Allosteric aptamers and aptazymes as probes for screening approaches

Michael Famulok

Address
Rheinische Friedrich-Wilhelms-Universität Bonn
Kekulé Institut für Organische Chemie und Biochemie
Gerhard-Domagk-Strasse 1
Bonn 53121
Germany
Email: m.famulok@uni-bonn.de

Current Opinion in Molecular Therapeutics 2005 7(2):137-143
 ISSN 1464-8431

Substantial effort is currently being devoted to engineering allosteric nucleic acids, aptamers and ribozymes for various applications in cellular and molecular biology, biotechnology and diagnostics. These molecular switches alter their different functional activities in response to specific binding molecules, including proteins, nucleic acids and small organic compounds. The interacting molecules trigger a response in the allosteric nucleic acid, which can be used for purposes such as real-time monitoring, high-throughput screening or gene expression control.

Keywords Allosteric ribozymes, aptamers, high-throughput screening, synthetic riboswitches

Introduction

Nucleic acids are increasingly being applied as tools to detect biomolecules or biomolecular interactions. The advantage of nucleic acids compared with protein-based technologies is that specific ligand-binding single-stranded DNAs, RNAs or chemically modified oligonucleotides can be easily obtained by evolutionary selection methods. These functional nucleic acids, also known as aptamers, are nucleic acid-based receptor molecules that are isolated from complex mixtures of trillions of nucleic acid sequences, entirely *in vitro*. Aptamers can bind to nearly every class of ligand molecule with high specificity and affinity. Moreover, functional aptamers can be readily adapted *in vitro* to meet certain criteria, such as increasing stability, affinity and specificity, or traceability. In addition, aptamers can be chemically synthesized in large quantities. These properties make aptamers suitable for a variety of applications that require the reliable, specific and sensitive detection of target compounds. Recently, novel methods based on functional nucleic acids, such as ribozymes and aptamers, were developed and applied for various purposes. At the heart of these methods are effector-regulated ribozymes, also known as aptazymes, reporter ribozymes or ribo-reporters. Aptazymes are chimeric molecules that consist of an aptamer domain and a ribozyme module. Just like allosteric enzymes, their catalytic activity is regulated by binding of a small ligand or protein to the aptameric domain of the aptazyme, distant from the active site.

The basis for this concept lies in an intrinsic property of aptamers to form ligand complexes, specifically adaptive binding, which involves different conformational ordering processes. Since the first nuclear magnetic resonance structures of aptamer small-molecule complexes were elucidated, it became clear that the complex formation between a ligand and an aptamer normally occurs by adaptive recognition. For example, aptamers often contain unpaired loop or bulge regions, which are disarranged in the free nucleic acid and achieve a defined conformation by adaptive folding around the ligand. As a consequence, binding of a ligand to its aptamer stabilizes folding at the binding site, reinforcing the stability of adjacent helical domains. When connected to a weakened but essential helix of a ribozyme or sensor module, ligand binding by the aptamer induces helix stabilization and triggering occurs only in the presence of the corresponding ligand.

There are several strategies for the construction of aptazymes [1]. The rational approach, by fusing a known aptamer to a ribozyme, was first shown for the hammerhead ribozyme and an ATP-binding aptamer, which led to an aptazyme regulated by ATP [2,3]. This strategy necessitates detailed knowledge of the secondary and tertiary structures of the RNA molecules to be fused. A critical issue is the design of the joint between ribozyme and aptamer, the communication module, which mediates the binding of the ligand from the aptamer to the ribozyme. A method to optimize the communication between aptamer and ribozyme is to fuse the two motifs via a randomized region and to evolve the optimal communication module by *in vitro* selection methods [4]. Considerably versatile RNA elements that function as communication modules, rendering disparate RNA folding domains interdependent, have been described [5]. Communication modules can easily be applied to engineer aptazymes with new ligand specificities. For example, when an aptazyme regulated by a flavin mononucleotide (FMN) aptamer module [6] was replaced with previously identified ATP- and theophylline-binding aptamer motifs [7,8], new aptazymes regulated by ATP and theophylline, respectively, were obtained [4]. An alternative option is the direct evolution of a new aptameric domain attached to a known ribozyme. This is useful in cases in which no aptamer is available for a given ligand. In this strategy, termed 'allosteric selection', aptamer and communication module are evolved together using selection schemes favoring the emergence of ribozymes that self-cleave only in the presence of their corresponding effector compounds [9-11].

