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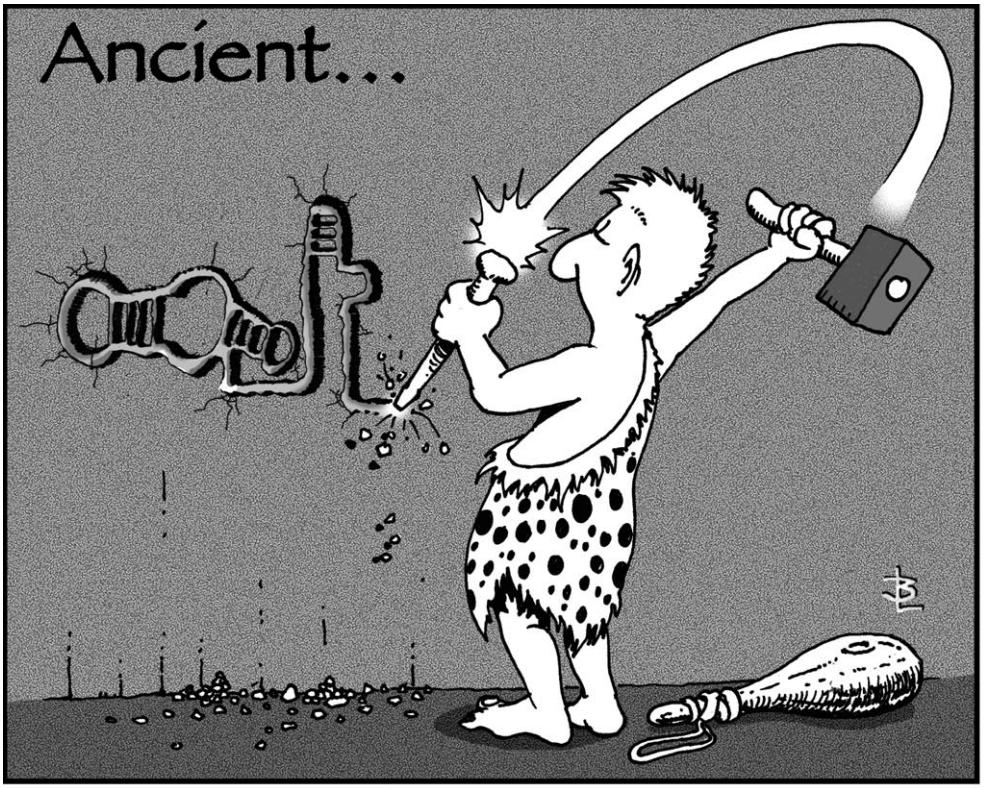
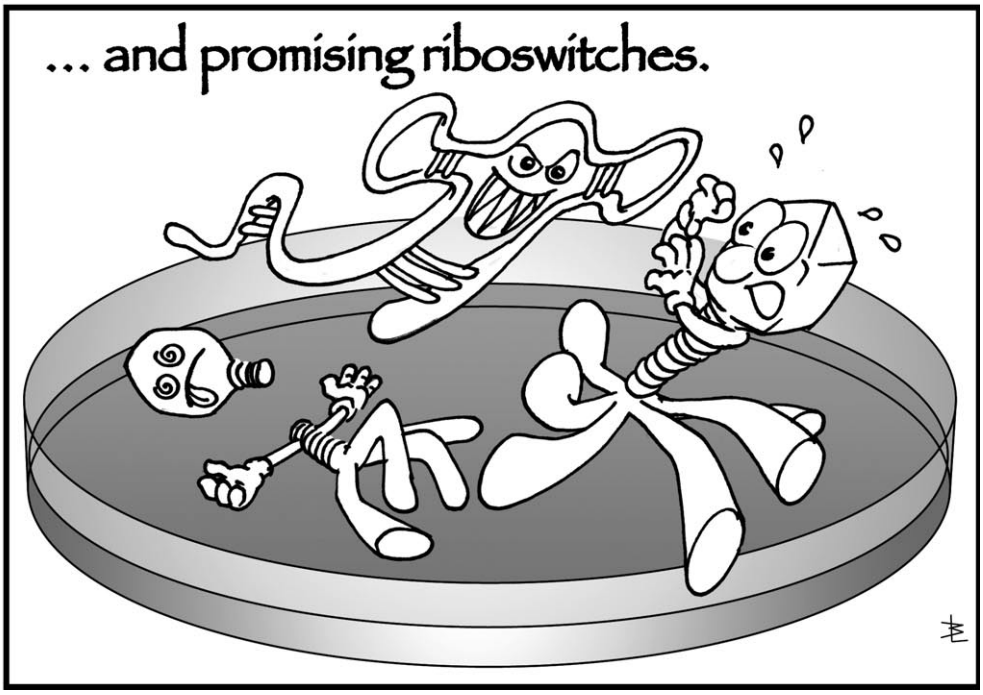
Riboswitches: Ancient and Promising Genetic Regulators

Simon Blouin,[a] Jérôme Mulhbacher,[a] J. Carlos Penedo,[b] and Daniel A. Lafontaine*[a]





Newly discovered metabolite-sensing riboswitches have revealed that cellular processes extensively make use of RNA structural modulation to regulate gene expression in response to subtle changes in metabolite concentrations. Riboswitches are involved at various regulation levels of gene expression, such as transcription attenuation, translation initiation, mRNA splicing and mRNA processing. Riboswitches are found in the three kingdoms of life, and in various cases, are involved in the regulation of essential genes, which makes their regulation an essential part of cell survival. Because riboswitches operate without the assistance of accessory proteins, they are believed to be remnants of an ancient time, when gene regulation was strictly based on RNA, from which are left numerous "living molecular fossils", as exemplified by ribozymes, and more spectacularly, by the ribosome. Due to their nature, riboswitches hold high expectations for the manipulation of gene expression and the detection of small metabolites, and also offer an unprecedented potential for the discovery of novel classes of antimicrobial agents.

1. Introduction

Only twenty-five years ago, RNA was mainly considered as a cellular messenger that carries genetic information from the genome to the ribosome for the production of proteins. Only protein enzymes were then considered to be truly "molecular effectors", which were involved in essential biological catalytic processes. The discovery of RNA enzymes, or ribozymes,[1,2] changed the way that scientists perceived the role of RNA as a cellular component. Ribozymes, by playing both informational and catalytic roles, inspired the RNA World hypothesis, which was put forward to address the "chicken and egg" problem of how a translation system could emerge without proteins already in place. Thus, if RNA or some RNA-related molecules could encode information and perform catalytic reactions, then protein-driven chemical reactions could have evolved from those molecules to yield the complexity observed in modern cellular processes. However, for such a primitive world to be viable, the large collection of catalytic reactions would require feedback regulatory loops as a way to regulate gene expression. These mechanisms would need to be highly specific to prevent unwanted regulatory cofactors from perturbing/altering metabolic regulation by acting on unrelated biochemical pathways. Because RNA has only a handful of functional groups compared to proteins, it can be hard to imagine that RNA can build tertiary structures complex enough to form specific ligand-binding sites. However, it was repeatedly shown by using SELEX (Systematic Evolution of Ligands by EXponential enrichment) that RNA can be a "good contortionist" at building complex architectures.[3] A large number of artificial RNA aptamers have been isolated that respond to various small molecules, such as theophylline, adenosine triphosphate, amino acids and organic dyes (recently reviewed[4]). By taking advantage of the fact that formation of the RNA–ligand complex increases the stability of an RNA structure compared to that of the RNA by itself, several gene regulation systems were engineered in which Shine–Dalgarno (SD) and initiation codon sequences were selectively sequestered depending on the presence of an exogenously added ligand, which altered the structure of the mRNA upon binding.[4] Such results strongly suggested that the RNA World could have benefited from feedback regulatory mechanisms to regulate gene expression.

Additional evidence for an RNA-based primitive world came from recent work showing that naturally occurring RNA motifs can act as specific regulatory switches. Strikingly, it was found that these RNA switches were able to use feedback regulatory loops to monitor and control their own expression, apparently all of this in the absence of any helper protein. These RNA elements, called riboswitches, are literally "molecular switches" that can sense the presence of small cellular metabolites and fold into a different conformation to either promote or inhibit expression of the protein encoded downstream. Given that a number of excellent recent reviews have covered in great detail riboswitch molecular structures,[5–24] this review will mainly focus on riboswitch regulation mechanisms. We will also explore the growing potential of riboswitches for use as antibacterial cellular targets, molecular biosensors and as ligand-inducible effectors. In a nutshell, this review will attempt to show the current knowledge about riboswitches and the vast possibilities that they offer as molecular tools.

2. Riboswitches as Novel Gene Control Elements

Riboswitches are located in untranslated regions (UTRs) of mRNA and are able to sense the concentration of a target cellular metabolite, which is almost always related to the gene product encoded by the riboswitch downstream sequence. To be able to sense various types of cellular changes, riboswitches use different molecular architectures that are highly adapted to monitor biological cues. The variety of riboswitch architectures spans from single temperature-responsive stem–loops to complex structures able to specifically bind large metabolites, such as coenzyme B_{12}. In all cases studied, this cis-acting mechanism enables a tight regulation control that allows the sensing RNA molecule to be directly regulated. This is a different strategy compared to RNAi-based trans-acting system, in which a short interfering RNA molecule acts as a guide to target complementary sequences within mRNAs, and leads to the activation of the RNA-induced silencing complex (RISC), and ultimately to mRNA silencing.[25] With the exception of the *glmS* ribozyme,[26–28] all reported riboswitch representatives control gene expression by modulating the formation of at

[a] *S. Blouin, Dr. J. Mulhbacher, Prof. D. A. Lafontaine*
Département de biologie, Université de Sherbrooke
2500, Boulevard Université, Sherbrooke, J1K 2R1 (Canada)
Fax: (+1) 819-821-8049
E-mail: daniel.lafontaine@usherbrooke.ca

[b] *Dr. J. C. Penedo*
University of St. Andrews, School of Physics and Astronomy
North Haugh, St. Andrews, KY16 9SS (UK)
Fax: (+44) 1334-463104

least one helical domain. Surprisingly, only by performing such a minimal structure alteration, riboswitches are able to modulate a large array of diverse biological processes, such as transcription attenuation, translation initiation, mRNA splicing and mRNA processing.

