Gene Ther Mol Biol Vol 9, 417-422, 2005

Synthetic riboregulators – an alternative means to control gene expression

Review Article

Christian Berens and Beatrix Suess*

Lehrstuhl für Mikrobiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Staudtstr. 5, D-91058 Erlangen, Germany

---

***Correspondence**: Suess, Beatrix, Lehrstuhl für Mikrobiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Staudtstrasse 5, 91058 Erlangen, Germany; Tel: +49 9131 8528818; Fax: +49 9131 8528082; Email: bsuess@biologie.uni-erlangen.de

Key words: engineered riboswitches, aptamer, ribozyme, antisense, conditional gene expression

Abbreviations: binding site, (SD); glucosamine-6-phosphate, (GlcN6P); microRNAs, (miRNAs); short interfering RNAs, (siRNAs); tetramethylrosamine, (TMR)

Received: 3 September 2005; Accepted: 26 September 2005; electronically published: November 2005

Summary

During the last years, the important role RNA plays for regulating gene expression in all organisms has become obvious. Consequently, several recent approaches aim to utilize the outstanding chemical properties of RNA in developing artificial RNA regulators for conditional gene expression systems. Rational design, *in vitro* selection and *in vivo* screening systems have been combined to create a versatile set of RNA based molecular switches. These tools rely on diverse mechanisms and exhibit activity in several organisms. In this review, we summarize recent developments in the application of synthetic riboswitches for gene regulation *in vivo*.

I. Introduction

The functional role of RNA had long been thought to be restricted to the transfer of genetic information from DNA to proteins. However, an emerging awareness of the role of RNA in gene control and catalysis of chemical reactions has changed this view dramatically. In many ways, RNA seems to be more akin to proteins than to the chemically related DNA. Like proteins, RNA can adopt complex three-dimensional structures for the precise presentation of chemical moieties which are essential for its function as a biological catalyst, regulator or structural scaffold.

In the course of the last few years, a wide set of regulatory RNAs were discovered. Short non-coding RNAs known as microRNAs (miRNAs) and the related short interfering RNAs (siRNAs) can negatively regulate gene expression in many eukaryotes. They work by recruiting diverse protein factors which process ~ 22-nucleotide long RNA fragments that form base-paired complexes with their target mRNA. These interactions cause inactivation of the targeted gene by different mechanisms (Meister and Tuschl 2004; Kawasaki and Taira, 2005). RNA silencing mechanisms were first recognized as antiviral mechanisms defending plants against RNA viruses or preventing the random integration of transposable elements. Today it is known that miRNAs have diverse expression patterns and regulate various developmental and physiological processes in plants and animals (Baulcombe, 2004; He and Hannon, 2004). Furthermore, small non-coding RNAs as regulators of translation and message stability have also been identified in bacteria. They are involved in the regulation of replication, the maintenance of prokaryotic extra chromosomal elements and in bacterial responses to changing environments (Gottesman, 2004; Storz et al, 2004, 2005). Although protein factors are essential for the gene-silencing pathways in eukaryotes and in bacteria, the initial trigger for all of these critical regulatory tasks is RNA.

Recently, a novel mode of RNA-mediated gene regulation was detected. RNA sequences located in the 5' leader region of mRNA serve as a molecular switch able to modulate transcription, translation or mRNA processing through conformational changes within the RNA structure prompted by direct interaction with a specific cellular metabolite. These *cis*-acting regulatory elements, termed riboswitches, control a diverse set of basic metabolic pathways in prokaryotes (Nudler and Mironow, 2003; Mandal and Breaker 2004; Soukup and Soukup 2004; Tucker and Breaker 2005; Winkler, 2005;). They consist solely of RNA, sense their ligand in a binding pocket which is an integral part of the riboswitch and undergo

restructuring upon metabolite binding whereby one of the two conformations efficiently interferes with gene expression. Their specific characteristic is that RNA accomplishes both sensor and effector functions thereby demonstrating that a protein cofactor is not an obligate requirement for regulation.