Regulation of aptazymes by small molecules

The aptazyme approach was further broadened by applying other catalytic RNAs or different effector molecules. An interesting approach involved the engineering of allosteric ligase ribozymes that had been previously isolated by *in vitro* selection [12]. In these ribozymes, the equilibrium between conformers strongly depends on the presence of ATP, the ligand that binds to the regulatory aptamer domain. The ribozyme thus only generates a novel oligonucleotide template by ligation in the presence of ATP. These new oligonucleotides could be amplified by RT-PCR, providing an interesting example of how a signal can be amplified when triggered by a small molecule. In a follow-up study, allosteric ligase ribozymes were constructed that were switched on by adenosine or theophylline by appending the corresponding aptamers [7,8] to the core motif of the ligase ribozyme [13]. When the FMN aptamer

[6] was used instead of the other two motifs, response to flavine was minimal. After randomization of the joining region between the aptamer and the ribozyme, a series of selection steps yielded 'communication modules' that could render the FMN aptamer and the ribozyme interdependent, resulting in up to 260-fold activation, triggered by FMN. To engineer an aptazyme, group I self-splicing introns were also used as the catalytic partner for the theophylline aptamer. The self-splicing activity of the group I aptazyme was specifically controlled by exogenously added theophylline *in vivo*. In accordance with the specificity exhibited by the aptamer module, caffeine, which differs from theophylline by only a single methyl group, was inactive as a small-molecule regulator of this aptazyme [14].

Synthetic DNAzymes were also applied as catalytic modules to construct RNA-cleaving DNA aptazymes whose catalytic activity can be controlled by the binding of activator molecules. Variants of the RNA-cleaving 8-17 DNAzyme [15-18] and 10-23 DNAzyme [17] were created, and their catalytic activity improved up to approximately 35-fold by the binding of the effector adenosine [19]. In addition, aptazymes based on the hammerhead ribozyme were constructed in the same study to respond to the effectors ATP and FMN [19]. In this approach, activation of the aptazymes by the effector molecules was achieved by stabilizing the enzyme-substrate complex rather than by modulating the chemical step of catalysis, a mechanism exerted by most of the aptazymes discussed above. As the researchers noted, their approach to controlling the activity of DNAzymes or ribozymes requires no prior knowledge of the enzyme's secondary or tertiary folding if substrate recognition occurs by Watson-Crick pairing. This regulatory strategy should be generally applicable to any RNA-cleaving ribozyme or DNAzyme.

The same DNA aptazyme was used to direct the assembly of gold nanoparticles to generate colorimetric biosensors that detect the analyte adenosine. The basis for this sensor principle is that the aptazyme is inactive in the absence of adenosine. In this case, the uncleaved substrate strands can act as linkers for DNA-activated gold nanoparticles to assemble into blue aggregates. When the aptazyme becomes active in the presence of adenosine, the substrate strand is cleaved and the nanoparticle aggregates disassemble, resulting in a red color [20•]. A similar strategy was used to construct aptazyme-sensors for Pb(II)-detection [21].

In another example, a non-catalytic, aptamer-based allosteric system can detect the presence of a low-molecular-weight analyte [22•]. The reporter is based on a short synthetic RNA sequence that specifically recognizes the triphenylmethane dye malachite green (MG). This dye is non-fluorescent in solution, but in complex with the aptamer, the fluorescence of MG increases 2360-fold [23•]. When fused to an aptamer sequence (which serves as the receptor domain for a small molecule to be detected) via an appropriate communication module, the MG-aptamer module can only bind to the dye, and hence generate a fluorescence signal, if a ligand is bound. These reporters represent an all-RNA equivalent of green fluorescent protein or luciferase with the advantage that a covalent attachment to a protein is not necessary and the effector molecules are extremely small.

Allosteric ribozymes can also report the presence of oligonucleotides [12,24-26]; these include extremely sensitive probes for viral nucleic acids, which are compatible with highly parallel detection formats [25,26]. Another example describes probes for microRNAs (miRNAs), an important class of small regulatory RNAs. Reporter ribozymes for the sensitive and sequence-specific detection of various miRNAs were engineered by introducing a binding domain specific for the miRNA into the hairpin ribozyme [27]. When the domain is bound by the cognate miRNA, the reporter ribozyme undergoes conformational changes enabling it to cleave a substrate oligonucleotide, which has a fluorophore at one end and a quencher at the other. Fluorescence is detected only when the ribozyme is active. Nine reporter ribozymes were designed that were activated by different miRNAs. Each of them detected its cognate miRNA reliably and sensitively in a mix of other miRNA sequences. A concentration of 5 nM of the cognate microRNA could be detected, making this system superior to molecular beacons [28,29]. Like the MG-aptamer-based system [23•], reporter ribozymes are entirely RNA-based and thus could be expressed endogenously, requiring only the addition of the short substrate oligonucleotide. These probes will be useful for the direct detection of minute amounts of nucleic acids, although for intracellular applications, further enhancement of the stability of the modified substrate oligonucleotide will be necessary.

