RNA Switches

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Improved Aptazyme Design and In Vivo Screening Enable Riboswitching in Bacteria**

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The development of artificial RNA-based switches of gene expression is inspired by a wealth of recently discovered, naturally occurring riboswitches.[1] Among these, only one example, the *glmS* riboswitch, operates by ribozyme-mediated cleavage of the RNA message upon activation by a small metabolite in bacteria.[2] On the other hand, several examples of so-called aptazymes based on the hammerhead ribozyme (HHR) have been generated that can be controlled by ligands interacting with introduced aptamer domains.[3,4] Recent results have shown that naturally occurring hammerheads comprise a tertiary interaction between stems I and II, thereby stabilizing the catalytically active conformation.[5–7] Such extended hammerhead ribozymes display much higher activities, enabling efficient cleavage even when low magnesium concentrations are present such as inside cells.[5] Here, we present a novel design strategy for ligand-controlled hammerhead ribozymes that enables the construction of artificial riboswitches that function in bacteria.

Fast-cleaving hammerhead ribozymes containing stem I/stem II interactions have been used before to down-regulate gene expression in mammalian cell culture as well as in animals by Mulligan and co-workers.[8,9] The HHR efficiently switches off gene expression when inserted into an mRNA. In addition, nucleoside analogues were shown to inhibit the ribozyme by getting incorporated into cellular RNA and hence into the encoded ribozyme. The modifications result in inactivated hammerheads which lead to elevated expression levels of the affected genes.[8,9] Besides this interesting finding, the quest for nontoxic compounds that can be used in a variety of organisms such as bacteria requires the generation of aptazymes operating by more specific mechanisms. For this purpose, RNA switches were constructed by introducing aptamers instead of aptazymes into mRNAs. Upon addition of the aptamer-specific ligand, gene expression was reduced.[10] More recently, an aptamer specifically binding the xanthine analogue theophylline[11] was inserted into the mRNA of bacteria allowing eightfold inhibition of gene expression.[12] Using an advanced approach, Gallivan and co-workers screened for variants of mRNAs containing the theophylline aptamer enabling efficient up-regulation of gene expression.[13–15] The selected modules trigger liberation of the ribosomal binding site (RBS) upon theophylline binding. An accessible Shine–Dalgarno (SD) sequence within the RBS is essential for efficient initiation of translation,[16] and ligand-dependent masking of the SD is a common mechanism in naturally occurring riboswitches.[17]

To construct a HHR-based switch of gene expression that operates in vivo, we have chosen a ribozyme that comprises tertiary contacts of stems I and II enabling fast cleavage kinetics. In addition, we engineered the ribozyme such that liberation of the RBS occurs upon mRNA cleavage. This novel design was necessary since cleavage of bacterial mRNA by a ribozyme positioned in the 5′-untranslated region does not efficiently affect translation of the message. We extended stem I of the HHR such as it masks the SD sequence (see SD/anti-SD region, Figure 1 A). Secondary structure results in efficient inhibition of gene expression, as was shown by introducing a single point mutation to the ribozyme core that renders the HHR inactive (see inactive HHR, Figure 1 B). An active HHR cleaves off the strand that pairs with the SD, resulting in reporter gene expression (see Figure 1 B, HHR). The developed strategy of blocking accessibility of the ribosome binding site in combination with ligand-dependent liberation of the latter upon cleavage of the message should make it possible to use ribozymes to control gene expression in bacteria by a well-defined mechanism. One should note that the only ribozyme known to act as a regulator of gene expression in nature, the *glmS* riboswitch, has the opposite reactivity, namely it has decreasing gene expression upon activation by glucosamine-6-phosphate. The exact mechanism of action seems to involve a specific nuclease that degrades the cleaved message.[2,18]

With the new setup of a fast-cleaving hammerhead acting on an mRNA established, we aimed at rendering the ribozyme ligand-dependent. In previous studies, stem II was used to fuse suited aptamers to a minimal version of the HHR, resulting in switchable but catalytically impaired ribozymes. In a recent report by Smolke and co-workers the aptamer was placed in stem II in a manner that preserved the tertiary interactions.[19] We have chosen to insert the aptamer at a different position. In order to both retain the essential tertiary contacts for fast cleavage kinetics as well as enable regulation of the activity of the HHR, we connected a theophylline aptamer to stem III of the HHR (Figure 2 A). For optimizing the connecting sequence that communicates ligand binding to the catalytic core, we randomized three nucleotides positioned at the interconnection of the aptamer and ribozyme core (see positions in red boxes in Figure 2 A).