These properties predestine RNA based regulators to act as archetype for the development of artificial regulators. In fact, there are several examples of how engineered RNAs can be utilized as molecular switches in vitro or change the level of gene expression of a specific gene product by ribozyme or siRNA mediated knock-down (Silverman, 2004; Akashi et al, 2005). Recently, the development of these switches has reached a new level of quality by exhibiting activity *in vivo* to control gene expression not just in bacteria but also in higher organisms. The subject of this review is to highlight recent developments performed by molecular engineers to create a novel variety of engineered riboswitches.

II. Aptamer based molecular switches

RNA-based gene-regulation switches can be designed by inserting engineered RNA modules into sensitive regions of the mRNA. For this purpose aptamers are promising tools. Aptamers are synthetic, *in vitro* selected RNA molecules which show high binding affinity and specificity for a respective ligand. Upon its binding they adopt a unique conformation wherein the ligand becomes an integral part of the complex (Patel et al, 1997; Hermann and Patel, 2000). Thus, in the presence of the ligand aptamers inserted into the 5'UTR of a reporter mRNA can principally interfere with initial stages of translational initiation thereby acting as molecular switch to turn off the expression of a downstream gene (schematically shown in **Figure 1B, C**).

This was first shown by Werstuck and Green by selecting aptamers that bound specifically to a Hoechst dye and inserting a pair of them into the 5′UTR of a luciferase reporter gene (Werstuck and Green, 1998). Gene expression in mammalian cells decreased 10-fold in the presence of the ligand. These results showed that a small molecule and its aptamer are capable of controlling gene expression.

A similar mechanism for the inhibition of translation in yeast was reported by Grate and Wilson, 2001. They inserted an *in vitro* selected malachite green binding motif into the 5′UTR of the *CLB2* cyclin gene. In this system addition of the malachite green analogue TMR inhibits translation 10-fold. As a consequence progression through the cell cycle is slowed and the cell morphology is affected. These results confirm that this technique can alter cellular processes and therefore serve as genetic tool for understanding cellular pathways.

We have identified a tetracycline (tc)-binding aptamer capable of controlling translation in yeast up to 40-fold (Berens et al, 2001; Suess et al, 2003). When inserted into the 5′UTR of several reporter genes this aptamer leads to reversible and dose-dependent reduction of reporter activity *in vivo* (Hanson et al, 2003). *In vitro* translation and subsequent sucrose gradient analysis of these constructs demonstrates that the aptamer in its tc-bound form interferes with the formation of the 80S ribosome, probably by blocking scanning (Hanson et al, 2003; **Figure 1B**). The aptamer is also active when placed directly downstream of the cap structure. Under these conditions, formation of the tc-aptamer-complex prevents binding of the small ribosomal subunit to the cap structure (**Figure 1C**). Cap proximal insertions were less active than cap distal ones (Hanson et al, 2003). Biochemical and genetic analyses of the aptamer revealed a complex tc-binding pocket and suggest that ligand-binding connects two distinct regions of the aptamer by a pseudoknot-like intramolecular linkage which may then be able to interfere with translation initiation (Hanson et al, 2005).

Pelletier and coworkers present another example for aptamer-based control of gene expression in eukaryotes (Harvey et al, 2002). They inserted one to three copies of the theophylline-aptamer (Jenison et al, 1994) or the biotin-aptamer (Wilson et al, 1998), respectively, into the 5′UTR of a CAT reporter gene. In the ligand′s presence they observed more than 10-fold inhibition of reporter activity both *in vitro* using different translation systems and *in vivo* in *Xenopus* oocytes. They showed that the regulation efficiency increases with the aptamer copy number. Interestingly, the cap proximal insertions were more active than cap distal ones. This is in contrast to the results obtained in yeast where regulation is more effective at a cap-distal position (Hanson et al, 2003). However, both results agree with data obtained from the analyses of the inhibitory potential of rigid RNA stem-loop structures inserted into the 5′UTR. In higher eukaryotes they are also more active when located in a cap-proximal position (Kozak, 1986) whereas in yeast there are only minor differences in the inhibitory capacity of a stem-loop structure at either of these positions (Oliveira et al, 1993; Vega Laso et al, 1993) suggesting mechanistic differences between yeast and other eukaryotic systems and a simple way to test positioning of a regulatory element.

