A riboswitch-containing sRNA controls gene expression by sequestration of a response regulator
Sruti DebRoy et al.
Science 345, 937 (2014);
DOI: 10.1126/science.1255091

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occurred normally in nen4-1 (n = 4 sieve element files) and nen4-2 (n = 2) (Fig. 4E and figs. S1 and S10). However, agglomerated structures abutting on the inner side of the nuclear envelope were observed after stage three (Fig. 4G and fig. S10), which was not observed in either Ws (n = 2) (Fig. 4F) or the Columbia (Col) wild type (n = 3) (Fig. 1A and fig. S3). The amorphous H2B-YFP remnants were located in the peripheral region of the nucleus in nen4-1 and nen4-2 (Fig. 4G and fig. S10). Furthermore, the defective enucleation in nen4-1 was transgenically complemented (fig. S10). Taken together, this indicates that NEN4 is required for completing the NAC45/86-regulated enucleation process.

We have outlined the process by which sieve element cells in Arabidopsis differentiate and divest themselves of a nucleus. NAC45/86 orchestrate the enucleation, perinuclear organelle clustering, shift in NEN1 and NEN2 localization, and cytosol degradation by regulating the expression of a set of target genes, including NEN1, NEN2, and NEN4. The interdependence of these various NAC45/86-mediated subcellular processes remains to be investigated. We also observed a set of NAC45/86 independent processes, such as the formation of cell wall architecture and callose accumulation, the latter of which appears to be dependent on the upstream transcription factor APL. Our results add to the diversity of autolytic processes by which eukaryotic cells restructure their contents.

## References and Notes


## Acknowledgments

We thank K. Kanaižiene, M. Herpol, M. Lindman, A. Salminen, and I. Sevilem for technical assistance; R. Siljago, A. Möhönen, the University of Ghent, the Arabidopsis Biological Resource Centre, and the Nottingham Arabidopsis Stock Centre for published materials; A. Möhönen, E.-L. Eskelinen, A. Suomalainen-Wartiovaara, and P. Runeberg-Ros for discussions; and P. Benley, K. Nakajima, and S. El-Shawky for their critical comments. The serial block-face electron microscopy was supported by Biocenter Finland. S.M. was financially supported by the Japanese Society for the Promotion of Science. Work in the lab of T.B. was partly financed by grants of the Research Foundation-Flanders (project FWO – G030011N) and the Interuniversity Attraction Poles Programme from the Belgian Federal Science Policy Office. Work in the E.J. laboratory is funded by the Academy of Finland, Biocenter Finland, and the University of Helsinki. Financial support for the lab of Y.H. was provided by the Academy of Finland Centre of Excellence programme, the University of Helsinki, the European Research Council Advanced Investigator Grant Symdev, Telkes (the Finnish Funding Agency for Technology and Innovation), and the Gatsby Foundation. The supplementary materials contain additional data.

## Supplementary Materials

www.sciencemag.org/content/345/6199/933/suppl/DC1

Materials and Methods

Supplementary Text

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21 March 2014; accepted 3 July 2014

Published online 31 July 2014,

10.1126/science.1253736

## RIBOSwitches

### A riboswitch-containing sRNA controls gene expression by sequestration of a response regulator

**Sruti DebRoy,**1,∗ Margo Gebbie,2,∗ Arati Ramesh,3 Jonathan R. Goodson,2 Melissa R. Cruz,1 Ambro van Hoof,1 Wade C. Winkler,2† Danielle A. Garsin1†

The ethanolamine utilization (eut) locus of Enterococcus faecalis, containing at least 19 genes distributed over four polycistronic messenger RNAs, appears to be regulated by a single adenosyl cobalamine (AdoCbl)–responsive riboswitch. We report that the AdoCbl-binding riboswitch is part of a small, trans-acting RNA, EutX, which additionally contains a dual-hairpin substrate for the RNA binding–response regulator, EutV. In the absence of AdoCbl, EutX uses this structure to sequester EutV. EutV is known to regulate the eut messenger RNAs by binding dual-hairpin structures that overlap terminators and thus prevent transcription termination. In the presence of AdoCbl, EutV cannot bind to EutX and, instead, causes transcriptional read through of multiple eut genes. This work introduces riboswitch-mediated control of protein sequestration as a posttranscriptional mechanism to coordinately regulate gene expression.

