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# Artificial Ribozyme Switches Containing Natural Riboswitch Aptamer Domains**

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Artificial RNA-based switches enable the control of gene expression upon the external addition of a ligand. A number of such systems have been generated by inserting aptamers into messenger RNA molecules, which then respond to the presence of the respective ligand with changes in gene expression.[1,2] These man-made tools should prove valuable in future applications of synthetic biology, such as targeted recruitment, reduction of complexity, and the implementation of novel regulatory circuits in biological entities.[3,4] Although several proof-of-principle studies have shown that artificial riboswitches can be generated by using various strategies, most of these riboswitches have been demonstrated in bacteria with an aptamer for theophylline.[5–8] These systems nicely demonstrate the potential of the artificial-riboswitch strategy; however, the use of theophylline is often problematic, as it has to be administered at high concentrations. Taking into account the small therapeutic window,[9] alternatives to the small-molecule trigger theophylline are urgently needed. Furthermore, for the construction of advanced RNA-based regulatory networks, more than one chemical stimulus needs to be available for triggering specific responses in gene expression. Although there have been a few studies on the use of antibiotics as regulatory agents,[10–12] this approach is not feasible in bacteria owing to the intrinsic toxicity of these compounds.

Nature has invented a diversity of RNA sequences, which bind to a variety of different ligands, such as amino acids, cofactors, and nucleobases. These aptamer sequences are found within natural riboswitch systems.[13,14] There have been only very few attempts to reprogram natural riboswitches for the artificial control of gene expression.[15,16] These manipulations were all based on the architectures of the natural riboswitches. The fact that the mechanisms of riboswitches are complex and well adapted to the genetic apparatus of the host may explain why so few attempts have been made so far to reprogram natural RNA switches. For example, in the case of transcriptional control, the relationship between ligand binding and changes in gene expression is not well understood, as kinetic effects rather than equilibrium conformational changes seem to play an important role in termination/antitermination switching in natural systems.[17,18] Nevertheless, mechanistic insight into some well-characterized riboswitches suggests an induced fit of the corresponding aptamer domains upon ligand binding.[19–22] This property makes natural aptamer domains interesting tools for the generation of artificial gene-regulation systems.

Herein, we demonstrate that natural aptamer domains can be coupled to a synthetic expression platform, namely, a self-cleaving ribozyme, to create efficient ligand-dependent switches. Previously, we introduced a novel format for artificial riboswitches that act as genetic regulators in *Escherichia coli*.[5] The system is believed to be independent of the host genetic mechanism, as sensing of the regulatory ligand triggers self-cleavage of the respective mRNA molecule. We demonstrate that the natural aptamer domain of the thiamine pyrophosphate (TPP) riboswitch can be used to construct very efficient ribozyme-based artificial switches of gene expression. The construction of both on and off switches that react very sensitively to small amounts of the natural cofactor thiamine in *E. coli* is possible.

Among the first riboswitches discovered were the TPP-responsive elements found in the thiM and thiC genes.[24,26] Since these initial discoveries, the TPP switch has been found in various organisms of all kingdoms. It is the most widespread riboswitch known to date. Besides its frequent occurrence in 5′-untranslated regions of bacterial operons that code for genes associated with thiamine biosynthesis,[27,28] the TPP switch has been identified in various plants[29] and in fungi,[30] in which it regulates splicing and alternative 3′-end processing of mRNA. Currently, there are two crystal structures available for the aptamer-domain/ligand interaction in *E. coli*[23] and *Arabidopsis thaliana*.[31] In bacteria, the aptamer domain is coupled to an expression platform that controls either transcription or translation.[24,26]

Fast-cleaving variants of the hammerhead ribozyme (HHR) with stem I/stem II tertiary interactions[25,32] have been used in eukaryotes for controlling gene expression. Mulligan and co-workers demonstrated that ribozyme inhibitors can be used to control the self-cleavage reaction of HHR and hence gene expression in mammals.[33] Win and Smolke demonstrated ligand-responsive HHRs for the regulation of gene expression in yeast.[34] We recently introduced a novel design that enables the liberation of the ribosomal binding site in bacteria by using a theophylline-dependent, fast-cleaving HHR.[5] A slightly different system based on minimal HHRs has been shown to enable moderate regulation under certain conditions.[8]

