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Riboswitch-Mediated Gene Regulation: Novel RNA Architectures Dictate Gene Expression Responses

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Abstract

Riboswitches are RNA elements that act on the mRNA with which they are cotranscribed to modulate expression of that mRNA. These elements are widely found in bacteria, where they have a broad impact on gene expression. The defining feature of riboswitches is that they directly recognize a physiological signal, and the resulting shift in RNA structure affects gene regulation. The majority of riboswitches respond to cellular metabolites, often in a feedback loop to repress synthesis of the enzymes used to produce the metabolite. Related elements respond to the aminoacylation status of a specific tRNA or to a physical parameter, such as temperature or pH. Recent studies have identified new classes of riboswitches and have revealed new insights into the molecular mechanisms of signal recognition and gene regulation. Application of structural and biophysical approaches has complemented previous genetic and biochemical studies, yielding new information about how different riboswitches operate.

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INTRODUCTION

The importance of RNA as a regulatory molecule in bacterial systems has become increasingly obvious. Many regulatory RNAs act in *trans*, either by base-pairing with a complementary region in the target mRNA or by sequestration of an RNA-binding protein (17, 59). Riboswitches, in contrast, are encoded as part of the mRNA for the gene they regulate and act in *cis*. These novel RNA elements directly sense a physiological signal and transmit that information to the gene expression machinery via a signal-dependent change in RNA structure (53, 57). Riboswitches are therefore defined by both specific signal recognition and a signal-dependent RNA structural shift, usually between two distinct states, i.e., an “on” state that allows expression of the gene and an “off” state in which expression is repressed.

The majority of riboswitches specifically bind a small-molecule ligand. The ligand is often the end product of a biosynthetic pathway, and the genes involved in its acquisition or synthesis are repressed when the ligand concentration is high, signaling to the cell that further biosynthetic capacity is unnecessary. Less frequently, riboswitches may respond to metabolites that serve as substrates for the regulated pathway, and expression of the pathway is induced when substrate concentration is high. Molecules as small as metal ions also can serve as ligands for riboswitches, which turn off pathways for their import or turn on genes that encode exporters to prevent toxic effects (3, 10, 11, 15, 55). An unusual riboswitch class monitors the aminoacylation status of a specific tRNA to regulate genes involved in amino acid metabolism (20, 24, 26). The simplest riboswitches, termed RNA thermosensors, utilize the temperature sensitivity of hydrogen bonds in the RNA secondary structure to regulate gene expression in response to changes in temperature (4, 30, 34). In each case, sensitivity of the riboswitch to its signal, and the resulting regulatory response, is tightly coupled to the cellular requirements for the products of the regulated genes.

Variations in riboswitch features affect specific ligand recognition, kinetics of ligand binding, ligand affinity, and the level at which gene expression is regulated. These variations increase the versatility of these elements and allow them to be combined with other regulatory systems to fine-tune gene expression or to integrate multiple signals. The riboswitch field is rapidly expanding, and investigation of new classes of riboswitches, coupled with in-depth analysis of previously