Riboswitches are an important class of regulatory RNA; they typically regulate expression in cis of downstream open reading frames in response to binding of metabolic ligands, usually by affecting the transcription or translation (1–3). Small regulatory RNAs (sRNAs) are another class, and they commonly regulate gene expression by interacting with target mRNAs to affect translation or stability (4). Herein, we report the discovery of an sRNA, EutX, that contains a riboswitch aptamer and a EutV-binding site named P3/P4 (Fig. S1A). The presence of ethanolamine (EA) causes the sensor histidine kinase EutW to phosphorylate the response regulator, EutV, which thereby converts it into an activator RNA binding protein (4, 5). Thus, in the absence of EA, EutV is unphosphorylated and inactive. Adenosyl cobalamin (AdoCbl) is a cofactor required in the first enzymatic step of EA catabolism (6). Under conditions in which EA is present, but AdoCbl is not, our model states that EutX uses P3/P4 to bind and sequester active EutV–P, which prevents its antitermination activity (fig. S1A). When AdoCbl is also present, it binds to the AdoCbl riboswitch and induces termination before generating the P3/P4 loops, which prevents sequestration. Unhindered, EutV is then able to promote antitermination at the four eut polycistronic RNAs to drive gene expression. By this mechanism, the Eut system demands the presence of both the catabolic substrate (EA) and the key cofactor (AdoCbl) (fig. S1A).

To gain a more complete understanding of the effect of AdoCbl and EA on eut gene expression, Enterococcus faecalis cells were cultured to exponential growth phase in modified minimal medium in the presence or absence of EA and AdoCbl. Total RNA was then extracted and subjected to RNA-sequencing (RNA-seq) analysis (Fig. 1 and table S1). This revealed increased expression across the eut locus for cells that had been cultured with EA and AdoCbl compared with those cultured only in minimal medium. Metabolism of EA is believed to occur within a protein-bound organelle called a microcompartiment [reviewed by (7)]. Electron microscopy revealed microcompartments only for cells cultured in EA and AdoCbl (fig. S1B). These aggregate data demonstrate that eut gene...
expression and microcompartment formation can indeed be induced in the presence of both EA and AdoCbl.

Of the four constitutive promoters in the eut locus, three are followed by a EutV RNA binding site (P1/P2, P5/P6, and P7/P8). The distal hairpin in each case overlaps the 5′ sequence of an adjacent, intrinsic terminator. Phosphorylated EutV dimersize, which increases the affinity for the dual hairpin, which prevents the formation of the terminator structure and thereby induces gene expression (fig. S1A) (4, 5, 6, 9). A fourth promoter was detected just upstream of the AdoCbl riboswitch (8). Moreover, we discovered that another two-hairpin motif (P3/P4) is located downstream of the AdoCbl riboswitch (fig. S1A). To more closely examine the transcripts in this region, we analyzed total RNA by Northern blotting. An antisense RNA probe that spanned 301 nucleotides (nt) of the eutT-eutG intergenic region (Fig. 2A) revealed an RNA species of ~300 nt in length that was not present in medium containing AdoCbl, detectable in minimal medium, and notably enhanced (about fivefold) upon addition of EA without AdoCbl (Fig. 2B). A second RNA of ~150 nt was produced upon addition of AdoCbl. To resolve which parts of the eutT-eutG intergenic region corresponded to these different RNA species, the membranes were hybridized with antisense probes corresponding to the left and right halves of the 301-nt antisense probe (Fig. 2A). This analysis revealed that the ~300-nucleotide and ~150-nucleotide RNAs both included the AdoCbl riboswitch. Quantitative reverse transcription polymerase chain reaction (qRT-PCR), using primers within and outside of

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**Fig. 1.** EA and AdoCbl induce eut gene expression. By RNA-seq, all the eut genes were expressed in the presence of EA and AdoCbl, whereas little or no expression was observed in minimal medium.

**Fig. 2.** AdoCbl binding to the riboswitch causes premature termination within a sRNA, preventing generation of the P3/P4 hairpins that bind EutV. (A) Location of 32P-radiolabeled RNA antisense probes used to analyze the eutT-eutG intergenic region. (B) Total RNA was isolated from cells grown in the indicated medium conditions, resolved by denaturing polyacrylamide gel electrophoresis, transferred to a membrane, and hybridized with different antisense probes (1, 2 and 3). (C) The mapped 5′ and 3′ termini and the M3 mutation are indicated on a secondary structure depiction of EutX. A putative intrinsic terminator is highlighted by a gray-shaded box. (D) In vitro transcription termination assays with wild-type EutX template display a premature termination product only in the presence of AdoCbl. Termination is lost in the M3 mutant template. (E) EMSA of binding of EutV to EutX. A 117-nt fragment of EutX (117EutX) and EutVD54E protein [constitutively active for RNA binding (5)] were used. Controls included addition of 30 μM of an unlabeled competitor P1/P2 RNA and an unrelated RNA. (F) DracALa to assess EutV binding to the 117EutX RNA by using the same reaction conditions and control RNAs as in (E). (G) EMSA to investigate whether EutX could compete for EutV binding to P1/P2.
of EutX, detected full-length EutX primarily in medium containing only EA (fig. S2). Together, these data demonstrate that an sRNA, coined EutX, is produced from the eutT-eutG intergenic region.

