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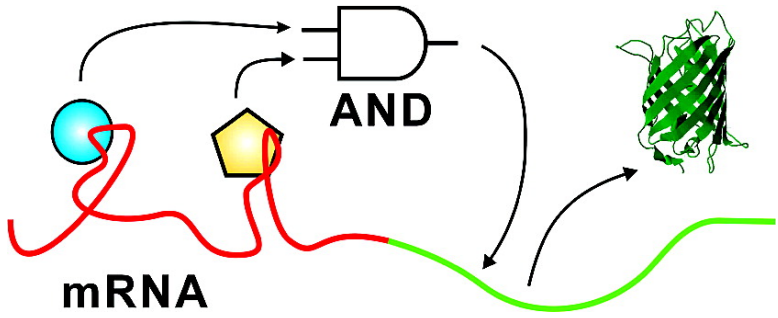

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Engineering Complex Riboswitch Regulation by Dual Genetic Selection

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Abstract: The recent discovery of riboswitches in diverse species of bacteria and few eukaryotes added metabolite-responsive gene regulation to the growing list of RNA functions in biology. The natural riboswitches have inspired several designs of synthetic analogues capable of gene regulation in response to a small molecule trigger. In this work, we describe our efforts to engineer complex riboswitches capable of sensing and responding to two small molecules according to Boolean logics AND and NAND. Two aptamers that recognize theophylline and thiamine pyrophosphate were embedded in tandem in the 5′ UTR of bacterial mRNA, and riboswitches that function as logic gates were isolated by dual genetic selection. The diverse phenotype of the engineered logic gates supports the versatility of RNA-based gene regulation which may have preceded the modern protein-based gene regulators. Additionally, our design strategy advances our ability to harness the versatile capacities of RNA to program complex behavior in bacteria without the use of engineered proteins.

Riboswitches are noncoding *cis*-regulatory elements found in the 5′ untranslated regions (UTRs) of bacterial messenger RNAs (mRNAs) that undergo metabolite-dependent conformational changes and modulate gene expression.[1−3] A typical riboswitch contains an aptamer domain that is responsible for metabolite sensing and an expression platform that mediates a local structural change in mRNA upon metabolite binding. Metabolite-induced structural shift affects premature transcription termination or accessibility of the Shine−Dalgarno (SD) sequence to the ribosome.

In addition to their fundamental biological significance in the early evolution of life,[4] riboswitches serve as inspiring models of synthetic gene switches for applications in biotechnology.[5−8] RNA aptamers capable of recognizing diverse classes of molecules have been obtained by *in vitro* selection.[9] One such aptamer that selectively binds theophylline[10] has been successfully used to engineer synthetic riboswitches through semirational design[11−13] and high-throughput screening or selection[14−17] in bacteria.

Previously, our group developed a dual genetic selection method that allows fast and efficient selection of gene switches from a large pool of variants in *Escherichia coli*.[18] The selection employs a single selection marker gene *tetA* that encodes the tetracycline/H^+ antiporter derived from pBR322.[19] Gene switches that turn ON or turn OFF TetA expression can be selected by resistance to an antibiotic tetracycline, or a toxic nickel salt ($NiCl_2$),[20] respectively. We successfully applied this method to reverse the ligand response of a natural thiamine pyrophosphate (TPP) riboswitch from *E. coli*.[21]

Many prokaryotic promoters are combinatorially regulated by multiple transcription factors that are either directly or indirectly regulated by small molecule metabolites. For example, the *E. coli lac* promoter is positively regulated by cAMP receptor protein (CRP) and LacI which respond to cAMP and lactose, respectively. Setty and colleagues showed that the *lac* promoter responds to the two metabolites in a fashion that is intermediate between Boolean AND and OR logic.[22] Synthetic logic gates incorporating such regulatory factors are expected

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to be useful for programming cellular behavior and engineering metabolic pathways.[23,24] These motivations have led some researchers to implement various synthetic logic gates in bacteria[25−27] and in other organisms. [28−31] Synthetic logic gates that use RNA to both sense molecules and regulate gene expression could offer simpler and flexible alternatives to the protein-based sensors and logic gates.[5−8] To our knowledge, the only such logic gate that has been experimentally confirmed is found in the 5′ UTR of *metE* mRNA in *Bacillus clausii* that contains aptamers for S-adenosylmethionine (SAM) and coenzyme B_{12} in tandem.[32] This atypical riboswitch exhibits premature transcription termination when either one or both of the metabolites are present, behaving as a genetic NOR gate.

