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Engineering a Ligand-Dependent RNA Transcriptional Activator

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Summary

RNA has recently been shown to play diverse roles in gene regulation, including the small molecule-dependent inhibition of translation in prokaryotes. To create an artificial genetic switch that acts at the level of transcription, we fused a small molecule binding aptamer to a previously evolved RNA that activates transcription when localized to a promoter. We designed a conformational shift in which a helical element required for transcriptional activation was stabilized upon ligand binding. Selection and screening in *S. cerevisiae* optimized the linker region, generating an RNA that is 10-fold more active in the presence of tetramethylrosamine (TMR). TMR increases the activity of this evolved RNA in a graded, dose-dependent manner. Our results exemplify a strategy for controlling the activity of laboratory-evolved RNAs in living cells.

Introduction

RNA plays multiple roles in the cell as a carrier of genetic information, a catalyst of several crucial biological reactions [1], and a regulator of gene expression [2, 3]. The recent discovery of natural RNA sequences that bind to metabolites in vivo identified a new mechanism of regulation: small molecule-dependent translational inhibition mediated by RNA "riboswitches" [4]. For example, Breaker and coworkers identified a riboswitch element in the 5′-untranslated region of several mRNAs coding for genes involved in thiamine biosynthesis in *E. coli* [5]. The TPP riboswitch consists of two domains: a TPP binding aptamer, and an "expression platform" that couples TPP binding to a conformational rearrangement that blocks the initiation of translation. The conservation of aptamer domains across species and the ability of these domains to bind ligand in the absence of the expression platform suggest that natural RNA aptamer domains are modular [4].

The creation of genetic switches can also benefit from the combination of modular components; eukaryotic transcription factors, for example, are amenable to engineering largely because their DNA binding and transcriptional activation domains are independent [6]. The ability of RNA to form functional modules suggests an analogous approach to RNA-based switches that would regulate gene expression in response to the presence of a small molecule. The creation of such artificial switches requires two elements: the ability to bind a small molecule, and the ability to transmit that binding into downstream functional changes.

RNA possesses a number of attractive properties for use as a tool to regulate cellular function. Powerful in vitro evolution methods can rapidly identify RNA aptamers for a wide variety of proteins or small molecules of interest [7]. Aptamers are capable of binding to their targets with very high specificity and affinity, and can be expressed in vivo [8]. For example, RNA aptamers against RNA polymerase II [9], NF-κB [10], RNA splicing factor B52 [11], and the β2 subunit of human integrin [12] have been expressed in situ and have been shown to inhibit protein function in living eukaryotic cells. These developments suggest that RNA aptamers evolved in vitro can be used as probes to control and study biological function.

Breaker and coworkers have elegantly shown that catalytic RNAs can be evolved to acquire ligand dependence [13]. For example, the hammerhead ribozyme was engineered to cleave over a thousand times faster in the presence of theophylline [14]. Similarly, Beal and coworkers evolved RNA aptamers that bind and inactivate the bacterial DNA-repair protein MutM in vitro in a manner that is blocked by neomycin [15]. Suess and coworkers recently used the linker region evolved by Breaker to design a theophylline-dependent translational switch in *B. subtilis* [16]. These examples suggest that evolutionary approaches are capable of generating and optimizing complex conformational shifts in RNA that link ligand binding to changes in function.

We previously evolved an RNA aptamer in *S. cerevisiae* that strongly activates transcription when tethered upstream of a reporter gene [17]. Here we report the development of a ligand-dependent transcriptional switch generated by fusion of the RNA transcriptional activator to a known small molecule aptamer, followed by the design and selection of a small library of linker regions. The basic mechanism of ligand dependence was confirmed by assaying a series of site-directed mutants. These findings demonstrate that RNA aptamers can be engineered to control biological function with synthetic small molecules in living cells.