To map the difference between the 300-nucleotide and 150-nucleotide RNAs, we used 3′ rapid amplification of cDNA ends (3′ RACE). By using cells grown in EA without AdoCbl, 3′ RACE located the 3′ terminus within an oligouridylate tract that occurs downstream of P3/P4 and just before the promoter of eutG, a length consistent with the Northern blot analysis (301 nts) (Fig. 2C). Using RNA from cells grown with just AdoCbl revealed a 3′ terminus at the end of the riboswitch aptamer where another stretch of U’s exists. The latter sequence appears to be part of an intrinsic terminator hairpin, which is formed from sequences at the 3′ end of the AdoCbl riboswitch aptamer (Fig. 2C), consistent with previous in vitro studies (8). Indeed, addition of low-micromolar AdoCbl promoted transcription termination at this site in vitro (Fig. 2D and fig. S3). AdoCbl riboswitches have a small loop containing three cytosines that forms a pseudoknot with downstream guanosines and is necessary for ligand binding (10, 11). The eut AdoCbl riboswitch is predicted to contain this characteristic L5 loop (Fig. 2C) (4). Consistent with a role in sensing AdoCbl, when we mutated these residues, it disrupted termination in response to AdoCbl (Fig. 2D).

Also, structural probing revealed that AdoCbl-induced changes were limited to the riboswitch portion of EutX (fig. S4). In addition, a construct in which lacZ is fused downstream of the terminator resulted in moderately decreased expression upon addition of AdoCbl (fig. S5).

To further characterize the function of EutX, the ability to bind EutV was confirmed by electrophoretic mobility assays (EMSA) and differential radial capillary action of ligand assays (DRAcALA) (12). Upon addition of micromolar concentrations, purified EutV bound to a portion of EutT that included P3/P4, as shown in Fig. 2, E and F. On the basis of comparative data of EutV binding to the P1/P2 hairpins, we speculate that the binding affinity to EutX may be moderately better (Fig. 2C) (9). Unlabeled 30 μM competitor RNA (EutX or P1/P2) could fully compete for binding to EutV in the context of radiolabeled EutX or P1/P2 (Fig. 2, E to G). However, an unrelated RNA of similar length was unable to compete for EutV, which demonstrated that binding of EutV to EutX is specific.

On the basis of the observation that the riboswitch exerts transcription attenuation–mediated control over a EutV binding site, we hypothesized that the EutX sRNA affects eut expression in trans. We therefore generated a eutG-lacZ fusion that contained the entire intergenic region between eutT and eutG (Fig. 3, A and B, blue construct), as well as fusions that either deleted P3/P4 (green), the riboswitch (red), or both (orange). These plasmids were transformed into a strain lacking the endogenous eutX or a wild-type strain. Expression of wild-type eutG-lacZ (blue) was induced by the presence of EA and AdoCbl when cells were grown in a modified minimal medium (Fig. 3B). No expression was observed for this construct in minimal medium, medium containing only AdoCbl, or in a background lacking EutV and EutW (fig. S6). The following data indicate that EutX acts in trans. First, the constructs that lacked P3/P4 were normally regulated in a wild-type strain that still contains P3/P4 in the chromosome (Fig. 3B), which suggested that P3/P4 is not needed in cis. Second, in the strain that lacked endogenous EutX, these same constructs were induced both in +EA and +EA+AdoCbl conditions (Fig. 3B), which suggested that the endogenous EutX prevents eut mRNA expression by sequestering EutV. Third, the construct that lacked the riboswitch aptamer, but still contained P3/P4, remained uninduced even in the presence of EA and AdoCbl, consistent with constitutive sequestration of EutV by P3/P4 (Fig. 3B). Because the regulation of the lacZ constructs could be altered by the presence or absence of EutX in the chromosome, these data demonstrate that the sRNA can regulate in trans and are consistent with the sequestration model (fig. S1A). Remarkably, in addition to inducing eutG-lacZ expression with only EA, the eutX strain has the additional property of supporting the formation of visually normal microcompartments in this medium, unlike the wild-type strain (fig. S7). Together, these data show that, in the absence of EutX, EA is sufficient to induce functional expression of the entire eut locus.

