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RNA as a Versatile and Powerful Platform for Engineering Genetic Regulatory Tools

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Introduction

RNA has traditionally been recognized for its role as a passive messenger of genetic information between the genome and the proteome in all living organisms. However, there are an increasing number of discoveries of naturally-occurring RNA molecules that act as regulatory elements, performing various cellular functions including gene expression regulation through sophisticated mechanisms, thereby expanding its traditional role as a genetic messenger and revealing it as a functionally versatile molecule. Antisense RNAs (Simons, 1988, Inouye, 1988), ribozymes (Doudna and Cech, 2002, Fedor and Williamson, 2005), riboswitches (Mandal and Breaker, 2004b, Winkler and Breaker, 2005, Lai, 2003, Sudarsan *et al*., 2003, Winkler and Breaker, 2003, Tucker and Breaker, 2005), and small interfering and microRNAs (siRNAs and miRNAs, respectively) (Meister and Tuschl, 2004, Dykxhoorn *et al*., 2003, Novina

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Abbreviations: RNA: ribonucleic acid, siRNA: small interfering RNA, miRNA: microRNA, mRNA: messenger RNA, ncRNA: non-coding RNA, DNA: deoxyribonucleic acid, UTR: untranslated region, RNAi: RNA interference, GC: guanine and cytosine, RBS: ribosomal-binding site, SD: Shine-Dalgarno, TPP: thiamine pyrophosphate, SELEX: Systematic Evolution of Ligands by EXponential enrichment, PCR: polymerase chain reaction, GUC: guanine, uridine, and cytosine, ATP: adenosine triphosphate, FMN: flavin mononucleotide, GlcN6P: glucosamine-6-phosphate, PTGS: post-transcriptional gene silencing, dsRNA: double-stranded RNA, RISC: RNAi-induced silencing complex, pre-miRNA: precursor hairpin transcript encoding microRNA, pri-miRNA: primary hairpin transcript encoding microRNA, RNP: ribonucleoprotein, miRNP: microribonucleoprotein, pol III: RNA polymerase III, shRNA: short hairpin RNA, pol II: RNA polymerase II, VEGF: vesicular endothelial growth factor, ELISA: enzyme-linked immunosorbent assay, HIV-1: human immunodeficiency virus 1, SPR: surface plasmon resonance, FRET: fluorescence resonance energy transfer, HCV: hepatitis C virus, NS3: non-structural protein 3, CTLA-4: cytotoxic T lymphocyte antigen-4, PSMA: prostate-specific membrane antigen.

and Sharp, 2004, Tang, 2005) are all examples of regulatory RNA elements that play diverse roles in mediating gene expression. Depending on the nature of the regulatory element, the control event may occur at different levels of gene expression such as transcription, translation, splicing, or decay. Unlike messenger RNAs (mRNAs), these molecules are non-coding RNAs (ncRNAs), or do not encode protein-coding information. Each of these regulatory elements employs a unique mechanism for controlling gene expression implemented through diverse physical platforms that can be grouped generally into *cis*- and *trans*-acting platforms. In the former platform, the regulatory domain is present within the same transcript as the target gene and the regulation event occurs through an intramolecular mechanism, whereas in the latter platform the regulatory element is a separate RNA molecule and acts on a transcript encoding a target gene through RNA-RNA interactions, or intermolecular binding events.

RNA exhibits a wide variety of functional properties, including catalytic, gene regulatory, and ligand-binding activities. In addition, RNA molecules achieve precise control over the expression of diverse gene sets through a combination of these unique properties. They adopt different conformations by folding into complex secondary and tertiary structures, which allow them to interact with various cellular constituents such as DNA, proteins, small molecules, and other RNA molecules (Breaker, 2004, Schroeder *et al*., 2004). Furthermore, RNA possesses the ability to undergo dynamic conformational changes between different adoptable conformations due to its structural flexibility. The binding of cellular and environmental molecules to a particular conformation has been demonstrated to regulate the equilibrium distribution between these stable conformational states (Soukup and Soukup, 2004, Bauer and Suess, 2006). These functional properties are encoded within the nucleotide sequence of an RNA molecule, which subsequently dictates its secondary and tertiary structure and ultimately its function. Furthermore, unlike larger biomolecules such as proteins, the functional activity of RNA is more directly defined by its secondary structure. These two properties, the interdependence between RNA sequence, structure, and function (Isaacs *et al*., 2006) and the connection between RNA secondary structure and function, has been recently used by molecular engineers to construct novel 'designer' regulatory RNA elements (Isaacs *et al*., 2006, Bauer and Suess, 2006, Davidson and Ellington, 2005). Therefore, by altering the nucleotide composition of an RNA molecule, the existing functional information can be reprogrammed to yield a wide array of regulatory properties. In addition, advances in nucleic acid engineering have made it possible to generate these sophisticated properties through rational and/or combinatorial design strategies (Davidson and Ellington, 2005, Bauer and Suess, 2006, Isaacs *et al*., 2006).

Recent research in both biological sciences and molecular engineering supports the model of RNA as a versatile molecule possessing sophisticated functional properties and also demonstrates that the design strategies connecting RNA structure and function are relatively well-developed. This review article provides an overview of several classes of naturally-occurring RNAs that are involved in gene expression regulation and their synthetic counterparts that are generated through various biomolecular design principles. Advances in RNA technology and nucleic acid engineering have allowed researchers to apply these naturally-occurring RNAs as basic regulatory platforms and to develop more sophisticated regulatory RNA devices

that involve integrated designs of multiple platforms. These synthetic riboregulators enable gene expression to be regulated in a more controlled manner and represent powerful tools for fundamental research and exhibit important applications in biotechnology and medical research. In addition to applications involving gene expression regulation, RNA is an attractive platform for the construction of designer molecules for applications such as targeted therapeutics, *in vivo* biosensors, and diagnostic devices.

Naturally-occurring RNA regulatory elements and their engineered counterparts for applications in gene expression control

RNA exhibits many unique functional properties and plays a critical role in the control of gene expression in various organisms. Many regulatory RNAs are *cis*-acting elements that are located in the 5' or 3' untranslated regions (UTRs) of transcripts harboring a target gene(s), whereas others are *trans*-acting elements that are transcribed independently and act on appropriate mRNA targets through intermolecular binding events (Davidson and Ellington, 2005). These regulatory RNA elements usually lack a protein-coding capacity and thus appear to act as genetic regulators mainly or exclusively at the post-transcriptional level (Erdmann *et al*., 2001). An increasing number of these regulatory RNAs have been discovered over the past few years, and their specific roles in the control of gene expression have been demonstrated in both prokaryotes and eukaryotes, including mammals (Erdmann *et al*., 2001). Four major functional classes of regulatory RNAs - riboswitches, antisense RNAs, ribozymes, and RNA interference (RNAi) substrates such as siRNAs and miRNAs – are described in this section. These classes are selected for their utility and prevalence in use in synthetic riboregulator systems. Among these four classes, riboswitches exhibit the most sophisticated regulatory properties since they function as allosteric regulators of gene expression through the interaction of integrated ligand-binding and regulatory domains. This also allows them to autonomously control their own regulatory activity in response to small molecule metabolite binding with no direct aid from proteins. Consequently, many recent engineering efforts have focused on the construction of regulatory RNA molecules with integrated ligand-binding domains such that these synthetic molecules are able to control their own gene regulatory activity through a binding event to a specific target molecule.

RIBOSWITCHES AS AUTONOMOUS ALLOSTERIC REGULATORS OF GENE EXPRESSION

(i) Mechanisms of regulation by riboswitches

All living organisms must direct the expression of extensive sets of genes in a precise manner in response to different biochemical signals such as metabolic demand and environmental changes (Winkler and Breaker, 2003). This degree of genetic control requires highly responsive sensors that accurately measure the magnitude of a particular molecular signal and modulate the levels and activities of appropriate gene products in order to control information flow through cellular networks in response to changing cellular environments. Although proteins have traditionally been

recognized as being the molecular sensors in biological control systems, recent discoveries have demonstrated that elements within mRNAs, termed riboswitches, are also capable of performing sensor-actuation functions.

Riboswitches are naturally-occurring, ligand-binding genetic control elements primarily located within the 5' UTRs of their target transcripts (Winkler and Breaker, 2003). While the majority of the elements characterized thus far have been discovered in prokaryotes, it has recently been shown that these allosteric RNA elements are also present in eukaryotes (Sudarsan *et al*., 2003). A riboswitch is comprised of two domains: the ligand-binding domain, referred to as the aptamer domain, and the gene regulatory domain, referred to as the expression platform domain (Figure 1) (Winkler and Breaker, 2003). The aptamer domain can adopt a secondary and associated tertiary structure inside which the binding pocket for a target ligand resides. Riboswitches achieve ligand-controlled regulation of gene expression by directing targeted dynamic conformational change between two primary conformations at equilibrium: one in which the regulatory domain is active and the other in which the regulatory domain is inactive. One of these conformational states is associated with the formation of the ligand-binding pocket within the aptamer domain, whereas the other is not. The binding of the ligand to the riboswitch shifts the equilibrium distribution between these stable equilibrium conformations to favor the ligand-bound form, thereby resulting in an allosteric gene regulation event. By altering the combination of active and inactive regulatory domain state with the binding-pocket formed and unformed aptamer domain state, riboswitches can either repress or activate the expression of the associated gene product. Most of the riboswitches characterized to date down-regulate the expression of the target gene; however, in a few exceptions such as the glycine (Mandal *et al*., 2004) and adenine riboswitches (Mandal and Breaker, 2004a), target gene expression is activated upon binding of the metabolite-ligand to the riboswitch.

