

Dual-acting riboswitch control of translation initiation and mRNA decay

Marie-Pier Caron^{a,b}, Laurène Bastet^a, Antony Lussier^a, Maxime Simoneau-Roy^{a,b}, Eric Massé^{b,1}, and Daniel A. Lafontaine^{a,1}

^aRNA Group, Department of Biology, Faculty of Science, Université de Sherbrooke, Sherbrooke, QC, Canada J1K 2R1; and ^bRNA Group, Department of Biochemistry, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada J1E 4K8

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Riboswitches are mRNA regulatory elements that control gene expression by altering their structure in response to specific metabolite binding. In bacteria, riboswitches consist of an aptamer that performs ligand recognition and an expression platform that regulates either transcription termination or translation initiation. Here, we describe a dual-acting riboswitch from *Escherichia coli* that, in addition to modulating translation initiation, also is directly involved in the control of initial mRNA decay. Upon lysine binding, the *lysC* riboswitch adopts a conformation that not only inhibits translation initiation but also exposes RNase E cleavage sites located in the riboswitch expression platform. However, in the absence of lysine, the riboswitch folds into an alternative conformation that simultaneously allows translation initiation and sequesters RNase E cleavage sites. Both regulatory activities can be individually inhibited, indicating that translation initiation and mRNA decay can be modulated independently using the same conformational switch. Because RNase E cleavage sites are located in the riboswitch sequence, this riboswitch provides a unique means for the riboswitch to modulate RNase E cleavage activity directly as a function of lysine. This dual inhibition is in contrast to other riboswitches, such as the thiamin pyrophosphate-sensing *thiM* riboswitch, which triggers mRNA decay only as a consequence of translation inhibition. The riboswitch control of RNase E cleavage activity is an example of a mechanism by which metabolite sensing is used to regulate gene expression of single genes or even large polycistronic mRNAs as a function of environmental changes.

gene regulation | RNA degradosome | translational control

Since the first demonstration that translation attenuation regulates the expression of the tryptophan operon (1), accumulating evidence has revealed the importance of posttranscriptional regulation in prokaryotes and eukaryotes alike. Posttranscriptional regulators include RNA molecules that operate through several mechanisms to control a wide range of physiological responses (2). Among newly identified RNA regulators are riboswitches, which are located in untranslated regions of several mRNAs and that modulate gene expression at the level of transcription, translation, or splicing (3). Riboswitches are highly structured regulatory domains that directly sense cellular metabolites such as amino acids, carbohydrates, coenzymes, and nucleobases. These genetic switches are composed of two modular domains consisting of an aptamer and an expression platform. The aptamer is involved in the specific recognition of the metabolite, and the expression platform is used to control gene expression by altering the structure of the mRNA. Recent bioinformatic analyses have reported the existence of several new RNA motifs exhibiting unconventional expression platforms (4, 5), suggesting that riboswitches using different regulation mechanisms are still likely to be discovered (6).

The lysine riboswitch was first characterized in *Bacillus subtilis*, where it is located upstream of a lysine transporter (*yvsH*) and a lysine-sensitive aspartokinase (*lysC*) (7–9). Sequence alignments predicted that the secondary structure of the aptamer domain is arranged around a five-way junction that is important for aptamer architecture (7, 9). Lysine binding to the riboswitch

results in the stabilization of the anti-antiterminator, the P1 stem, which induces premature transcription termination (7, 9, 10). Helices P2–P5 are involved in the formation of the global riboswitch structure that is required in the formation of the binding pocket specifically recognizing lysine (11, 12). Sequence alignments also predicted that, in contrast to *B. subtilis*, the *Escherichia coli lysC* riboswitch could control gene expression at the translational level (8). According to this model (Fig. 1A), the *E. coli lysC* riboswitch adopts an ON state in the absence of lysine to allow ribosome access to the ribosome-binding site (RBS). However, it is predicted that, when bound to lysine, the riboswitch folds into an OFF state that sequesters the RBS into a stem-loop structure, thereby inhibiting translation initiation.

Despite recent efforts dedicated to characterizing in vivo riboswitch regulation, very little is known about the actual cellular processes involved in riboswitch control mechanisms. In this work, we have studied the in vivo regulation mechanism of the *E. coli lysC* riboswitch. As expected from earlier predictions, our results are consistent with the riboswitch modulating translation initiation as a function of lysine. However, we observed that lysine binding to *lysC* also targets RNase E cleavage in the riboswitch, significantly reducing the *lysC* mRNA level. RNase E cleavage sites were mapped within the riboswitch expression platform, which become accessible to RNase E uniquely when the riboswitch is bound to lysine. The conformation of the lysine-free riboswitch prevents mRNA cleavage, thereby allowing *lysC* mRNA accumulation and translation. We also found that both regulatory mechanisms can be individually inhibited, directing the riboswitch to regulate expression at the level of either translation initiation or mRNA decay. These results are in contrast to other riboswitches, such as *thiM* and *btuB*, for which mRNA decay occurs only as a consequence of the inhibition of translation initiation. Our study provides a clear molecular mechanism in which riboswitches directly control mRNA decay by selectively modulating the access to RNase E cleavage sites as a function of metabolite binding.

Results

***lysC* Riboswitch Controls Gene Expression at the mRNA Level.** We first investigated the regulatory mechanism of the *lysC* riboswitch using transcriptional and translational *lacZ* chromosomal fusions containing the riboswitch domain with the first 57 nucleotides (19 codons) of *lysC* ORF (see constructs in *SI Appendix, Fig. S1A*). In these experiments, although the LysC-LacZ trans-

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¹To whom correspondence may be addressed. E-mail: eric.masse@usherbrooke.ca or Daniel.Lafontaine@USherbrooke.ca.

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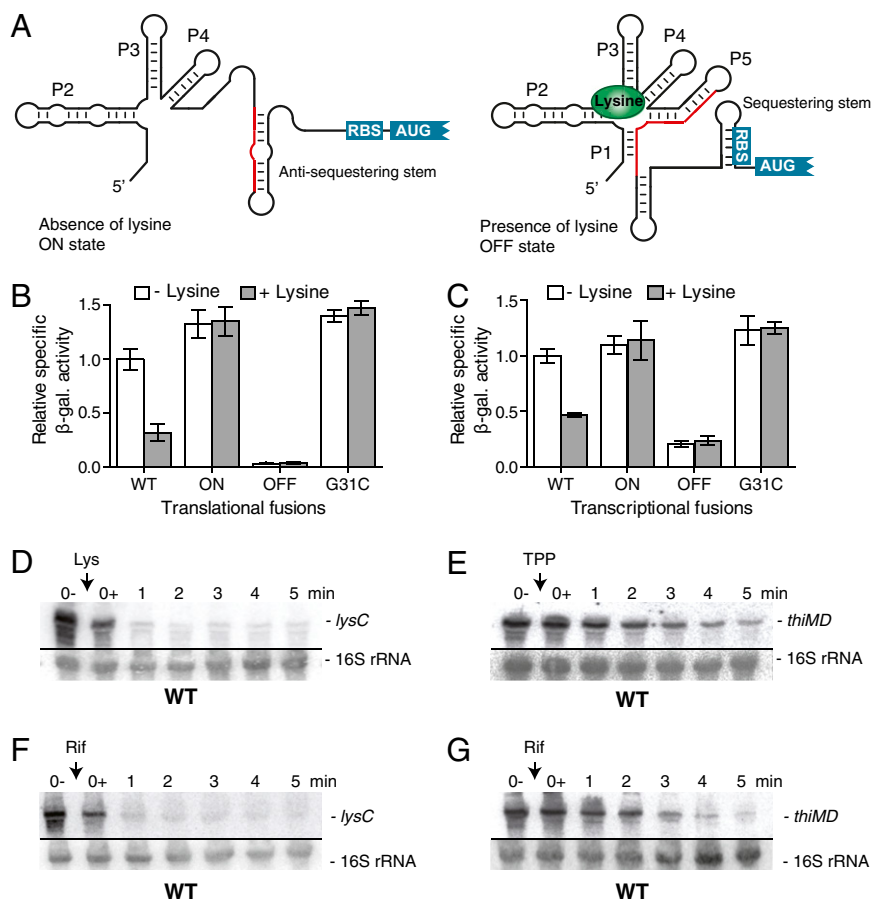


