The riboswitch control of bacterial metabolism

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Aptamers are artificial nucleic acids that selectively bind small molecules. In the past two years, it has become clear that nature has already devised its own aptamers that play important regulatory roles. RNA sensors have been discovered in both Gram-positive and Gram-negative bacteria that function as molecular switches in response to direct binding of structurally diverse metabolites. These natural RNA aptamers, called 'riboswitches', are imbedded in the leader sequences of numerous metabolic genes. Riboswitches are able to repress or activate their cognate genes at both transcriptional and translational levels. Here, we summarize the recent progress in the identification and characterization of riboswitches and discuss their evolution and distribution.

Organisms use RNA in a wide range of regulatory mechanisms to control gene expression. The classical examples of such regulation are transcription and translation attenuation in bacteria. The common principle behind these mechanisms is that some external event, usually associated with the metabolic status of the cell, controls the formation of a stem–loop RNA structure that either terminates transcription prematurely or sequesters the Shine–Dalgarno (SD) sequence and inhibits translation initiation. Traditionally, protein factors have been implicated in regulation of these processes. For example, in *Bacillus subtilis* the *trp* RNA-binding attenuation protein (TRAP) regulates tryptophan biosynthesis by participating in both transcription attenuation and translational control [1]. In transcription attenuation, TRAP is responsible for the decision to terminate transcription of the *trpEDCFBA* operon leader region or to allow transcription to proceed to the structural genes by sensing the level of tryptophan in the cell [2]. TRAP also regulates translation of *trpE* and *trpG* genes. In the case of *trpE*, TRAP binding promotes formation of an RNA structure that sequesters SD, whereas in trpG, it binds to a portion of the RNA that contains the SD sequence [3]. In all three situations, TRAP functions by binding to RNA targets in a tryptophan-dependent manner. The Bgl family of antitermination factors is another classical example of RNA-binding proteins that regulate expression of various catabolic operons in Gram-positive and Gram-negative bacteria [4].

There are many other specific regulatory proteins that bind RNA in the metabolite-depended manner to control transcription elongation or translation initiation [5,6]. Like TRAP and Bgl, such proteins modulate the formation of alternative RNA structures that act as the intrinsic transcription terminator or SD sequestor, or alternatively, as the antiterminator or antisequestor. Often, the portion of the leader that binds the factor can form a third structure – the anti-antiterminator or anti-antisequestor – which enables the protein to control transcription termination or translation initiation at a large (100–300 nucleotide) distance from the terminator or ribosome-binding site (RBS), respectively. For example, in *Escherichia coli*, the translating ribosome functions as a tryptophan sensor instead of TRAP. It pauses within the leader message in the absence of tryptophan and prevents the formation of the anti-antiterminator, which would otherwise promote the formation of the terminator stem–loop structure [7].

More recently, it was found that uncharged tRNA might substitute for the protein in controlling transcription attenuation in *B. subtilis*. Many aminoacyl-tRNA synthetase genes and several operons involved in amino acid biosynthesis possess the so-called T-box module within their untranslated leader transcript. The read-through of the intrinsic terminator of this leader occurs when the charged to uncharged ratio of a particular tRNA is decreased, signaling the deficiency of the corresponding amino acid. It is presumed that uncharged tRNA makes two specific contacts with the T-box. Codon–anticodon pairing with the upstream segment of the T-box assures specificity, whereas the antiterminator–acceptor pairing stabilizes the antiterminator and prevents formation of the competing terminator [8].

In this review, we describe a new class of regulatory RNA that needs the same principles of alternative structure formation to control transcription elongation and translation initiation depending on the metabolic status of the cell. The uniqueness of these RNA systems is that they do not require any intermediary sensory molecules (i.e. protein factors or tRNA) to govern the attenuation process; they behave as sensors of small molecules themselves. Such natural RNA aptamers, also known as riboswitches, appear to control expression of a wide spectrum of metabolic genes in bacteria and possibly in higher organisms as well.

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http://tibs.trends.com 0968-0004/$ - see front matter doi:10.1016/j.tibs.2003.11.004

The riboswitch control of vitamin metabolism

The first evidence for the existence of riboswitches came from genetic and biochemical studies on the feedback regulation of vitamin biosynthetic operons in *B. subtilis*, *E. coli* and *Rhizobium etli*.

