RIBOSWITCHES: CLASSIFICATION, FUNCTION and *INSILICO* APPROACH.

MEHTA NEEL B.[1*] and PRASHANNA BALAJI [1]

[1] Final year B.Tech student, Department of Bioinformatics, Maulana Azad National Institute of Technology – Bhopal (M.A.N.I.T.), Madhya Pradesh, India

ABSTRACT:

Extensive study on RNA in recent times has resulted into discovering a type of genetic regulatory elements which are found in number of organism and undergoes different folding and functional process leading to repression of gene expression, termed as Riboswitches. Riboswitches are present in three kingdoms of life and have been identified in the genomes of plants, archaea and fungi. They are highly structured cis-acting elements located in the 5'-UnTranslated Region (UTR) of mRNA in the organism and the most important characteristic of riboswitches is that they fold into complex three dimensional structures to serve as precise receptors for their target molecules, organized around helical junctions with varying degrees of complexity. The combinations of computational and molecular approaches have significantly led to the identification of an increasing number of existing and novel riboswitches. In this paper we have classified all main types of riboswitches according to their functional characteristics and various tools (software) are mentioned to identify them through *in silico* approach.

KEYWORDS:

Cis-acting elements, Gene expression, Riboswitches and UnTranslated Region (UTR)

INTRODUCTION:

Precise genetic control is an essential feature of living systems, as cells must respond to a multitude of biochemical signals and environmental cues by varying genetic expression patterns. Most known mechanisms of genetic control involve the use of protein factors that sense chemical or physical stimuli and then modulate the gene expression by selectively interacting with the relevant DNA or messenger RNA sequences. Recent studies have begun to reveal the substantial role that small non-coding RNAs play in selectively targeting mRNAs for gene expression and it has been discovered that certain natural mRNAs serve as metabolite-sensitive genetic switches wherein the RNA directly binds a small organic molecule. The term "riboswitch" was coined by Dr. Ronald Breaker in 2002 when he reported that mRNA-encoding enzymes involved in vitamin B1 and B12 biosynthesis in E. coli could bind associated metabolites without helper proteins being involved and they are RNA based components that integrate ligand binding and gene regulation to dynamically respond to molecular signals within cells [1]. *Figure1* is an example of a riboswitch that controls transcription [2]. When metabolite is not bound (-M), the expression platform incorporates the switching sequence into an anti-terminator stem-loop (AT) and transcription proceeds through the coding region of the mRNA. When metabolite binds (+M), the switching sequence is incorporated into the aptamer domain, and the expression platform folds into a terminator stem-loop (T), causing transcription to abort.

Riboswitches are divided in two parts: i) evolutionary conserved sensor domain (an aptamer) which directly binds small molecules and ii) an expression platform which undergoes structural changes in response to changes in aptamer. [3] Riboswitches regulate several metabolic pathways including the biosynthesis of vitamins (e.g. riboflavin, thiamin and cobalamin) and the metabolism of methionine, lysine and purines and alter gene-expression processes, including transcription termination and translation initiation by involving the formation of alternative structures. They are also metabolic binding domains within mRNAs to sense concentration of their corresponding ligands and upon a ligand binding, allosteric rearrangement of mRNA structure modulates gene expression. [4] These riboswitches have a specific pattern of ligand binding and its aptamer act in such a way that the production of the metabolite (usually protein) gets terminated by self destruction (as in the case of a specific gram positive bacterium that controls the sugar production for its cell wall by using aptamer that bind to their mRNA and self destructs). Comparing the taxonomic diversity of riboswitches and molecular mechanisms of regulation suggests that riboswitches represent one of the oldest regulatory systems. [5]

RIBOSWITCHES

Structure and Categorization of Riboswitch

A typical bacterial mRNA transcript controlled by a ribo-regulatory element such as a riboswitch is composed of three sections: the 5' UnTranslated region (5' UTR), the protein-coding region beginning with the start codon (AUG) and ending with a stop codon (UAA) and the 3' UnTranslated region (3' UTR) as shown in *figure2*. [6] Riboswitches are organized into families and classes according to two features: the type of ligand they bind, and their secondary structure (the arrangement of Watson-Crick paired helices). A family of riboswitches is typically a group of RNAs related by the ligands they recognize. Most common mechanisms of naturally occurring riboswitches are through regulation of transcription termination i.e. a metabolite binding to the aptamer domain of the riboswitch triggers changes in the expression platform, folding of a transcriptional terminator is stabilized as shown in *figure3*. [7]

According to **Rfam database**, there are 10 main Riboswitch classes as mentioned in *TABLE1*. [8, 9] Each of these riboswitches is classified and mentioned briefly below:

Enzymes cofactor group

TPP (THI Element): Thiamine pyrophosphate (TPP) represents one of the most intensively studied Riboswitch groups and is an essential cofactor in bacteria, archaea, and eukaryotes (*figure4)* [10]. TPP is the active form of thiamine (vitamin B_1), an essential coenzyme synthesised by coupling of pyrimidine and thiazole moieties in bacteria. Its production is tightly regulated by TPP-binding riboswitches, which have been identified in thiamine-biosynthetic genes in all kingdoms. Thiamine pyrophosphate-dependent Riboswitch from *E. coli thiM* RNA is comprised of an aptamer domain that binds the effector and an ‘‘expression platform’’ that is directly responsible for altering gene expression. Notably, it is also involved in the 3’-end processing of some plant mRNA. TPP-binding riboswitches were first identified in *Bacillus subtilis* and *Escherichia coli*. [11]

AdoCbl (Adenosine Cobalamin): It is one of the first to be discovered and one of the largest aptamer with many connecting points and it is a cis-regulatory element which is widely distributed in 5' UnTranslated regions of vitamin B_{12} (Cobalamin) related genes in bacteria. [12] Cobalamin in the form of adenosyl cobalamin (Ado-CBL) is known to repress expression of proteins for vitamin B_{12} biosynthesis via a post - transcriptional regulatory mechanism that involves direct binding of Ado-CBL to 5' UTRs in relevant genes, preventing ribosome binding and translation of those genes [13] *(figure5).* Its main functions are Cobalamin synthesis, aerobic and anaerobic ribonucleotide reductase, glutamate succinate fermentation and gene control.

SAM (S-adenosyl methionine: SAM riboswitch classes have been identified and characterized by using various genetic biochemical and bioinformatics methods. The first class of RNAs that was proven to be SAM-sensing riboswitches was SAM-I. Members of this riboswitch class were designated as S-box RNAs as they respond to small molecule effector via an unknown regulatory factor. Conserved structure features of some S-box RNAs suggest using them as a transcription termination mechanism. Moreover, genetic and transcriptional profile studies pointed to SAM as the most likely effector molecule. SAM-I riboswitches are exceedingly common in Firmicutes. SAM riboswitch is found upstream of a number of genes which code for proteins involved Cysteine biosynthesis in Gram-positive bacteria. Many SAM riboswitches are likely to regulate gene expression at the level of translation. The structure of the SAM riboswitch has been determined with X-ray crystallography *in figure6.* [14, 15]

SAM-IV: SAM-IV riboswitches are a kind of riboswitch that specifically binds S-adenosyl methionine (SAM), a cofactor used in methylation reactions. SAM-IV riboswitches are largely confined to the Actinomycetales and conserved features of SAM-IV riboswitch imply that they share a similar SAM-binding site to another class of SAM-binding riboswitches called SAM-I riboswitches. However, the scaffolds of these two types of riboswitch appear to be distinct. [16]

FMN (flavin mononucleotide): The FMN riboswitch in *figure7* [17] is a highly conserved RNA element that is found in the 5'-untranslated regions of prokaryotic mRNAs that encode for FMN biosynthesis and transport proteins. In Bacillus subtilis, the FMN riboswitch controls gene expression by causing premature transcription termination within the 5' UnTranslated region of mRNA.

Amino acid group

LYSINE: The Lysine riboswitch as shown in *figure8* is a metabolite binding RNA element found within certain messenger RNAs that serve as a precision sensor for the amino acid lysine. [18] Allosteric rearrangement of mRNA structure is mediated by ligand binding, and this result in modulation of gene

expression. This riboswitch is found in a number of genes involved in lysine metabolism and has also been identified independently and called the L box. [19]

GLYCINE: The bacterial Glycine riboswitch is an RNA element that can bind the amino acid Glycine. Glycine riboswitches usually consist of two metabolite-binding aptamer domains with similar structures. The aptamer cooperatively bind Glycine to regulate the expression of downstream genes. In Bacillus subtilis, this riboswitch is found upstream of the gcvT operon which controls Glycine degradation. [20] Glycine levels in a bacterial cell must be maintained at a certain baseline level to support protein synthesis.

PURINE: Purine riboswitches *(figure9)* consist of a highly conserved aptamer domain and an adjoining expression platform of about the same size. Sequences of the expression platform are more diverse to fulfil the specific requirements that arise from different functional determinants, such as transcriptional vs. translational regulation. Purine riboswitches are RNA structures that regulate protein biosynthesis in response to purines [21, 22]. Purine riboswitches are a class of riboswitches that recognises guanine and adenine. In Bacillus Subtilis, this mRNA motif is located on at least five separate transcriptional units that together encode many genes that are involved in Purine nucleotide synthesis.