Fig. 1. The *E. coli* *lysC* riboswitch modulates the mRNA level upon lysine binding. (A) Schematic representation of the predicted lysine riboswitch translational control. In the absence of lysine, the ON state exhibits an antisequestering stem, which exposes the RBS and allows translation initiation. However, when bound to lysine, the riboswitch adopts the OFF state in which the presence of a sequestering stem masks the RBS and inhibits gene expression. The region shared by the antisequestering stem (ON state) and the aptamer (OFF state) is represented in red. (B) β -Galactosidase assays of translational *LysC*-*LacZ* fusions for wild type and ON, OFF, and G31C mutants (Tables S1–S3). Enzymatic activities were measured in the absence and presence of 10 μ M lysine. Values were normalized to the enzyme activity obtained for the wild type in the absence of lysine. (C) β -Galactosidase assays of transcriptional *lysC*-*lacZ* fusions for the wild type and for the ON, OFF, and G31C mutants. Enzymatic activities were measured in the absence and presence of 10 μ M lysine. Values were normalized to the activity obtained for the wild type in the absence of lysine. (D) Northern blot analysis of *lysC* mRNA level. Wild-type *E. coli* strain MG1655 was grown to midlog phase in M63 minimal medium with 0.2% glucose at 37 °C, and total RNA was extracted at the indicated times immediately before (0–) and after (0+) the addition of lysine (10 μ M). The probe was designed to detect the riboswitch region (positions 42–201) of the *lysC* mRNA (SI Appendix, Fig. S4A). 16S rRNA was used as a loading control. (E) Northern blot analysis of the *thiMD* mRNA level. Bacterial growth culture and RNA extractions were performed as described in D. TPP was added at a final concentration of 500 μ M. The probe was designed to detect the riboswitch region (positions 4–141) of *thiMD* mRNA. 16S rRNA was used as a loading control. (F) Determination of the *lysC* mRNA stability. Bacterial growth culture and RNA extractions were performed as described in D, but without the addition of lysine. Rifampicin was added at a final concentration of 250 μ M. 16S rRNA was used as a loading control. (G) Determination of *thiMD* mRNA stability. Bacterial growth culture and RNA extractions were performed as described in D. Rifampicin was added at a final concentration of 250 μ M. 16S rRNA was used as a loading control.

lational fusion reports on the *LysC* protein level, the transcriptional *lysC*-*lacZ* fusion has two independent RBS for *lysC* and *lacZ*, which monitor the *lysC* mRNA level. Because the *lysC* promoter is regulated, to a small extent, by lysine (SI Appendix, Fig. S1 B and C) (13), we used an arabinose-inducible promoter to prevent lysine-dependent promoter regulation.

In the context of a translational construct, we observed that the β -galactosidase activity of the wild-type riboswitch was reduced by 70% in the presence of 10 μ M lysine (Fig. 1B, WT). The extent of regulation is significantly larger than that obtained at the promoter level (SI Appendix, Fig. S1C), suggesting that genetic control is performed mostly via the *lysC* riboswitch. To verify that the regulation was dependent on riboswitch conformational changes, we used reporter constructs in which the riboswitch secondary structure was stabilized in either the ON or OFF state (see constructs in SI Appendix, Fig. S2). As expected, β -galactosidase activities were consistent with predicted struc-

tural states, in which ON and OFF constructs showed elevated and reduced activities, respectively (Fig. 1B). The introduction of a single-point mutation (G31C) in the core domain of the riboswitch completely abolished the lysine-induced regulation (Fig. 1B), in agreement with ligand binding being important for riboswitch activity (9). We next used transcriptional reporter constructs to establish whether the *lysC* riboswitch controls gene expression by modulating the mRNA level. Unexpectedly, we found a strong decrease in β -galactosidase activity when cells were grown in the presence of lysine (Fig. 1C, WT). Furthermore, when using constructs stabilizing the riboswitch in either the ON or OFF state or having the G31C mutation, we observed a clear correlation between reporter gene expression and riboswitch conformation, clearly indicating that the *lysC* riboswitch modulates the *lysC* mRNA level upon lysine binding. To determine whether the *lysC* ORF region is required to modulate the mRNA level, we designed a transcriptional fusion containing only

the riboswitch domain up to the AUG start codon (*SI Appendix, Fig. S1D*). A very similar lysine-dependent decrease in β -galactosidase activity was obtained (*SI Appendix, Fig. S1E*), indicating that the *lysC* riboswitch is sufficient to modulate the mRNA level as a function of lysine binding.

Because mRNA stability may be decreased when translation initiation is inhibited (14, 15), we next verified whether a decrease in the mRNA level (Fig. 1C) is observed generally among riboswitches that control translation when inhibiting translation initiation. To address this question, we used the *E. coli thiM* thiamin pyrophosphate (TPP) riboswitch that previously was shown to act at the translational level (16, 17). Although a strong TPP-dependent decrease in β -galactosidase activity was observed for the translational fusion in the presence of 500 $\mu\text{g}/\text{mL}$ TPP, no such decrease was detected when the transcriptional construct was used (*SI Appendix, Fig. S3*). However, when a transcriptional fusion containing a significant portion of the *thiM* ORF (100 codons) was used, a large reduction of β -galactosidase activity was obtained in the presence of TPP (*SI Appendix, Fig. S3B*). These results show that the *thiM* riboswitch requires a section of the ORF to decrease the mRNA level upon ligand binding, most probably because of putative regulatory elements, such as ribonuclease sites or transcription terminators, that are located in the coding region (14, 15). Thus, these results support our data indicating that the *lysC* riboswitch domain contains a regulatory signal that is modulated in a lysine-dependent manner to control *lysC* mRNA decay.

Level of *lysC* mRNA Decreases Rapidly in the Presence of Lysine. To analyze further the influence of lysine on the *lysC* mRNA, we performed Northern blot experiments using a probe directed against the riboswitch domain of *lysC* (see probe details in *SI Appendix, Fig. S4A*). In these experiments, cells were grown in minimal medium, and total RNA was extracted before and after the addition of lysine at several time points. A very rapid decrease in the *lysC* mRNA level was observed upon the addition of ligand (Fig. 1D), in agreement with lysine modulating the expression of a transcriptional *lacZ* fusion (Fig. 1C). A similar lysine-induced rapid decrease in the mRNA level also was obtained when using a probe directed against the *lysC* coding region (*SI Appendix, Fig. S4B*), supporting the idea that lysine binding to the riboswitch modulates the *lysC* mRNA level. However, in contrast to *lysC*, Northern blot experiments showed that the addition of TPP results in a slower decrease of the *thiMD* mRNA level (Fig. 1E). These results show that ligand binding to the *lysC* and *thiM* riboswitches does not modulate the mRNA level to the same extent, as is consistent with the two riboswitches exhibiting mechanistic differences in regulating gene expression.