Flavin mononucleotide-sensing riboswitches

Riboflavin (vitamin B2) is a precursor of the essential coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). In Bacilli, the riboflavin operon (*ribGBAHT*) consists of five genes that encode enzymes for riboflavin synthesis from GTP [9]. The operon has an untranslated regulatory leader region of ~300 base pairs in front of its first gene. The leader is responsible for negative regulation of the operon by flavins. Intensive genetic and genomic studies of the *rib* leader in *B. subtilis* and other Gram-positive bacteria revealed the evolutionarily conserved regulatory element called *rfn* [10]. It folds into a characteristic conserved structure with five hairpins. Mutations in the *rfn*-box prevent the repression by flavins and lead to riboflavin overproduction [11]. The *rib* leader also contains a classical intrinsic terminator (a stable hairpin followed by a stretch of U residues) and two additional elements within the *rfn*-box – the 5′-proximal antiterminator and anti-antiterminator, which prevents the antiterminator from interfering with the terminator (Figure 1a). A separate gene *ribC*, which encodes a bifunctional flavokinase/FAD synthetase, was also implicated in the *rib* operon regulation, suggesting that FMN and/or FAD serves as a co-repressor [12,13]. Because no protein candidate has been found that binds the *rfn*-box in a FMN- or FAD-dependant manner, a model was proposed whereby direct binding of FMN or FAD to the *rfn*-box promotes the formation of the terminator by changing the structure of *rfn* [9,10]. Recently, this model was directly confirmed by using a reconstituted transcription system with highly pure *B. subtilis* and *E. coli* RNA polymerases lacking any additional factors [14]. The presence of FMN in low micromolar concentration was sufficient to potentiate transcription termination *in vitro*. FMN-mediated termination was strictly dependent on the intact *rfn*-box because various point mutations in the *rfn* region abolished this effect. The predicted conformational change in the pure *rib* leader RNA in response to FMN, but not to riboflavin, was demonstrated with the use of oligonucleotides that were complementary to RNA and ribonuclease H (RNase H) probing [14]. Independent evidence of direct binding of FMN to *rfn* came from the observation that the intrinsic fluorescence of FMN was quenched upon RNA synthesis [14]. In addition, it was shown that FMN altered the spontaneous cleavage pattern of *rib* leader RNA in the so-called 'in-line RNA-probing' assay [15], which is also indicative of the conformational change in the RNA structure induced by the metabolite. As similar methods have been used for characterization of other riboswitch systems, we will refer to them as 'RNase H-probing' and 'in-line RNA-probing' assays.

In contrast to Gram-positive bacteria, in which most *rib* genes are clustered in a single operon, in Gram-negative bacteria they are scattered around the chromosome. Usually, two of those genes – *ribB* and *ribH2* – are regulated by an *rfn*-box [16]. Interestingly, in this case, the riboswitch operates at the translational rather than the transcriptional level. For instance, in *E. coli*, the translational repression of *ribB* by FMN via the *rfn*-box reaches two orders of magnitude, but the transcriptional repression is only fivefold (A.S. Mironov and E. Nudler, unpublished observations). As shown in Figure 1b, the stem–loop structure downstream of the *rfn*-box acts as the sequestor of SD and the start codon rather than an intrinsic transcription terminator. In the presence of FMN, the *rfn* riboswitch prevents the formation of the anti-sequestor, thus, enabling the sequestor to interfere with translation initiation. Such differences between Gram-negative and Gram-positive bacteria, with respect to the target of riboswitch-mediated regulation, are common (Table 1). In some cases, for example the *ypaA* gene, the *rfn*-box is predicted to act at both transcriptional and translational levels because the intrinsic terminator hairpin overlaps with SD [16].

Thiamin pyrophosphate-sensing riboswitches

Thiamin, also known as vitamin B1, is a precursor of thiamin pyrophosphate (TPP) – a cofactor of key enzymes of carbohydrate metabolism. The regulation of thiamin genes has been well documented in *E. coli*, *R. etli* and *B. subtilis*. In most cases, thiamin gene expression is negatively controlled by thiamin and TPP [9,17,18]. Those *thi* operons that are subjected to feedback regulation (e.g. *thiCEFSGH, thiMD* and *sfuABC* in *E. coli*) possess untranslated leaders that contain an evolutionarily conserved 39-nucleotide region referred to as the *thi*-box [19]. A phylogenetic comparative analysis has shown that all *thi*-box sequences from various bacteria species are folded into similar hairpin structures [20,21]. The detailed functional analysis of the *thi* leader RNA box of the *thiCOGE* operon from *R. etli* revealed that repression by thiamin is a post-transcriptional event and that the *thi*-box is indispensable for such regulation [21]. As the putative regulatory factor has not been detected either genetically or via titration of the leader RNA, it has been proposed that TPP might interact with the *thi*-box directly to modulate the SD-sequester structure in *R. etli* [20,22]. This model was tested directly *in vitro* using the *thi*-box leader from *B. subtilis* that controls the *tenAI–goxB–thiSGF–yjbV–fabI* polycistronic locus [14]. The *tenA* leader is organized as a terminator–antiterminator–anti-anti-terminator system with the *thi*-box positioned to stabilize the anti-antiterminator stem–loop structure and prevent transcription of downstream genes (Figure 1a). The strong stimulating effect of low micromolar amounts of TPP on transcription termination of the *tenA* leader was detected in a single round run-off assay with the pure RNA polymerase lacking any additional factors. The effect was strictly *thi*-box-dependant and highly specific because the thiamin, at physiological concentrations, had no effect on transcription termination [14]. Independently, an in-line RNA-probing assay was carried out on *thiM* and *thiC* leader RNAs from *E. coli* to demonstrate direct TPP binding and conformational change of the RNA in response to TPP [23]. Taken together, these results established the *thi*-box as a TPP-sensing riboswitch. Similar to the FMN-sensing