To further test the sequestration model, we introduced point mutations into the eutG-lacZ construct instead of deletions. An adenosine (A) at the first position and a guanine (G) at the fourth position of the six-nucleotide loops were

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**Fig. 3.** Both the riboswitch and the P3/P4 hairpins are required for inducible expression in vivo. (A) A diagram of the experimental design. A plasmid containing a eutG-lacZ fusion was manipulated to contain a deletion of the riboswitch, the P3/P4 hairpin, or both. The plasmid was introduced into E. faecalis strains containing either wild-type EutX or a deletion. (B) β-Galactosidase assays of strains shown in (A) when grown in modified minimal medium containing EA or both EA and AdoCbl. (C) β-Galactosidase assays of strains missing EutX but carrying eutG-lacZ plasmids that contain either the M1 or M2 mutations. The M1 mutations are A249U and G252U and the M2 mutations are A273U and G276U site-directed changes. The data are presented as the average of three or more independent experiments, and the error bars represent the standard deviation.
Riboswitches are ligand-binding elements contained within the 5′ untranslated regions of bacterial transcripts, which generally regulate expression of downstream open reading frames. Here, we show that in *Listeria monocytogenes*, a riboswitch that binds vitamin B₁₂ controls expression of a noncoding regulatory RNA, Rli55. Rli55, in turn, controls expression of the *eut* genes, whose products enable ethanolamine utilization and require B₁₂ as a cofactor. Defects in ethanolamine utilization, or in its regulation by Rli55, significantly attenuate *Listeria* virulence in mice. Rli55 functions by sequestering the two-component response regulator EutV by means of a EutV-binding site contained within the RNA. Thus, Rli55 is a riboswitch-regulated member of the small group of regulatory RNAs that function by sequestering a protein and reveals a distinctive mechanism of signal integration in bacterial gene regulation.

Ethanolamine is an abundant molecule in the vertebrate intestine (1, 2), and genes of the ethanolamine utilization pathway (*eut*) are widely conserved in pathogenic bacteria (3–5). This includes the Gram-positive intracellular human pathogen *Listeria monocytogenes*, in which *eut* expression has been shown to be up-regulated in the intestine during infection of mice (6), which suggests that ethanolamine is important for *Listeria* pathogenesis. In *Enterococcus faecalis*, *eut* expression is activated in response to ethanolamine by a two-component response regulator, EutVW (7, 8). In *Salmonella enterica*, ethanolamine utilization requires vitamin B₁₂ as a cofactor (9), and we noted the presence of a B₁₂-binding riboswitch located upstream of the first gene in the *eut* locus of *L. monocytogenes* (Fig. 1A) (10), which suggested that *eut* expression might also be regulated in response to B₁₂ availability.

To investigate a role for B₁₂, we examined expression of the *eut* locus in response to B₁₂ and ethanolamine by RNA sequencing (RNA-seq) (Fig. 1B) and quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. S1). We observed expression of the *eut* locus under all conditions, albeit at low levels, which suggested that the cell maintains a pool of EutVW to sense and respond to ethanolamine. In contrast, higher-level expression of EutVW and expression of other *eut* genes require both B₁₂ and ethanolamine (Fig. 1B). These data indicated B₁₂ is required to activate *eut* expression and suggested that the B₁₂ riboswitch does not prevent transcription of the *eut* locus in the presence of B₁₂, as might be expected for a classical riboswitch (11).

To clarify whether the riboswitch has a role in the B₁₂-dependent regulation of *eut* expression, we examined transcription of the riboswitch locus (Fig. 1C) in response to B₁₂ and ethanolamine. We were unable to detect any long transcript, which might extend into the downstream *eut* locus. However, we did detect a ~450-nucleotide (nt) transcript, Rli55 (10, 12), in the absence of B₁₂ (Fig. 1D), and a smaller (~200-nt) transcript that accumulated in the presence of B₁₂ (Fig. 1D). This result suggested that the riboswitch mediates transcription termination of the ~450-nt Rli55 transcript in response to B₁₂, and any remaining long transcript is rapidly degraded (fig. S2A). A strain with a deletion in the B₁₂ riboswitch (Δribos) constitutively expressed the long Rli55 transcript under all conditions (Fig. 1E and fig. S2B), which confirmed B₁₂-dependent regulation by the riboswitch. Conversely, neither the long nor short Rli55 transcripts were detected in a strain (Δrli55) in which the entire *rli55* locus was deleted (Fig. 1E and fig. S2B). Thus, the B₁₂ riboswitch determines whether Rli55 is expressed as a long or short transcript in response to B₁₂.

We reasoned that Rli55 might act as a regulatory RNA controlling expression of the *eut* locus. To test this hypothesis, we examined expression of *eut* genes by qRT-PCR in the wild-type, Δribo, and Δrli55 strains. In the wild-type strain, *eut* expression was elevated only when both ethanolamine and B₁₂ were present in the media (Fig. 1F and fig. S3, A and B), whereas in the Δribo strain, which constitutively expresses the long Rli55, *eut* genes were never expressed under any conditions. In contrast, in the Δrli55 strain, expression of the *eut* genes was high in the presence of ethanolamine alone in addition.