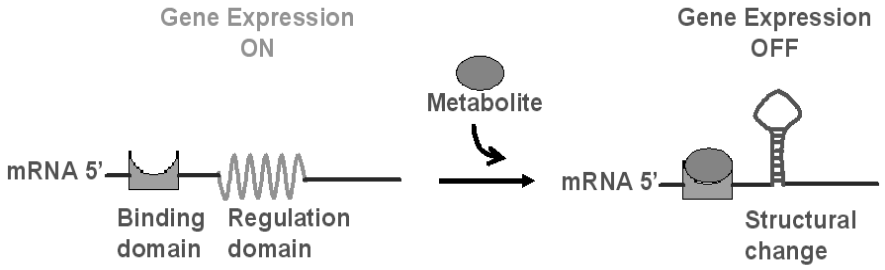

Figure 1. A schematic diagram of a riboswitch comprised of two distinct domains: the aptamer or ligand-binding domain and the expression platform or regulatory domain (adapted from Winkler and Breaker (Winkler and Breaker, 2003)). In this example, metabolite binding to the aptamer domain stabilizes the altered conformation of the regulatory domain, resulting in a shift in the equilibrium distribution between the two regulatory conformations that leads to mediation of expression of the target gene.

The presence of the ligand-binding domain enables riboswitches to sense intracellular target concentrations through specific binding interactions and subsequently regulate expression levels of the associated gene product. Often times this gene product is an enzyme that plays a direct role in the biosynthesis or biocatalysis of the target molecule. In this manner, riboswitches are able to serve as autonomous

RNA sensor-actuator systems. Therefore, unlike other regulatory systems riboswitches function as autonomous control systems that do not require additional aid from proteins as reporter sensors to exert their regulatory effects over gene expression. This enables a direct dynamic relationship between the intracellular concentration of the ligand metabolite and the levels of the target enzyme involved in the metabolite's synthesis or breakdown. In addition, riboswitches are capable of binding their target metabolites with high specificity and affinity comparable to that of protein-ligand interactions.

Riboswitches regulate gene expression in response to changing metabolite levels through different mechanisms involving transcription termination, translation initiation, mRNA processing, and potentially splicing (Figure 2) (Winkler and Breaker, 2005, Sudarsan *et al*., 2003). Regulation through transcription termination involves the mediated formation of a rho-independent terminator stem, which is usually GC rich, thereby destabilizing the transcription elongation complex (Winkler *et al*., 2003). In another example, a terminator stem of a riboswitch is disrupted upon binding of the target metabolite, thereby allowing proper transcription and thus up-regulation of the target gene expression (Mandal and Breaker, 2004a). Translation initiation is inhibited through a change in the secondary structure that interferes with ribosomal access to the gene, such as sequestering the ribosome-binding site (RBS) or Shine-Dalgarno (SD) sequence in prokaryotic cells. Mechanisms targeting deactivation of the target transcript or mRNA processing can be achieved through self-cleaving ribozymes (Winkler *et al*., 2004) where the transcript is subject to rapid decay following the ribozyme cleavage event. In addition, metabolite-binding domains have also been characterized within intronic sequences and 3' UTRs in certain organisms, suggesting that riboswitch-mediated control over gene expression may also occur through the regulation of splicing and mRNA stability, respectively (Sudarsan *et al*., 2003).

Riboswitches exhibiting unique mechanistic properties have also been recently identified. For example, the glycine riboswitch (Mandal *et al*., 2004) exhibits cooperative binding to its metabolite product, in which the metabolite binding turns on the gene expression of the enzyme responsible for the glycine cleavage system. This cooperative-binding feature is present in the glycine riboswitch and is proposed to ensure that the metabolite is indeed in excess after consumption to provide carbon flux through the citric acid cycle while maintaining sufficient amounts of the amino acid available for protein synthesis. In another example, a tandem riboswitch system (Sudarsan *et al*., 2006) was characterized that exhibits a Boolean logic ability and functions as a two-input NOR logic gate, in which the two ligands (S-adenosylmethionine and coenzyme B_{12}) can independently suppress the target gene expression by binding their corresponding aptamers located upstream of a structure resembling an intrinsic transcription terminator (Sudarsan *et al*., 2006). Another tandem riboswitch system (Welz and Breaker, 2007) was very recently discovered that consists of two distinct riboswitches. Unlike the glycine riboswitch and the two input-responsive logic-gate riboswitch systems, this riboswitch system does not exhibit cooperative ligand binding or detect two different metabolites, respectively. This tandem riboswitch system responds independently to the same metabolite, thiamine pyrophosphate (TPP), and is predicted to function in concert to yield a more 'digital' gene control output response than a single riboswitch system.

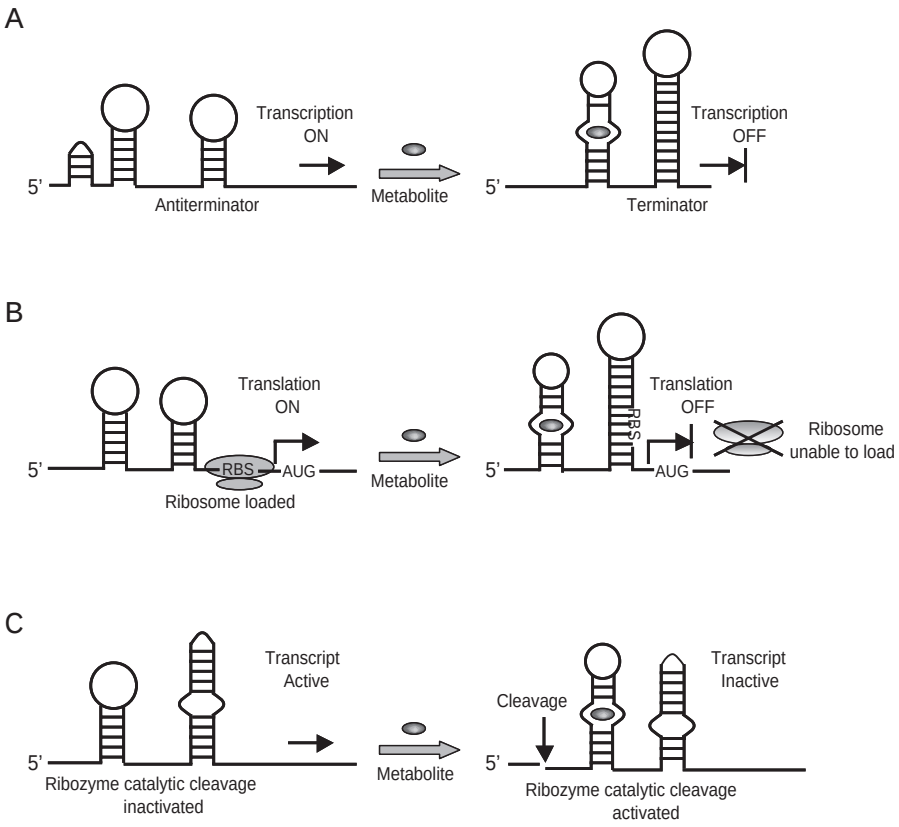

Figure 2. A schematic illustration of several examples of mechanisms by which riboswitches achieve gene expression regulation in response to binding their metabolite targets (A and B adapted from Nudler and Mironov (Nudler and Mironov, 2004)). Ligand-regulated mechanisms are illustrated through the (A) formation of a transcription terminator stem, (B) sequestering the RBS and blocking translation initiation, and (C) mRNA processing via catalytic cleavage of the transcript.

(ii) Ligand-binding domains of riboswitches

Riboswitches have garnered great interest in their application as genetic control elements for various biotechnological and basic research applications. They represent a class of sophisticated riboregulator elements involved in the dynamic control of many key metabolic pathways through specific aptamer-ligand binding events. However, the accuracy of riboswitch-mediated gene expression events depends on the specificity and affinity of the aptamer domain to its target ligand. Consequently, the ligand-binding or aptamer domains of riboswitches are critical components that enable these molecules to function as autonomous sensor-regulator systems within the complex chemical environment of the cell. Aptamers to novel ligands have been synthetically generated and employed in the construction of artificial riboswitches.

Synthetic aptamers are generated through *in vitro* selection or SELEX (Systematic Evolution of Ligands by EXponential enrichment) processes (Tuerk and Gold, 1990,

Ellington and Szostak, 1990). SELEX provides a very powerful selection method whereby nucleic acid molecules exhibiting rare and specific binding properties to a ligand of interest can be generated *de novo* by selecting for function binding activities from large randomized nucleic acid pools through iterative *in vitro* selection and amplification cycles of the selected pools. *In vitro* aptamer selection schemes start with a large pool of single-stranded RNA molecules generated through *in vitro* transcription from a DNA library. Aptamer pools are usually comprised of 30-70 randomized nucleotides, in order to generate an initial sequence diversity between 10^{14} to 10^{15} molecules (Rimmele, 2003). The pool is incubated with the target ligand of interest and subject to a subsequent partitioning event to separate bound members from unbound members. The most common partitioning schemes are those that use affinity chromatography-based separation schemes. Bound (functional) members are recovered and then amplified through reverse transcription and polymerase chain reaction (PCR) to yield a pool enriched for target binding. This enriched pool will become the input pool for the next round of selection.