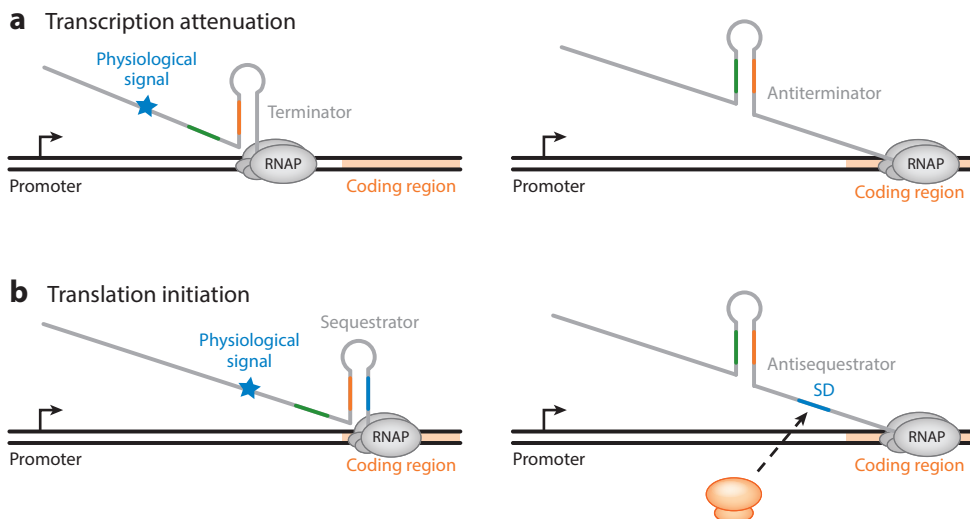


Figure 1

General mechanism of riboswitch gene regulation. (a) Regulation of transcription attenuation. (b) Regulation of translation initiation. DNA is shown as a black double line; the green and orange lines are RNAs that base-pair to form (a) the antiterminator and (b) the antisequester structures in the absence of the physiological signal. The blue line represents the Shine-Dalgarno sequence, which binds to the ribosome (two orange ovoids) in the absence of the physiological signal (blue star). Abbreviations: RNAP, RNA polymerase; SD, Shine-Dalgarno.

recognized elements, provides new insights into how these elements operate. This review describes some of the most common classes of elements and their impact on gene regulation.

REGULATORY MECHANISM

Most riboswitches control gene expression at the level of transcription attenuation or translation initiation (**Figure 1**). For transcriptional riboswitches, the physiological signal usually promotes the activity of an intrinsic transcriptional terminator, which consists of a G + C-rich helix, followed by a run of uridines in the nascent RNA that form a U-A RNA-DNA hybrid within the transcription elongation complex. Stabilization of the terminator helix in response to the signal triggers dissociation of RNA polymerase (RNAP) from the DNA template, which leads to transcription termination at a site upstream of the regulated downstream coding sequence. In the absence of the signal, the RNA folds into an alternate antiterminator, which sequesters sequences that would otherwise form the terminator helix; formation of the antiterminator structure therefore allows transcription of the downstream gene.

Some transcriptional riboswitches utilize Rho-dependent terminators instead of intrinsic terminators (27). Rho is a transcription termination factor that releases RNAP from the DNA template by binding to a Rho-utilization (*rut*) site in the nascent transcript. Binding to the *rut* site increases the ATPase activity of the Rho protein, which promotes translocation along the RNA toward RNAP, which is paused at a downstream site. Rho then unwinds the DNA-RNA hybrid at the active site of RNAP, terminating transcription. In riboswitches that utilize Rho-dependent terminators, interaction with the physiological signal results in structural rearrangements of the transcript that increase availability of the *rut* site for Rho factor binding, which ultimately leads to transcription termination.

Translational riboswitches replace the terminator helix of typical transcription attenuator riboswitches with a sequestrator helix that occludes the Shine-Dalgarno (SD) sequence by base-pairing with a complementary sequence and prevents ribosomal binding (**Figure 1b**). Formation of the sequestrator helix is usually stimulated by the physiological signal. In the absence of the regulatory signal, an alternate antisequestrator structure (analogous to the antiterminator in the transcriptional riboswitches) forms, which increases the availability of the SD region for ribosomal binding and allows initiation of translation.

Transcriptional riboswitches require cotranscriptional interaction with the signal, whereas signal interaction with translational riboswitches can be either co- or posttranscriptional (45). The time frame for this interaction is shorter in transcriptional riboswitches compared to translational riboswitches and depends on the rate of transcription (32, 45, 67). The regulatory decision in transcriptional riboswitches must be made prior to stabilization of the terminator because formation of this element is irreversible. Translational riboswitches can function as true reversible switches because the regulatory signal can potentially associate with the riboswitch multiple times during the lifetime of the transcript (58). Translational regulation provides a faster response to environmental stimuli owing to availability of the gene transcript for immediate translation. However, this advantage comes at an energy cost, as unnecessary transcripts may be generated that are inactive in translation.

Rarer classes of riboswitches regulate gene expression at the levels of mRNA stability or splicing. These can act via structural changes in the transcript that affect accessibility of the RNA to RNases or (in the case of the *glmS* ribozyme) via ligand-mediated activation of ribozyme activity, which in turn results in susceptibility to RNase J1 degradation from the newly generated 5' end (8). In the few eukaryotic riboswitches that have been identified to date, ligand-mediated structural rearrangements in a riboswitch element in the 3' end of the transcript or overlapping a splicing site internal to the transcript can affect mRNA stability or splicing, respectively (6, 31, 64).

