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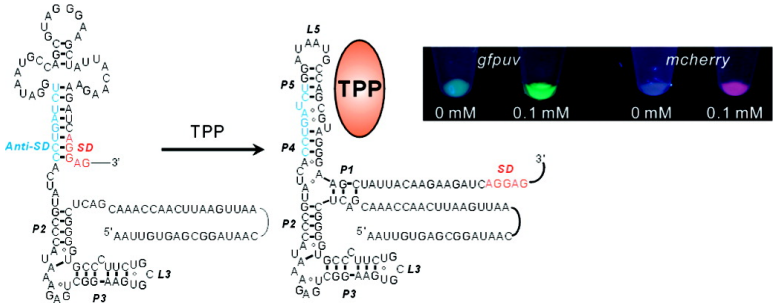

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Reengineering a Natural Riboswitch by Dual Genetic Selection

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Riboswitches are noncoding genetic elements found widely in prokaryotic mRNAs that specifically interact with small molecule metabolites and modulate gene expression.[1] Binding of a metabolite to a natural RNA aptamer located within the 5′ untranslated region (UTR) of mRNA induces a structural change mediated by an expression platform, resulting in a transcriptional or translational modulation of gene expression.

Most naturally found riboswitches downregulate gene expression on metabolite binding, probably because of their roles in negative feedback regulation within the metabolic pathways. Some classes of riboswitches regulate gene expression by both transcriptional and translational control using essentially the same aptamer, suggesting the flexibility of the expression platforms to achieve gene regulation through multiple mechanisms. Moreover, the thiamine pyrophosphate (TPP) aptamer regulates mRNA splicing in eukaryotes[2] in addition to the negative transcriptional and translational control in bacteria.[3]

We examined whether it is possible to reverse the ligand response of a natural TPP riboswitch which downregulates gene expression upon binding TPP. The process of engineering the riboswitch mimicked the natural selection. Selective pressure was applied to favor the survival or growth of the bacteria harboring functional (upregulated) riboswitches whose expression platform regions were randomized. We discovered a number of riboswitches that activate gene expression in response to TPP, underscoring the plasticity of the expression platform. Furthermore, these artificial riboswitches may be useful for in vivo monitoring of intracellular metabolites and engineering metabolic pathways.

The TPP riboswitch encoded in the 5′ UTR of *Escherichia coli thiM*[3a] was subcloned in the 5′ UTR of *tetA* which encodes the class C tetracycline/H^+ antiporter derived from the plasmid pBR322.[4] A riboswitch plasmid library was constructed by incorporating up to 30 degenerate bases between the TPP aptamer and the Shine−Dalgarno (SD) sequence using polymerase chain reaction (PCR) (Figure 1A, also see Supporting Information) and transformed into *E. coli* TOP10 cells, which yielded approximately 75 000 unique riboswitch clones.

First, the cells with riboswitches that were turned ON in the presence of thiamine (which is converted to TPP intracellularly) were selected by plating the cell population on M9 minimal media plates containing tetracycline (20 μg/mL) and thiamine (0.1 mM). Subsequently, the surviving cells were harvested and plated on M9 minimal media plates containing $NiCl_2$ (0.3 mM) without thiamine to select for cells with attenuated expression of *tetA*.[5] The selection was repeated one more cycle and the selected clones were individually screened for thiamine-dependent growth on M9 mimimal media plates containing tetracycline (20 μg/mL).

