RNA Technology

DOI: 10.1002/anie.201001482

Posttranscriptional Signal Integration of Engineered Riboswitches Yields Band-Pass Output**

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Recent progress in synthetic and systems biology has advanced our ability to program complex cellular behaviors using multiple genetic elements with promising applications in biotechnology and medicine.[1] A number of genetic elements such as transcription factors, promoters, and reporter genes that individually perform simple tasks can be combined to engineer circuits with complex functions. One of the striking examples that illustrate the promise of this strategy is the bacterial band-detect (band-pass) circuit constructed from a set of transcription factors and promoters. Basu et al. have designed a circuit in *Escherichia coli* that expresses GFP within a limited concentration range of the small molecule quorum sensing signal produced by neighboring cells.[2] This behavior was accomplished by integration of the high-pass and low-pass modules with a synthetic promoter that functions as a NOR logic gate (Figure 1 a). An alternative design by Sohka et al. employed a number of genes involved in antibiotic resistance to enable cell growth within a limited range of ampicillin concentration (Figure 1 b).[3] Both research groups elegantly demonstrated the synthesis of a complex function (band-pass) through integration of simpler functional modules (low-pass and high-pass modules).

Networks of transcription factors and promoters play a central role in many of the synthetic circuits reported to date, including the band-pass circuit by Basu et al.[2] which contains four transcription factors. However, there are several challenges and limitations in scaling up the strategy to build circuits with higher complexity and more components. For example, expression of many exogenous transcription factors may burden or interfere with the host cell machinery. Moreover, cascades of transcription factors as seen in Basu et al.[2] would be expected to result in a slower circuit response. Consequently, we believe that new genetic circuit elements other than transcription factors and promoters need to be explored for practical applications of synthetic genetic circuits—especially as their complexity increases. To illustrate the advantage of utilizing diverse gene regulatory mechanisms to engineer complex cellular behaviors, herein we describe a band-pass circuit based on genetic elements other than transcription factors.

Our band-pass circuit design is depicted in Figures 1 c,d. Riboswitches are noncoding gene regulatory elements found in the 5′ untranslated regions (UTRs) of prokaryotic mRNAs that modulate gene expression by directly binding small molecule metabolites.[4] Engineered riboswitches that respond to thiamine pyrophosphate (TPP) were used as the chemical sensor and to implement high/low-pass functions. The signals from the two riboswitches were integrated by the split GFP, developed by the Waldo research group.[5] The low-pass riboswitch (TPP-OFF) controls the expression of the $GFP_{1–10}$ fragment while the high-pass riboswitch (TPP-ON) regulates the 15-residue GFP_{11} peptide fragment fused to maltose binding protein (MBP). The host *E. coli* cells fluorescence through protein complementation only when both GFP fragments are expressed at thiamine concentrations above and below the thresholds of the two riboswitches (Figure 1 d).

First, we characterized the engineered riboswitches fused to the reporter gene *gfpuv*. As the high-pass riboswitch (TPP-ON), we chose one of the synthetic riboswitches we previously discovered through dual genetic selection ($+thiMN_{15}$#19).[6] The riboswitch $+thiMN_{15}$#19 was chosen for its superior ON/OFF ratio and its low thiamine concentration needed to induce gene expression (Figure 2). A natural *E. coli* riboswitch derived from the 5′ UTR of *thiC* (−thiCwt)[7] was first tested as the low-pass riboswitch (TPP-OFF). As evident from Figure 2, however, −thiCwt displaed low fluorescence at low thiamine concentrations (low ON level) and the gene expression was essentially turned off at approximately 10 μM thiamine, where $+thiMN_{15}$#19 starts to turn on. To engineer a low-pass riboswitch with more desirable characteristics, we screened mutants of the *thiC* riboswitch generated by error-prone PCR. One of the −thiCwt mutants (−thiC#19) exhibited a high ON level at low thiamine concentrations and retained significant gene expression level at approximately 10 μM thiamine (Figure 2).

Next, the riboswitches −thiC#19 and $+thiMN_{15}$#19 were used to regulate expression of $GFP_{1–10}$ and MBP-GFP_{11}, respectively, in separate but compatible plasmids (Figure S1 in the Supporting Information). *E. coli* cells cotransformed with the two plasmids and exhibited a band-pass response to thiamine as shown in Figure 3. The cells displayed the maximum fluorescence at approximately 6 μM thiamine, the concentration at which the two riboswitches express at similar levels (Figure 2). The cellular fluorescence is clearly visible under UV excitation (Figure S2 in the Supporting Information). In contrast, cells in which −thiC#19 was replaced with

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[**] This research was supported by the National Science Foundation (CCF0621523 and CCF0829536). We thank Prof. Ronald Breaker for the gift of *thiC* gene, and Dr. Geoffrey Waldo for the split GFP gene.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201001482.

