Fluorescence monitoring of riboswitch transcription regulation using a dual molecular beacon assay

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ABSTRACT

Riboswitches are mRNA elements that specifically bind cellular metabolites and control gene expression by modifying their structure. As riboswitches often control essential genes in pathogenic bacteria, riboswitches have been proposed as new targets for antibiotics. High-throughput screening provides a powerful approach to identify riboswitch ligand analogs that could act as powerful antibacterial drugs. Biochemical assays have already been used to find riboswitch-binding analogs, but those methods do take into account the transcriptional context for riboswitch regulation. As the importance of co-transcriptional ligand binding has been shown for several riboswitches, it is vital to develop an assay that screens riboswitch-binding analogs during the transcriptional process. Here, we describe the development of a dual molecular beacon system monitoring the transcriptional regulation activity of the Bacillus subtilis pbuE adenine riboswitch. This system relies on two molecular beacons that enable the monitoring of transcription efficiency, as well as the regulatory activity of the riboswitch. Different analogs were tested using our system, and a good correlation was observed between riboswitch activity and reported metabolite affinities. This method is specific, reliable and could be applied at the high-throughput level for the identification of new potential antibiotics targeting any riboswitch-regulating gene expression at the mRNA level.

INTRODUCTION

Riboswitches are gene regulatory elements located within mRNA untranslated regions that modulate gene expression through the specific binding of small metabolites (1). The ligand-binding activity of riboswitches is performed by the aptamer domain consisting of a defined structure involved with most of the ligand atomic groups, ensuring high-binding affinity and specificity of recognition (2). The formation of the metabolite–aptamer complex is coupled to the modulation of the downstream expression platform that controls gene expression through various mechanisms, such as transcription termination, translation initiation and mRNA splicing (3). The versatility of riboswitches is well illustrated by purine riboswitches. They comprise the adenine- and guanine-sensing variants, which have been shown to activate or repress gene expression through metabolite binding, respectively (4,5). Although the guanine-specific riboswitch regulates only at the level of transcription by modulating the formation of a transcription terminator, the adenine riboswitch is involved in both the control of transcription elongation and in the modulation of translation initiation by sequestering the Shine–Dalgarno and AUG start codon sequences (6–8).

As pathogenic bacteria are increasingly found to acquire resistance to a large variety of antibiotics (9), riboswitches have been considered to be promising targets for the development of novel antibacterial drugs (10–12). The large-scale exploration of new riboswitch-targeting analogs requires the development of novel assays easily applicable to high-throughput screening approaches. Although initial efforts using high-throughput screens have been put forward by engineering allosteric ribozymes having the ability to be regulated via molecular effectors (13), recent studies have reported the use of the naturally occurring glmS ribozyme that can be specifically activated by the binding of glucosamine-6-phosphate (GlcN6P) (14,15). However, for most other riboswitches that are putative candidates as drug targets but do not have ribozyme activity, it may be challenging to identify an antimicrobial compound that is specific towards the targeted riboswitch rather than to the ribozyme-derived riboswitch construct.

In this work, we report the development of a straightforward detection assay based on molecular beacons that...
enables the identification of drug compounds targeting any riboswitch that regulates expression at the mRNA level. Molecular beacons are nucleic acid molecules possessing a stem–loop structure in which the loop portion of the beacon is complementary to a pre-determined sequence in a target mRNA (16). These fluorescent reporters contain a fluorophore and a quencher that are attached to both extremities of the molecule. Although a beacon does not fluoresce when folded into the stem–loop conformation, it emits fluorescence when hybridized to its target mRNA because the fluorophore and the quencher are too far apart from each other. Recent studies have shown that because the fluorophore and the quencher are too far apart from each other, the conformation, it emits fluorescence when hybridized to its beacon does not fluoresce when folded into the stem–loop domain, and transcription reactions were performed using Escherichia coli RNA polymerase from Epicenter Biotechnologies as previously described (8). Transcription products were migrated on a 10% polyacrylamide gel at 18 W for 2 h in 1× TBE (90 mM Tris-borate, pH 8.3, 2 mM EDTA) buffer. Experiments were conducted at least three times, and all exhibited similar uncertainties (<10%). Additional mutations introduced are indicated in the text.