Binding properties, such as affinities and specificities, of the synthetic aptamers can be programmed by tailoring the stringency and counter-selections for each selection. Aptamer affinities are tailored through the stringency of each selection cycle, normally by modifying wash volumes and target concentrations. Aptamer specificities are tailored through the counter-selections performed for each selection. Later selection cycles can be performed on enriched pools with structural analogs to the target, where the collected pool includes those molecules that do not bind the ligand used in these counter-selection cycles. Typically eight to fifteen selection cycles are required to generate aptamers with high binding affinities and specificities. Recent work has demonstrated that protein aptamer selection schemes can be automated using standard robotics (Cox *et al*., 2002). In addition, newer partitioning schemes for protein aptamer selections based on capillary electrophoresis have been recently developed that introduce several advantages over conventional affinity-based partitioning schemes (Berezovski *et al*., 2005). In particular, the efficiency of separation between the bound and unbound pools is significantly greater such that comparable aptamer pools can be generated in one to three cycles of selection. Numerous synthetic aptamers have been generated to a wide range of target ligands including small molecules, antibiotics, carbohydrates, amino acids, peptides, proteins (Hermann and Patel, 2000), and even organelles such as phospholipid bilayers (Khvorova *et al*., 1999, Vlassov *et al*., 2001), proving that aptamers can be synthetically generated against a wide range of target molecules and thus potentially any targets of interest for specific applications.

(iii) Synthetic riboswitches that control target gene expression levels

Synthetic riboswitches can be engineered by incorporating an aptamer(s) directly into the 5' UTR of a target mRNA. This should be done such that the insertion of the aptamer itself and its corresponding secondary structure do not interfere with translation in the absence of the target ligand. In the presence of the target ligand, the binding of the aptamer to its target results in structural stabilization due to the molecular binding interaction between the aptamer and its target (Patel *et al*., 1997, Hermann

and Patel, 2000). Similar to binding of a protein to the 5' UTR (Stripecke *et al*., 1994, Paraskeva *et al*., 1998), this stabilized secondary structure can repress translation (Pelletier and Sonenberg, 1985) presumably by interfering with ribosomal scanning or the ribosome-mRNA interaction. The first synthetic riboswitches based on this design strategy were constructed by Werstuck and Green (Werstuck and Green, 1998), who employed small molecule-binding RNA aptamers in their engineered riboswitches. They inserted different aptamers into the 5' UTR of a reporter mRNA and translation of the mRNA was shown to be repressed upon addition of the corresponding ligand both *in vitro* and *in vivo* in bacterial and mammalian cells. Following this initial work, theophylline (Jenison *et al*., 1994)-, biotin (Wilson *et al*., 1998)-, and tetracycline (Berens *et al*., 2001)-binding aptamers were utilized by different groups in a similar manner and demonstrated similar ligand-controlled gene expression patterns in different systems including *Xenopus oocyte* (Harvey *et al*., 2002) and the budding yeast *Saccharomyces cerevisiae* (Suess *et al*., 2003, Hanson *et al*., 2003).

Synthetic riboswitches have also been constructed to regulate translation of target genes in prokaryotes. Although still located in the 5' UTR of the target transcript, prokaryotes do not exhibit the same type of ribosomal scanning present in eukaryotic organisms and therefore the physical implementation of these switches require slightly different design strategies. In bacteria, the sequence distance between the RBS and the start codon is relatively short and varies between 5 to 13 nucleotides (Kozak, 1999). As such, insertion of an aptamer targeted to interfere with ribosomal scanning through the formation of a ligand-induced secondary structure in the 5'UTR is generally not applicable in prokaryotes. In many bacteria, translation initiation relies on ribosomal accessibility to the RBS and the start codon located nearby, such that mRNA secondary structure in the translational initiation region may often times dictate the efficiency of translation (de Smit and van Duin, 1990a, de Smit and van Duin, 1990b, de Smit and van Duin, 1994). Accordingly, Suess *et al*. (Suess *et al*., 2004) engineered a synthetic riboswitch composed of a theophylline aptamer (Jenison *et al*., 1994) and a previously developed 'communication module' (Soukup and Breaker, 1999c), placed at a position proximal to the RBS. In earlier work this communication module was proposed to perform helix slipping by 1 nucleotide between the ligand-bound and unbound states (Soukup and Breaker, 1999c). In this design, the communication module served as a helix bridge between the aptamer and the RBS. This engineered riboswitch regulates gene expression in such a way that binding of theophylline to its aptamer causes a single-nucleotide shift in the communication module, which allows ribosome binding to the RBS without steric interference and thereby enables efficient translation in the presence of theophylline. Similar to this work, Desai and Gallivan (Desai and Gallivan, 2004) developed a synthetic riboswitch system in *Escherichia coli* targeting the RBS. In this system, the theophylline aptamer was placed at a location five base-pairs upstream of the RBS in a reporter gene and the theophylline-dependent gene expression regulation was screened through plate-based assays. A synthetic riboswitch activity that enhances the reporter gene expression was demonstrated in this study.

Regulation of gene expression at levels other than translation has also been demonstrated by using synthetic riboswitches. In a recent example, a riboswitch was designed to control pre-mRNA splicing by insertion of the theophylline aptamer

(Jenison *et al*., 1994) within the 3' consensus splice site region (Kim *et al*., 2005b) of a model pre-mRNA. With this system, the addition of theophylline was shown to repress i*n vitro* splicing of a model pre-mRNA harboring the aptamer. Furthermore, this theophylline-mediated splicing control system was demonstrated to be very specific to the target ligand. In another example, a riboswitch system was engineered to target transcriptional regulation (Buskirk *et al*., 2004), by coupling a tetramethylrosamine aptamer (Grate and Wilson, 1999, Baugh *et al*., 2000) to an RNA element or activation domain, previously evolved and demonstrated to activate transcription in *S. cerevisiae* (Buskirk *et al*., 2003). When localized to the promoter region, this riboswitch activates transcription *in vivo* in a *trans*-acting manner, where binding of the ligand to its aptamer facilitates the formation of the activation domain that can subsequently interact with a protein transcriptional activator, and leads to transcriptional activation.

Recent research has described engineered riboswitches that integrate aptamer domains with different regulatory platforms such as antisense RNAs and siRNAs. These synthetic riboswitches extend the mechanisms from which naturally-occurring riboswitches have been demonstrated to function thus far. These riboswitches and their corresponding design platforms are discussed in the following sections.

ANTISENSE RNAS REGULATE GENE EXPRESSION THROUGH BASE-PAIRING TO THEIR TARGET TRANSCRIPTS

(i) Mechanisms of regulation by antisense RNAs

The regulation of gene expression by an antisense RNA was first discovered as a natural process in prokaryotes (Green *et al*., 1986). Antisense RNA molecules are single-stranded, *trans*-acting ncRNAs, whose sequences are complementary to target transcripts and usually consist of 12-20 complementary nucleotides (Crooke, 2004). These complementary strands bind to their target mRNAs by base-pairing in a sequence-specific manner, thereby interfering with the expression of the gene encoded by the bound mRNA through one of the two mechanisms (Kurreck, 2003). The first mechanism involves targeting by double-stranded RNA-cleaving enzymes, such as the RNase III family of RNases (Figure 3A). The second mechanism involves the binding of the antisense molecules to their target mRNAs around the translation start site or the 5' cap region so that access or scanning of the ribosome to the target mRNA is inhibited, thereby interfering with the translation initiation process (Figure 3B).

(ii) Synthetic antisense RNAs and antisense RNA-based riboswitches

The discovery that antisense RNAs can inhibit gene expression in natural systems has lead to the development of synthetic control systems designed to regulate gene expression based on this mechanism of regulation. Antisense RNAs can be synthesized *in vivo* from a plasmid and have been shown to selectively target and inhibit a variety of genes of interest (Gottesman, 2002). For example, Blomberg *et al*. (Blomberg *et al*., 1992) and Gerdes *et al*. (Gerdes *et al*., 1997) separately demonstrated inhibition

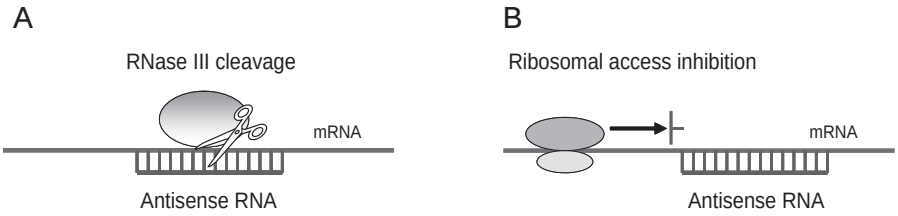

Figure 3. Two major mechanisms through which antisense RNA-mediated inhibition of gene expression can occur (adapted from Kurreck (Kurreck, 2003)). (A) Cleavage by an RNase III type nuclease induced by double-stranded RNA molecules. (B) Antisense-directed inhibition of translation by sterically impeding ribosomal loading or scanning on the target mRNA for proper translation.

of translation of a leader gene using antisense RNAs in bacteria. While antisense RNAs are normally designed to be shorter than 20 nucleotides, Bonoli *et al*. (Bonoli *et al*., 2006) employed a longer antisense RNA molecule complementary to the 5' UTR of a target mRNA and demonstrated effective antisense-based gene silencing in *S. cerevisiae*. Antisense RNAs have also been designed to silence gene expression through different mechanisms. For example, Novick *et al*. (Novick *et al*., 1989) demonstrated regulation of target gene expression by employing antisense RNAs that cause premature termination of the target transcripts by promoting the formation of a terminator hairpin upstream of the start codon. In the absence of the antisense RNA in this system, an upstream sequence hybridizes with part of the terminator hairpin, thereby preventing termination and enabling transcription of the target gene.

