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Regulation of gene expression by effectors that bind to RNA

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Recent studies have revealed several genetic systems in bacteria that use complex RNA structural elements to monitor regulatory signals and control expression of downstream genes. These include RNA thermosensors, in which an inhibitory structure melts at high temperature, and systems where binding of small RNAs or cellular metabolites modulates the structure of the RNA. The remarkable feature of these systems is the ability of the regulatory RNA elements to specifically sense the regulatory signal, without accessory components, and convey that information to the gene expression machinery via a structural change in the nascent RNA.

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Abbreviations

AdoCbl	adenosylcobalamin
FMN	flavin mononucleotide
RNAP	RNA polymerase
SAM	*S*-adenosylmethionine
TPP	thiamin pyrophosphate

Introduction

Regulation of gene expression by modulation of RNA structure was discovered over 25 years ago, and a variety of molecular mechanisms have been uncovered (reviewed in [1]). Early examples included the *Escherichia coli trp* and *Salmonella typhimurium his* operon attenuation systems, where readthrough of a transcription termination signal in the 5′ region of an mRNA (the 'leader region') is determined by the progression of a ribosome translating a segment of the leader RNA upstream of the terminator; stalling of the ribosome results in formation of an antiterminator structure, which prevents formation of the terminator helix [2]. Leader RNA translation can also control expression of a downstream coding sequence by affecting the accessibility of the translation initiation region, in systems such as the *Bacillus subtilis cat* gene [3]. In the first type of system, availability of specific charged tRNAs determines the efficiency of leader peptide translation, thereby altering the relative positions of the ribosome and RNA polymerase (RNAP). In the second type of system, the leader peptide itself acts in conjunction with an inducer molecule such as chloramphenicol to stall the ribosome on the mRNA. In both cases, the translating ribosome monitors the key physiological signal (amino acid availability or antibiotic concentration), and the resulting effects on ribosome position determine the structure of the nascent RNA, and its fate.

A second mechanism for the use of RNA structures to control gene expression involves a specific RNA binding protein, binding of which determines whether the RNA will fold to form an intrinsic terminator helix, or a structure that occludes the translation initiation site; alternatively, binding of the protein can directly block access of the initiating ribosome. The concentration of the RNA binding protein can be monitored directly, for example in ribosomal protein operons, or its RNA binding activity can be controlled by interaction with an effector molecule (e.g. tryptophan in the *B. subtilis* TRAP system), or second regulatory protein (e.g. BglG phosphorylation by BglF). Systems of this type are reviewed in this issue by Babitzke [4].

Environmental signals can also directly alter RNA structure. The mRNAs for several genes, including *E. coli rpoH*, encoding the heat-shock sigma factor, and *Listeria monocytogenes prfA*, encoding a key regulator of pathogenesis, contain structures that prevent binding of the ribosome to the translation initiation region. Melting of these structures in response to an increase in temperature allows induction of gene expression under appropriate conditions, so that the mRNAs act as thermosensors [5,6]. RNA structure can be modulated by binding of antisense RNAs to control translation, mRNA stability or premature termination of transcription (reviewed by Storz *et al.* [7] in this issue and [8]); in these systems, the concentration of the regulatory RNA is monitored.

Recent studies have identified a variety of systems where leader RNAs directly sense physiological signals by binding an effector molecule, without a requirement for 'interpretation' of the signal by a regulatory protein or translating ribosome. In this review, we focus on systems of this type, which illustrate the ability of RNA to specifically recognize effector molecules and to transmit information to the gene expression machinery via RNA structural rearrangements. Because the data indicate that these RNAs monitor a range of effector concentrations, and give intermediate responses to intermediate

concentrations, we use the term 'RNA sensors'; the term 'riboswitch' is more appropriately used for any RNA that exhibits a structural switch in response to binding of any ligand, including a protein.