The interesting aspect remains that only a few of the aptamers tested are able to act as a regulatory element and it will be exciting to unravel the determinants which allow an aptamer to act as a riboswitch.

III. Engineered riboswitches based on helix slippage modulate accessibility of the ribosomal binding site in bacteria

Control of gene expression by inserting synthetic aptamers into the untranslated region is not feasible in prokaryotes due to the spatial coupling between the ribosomal binding site (Shine-Dalgarno sequence, SD) and the start codon. This distance must not exceed 13 nucleotides and is therefore too short for general aptamer insertion or requires the specific selection of novel aptamers accommodating these two elements. We rather developed an alternative strategy which makes use of the inhibitory potential of placing a stem-loop close to the ribosomal binding site (de Smit and van Duin 1990). For that purpose we combined an RNA aptamer for theophylline (Jenison et al, 1994) and a structural bridge module (Soukup and Breaker 1998). Ligand binding to the aptamer domain shifts the double stranded bridge by one

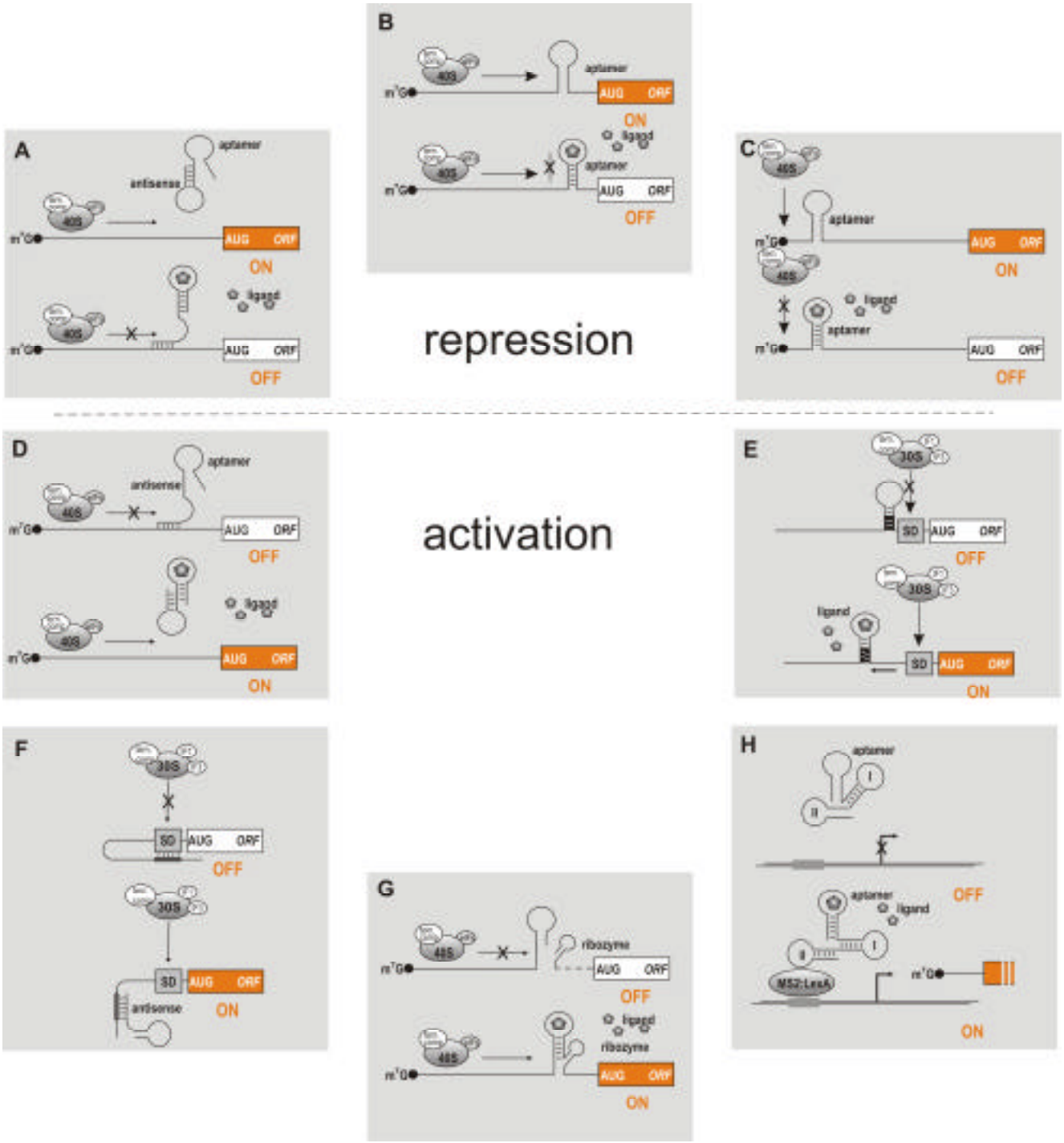

Figure 1. Different strategies for designer RNA switches: Antisense-based RNA switches (A) An antisense domain that targets the 5′UTR of an mRNA is fused to an aptamer domain. In the presence of the ligand, a conformational change occurs that frees the antisense domain which was sequestered in a stem-loop and switches gene expression off. **D)** Alternatively, the antisense domain is free and suppresses gene expression. Ligand binding leads to sequestration of the antisense domain permitting target gene expression. **F)** The bacterial ribosomal binding site (SD) of a bacterial translation initiation region is sequestered within a stem-loop structure which abolishes gene expression. An antisense molecule was designed to interfere with this region thereby freeing the SD. **Aptamer-based RNA switches** The aptamer is located in the 5′UTR of an eukaryotic mRNA. The addition of the ligand facilitates the formation of a ligand-aptamer