Antisense RNA molecules are powerful control elements for targeted gene expression regulation and have been demonstrated to function *in vivo*. Antisense technology is therefore potentially a useful tool for various biotechnological applications. Consequently, novel regulatory RNA elements that act as riboswitches have been developed using the antisense RNA platform to provide a more effective means of controlling gene expression. In one example, a bacterial riboswitch system (Figure 4A) was constructed in which the RBS or SD sequence was sequestered through a stem-loop structure encompassing an anti-SD sequence located 5' to the RBS within the same transcript, thus preventing ribosomal accessibility to the RBS (Isaacs *et al*., 2004). This *cis*-repression of translation was alleviated by introducing a small ncRNA transcribed from a separate inducible promoter and containing a complementary sequence. This *trans*-acting ncRNA targets and unfolds the stem-loop structure, thereby removing RBS sequestration from the *cis*-system and activating translation in response to the presence of the inducer molecule for the promoter regulating the expression of the *trans*-acting regulatory RNA.

More sophisticated antisense-based riboswitch systems, in which the actuator and sensor elements are located within a single molecular platform, have also been described. An autonomous, synthetic antisense-based riboswitch system (Figure 4B) was recently demonstrated by Bayer and Smolke (Bayer and Smolke, 2005). This synthetic riboswitch platform, termed the antiswitch, acts *in trans* to regulate expression of the target gene, and contains two domains: a ligand-binding domain and a gene regulatory domain. In this work, a switch platform was rationally constructed, in which the ligand-binding domain was coupled to the regulatory domain

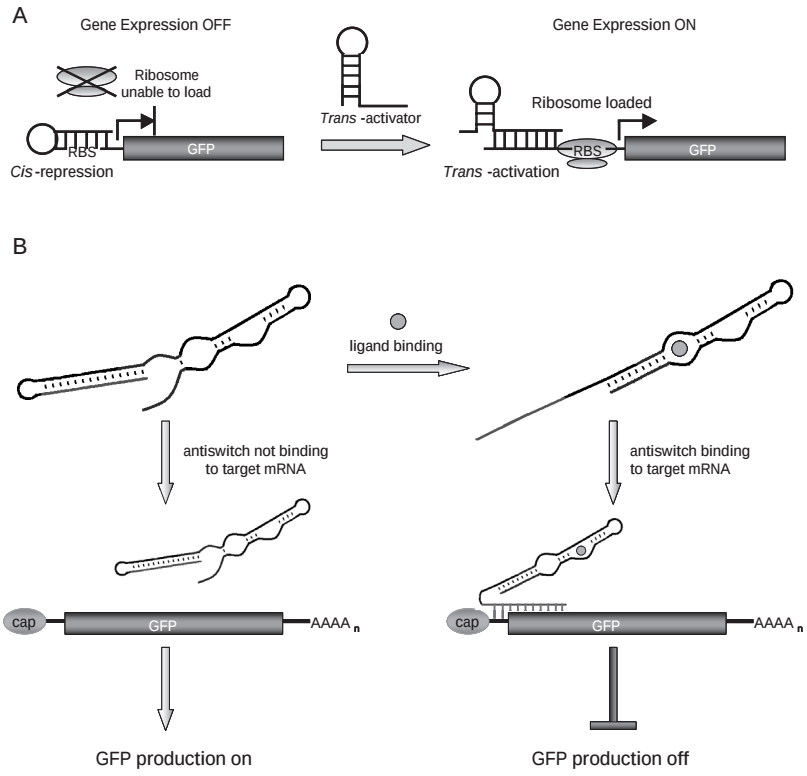

Figure 4. Examples of synthetic antisense-based riboswitch systems (adapted from Isaacs *et al*. (Isaacs *et al*., 2004), and Bayer and Smolke (Bayer and Smolke, 2005), respectively). (A) Target gene expression in bacteria, in this example GFP, is repressed in cis through sequestering the RBS by a complementary sequence segment forming a stem loop. This cis-repression is removed by introducing a trans-acting RNA molecule containing a sequence complementary to the cis-repressing sequence segment. Binding of the trans-acting RNA to its target sequence results in unfolding of the stem loop, thereby freeing the RBS to ribosomal loading. (B) Target gene expression in yeast, in this example GFP, is regulated by an OFF antiswitch molecule. In the absence of ligand the antisense domain is bound in the double-stranded region within the switch molecule referred to as the antisense stem and unable to bind to its target mRNA. In the presence of ligand, the antisense stem is disrupted due to ligand binding to the aptamer domain, enabling the antisense strand to become available to bind to the target mRNA and subsequently turn off the target gene expression. The ON antiswitch molecule employs a reverse mechanism to that of the OFF switch.

through a linker that translates the binding event within the former domain to a standardized, larger conformational change within the latter domain through a strand displacement strategy. The conformational change within the regulatory domain controls its ability to bind to its target mRNA. This standardized linking system provides a modular platform in which the aptamer and regulatory domains are uncoupled from one another to enable a plug-and-play type capability for the design of new switches. The modularity of the platform was demonstrated with theophylline and tetracycline aptamers as the ligand-binding domains and antisense strands complementary to different target reporter genes as the regulatory domains.

In addition to the demonstrated modularity of this system, this platform also provides flexibility and programmability in tailoring the properties of new switches. Two regulatory platforms, so-called OFF and ON switches, were characterized. In an OFF platform, in the absence of ligand the antisense strand is sequestered in a hairpin antisense stem within the switch molecule and therefore unable to bind its target mRNA. In the presence of ligand the antisense stem is disrupted as the ligand binds to the aptamer, releasing the antisense strand such that it can bind the target mRNA and subsequently 'turn off' gene expression. The ON switch design employs a reverse mechanism to that of the OFF switch. The antisense strand of an ON switch is available to bind its target mRNA in the absence of the ligand, whereas in the presence of the ligand most of the antisense domain is sequestered in the formation of the aptamer base stem, thereby 'turning on' gene expression. In addition, the concentration-responsiveness of these switch platforms was demonstrated to be tunable across wide concentration ranges. This work presented some of the first efforts to build standardized synthetic riboswitch platforms that due to their modularity, programmability, and tunability can be used as general platforms on which to build tailor-made synthetic riboswitches to particular applications.

RIBOZYMES ARE CATALYTIC RNAS CAPABLE OF REGULATING GENE EXPRESSION

Functional characteristics of ribozymes

Ribozymes are catalytic RNAs that were first discovered over twenty years ago (Zaug and Cech, 1980, Cech *et al*., 1981, Kruger *et al*., 1982, Guerrier-Takada *et al*., 1983, Symons, 1992). They are naturally found in plant RNA viruses, satellite RNAs, and viroids (Forster *et al*., 1987) and most commonly catalyze the cleavage and/or ligation of RNA molecules (Sun *et al*., 2000). There are several types of natural ribozymes, and four of them are classified as self-cleaving ribozymes (Doudna and Cech, 2002): hammerhead, hairpin, hepatitis delta virus, and Varkud satellite. Self-cleavage occurs at a specific phosphodiester bond via an internal phosphoester transfer reaction (Tang and Breaker, 2000) in a divalent metal ion-dependent manner where Mg^{2+} is commonly used in most studies (Birikh *et al*., 1997). Among these naturally-occurring ribozymes, the hammerhead ribozyme has been extensively studied and most commonly used for its potential significance in targeting messages in biotechnological and medical applications due to its small size, ease of design, and rapid kinetics (Jen and Gewirtz, 2000, Sun *et al*., 2000). Each class of natural ribozymes exhibits a highly conserved catalytic core sequence (Figure 5). Hammerhead ribozymes are generally known to cleave any 5'-NUX-3' triplets of an RNA sequence 3' to the X where N is any nucleotide, U is uridine, and X is any nucleotide except guanosine. The sequence 5'-GUC-3' has been the conventional and most commonly found cleavage triplet in nature (Vaish *et al*., 1998, Birikh *et al*., 1997).

In natural contexts, two of the hammerhead ribozyme's stems are each closed by a loop of nucleotides, and the majority of the hammerhead ribozymes are embodied within the viral RNA sequences through stem III (Khvorova *et al*., 2003) (Figure 5A). The presence of loops closing stem I and stem II has been shown to be critical to the *in vivo* catalytic activity of the hammerhead ribozymes in native sequences, suggesting possible loop I-loop II interactions (Khvorova *et al*., 2003, De la Pena *et*

al., 2003, Blount and Uhlenbeck, 2005). Therefore, hammerhead ribozymes catalyze self-cleavage intramolecularly or *in cis* in nature. However, genetic manipulations have been performed to generate ribozymes that cleave intermolecularly or *in trans* by eliminating one of the two stem loops. Such manipulations result in three possible designs, depending on which strands of the stems are targeted to bind the substrate transcript for cleavage (Birikh *et al*., 1997). For instance, one of these designs may be constructed, in which the loop sequence of stem I is removed, and stem I and stem III strands are used as targeting arms to bind a transcript (Figure 5B).

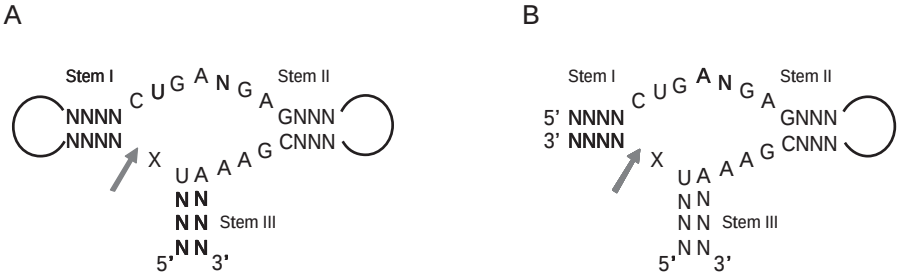

Figure 5. Structures of (A) a self- or cis-cleaving hammerhead ribozyme (adapted from Salehi-Ashtiani and Szostak (Salehi-Ashtiani and Szostak, 2001)) and (B) a trans-cleaving ribozyme. The conserved catalytic core sequence is shown and N represents any nucleotide. The arrow indicates the cleavage site. The self-cleaving ribozyme contains stems I and II enclosed by loop sequences and therefore the cleavage is intramolecular, whereas the trans-cleaving ribozyme contains only one of the stems enclosed by a loop and therefore the cleavage is intermolecular. Non-conserved loop sequences are illustrated in black circular lines.