Many riboswitches were first identified because of the presence of conserved sets of sequence and structural motifs upstream of genes that were either predicted to be coordinately regulated owing to a related function (e.g., in a particular biosynthetic pathway) or for which no function was known. Initial tests of riboswitch function have utilized the demonstration of ligand binding to purified RNA in vitro, ligand-dependent changes in the RNA structure using a variety of structural mapping techniques, or regulatory effects in vitro. For example, demonstration of termination at the predicted site in response to addition of the ligand (but not related compounds) to an in vitro transcription system provides clear evidence of the activity of a riboswitch that acts at the level of transcription attenuation. Similarly, inhibition of ribosome binding in primer extension inhibition (toe print) assays provides evidence of effects on translation initiation. In contrast, demonstration of effects on mRNA stability and splicing has relied primarily on in vivo assays.

PHYSIOLOGICAL SIGNALS

A wide range of signals can be utilized by riboswitches to modulate gene expression, including physical parameters (temperature, pH), small molecules, or specific tRNAs.

RNA Thermometers

The simplest riboswitches are RNA thermometers (RNATs), which respond to temperature changes to regulate gene expression at the level of translation initiation (30). The basic mechanism involves formation of a sequestrator helix at lower temperatures, which melts at higher temperatures to expose the SD region for ribosome binding and translation initiation

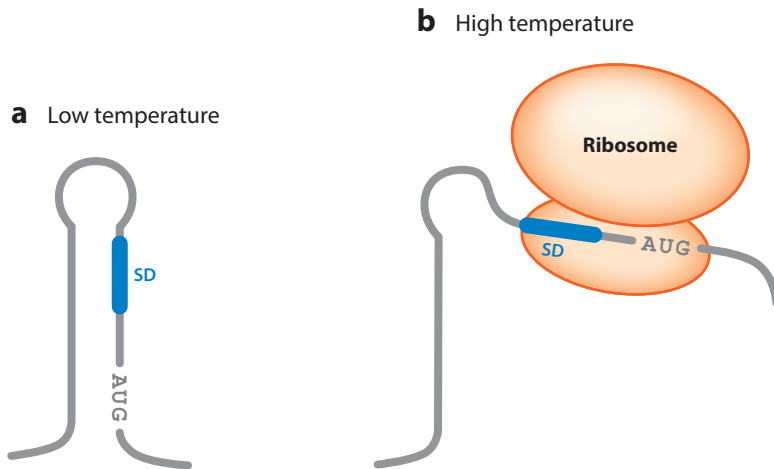


Figure 2

Regulation of gene expression by RNA thermometers. The Shine-Dalgarno (SD) region (blue) is unavailable for binding of the ribosome (shown as two orange ovals) at (a) lower temperatures but becomes accessible upon RNA helix melting at (b) higher temperatures.

(Figure 2). RNAT gene regulation depends on the balance between stabilizing (G-C base pairs) and destabilizing (internal loops and mismatches) elements in the sequesteror.

Although mechanistically simple, RNATs are important regulatory mechanisms for bacteria. For instance, human pathogens like *Yersinia pseudotuberculosis* and *Neisseria meningitidis* rely on RNATs to control their virulence (4, 34). Translation of the *Y. pseudotuberculosis* *lcrF* gene, which encodes a general transcriptional activator of virulence genes, is inhibited at 25°C but is induced at 37°C, allowing bacteria to sense entry into the mammalian host (4). Mutations that stabilize the sequesteror in the RNAT result in loss of virulence. Interestingly, mutations that destabilize the helix and allow continuous gene expression also result in reduced virulence, which demonstrates the importance of fine-tuned RNAT regulation for the *lcrF* gene.

pH Sensors

Several riboswitches that respond to pH have been identified. For example, the 5' region of the *Escherichia coli* *alk* gene, expression of which is induced under alkaline conditions, was shown to exhibit a pH-dependent shift in structure that affects downstream gene expression (43). At neutral pH, the RNA folds into a structure in which the SD sequence is sequestered, whereas high pH results in an alternate antisequesteror structure that allows ribosome binding and translation of the *alk* coding sequence. In this RNA, pH is proposed to affect RNA folding dynamics during transcription via effects on transcriptional pausing by RNAP. It is interesting to note that a sequence in the *yybP-ykoY* family, which has been proposed to be responsive to both pH and ATP (66), is found within the *alk* RNA.

