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Emerging Application of Riboswitch

Moammar Azeez

Biochemistry Department University of Health Sciences Lahore, Pakistan

ABSTRACT

Riboswitches were discovered in 2002 in bacteria as RNA-based intracellular sensors of vitamin derivatives. During the last decade, naturally occurring RNA sensor elements have been found to bind a range of small metabolites and ions and to exert regulatory control of transcription, translation, splicing, and RNA stability. Extensive biochemical, structural, and genetic studies have established the basic principles underpinning riboswitch function in all three kingdoms of life with implications for developing antibiotics, designing new molecular sensors, and integrating riboswitches into synthetic circuits. An expanding number of metabolite-binding riboswitch classes are being discovered in the non-coding portions of bacterial genomes. Findings over the last decade indicate that bacteria commonly use these RNA genetic elements as regulators of metabolic pathways and as mediators of changes in cell physiology. Some riboswitches are surprisingly complex, and they rival protein factors in their structural and functional sophistication. Each new riboswitch discovery expands our knowledge of the biochemical capabilities of RNA, and some give rise to new questions that require additional study to be addressed. This review analyzes the contemporary applications of riboswitch.

Keywords: Gene expression, Riboswitch, Aptamer, Gene regulation, Ligand.

INTRODUCTION

For many years, bacteria and phages were the source of RNAbased paradigms of gene regulation, ranging from transcription attenuation and thermosensing to small regulatory RNAs (sRNAs). Even after seemingly exhaustive genetic and genomic explorations, bacteria continue to instigate some major advances in RNA biology. A case in point are the regulatory networks in which specific regions of mRNAs termed riboswitches directly sense cellular metabolites to modulate transcription or translation of mRNAs, which typically encode proteins involved in the biogenesis or transport of the metabolites. Following their first description in 2002 [1] [2] [3], riboswitches have become recognized as important and widespread elements in the control of gene expression in numerous evolutionarily distant bacteria, with counterparts in archaea, plants, fungi, and algae.

The discovery of riboswitches showed that organisms had capitalized on this ability of RNA and put it to good use. Riboswitches are regions of mRNAs that contain specific evolutionarily conserved ligand-binding (sensor) domains along with a variable sequence, termed the expression platform that enables regulation of the downstream coding sequences. The term ‘‘riboswitch’’ reflects the ability of these noncoding RNAs to function as genetic switches. When the metabolite abundance exceeds a threshold level, binding to the riboswitch sensor induces a conformational change in the expression platform, leading to modulation of downstream events (Figure 1). Switching RNA states as a way to control gene expression is shared by other mRNA-based regulators (attenuators) associated with metabolite sensors in a form of proteins [4] or tRNA [5]. However, riboswitches are unique in their ability to directly bind diverse small ligands without intermediate molecules.

The known collection of riboswitch classes probably constitute only a tiny portion of the total that exist in the biosphere, and so many new classes are likely to be discovered in the future [6] [7]. Some of the characteristics established in published studies on riboswitches are sure to be very general, whereas other properties are noteworthy for their rarity and for the interesting capabilities they offer to the cells that carry these unique riboswitches. One of the most understandable features of a riboswitch is that the ligand-binding ''aptamer'' domain is its most conserved component. This makes sense because the aptamer is formed from only four types of nucleotides, and these must fold to form a precise sensor for a ligand (Figure 1A, top) that never changes through evolution. In contrast, the sequence and structure of the regulatory ''expression platform'' of each riboswitch can vary greatly [8] because there are far more ways in which simple RNA structures can influence such processes as transcription, translation, and RNA processing.

Another readily understood feature of bacterial riboswitches is that they are almost exclusively located in the 50 untranslated regions (UTRs) of the mRNAs whose expression they control. This arrangement permits the riboswitch to be synthesized first and gives time for the riboswitch to respond to metabolite binding before the full-length mRNA (or entire operon) is produced. The foremost mechanism for riboswitch-mediated gene control appears to be transcription termination. Bacteria favor this arrangement probably to save time and to avoid wasting energy producing full-length mRNAs that are not needed. Thus, RNA structural diversity, the relative ease of regulating mRNA expression, and the requirement for biochemical efficiency all influence the structures, genomic locations, and mechanisms of riboswitches. These and many other common characteristics of metabolite sensing riboswitches [9] [10] [11] have been revealed over the last decade. However, there are some very interesting puzzles and paradoxes that lie where knowledge becomes sparse. While the strange characteristics of some riboswitches are believed to have been resolved, others remain mysterious and await future resolution.