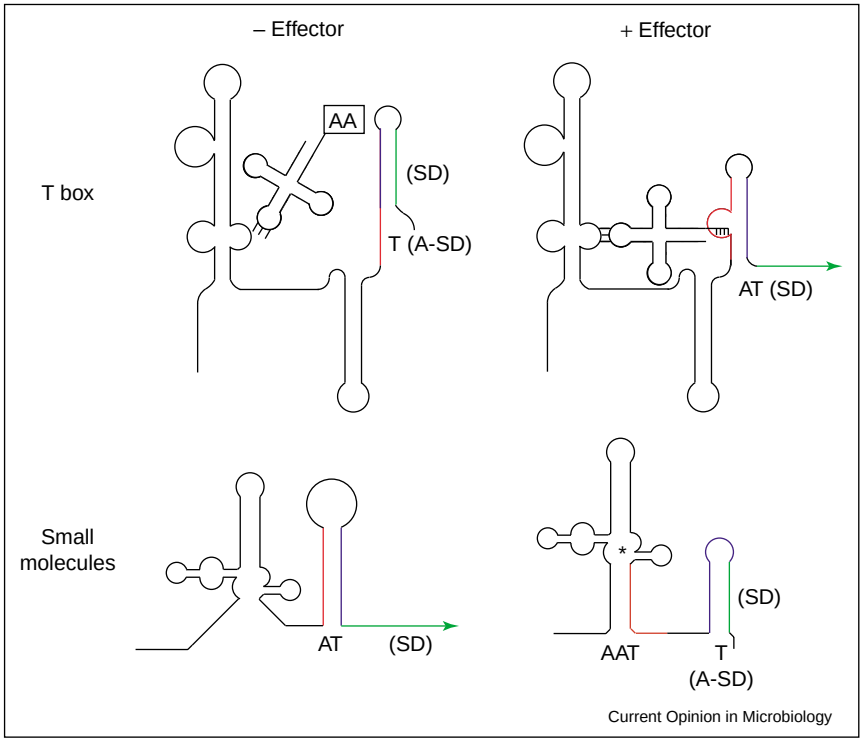
Leader RNA-tRNA recognition

tRNA plays a crucial role in protein synthesis, by conveying each amino acid in turn to a growing peptide chain. Each tRNA is selected by codon-anticodon pairing within the ribosome, and the specificity of the tRNA-amino acid linkage, determined by the cognate aminoacyl-tRNA synthetase, ensures that codons will be correctly interpreted. The charging ratio of tRNA can also be used as a regulatory signal, allowing the cell to recognize amino acid starvation via the stringent response, or to measure availability of a specific amino acid during leader peptide translation, as described above. tRNA charging can also be monitored directly by a leader RNA, in the absence of translation.

The T box system

Many amino-acid-related genes in Gram-positive bacteria are regulated at the level of transcription termination by the T box mechanism [9]. Transcriptional units in this family exhibit a set of conserved primary sequence and structural elements in their 5′ regions, with gene expression controlled by competition between mutually exclusive terminator and antiterminator structures. The decision between the alternate leader RNA structures is determined by interaction of the nascent transcript with an uncharged tRNA specific for the amino acid class of the downstream coding sequence, so that a tyrosyl-tRNA synthetase gene responds to $tRNA^{Tyr}$, and so on. The specificity of the tRNA response is dependent on pairing of the anticodon of the tRNA with a single codon, designated the 'specifier sequence', in the leader sequence. The acceptor end of the uncharged tRNA also pairs with residues in the antiterminator element to stabilize the antiterminator, and prevent formation of

Figure 1



RNA elements that directly bind effector molecules. For the T box system, the cognate uncharged tRNA (shown in cloverleaf form) serves as the effector. In the absence of the effector (or in the presence of aminoacylated tRNA, indicated by the boxed AA), the leader RNA folds into the terminator form (T, blue paired with green). In the presence of the effector, the antiterminator (AT, red paired with blue) is stabilized, preventing formation of the terminator helix and allowing transcription of the downstream coding regions (green arrow). This arrangement can also be used to regulate gene expression at the level of translation initiation, in which case the 'terminator' helix (A-SD) sequesters the Shine-Dalgarno sequence (SD) of the downstream gene. For the small molecule binding RNAs, the antiterminator structure forms in the absence of effector, allowing expression of the downstream coding regions (green arrow); addition of the effector (*) promotes stabilization of an anti-antiterminator element (AAT) that sequesters sequences (red) required for formation of the antiterminator (AT, red–blue), permitting formation of the less stable terminator helix (T, blue-green). As described above, the terminator helix can be replaced with a structure that occludes the Shine-Dalgarno sequence (SD) of the downstream gene to permit regulation at the level of translation initiation. A simplified version of the S box leader format is shown as an example; the region above the anti-antiterminator helix varies in different systems, presumably to confer ligand binding specificity.

the competing terminator helix [10] (Figure 1). The leader RNA-tRNA interaction was demonstrated for the *B. subtilis glyQS* gene in a purified *in vitro* transcription system; antitermination is dependent on addition of $tRNA^{Gly}$, in the absence of any additional factors [11••]. This result indicates that the *glyQS* nascent transcript can directly monitor uncharged $tRNA^{Gly}$. The ability to use unmodified tRNA in this system facilitated detailed analysis of tRNA requirements [12]. By contrast, $tRNA^{Thr}$-dependent antitermination of the *thrS* gene required addition of either a cellular fraction or high concentrations of spermidine, possibly to facilitate proper folding of the leader RNA [13•]. It is clear from these and related biochemical studies of the isolated antiterminator domain [14,15•] that the tRNA can interact directly with the leader RNA, without a translating ribosome.