Metabolite-Binding Riboswitches

The largest class of riboswitches identified to date binds to small molecules, and a wide variety of regulatory ligands has been identified (Table 1). The most common architecture of these

Table 1 Regulatory ligands of metabolite-binding riboswitches

Ligand class	Examples	References
Coenzymes and related compounds	Adenosylcobalamin	43
	Flavin mononucleotide	41
	Molybdenum/tungsten cofactors	48
	S-adenosylmethionine	9, 13, 40
	S-adenosylhomocysteine	65
	Tetrahydrofolate	2
Nucleotides and their derivatives	Thiamine pyrophosphate	42
	Adenine	37
	Adenosine triphosphate	66
	Cyclic diguanosyl-5' monophosphate	61
	Cyclic diadenosyl-5' monophosphate	44
	Deoxyguanosine	29
	Guanine	38
Amino acids	Prequeuosine-1	49
	Glutamine	1
	Glycine	39
	Lysine	23
Sugars	Glucosamine-6-phosphate	71
Ions	Fluoride	3
	Magnesium	10, 11
	Manganese	55
	Nickel/cobalt	15

riboswitches utilizes separate domains for ligand recognition (the aptamer domain) and the regulatory response (the expression platform) (5, 45, 53). The two domains are usually coupled by a shared region, and binding of the regulatory ligand to the aptamer domain results in stabilization of that domain, which affects the structure of the expression platform and therefore modulates expression of the downstream coding sequences.

A second less common architecture utilizes a single domain for both ligand binding and gene regulation. For example, the binding pocket for S-adenosylmethionine (SAM) in the S_{MK} box (SAM-III) riboswitch includes the SD sequence; the ligand makes base-specific contacts with residues within the SD sequence and thereby stabilizes a structure that blocks translation initiation (35). Riboswitches of this type are often smaller and simpler in structure than the two-domain riboswitches.

Binding to the regulatory ligand must be highly selective to prevent interaction with nonspecific small molecules in a sea of structurally similar cellular metabolites. High selectivity can be achieved by a combination of mechanisms, including shape complementarity, direct hydrogen bonding, stacking interactions, and recognition of both negatively and positively charged groups of the ligand (45). For example, riboswitches that respond to glycine contain a very small ligand-binding pocket that is not accessible to larger amino acids (28). In purine riboswitches, discrimination between guanine and adenine is achieved by a single nucleotide in the riboswitch that makes a direct Watson-Crick base pair with the regulatory adenine or guanine ligand (51). The regulatory ligand thiamine pyrophosphate (TPP) intercalates its aromatic ring in the bases of the TPP-responsive riboswitch, providing selectivity for the presence of the aromatic ring (52). The sulfonium group of SAM participates in electrostatic interactions with the O_2

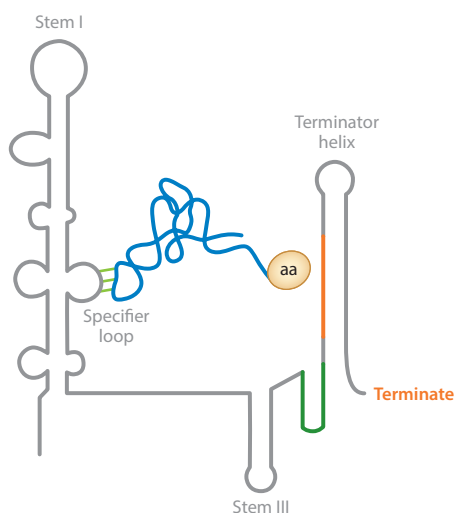
carbonyl oxygen atoms of uridines found in the binding pocket of SAM-binding riboswitches (16). This interaction is essential for differentiation between SAM and the biologically relevant *S*-adenosylhomocysteine, which lacks the sulfonium group. Although three structurally and evolutionary distinct SAM-binding riboswitches have been identified, all three utilize the sulfonium ion to discriminate against *S*-adenosylhomocysteine (46).

