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An RNA Aptamer that Induces Transcription

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SUMMARY

We identified an RNA aptamer that induces TetR-controlled gene expression in *Escherichia coli* when expressed in the cell. The aptamer was found by a combined approach of in vitro selection for TetR binding and in vivo screening for TetR induction. The smallest active aptamer folds into a stem-loop with an internal loop interrupting the stem. Mutational analysis in vivo and in-line probing in vitro reveal this loop to be the protein binding site. The TetR-inducing activity of the aptamer directly correlates with its stability and the best construct is as efficient as the natural inducer tetracycline. Because of its small size, high induction efficiency, and the stability of the TetR aptamer under in vivo conditions, it is well suited to be an alternative RNA-based inducer of TetR-controlled gene expression.

INTRODUCTION

The last decade has revealed a fundamental role for RNA in controlling gene expression. Numerous small noncoding RNA (sRNA) molecules with regulatory activity have been identified in both prokaryotes and eukaryotes. Short noncoding RNAs known as microRNAs and the related short interfering RNAs negatively regulate gene expression in many eukaryotes. They act by recruiting diverse protein factors that process precursors to ∼22-nucleotide-long RNA fragments that form base-paired complexes with their target mRNA (Eulalio et al., 2008; Farazi et al., 2008). Bacterial regulation of gene expression by sRNAs does not follow a common principle. These untranslated molecules are heterogenic in size and structure and act via different mechanisms (Storz et al., 2005). Most bacterial sRNAs serve as antisense RNAs that bind to *trans*-expressed mRNAs and compete with initiating ribosomes, resulting in translational inhibition. However, there are also examples of sRNAs functioning as translational activators by base-pairing to the untranslated region of an mRNA that otherwise sequesters a ribosome binding site; as a result, the poorly translated mRNA becomes translationally active. Further on, sRNAs were found to regulate gene expression by binding to regulatory proteins.

Here, we ask if a bacterial repressor like TetR, which is not known to be regulated by RNA, can be controlled by a small non-coding RNA. This would be a first example of an in vitro selected RNA which acts in the same manner as endogenously expressed sRNAs and which can be used as an efficient inducer for conditional control of gene expression (Berens and Hillen, 2004). Tet repressor (TetR) is one of the most intensely studied transcriptional regulators. It is genetically, biochemically and structurally well characterized and belongs to the Tet/Cam family of bacterial regulators. TetR is a 46.6 kDa homodimer with N-terminally located helix-turn-helix motifs (Orth et al., 2000). Its activity is controlled by tetracycline (tc) or several of its derivatives and tc-dependent gene regulation is nowadays widely used as a tool to control the expression of single genes in many different organisms. Tc-binding to the protein is very specific and occurs in a binding pocket within the protein core. In the absence of ligand, TetR binds to operator sequences, repressing gene expression. Upon ligand binding, the repressor undergoes a conformational change and operator binding is abolished leading to gene expression. Thus, allostery can be triggered upon tc-binding and the concomitant conformational changes are essential for small molecule dependent induction of transcription (Tiebel et al., 2000).

We describe here the isolation and characterization of an RNA aptamer which is able to induce TetR-controlled gene expression. TetR-binding aptamers were isolated by in vitro selection and an additional screening step that was key to identifying aptamers active inside the cell. Mutational analyses define the minimal active sequence and highlight the aptamer bases involved in induction. Taken together, we have created a small noncoding RNA that is able to activate gene expression in vivo.

