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Intracellular light-activation of riboswitch activity

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Abstract

By combining a riboswitch with a cell-permeable photocaged small molecule ligand, an optochemical gene control element was constructed, enabling spatial and temporal control of gene expression in bacterial cells. Because of the simplicity of this strategy, coupled with the ability to create synthetic riboswitches with tailored ligand specificities and output in a variety of microorganisms, plants, and fungi, this approach may afford a general strategy to photo-control gene expression in vivo. The ability to activate riboswitches using light enables the interrogation and manipulation of a wide range of biological processes with high precision, and will have broad utility in regulation of artificial genetic circuits.

Keywords

riboswitch; photocontrol; RNA; synthetic biology; chemical biology

Introduction

Riboswitches are a class of naturally occurring genetic regulatory devices, found in all three domains of life and widely distributed throughout bacteria. Riboswitches respond to a range of metabolites, including protein cofactors, amino acids, nucleobases, metal ions, and some natural products, often with exquisite specificity.^[1] Most riboswitches are located in the untranslated regions of metabolic genes and are usually involved in regulation of the associated pathway.^[2] In general, riboswitches consist of an aptamer domain, required for ligand recognition, and an expression platform. Ligand binding results in a conformational change in the expression platform that subsequently either switches gene expression from 'ON' to 'OFF' or from 'OFF' to 'ON'. Naturally occurring riboswitches control gene expression via transcriptional control, translational control, or by acting as cis-acting ribozymes.^[3] To date, only the *glmS* ribozyme is known to operate via the latter mechanism,^[4] although riboswitches that affect splicing in fungi, plants, and algae, are also known.^[5]

Given the remarkably small size of naturally occurring riboswitches, which do not require protein components for function, and the ability to easily insert them into untranslated regions of genes, there has been much interest in applying riboswitches to the conditional control of gene expression and to the sensing of small molecules. For example, riboswitches

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could be used in synthetic biology as parts of artificial genetic circuits for controlling cellular behaviour,^[6] and could be used to probe, interrogate, and manipulate biological processes in vivo for a variety of chemical biology applications.^[7] In order to expand the applications of riboswitches, synthetic riboswitches with tailored ligand specificities and output functions have been engineered.^[6b, 8] However, the ability to control riboswitch activity inside cells in a spatial and temporal fashion remains severely limited. Light is an external input signal that can be used to control a broad array of biological processes with high spatio-temporal resolution, complete bioorthogonality, and simple equipment.^[9] Accordingly, light is a potentially powerful input that in combination with small molecule ligands could be used to control the activity of natural or engineered riboswitches in vivo with minimal invasion. Typically, photocaging groups are used to render small molecule ligands or biological macromolecules photosensitive. Upon irradiation with light, the caging group is removed, thus revealing the active small molecule or macromolecule and activating its function. Notably, while several ribozymes have been controlled using photocaging technologies,^[10] there are no reports of using light to control the activity of other types of riboswitches, such as those that operate at the transcriptional or translational level. Here, we used a photocaged analogue of a riboswitch ligand to afford spatial and temporal control of gene expression (Figure 1). Because the caged ligand is cell permeable,^[10d] non-toxic at active concentrations, and completely orthogonal to the host organism, this approach affords a convenient strategy to control gene expression in vivo. Furthermore, we hypothesize that the simplicity and potential adaptability of this strategy might lead to the development of general tools for spatial and temporal control of gene expression in a wide variety of organisms.