tRNA-Binding Riboswitches

An unusual class of riboswitches, designated the T box riboswitch, utilizes specific tRNA molecules as the regulatory ligand (20, 24, 26). These riboswitches regulate a variety of amino acid–related genes, expression of which is induced by binding of the cognate uncharged tRNA. Unlike most riboswitches, which bind a single ligand, T box RNAs also bind the aminoacylated form of the same tRNA, but only the uncharged tRNA can function as the inducer. The aminoacylated form acts as a competitive inhibitor of uncharged tRNA binding, and the regulatory system therefore monitors the ratio between aminoacylated and unaminoacylated forms of the tRNA, rather than the absolute amount of uncharged tRNA (72). The T box mechanism is widely used in gram-positive bacteria, and regulation is primarily at the level of transcription attenuation. However, variants that regulate at the level of translation initiation have been identified in *Actinobacteria* (54), indicating that either level of regulation is possible.

Specific tRNA recognition by T box riboswitches relies primarily (but not exclusively) on base-pairing between a triplet sequence, designated the Specifier Sequence, embedded in the riboswitch with the tRNA anticodon (**Figure 3**). The Specifier Sequence corresponds to the amino acid class of the downstream regulated gene and therefore dictates binding of the cognate tRNA (20). A

a High tRNA charging



b Low tRNA charging

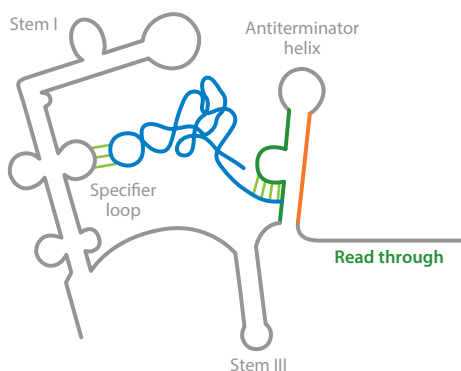


Figure 3

T box riboswitch gene regulation at the level of transcription attenuation. (a) During high tRNA aminoacylation, charged tRNA binds to the T box riboswitch RNA (blue strand) only at the Specifier Loop, and the intrinsic terminator forms, stopping transcription. (b) When tRNA aminoacylation is low, the uncharged tRNA (blue strand) interacts with the T box sequence and stabilizes a competing antiterminator structure, which allows continuation of transcription. Abbreviation: aa, amino acid.

second base-pairing interaction between the acceptor end of the tRNA (5'-NCCA-3') and the conserved residues in a seven-nucleotide bulge in the antiterminator (5'-UGGN-3', where the N residues co-vary to maintain base pairing) stabilizes the antiterminator and prevents formation of the more stable terminator helix, thereby allowing synthesis of the full-length transcript. In translational systems, this interaction stabilizes an antisequester that prevents formation of the sequester and allows translation of the downstream coding sequence. Aminoacylation of the tRNA prevents the stabilizing interaction via steric hindrance (74).

T box riboswitches are unique both in the nature of the ligand and in the ability to shift specificity of ligand recognition by a few nucleotide substitutions. This has resulted in the utilization of this mechanism for multiple different transcriptional units in the same organism, with the number per genome ranging from 1 to 40; they are especially abundant in the *Firmicutes* (25). The versatility of this riboswitch in terms of ligand recognition results in a major impact on the gene expression machinery in the cells in which they are widely used.

However, although Specifier Sequence–tRNA anticodon pairing is the primary determinant for tRNA recognition, in many cases it is not sufficient for interaction with a noncognate tRNA (22, 33). This indicates that determinants other than the known base-pairing interactions are important for tRNA recognition. These are likely to include both determinants in the riboswitch, including residues above the Specifier Sequence that interact with the tRNA elbow (18, 73), and determinants in the tRNA that may influence global tRNA structure (33).