RESULTS

Identification of an RNA Aptamer that Induces TetR-Controlled Gene Expression

We used a two-step approach to identify RNA aptamers that induce the transcriptional regulator TetR in vivo. First, an automated in vitro selection protocol was followed to isolate RNA aptamers that bind TetR. After 12 rounds of selection, a significant enrichment of TetR-binding RNA was detected and its specificity of binding was verified by a filter-retention assay (Figure 1A).
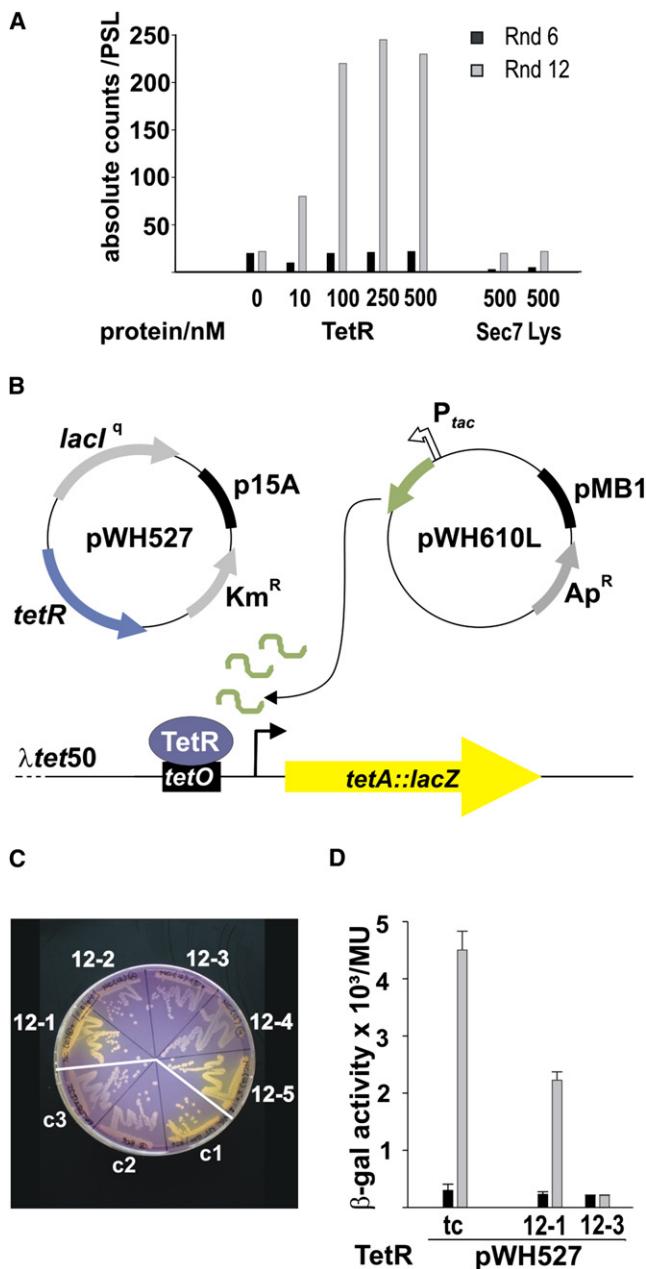
Figure 1. In Vitro Selection and In Vivo Screening System for TetR Inducing Aptamers

(A) Filter-retention analysis to analyze the progression of the in vitro selection procedure. The amount of TetR bound radioactively labeled RNA (absolute amount [PSL]) is given. For specificity analysis the control proteins Sec7 and lysozyme were included. Data resulting from RNA obtained after 6 (black bars) and 12 (gray bars) rounds of in vitro selection are shown.

(B) Screening was done in *E. coli* WH207(λ*tet*50) containing *lacZ* under Tet control. Plasmid pWH527 expresses both TetR and LacI constitutively. TetR is shown as blue oval and controls transcription of *lacZ* gene. The aptamer-expressing plasmid pWH610L encodes an aptamer (green) under control of IPTG/LacI via the P$_{tac}$-promoter. Resistance markers and the respective origins of replication of the compatible plasmids are indicated.

(C) Five individual colonies of *E. coli* WH207(λ*tet*50) pWH527/pWH610L are shown on McConkey indicator plates. Control c1–c3: *E. coli* WH207(λ*tet*50) without any plasmid (c1, β-galactosidase is fully expressed), *E. coli* WH207(λ*tet*50) pWH527 (c2, β-galactosidase is repressed by TetR), and

We then used an *E. coli* strain to screen for aptamer-mediated induction of TetR in vivo (Figure 1B). Aptamers from the in vitro selection were flanked with 5′ and 3′ stabilizing stem loop structures (Blind et al., 1999) and expressed under P$_{tac}$ control from the plasmid pWH610L. Constitutively expressed Lac and Tet repressors are encoded on the compatible low-copy plasmid pWH527. Both plasmids were introduced into *E. coli* WH207(λ*tet*50) carrying a single copy *tetA::lacZ* transcriptional fusion under TetR control (Smith and Bertrand, 1988). Expression of the aptamers is induced by adding isopropylthiogalactoside (IPTG) and only leads to β-galactosidase expression if they, in turn, induce TetR.