(ii) Synthetic ribozymes and design strategies for ribozyme-mediated gene expression regulation

Ribozymes are attractive tools for their potential applications in silencing gene expression *in vivo* through their catalytic activities. For such applications, a target gene may be regulated through one of two different mechanisms: *in cis* or *in trans*. For *cis*-acting regulation systems, a hammerhead ribozyme can be integrated into a non-coding region of the target transcript, resulting in self-cleavage of the transcript and thereby inactivating expression of the target transcript by significantly lowering its half-life through rapid degradation by nucleases. Alternatively, *trans*-acting ribozymes can regulate gene expression through identical mechanisms, but they must be engineered through design strategies that satisfy the natural requirements for their catalytic activities. The basic strategy is to target regions of specific transcripts that harbor an 'NUX' triplet to define the cleavage site on the transcript and to design a ribozyme carrying the catalytic core and targeting arm sequences that are complementary to the appropriate region of the target transcript for sequence-specific base-pairing (Symons, 1992) (Figure 5B). Self-cleaving hammerhead ribozymes with different catalytic activities have been generated through *in vitro* selection processes (Eckstein *et al*., 2001, Salehi-Ashtiani and Szostak, 2001). These ribozymes may be potentially useful for regulating the expression level of certain heterologous genes, as their use may result in different levels of gene expression due to their different

cleavage activities. Similarly, *trans*-cleaving hammerhead ribozyme variants exhibiting varying cleavage activities have been developed *in vitro* (Ishizaka *et al*., 1995, Conaty *et al*., 1999, Persson *et al*., 2002). The *in vivo* activities of both *cis*- and *trans*-cleaving hammerhead ribozymes have been demonstrated where gene expression of various target genes was regulated through cleavage (Marschall *et al*., 1994, Fujita *et al*., 1997, Kawasaki *et al*., 1996, Khvorova *et al*., 2003, Sakamoto *et al*., 1996, Gavin and Gupta, 1997, Kijima *et al*., 1998, Kim *et al*., 1999).

(iii) Ligand-responsive ribozymes and ribozyme-based riboswitches

As ribozymes have proven to be a powerful platform for controlling gene expression, several research groups have engineered a special class of synthetic ribozymes called allosteric ribozymes (Tang and Breaker, 1997, Araki *et al*., 1998, Soukup and Breaker, 1999a, Soukup and Breaker, 1999b, Soukup *et al*., 2000, Kertsburg and Soukup, 2002). This class of synthetic ribozymes may represent tools that can provide a better control over gene expression. Allosteric ribozymes resemble allosteric enzymes in that the cleavage activities of the former are also modulated through binding of specific effectors, often small molecule ligands (Soukup and Breaker, 1999c). An allosteric ribozyme must contain two separate domains, a catalytic domain and a ligand-binding domain, or an aptamer domain, which interact in a ligand-dependent manner to control the catalytic activity of the molecule (Soukup and Breaker, 1999c). Thus, the allosteric property of these ribozymes enables their catalytic activity to be regulated through specific ligands and thus may represent a modular design platform, which can directly make use of different ligand-aptamer pairs. The hammerhead ribozyme has been the most widely used catalytic motif for the development of allosteric ribozymes (Soukup and Breaker, 1999c).

Allosteric ribozymes have been generated using different engineering strategies that include rational design strategies, library screening design strategies, and combinations thereof (Soukup and Breaker, 1999c, Wilson and Szostak, 1999). Rational design strategies involve integration of a known ligand-binding or aptamer domain directly to the catalytic domain of the ribozyme through different linkers followed by examination of their resulting activities (Tang and Breaker, 1997, Araki *et al*., 1998, Soukup and Breaker, 1999a), whereas library screening design strategies utilize *in vitro* selection procedures and screening for novel ligand-binding domains that function allosterically with the attached catalytic domain (Koizumi *et al*., 1999, Piganeau *et al*., 2001). Finally, allosteric ribozymes have been developed with a combined approach in which an existing ligand-binding domain is coupled to the catalytic domain of the ribozyme through a randomized linker, and allosterically functional linkers, so-called 'communication modules' (Figure 6), are then screened from a combinatorial library of sequences (Soukup and Breaker, 1999b, Soukup *et al*., 2000, Kertsburg and Soukup, 2002). The majority of the synthetic allosteric ribozymes constructed to date are responsive to small molecule ligands that bind specifically to the aptamer domains within these molecules such as theophylline (Jenison *et al*., 1994, Soukup and Breaker, 1999b), adenosine triphosphate (ATP) (Sassanfar and Szostak, 1993, Tang and Breaker, 1997), and flavin mononucleotide (FMN) (Burgstaller and Famulok, 1994, Araki *et al*., 1998). While tremendous success

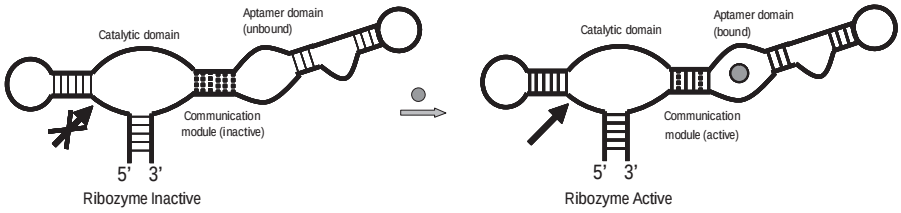

Figure 6. A schematic diagram of an allosteric ribozyme whose activity is regulated by a specific ligand. The ligand-binding or aptamer domain is coupled to the catalytic domain through a communication module shown in dotted lines. In the absence of ligand the ribozyme primarily adopts the inactive conformation (left), whereas binding of the ligand to the aptamer, translated to the catalytic domain through the communication module via a helix slipping event, favors the ribozyme-active conformation (right), such that the observed cleavage activity of the allosteric ribozyme increases as a function of the ligand concentration.

has been achieved in engineering these allosteric hammerhead ribozymes, their *in vitro* functional activities have not yet been translated to *in vivo* environments to date. Allosteric hammerhead ribozymes with *in vivo* functionality are highly attractive gene control elements for their general applicability to a broad range of organisms, as their regulatory mechanism through cleavage does not require cell-specific machinery and thus represent a universal platform for controlling gene expression.

While most allosteric ribozymes are synthetic and have been generated through various engineering approaches, recently a naturally-occurring allosteric ribozyme was discovered in gram-positive bacteria (Winkler *et al*., 2004). The ribozyme is embedded within the *glmS* gene, and its self-cleavage activity is induced by binding of the metabolite glucosamine-6-phosphate (GlcN6P), which is the metabolic product of the GlmS enzyme. This ribozyme functions to repress expression of the *glmS* gene product in response to increasing concentrations of GlcN6P, thereby serving as a metabolite-responsive genetic switch that exhibits metabolite sensing-actuation capabilities.

Shortly after the discovery of the naturally-occurring GlcN6P-responsive ribozyme-based riboswitch in bacteria, a drug-responsive ribozyme-based regulatory system was demonstrated in mammalian cells and animal models (Figure 7) (Yen *et al*., 2004). This system was constructed with a series of different hammerhead ribozymes incorporated into the 5' or 3' UTRs of a reporter gene. These constructs were screened for cleavage inhibition against a variety of small molecule and oligonucleotide effectors. Although this ribozyme system does not contain a distinct ligand-binding domain as opposed to its natural counterpart, several small molecules were shown to suppress the self-cleavage activity of the ribozyme and thereby allow expression of the reporter gene in the presence of these effector molecules (Yen *et al*., 2004, Yen *et al*., 2006). This early example has demonstrated that ribozymes can be used to modulate gene expression in a ligand-controlled manner *in vivo* and represents an initial step towards the development of allosteric ribozyme platforms that contain distinct, modular ligand-binding domains.

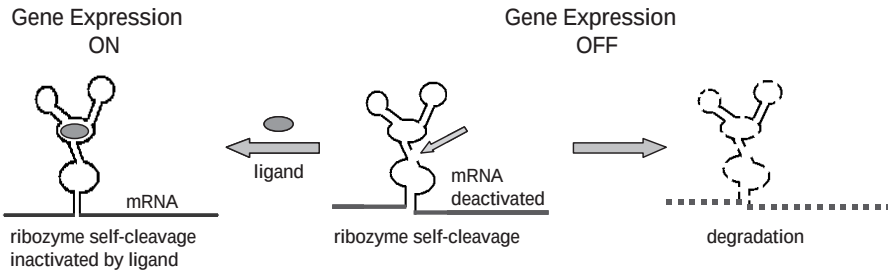

Figure 7. A schematic diagram of a self-cleaving ribozyme, inserted into the 5' UTR of an mRNA, whose cleavage activity is regulated by a specific drug molecule (adapted from Yen *et al*. (Yen *et al*., 2004)). In the absence of the drug molecule, the ribozyme is active and resulting in cleavage of the transcript (middle), thereby inhibiting the expression of the target gene through subsequent mRNA decay (right). This cleavage activity is inhibited in the presence of the small molecule drug (left).