complex which interferes with translational initiation either by hindering successful scanning **(B)** or binding of the ribosomal subunits **(C)**. (**E)** A structural element composed of an aptamer domain and a helical bridge module (black box) is inserted close to the SD and interferes with binding of the small ribosomal subunit. Binding of the ligand to the aptamer domain mediates helix slippage within the bridge module. This shifts the element away allowing the ribosome to bind. **Ribozyme-based RNA switches G)** A ribozyme inserted in the 5′UTR of an eukaryotic reporter self-cleaves and destabilizes its mRNA. Ligand binding switches gene expression on by inhibiting ribozyme function. **RNA switches activate transcription H)** An RNA domain that functions as a transcriptional activator **(I)** is recruited to the promoter of a reporter gene via the interaction of the MS2 binding domain **(II)** with a MS2 coat protein-LexA fusion protein. An aptamer is integrated into this construct in such a manner that ligand binding promotes formation and function of the activation domain.

base into a position that enables binding of the small ribosomal subunit to the SD-sequence (schematically shown in Figure 1E). Consequently, upon addition of theophylline reporter gene activity increases 8-fold (Suess et al, 2004).

IV. Antisense based riboswitches

Another means for post-transcriptional regulation in prokaryotes is to sequester the ribosomal binding site within a stem loop structure. Several natural riboswitches make use of this mechanism (Winkler et al, 2002). Collins and coworkers developed artificial RNA switches which control gene expression by blocking the ribosomal binding site. They inserted a complementary *cis*-sequence directly upstream of the SD-sequence in a reporter gene. After transcription this *cis*-element causes the formation of a stem-loop structure interfering with translation initiation (Isaacs et al, 2004). Expression of a trans-activating RNA leads then to an 8-fold derepression of the reporter by base-pairing with one arm of the sequestration helix (**Figure 1F**).