Nucleotide bases group

GlmS Riboswitch: The Glucosamine-6-phosphate activated ribozyme is a RNA structure that regulates cellular production of Glucosamine-6-phosphate (GlcN6P) by controlling the production of the GlmS enzyme. When levels of GlcN6P are high, the ribozyme uses these molecules to catalyze its own cleavage which leads to the degradation of the mRNA that contains the ribozyme, and lowers the production of GlmS enzyme. When GlcN6P levels are low, the ribozyme does not cleave, and GlmS enzymes are produced at higher levels. GlmS ribozyme controls gene expression and its structure *(figure10)* has been determined by X-ray Crystallography [23, 24, 25].

PreQ1 riboswitch (pre-queuosine$_1$): The PreQ1 riboswitch as shown in *figure11* is a cis-acting element identified in bacteria which regulates expression of genes involved in biosynthesis of the nucleoside queuosine. This riboswitch binds $preQ_1$, an intermediate in the queuosine pathway. [26] The $preQ_1$ riboswitch is distinguished by its small aptamer, compared to other riboswitches.

Now, these 10 main types of riboswitches are predicted with the following main tools as mentioned below.

APPROACHES (TOOL and SOFTWARE) FOR PREDICTION OF RIBOSWITCHES

There are many computational tools (software) available for finding sequence alignment, structure prediction, sequence homology, phylogenetic analysis, etc. The tools which are mentioned below are commonly used to predict the riboswitches.

1) **Riboswitch Finder**

Riboswitch finder analyzes a given sequence using the web interface and then checks specific sequence elements and secondary structure, calculates and displays the energy folding of the RNA structure. It has batch-mode determination and the program is available on http://www.biozentrum.uniwuerzburg.de/bioinformatik/Riboswitch/. [27]

2) **RibEx** (Riboswitch Explorer)

RibEx (Riboswitch explorer) is a web server which searches any sequence for known riboswitches and for other predicted ones, but they are highly conserved, bacterial regulatory elements. Visual inspection of the identified motifs in relation to attenuators and open reading frames (ORFs) is made possible. RibEx is available at www.ibt.unam.mx/biocomputo/ribex.html. [28]

3) **CMfinder** (Covariance model based RNA motif finding algorithm)

This tool performs well on unaligned sequences and also when the motif is only present in a subset of sequences. It is an expectation maximization algorithm using covariance models for motif description, carefully crafted heuristics for effective motif search, and a novel Bayesian framework for structure prediction combining folding energy and sequence co variation. CMfinder also integrates directly with genome-scale homology search, and can be used for automatic refinement and expansion of RNA families. The webpage of CMfinder is http://wingless.cs.washington.edu/htbin-post/unrestricted/CMfinderWeb/CMfinderInput.pl [29]

4) **RiboSW**

RiboSW is a tool for searching putative riboswitches in a sequence. The tool characterizes each riboswitch as two crucial parts, the RNA secondary structure and the functional region to search putative riboswitches. RiboSW is capable of dealing with the compensatory mutation of the base-paired regions. The program is available at http://ribosw.mbc.nctu.edu.tw/. [30]

Kinetic Control of Riboswitches:

Riboswitches can reversibly switch back and forth between an 'on' state and an 'off' state, depending on the concentration of the ligand. When the ability to repress (or activate) gene expression is dictated by its inherent affinity for the ligand, a riboswitch is said to be thermodynamically controlled. In contrast, functional studies of a number of riboswitches have revealed that they require a much higher ligand concentration to be activated, and alter the level of gene expression. Evidently because riboswitches do not reach equilibrium with the ligand, the analogy is more like that of a fuse than a switch termed as '*ribofuse'*. [31] Given these observations about ligand concentration, ligand-riboswitch binding, and riboswitch activation, scientists have proposed a more plausible model, stating that riboswitches are under kinetic control. Recall that a kinetically controlled event is one that depends largely or entirely on the rate-limiting step of a chemical process. For riboswitches, we must understand the relationship between the rates of transcription, RNA folding, and ligand binding. During transcription, the aptamer domain is always the first part of the RNA to be synthesized and fold into a shape capable of binding ligand. After completion of the aptamer domain, often a programmed pause site in the mRNA causes the polymerase to temporarily stall *(Figure12)*. [32] This short pause (actually measured to be 2–10 seconds) gives the aptamer domain time to interrogate the cellular environment for the presence of ligand. If a sufficient concentration of ligand is present, then the ligand will occupy the binding pocket prior to resumption of transcription. Often a second pause site midway through the expression platform gives the RNA time, if ligand has not bound to reconfigure the RNA secondary structure.