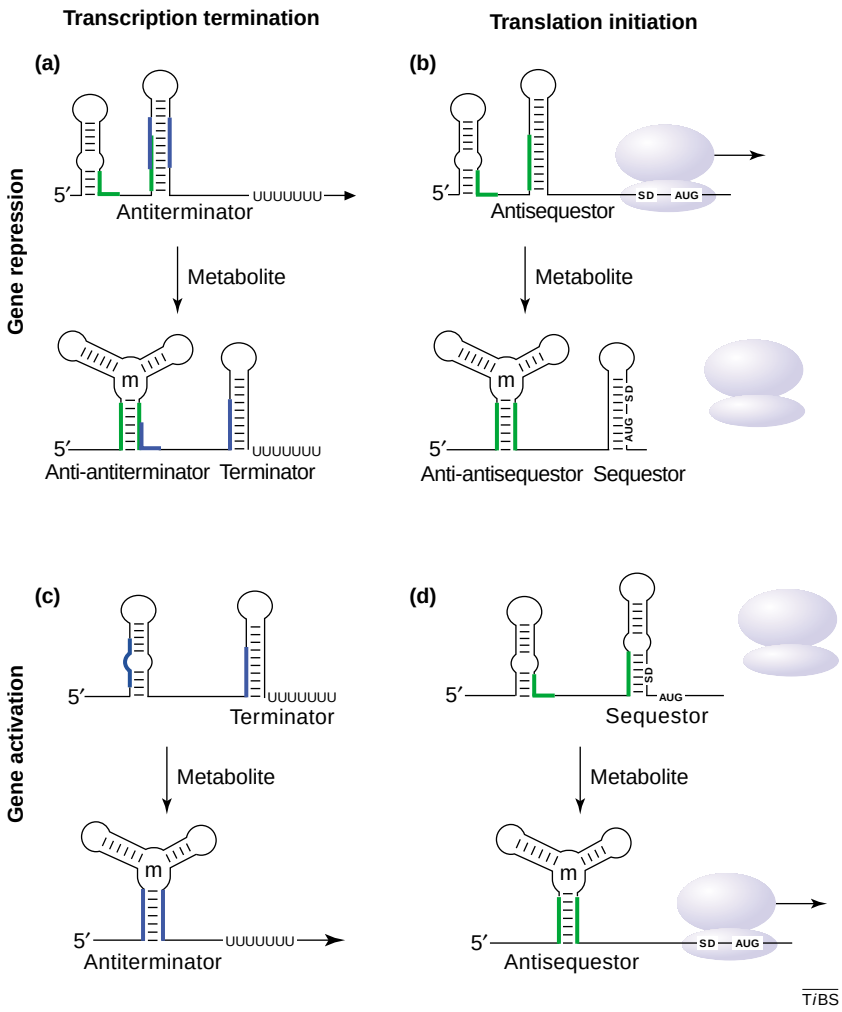

Figure 1. Riboswitch-mediated control of gene expression. Bacterial riboswitches repress or activate their genes depending on the configuration of the corresponding leader RNA sequence (shown by the line growing from the 5′ end). In response to the change of the metabolite concentration they control transcription termination **(a,c)** or translation initiation **(b,d)**, or both if the stem–loop structure of the terminator also serves as a sequestor of the ribosome-binding site (RBS). In each case, the binding of a specific metabolite (m) to the conserved RNA-sensor domain stabilizes the riboswitch structure (shown as a hypothetical three-stem structure) thus preventing the formation of an alternative RNA structure that could be an antiterminator (a), antisequestor (b), terminator (c) or sequestor of RBS (d). The ribosome is shown in pale blue. The complementary RNA regions are indicated in green and blue.

riboswitch, the *thi*-box riboswitch appeared to control its own genes by inducing transcription termination in Gram-positive bacteria or inhibiting translation initiation in Gram-negative bacteria (Table 1).

Remarkably, the *thi*-box sequences have been found, not only in genomes of nearly 100 bacterial species from every taxonomic group, but also in the 5′ and 3′ untranslated regions (UTRs) of archaea, fungi and plant species [21,24]. This suggests that the *thi*-box riboswitch might also control B1 metabolism and transport in eukaryotic organisms. However, the target for such regulation is likely to be different from intrinsic transcription termination or translation initiation. The *thi*-boxes have been located near the splice sites within intron sequences or close to the polyA tail, thus, they might control RNA processing and/or stability in response to TPP concentration [24].