RNA INTERFERENCE IS AN RNA-GUIDED SEQUENCE-SPECIFIC GENE-SILENCING MECHANISM

(i) Mechanisms of the RNA interference pathway

RNA interference (RNAi) is a highly evolutionally conserved gene-silencing pathway (Sharp, 2001) and is a mechanism for RNA-directed silencing of target gene expression. This pathway was originally observed in plants (Napoli *et al*., 1990, van der Krol *et al*., 1990), where it was referred to as post-transcriptional gene silencing (PTGS), but first demonstrated by Fire and Mello in the nematode worm *Caenorhabditis elegans* (Fire *et al*., 1998), and later found in a wide variety of organisms, including mammals (Meister and Tuschl, 2004). This silencing pathway is triggered by double-stranded RNAs (dsRNAs), which cause sequence-specific degradation of target transcripts when introduced into a cell (Figure 8) (Dykxhoorn *et al*., 2003). dsRNAs can be generated from numerous natural sources including viruses, overlapping transcripts, and transposons (Meister and Tuschl, 2004, Bartel, 2004). Once in the cell, these dsRNAs, the substrates of RNAi, are recognized by a dsRNA-specific RNase III-type enzyme called Dicer (Bernstein *et al*., 2001) and cleaved into short RNA duplexes known as small-interfering RNAs (siRNAs). siRNAs are generally 21-23 nucleotide-long dsRNAs with 2-nucleotide overhangs at the 3' ends with 5' phosphate and 3' hydroxyl groups (Dykxhoorn *et al*., 2003, Meister and Tuschl, 2004). The cleaved products of dsRNAs are subsequently unwound and incorporated into endoribonuclease-containing complexes known as RNAi-induced silencing complexes (RISCs) (Hammond *et al*., 2000). RISC is a multiple-turnover RNAi enzyme complex (Hutvagner and Zamore, 2002), and the functional RISC contains only the antisense strand in the complex. This bound antisense strand is then guided to the target mRNA by RISC where the two RNA strands hybridize in a sequence-specific manner, which subsequently leads to the ATP-dependent cleavage of the target mRNA by endonucleases at a single site near the center of the siRNA strand with regular intervals of 21-23 nucleotides (Elbashir *et al*., 2001b, Zamore *et al*., 2000).

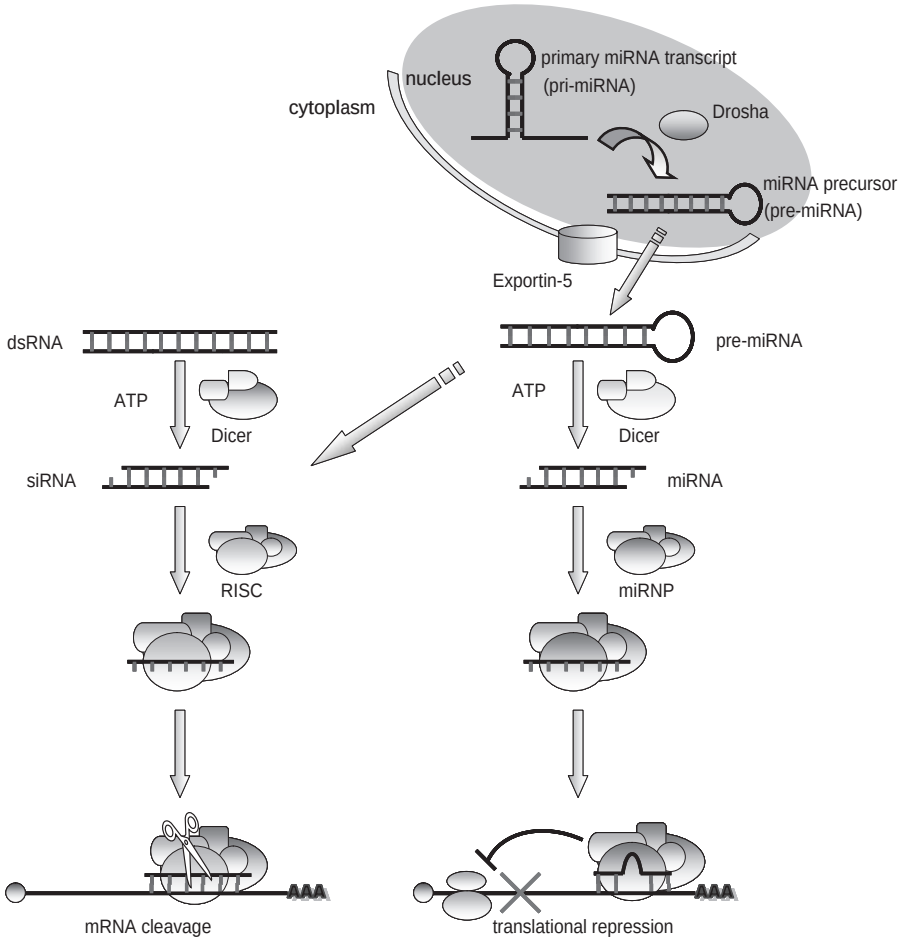

Figure 8. Overview of the RNA interference pathway (adapted from Meister and Tuschl (Meister and Tuschl, 2004)). dsRNAs introduced artificially or generated inside the cell can induce sequence-specific gene silencing through the RNAi pathway. These molecules are initially processed into siRNA duplexes by Dicer. This processing event is followed by unwinding of these duplexes and incorporation of one of the duplex strands into RISC. The functional RISC contains the antisense strand of the duplex, which mediates gene silencing of the target mRNA through cleavage. Primary miRNA transcripts (pri-mRNAs) are processed by Drosha in the nucleus to yield miRNA precursors (pre-miRNAs), which are exported into the cytoplasm by Exportin-5. Once in the cytoplasm, the pre-miRNA is further processed by Dicer to yield siRNA-like duplexes known as miRNAs. The duplex is unwound, assembled into miRNP/RISC, and regulates translational repression or cleavage of the target mRNA depending on the degree of complementarity between the miRNA strand and its targeting transcript sequence.

(ii) The RNAi-associated microRNA (miRNA) pathway

RNAi was initially recognized as an innate defense mechanism of host organisms to RNA viruses and transposable elements (Waterhouse *et al*., 2001, Meister and Tuschl, 2004). However, certain genes in plants and animals naturally encode RNA hairpins

(Lee *et al*., 1993, Reinhart *et al*., 2000, Mello and Conte, 2004) that are about 70 nucleotides in length (Dykxhoorn *et al*., 2003, Bartel, 2004) and composed of complementary or nearly complementary inverted repeats (Meister and Tuschl, 2004, Novina and Sharp, 2004). These endogenous RNA hairpins are the precursors of another class of small RNA molecules known as microRNAs (miRNAs) and have been shown to be processed by Dicer and lead to sequence-specific gene silencing (Figure 8) (Hutvagner *et al*., 2001, Ketting *et al*., 2001, Grishok *et al*., 2001). Prior to processing by Dicer in the cytoplasm, the first step in maturation of miRNAs is the nuclear cleavage of the primary hairpin transcript (pri-miRNA) (Lee *et al*., 2002b, Lee *et al*., 2003) by a second RNase III-type endonuclease called Drosha (Lee *et al*., 2003). Drosha cleaves both strands of the hairpin stem at sites close to the base, and the cleaved hairpin has a 5' phosphate and a 2-nucleotide 3' overhang remaining at the base of the stem (Lee *et al*., 2003, Basyuk *et al*., 2003). This cleaved hairpin product is known as the pre-miRNA, which is then exported to the cytoplasm by the RanGTP-dependent nuclear export receptor, Exportin-5 (Yi *et al*., 2003, Lund *et al*., 2004, Bohnsack *et al*., 2004). Once in the cytoplasm, the pre-miRNA is processed by Dicer, yielding an siRNA-like imperfect duplex of about 21-22 nucleotides in length that has 5' phosphates and 2-nucleotide 3' overhangs (Meister and Tuschl, 2004, Bartel, 2004). Similar to siRNAs, these miRNA duplexes are subsequently unwound and loaded into ribonucleoprotein (RNP) complexes, denoted as miRNPs (Mourelatos *et al*., 2002). Once loaded into the miRNP, the single-stranded miRNA can direct silencing of gene expression through two different mechanisms: mRNA cleavage or translational repression (Bartel, 2004, Meister and Tuschl, 2004). The miRNA-guided cleavage mechanism is similar to that of siRNAs, whereas the miRNA-directed inhibition of productive translation occurs through imperfect complementary binding of miRNAs to their target mRNAs in the 3' UTR (Meister and Tuschl, 2004).

(iii) Synthetic siRNA and miRNA-based control systems for gene silencing

Researchers have taken advantage of the effectiveness of RNAi-mediated gene silencing and the conserved nature of the pathway to build synthetic regulators that silence genes through this endogenous pathway. Small dsRNAs have been engineered through a variety of methods (Dykxhoorn *et al*., 2003) to down-regulate expression of various target genes. *In vitro*-synthesized dsRNAs have been introduced into cells and shown to be processed by Dicer into siRNAs, which subsequently caused silencing of target gene expression (Zamore *et al*., 2000, Elbashir *et al*., 2001b). It has also been demonstrated that when chemically-synthesized siRNAs are directly introduced into cells they effectively suppress gene expression by bypassing the Dicer cleavage event and loading directly into the RISC complex (Elbashir *et al*., 2001a, Caplen *et al*., 2001). This strategy eliminates potential induction of the interferon response by dsRNAs longer than 30 nucleotides (Elbashir *et al*., 2001a). However, other studies have demonstrated that slightly larger RNA duplexes (~27-mers) can effectively target sites that are refractory to silencing by 21-mer siRNAs and silence gene expression without inducing the interferon response (Kim *et al*., 2005a). Synthetic siRNAs can also be expressed *in vivo* from RNA polymerase III (pol III) promoters in tandem, where individual sense and antisense strands of each siRNA are separately produced and associate *in trans* thereafter. Such siRNAs, synthesized and assembled

intracellularly, have been demonstrated to silence target gene expression *in vivo* (Miyagishi and Taira, 2002, Lee *et al*., 2002a).