Native gel electrophoresis

[5'-32P]-labeled adenine riboswitches were incubated in absence or in presence of molecular beacons at 70°C for 3 min and allowed to slowly cool to room temperature to ensure hybridization. Samples were separated in 1× TB (90 mM Tris-borate, pH 8.3) and 1 mM MgCl2 in 8% acrylamide:bisacrylamide (29:1) gel in Tris–borate buffer containing 1 mM MgCl2 at room temperature at 150 V for 8 h with the running buffer circulated during the electrophoresis.

Fluorescence analysis

Fluorescence spectroscopy was performed on a Quanta Master fluorometer, and spectra were corrected for lamp fluctuations and instrument variations as described previously (21). All data were collected at 37°C in a buffer containing 40 mM Tris, pH 7.5, 20 mM MgCl2 and 10 mM DTT in a final volume of 100 μl. Each reaction is composed of 5 pmoles template, 65 μM rNTP and 10 pmoles of each 5′-OMB and 3′-OMB. The solution was kept at 37°C for 5 min before 5 U of E. coli RNAP were added. Tetramethyl-rhodamine (TMR) was excited at 558 nm, and the emission values were collected from 568 to 595 nm. Fluorescein was excited at 494 nm, and the emission values were collected from 505 to 530 nm. For each point, the initial fluorescence intensity (I0) is subtracted to the fluorescence intensity of both 5′-OMB and 3′-OMB (I) and divided by I0, given the equation \((I - I0)/I0\). The riboswitch activity is measured by normalizing the fluorescence intensity of the 3′-OMB with the corresponding 5′-OMB value. Experiments were performed in absence or presence of 10 μM ligand.

**MATERIALS AND METHODS**

**Molecular beacons**

The sequence of 2′-O-methyl molecular beacons are DABCYL-GCCUUUUUUUUUCGC–Fluorescein (5′-OMB) and DABCYL-GCGUUGAUUUUCUCG C-5′-TMR (3′-OMB), respectively, for the 5′ and 3′ molecular beacons.

**Synthesis of RNA molecules**

Templates for transcription were made by polymerase chain reaction from synthetic DNA oligonucleotides. Synthetic DNA templates included an xpt-pbuX bacterial promoter (19), followed by the B. subtilis pbuE riboswitch sequence, including 25 nt after the terminator stem. Where indicated, the T7 RNAP promoter (20) was used instead of the xpt-pbuX promoter. DNA sequences are shown for wild-type and mutant constructs. The 5′-OMB and 3′-OMB target sites are underlined.

WT

ATCGCAACAAATTAAAAAGCG
ATTTATTACATGTGGTACACTCATCAACGGAAA

OFF Mutant

CAGCCTATGCAAGAGATTAGAATCTTGATATA
ATTATTACATGTGGTACACTCATCAACGGAAA

Single-rounds *in vitro* transcription assays

DNA templates for single-round *in vitro* transcriptions were prepared as described previously (8). Briefly, the xpt-pbuX promoter sequence was used to generate a transcription start site ~46 nt upstream of the aptamer domain, and transcription reactions were performed using *Escherichia coli* RNA polymerase from Epicenter Biotechnologies as previously described (8). Transcription products were migrated on a 10% polyacrylamide gel at 18 W for 2 h in 1× TBE (90 mM Tris-borate, pH 8.3, 2 mM EDTA) buffer. Experiments were conducted at least three times, and all exhibited similar uncertainties (<10%). Additional mutations introduced are indicated in the text.
Experiments were performed at least three times, and all exhibited similar uncertainties (<5%).

RESULTS

General strategy for monitoring riboswitch activity using molecular beacons

The *B. subtilis* adenine riboswitch modulates transcription elongation of the *pbuE* gene encoding a purine efflux pump involved in the export of adenine (22,23). In presence of low adenine concentrations, the riboswitch adopts an OFF state-inhibiting gene expression by inducing the formation of a transcription terminator (Figure 1A). However, in presence of high-adenine concentrations, the riboswitch folds into an ON state that allows transcription elongation and synthesis of a full-length *pbuE* transcript. Although the riboswitch domain is transcribed independently of the adenine concentration, the elongated mRNA is only produced when the intracellular concentration of adenine is high (5).