Adenosylcobalamin-sensing riboswitches

B12, or cyanocobalamin, is yet another vitamin whose genes appear to be under riboswitch control. In *Salmonella* and *E. coli*, the *btuB* gene encodes an outer-membrane cobalamin transport protein and the *cob* operon [25,26] encodes cobalamin biosynthetic enzymes. Regulation of these genes shows strong similarities to that of the *thi and rib* genes. *btuB* and *cob* are transcribed with long regulatory leaders of 241 and 468 nucleotides, respectively, that contain an evolutionarily conserved 25-nucleotide element called the B_{12}-box [27–29]. The B_{12}-box has been shown to be required for negative control of *btuB* expression by the effector molecule adenosylcobalamin (Ado-Cbl) [30,31]. The primary control of *btuB* expression by Ado-Cbl occurs at the level of translation initiation where a hairpin structure sequesters the SD sequence [30,31]. Similar to the case for thiamin and riboflavin gene expression, no *trans*-acting protein factors involved in cobalamin repression have been found. Instead, it has been shown that Ado-Cbl, but not cyanocobalamin, inhibits the binding of the 30S ribosomal subunits to purified *btuB* RNA, and that such inhibition is strictly dependent on the intact B_{12}-box [32]. In addition, in-line RNA probing of *E. coli btuB* RNA confirmed that

Table 1. Riboswitches and their role in regulation of bacterial metabolism[a,b]

Metabolite (ligand)	Precursor	RNA sensor	Target process	Target genes	Where found	Refs
FMN	B2	*rfn*-box	Transcription termination or translation initiation	B2 synthesis and transport	Gram(+) and Gram(−) bacteria	[14–16]
TPP	B1	*thi*-box	Transcription termination or translation initiation	B1 synthesis and transport	Gram(+) and Gram(−) bacteria, some archaea, fungi and plants	[14,20,21,23]
Ado-Cbl	B12	B_{12}-box	Transcription termination and/or translation initiation	B12 synthesis and transport	Gram(+) and Gram(−) bacteria	[32,33]
SAM	Met	S-box	Transcription termination	Sulfur metabolism	Gram(+) bacteria	[40–42]
Lysine	N/A	L-box	Transcription termination	*lysC*	Gram(+) and Gram(−) bacteria	[44,46,47]
Guanine, HX	N/A	G-box	Transcription termination and antitermination	Purine metabolism and transport	Gram(+) bacteria	[44][c]

[a]The table provides an up-to-date list of natural RNA sensors of small molecules.
[b]Abbreviations: Ado-Cbl, adenosylcobalamin; B2, riboflavin; B1, thiamine; B_{12}-box, G-box, L-box, *rfn*-box and *thi*-box, evolutionarily conserved RNA structures that bind Ado-Cbl, guanine, lysine, FMN and TPP, respectively; FMN, flavin mononucleotide; Gram(+), Gram positive; Gram(−), gram negative; HX, hypoxanthine; Met, methionine; N/A, not applicable; SAM, *S*-adenosyl-L-methionine; TPP, thiamin pyrophosphate.
[c]A.S. Mironov *et al.*, unpublished observations.

specific binding of Ado-Cbl induces a conformational change in this RNA [33]. In the *cob* leader RNA from *Salmonella*, it was also shown that in the absence of cobalamin, the anti-sequestor structure located upstream of the SD sequestor is required to unmask the ribosome-binding site and de-repress the operon [34] (Figure 1b). As an intrinsic terminator structure that could serve as a transcriptional attenuator resides in the 5′ portion of the coding sequence of *btuB* [35], the B_{12}-box riboswitch is likely to operate in *E. coli* and *Salmonella* at the transcriptional level as well. This mode of regulation by Ado-Cbl is predicted to be predominant in Bacilli and some other Gram-positive bacteria [36] (Table 1).

The riboswitch control of amino acid metabolism

The second group of riboswitches that has been characterized appears to regulate metabolic pathways involving at least three amino acids – methionine (Met), cysteine (Cys) and lysine (Lys) – in Gram-positive bacteria.

S-adenosyl-methionine-sensing riboswitches

At least 60 transcription units from a variety of bacteria species are members of the S-box regulon [8]. This family of genes is characterized by the presence of an evolutionarily conserved regulatory leader sequence (S-box) [37]. Most of these genes are directly involved in sulfur metabolism, Cys and Met biosynthesis, and biosynthesis of *S*-adenosyl-methionine (SAM). SAM is an essential coenzyme in all organisms. It is synthesized directly from Met by SAM synthetase and serves as a source of methyl groups for protein and nucleic acid modification. In *B. subtilis*, there are 11 operons with a total of 26 genes under S-box control [37]. The leader sequences of these genes include an intrinsic transcription terminator, competing antiterminator and the S-box that functions as an anti-antiterminator (Figure 1a). Genetic and physiological studies on the *yitJ* gene support the attenuation model and indicate that the S-box serves as a target for repression during growth in the presence of Met [37]. Although an unknown factor was proposed to interact with the S-box RNA upon binding to Met and to attenuate transcription of downstream genes [8], no such factor has been found. *In vivo* analysis of the *metIC* operon also points to transcription attenuation control; the induction of *metIC* is independent of the promoter [38]. Furthermore, northern blot analysis shows two transcripts: a small one corresponding to early termination at the end of the S-box leader sequence and a large one corresponding to the transcript of the entire *metIC* operon. The ratio between the two transcripts depends on Met availability [38]. It has also been shown that overexpression of SAM synthase leads to Met auxotrophy in *B. subtilis,* suggesting that SAM, not Met, is an effector molecule of Met biosynthesis *in vivo* [39].