The term "riboswitch" was coined by Ronald Breaker in 2002 when he reported that mRNA-encoding enzymes involved in vitamin B_1 and B_{12} biosynthesis in *E. coli* could bind associated metabolites without helper proteins being involved.[29,30] Similar findings were also obtained from other groups when they observed that ligand-dependent feedback regulation mechanisms could rely entirely on RNA structural changes.[31–34] However, before the riboswitch concept was generally accepted, several hints came from previous biochemical studies, which paved the way to a significant conceptual progress, and ultimately to the proposition that RNA structures can directly bind cellular metabolites to modulate gene expression.[35–37] For instance, based on mutational analyses, it was proposed that elements found in the 5′ UTR of mRNA involved in the synthesis of thiamine pyrophosphate (TPP), riboflavin and adenosylcobalamin were required for gene regulation of downstream gene products.[32,38,39] These studies provided a clear answer to why several attempts to identify regulatory proteins involved in gene regulation did not reveal expected protein cofactors. After the riboswitch concept was put forward, a "minirevolution" took place among the RNA community, which led to the discovery of many distinct classes of RNA switches. Natural cis-acting riboswitches have been shown to respond to various metabolites, such as adenine,[40] adenosylcobalamin (B_{12}),[30] flavin mononucleotide (FMN),[31,41] guanine,[42] 2′-deoxyguanosine,[43] glucosamine-6-phosphate,[26] glycine,[44] lysine,[33,45] molybdenum,[46] 7-aminomethyl-7-deazaguanine (preQ1),[47] *S*-adenosylmethionine (SAM),[34,48–51] *S*-adenosylhomocysteine (SAH)[52] and TPP.[29,31]

The sensing of target metabolites by riboswitches is performed by the aptamer domain, which is responsible for the high affinity and specificity of riboswitch–ligand complexes (Figure 1).[6] Where studied, it has been shown that the aptamer folds into a defined structure that produces specific contacts with most of the atomic groups of the ligand, which en-

Simon Blouin completed his MSc at the Université de Sherbrooke in 2007. He received a Graduate Scholarship from the National Sciences and Engineering Research Council of Canada (NSERC) to pursue his research at the PhD level on the structure–function of riboswitches in the laboratory of Prof. Daniel Lafontaine. His current research interests include the structural and functional characterization of the lysine riboswitch and the influence of small RNA motifs on riboswitch function.

Carlos Penedo received his PhD from the University of Santiago de Compostela (Spain). Following a postdoctoral position with Prof. John Kelly at Trinity College Dublin, he joined the Analytical Division of Edinburgh Instruments, Ltd., as Project Manager and Development Engineer. He subsequently moved to the University of Dundee to work with Prof. David Lilley on the implementation of single-molecule fluorescence techniques to study RNA folding. He has recently been awarded a Scottish Universities Physics Alliance (SUPA) Advanced Research Fellowship at the University of St. Andrews. His current research interests are at the interface between chemistry, physics and biology, and mainly focus on the application of fluorescence and single-molecule detection techniques to study the dynamics of biomolecular interactions.

Jérome Mulhbacher obtained a PhD in biochemistry under the supervision of Prof. Mircea-Alexandru Mateescu at the Université du Québec à Montréal where he worked on controlled-release drug properties and mechanisms of starch matrices for pharmaceutical applications. In 2005, he moved to the Université de Sherbrooke where he obtained a postdoctoral NSERC fellow in the laboratory of Prof. Daniel Lafontaine. His main research interest is in the ligand recognition determinants of guanine riboswitches and riboswitch therapeutical application.

Daniel Lafontaine completed his PhD at the Université de Sherbrooke and carried out a postdoctoral research at the University of Dundee as an EMBO Postdoctoral Fellow where he worked on the structural and functional characterization of the Varkud satellite ribozyme in the laboratory of Prof. David Lilley. In 2003, he joined the Université de Sherbrooke where he is mainly interested in the study of RNA structure–function relationships and their involvements in gene expression regulation. He has recently received a New Investigator award by the Canadian Institutes of Health Research as well as a Chercheur-boursier Junior 2 from the Fonds de la recherche en Santé du Québec.

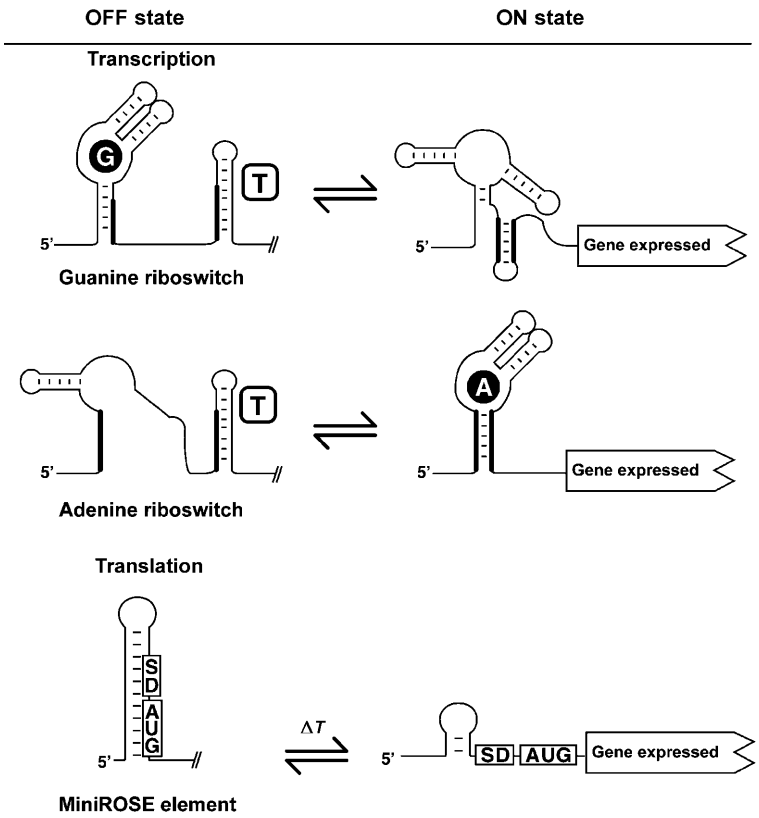


Figure 1. Riboswitch gene regulation mechanisms. The guanine riboswitch is shown as an example of a negatively controlled riboswitch since premature transcription termination is favored in the presence of guanine. The adenine riboswitch positively controls gene expression since the presence of adenine is associated with transcription elongation. The miniROSE element positively modulates translation initiation when temperature is high enough. Guanine, adenine and transcription terminators are shown as G, A and T, respectively; SD is the Shine–Dalgarno sequence.

sures a very high ligand-binding specificity. Importantly, the formation of the ligand–aptamer complex has a direct influence on the folding of the downstream expression platform, which is used to control gene expression. The binding site recognition properties appear to be highly adaptive as a large spectrum of ligand-binding riboswitches has been found so far.[6] The expression platform also shows an important level of plasticity since it can adopt a large array of secondary structures that can be used to regulate most of the important biological processes, and this either by up- or down-regulating gene expression. The modularity of the aptamer and the expression platform makes riboswitches the "Swiss-army knife" of cellular regulation, and even more remarkably, all of this without the absolute requirement for protein cofactors. The genetic decision relying on the folding status of the riboswitch is dictated by the binding of the target metabolite to the aptamer domain. In most cases, when the concentration of the target metabolite is high in the cell, its binding to the riboswitch aptamer results in the repression of its synthesis (Figure 1). However, gene expression is stimulated by ligand binding in cases in which it is involved either in metabolite cellular transport,[40] or as an energy source.[44]

The paradigm of riboswitch regulation is well represented by the purine riboswitch class, which consists of guanine and adenine riboswitches that negatively and positively regulate gene expression upon ligand binding, respectively (Figure 1).[40,42] In the case of the guanine riboswitch, a transcription terminator element is formed only in the presence of guanine; this results in gene repression,[42] which can vary according to the riboswitch representative.[53] For the adenine riboswitch, presence of adenine is associated with the disruption of the transcription terminator; this allows gene expression to take place (Figure 1). Crystal structures show that despite their high structural similarities, both riboswitch aptamers display very high specificity toward their respective ligand;[40,42] this is essentially achieved by the formation of a Watson–Crick base pair between the ligand and a nucleobase located in the aptamer domain.[54,55] The adenine riboswitch is also thought to modulate at the translational level,[55,56] which is another common regulation mechanism used by bacterial riboswitches (see Section 4.2). Translational riboswitch control has also been characterized in detail in the case of the ROSE (repressor of heat-shock gene expression) element, which controls translation initiation by thermosensing; this makes the miniROSE element one of the simplest riboswitch structures (Figure 1).[57] This control mechanism is achieved with only a simple helical domain that sequesters the SD sequence and the AUG start codon, both of which are exposed when the temperature is increased to allow expression of heat-shock gene products.[57] A similar thermosensible regulation mechanism is involved in infections caused by the food-borne pathogen *Listeria monocytogenes*.[58] It has been shown that invasion proteins and phospholipases involved in the infectious process are controlled by *prfA*, which is not expressed below 30 °C; this results in low expression of virulence. However, when *L. monocytogenes* enters an animal host, the temperature shifts to 37 °C; this results in *prfA* expression and production of proteins involved in the infectious process. Interestingly, a thermosensor RNA structural element has been shown to control *prfA* expression by masking the ribosomal-binding region at 30 °C but allowing ribosome access at 37 °C.[58]

