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Developing RNA Tools for Engineered Regulatory Systems

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Introduction

RNA has been shown recently to play a prominent role in regulating gene expression in a wide variety of organisms. Natural examples of RNA-dependent gene regulation include modulation of transcription (Wassarman and Storz, 2000; Winkler *et al.*, 2002), translation (Moller-Jensen *et al.*, 2001; Moller *et al.*, 2002; Bartel, 2004), and the temporal stability of mRNA (Masse *et al.*, 2003; Meister and Tuschl, 2004; Winkler *et al.*, 2004). Environmental perturbations, such as temperature (Hoe and Goguen, 1993; Johansson *et al.*, 2002), osmolarity (Andersen *et al.*, 1987; Chen *et al.*, 2004), oxidative stress (Altuvia *et al.*, 1997), and chemistry (such as the presence of metabolites or toxins, reviewed in Mandal and Breaker, 2004) can all trigger RNA-mediated changes in gene expression.

In parallel, researchers have begun to use engineered RNAs as regulatory molecules for artificial genetic circuits. The same mechanisms (base-pairing and ligand-induced conformational change) that make RNA a 'natural' choice for gene regulation are also amenable to rational engineering or directed evolution efforts. Nonetheless, the clever machines that nature has crafted have accelerated the use of artificial regulatory RNAs. Therefore, we will initially consider the natural RNA tools and mechanisms that are available to researchers, then examine how these tools are being applied in artificial genetic circuits. Ultimately, though, both the adaptation of natural, and the *de novo* development of artificial, RNA-based regulatory mechanisms should foment the production of much more complex artificial genetic circuits, potentially including 'smart' nucleic acid drugs that regulate their own production and release.

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Abbreviations: crRNA, *cis*-repressing RNA; dsRNA, double-stranded RNA; mRNA, messenger RNA; miRNA, microRNA; N40, a region of random RNA 40 nucleotides in length; RBS, ribosome binding site; RISC, RNA induced silencing complex; RNAi, RNA interference; siRNA, small interfering RNA; taRNA, *trans*-activating RNA; UTR, untranslated region.

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Natural RNAs as regulatory tools

RNA regulation is, in general, based on two major transduction mechanisms: base-pairing (antisense) and shape-based (operator, riboswitch) systems. Most natural antisense systems act by inhibiting translation or by promoting RNase cleavage of a target mRNA. Antisense RNA regulators include small non-translated RNA in bacteria and small interfering (siRNA) and micro (miRNA) RNA in eukaryotes. These RNA regulators can bind RNA targets tightly and in a sequence specific manner, often guiding a protein-based regulatory system to perform a downstream regulatory function (i.e. mRNA cleavage). The simplicity of Watson–Crick base-pairing and the ability to accurately predict nucleic acid secondary structures has aided creative rational design efforts to adapt and generate artificial RNA regulators (Isaacs *et al.*, 2004) and opened the potential to design regulatory circuits based on these regulators.

RNA has been shown previously to adopt structures that can bind tightly and specifically to a variety of non-nucleic acid targets, from small ions to metabolites to proteins to supramolecular structures, such as viruses (Xu and Ellington, 1996; Weiss *et al.*, 1997; Convery *et al.*, 1998; Famulok and Mayer, 1999; Hesselberth *et al.*, 2000; Marshall and Ellington, 2000; Homann and Goringer, 2001). Importantly, ligand binding often produces a change in the secondary and/or tertiary structure of the RNA. Such 'shape-shifting' RNAs potentially can be exploited to alter regulatory function in natural mRNAs. For example, naturally occurring 'riboswitches' in bacteria are natural aptamers that undergo ligand-mediated conformational changes and, in turn, modulate translation initiation and transcription termination. As with antisense mechanisms, the ability to engineer base-pairing and secondary structural changes (Kertsburg and Soukup, 2002) allows artificial riboswitches and other shape-based regulators to be designed and implemented in artificial genetic circuits (see Werstuck and Green, 1998; Suess *et al.*, 2004 below).

ANTISENSE-BASED RNA REGULATION

Until recently, little was known about the presence or function of small RNAs in bacteria. Now, however, there are a handful of well-studied, small regulatory RNAs in *E. coli*, such as RhyB, DsrA, and Spot 42. There are many more small RNAs that have been predicted based on computational approaches but which currently have no known function (Argaman *et al.*, 2001; Carter *et al.*, 2001; Rivas *et al.*, 2001; Wassarman *et al.*, 2001; Chen *et al.*, 2002). The small RNAs with known function act as post-transcriptional regulatory switches by either activating or, more commonly, inhibiting translation of a target gene or genes. It is common for these small RNAs to base pair with their target mRNA in the 5' UTR and sequester the ribosome binding site, turning off translation. For example, RhyB is a small regulatory RNA that helps regulate iron metabolism in *E. coli* (Masse and Gottesman, 2002). The RhyB RNA targets a small group of iron binding and iron storage mRNAs, and inhibits their translation while also targeting them for degradation. Under conditions of low iron availability, RhyB is expressed to keep these non-essential iron-binding genes from being expressed. Under conditions of excess iron, the Fur repressor is active and stops transcription of, among other genes, *rhyB*. The RhyB RNA is one of the many

small RNAs that acts in conjunction with the RNA chaperone Hfq (Zhang *et al.*, 2003). The Hfq protein does not have a precise sequence that it recognizes, but tends to bind AU rich sequences (Vytvytska *et al.*, 2000; Zhang *et al.*, 2002; Brescia *et al.*, 2003). This sequence can, at times, overlap with RNaseE cleavage sites (Moll *et al.*, 2003), since RNaseE also recognizes an AU rich sequence (Mackie, 1991; Mackie and Genereaux, 1993; McDowall *et al.*, 1994). The RhyB binding event triggers RNaseE-dependent degradation of both the target mRNA and the RhyB small RNA (Masse *et al.*, 2003). These authors propose that Hfq mediates binding of RhyB to its target mRNA and then dissociates, allowing RNaseE to initiate degradation of both RhyB and bound mRNA.

Another small RNA that inhibits translation, but that does so without triggering RNase degradation, is the Spot42 small RNA. Spot42 allows for discoordinate expression of the *E. coli* galactose operon (Moller *et al.*, 2002). Even though the galETKM operon is transcribed as a polycistronic mRNA, some environmental conditions result in a decrease in GalK protein levels relative to the rest of the operon. This regulation is due to Spot42 RNA binding and concomitant inhibition of translation of, the *galK* gene; surrounding genes in the mRNA (*galE* and *T* upstream and *galM* downstream) are not affected. The reasons for this unusual type of genetic regulation are not yet fully understood. However, when galactose is present as a primary carbon source, the entire gal operon is needed to transport galactose into the cell and convert it to a glycolysis intermediary (glucose 6-phosphate). When other carbon sources are used for growth (such as glucose), only a part of the operon is needed (especially *galE* and *galM*) to produce substrates for biosynthetic glycosylations. It is likely that the Spot42 RNA acts as a very specific regulator, inhibiting only one region of this polycistronic operon, and thereby tightly rationing cellular resources (Moller *et al.*, 2002).