Over 300 transcriptional units have been identified as likely members of the T box family by genomic analyses ([16]; FJ Grundy, SM Rollins and TM Henkin, unpublished). tRNA-dependent antitermination has been demonstrated for several of these genes, and can be inferred for many others based on identification of competing terminator and antiterminator helices. In addition, there are several putative T box leaders in which it appears that regulation occurs at the level of translation initiation, because the helix that competes with the antiterminator-like domain sequesters the Shine-Dalgarno sequence of the downstream coding region. However, translational control has not yet been demonstrated.

Leader RNA-metabolite interactions

Several regulatory systems in bacteria that involve direct interaction of small molecules with leader RNA elements have recently been uncovered. Discovery of systems of this type hinged on identification of conserved regulatory elements in the leader regions of sets of genes with related function, and the failure to identify a *trans*-acting regulatory protein. This possibility was raised in several reports [17–20], and has now been confirmed by biochemical studies. Regulation by either premature termination of transcription, primarily in Gram-positive bacteria, or inhibition of translation initiation, primarily in Gram-negative bacteria, has been found or proposed. The RNA elements fit a general pattern where formation of the helix of the intrinsic terminator (or the helix that sequesters the translation initiation region) is, in the absence of effector, prevented by formation of a more stable competing antiterminator structure. Effector binding is proposed to stabilize a third competing structure that serves as an anti-antiterminator, which allows formation of the terminator (Figure 1).

Related RNA elements have been identified in eukaryotic cells, where they have been predicted to affect RNA splicing or stability [21]; this has been confirmed for the *Aspergillus oryzae thiA* gene, where thiamin availability *in vivo* appears to inhibit splicing [22]. Synthetic regulatory elements designed to allow modulation of RNA structure, and therefore translation, in response to binding of tetracycline, have also been designed for eukaryotic systems, illustrating the practical use of this type of mechanism [23].

The B_{12} element

Genes involved in biosynthesis and transport of vitamin B_{12} (cobalamin) contain conserved features in their upstream regions. The first element identified, designated the B_{12} box [24], was subsequently expanded by a more comprehensive phylogenetic analysis and termed the B_{12} element [25•,26•]. Addition of adenosylcobalamin (AdoCbl) to *E. coli btuB* mRNA inhibited ribosome binding, and primer extension analysis revealed a structural change in the RNA [18]. A more detailed analysis of the *btuB* mRNA structure yielded a model somewhat different from that derived from phylogenetic analysis, and showed several AdoCbl-dependent structural changes [27•]. Together, these results suggest that binding of AdoCbl to the *btuB* mRNA causes a rearrangement in the RNA that results in sequestration of the translation initiation region; by contrast, binding of AdoCbl to the B_{12} element in genes from low G+C Gram-positive bacteria is proposed to trigger formation of an intrinsic terminator helix [25•], although experimental confirmation has not yet been reported.

The THI box

The presence of a conserved structural element upstream of genes involved in biosynthesis and transport of thiamin (vitamin B_1) suggested regulation by a common mechanism [19,28,29•]. Analysis of *Rhizobium etli thiC* expression *in vivo* revealed that regulation occurs at the level of premature termination of transcription; a thiamin-dependent structural alteration in the RNA was proposed to block binding of the ribosome to the Shine-Dalgarno sequence, allowing formation of the terminator helix in the transcript [19]. Addition of thiamin pyrophosphate (TPP) to *E. coli thiC* RNA synthesized *in vitro* resulted in structural alterations consistent with the model; by contrast, TPP-dependent sequestration of the Shine-Dalgarno region of the *E. coli thiM* gene appears to cause regulation at the level of translation initiation, without transcription termination [30••]. Addition of TPP to an *in vitro* transcription system using purified *B. subtilis* RNAP and a *B. subtilis tenA* template resulted in termination within the leader region [31••]. In each of these genes, mutation of conserved elements in the THI box confers loss of response to TPP. These results indicate that the leader RNA THI box element is responsible for specific regulation in response to TPP. Detailed phylogenetic analysis of thiamine-related genes [29•] supports the initial observation that, as in the B_{12} genes, regulation is primarily at the level of translation in Gram-negative

organisms, and at the level of transcription termination in low G+C Gram-positive species.