3. Riboswitch Structural Diversity

Riboswitches fold into diverse secondary structures that are often organized around helical junctions with varying degrees of complexity. For instance, in the case of purine riboswitches, and most probably also for the recently discovered 2′-deoxyguanosine riboswitch (Figure 2), the aptamer folds around a three-way junction and displays a loop–loop interaction, which is important for folding and activity.[40,42] The junction also contains a helical-stacking interaction, which is frequently found in three-way RNA junctions, between stems P1 and P3.[14] The

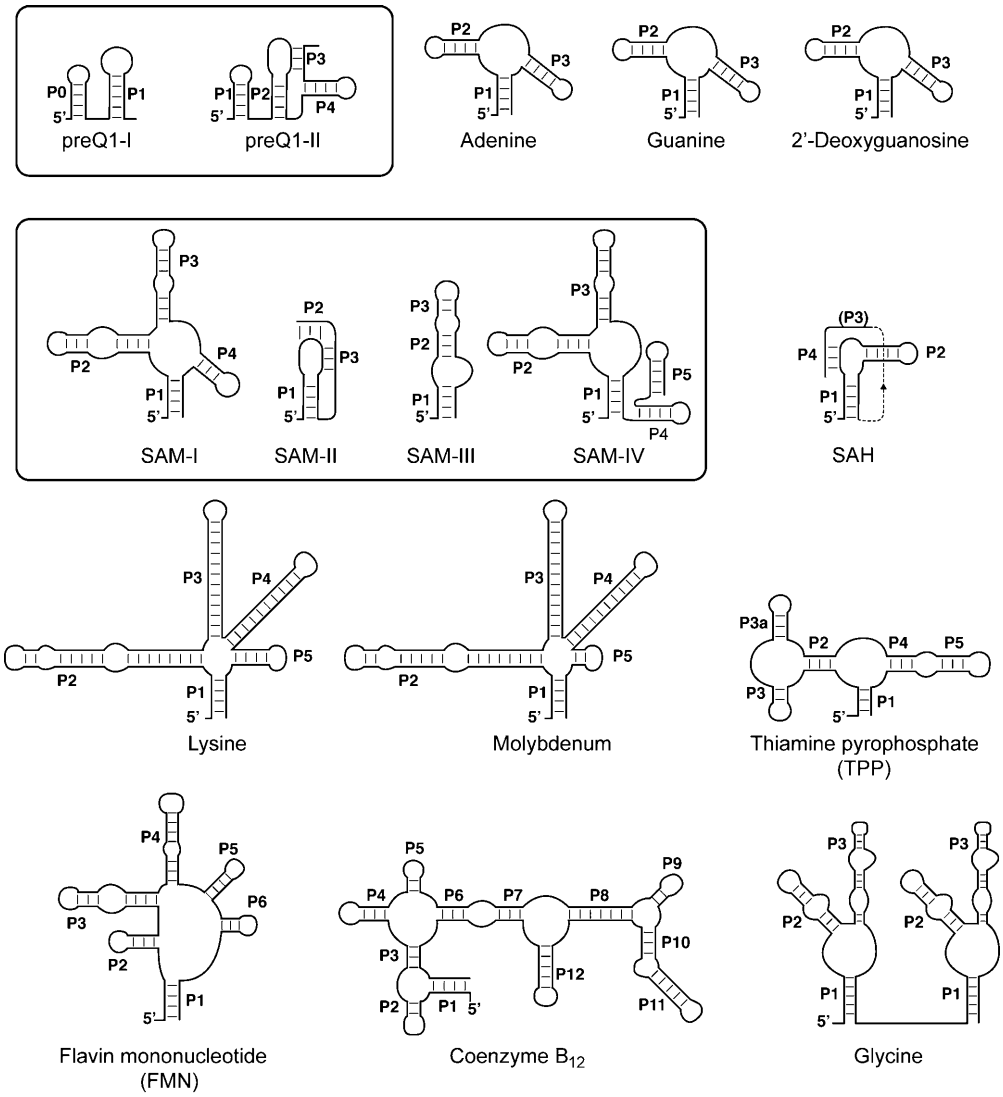


Figure 2. Secondary structures of metabolite-binding riboswitch aptamers. The sensed metabolite is indicated for each riboswitch class. Aptamers sensing an identical ligand are grouped in a rectangle. An additional P3 stem–loop located between P2 and P4 might be present in some adenosylhomocysteine (SAH) natural representatives and is indicated in parentheses. A zero-length linker region is drawn for the SAH riboswitch by a dashed line and the strand polarity is indicated by an arrow. Aptamer secondary structures should be considered only as artistic representations since known tertiary interactions are not represented and do not contain the exact number of base pairs. The *glmS* ribozyme is shown in Figure 3.

hammerhead ribozyme shows a highly similar global tertiary fold as it also contains a loop–loop interaction as well as an equivalent helical-stacking interaction. In the case of the hammerhead, the presence of the loop–loop interaction was only recently appreciated when it was shown that this distal interaction is very important for the ribozyme function under physiological conditions.[59–62] The presence of the loop–loop interaction has been shown to be also important for purine riboswitches.[54,63–66] As opposed to the hammerhead ribozyme, a minimal adenine riboswitch aptamer lacking the loop–loop interaction does not readily fold into a similar global tertiary structure as compared to the wild type (Lemay and Lafontaine, unpublished observations); this most probably indicates that the aptamer core is less rigid than the hammerhead ribozyme and cannot by itself drive the riboswitch to the active conformation. A naturally occurring variant of the purine riboswitch has recently been reported in only one bacterial species that exhibits selective and high-affinity binding of 2′-deoxyguanosine;[43] this indicates that the ligand specificity of existing riboswitches can be reprogrammed without significantly altering the global aptamer architecture.

Riboswitches have found different molecular solutions to create ligand-binding aptamers for both preQ1 and SAM (Figure 2). Whilst aptamers preQ1-I and preQ1-II exhibit relatively similar secondary structures, those specific to SAM display very different structures ranging from a simple stem–loop to complex three- or four-way junctions; this suggests that evolutionary distinct RNA aptamers can converge to the same function (Figure 2). Crystal structures of SAM-I, SAM-II and SAM-III aptamers show that although the ligand is bound in distinct ways,[67–69] high affinity and specificity is preserved in both aptamers; this enables a strong discrimination against

most analogues of SAM, such as SAH.[70] For instance, to recognize the positively charged sulfur group as well as the activated methyl group, the SAM-II aptamer makes use of carbonyl oxygen groups from uracils—a very similar strategy to the one employed by SAM-I, which employs two universally conserved A–U pairs to provide the carbonyl oxygen atoms. Interestingly, a particular SAH-binding aptamer, which shares structural similarities with the SAM-II aptamer,[52] suggests a common ligand-binding site for both ligands as SAM and SAH only differ by the presence of a single methyl group and a positive charge. The existence of several different aptamers for preQ1 and SAM is consistent with the idea that riboswitches exploit the structural versatility inherent to RNA molecules and suggests that other RNA-binding aptamers sharing similar ligand binding strategies are bound to be discovered.

The lysine riboswitch is centered on a unique five-way junction that is most probably important for lysine binding.[33,45,71] A long-range loop–loop interaction occurring between stems P2 and P3 has been shown to be important for the aptamer global folding, ligand binding and riboswitch transcriptional control activity.[71] This study also found that the correct folding of a kink–turn (K-turn) element present in the P2 stem is crucial for the loop–loop interaction to take place. The simultaneous presence of a K-turn element together with a long-range tertiary interaction is not unique to the lysine riboswitch since a similar architectural arrangement is also observed in the SAM-I aptamer. From the SAM aptamer crystal structure, it is clear that the presence of the K-turn is critical for the long-range pseudoknot interaction to form, which in turn is vital for the ligand-binding activity of the aptamer.[72] In addition, in the 23S ribosomal RNA, a three-way junction also exhibits a loop–loop interaction in which one of the interacting arms contains a K-turn motif.[73,74] In the case of the ribosomal junction, the crystal structure shows that ribosomal proteins L24 and L29 interact with the contained K-turn (Kt-7); this suggests that their presence could be important for the correct folding of the K-turn motif and, consequently, for the loop–loop structure.

The glycine-specific riboswitch is unique among known riboswitches since it is the only one that employs cooperative binding to control gene expression.[44] The secondary structure of the aptamer encompasses two similar domains joined by a linker sequence (Figure 2). Because these two domains are interrelated, cooperative binding can be used to efficiently respond to ligand within a small range of concentrations. A recent study employing nucleotide analogue interference mapping (NAIM) has identified two sites that are involved in cooperative tertiary interactions.[75] In this study, Kwon and Strobel have found that the minor groove of the P1 stem of the first aptamer as well as the major groove of the P3 stem of both aptamers are likely to be important for interdomain docking. These findings suggest that the glycine riboswitch uses the P1 stem of the first aptamer not to modulate the expression platform as done in other riboswitches, but to contribute to tertiary interactions that are important for ligand-binding cooperativity. This finding is a striking example that riboswitches can exhibit complex regulation characteristics that were previously thought to belong only to protein cofactors. As if the remarkable riboswitch versatility already discussed was not enough to control gene expression, recently discovered riboswitches exhibit a further degree of complexity.[44,76,77] For instance, molecular arrangements consisting of two independent TPP riboswitch units have been reported in *Bacillus anthracis*.[77] The riboswitch duplication is most probably important to provide a greater dynamic range for ligand binding and thus, a higher sensitivity for the control of gene regulation. A further level of complexity is shown by the SAM–B_{12}-dependent dimeric riboswitch,[76] which exhibits two aptamers that are dissimilar in their ligand-binding specificity. Such a combination enables gene expression to be controlled in a Boolean NOR logic gate fashion, in which the binding of any ligand produces down-regulation of gene expression. Similar dimeric riboswitches responding to different ligands are also very likely to exist since a dimeric riboswitch containing a guanine aptamer as well as an additional domain with unknown ligand specificity was recently described.[76] Riboswitch-mediated gene regulation is not only performed by using metabolite-binding aptamer since other cellular signals, such as temperature,[78,79] magnesium[80] and tRNA,[18] have been shown to influence RNA structure and to modulate gene expression. For instance, it is interesting to note that tandem arrangements are also observed on a higher frequency for tRNA-sensing T-boxes, which are likely to require tighter regulation control over tRNA charging.[77,81]

