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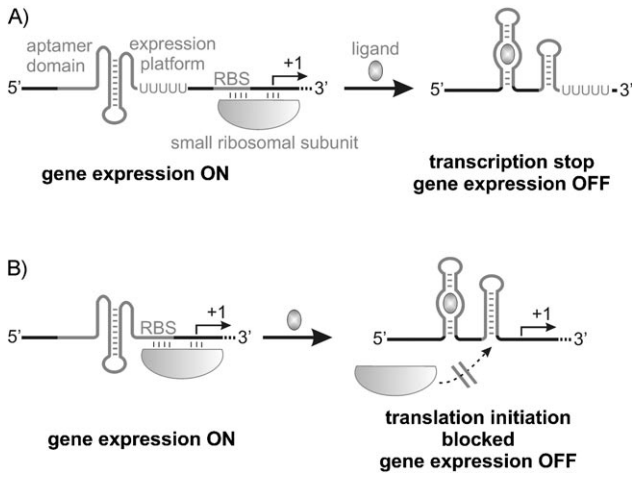
Artificial Riboswitches: Synthetic mRNA-Based Regulators of Gene Expression

Markus Wieland and Jörg S. Hartig*[a]

Introduction

For half a century, bacterial regulation of gene expression has been known to be dominated by proteins that interact with metabolites, which results in altered transcription initiation. Although the expression of the majority of genes is controlled by protein-based mechanisms, the discovery of RNA-based feedback devices that enable regulation of expression without the need for engaged proteins came as a surprise. Breaker and co-workers initially discovered that the use of such mechanisms, termed riboswitches, is widespread in bacteria. For excellent reviews that highlight naturally occurring riboswitches, we refer to the recent literature.[1–4] Riboswitches are typically located in the 5′-untranslated region (5′-UTR) of bacterial mRNA, and consist mainly of a first domain (called aptamer domain) that specifically senses a metabolite, and a second domain (the expression platform) that facilitates control over transcription termination or translation initiation by a structural rearrangement (see Scheme 1).

With respect to the revolutionary findings of Breaker and co-workers, it is very intriguing that researchers have successfully constructed similar, artificial systems even several years before naturally occurring riboswitches were discovered.[5] The generation of such man-made, RNA-based regulators was possible by using aptamer technology for the recognition of ligands by RNAs. Aptamers are in-vitro-selected nucleic acid sequences that specifically bind to a ligand of choice.[6–8] Such artificial, RNA-based switches enable the control of gene expression, uncoupled from the intrinsic metabolism. Although natural riboswitches are mainly found in bacteria, artificial systems have been constructed for eukaryotic organisms as well. Such tailor-made regulatory devices should prove of value as tools in biotechnology as well as synthetic biology applications. Here, we give an overview of the different concepts that are based on the insertion of ligand-sensing elements into mRNAs, thereby enabling the regulation of expression of the respective message. Due to space restrictions we will neither discuss artificial *trans*-acting mechanisms such as small-molecule-regulated, RNA-based transcriptional activators,[9] nor ligand-controlled antisense constructs for the regulation of gene expression.[10]



Scheme 1. Most common mechanisms of naturally occurring riboswitches. A) Regulation of transcription termination: A metabolite binding to the aptamer domain of the riboswitch triggers changes in the expression platform, folding of a transcriptional terminator is stabilized. B) The same general architecture can also be used for translational control by masking the ribosomal binding site (RBS) upon ligand binding. Note: Opposite reactivity (that is, transcription anti-termination as well as translational initiation) upon presence of the metabolites is also found in some riboswitches.