We have designed a reporter system in which two OMBs are used to monitor the promoter efficiency, as well as the control of the *pbuE* riboswitch transcription elongation (Figure 1A). We first designed a molecular beacon (5'-OMB) that binds upstream of the aptamer domain so that the promoter transcription activity could be monitored. A second molecular beacon (3'-OMB) was engineered to hybridize downstream of the transcription terminator; therefore, it only detects the elongated riboswitch mRNA. Although the 5'-OMB reporter was labeled with fluorescein and a DABCYL quencher, tetramethylrhodamine (TMR) and DABCYL were used for the 3'-OMB molecule. Riboswitch target sequences were modified to allow efficient binding of molecular beacons without affecting riboswitch activity. By using such a dual molecular beacon system, it is possible to simultaneously monitor both transcription efficiency and riboswitch regulation control.

The transcriptional regulation activity of the *pbuE* riboswitch was first assessed using single-round *in vitro* transcription assays (19). In these assays, we used a DNA template, including the *B. subtilis xpt* promoter.
fused to the pbuE riboswitch to allow transcription of a readthrough product terminating 40 nt after the AUG start codon. In our experimental conditions, a low readthrough efficiency of 23% was obtained when performing a transcription reaction in absence of ligand (Figure 1B; WT). However, in presence of 10 µM 2:6-diaminopurine (DAP), which is known to bind the pbuE riboswitch with high affinity (5), the readthrough efficiency was increased to a high level of 84%, consistent with ligand binding, promoting pbuE transcription elongation in vivo (5,8,21). As expected, the introduction of mutations in the P1 stem stabilizing the riboswitch ON state (ONMutant) promoted a high level of mRNAs elongation, regardless of the presence of the ligand (>80% readthrough efficiency; Figure 1B). In contrast, destabilization of the P1 stem to favor the OFF state (OFFMutant) resulted in the constitutive production of a high fraction of prematurely terminated mRNA species (22% readthrough efficiency, Figure 1B). Thus, our data are in agreement with the pbuE riboswitch transcription modulation being controlled by a ligand-induced conformational change.

To determine whether both molecular beacons can efficiently bind to the pbuE transcript, RNA molecules corresponding either to the readthrough or terminated mRNA were transcribed using the T7 RNAP and were assessed for their ability to bind molecular beacons using electrophoretic mobility shift assay (EMSA) (Figure 1C). When transcripts corresponding to the readthrough species were migrated in native conditions in presence of either 5′-OMB or 3′-OMB molecules, a similar gel retardation was observed in both cases, suggesting the formation of a transcript-beacon complex (Figure 1C, lane 1, respectively). Furthermore, a slower migrating complex was observed when both beacons were present, indicating that riboswitch transcripts hybridized simultaneously to both OMBs (Figure 1C, lane 4). When the EMSA assay was repeated using the terminated transcript (Figure 1C, lanes 5–8), complex formation was observed only when the 5′-OMB was present (Figure 1C, lanes 6 and 8), agreeing with the absence of a 3′-OMB target sequence in the terminated transcript. These results suggest that both beacons can efficiently bind to their respective target sequence in the context of the adenine riboswitch.

**Fluorescence detection of pbuE riboswitch transcription**

Using the transcription conditions previously established (Figure 1B), we used the 5′-OMB reporter to monitor E. coli RNAP transcription activity. The fraction of bound 5′-OMB was obtained by exciting fluorescein at 494 nm and by measuring the emitted fluorescence at 517 nm at 1-s intervals. When the transcription was performed using 10 µM rNTP, the 5′-OMB fluorescence intensity was recorded as a function of time by varying the rNTP concentrations to 10, 20, 65 and 100 µM.