Recently, it was reported that two riboswitches control gene expression via a Rho-dependent transcription termination mechanism (18). Because no obvious Rho-independent terminator could be identified within the *lysC* riboswitch, we wondered if the riboswitch could rely on Rho to terminate transcription prematurely upon lysine binding. We thus performed Northern blot assays in the presence of bicyclomycin, which is known to inhibit specifically the Rho ATP-dependent RNA-binding activity (19), and assessed whether the *lysC* mRNA level still would be decreased as a function of lysine. No effect of bicyclomycin was observed on the lysine-dependent decrease in the *lysC* mRNA level (*SI Appendix, Fig. S5*), suggesting that Rho is not involved in the *lysC* mRNA regulation. In contrast, we observed that bicyclomycin had a strong effect on the level of *rho* mRNA (*SI Appendix, Fig. S5*), which is known to be autoregulated via a Rho-dependent mechanism (20). Therefore, our results show that the level of the *lysC* mRNA is not controlled via a Rho-dependent transcription regulation mechanism.

The rapid decrease of the *lysC* mRNA level in the presence of lysine suggests an active mechanism of mRNA destabilization.

To determine whether lysine binding to *lysC* mRNA alters transcript stability, we used rifampicin to inhibit transcription initiation (21). Upon the addition of rifampicin, the level of *lysC* mRNA decreased drastically, with only trace amounts being detected after 1 min (Fig. 1F). This result indicates that the *lysC* mRNA half-life is too short to be determined experimentally, unlike most *E. coli* mRNAs, which exhibit half-lives between 3 and 9 min (22). However, a half-life of ~ 2 min was observed for *thiMD* mRNA (Fig. 1G), indicating that it is more stable than *lysC* mRNA. Therefore, because the *lysC* mRNA is a highly unstable transcript, our data suggest that *lysC* could be a target for a ribonuclease in which lysine binding to the riboswitch could modulate the rate of mRNA cleavage and degradation.

RNase E and RNA Degradosome Are Involved in *lysC* mRNA Decay.

In *E. coli*, the initiation of mRNA decay is caused primarily by rate-limiting endonucleolytic cleavage, generally mediated by the essential enzyme RNase E (23). RNase E is part of a multi-protein complex, the RNA degradosome, which also contains an exoribonuclease (PNPase), an RNA helicase (RhlB), and an enolase (24). We speculated that RNase E and/or the RNA degradosome could be involved in the rapid degradation of *lysC* mRNA. To verify the implication of the degradosome, we used the bacterial strain *rne-131*, which retains the ribonucleolytic activity of RNase E while preventing the formation of the RNA degradosome (25). We observed that, as compared with the wild-type strain (Fig. 1D), the level of *lysC* mRNA in the *rne-131* strain was increased significantly in the presence of lysine (Fig. 2A and B), suggesting that the RNA degradosome is involved in the lysine-dependent degradation of *lysC* mRNA. In agreement with this notion, we found that the half-life of *lysC* mRNA was increased to ~ 3 min in *rne-131* cells, significantly longer than in the wild-type strain (*SI Appendix, Fig. S6*). In contrast, the *thiMD* mRNA level remained very similar in both wild-type and *rne-131* strains (Fig. 2C and D), indicating that the RNA degradosome is not implicated in the regulation of *thiMD*.

To determine whether the endoribonucleolytic activity of RNase E is involved in the *lysC* mRNA decay, we used the mutant strain *rne-307I*, which carries a thermosensitive RNase E [RNase E(TS)] that is inactivated at 44 $^{\circ}\text{C}$ (26). As expected, when cells were grown at 30 $^{\circ}\text{C}$, very efficient *lysC* mRNA degradation was observed in the presence of lysine (Fig. 2E). However, when RNase E(TS) cells were incubated with lysine and shifted from 30 $^{\circ}\text{C}$ to 44 $^{\circ}\text{C}$, the level of *lysC* mRNA recovered to $\sim 85\%$ after 24 min (Fig. 2F). These results show that heat inactivation of RNase E(TS) resulted in the accumulation of *lysC* mRNA, even in the presence of lysine. For comparison, we performed similar experiments using the wild-type strain (*SI Appendix, Fig. S7*) and observed a slight accumulation of the *lysC* mRNA when cells were grown at 44 $^{\circ}\text{C}$ ($\sim 22\%$ at 24 min; *SI Appendix, Fig. S7B*). The significantly higher recovery yield of *lysC* mRNA at 44 $^{\circ}\text{C}$ in the RNase E(TS) strain ($\sim 85\%$) as compared with wild-type ($\sim 22\%$) (Fig. 2G) strongly suggests that RNase E is involved in lysine-dependent degradation of *lysC* mRNA.

***lysC* Riboswitch Exposes RNase E Cleavage Sites When Bound to Lysine.**

Because the *lysC* mRNA level depends on both the riboswitch conformational changes (Fig. 1C) and RNase E activity (Fig. 2G), we reasoned that the riboswitch could modulate the *lysC* mRNA level by selectively controlling the access to critical RNase E cleavage sites located in the riboswitch expression platform. We therefore set out to map cleavage sites precisely by using an in vitro cleavage assay using purified RNA degradosomes (27). As an effort to use minimal substrate sequences for RNase E activity, we first used an RNA molecule corresponding to the expression platform domain, comprising positions 212–309 (Fig. 3A and B). When incubating the 3'-end-radiolabeled expression platform in the presence of increasing

