Engineering a Direct and Inducible Protein−RNA Interaction To Regulate RNA Biology

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Many molecular mechanisms underlying post-transcriptional regulation have been described in recent years, and their direct relevance in controlling various cellular processes is being realized. An especially common regulatory scheme is built on the interaction of RNA-binding proteins with unique sequences located in the target transcript (1, 2). Once bound to a transcript, these RNA-binding proteins can directly or indirectly mediate specific functions. In the latter case, accessory proteins are recruited to the foundational RNA−protein interaction site *via* specific protein−protein contacts. Collectively, these mechanisms regulate diverse aspects of mRNA fate within the cell, including its translation, degradation, and subcellular localization (3−5). Modulating RNA translation and degradation *via* small molecule−RNA interactions has been recently emphasized (6). However, these approaches cannot readily take advantage of endogenous RNA regulatory networks that mainly utilize protein-based interactions to achieve, for example, regulated mRNA subcellular localization. Therefore, it can be advantageous to engineer exogenously controlled protein-based RNA regulation that simultaneously exploits the diverse endogenous mechanisms evolved to achieve fine-tuned regulation over multiple facets of RNA biology.

The biological utility of naturally occurring protein-based RNA regulation is inextricably linked to its reversibility. Despite some notable efforts (7−10), orthogonally controlled and directly inducible protein-based RNA regulation has not previously been fully reconstituted in living cells. The objective of the present work is to broaden the landscape of approaches available for manipulating multiple aspects of RNA cell biology. A model prominently motivating this work is the system

ABSTRACT The importance and pervasiveness of naturally occurring regulation of RNA function in biology is increasingly being recognized. A common mechanism uses inducible protein−RNA interactions to shape diverse aspects of cellular RNA fate. Recapitulating this regulatory mode in cells using a novel set of protein−RNA interactions is appealing given the potential to subsequently modulate RNA biology in a manner decoupled from endogenous cellular physiology. Achieving this outcome, however, has previously proven challenging. Here, we describe a ligand-responsive protein−RNA interaction module, which can be used to target a specific RNA for subsequent regulation. Using the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method, RNA aptamers binding to the bacterial Tet Repressor protein (TetR) with low- to subnanomolar affinities were obtained. This interaction is reversibly controlled by tetracycline in a manner analogous to the interaction of TetR with its cognate DNA operator. Aptamer minimization and mutational analyses support a functional role for two conserved sequence motifs in TetR binding. As an initial illustration of using this system to achieve protein-based regulation of RNA function in living cells, insertion of a TetR aptamer into the 5′-UTR of a reporter mRNA confers post-transcriptionally regulated, ligand-inducible protein synthesis in *E. coli*. Altogether, these results define and validate an inducible protein−RNA interaction module that incorporates desirable aspects of a ubiquitous mechanism for regulating RNA function in Nature and can be used as a foundational interaction for functionally and reversibly controlling the multiple fates of RNA in cells.

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underlying mammalian iron storage and metabolism. This mechanism is based on the interaction between a specific RNA sequence, the iron responsive element (IRE), and an IRE-Binding Protein (IRE-BP) (11). An IRE is found within the 5′-UTR of ferritin mRNA, and under low intracellular iron conditions, the IRE-BP associates with the IRE and represses translation initiation and, consequently, ferritin protein synthesis. When intracellular iron concentrations increase, the IRE-BP binds iron and undergoes a conformational change (12), thus lowering its affinity for the IRE. In the absence of bound IRE-BP, translation initiation and ferritin protein synthesis occur more efficiently.

This system can be dissected into three critical biochemical components, namely, (i) an RNA element "marking" a given transcript for regulation, (ii) a sensor protein that can reversibly interact with the RNA element, and (iii) an appropriate chemical stimulus controlling the RNA−protein interaction. With this framework in mind, a strategy we refer to as transcription factor reprogramming was implemented. As part of this approach, a ligand-inducible DNA-binding transcription factor is effectively converted into a ligand-inducible RNA-binding protein to achieve post-transcriptional control over a target RNA. This efficiently achieves the three key requirements outlined above. For this study, the bacterial transcription factor TetR was selected as a sensor protein. This choice was influenced by several considerations. First, this protein has been extensively characterized and is easily recombinantly expressed and purified (13, 14). Second, it can be expressed at suitable levels and functions in a broad variety of prokaryotic and eukaryotic contexts (15−17). Third, TetR specifically interacts with several cell-permeable tetracycline (Tc) analogues with high affinity, permitting regulation that is independent of cellular metabolites. Last, TetR undergoes a conformational change in response to Tc binding, which significantly decreases its affinity for its cognate DNA operator tetO (18). Thus, by discovering RNA aptamers capable of reversibly interacting with TetR in a Tc-dependent manner, a system analogous to the IRE/IRE-BP can be attained.

The isolation and characterization of RNA aptamers that bind TetR in a Tc-dependent manner are reported here. These aptamers bind TetR with low- or subnanomolar affinity and share conserved sequence and predicted structural motifs. We demonstrate that the in vitro discovered TetR−aptamer system can be adapted to function in vivo to post-transcriptionally control prokaryotic protein synthesis in an anhydrotetracycline (aTc)-inducible manner. The experiments described here define both the approach and requisite elements for recreating the fundamental interaction underlying a biologically pervasive RNA regulatory mechanism. Furthermore, our system consists of cell-orthogonal components, which should enable experimenter control over specific RNA-related processes.

RESULTS AND DISCUSSION

Discovering RNA Aptamers Binding TetR in a Tc-Dependent Manner. RNA aptamers with these desired properties were obtained using in vitro SELEX (19, 20). Recombinantly expressed His$_6$-tagged TetR was immobilized on Ni-NTA magnetic beads, and the starting library contained $\sim 10^{13}$ distinct RNA molecules. First, aptamers having affinity for TetR were enriched by eluting His$_6$-tagged TetR−RNA complexes during the first four selection rounds with imidazole. Next, the aptamer subset binding TetR in a Tc-dependent manner was eluted using a low Tc concentration during the fifth and final selection round. The rationale for this approach is based on knowledge that Tc induces a significant conformational change in TetR that abolishes its affinity for its cognate DNA operator sequence, tetO (16). We reasoned that some fraction of the RNA surviving earlier selection rounds could be binding TetR via critical molecular contacts that are disrupted by the Tc-induced conformational change. Therefore, eluting with Tc would selectively enrich this RNA subset.