![Figure 2](https://academic.oup.com/nar/article-abstract/41/10/e106/1074346)

Figure 2. Characterization of 5′-OMB and 3′-OMB reporter activities in the context of the pbuE riboswitch. (A) 5′-OMB fluorescence intensity monitored as a function of time by varying the rNTP concentrations to 10, 20, 65 and 100 µM. (B) The 5′-OMB fluorescence emission recorded when transcribing the pbuE riboswitch using either T7 or E. coli RNA polymerase. The fluorescence intensity of the 5′-OMB is represented as a function of the transcription reaction time. (C) The 5′-OMB fluorescence intensity measured at various temperatures in presence of its target sequence. A control, the 5′-OMB was incubated alone at 45°C and showed no significant fluorescence emission because of temperature destabilization. (D) Effect of DAP concentration on the pbuE riboswitch transcriptional regulation monitored using the 3′-OMB fluorescence emission. DAP was added to the transcription reaction at concentrations ranging from 0 to 25 µM, and the fluorescence emission of the 3′-OMB was measured as a function of time.
emission was observed to increase steadily over the course of the transcription reaction (Figure 2A), consistent with an accumulation of pbuE transcripts. As we previously observed that the rNTP concentration influences the rate of transcription (8), we repeated the experiment using a different rNTP concentration to determine whether the fluorescence increase is proportional to the transcriptional activity. As expected, the 5'-OMB fluorescence signal increased proportionally to the concentration of rNTPs (Figure 2A), consistent with fluorescence emission being related to transcription efficiency. However, signal saturation was obtained in presence of 65 μM rNTP, suggesting either that the maximal rate of transcription was attained, or that the binding of the molecular beacon was limiting. To distinguish between these alternatives, we repeated the transcription reaction assay using T7 RNAP, which is known to exhibit ~10–20 times faster transcription rates (24). When using T7 RNAP, the 5'-OMB fluorescence signal was dramatically increased over the first 5 min of the reaction and then reached a plateau for the rest of the time course (Figure 2B). Thus, because our assay can monitor the higher transcriptional activity of T7 RNAP, it suggests that the 5'-OMB reporter can readily detect the transcriptional activity of the E. coli RNAP. The signal saturation obtained when increasing rNTP concentrations also suggests that the transcriptional activity does not significantly change at rNTP concentrations higher than 65 μM.

Although molecular beacons are designed to fluoresce only when bound to their target, it is possible that unbound beacons may emit fluorescence because of an equilibrium existing between open (fluorescent) and closed (non-fluorescent) conformations (16). For example, given that the transcription reaction is performed at 37°C, the partial melting of the unbound beacon could give rise to fluorescence emission. To rule out this possibility, the 5'-OMB was incubated at 45°C in absence of the target transcript, and its fluorescence was recorded over time (Figure 2C). No significant fluorescence emission was detected, suggesting that the beacon remains folded in its non-fluorescent conformation. In contrast, significant fluorescence emission was observed when incubating the beacon in presence of the target transcript at all tested temperatures (Figure 2C), consistent with the hybridization of 5'-OMB being required for fluorescence emission.

The riboswitch transcriptional control was next monitored by measuring the fluorescence signal of the 3'-OMB as a function of DAP concentration (Figure 2D). As expected from single-round transcription assays (Figure 1B), we found that the 3'-OMB fluorescence signal was increased proportionally to DAP concentrations (Figure 2D). No significant 3'-OMB fluorescence increase was observed >10 μM DAP, suggesting that ligand saturation was attained in these conditions. This result agrees well with a previous single-round in vitro transcription study in which a T50 value of ~0.5 μM was reported for DAP (8). Taken together, these data suggest that our molecular beacon assay can be used to accurately monitor the pbuE transcription efficiency, as well as the ligand-dependent riboswitch transcriptional regulatory control. For all of the following experiments, transcription reactions were performed at 37°C in presence of 10 μM DAP and 65 μM rNTP, which represents the lowest concentrations showing the highest riboswitch transcriptional control (Figure 2A).