The kinetic control component of this process is the balance of rates of several processes: (1) speed of riboswitch transcription, including time spent residing at any potential pause sites; (2) ligand binding; and (3) a possible secondary structural rearrangement. Because several studies have shown that the rate of ligand binding to the aptamer domain is slow, the cellular concentration of ligand must be far greater than the dissociation constant (K_D) in order for binding to be sufficiently rapid to beat the kinetics of transcription. [33] In *figure12* it shows a kinetically controlled riboswitch that regulates mRNA transcription.

Riboswitches: Sensing Metabolic Signals

Riboswitches interact with the cognate regulatory signal which determines whether the RNA folds into the helix of an intrinsic terminator, resulting in premature termination of transcription. Similar RNA rearrangements mediate translational regulation by sequestration of the ribosome binding site. We have identified several systems of this type, including the T box system, which monitors the charging ratio of a specific tRNA, the S box and SMK box systems, which respond to S-adenosyl methionine (SAM), and the L box system, which responds to lysine. Each class of riboswitch RNA recognizes its signal with high specificity and an affinity appropriate to the in vivo pools of the effector. [34, 35] Characterization of the RNA-effector interaction in these systems has provided new information about how different classes of effectors are recognized, and about the impact of these regulatory mechanisms on the cell.

CONCLUSION and FUTURE PROSPECTS:

Riboswitches (genetic regulation networks) can be applied to biosensors, metabolic engineering of organisms and in gene therapy treatments. It has shown the potential to act as a drug target against various bacterial diseases (e.g. Tuberculosis, which is caused by mycobacterium tuberculosis bacterium). Here, Riboswitches acts as a metabolite – sensing switch, turning 'on' and 'off' depending on the cellular concentration of the ligand. A powerful antibacterial strategy would be to design metabolite mimics that bind to a riboswitch receptor, shut off expression of the adjoining genes, and starve the cell of important metabolites generating analogues of the ligands that bind to riboswitches and regulate key metabolic pathways may become a new approach for developing antimicrobial agents.

Modified versions of these natural "riboswitches" (created by using various nucleic acid engineering strategies) can be employed as designer genetic switches that are controlled by specific effector compounds. A priority for future studies is to characterize the self – cleavage activities of recently identified genome encoded riboswitches, as well as candidate ribozymes and RNA sensor sequences, that might reveal new aspects of genetic regulation by RNA elements. More research and projects can be done on finding riboswitches in 3'

region of mRNAs which can be involved in novel types of regulation. These biochemical and genetic studies should be supplemented by searches for novel RNA sensors and ribozymes using new computational approaches that can identify candidate RNA sequences with low sequence homology. Despite considerable progress in studies of ribozymes and riboswitches at the atomic level, future research must resolve the uncertainties in three dimensional structures of various riboswitches. [36, 37, 38, 39] Comprehensive research in this area would enhance our mechanistic understanding of the biological function of these molecules and more complex RNA-containing cellular machineries such as the spliceosome, the ribosome and telomerase.

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TABLE1: Classification and distribution of different Riboswitch classes.

Group	Member	Main functions	Natural ligand	Size (nucleotides)	Distribution
Enzyme cofactor	TPP	Thiamine synthesis, phosphorylation, gene control	TPP	100-120	Alpha-proteobacteria, Beta-proteobacteria, Gamma-proteobacteria, Epsilon-proteobacteria, Delta-proteobacteria, Archaea, Eukaryotes (fungi, plants)
	AdoCbl	Cobalamin synthesis, aerobic and anaerobic ribonucleotide reductase, glutamate and succinate fermentation, gene control	AdoCbl	200-220	Proteobacteria, Cyanobacteria and Archaea
	SAM (I)	Methionine and Cysteine biosynthesis, SAM synthesis, Metabolite transport, methylene tetrahydrafolate reductase and uncharacterized genes	SAM	105-125	Mostly gram+ bacteria, Gamma-proteobacteria and Delta-proteobacteria.
	SAM Alpha	Gene control	SAM Alpha	70-90	Alpha proteobacteria
	SAM - IV	Gene control	SAM – IV	115-135	Bacteria
	FMN	Riboflavin biosynthesis, gene control	FMN	120-140	Alpha-proteobacteria, Beta-proteobacteria, Gamma-proteobacteria,
Amino acid	Lysine	Lysine Synthesis and lysine catabolism	Lysine	165-190	Gamma-proteobacteria, Thermotogales, Firmicutes
	Glycine (I+II)	Glycine catabolism and efflux and gene control	Glycine	100-120	Alpha-proteobacteria, Beta-proteobacteria, Gamma-proteobacteria
Nucleotide bases	$preQ_1$	Gene control	$preQ_1$	25-45	Bacteria
	Purines	Purine synthesis and transport and gene control	Guanine, Adenine	60-80	Gram+ bacteria, Gamma-proteobacteria and Delta-proteobacteria.