Although small RNAs that activate translation are rarer, the DsrA RNA uses antisense pairing to increase the production of one of its known targets, RpoS (the stationary phase/stress response sigma factor). The DsrA RNA is expressed in response to environmental stresses, such as low pH (Lease *et al.*, 2004). Once expressed, it binds to both RpoS mRNA, activating translation, and to Hns mRNA (a nucleoid-structuring protein that acts as a global transcriptional repressor), repressing translation (Lease and Belfort, 2000). This combination acts to change the expression patterns of a number of genes involved in stress response (Lease *et al.*, 2004). The DsrA RNA activates translation of the RpoS mRNA, since the nascent RpoS mRNA transcript is not translatable; it contains a hairpin that occludes ribosome binding. When DsrA is co-expressed, it binds to a portion of the hairpin sequence, leading to a structural rearrangement within the 5' UTR that frees the ribosome binding site from base-pairing and allows translation to begin (Lease *et al.*, 1998; Lease and Belfort, 2000). This natural strategy shall later prove useful in engineering regulatory RNAs, as well (see Suess *et al.*, 2004 below).

The closest eukaryotic equivalents of bacterial small regulatory RNAs are microRNAs (for review, see Bartel, 2004). Much like their bacterial counterparts, they are expressed and encoded separately from the genes they target, and imperfectly base-pair to the target sequences. In plants, miRNAs generally base pair perfectly with their target mRNAs and direct ribonucleolytic cleavage of the mRNA (Tang *et al.*, 2003). There are some examples of animal miRNAs that repress their

targets via this mechanism (Yekta *et al.*, 2004), but most animal miRNAs appear to inhibit translation via base-pairing to sites in the 3' UTRs of target mRNAs. The exact mechanism of translation inhibition is currently unknown, but it involves a substantial protein component. It is likely that miRNAs act as guides for the proteins that actually regulate translation, just as bacterial regulatory RNAs can serve as 'guides' for RNaseE degradation.

Though less related to the small RNAs of prokaryotes, the small interfering RNAs (siRNAs), which trigger RNA interference (RNAi), have proven to be excellent tools for the manipulation of eukaryotic gene expression. In 1998, Fire and co-workers discovered that double-stranded RNA molecules bearing sequence identity to mRNAs resulted in strong silencing of the cognate mRNA (Fire *et al.*, 1998). This phenomenon, now widely known as RNAi, is mediated by small (~21 bp) double-stranded RNA molecules, siRNAs, which guide a ribonuclease complex to cleave a target mRNA, thereby down-regulating the expression of that gene (for a recent review, see Meister and Tuschl, 2004). The end result of RNA interference is the strong, in some cases systemic, or even epigenetic, silencing of specific genes. Proposed natural functions of the RNAi pathway are the silencing of selfish and unstable genomic elements, such as transposons and tandem repeats, as well as inhibition of infection by RNA viruses (Aravin *et al.*, 2004).

RNA interference also differs from other regulatory RNA phenomena in that the sequences involved in regulation are not encoded as separate regulatory RNAs. Instead, longer, double-stranded RNAs are recognized and processed into siRNA duplexes by an RNaseIII-like enzyme, known as Dicer (Bernstein *et al.*, 2001). These siRNAs then pass through a series of complexes consisting of different proteins involved in the RNAi pathway, ultimately associating with a ribonucleoprotein complex known as the RISC (RNA induced silencing complex). Though RISC contains many undescribed components, the nuclease component within RISC that associates directly with siRNAs and cleaves target mRNAs is a highly conserved protein called Argonaute2 (Liu *et al.*, 2004).

RNAi has seen greatly increased use as a biotechnology tool since its discovery seven years ago. Researchers now routinely use RNAi as the gold standard for gene silencing in model organisms. Defined siRNAs are used to knock out known genes, while libraries of siRNAs can be used to create phenotypes equivalent to classical genetic knockouts (Hsieh *et al.*, 2004; Kittler *et al.*, 2004). There is obviously great therapeutic potential in RNAi, as there would be in any sequence-specific method for inhibiting mammalian gene expression. It has been estimated that up to 10% of the American drug market could eventually be occupied by siRNA-based therapeutics (Howard, 2003). Because siRNAs can turn over substrates, they are estimated to be up to 1000-fold more potent than antisense RNAs, and are therefore more attractive therapeutics as less compound would presumably have to be administered to a patient (Howard, 2003). However, the efficient delivery of nucleic acid drugs remains a great obstacle, especially given the ubiquitous presence of nucleases *in vivo*. Recently, Soutschek and colleagues were able to surmount this obstacle and systemically introduce siRNAs via intravenous delivery into mice (Soutschek *et al.*, 2004). To aid stability, the siRNAs were modified with several nuclease-resistant modifications, and to aid cellular uptake, a cholesterol group was tagged onto the end of the sense (non-functional) strand of the siRNA duplex. The modified siRNAs

were efficiently delivered across cellular membranes and proved to be functional in multiple organs within the mice.

SHAPE-BASED RNA REGULATION

Naturally occurring RNA binding sites within proteins have long been known to be involved in gene regulation. For example, the MS2 viral coat protein binds to an RNA hairpin (operator) in the viral genome, inhibiting translation of the MS2 replicase (Ling *et al.*, 1970; Stockley *et al.*, 1995). However, for the most part, such shape-based RNA regulators have been thought of as passive actors in signal transductions, mere docking sites for their more active protein counterparts. More recently, though, RNA regulators have been described that broadly regulate gene expression in both bacteria and eukaryotes, by binding to and modulating the activities of proteins. The best characterized example in bacteria is 6S RNA (Wassarman and Storz, 2000). The 6S RNA is transcribed as cells exit logarithmic growth and enter stationary phase. It inhibits transcription by the *E. coli* RNA polymerase responsible for normal growth by binding to the sigma 70 subunit and to the B/B' subunits of the core polymerase. The production of this small regulatory RNA helps stop transcription of normal 'housekeeping' genes as the cell makes the transition into stationary phase. Similarly, B2 RNA in eukaryotic cells has been shown to repress transcription in response to heat shock by binding and inhibiting RNA polymerase II (Allen *et al.*, 2004; Espinoza *et al.*, 2004).

Natural RNA regulators have also been found that bind small metabolites rather than proteins. These so-called 'riboswitches' undergo metabolite-dependent structural changes that in turn modulate gene expression (reviewed in Winkler and Breaker, 2003 and Mandal and Breaker, 2004). Separate functional domains within a riboswitch make up the metabolite binding (aptamer) and regulatory regions. The two most common mechanisms for the modulation of gene expression are the regulation of translational initiation and transcription termination (*Figure 2.1*). To modulate translation initiation, riboswitches commonly occlude the ribosome binding site. Generally, in the absence of a metabolite, the Shine–Dalgarno sequence is base-paired to a complementary sequence within a stem structure, prohibiting ribosome access and the initiation of translation. However, in the presence of the target metabolite, the riboswitch undergoes a conformational change that frees the Shine–Dalgarno sequence, allowing ribosome binding and active translation (*Figure 2.1a*). For example, coenzyme B12-dependent riboswitches are present in the 5' UTR of both the *E. coli* and *S. typhimurium btuB* gene (cobalamin-transport protein; Winkler and Breaker, 2003), and regulate the intracellular concentration of coenzyme B12 by inhibiting the translation of B12 biosynthetic and transport genes. When coenzyme B12 binds the riboswitch, it alters the secondary structure of the mRNA, leading to occlusion of the ribosome binding site. In the absence of coenzyme B12, the 5' UTR adopts a structure that allows ribosome binding, and therefore translation.