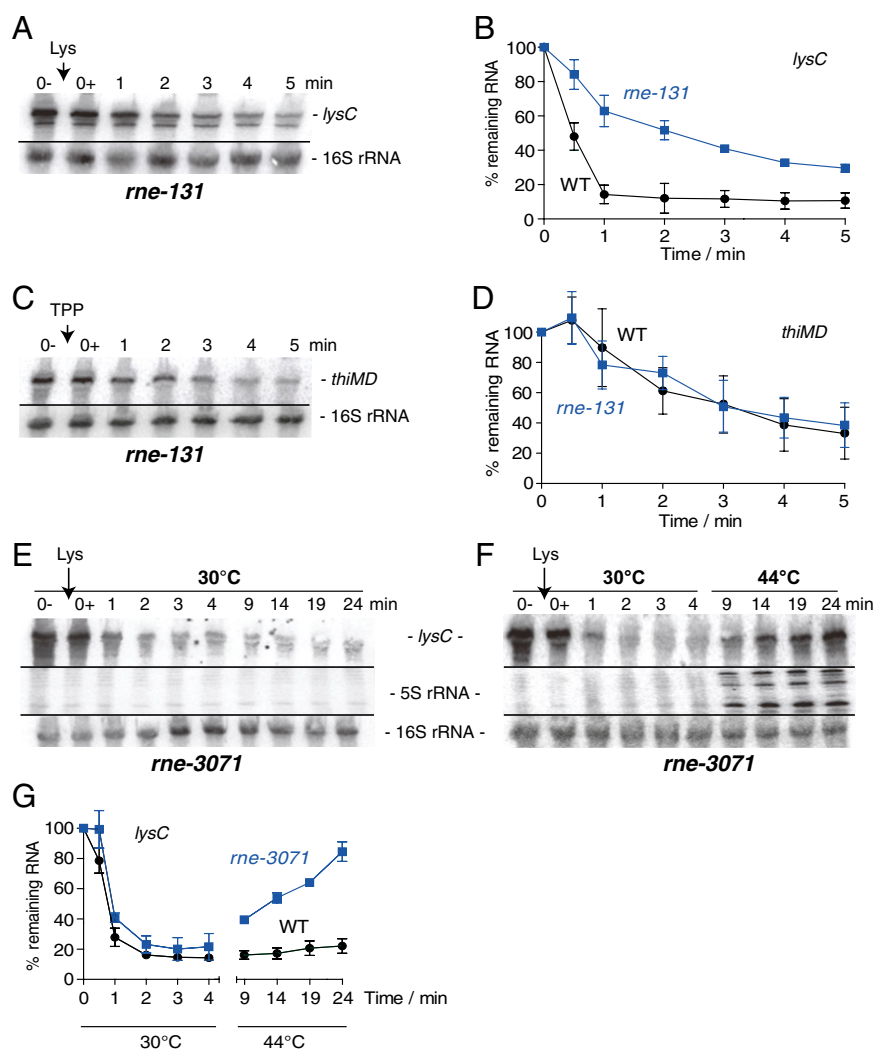


Fig. 2. RNase E and the RNA degradosome are involved in the rapid decrease of *lysC* mRNA. (A) Northern blot analysis of the *lysC* mRNA level in the context of the *rne-131* strain. The *E. coli* strain *rne-131* was grown to midlog phase in M63 minimal medium with 0.2% glucose at 37 °C, and total RNA was isolated at the indicated times immediately before (0–) and after (0+) the addition of lysine (10 μg/mL). 16S rRNA was used as a loading control. (B) Quantification analysis of Northern blots shown in A and Fig. 1D. The average values of three independent experiments with SDs are shown. (C) Northern blot analysis of the *thiMD* mRNA level in the context of the *rne-131* strain. Bacterial growth culture and RNA extractions were performed as described in A. Total RNA was isolated at the indicated times immediately before (0–) and after (0+) the addition of TPP (500 μg/mL). 16S rRNA was used as a loading control. (D) Quantification analysis of Northern blots shown in C and Fig. 1E. The average values of three independent experiments with SDs are shown. (E) Northern blot analysis of *lysC* mRNA in the context of the *rne-3071* [RNase E(TS)] strain grown at 30 °C (permissive temperature). The *E. coli* strain *rne-3071* was grown to midlog phase in M63 minimal medium with 0.2% glucose at 30 °C, and total RNA was isolated at the indicated times immediately before (0–) and after (0+) the addition of lysine (10 μg/mL). 16S rRNA was used as a loading control. (F) Northern blot analysis of *lysC* mRNA in the context of the *rne-3071* strain grown at 30 °C followed by a temperature shift at 44 °C (restrictive temperature). Bacterial growth culture and RNA extractions were performed as described in E. Total RNA was isolated at the indicated times immediately before (0–) and after (0+) the addition of lysine (10 μg/mL). Cells were incubated at 30 °C from 0–4 min and at 44 °C from 4–24 min. 16S rRNA was used as a loading control. Note that unprocessed 5S rRNA intermediates are detected at 44 °C, as is consistent with the inactivation of RNase E (26). (G) Quantification analysis of Northern blots shown in F and *SI Appendix*, Fig. S7B. The quantification represents the extent of recovery of *lysC* mRNA in the context of the *rne-3071* strain when grown at 44 °C. Such a recovery is not observed in wild-type cells. The average values of three independent experiments with SDs are shown.

amounts of purified RNA degradosomes, two cleavage sites were observed corresponding to nucleotides 249–250 and 257–258 (Site1 and Site2; Fig. 3C). Close examination of surrounding sequences showed that both cleaved regions exhibit an identical sequence, UCUUCC, in which the two cleavage sites flank the first uracil (Fig. 3A). The cleaved sequence is similar to previously reported RNase E cleavage sites, which are often, but not always, restricted to AU-rich sequences (14).

To determine whether the complete riboswitch sequence could be a substrate for the RNA degradosome, we next used the lysine riboswitch in our degradation assays and used primer extension

to detect cleavage sites. In the absence of lysine, a low cleavage activity was observed for regions Site1 and Site2 (Fig. 3D, lane 6), which correspond to determined positions using the expression platform sequence (Fig. 3C). However, a significant increase in RNase E cleavage was observed for both regions in the presence of lysine (Fig. 3D, lane 8), suggesting that lysine binding promotes RNase E cleavage. No such lysine-dependent increase was observed when using the G31C mutant (Fig. 3D, compare lanes 10 and 12), confirming that lysine binding to the riboswitch is important to facilitate RNase E cleavage. Moreover, when using constructs corresponding to OFF and ON riboswitch mutants, we