Confirmed riboswitches were sequenced and the *tetA* gene was replaced with *lacZ*, which encodes β-galactosidase, to enable quantitative measurements of the riboswitches.[6] We identified and characterized 11 variants which exhibited >3 fold upregulation of

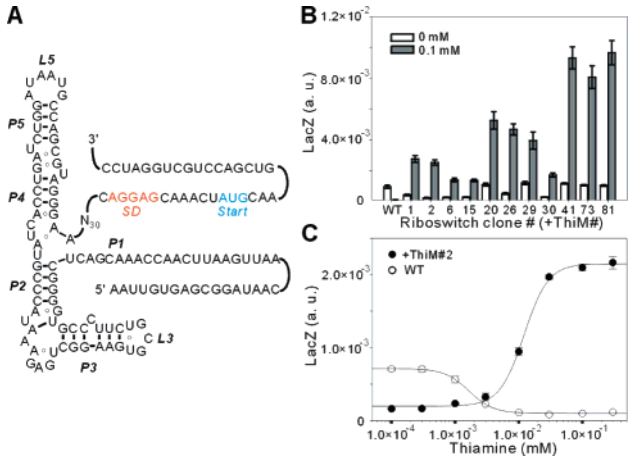

Figure 1. Reengineered *thiM* riboswitches. (A) Partial sequence and the expected TPP aptamer secondary structure[3] of the riboswitch library. The transcription is driven by the *E. coli lac* promoter. (B) LacZ expression of the wild-type (WT) and the selected riboswitches (#1−81) in the absence and in the presence of 0.1 mM thiamine. Data are averages of five independent measurements, and the error bars indicate standard deviations (see Supporting Information for details). (C) Dose-dependent LacZ expression of the wild-type (WT) and +ThiM#2. Each data point represents an average of four independent measurements, and the error bars indicate standard deviation. Curves are shown to guide the eye only.

LacZ activity in the presence of 0.1 mM thiamine (Figure 1B and Table S1, Supporting Information).

+ThiM#2 exhibited the greatest dynamic range among these riboswitches with 11-fold activation, compared to the 9-fold downregulation by the wild-type *thiM* riboswitch measured under the same conditions. The dose-dependent gene expression profiles of the wild-type and +ThiM#2 riboswitches are shown in Figure 1C.

Examination of the nucleotide sequences of +ThiM#2 and other riboswitch clones (Table S1, Supporting Information) suggested a potential mechanism of the selected riboswitches. We speculate that the sequence neighboring the SD region hybridizes with a part of the TPP aptamer (anti-SD) in the absence of the ligand, resulting in a translationally repressed state (Figure 2A). Upon ligand binding, the anti-SD engages in TPP binding, making the SD sequence accessible to the ribosome. The tentative mechanism is reminiscent of the natural and synthetic riboswitches that exhibit translational regulation.[1,7]

To support the proposed mechanistic model, we altered two bases that are expected to destabilize the SD-anti-SD helix in +ThiM#2 (*mut1*, Figure 2A). As expected, this resulted in a 10-fold higher background expression accompanied by a significantly reduced (12.7−3.5-fold) relative activation by 0.1 mM thiamine (Table 1). A compensatory mutation within the TPP aptamer (*mut2*) which restores the 8-bp SD-anti-SD helix reduced the background expression back to a level similar to +ThiM#2. However, the mutation

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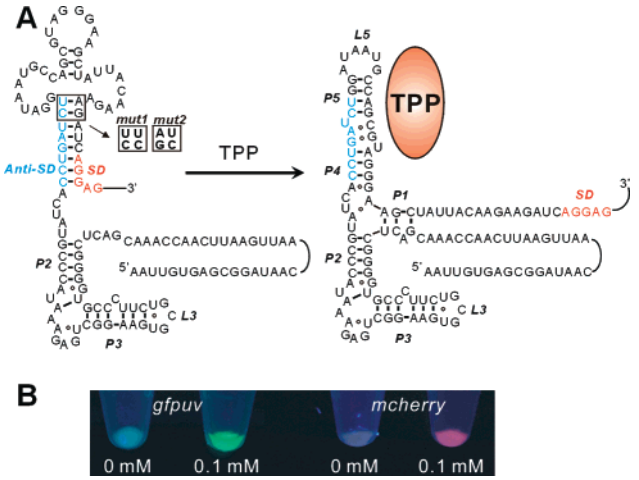