Riboswitches can also modulate transcription termination by undergoing a metabolite-dependent change in secondary structure that either allows or prevents a rho-independent transcription terminator from forming (*Figure 2.1b*). The most commonly observed mechanism is simple feedback inhibition, although there are

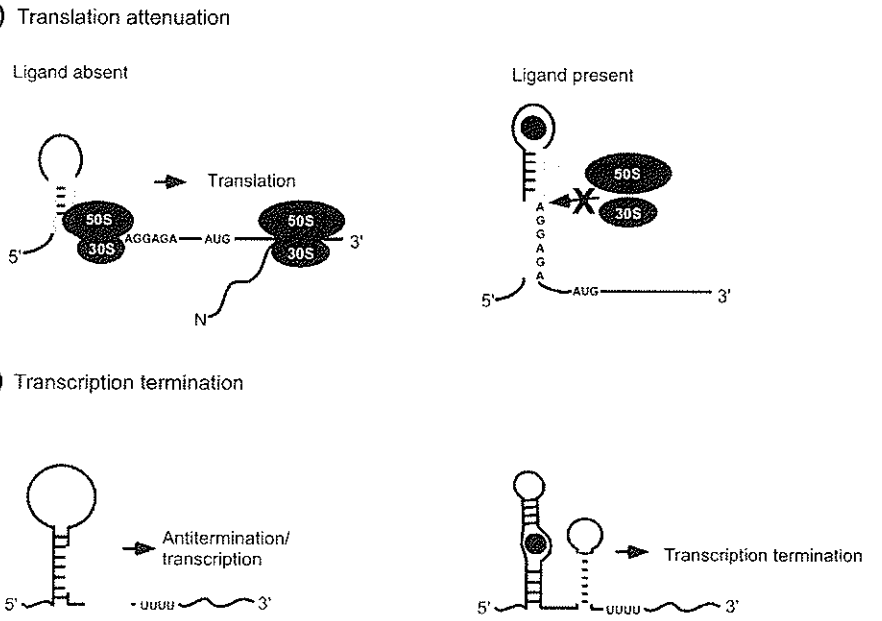

Figure 2.1. Mechanisms of riboswitch-based genetic regulation. a) Translational attenuation. In the absence of ligand (left), the riboswitch folds such that the Shine–Dalgarno sequence is available to the ribosome, and translation proceeds. In the presence of effector ligand (right), the Shine–Dalgarno sequence folds into a stem structure within the riboswitch, and is occluded from ribosome recognition, thereby inhibiting translation. b) Transcription termination. An antiterminator structure is formed in the absence of target ligand, prohibiting the formation of the hairpin transcription terminator (two grey boxes). In the presence of ligand, the riboswitch folds into an alternate conformation, freeing the occupied half of the terminator stem to base-pair with the other half-terminator stem, forming a transcription terminator and stopping transcription of the downstream mRNA.

two known examples of riboswitches which activate gene expression by similar mechanisms (Mandal and Breaker, 2004; Mandal *et al.*, 2004). For example, the glycine riboswitch in the 5' UTR of the *Bacillus subtilis gcvT* operon (which allows glycine utilization as an energy source) binds glycine and activates transcription (Mandal *et al.*, 2004). In the absence of glycine, a rho-independent transcription terminator forms in the 5' UTR, leading to termination before the operon can be transcribed. In the presence of glycine, an alternative structure forms that allows transcription of the complete operon. This riboswitch is unique among currently characterized RNA regulatory elements in that it is known to cooperatively bind two glycine molecules. Interestingly, a cooperative ligand-responsive ribozyme was evolved in the laboratory (Jose *et al.*, 2001) prior to the discovery of the similarly acting riboswitch in nature; that is, biotechnology predicted this natural phenomenon.

Recently, a natural allosteric ribozyme that regulates gene expression of the *glmS* gene was discovered (Winkler *et al.*, 2004). It is similar to most riboswitches in that it is located in the 5' UTR of *glmS* and its function is dependent upon its ability to bind glucosamine-6-phosphate (GlcN6P), the product of the GlmS enzyme.

However, instead of affecting transcription or translation, this ribozyme affects gene expression by cleaving the *glmS* mRNA, decreasing transcript levels. In the absence of the GlcN6P effector, the ribozyme cleavage occurs with a half-life of approximately 4 hours. The presence of the effector decreases the half-life to approximately 15 seconds. This increase in the rate of reaction is highly specific for GlcN6P as compared to the close structural analogs tested (Winkler *et al.*, 2004). This discovery stands as a unique landmark, a possible relic of an RNA world wherein RNA acted as a carrier of genetic information, a regulator of metabolism, and a catalytic entity subject to direct regulation via metabolic feedback. While similar allosteric ribozymes have not yet been discovered in eukaryotes, a new ribozyme has been observed in the 3' UTR of the human beta-globin gene that is thought to function as an (apparently unregulated) transcription terminator (Teixeira *et al.*, 2004).

As noted above, the discovery of 'shape based' RNA regulation has been preceded in many cases by descriptions of similar, engineered mechanisms *in vitro* (Knudsen and Ellington, 2004). For example, natural protein-binding aptamers have been known for many years, while the natural RNAs that regulate polymerases have been described more recently (see 6S and B2, described above). The description of these natural aptamers was predated by the *in vitro* evolution of RNAs, which inhibit the function of nucleic acid polymerases (Tuerk *et al.*, 1992; Chen and Gold, 1994; Chen *et al.*, 1996; Thomas *et al.*, 1997). The fact that natural strategies of genetic regulation are so frequently predicted by laboratory evolution methods is striking. It suggests either that both natural and *in vitro* selection methods can thoroughly search fitness landscapes and come up with similar, optimal solutions, or that the chemical rules and mechanisms that underlie the evolution of functional nucleic acids strongly constrain the outcome of evolutionary experiments, natural or directed. Nonetheless, to the extent that such congruence continues to hold, *in vitro* evolution can be seen as an increasingly powerful tool for studying biological evolution.

In vitro engineering strategies for generating and optimizing RNA tools

The natural mechanisms for RNA-mediated regulation of metabolism provide a wealth of RNA tools for further engineering efforts. The simplicity of base-pairing rules in general means that engineering antisense mechanisms is a relatively straightforward enterprise. However, there is no easy map from sequence to shape, and to bridge this gap, researchers have developed a variety of directed evolution strategies to create functional ligand-binding and catalytic RNAs. Beyond using such strategies to engineer tools which mimic those found in nature, biotechnology inventions frequently precede the discovery of natural regulatory mechanisms, and may lead to the development of novel tools that nature has not yet chanced upon.

The *in vitro* or directed evolution of nucleic acids follows the same principles that apply in natural selection. A pool of heritable diversity is generated, typically by partial or complete randomization of a nucleic acid strand, rather than by point mutation. The pool is selected for some binding or catalytic function. For example, aptamers are selected by sieving ligand-binding nucleic acids from the general population by methods such as filtration or affinity chromatography. The winning species are then amplified by some combination of reverse transcription, PCR, and

in vitro transcription. Multiple cycles of selection and amplification yield nucleic acids with greatly improved (if not optimal) functionalities.

