bacterial representatives. For a more comprehensive overview of this research area, the reader is directed to several recent reviews [13–15,28–30].

Structural and functional domains of riboswitches

Most riboswitches can be divided roughly into two structural domains: an aptamer [31,32] and an expression platform (Figure 1) [26]. The aptamer domain is a highly folded structure that selectively binds to the target metabolite. The expression platform converts metabolite binding events into changes in gene expression by harnessing changes in RNA folding that are brought about by ligand binding. It is the distinctive sequence and structural features of each aptamer that are used to classify each new type of riboswitch. These metabolite-binding domains are conserved amongst widely divergent organisms, indicating that they have long persisted through evolution despite the fact that protein factors should have been strong competition for billions of years. In contrast, the sequences and structures that comprise each expression platform vary considerably among different riboswitch classes and also among representatives of the same riboswitch class, even when the regulatory mechanisms employed are similar [25•,26,33].

The genes controlled by riboswitches often encode proteins involved in the biosynthesis or transport of the metabolite being sensed [30]. Therefore, the riboswitch is used as a form of feedback inhibition, whereby binding of the metabolite to the riboswitch decreases the expression of the gene products used to make the metabolite. In most instances, repression is accomplished either by terminating transcription to prevent the production of full-length mRNAs or by preventing translation initiation once a full-length mRNA has been made. For transcription control, an anti-terminator structure is formed in the absence of a bound metabolite. When a metabolite binds, a competing stem structure is formed that serves as an intrinsic transcription terminator [22,34,35]. For control of translation initiation, the bound aptamer precludes translation by rendering inaccessible the ribosome-

Figure 1



Common mechanisms of riboswitch gene control. Transcription control involves metabolite binding and stabilization of a specific conformation of the aptamer domain that precludes formation of a competing anti-terminator stem. This allows formation of a terminator stem, which prevents the full-length mRNA from being synthesized. In contrast, control of translation is accomplished by metabolite-induced structural changes that sequester the ribosome-binding site (RBS), thereby preventing the ribosome from binding to the mRNA.

binding site or Shine–Dalgarno sequence (Figure 1) [26]. This mechanism is consistent with the observation that ribosomes do not bind to an mRNA carrying a coenzyme B_{12} riboswitch that controls gene expression at the level of translation [18].

The above descriptions of riboswitch architecture and function had represented the extent of what was known about the gene control mechanisms of riboswitches. However, research over the past year has revealed new riboswitch classes and some new mechanisms by which metabolite binding leads to changes in gene expression. Newly discovered riboswitches have deviated, in some cases surprisingly, from the more common regulatory mechanisms described previously. Moreover, new X-ray crystal structures have given us a first glimpse at the molecular details of riboswitches that, until recently, could only be inferred from biochemical data.