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To investigate whether naturally occurring aptamer riboswitch motifs can be utilized in a ribozyme-dependent mechanism, we introduced the thiM aptamer domain from *E. coli*[23,24] into stem III of a fast-cleaving HHR (Figure 1). Next, we randomized six nucleotides of the region connecting the aptamer and ribozyme domains to perform an in vivo screen for functional RNA switches.[5] We screened 4000 clones (representing 50% coverage of the total sequence space; see the Supporting Information for calculation of the sampling factor) for differential expression of the reporter gene *eGFP* upon the addition of thiamine to the growth medium and found that a surprisingly large fraction (approximately 2% of all clones) showed clearly detectable changes in expression. A control clone containing the HHR without the TPP aptamer did not display changes in response to thiamine. Importantly, switches that inhibited and switches that activated gene expression upon the addition of thiamine to the medium were both found frequently (see Figure 2 for examples). The discovered switches covered a broad distribution of switching ratios and operational ranges. The highest activation and inhibition ratios were observed with switches that operate at lower absolute expression levels. Accordingly, the highest absolute changes in reporter-gene expression resulted in only moderate on/off ratios of between 3:1 and 4:1 (see Figure 2).

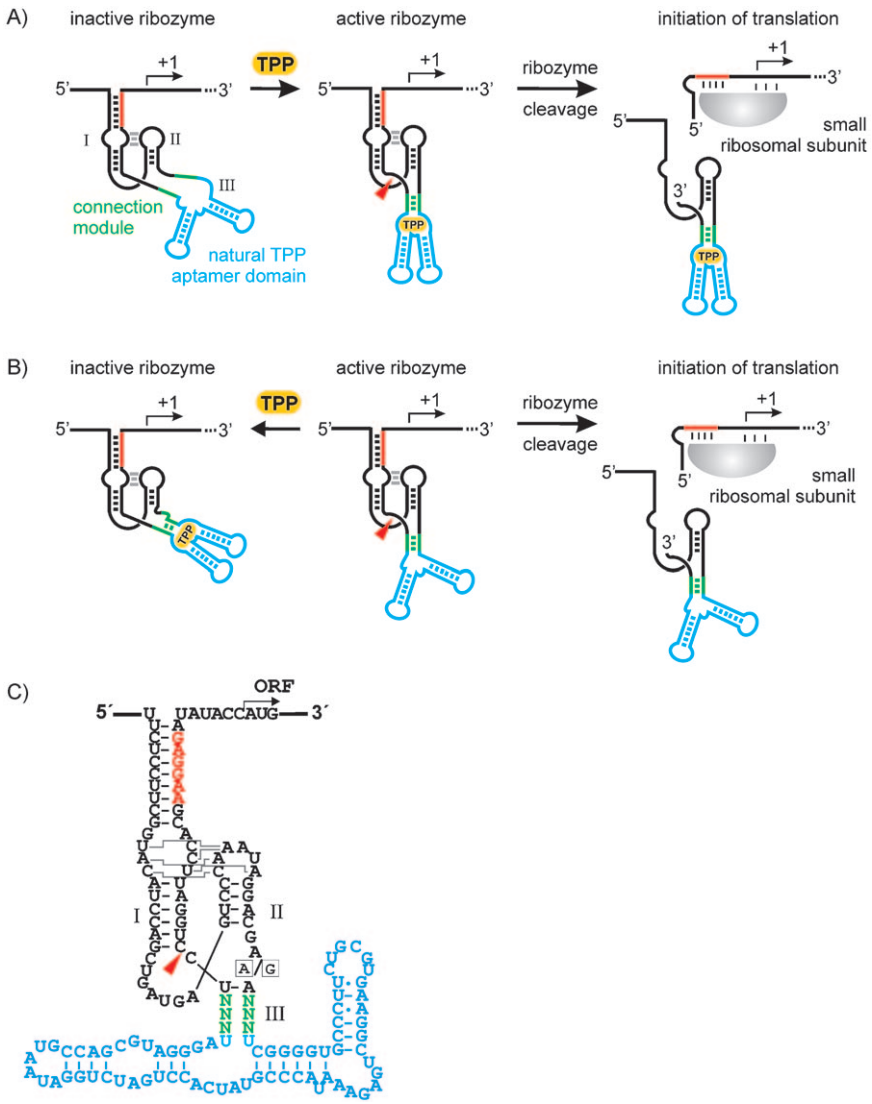