Using such methods, aptamers have been selected against a wide variety of targets (Famulok and Mayer, 1999; Hesselberth *et al.*, 2000; Marshall and Ellington, 2000), including many nucleic acid binding proteins, such as T4 DNA polymerase (Tuerk and Gold, 1990) and HIV-1 Rev (Giver *et al.*, 1993), and a surprising number of non-nucleic acid binding proteins. Aptamers typically bind with extremely high affinity and specificities that rival those of antibodies. For example, an aptamer that binds theophylline discriminates approximately 10 000-fold against the highly similar molecule caffeine, which differs by only a single methyl moiety (Jenison *et al.*, 1994).

The extraordinary recognition properties of aptamers have led to their development and application as therapeutics. For example, aptamers against tumour-related proteins have been shown to significantly target and inhibit the growth of tumours *in vivo* (Huang *et al.*, 2001; Kim *et al.*, 2002; Farokhzad *et al.*, 2004). However, given the instability of RNA within a biological context, the exogenous delivery of aptamers for *in vivo* applications is obviously a challenge. The half-life of a single-stranded RNA molecule in the presence of serum nucleases is extremely low. To get around this problem, modified nucleotides and conjugates can be incorporated that significantly increase stability to the point where modified RNAs can circulate for days. In addition, specialized aptamer expression vectors have been developed that can be used for the expression of intracellular aptamers in gene therapy approaches. For example, Joshi and Prasad expressed anti-HIV reverse transcriptase aptamers from a vector and flanked them with *cis*-cleaving hammerhead ribozymes in order to release the aptamer from the transcript (Joshi and Prasad, 2002). The aptamers were able to reduce HIV replication up to 99.5% *in vivo*. The fact that aptamers have gone from curiosities to therapeutics bodes well for the future applications of the tools and circuits described in this review.

Of course, the functionality of RNA is not limited to ligand binding, and structured nucleic acids also boast significant catalytic ability (albeit still thousands of times less than that of their more chemically adept protein counterparts). Researchers have been able to use rational design and directed evolution to modify and refine RNA catalysts. For example, the naturally occurring hammerhead ribozyme is a *cis*-cleaving RNA that has been re-engineered to function in *trans*, and to cleave nearly any sequence containing a UH (U followed by any base except G) dinucleotide cleavage site (Kore *et al.*, 1998). To expand the range of RNA targets, researchers have used directed evolution to modify the hammerhead's cleavage site specificity. In particular, Eckstein's group was able to modify the strict requirements for a UH target site to a GAC target site (Kore *et al.*, 2000). Following *in vitro* selection of a hammerhead library, only one mutation in the catalytic core was required to confer this new target specificity to the hammerhead, though the rate of cleavage was significantly depressed relative to the wild-type sequence.

Like aptamers, ribozymes can be employed *in vivo*. In a particularly interesting application, a library of randomized hammerhead ribozymes was used to identify genes involved in tumour metastasis in mice (Suyama *et al.*, 2004). In short, a library of hammerhead ribozymes in which the substrate recognition arms were randomized was cloned into an expression vector and transformed into a pre-metastatic cell

strain. The cell strain expressing the ribozyme library was then injected into mice. Individual cells that became metastatic were recovered from different organs in the mice, and the ribozymes in those cells were sequenced. The binding arms of the ribozymes were then compared with the genome, and the targets whose inhibition influenced metastasis were identified and then confirmed via alternative gene knock-down methods. In all, 8 genes involved in metastasis were found by this remarkable method; 5 of which were previously unknown. The success of this method affirms that one of the most useful aspects of nucleic acid tools is that base-pairing can be readily deciphered and engineered; similar searches for novel target specificities in protein enzymes might now also be possible, but require extraordinary computational power (Looger *et al.*, 2003).

Given that RNA can bind ligands and carry out reactions, it should not be surprising that these two characteristics can be fused to generate ligand-responsive catalysts (aptazymes). Initially, Breaker and co-workers appended aptamers to the core catalytic domain of the hammerhead ribozyme and generated a chimeric structure in which ligand-dependent conformational changes in the aptamer modulated the activity of the ribozyme catalytic domain (Tang and Breaker, 1997). This strategy was largely successful because both classes of molecules could be engineered in 'flatland'; that is, based almost solely on knowledge of their secondary structures. The general method for the design of aptazymes has been the replacement of non-critical, structure-stabilizing, secondary structures of ribozymes with aptamer secondary structures and a connecting stem that can serve as a 'communication module' (Koizumi *et al.*, 1999; Robertson and Ellington, 1999, 2000; Jose *et al.*, 2001; Soukup *et al.*, 2001). As an example, ATP aptamers have been incorporated as allosteric domains into both ribozyme cleavases (Tang and Breaker, 1997) and ligases (Robertson and Ellington, 2000). Interestingly, depending on the nature of the construct, either inhibition or activation could be engineered into the resultant aptazymes; the cleavase was repressed 180-fold by ATP, while the ligase was activated 800-fold. The range of effectors that can be used to regulate aptazymes is not limited to small organics, but includes metals, nucleic acids, peptides, and proteins. Examples include RNA-dependent ribozymes (Kuwabara *et al.*, 1998; Robertson and Ellington, 1999), a HIV REV peptide-dependent ribozyme (Robertson *et al.*, 2004), and ribozymes specifically activated by the different phosphorylation states of ERK2 (Vaish *et al.*, 2002). Several groups have shown that modulation by nucleic acid sequences, rather than more structured organic or protein effectors, is also possible. One example is the replacement of the stable stem II structure of the hammerhead ribozyme with an RNA sequence capable of forming a stable pseudo-half-knot structure only in the presence of an added oligonucleotide effector (Komatsu *et al.*, 2000). In this case, hammerhead cleavage activity was negligible in the absence of, and up to 250-fold activated by the addition of, an effector oligonucleotide. Though aptazymes primarily have been generated and optimized *in vitro*, further adaptation of these catalysts to an *in vivo* milieu is straightforward, and it should be possible to engineer aptazymes to elicit defined behaviours in a cellular context.

Some complex RNA catalysts are capable of much more than mere cleavage or ligation activities, and already function in gene regulation. In particular, Group I and Group II introns are capable of undergoing a cascade of transesterification

reactions and excising themselves from transcripts in the absence of protein catalysts. The utility of a ligand-regulated, self-splicing intron for gene regulation is manifest. Fortunately, the same flatland engineering efforts that have proven successful with simple RNA catalysts should also be amenable to the larger, more complex self-splicing introns. Kertsburg and Soukup used strategies previously shown to be successful for hammerhead aptazyme engineering to select for an RNA 'communication module' between an anti-theophylline aptamer and the *Tetrahymena* Group I ribozyme (Kertsburg and Soukup, 2002). The resultant Group I aptazyme was able to regulate self-splicing *in vitro*. Even though the engineered aptazyme spliced less efficiently than the wild type Group I intron, it was activated ~26-fold by theophylline *in vitro*. Thompson and colleagues also inserted a theophylline-binding aptamer at various locations within a *thymidylate synthase* (td) Group I self-splicing ribozyme from bacteriophage T4 and tested the constructs for allosteric activation *in vitro*. Those that showed strong activation with low background were subsequently tested *in vivo* for their ability to rescue a thymidine auxotroph in the presence or absence of the regulator, theophylline. A construct was found which increased growth when spotted on minimal media plates in the presence of the effector (Thompson *et al.*, 2002). Other attempts to engineer regulatable self-splicing introns have used arginine-rich motif (ARM) peptides as activators (Atsumi *et al.*, 2001, 2003). Knowledge of the complex tertiary structure of the *Tetrahymena* self-splicing Group I intron (Cate *et al.*, 1996) and of two different ARM-RNA interactions (Battiste *et al.*, 1996; Legault *et al.*, 1998) were used to re-engineer the intron for peptide-dependent catalysis. Two regions of the intron which were known to interact in the natural ribozyme (P5b and P6) were engineered to contain the natural binding sites for two ARMs, the bacteriophage lambda box B and the HIV RRE, respectively. A heterodimeric peptide activator that contained ARMs from bacteriophage lambdaN (amino acids 1–19) and HIV Rev protein (amino acids 34–50) connected by a short linker region functioned as a bridge to link the two regions, resulting in the formation of the active tertiary structure of the ribozyme. Splicing was activated *in vivo* approximately 3-fold over a non-functional control peptide that did not contain one of the two ARMs. In order to improve function of the peptide-activated intron *in vivo*, the linker region was randomized, the resultant library was inserted into the beta-galactosidase alpha-fragment, and fast-growing cells were selected by use of minimal media containing lactose as the carbon source. The peptide with the selected linker region activated splicing 7.5-fold higher *in vivo* than when a non-functional peptide was used.