The aptamer pool from the 12th round of the in vitro selection was inserted into pWH610L behind the P$_{tac}$ promoter and amplified in *E. coli* providing a pool with 5×10^4 independent clones. Screening of about 2.5×10^5 candidates resulted in ~5% yellow colonies on McConkey agar plates (Table 1, Figure 2C). Sequencing of 30 candidates resulted in only one unique sequence called 12-1 (Table 1). This aptamer shows 7-fold induction of TetR (Figure 2D and Table 2). Sequencing analysis of 18 aptamers that did not phenotypically show induction (white colonies [e.g., 12-2–12-4]; see Figures 2C and 2D) yielded five different sequences, demonstrating that although the complexity of the inserted pool was low it still contained individual sequences (Table 1).

The RNAs expressed are composed of a 50-nt-long random region flanked by the constant regions C1 and C2 required for in vitro selection. To improve the stability of the aptamer sequences in vivo, we attached stabilizing stem loop structures 5′ and 3′ to the aptamer pool (depicted schematically in Figure 2). We constructed a series of truncations to identify the minimal sequence of the 12-1 aptamer that is still able to induce TetR (Figure 2). Activation of β-galactosidase expression of these constructs is summarized in Table 2. In the absence of IPTG, the TetR-repressed β-galactosidase activity of all truncated constructs was identical to that of the parent construct 12-1. 12-1H and 12-1K are the constructs with the largest 3′ and 5′ truncations, respectively, that retained activity. Their combination in 12-1M defines a 49-nt-long sequence as minimal sequence sufficient to induce TetR.

The Induction Efficiency Depends on the Concentration of the Aptamer and TetR

Secondary structure calculation of the TetR inducing aptamer 12-1 using the program Mfold (Zuker, 2003) predicts a stem-loop structure with two stems P1 and P2 separated by an extended internal loop region (Figure 3A). The comparison between 12-1E and M (Table 2) led us to assume that the stability

E. coli WH207(λ*tet*50) pWH527/pWH610L expressing a nonsense aptamer (c3). 12-1–12-5 are individual colonies expressing different aptamers from pWH610L after 12 rounds of in vitro selection.

(D) β-Galactosidase activity was quantified with cells grown at 37°C. The left bar doublet represent the controls in which β-galactosidase activity is repressed by TetR (black bar) or induced with 0.2 μg tc (gray bar). The following bar doublets represent β-galactosidase induced by the individual aptamer clones 12-1 and 12-3 expressed on pWH610L. Aptamer expression is induced with no IPTG (black bars) and 1 mM IPTG (gray bars), respectively. Three independent clones were assayed and experiments were repeated twice.

Table 1. Sequence Distribution of the In Vitro Selected Aptamer Pool

				Sequenced Candidates	
Clone No.	Phenotype	Sequence of the N$_{50}$ Region	Distribution[a]	Y: 30[b]	W:18[c]
12-1	Yellow	AGCAGCATGTTATGGGTCATCACAGACCAGAGAAAAGCTTGATAGTAAAG	5%	30	
12-2	White	TATTGGGAAACCTGTAGAGCGGACGAACGGGCATGGAGTTAGGGCGGAGG	26.3%		5
12-3	White	GCATATAGGCCAGAATGAGTGTACGCAAGTTAGTCCCTAGTATACGCAGC	5.3%		1
12-4	White	TTGCGTATACAAGCGGCACCACAAACGCGGGATAGGATTTAGAGCGGAGG	31.6%		6
12-9	White	ACCAGAGGATAAATACGCAATTGAGAGGCACCTGAAGGCCTAGACACGGT	21.0%		4
12-11	White	TATAACTCGGGTAAACCGATGATAGCAGCCTTCCCGGAAAGGGCGGAGGG	10.5%		2

[a] 5% yellow colonies were obtained, distribution of white colonies were calculated using the sequences of 18 sequenced candidates which correspond to 95% of the total population.
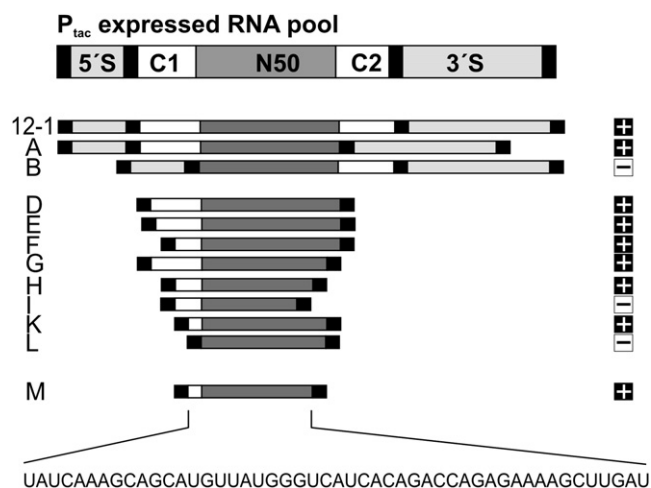[b] Thirty yellow colonies were sequenced and yielded one unique sequence.
[c] Eighteen white colonies were sequenced and yielded five different sequences.