***Figure 1.*** Representation of artificial thiamine pyrophosphate (TPP) riboswitches. The natural TPP aptamer domain (blue)[23,24] was fused to stem III of the *Schistosoma mansonii* hammerhead ribozyme.[25] Stems are indicated with roman numerals; rate-enhancing stem I/stem II interactions are shown as gray lines; the cleavage site is marked by a red arrowhead. The extended stem I of the ribozyme masks the Shine–Dalgarno sequence (red). Upon self-cleavage of the activated ribozyme, the ribosome-binding site is liberated, and gene expression is turned on. A) TPP-activated riboswitches increase ribozyme cleavage and hence gene expression upon the external addition of thiamine. B) The same screen revealed sequences that shut off self-cleavage in the presence of TPP, which results in repressed gene expression. C) Sequence of the artificial TPP-responsive riboswitches; red: Shine–Dalgarno sequence, blue: TPP aptamer, green: nucleotide positions randomized for the screening of TPP-responsive sequences, boxed nucleotides: position of the ribozyme-inactivating mutation (A→G).

Next, we investigated the sensitivity of the switches by measuring gene expression in response to varying concentrations of thiamine in the growth medium (Figure 3B,C). Surprisingly, all investigated switches showed very high sensitivities with half-maximum expression at thiamine levels well below 1 μM. This result represents a significant advancement, as most artificial switches need very high effector levels. For example, the most widely used theophylline-based systems require ligand concentrations of 1 mM or more, although the aptamer selected in vitro also has a dissociation constant of 100 nM.[9,35]

We carried out important control experiments to validate the hypothesis that the switches operate through ribozyme-dependent initiation of translation. A point mutation known to render the ribozyme inactive was introduced in the catalytic core (A→G, see Figure 3A).[33] If the proposed mechanism

  