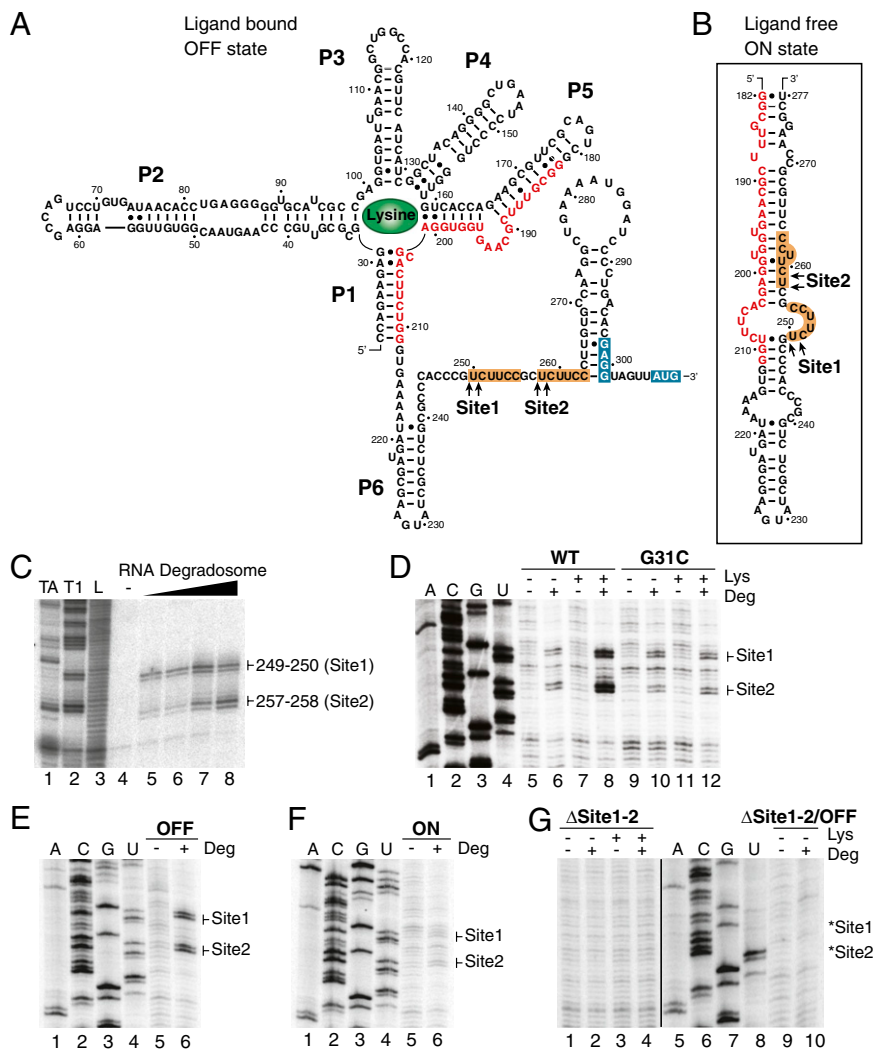


Fig. 3. The RNase E cleavage sites are located in the *lysC* riboswitch expression platform. (A) Predicted secondary structure of the *lysC* riboswitch in the presence of lysine (OFF state). Nucleotides shared by the antisequestering stem (ON state) and the aptamer (OFF state) are shown in red. RNase E cleavage Site1 (positions 249–250) and Site2 (positions 257–258) determined in vitro are indicated by arrows. Identical sequences surrounding both cleavage sites are in orange boxes. The RBS and AUG start codon are in blue boxes. (B) Predicted secondary structure of the antisequestering stem of the *lysC* riboswitch (positions 182–277). RNase E cleavage sites 1 and 2 are shown by arrows. (C) In vitro mapping of the RNA degradosome cleavage sites using 3'-end-radiolabeled riboswitch expression platform (positions 212–309). Increasing amounts of purified RNA degradosomes (0, 0.5, 0.76, 1.0, and 1.5 ng/μL) were used in the cleavage buffer (lanes 4–8). RNase TA (lane 1), RNase T1 (lane 2), and alkaline hydrolysis (L; lane 3) were used to generate molecular markers for gel migration. (D) In vitro mapping of the RNA degradosome cleavage sites using wild-type (lanes 5–8) or G31C riboswitch mutant (lanes 9–12). Riboswitch molecules were incubated in the absence and in the presence of lysine (68 μM) and/or RNA degradosomes (1 ng/μL). Reaction products were analyzed by reverse transcription using the EM1444 oligonucleotide (SI Appendix, Table S2). Lanes 1–4 represent the sequencing ladder. (E) In vitro mapping of the RNA degradosome cleavage sites using the OFF state riboswitch mutant in the absence (lane 5) and presence (lane 6) of RNA degradosomes (1 ng/μL). Cleavage sites were detected using reverse transcription as indicated in D. See SI Appendix, Fig. S2 for details about the OFF state riboswitch mutant. (F) In vitro mapping of the RNA degradosome cleavage sites using the ON state riboswitch mutant in the absence (lane 5) and presence (lane 6) of RNA degradosomes (1 ng/μL). Cleavage sites were detected using reverse transcription as indicated in D. See SI Appendix, Fig. S2 for details about the ON state riboswitch mutant. (G) In vitro mapping of the RNA degradosome cleavage sites using the ΔSite1-2 (lanes 1–4) and the ΔSite1-2/OFF state (lanes 9 and 10) riboswitch mutants. Experiments were performed in the absence and presence of lysine (68 μM) and/or RNA degradosomes (1 ng/μL). Cleavage sites were detected using reverse transcription as indicated in D. The expected positions of cleaved products are indicated on the right of the gel by asterisks. See SI Appendix, Fig. S2 for details about the ΔSite1-2 riboswitch mutant. The ΔSite1-2/OFF construct contains mutations used to obtain the ΔSite1-2 and OFF riboswitch mutants. Note that the gels shown for ΔSite1-2 and ΔSite1-2/OFF cleavage reactions were taken from different experiments.

observed high and low cleavage levels for OFF (Fig. 3E) and ON (Fig. 3F) state mutants, respectively, indicating that RNase E cleavage is modulated by *lysC* riboswitch conformational changes.

Because no clear consensus sequence has been determined for RNase E cleavage sites (24), we performed additional mutations to determine the specificity of the cleavage reaction. To avoid unforeseen production of cleavage sites caused by site-directed mutagenesis, we engineered a riboswitch mutant in which both

sites were partially deleted (ΔSite1-2; SI Appendix, Fig. S2). As expected, no cleavage was detected for both sites when this construct was used in our in vitro degradation assays, suggesting that the identity of Sites 1 and 2 is important for RNase E cleavage (Fig. 3G, lanes 1–4). In principle, because sequences surrounding both cleavage sites were removed, it is possible that the mutant inherently folds into the ON state, thus precluding the access to cleavage sites. To verify this possibility, we introduced

the Δ Site1-2 mutation in the context of an OFF state mutant that should prevent the riboswitch from folding into the ON structure. As expected, no cleavage activity was observed when using this mutant (Fig. 3G, Δ Site1-2/OFF, lanes 9 and 10), indicating that the inability of RNase E to cleave is caused by the removal of the cleavage site. Thus, our data clearly suggest that lysine binding induces the riboswitch to adopt the OFF state secondary structure in vitro, in turn allowing RNase E to cleave the riboswitch expression platform. Interestingly, when performing 3' RACE experiments, we detected *lysC* mRNA species that were truncated at Site1, consistent with the *lysC* riboswitch being targeted by RNase E in vivo (The cleavage site represented by an arrow, CCCGU↓CUUCC, was detected at least three times in our conditions).

Coupling Between Translation and mRNA Decay Is Not Required for *lysC* Riboswitch Regulation. To evaluate the in vivo importance of RNase E cleavage sites in the riboswitch expression platform, we engineered *lacZ* constructs in which both RNase E cleavage sites were removed (Δ Site1-2). Although the β -galactosidase activity of the translational fusion (trL) construct was reduced strongly in the presence of lysine, no such effect was observed with the transcriptional fusion (trX) construct (Fig. 4A), indicating that

the mRNA level no longer is modulated as a function of lysine. Furthermore, to establish the relative importance of Site1 and Site2, we made reporter constructs in which both sites were mutated (*SI Appendix*, Fig. S2). When the Site1 sequence was altered, the effects observed were very similar to those observed with the Δ Site1-2 mutant (Fig. 4A), as is consistent with Site1 being essential for the lysine-induced mRNA decay. In contrast, the removal of Site2 still allowed the mRNA level to be reduced (Fig. 4A, Δ Site2, trX construct), suggesting that Site2 is less important than Site1 in modulating the mRNA level. Therefore, although our results clearly suggest that the lysine riboswitch directly controls the mRNA decay by selectively allowing RNase E cleavage at Site1, they also indicate that the *lysC* riboswitch does not require RNase E cleavage for regulation at the translational level.

We next evaluated whether the *lysC* riboswitch could control gene expression exclusively by modulating mRNA decay. To do so, we first altered the AUG initiation codon sequence by introducing a U308A mutation (AAG mutant; *SI Appendix*, Fig. S2). When this mutation was introduced in a translational fusion, virtually no β -galactosidase activity was detected (Fig. 4B, AAG mutant, trL construct), in agreement with the inability of ribosomes to initiate translation. However, a significant decrease in gene expression was observed in the presence of lysine in the context of a transcriptional fusion (Fig. 4B, AAG mutant, trX construct), suggesting that the riboswitch is able to modulate the *lysC* mRNA level even in the absence of efficient translation regulation. Similar results were obtained when a G300C mutation was introduced into the RBS to disrupt ribosome binding (Fig. 4B, GACG mutant). These results concur with the *lysC* riboswitch being able to modulate mRNA decay even in the absence of translational control, in stark contrast to results for the *thiM* riboswitch (*SI Appendix*, Fig. S3B).