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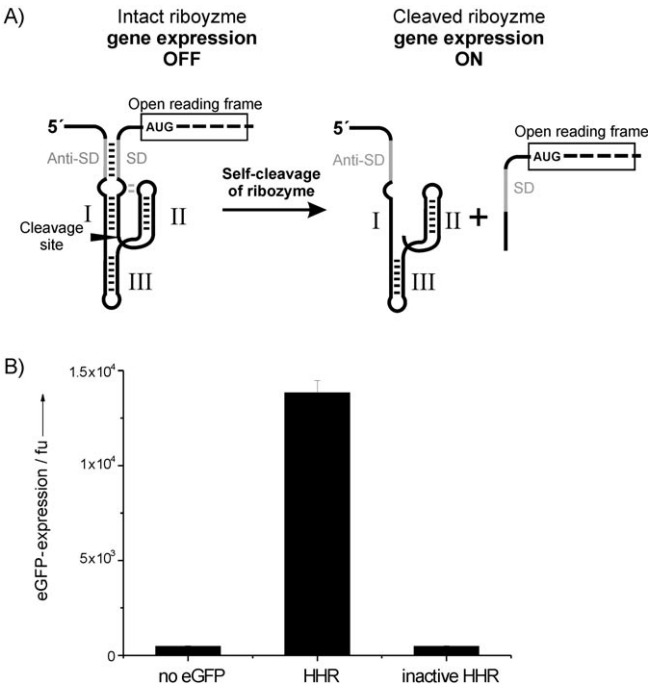

Figure 1. Design of a fast-cleaving hammerhead ribozyme (HHR) that activates reporter gene expression (eGFP) upon cleavage of mRNA in bacteria. A) A novel design was applied to obtain a HHR that mediates expression in bacteria. Important features include stem I/stem II contacts as well as blockage of the Shine–Dalgarno sequence (SD/Anti-SD, gray). Ribozyme cleavage liberates the SD resulting in gene expression. B) In vivo expression levels of eGFP in *Escherichia coli*: Control plasmid lacking the eGFP reporter (no eGFP); an active HHR with stem I/II contacts cloned into the 5′-UTR of an eGFP reporter as depicted in (A), and an inactive variant HHR in the 5′-UTR containing a ribozyme-inactivating point mutation (see the Supporting Information for sequence and mutation).