In this work, we demonstrate that the riboswitch mechanism can be harnessed to yield other biologically relevant gene expression logics in response to two distinct aptamer ligands. Specifically, we adapted *tetA* dual genetic selection to isolate AND and NAND logic gates in response to two small molecules, theophylline and TPP. The results underscore the versatile capacity of the riboswitch mechanism to effect complex gene regulation carried out by modern protein transcription factors.

Materials and Methods

Bacterial Strains and Growth Conditions. *E. coli* TOP10 cells (Invitrogen, F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80*lac*ZΔM15, Δ*lac*X74 *rec*A1, *ara*D139 Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L (Str^R), *end*A1, *nup*G) were used in all experiments. The cells were grown at 37 °C from single-colony isolates or diluted from overnight cultures in M9 minimal medium[33] supplemented with 0.1% casamino acids (Fisher Scientific) and 0.8% glycerol as the carbon source or in LB medium[33] (BD Biosciences). Unless otherwise indicated, thiamine hydrochloride and theophylline (Acros Organics) were used at concentrations of 0.1 mM and 1 mM, respectively. Nickel chloride (Fisher Scientific) was used at a final concentration of 0.3 mM. Ampicillin (Fisher Scientific) (for plasmid maintenance) and tetracycline (MP Biomedicals) were used at concentrations of 100 μg/ml and 20 μg/ml, respectively. When necessary, *E. coli* cells were grown on 1.5% agar (Fisher Scientific) plates in M9 minimal medium or LB medium.

Plasmid and Library Construction. Library for AND gate selection was constructed as follows. First, the theophylline aptamer was cloned at the *Eco*RI site of the plasmid encoding the +ThiM#2 riboswitch[21] fused to *tetA* (pLac+ThiM#2tetA2, Figure S1 [Supporting Information]) by inserting preannealed oligo DNAs (Theo28+ and Theo28−, Table S2 [Supporting Information]). The resulting plasmid was used as a template to generate the AND gate library by PCR using the primers 20-TPP-f/10-TPP-f and Theo28r (Table S2 [Supporting Information]), as reported previously.[21] The NAND gate library was similarly constructed using the analogous wild-type TPP riboswitch[34] construct (pLacThiMtetA2) as the PCR template. Approximately 2.0×10^5 and 1.9×10^5 independent clones were obtained in AND gate and NAND gate libraries, respectively. For fluorescence measurements, *tetA* in the selected logic gate clones was replaced with *gfpuv* cloned from pGFPuv (Clontech). Site-directed mutagenesis was performed by PCR using Phusion High-Fidelity DNA Polymerase (New England Biolabs/Finnzymes) using the mutagenic primers listed in Table S2 (Supporting Information). All mutations were confirmed by DNA sequencing.

Dual Genetic Selection of AND and NAND Gates. For each positive (ON) and negative (OFF) selection steps, the libraries containing TOP10 cells expressing the logic gate variants were first washed with M9 medium. The cells were diluted into fresh M9 medium supplemented with ampicillin (M9-A) with appropriate combination of the input molecules and incubated at 37 °C in a shaker for approximately 90 min without selective agents before being plated onto M9-A agar plates containing appropriate inducer(s) and selective agent (tetracycline for ON selection and $NiCl_2$ for OFF selection). Cells that grew on the plates were harvested by overlaying liquid M9 medium and used for the next selection step. For both AND and NAND gate selections, two cycles of four ON or OFF selection steps were carried out in the sequence described in Table S3 (Supporting Information). After the completion of two selection cycles, AND gate cells were plated on M9-A tetracycline plates supplemented with thiamine and theophylline, and the NAND gate cells were plated on M9-A tetracycline plates supplemented with thiamine. Individual colonies were screened for the logic gate phenotypes by growth assay on tetracycline-containing media (see section following). The number of bases in the randomized linker was often found to be fewer than expected probably due to the suboptimal purity of the synthetic DNA primers.