Monitoring pbuE riboswitch activity using a dual molecular beacon assay

We next modified our method to monitor both molecular beacons within a single experiment to ensure a more robust and reliable assay across experimental conditions. In these assays, the ratio of bound 5'-OMB and 3'-OMB were obtained by selectively exciting fluorescein at 494 nm and collecting the emitted fluorescence from 505 to 530 nm, and by exciting TMR at 558 nm and measuring the emitted fluorescence signal from 568 to 595 nm. The sensitivity of the method was first determined by varying the concentration of DNA template from 2 to 20 pmole (Figures 3A and 3B). From the emission spectra, we observed that the 5'-OMB fluorescence increased proportionally to concentration of the template (Figure 3A). Furthermore, when analyzing the 3'-OMB fluorescence, a signal increase was also observed as a function of the concentration of the template (Figure 3B), even if the transcription reaction was performed in absence of ligand. These results are expected according to our single-round transcription data because a small fraction of elongated RNA is still produced in these conditions (23% readthrough product; Figure 1B).

Next, we monitored the transcription reaction as a function of DAP when using both beacons. Because the 5'-OMB target sequence is located upstream of the transcription terminator (Figure 1A), the 5'-OMB fluorescence should not be modulated by the riboswitch transcriptional control and thus could be used as an ‘internal reporter’ to normalize the 3'-OMB fluorescence signal. In agreement with this, no signal variation was observed when monitoring the 5'-OMB fluorescence in absence or presence of DAP (Figure 3C). In contrast, a significant fluorescence increase was detected for the 3'-OMB in presence of DAP (Figure 3D), consistent with the riboswitch-promoting transcriptional readthrough in presence of ligand (Figure 1B). By combining data obtained for both 5'-OMB and 3'-OMB beacons (Figures 3C and D), a plot of transcription reactions was generated in which the 3'-OMB fluorescence signal is normalized to provide a direct measure of the ligand-induced riboswitch transcriptional control (Figure 3E). In our conditions, we observed that the presence of 10 μM DAP resulted in an ~5-fold increase in transcription readthrough (Figure 3E; compare time points at 60 min), in agreement with single-round transcription reactions (Figure 1B). Control experiments were also performed to establish the sensitivity and robustness of the fluorescent reporters in the context of the transcription reaction. As expected, when using ONMutant and OFFMutant templates, we obtained elevated and reduced riboswitch transcriptional activities for the ONMutant and OFFMutant constructs, respectively (Figure 4A). Our results suggest that the extent of transcription elongation is slightly higher for the ONMutant
We next engineered a dual beacon target construct containing both 5'-OMB and 3'-OMB-binding sites (Figure 4A). Importantly, this construct lacks the metabolite-binding aptamer domain and was engineered to estimate the maximum signal that can be obtained in conditions where all transcribed riboswitch mRNAs are in their elongated form, thus providing binding sites for both OMBs. Using this construct, we observed an increase of the fluorescence signal which was higher than what was obtained using the riboswitch ONMutant template (Figure 4B). This result indicates that even in the context of the ONMutant riboswitch (Figure 4B), where the P1 stem is stabilized, there is still a fraction of premature transcription termination.

To estimate the maximum efficiency of premature termination in our conditions, we designed an additional template control lacking the aptamer domain in which the transcription terminator of the riboswitch was inserted between both 5'-OMB and 3'-OMB target sequences (Figure 4A; terminator construct). Using this control, transcription reactions yielded a small fraction of transcripts exhibiting 3'-OMB-binding site (Figure 4B), which is reminiscent of what we obtained for the wild-type riboswitch in absence of ligand (Figure 3E) or for the OFFMutant construct (Figure 4A).

These data allowed us to determine the dynamic range of the adenine riboswitch regulation usable to screen

Figure 3. Monitoring riboswitch activity using a dual molecular beacon assay. (A) 5'-OMB fluorescence emission monitored from 505 to 530 nm with increasing concentrations of DNA template ranging from 2 to 20 pmoles. (B) Fluorescence emission of 3'-OMB measured from 565 to 595 nm using increasing DNA template concentrations from 2 to 20 pmoles. (C) Transcription reaction monitored using 5'-OMB fluorescence. The fraction of 5'-OMB fluorescence emission was determined in absence (squares) and in presence (circles) of 10 μM DAP. (D) Transcriptional control of the riboswitch monitored using 3'-OMB fluorescence. The fraction of 3'-OMB fluorescence was determined for each time point in absence (squares) and in presence (circles) of 10 μM DAP. (E) Normalized pbuE riboswitch activity obtained in absence (squares) and in presence (circles) of 10 μM DAP. The riboswitch activity is normalized by using the 5'-OMB fluorescence emission, which is not dependent on the riboswitch transcriptional control.
riboswitch analogs. Together, our experimental controls indicate that our fluorescent assay is robust and sensitive to detect riboswitch conformational states.