The spectrum of designer RNA molecules is expanded by work done by Bayer and Smolke, 2005. They designed an antisense RNA domain controlled by an aptamer domain able to recognize specific ligands and expressed this regulator RNA module in *trans*. The antisense domain is capable of base-pairing with a target mRNA, thereby inhibiting translation. Upon binding of a specific ligand to the aptamer domain, the RNA molecule – termed antiswitch - undergoes a conformational change that affects the ability of the antisense domain to recognize the target mRNA (**Figure 1A, D**). Smolke and coworker designed two types of antiswitches: an off and an on switch. In the absence of the ligand, the off antiswitch has a sequestered antisense domain, blocking its ability to repress expression of luciferase mRNA transcript in yeast. Upon ligand binding to the aptamer domain, the RNA element undergoes conformational changes which expose the antisense domain and target gene expression is repressed. The on antiswitch possesses an antisense domain which is free to bind a target mRNA. After ligand binding to the aptamer domain, the switch undergoes an allosteric transition that causes the antisense domain to form a stem loop, rendering it inactive. Consequently, target gene expression is upregulated (Bayer and Smolke, 2005).

The authors show that switching only occurs in the presence of specific ligands and that the antiswitch sequences are exchangeable as modules. Among other applications this technique provides a tool for analyzing cellular pathways by sensing and responding to intracellular metabolite levels.

V. Regulatable ribozymes

Catalytic RNAs, also known as ribozymes, can be engineered to optimize their activities in the intracellular environment. The introduction of a library of active ribozymes into cells, and the subsequent screening for phenotypic changes, allows the rapid identification of gene function. Thus, ribozyme technology represents a valuable RNA-based tool for the determination of gene function (Akashi et al, 2005). Furthermore, a trans-splicing ribozyme has been developed which can be efficiently used for gene repair or replacement (Byun et al, 2003; Baum and Testa, 2005).

Recently, these ribozyme-based genetic control elements have further been improved by making them controllable with a small molecule. Thereby, Mulligan and coworkers display an exciting advance in the quest to create designer control elements (Yen et al, 2005). They screened natural and engineered ribozymes for activity *in vivo*. After optimization they obtained a highly efficient ribozyme sequence which is constitutively active in mammalian cells. In a second step, they performed a high throughput screening to identify a small molecule compound or an oligonucleotide complementary to the ribozyme sequence that could interfere with ribozyme activity. When inserted into a reporter mRNA immediately upstream of the translational start, the optimized ribozyme self-cleaves before translation can occur, resulting in degradation of the mRNA and loss of gene expression. Addition of the nucleoside analog toyocamycin which acts as an inhibitor of the ribozyme prevented mRNA cleavage and gene expression was restored 250-fold (**Figure 1G**).

Actually, regulation of ribozyme activity by small molecules has already been observed in nature. The mRNA of the *glmS* gene in Gram-positive bacteria is cleaved by a ribozyme. This ribozyme is activated by glucosamine-6-phosphate (GlcN6P), which is the metabolic product of the GlmS enzyme. Therefore the ribozyme serves as a metabolite-responsive genetic regulator that represses the *glmS* gene in response to rising GlcN6P concentrations (Winkler et al, 2004).

Before this natural switch was discovered, several allosterically regulateable ribozymes had already been developed by rational design and *in vitro* selection (Silverman 2003; Breaker 2005). However, Mulligan and coworkers are now the first to present a ribozyme-based regulator that is active in higher organisms. Further combinations of the now established ribozyme platform with other ligand binding domains should yield a novel set of RNA switches based on ribozyme activity (Yen et al, 2005).