Regulation of aptazymes by proteins

The ligase ribozyme was also used to obtain a protein-dependent aptazyme by *in vitro* selection [12]. The ligase was attached to a 50-nucleotide randomized region, and aptazyme variants that required either a tyrosyl tRNA synthetase from *Neurospora crassa* mitochondria, or hen egg white lysozyme as protein co-factors for ligase activity were selected [30]. In a second approach, the same ligase library was used to isolate HIV-1 Rev-dependent aptazyme ligases by *in vitro* selection [31]. A ligase aptazyme was isolated that was activated by > 18,000-fold by the arginine-rich motif (ARM) peptide epitope of the HIV-1 Rev protein without responding to other ARMs, except for the closely related ARM of the HIV-1 Tat protein. Interestingly, these protein-dependent aptazymes did not closely resemble natural or selected sequences or secondary structures that bind either the HIV-1 Rev ARM peptide or the Rev protein.

In a further example, *in vitro* and *in vivo* selection were applied to isolate new arginine-rich RNA-binding motifs and corresponding peptide-binding motifs in RNA, which replace the Rev protein and its target RNA motif, the Rev-responsive element (RRE), respectively [32]. The catalytic domain consisted of M12 RNA, a derivative of the Tetrahymena group I intron. Both pep S, a designed arginine-rich RNA-binding motif [33], and its target RNA motif in the aptazyme were substituted with randomized sequences and used for the selection experiments. Newly established RNA-peptide interactions effectively regulated splicing both *in vivo* and *in vitro*. The affinities between the peptide motifs and the corresponding RNA motifs were comparable to that between the Rev and RRE [32]. In this manner, alternative forms of self-splicing intron RNA were obtained whose activities were regulated by a protein component.

Another class of protein-regulated aptazymes was obtained by constructing hammerhead ribozymes activated by protein kinases [34•]. Two different aptazymes were constructed, one regulated by the unphosphorylated MAP-kinase ERK2, and the other triggered by the phosphorylated version. Using a fluorophore-carrying nuclease-stabilized oligonucleotide substrate, the aptazyme quantitatively detected unphosphorylated ERK2 in a multiplexed solution-phase assay. Thus, these protein detection reagents can be applied to monitor the activation state of signal transduction components.

Reporter ribozymes for screening purposes

These results show that aptazymes are powerful reagents for the specific detection of proteins. Beyond that, it would be extremely valuable if these reporters could also be used to identify the interaction partners of a target protein. This might be particularly useful in cases in which an aptamer has been selected for a given target protein and its inhibitory activity has been verified. In such cases, aptazymes could be set up for applications in screening assays that search small-molecule libraries for lead compounds that displace the aptamer from its target and adopt its inhibitory activity. This would allow direct translation of information stored within an aptamer into a small molecule, which likely will be an inhibitor itself (Figure 1A).

This issue was addressed by constructing hammerhead ribozyme-based reporter ribozymes that are either active or inactive upon binding to the HIV-1 Rev protein or its ARM peptide epitope [35•]. A similar setting was used for reporting the interaction of HIV-1 reverse transcriptase with its specific pseudoknot aptamer [36]. The Rev-responsive reporter-ribozymes were used to screen a 96-sample library of antibiotics for molecules that could disrupt the interaction between Rev and its cognate RNA. Substrate RNA oligonucleotides labeled with a fluorescence donor at the 5'-end and a quencher molecule at the 3'-end, that emit a fluorescence signal only after cleavage by the ribozyme, were used for monitoring the activity of the reporter ribozymes [37,38]. This kind of reaction setup allows the efficient real-time analysis of aptazyme performance as a function of protein binding to its cognate RNA module. The screen identified the gyrase inhibitor coumermycin A1 as a hit that bound Rev with a K_d value of 7.5 μM and inhibited HIV-1 virus replication in a concentration-dependent manner. Thus, the small molecule possesses the same characteristics as the aptamer from which it was derived. This study established that it is possible to identify novel small-molecule inhibitors for a given protein using interference with RNA/protein interactions as a basis for screening (Figure 1B).

The same approach was expanded to using hairpin ribozyme variants regulated by α-thrombin [35•]. These constructs contained sequences complementary to the anti-thrombin aptamer [39]. Upon addition of the aptamer sequence, the aptazymes become specifically inactivated. α-Thrombin sequesters the hybridized aptamer, inducing proper folding of the ribozyme into its active conformation, which results in substrate cleavage and, therefore, generation of a fluorescent signal. In the presence of hirudin, an oligopeptide that binds thrombin and competes with aptamer binding, the signal was reverted, demonstrating that the aptazymes can report the thrombin-hirudin interaction and thus provide an effective tool for the *in vitro* analysis of protein-protein interactions.

Additional modularity of reporter ribozymes was achieved in hairpin ribozyme variants that can be induced or repressed by external oligonucleotides. A new domain was incorporated into the ribozymes to form alternate stable RNA motifs, allowing directed switching of cleavage activity; the same effector oligonucleotide can either serve as an inducer or repressor [40]. These hairpin ribozyme derivatives were engineered for detecting trp leader mRNA, the RNA sequence tightly bound by L-tryptophan-activated trp-RNA-binding attenuation protein (TRAP) of *Bacillus subtilis*. When the amending domain is complementary to this mRNA, ribozyme activity can be switched by hybridizing to the trp leader mRNA, then specifically reverted by the TRAP-L-tryptophan complex via sequestration. These reporter ribozymes can thus sense the activity status of a protein controlled by its metabolite molecule and could potentially be applied for the screening of TRAP-binding small molecules.