Three recent *in vitro* studies used different techniques to unambiguously demonstrate that the S-box RNA from *B. subtilis* directly senses the level of SAM and functions as a SAM-dependent riboswitch [40–42]. The attenuation mechanism relies on tight binding of SAM to the nascent S-box transcript leading to stabilization of its anti-antitermination structure, which, in turn, enables the formation of the stem–loop of the attenuator and early transcription termination (Figure 1a). SAM–RNA binding is highly specific; alterations in the S-box sequence that result in constitutive expression of S-box operons *in vivo* prevent SAM binding and SAM-dependent intrinsic termination. Binding of SAM to S-box RNA *in vitro* occurs at physiological concentrations; the close analogs of SAM, such as Met or *S*-adenosylhomocysteine, do not bind or affect transcription even at much higher concentrations [40–42]. However, it should be noted that several genes containing the S-box do not respond to methionine limitation *in vivo* [43]. For example, a well-characterized cluster of genes involved in cysteine biosynthesis in *B. subtilis*, the *cysH* operon, contains S-box and responds to SAM *in vitro* [40]. However, the physiological relevance of the leader RNA in the expression of these genes has not been demonstrated. The expression of *cysH* is regulated at the level of transcription initiation in response to the intracellular levels of *O*-acetyl-L-serine [43] Thus, although many bacterial genes could contain a putative

RNA sensor, some of those genes might not necessarily be regulated by a riboswitch-type mechanism.

Lysine-sensing riboswitches

It has been noted recently that the *lysC* gene in *B. subtilis,* which encodes the first specific enzyme of lysine biosynthesis, is under lysine-sensing riboswitch control [44]. Although the details of this mechanism have not been published, sequence organization of the *lysC* leader transcript [45] and previous genetic and physiological data favor the riboswitch mode of regulation via a transcription attenuation mechanism (Figure 1a).

lysC is negatively controlled by the availability of the end product, lysine [46,47]. Northern blot analysis has demonstrated that the full-length *lysC* mRNA forms only when cells are starved of lysine. In the presence of excess lysine, a truncated 270-nucleotide RNA is generated in place of the full-length product [46]. Furthermore, a point substitution in the presumed anti-antiterminator domain (also a putative lysine sensor) de-repressed *lysC* full-length mRNA, even during growth with lysine. Mapping of the truncated RNA has shown that it corresponds to the upstream portion of the *lysC* leader transcript, which extends from the transcription initiation site to a putative intrinsic terminator. Quantitative transcript analysis has also shown that lysine does not affect the number of *lysC*-specific RNA molecules, but stoichiometrically replaces the full-length mRNA with truncated transcripts. Neither a protein factor nor tRNA has been found to be responsible for sensing lysine concentration. These results indicate that lysine regulates the expression of the *lysC* gene by an attenuation mechanism, which is likely to be mediated by the antiterminator and the evolutionarily conserved Lys-box, which serves as the ligand-sensing anti-antiterminator. Mutations within the Lys-box of the *E. coli* homolog of *lysC* lead to the constitutive expression of this gene [47], suggesting that it is also probably feedback-regulated by a lysine-sensing riboswitch.

The riboswitch control of purine metabolism

The most recently described group of riboswitches regulates five operons involved in purine biosynthesis, interconversion and transport in *B. subtilis* [44] (A.S. Mironov *et al.*, unpublished observations). The *pur* operon (*purEKBCSQLFMNHD*), which encodes the enzymes for *de novo* synthesis of IMP (inosine monophosphate) and the *xpt–pbuX* operon, encoding xanthine phosphoribosyltransferase and a xanthine transporter, are regulated at both transcription initiation and attenuation [48]. For the *xpt-pbuX* operon, evidence has been obtained that an attenuation mechanism is activated *in vivo* by the free bases hypoxanthine and guanine [49]. In the presence of these purines, transcription of *pur* and *xpt-pbuX* terminates in front of the first structural gene. Otherwise, it proceeds to the end of each operon [49,50]. Analysis of the leader sequence of both operons reveals a typical terminator–antiterminator–anti-antiterminator configuration (Figure 1a). The most conserved portion of the leader – the G-box – has also been found in the leader of three other operons: *pbuG*, *nupG* (*yxjA*) and *pbuE* (*ydhL*), which encode a hypoxanthine–guanine transporter, purine nucleoside transporter and purine efflux pump, respectively [44,51]. The latter two genes were noticed and functionally characterized owing to the presence of the G-box in their 5′ UTRs. Various *in vitro* approaches, including in-line RNA probing and equilibrium dialysis, have demonstrated that the G-box is a direct sensor of guanine and hypoxanthine, which tightly and specifically bind G-box RNA at nanomolar and low micromolar concentrations, respectively [44]. Other purine analogs and nucleosides fail to bind the G-box RNA at physiologically relevant concentrations. It has also been shown by in-line RNA probing that the G-box of *pbuE* is specific for adenine rather than guanine [44]. Except for *pbuE*, the G-box serves as a guanine-dependent anti-antiterminator, which promotes intrinsic termination of the corresponding leader RNA both *in vivo* [51] and in the reconstituted system *in vitro* (A.S. Mironov *et al.*, in preparation). Remarkably, in the case of *pbuE*, the G-box plays the part of the antiterminator *in vitro*, not the anti-antiterminator, and thus serves as a purine-dependent activator rather than suppressor of its gene (Figure 1c; A.S. Mironov *et al.*, in preparation). Taking into account the role of *pbuE* as an efflux pump of purines, this explains why other *pur* regulon genes are expressed at elevated levels in the mutant that overproduces *pbuE* [51].