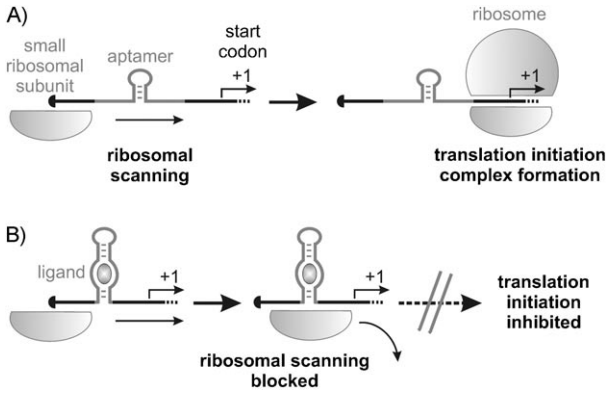
Artificial Riboswitches

Aptamer-based RNA switches in eukarya

Aptamers are short RNA sequences that bind specifically to ligands such as small molecules or proteins.[8] Ten years ago, Werstuck and Green inserted aptamers that were specific for the dye H33258 into the 5′-UTR of a reporter gene in eukaryotes.[5] Upon addition of the dye, which binds to the aptamer, gene expression of the reporter gene was significantly reduced in Chinese hamster ovary cells while a second unmodified reporter gene was unaffected. Since then it has been shown that upon insertion of theophylline, biotin,[11] as well as tetracycline[12] aptamers into the 5′-UTR in eukaryotic cells comparable effects can be observed; this results in reduced gene expression levels in the presence of the corresponding small molecule. Ribosomal binding studies on mRNA revealed that the aptamer in the presence of the ligand interferes with ribosomal scanning of the 5′-UTR for the start codon, and therefore with the formation of the translation initiation complex (see

[a] *M. Wieland, Prof. Dr. J. S. Hartig*
Department of Chemistry and
Konstanz Research School Chemical Biology (KoRS-CB)
University of Konstanz
Universitätsstrasse 10, 78457 Konstanz (Germany)
Fax: (+49) 7531-88-5140
E-mail: joerg.hartig@uni-konstanz.de

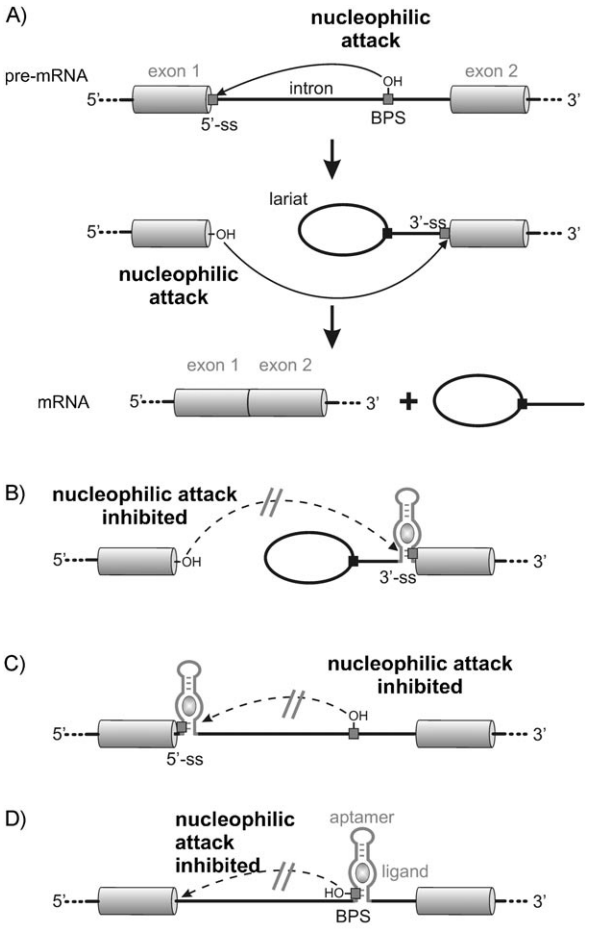
Scheme 2).[11] This result is in accordance with the observation that multiple aptamers that are cloned into the 5′-UTR show a more pronounced effect than a single aptamer.[5,11] Also, gene expression seemed to be unaffected if the aptamer was located within the coding sequence (ORF) or the 3′-UTR of the reporter gene.[11]



Scheme 2. Artificial riboswitches in eukaryotes were constructed by inserting aptamers into 5′-untranslated regions of mRNAs (5′-UTRs). A) In eukaryotes, the small subunit recognizes the first start codon after scanning the mRNA from its 5′-end. B) Ligand binding to the aptamer sequence disturbs the scanning process, and results in decreased translation initiation.[5,11,12]