As synthetic RNAi substrates gain recognition as powerful research and application-based tools in regulating gene expression and as our understanding of the mechanism of this pathway deepens, the molecular design strategies guiding the construction of synthetic RNAi substrates have evolved. As an example, short hairpin RNAs (shRNAs) with perfect base-pairing within the stems have been constructed to produce Dicer-processable substrates through exogenous or endogenous transcription events, and have been demonstrated to induce efficient gene silencing *in vivo* (Brummelkamp *et al*., 2002, Paddison *et al*., 2002, Yu *et al*., 2002, Paul *et al*., 2002). In addition, shRNAs that contain imperfect base-pairing or nucleotide bulges within their stems have been constructed to mimic precursors of the naturally-occurring miRNAs. These imperfect hairpins are processed by Dicer in the cell to duplexes, the antisense strands of which are fully complementary to the target genes. When expressed in cells from a pol III promoter, these miRNA precursor-like hairpins are capable of down-regulating the expression of targeted genes (McManus *et al*., 2002, Paddison *et al*., 2002). Finally, miRNA-based hairpins encompassing target sites with either perfect or imperfect complementarity to their target transcripts have been constructed and expressed *in vivo* as part of longer transcripts from an RNA polymerase II (pol II) promoter. Compared to earlier designs, this implementation more closely mimics that of natural miRNAs, as miRNA precursors are usually encoded within the context of longer transcripts. These synthetic RNAi substrates have been shown to silence the expression of target genes, indicating that synthetic miRNA precursors can be engineered to be excised from longer transcripts, resulting in the production of regulatory miRNAs that target desired gene sequences (Zeng *et al*., 2002).

(iv) Synthetic ligand-regulated RNAi substrates

Naturally-occurring riboswitches that control gene expression by regulating the efficiency of post-transcriptional processes by mediating mechanisms that act through RNA-RNA base-pairing interactions, such as formation of a terminator stem, sequestering the RBS (antisense), or transcript processing through RNA cleavage (ribozyme) upon binding of small molecule ligands have been described. However, naturally-occurring riboswitch or riboswitch-like systems have not yet been identified that regulate gene silencing through the RNAi pathway. A ligand-regulated shRNA molecule (An *et al*., 2006) was recently described to modulate RNAi-based gene silencing in mammalian cells through a small molecule ligand-aptamer interaction within the RNAi substrate molecule. In this example, the theophylline aptamer (Jenison *et al*., 1994) was directly incorporated into the loop region of an shRNA molecule targeting a fluorescent reporter protein to regulate the Dicer processing step of this shRNA. When this shRNA construct was expressed in mammalian cells, silencing of the reporter gene was inhibited in a theophylline dose-dependent manner. Cleavage inhibition of the aptamer-fused shRNA for subsequent generation of the siRNAs was also demonstrated *in vitro* and observed *in vivo* to be regulated by theophylline. Although this regulatory molecule does not involve an apparent conformational change within the molecule itself through previously described mechanisms such as strand displacement or helix slipping, theophylline-mediated Dicer-cleavage inhibition was

likely achieved due to locating the ligand-binding site of the aptamer sufficiently close to the Dicer processing site such that binding of theophylline to its aptamer blocks Dicer cleavage, thereby demonstrating a regulatable siRNA-directed gene silencing in mammalian cells.

Synthetic RNA molecules for applications in biosensing and therapeutic strategies

The successful application of various engineered RNA-based control systems in regulating gene expression events in diverse organisms has demonstrated that synthetic RNA molecules can provide very powerful regulatory tools. The employment of diverse regulatory platforms and the implementation through different mechanisms to achieve specific control over expression levels have exhibited the design flexibility and functional versatility of RNA in biological design. These properties underlie the utility of RNA-based devices in other biotechnological applications such as biosensors and therapeutic molecule design.

BIOSENSOR APPLICATIONS OF SYNTHETIC RNA APTAMERS AND RNA-BASED GENETIC SWITCHES

RNA aptamers are excellent candidates for sensing domains in the design of molecular switches for both *in vitro* and *in vivo* biosensing applications (Rimmele, 2003). As previously described RNA possess unique sensing capabilities in the aptamer domains of riboswitches, which exhibit precise target recognition and high binding affinities for their specific target ligands. Advances in nucleic acid screening technologies have enabled researchers to synthetically generate RNA aptamers to novel ligands with binding properties comparable to those of the naturally-occurring aptamer domains through standard *in vitro* selection strategies. In addition, aptamers have been selected to a wide range of target ligands and have been demonstrated to exhibit binding affinities and specificities that rival those of antibodies (Rimmele, 2003, Jayasena, 1999, Bunka and Stockley, 2006). As a result, aptamers have been proposed as alternatives to antibodies in a variety of diagnostic applications (Jayasena, 1999, Bunka and Stockley, 2006, Rimmele, 2003).

Synthetic RNA aptamers have been implemented as sensing elements in various *in vitro* diagnostic assay formats (Jayasena, 1999). In one recent example, the vesicular endothelial growth factor (VEGF)-binding aptamer labeled with fluorescein at its 5' end was used in an enzyme-linked immunosorbent assay (ELISA) in the place of the primary antibody to capture the analyte growth factor and detected with an anti-fluorescein Fab fragment conjugated to alkaline phosphatase, which serves as a reporter enzyme (Drolet *et al*., 1996). RNA aptamers have also been used as analytical probes in flow cytometry, where fluorescein-labeled human CD4-binding RNA aptamers were demonstrated to stain human CD4 expressed on the cell surface in a manner similar to anti-CD4 antibodies (Davis *et al*., 1998). An RNA aptamer with affinity and specificity for human immunodeficiency virus 1 (HIV-1) Tat protein has also been used as a biorecognition element in the development of novel biosensor assays, which make use of piezoelectric quartz crystals or surface plasmon resonance

(SPR) detection technologies (Tombelli *et al*., 2005). RNA aptamers labeled with 'smart dyes' have also been examined for biosensor applications as 'signaling aptamers' that change fluorescence intensity levels through structural rearrangements upon binding of an analyte (Jhaveri *et al*., 2000, Rajendran and Ellington, 2002). Other research has also described RNA aptamer-based biosensor arrays for the detection, quantification, and screening of diverse biological macromolecules (McCauley *et al*., 2003, Collett *et al*., 2005).

Another class of biosensors has been described based on the application of allosteric ribozymes as the sensing components of the biosensor. In the design of this class of biosensors, termed aptazymes, aptamers are coupled to ribozymes in a manner that provides concentration-dependent ligand regulation over the catalytic activity of the ribozyme domain (Hesselberth *et al*., 2003). An array of aptazyme ligases was recently described in which the sensors were activated in the presence of their specific analytes (Hesselberth *et al*., 2003). In another example, a small sensor array was constructed with seven different aptazymes using the hammerhead ribozyme and employed for the detection of corresponding target analytes present in a complex mixture (Seetharaman *et al*., 2001). Recent work has also described that catalytic signals received from such aptazyme sensors can be further amplified into fluorescence signals by exploiting fluorescence resonance energy transfer (FRET) (Rueda and Walter, 2006). All these studies described above highlight the success of using RNA molecules for *in vitro* biosensor applications.

In addition to their successful application as *in vitro* biosensor components, RNA molecules also have potential utility for *in vivo* biosensor applications. The various engineered riboswitches discussed previously provide examples of potential candidate molecules that can be employed as *in vivo* biosensors. In these engineered systems, different actuation domains such as antisense RNAs and siRNAs have been utilized to translate a target recognition event (an input) at the sensor domain of the biosensor to the modulation of specific gene expression events, which can then be detected as an output signal. In other words, target sensing events can be detected as changes in the expression patterns of specific reporter genes through synthetic riboswitch systems. Therefore, engineered riboswitches may represent platforms for the development of RNA-based *in vivo* biosensors.

APPLICATIONS OF RNA MOLECULES IN NEW THERAPEUTIC STRATEGIES

RNA is attractive as a therapeutic molecule since it is non-immunogenic, unlike its protein counterparts, monoclonal antibodies, which are often immunogenic (Jayasena, 1999). In addition, inherently less immunogenic antibodies against particular targets of interest are difficult to raise, while aptamers can be selected against potentially any target ligand using a standardized procedure. RNA aptamers have been generated against numerous disease-related targets and employed to inhibit the activities of these targets (Rimmele, 2003). For example, RNA aptamers have been selected against various HIV-associated proteins and used as antiviral agents in blocking the functional activities of these proteins and thus viral replication (Joshi *et al*., 2003, Held *et al*., 2006). A novel RNA aptamer with high binding affinity and specificity for the Tat protein of HIV-1, a protein responsible for the expression of HIV-1 genes, was

demonstrated to compete with the virally-encoded TAR RNA sequence in binding Tat and thereby prevent Tat-dependent *trans*-activation of the viral RNA both *in vitro* and *in vivo* (Yamamoto *et al*., 2000). In separate examples, RNA aptamers against HIV-1 reverse transcriptase has been demonstrated to inhibit the activity of this protein in *E. coli* (Nickens *et al*., 2003) and serve as powerful antiviral activity in human T lymphoid cells by binding to the protein target and interfering with its replication activity (Chaloin *et al*., 2002). A number of RNA aptamers have also been generated against the HIV-1 nucleocapsid protein, a protein that plays an important role in the encapsidation of viral RNA and assembly of viral particles (Kim *et al*., 2002, Kim and Jeong, 2003). When one of these aptamers was expressed in a T cell line, the aptamer was shown to inhibit the packaging of viral genomic RNA (Kim and Jeong, 2004). Another pool of RNA aptamers that bind gp120, the viral surface glycoprotein that facilitates virus entry into cells, were also shown to neutralize HIV-1 infectivity in human peripheral blood mononuclear cells (Khati *et al*., 2003).