Concluding remarks and future directions

Traditionally, the ability to monitor the metabolic status of the cell has been associated with proteins. New studies, however, demonstrate that untranslated RNA messengers (riboswitches) can also sense metabolite level and turn the corresponding genes on and off accordingly. Riboswitches reside in the leader sequences of numerous bacterial operons and control both transcription and translation by adopting alternative RNA structures, which can induce or prevent the formation of intrinsic terminators or RBS sequesters. Thus, depending on the configuration of the leader transcript, the same riboswitch can either be a repressor or an activator of a cognate gene (Figure 1). So far, only one riboswitch activator has been found – it suppresses premature termination of *pbuE* gene transcription (Figure 1c). By analogy with riboswitch repressors, one can predict that riboswitch activators exist that can also modulate translation initiation (Figure 1d). Interestingly, in Gram-negative bacteria riboswitches tend to function via modulation of translation initiation, whereas in Gram-positive bacteria they predominantly function via transcription termination (Table 1). It is possible that because Gram-positive bacteria have their genes clustered in larger operons, it is easier to control them by prematurely terminating polycistronic RNA messages.

In comparison with artificial RNA aptamers that have been selected *in vitro* to bind particular small molecules (e.g. FMN and guanine) [52,53], the riboswitches are much more selective and sensitive. Yet the principles of molecular recognition by these natural RNA aptamers remain unknown. There seem to be no common structural features between RNA-sensor-binding metabolites (e.g. L-lysine and FMN); the high resolution X-ray

structure of a riboswitch with its ligand should address this issue.

The known riboswitches are highly conserved among bacteria. In fact, based on the presence of certain riboswitch motifs in the leader sequences, several unknown genes were assigned to the corresponding regulons and their function predicted [10,51]. The presence of potential riboswitch sequences in 5′ and 3′ UTRs of eukaryotic genes suggest that they could also control the fate of mRNA [24] (A.S. Mironov and E. Nudler, unpublished observations). It will be important to test this hypothesis.

The evolutionary conservation of known riboswitches argues for their ancient origin. It has been suggested that they represent 'molecular fossils' – a holdover from the RNA world [23]. By contrast, because riboswitches are the most 'economical' and fast-reacting regulatory systems (no intermediate factors involved), various organisms could develop their own 'modern' riboswitches for various purposes, not necessarily for regulation of biosynthesis and transport of a cognate metabolite. In principle, any process associated with RNA (not only transcription and translation, but also processing, transport and degradation) could be modulated by a riboswitch-type mechanism. If 'modern' riboswitches exist, it will be a challenge to find them because they have not been evolutionarily conserved. However, the proof of their existence would expose a new level of complexity in gene regulation.

Note added in proof

We would like to direct the readers to the following references, which were published since the writing of this article

(i) Grundy, F. J. *et al.* (2003) The L box regulon: lysine sensing by leader RNAs of bacterial lysine biosynthesis genes. *Proc. Natl. Acad. Sci. U. S. A.* 100, 12057–12062

(ii) Sudarsan, N. *et al.* (2003) An mRNA structure in bacteria that controls gene expression by binding lysine. *Genes Dev.* 17, 2688–2697

These papers provide a detailed characterization of the lysine-sensing riboswitch.

Acknowledgements

We thank Per Nygaard for sharing results before publication. This work was supported by National Institutes of Health (GM58750) and Fogarty International Collaboration Research Award TW06122 (E.N.).

References

1 Yanofsky, C. *et al.* (1996) Some novel transcription attenuation mechanisms used by bacteria. *Biochimie* 78, 1017–1024

2 Babitzke, P. *et al.* (1994) TRAP, the *trp* RNA-binding attenuation protein of *Bacillus subtilis*, is a multisubunit complex that appears to recognize G/UAG repeats in the *trpEDCFBA* and *trpG* transcripts. *J. Biol. Chem.* 269, 16597–16604

3 Babitzke, P. and Gollnick, P. (2001) Posttranscription initiation control of tryptophan metabolism in *Bacillus subtilis* by the trp RNA-binding attenuation protein (TRAP), anti-TRAP, and RNA structure. *J. Bacteriol.* 183, 5795–5802

4 Rutberg, B. (1997) Antitermination of transcription of catabolic operons. *Mol. Microbiol.* 23, 413–421

5 Stulke, J. (2002) Control of transcription termination in bacteria by RNA-binding proteins that modulate RNA structures. *Arch. Microbiol.* 177, 433–440