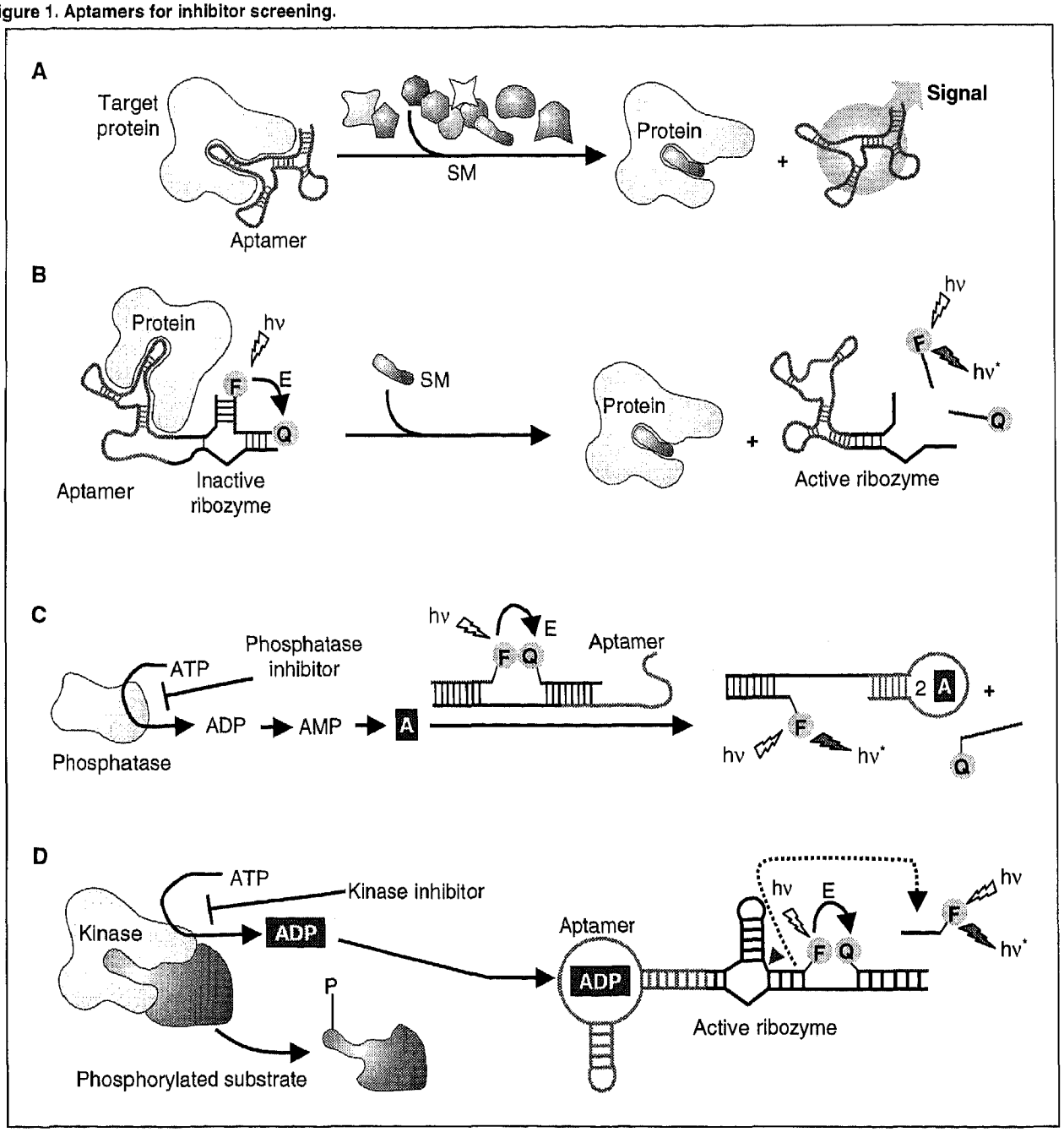
Nutiu *et al* also examined allosteric aptamers as real-time reporters for small-molecule metabolites to monitor enzyme activities [41•]. They used an allosteric DNA aptamer [42] that binds adenosine better than 5'-adenosine monophosphate (AMP) as fluorescence reporter for quantifying the yields of the alkaline phosphatase (ALP)-catalyzed cleavage of AMP to adenosine in real time (Figure 1C). The aptamer reporters were used as probes, compatible with high-throughput technology, for the indirect identification of known small-molecule inhibitors of ALP.

Conversely, aptamer-based sensors, or ribo-reporters that also discriminate between closely related adenosine derivatives, namely adenosine diphosphate (ADP) and ATP, were applied to monitor protein kinase activities [43•]. As in the studies described above [35•,36,40], detection relies on fluorescence and quencher-labeled oligonucleotides and their cleavage by an aptazyme. Again, these reporters are potentially compatible with high-throughput approaches, as demonstrated by the re-identification of previously known kinase inhibitors in a proof-of-concept study (Figure 1D).