Structure of Riboswitches

Structurally, riboswitches are composed of two regions: (i) evolutionary conserved aptamer domain, which is responsible for ligand sensing and (ii) variable expression platform, controlling the gene expression. Genes controlled by the concentration of a given metabolite are usually engaged in its biosynthesis, metabolism or transport. A plethora of ligands binding to riboswitches, each recognized in accurate and specific manner requires a unique architecture perfectly matched to a given molecule. Additionally, different riboswitches belonging to one class may regulate the expression of more than one gene. For this reason, riboswitches reveal a huge diversity of structures. On the other hand, genes controlled by riboswitches are often involved in crucial metabolic pathways and cellular processes, thus the ligand-specific aptamer domains are usually strongly evolutionary conserved. Based on structural features, riboswitches can be divided into two large groups: (i) pseudoknoted and (ii) junctional riboswitches [12].

In the case of pseudoknoted riboswitches, the RNA chain is predominantly folded into a single knot-like structure, composed of two stem-loops, where the part of one loop is engaged in base paring with the second loop. Such structures are found, among others, in pre-queosine, SAM-II and fluoride riboswitches [13]; [14]; [15]; [16]. The ligand may interact with either the junctional region between helices or along the groove of the helix stabilized by pseudoknot. The second group, the junctional riboswitches, is composed of a central loop playing a role of multihelical junction and several radial helices. The number of helices is variable and ranges from three for purine and thiamine pyrophosphate (TPP) riboswitches [17] up to six for flavin mononucleotide (FNM) riboswitch [18]. The ligand binding site is usually located

within the junction or regions adjacent to it, however, in some cases, the ligand may interact with distant regions of the riboswitch.

Riboswitch structure-function

Riboswitches have two main components: an 'aptamer domain' and an 'expression platform'. The aptamer domain binds tightly to a specific metabolite (or ligand). Riboswitches are named based on their target ligands. For example, the theophylline and S-adenosyl methionine (SAM) II riboswitches bind specifically to theophylline and SAM, respectively. A riboswitch is a cis-acting RNA structure that is generally found in the 5^1 untranslated region (5^1 UTR) of an mRNA, with only the thiamin pyrophosphate (TPP) riboswitch known to exist in the 3^1 UTR [19]. These elements are divided into two groups based on their structural features: (i) pseudoknot-like SAM II riboswitches [20] and (ii) three-way junctions with distal tertiary contacts, such as the TPP riboswitch [21].

However, other exceptional tertiary structures have been discovered in some riboswitches, such as a four-way junction in SAM I [22], a tertiary docking interface in yybP-ykoy [23], an inverted junction that has a single aptamer in tetrahydrofolate (THF) with two metabolite binding sites [24]; [25], and interdomain interactions in glycine riboswitches [26]. All members of a riboswitch family recognize the same ligand and the family can be classified based on their folding features. For example, the SAM riboswitch family members all bind the SAM ligand, but the SAM I [27], SAM II [28] and SAM III [29] classes have a four-way helical junction, classic pseudoknot and three-way folding features, respectively.

The expression platform changes its structural conformation in response to binding of the metabolite to the aptamer domain. The refolding of the expression platform in response to the binding of the specific ligand is dose dependent and can either activate or repress the expression of the downstream open reading frame (ORF). This regulation can be exerted at the level of transcription [30], translation or (less often) alternative splicing [31]. Most riboswitches regulate the transcription of a gene either they prematurely terminate transcription by forming an intrinsic (Rho-independent) terminator structure, or they form an alternative 'anti-terminator' structure to enable transcription of the full mRNA [32]. Other riboswitches control the initiation of translation by sequestering (e.g. SAM II riboswitch) [33] or exposing (e.g. theophylline riboswitch) [34] the Shine–Dalgarno (SD) ribosome-binding site (RBS) to repress (Fig. 1c) or activate (Fig. 1d) gene expression, respectively.