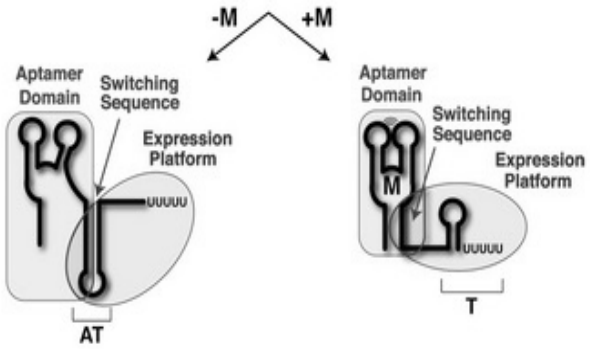

Figure1 - Riboswitch domains

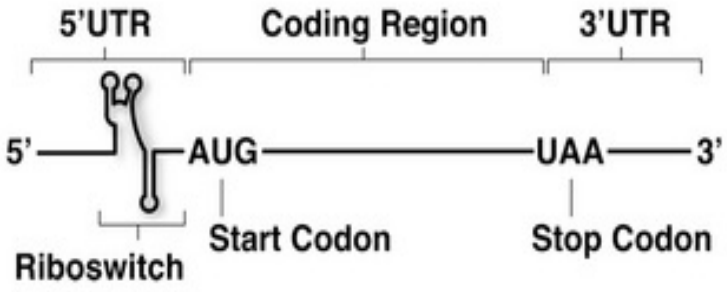

Figure2: mRNA anatomy

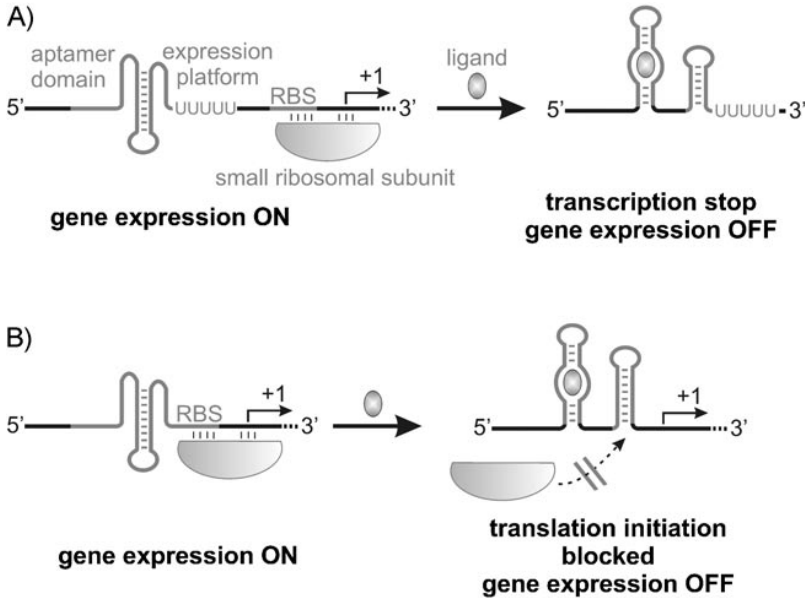

Figure3: Mechanism of Riboswitch