Results

Design of a Ligand-Dependent Conformational Shift

The creation of an RNA-based transcriptional switch requires that a functional RNA acquire both small molecule binding activity and a small molecule-dependent conformational equilibrium that transduces binding into altered function. As the starting point for our engineering efforts, we used an RNA that we previously evolved in *S. cerevisiae* to activate transcription [17]. Characterization of one of the strongest activators, m26-11, suggested three regions that participate in base pairing (bases 17–19, 23–33, and 35–38) and are required for

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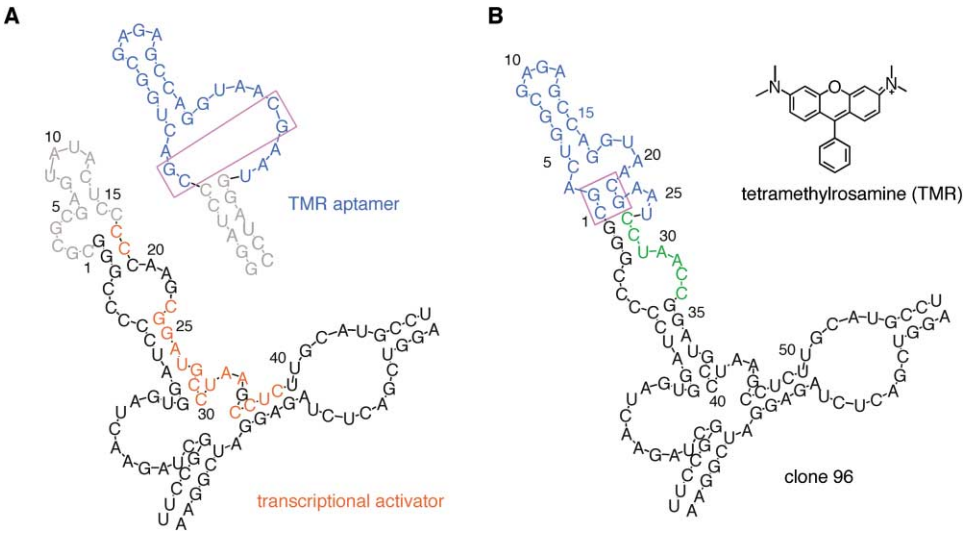

Figure 1. Design of a Ligand-Dependent Transcriptional Activator

(A) Critical positions in the m26-11 transcriptional activator are shown in red [17]. Nonessential bases 1–16 (gray) were replaced with bases 7–32 of the TMR (tetramethylrosamine) binding aptamer [18] (blue). Two C:G base pairs (boxed in purple) within the TMR binding aptamer are stabilized upon TMR binding.

(B) Secondary structural prediction for clone 96 when base pairing between C1 and G23 and between G2 and C22 is enforced. The formation of these base pairs upon TMR binding stabilizes the base pairing of bases 27–29. The randomized linker region is green. Base numbering from clone 96 is used throughout the text.

transcriptional activation (Figure 1A). In contrast, bases 1–16 are predicted to not interact with essential secondary structural elements, are not conserved among related activators, and can be mutated with little effect on function.

We sought a well-characterized small molecule binding aptamer to insert into the nonconserved region of m26-11. An aptamer selected by Wilson and coworkers [18] binds tetramethylrosamine (TMR), an aromatic intercalator (Figure 1B), with high affinity (K_d = 40 nM) [19]. TMR is known to cross the *S. cerevisiae* cell wall and is nontoxic to yeast at concentrations up to 1 μM [20]. Positions in the aptamer crucial for ligand binding have been identified by site-directed mutagenesis and X-ray crystallography [19]. The structure reveals that the TMR binding loops form two C:G base pairs that stack on either side of the bound ligand (Figure 2). Ligand binding stabilizes these base pairs, orders the loops, and increases the stability of the helices at either end of the aptamer. These conformational changes suggest a strategy for linking TMR binding to the activity of the m26-11 transcriptional activator.

We previously showed that mutation of m26-11 bases 17 or 18 from C to A led to a 10- to 20-fold reduction in transcriptional activation [17]. Based on structural models, we proposed that these two conserved bases, together with C19, form key C:G base pairs that stabilize a secondary structure required for activity (Figure 1A) [21]. We hypothesized that when bases 1–16 of m26-11 are replaced with the core of the TMR aptamer (bases 7–32), the flexibility of the unliganded loops would destabilize these three C:G base pairs and render the RNA inactive. Upon TMR binding, the loops may become more ordered, stabilizing these key base-pairing interactions within m26-11 (Figure 1B) and restoring transcriptional activation activity.