Lastly, it appears that riboswitches could show even more diversity for ligand-binding effectors as compelling evidences for this were recently obtained.[82,83] For instance, using a computational pipeline, Weinberg et al. used bacterial comparative genomics and identified 22 novel candidate RNA motifs, some of which are apparently involved in transport, the citric acid cycle, molybdenum cofactor biosynthesis and *Vibrio cholerae* natural competence.[82] Also, three other widespread candidates, which exhibit a conserved secondary structure, are associated with homologous genes, and thus share most characteristics that are usually associated with typical riboswitches. However, three other candidates show a narrower distribution since they are only found in a single taxonomic order; this suggests that many riboswitches remaining to be discovered might exhibit narrower phylogenetic distribution than those already known.[82]

4. Biological Processes Regulated

4.1 Premature transcription termination

Bacteria primarily use two mechanisms for termination of transcription. Some genes incorporate a termination signal that is dependent upon Rho protein,[84] while others make use of Rho-independent terminators (intrinsic terminators) to destabilize the transcription elongation complex.[85–87] These latter RNA elements are composed of a GC-rich stem–loop followed by a stretch of 6–9 uridyl residues. Intrinsic terminators are widespread throughout bacterial genomes,[88] and are typically located at the 3′ termini of genes or operons. However, they are also found in riboswitch expression platforms where ligand binding can either produce the formation or disruption of the

terminator; this results in abortive transcription or elongation, respectively (Figure 1). The affinity of the aptamer–ligand complex is presumably one of the most important factors in the riboswitch gene regulation mechanism. Indeed, provided that the ligand is present at sufficient concentration, it is predicted that the aptamer–ligand complex is formed and gene regulation ensued. As long as ligand binding is fast compared to the transcription rate, riboswitches operate under a thermodynamic regime with the equilibrium between the bound- and unbound-ligand aptamer, which dictates the outcome of the genetic expression.

A further notion was discovered when it was observed that for some riboswitches, the rate of ligand binding (k_{on}) is important for the regulation process.[89,90] Indeed, compared to RNA polymerase (RNAP) transcription rate, slow ligand-binding kinetics implies that formation of the aptamer–ligand complex might not attain equilibrium before the expression platform is transcribed. Thus, under conditions in which ligand binding is slow compared to RNAP transcription rate, ligand binding to the aptamer domain might not occur before the expression platform is transcribed; this indicates that aptamer stabilization might not be achieved by the time the genetic decision is made, which corresponds to a kinetic regime regulation control. For riboswitches that are kinetically controlled, it is expected that the concentration of ligand required to trigger transcription termination is higher than the apparent K_D value determined for the riboswitch–ligand complex, which has been observed in many cases.[34,45,50,90] However, it should be noted that the apparent discrepancy between these values could also result in part from the presence of alternative RNA structures that do not produce ligand-binding competent riboswitches. In addition, because the concentration of ribonucleotide triphosphates (NTPs) modulate RNAP transcription rate, it is expected that NTP concentration can affect the concentration of ligand required to mediate termination, which has been observed for the *ribD* FMN[90] and the *gcvT* glycine[44] riboswitches. These results are consistent with the idea that the transcription rate can influence transcription termination by allowing more or less time for ligand binding to occur. In addition, in the context of the *pbuE* adenine riboswitch, it was recently shown[66] that the presence of the expression platform negatively influences the ability of the aptamer to perform ligand binding, which is consistent with kinetic control under certain conditions.[89]

Under conditions in which a given riboswitch operates under a kinetic regime, and therefore when the rate of RNAP transcription is determining, pausing might be a significant factor in riboswitch regulation. Because transcriptional pauses are known to decrease transcription elongation rate by as much as 10000-fold,[91] it is very likely that they could be of vital importance for kinetically driven riboswitches by providing additional "breathing time" for riboswitch–ligand complex formation. For instance, in the case of the FMN riboswitch, it has been found that two major pause sites present in the riboswitch sequence are characterized by lifetimes of ~10 s and ~1 min.[90] In agreement with the idea that pause sites provide more time for ligand binding, the mutation of either pause site increased transcription termination, and an increase in the ligand concentration was required to obtain 50% of the full extent of transcription termination. Also, the addition to transcription reactions of the RNA-binding protein, NusA, which is known to alter transcription termination levels,[92] resulted in increased lifetimes at the pause sites whereas the increase in NTP concentration resulted in the decrease of pause lifetimes, as expected from their respective influences on transcription rates. Together, these results suggest that rates of ligand binding as well as transcription are in some cases more important than the affinity of the riboswitch–ligand complex.

4.2 Translation initiation

Whereas Gram-positive bacterial riboswitches mainly control transcription attenuation, Gram-negative riboswitches tend to modulate translation initiation levels.[19,93] This functional difference can be explained by the relative prevalence of polycistronic mRNA found in both bacterial types. For instance, given that Gram-positive bacteria have larger gene clusters, they can benefit from premature transcription attenuation control.[19,93] Among known riboswitch representatives, those responding to TPP,[29] FMN,[41] coenzyme B_{12}[36] and SAM[94] have been shown to be involved in translational control. Translation initiation is also controlled by making use of secondary-structure changes by which access to the SD and AUG sequences is modulated to control ribosome binding, and hence translation initiation. The modulated structure can be considered as a sequestering helix given that it is involved in the base pairing of the SD and AUG sequence segments. In contrast to transcriptionally regulated mRNA, translationally controlled transcripts allow the mRNA molecule to be full length when ligand binding occurs. Whether or not translation-controlling riboswitches operate under various regulation modes (kinetic vs. thermodynamic) is not readily apparent. In contrast to transcription-regulating riboswitches, those involved at the translational level do not appear to need to perform their regulation during the transcription process. However, this will need to be further investigated by using appropriate methods since those already developed for riboswitches regulating transcription might not be readily transposable.

One of the first riboswitches identified in translational control is the TPP-responding riboswitch.[29] It was previously observed that a conserved cis-acting element located in the 5′ UTR of an mRNA associated with thiamine biosynthesis and transport is important for TPP-dependent regulation.[32,95] Importantly, no protein regulatory factor was identified with experimental assays; this suggests that the *E. coli thi* box element acts as a potential riboswitch motif. Its riboswitch-dependent regulation was established by using the *lacZ* reporter gene, which showed gene repression when bacteria were grown in the presence of thiamine.[29] The repression mechanism was attributed to direct binding of TPP onto the *thi* box element, which caused structural alterations in the aptamer domain. The apparent K_D value obtained for the formation of the RNA–TPP complex is ~500 nM, while other thiamine phosphorylated forms lead to lower affinities; this shows that the TPP ribo-

switch is highly specific for TPP. This molecular specificity was further supported by using in-line probing assays; these showed that the RNA is able to make specific contacts with two different regions of the ligand, which consist of the purine and phosphate moieties. More specifically, in-line probing also revealed that the SD sequence becomes more structured upon TPP addition; this is consistent with the formation of a helical structure—involving the SD element—that does not allow ribosome binding and hence prevents translation.