The Tc-dependent binding properties of the bulk Round 5 selected library were qualitatively assessed by measuring whether His$_6$-tagged TetR bound to Ni-NTA beads could extract RNA aptamers from bulk solution. As a positive control, tetO binding to TetR in this format was tested. About 48% of the incubated tetO bound in the absence of Tc, and adding Tc caused a significant decrease in the fraction of bound tetO to $\sim 14\%$ (Figure 1), consistent with the fact that Tc will induce tetO release. Extending this analysis to the bulk Round 5 library showed that 55% bound TetR in the absence of Tc, and adding Tc significantly reduced the fraction of bound library to $\sim 18\%$. These data confirm that the Round 5 library contains aptamers capable of binding TetR and that this binding is Tc-dependent. Interestingly, TetR-binding aptamers have recently been reported, but Tc-dependent binding was not demonstrated (21).
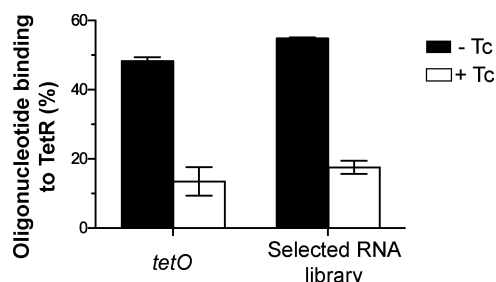
Figure 1. Bulk Round 5 selected library binds TetR in a Tc-dependent manner. *tetO* is the dsDNA corresponding to the *tet* operator sequence. Error bars show standard deviation from three independent experiments.

Characterizing Individual TetR-Binding Aptamers. Individual aptamers (26 clones) from the Round 5 selected library were characterized (Figure 2, panel a). Sequence analysis revealed that 11 distinct aptamers were represented in this group. Several aptamers were represented multiple times, indicating that the selection

strategy very efficiently led to convergence on a library having the desired binding properties in only five rounds. To better understand the TetR-binding properties of the 11 isolated aptamers, qualitative binding assays were performed as before. The majority of these aptamers exhibited Tc-dependent binding (Figure 2, panel b). For aptamer 5−2, although detectable binding is observed, its interaction with TetR appears to be poorly modulated by Tc. For aptamer 5−29 binding could not be unambiguously established, which might indicate that it is a weaker binder relative to the other aptamers or its level of binding cannot be discerned using this assay. Thus, overall, while the selected library comprises mostly aptamers binding TetR in a Tc-dependent manner, both non-inducible but strong binders and poor binders are present at some frequency.

For a more quantitative assessment of aptamer binding affinity for TetR, a cytometry-based binding assay was used. We first established the suitability of this as-
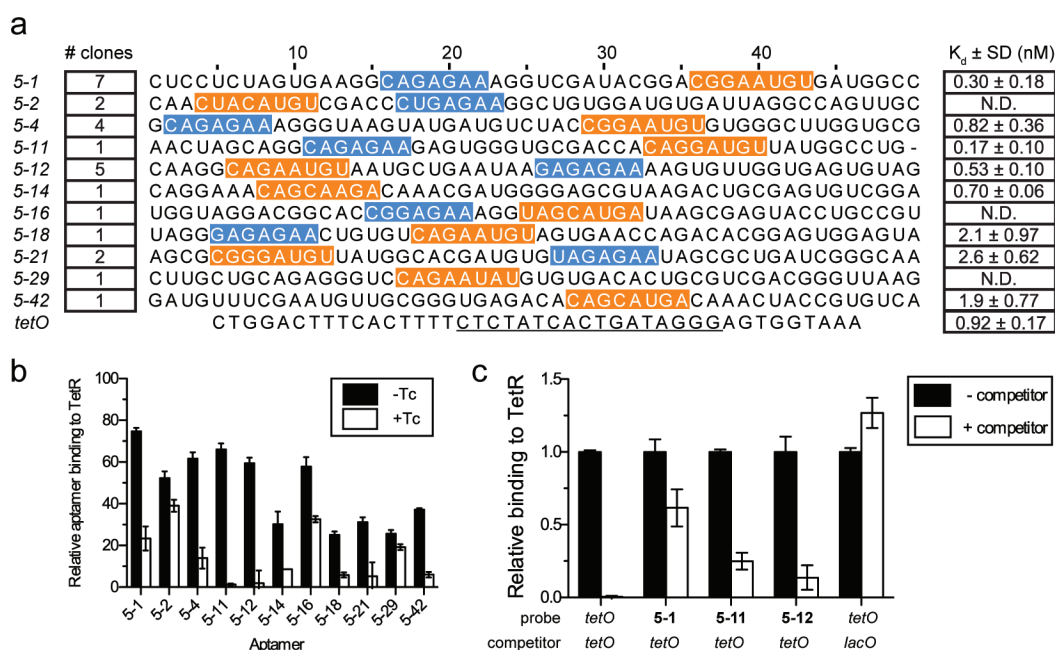