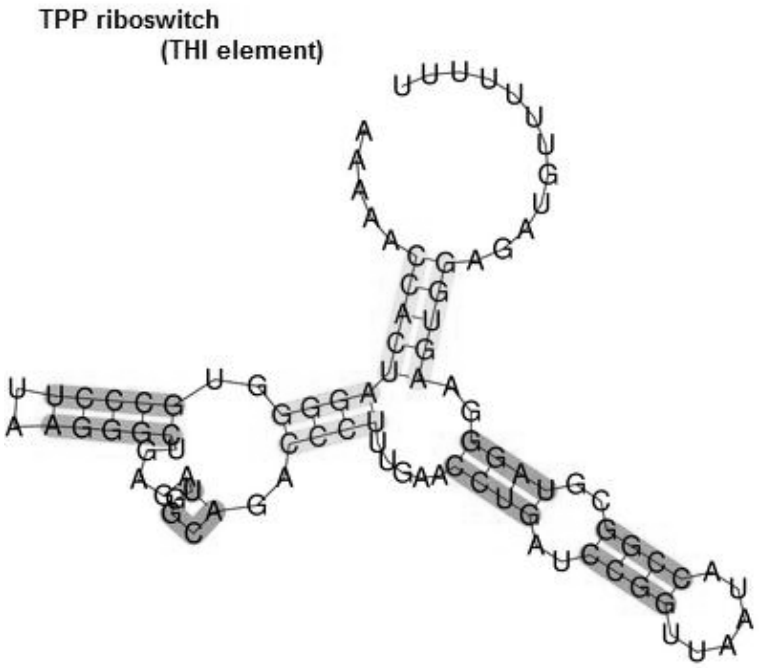

Figure4: TPP riboswitch

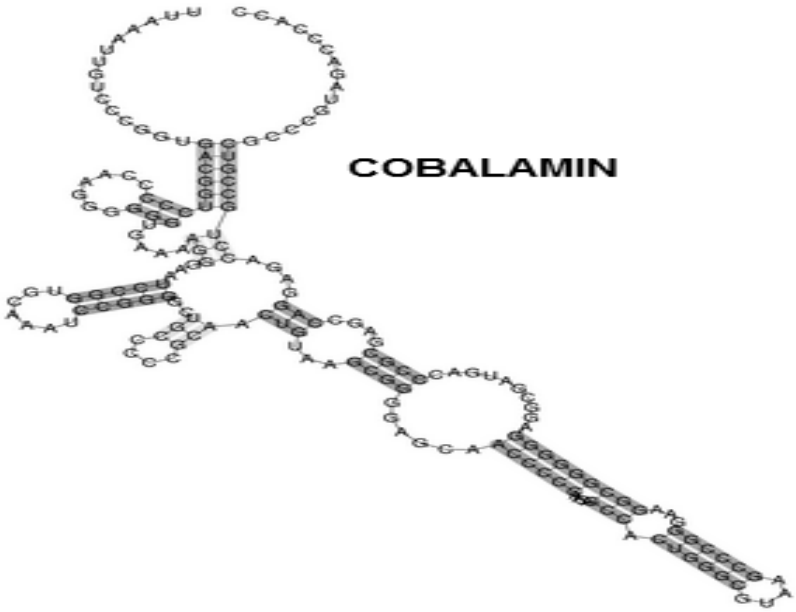

Figure5: Structure of Cobalamin Riboswitch

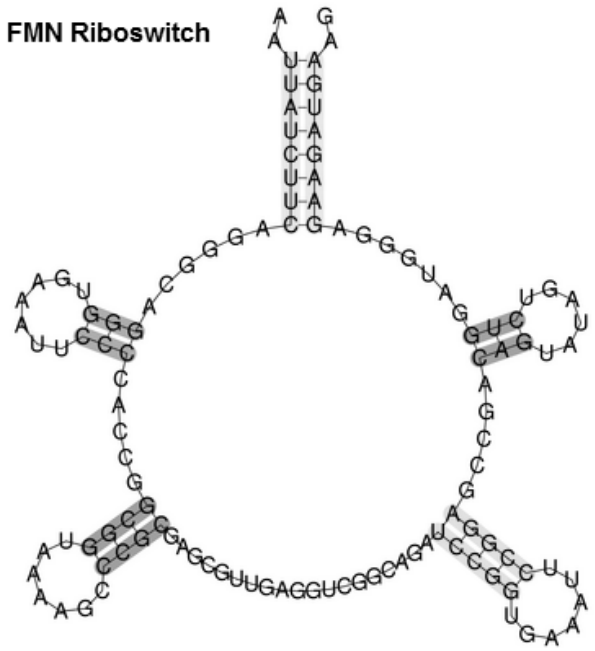

Figure6: SAM Riboswitch