Besides these initial reports, control of gene expression by placing aptamers into mRNAs was successfully demonstrated by using mechanisms that differ from interfering with translational initiation. For this purpose, splicing of pre-mRNA can be a valuable target. During the splicing event, the newly transcribed pre-mRNA of eukaryotes is altered by removing non-coding intron sequences and rejoining the coding exons. Simplified, the 2′-OH of a nucleotide in the branch point sequence (BPS) performs a nucleophilic attack on the first nucleotide of the intron at the 5′- splice site (5′-ss) to form a lariat and release the 5′-exon. This exon subsequently performs a nucleophilic attack on the last nucleotide of the 3′-splice site (3′-ss) of the intron. Consequently, the 5′- and 3′-exons are rejoined, and the intron is released (see Scheme 3 A). Because inefficient splicing reduces gene expression, the mRNA sequences that are essential for splicing (BPS, 5′-ss and 3′-ss) can be targeted by an aptamer to control expression levels.

Gaur and co-workers inserted a theophylline aptamer into the 3′-ss of a pre-mRNA in vitro. The last nucleotide of the 3′-ss is crucial for the nucleophilic attack on the 5′-exon to form the spliced mRNA. The addition of theophylline to the reaction stabilizes the aptamer and therefore sequesters this last nucleotide of the 3′-ss (see Scheme 3 B). Thus, addition of the aptamer-specific ligand results in inhibition of splicing, and hence gene expression.[13] A similar approach was chosen by Suess and co-workers by introducing a theophylline aptamer into the 5′-ss of an intron of a reporter gene (see Scheme 3 C). Splicing and eventually gene expression is reduced in the presence, but unaffected in the absence of theophylline. Interestingly, the effectiveness of gene expression control can be further increased if theophylline aptamers are inserted in several



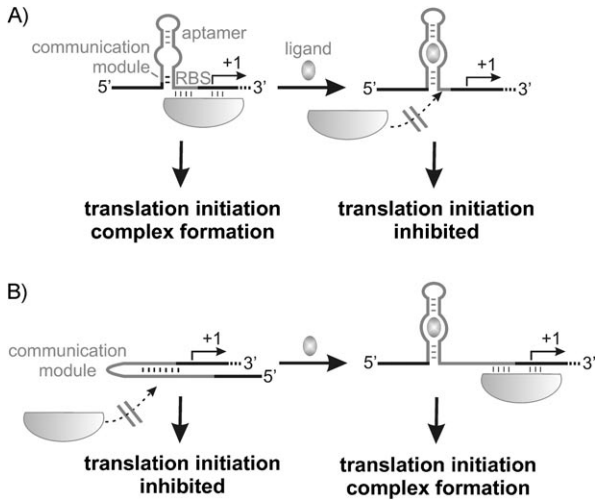
Scheme 3. Control of gene expression by inhibition of splicing in eukaryotes: A) Simplified splicing mechanism: Branching point sequence (BPS) performs nucleophilic attack on 5′-splice site (5′-ss) and releases exon **1**. Subsequently, exon **1** performs nucleophilic attack on 3′-splice site (3′-ss), which results in the joining of exons **1** and **2**. Splicing can be regulated by insertion of an aptamer into B) the 3′-ss,[13] C) the 5′-ss[14] or D) the BPS.[15] Blocking of essential elements for nucleophilic attack results in inhibition of splicing, eventually leading to reduced expression levels.

5′-ss of one gene.[14] Recently, a theophylline aptamer was placed around the BPS of an intron. This design is similar to the previous design of splicing regulation because the aptamer sequesters an essential sequence if theophylline is added; this results in inhibition of splicing (see Scheme 3 D). Furthermore, this design even allows, to a certain extent, the modulation of alternative splicing, which opens fascinating possibilities for synthetic biology applications.[15] In addition to controlling the spliceosome-mediated mRNA procession, Ellington and co-workers have rendered the group I intron ribozyme ligand dependent in bacteria. The group I intron RNA catalyzes a self-splicing reaction and was engineered to be controlled by theophylline upon insertion of the aptamer into the ribozyme.[16]

Aptamer-based RNA switches in bacteria

Although these ways of regulating gene expression by aptamers in eukaryotes are very impressive, transfer to bacterial systems is not feasible because the genetic mechanisms differ

strongly in eukaryotes and bacteria. Therefore, in bacteria other essential features of gene expression have to be targeted. Inspired by naturally occurring riboswitches, sequestering and liberating of the ribosomal binding site (RBS), which needs to be single stranded for a successful translation initiation seemed to be an appealing task. For that reason, a theophylline aptamer that was attached to a so-called communication module was inserted directly upstream of the RBS.[17] The communication module, which originates from an in-vitro-selected ligand-dependent ribozyme[18] facilitates a structural rearrangement of the RNA secondary structure upon theophylline binding, and eventually leads to a blocked RBS, and hence, reduced gene expression (see Scheme 4A).