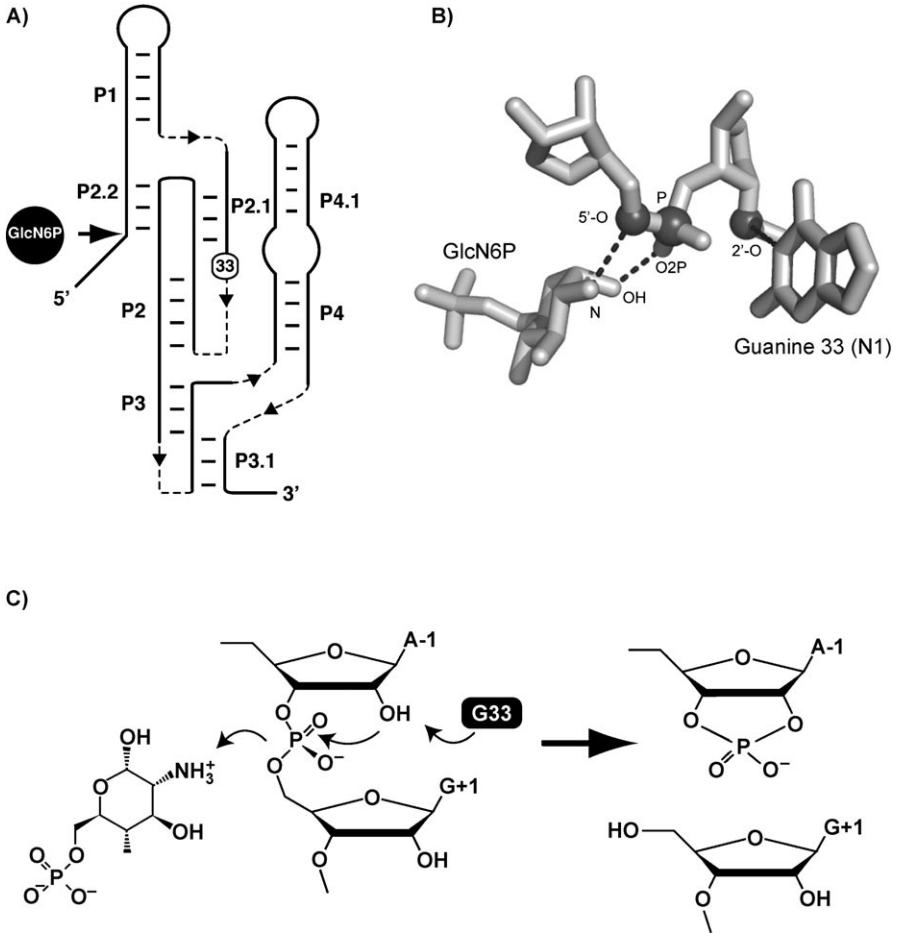


Figure 3. The *glmS* ribozyme. A) The secondary structure arrangement of the *glmS* ribozyme is shown. The cleavage site is indicated by a large arrow and the key nucleobase, G33, is shown. Discontinuous lines represent zero-length linker regions. B) The cleavage site of the *glmS* ribozyme. The 2′OH (2′-O), the phosphate (P) and the 5′OH (5′-O) leaving group are positioned for an in-line attack. The N1 functional group of G33 is shown very close to the 2′OH as an activator of the catalytic reaction. The bound GlcN6P is shown to make interactions with the 5′OH and the pro-Sp (O2P) oxygen groups. The C1–OH group of GlcN6P is also shown to interact with the phosphate center to stabilize the charge developing on the scissile phosphate. C) The cleavage mechanism of the *glmS* ribozyme. G33 acts as a general base by deprotonating the 2′OH nucleophile and GlcN6P plays the role of a general acid by protonating the 5′OH leaving group. The cleavage reaction yields 2′,3′-cyclic phosphate and 5′OH termini, which is typical of other small nucleolytic ribozymes.

4.3 mRNA processing

In addition to transcription and translation mechanisms, riboswitches have also evolved to control ligand-dependent mRNA processing. The *glmS* gene, which encodes the enzyme glutamine-fructose-6-phosphate synthetase, converts fructose-6-phosphate and glutamine into GlcN6P. Importantly, upon binding to GlcN6P, the *glmS* mRNA undergoes autocatalytic cleavage that generates 5′-OH and 2′,3′-cyclic phosphate moieties;[26] this suggests a catalytic mechanism similar to that previously described for small nucleolytic ribozymes. The *glmS* secondary structure is quite different from other ribozymes (Figure 3 A) and shows a double pseudoknot structure (P2.1 and P2.2)—a feature that is also found in other ribozymes, such as the hepatitis delta virus and Diels–Alderase ribozymes.[96,97] It has been recently reported that both pseudoknots are involved in the formation of the ribozyme core as well as the ligand-binding site and that a long-range tertiary interaction is formed between a GNRA tetraloop located in the P4.1 stem and the minor groove of the P1 helix, all of which are important for the compact fold of the ribozyme.[27,28]

The ligand-binding specificity of the *glmS* ribozyme has recently been studied in detail by using a variety of ligands.[98] In their work McCarthy et al. established that several functional groups of the ligand are important for the cleavage reaction. Among them, the amine group of the GlcN6P is crucial given that although glucose-6-phosphate (Glc6P) does not support catalytic activity, metabolites such as serinol, L-serine and ethanolamine yield to catalysis. However, the presence of the phosphate group is not as important since efficient catalytic activity is observed in the presence of D-glucosamine. By examining all potent metabolites, common features were found, which consist of an ethanolamine moiety as well as a vicinal hydroxyl group,[26] and because Glc6P inhibits *glmS* activity this suggests that the presence of the amine is dispensable for ribozyme binding. The essential role of GlcN6P was recently highlighted in an in vitro selection study,[99] in which it was not possible to alter the range of compounds supporting catalytic activity; this is consistent with the hypothesis that GlcN6P is used as a coenzyme for *glmS* self-cleavage.[100]

By using a trans-acting form of the ribozyme, the role of GlcN6P on the *glmS* structure was investigated with the aid of fluorescence resonance energy transfer (FRET) analysis.[101] Interestingly, the authors found that the complex formed between the ribozyme and the substrate does not undergo a

global conformational change following GlcN6P binding, which was confirmed by using terbium and enzymatic probing. Another study that used hydroxyl radical protection and UV cross-linking assays showed that the riboswitch binds the ligand with a prefolded active-site pocket.[102] Together with previous studies, this suggests that GlcN6P assists *glmS* catalysis by directly participating at the chemical step. The role of metal ions in the *glmS* catalytic activity was also recently studied.[103] It was shown that significant levels of activity are supported in the presence either of the exchange-inert cobalt(III) complex or monovalent ion molar concentrations. These results suggest that metal ions are not directly involved in *glmS* catalysis but instead are used for the correct folding of the RNA structure. Supporting results were obtained from NAIM experiments, which indicated that phosphate oxygens, 2′-hydroxyl and particular nucleobase functional groups are essential for ribozyme catalysis.[104] In this study, it was observed that metal-ion contacts are found in the catalytic core when Co^{III} is used; this suggests that these metal ion–RNA interactions are not directly involved in the *glmS* catalytic reaction.

Recent crystal structures have shed light on the *glmS* riboswitch catalytic activity and the role of GlcN6P in the cleavage mechanism.[27,28] Crystal structures obtained for a ribozyme lacking a 2′-hydroxyl at the cleavage site in the presence or absence of the competitive inhibitor Glc6P, and for the cleaved form, revealed that the ribozyme is a very rigid RNA molecule that exhibits a preformed ligand-binding site.[28] A crystal structure solved for a trans-acting form of the ribozyme with a 2′*O*-methyl substitution at the cleavage site, in the presence of GlcN6P, confirmed previous findings as well as offered new insights into the catalytic mechanism.[27] For instance, as suggested by other structures,[28] this study showed that GlcN6P directly participates in the reaction as a catalytic cofactor by using the ethanolamine moiety on the glucosamine sugar moiety. This work proposes that while the C1–OH group stabilizes developing charge on the scissile phosphate, the C2–NH_2 group acts as a general acid to activate the 5′-*O*-leaving group (Figure 3B). Moreover, because there is no functional group (and no metal ion) other than the strongly conserved G33, which is sufficiently close to the nucleophile to activate the 2′-hydroxyl at the cleavage site, it was suggested that G33 could be involved as a general base (Figures 3B and 3C).[27,28] The *glmS* ribozyme is thus not like a conventional riboswitch that experiences structural reorganization upon ligand binding, but rather uses ligand as cofactor to achieve catalytic activity, much like protein enzymes.

So, what is the outcome of *glmS* processed mRNAs? Because of the ribozyme self-cleavage reaction, the *glmS* downstream transcript exhibits an unusual 5′-hydroxyl terminus, which specifically targets the molecule for intracellular degradation.[105] The degradation pathway makes use of the widespread RNase J1, which has been proposed to be the functional homologue of RNase E found in *E. coli*. However, although RNase E poorly degrades mRNA transcripts exhibiting a 5′-hydroxyl group, RNase J1 specifically processes mRNA carrying 5′OH molecules. Importantly, the presence of the 5′OH-specific RNase J1 in *B. subtilis* strongly suggests that additional gene regulation mechanisms might use similar degradation strategies.

4.4 mRNA splicing and stability

Thiamin pyrophosphate (TPP) is a coenzyme derived from thiamin, which is synthesized by most bacteria, fungus and plants.[106] It is an important cofactor in amino acid and carbohydrate metabolic routes.[37] The TPP riboswitch is the most widespread riboswitch known to date and it is so far the only riboswitch found in archaea and eukaryotes.[107] The TPP riboswitch is involved in different types of gene regulation mechanisms ranging from transcription and translation control in bacteria[31,32] to splicing modulation in fungi.[108,109] Notably, it is also involved in the 3′-end processing of some plant mRNA,[110,111] where its presence in ancient plant taxa has been speculated to indicate that this riboswitch mechanism exists since the emergence of vascular plants, some 400 million years ago.[110]

The complexity and versatility that riboswitches are able to achieve is well illustrated by the TPP-riboswitch-mediated alternative splicing of mRNA in *Neurospora crassa*.[109] It has been observed that the TPP riboswitch is flanked on one side by two 5′ splice sites and on the other side by a single 3′ splice site. When TPP concentration is low, the TPP aptamer masks the proximal 5′ splice site; this results in the use of the distal 5′ splice site, which ultimately leads to the production of *NMT1* mRNA. However, when TPP binds the aptamer, an mRNA conformational change takes place that sequesters both the distal 5′ splice site and the splicing branch site. The net effect of the conformational change is a reduction in splicing efficiency of the former mRNA species as well as an increase in an alternatively spliced mRNA that results from the use of the proximal 5′ splice site. Importantly, this TPP-dependent mRNA contains μ-open reading frames (μORFs), which compete with the translation of the main ORF and thus repress *NMT1* expression. Interestingly, Breaker and co-workers have also identified a TPP riboswitch in an intron of another mRNA that interrupts the main ORF.[109] They have observed that stop codons present in the intron prevent mRNA translation. However, in the presence of thiamine, the splicing of the intron is favored; this suggests that the TPP riboswitch is also acting as a positively regulating riboswitch.

TPP-dependent alternative splicing is also present in plants, where it has been observed to differentially process the mRNA 3′ end.[110,111] This post-transcriptional regulation mechanism relies on the conditional presence of a poly A signal retained in the absence of TPP and associated with the production of a stable mRNA encoding the thiamin biosynthetic gene *THIC*. However, when TPP is abundant in the cell, a splicing reaction removes the poly A signal and produces a longer transcript carrying various polyadenylation signals. This latter mRNA is highly unstable and is associated with a reduction of *THIC* expression. Together, these findings suggest that riboswitches are clearly important players in eukaryotic gene expression mechanisms.