VI. Engineered riboswitches acting as transcriptional regulator

Besides the development of RNA based regulators which target post transcriptional steps of gene expression, there are also alternative approaches of regulators that act at the level of transcription.

Liu and coworkers engineered an artificial ligand dependent RNA transcriptional activator. They screened a random RNA library in yeast and identified a short RNA sequence that activates transcription with a potency rivaling some of the strongest natural protein activation domains using genetic selection in yeast (Buskirk et al, 2003, 2004). The crucial sequence and structural elements were identified and lengthened by a short RNA sequence previously selected to bind the small molecule dye tetramethylrosamine (TMR; Grate and Wilson 1999).

Screening and selection of a library of linker sequences optimized a conformational shift in the RNA activator such that in the presence of TMR, transcription is enhanced 10-fold in yeast (**Figure 1H**). The ability of RNA to activate transcription in a ligand dependent manner is an additional example for the versatility of RNA evolution and design methods.

VII. RNA as novel tool box in molecular biology

The diversity of these novel molecular RNA-based switches demonstrates the power of combining *in vitro* selection, *in vivo* screening and rational design. The RNA switches summarized here are of small size and respond in a dose-dependent manner to small molecules which are nontoxic, not cellular metabolites and display good cell permeability. In addition, they have the advantage of being composed of only one component with no need of further components like proteins. Currently, the only limitation and biggest obstacle of RNA based systems in comparison with classical transcriptional regulatory systems is the need for a high ligand concentration which is 100-1000 fold higher than expected by the respective dissociation constants. The reasons are poorly understood and may be due to alternative RNA conformations or competition with the binding of proteins and other RNAs. However, the mechanistic diversity with which the applied strategies interfere at almost every step of gene expression including transcription activation, ribosomal binding or scanning shows the enormous potential inherent in RNA based technologies to generate excellent molecular switches for conditional gene expression and promises further increases in number, diversity and sophistication of such switches.

This sets the stage for customized switches that could be designed to either repress or activate the expression of any target gene in response to selected, membrane-permeable small molecules in the environment. By assembling various combinations of antisense and aptamer domains, multiple artificial riboswitches can be designed and coexist in the same cell which responds independently to different small molecules regulating different mRNA targets. Thereby, probing and reprogramming of entire regulatory networks should be possible.

The success of engineered riboswitches provides additional evidence that RNA molecules can serve as regulatory elements and promises to bring custom-designed RNA molecules as a novel and versatile tool box to molecular biology.

References

Akashi H, Matsumoto S, Taira K **(2005)** Gene discovery by ribozyme and siRNA libraries. **Nat Rev Mol Cell Biol.** 6, 413-22.

Baulcombe D **(2004)** RNA silencing in plants. **Nature** 16**,** 356-363.

Baum DA and Testa SM **(2005)** *In vivo* excision of a single targeted nucleotide from an mRNA by a trans excision-splicing ribozyme. **RNA**, 11, 897-905.

Bayer TS and Smolke CD **(2005)** Programmable ligand-controlled riboregulators of eukaryotic gene expression. **Nat Biotechnol** 23, 337-343.

Berens C, Thain A and Schroeder R **(2001)** A tetracycline-binding RNA aptamer. **Bioorg Med Chem** 9, 2549-2556.

Breaker RR **(2005)** Harnessing RNA switches. **Gene Therapy** 12, 725-726.

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

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

Byun J, Lan N, Long M and Sullenger BA **(2003)** Efficient and specific repair of sickle β-globin RNA by trans-splicing ribozymes. **RNA** 9, 1254-1263.

de Smit MH and van Duin J **(1990)** Control of prokaryotic translational initiation by mRNA secondary structure. **Prog Nucleic Acid Res Mol Biol**, 38, 1-35.

Gottesman S **(2004)** The small RNA regulators of Escherichia coli: roles and mechanisms. **Annu Rev Microbiol** 58, 273–301.