of stem P1 might affect aptamer activity. To test this, we deleted the bulged nucleotide A20 (marked with ΔA in Figure 3A) and successively elongated the closing stem P1 (constructs P-U with 1, 3, 4, 6, 8, and 10 additional GC base pairs, respectively, see Figure 3A). The induction efficiency of these new constructs is shown in Figure 3B and Table 2. The results show a clear correlation between the stability of the closing stem and the induction efficiency with a maximum for 12-1R (Figures 3 and 4; Table 2). A further increase in stem length does not improve the aptamer's ability to induce TetR in this regulatory system.

Stem regions are most often active as stabilizing scaffolds for the respective binding pockets and of minor importance with respect to sequence and length. To prove this, we completely altered P1 and P2 (12-1X and 12-1Y). We thereby exchanged

Figure 2. Schematic Aptamer Design and Activity Profile of Truncated Variants

Graphical representation of the expressed aptamer pool with small black boxes representing the respective restriction sites XbaI, NcoI, XhoI, and NdeI (from left to right) and light gray boxes marked with 5′S and 3′S indicating the stabilizing stem loop structures flanking the aptamer pool. White boxes (C1 and C2) are the constant regions of the aptamer pool and the randomized region is shown as a dark gray box (N50). All boxes are drawn to scale. The aptamer truncations are displayed with the sequence of the minimum inducing aptamer 12-1M. Truncated aptamers that are able to induce TetR 5-fold and higher are marked with "+" and inactive once with a "−" on the right side of the diagram. Induction properties of all constructs are given in detail in Table 2.

AU into GC pairs and replaced the terminal loop with a stable tetraloop, which leads to further stabilization of the aptamer (data are shown in Figure 3A and Table 2). Both exchanges significantly increase activity, which supports the hypothesis that stability increases activity.

We then performed titration experiments with concentrations of IPTG between 0.1 μM and 2 mM to express increasing amounts of the aptamer 12-1. Induction of TetR steeply ascends reaching maximum β-galactosidase activities at 0.1 mM IPTG (Figure 4A; see Table S1 available online). This high efficiency encouraged us to characterize the aptamer in a system in which TetR is expressed at an ~8-fold higher level (plasmid pWH1413). As expected, the higher level of TetR led to reduced efficiency of induction (2500 MU instead of 4000 MU) and a shift to higher concentrations of IPTG (30 μM instead of 1 μM for the first observed increase in β-galactosidase activity). As for the IPTG-titration with the lower expression level of TetR from pWH527, once induction is observed it increases steadily with the IPTG concentration until it reaches a plateau. Thus, different steady-state levels of TetR can be used to resolve the amount of aptamer-containing RNA present in the cell. Low steady-state levels of TetR can be used to sensitively detect small amounts of aptamer-containing RNA (1–10 μM IPTG), whereas medium amounts of TetR can resolve differences at intermediate to high levels of RNA aptamer expression (60–500 μM IPTG). We then repeated the experiments at 28°C, which would lead to further stabilization of the aptamer. The higher β-galactosidase activities determined for all concentrations of IPTG at 28°C compared with 37°C further indicates that the stability of the RNA leads to increased induction efficiency (Figure 4B and Table S1).