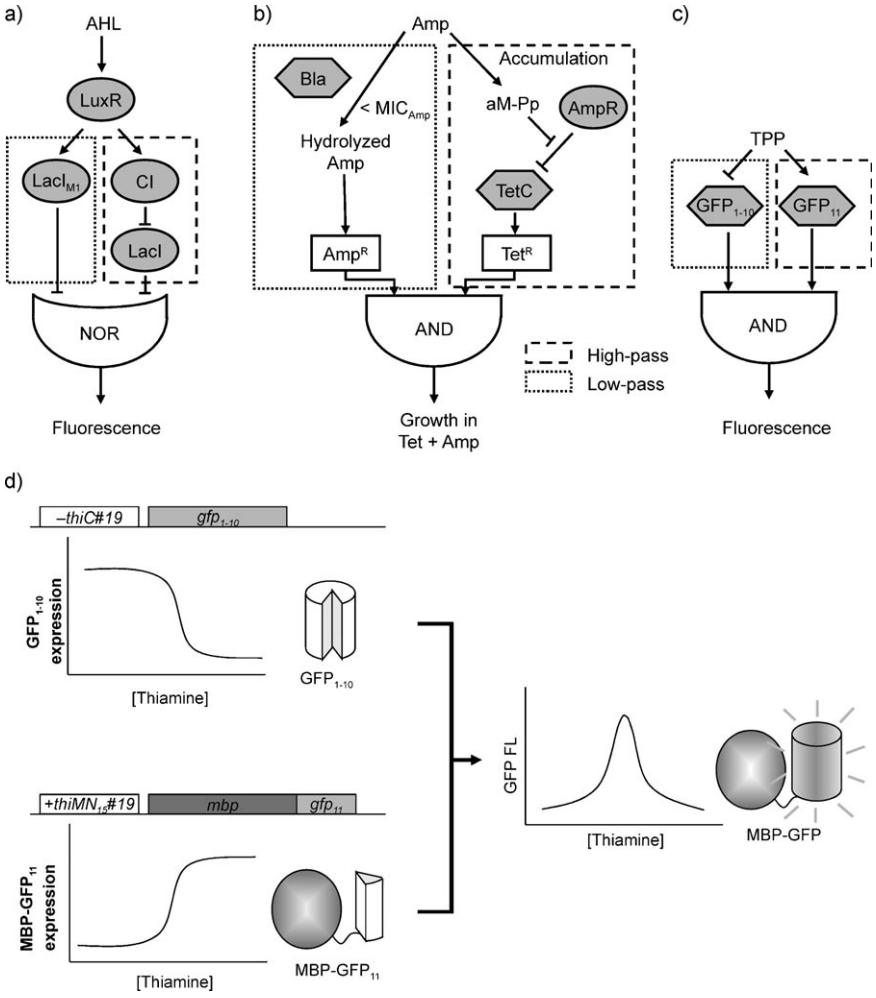

Figure 1. Schematic illustrations of *E. coli* band-pass circuits. a) Transcriptional cascade network by Basu et al.[2] LuxR senses *N*-acyl homoserine lactone (AHL) and activates the repressor LacI$_{M1}$ (low-pass) and CI/LacI transcriptional cascade (high-pass). The low-pass and high-pass signals are integrated by a synthetic promoter that operates as a NOR logic gate. b) Tunable band-pass circuit by Sohka et al.[3] Cell growth in the presence of tetracycline (Tet) is only observed below the ampicillin (Amp) concentration (MIC$_{Amp}$) at which β-lactamase (Bla) can efficiently hydrolyze Amp, and above the Amp concentration at which TetC expression is activated. Amp promotes accumulation of the murein breakdown product aM-pentapeptide (aM-Pp) that induces TetC expression from the AmpR-regulated promoter. c) and d) Band-pass circuit design described in this work. TPP regulates two engineered riboswitches. One riboswitch (−thiC#19) functions as a low-pass filter by repressing the expression of the split GFP fragment GFP$_{1-10}$. Another riboswitch (+thiMN$_{15}$#19) activates expression of the other split GFP fragment GFP$_{11}$ fused to MBP and functions as the high-pass filter. The two signals are integrated by protein complementation.

the wild-type *thiC* riboswitch did not show a band-pass response (Figure 3).

In this study, we employed engineered riboswitches as both the chemical sensor and high/low-pass modules which are functionally equivalent to the four transcription factors used in the circuit developed by Basu et al. We and others have shown that riboswitches can be engineered relatively easily through genetic selection, screening, or rational design.[6,8] For example, we recently engineered synthetic riboswitches that respond both positively[6,8e,f] and negatively[8g] to TPP, and logic gates[8h] that respond to multiple chemical inputs using dual genetic selection. As hundreds of RNA aptamers have been isolated through in vitro selection,[9] one can expect to engineer synthetic riboswitches that sense and respond to a diverse class of molecules that are of practical interest. Moreover, riboswitches may exert a lower metabolic burden on the host cells compared to protein-based components. Another advantage of riboswitches is their small genetic size compared to transcription factors.