RNase E Cleavage of the *lysC* Riboswitch Is Limiting for *lysC* mRNA Decay. Our results suggest that RNase E cleavage at Site1 is essential to modulate the decay of the *lysC-lacZ* mRNA construct (Fig. 4A). To verify whether cleavage at Site1 also is important to control the decay in the context of the complete *lysC* mRNA, we engineered plasmids carrying an arabinose-inducible wild-type *lysC* or a *lysC* Δ Site1 gene. Both wild-type and Δ Site1 plasmids were introduced into a Δ *lysC* strain (see *SI Appendix*, *SI Materials and Methods* for details). According to our results, the *lysC* Δ Site1 mRNA exhibited a significantly (30–50%) stronger steady-state signal than the wild-type *lysC* mRNA after 15 min of arabinose induction (Fig. 5A and B, compare lanes 0–). In addition, although both mRNAs responded to lysine (added at time 0+), the *lysC* Δ Site1 mRNA was significantly less sensitive than wild-type *lysC* mRNA to lysine addition. Quantification of our results indicated that *lysC* Δ Site1 mRNA was consistently more abundant (20–40%) than wild-type mRNA in the presence of lysine (Fig. 5C). Thus, the increased steady state and reduced turnover of the *lysC* Δ Site1 mRNA suggest that RNase E cleavage of the *lysC* riboswitch is rate limiting for *lysC* mRNA degradation, as is consistent with the cleavage at Site1 being important to initiate the decay of the *lysC* mRNA.

Discussion

Our work has revealed a regulation mechanism involving a lysine-sensing riboswitch that controls mRNA decay directly through the selective folding of its expression platform. The large substrate recognition spectrum of RNase E suggests that riboswitch control of mRNA decay may be a widespread mechanism for regulating gene expression. Moreover, the *lysC* riboswitch modulates gene expression at two different genetic levels, namely translation initiation and mRNA decay.

Riboswitch Modulation of Translation Initiation and mRNA Decay in *E. coli*. Various studies have shown that riboswitches repress the

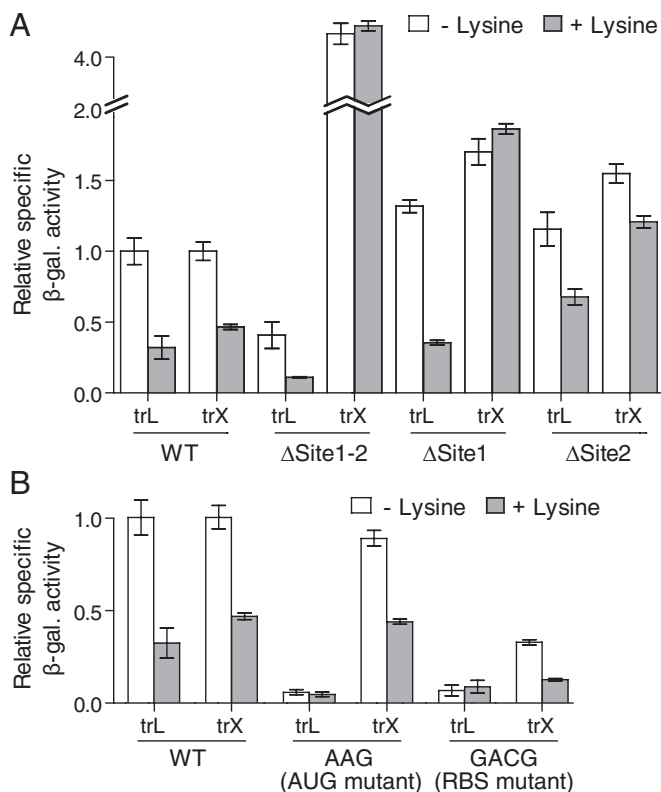


Fig. 4. The *lysC* riboswitch can be directed to control either translation initiation or mRNA decay. (A) β -Galactosidase assays using translational (trL) and transcriptional (trX) fusions of the *lysC* riboswitch for the wild-type, Δ Site1, Δ Site2, and Δ Site1-2 constructs. β -Galactosidase enzymatic activities were measured in the absence and presence of 10 μ g/mL lysine. Values obtained for translational and transcriptional fusions were normalized to enzymatic activities obtained for WT translational and transcriptional fusions, respectively, in the absence of lysine. See *SI Appendix*, Fig. S2 for details about riboswitch mutant constructs. (B) β -Galactosidase assays using translational and transcriptional fusions of the *lysC* riboswitch for the wild-type, AAG, and GACG constructs. Values obtained for translational and transcriptional fusions were normalized to enzymatic activities obtained for WT translational and transcriptional fusions, respectively, in the absence of lysine. See *SI Appendix*, Fig. S2 for details about riboswitch mutant constructs.

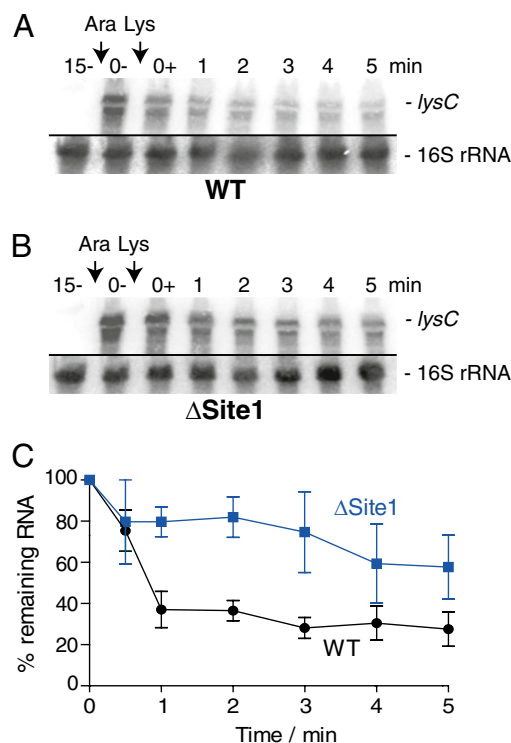


Fig. 5. Deletion of Site1 affects the lysine-dependent turnover of the *lysC* mRNA. (A) Northern blot analysis of the *lysC* mRNA expressed from a pBAD-*lysC* plasmid in the context of a Δ *lysC* strain. The plasmid was transformed into a strain in which the endogenous *lysC* gene was deleted (EM1055 Δ *lysC*::*cat*; *SI Appendix, SI Materials and Methods*). The resulting MPC70 strain was grown at 37 °C to midlog phase in M63 medium with 0.2% glycerol. Total RNA extractions were performed 15 min (lane 15-) before *lysC* mRNA induction with 0.1% arabinose (Ara) and before (lane 0-) and after (0+) the addition of lysine (10 μ g/mL). The probe was designed to detect the riboswitch region (positions 42–201) of the *lysC* mRNA. 16S rRNA was used as a loading control. (B) Northern blot analysis of the *lysC* Δ Site1 mRNA expressed from a pBAD-*lysC*- Δ Site1 plasmid. Bacterial growth cultures and RNA extractions were performed as described in A. 16S rRNA was used as a loading control. (C) Quantification analysis of Northern blots shown in A and B. The average values of three independent experiments are shown with SD.

initiation of translation upon ligand binding (17, 28–31). As expected for most translationally repressed mRNAs, the absence of translating ribosomes can reveal critical cleavage site(s) located in the ORF domain that are prone to nuclease attack, ultimately leading to mRNA degradation (32). A clear example has been described for the translationally acting *btuB* riboswitch from *E. coli*, for which transcriptional fusions containing only the riboswitch domain showed no modulation as a function of ligand (33). However, the β -galactosidase activity was decreased in the presence of ligand only when using fusions containing significant portions of the coding region (34), suggesting the presence of regulatory element(s) within the *btuB* ORF that negatively modulate the mRNA level in the absence of translating ribosomes (34). Together with recent studies (16, 17), our data suggest that the *thiM* riboswitch uses a similar genetic regulating mechanism (*SI Appendix, Fig. S3*). There is considerable precedence for such an interplay between translation and degradation in which mRNA lifetime is influenced mostly by the time during which it can support protein synthesis (32). In such cases, mRNA decay occurs only as a consequence of translation inhibition and is involved only as a scavenging process, which is referred to as a “nonnucleolytic repression mechanism” (15). Therefore, for both *btuB* and *thiM* riboswitches, available data are consistent with the translational process being central to mRNA

regulation in which ligand binding results in the nonnucleolytic repression of the regulated gene (Fig. 6A).