Scheme 4. Aptamers can regulate gene expression in bacteria. For successful translation initiation, the ribosome binding site (RBS) has to be accessible for the small subunit of the ribosome. Ligands that interact with inserted aptamer sequences can sequester the RBS by either A) small structural changes (slipping mechanism)[17] or B) global changes. The communication module supports the structural rearrangement.[19,20]

Using a similar approach, Gallivan and co-workers placed a theophylline aptamer several nucleotides upstream of the RBS. In contrast to the above-described design by Suess and co-workers, gene expression is induced in the presence of theophylline by liberating the RBS upon ligand binding.[19] Subsequently, the nucleotides in between the aptamer and the RBS were randomized to screen in vivo for better activation ratios. By using this approach several clones were identified that show enhanced reporter gene expression levels.[20] Mechanistic studies revealed that global changes in the secondary structure are initiated upon theophylline binding, which resulted in liberation of the RBS (see Scheme 4B). Gallivan and co-workers nicely demonstrated the potential of such RNA switches by implementing it in recombinant strains of *Escherichia coli* that contain an impaired regulation of the chemotactic system.[21,22] Only if the bacteria encounter theophylline, do they start to move into random directions, otherwise they tumble in place. By placing the expression of cellular factors that regulate bacterial movement under control of the theophylline-dependent RNA switch, *E. coli* was enabled to trace tracks of the before unrecognized chemoattractant theophylline. The artificial theophylline-dependent movement of a bacterium was termed pseudotaxis.

Aptamer-mediated control of gene expression was also realized at the transcriptional level. The synthesis of subgenomic mRNAs of the Tomato bushy stunt virus (TBSV) seems to involve premature termination of transcription of the viral RNA genome.[23] An essential stem loop of the attenuation signal in the genome was replaced by the theophylline aptamer.[23] The folding stability of the aptamer-containing stem loop in the absence of theophylline is reduced compared to the stability of the original stem loop. As a consequence, the attenuation signal cannot form, and run-off transcripts are synthesized. In the presence of theophylline the attenuation signal is stabilized; this results in transcription termination. The small, terminated RNAs are crucial for the further production of the subgenomic mRNAs.[23] Similarly, the incorporation of the theophylline aptamer into the stem loop of a regulatory element of the tombus virus enabled Wang and White to switch replication of the virus by reconstituting the functional regulatory element upon ligand binding.[24]

Aptamer-based artificial riboswitches have been demonstrated to function in a variety of species, ranging from viral to prokaryotic as well as eukaryotic organisms. Although there are several known examples of these aptamer-based RNA switches, each has to be adapted to the genetic background of the specific organism. Because eukaryotes and prokaryotes differ significantly in their genetic mechanisms, to date there is no example of an RNA switch that can simply be transferred from one kingdom to another. In the next section we will discuss ribozyme-based switches that, in our opinion, show an increased potential for species-independent control of gene expression.

Ribozyme-based RNA switches

In analogy to enzymes, RNA sequences that catalyze reactions are called ribozymes. Naturally occurring ribozymes are—besides other important tasks—involved in gene regulation, and therefore represent a special class of natural riboswitches. The *glmS*-ribozyme was the first ligand-dependent regulator of gene expression to be discovered that acts via cleavage of the message. Upon binding of its specific ligand (the *glmS*-metabolite glucosamine-6-phosphate), autocatalytic cleavage of the mRNA is activated by glucosamine-6-phosphate acting as a cofactor rather than by inducing structural rearrangements.[25] The cleaved mRNA is then recognized by the specific ribonuclease RNase J1 and is subsequently degraded; this results in reduced gene expression.[26] Because all other known bacterial riboswitches control either transcription elongation or translation initiation, the *glmS* riboswitch mechanism is unique.