4.5 Increasing complexity of riboswitch genetic regulation

Most riboswitches have been found to control gene products that are involved either in the biosynthesis or transport of the associated metabolite. However, some riboswitches have also been predicted to regulate genes that are not related to their regulating metabolite.[112] For instance, in some Alphaproteobacteria, Actinobacteria, *Bacillus* species, *B. fragilis* and *Thermosynechococcus elongatus*, the isozymes methionine synthase MetE and ribonucleotide reductases NrdDG and NrdAB have B_{12}-specific riboswitches located in their upstream regions (Figure 4). While these gene products operate in a B_{12}-independent fashion, a group of isozymes (MetH and NrdJ) mostly found in the same organisms are not regulated by a riboswitch-based mechanism but employ vitamin B_{12} as coenzyme. It was thus proposed that when vitamin B_{12} is present at a sufficient cellular concentration, the expression of B_{12}-independent isozymes MetE and NrdDG-NrdAB is repressed by a riboswitch regulation mechanism, and only the more efficient B_{12}-dependent isozymes are used to perform the biological reactions.[112] In addition, a slightly different situation has recently been described in *B. clausii* in which the expression of MetE and MetH are also controlled in a riboswitch-dependent manner.[76] However, in this case, it has been observed that the *metH* mRNA contains a SAM-specific riboswitch while the *metE* mRNA exhibits an unusual riboswitch tandem configuration containing two complete riboswitch units that respond to SAM and B_{12}. Because both enzymes are involved in the biosynthesis of SAM, it appears logical that both mRNA can be regulated by SAM cellular concentration. The rationale for the presence of a B_{12} riboswitch element in *metE* stems from the lower efficiency of the MetE enzyme to catalyze the formation of homocysteine compared to MetH, which uses a derivative of adenosylcobalamin, methylcobalamin, as a cofactor. Thus, when adenosylcobalamin is available in cells, expression of MetE is down-regulated to favor the use of the more efficient MetH enzyme in SAM production.[76]

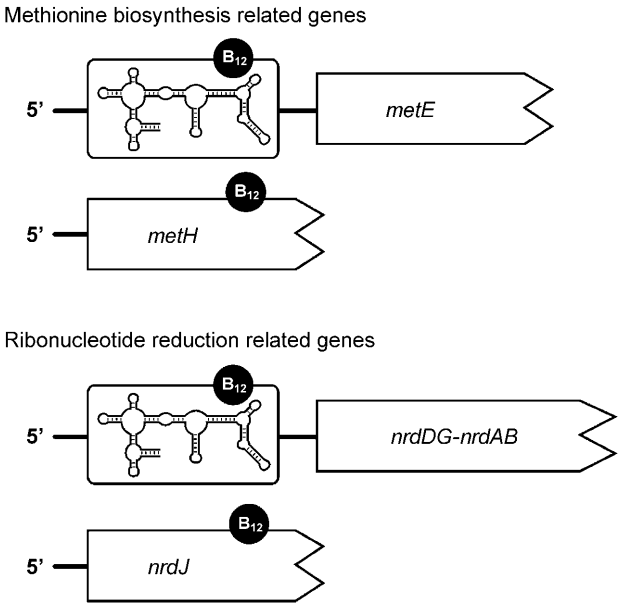


Figure 4. Riboswitches act as gene regulation elements and favor particular biochemical pathways. The MetE and NrdDG-NrdAB enzymes are regulated by B_{12}-specific riboswitches, which are expected to inhibit the expression of enzymes at an elevated intracellular B_{12} concentration. However, the expression of MetH and NrdJ B_{12}-dependent enzymes should not be affected.

Furthermore, the B_{12}-dependent riboswitch-driven regulation is not restrained to SAM and B_{12} biochemical pathways. A recent comparative genomic study in *Treponema denticola* has predicted two alternative pathways of glutamate utilization and identified B_{12}-specific riboswitches upstream of genes involved in glutamate metabolism.[112] It was reported that a B_{12}-independent catabolic glutamate dehydrogenase, rocG, is controlled by a B_{12} riboswitch. Interestingly, it was observed that an orthologue of MutSL, which catalyzes the first step of the B_{12}-dependent pathway of glutamate catabolism, is also present in the same bacterium. These observations suggest that an excess of vitamin B_{12} would repress the expression of rocG while still allowing MutSL to catabolize glutamate. These findings suggest that B_{12}-independent isozymes are regulated by B_{12}-specific riboswitches in organisms containing both B_{12}-dependent and B_{12}-independent isozymes. Although these observations need to be experimentally verified, they nevertheless suggest that riboswitch elements can be used to add a further level of regulation complexity by favoring more efficient biochemical pathways under certain cellular conditions.

5. Naturally and Artificially Occurring Aptamers

Thanks to their nucleic acid architecture, riboswitches exhibit a surprisingly high capacity and plasticity to create binding sites for targeted cellular ligands. Riboswitch plasticity, which is central to the ligand-binding specificity, is best shown in the case of the SAM riboswitches for which four different aptamers are known.[34,48–51] However, the plasticity of the ligand-binding site can also be evaluated for other riboswitch classes given that related artificial aptamers have been selected by SELEX.[113] In general, natural riboswitches tend to be larger than their in vitro evolved counterparts.[114,115] We refer the reader to a recent review covering artificial aptamers that bind AMP, GTP, vitamin B_{12} and FMN,[114] and describes their structural basis for ligand binding, which are not covered here. Instead, we will focus on the XBA RNA aptamer selected by using affinity chromatography on a xanthine agarose column in which xanthine was linked to the agarose through its C8 position.[113] The aptamer secondary structure is composed of a single stem–loop structure containing an asymmetric internal loop (Figure 5 A). By comparing the different sequences obtained, Yokoyama and co-workers observed that most of the internal loop is highly conserved; this suggests that it is involved in the recognition of the ligand.[113] From a survey of various metabolites, the authors found that xanthine, guanine and hypoxanthine bind with similar affinities ($K_D \sim \mu M$) but that adenine is strongly discriminated (Figure 5 B). Interestingly, such a molecular discrimination is highly reminiscent of the guanine riboswitch

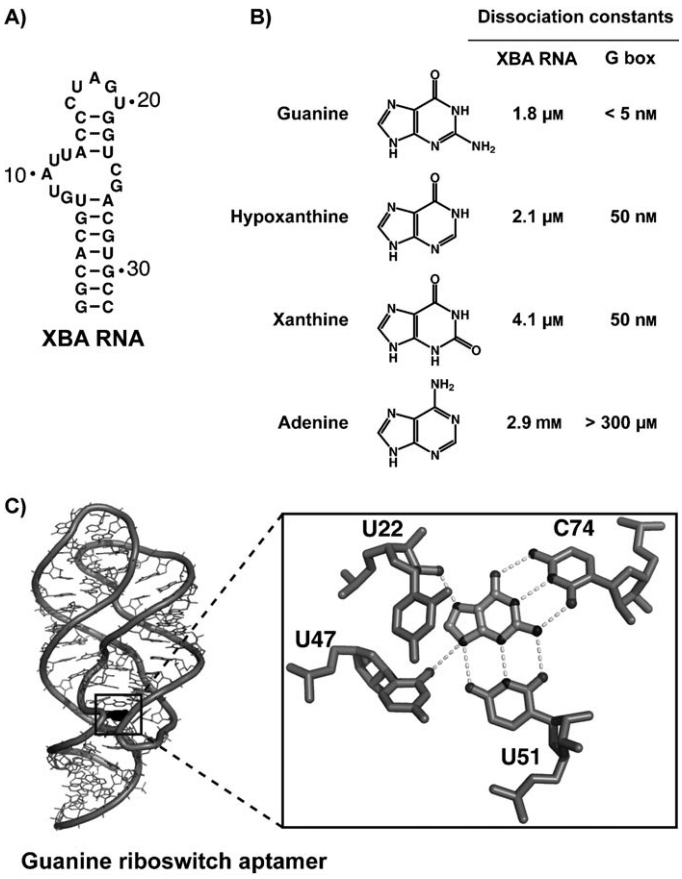


Figure 5. The XBA RNA and the guanine riboswitch aptamers. A) The secondary structure of the XBA RNA is shown and the numbering is according to Kiga et al.[113] B) Dissociation constants are indicated for guanine, hypoxanthine, xanthine and adenine for both the XBA RNA[113] and the guanine riboswitch aptamer.[54] The chemical structures of the ligands are also shown. C) The ligand binding site of the *xpt* guanine riboswitch aptamer (PDB ID: 1Y27). The guanine aptamer and the ligand binding site (inset) are shown. Nucleotides making direct H-bond interactions with the bound guanine are indicated.

aptamer.[42] While the asymmetric internal loop of the XBA aptamer is likely to be involved in ligand binding,[113] the *xpt* riboswitch achieves binding by using a series of stacking and hydrogen-bonding interactions with guanine. Moreover, the ligand specificity is achieved by the formation of a Watson–Crick base pair between the guanine ligand and a conserved cytosine (C74) located in the aptamer core (Figure 5 C). Extensive riboswitch–ligand interactions ensure that high affinity is achieved for the formation of the complex; this leads to dissociation constants in the low nanomolar range.[42] However, typical in vitro selection methods that make use of a ligand bound to a solid support represent a potential problem for the detection of high affinity aptamers since they do not allow the RNA to interact with all ligand functional groups, which is not the case for natural riboswitch aptamers.

Nevertheless, other ways that circumvent the use of a solid support for aptamer selection have been put forward. For instance, Nomura and Yokobayashi have developed a selection scheme that allows the re-engineering of the TPP riboswitch through an in vivo SELEX procedure.[116] In this work, the authors randomized a part of the expression platform of a negatively regulating riboswitch and selected for a riboswitch that allows gene expression in the presence of TPP. A very similar approach was previously employed by Lynch et al. to select for synthetic riboswitches that activate protein translation in the presence of theophylline.[117] Such approaches are very powerful in harnessing the huge possibilities offered by riboswitches as gene-regulation elements and could represent an improved way to develop artificial aptamers that mimic nature's work more efficiently.