Integrating tools into circuits: synthetic biology

The various RNA tools, mechanisms, and methods described above can potentially be combined into regulatory circuits of great complexity. In the past, this would have been called biotechnology, but there is an emerging field (or buzzword) known as synthetic biology that has arisen, in part because of new perspectives on the older discipline. Just as physicists once 'invaded' molecular biology, electrical and other engineers have begun to bring their perspectives to biological research, and have gone about putting biology on more 'logical' grounds. As opposed to biology's traditional dissective mentality of discovering genes via genetics, describing

behaviours through biochemistry, and then drawing qualitative cartoon diagrams of how it all works, synthetic biologists have tried to establish quantitative rules for the behaviour of genes and proteins, and to thereby use these components to assemble genetic circuits whose behaviours can be predictively modelled and manipulated (Elowitz and Leibler, 2000; Gardner *et al.*, 2000; Guet *et al.*, 2002; Hasty *et al.*, 2002; Yokobayashi *et al.*, 2002; Atkinson *et al.*, 2003; Basu *et al.*, 2004; Kobayashi *et al.*, 2004; You *et al.*, 2004).

One of the most innovative implementations of a synthetic biological circuit was the 'repressilator' built by Elowitz and Leibler in 2000. The repressilator was a genetic oscillator that was built as an independent circuit in *E. coli* and consisted of three repressor proteins connected in series. In this scheme, A repressed B, B repressed C, and C repressed A. Such a device is known as a ring oscillator in electrical engineering, and in general, consists of any odd number of inverters (analogous to biological repressors) in series. This circuit inherently exhibits oscillatory behaviour. In electronics, the oscillating signal is current, while in the case of the repressilator, the oscillating signal was the concentration of GFP (green fluorescent protein), which was under the control of one of the repressors. As the genetic circuit began to function, GFP levels within single cells oscillated over several cell divisions with a period of several hours. Interestingly, only ~40% of the cells in a given population exhibited the oscillatory phenotype. This phenomenon is indicative of the challenge that permeates synthetic biology: even though the population was genetically identical, its behaviour was not fully predictable. This was no doubt due to the unique challenges associated with thinking of biological systems as logical devices. Among other factors, cross-talk between the engineered elements and other components of the cell, noise associated with gene expression, differences in the cellular concentrations of proteins or other relevant factors, and variability in plasmid copy number likely played a significant role in making the designed behaviour somewhat unpredictable *in vivo*. Undaunted, the authors have since used this system as inspiration to study some of these phenomena, including elegant studies on noise in gene expression (Elowitz *et al.*, 2002; Swain *et al.*, 2002). One of the most important aspects of synthetic biology may be that we will finally learn about both key and subtle parameters that influence the behaviour of natural biological systems.

The development of nucleic acid-based circuits is in its infancy, but a number of impressive results have already been obtained. Circuits that use nucleic acid inputs, provide nucleic acid outputs, and compute completely with nucleic acid logic devices have been designed and implemented (Stojanovic *et al.*, 2002; Benenson *et al.*, 2003, 2004; Stojanovic and Stefanovic, 2003a,b). In the most encompassing endeavour to date, Stojanovic and Stefanovic designed a series of deoxyribozymes that could effectively play a game of tic-tac-toe with flawless logic (Stojanovic and Stefanovic, 2003b). By again using easily engineered base-pairing rules, these authors modified an RNA-cleaving deoxyribozyme originally selected by Breaker and Joyce to be responsive to up to three DNA oligonucleotide effectors. The oligonucleotide effectors acted as activators for some deoxyribozyme variants and as inhibitors for others. Different combinations of oligonucleotide-binding, allosteric domains within single deoxyribozymes yielded logic gates that represented all possible Boolean logic functions. In all, different subsets of 23 different deoxyribozymes were placed in nine different wells (squares of the tic-tac-toe

board), and eight different oligonucleotide effectors were used to signal the moves made by the human player at each step. After the human player made their move, the DNA-based automaton would respond by processing the input and cleaving an RNA substrate to yield a fluorescent product in only one well, signalling automaton's move. This tour de force is obviously merely a toy, yet demonstrates the complex information processing capabilities available to nucleic acid regulators. Should similarly complex schemes be implemented in cells, it may be possible to generate programmable drugs that respond to their environment, as described below.

The drawback to an engineering mentality, though, is that it generally does not work as well as the engineers would like (Yokobayashi *et al.*, 2002). This is in part because biological parts are far from standardized and, more importantly, the vehicles in which the parts typically function, organisms, are extremely complex and idiosyncratic. The ancillary or unwanted interactions that occur between even the most well characterized biological 'parts' (i.e. the Lac repressor in *E. coli*) and the cell generally lead to a failure of the engineered system, or at best, woefully sub-optimal performance. Despite this handicap, engineers have unquestionably had success in specifying designer genetic circuits, with a remarkable ability to predict and manipulate behaviours (for an elegant example, see Atkinson *et al.*, 2003).

It is telling that nature (the supreme optimizer) has always relied on a protocol that is, in general, far superior to rational design: evolution. However, to date there has been only one example of the directed evolution of a complex genetic circuit (Yokobayashi *et al.*, 2002). The authors designed a system whereby the Lac repressor (LacI) repressed expression of a second protein, lambda repressor (cI), which in turn repressed expression of a reporter, EYFP. In this system, IPTG should disable LacI repression, which should allow expression of cI (an inverter), which should turn off EYFP. However, this very simple circuit design failed due to a quantitative mismatch between the two regulatory elements. That is, background expression of cI was so high that EYFP was constitutively repressed. To redress this deficiency, the authors mutagenized the cI protein and evolved it in the context of the circuit. The simplistic solution that was selected was a mutation in the cI protein that resulted in lower affinity for its operator, thereby better tuning the output of the first regulatory element relative to the second regulatory element, and achieving dynamic levels of proteins that allowed signals to be passed between the elements.

So far, the circuits that have been considered were composed of protein- or DNA-based biological parts. Keeping in mind that RNA parts are lined up (as described above), and that the protocols for their use are just beginning to be established, we will now discuss nascent examples of the design and the evolution of genetic regulatory circuits composed of RNA.