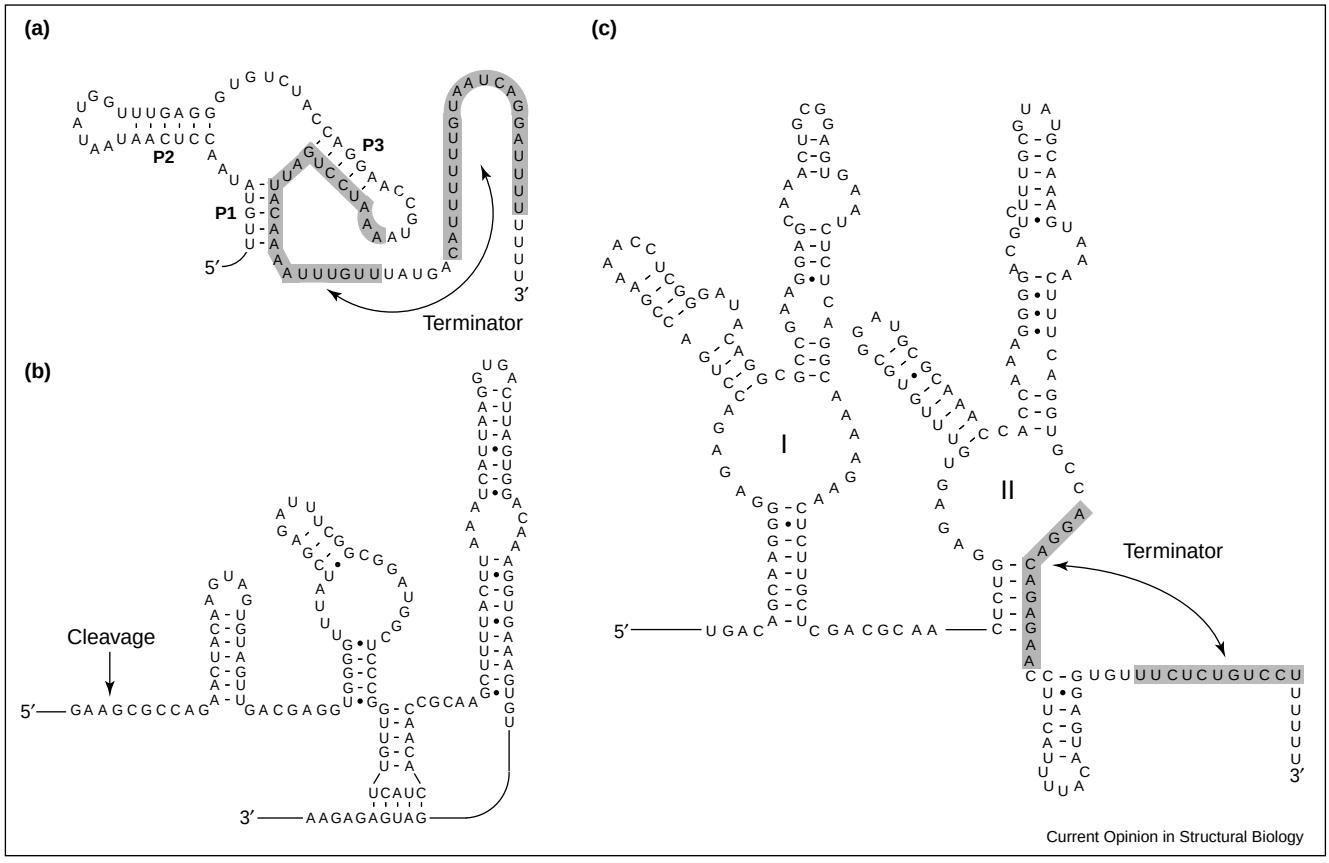
New riboswitch classes

In the past year, three newly confirmed riboswitch classes have been reported (Figure 2). In all three cases, differences are observed compared to the prototypic riboswitch mechanisms described above. The first of these, an adenine-sensing riboswitch, is remarkably similar to a previously reported riboswitch that binds guanine [20,36•,37]. The guanine riboswitch had been shown to repress purine biosynthetic and salvage genes upon binding directly to guanine [20,37]. However, certain RNA elements that conform almost perfectly to the consensus sequence and structure of guanine riboswitches were found in front of genes that were defined as coding for adenine deaminase enzymes or identified as encoding a purine efflux pump [37]. Additionally, these representative RNA elements were distinguished by the presence of a uracil residue in place of an otherwise strictly conserved cytosine residue.

This led to speculation that this single base change might alter ligand specificity. Specifically, if the cytosine (corresponding to position 74 in constructs described below) forms a Watson–Crick base pair with guanine, then mutation to a uracil would change the ligand specificity to adenine. As predicted, representative riboswitches that carry this C→U mutation are uniquely specific for adenine [36•,37].

Furthermore, these riboswitches activate the expression of a reporter gene *in vivo* upon the addition of adenine to cultured bacterial cells [36•]. In this case, it is believed that the transcription terminator stem is allowed to form only when the ligand-receptive fold of the aptamer is not

Figure 2



Recently characterized riboswitch classes. **(a)** Adenine riboswitch (*ydhL*). In contrast to previously characterized riboswitches, binding of adenine promotes mRNA transcription by preventing formation of a terminator stem. **(b)** GlcN6P riboswitch (*glmS*). Binding of GlcN6P induces the riboswitch to self-cleave (site identified by arrow). RNA cleavage results in decreased gene expression through an unknown mechanism. **(c)** Glycine riboswitch (*gcvT*). Binding of glycine allows mRNA transcription using a mechanism similar to that of the adenine riboswitch. However, two glycine molecules are bound cooperatively to two aptamer domains (labeled I and II). The sequence that forms the terminator stem is shaded.

formed (Figure 2a). This example of gene activation upon riboswitch–metabolite complex formation is quite rare. With all previously studied riboswitch mechanisms that control transcription termination, the terminator stem forms only when the aptamer is stabilized by metabolite binding [12]. Regardless of whether a riboswitch activates or deactivates gene expression upon ligand binding, they harness the same types of RNA folding changes. Therefore, the strong bias in favor of genetic 'OFF' switches is not due to any inherent limitations of RNA. Rather, this distribution of mechanisms most likely reflects the greater need that cells have for repressing metabolic gene expression when specific metabolites are in abundance.