6 Romby, P. and Springer, M. (2003) Bacterial translational control at atomic resolution. *Trends Genet.* 19, 155–161

7 Landick, R. *et al.* (1996) Transcription attenuation. In *Escherichia coli and Salmonella Typhimurium* (Neidhardt, ed.), pp. 1263–1286, American Society for Microbiology

8 Grundy, F.J. and Henkin, T.M. (2003) The T box and S box transcription termination control systems. *Front. Biosci.* 8, d20–d31

9 Perkins, J.B. and Pero, J. (2002) Biosynthesis of riboflavin, biotin, folic acid, and cobalamin. In *Bacillus Subtillis and Its Closest Relatives: from Genes to Cells* (Sonenshein, A.L., Hoch, J.A. and Losick, R., eds), pp. 271–286, ASM Press

10 Gelfand, M.S. *et al.* (1999) A conserved RNA structure element involved in the regulation of bacterial riboflavin synthesis genes. *Trends Genet.* 15, 439–442

11 Kreneva, R.A. and Perumov, D.A. (1990) Genetic mapping of regulatory mutations of *Bacillus subtilis* riboflavin operon. *Mol. Gen. Genet.* 222, 467–469

12 Gusarov, I. *et al.* (1997) Primary structure and functional activity of the *Bacillus subtilis ribC* gene. *Mol Biol (Mosk)* 31, 820–825

13 Mack, M.A. *et al.* (1998) Regulation of riboflavin biosynthesis in *Bacillus subtilis* is affected by the activity of the flavokinase/flavin adenine dinucleotide synthetase encoded by ribC. *J. Bacteriol.* 180, 950–955

14 Mironov, A.S. *et al.* (2002) Sensing small molecules by nascent RNA: a mechanism to control transcription in bacteria. *Cell* 111, 747–756

15 Winkler, W.C. *et al.* (2002) An mRNA structure that controls gene expression by binding FMN. *Proc. Natl. Acad. Sci. U. S. A.* 99, 15908–15913

16 Vitreschak, A.G. *et al.* (2002) Regulation of riboflavin biosynthesis and transport genes in bacteria by transcriptional and translational attenuation. *Nucleic Acids Res.* 30, 3141–3151

17 Begley, T.P. *et al.* (1999) Thiamin biosynthesis in prokaryotes. *Arch. Microbiol.* 171, 293–300

18 Petersen, L.A. and Downs, D.M. (1997) Identification and characterization of an operon in *Salmonella typhimurium* involved in thiamine biosynthesis. *J. Bacteriol.* 179, 4894–4900

19 Miranda-Rios, J. *et al.* (1997) Expression of thiamin biosynthetic genes (*thiCOGE*) and production of symbiotic terminal oxidase cbb3 in *Rhizobium etli*. *J. Bacteriol.* 179, 6887–6893

20 Miranda-Rios, J. *et al.* (2001) A conserved RNA structure (*thi* box) is involved in regulation of thiamin biosynthetic gene expression in bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 98, 9736–9741

21 Rodionov, D.A. *et al.* (2002) Comparative genomics of thiamin biosynthesis in procaryotes. New genes and regulatory mechanisms. *J. Biol. Chem.* 277, 48949–48959

22 Stormo, G.D. and Ji, Y. (2001) Do mRNAs act as direct sensors of small molecules to control their expression? *Proc. Natl. Acad. Sci. U. S. A.* 98, 9465–9467

23 Winkler, W. *et al.* (2002) Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. *Nature* 419, 952–956

24 Sudarsan, N. *et al.* (2003) Metabolite-binding RNA domains are present in the genes of eukaryotes. *RNA* 9, 644–647

25 Wei, B.Y. *et al.* (1992) Conserved structural and regulatory regions in the *Salmonella typhimurium btuB* gene for the outer membrane vitamin B12 transport protein. *Res. Microbiol.* 143, 459–466

26 Escalante-Semerena, J.C. and Roth, J.R. (1987) Regulation of cobalamin biosynthetic operons in *Salmonella typhimurium*. *J. Bacteriol.* 169, 2251–2258

27 Lundrigan, M.D. *et al.* (1991) Transcribed sequences of the *Escherichia coli btuB* gene control its expression and regulation by vitamin B12. *Proc. Natl. Acad. Sci. U. S. A.* 88, 1479–1483

28 Richter-Dahlfors, A.A. *et al.* (1994) Vitamin B12 repression of the cob operon in *Salmonella typhimurium*: translational control of the *cbiA* gene. *Mol. Microbiol.* 13, 541–553

29 Richter-Dahlfors, A.A. and Andersson, D.I. (1992) Cobalamin (vitamin B12) repression of the Cob operon in *Salmonella typhimurium* requires sequences within the leader and the first translated open reading frame. *Mol. Microbiol.* 6, 743–749