By employing three different fluorophores, two on the RNA and one on the protein, Williamson *et al* were able to monitor conformational changes that occur in the central domain of 16S rRNA upon binding to its ribosomal target protein S15, and the binding of S15 itself, in a single assay [44]. This system was used to identify members of a 34-compound library that bound to the three-way junction of the 16S rRNA central domain. While some compounds bound and promoted the folding of this RNA domain, none of them interfered with complexation of the S15 protein.

These approaches show that reporter ribozymes have three distinct advantages over many other assays or sensors, detection occurs in real time, none of the actual reaction partners needs to be labeled, and the format is highly modular and can be configured for any kind of protein for which aptamers can be selected or for which a natural RNA target is available.

Figure 1. Aptamers for inhibitor screening.



(**A**) Illustration of how a functional aptamer-target complex might be used to develop screening assays that allow identification of small molecules (SMs) that displace the aptamer from the target, resulting in a signal. (**B**) Reporter ribozymes for high-throughput screening are shown. If a small molecule can compete with a protein/RNA interaction, the ribozyme becomes active and cleaves a substrate labeled with a fluorescence tag (**F**) and a quencher (Q). In the uncleaved state, the substrate has low fluorescence quantum yield, due to fluorescence resonance energy transfer. In the cleaved state, the two product oligonucleotides rapidly dissociate off the ribozyme, resulting in a fluorescence signal (hν*). These reporter ribozymes were used to identify a novel small-molecule inhibitor for the HIV-1 Rev protein. (**C**) Signaling aptamers for monitoring enzyme activity are illustrated. The DNA aptamer reports the presence of two molecules of adenosine (A), generated by dephosphorylation of ATP, ADP or AMP with alkaline phosphatase. In the presence of A, the aptamer folds so that the Q-labeled oligonucleotide can no longer bind to the signaling aptamer construct. In the absence of A, the signaling aptamer has low fluorescence quantum yield, resulting from the close proximity of the hybridized F- and Q-labeled DNA. These probes can report known inhibitors of alkaline phosphatase. (**D**) ADP-specific ribo-reporters are shown. ADP is generated by a kinase from an ATP co-factor and specifically bound by the ADP aptamer module, which renders the ribozyme module active to cleave off the F-labeled product oligonucleotide (the cleavage site is indicated by a triangle). In the inactive form in the absence of ADP, the ribo-reporter has a low fluorescence quantum yield because of the close proximity of the F-label to a Q-labeled DNA. After cleavage, the F-labeled product dissociates off the ribozyme (dotted arrow) emitting fluorescence, hν*. **E** energy.

Synthetic riboswitches

Recently, a novel class of bacterial mRNA elements, called riboswitches, were described that contain allosteric aptamer domains that regulate the expression of metabolic genes and are controlled by small-molecule regulators [45,46]. These naturally occurring aptamers bind highly selectively to small molecules, which induce conformational changes in the RNA that interfere with transcription or translation.

Prior to the discovery of riboswitches in nature, the underlying principle had already been realized in artificial systems. By introducing aptamers that bind to Hoechst dyes H33258 and H33342 into the 5'-UTR of a β-galactosidase reporter, small molecule-mediated repression of translation was achieved in mammalian cells [47]. Translational control elements based on tetracycline- and theophylline-binding aptamers have been realized recently in yeast [48-50]. In these studies, the potential of aptamer-ligand interactions that suppress translation was demonstrated using reporter genes. In contrast, Grate and Wilson inserted the MG aptamer into the 5'-UTR of a cyclin transcript in *Saccharomyces cerevisiae*, thereby rendering the cell-cycle control responsive to the triphenylmethane dye [51]. These approaches of inserting small-molecule-binding aptamers into transcripts represent an interesting way to specifically regulate gene expression on the translational level.

An exogenous control system for regulating gene expression in several mammalian cell lines was developed by Mulligan and colleagues [52•]. The researchers positioned a modified version of the Sm1 hammerhead ribozyme from the trematode *Schistosoma mansoni*, termed N79, into different untranslated regions upstream and downstream of a *LacZ* reporter gene. The constitutive cleavage activity of the ribozyme represses gene expression. However, interfering with the self-cleavage activity of N79 by adding either an oligonucleotide complementary to the ribozyme or a small-molecule inhibitor of ribozyme activity, switches gene expression on. Thus, the oligonucleotide or the small molecule may serve as broadly applicable tailor-made inducers of gene expression that even function in mice. Together with other methods, such as intramers or aptazymes [53•,54], it may be possible to tailor gene-regulation systems to respond to virtually any small molecule or metabolite.