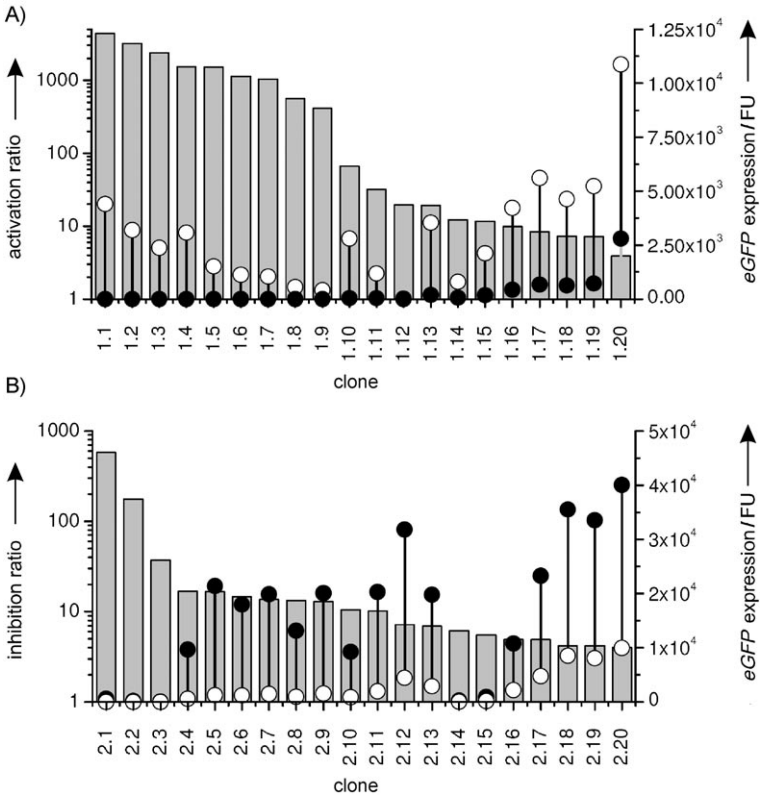


***Figure 2.*** Screening results for TPP-dependent gene expression. A) Clones found to be activated upon the addition of thiamine. B) Clones that displayed inhibited gene expression when thiamine was added. Gray bars show the activation or inhibition ratio, which was generated by dividing the fluorescence in the activated state by the fluorescence in the inactivated state. Circles show the absolute fluorescence (arbitrary units FU) measured in both states (open circles: in the presence of thiamine (1 mM), closed circles: in the absence of thiamine).

of self-cleavage necessary to free the Shine–Dalgarno sequence is valid, an inactivated variant of the ribozyme should not display gene expression at all owing to the permanently blocked ribosome-binding site. Indeed, all switches inactivated by the mutation showed no gene expression, irrespective of the thiamine concentration (Figure 3B,C).

We investigated the kinetics of in vitro cleavage with the isolated hammerhead ribozymes. In the case of the activating switches, ribozymes transcribed in vitro showed significant rate enhancement upon the addition of TPP (Table 1). In the case of the inactivating sequences, only marginal changes in the cleavage rates were observed.

The surprising finding of the highly sensitive onset of changes in gene expression demands a more thorough discussion of the intracellular concentration and metabolism of thiamine derivatives. First of all, the presented data demonstrate that naturally occurring RNA ligands can be utilized as external triggers of synthetic switches despite the fact that there are intrinsic mechanisms in place for the metabolism of these compounds.[36,37] The *E. coli* strain BL21 used in this study was grown in minimal medium (excluding thiamine derivatives) and is proficient in TPP biosynthesis. Nevertheless, the intrinsic levels of TPP seem to be low enough to prevent a permanent stimulation of the switches. In contrast, upon the external addition of thiamine, TPP seems to be synthesized (through import into the cells, followed by the action of thiamine kinase and thiamine phosphate kinase)[37] in amounts that enable the triggering of the synthetic switches. Intracellular thiamine levels have been reported to be in the range of 0.25 to 4.5 μM in *Bacillus subtilis*,[38] whereas older references mention that the total amount of thiamine and its pyrophosphate is as high as 40 μM in *E. coli*.[36] However, the concentration of freely available TPP is probably much lower, as suggested by the apparent dissociation constant of the aptamer/TPP complex of 0.1 μM.[24] This value is in agreement with the observation that synthetic switches containing this aptamer domain are able to sense the ligand at concentrations as low as 1 μM. The intrinsic biosynthesis is unlikely to interfere with the switch after external thiamine has been added to the medium, as it has been shown that biosynthesis shuts down drastically upon the addition of thiamine. (A thiamine concentration of 0.1 μM in the growth medium resulted in a decrease in novo synthesis to 2% of the initial amount).[36] After the addition of thiamine to the growth medium, the intracellular concentrations of thiamine and its phosphorylated derivatives should be much higher than the extracellular concentrations as a result of the active import of thiamine. The applicability of synthetic switches triggered by natural ligands would certainly benefit from such mechanisms.

In conclusion, we have shown the validity of utilizing intrinsic metabolites to trigger synthetic switches containing natural aptamer modules. We were able to identify several inactivating and activating switches with significant advantages over completely artificial systems, such as those based on theophylline aptamers. A broad spectrum of switching parameters was found for the new systems, which are characterized by very sensitive onset concentrations. Nevertheless, more examples of synthetic switches constructed from natural systems are required to generalize the findings. The use of synthetic biology to assemble natural structural moieties in novel ways enables the engineering of devices with properties that differ from those of the natural systems. The results of the present study support recent hypotheses that aptamers selected in vitro are often not suited for in vivo applications.[2,39] Hence, the approach of exploiting natural ligand-binding RNA sequences should gain importance in the engineering of designer organisms. In future studies, we will