Structural Probing Indicates the Protein Binding Site on the RNA

The mutagenesis studies indicate that the stems P1 and P2 form the scaffold of the aptamer and are important for the stability in the cell. We performed inline probing to verify the predicted secondary structure of the aptamer and to identify the interaction site with TetR. This method analyses changes in the pattern of spontaneous RNA cleavage that occur upon ligand binding. The aptamer exhibits substantial changes in spontaneous cleavage mainly in the 5′ part of the internal loop and in the terminal loop. Only one cleavage site is detectable in the 3′

Table 2. Efficiency of Induction of TetR by Various Aptamer Species

Low-Level Expression of TetR (pWH527)

Effector	IPTG/mM	β-Gal/MU	SD/MU	Induction (-fold)	Effector	IPTG/mM	β-Gal/MU	SD/MU	Induction (-fold)
—	—	300	107	—	12-1K	1	2198	103	7
0.4 μM Tc	—	4503	328	15	12-1L	1	254	16	1
12-1	0	231	44	—	12-1M	1	1567	30	5
12-1	1	2226	145	7	MΔA	1	3089	343	10
12-3	0	214	2	—	12-1P	1	3389	277	11
12-3	1	213	5	1	12-1Q	1	3701	537	12
12-1A	1	2963	244	12	12-1R	1	4763	171	16
12-1B	1	311	23	1	12-1S	1	4604	240	15
12-1D	1	2957	107	10	12-1T	1	4485	359	15
12-1E	1	2686	300	9	12-1U	1	4481	338	15
12-1F	1	2359	277	8	12-1X	1	4436	336	15
12-1G	1	2653	66	9	12-1Y	1	4166	769	14
12-1H	1	1541	62	5	12-1K 14CA	1	2045	45	7
12-1I	1	285	95	1	12-1K 21UC	1	1502	23	5

part of the internal loop, which is probably caused by protection of spontaneous cleavage of the stacked A nucleotides. A clear protection of the 5′ part of the internal loop is then observed with increasing concentrations of TetR (Figure 5). To quantify TetR binding to the aptamer, we performed a gel retardation experiment (Figure 5C) and determined a dissociation constant K_D of 30 nM.

Taken together, the probing data nicely support the data obtained for the stem modifications, indicating that the stem regions form a scaffold that presents the asymmetrical loop region as the protein binding site. The stability of the stem regions can then modulate the activity of induction.

### The Internal Loop of the Aptamer Is the TetR Contacting Surface

Probing data indicate that the internal asymmetrical loop participates in TetR binding and/or activity. To further prove this, we performed a saturating mutagenesis and randomized seven bases belonging to the 5′ side of this element. All 21 mutants, except for 2 (14CA and 21UC, for data see Table 2 and Figure 5B), lead to a complete loss of activity, indicating the importance of this region for activity. The right part of the proposed binding pocket was subjected to genetic screening. Thereby, an oligonucleotide containing the aptamer sequence with completely randomized nucleotides 38–44 was cloned into pWH610L and the resulting aptamer pool was screened for candidates with detectable β-galactosidase activity on McConkey plates. The screen resulted in only five yellow colonies (out of 5000) from which plasmids were prepared and sequenced. All candidates exclusively showed the parental aptamer sequence indicating that no variability at all is allowed in the loop region.

DISCUSSION

In vitro selection of RNA aptamers is a powerful method of isolating and evolving molecules that bind with high affinity and specificity to a target (Ellington and Szostak, 1990; Tuerk and Gold, 1990). This method allows us to create molecules de novo that are able to modulate or inhibit specific functions of proteins and, therefore, has an enormous potential for various applications. In the last few years, a plethora of aptamers that bind to distinct regions of proteins have been described (Famulok et al., 2007). However, problems can arise if in vitro selected aptamers are expressed within the cellular environment in which different ionic conditions and the presence of cellular proteins might lead to RNA misfolding or even degradation. As a consequence, many aptamers lose their activity in vivo. One attempt to circumvent this problem is to express the aptamers flanked with stabilizing stem loop structures, which increases the intracellular stability and reduces their potential for misfolding. This strategy has successfully been applied for aptamers binding integrin or cytohesin-1 (Blind et al., 1999; Mayer et al., 2001). In our case, such stabilizing 5′and 3′ stem loops that were attached to the originally expressed aptamer pool were not needed for TetR induction by the aptamers.