30 Ravnum, S. and Andersson, D.I. (1997) Vitamin B12 repression of the *btuB* gene in *Salmonella typhimurium* is mediated via a translational control which requires leader and coding sequences. *Mol. Microbiol.* 23, 35–42

31 Nou, X. and Kadner, R.J. (1998) Coupled changes in translation and

transcription during cobalamin-dependent regulation of *btuB* expression in *Escherichia coli*. *J. Bacteriol.* 180, 6719–6728
32 Nou, X. and Kadner, R.J. (2000) Adenosylcobalamin inhibits ribosome binding to *btuB* RNA. *Proc. Natl. Acad. Sci. U. S. A.* 97, 7190–7195
33 Nahvi, A. *et al.* (2002) Genetic control by a metabolite binding mRNA. *Chem. Biol.* 9, 1043–1049
34 Ravnum, S. and Andersson, D.I. (2001) An adenosyl-cobalamin (coenzyme-B12)-repressed translational enhancer in the cob mRNA of *Salmonella typhimurium*. *Mol. Microbiol.* 39, 1585–1594
35 Franklund, C.V. and Kadner, R.J. (1997) Multiple transcribed elements control expression of the *Escherichia coli btuB* gene. *J. Bacteriol.* 179, 4039–4042
36 Vitreschak, A.G. *et al.* (2003) Regulation of the vitamin B(12) metabolism and transport in bacteria by a conserved RNA structural element. *RNA* 9, 1084–1097
37 Grundy, F.J. and Henkin, T.M. (1998) The S box regulon: a new global transcription termination control system for methionine and cysteine biosynthesis genes in gram-positive bacteria. *Mol. Microbiol.* 30, 737–749
38 Auger, S. *et al.* (2002) The *metIC* operon involved in methionine biosynthesis in *Bacillus subtilis* is controlled by transcription antitermination. *Microbiology* 148, 507–518
39 Yocum, R.R. *et al.* (1996) Cloning and characterization of the *metE* gene encoding *S*-adenosylmethionine synthetase from *Bacillus subtilis*. *J. Bacteriol.* 178, 4604–4610
40 McDaniel, B.A. *et al.* (2003) Transcription termination control of the S box system: direct measurement of S-adenosylmethionine by the leader RNA. *Proc. Natl. Acad. Sci. U. S. A.* 100, 3083–3088
41 Epshtein, V. *et al.* (2003) The riboswitch-mediated control of sulfur metabolism in bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 100, 5052–5056
42 Winkler, W.C. *et al.* (2003) An mRNA structure that controls gene expression by binding *S*-adenosylmethionine. *Nat. Struct. Biol.* 10, 701–707
43 Mansilla, M.C. *et al.* (2000) Transcriptional control of the sulfur-regulated *cysH* operon, containing genes involved in L-cysteine biosynthesis in *Bacillus subtilis*. *J. Bacteriol.* 182, 5885–5892
44 Mandal, M. *et al.* (2003) Riboswitches control fundamental biochemical pathways in *Bacillus subtilis* and other bacteria. *Cell* 113, 577–586
45 Lu, Y. *et al.* (1992) Fine-structure mapping of *cis*-acting control sites in the *lysC* operon of *Bacillus subtilis*. *FEMS Microbiol. Lett.* 71, 23–27
46 Kochhar, S. and Paulus, H. (1996) Lysine-induced premature transcription termination in the *lysC* operon of *Bacillus subtilis*. *Microbiology* 142, 1635–1639
47 Patte, J.C. *et al.* (1998) The leader sequence of the *Escherichia coli lysC* gene is involved in the regulation of LysC synthesis. *FEMS Microbiol. Lett.* 169, 165–170
48 Switzer, R.L. *et al.* (2002) Purine, pyrimidine, and pyridine nucleotide metabolism. In *Bacillus Subtillis and Its Closest Relatives: from Genes to Cells* (Sonenshein, A.L., Hoch, J.A. and Losick, R., eds), pp. 255–269, ASM Press
49 Christiansen, L.C. *et al.* (1997) Xanthine metabolism in *Bacillus subtilis*: characterization of the *xpt-pbuX* operon and evidence for purine- and nitrogen-controlled expression of genes involved in xanthine salvage and catabolism. *J. Bacteriol.* 179, 2540–2550
50 Ebbole, D.J. and Zalkin, H. (1988) Detection of *pur* operon-attenuated mRNA and accumulated degradation intermediates in *Bacillus subtilis*. *J. Biol. Chem.* 263, 10894–10902
51 Johansen, L.E. *et al.* (2003) Definition of a second *Bacillus subtilis pur* regulon comprising the *pur* and *xpt-pbuX* operons plus *pbuG*, *nupG* (*yxjA*), and *pbuE (ydhL)*. *J. Bacteriol.* 185, 5200–5209
52 Fan, P. *et al.* (1996) Molecular recognition in the FMN–RNA aptamer complex. *J. Mol. Biol.* 258, 480–500
53 Kiga, D. *et al.* (1998) An RNA aptamer to the xanthine/guanine base with a distinctive mode of purine recognition. *Nucleic Acids Res.* 26, 1755–1760