In contrast to *btuB* and *thiM*, our results show that the *lysC* riboswitch employs a different regulation mechanism, by directly modulating the cleavage of RNase E (Fig. 6B). Our data indicate that, in the absence of ligand, the riboswitch folds into the ON state that not only allows ribosome binding but also sequesters RNase E cleavage sites, thus ensuring efficient mRNA translation. However, in its ligand-bound form, the *lysC* riboswitch adopts the OFF state that concomitantly sequesters the RBS and exposes RNase E cleavage sites, thus effectively inhibiting translation and initiating mRNA decay. The location of RNase E cleavage sites in the riboswitch expression platform, and not in the ORF, strongly suggests that the *lysC* riboswitch directly controls the cleavage of the mRNA as a function of ligand binding. In such cases, the transcript stability and concomitant translation are reduced directly through endoribonucleolytic action, a situation referred to as “nucleolytic repression” (Fig. 6B) (15). In addition to the various characterized systems using such a regulation mechanism, it has been observed recently that small RNAs (sRNAs) also can use nucleolytic repression to control gene expression (27). However, although these observations suggest that riboswitches and sRNAs may exhibit similarities in their regulatory mechanisms, it also is likely that they possess fundamental differences such as the requirement for Hfq, which often is found in complex with RNase E. Indeed, when the lysine-induced *lysC* mRNA decay in the context of an *hfq* mutant strain was assayed, no apparent change in the decay was observed compared with a wild-type strain, suggesting that Hfq is not involved in *lysC* riboswitch-dependent mRNA decay (*SI Appendix, Fig. S8A*).

For the *lysC* riboswitch, it is possible that the presence of a bound 30S ribosome subunit at the RBS would prevent RNase E cleavage at Site1 by steric hindrance, thereby increasing the *lysC* mRNA level. Accordingly, a transcriptional *lysC-lacZ* fusion blocking translation elongation (AUG mutant) is expressed significantly more than a construct inhibiting translation initiation (RBS mutant) (Fig. 4B). Similarly, we found that the steady-state level of *lysC* mRNA is increased significantly in the presence of the antibiotic kasugamycin (*SI Appendix, Fig. S8B*), which specifically blocks initiating ribosomes at the RBS (35). However, even in the complete absence of ribosome binding (RBS mutant), a similar lysine-dependent reduction of the mRNA level is still observed (approximately twofold as compared with the wild-type) (Fig. 4B). These results suggest that the riboswitch does not rely mainly on ribosome binding to alter the *lysC* mRNA level but rather relies on conformational changes to modulate RNase E cleavage activity selectively. In addition, because the initiation of mRNA decay occurs within seconds after the addition of lysine to the medium (Fig. 1D, compare lanes 0- and 0+), it is not immediately clear why the *lysC* riboswitch also performs translational control. Most likely, the repression of initiating ribosomes may unmask ribonuclease cleavage sites located in the ORF, thereby allowing efficient mRNA degradation as observed in nonnucleolytic inactivation. Moreover, it is interesting to consider why the *lysC* riboswitch regulates mRNA decay in addition to translation initiation (Fig. 6B). One obvious explanation may be that it is important to shut down *lysC* expression irreversibly in the presence of lysine. For instance, we have shown previously that translation regulation is performed under thermodynamic control for the *add* adenine riboswitch (36, 37), allowing structural reversibility between both ligand-free and ligand-bound riboswitch conformations. However, in contrast to *add*, ligand binding to the riboswitch ensures rapid mRNA degradation and consequently definitive repression of gene expression, a function we consider to be dominant over translation inhibition. Moreover, our study strongly suggests that RNase E cleavage at Site1 is crucial for the rapid decay of the *lysC* mRNA

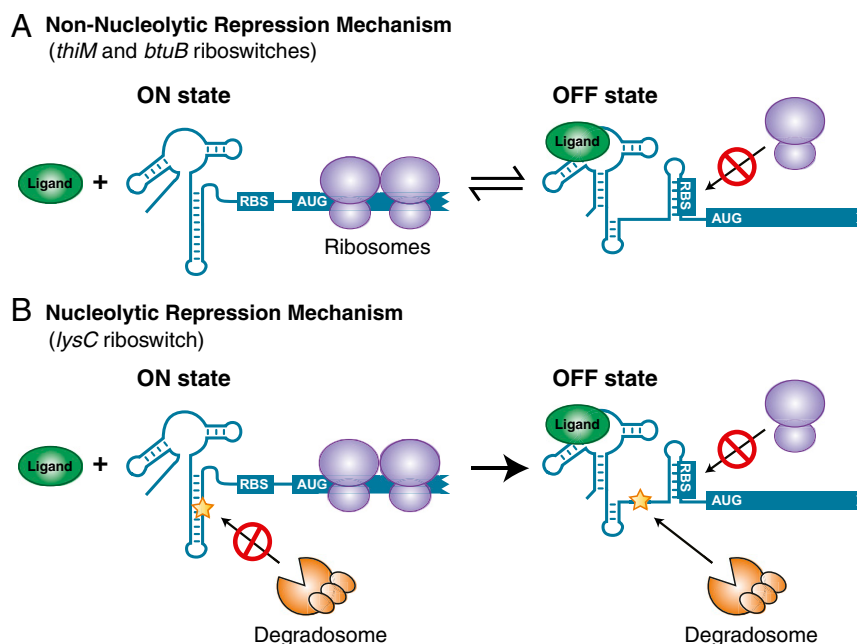


Fig. 6. Regulation mechanisms of riboswitches controlling at the levels of translation initiation and mRNA decay. (A) Regulation mechanism of *btuB* and *thiM* riboswitches controlling translation initiation (nonnucleolytic repression). In the absence of ligand, the riboswitch adopts the ON state in which translation initiation is allowed and gene expression ensues. However, ligand binding to the riboswitch leads to the adoption of the OFF state in which the RBS is sequestered, resulting in the inhibition of translation initiation and ultimately to mRNA degradation. (B) Regulation mechanisms of the *lysC* riboswitch controlling translation initiation and mRNA decay (nucleolytic repression). In the absence of ligand, the riboswitch folds into the ON state that sequesters the RNase E cleavage site (indicated by a star) and that allows translation initiation for gene expression. Upon ligand binding, the riboswitch adopts the OFF state that exposes an RNase E cleavage site and sequesters the RBS, both ensuring definitive gene repression.

(Figs. 4A and 5B), in agreement with the RNase E cleavage in the riboswitch expression platform being rate limiting and probably among the initial steps involved in the *lysC* mRNA degradation process, as previously observed for other RNase E targets (14). Additional cleavage sites are likely to be present in *lysC* mRNA, because the deletion of Site1 does not completely abolish the mRNA decay.