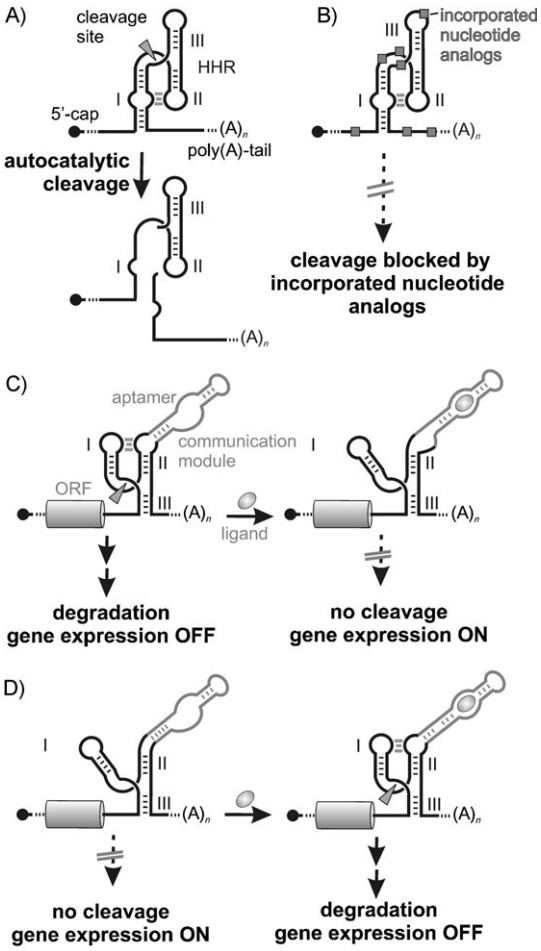
Phosphodiester-cleaving ribozymes such as the hammerhead motif (HHR) have been used for a long time to control gene expression, mostly by targeting mRNAs by *in trans* cleavage.[27] Since its first realization around ten years ago, there are several examples of ribozymes that have been engineered to cleave in a ligand-dependent fashion.[18,28,29] For this purpose,

the ribozymes were combined with a variety of aptamers in vitro. The activity of the resulting aptazymes can be controlled by the addition of the specific ligand of the aptamer. Such aptazymes have been used as molecular switches as well as sensors of molecular interactions.[18,28,29] However, the minimal hammerhead motifs that are frequently used for these studies required higher Mg^{2+} concentrations than are present under physiological conditions inside cells. Recent biochemical findings along with a crystallographic structure of a full-length HHR, which was resolved by Scott and co-workers clarified the importance of loop sequences that are distant from the catalytic core.[30–32] Surprisingly, it was found that tertiary interactions of stem I and stem II of the ribozyme significantly enhance its activity; this enables cleavage even at low, physiological Mg^{2+} concentrations.

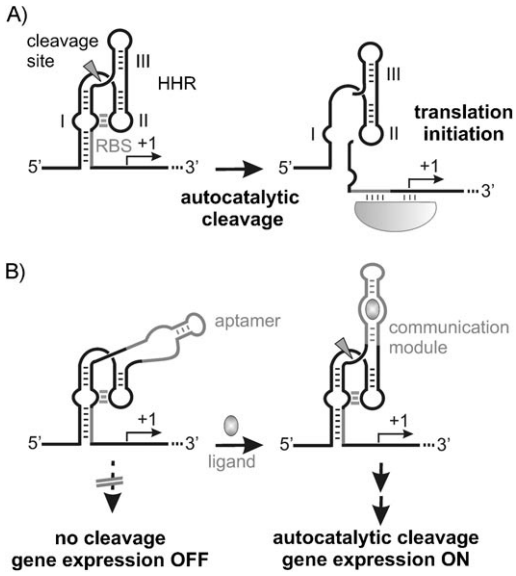
These important findings paved the way for a novel kind of regulators that are based on full-length hammerhead ribozymes. Mulligan and co-workers inserted a sequence-optimized version of the full-length HHR at different positions into eukaryotic mRNA, namely into the 5′-UTR, the 3′-UTR and an intronic region. Upon autocatalytic cleavage of the inserted HHR, the mRNA is sliced. The cleavage results in mRNA destabilization by degradation, which results in decreased gene expression (see Scheme 5 A). Interestingly, down-regulation is much more effective if the ribozyme is located in the 5′-UTR compared to the 3′-UTR or intron location. Also, gene expression can be inhibited even stronger by introducing two successive ribozymes instead of a single one.[33] To turn these HHRs into artificial riboswitches, nucleotide analogues were added; this resulted in a dose-dependent increase of gene expression levels. The underlying mechanism is based on the incorporation of the analogues into the mRNA, and consequently in the HHR sequence, whereby the cleavage activity is reduced (see Scheme 5 B).[34] However, the action of the nucleoside analogues is not ribozyme-specific and is likely to show cytotoxic side effects, especially in prokaryotes.