Selection and Screening to Optimize Ligand Dependence

Since it is difficult to predict the optimal linker sequence that would efficiently transduce TMR binding to the restoration of m26-11 function, we randomized seven nucleotides on one strand in the linker region (shown in green in Figure 1B), including the bases predicted to be involved in the key secondary structure (27–29). DNA

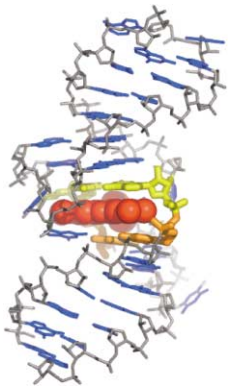

Figure 2. Structure of TMR Binding Aptamer Bound to TMR

TMR is shown in red, the C1-G23 base pair in orange, and the G2-C22 base pair in yellow (Figure 1B numbering) [19]. Rendered with Pymol.

oligonucleotides containing the TMR aptamer sequence fused to the activator through seven randomized linker nucleotides were cloned into an m26-11 RNA expression vector as previously described [17]. The library was amplified in *E. coli* (1×10^6 transformants providing full coverage of the theoretical diversity of 1.6×10^4 linkers) and used to transform *S. cerevisiae* selection strain YBZ-1. This yeast strain contains *HIS3* and *lacZ* reporter genes downstream of lexA operator sites and expresses a LexA-MS2 coat protein fusion that localizes our RNA construct to these reporter genes [22]. RNAs that are capable of transcriptional activation enable the cells to grow in the absence of histidine and to express β-galactosidase.

YBZ-1 cells expressing the RNA library (1×10^5 transformants) were plated onto media lacking histidine and containing 1 μM TMR. Selection plates also contained varying amounts of 3-aminotriazole (0, 0.2, or 1.0 mM 3-AT), a competitive inhibitor of His3p activity, to increase the stringency of the selection. Several thousand colonies grew without any 3-AT, several hundred colonies grew in the presence of 0.2 mM 3-AT, and ~50 colonies grew robustly at the highest stringency (1 mM 3-AT).

To assess whether these surviving clones encoded ligand-dependent transcriptional activators, 50 robust colonies and 60 smaller colonies surviving the selection with 1 mM 3-AT were replated onto fresh selection media in the presence or absence of TMR. Of the 110 colonies screened in this manner, 17 colonies (9 from the 60 smaller colonies) displayed promising phenotypes by growing more readily in the presence of TMR than in its absence. After confirmation of positives by retransformation and plating, four unique clones (clones 19, 32, 77, and 96) exhibited significantly faster growth in the presence of TMR, further suggesting that transcriptional activation was increased by ligand binding. While clones 19, 32, and 77 each showed modest ligand dependence, as characterized by differences in the number and size of colonies, clone 96 showed a more dramatic phenotype: no growth in the absence of TMR in media lacking histidine and containing 1 mM 3-AT and robust growth on the same media containing 1 μM TMR (Figure 3A).

To further characterize the selected clones, cultures of clones 19, 32, 77, 96, and m26-11 (without the TMR aptamer as a control) in YBZ-1 were assayed for β-galactosidase activity after growing 24 hr either in the absence or presence of 1 μM TMR. The presence of TMR did not affect transcriptional activation by m26-11, confirming that TMR alone does not influence transcription of the reporter gene (Figure 3B). Clones 19 and 32 exhibited only modest ligand-dependent activation (≤1.6-fold higher β-galactosidase activity in the presence of TMR). Clone 19 maintained the level of activity seen with m26-11 and was slightly enhanced upon TMR binding. Clone 32, however, showed much weaker activity even in the presence of TMR (data not shown), consistent with its poor survival in the histidine selection system. Two clones demonstrated stronger levels of TMR dependence: clone 77 was activated 2.3-fold, while clone 96 was 10.3-fold more active in the presence of TMR (Figure 3B). Both of these clones exhibited reduced activity in the absence of TMR and potent transcriptional activation, comparable to that of m26-11, upon addition of TMR.