Growth Assay on Tetracycline-Containing Media. TOP10 cells harboring logic gate clones (*tetA* fusion) selected by dual genetic selections were individually cultured overnight at 37 °C in M9-A. The cells were washed with M9 medium and diluted 10-fold into fresh M9-A supplemented with appropriate combinations of thiamine (0.1 mM) and theophylline (1 mM). After 90 min incubation at 37 °C, the cells were streaked onto M9-A agar plates containing tetracycline and appropriate effectors. The plates were incubated at 37 °C for approximately 24 h.

Fluorescence Assays for GFPuv Expression in *E. coli*. TOP10 cells transformed with a plasmid encoding a *gfpuv*-fused logic gate were inoculated into M9-A and grown overnight at 37 °C. The following day, cells were washed and diluted 100-fold (v/v) into fresh M9-A (1 mL) supplemented with thiamine and/or theophylline as appropriate and grown until early log phase at 37 °C in glass culture tubes. The cells were then harvested by centrifugation and washed once with phosphate-buffered saline (PBS). Next, the cells were resuspended in 200 μL of PBS and transferred to a 96-well plate. Optical density at 600 nm (OD_{600}) and GFPuv fluorescence intensity (excitation wavelength: 395 nm; emission wavelength: 509 nm) were measured using Safire2 microplate reader (Tecan). The control TOP10 cells transformed with a plasmid encoding AND-152 *lacZ* fusion were used to subtract the background fluorescence. The corrected fluorescence values were further normalized by OD_{600}. The transfer function of the AND gates were measured similarly except that the cells were cultured in 96 deep-well plates in 750 μL volume. GFPuv induction kinetics using AND-152 was measured in glass culture tubes after the addition of thiamine and/or theophylline to the cells in early log phase ($OD_{600} = 0.15$).

Results and Discussion

We envisaged that dual genetic selection would be an ideal method to design novel logic gates using existing RNA

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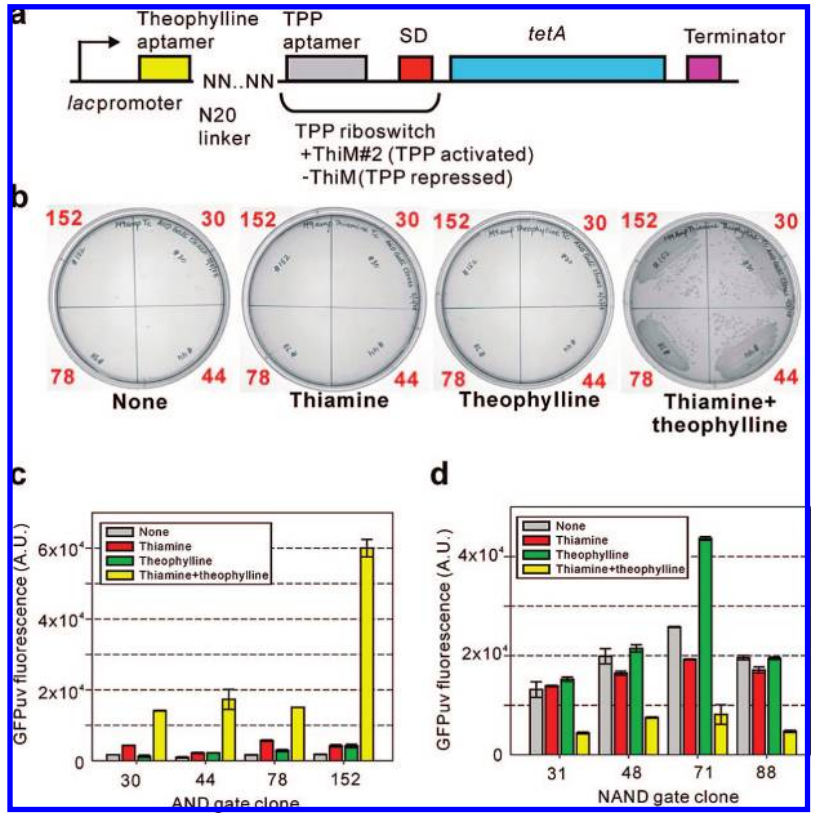