Results and Discussion

Photo-control strategy

An engineered riboswitch designed to respond to theophylline (**1**, Figure 1A), designated '12.1', was chosen as the prototype for this study because the switch is predicted to operate at the translational level via a simple RBS sequestration mechanism (Figure 1B).^[11] Accordingly, we reasoned that the switch could potentially be utilized for the photo-control of biological processes in a wide variety of bacteria.^[12] The solution structure of the parent aptamer used to create the 12.1 riboswitch indicates that a uracil residue (U24) in the ligand-binding site of the aptamer is hydrogen bonded to N9 of theophylline.^[13] Disruption of this intermolecular bond likely destabilizes a set of stacking interactions that constitute the core of the aptamer structure, and could explain the remarkable discrimination that the aptamer displays between closely related small molecules. Accordingly, we reasoned that a nitrobenzyl photocaging moiety located at N7 of **1** would provide an analogue (**2**, Figure 1A)^[10d] that would not be recognized by the aptamer portion of 12.1 and would therefore fail to turn on gene expression. Conversely, irradiation should remove the caging moiety, revealing the active ligand and switching on gene expression (Figure 1B). The synthetic riboswitch is housed in the plasmid pSAL, which includes the *IS10* promoter and terminator sequences, in addition to a β -galactosidase reporter gene, *lacZ*.^[11a] Determination of the activation ratio of 12.1 in response to **1** using a modified Miller assay in liquid cultures of *E. coli* TOP10 verified the expected high activation response of this synthetic switch (Figure

2A). Indeed, galactosidase activity of the theophylline-activated riboswitch was similar to that obtained by constitutive LacZ expression from a control plasmid that lacked the riboswitch. Interestingly, the activation ratio of 12.1 in response to theophylline determined using other common laboratory strains of *E. coli* proved less impressive (Figure 2A), largely due to reductions in the 'ON' activity (presence of ligand), but nonetheless represented some of the most robust riboswitches characterized to date. By varying the concentration of **1**, the dose-dependency of the 12.1 riboswitch was evaluated. As expected, the induction of LacZ expression increases in a dose-dependent manner (Figure 2B) in response to increasing concentrations of **1**. Next, we investigated the ability of the caged theophylline **2** (Figure 1A) to induce gene expression under the control of the 12.1 riboswitch. In the absence of **2**, a very low level of β -galactosidase expression is detected in the presence or absence of UV irradiation (Figure 2C), consistent with the low 'off' activity of 12.1 – indicating that UV light alone does not have an effect on reporter gene expression. Similarly, in the presence of **2** and the absence of UV irradiation, essentially no β -galactosidase activity is detected, demonstrating that the caged ligand is unable to induce a conformational change that exposes the 12.1 RBS to allow gene expression. Notably, in the presence of **2** and UV irradiation, LacZ expression is activated to levels similar to that of natural theophylline (Figures 2A/C). Gratifyingly, 12.1-controlled LacZ expression in the presence of UV-irradiation and **2** was also linearly dependent on the concentrations of **2** (Figure 2D). Cumulatively, this data confirms the expected UV dependency of the 12.1 riboswitch in response to the photocaged ligand and demonstrates for the first time the ability to photo-regulate the activity of a simple riboswitch in vivo.

Versatility of the photo-controlled riboswitch as a gene control element

Several riboswitches have been described as modular with regard to the reporter protein, and in at least some examples, with respect to the promoter system. To determine whether **2** in combination with the 12.1 riboswitch could be used to photo-control the expression of other reporter proteins, we substituted the *lacZ* gene with the gene encoding GFP, yielding the plasmid pSAL/*gfp*. Subsequent quantification of GFP expression by fluorescence measurements in the absence or presence of theophylline revealed an activation ratio of only four, an unexpected 20-fold lower than that with LacZ as the reporter (Table 1, entries 1 and 2). Similarly, when *DsRed* was used as the reporter gene (pSAL/*DsRed*), the activation ratio was low (Table 1, entry 3). We hypothesized that the loss in activation efficiency when *lacZ* is replaced with another reporter gene is a result of interactions between the *lacZ* encoding sequence and the 12.1 riboswitch in the corresponding transcript. To test this hypothesis, we fused the first 42 nucleotides of the *lacZ* gene sequence directly upstream of the *gfp* gene in the plasmid pSAL/*gfp*, to afford the construct pSAL/*lacZ-gfp*. Subsequent determination of the activity ratio of this *lacZ-gfp* fusion indicated it was unable to rescue activation of the 12.1 riboswitch to efficiencies as high as that with full-length *lacZ* (Table 1, compare entry 1 and 4), even though a purified LacZ-GFP fusion protein retained 95% the fluorescent output of GFP alone (data not shown). In a further effort to overcome the apparent dependency of gene reporter and activation ratio, the relatively weak IS10 promoter of each of the pSAL series of vectors was replaced with the stronger T7 promoter, while the IS10 terminator was substituted with the T7 terminator sequence. The resulting *lacZ*, *gfp*, and *DsRed* constructs (pET17b/T7/*lacZ*, pET17b/T7/*gfp*, and pET17b/T7/*DsRed*, respectively) were used to