Sequence analysis revealed that the conserved CCC sequence (bases 17–19 in m26-11; bases 27–29 in clone 96) in three of the above selectants (clones 19, 77, and 96) was mutated to CCU (Figure 4). Secondary structure prediction using the mfold algorithm [21] suggests that CCU can replace the conserved CCC sequence and pair with GGG (bases −3 to −1; hereafter all base numbers are from clone 96, Figure 1B) to maintain the key interaction present in the original m26-11 aptamer. We hypothesize that the introduction of the G-U wobble pair destabilizes this pairing enough to significantly lower transcriptional activation potency, as evidenced by the lower activity of the selected clones in the absence of TMR compared with that of m26-11. In the presence of TMR, the loops of the aptamer are ordered by the formation of two C:G base pairs (C1:G23 and G2:C22) surrounding TMR, stabilizing duplex structure in this region (Figure 1B). This stabilization enhances pairing of the CCU-containing region, restoring function. The single nucleotide change of C29 to U is not sufficient for maximum observed ligand dependence since clones 96, 77, and 19 each contain this mutation but display varying degrees of activity and ligand dependence. These results highlight the advantages of an evolutionary approach that can simultaneously optimize complex and interconnected conformational changes that occur upon small molecule binding.

Mechanism and Properties of Ligand-Dependent Activators

To test our hypothesis about the importance of the predicted wobble pair and to demonstrate that TMR binding is necessary for the observed ligand dependence, we prepared three site-directed mutants of clone 96, the most highly ligand-dependent transcriptional activator. Mutant m96-1 reverts U29 (in CCU) to CCC to test the role of the wobble pair. The above model predicts that m96-1 should be highly active but much less dependent on TMR. Indeed, β-galactosidase assays of m96-1 revealed that its absolute activity is similar to that of activated clone 96, but that it is much less dependent on ligand (1.8-fold instead of 10.3-fold for clone 96, Figure 3B).

Mutant m96-2 contains a C22 to A mutation in the TMR binding loop that is predicted to abolish TMR binding [18, 19]; similarly, mutant m96-3 mutates a critical A (base 21) in this binding loop to C. If TMR binding occurs in a manner similar to that of the isolated aptamer and is necessary for the observed ligand dependence, then both of these mutants should not be activated upon addition of ligand. Mutants m96-2 and m96-3 indeed exhibit the same low activity as clone 96 in the absence of ligand, and addition of TMR does not further activate transcription (Figure 3B). These findings indicate that TMR binding is required for the observed ligand dependence of the clone 96 RNA.

To explore further the properties of clone 96, we determined the dose dependence of transcriptional activation by growing cultures expressing clone 96 in the presence of varying concentrations of TMR ranging from 1 nM to