The size of natural and artificial aptamers, which seems to be the most obvious difference, can also be explained by their respective aim. For instance, artificial aptamers are isolated solely by their ability to bind a particular ligand. However, riboswitches have a more complex role to play by achieving gene regulation upon ligand binding. Riboswitch regulation is possible through the global RNA reorganization and P1 stem stabilization induced by ligand binding, which require essential RNA–ligand interactions. This could explain the high variability in size and structure of natural riboswitches, which evolved to encompass their respective ligand. The extended conformation of the TPP riboswitch represents well this ligand–riboswitch coevolution: the RNA conformation allows two binding pockets to be formed, which interact with the 4-amino-5-hydroxymethyl-2-methylpyrimidine and the pyrophosphate moieties of the ligand.[118] However, recently discovered preQ1[47] and metal sensing[119] riboswitches seem to head off this generally observed correlation that exists between the size of riboswitches and their cognate ligand. Whilst the modified purine preQ1 binds to a single stem–loop riboswitch with great affinity, the M-box is composed of a more complex structure that binds a ligand as small as six magnesium ions; this suggests that many unexpected metabolite-binding RNA structures wait to be discovered and they most probably display higher affinity than known in vitro evolved aptamers.

6. Riboswitch Differential in vivo Gene Regulation

Each riboswitch class shows a high level of sequence and secondary structure conservation among its representatives. However, some differences are apparent in the aptamer regions, which could be linked to a different ligand-dependent gene regulation response as observed in two recent studies performed in *B. subtilis*.[53,120] In one of the studies aimed at analyzing differences in gene regulation for SAM riboswitches, it was shown that expression levels can vary for each riboswitch representative as a function of concentration and time.[120] When *B. subtilis* cells were put under starvation conditions, by using quantitative RT-PCR it was observed that mRNA levels of most riboswitches showed an increase after 30 min starvation period. However, in the case of *metK*, it was observed that gene expression initially increased during the first 30 min and then returned to prestarvation levels after 60 min. In addition, when gene expression was monitored by using *lacZ* transcriptional fusions, it was found that all but two genes are positively regulated in starvation conditions. The two candidates that

did not experience riboswitch regulation, *metK* and *cysH*, showed no induction but one of them exhibited an increase in gene expression during the first part of the methionine starvation, followed by a return to its basal expression. This observation could also suggest that this particular gene might be controlled at the transcriptional level by other mechanisms. From this in vivo study, the authors conclude that ten of the eleven SAM riboswitch variants found in *B. subtilis*, although functional, exhibit a high variability in gene expression levels, which is probably related to the gene function itself.

An additional study was recently performed on guanine riboswitches for which 89 aptamer representatives were analyzed by using sequence alignment and a representative subset was also studied biochemically.[53] By using in-line probing and 2-aminopurine fluorescence assays, it was shown that guanine aptamers exhibit a large binding affinity spectrum for which variations in affinity could be as much as 800-fold; this suggests that although a high homology in secondary structure is present, subtle sequence differences in the aptamer core domain have important consequences for the aptamer ligand binding activity. More precisely, when examining binding affinities for *xpt*, *purE* and *yxjA* aptamers present in *B. subtilis*, it was observed that whilst *xpt* and *purE* exhibit similar binding affinities, *yxjA* shows a 13-fold higher binding affinity. As a way to directly monitor the *B. subtilis* riboswitch gene regulation control in a biological context, a reverse transcript quantitative PCR (RT-qPCR) strategy was employed in which the ratio of the full-length mRNA versus the prematurely truncated transcript was determined. The rationale behind these experiments is that the riboswitch mRNA should be prematurely truncated when cells are grown in the presence of a sufficiently high guanine concentration, and inversely, the presence of the full-length species should be favored when cells are grown under conditions in which guanine is at low concentration. When growing cells are exposed to different concentrations of guanine, *xpt*, *purE* and *yxjA* representatives show a unique gene expression profile. For instance, while the *xpt* riboswitch does not inhibit gene expression in the presence of 0.25 mg mL^{-1} guanine, the *purE* representative shows a significant premature transcription termination (~20%), which is further enhanced (~80%) when the cells are incubated under saturating guanine conditions. Higher transcription termination efficiencies were obtained with the *yxjA* variant, which yielded ~55 and ~98% efficiencies at 0.25 mg mL^{-1} and saturating conditions, respectively. This suggests that guanine riboswitches show differential regulation in their gene control activity.

Since all three guanine riboswitch representatives are present in the same cellular environment, it might be informative to compare their thermodynamic properties and to correlate them with their gene-regulation activity. When estimating the relative free energies for both terminator and antiterminator structures,[53] the tendency to adopt the terminator structure ($\Delta G^{term}/\Delta G^{anti}$) can be established for each riboswitch (Figure 6). In principle, this value is an indication of the propensity of premature transcription termination under conditions in which the aptamer domain is fully bound, that is, when the intracellular concentration of guanine is high. It was observed that this

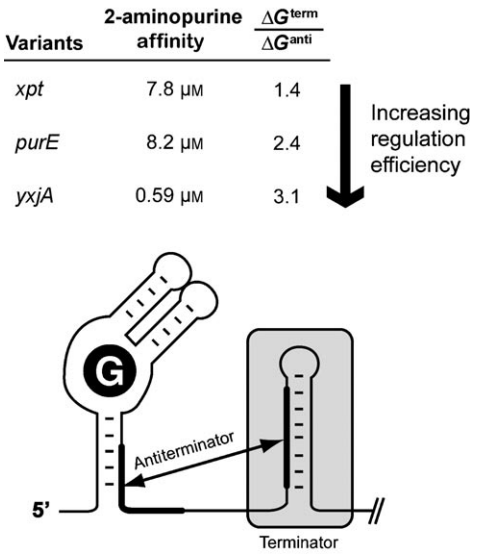


Figure 6. Gene regulation activity of selected *B. subtilis* guanine riboswitches. The ligand-binding affinity of the aptamer domain as well as the propensity to terminate transcription, are shown for *xpt*, *purE* and *yxjA* representatives. The increasing gene regulation control, as determined from the increase of both the ligand-binding affinity and the propensity for premature transcription termination, is shown by an arrow. A secondary structure representation of a guanine riboswitch is shown and both the terminator and the antiterminator structures are indicated. The relative free energies were calculated with the program mfold.[153]

propensity increases in the order *xpt* < *purE* < *yxjA*; this suggests that *yxjA* is the riboswitch representative that should be associated with the most efficient termination efficiency at saturating guanine concentrations. RT-qPCR data are in excellent agreement with this observation, which is consistent with the idea that the gene regulation process is mainly controlled by the RNA–ligand binding affinity. In addition, it appears that the affinity is directly proportional to the transcription termination propensity (Figure 6). Whether or not the ligand-binding affinity is an indicator of the gene regulation efficiency remains to be determined in other systems.

Lastly, of all guanine riboswitches found in *B. subtilis*, the *yxjA* representative is the only one that is not under the control of the PurR repressor, which is involved in transcription initiation regulation of several genes involved in purine synthesis, metabolism, transport and cofactor synthesis.[121,122] The affinity of PurR for its DNA control region is influenced by 5-phosphoribosyl-1-pyrophosphate (PRPP), which inhibits PurR–DNA binding in vitro and could thus be a transcription inducer in vivo.[123] Because *yxjA* gene regulation control does not rely on PurR transcription initiation control, it is tempting to speculate that a tighter riboswitch regulation control must be in place to ensure proper expression.

7. Riboswitch-Based Applications

7.1 Riboswitches as biosensor molecular tools

In principle, because the riboswitch binding site and expression platform domains are modular, one could envisage genetically altering one domain without affecting the function of the other. Doing so could enable the alteration of the ligand-binding specificity of the riboswitch while keeping the gene regulation control unaltered. Thus, by combining the possibility to develop novel aptamers by SELEX and the ability of riboswitches to control gene expression in a ligand-dependent manner, the generation of novel biosensors becomes readily attainable. By exploiting biological reporters, such as the green fluorescent protein, β-galactosidase or luciferase, synthetic riboswitches can be used to monitor the presence of virtually any metabolites in solution and thus provide molecular tools that can be tailored for very specific needs. One of the first examples for such a control in vivo was provided in an eukaryotic system where a small molecule aptamer was developed against the cell-permeable dye, Hoechst 33258.[124] By inserting the aptamer between the 5′-m^7G cap and the start codon, it was shown that the formation of a stable complex between the RNA and the dye precludes the ribosome-scanning process required for protein translation. Since then, various studies have explored the potential of synthetic riboswitches in bacteria as well as in eukaryotes.[117,125–136]

A high-throughput compatible assay was recently developed[136] with the *glmS* ribozyme,[26] which is specifically activated in the presence of glucosamine-6-phosphate (GlcN6P). In this study, Mayer and Famulok performed a model screening study using commercially available compounds to detect metabolites able to support the ribozyme self-cleavage activity, which would reveal to be a powerful approach against microbial agents carrying the *glmS* ribozyme element. For this purpose, the authors used a 5′-fluorescein labeled ribozyme and measured the fluorescence polarization (FP) under various experimental conditions. The principle of fluorescence polarization relies on the fact that low molecular weight molecules rotate and tumble at a faster rate than high molecular weight molecules.[137] It is expected that if the fluorescent ribozyme is cleaved in the presence of a competent metabolite, the tetramer reaction product carrying the fluorescence reporter will exhibit a significant reduction in the observed FP. However, no change in polarization should be detected in the presence of a metabolite unable to support catalytic activity. Of all the 88 tested compounds, none was found to support catalytic cleavage; this indicates that none could replace GlcN6P as a *glmS* ribozyme cofactor. Nevertheless, this study clearly demonstrates that high-throughput screening assays can be successfully adapted to identify novel antimicrobial drugs that target riboswitches, as well as any novel metabolites used for controlling gene expression.