Desai and Gallivan demonstrated that it is possible to use *in vitro*-selected aptamer sequences to create strains of *Escherichia coli* that activate protein translation only in the presence of the cognate small molecule [55]. They transformed the theophylline aptamer [7] into a synthetic riboswitch by subcloning it five basepairs upstream of the ribosome binding site of a β-galactosidase reporter gene that allowed accurate measurement of small changes in β-galactosidase activity in response to theophylline binding. Interestingly, cells that harbored the aptamer could be specifically amplified from a pool containing a 10^6-fold excess of cells expressing a mutant aptamer that does not respond to theophylline. This suggests that this method might be further used to perform *in vivo* selections for aptamers with riboswitch activities that respond to closely related small molecules from a pool of mutant sequences.

Finally, Buskirk *et al* [56•] created a synthetic genetic switch for small-molecule-induced transcription activation by combining the malachite green (MG) aptamer [57] with an RNA-based transcriptional activation domain [58]. The RNA domain for transcriptional activation displayed 10-fold increased activity when the cell-permeable MG was present.

Conclusion

All these studies demonstrate that small-molecule-dependent regulators can be engineered that have the potential to respond to any kind of ligand for which an aptamer can be selected. These systems function well in living cells, and thus should have broad utility in diverse settings in which time-resolved, ligand-induced gene expression is desired.

References

•• of outstanding interest
• of special interest

1. Soukup GA, Breaker RR: **Nucleic acid molecular switches.** *Trends Biotechnol* (1999) **17**(12):469-476.
2. Tang J, Breaker RR: **Rational design of allosteric ribozymes.** *Chem Biol* (1997) **4**(6):453-459.
3. Tang J, Breaker RR: **Mechanism for allosteric inhibition of an ATP-sensitive ribozyme.** *Nucleic Acids Res* (1998) **26**(18):4214-4221.
4. Soukup GA, Breaker RR: **Engineering precision RNA molecular switches.** *Proc Natl Acad Sci USA* (1999) **96**(7):3584-3589.
5. Kertsburg A, Soukup GA: **A versatile communication module for controlling RNA folding and catalysis.** *Nucleic Acids Res* (2002) **30**(21):4599-4606.
6. Burgstaller P, Famulok M: **Isolation of RNA aptamers for biological cofactors by *in vitro* selection.** *Angew Chem Int Ed* (1994) **33**(10):1084-1087.
7. Jenison RD, Gill SC, Pardi A, Polisky B: **High-resolution molecular discrimination by RNA.** *Science* (1994) **263**(5152):1425-1429.
8. Sassanfar M, Szostak JW: **An RNA motif that binds ATP.** *Nature* (1993) **364**(6437):550-553.
9. Koizumi M, Soukup GA, Kerr JN, Breaker RR: **Allosteric selection of ribozymes that respond to the second messengers cGMP and cAMP.** *Nat Struct Biol* (1999) **6**(11):1062-1071.
10. Piganeau N, Jenne A, Thuillier V, Famulok M: **An allosteric ribozyme regulated by doxycycline.** *Angew Chem Int Ed* (2000) **39**(23):4369-4373.
11. Piganeau N, Thuillier V, Famulok M: ***In vitro* selection of allosteric ribozymes: Theory and experimental validation.** *J Mol Biol* (2001) **312**(5):1177-1190.
12. Robertson MP, Ellington AD: ***In vitro* selection of an allosteric ribozyme that transduces analytes to amplicons.** *Nat Biotechnol* (1999) **17**(1):62-66.
13. Robertson MP, Ellington AD: **Design and optimization of effector-activated ribozyme ligases.** *Nucleic Acids Res* (2000) **28**(8):1751-1759.
14. Thompson KM, Syrett HA, Knudsen SM, Ellington AD: **Group I aptazymes as genetic regulatory switches.** *BMC Biotechnol* (2002) **2**(1):21.
15. Faulhammer D, Famulok M: **The Ca^{2+} ion as a cofactor for a novel RNA-cleaving deoxyribozyme.** *Angew Chem Int Ed* (1996) **35**(23-24):2837-2841.

16. Faulhammer D, Famulok M: **Characterization and divalent metal-ion dependence of *in vitro* selected deoxyribozymes which cleave DNA/RNA chimeric oligonucleotides.** *J Mol Biol* (1997) **269**(2):188-202.

17. Santoro SW, Joyce GF: **A general purpose RNA-cleaving DNA enzyme.** *Proc Natl Acad Sci USA* (1997) **94**(9):4262-4266.

18. Peracchi A: **Preferential activation of the 8-17 deoxyribozyme by Ca^{2+} ions. Evidence for the identity of 8-17 with the catalytic domain of the Mg5 deoxyribozyme.** *J Biol Chem* (2000) **275**(16):11693-11697.

19. Wang DY, Lai BH, Sen D: **A general strategy for effector-mediated control of RNA-cleaving ribozymes and DNA enzymes.** *J Mol Biol* (2002) **318**(1):33-43.