Figure 2. Individual aptamers bind TetR with high affinity and in a Tc-dependent manner and compete with *tetO* binding to TetR. a) Sequence alignment of the variable regions of TetR-binding aptamers. Conserved sequence motifs (see Figure 3, panel a) are highlighted in orange (Motif 1) and blue (Motif 2). The TetR-binding region of *tetO* is underlined. Dissociation constants with standard deviations are shown for selected aptamers. N.D. = not determined. **b)** Tc-dependence of individual aptamers binding to TetR. A threshold of ≥20% was used to define a positive binding interaction based on the residual signal observed during *tetO* binding experiments done in the presence of Tc. **c)** Excess (1 μM) competitor *tetO* reduces TetR binding of fluorescently labeled probe (10 nM). A control oligonucleotide (*lacO*) does not interrupt the specific TetR−nucleic acid interaction.
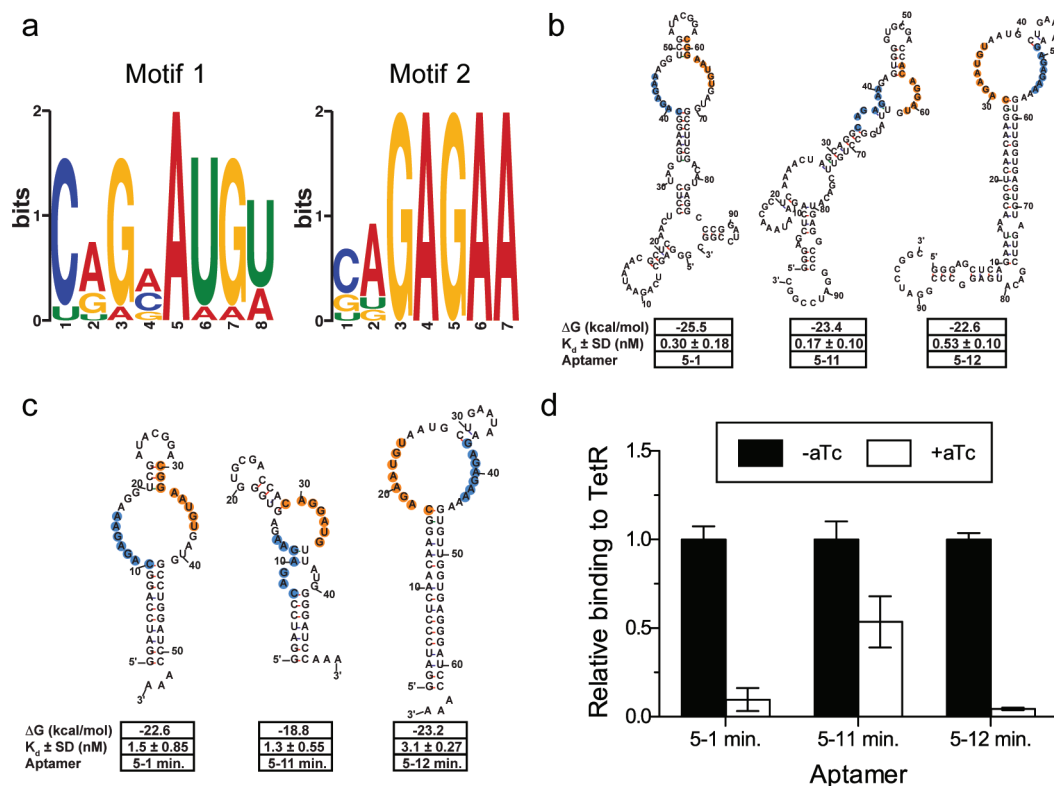
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Figure 3. Conserved sequence motifs are predicted to occupy the loop regions of stem-loop structures. a) Sequence logos for motifs 1 and 2 identified using the MEME algorithm. b) Secondary structure predictions using Mfold for the parent aptamers 5−1, 5−11, and 5−12. c) Minimized versions of aptamers 5−1, 5−11, and 5−12. In both panels b and c, Motif 1 (orange) and Motif 2 (blue) are highlighted. The predicted folding free energies and experimentally determined dissociation constants are also shown. d) Minimized aptamers bind TetR in an aTc-dependent manner. Error bars show standard deviations from three independent experiments.

say to our experimental purposes by determining the K_d for TetR binding to *tetO*. A K_d of 0.92 ± 0.17 nM for this interaction was measured (Figure 2, panel a and Supplementary Figure 1), which compares favorably with the previously reported K_d of 0.17 nM obtained using untagged TetR and surface plasmon resonance (*22*). The discrepancy between the reported K_d and that measured in this study may reflect inherent differences between the binding assays used and/or the *tetO*-binding properties of untagged versus the tagged TetR used in this study. K_d values for selected TetR binding aptamers in the absence of Tc were determined for those exhibiting unambiguous Tc-inducible binding. These were all in the sub- to low-nanomolar range (Figure 2, panel a). Overall, we concluded that our selection strategy had permitted efficient recovery of high-

affinity RNA aptamers exhibiting Tc-dependent binding to TetR.

Though sequence analysis indicated no discernible similarities between the RNA aptamers and *tetO*, we examined whether *tetO* competed with selected RNA aptamers binding to TetR. Previously, RNA aptamers to DNA binding proteins such as NF-κB, Heat Shock Factor (HSF), and TATA Binding Protein (TBP) have been shown to interact with the nucleic acid binding domains in competition with their cognate DNA binding sequence (*23–25*). Using the cytometry-based binding assay, we confirmed that unlabeled *tetO* competed with fluorescently labeled *tetO* for binding to TetR in a positive control experiment (Figure 2, panel c). Similarly, unlabeled *tetO* competed with the labeled RNA aptamers **5−1**, **5−11**, and **5−12** for binding to TetR. However, an unlabeled oligonucleotide corresponding to the *lac* operator sequence, which does not bind TetR, did not compete with binding of labeled *tetO*. These results suggest that the tested aptamers likely interact with the TetR nucleic acid binding domain to interfere with *tetO* binding and that TetR induction by Tc disrupts this interaction.