Lastly, some riboswitches have been found to regulate splicing in prokaryotes or eukaryotes [35]; [36]; [37]. In bacteria for instance, in Clostridium difficiles strains the cyclic-di-guanosine monophosphate (GMP) II riboswitch responds to GMP II in a dose-dependent manner to promote the self-splicing of group I introns [38]; [39]. To our knowledge, only the TPP riboswitch has been discovered in eukaryotes so far [40]. The TPP riboswitch modulates alternative splicing in a fungus (Neurospora crassa), leading to the removal of an inhibitory upstream ORF (uORF) from the 5^1 UTR [4]. In plants, the TPP riboswitch alters splicing and alternative 3^1 UTR lengths of its mRNA by either occluding or exposing a splicing site at a low or high concentration of TPP, respectively [12].

Riboswitches as Tools For Regulated Gene Expression

Ligand-inducible expression systems are important genetic tools for common laboratory organisms such as *E. coli* and *B. subtilis*. However, many of these inducers (such as IPTG) are too expensive to be useful on the industrial scale. Natural riboswitches that are activated by amino acids may therefore represent an affordable alternative for such applications. Toward this goal, a tandem glycine riboswitch from *B. subtilis* was used for glycine-inducible production of β-galactosidase in *B. subtilis* cells. Although this system provided only 6-fold induction when glycine was added, it may be worthwhile to revisit this general

strategy if riboswitches with more ideal induction parameters can be developed.

Jin et al. proposed in a report that ligand-sensitive riboswitches may be useful genetic tools for the production of conditional hypermorphic mutants, particularly in cases where null mutants are lethal. To test this hypothesis, a theophylline-sensitive synthetic riboswitch was designed and used to regulate the chromosomal copy of an essential *E. coli* gene that is known to modulate motility (*csrA*). The creation of a *csrA-lacZ* fusion at this chromosomal position verified that expression levels were very low in the absence of theophylline, but approached wild type expression levels in the presence of theophylline. The authors further showed that the cells were non-motile in the absence of the inducer, but regained their motility phenotype in the presence of theophylline. These experiments fortuitously revealed that CsrA is a negative regulator of auto aggregation in *E. coli*. The ability of a synthetic riboswitch to permit reversible and tunable ligand-dependent gene expression of a protein over its native expression range suggests that synthetic riboswitches may find broad use in studying microbial genetics.

Riboswitches as Antimicrobial Targets

RNA is a primary target of many antibacterial compounds. While it has been known for quite some time that ribosomal RNA is an important antimicrobial target, it was recently discovered that some antibiotics whose mechanisms of actions were previously unknown may act, in part, by targeting riboswitches. Following up on observations that some roseoflavin-resistant strains of *B. subtilis* featured mutations within an FMN aptamer sequence, Lee et al. demonstrated that this naturally-occurring antimicrobial binds the FMN riboswitch as a major target. Additionally, it has been shown that two lysine analogs that repress the growth of some Grampositive bacteria (L-aminoethylcysteine and DL-4-oxalysine) also bind the *lysC* riboswitch of *B. subtilis*. Although the primary antimicrobial mechanism of these lysine analogs may not involve riboswitch-binding, Breaker and coworkers were inspired by this discovery and asked if they could identify new antimicrobials that target natural riboswitches. With the goal of identifying an antimicrobial compound that could specifically repress bacterial purine metabolism, they tested a panel of 16 guanine analogs for the ability to bind the *B. subtilis* guanine riboswitch. By pairing *in vitro* in-line probing assays with *in vivo* growth inhibition and reporter gene expression assays, they identified a specific analog that may inhibit *B. subtilis* growth by the intended mechanism. Although *B. subtilis* is not pathogenic, these advancements serve as a guide for future efforts to screen for riboswitch-binding antimicrobial agents in pathogenic bacteria.