20. Liu J, Lu Y: **Adenosine-dependent assembly of aptazyme-functionalized gold nanoparticles and its application as a colorimetric biosensor.** *Anal Chem* (2004) **76**(6):1627-1632.

• *This paper describes a colorimetric biosensor based on aptazyme-directed assembly of gold nanoparticles that change from blue to red in the presence of the analyte adenosine. The beauty of this sensor is its simplicity, the color difference can be observed with the naked eye by spotting the resulting sensor solution onto an alumina TLC plate.*

21. Liu J, Lu Y: **A colorimetric lead biosensor using DNAzyme-directed assembly of gold nanoparticles.** *J Am Chem Soc* (2003) **125**(22):6642-6643.

22. Stojanovic MN, Kolpashchikov DM: **Modular aptameric sensors.** *J Am Chem Soc* (2004) **126**(30):9266-9270.

• *An entirely RNA-based probe is described that can detect small organic molecules by fluorescence signals, with no requirement for labeling. This probe potentially requires only the exogenous addition of a simple dye molecule to the growth medium to become an intracellular sensor.*

23. Babendure JR, Adams SR, Tsien RY: **Aptamers switch on fluorescence of triphenylmethane dyes.** *J Am Chem Soc* (2003) **125**(48):14716-14717.

• *This paper provides the basis for the detailed study in reference [22•].*

24. Vauleon S, Muller S: **External regulation of hairpin ribozyme activity by an oligonucleotide effector.** *Chembiochem* (2003) **4**(2-3):220-224.

25. Vaish NK, Jadhav VR, Kossen K, Pasko C, Andrews LE, McSwiggen JA, Polisky B, Seiwert SD: **Zeptomole detection of a viral nucleic acid using a target-activated ribozyme.** *RNA* (2003) **9**(9):1058-1072.

26. Kossen K, Vaish NK, Jadhav VR, Pasko C, Wang H, Jenison R, McSwiggen JA, Polisky B, Seiwert SD: **High-throughput ribozyme-based assays for detection of viral nucleic acids.** *Chem Biol* (2004) 11(6):807-815.

27. Hartig JS, Grune I, Najafi-Shoushtari SH, Famulok M: **Sequence-specific detection of microRNAs by signal-amplifying ribozymes.** *J Am Chem Soc* (2004) **126**(3):722-723.

28. Fang X, Mi Y, Li JJ, Beck T, Schuster S, Tan W: **Molecular beacons: Fluorogenic probes for living cell study.** *Cell Biochem Biophys* (2002) **37**(2):71-81.

29. Tsourkas A, Behlke MA, Rose SD, Bao G: **Hybridization kinetics and thermodynamics of molecular beacons.** *Nucleic Acids Res* (2003) **31**(4):1319-1330.

30. Robertson MP, Ellington AD: ***In vitro* selection of nucleoprotein enzymes.** *Nat Biotechnol* (2001) **19**(7):650-655.

31. Robertson MP, Knudsen SM, Ellington AD: ***In vitro* selection of ribozymes dependent on peptides for activity.** *RNA* (2004) **10**(1):114-127.

32. Atsumi S, Ikawa Y, Shiraishi H, Inoue T: **Selections for constituting new RNA-protein interactions in catalytic RNP.** *Nucleic Acids Res* (2003) **31**(2):661-669.

33. Atsumi S, Ikawa Y, Shiraishi H, Inoue T: **Design and development of a catalytic ribonucleoprotein.** *EMBO J* (2001) **20**(19):5453-5460.

34. Vaish NK, Dong F, Andrews L, Schweppe RE, Ahn NG, Blatt L, Seiwert SD: **Monitoring post-translational modification of proteins with allosteric ribozymes.** *Nat Biotechnol* (2002) **20**(8):810-815.

• *This paper describes a generally applicable ribozyme-based approach for monitoring post-translational modifications in proteins.*

35. Hartig JS, Najafi-Shoushtari SH, Grune I, Yan A, Ellington AD, Famulok M: **Protein-dependent ribozymes report molecular interactions in real time.** *Nat Biotechnol* (2002) **20**(7):717-722.

• *This paper uses allosteric ribozymes to monitor the presence and concentration of molecules that modulate protein function. Reporter ribozymes are used for the screening of a small molecule that disrupts binding of the HIV-1 Rev protein to its nucleic acid target, the Rev-responsive element, and for monitoring protein-protein interactions.*

36. Hartig JS, Famulok M: **Reporter ribozymes for real-time analysis of domain-specific interactions in biomolecules: HIV-1 reverse transcriptase and the primer-template complex.** *Angew Chem Int Ed Engl* (2002) **41**(22):4263-4266.

37. Jenne A, Gmelin W, Raffler N, Famulok M: **Real-time characterization of ribozymes by fluorecence resonance energy transfer (FRET).** *Angew Chem Int Ed* (1999) **38**(9):1300-1303.