Additional riboswitch classes have been identified by a bioinformatics search using intergenic sequences from the genome of *Bacillus subtilis* [38]. The sequence of each intergenic region (IGR) from *B. subtilis* was compared to the IGR sequences of 90 other bacteria to identify highly conserved RNA elements. Eight RNA motifs with at least some characteristics that are suggestive of riboswitch function were identified. Of these candidates, two have subsequently been shown to be riboswitches. One of these riboswitches always occurs adjacent to the *glmS* gene, which encodes the enzyme glutamine-fructose-6-phosphate amidotransferase. This enzyme produces glucosamine-6-phosphate (GlcN6P) and it is this compound that triggers riboswitch function. Interestingly, members of the GlcN6P class of riboswitch are also self-cleaving ribozymes that are activated when the sugar-phosphate compound is bound (Figure 2b) [39••]. Mutations that diminish or abolish ribozyme self-cleavage activity *in vitro* similarly cause a reduction or loss of gene regulation *in vivo*, which suggests that ribozyme self-cleavage activity is necessary for down-regulation of gene expression. The site of RNA cleavage is located upstream of the *glmS* open reading frame. Therefore it is not clear how cleavage activity causes a reduction of gene expression. One

possible explanation is that the truncated mRNA produced by the cleavage event is subsequently targeted for degradation by RNases.

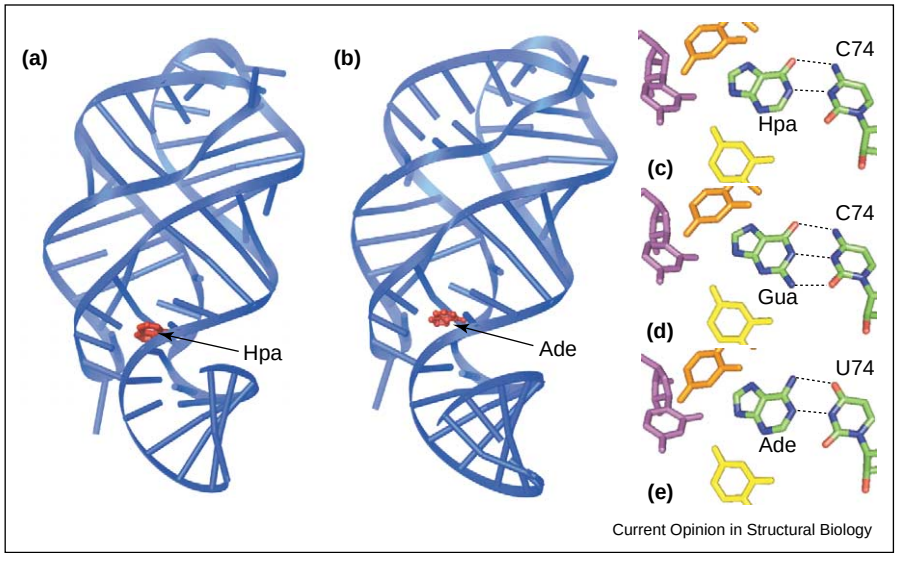
Another riboswitch candidate identified by the bioinformatics effort described above has recently been shown to function as a glycine-dependent riboswitch [40••]. Glycine riboswitches are unique in that they possess two similar aptamer structures that reside adjacent to each other, separated only by a short conserved linker sequence (Figure 2c). In many instances, these structures are located upstream of genes encoding proteins that form the glycine cleavage system, which catalyzes the initial reactions for the use of glycine as an energy source. It was shown both *in vitro* and *in vivo* that the riboswitch from *B. subtilis* activates transcription upon exposure to glycine [40••]. Thus, like the adenine riboswitch described above, members of this class serve as a rare form of genetic 'ON' switch.