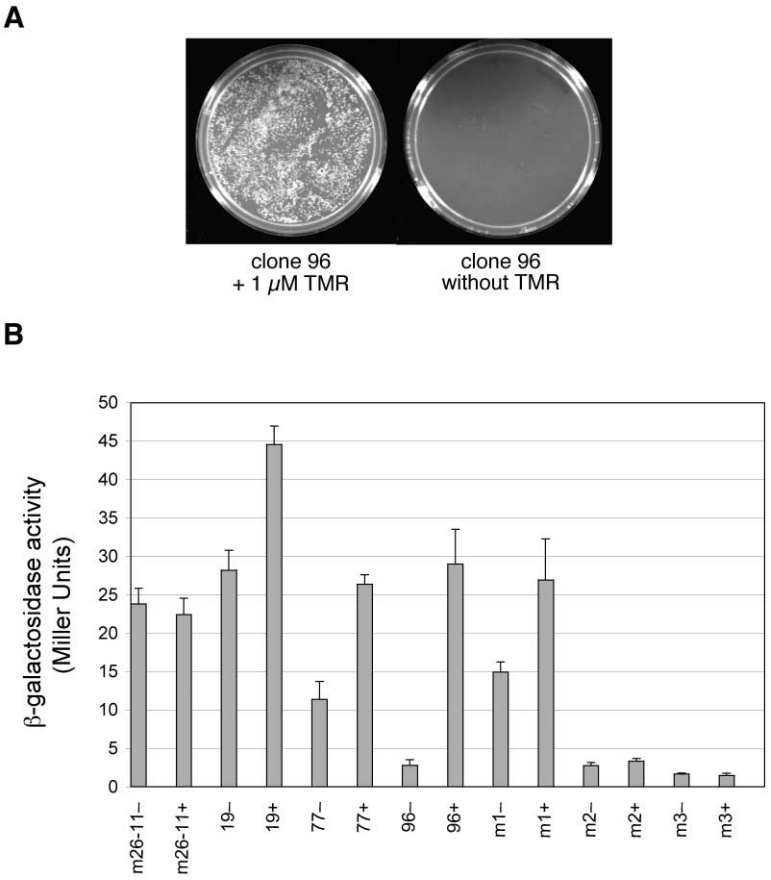

Figure 3. Transcriptional Activation and Ligand Dependence of RNAs in This Study

(A) *S. cerevisiae* strain YBZ-1 cells transformed with clone 96 were plated on media lacking histidine and containing 1 mM 3-AT, a competitive inhibitor of His3p activity. Transcriptional activation of the *HIS3* reporter conveys survival on media containing 1 μM TMR (left), but no survival in the absence of TMR (right).

(B) Quantitative β-galactosidase assays of lysates from cells expressing various RNAs described in this work grown in the presence or absence of 1 μM TMR. Error bars reflect standard deviations of values from independent assays performed in triplicate.

10 μM. As seen in Figure 5, transcriptional activation increases gradually with higher concentrations of TMR. These results further support the conclusion that TMR increases levels of transcriptional activation through specific binding to the RNA activation domain and modulation of its function.

Discussion

The creation of small molecule-dependent transcriptional switches provides researchers with the ability to regulate biological function with precisely chosen inputs. By appending a known RNA aptamer to a functional RNA and using in vivo selection methods to evaluate a library of possible linker sequences, we created an entirely artificial ligand-activated transcriptional activation domain. This work represents, to our knowledge, the first example of engineering the regulation of a functional RNA by a small molecule ligand to modulate biological function in vivo.

In vivo selection and screening for ligand dependence yielded a transcriptional activator that displays a 10-fold

Clone #	Sequence
m26-11	CGCGCGAGUAUACUCC----------CCCAAGC-GGAUGCCUAAGCCUCUU
19	1 5 10 15 20 25 30 35 40 45 50 CGACUGGCGAGAGCCAGGUAACGAAUCCUGGGU-GGAUGCCUAAGCCUCUU
32	CGACUGGCGAGAGCCAGGUAACGAAUCCGGGCA-GGAUGCCUAAGCCUCUU
77	CGACUGGCGAGAGCCAGGUAACGAAUCCUUACGCGGAUGCCUAAGCCUCUU
96	CGACUGGCGAGAGCCAGGUAACGAAUCCUAACC-GGAUGCCUAAGCCUCUU
96 mut-1 U29C	CGACUGGCGAGAGCCAGGUAACGAAUCCCAACC-GGAUGCCUAAGCCUCUU
96 mut-2 C22A	CGACUGGCGAGAGCCAGGUAAAGAAUCCUAACC-GGAUGCCUAAGCCUCUU
96 mut-3 A21C	CGACUGGCGAGAGCCAGGUACCGAAUCCUAACC-GGAUGCCUAAGCCUCUU

Figure 4. Sequences of TMR-Dependent Activators

Nucleotides 1–16 of m26-11 were replaced with bases 7–32 of the TMR aptamer (blue). Essential positions in m26-11 are colored red. The randomized linker region (bases 27–33) is shown in green. Site-directed mutations introduced into clone 96 are underlined.