Defining Minimal Aptamers Retaining Tc-Inducible TetR Binding. To obtain compact, functional aptamers, the sequence elements indispensable to binding were defined. *In silico* sequence motif searching and secondary structure predictions were used in combination with quantitative binding experiments to efficiently explore the possibilities. Using MEME (*26, 27*), two highly

a

Motif 2   Motif 1

```
                  .       10        .        20        .        30        .        40        .
5-1     C U C C U C U A G U G A A G G C A G A G A A A G G U C G A U A C G G A C G G A A U G U G A U G G C C
mut 1   C U C C U C U A G U G A A G G C C A U U C G A G G U C G A U A C G G A C G G A A U G U G A U G G C C
mut 2   C U C C U C U A G U G A A G G C A G A G A A A G G U C G A U A C G G A G A U A G G U C G A U G G C C
mut 1,2 C U C C U C U A G U G A A G G C C A U U C G A G G U C G A U A C G G A G A U A G G U C G A U G G C C
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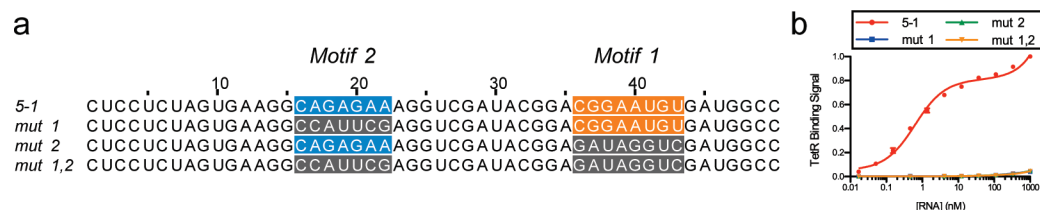
b

Figure 4. Mutating the conserved motifs abrogates aptamer binding to TetR. a) Variable region sequences of the original and mutated 5−1 aptamers. The original motifs are highlighted as in Figure 2, while mutated regions are shown in gray. b) TetR binding curves are shown for the parent 5−1 aptamer and three variants. mut 1 = Motif 1 mutated; mut 2 = Motif 2 mutated; and mut 1,2 = Motif 1 and Motif 2 co-mutated. Curves were fit to eq (1) in Methods.

conserved sequence motifs (Motif 1 and Motif 2) were detected (Figure 3, panel a). Motif 1 is present in all of the sequenced aptamers and is heterogeneous in base composition. Motif 2 is also prevalent but purine-rich. Neither motif shared significant MEME-detected sequence homology with *tetO*, indicating that the molecular interactions responsible for DNA operator and RNA aptamer binding are distinguishable at the nucleotide sequence level. However, the consensus regions do not share common position, order, or spacing within the aptamer sequences, which might suggest that binding is less dependent on primary structure and perhaps more so on factors such as relative positioning of the motifs within the aptamers' secondary and/or tertiary structures.

To explore this possibility further, Mfold (*28, 29*) was used to predict the secondary structures of the RNA. Three representative aptamers were predicted to fold into stem-loop structures (Figure 3, panel b). In each case, the conserved MEME-detected motifs were predicted to occur predominantly in the single stranded loop regions of these stem-loop structures. Together, these findings suggested that preserving both the overall predicted stem-loop structure and the relative positioning of the conserved motifs could be a straightforward approach for defining minimal aptamers.

To test this, aptamers 5−1, 5−11, and 5−12 were selected for further analysis. These aptamers were minimized, while retaining the following regions from the respective parent aptamers: 5−1 (bases 36−74), 5−11 (bases 34−68), and 5−12 (bases 19−68). Furthermore, the stem base sequence was altered while retaining base-pairing in order to explore dispensability of the parental stem sequence. The predicted secondary structures indicated that the respective parental con-

served motif locations and stem-loop structures were retained after these modifications (Figure 3, panel c). These minimized aptamers bound TetR with low nanomolar dissociation constants, albeit ∼5−10-fold higher than the respective full-length parent aptamers. Minimized aptamers 5−1 and 5−12 retained Tc-inducible binding to TetR, comparable to that of the parents. However, the 5−11 truncation displayed induction efficiency significantly lower than that of its parent (Figure 3, panel d). The decrease in affinity could arise as a result of less favorable folding thermodynamics of the truncated aptamers, as indicated by diminished calculated folding ΔG values for two of the three tested truncations (Figure 3, panels a and b). Alternatively, it is possible that poorly conserved aptamer elements outside the conserved regions interact weakly with TetR to provide important stabilization, and removal negatively affects binding and Tc-dependent binding in the case of 5−11 min. Identifying such putative elements will likely require detailed structural analysis of the TetR−TetR aptamer complex.

To further assess the contribution of the two conserved motifs to the TetR interaction, each was mutated individually and both simultaneously within the context of aptamer 5−1 (Figure 4, panel a). Motif 1 was scrambled, whereas Motif 2 was replaced with a randomly generated sequence of the same length due to its high purine content. Mutating one or both of these conserved regions completely abolished aptamer binding to TetR (Figure 4, panel b), suggesting that these regions contribute significantly to the TetR−RNA aptamer interaction. This contrasts significantly with the minimal impact that swapping the stem sequence within the truncation has on binding to TetR. Overall, by using *in silico* primary sequence motif identification and second-
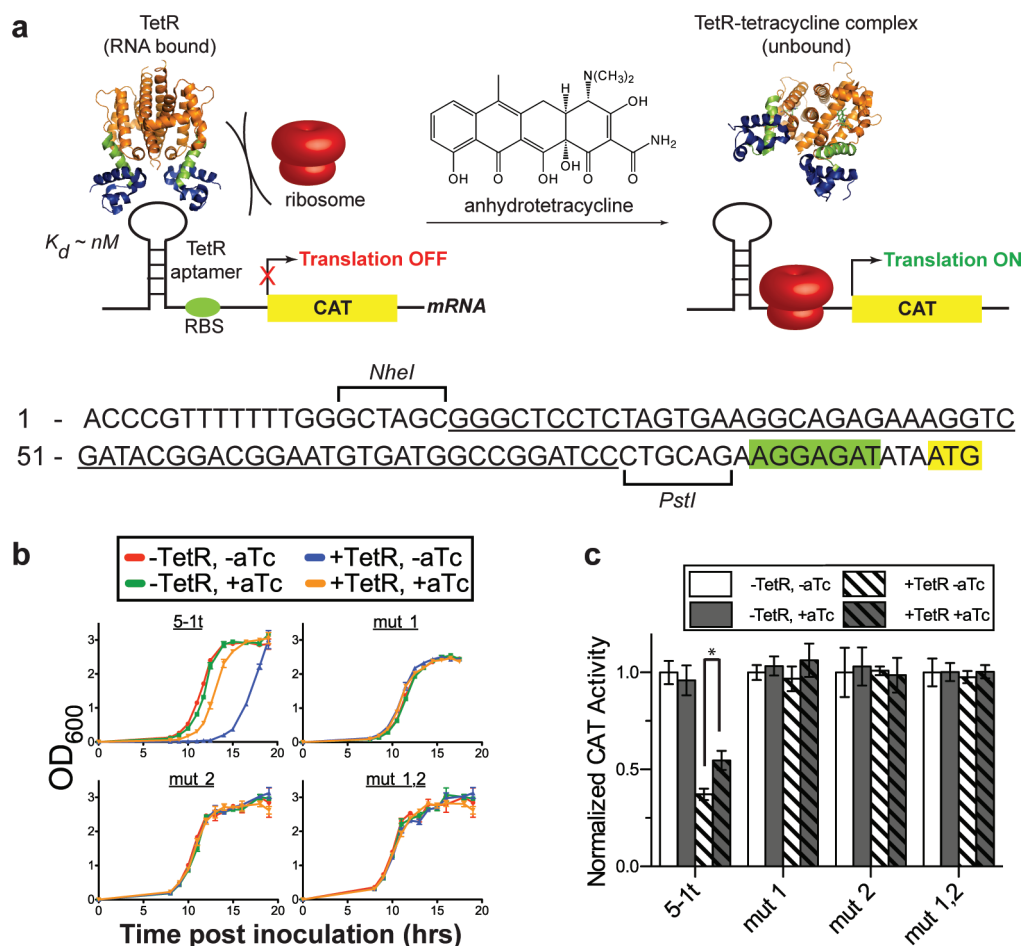
a

TetR
(RNA bound)

TetR-tetracycline complex
(unbound)

anhydrotetracycline

$K_d \sim nM$ TetR aptamer

ribosome

Translation OFF

RBS   CAT   *mRNA*

Translation ON

CAT

*Nhe*I