RNA aptamers isolated by in vitro selection for binding to a target molecule can interact with the target without altering its biological activity. They can also mimic the biological activity of natural ligands or even interfere with the target's biological activity. Because of the large number of potential binding sites on a given target molecule, it is highly likely that the majority of the selected aptamers will not affect the target's biological activity. To be able to identify those RNA molecules that display a certain biological function out of the entire aptamers pool might, therefore, require adding an additional screen or selection step for this property. Because our aim was to identify TetR-inducing aptamers, we subjected the in vitro selected pool to an in vivo genetic screen for induction of TetR and isolated one active aptamer. Table 1 clearly shows that this aptamer, 12-1, was not the dominant species in the pool after 12 rounds of in vitro selection. Species like 12-2 and 12-10 with 26.3% and 31.6% are more highly represented within the aptamer pool but show no inducing activity in vivo (Table 1 and Figure 2C, white colonies). A similar result was obtained for an approach that aimed to identify engineered riboswitches. Thereby, aptamers that bind the antibiotic
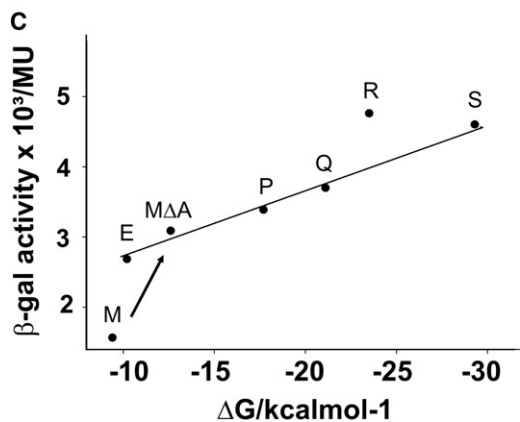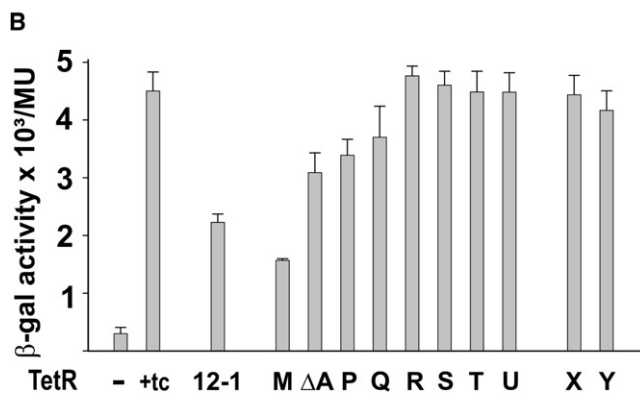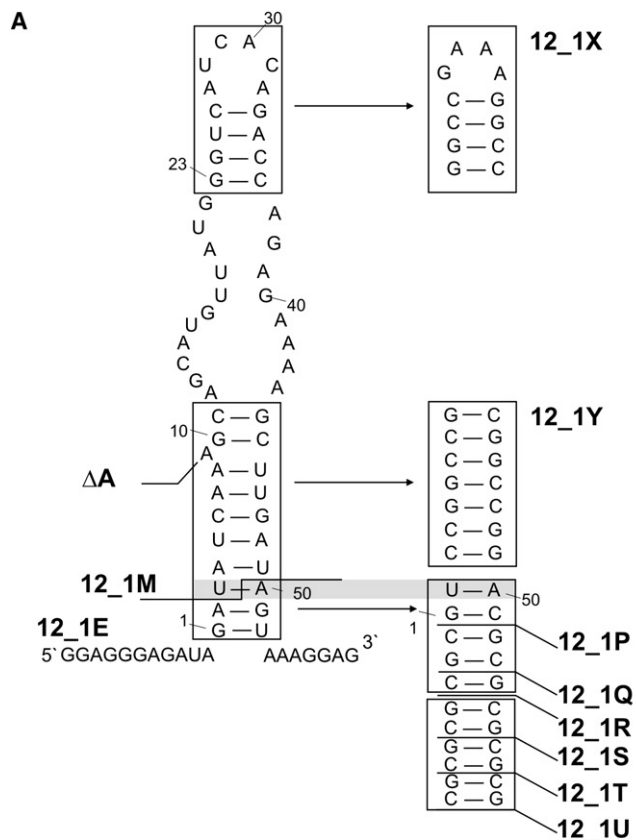
Figure 3. Dependence of Aptamer-Mediated Induction of TetR on the Stability of the Stem Regions