**Low-throughput analysis of the ligand-induced pbuE riboswitch transcriptional activity**

To demonstrate the suitability of our system for the search of riboswitch-binding small molecules, we performed a low-throughput analysis of various purine-related compounds that have been previously tested to bind purine riboswitch aptamers (4,5). The concentration of reagents added to our assay was optimized to use a minimal ligand concentration giving a reproducible and significant fluorescence signal. Individual experiments were monitored at the beginning and after 1 h of transcription, and results were normalized according to the dual beacon target construct (Figure 5). Without ligand, normalized transcription efficiencies were low, whereas reactions performed in presence of DAP, 2AP, adenine (Ade), guanine (Gua), 9-methylguanine (9-Me-Gua) and O₆-thioguanine (Thio-Gua). Only DAP, 2AP and adenine allow significant transcription elongation. Control experiments were performed with the dual beacon target and the ON₉Mutant transcripts, both enabling transcription elongation without added ligand.

**DISCUSSION**

The system developed herein addresses the need for a screening assay able to identify new riboswitch analogs that could potentially be used as antimicrobial agents (11). This method is the first one designed to directly measure the riboswitch transcriptional regulatory activity using fluorescence detection, thus substantially facilitating its implementation in a high-throughput context. Although RNA misfolding could influence the outcome of the assay, it should not occur in a ligand-dependent fashion and, therefore, should not introduce a bias in the detection of binding analogs. The development of such a system strongly depends on the efficient binding of molecular beacons to accurately report on the riboswitch transcriptional control...
activity. For instance, a recent study on the
in a high-throughput fashion, they do not address the
ligand-binding activity of riboswitches.

variants, it could also be used to screen for potential
measure the SAM-binding affinity for many aptamer
stabilized and does not efficiently interact with the fluor-
escence. However, on SAM binding, the aptamer is
the riboswitch ON state, which does not allow 2-AP fluor-
is free to interact with the 2-AP–containing strand to form
base (26). In absence of the ligand, the SAM-I aptamer
an RNA oligonucleotide containing a 2-AP fluorescent
molecularly by the hybridization of the aptamer and of
switch in which the terminator helix is formed inter-

...ction reaction. Indeed, because large concentrations of
compounds are used in our assays (10 μM), it is expected
that low-binding affinity metabolites, such as guanine and
related compounds, may be detected (Figure 5). However,
more stringent conditions can readily be applied by
decreasing the ligand concentration to detect compounds
having higher binding affinity.

Fluorescent nucleic acids have already been described
as tools to measure riboswitch ligand-binding affinities.
For example, two recent studies have used the ligand-
dependent glmS ribozyme cleavage activity to identify
new potential ligands (14,15). In both cases, fluorescent
versions of the RNA were engineered in which ligand-
induced ribozyme cleavage would alter the fluorescence
signal. As the modulation is directly related to the
ligand-induced ribozyme activity, analogs exhibiting high
affinity towards the ribozyme can easily be detected.
Another method recently described uses a synthetic
aptamer responsive to the aminoglycoside antibiotic
tobramycin (25). The system consists of a fluorescein-
labeled aptamer exhibiting a sequence complementary
to a DNA oligonucleotide attached to magnetic beads.
On ligand binding, the aptamer undergoes structural
to prevent its pairing to DNA. After purification
of RNA bound to the magnetic beads, the binding
specificity for various molecules is evaluated by measuring
the fluorescence emission. Finally, Sanbonmatsu and
coworkers monitored the SAM-I riboswitch-binding
activity by designing a trans-acting version of the ribo-
switch in which the terminator helix is formed inter-
molecularly by the hybridization of the aptamer and of
an RNA oligonucleotide containing a 2-AP fluorescent
base (26). In absence of the ligand, the SAM-I aptamer
is free to interact with the 2-AP–containing strand to form
the riboswitch ON state, which does not allow 2-AP fluor-
escence. However, on SAM binding, the aptamer is
stabilized and does not efficiently interact with the fluo-
rescent strand, thus effectively allowing fluorescence to be
emitted from 2-AP. Although this construct was used to
measure the SAM-binding affinity for many aptamer
variants, it could also be used to screen for potential
ligand analogs binding to the SAM-I riboswitch.