```
1  -  ACCCGTTTTTTTGGGCTAGCGGGCTCCTCTAGTGAAGGCAGAGAAAGGTC
51 -  GATACGGACGGAATGTGATGGCCGGATCCCTGCAGAAGGAGATATAATG
```

*Pst*I

b

- ■ -TetR, -aTc   ■ +TetR, -aTc
- ■ -TetR, +aTc   ■ +TetR, +aTc

5-1t

mut 1

mut 2

mut 1,2

OD$_{600}$

Time post inoculation (hrs)

c

□ -TetR, -aTc   ▨ +TetR -aTc
▦ -TetR, +aTc   ▨ +TetR +aTc

Normalized CAT Activity

*

5-1t   mut 1   mut 2   mut 1,2

Figure 5. A TetR–aptamer interaction post-transcriptionally regulates protein synthesis in *E. coli*. a) Schematic of the reporter construct used to test functionality of the TetR–TetR aptamer module. TetR binding to the aptamer element in the 5′-UTR of the reporter mRNA can sterically interfere with ribosome access and/or affect mRNA turnover rates (see text), thereby inhibiting CAT synthesis and bacterial growth in *Cm*-containing media. Reversal of the TetR-aptamer interaction with aTc is expected to induce CAT synthesis and favor bacterial growth in *Cm*-containing media. The DNA sequence encoding the CAT transcript 5′-UTR is shown. The 5−1t aptamer is underlined, the RBS and CAT start codon are highlighted in green and yellow, respectively. Restriction sites used for cloning are also indicated. b) *Cm*-dependent growth curves for *E. coli* expressing the CAT construct with the 5−1t aptamer (parental Motifs 1 and 2 present) and 5−1t variants: mut 1 = Motif 1 mutated; mut 2 = Motif 2 mutated; and mut 1,2 = Motif 1 and Motif 2 co-mutated. c) CAT activity measurements in *E. coli* cell lysates derived from cells co-expressing the various reporter constructs and TetR. Data is relative to the −TetR/−aTc condition for each construct tested. Error bars represent standard deviation from three independent experimental replicates. *p-value = 0.006.

aptamer module could be used to functionally modulate the activity of a target mRNA in cells in a post-transcriptional and Tc-responsive manner.

Using a TetR Aptamer for Tc-Dependent Post-transcriptional Regulation. Translation in *E. coli* was selected as a model process for evaluating the TetR/TetR aptamer module. Chloramphenicol acetyl transferase (CAT) was used as a reporter gene. Plasmids encoding TetR (IPTG-inducible) and the CAT reporter were co-transformed into *E. coli*. The CAT reporter transcript contained a TetR-binding aptamer seven bases upstream of the ribosome binding site (RBS) within the 5′-UTR (Figure 5, panel a). This configuration was selected on the basis of previous work demonstrating that naturally occurring or synthetic post-transcriptional regulation is achievable using this relative positioning between small molecule- or protein-binding RNA elements and the RBS (30–32). A model of how this system might work involves TetR binding to the aptamer region within the reporter transcript to inhibit CAT synthesis (Figure 5, panel a). This could occur if

ary structure predictions in conjunction with quantitative binding assays, minimal TetR binding aptamers retaining high affinity binding to TetR were efficiently defined. Given the Tc-inducible interaction of these aptamers with TetR and their minimization to more compact sizes, we next investigated whether a TetR/TetR

the assembled TetR–TetR aptamer complex sterically hinders ribosome access to the RBS (*31, 32*), induces changes in the target mRNA turnover kinetics (*33*), or causes both simultaneously (*33, 34*). Since aTc disrupts the interaction of TetR with its aptamer, adding aTc is expected to increase CAT synthesis and allow cells to

grow in media containing chloramphenicol (*Cm*). To evaluate this, CAT levels and bacterial growth in the presence of *Cm* were measured, both as a function of TetR expression and the presence or absence of aTc.

Several full length and truncated aptamers were inserted between the *Nhe*I and *Pst*I sites within the 5′-UTR of the CAT gene (Figure 5, panel a). Screens identified a truncated version of aptamer **5−1** (**5−1t**; $K_d = 14$ nM) as exhibiting the anticipated behavior in *E. coli*. This relatively low yield of functional candidates was not surprising given the challenges associated with preventing misfolding when adapting *in vitro* derived aptamers for intracellular applications (*35, 36*). During growth experiments in *Cm*-containing media, cells co-expressing TetR and the CAT reporter construct (+TetR/−aTc) grew significantly more slowly, exhibiting a lag time ∼5 h longer than that for control cells (−TetR/ ± aTc) (Figure 5, panel b). Adding aTc to TetR-expressing cells (+TetR/+aTc) significantly reduced this lag time to ∼1 h relative to the controls. Importantly, aTc had no effect on growth in the absence of TetR, indicating that there was no observable regulatory outcome mediated by interaction of aTc with the TetR aptamer element or aTc toxicity. Mutating both conserved motifs individually or simultaneously restored growth to wild-type levels, independent of TetR co-expression and aTc status. Cells grown under repressed conditions (+TetR/−aTc) eventually grew to saturation. A mutation(s) deleterious to the TetR−TetR aptamer interaction were ruled out as an explanation by sequencing the 5′-UTR region of reporter plasmids rescued from these cells. Instead, it is likely that the low residual CAT levels permit some growth, albeit at rates lower than that of the induced (+TetR/+aTc) condition. Once a threshold cell density is attained, these cultures are able to overcome the applied bacteriostatic *Cm* pressure.