The RFN element

A conserved element found upstream of genes involved in riboflavin biosynthesis was designated the RFN element [17], and sequence analysis suggested that this element controls gene expression at the levels of transcription termination or translation initiation [32•]. Binding of flavin mononucleotide (FMN) to the *B. subtilis rib* operon leader RNA, FMN-dependent transcription termination and structural changes in the leader RNA were demonstrated [31••,33••]. The predicted regulation of other riboflavin genes at the level of translation initiation has not yet been tested.

The S box

Genes involved in methionine metabolism in low G+C Gram-positive bacteria were found to contain a conserved leader RNA element designated the S box [34]. Addition of *S*-adenosylmethionine (SAM) is sufficient to promote termination *in vitro* by *B. subtilis* RNAP, and a SAM-dependent structural rearrangement consistent with the model was observed [35••]. *In vitro* transcribed leader RNA exhibited specific binding of SAM, and mutations that cause constitutive expression *in vivo* confer loss of SAM-dependent termination and SAM binding *in vitro* [35••–37••]. Very few S box leaders have been identified in Gram-negative organisms; however, consistent with the pattern for other systems, these genes appear to be regulated at the level of translation initiation (FJ Grundy, BA McDaniel and TM Henkin, unpublished).

The G box

Several genes involved in guanine biosynthesis, primarily in low G+C Gram-positive organisms, contain a conserved 5′ element designated the G box [38•,39••]. This element in the *B. subtilis xpt-pbuX* mRNA binds guanine and exhibits a structural alteration consistent with the model that binding of guanine promotes formation of a terminator helix. However, guanine-dependent transcription termination has not been demonstrated *in vitro*.

The L box

Expression of the *B. subtilis lysC* gene, encoding aspartokinase II, is repressed during growth in lysine at the level of premature termination of transcription, and leader region mutations result in constitutive expression [40]. Sequence similarity between the *B. subtilis* and *E. coli lysC* upstream regions was noted, but no transcriptional terminator was observed in the *E. coli* gene, suggesting that the regulatory mechanism differs in the two organisms [41]. A pattern of conserved elements, designated the L box, was identified upstream of several lysine biosynthesis genes in both Gram-positive and Gram-negative bacteria, and this element was shown to be necessary for lysine-dependent transcription termination *in vitro* [42••]. Further phylogenetic analyses identified this element upstream of additional lysine related genes [43•]. Binding of lysine to the *lysC* RNA causes structural changes consistent with the model that lysine binding promotes termination by preventing formation of an alternate antiterminator structure [42••,44••].

Conclusions

Specificity and sensitivity

A crucial element of any regulatory system is the ability to differentiate the relevant effector molecule from related molecules. For example, in the S box system it is essential to distinguish SAM, the true effector, from *S*-adenosylhomocysteine, the byproduct of SAM as a methyl donor. S box RNAs exhibit 200-fold discrimination between these molecules, although they differ by a single methyl group [35••]. Similar specificity has been observed for other regulatory RNAs in this group. For the metabolite binding RNAs, the specificity determinants have not yet been uncovered. In the T box system, the leader RNAs make use of the same identity determinants used by many aminoacyl-tRNA synthetases to recognize their cognate tRNAs (i.e., the anticodon and the discriminator base).

A second major issue for biological function is sensitivity to effector concentration. Do the RNAs respond to physiologically relevant concentrations? For each system where this information is available, the concentration of effector required for a response *in vitro* reflects the intracellular pools. As the effective concentration varies widely for different systems, for example 5 μM SAM versus 3 mM lysine, each RNA must be calibrated to have the appropriate affinity for its ligand.

How widespread are RNA sensor systems?

The increasing availability of genomic data, and the tools for recognition of conserved patterns upstream of related genes, have resulted in the discovery of an amazing array of systems of this type within a very short time period (Table 1). In each case, an understanding of the regulatory properties of these gene sets and likely effectors, based on classical microbial genetic and physiological

Table 1

Regulatory systems using direct RNA measurement of effector molecules.

System	Genes	Effector	Regulatory response
T box	Amino acid	Uncharged tRNA	Induced
B_{12} element	Cobalamin	AdoCbl	Repressed
THI box	Thiamin	TPP	Repressed
RFN element	Riboflavin	FMN	Repressed
S box	Methionine	SAM	Repressed
G box	Purine	Guanine	Repressed
L box	Lysine	Lysine	Repressed

analyses, has provided the foundation for the biochemical analyses that establish the role of the RNA itself. Given the rapid pace of work in this field, it is highly likely that many more systems remain to be uncovered.

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