Integration of the two riboswitch signals was achieved by complementation of the two split GFP fragments, which functions as an AND gate because both fragments must be expressed for the output (GFP fluorescence) to be ON (Figure 1c). Protein complementation[10] has been utilized extensively to study biomolecular interactions some of which may be adapted to synthetic genetic circuits as shown here. More importantly, the use of split inteins[11] should allow arbitrary proteins to be used as the output of the posttranscriptional AND gates, possibly interfacing the circuit with another circuit or the host cellular machinery.

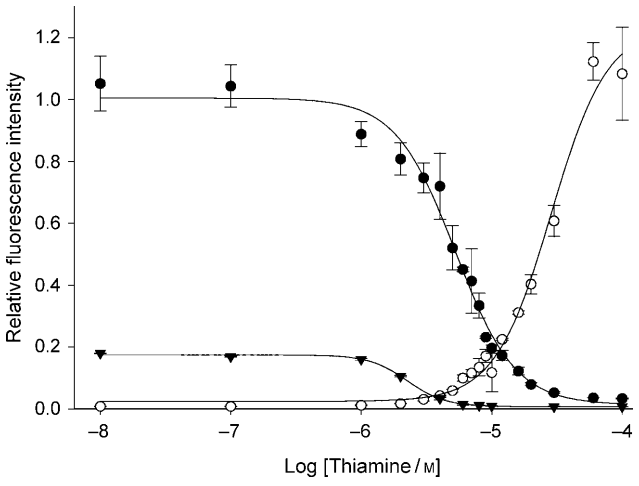

Figure 2. Thiamine-dependent GFPuv expression of −thiC#19 (closed circles), +thiMN$_{15}$#19 (open circles) and −thiCwt (closed triangles). All fluorescence data are averages of measurements from two independent cultures (8 h after dilution) and the error bars represent the range of the two measurements. The fluorescence intensities were normalized to the observed fluorescence of −thiC#19 grown without thiamine (equal to 1.0). Curves are shown to guide the eye only.

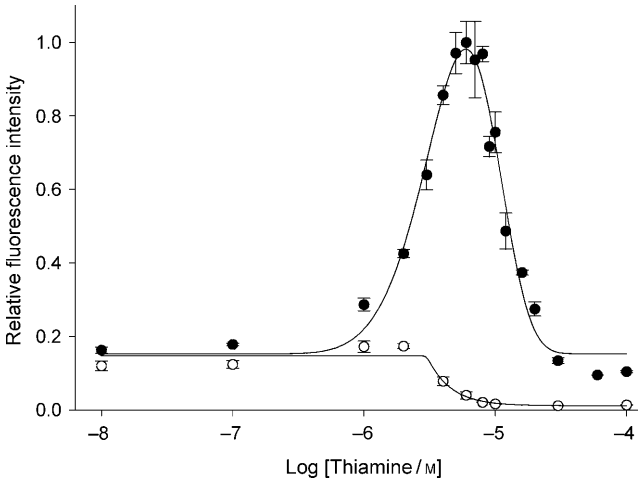

Figure 3. Characterization of the band-pass circuit. Thiamine-dependent GFP fluorescence of the cells cotransformed with plasmids expressing +thiMN$_{15}$#19-mbp-gfp$_{11}$ with −thiC#19-gfp$_{1-10}$ (closed circles) or −thiCwt-gfp$_{1-10}$ (open circles). All fluorescence data are averages of measurements from three independent cultures (8 h after dilution) and the error bars represent standard deviations. The fluorescence intensities were normalized to the observed fluorescence of the cells containing +thiMN$_{15}$#19-mbp-gfp$_{11}$ and −thiC#19-gfp$_{1-10}$ grown in the presence of 6 μM thiamine (equal to 1.0). Curves are shown to guide the eye only. Note that the fluorescence intensities shown in Figures 2 and 3 are not equivalent due to the different GFP variants and excitation/emission wavelengths used (see the Supporting Information).

In summary, we have demonstrated that the use of engineered biomolecules such as riboswitches and split proteins can greatly facilitate and simplify the design of genetic circuits with complex functions. Harnessing the rich posttranscriptional regulatory mechanisms in the cell should accelerate design of future synthetic genetic circuits with complex and practical functions.

Experimental Section

All plasmids encoding the riboswitches and split GFP fragments were constructed using standard molecular cloning techniques and the sequence information is provided in Supporting Information. Detailed procedures for cell culture and GFP fluorescence assay are provided in Supporting Information. Briefly, *E. coli* TOP10 cells (Invitrogen) transformed with appropriate plasmids were grown at 37 °C for 8 h ($OD_{600} \approx 0.3$) in M9 minimal medium containing 0.8 % glycerol as the carbon source supplemented with appropriate antibiotics, casamino acids (0.1 %), and thiamine. Cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS) and resuspended in PBS. The GFP fluorescence was measured using Safire2 microplate reader (Tecan) and normalized by OD_{600}. Fluorescence data obtained from PBS-filled wells were used as a background.

Received: March 11, 2010
Published online: May 17, 2010

Keywords: band-pass circuits · genetic engineering · protein complementation · riboswitches · RNA

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