Figure 1. Design and selection of riboswitch logic gates. (a) Design of the logic gate libraries. The libraries were based on the previously described TPP riboswitches fused to *tetA*.[21] For AND gate selection, the reengineered TPP riboswitch, +ThiM#2, that activates gene expression in the presence of TPP was used. For NAND gate selection, the *E. coli* wild-type TPP riboswitch, -ThiM, that represses gene expression upon binding TPP[34] was used. The theophylline aptamer[10] was attached to the 5′ end of the TPP aptamer via a randomized nucleotide linker up to 20 nucleotides. The expression of the riboswitch-*tetA* transcript is driven by the *E. coli lac* promoter. (b) Growth of the selected AND gate clones fused to *tetA* on tetracycline-containing agar plates supplemented with theophylline (1 mM) and thiamine (0.1 mM) as indicated. (c) Fluorescence measurements of the AND gate clones fused to *gfpuv* grown in liquid media supplemented with theophylline (1 mM) and thiamine (0.1 mM) as indicated. The data represent averages of at least 3 independent measurements, and the error bars indicate ± s.d. (d) Fluorescence measurements of the NAND gate clones fused to *gfpuv* grown in liquid media supplemented with theophylline (1 mM) and thiamine (0.1 mM) as indicated. The data represent averages of at least three independent measurements, and the error bars indicate ± s.d.

aptamers. As an initial step, we decided to construct the AND and NAND logic gates because analogous riboswitches have not been discovered in nature. In order to construct a library that would enable us to select AND gates, we started with the TPP riboswitch that was reengineered to activate gene expression in the presence of TPP (+ThiM#2, Figure S1 [Supporting Information]).[21] Another aptamer that binds theophylline[10] was fused to the 5′ end of +ThiM#2 *via* a stretch of degenerate bases up to 20 nucleotides (Figures 1a, S2 [Supporting Information]). We reasoned that the chance of discovering AND gates could be maximized by using the TPP-activated riboswitch (+ThiM#2) that embodies a partial characteristic of the desired AND gate. Similarly, we constructed a library using the wild-type TPP riboswitch[34] (-ThiM) for the selection of NAND gates that repress gene expression only when both theophylline and TPP are present (Figure 1a, S3 [Supporting Information]).

To select for two-input logic gates, the libraries were subjected to four different selections representing all possible input states. For AND gate selection, the library cells were successively plated on M9 minimal medium agar plates containing tetracycline (ON) or $NiCl_2$ (OFF) supplemented with appropriate combinations of the two input molecules (Table S3, Supporting Information). After the completion of selection, individual clones were screened for growth on tetracycline-containing media with and without the input molecules. The four AND gate clones shown in Figure 1b grew robustly only on the medium containing both thiamine and theophylline, consistent with the expected AND logic. To obtain more quantitative characteristics of the selected AND gates, the selection marker *tetA* was replaced with a fluorescent reporter gene *gfpuv* to assay gene expression by fluorescence measurement. All four clones exhibited the expected GFPuv expression responses to the two input molecules when cultured in liquid media (Figure 1c). Next, the library based on the wild-type (-ThiM) TPP riboswitch (Figure 1a) was selected similarly to isolate NAND gates (Figure S4, Supporting Information). The NAND gate clones that exhibited the expected tetracycline resistance pattern (Figure S4, Supporting Information) were further analyzed as *gfpuv* fusions. As expected, GFPuv expression was significantly repressed only in the presence of both thiamine and theophylline (Figure 1d). The relative ON/OFF ratios of the gene expression levels in NAND gates appear to be less striking compared to those of the AND gates. A possible explanation is that the expression level of the ON states of the parental TPP riboswitches were different; the wild-type *thiM* riboswitch on which the NAND gates were based exhibited a lower ON expression level compared to the +ThiM#2 riboswitch,[21] which may have limited the ON levels of the selected NAND gates.