determine theophylline activation ratios in *E. coli* BL21(DE3). However, none of these T7-based constructs delivered activation ratios greater than five (Table 1, entries 5-7) and were 10- to 50-fold lower than pSAL-based activation in the same strain (compare to Fig 2A). The lacZ-gfp fusion also led to a poor activation ratio (Table 1, entry 8). Increasing or decreasing the number of nucleotides between the promoter sequence and RBS failed to improve the activation ratio when either GFP and IS10 were used as the reporter and promoter, respectively (Table 1, entries 10-11). Similarly, sequence insertions or deletions failed to improve activation ratios when combinations of lacZ/GFP and T7 were used as the reporter and promoter, respectively (Table 1, entries 12-14). In summary, the activation ratio of the 12.1 riboswitch is dependent on both the reporter gene and promoter sequence identity. Intriguingly, Mfold calculations of the 12.1 transcript do not support structural interaction between the promoter and 12.1 riboswitch sequence (data not shown). Our data suggests that a more complex mechanism might be responsible for the poor activation ratio of the *gfp*- and/or T7-based constructs. Perhaps the in vivo concentration of RNA transcript affects the riboswitch behaviour, a mechanism that could be highly dependent on the vector copy number, promoter strength, and reporter sequence identity. Some dependency between riboswitch activation ratio and promoter identity has been reported previously for a theophylline riboswitch,^[14] although activation ratios differed only ~10-fold with three different promoters in that study. In the context of parts for synthetic biology applications, riboswitches possess a certain degree of portability in terms of combining them with different promoters and gene reporters, however, they may require at least some optimization for specific applications that were not originally designed for.

Spatial control of riboswitch activity

Based on the successful light-activation of riboswitch activity using bulk in vivo reactions, the effectiveness of using light to control riboswitch activity in a spatial fashion was investigated using liquid cultures. *E. coli* TOP10 cells harboring the plasmid pSAL were grown in each well of a microplate in the presence of **2**. Selected wells of the 96-well microplate were irradiated with UV light using a mask. LacZ expression was only observed in the wells that were exposed to UV light, as judged by the distinct coloration of those wells as a result of the chromogenic galactosidase substrate X-gal (Figure 3). This data clearly demonstrates the ability to control riboswitch activity in a spatial and temporal fashion.

Conclusions

The ability to dissect and manipulate biological processes in a spatial and temporal manner is greatly facilitated by the use of light. Here, we have demonstrated the ability to photo-control gene expression by combining a translational-controlled riboswitch with a light-activated version of its ligand. This strategy should prove a general approach for photo-controlling gene expression in vivo given (1) the breadth of ligands recognized by naturally occurring riboswitches,^[1b] (2) the emergence of efficient strategies for creating synthetic riboswitches,^[1a] and (3) the ease of installing photocaging moieties into diverse small molecules.^[9a] Moreover, riboswitches have been engineered to function in several Gram-negative and Gram-positive bacterial hosts,^[12, 14] as well as plants,^[15] thus our photocaging

strategy could potentially be applied in a broad range of organisms. Accordingly, we expect this photo-control strategy will be widely adopted for controlling gene expression, and will provide a valuable device for artificial genetic circuits.