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

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

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

Hanson S, Bauer G, Fink B and Suess B **(2005)** Molecular analysis of a synthetic tetracycline-binding riboswitch. **RNA** 11, 503-511.

Harvey I, Garneau P and Pelletier J **(2002)** Inhibition of translation by RNA-small molecule interactions. **RNA** 8, 452-463.

He L and Hannon GJ **(2004)** MicroRNAs: small RNAs with a big role in gene regulation. **Nat Rev Genet** 5, 522-531.

Hermann T and Patel DJ **(2000)** Adaptive recognition by nucleic acid aptamers. **Science** 287, 820-825.

Isaacs FJ, Dwyer DJ, Ding C, Pervouchine DD, Cantor CR and Collins JJ **(2004)** Engineered riboregulators enable post-transcriptional control of gene expression. **Nat Biotechnol** 22, 841-7.

Jenison RD, Gill SC, Pardi A and Poliski B **(1994)** High-resolution molecular discrimination by RNA. **Science** 263**,** 1425-1429.

Kawasaki H and Taira K. **(2005)** Transcriptional gene silencing by short interfering RNAs. **Curr Opin Mol Ther**. 7, 125-31.

Kozak M **(1986)** Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. **Proc Natl Acad Sci USA** 83, 2850-2854.

Mandal M and Breaker RR **(2004)** Gene regulation by riboswitches. **Nat Rev Mol Cell Biol** 5, 451-463.

Meister G and Tuschl T **(2004)** Mechanisms of gene silencing by double-stranded RNA. **Nature** 16, 343-349.

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

Oliveira CC, van den Heuvel JJ and McCarthy JE **(1993)** Inhibition of translational initiation in *Saccharomyces cerevisiae* by secondary structure: the roles of the stability and position of stem- loops in the mRNA leader. **Mol Microbiol** 9, 521-532.

Patel DJ, Suri AK, Jiang F, Jiang L, Fan P, Kumar RA and Nonin S **(1997)** Structure, recognition and adaptive binding in RNA aptamer complexes. **J Mol Biol** 272, 645-664.

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

Soukup GA and Breaker RR **(1999)** Engineering precision RNA molecular switches. **Proc Natl Acad Sci USA** 96**,** 3584-3589.

Soukup JA and Soukup GA **(2004)** Riboswitches exert genetic control through metabolite-induced conformational change. **Curr Opin Struct Biol** 14, 344-349.

Storz G, Obdyke JA and Zhang A **(2004)** Controlling mRNA stability and translation with small, noncoding RNAs. **Curr Opin Microbiol** 7, 140-144.

Storz G, Altuvia S and Wassarman KM **(2005)** An abundance of RNA regulators. **Annu Rev Biochem** 74, 199-217.

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

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

Tucker BJ and Breaker RR **(2005)** Riboswitches as versatile gene control elements. **Curr Opin Struct Biol** 15, 1-7.

Vega Laso MR, Zhu D, Sagliocco F, Brown AJ, Tuite MF and McCarthy JE **(1993)** Inhibition of translational initiation in the yeast *Saccharomyces cerevisiae* as a function of the stability and position of hairpin structures in the mRNA leader. **J Biol Chem** 268, 6453-6462.

Wilson C, Nix J and Szostak J **(1998)** Fundamental requirements for specific ligand recognition by a biotin-binding RNA pseudoknot. **Biochemistry** 37, 14410-14419.

Winkler WC, Nahvi A and Breaker RR **(2002)** Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. **Nature** 419, 952-956.

Winkler WC, Nahvi A, Roth, A., Collins JA and Breaker RR **(2004)** Control of gene expression by a natural metabolite-responsive ribozyme. **Nature** 428, 281-286.

Winkler W **(2005)** Metabolic monitoring by bacterial mRNAs. **Arch Microbiol** 183, 151-159.

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

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

Beatrix Suess