The AND gates were further analyzed by mapping the transfer functions for the two inputs. The transfer functions reveal that the logic gates exhibit varying characteristics even though they

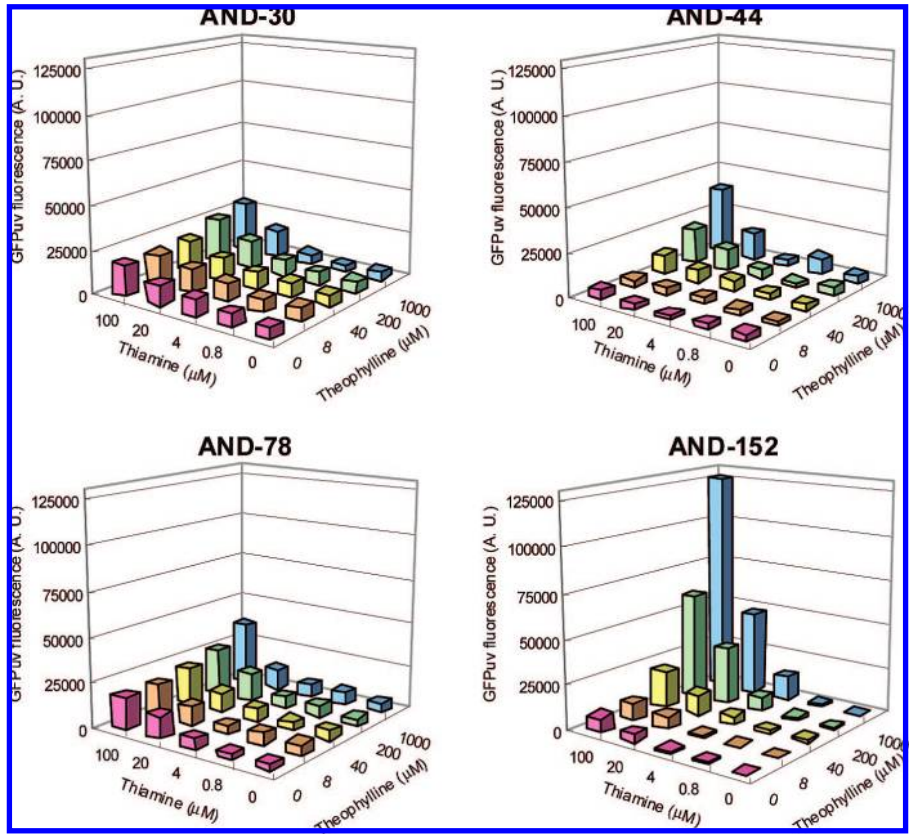

Figure 2. Transfer functions of the AND logic gates for the two inputs. The data represent averages of at least two independent cultures grown in 96-deepwell plates.

were all isolated in a single selection experiment. As expected from Figure 1c, AND-44 and AND-152 display sharper and more ideal (digital) AND gate transfer functions albeit with different ON expression levels (Figure 2). Both AND-30 and AND-78 exhibit a partial response to thiamine even in the absence of theophylline. The diversity of transfer functions of the AND gates shown in Figure 2 is not apparent from the tetracycline-growth assays which were used to screen the library after dual genetic selection (Figure 1b). This is probably due to the fact that the tetracycline growth assay has a sharp threshold (and a low dynamic range) in response to *tetA* expression level. In other words, a small change in the TetA level near a threshold can dramatically affect cell growth, whereas the more subtle growth phenotypes are difficult to distinguish at conditions away from the threshold. Efficient screening methods that can more quantitatively evaluate the expression levels of the selected clones could further facilitate the discovery of complex gene switches by dual genetic selection.

One of the expected features of translationally regulated riboswitches is their rapid response to the cognate metabolites compared to other regulatory mechanisms that operate at the transcriptional level. Accordingly, we investigated the dynamic response of AND-152 to the addition of the effectors. As evident from Figure 3, induction of GFPuv expression was observed after 30 min, increasing linearly for the first 3 h after the addition of thiamine and theophylline. The relatively slow induction of gene expression could be due to the rate-limiting transport of thiamine or its intracellular conversion to TPP by *thiK*[35] and