Experimental Section

General

All plasmids were verified by DNA sequencing. Purifications of all DNA were performed with kits from BioBasic. All reagents, including **1**, *o*-nitrophenyl- β -D-galactopyranoside (ONPG), and ampicillin were purchased from Sigma. X-gal was purchased from MP Biomedicals. Synthetic oligonucleotides were purchased from IDT. The caged analog **2** was synthesized according to published protocols.^[10d]

Construction of pSAL plasmids

The pSAL-based plasmids containing various reporter genes were constructed by PCR amplification of each *gfp*, *lacZ*, and *DsRed* reporter gene using forward primer oligonucleotides that included the 12.1 riboswitch sequence (aptamer domain, expression platform, and start codon) at the 5'-terminus of the oligonucleotide (see Supplementary Table S1 for oligonucleotide sequences and template reporter plasmids). Each PCR product was digested with *Kpn*I and *Hind*III and ligated into pSAL/12.1 that had been similarly digested, placing the riboswitch-reporter sequence immediately downstream to the IS10 promoter (see Supplementary Table S2 for DNA sequences of each construct). The ligated DNA was then transformed into chemically competent *E. coli* Top10 cells.

Construction of pET17b plasmids

The pET17b series of riboswitch vectors containing various reporter genes were constructed by PCR amplification of each riboswitch-reporter fusion using forward primer oligonucleotides designed to replace the IS10 promoter sequence with the T7 promoter sequence (see Supplementary Table S1 for oligonucleotide sequences). The template for each PCR amplification reaction was the corresponding pSAL vector, described above. Each PCR product was digested with *Bgl*II and *Hind*III and ligated into pET17b that had been similarly digested (see Supplementary Table S2 for DNA sequences of each construct). The ligated DNA was then transformed into chemically competent *E. coli* TOP10 cells.

Insertion/deletion nucleotides

Insertion and deletion of nucleotides between the promoter and riboswitch sequence was accomplished using the QuickChange II mutagenesis kit (Stratagene) per the manufacturer's instructions (see Supplementary Table S1 for sequences of mutagenic oligonucleotides).

Synthesis of caged theophylline **2**

The synthesis and structural characterization of **2** was carried out as previously described.^[10d]

Measurement of β -galactosidase activity and light-activation

For LacZ reporter assays, each relevant pSAL- or pET17b-derived plasmid (see Table 1) was transformed into *E. coli* TOP10 or *E. coli* BL21(DE3), respectively. Single colonies were picked and grown overnight in LB media (3 mL) supplemented with ampicillin (100 μ g/mL) at 37 °C, with shaking at 250 rpm. An aliquot (50 μ L) of the overnight culture was used to inoculate fresh LB media (7 mL) supplemented with ampicillin (100 μ g/mL) at 37 °C, with shaking at 250 rpm. The culture was grown until the OD₆₀₀ was 0.1, at which time the culture was divided into two portions (3 mL each). To one of these portions, **1** (10 mM stock in DMSO) or **2** (10 mM stock in DMSO) was added (to a maximum final concentration of 1 mM). IPTG was added to cells containing the pET17b constructs (to a final concentration of 1 mM). Samples to be treated with light were irradiated for 3 min at 365 nm, using a hand-held 25 W UV lamp placed 10 cm from the samples. Cultures were then incubated in the dark for 5 h at 20 °C, with shaking at 250 rpm, and a sample (20 μ L) was removed for determination of β -galactosidase activity using a protocol adapted from a previously described Miller assay. The culture aliquot was mixed with permeabilization solution (80 μ L total volume, 100 mM sodium phosphate dibasic, 20 mM potassium chloride, 2 mM magnesium sulfate, 0.8 mg/mL hexadecyltrimethylammonium bromide, 0.4 mg/mL sodium deoxycholate, 5.4 μ L/mL β -mercaptoethanol pH 7.0). The lysed culture sample was then mixed with the substrate solution (600 μ L total volume, 60 mM sodium phosphate dibasic, 40 mM sodium phosphate monobasic, 1 mg/mL *o*-nitrophenyl- β -D-galactoside, 2.7 μ L/mL β -mercaptoethanol, pH 7.0). The galactosidase was allowed sufficient reaction time to hydrolyze such that a slight yellow color is observed (typically between 2 and 60 min, at which point hydrolysis is linear over time) and quenched by the addition of 1 M sodium carbonate (700 μ L) and centrifuged at 10,000 g. The absorbance was then read on a plate reader (Hybrid Synergy IV, BioTek) at 420 nm and another sample was used to read the cell densities at 600 nm. The Miller units for each sample were then found using the following equation:

$$\text{Miller units} = 1000 \times \text{Abs}_{420} / (\text{reaction time in min's} \times \text{mL of reaction volume} \times \text{OD}_{600})$$

Background LacZ activity was obtained by calculating the β -galactosidase activity of the host strain harboring empty vector. An average of the background was subtracted from the experimental data derived from the various riboswitch constructs. Activation ratios were calculated by dividing the Miller units in the presence of **1** or **2**, by that in the absence of ligand. Each assay was performed with three independent cultures.

Measurement of GFP expression and light-activation

For GFP reporter assays, each relevant pSAL- or pET17b-derived plasmid (see Table 1) was transformed into *E. coli* TOP10 or *E. coli* BL21(DE3), respectively. Single colonies were picked and grown overnight in LB media (3 mL) supplemented with ampicillin (100 μ g/mL) at 37 °C, with shaking at 250 rpm. An aliquot (50 μ L) of the overnight culture was used to inoculate fresh LB media (7 mL) supplemented with ampicillin (100 μ g/mL) at 37 °C, with shaking at 250 rpm. The culture was grown until the OD₆₀₀ was 0.1, at which time the culture was divided into two portions (3 mL each). To one of these portions, **1** (10 mM stock

in DMSO) or **2** (10 mM stock in DMSO) was added (to a maximum final concentration of 1 mM). IPTG was added to cells containing the pET17b constructs (to a final concentration of 1 mM). Samples to be treated with light were irradiated for 3 min at 365 nm, using a hand-held 25 W UV lamp placed 10 cm from the samples. Cultures were then incubated in the dark for 2.5 h at 37 °C, with shaking at 250 rpm, and a sample (200 µl) was removed for determination of OD₆₀₀ and fluorescence intensity in a plate reader (Hybrid Synergy IV, BioTek). Fluorescence measurements were taken at an excitation and emission wavelength of 480 nm and 520 nm, respectively, and fluorescence intensities were normalized to the culture optical density. Background fluorescence was obtained by calculating the OD₆₀₀ normalized fluorescence of the host strain harboring empty vector. An average of the background was subtracted from the experimental data derived from the various riboswitch constructs. Activation ratios were calculated by dividing the OD₆₀₀-normalized and background-corrected fluorescence intensity in the presence of **1** or **2**, by that in the absence of ligand. Each assay was performed with three independent cultures.

Spatial control of gene expression

An aliquot (10 µl) of *E. coli* TOP10 pSAL/12.1 starter culture was used to inoculate each well of a 96-well microplate that contained LB media (190 µl) supplemented with ampicillin (100 µg/mL). The microplate was incubated at 37 °C with shaking at 300 rpm for 1 h, at which point **2** was added (to a final concentration of 1 mM). The microplate was then covered with a mask and irradiated with UV light at 365 nm for 3 min, as described above. The cultures were incubated in the dark for 4 h at 20 °C, with shaking at 200 rpm. Then, the chromogenic reagent 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 2 µl of 20 mg/ml in DMSO) was added to each well. Following incubation for 10 min at room temperature in the dark, the microplate was photographed using a digital camera.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

General strategy for riboswitch photo-control. A) Structures of theophylline (**1**) and the photocaged analogue **2**. B) Schematic representation of the theophylline riboswitch 12.1. In the absence of UV irradiation, the caged ligand is unable to bind to the riboswitch aptamer domain, and gene expression is turned OFF. Upon UV irradiation, the ligand is now recognized by the riboswitch aptamer domain, and gene expression is turned ON. RBS, ribosome binding site.