Gallivan and co-workers have also employed an automated screening method to identify riboswitches exhibiting better performances in the signal to noise ratio.[117] They used a previously described theophylline aptamer,[138] which was fused to a β-galactosidase reporter gene in order to control the access of the ribosomal binding site. In this study, theophylline riboswitch mutants were developed by high-throughput screening in 96-well microplates by using randomized variants of theophylline riboswitches coupled to *lacZ* reporter in an *E. coli* expression system. This mutational study focused on the segment located between the aptamer and the ribosome binding site (RBS), and one mutant was found to increase the signal to background ratio from eight- to 36-fold. One of the best clones obtained, clone 8.1, displayed a predicted secondary structure for the sequestered RBS that was more stable than the clones in which the RBS was unpaired ($\Delta\Delta G = 5.5$ kcal mol^{-1}); this is consistent with the reduced background riboswitch activity that the authors obtained in their study. Moreover, when determining the free energy of theophylline binding, it was found that the free energy was lowered by 9.2 kcal mol^{-1}, which supports the high activation ratio that the riboswitch displays upon ligand binding. This study shows that in vitro selected aptamers can be readily converted into efficient riboswitches that perform gene regulation in a cellular environment.

7.2 Riboswitches as antibacterial targets

Bacterial resistance to antibiotics has been a growing problem during the last ten years, particularly in hospitals where the excessive and prolonged use of antibiotics has allowed some bacteria to acquire multiple drug resistance (MDR). A second cause that can explain the dramatic increase of bacterial resistance is that during the last 30 years, only one new antibiotic chemical scaffold was produced;[8] this has given pathogenic microorganisms an opportunity to circumvent the action mechanism of antibiotics. As the pharmaceutical industry chose to develop new antibiotics mostly by modifying existing ones, bacteria became more resistant to new compounds given their similarity to already existing ones. The limited number of cellular processes targeted further promotes this increase in bacterial survival.[8,139]

Riboswitches offer a tantalizing solution to the alarming and fast growing MDR problem. Given the high specificity of riboswitches towards ligands that do not change significantly during evolution, the aptamer domain to which they bind is highly conserved. Therefore, it should be possible to design ligand analogues that bacteria cannot metabolize, and thus shut down the expression of a particular riboswitch's regulated gene. However, mutations could be acquired that might disrupt the riboswitch gene expression control and therefore promote bacterial resistance. Precedents for this have been observed in the past for the adenine riboswitch.[122] *B. subtilis* mutant strains found to be resistant to the mutagen 2-fluoroadenine exhibited mutations that caused over-expression of the *pbuE* gene product. The over-expressing mutations were located in the *pbuE* adenine riboswitch where portions of the terminator stem were deleted, which prevented the OFF state of the riboswitch to be adopted and favored the constitutive expression of *pbuE*.

The compound *S*-(2-aminoethyl)-L-cysteine (AEC) has for a long time been known as an antimicrobial agent.[140] It was also established that AEC is incorporated into proteins, which could have toxic effects on the cell.[141,142] Various *E. coli* and *B. subtilis* mutant strains[143–145] resistant to AEC were found to cause derepression of *lysC* expression. The mutations were located in the lysine aptamer domain;[45] this suggests that the toxic effects might be due to the repression of aspartokinase expression following binding of AEC to lysine riboswitch. A *lacZ* reporter gene construct was used to monitor the lysine riboswitch control on expression when *B. subtilis* is grown in the absence or presence of lysine. Whilst β-galactosidase expression was inhibited in the presence of lysine in wild-type bacteria, no such inhibition was observed when experiments were carried out with AEC resistant mutants.

In an attempt to target the lysine riboswitch for antibacterial therapy, a recent study has explored the possibility to design new compounds that bind the riboswitch and suppress lysine biosynthesis and transport genes.[146] Four compounds were found to exhibit high binding affinity: L-3-[(2-aminoethyl)-sulfonyl]-alanine, L-4-oxalysine, L-homoarginine and D,L-*trans*-2,6-diamino-4-hexenoic acid, for which the dissociation constants of 2.5, 13, 7, 0.97 μM were found, respectively. Compared to values of 1 and 30 μM for lysine and AEC, respectively, these high-binding-affinity molecules show a high potential to be used as antibiotic compounds. Of these four compounds only three were able to inhibit bacterial growth.[146] These compounds were then tested for their ability to repress the expression of a β-galactosidase reporter and were found to be as efficient as lysine in inhibiting gene expression. To confirm that the antibacterial effects are due to the inhibition of *lysC* by the compounds, bacteria were selected for their resistance to L-4-oxalysine by serial passage. The *B. subtilis lysC* and *yvsH* lysine riboswitches present in L-4-oxalysine-resistant bacteria were sequenced and the observed mutations in the *lysC* riboswitch domain suggest that the effects were caused by the specific interaction between the antibiotic compound and the lysine riboswitch. Moreover, two resistant clones were characterized for their resistance to L-3-[(2-aminoethyl)-sulfonyl]-alanine and D,L-*trans*-2,6-diamino-4-hexenoic acid and for their ability to express β-gactosidase. As expected, clones were resistant to both molecules as no significant inhibition of β-galactosidase production was observed either in the absence or presence of ligands. Together, these results are consistent with the idea that the toxic effects are due to the inhibition of aspartokinase expression following the binding of the lysine analogues to the lysine riboswitch.

A second example is the elucidation of the pyrithiamine (PT) action mechanism.[147] PT is an isosteric pyrimidine analogue of thiamine known to be toxic for fungi and bacteria,[148,149] and is phosphorylated in cells to pyrithiamine pyrophosphate (PTPP).[150] PT was known to be an inhibitor of thiamine pyrophosphorylase and PTPP to be an inhibitor of enzymes that use TPP as coenzyme.[150] Nevertheless, this knowledge cannot explain the toxic effect of PT and its derivative on bacteria, which are able to synthesize TPP de novo and might compensate the inhibitory effect by up-regulating thiamine production.[151] The TPP riboswitch binds TPP with a dissociation constant of 100 nM whereas thiamine phosphate (TP), which only differs from TPP by one missing phosphate, exhibits a constant of at least 100-fold higher.[29] The riboswitch was shown to be able to prematurely stop transcription[31] and to repress the expression of β-galactosidase reporter under the control of TPP riboswitch.[29] It was also found that PT, and more efficiently PTPP, were able to bind the TPP riboswitch with a dissociation constant of 6 μM and 100 nM, respectively,[147] the latter being comparable to values obtained with TPP. The use of a β-galactosidase reporter has shown that PT is able to inhibit the expression of a gene under the control of the TPP riboswitch; this highlights its potential use as an antimicrobial compound. The DNA sequences coding for TPP riboswitches in resistant clones, which were obtained by serial passage, were sequenced and mutations were located. The introduction of these mutations in the context of a wild-type riboswitch, rendered the riboswitch inactive in response to elevated ligand concentrations. *ok?*

Another example was found for roseoflavin, which is an antibiotic involved in the inhibition of riboflavin biosynthesis.[152] Because of its similarity in structure with FMN, roseoflavin might potentially target the FMN riboswitch and control the operon involved in riboflavin biosynthesis.[35,41] It was recently reported that roseoflavin is able to bind FMN riboswitch in vitro.[8] As found in the other cases, when the bacteria were grown in the presence of analogues, mutations were found in FMN riboswitch domains; this is again consistent with the idea that riboswitches can be used as antimicrobial cellular targets.

8. Outlook

The fascinating discovery that certain structures found in mRNAs can recognize small metabolites by using a spectrum of biochemical interactions, and thus control gene regulation at different stages, has greatly contributed to the placement of RNA at the forefront of modern biology, and has highlighted its active role in many cellular processes and further expanded its apparently unlimited functional possibilities. Furthermore, because riboswitches appear to strictly rely on RNA to regulate gene expression, they have been proposed to be remnants of an ancient biological environment where RNA was the predominant functional species, before proteins emerged as main effectors. Several features of RNA switches, including their evolutionary conserved architecture, which has probably derived from the need to recognize metabolites that have remained unchanged through evolution, together with the presence of riboswitches in diverse organisms have led to a renaissance of the RNA World hypothesis.

Regardless of whether these structures represent relics of an ancient RNA-based world or are more recent additions to the biochemical repertoire, riboswitches constitute an emerging field with many exciting tasks to be accomplished in the future. Among these, it is crucial to elucidate in detail the interplay between RNA folding, ligand recognition and function in existing and novel structures, both at the post- and cotranscriptional levels. It is vital to establish the mechanistic basis

for riboswitch function and to determine how RNA polymerase pausing sites and perhaps, DNA template and RNA secondary structure features, can modulate whether a riboswitch is kinetically or thermodynamic controlled. Comprehensive research in this area would certainly enhance our understanding of RNA folding and function in general and will pave the way to successfully approach more complex RNA-containing structures, such as the ribosome.

One of the most fundamental questions that must also be addressed is whether or not additional riboswitches are present in eukaryotes. The fact that few eukaryotic riboswitches have been discovered should not be taken as a negative answer, but rather as an indicator that they are most probably involved in the control of different molecular processes than those found in bacteria. Striking examples of this have already been reported for splicing-controlling riboswitches; these suggest that even more complex structures wait to be discovered in eukaryotic organisms.

Given the increasing bacterial resistance to existing antibiotics, it is also a priority to explore the feasibility of riboswitch-based therapeutics. Because of their conservation, it might be possible to use the presence of riboswitches in pathogenic bacteria as a way to develop novel types of antibiotic compounds aimed at disrupting riboswitch cellular activity, provided that these representatives are not found in humans. Lastly, riboswitches can be viewed as promising gene-controlling elements that can be used to develop novel bimolecular tools or modules that could trigger specific cellular mechanisms in response to natural or artificial cellular metabolites.

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