In order to realize a specific ligand–RNA interaction within the ribozyme context, the concept of long-known in-vitro-inducible aptazymes was evolved for regulation of gene expression in vivo by Smolke and co-workers, as well as by our group.[35,36] For this purpose, Win and Smolke inserted a HHR into the 3′-UTR of a eukaryotic reporter gene to ensure that regulation is based solely on mRNA cleavage, in contrast to the possible structural inhibition of translation initiation if inserted into the 5′-UTR. Subsequently, aptamers were attached to an extended stem II which likely results in control of the formation of tertiary interactions by ligand binding. Two opposite formats were realized, in the first one, gene expression is turned on upon ligand-dependent inhibition of the ribozyme, in the second one, gene expression shuts down by activating ribozyme-mediated mRNA cleavage (see Schemes 5 C and D). The modularity of the approach was demonstrated by realizing the concept with two different aptamers, namely for theophylline as well as for tetracycline.[35]

In contrast to eukaryotes, the stability of prokaryotic mRNA is not regulated by 5′-cap or 3′-poly(A) tail elements. In addition, transcription and translation are spatially as well as temporally coupled in bacteria. These circumstances do not allow for an easy transfer of an aptazyme-based RNA switch from eukaryotes to prokaryotes because messages that are cleaved in the 5′- or 3′-UTR are still efficiently translated. In order to develop an aptazyme-based RNA switch in prokaryotes, our group envisioned an advanced aptazyme design.[36] For this purpose, we incorporated the RBS into the ribozyme fold. As mentioned above, the RBS needs to be single stranded for efficient translation initiation. In our system, the RBS is part of an extended stem I of the HHR and initially sequestered, see Scheme 6 A). Upon autocatalytic cleavage, the RBS is liberated by dissociation of the two hammerhead fragments and translation proceeds. In contrast to the consequences of ribozyme cleavage in eukaryotes, activation of ribozyme catalysis in prokaryotes results in activation of gene expression. Ogawa and Maeda developed a similar design by incorporating the RBS into stem III of a theophylline-dependent minimal motif HHR aptazyme that was obtained by in vitro selection by Breaker



Scheme 5. A) Active HHRs that have been inserted into eukaryotic mRNA result in removal of mRNA-stabilizing elements (5′-cap or 3′-poly(A) tail) and eventually in degradation. Gene expression is inhibited. B) Gene expression can be recovered if nucleotide analogues are incorporated into the mRNA. This leads to nonspecific inhibition of the HHR activity.[33,34] C) and D) Specific HHR activity control can be obtained by attaching aptamers to stem II. Ligand binding thereby affects essential tertiary interactions and finally ribozyme activity.[35]



Scheme 6. Fast-cleaving HHRs as RNA switches in bacteria. A) The RBS is integrated into stem I of the HHR and therefore blocked. Only after self-cleavage of the HHR the RBS gets accessible for the ribosome and gene expression can occur. B) Replacing stem III with an aptamer that is connected by a communication module results in artificial, ligand-dependent control of gene expression. If the ligand is bound, the HHR adopts the active conformation and the RBS is freed by self-cleavage.[36]

and co-workers.[37,38] However, regulation of gene expression upon addition of theophylline in vivo is observed only at very high Mg^{2+} concentrations and low temperature, probably due to the missing tertiary interactions between stems I and II.[39] In contrast, our aptazyme design preserves the rate-enhancing tertiary interactions of stems I and II by attaching a theophylline aptamer to stem III of the HHR (see Scheme 6 B).