Similar to these HIV-targeting aptamers, a variety of RNA aptamers have been developed against regulatory proteins of hepatitis C virus (HCV), such as the non-structural protein 3 (NS3) (Fukuda *et al*., 1997, Urvil *et al*., 1997, Kumar *et al*., 1997, Fukuda *et al*., 2000, Hwang *et al*., 2004, Nishikawa *et al*., 2004). Nishikawa *et al*. (Nishikawa *et al*., 2003) demonstrated inhibition of the functional activity of the HCV NS3 protease *in vivo* by expressing NS3 protease-specific aptamers in HeLa cells. These examples have demonstrated that synthetic RNA aptamers have the potential to serve as effective antiviral agents, capable of interfering with the functional activities of various viral proteins critical to viral replication and infection.

In addition to potentially serving as potent antiviral agents, RNA aptamers have been demonstrated to function as potential therapeutic agents for other diseases including cancer. For example, NX 1838 is a nuclease-resistant RNA aptamer against VEGF-165, a major factor in angiogenesis, tumor proliferation, and vascular permeability (Bell *et al*., 1999). This aptamer was observed to exhibit an inhibitory effect on VEGF-induced cell proliferation (Bell *et al*., 1999). When the VEGF-165-specific aptamer was delivered to mice injected with Wilms tumor cells, this aptamer was shown to efficiently suppress tumor growth in the aptamer-treated mice compared to the non-treated animals with no observed adverse effects (Huang *et al*., 2001).

RNA aptamers have also been developed for cancer immunotherapy applications. In one example, high affinity and specificity aptamers were generated against cytotoxic T lymphocyte antigen-4 (CTLA-4) expressed on activated T cells (Santulli-Marotto *et al*., 2003). CTLA-4 is involved in the attenuation of signals required for T-cell activation. In this study, these aptamers were demonstrated to inhibit the antigen's function *in vitro* and enhance tumor immunity in mice, serving as potent agents for *in vivo* immunogenic therapeutic applications. In another example, RNA aptamers that bind prostate cancer cells through the extracellular portion of the prostate-specific membrane antigen (PSMA) have also been selected and proposed as prostate-specific cell markers (Lupold *et al*., 2002). Such aptamers have been employed in cell-type specific delivery applications, in which a PSMA-binding aptamer was fused to an siRNA targeting the expression of survival genes (McNamara *et al*., 2006). When applied to cells expressing PSMA, these fused RNA molecules were internalized and processed by Dicer, thereby releasing siRNAs and subsequently down-regulating the survival gene expression levels, resulting in increased cell death. No increases in cell death were observed in cell populations that do not express PSMA.

Many other examples exist of RNA aptamers that have been generated and examined for potential therapeutic effects. One example is the development of RNA aptamers against coagulation factor IXa by Rusconi *et al*. (Rusconi *et al*., 2002). Factor IXa is a protein involved in the production of thrombin, which converts soluble fibrinogen to fibrin, the protein that polymerizes to form a blood clot (Tuddenham, 2002). These aptamers have been shown to act as potent anticoagulants. Rusconi and colleagues have also demonstrated that this anticoagulant effect is reversible by applying oligonucleotides complementary to these aptamers, which act as efficient antidotes in human plasma. This work also provides an example of utilizing synthetic RNA aptamers as regulatable therapeutic molecules.

The application of RNA aptamers as therapeutic agents is not limited to the examples provided above. There are numerous RNA aptamers that have been selected for therapeutic applications, and RNA aptamer-based therapeutic and clinical applications have been described in numerous reviews (Read *et al*., 2003, Proske *et al*., 2005, Pestourie *et al*., 2005, Ulrich, 2005, Becker and Becker, 2006). There are examples of ribozymes and antisense RNAs that are also in preclinical and clinical trials (Kurreck, 2003), demonstrating the considerable potential of these RNA molecules in therapeutics. The more-recently discovered siRNAs have also gained tremendous interest as therapeutic molecules and have been demonstrated as potential therapeutic agents targeting various cancerous genes (Butz *et al*., 2003, Liu *et al*., 2004, Ito *et al*., 2005, Wang *et al*., 2005, Kakar and Malik, 2006, Duxbury *et al*., 2004, Ameyar-Zazoua *et al*., 2005, Devi, 2006). Engineered RNA devices have broad applications in biotechnology, beyond the examples in therapeutics and biosensors cited here, including metabolic engineering (Smolke *et al*., 2001, Allen *et al*., 2004, Pfleger *et al*., 2006), antibiotics research (Patel and Suri, 2000, Tereshko *et al*., 2003, Jiang *et al*., 1999, Cowan *et al*., 2000), and studies of cellular signal transduction pathways (Seiwert *et al*., 2000, Kimoto *et al*., 2002).

Challenges and future directions

As an increasing number of functional roles of RNA as a regulatory element have been discovered over the past several years, the engineering of RNA regulators has expanded as a field, in large part due to its broad potential for widespread application in medicine and biotechnology. However, RNA engineering is still an emerging field and thus presents a number of challenges, in particular in translating the instances of RNA devices that have been demonstrated by various research laboratories into frameworks and infrastructures for their widespread implementation for *in vivo* applications.

First, research that focuses on elucidation of the general design principles for constructing ligand-regulated RNA devices is necessary for building a framework for researchers to work in this area. Research to date has largely focused on the generation of specific instances of RNA devices, which do not necessarily guide researchers in how to translate that particular instance into other instances useful for specific systems or applications. Often times the activity of these RNA devices, is dependent on the particular system in which it was developed, including sequences immediately surrounding the regulator element, and modularity is not maintained. In order to translate RNA engineering into a broadly applicable field, modular molecular

platforms, on which libraries of instances of RNA regulators can be rapidly constructed and which are functionally independent of the particular system in which they are employed, are required.

Second, large libraries of well-characterized aptamers to molecular targets useful for *in vivo* sensing and control applications must be generated. While the synthetic RNA aptamers generated to date exhibit powerful sensing capabilities and target specificities, they are usually generated *in vitro*, under substantially different environments from that which would be found *in vivo*. Therefore, the binding and specificity properties of a significant portion of the available RNA aptamers may not be translated directly when employed for *in vivo* applications. In addition, although there are numerous aptamers generated to bind a wide variety of ligands, many of their target ligands, particularly small molecules, are not well-suited for therapeutic use or other *in vivo* applications. Specifically, many of the most well-characterized aptamers are to ligands that are toxic to cells at relatively low concentrations or do not cross the cell membrane efficiently such as theophylline (Jenison *et al*., 1994), tetracycline (Berens *et al*., 2001), codeine (Win *et al*., 2006), and tobramycin (Wang and Rando, 1995). Consequently, high-throughput selection schemes that optimize the generation of aptamers with *in vivo* functional activity are desired. In addition, standardized high-throughput characterization schemes that provide both equilibrium and kinetic binding properties of new aptamer sequences are desired (Win *et al*., 2006).

Third, new RNA devices must be integrated into cellular networks as control systems, both in the detection and regulation of endogenous cellular targets. The bulk of the synthetic riboswitch elements generated and characterized to date have been examples of instances in which exogenous effector molecules are added to cell culture and the RNA regulator is demonstrated to regulate a reporter gene. These instances must be translated to the regulation of genes involved in cellular networks, either synthetic or endogenous, in response to molecular inputs of interest for a given application. This translation from demonstrative instances of a device function to implementation in cellular engineering applications is critical to the advancement of this technology and a significant challenge in this field. Many of the barriers in employing these devices in specific applications will be reduced as the first and the second challenges are addressed. Nevertheless, the challenges presented here associated with widespread implementation of engineered RNA devices to fit specific applications of interest may soon be overcome, as engineering strategies in RNA technology have been advancing rapidly.

Conclusions

RNA is a functionally diverse and versatile molecule that plays a wide variety of roles in regulating gene expression in various organisms. Regulation can occur through different mechanisms including RNA-RNA base-pairing interactions and self-cleavage. In addition, RNA can actuate at each level in the gene expression pathway, including transcription, post-transcription, and translation. These regulatory mechanisms can occur through different physical implementations that can be grouped into *cis*- and *trans*-acting interactions. RNA molecules can exhibit ligand recognition properties, through which they can sense their environment, and allosteric binding

properties, through which they can self-regulate their own activity. These properties enable RNA to function as precise molecular sensors and autonomous control systems that require no additional aid from proteins as intermediate sensor or actuator elements. In addition, RNA can exhibit unique and sophisticated properties such as cooperativity and logical signal processing. Furthermore, RNA molecules can adopt different conformations and fold into precise secondary and tertiary structures, and yet still possess the ability to undergo dynamic conformational and structural changes, demonstrating the flexible nature of RNA molecules.

Due to its unique array of functional properties, RNA is a powerful platform for the design of regulator molecules for a wide range of biotechnological applications such as gene expression regulation, biosensors, therapeutic molecule design, metabolic reprogramming, studies of antibiotic-RNA interactions, and tools for elucidating cellular functions. Riboswitches, antisense RNAs, ribozymes, and siRNAs and miRNAs are recently-discovered regulatory RNA molecules that are widespread in nature. The range of sophisticated properties and versatility of RNA represents a resource that can be exploited in conjunction with emerging biomolecular design strategies to enable researchers to manipulate complex biological processes at the molecular level with greater flexibility and reliability.

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