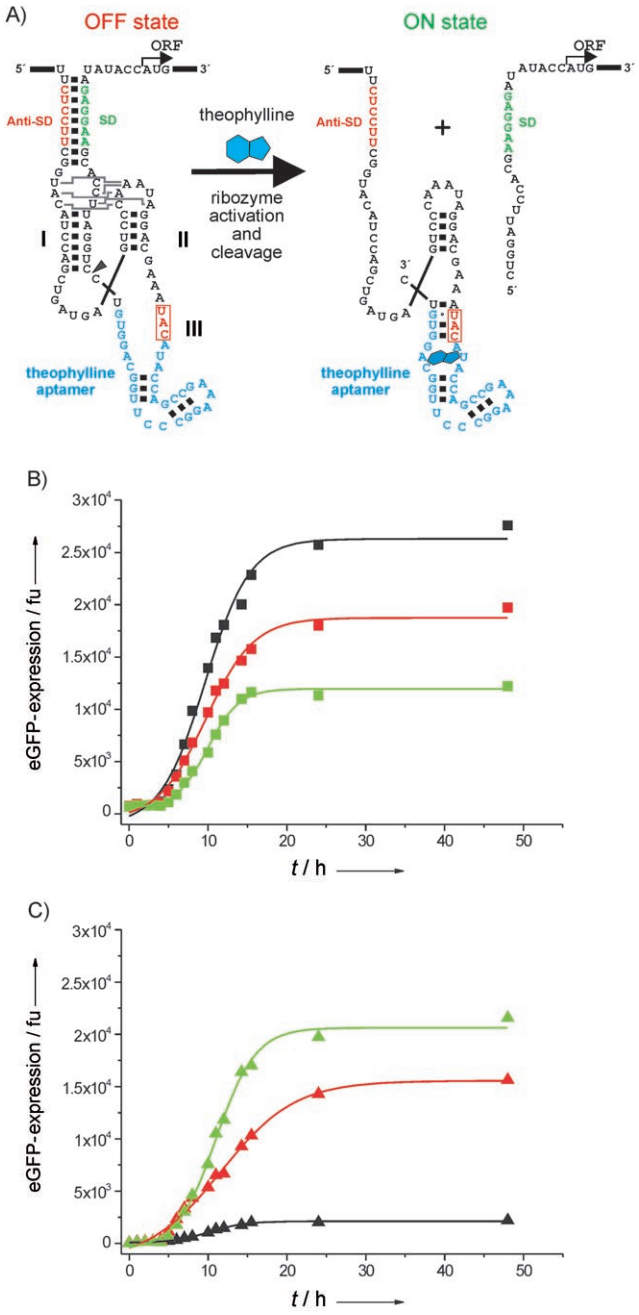

Figure 2. A theophylline-dependent ribozyme acts as an artificial riboswitch. A) Design of the theophylline-dependent hammerhead aptazyme (theo-HHAz): a theophylline aptamer is inserted in stem III of the HHR, allowing stem I/stem II interactions to occur (gray lines). Addition of theophylline activates ribozyme cleavage and hence gene expression. theo-HHAz was obtained by screening a library of clones that were randomized at the positions marked by red boxes. B) In vivo eGFP expression of clone HHR (see Figure 1 for sequence) in response to theophylline. Black: absence of theophylline, red: 800 μM theophylline, green: 4 mM theophylline. C) eGFP expression of the clone theo-HHAz in response to theophylline (color scheme same as in B). While theophylline reduces expression by about twofold in the HHR clone, tenfold activation is observed with the theo-HHAz variant.

The randomized ribozyme was cloned into the eGFP expression plasmid, and isolated *E. coli* clones were screened for changes in gene expression in response to theophylline. By employing such an in vivo screening approach, we obtained sequences directly that are able to act as switches in vivo. We identified one clone that displayed strong induction of eGFP by theophylline: In the presence of theophylline, eGFP expression of the respective clone (theo-HHAz) was induced tenfold, whereas a clone harboring the parental HHR sequence (shown in Figure 1) displayed decreased expression in the presence of theophylline (Figure 2B and C). Importantly, gene expression was not induced upon addition of the analogue caffeine, which differs by only one additional methyl group in the 7-position of the purine (see the Supporting Information). Hence, the high specificity of the aptamer was retained in the ribozyme context. In order to investigate the mechanism of switching, we introduced site-directed mutations that weaken as well as strengthen the selected communication module in stem III. The gene expression in response to these mutations was observed and indicates that stability of stem III is the molecular mechanism of ribozyme switching. As expected, by weakening stem III, the ribozyme is inactivated, thereby resulting in inhibition of gene expression, the opposite outcome was observed when a mutation stabilizing stem III was characterized (see the Supporting Information).

In order to evaluate whether the changes of gene expression observed in vivo result from theophylline-dependent changes of ribozyme activity, the sequence of the clone theo-HHAz was determined and *in-cis*-cleaving ribozymes were synthesized by in vitro transcription.[20] In vitro cleavage assays were carried out at low Mg^{2+} concentrations (200 μM) in order to replicate the low concentrations of free intracellular magnesium ions.[21,22] The observed cleavage rates (k_{obs}) of HHR and theo-HHAz in the absence of theophylline was $> 8\ min^{-1}$ and $1.3\ min^{-1}$, respectively. In the presence of 1 mM theophylline the activity of the unmodified HHR remains unchanged, but theo-HHAz was enhanced almost threefold to $3.6\ min^{-1}$ (Figure 3). In accordance with the in vivo data, caffeine did not enhance theo-HHAz ribozyme activity in vitro (see the Supporting Information). The observed cleavage rates correspond to ribozymes with fast kinetics that are able to cleave efficiently at low magnesium concentrations.[5,7] Almost complete cleavage (90%) was observed in our setup for both the unmodified and the theo-HHAz ribozyme in contrast to previous reports of fast-cleaving HHRs.[5,7]