KINETICS OF LIGAND BINDING

As noted above, riboswitches that act at the level of transcription attenuation have a very short window for interaction with the ligand, whereas translational riboswitches (and those that operate at the level of transcript stability or splicing) can operate either cotranscriptionally or posttranscriptionally. Ligand binding can be driven either by kinetic or thermodynamic parameters, and this has the potential to influence whether regulation can occur cotranscriptionally or posttranscriptionally. The flavin mononucleotide (FMN) riboswitch may be under kinetic control (67), whereas the adenine riboswitch appears to be affected by both kinetic and thermodynamic factors (32, 68). It is also likely that transcriptional pausing *in vivo* may contribute to the RNA folding pattern and ligand-binding interactions.

A variety of biophysical approaches, including fluorescence labeling (7), small-angle X-ray scattering (SAXS) analyses, and calorimetry (75), have provided new information about the RNA transitions induced in riboswitch structures by ligand binding. Single-molecule studies have been particularly valuable because of the ability to tease out sequential events that occur during folding of the RNA in the presence or absence of the ligand and ligand-mediated structural shifts (50). Application of these approaches, in conjunction with biochemical studies and structural analyses that provide information not only about the ligand-bound state (usually by X-ray crystallography) but also about the more flexible ligand-free state (often by NMR), is providing new insight into the mechanistic basis for ligand recognition.

An example of how these types of approaches can converge to illuminate new principles is provided by the SAM-responsive S_{MK} box (SAM-III) riboswitch. This small riboswitch, which contains a single module responsible for both ligand binding and gene regulation, has been analyzed by a variety of approaches (13, 14, 35, 36). It is notable that, in this translational riboswitch, SAM binding is highly unstable, leading to rapid transitions between the bound and unbound states (58, 69). Crystal structure analysis revealed a very compact structure, which was more open in the unbound state. NMR and isothermal calorimetry studies suggested the existence of intermediate structure(s) that prime the RNA for ligand binding (36, 69). This is suggestive of a

conformational selection mechanism, whereby the RNA transitions between a free state and one or more intermediate states more closely resemble the bound state. Interaction between the ligand and the RNA when it is in the intermediate state then results in binding. Locking the RNA into the bound state may then involve additional ligand-induced conformational changes that are consistent with an induced-fit mechanism, so that both conformational selection and induced fit are involved in formation of the final bound complex.

The proposed existence of intermediate structures is appealing as it provides an explanation of how residues that are physically distant in the unbound state can come together to assemble a binding site for a small ligand. This type of mechanism may also provide a rationale for the observation that different riboswitches of the same class, which retain all of the known determinants for recognition of the cognate ligand, may have very different affinities, as was observed for the S box (SAM-I) riboswitch (62). It is reasonable to suggest that residues outside the core-binding domain can contribute to affinity via effects on the formation or stability of the intermediate state.

VARIATIONS WITHIN A RIBOSWITCH CLASS

The abundance of riboswitches that have now been identified, and the development of bioinformatic tools (62), has revealed both variations across the riboswitch landscape and variability within a single riboswitch class.

Ligand-Binding Specificity

As noted above, subtle changes in riboswitch structure can result in major changes in ligand recognition. This is obvious in cases where ligand binding utilizes Watson-Crick base-pairing (e.g., the T box and purine riboswitches) and where changes in a small number of nucleotides are sufficient to alter the specificity of ligand binding. Changes in the ligand-binding pocket also can affect the ability of metabolite-binding riboswitches to discriminate between the cognate ligand and related analogs, for example, in the lysine riboswitch (70). Similar results have been observed when riboswitches have been studied as possible drug targets. The native riboswitch often discriminates effectively against related compounds, but subtle mutational changes can affect that selectivity (12). Analyses of this type can also be applied to development of synthetic riboswitches that are generated completely de novo using in vitro-derived ligand-binding aptamers and synthetic gene expression platforms or that combine natural and synthetic components to target gene expression in a variety of experimental systems (19).