CAT activity measurements in cell lysates followed the trend predicted from the growth assay results. Specifically, TetR expression in the absence of aTc decreased CAT activity levels to ∼37% of that measured in the −TetR/± aTc control cells (Figure 5, panel c). Although aTc induction did not fully restore CAT activity to control levels, there was a reproducible and statistically significant ($p = 0.006$) increase in CAT activity to ∼55% of that detected in control cells. In accordance with the results of the growth experiments, all three aptamer mutants exhibited similar relative CAT activity profiles independent of the presence or absence of TetR
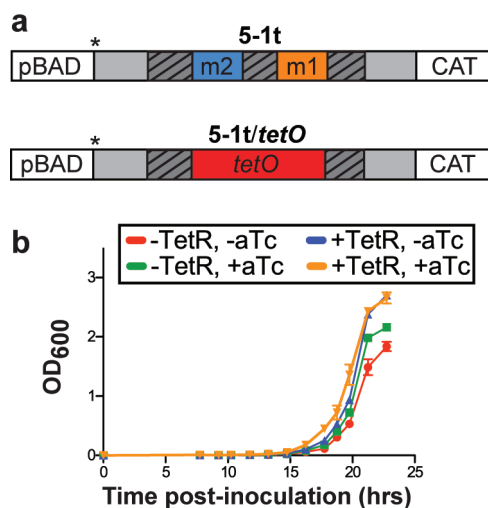

Figure 6. TetR-mediated transcriptional regulation is not responsible for the observed growth phenotype. a) Schematic of the 5′-UTR for reporter constructs with 5−1t and 5−1t/*tetO*. The asterisk indicates the transcription start site. b) *Cm*-dependent growth curves for *E. coli* co-expressing TetR and the 5−1t/*tetO* construct.

and aTc. Thus, the interaction between TetR and aptamer **5−1t** modulated CAT activity level in response to aTc, and the ∼48% increase in CAT activity between the repressed and aTc-induced condition is sufficient to directly mediate a significant change in growth phenotype.

As TetR is a transcriptional regulator, it is important to establish that the measured changes in CAT expression and growth phenotype are not arising transcriptionally. To exclude this possibility, we first determined that TetR did not specifically interact with the DNA encoding the aptamer sequence. While the measured K_d for the TetR/*tetO* interaction is 0.92 nM, the K_d for the interaction between TetR/**5−1t** aptamer DNA is >500 nM. This suggested that an interaction between TetR and the **5−1t** DNA might be too weak to directly mediate robust transcriptional repression.

To further support this reasoning, a hybrid **5−1t**/*tetO* reporter was constructed by replacing both conserved aptamer motifs and the intervening sequence in the CAT reporter construct with *tetO* (Figure 6, panel a). This construct placed a high affinity DNA sequence with known transcriptional regulation function in the exact location as the conserved MEME-detected motifs. Thus, a lack of transcriptional regulation using the **5−1t**/*tetO* (high-affinity TetR binding) construct would

argue strongly against the possibility that the **5−1t** aptamer DNA (low-affinity TetR binding) can do so. Co-expressing the **5−1t**/*tetO* CAT reporter construct with TetR in experiments identical to those described above revealed no differences in growth phenotype as a function of either TetR expression or aTc (Figure 6, panel b). Altogether, these results support a model whereby TetR interacts specifically with the **5−1t** aptamer within the CAT reporter mRNA, and a significant role for transcriptional regulation is ruled out. This strongly supports the conclusion that our TetR/Tc/TetR aptamer module represents a chemically controlled, foundational protein−RNA interaction capable of regulating a biologically important process directly at the level of the target RNA.

Discussion. In this work, we have demonstrated a straightforward approach for effectively converting the transcription factor TetR into a tetracycline-inducible RNA binding protein by selecting for RNA aptamers capable of binding TetR in a Tc-dependent manner. Using *in silico* sequence and secondary RNA structure analyses in combination with quantitative binding assays provided an efficient strategy for rapidly defining functional minimized RNA aptamers considered more desirable for intracellular-based applications. We have shown that the *in vitro*-selected aptamers can be functionally implemented in cells to achieve regulation of protein synthesis as a model process, and that the Tc-inducible interaction between TetR and its RNA aptamer directly mediates the observed regulation.

The inducible protein−RNA interaction we report is an important culmination of significant prior efforts toward the goal of discovering such a system that can be implemented in a manner orthogonal to host cell metabolites. In earlier work, bifunctional tobramycin-biotin compounds were used to force a bridging interaction between a tobramycin-binding aptamer in a target transcript and streptavidin as the repressor protein (*7*). While this approach could be used to regulate translation *in vitro*, its effectiveness in cells was not demonstrated, probably because of the inherent cell-impermeability of the tobramycin-biotin compounds. A more recent alternative approach used a mutant FKBP as a "presenter" protein designed to interact with a bi-functional and cell-permeable ligand, AP1867-guanine (*8*). RNA aptamers that selectively bound the mFKBP-AP1867-guanine complex with high affinity were obtained. However, the functionality of this system in cells has not been reported. In this present work, we have

emphasized using a naturally evolved transcription factor-ligand pair, an approach that can be readily extended by taking advantage of the many examples of these naturally occurring pairs. Indeed, discovering RNA aptamers capable of interacting with transcription factors with high affinity is not an incidental occurrence for TetR, as other examples, both naturally occurring and synthetic, have been reported for NF-κB (*23*), TFIIIA (*37*), and bicoid from *Drosophila* (*38, 39*), for example. Therefore, the selection strategy used in our study can potentially be used to take advantage of other ligand-regulated transcription factors in expanding the functional set of orthogonally regulated protein−RNA interactions.