Riboswitches as Boolean Logic Gates

While most natural riboswitches consist of a single aptamer domain and an expression platform, some natural riboswitches are more complex. Some riboswitches, such as the tandem TPP riboswitch from *B. anthracis* and the tandem glycine riboswitch from *B. subtilis*, have two aptamers that work together to produce a more digital response than can be achieved by riboswitches that employ only a single aptamer. Other complex riboswitches, such as the *metE* tandem riboswitches (SAM and AdoCbl) from *B. clausii*, function independently of one another to constitute a two-input Boolean NOR logic gate. In this system, high concentrations of SAM repress the *metE* operon, as well as other related operons, in a widespread effort to prevent further SAM biosynthesis. Additionally, AdoCbl independently represses the *metE* operon because this cofactor enables MetH to synthesize methionine more efficiently than MetE. Thus, high concentrations of either SAM or AdoCbl cause transcriptional termination of *metE* RNA. The net result is a NOR logic gate, whereby MetE is produced at high levels only when both SAM and AdoCbl are present at low concentrations.

These complex riboswitches have inspired others to develop synthetic logic gates to reprogram cell behavior and to engineer metabolic pathways. Shortly before the first riboswitch was reported, Jose et al. recognized that the catalytic benefits gained by cooperative binding in allosteric proteins might be harnessed in the RNA realm by constructing binary allosteric ribozymes. In an impressive demonstration of modular rational design, these researchers engineered a binary ribozyme that would self-cleave only in the presence of two effectors (theophylline and FMN). *In vitro* studies demonstrated that this binary ribozyme responds in a digital fashion, exhibiting little cleavage in the presence of a single effector, but providing a ~300-fold rate enhancement when both effectors are present.

Building upon these earlier efforts, Win and Smolke recently engineered several versions of binary ribozymes to obtain genetic logic gates that operate by Boolean logic. By incorporating the previously reported theophylline and tetracycline aptamers in various positions relative to a self-cleaving ribozyme, the researchers sought to construct AND, NOR, NAND, and OR genetic gates that might function within living cells. When tested in yeast, however, it was found that each of these allosteric ribozymes exhibited less than 3-fold modulation of reporter gene expression when the appropriate ligands were present. The modest performance of these ribozymes in cells compared to their *in vitro* counterparts might be improved by subjecting the ribozymes to an *in vivo* selection. Wieland et al. recently performed *in vivo* selections for allosteric ribozymes featuring improved modulation of reporter gene expression in bacteria, suggesting that this strategy might be extended to optimize binary riboswitches for *in vivo* applications.

With a similar strategy in mind, Yokobayashi and coworkers used their TetA dual genetic selection system to perform *in vivo* selections for complex riboswitches, which function in *E. coli* as AND or NAND Boolean logic gates. The AND gates exhibited particularly good properties, as gene expression remained low unless both theophylline and thiamine were present, which then enabled up to 18-fold induction of gene expression *in vivo*. These impressive results suggest that dual genetic selection may be a useful approach for creating Boolean logic gates in living cells.

Riboswitch-based Control of Bacterial Behavior

Because riboswitches are versatile tools for controlling gene expression, they can be used to reprogram a variety of bacterial behaviors. Bacterial chemotaxis has been studied extensively and is well understood at the genetic level. The ability to modulate bacterial motility in response to arbitrary chemical signals would provide new tools for bioremediation and drug delivery. *E. coli* chemotaxis system could be reprogrammed by placing a key chemotaxis signaling protein (*cheZ*) under the control of a theophylline-sensitive riboswitch. Reprogrammed cells would then migrate up gradients of this ligand and autonomously localize to regions of high theophylline concentration, which is a behavior that cannot be accomplished by the natural *E. coli* chemotaxis system.

Reprogrammed cells were then spotted at the bottom of the theophylline path. The population of reprogrammed cells migrated up the first portion of the theophylline path, and then made a right turn to continue following this ligand, without migrating off the path. This precise localization to the ligand represents a sharp contrast to the behavior of wild type *E. coli* (which do not stop moving, and thus cannot localize to a chemical signal), and such behavior may prove useful for targeting cells.

CONCLUSION

Many bacterial species, including well-studied organisms such as Bacillus subtilis and Escherichia coli, have a variety of riboswitch classes controlling key metabolic pathways. Since these bacteria grow rapidly and have the

potential to evolve rapidly, the presence of so many riboswitches suggests that these RNA domains are not primitive and ineffectual genetic elements, but rather are highly refined sensors and switches that are competitive with protein factors through evolution. Several interesting ideas have appeared so far to explore naturally existing or artificial riboswitches. The development of different analogs of native ligands finds potentially broad application in the field of medicine.

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