38. Jenne A, Hartig JS, Piganeau N, Tauer A, Samarsky DA, Green MR, Davies J, Famulok M: **Rapid identification and characterization of hammerhead-ribozyme inhibitors using fluorescence-based technology.** *Nat Biotechnol* (2001) **19**(1):56-61.

39. Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ: **Selection of single-stranded DNA molecules that bind and inhibit human thrombin.** *Nature* (1992) **355**(6360):564-566.

40. Najafi-Shoushtari SH, Mayer G, Famulok M: **Sensing complex regulatory networks by conformationally controlled hairpin ribozymes.** *Nucleic Acids Res* (2004) **32**(10):3212-3219.

41. Nutiu R, Yu JM, Li Y: **Signaling aptamers for monitoring enzymatic activity and for inhibitor screening.** *Chembiochem* (2004) **5**(8):1139-1144.

• *In this elegant report, allosteric aptamers were engineered as fluorescent reagents for reporting the progress of an enzyme-catalyzed chemical reaction in real-time. There is also potential to use this principle for inhibitor screening.*

42. Nutiu R, Li Y: **Structure-switching signaling aptamers.** *J Am Chem Soc* (2003) **125**(16):4771-4778.

43. Srinivasan. J, Cload ST, Hamaguchi N, Kurz J, Keene S, Kurz M, Boomer RM, Blanchard J, Epstein D, Wilson C, Diener JL: **ADP-specific sensors enable universal assay of protein kinase activity.** *Chem Biol* (2004) **11**(4):499-508.

• *This paper is also worthy because, as in references [35•,41•], allosteric ribozymes have demonstrated potential for screening protein inhibitors.*

44. Klostermeier D, Sears P, Wong CH, Millar DP, Williamson JR: **A three-fluorophore FRET assay for high-throughput screening of small-molecule inhibitors of ribosome assembly.** *Nucleic Acids Res* (2004) **32**(9):2707-2715.

45. Winkler WC, Breaker RR: **Genetic control by metabolite-binding riboswitches.** *Chembiochem* (2003) **4**(10):1024-1032.

46. Nudler E, Mironov AS: **The riboswitch control of bacterial metabolism.** *Trends Biochem Sci* (2004) **29**(1):11-17.

47. Werstuck G, Green MR: **Controlling gene expression in living cells through small molecule-RNA interactions.** *Science* (1998) **282**(5387):296-298.

48. Hanson S, Berthelot K, Fink B, McCarthy JE, Suess B: **Tetracycline-aptamer-mediated translational regulation in yeast.** *Mol Microbiol* (2003) **49**(6):1627-1637.

49. Suess B, Hanson S, Berens C, Fink B, Schroeder R, Hillen W: **Conditional gene expression by controlling translation with tetracycline-binding aptamers.** *Nucleic Acids Res* (2003) **31**(7):1853-1858.

50. Suess B, Fink B, Berens C, Stentz R, Hillen W: **A theophylline responsive riboswitch based on helix slipping controls gene expression *in vivo*.** *Nucleic Acids Res* (2004) **32**(4):1610-1614.

51. Grate D, Wilson C: **Inducible regulation of the *S. cerevisiae* cell cycle mediated by an RNA aptamer-ligand complex.** *Bioorg Med Chem* (2001) **9**(10):2565-2570.

52. Yen L, Svendsen J, Lee JS, Gray JT, Magnier M, Baba T, D'Amato RJ, Mulligan RC: **Exogenous control of mammalian gene expression through modulation of RNA self-cleavage.** *Nature* (2004) **431**(7007):471-476.

• *This study demonstrates that regulation of ribozymes by an exogenous small-molecule inhibitor can be used to modulate gene expression by controlling whether or not the corresponding mRNA is cleaved by the ribozyme.*

53. Silverman SK: **Rube Goldberg goes (ribo)nuclear? Molecular switches and sensors made from RNA.** *RNA* (2003) **9**(4):377-383.

• *This is one of the best reviews on allosteric functional RNAs.*

54. Famulok M, Verma S: ***In vivo*-applied functional RNAs as tools in proteomics and genomics research.** *Trends Biotechnol* (2002) **20**(11):462-466.

55. Desai SK, Gallivan JP: **Genetic screens and selections for small molecules based on a synthetic riboswitch that activates protein translation.** *J Am Chem Soc* (2004) **126**(41):13247-13254.

56. Buskirk AR, Landrigan A, Liu DR: **Engineering a ligand-dependent RNA transcriptional activator.** *Chem Biol* (2004) 11(8):1157-1163.

• *This paper presents an impressive application of allosteric activation of a functional RNA for transcription activation, induced by a small, cell-permeable molecule.*

57. Grate D, Wilson C: **Laser-mediated, site-specific inactivation of RNA transcripts.** *Proc Natl Acad Sci USA* (1999) **96**(11):6131-6136.

58. Buskirk AR, Kehayova PD, Landrigan A, Liu DR: ***In vivo* evolution of an RNA-based transcriptional activator.** *Chem Biol* (2003) 10(6):533-540.