The glycine riboswitch is remarkable for two other reasons. First, most glycine riboswitches carry two aptamers that each sense a ligand that has only ten atoms. Second, the two aptamers bind glycine cooperatively, such that ligand binding by one aptamer improves the binding affinity of the other by ~1000-fold and vice versa. This characteristic was demonstrated by conducting binding assays and *in vitro* transcription termination assays using constructs based on the riboswitch from *Vibrio cholerae* carrying either single or tandem aptamer configurations. For example, single and tandem aptamer constructs bind glycine with Hill coefficients of 0.97 and 1.64, respectively [40••]. Hill coefficients represent degrees of cooperativity; values near 1 represent little or no cooperativity, whereas values greater than 1 represent positive cooperativity [41]. The Hill coefficient of 1.64 supports the hypothesis that the tandem aptamer construct binds two glycine molecules cooperatively to serve as a more digital genetic switch. Furthermore, the level of cooperativity exhibited by the two aptamers of this riboswitch is comparable to that exhibited by each domain of the tetrameric protein subunits of hemoglobin [42]. Glycine riboswitches are far more sensitive to small changes in metabolite concentration than riboswitches that carry single aptamers. This more sophisticated riboswitch design is most probably required by the cell to maximize the expression of glycine cleavage system proteins when excess glycine is present. Likewise, this cooperative riboswitch can rapidly turn off the expression of these proteins as glycine levels modestly decline to assure sufficient amounts of this amino acid are available for protein synthesis.

High-resolution riboswitch structures

Recently, three high-resolution structural models have detailed the structural basis of ligand recognition by purine-binding riboswitches (Figure 3a,b) [43••,44••]. One of these structures is the aptamer domain of the guanine riboswitch from the *xpt-pbuX* operon of *B. subtilis* [43••] (Figure 3a). The aptamer was crystallized in

Figure 3



Structural features of the purine-responsive riboswitch aptamers. Structures of **(a)** the guanine riboswitch aptamer (*xpt-pbuX*) and **(b)** the adenine riboswitch aptamer (*add*) reveal a similar tertiary fold, despite only 59% sequence identity. Bound metabolites, hypoxanthine (Hpa) and adenine (Ade), respectively, are shown in red. Discrimination at the binding site of the guanine aptamer **(c,d)** and the adenine aptamer **(e)** results from the identity of nucleotide 74 (using the numbering system of the *B. subtilis* construct), which makes Watson–Crick hydrogen bonds to the appropriate metabolite. Nucleotides U22, U47 and U51 are colored orange, magenta and yellow, respectively. The oxygen and nitrogen atoms of the metabolite and nucleotide 74 are colored red and blue, respectively.

complex with hypoxanthine, a metabolite that is similar in structure to guanine, and has previously been shown to be bound by the riboswitch and to regulate gene expression [20,37,45]. In a similar study [44••], the same riboswitch was crystallized in complex with guanine.

These structural models add new insight into how RNA molecules, either natural or engineered, selectively bind to nucleotide-like compounds [46–49]. Similarities exist between this model and the structures of engineered aptamers, particularly in the use of base triples to form the binding pocket and in the use of base stacking to stabilize the bound aptamer state [43••,44••,49]. A unique feature of the riboswitch structure is a novel loop–loop interaction that stably bridges two stems of the aptamer. Mutations of these loop sequences were known to eliminate metabolite binding *in vitro* and this effect probably explains why the identities of these nucleotides are strictly conserved in all examples identified to date [20]. This loop–loop interaction involves the formation of a web of hydrogen bonds that appears to be essential for constraining the tertiary structure of the ligand-bound state [43••,44••,48] (Figure 3a).

Another notable feature of this structure is that the ligand is almost entirely engulfed by the binding pocket of the aptamer [43••,44••]. The side walls of the binding pocket or 'compartment' are formed by three nucleobases and a ribose (Figure 3c,d), whose functional groups form as many as eight hydrogen bonds with guanine. This extensive network of contacts would be difficult to achieve with most engineered aptamers, which have more open binding pockets, and typically exhibit weaker and less selective interactions with their target ligands.