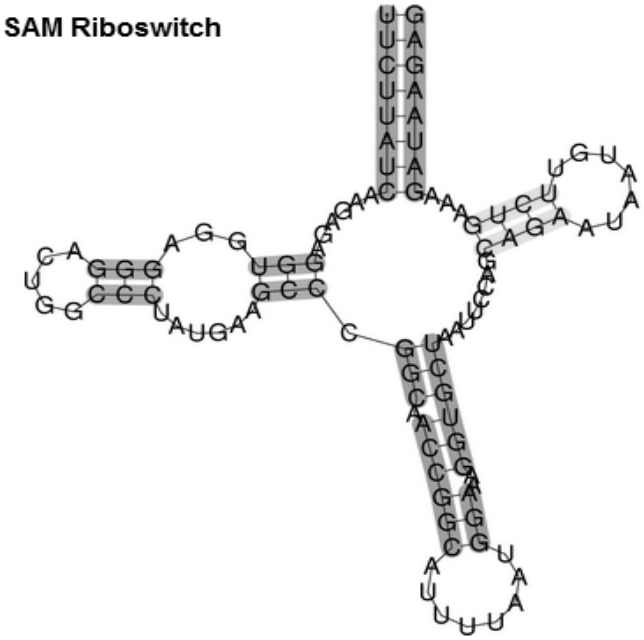

Figure7: FMN Riboswitch

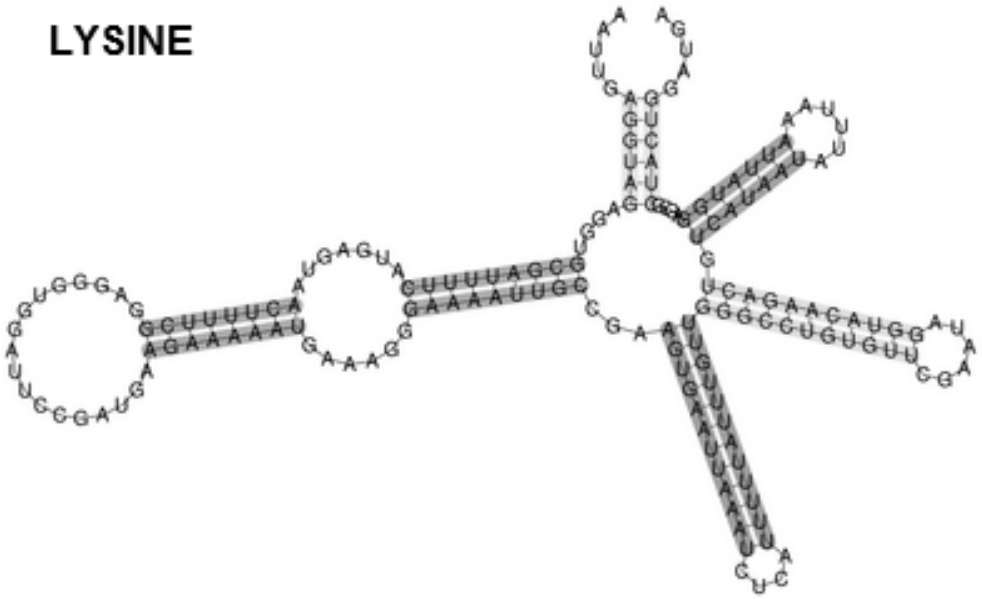

Figure8: LYSINE

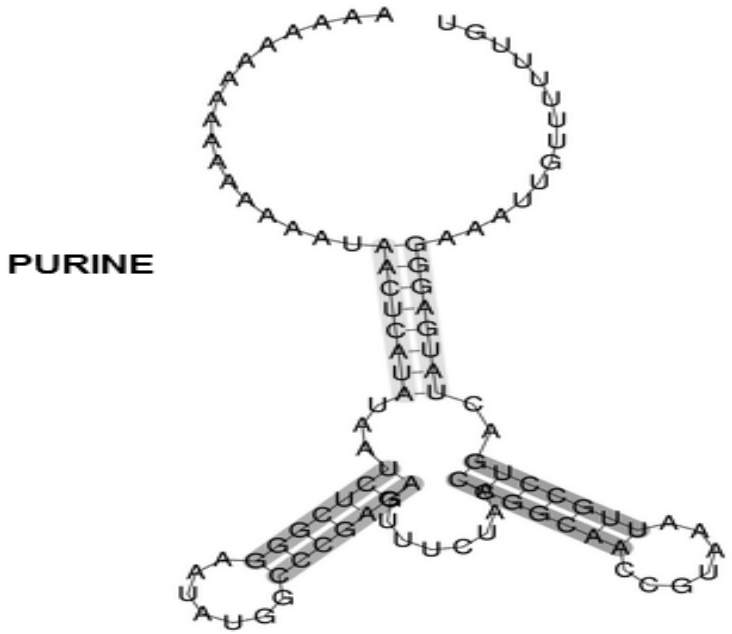

Figure9: PURINE

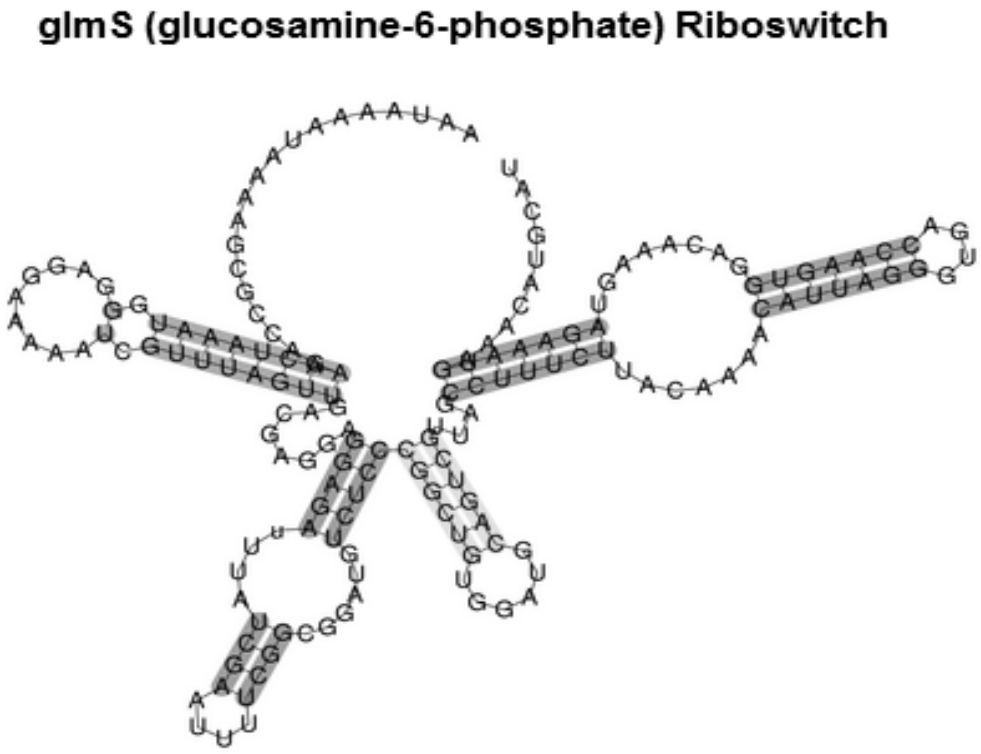