Rational engineering strategies for translational control

Just as small regulatory RNAs and riboswitches can be used to regulate translation in organisms, it has proven possible to engineer effector-modulated control of RNA conformation that in turn regulates translation. Before riboswitch-like mechanisms of translation regulation were known to exist naturally, Werstuck and Green placed dye-binding aptamers in the 5' UTR of genes to regulate translation (Werstuck and Green, 1998). These authors were then able to disrupt active translation with dye

molecules, due to the formation of an aptamer-stabilized structure near the Shine–Dalgarno region of an mRNA. This strategy proved to be somewhat generalizable, as it also proved possible to place anti-tetracycline aptamers near the cap structure of the 5' UTR of a luciferase mRNA in yeast to disrupt cap-binding complex formation, and therefore translation (Hanson *et al.*, 2003; Suess *et al.*, 2003).

In a more recent report, Mulligan and co-workers engineered the translational control of a mammalian target gene using a *cis*-cleaving hammerhead ribozyme, rather than an aptamer (Yen *et al.*, 2004). Interestingly, this engineering approach mirrored the natural, *cis*-cleaving aptazyme identified by Breaker's group at about the same time (Winkler *et al.*, 2004). In this scheme, the authors cloned several hammerhead ribozyme variants into multiple locations in the 5' UTR of a reporter gene, *lacZ*, in order to determine which would give the greatest *cis*-cleavage rate, and hence the strongest silencing of LacZ expression. By manipulating constructs, the authors were able to achieve 1400-fold repression of expression versus a catalytically inactive ribozyme control. While this was impressive in and of itself, the authors then employed two strategies to reverse the ribozyme-mediated quenching of gene expression. First, they introduced antisense morpholino oligonucleotides that targeted the ribozyme and were able to recover LacZ expression up to ~50% of maximal activity. Second, they stably transfected a cell line with an engineered ribozyme-luciferase construct (rather than a LacZ construct) and screened a library of small molecules for those that could inhibit cleavage by the ribozyme. Toyocamycin, a nucleoside analog, was found to inhibit ribozyme cleavage almost completely. The luciferase construct was cloned into an adeno-associated virus genome and maintained functionality when delivered to differentiated adult mouse tissue. Impressively, toyocamycin then significantly inhibited ribozyme-mediated reductions in gene expression (up to 190-fold) when delivered locally to the mice. This work represents one of the most elegant and thorough implementations of synthetic post-transcriptional control to date, and is a harbinger of the development of synthetic genetic circuits that can be transferred from laboratory systems to complex organisms. For example, it may prove possible to develop regulatable gene therapies using drug-activated ribozymes or self-splicing introns.

Recently, Collins and co-workers have reported the rational engineering of a more complex RNA-based translation regulation scheme (Isaacs *et al.*, 2004). With inspiration from naturally occurring translation regulatory systems, such as the DsrA-RpoS *trans*-activation system (Franch *et al.*, 1999), a generalizable, two-component translational regulatory system was developed. A *cis*-repressing (cr)RNA structure was first appended to the 5' terminus of a GFP transcript. The crRNA was a hairpin that base-paired with, and thereby occluded the ribosome binding site of the downstream gene, inhibiting translation (*Figure 2.2*). At a separate locus, a *trans*-activating (ta)RNA molecule was expressed that could initiate binding in the loop of the crRNA and free the RBS. Initial designs yielded only modest translational activation, so the authors assiduously re-engineered their constructs in an attempt to find the most favourable balance between stable repression and high activation. The final crRNA/taRNA riboregulator pairs were highly sequence specific and displayed little cross-talk, and translational activation proved to be significant; GFP levels increased up to 19-fold. As the authors point out, the generalizability and dose-dependent activity of the designed riboregulators may allow them to be employed as

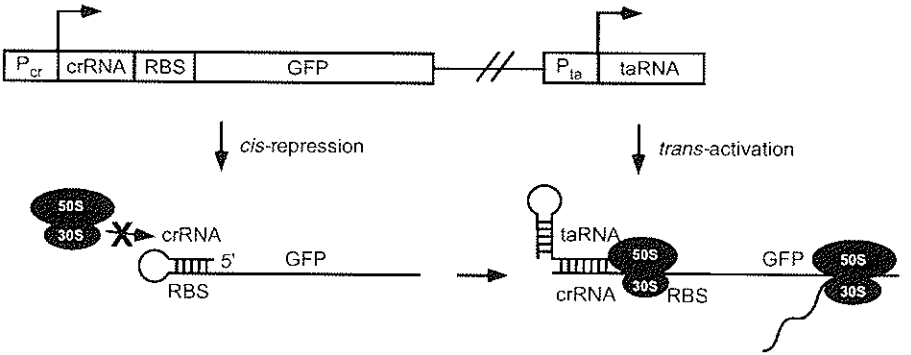

Figure 2.2. Engineered two-component riboregulator. A *cis*-repressing RNA (crRNA) is cloned upstream of the ribosome binding site of a reporter molecule (GFP). The crRNA folds into a stable stem-loop structure involving the RBS, and thereby makes the RBS inaccessible to the ribosome. A separate *trans*-activating RNA (taRNA) forms a more stable stem structure with the crRNA, causing it to change conformations and free the RBS, allowing translation of GFP to proceed. (Adapted from Isaacs *et al.*, 2004.)

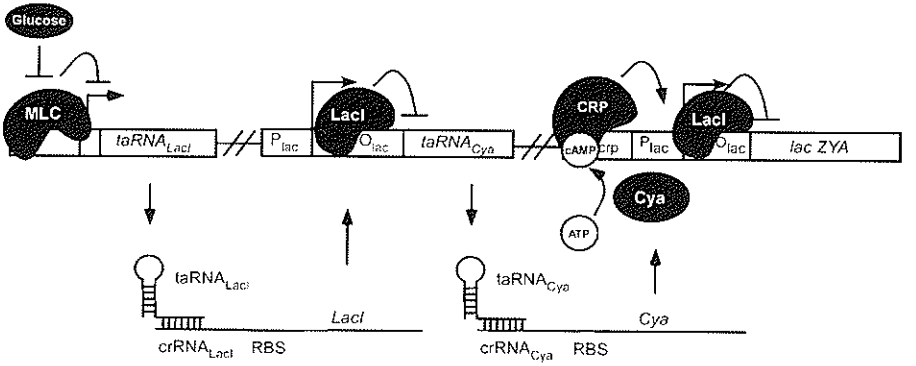

Figure 2.3. Proposed riboregulator-based catabolite repression system. Glucose enters the cell, causing the sequestration of the repressor, MLC. This activates expression of an engineered taRNA ($taRNA_{LacI}$) which relieves repression of LacI by an engineered crRNA ($crRNA_{LacI}$). LacI represses expression of the *lac* operon (*lacZYA*), as well as a second taRNA responsible for activating expression of adenylate cyclase (Cya). Adenylate cyclase converts cellular ATP to cAMP, which associates with the cAMP binding protein, CRP, to activate transcription from the *lac* operon. Therefore, the presence of glucose represses the expression of lactose catabolic genes via two strategies, via activation of an inhibitor and inhibition of an activator.

tools for manipulating the expression and studying the function of natural genes. Moreover, it seems likely that multiple pairs of riboregulator modules could be designed to function orthologously *in vivo*, ultimately creating more complex, even hierarchical genetic regulatory circuits (*Figure 2.3*).