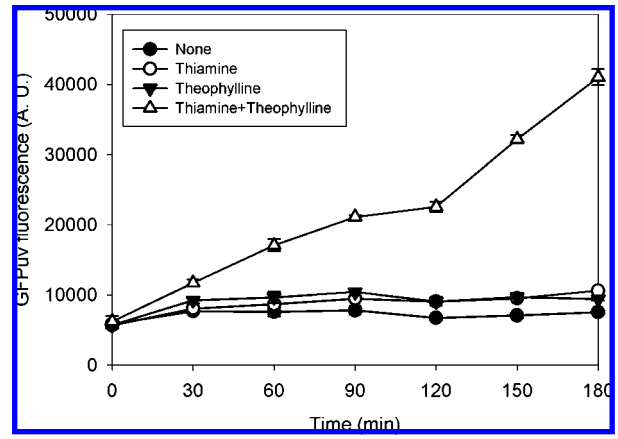

Figure 3. Induction dynamics of AND-152 upon addition of the effectors. Thiamine (0.1 mM) and/or theophylline (1 mM) were added after the OD_{600} of the cultured cells reached 0.15. Each data point represents an average of three independent cultures, and the error bars indicate ± s.d.

thiL[36] gene products. Additionally, passive diffusion of theophylline into *E. coli* cells is known to be relatively inefficient.[11]

In the absence of comparable natural riboswitches, we attempted to propose a mechanistic model of the AND gate clone 152 (AND-152) that displayed the best ON/OFF gene expression ratio (~18-fold in Figure 1c, OFF = in the absence of both inputs) among the engineered logic gates. Examination of the linker sequence and the predicted secondary structures[37] led us to the putative mechanistic model depicted in Figure 4a.

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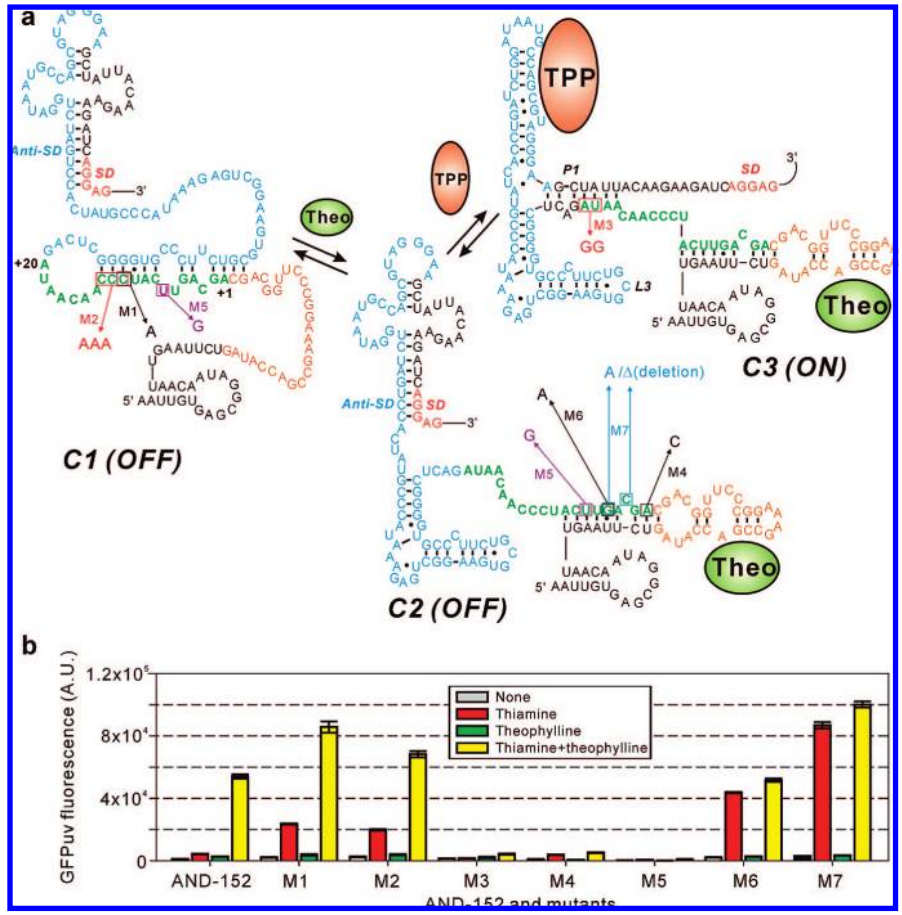

Figure 4. Proposed mechanism and mutational analysis of AND-152. (a) Sequential binding model of AND-152. Nucleotides are numbered with the first base in the linker designated as +1. Bases −27 to −1 (orange): theophylline aptamer, +1 to +20 (green): linker, +21 to +75 (blue): TPP aptamer, +93 to +97 (red): SD sequence. (b) Fluorescence measurements of AND-152 and its mutants (M1−M7 depicted in Figure 4a) fused to *gfpuv* grown in liquid media supplemented with theophylline (1 mM) and thiamine (0.1 mM) as indicated. The data represent averages of at least three independent measurements, and the error bars indicate ± s.d.