Figure10: glmS Riboswitch

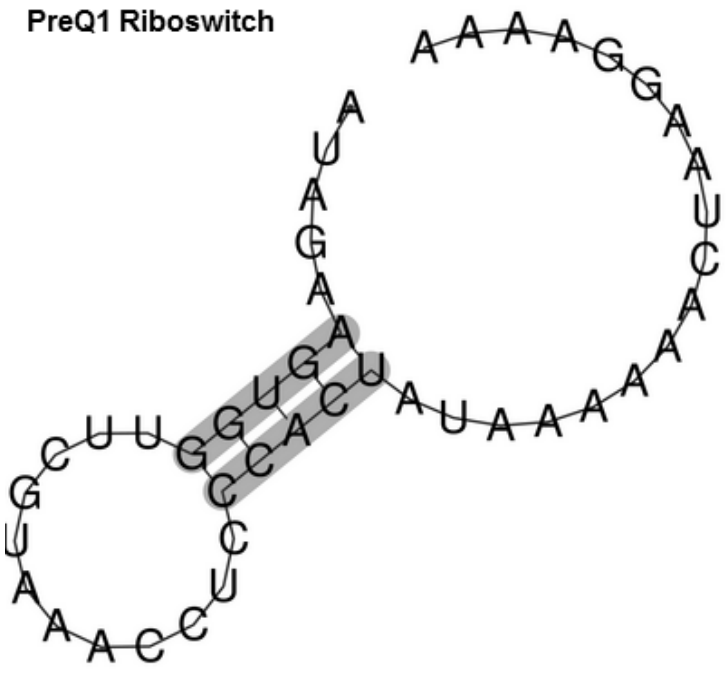

Figure11: PreQ1 Riboswitch

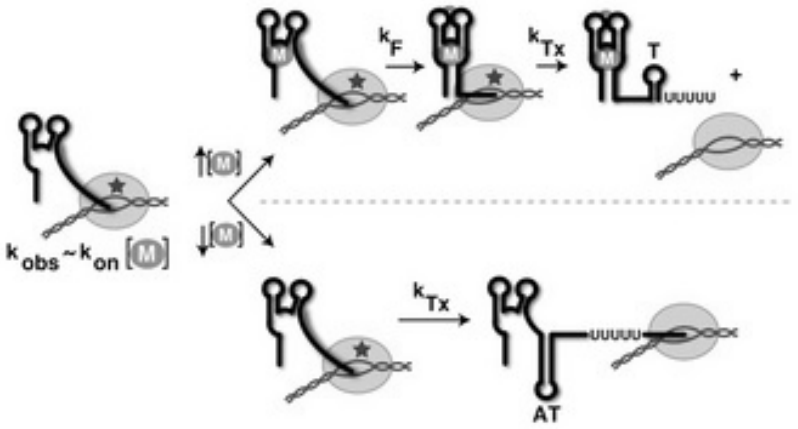

Figure12: A model of kinetically controlled riboswitches.