Although the action of RNase E has been shown previously to be favored by an accessible 5'-end terminus carrying a monophosphate residue (38), it also was suggested that RNase E cleavage might occur through internal entry (39). The sequence upstream of the *lysC* riboswitch contains 22 nucleotides and does not exhibit any strong secondary structure, suggesting that RNase E may recognize this sequence as a recruitment site. However, in addition to region containing Site1, the *lysC* riboswitch contains a single-stranded region (positions 276–288) in the expression platform that could constitute an RNase E entry site in the context of the OFF state (Fig. 3A). However, deletion of a major part of this region ($\Delta 279$ –283 mutant) did not significantly alter the lysine-dependent transcriptional repression (*SI Appendix, Fig. S8C*), suggesting that the 5' region of the riboswitch may be important for RNase E recruitment.

Riboswitches and Control of mRNA Decay. No other translationally acting riboswitch has been described that selectively modulates the access of ribonuclease cleavage sites to regulate gene expression. Still, several studies have reported that ligand binding to riboswitches may be linked to the modification of mRNA stability. For example, tRNA binding to the *B. subtilis thrS* leader has been shown to result in transcription antitermination (40). However, tRNA binding subsequently was found also to produce a cleavage upstream of the leader terminator, ultimately promoting mRNA stabilization and gene expression (41). Moreover, a recent study in *B. subtilis* has shown that the transcriptionally acting *yjiJ* SAM riboswitch is targeted rapidly by RNase Y (42).

In contrast to *lysC*, the antiterminated full-length mRNA is not a substrate for RNase Y in vitro or in vivo, suggesting that RNase Y is not involved in the regulation of the expressed gene but rather in riboswitch turnover (42). Another study performed in *Salmonella enterica* has suggested that the *mgtA* transcript is targeted by RNase E in the presence of high magnesium ion concentrations (43). Although additional experiments are required to establish how the transcriptionally acting *mgtA* riboswitch controls ribonuclease cleavage and whether putative cleavage site(s) occur in the riboswitch domain, it is possible that the *mgtA* riboswitch may use a regulation mechanism similar to that of *lysC* to control gene expression. In addition, a very recent study has found that *ribB* and *mgtA* riboswitch regulation mechanisms are more complex than previously thought, because both were found to control transcription termination using the transcription cofactor Rho (18). Interestingly, the modulation of Rho-dependent termination exhibits some mechanistic similarity to the regulation performed by the *lysC* riboswitch. Indeed, for both mechanisms, alternate riboswitch conformations selectively sequester or exhibit sequences that are recognized by external protein cofactors. Last, it also is important to consider the *glmS* ribozyme/riboswitch performing autocatalytic mRNA cleavage using glucosamine-6-phosphate (44). Even if the *glmS* ribozyme does not rely on a protein complex such as RNase E to perform mRNA cleavage, it uses a mechanism similar to the *lysC* riboswitch, because it also relies on ligand-induced endonucleolytic activity to achieve mRNA cleavage, thus providing access to the 5'-3' exoribonuclease J1 (45).

Together with existing literature, our findings describe a probably widespread mechanism whereby riboswitches directly control the mRNA level as a function of ligand binding. From an evolutionary perspective, it is plausible that RNase E cleavage sites appeared in already existing riboswitches controlling only translation initiation, giving rise to more modern dual-acting riboswitches modulating at the levels of translation initiation and

mRNA decay. In this sense, dual-acting riboswitches may represent more evolved representatives than transcriptionally or translationally regulating riboswitches and could consist of intermediate riboregulators toward a protein-based world.

Materials and Methods

DNA Oligonucleotides, Bacterial Strains, and Plasmids. DNA oligonucleotides were purchased from Integrated DNA Technologies. All bacterial strains were derived from *E. coli* MG1655. Mutations performed in *lysC* were made essentially as described previously (46). Transcriptional and translational fusions were performed using a PCR method (47, 48) described in *SI Appendix, SI Materials and Methods*.

β -Galactosidase Assays. Kinetic assays for β -galactosidase experiments were performed using a SpectraMax 250 microtiter plate reader (Molecular Devices) as described previously (49). Briefly, an overnight bacterial culture grown in M63 0.2% glycerol minimal medium was diluted 50 \times into 50 mL of fresh medium. The culture was incubated at 37 °C until an OD₆₀₀ of 0.1 was achieved, and then arabinose (0.1% final concentration) was added to induce expression of the *lacZ* constructs. Lysine (10 μ g/mL final concentration) was added when indicated. For the *lysC*-promoter fusions, cells were grown in M63 0.2% glucose minimal medium at 37 °C until an OD₆₀₀ of 0.1 was achieved, and the lysine (10 μ g/mL) was added when indicated. For all experiments, specific β -galactosidase activities were calculated as described previously (27). Each specific β -galactosidase activity has been relativized to the specific activity of the WT construct obtained in the absence of lysine. Reported results represent data of at least three experimental trials.

In Vitro Degradation Assays. Reactions were performed with 5 nM of unlabeled or [³²P]-labeled RNA corresponding, respectively, to the complete riboswitch sequence or to the riboswitch expression platform (see *SI Appendix, Table S4* for oligonucleotides used to generate DNA templates). RNA molecules were mixed with tRNA (6.6 ng/ μ L), denatured at 70 °C, and slowly cooled to 30 °C before the addition of the degradation buffer (13 mM Tris-HCl, 0.33 mM DTT, 73 mM NH₄Cl, 3.33 mM MgOAc, 0.1 mM EDTA, 0.7% glycerol). Pu-

rified RNase E-FLAG degradosomes (27) or degradation buffer [5 mM Tris-HCl, 50% (vol/vol) glycerol, 75 mM NaCl] was added to the mixture, which was incubated at 37 °C for 30 min. Reactions using [³²P]-labeled RNA corresponding to the riboswitch expression platform were stopped with an equal volume of phenol. Reactions using unlabeled transcripts corresponding to the complete riboswitch sequence were extracted using phenol-chloroform-isomyl, precipitated and annealed with 0.4 pmol of 5'-end-radiolabeled oligonucleotide (EM1444). The mixture then was heated at 90 °C for 1 min and chilled on ice. Reverse transcription was performed using 50 U of SuperScript II (Invitrogen) according to the manufacturer's protocol. The reaction was stopped by adding the stop buffer [41 mM Tris-HCl (pH 8.0), 0.083% SDS, 8 mM EDTA], and the cDNA was extracted once with phenol-chloroform, pH 8.0. The aqueous phase was treated with 125 mM KOH, heated at 90 °C for 5 min for RNA degradation, and precipitated with ethanol. Reaction products were separated on 8% polyacrylamide 8 M urea gel. The gel was scanned with a Typhoon Trio (GE Healthcare) and analyzed by ImageQuant software (Molecular Dynamics). The sequencing ladder was performed with the DNA T7 template used for in vitro RNA synthesis and 5'-end-radiolabeled oligonucleotide EM1444. The generation of the RNA ladder has been described previously (27).

Northern Blot Analysis and mRNA Half-Life Determination. Bacterial cultures were grown at 30 °C or 37 °C in M63 0.2% glucose minimal medium to midlog phase. Cells then were centrifuged and resuspended with sterile water, and total RNA was extracted immediately by the hot phenol procedure (50). Lysine (10 μ g/mL), TPP (500 μ g/mL), bicyclomycin (20 μ g/mL), or kasugamycin (500 μ g/mL) was added at the indicated time. For half-life determination, RNA transcription was blocked by the addition of 250 μ g/mL rifampicin. Northern blot experiments were performed as described previously (27).

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