As described above, most riboswitches must alternate between two structural states: one that allows gene expression and one that precludes expression (Figure 1). The ligand-bound state of the guanine aptamer allows a terminator stem to form by preventing antiterminator formation [20]. Therefore, the formation of the aptamer structure that can receive guanine must be somewhat transitory to allow these alternate structures to form. The enclosed binding compartment of the structure prevents the aptamer from completely forming in the absence of metabolite, otherwise the compartment would not allow ligand access. Furthermore, it appears that the base contributes to the folding of the aptamer's ligand-bound state [48]. Therefore, the stabilization of the aptamer structure upon nucleotide binding provides an effective switching mechanism for gene control.

A structural model of the related aptamer domain from the adenine-specific riboswitch of the *Vibrio vulnificus add* gene also has been reported [44••]. One of the most striking features of the guanine and adenine riboswitches is that they adopt nearly identical tertiary structures despite sharing less than 60% sequence identity (Figure 3a,b). Likewise, the binding compartment formed by the adenine-specific aptamer is nearly identical to that observed for the *xpt-pbuX* aptamer (Figure 3e). However, the most important difference in nucleotide sequence between the two riboswitch classes occurs at one of the nucleotides that forms this compartment (position 74 of the *B. subtilis xpt-pbuX* RNA). The nucleotide at this position is a cytosine in guanine-binding riboswitches and a uracil in adenine-binding riboswitches (Figure 3c–e). As previously suggested [36•], each aptamer uses this nucleotide to selectively bind its target purine through the formation of standard Watson–Crick base pairing interactions.

Conclusions

Despite the recent discoveries of new RNA genetic elements, it is likely that the current collection of known elements reflects only a small fraction of the contribution that RNA makes to the regulation of modern cells. Furthermore, it seems likely that the diversity of RNA structure and function could have been harnessed by the earliest life forms to construct sensory and regulatory RNAs. Some riboswitches could be direct descendents of ancient metabolite sensors that first emerged in the RNA world [28]. This hypothesis is certainly intriguing, given that all riboswitches identified to date are triggered by compounds that are near universal in their evolutionary distribution, and that many of these ligands carry phosphate and nucleotide-like moieties, as would be expected of metabolites from an RNA world [50,51].

However, riboswitches need not be remnants of an ancient sensory system to have taken their current place in gene regulation systems. RNA could simply be best suited to perform many of the regulatory roles it serves in modern cells. Many riboswitches appear to be widespread in bacteria and therefore they might have been present in the last common ancestor of bacteria. This does not mean that they were present in a purely RNA world organism, but might have emerged after organisms of the RNA world gave way to protein-dominated life forms. Also, lateral transfer and repetitive re-invention of riboswitch classes could have caused riboswitches to be distributed widely despite possible recent emergence. What has been learned about riboswitches over the past several years is consistent with either hypothesis. Furthermore, it seems reasonable to speculate that there might be a mixture of lineages for riboswitches, whereby some are post RNA world representatives whereas others date back to a time before proteins were present.

Given that some riboswitches sense small molecules cooperatively and others self-cleave upon ligand binding, it is possible that future discoveries will expand our understanding of the capabilities of RNA. As for the known riboswitch classes, there remain many questions

of structure and mechanism to be addressed. The recent crystal structures have helped in this regard, lending insight into how metabolites are recognized and how the binding domains discriminate between similar molecules. For the guanine and adenine aptamers, recognition is the result of a simple Watson–Crick base pairing interaction. One might speculate that similar strategies are employed, at least in part, for the other nucleotide metabolites, such as FMN and SAM. However, it is less easily predicted how an aptamer will form a binding pocket for a simple molecule such as glycine or lysine. Additional structural and biophysical studies will be needed to address some of the more complex mechanistic questions, such as those concerning the interactions between aptamer and expression platform, between aptamer and aptamer when cooperative function is present, or between the expression platform and RNA polymerase.

One of the major open questions in the field is how and to what degree riboswitches function in eukaryotes. As mentioned earlier, riboswitches have been found in plants and fungi, where they are located near splice site junctions of introns and appear to be regulating mRNA splicing [27•,52•]. However, more detailed characterization is needed to determine the precise role of riboswitches in these processes. Although only one class of metabolite-binding RNA has been identified in eukaryotes [27•], it is possible that improved bioinformatics and genomics approaches will reveal new riboswitch candidates within eukaryotic genomes. Currently, there is no reason to assume that higher organisms cannot exploit the molecular recognition and allosteric properties of RNA, as do their bacterial counterparts.

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