Figure 2.

In vivo light-activation of the theophylline riboswitch. A) Activity of the 12.1 riboswitch in a series of laboratory *E. coli* strains. Left axis (grey bars) represents the activation ratio of the riboswitch as the ratio of activities in the absence and presence of **1** (1 mM). Right axis represents the β -galactosidase activity (Miller units) of the riboswitch in the absence (closed circle) or presence (open circle) of **1** (1 mM). Dashed lines indicate standard deviation of activity ($n=3$). B) Dose dependent LacZ expression under control of the 12.1 riboswitch in the presence of **1**. Dashed lines indicate standard deviation of activity ($n=3$). Axis as described for panel A. C) In vivo light-activation of 12.1-driven LacZ expression in liquid media. LacZ is only expressed in the presence of UV irradiation and **2** (1 mM in each case). Activation ratio is given above each grey bar. Error bars indicate standard deviation ($n=3$). D) Dose dependent LacZ expression under control of the 12.1 riboswitch in the presence of **2** and UV irradiation. Activation ratio is given above each grey bar. Error bars indicate standard deviation ($n=3$).

Figure 3.

In vivo spatial control of riboswitch activity. A mask was used to irradiate selected wells of a microplate with UV light. Each well contained an identical culture of *E. coli* TOP10 cells harbouring the plasmid pSAL/12.1. The photocaged ligand **2** was included in all wells at 1 mM and a colorimetric LacZ assay carried out using X-gal. See Experimental Section for details.

Table 1

Activation ratios of the 12.1 riboswitch with different reporters and promoters

Entry	Construct	Reporter/ Promoter	<i>E. coli</i> host strain	Activation ratio ^[a]
1	pSAL/12.1	LacZ/IS10	TOP10	275.7 ± 26
2	pSAL/ <i>gfp</i>	GFP/IS10	TOP10	3.6 ± 0.5
3	pSAL/ <i>DsRed</i>	DsRed/IS10	TOP10	6.6 ± 0.8
4	pSAL/ <i>lacZ-gfp</i>	GFP ^[b] /IS10	TOP10	5.9 ± 0.6
5	pET17b/T7/ <i>lacZ</i>	LacZ/T7	BL21 (DE3)	4.8 ± 0.5
6	pET17b/T7/ <i>gfp</i>	GFP/T7	BL21 (DE3)	1.8 ± 0.3
7	pET17b/T7/ <i>DsRed</i>	DsRed/T7	BL21 (DE3)	1.2 ± 0.2
8	pET17b/T7/ <i>lacZ-gfp</i>	GFP ^[b] /T7	BL21 (DE3)	1.8 ± 0.3
9	pSAL/ <i>tac-gfp</i>	GFP/Tac	BL21 (DE3)	4.2 ± 0.6
10	pSAL/ <i>gfp</i> +2 ^[c]	GFP/IS10	TOP10	3.2 ± 0.7
11	pSAL/ <i>gfp</i> -2 ^[c]	GFP/IS10	TOP10	2.9 ± 0.6
12	pET17b/T7/ <i>lacZ</i> +3 ^[c]	LacZ/T7	BL21 (DE3)	4.2 ± 0.5
13	pET17b/T7/ <i>lacZ</i> +1 ^[c]	LacZ/T7	BL21 (DE3)	4.1 ± 0.7
14	pET17b/T7/ <i>gfp</i> -3 ^[c]	GFP/T7	BL21 (DE3)	1.8 ± 0.6

^[a] Average activation ratio, ± standard deviation (*n*=3).

^[b] Includes N-terminal 14 amino acids of LacZ.

^[c] +/- *n* indicates insertion/deletion *n* nucleotides between promoter sequence and RBS of the riboswitch.