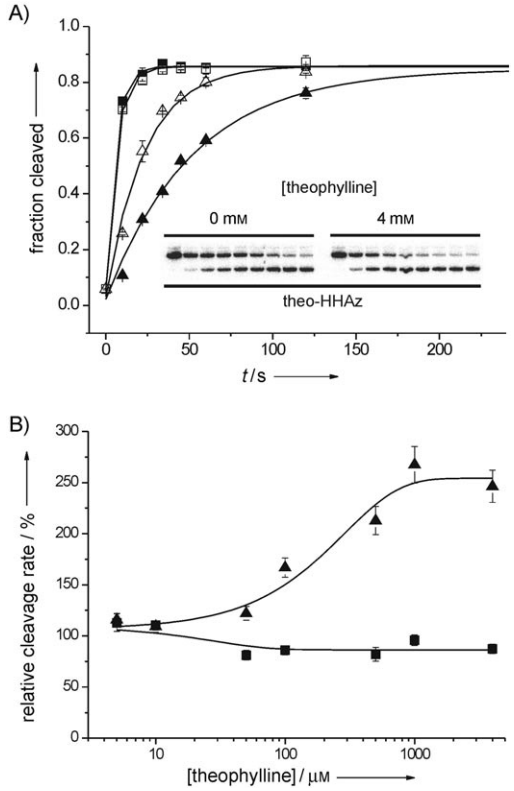

Figure 3. Cleavage kinetics of isolated ribozymes. A) Time course of ribozyme cleavage at 200 μM Mg^{2+}. Squares: HHR, triangles: theo-HHAz. Filled symbols: measured in the absence of theophylline; open symbols: measured at 4 mM theophylline. Insert: PAGE analysis of cleavage reactions of theo-HHAz, *in cis* cleaving ribozymes were obtained by in vitro transcription using a blocking strand that hybridizes to the catalytic core of the ribozymes, hence preventing self-cleavage during transcription. B) Concentration dependence of cleavage-rate activation normalized to absence of theophylline. Squares: HHR, triangles: theo-HHAz.

The observed activation of theo-HHAz by theophylline in vitro is much smaller than the activation ratios of stem II-based theophylline aptazymes selected previously.[3] Nevertheless, the aptazyme theo-HHAz represents a hammerhead ribozyme that can be regulated by ligand binding in vivo. Even the moderate induction ratio observed in vitro seems to be sufficient to activate gene expression in vivo. A possible explanation could be represented by the identification of a critical window of ribozyme activity ($k_{obs} = 1–3\ min^{-1}$), which, in our setup, enables regulation of expression. The discovery of such a window is expected since a reversed strategy of aptazyme discovery was applied; by in vivo screening rather than in vitro selection, one directly identifies clones with desired phenotypes.

In summary, we have presented a novel hammerhead aptazyme design that enables ribozymes to act as artificial riboswitches in bacteria. This approach has distinct advantages over approaches in which the corresponding aptamer alone is inserted into an mRNA. Since cleavage of the RNA is an irreversible and drastic modification of the message, pronounced effects on gene expression could be expected in all organisms even if the genetic mechanisms differ significantly between certain species. We are currently investigating the possibility of employing our design strategy to switch gene expression in eukaryotic organisms. The realization in bacteria was possible by developing a ribozyme that masks the RBS and switches on expression upon cleavage. In addition, attaching an aptamer at a novel position, that is, stem III, allowed tertiary contacts between stems I and II to occur, facilitating efficient cleavage at low magnesium concentrations. By screening a collection of mutants randomized at the connection between aptamer and ribozyme, we were able to identify a ligand-inducible ribozyme. The resulting aptazyme displays high cleavage activity even at low Mg^{2+} concentrations. In addition to providing a novel setup for artificial RNA-based control of gene expression, this study gives insights into the required span of catalytic activity of mRNA-cleaving ribozymes. More detailed studies concerning the catalytic requirements of regulatory hammerhead ribozymes cleaving in vivo with respect to different species are under way. Although only three nucleotides of the connecting communication module were randomized, we were able to discover a potent genetic switch in bacteria. Discovery of ribozymes with optimized in vivo activation ratios as well as variants that are inhibited upon ligand binding might be possible by screening more diverse libraries.

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