***Table 1:*** In vitro cleavage rates of TPP-dependent hammerhead ribozymes.[a]

| TPP | Clone 1.2[b] | Clone 1.20[c] | Clone 2.5[d] | Clone 2.12[b] | wt HHR[b] |
|---|---|---|---|---|---|
| – | 0.024 | 0.055 | 3.9 | 0.7 | 7.8 |
| 100 μM | 0.150 | 0.210 | 3.3 | 0.7 | 8.3 |

[a] The $k_{obs}$ values of in-*cis*-cleaving ribozymes are given (in $min^{-1}$). [b] $Mg^{2+}$: 2 mM. [c] $Mg^{2+}$: 10 mM. [d] $Mg^{2+}$: 0.2 mM.

  

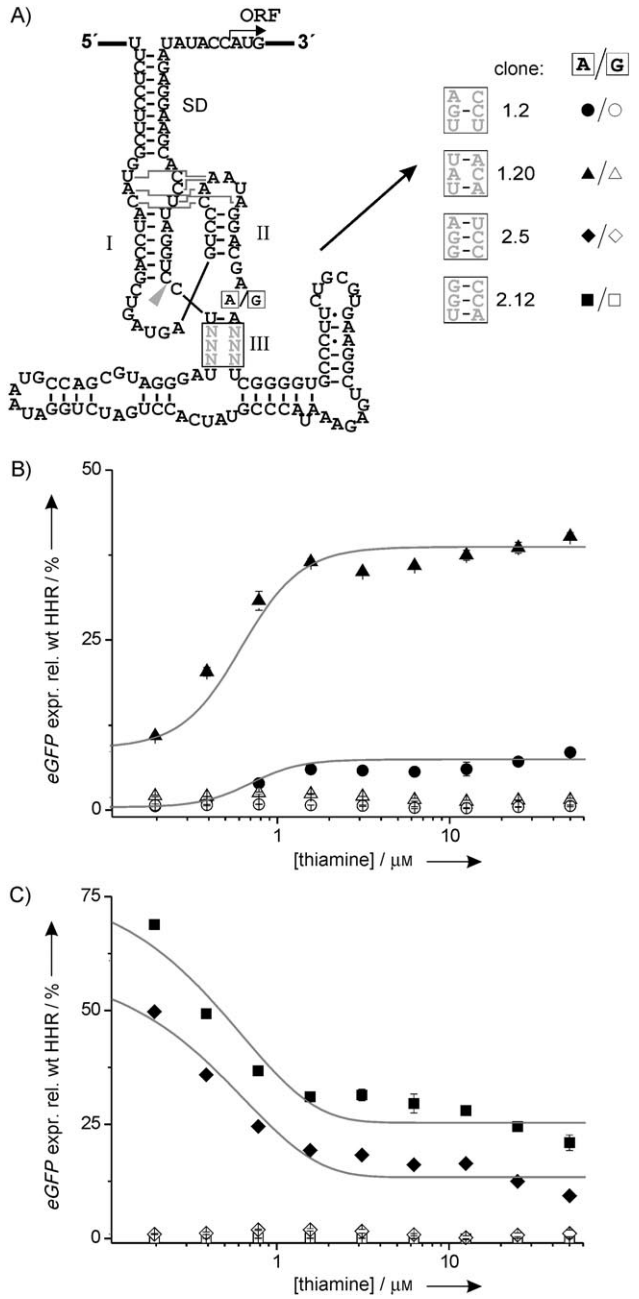


***Figure 3.*** Characterization of individual clones: A) Sequences of two activating and two inhibiting switches: clones 1.2, 1.20, 2.5, and 2.12 (SD = Shine–Dalgarno sequence). B,C) Dependence of gene expression on thiamine concentration (*y* axis: *eGFP* expression relative to that with the wt HHR). Thiamine was added to the growth medium, and *eGFP* was detected in outgrown cultures. Closed symbols: clones as depicted in A, open symbols: clones as in A with a ribozyme-inactivating mutation in the catalytic core (A→G).

utilize the platform based on synthetic hammerhead ribozymes to control RNA functions other than messaging.

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