Even though the techniques described earlier in the text
can monitor the ligand-binding activity of riboswitches
in a high-throughput fashion, they do not address the
transcriptional context of the riboswitch regulation
activity. For instance, a recent study on the B. subtilis
flavin mononucleotide (FMN) riboswitch has shown that
the riboswitch relies on RNAP transcription-pausing sites
to allow more time for ligand binding (27). Moreover,
it was recently demonstrated that two adenine-sensing
riboswitches differ by using different regulation regimes
(8). Although the transcriptionally acting B. subtilis
pbuE riboswitch was also shown to operate under a
kinetic regime, the translationally acting Vibrio vulniﬁcus
add riboswitch was found to use a different regulation
mechanism that seems to be thermodynamically driven
and where ligand binding can occur post-transcriptionally.
Furthermore, the btuB, mgtA and ribB riboswitches from
E. coli were also shown to depend on the transcriptional
process (28,29), suggesting that other riboswitches are
likely to rely on the transcriptional context to regulate
gene expression. Therefore, because riboswitch genetic
regulation cannot always be predicted from thermo-
dynamic characteristics derived from the aptamer–ligand
complex, it is important to seek alternative
high-throughput analogs screening methods that take
into account the riboswitch regulatory context.

The dual molecular beacon assay described here is
robust and can be fully automated to screen compounds
binding to any riboswitch-controlling expression at the
transcription level. The main advantage of our method
is that it directly measures the ligand effect on riboswitch
transcriptional regulation activity. Indeed, this system
facilitates the identiﬁcation of molecules that bind to the
riboswitch aptamer and induce the structural switch in the
expression platform. Because this assay relies on transcrip-
tion, it is obvious that any compound like rifampicin that
inhibits transcription would not be good candidates owing
to their likely large antibiotic spectrum (18). However,
because the 5'-OMB signal monitors the level of transcription,
such compounds will be readily detected and avoided.
Although the described method is useful and
informative, the molecular beacon assay has its own limi-
tations. First, some inherent physical characteristics of
screened analogs could potentially affect data analysis
and lead to misinterpretation. Precisely, any fluorescent
analogs could affect the fluorescence signal of the molecu-
lar beacons if both signals are emitted in the same range
of wavelengths. To avoid such false positives, it is advis-
able to perform control transcription reactions using an
OFF Mutant construct, which should not show any ligand-
dependent fluorescence increase. One possible solution
for those cases would be to label the problematic molecu-
lar beacon using alternative dyes. Second, the insolub-
ility of compounds in the reaction buffer could also be
a problem for this assay. For those molecules, different
buffer conditions should be tested to promote their
solubility.

In summary, we have used the adenine-sensing ribo-
switch as a model to validate a novel screening assay
using molecular beacons. However, this system could be
used with any riboswitch-regulating gene expression at the
transcriptional level. This system is also amenable to
high-throughput approaches and is promising for the dis-
covery of new potential inhibitors of riboswitch-regulated
genes. Moreover, similar approaches to the one described
here could be developed to screen molecules against
translational riboswitches. A simple method would use a molecular beacon complementary to the ribosome binding site (RBS) sequence. On metabolite recognition by the aptamer domain, the RBS would be sequestered and inaccessible for binding the molecular beacon. However, the absence of ligand binding would result in the binding of the molecular beacon and fluorescence emission. This general approach could provide a high-throughput platform for the discovery of novel riboswitch-binding analogs exhibiting powerful antimicrobial properties.

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