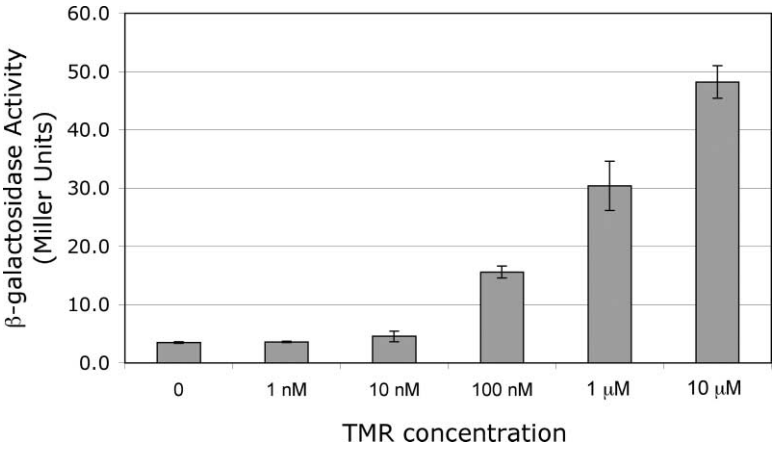

Figure 5. Dose-Dependent Response of a Ligand-Dependent RNA Transcriptional Activator

S. cerevisiae expressing the clone 96 RNA were grown in varying concentrations of TMR and assayed quantitatively for β-galactosidase activity. Error bars represent the range of values from two independent trials.

increase in activity in the presence of a cell-permeable small molecule, tetramethylrosamine. In this mutant, a critical 3 base pair structural element was mutated from CCC (bases 27–29) to CCU. We propose that the weaker wobble pair destabilizes this structure, allowing the unliganded TMR aptamer to force the activator sequence into a nonfunctional conformation. Upon binding of ligand, the TMR aptamer is ordered, with increased helical structure, forcing the CCU sequence at bases 27–29 to pair with GGG (bases −3 to −1) to form an active conformation. As predicted by this model, reversal of this critical CCU to the original CCC sequence increases activity but reduces ligand dependence.

The randomized bases at positions 30–33 also must play a role, since three clones with the CCU at positions 27–29 but differing at 30–33 were shown to have different degrees of ligand dependence. Consistent with this analysis, only 7% of the RNA subpopulation containing CCU at bases 27–29 survived the initial selection (110 survivors in $10^5 \div 64$ CCU-containing library members), underscoring the relevance of the other randomized bases. Positions 30–33 are predicted to form a bulge and can be mutated with little or no effect in the m26-11 context. In the TMR aptamer fusions, these bases may stabilize inactive conformations (through base pairing) in the unliganded structure and thereby influence the conformational equilibrium without altering the ligand-bound secondary structure. The use of in vivo selection techniques therefore identified both the wobble pair and additional optimal sequences that collectively couple ligand binding with increased transcriptional activation.

The success of our design strategy highlights the power of methods available for the manipulation of RNA. Mutagenesis studies and secondary structural prediction tools provided a model of both the structure and key functional determinants in our previously evolved RNA activation domains. This information suggested that a nonessential sequence element could be replaced with a small molecule aptamer, and that the structure could be disrupted in a predictable manner to regulate function. Together with directed evolution techniques, these tools provide powerful engineering capabilities for functional RNAs. The lack of structural prediction tools makes analogous engineering efforts for proteins more difficult.

These results suggest a general approach to the creation of RNA-based probes of biological function than can be regulated by small molecules. As methods exist to create RNA aptamers against a wide variety of proteins of interest, it may be possible to evolve RNA inhibitors of protein function and engineer them to be regulated by a ligand such as TMR [15]. There may be some situations in which it is easier to discover an RNA that inhibits protein function than a small molecule that does the same job directly. Small molecule modulators of protein function are difficult to find for proteins that lack natural small molecule binding sites or that participate in protein-protein interactions [23]. The ability of RNAs to bind to both small-molecule and protein targets with high affinity and specificity may allow their use as ligand-dependent switches to dissect genetic pathways and elucidate gene function. In addition, the observed dose dependence of clone 96 in response to TMR highlights an advantage of small molecule-based approaches over purely genetic approaches to studying biological function: activity can be fine tuned by varying the concentration of small molecule inducer.