In order to search for sequences that enable ligand-dependent control of gene expression, we screened for optimized communication modules in vivo.[36] A major task in the construction of aptazyme-based riboswitches is the search for optimum connection sequences between the aptamer and the ribozyme. The communication module couples the event of ligand binding at the aptamer domain to changes of the catalytic rate of the ribozyme domain. Early communication modules for in-vitro-switchable aptazymes were obtained by inserting a randomized stretch of nucleotides between the ribozyme and aptamer sequences followed by in vitro selection for switchable sequences.[18] On the other hand, recent results suggest that in-vitro-selected, fast-cleaving aptazymes cannot be simply transferred to an in vivo system.[40] However, in the rational aptazyme approach discussed above[35] it was possible to construct aptazyme-based switches by using communication modules that were previously selected in vitro.

In order to construct HHR-based switches that function in bacteria, we decided to search for new communication modules by screening for changes of reporter gene expression in the presence and absence of the effector molecule in vivo.[36] The evident advantage of this method in contrast to in vitro selection protocols is that all selected clones function as RNA switches in vivo. On the other hand, the maximum sequence space that can be searched in such pools in vivo is certainly much smaller compared to in vitro selection techniques. A method that was developed by Gallivan and co-workers could help to overcome this problem: the above-mentioned system of controlling the movement of bacteria in a ligand-dependent fashion can be used to select new artificial riboswitches.[21] Within such a selection setup, clones that covered a long distance in the presence of a specific effector and didn't move in the absence of the effector; this resulted in the discovery of activating RNA switches and vice versa.[21] In addition, in vivo selection protocols that are based on counter-selectable markers could provide even more powerful means to realize RNA-based switches in living cells. In a first example, Nomura and Yokobayashi used the *tetA* gene, which provides resistance to tetracycline but can be counterselected due to an increased sensitivity to Ni^{2+}. By using this in vivo selection strategy, it was possible to invert the reactivity of a natural *thiM* riboswitch to get activated instead of inhibited gene expression in the presence of thiamine.[41,42]

Temperature-sensitive mRNA switches

In contrast to other riboswitches, thermoresponsive switches are not dependent on a chemical but on a physical input, namely changes in temperature. Natural devices that are found in bacteria are based on an elegant but simple mechanism where the RBS is sequestered by a secondary hairpin structure, which is stable only at lower temperatures. Increasing temperature destabilizes the secondary structure and allows translation to be initiated. Most prominently, expression of heat shock and virulence genes in bacteria is regulated in this manner.[43,44] Imitating this concept, we inserted several G-quadruplexes (GQPs) with different stabilities around the RBS. GQPs are guanosine-rich nucleic acid sequences that can fold into stable, four-stranded structures. The stability of the quadruplexes that mask the RBS can be fine-tuned and thereby represents a novel means of modulating gene expression by masking the RBS. By doing so, we were able to identify artificial, thermo-responsive sequences based on the formation of quadruplexes that inhibit translation initiation.[45]

Conclusions

While naturally occurring riboswitches have implications for early regulatory networks within the context of a hypothetical RNA world, artificial riboswitches contribute to the emerging field of synthetic biology. The presented strategies should provide valuable tools for implementing complex regulatory functions even when only minimal knowledge about the genetic apparatus of less well characterized species is available. In this respect, especially ribozyme-based strategies seem to have the potential to act in many different organisms because cleavage of the message results in irreversible changes. Such a drastic processing is likely to interfere with gene expression regardless of the species-specific genetic mechanisms. A successful example is represented by the implementation of fast-cleaving hammerhead ribozymes for the regulation of gene expression in

the apicomplexan parasites *Toxoplasma gondii* and *Plasmodium falciparum*.[46] In addition, artificial riboswitches should be useful in approaches that aim at implementing novel functions in minimal organisms because no additional proteins are needed to control expression via external stimuli. The first steps of successful synthetic biology applications are represented by the implementation of artificial chemotaxis,[21] (termed pseudotaxis) as well as switching virus replication[24] by using artificial, RNA-based switches. In well-balanced contexts it might be possible to control global expression sets by RNAs acting on their own as well as other transcripts; this diminishes the need for most regulatory protein factors. Furthermore, RNA-only mechanisms could have the advantage of shorter onset times of the wanted effects since they represent a short-cut to protein-based control mechanisms.

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Keywords: aptamers · gene expression · ribozymes · RNA interactions · synthetic biology

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