Another engineered mechanism for translation regulation was based on riboswitches, rather than small regulatory RNAs. Hillen and co-workers described the rational design of a ligand-responsive regulatory element in *B. subtilis* (Suess *et al.*, 2004). Taking a cue from the abundant riboswitch elements present in nature (including those that have previously been described in *B. subtilis*), these authors

generated a synthetic riboregulator that could relieve repression of translation in the presence of the small molecule theophylline. As alluded to above, Breaker and co-workers have previously joined the theophylline aptamer to the hammerhead ribozyme, and randomized the joining region between the two domains in order to select for aptazymes that were activated by theophylline (Soukup *et al.*, 2000). Some of the selected communication modules could generally transduce ligand-induced conformational changes between the two RNA domains, irrespective of the identities of the ligand-binding or catalytic components. Therefore, Hillen and co-workers adapted a previously selected communication module to join an anti-theophylline aptamer to a stem-loop structure that was inserted immediately upstream of the RBS of a reporter gene (Suess *et al.*, 2004). The stem loop effectively inhibited translation from the downstream RBS. However, upon binding of theophylline to the aptamer, the composite structure underwent a small conformational change in which the two strands of the communication module moved with respect to one another, and translation was activated by 8-fold *in vivo*.

Directed evolution strategies for transcriptional control

Whereas the majority of engineered or evolved RNAs (antisense, ribozymes, aptamers) and natural regulatory RNAs (riboswitches, miRNAs) inhibit translation, RNAs can also be adapted to modulate transcription. Some of the best natural examples of RNA regulation of transcription are the RNA motifs that act as terminators, including ligand-mediated riboswitches, as described above. However, we have also seen that RNAs that recognize shape need not be confined to mRNA transcripts themselves, but can also assume a more structural role in transcription complexes. Along these lines, Cassiday and Maher used a combination of *in vitro* and *in vivo* selection to generate an RNA aptamer that could activate transcription in yeast (Cassiday and Maher, 2003). These authors used a yeast three-hybrid selection method to optimize a previously *in vitro* selected anti-NF-κB aptamer for *in vivo* function. Initially, the authors cloned a degenerate anti-NF-κB aptamer library into the three-hybrid system in an attempt to conserve core-binding features, while optimizing sequences for *in vivo* stability and specificity. Following selection, though, only a modest ~3-fold improvement in activity was obtained. This seemed to indicate that molecules optimized *in vitro* might not be capable of robust *in vivo* function. The authors returned instead to an intermediate (and presumably not fully optimized) RNA pool from the original *in vitro* selection and cloned it into the three-hybrid HIS3 selection system (*Figure 2.4*). This method combined *in vitro* enrichment of sequences bearing motifs with affinity for NF-κB with a concomitant reduction in the size of the random RNA pool that would have to be sieved *in vivo*. As a result, an RNA with ~20-fold improved NF-κB binding, as compared to the original aptamer, was discovered. Moreover, the new aptamer (RNA6) was shown to inhibit successfully NF-κB transcription activation activity *in vivo*, likely through competitive inhibition of the DNA binding domain. RNA6 bound to NF-κB with a 1:1 stoichiometry, and competitively inhibited the natural NF-κB target DNA from binding the DNA binding groove in the NF-κB protein, suggesting similar binding sites for the evolved RNA and the natural DNA sequence. RNA6 shared the core NF-κB binding sequence observed in the previous *in vitro* selection (RNA1), yet

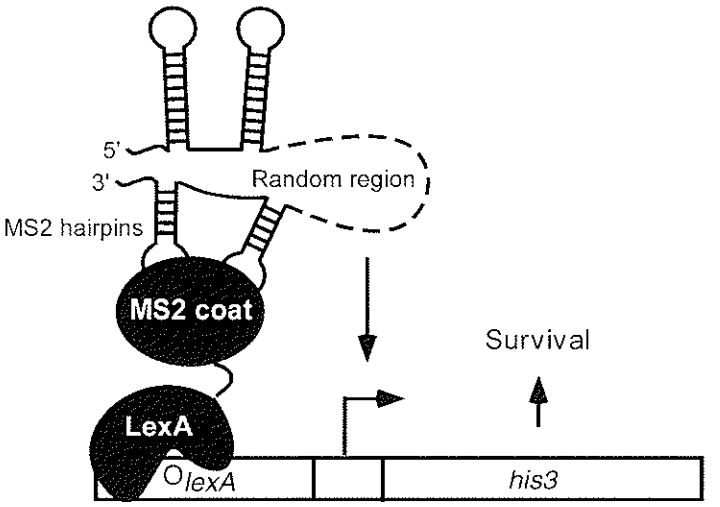

Figure 2.4. Selection strategy for a ribo-transcription factor. A random region of 40 or 80 nt (dashed line) was flanked on the 5'-end by structure-stabilizing hairpins and on the 3'-end by MS2-binding hairpins. The MS2-binding hairpins allow for localization of the RNA to the *his3* gene by associating with an MS2 coat protein fused to LexA, which in turn binds to its cognate operator upstream of *his3*. The pool is transformed into a histidine auxotroph, and if an RNA variant is capable of activating transcription of *his3*, that clone will survive on media plated without histidine. (Adapted from Buskirk *et al.*, 2003.)

acquired significant structural changes during its *in vivo* evolution. While the sequence and structural changes were crucial for *in vivo* functionality, these changes could never have been rationally designed. None of the changes bestowed increased stability on the aptamer (an issue when expressing functional RNAs *in vivo*), nor bolstered NF-κB binding affinity. Presumably, the changes increased affinity or specificity for NF-κB in the cellular milieu in a way that is not readily understood by studying protein–RNA interactions *in vitro*. The serial use of *in vitro* and *in vivo* selection methods should eventually prove to be an excellent method for quickly generating functional RNA elements with biological activities.

However, it may not even be necessary to first sieve pools *in vitro*. Liu and colleagues have recently directly selected an RNA regulator that functions as a transcriptional activator (Buskirk *et al.*, 2003). A random pool of RNA was expressed in yeast and localized upstream of a selective marker, the *his3* DNA (*Figure 2.4*). The pool was flanked by hairpin structures to increase stability in the transcribed RNA; some of the hairpin structures were also MS2 hairpins that could associate with MS2 coat protein fusions to LexA (*Figure 2.4*). The LexA–MS2 fusion in turn localized to the *lexA* DNA operator (a natural target for the LexA protein), which was cloned directly in front of *his3*. It was hoped that the physical proximity of some RNA structures to the promoter complex would either directly stimulate transcription or that the RNA structures would serve as docking sites for transcriptional activators, which would in turn act upon the proximal DNA sequence.