The ease with which two preexisting RNAs can be functionally linked has evolutionary implications for the creation of novel ligand-dependent RNAs. Small molecule- and macromolecule binding RNA structures can be modularly combined, analogously to domain swapping in protein evolution, to rapidly generate new functions. Breaker and coworkers recently characterized several natural translational riboswitches that contain modular small molecule binding and regulatory domains [4, 5]. Consistent with these findings, our results suggest that RNA functional elements may have combined in simple ways to serve as sophisticated genetic control systems before the advent of modern protein-based regulation. In addition, the creation of an activator of transcription that is dependent on a specific cell-permeable synthetic small molecule increases the scope of known RNA regulatory activity, which has been primarily limited to repression of gene expression [2, 4].

Significance

The development of small molecule-dependent switches can facilitate the regulation and study of biological

function. We engineered a transcriptional switch from two modular RNA elements: a known small molecule binding aptamer and an RNA-based transcriptional activation domain. Prior structure-function studies and secondary structural prediction enabled the successful design of a conformational shift upon ligand binding, which was functionally optimized by selection in living cells. The resulting RNA transcriptional activation domain displays 10-fold higher activity in the presence of the cell-permeable small molecule tetramethylrosamine. Our results highlight the strengths of tools available for engineering RNA structure and function. The method of generating aptamers to a protein target of interest and appending a small molecule binding aptamer may serve as a general approach to creating small molecule-dependent regulators of biological function in living cells. The ability of RNA to activate transcription in a ligand-dependent manner adds to the known repertoire of gene regulation by RNA and hints at the versatility of modular RNA elements that may have played a role in regulating macromolecular function in an ancient RNA world.

Experimental Procedures

Yeast Strains and Media

Media consisted of yeast nitrogen base (Sigma), 4% dextrose, and synthetic drop out supplements lacking histidine or uracil (Clontech). Yeast were cultured at 30°C. Strain YBZ-1 (*MATa, ura3-52, leu2-3,112, his3-200, trp1-1, ade2, LYS2::(LexA op)-lacZ, LexA-MS2-MS2 coat (N55K)*) was a gift from Prof. Marvin Wickens [22]. Tetramethylrosamine was purchased from Molecular Probes, and 3-aminotriazole was purchased from Sigma.

Construction of RNA Library and Mutants

Plasmids expressing the RNA activator m26-11 and its derivatives were based on the yeast shuttle vector pIIIa/MS2 [22]. Library-encoding sequences were cloned directly into plasmids using unique SphI and XmaI sites. Fusion of the TMR aptamer and m26-11 sequence connected by seven randomized nucleotides was accomplished using the following degenerate oligonucleotide: 5′-CGC GCGGCATGCAAGAGGCTTAGGCATCCNNNNNNNATTCGTTACCT GGCTCTCGCCAGTCGCCCGGGACGCCGACGCC-3′ synthesized on an Applied Biosystems Expedite 8909 DNA Synthesizer. Blunt-ended double-stranded library inserts were generated by primer extension using the Klenow fragment of DNA Pol I from a constant primer binding site on the library oligonucleotides, digested with SphI and XmaI, and ligated into precut pIIIa/MS2 backbone. Library-encoding plasmids were amplified in *E. coli* DH10B and isolated via plasmid purification.

Three mutants of clone 96 were generated by DNA synthesis of both strands incorporating the relevant mutation, followed by annealing, digestion, and ligation as described above. All constructs were verified by DNA sequencing using Applied Biosystems Big-Dye Terminator 3.0 kits. Molecular biology enzymes were purchased from New England Biolabs.

Selection and Assay Protocol

For the selection experiments, the RNA expression plasmid was transformed into YBZ-1 using a standard lithium acetate protocol. Transformants were selected on media lacking histidine and containing 0, 0.2, or 1 mM 3-aminotriazole (3-AT) to increase stringency. Plasmid DNA was extracted from selectants by glass bead lysis and phenol extraction, ethanol precipitated, and amplified in *E. coli*. Selection survivors were initially screened by replating on media lacking histidine, containing 1 mM 3-AT, and containing either no TMR or 1 μM TMR. Retransformed clones were grown on media lacking uracil with or without TMR and assayed in triplicate for β-galactosidase activity using liquid *o*-nitrophenyl-β-galactopyranoside (ONPG) as described [24]. Activity was calculated as Miller units. Growth was inhibited slightly at 10 μM TMR and more strongly at higher concentrations, preventing saturation of the dose-dependence response (see Figure 5).

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