Natural and synthetic aptamers can bind small molecules or proteins to effect post-transcriptional regulation in *E. coli* (*6, 40*). In comparing the regulation achieved using these systems with that using the TetR/TetR aptamer system, it is important to distinguish two regulatory steps. The first is regulation resulting from the primary interaction of the small molecule or protein with the aptamer target. All of these systems can be directly compared at this level. In this study, the interaction of TetR with its aptamer repressed reporter protein synthesis ∼3-fold. This compares favorably with the previously reported 3−4-fold regulation achieved using the theophylline aptamer (*30*) and 3−6-fold regulation of the *thrS* transcript by its tRNA^Thr ligase (*41*) protein product. In these cases, single regulatory aptamers were presented in contexts similar to that in Figure 5, panel a. Thus, the primary interaction of TetR with its aptamer achieves expected regulation levels. The second regulation step is induction achieved by reversal of the TetR−TetR aptamer interaction by aTc. To the best of our knowledge, the present work is the first report of this being achieved in cells, and thus a published benchmark is unavailable. The incomplete induction seen here could arise if under intracellular conditions the TetR−aTc complex retains some affinity for the TetR aptamer. Alternatively, at the aTc concentrations used, some free TetR may still be present leading to partial repression. Higher aTc concentrations were avoided to completely circumvent any direct aTc-induced cell toxicity. Nontoxic tetracycline mimetics capable of potently inducing TetR have been recently synthesized (*42*). Such compounds could therefore be extremely useful in the future for improving the induction dynamic range attainable with TetR-based systems in bacteria. In extending

this system to eukaryotes that are less susceptible to tetracycline-induced toxicity, this is expected to be less of a challenge.

In a broader context, defining this small molecule inducible protein−RNA interaction offers a unique opportunity for recapitulating and dynamically controlling fundamental aspects of RNA biology, including its translation, processing, subcellular localization, and turnover. Fusing TetR to trans-activating domains such as VP16 has been extensively used for inducibly regulating transcription based on the interaction between TetR and *tetO* at the DNA level (*43, 44*). Similarly, we envision that the TetR-aptamer module developed here will be an important foundational interaction through which experimentally controllable regulation of fundamental and diverse RNA biology can be directly achieved. This potentially permits obtaining a deeper understanding of how regulation of these processes shape basic cellular responses to biologically relevant stimuli. Additionally, in design-oriented disciplines such as synthetic biology this work provides an additional option for directly and controllably regulating intracellular RNA networks. Here, we have illustrated this inducible foundational RNA−protein in a prokaryotic context. However, we anticipate that analogous to the noninducible, bacterially derived MS2 coat protein−RNA interaction, which retains functionality in several eukaryotic contexts including yeast, *Drosophila*, and mammalian cells (*45−47*), the work described here has the potential to be developed into an important platform for more extensively and flexibly regulating various aspects of RNA biology in different organisms.

METHODS

Oligonucleotides were purchased from Integrated DNA Technologies. All chemicals used were analytical or molecular biology grade.

Systematic Evolution of Ligands by Exponential Enrichment (SELEX). The single stranded DNA library used has the sequence: CCGAAGCTTAATACGACTCACTATAGGGAGCTCAGAATAAACG-CTCAA[N$_{50}$] TTCGACATGAGGCCCGGATCCGGC, where N indicates a randomized base. The 5′− and 3′−primers used for RT and PCR amplification of this library are CCGAAGCTTAATACGACTCAC-TATAGGGAGCTCAGAATAAACGCTCAA and GCCCGGATCCGGGCCT-CATGTCGAA, respectively. This dsDNA library was made using PCR and, after agarose gel purification, used as a template for RNA synthesis using the AmpliScribe T7−Flash Transcription Kit (Epicentre Biotechnologies). RNA (500−700 pmol) in diethyl pyrocarbonate (DEPC)-treated water was denatured (70 °C 5 min), allowed to cool to room temperature, and refolded in binding buffer (BB): 50 mM Tris-HCl pH 8.0, 50 mM KCl, 5 mM MgCl$_2$, 5 mM β-mercaptoethanol, 5% glycerol (v/v), and 0.05% Tween-20 (v/v). For negative selection, RNA in 500 μL of BB was added to ∼15 μL pre-washed Ni-NTA magnetic beads (QIAGEN) and incubated at ambient temperature with gentle mixing for 30 min. The supernatant was added to Ni-NTA magnetic beads pre bound with TetR (19−25 pmol) for positive selection, and incubated for 1 hr at ambient termperature with gentle agitation. These beads were washed 5 times with 500 μL of BB. During the first four SELEX rounds, RNA was eluted with 500 mM imidazole in 20 μL BB incubated with the selection beads for 5 minutes. For the fifth and final SELEX round, 100 μM tetracycline in 20 μL BB for 10 minutes was used for elution. RNA was amplified using 4 × 40 μL Ready-To-Go RT-PCR tubes (Amersham) and ∼300 pmol each of the 5′− and 3′−primers. RT was carried out at 42 °C for 40 min. The reverse transcriptase was inactivated at 95 °C × 5 min, followed by 18 PCR cycles (94 °C × 30 s, 57 °C × 60 s, 72 °C × 60 s) and a 7 min final extension. The RT-PCR products were pooled, concentrated, and purified using 4% agarose gel electrophoresis, and the desired length product was extracted and ethanol precipitated. DNA was resuspended in DEPC-treated H$_2$O for the next round of *in vitro* transcription. After the fifth round of SELEX, the evolved library was cloned into the PCR2.1-TOPO vector (Invitrogen) and transformed into DH5α *E. coli*. Single colonies were used for mini-prep cultures from which plasmid encoding a single aptamer was isolated for sequencing and archiving.