Variability in Ligand-Binding Affinity

Along with changes in ligand-binding specificity, small changes in riboswitch sequence and structure also can affect affinity for the cognate ligand. A clear example of this is provided by the SAM-responsive S box (SAM-I) riboswitch, which is present in 11 copies in the *Bacillus subtilis* genome (21). Analysis of ligand-binding affinity in vitro, SAM-dependent transcription termination in vitro, and expression patterns in vivo demonstrated a 100-fold range in sensitivity to SAM concentrations (63). This is likely to result in hierarchical gene expression in vivo, where all of the transcriptional units are repressed when SAM pools are high and individual operons are induced in a stepwise manner as SAM pools begin to fall. It is interesting to note that genes involved in acquisition of methionine (a precursor for SAM biosynthesis) from the environment are induced first, whereas genes involved in methionine biosynthesis are induced only when SAM pools are very low. This allows the cell to gradually invest resources into the SAM biosynthesis pathway as

SAM levels are depleted. Tandem riboswitches, as are found for a subset of glycine riboswitches or in certain T box–regulated genes, also affect ligand sensitivity (39, 47).

Superimposing Regulatory Levels

Regulation of gene expression often utilizes multiple regulatory systems to monitor different regulatory signals. A classic example is provided by the carbon metabolism pathways, where both an operon-specific inducer (e.g., lactose for the *lac* operon) and a global regulator (e.g., CAP–cyclic AMP for carbon catabolite regulation) function together to ensure appropriate patterns of expression. Riboswitches can also function in concert with other regulatory systems. For example, the *B. subtilis ilv-leu* branched-chain amino acid biosynthesis operon monitors both leucine availability (via a tRNA^{Leu}-responsive T box) and isoleucine/valine (through the CodY transcriptional regulatory protein); this allows expression to be sensitive to levels of all three branched-chain amino acids (56).

There are a number of cases where riboswitches of distinct types are arranged in tandem. For example, the presence of both a SAM-responsive S box and a vitamin B₁₂-responsive AdoCbl riboswitch upstream of the *Bacillus clausii metE* gene, which encodes a B₁₂-independent form of methionine synthase, ensures that this gene is repressed when B₁₂ is available to serve as a cofactor for the alternate B₁₂-dependent form of the enzyme. The SAM-responsive riboswitch ensures that the gene is repressed by SAM even if B₁₂ is low, as SAM serves as a physiological signal of methionine abundance (60).

Structural Variants

Although most studies of a particular riboswitch class tend to focus on specific examples that are well behaved in vitro, it is not always clear that the properties of the model RNAs represent all members of the family. As noted above, SAM-responsive S box RNAs have a wide range of affinities for their ligand (63) and also show significant variability in structure, with insertion or deletion of structural elements surrounding the core ligand-binding elements (21). Similarly, S_{MK} box riboswitches vary in size from 80 to 500 nucleotides, with the extra residues present in one or two long helical extensions (13). These variations are likely to affect RNA folding dynamics and may therefore affect the kinetics of ligand binding.

The T box riboswitches, which are unique in that different subclasses utilize different tRNAs as their ligand, exhibit variations in structure that to a large extent correlate with the tRNA class. The largest class of T box RNAs exhibit the full set of features originally described (20, 25), including three large helices and a pseudoknot (in addition to the competing terminator and antiterminator). All tRNA^{Gly}-responsive genes lack one of these helices and the pseudoknot (24), whereas tRNA^{Ala}-responsive genes of both types have been identified (33). Genes that respond to tRNA^{Ile} also fall into multiple structural classes, including both RNAs that contain the full set of features and those in which a large segment of the helix containing the Specifier Sequence is absent. In these RNAs, the Specifier Sequence is located in a terminal loop rather than an internal loop, indicating that interaction with the tRNA anticodon must occur in a different structural context (54). It is interesting to note that the domain that is absent in this subclass of tRNA^{Ile}-responsive genes is responsible for a key interaction with the tRNA elbow domain in tRNA^{Gly}-responsive genes (18, 73). These structural variations suggest that different T box RNAs utilize diverse sets of interactions to achieve specific tRNA binding.

CONCLUSIONS

Riboswitches represent widespread mechanisms for gene regulation that have major impacts on bacterial cell physiology. Recent studies continue to identify new classes of these elements, as well as novel aspects of their biology and biophysical properties. Riboswitches are under development both as tools for manipulation of gene expression in a variety of biological systems and as targets for development of novel antimicrobial compounds. The amazing range of structural properties and ligand recognition properties illustrates the plasticity of RNA and the versatility of RNA-mediated regulatory systems. The riboswitch field has exploded over the past decade, and it is clear that new aspects of their activities remain to be discovered.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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