In the absence of the ligands, nucleotides −2 to +13 (the first base in the linker is designated +1) would form a stable imperfect stem with the 5′ part of the TPP aptamer (**C1** in Figure 4a). This structure could effectively inhibit the TPP aptamer from binding to its ligand even when TPP is available. When theophylline becomes available, however, bases +1 through +9 in the linker would shift to form a base stem structure of the theophylline aptamer, disengaging the inhibitory stem that blocks the TPP aptamer from binding its ligand (**C2** in Figure 4a). Only when both theophylline and TPP are bound to their cognate aptamers (**C3** in Figure 4a), does the Shine−Dalgarno (SD) sequence become accessible to the ribosome. It should also be noted that the bases +16 to +20 in the linker may contribute to form an extended P1 stem structure of the TPP aptamer to provide added stability to the TPP-bound configuration (**C3**).

Characterization of a number of AND-152 mutants has yielded results that are consistent with the proposed mechanistic model (Figure 4b, Table S1, Supporting Information). Mutations M1 and M2 are both expected to selectively destabilize **C1**, relaxing the inhibition of the TPP aptamer in the absence of theophylline. As expected, mutants M1 and M2 activate GFPuv expression in the presence of thiamine even when no theophylline is added. However, there seems to be some residual inhibitory effect by the **C1** conformation which is evident from the further 3- to 4-fold activation of gene expression upon theophylline addition when thiamine is present. Mutant M3 was designed to destabilize the extended P1 stem in the TPP aptamer as depicted in **C3** conformation, which resulted in a virtual loss of response to the effectors. Similar loss of response to the ligands was observed with M4, a single mutation that is expected to disrupt the putative base stem of the theophylline aptamer which presumably negatively affects theophylline binding. Another point mutant M5 is predicted to stabilize **C1** and destabilize **C2** which is apparently sufficient to effectively lock the mutant into **C1**, resulting in no response to the ligands. Mutants M6 and M7 were designed to stabilize the theophylline aptamer base stem structure depicted in **C2** and **C3**. Additionally, both mutants are also expected to destabilize the **C1** conformation. Consistent with the model, these mutants respond to thiamine regardless of theophylline concentration. The mutations presumably shift the equilibrium toward **C2** in the absence of the ligands. Notably, M7, which is expected to yield a more stable **C2** conformation in the absence of theophylline yields significantly higher GFPuv expression in the presence of thiamine compared to AND-152 and M6. While these mutational studies are supportive of the putative mechanism, further characterization at the molecular level will be necessary to validate the proposed model.

Conclusion

Besides the natural and synthetic bacterial riboswitches, various RNA aptamers have been adapted to mediate chemical

regulation of gene expression in diverse organisms. The observed diversity of mechanisms in which aptamers have been shown to control gene expression strongly supports the hypothesis that RNA played an important role in metabolite-regulated gene expression in primitive organisms. For example, aptamers have been shown to block translation initiation in eukaryotes,[38−40] to control the activity of ribozymes embedded in noncoding regions of mRNAs,[12,17,41−43] to inhibit RNA interference in mammalian cells,[44,45] and to modulate antisense effects in yeast.[46] RNA logic gates that use aptamer−ligand binding and oligonucleotide hybridization as inputs have been constructed *in vitro*,[47−49] but genetically encoded aptamers have not been engineered to function as logic gates in living cells, which we have demonstrated to be feasible for the first time. The remarkable flexibility of RNA to achieve simultaneous molecular recognition and complex gene regulation may also provide insights into the undiscovered roles of riboswitches in nature.

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Supporting Information Available: . Supporting figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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