Initial Aptamer Binding and Tc-Induction Assays. TetR (200 pmol) was immobilized on Ni-NTA magnetic beads (QIAGEN) in BB. Refolded RNA (100 pmol) was added to the protein−bead mixture (to a final volume of 500 μL) and incubated for 1 h. Supernatant aliquots (20 μL), removed both before and after Tc addition (100 μM for 15 min), were mixed with 200 μL of SYBR Gold solution (1:10,000 dilution SYBR Gold (Invitrogen) in 10 mM Tris-HCl, pH 8.0). Fluorescence signal was measured (excitation = 490 nm, emission = 537 nm) on a Fluoromax-2 fluorometer. Binding to TetR is indicated by a decrease in RNA levels in the supernatant.

Cytometric Bead-Binding Affinity Assays. Aptamers were transcribed *in vitro* (*48*) and fluorescently labeled at the 3′ end using fluorescein-5-thiosemicarbazide (Marker Gene Technologies) as described previously (*49*). Labeled RNA was purified by LiCl/isopropanol precipitation and desalted with a Micro Bio-Spin P-30 column (Bio-Rad). RNA integrity and purity were verified by denaturing urea-PAGE analysis. Labeling efficiency and RNA concentration were determined from A_{260} and A_{492} measurements.

Quantitative affinity measurements of aptamer interactions with TetR were performed as described previously (*50, 51*). Dynabeads TALON (Invitrogen) (1 μL) were washed with Affinity Binding Buffer (ABB) (BB + 10 μg mL^{-1} BSA) and centrifuged at 250 × *g* for 2 min. Beads were resuspended in ABB and incubated with 60 μg of purified His$_6$-tagged TetR for 1 h at RT. TetR-coated beads were washed twice and resuspended in ABB. Beads were counted by microscopy, and 70,000 beads were mixed with fluorescein-labeled RNA in ABB in a 96-well microplate. Nonspecific RNA interactions were reduced by adding 10 μg mL^{-1} yeast tRNA. The binding reaction proceeded with moderate shaking for 4 h at RT. Beads were washed once and resuspended in ABB. Cytometric analysis was performed on an Accuri C6 flow cytometer (Accuri Cytometers Inc.). The median fluorescence intensities of the samples were plotted against RNA or DNA concentration using GraphPad Prism 5 (GraphPad Software, Inc.). Dissociation constants were determined by fitting these data using nonlinear regression to eq 1:

$$Y = B_{max} \cdot \frac{K_d + [L]_o + [P]_o - \sqrt{(K_d + [L]_o + [P]_o)^2 - 4[P]_o[L]_o}}{2[P]_o} + NS \cdot [L]_o + A \qquad (1)$$

where B_{max} is the upper limit of the specific binding signal, K_d is the dissociation constant between TetR and the oligonucle-otide, $[L]_o$ is the initial oligonucleotide concentration, $[P]_o$ is the initial TetR concentration, NS is the nonspecific binding constant, and A is the background signal intensity of the beads.

For *tetO* competition experiments, TetR was initially incubated with 1 μM unlabeled competitor oligonucleotide for 30 min. Fluorescently labeled probe (10 nM) was then added, and the binding reaction was continued for 4 h before cytometric analysis.

For induction experiments with minimized aptamers, saturating levels of labeled RNA were incubated with TetR for 4 h prior to incubation with aTc (333 nM) for 30 min and subsequent cytometric analysis.

E. coli Growth Assays. *E. coli* HB101 cells harboring both the repressor and reporter vector were grown to saturation. These cells were diluted 1:400 and grown for 4 h at 37 °C in LB supplemented with 100 μg mL^{-1} ampicillin and 50 μg mL^{-1} kanamycin for plasmid selection, 0.2% arabinose (w/v), and 1 mM IPTG (to induce TetR expression) and/or 300 nM aTc where appropriate. OD$_{600}$ readings were taken for each culture, and all were diluted 1:500−1:1000 in LB, keeping the cell number the same across all inoculations. This LB was supplemented with antibiotics for plasmid selection, 0.2% arabinose (w/v), 10 μg mL^{-1} chloramphenicol (*Cm*) (or 5 μg mL^{-1} with **5−1t** mutant 1 to preserve comparable growth rates), and 1 mM IPTG and/or 300 nM aTc where appropriate. Growth rate was measured by taking OD$_{600}$ readings over time.

CAT Activity Assays. Cells were grown overnight and diluted 1:1000 into LB containing 100 μg mL^{-1} ampicillin and 50 μg mL^{-1} kanamycin for plasmid selection, 0.2% arabinose (w/v), and either 1 mM IPTG and/or 300 nM aTc where appropriate. Cultures were incubated at 37 °C for 6 h until harvesting. CAT activity was assayed using the fluorescent FAST-CAT substrate (Invitrogen). After extraction, products were separated on silica TLC plates, which were imaged using a Gel Logic 2200 (Kodak) and quantitated using the Kodak Molecular Imaging Software.

Supporting Information Available: Information on vector construction and TetR expression and purification is detailed in Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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