Figure 2. (A) Proposed gene activation mechanism of +ThiM#2 by TPP. In the absence of TPP, the anti-SD sequence within the TPP aptamer hybridizes with the SD region and its upstream bases, resulting in translation repression. Aptamer-TPP interaction frees the SD sequence, making it accessible to the ribosome. A two-base mutation *mut1* is expected to destabilize the SD-anti-SD helix, whereas *mut2* reconstructs an analogous helix disrupted by *mut1*. (B) Thiamine-activated expression of fluorescent proteins by +ThiM#2. Cells were grown in M9 minimal medium supplemented with indicated concentration of thiamine for 10 h. Cells were pelleted and imaged on a 360 nm UV transilluminator.

Table 1. Gene Expression by +ThiM#2 and Its Mutants

	β-galactosidase (LacZ) activity (10^4×a.u.)[a]		
clone	thiamine 0 mM	thiamine 0.1 mM	relative activation[b] (0.1 mM/0 mM)
+ThiM#2	1.61 ± 0.21	20.4 ± 2.54	12.7
+ThiM#2 *mut1*	16.1 ± 1.86	55.5 ± 5.31	3.5
+ThiM#2 *mut2*	1.37 ± 0.18	1.24 ± 0.13	0.9

[a] Data are averages of four independent measurements ± standard deviation. [b] Calculated as the ratio of LacZ activity in 0.1 mM and 0 mM thiamine.

in the aptamer also eliminated the thiamine response of +ThiM#2 *mut2*.

To further demonstrate that +ThiM#2 functions independently from the coding region, fluorescent protein genes *gfpuv*[8] and *mcherry*[9] were cloned in place of *tetA* and *lacZ*. The cells transformed with +ThiM#2 fused to the fluorescent reporter genes were grown in the absence and presence (0.1 mM) of thiamine and pelleted to visualize the expressed proteins (Figure 2B). The use of fluorescent reporters should enable noninvasive and real-time monitoring of intracellular metabolites, which may be useful for analyzing and/or engineering metabolic pathways.

Natural TPP riboswitches found in bacteria all repress gene expression in the presence of the ligand using the highly conserved aptamer domain.[3] However, the natural expression platform sequences that mediate gene repression display no significant conservation. Our results indicate that a single aptamer can mediate both positive and negative gene regulation in response to the ligand. Moreover, the lack of a strong consensus among the selected sequences (Table 1S, Supporting Information) implies that potential expression platforms occupy a relatively large proportion of the unbiased sequence space.

The observed functional flexibility of the riboswitches may reflect some of the characteristics of the ancient riboswitches. For example, riboswitches may have allowed the primitive cells to explore diverse regulatory circuits in a relatively small sequence space in the absence of sophisticated and complex proteins.

Other genetic selection methods adapted for gene switches use two or more separate genes as selective markers for ON and OFF states.[10] A spontaneous mutation in one marker gene could result in a false positive that cannot be removed by subsequent selections because the remaining marker gene is intact. To avoid accumulating false positives, it is necessary to periodically isolate the plasmid pool from the selection markers and retransform the library plasmids into *E. coli* harboring fresh selective markers.[10]

In contrast, *tetA* dual selection is robust against emergence of false positives, presumably because a single gene is responsible for both phenotypes (tetracycline and $NiCl_2$ resistance) selected for in ON and OFF selections.[5b] Loss of one phenotype by a spontaneous mutation is most likely accompanied by the loss of the other phonotype as well. As a result, we were able to perform the selection without plasmid isolation during the selection.

Genetic selection allows larger sequence space to be explored compared to high-throughput screening based on reporter gene assay.[7c] This advantage may be crucial for future engineering and discovery of more complex riboswitch functions, such as Boolean logic[11] and cooperative response,[12] which are currently under investigation.

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Supporting Information Available: Detailed description of the experimental procedures and materials used. This material is available free of charge via the Internet at http://pubs.acs.org.

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