In the first round of plating, a surprisingly large fraction, 0.2% of the library,

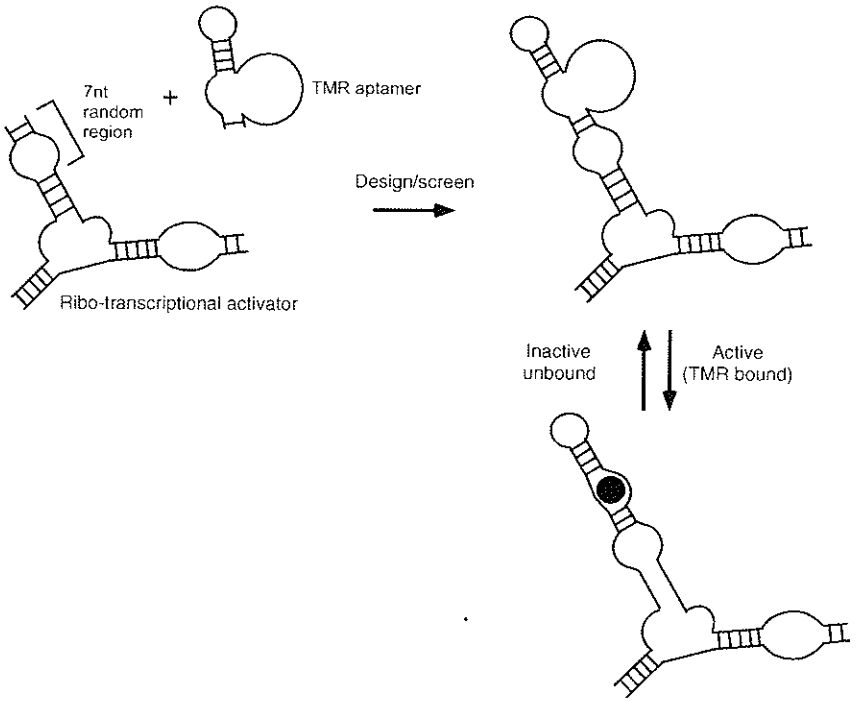

Figure 2.5. Engineering a ligand-dependent RNA transcriptional activator. An anti-tetramethylrosamine (TMR) aptamer was rationally appended to a selected transcriptional activator (left) at a non-critical region. A 7-nt 'communication module' adjacent to the anti-TMR aptamer was randomized, and variants dependent on TMR for activity were identified in a screen. The variants thus obtained were activated up to 10-fold by TMR. (Adapted from Buskirk *et al.*, 2004.)

proved to be functional, indicating that there may be many new ways in which RNA can supplant biology's choice regulatory macromolecules, proteins. In additional rounds, increased selection pressure (using a competitive inhibitor of His3) and biased randomization of the strongest initial clone led to the isolation of RNA variants with greatly increased activity. The most active clones contained several highly conserved structural elements that were required to activate transcription. The most functional clone activated transcription 53-fold better than the highly active protein transcriptional activator, Gal4, under the same conditions. Importantly, the RNA transcription activators acquired through this selection were not gene specific; they were also capable of activating LacZ expression under the same operator and promoter. Upon removal of the local MS2–LexA recruitment complex, the selected RNAs were no longer able to activate transcription. The fact that the selected RNAs were not ubiquitously active within the cell, but rather required spatial localization, should allow this tool to be used to program the transcription of individual target genes, just as specific interactions between transcription factors or repressors and DNA binding sites have been adapted previously to regulate heterologous genes.

From an engineering standpoint, one of the most striking advantages of selecting RNA regulators is that the secondary structures of functional molecules can be

predicted, and mutations can be introduced rapidly to test the predicted relationship of structure to function. Once a functional structure has been mapped, constructs can be engineered intelligently for more complex behaviour and control. For example, Liu and colleagues were able to remove a non-critical segment of the original transcription activator and replace it with an anti-tetramethylrosamine (TMR) aptamer (Buskirk *et al.*, 2004; *Figure 2.5*). They then randomized a 7-nucleotide segment immediately adjacent to the aptamer domain that spanned a stem structure required for activity (akin to a communication module in an aptazyme). Clones were screened for TMR-dependent transcriptional activation, and a variant that exhibited ~10-fold activation in the presence of TMR was isolated. As expected, this clone exhibited dose-dependent activation dynamics, a feature that may be useful for fine tuning gene expression.

Though *in vivo* selections and screens have been established as powerful methods for generating novel RNAs with desired biological functions, the rational design of genetic control elements, especially those inspired by nature, has been a productive avenue for the development of novel tools for the synthetic biological toolbox. As the above examples elucidate, the generation of novel transcriptional regulators is currently a problem best solved by directed evolution methods. This makes sense in that the mechanisms controlling transcription are generally complex protein–DNA interactions, which are not amenable to simple rational design strategies. To the contrary, translational control is often mediated by secondary structural features of RNA, which are extremely facile to predict, modify, and assay. These characteristics bode well for rational design efforts, and indeed, there are many examples of rational strategies for the development of translational control *in vivo*.

Conclusion: genetic regulatory circuits in medicine

RNA regulators have now been shown to remove control two of the most fundamental aspects of gene expression, transcription and translation. Since translational control is often mediated by predictable secondary structural features of RNA, it should be possible to rationally modify these features to be responsive to effectors or to interact with one another. This is also true for some types of transcriptional control, such as termination. For those mechanisms that involve complex protein–protein or protein–DNA interactions that are not as easily amenable to design rules, directed evolution methods can be used, as in the generation of novel transcriptional regulators Together, rational design and directed evolution have proved to be powerful methods for the development of novel RNA regulatory tools for the synthetic biological toolbox.

The challenge now is to apply these tools to making practical genetic regulatory circuits. The novel circuits described above are an interesting foray of engineering into biology (or vice versa), but might not seem to have much in the way of immediate practical value. However, to the extent that synthetic biology is just a new twist on biotechnology, the ability to design and evolve regulatory circuits should have important applications in the development of increasingly smarter therapeutics. The regulatory power represented by the tic-tac-toe game described above is an indication of what might be achieved (Tabor and Ellington, 2003). A less complex, but more relevant, example has been developed by Shapiro and co-

workers (Benenson *et al.*, 2004). These authors have designed a DNA 'computer' that relies on a series of *trans* DNA regulators and hybridization to an mRNA from a pathological allele to create an ordered set of restriction cleavages *in vitro*. The DNA computer ultimately releases an antisense drug against the pathological allele. While the computer is ultimately impractical (given the lack of appropriate restriction enzymes in a human cell, and the inherent problems associated with trying to keep *trans* regulators and their targets together), it is, nonetheless, a working example of how combinations of molecular diagnostics and molecular therapeutics might be brought together in the future to create smart drugs.

The RNA tools described throughout may soon yield far more practical examples of how sequence- and analyte-dependent modulation of activity can lead to smart therapeutics. For example, ribozymes could potentially be regulated by mRNAs, other ribozymes, or cleavage products, leading to interesting hierarchies of regulation and complex behaviour or computational ability. The first taste of such dreams has been provided by Taira and co-workers, who modified a hammerhead ribozyme to be allosterically activated by a disease-related mRNA, and to subsequently cleave and down-regulate that mRNA in human tissue culture cells (Kuwabara *et al.*, 1998). The hammerhead was designed to base-pair with a ~20 nucleotide region of the target mRNA in order to elicit allosteric activation. The RNA trigger was expressed as part of a luciferase reporter mRNA, and the ribozyme specifically down-regulated this fusion mRNA to almost negligible levels in the cell. Again, the engineering of base-pairing specificity was so simple and straightforward that presumably this, or another ribozyme, could be engineered to be activated by, and subsequently cleave, virtually any RNA sequence. By rationally tuning base-pairing, it should also be possible to rationally fine tune ribozyme activation and catalysis, and thus the dynamics of regulatory circuits in otherwise extremely complex and poorly defined cellular environments.

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