(A) RNA secondary structure prediction of the aptamer 12-1E. The minimal active aptamer 12-1M is also indicated. The bulged nucleotide A9 was deleted in 12-1M and named ΔA. Successive elongation of the stem 12-1MΔA results in 12-1P–12-1U. For 12-1X and 12-1Y, the complete upper or lower stem region (boxed) was exchanged.
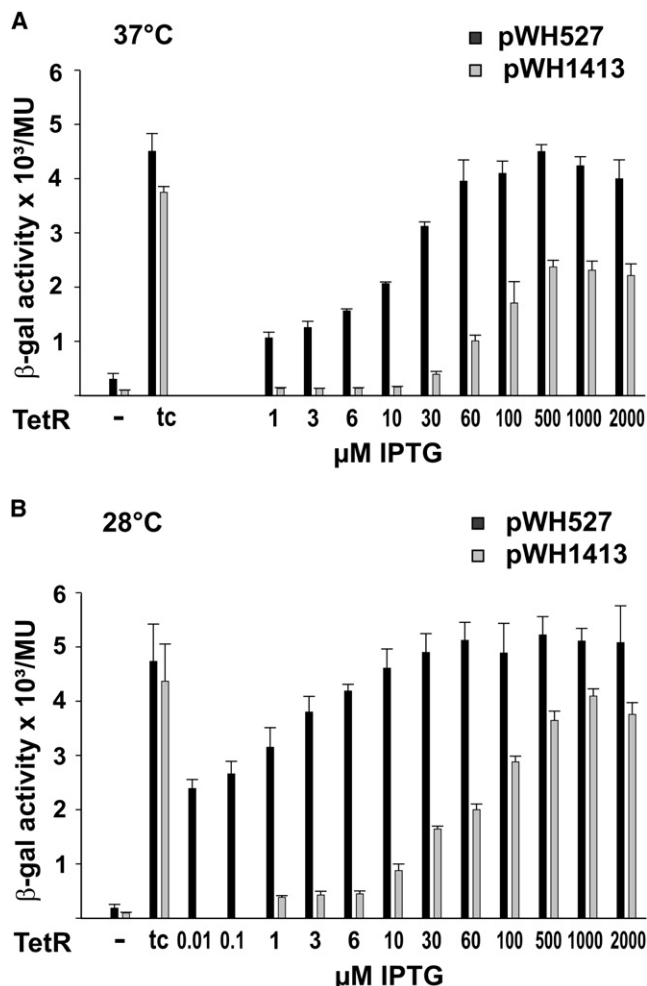
(B) β-Galactosidase activity was determined in *E. coli* WH207(λ*tet*50). The left two bars show β-galactosidase activity repressed by TetR (expressed at a low level from pWH527) and induced with 0.2 μg tc. The next bars show induction of the RNA aptamers 12-1 and respective stem variants. The data are summarized in Table 2.

(C) Correlation between induction in vivo and the calculated thermal stability of the closing stem. The increase in induction by deletion of the bulged adenine in the constructs 12-1M and 12-1ΔA is indicated by an arrow.

neomycin were analyzed for their potential to control gene expression in a ligand-dependent manner when the aptamer was inserted into the untranslated region of a reporter gene. One aptamer was identified using a combined approach of in vitro selection and in vivo screening (Weigand et al., 2008), but it was not among the sequences isolated from in vitro selected neomycin-binding aptamers (Wallis et al., 1995). Another example is an aptamer that binds Rrm4, a protein involved in pathogenic development of *Ustilago maydis*. Here, the authors performed a yeast three-hybrid screen after 11 cycles of in vitro selection and showed that two of four aptamer classes revealed in vivo binding (Konig et al., 2007). Interestingly, aptamers from class A, which were most frequently found in vitro, did not show any in vivo activity. These examples clearly demonstrate that a suitable genetic screen is essential for identifying aptamers with biological activity from in vitro selected pools, because these mostly represent only a minor fraction of all aptamers.

The TetR-inducing aptamer is functionally divided into two stem regions capped by a terminal loop and interrupted by an internal asymmetrical loop. Loop structures very often form functionally important regions like binding pockets or contact surfaces. Our studies support this for the TetR-binding aptamer. Nearly all mutations within the internal loop region of the aptamer lead to a complete loss of its activity as an inducer of TetR, and the protection of the entire region in the in-line probing experiment proved it as binding surface for TetR.

Both stems and the terminal loop have no importance for ligand recognition because they are fully exchangeable (see Figure 3, construct X and Y). However, the activity of the aptamer can be modulated by stem variations. We observed a clear correlation between TetR induction efficiency and the stability of the closing stem (Figure 3C). The deletion of the bulged adenine in the closing stem that results in a canonical double helix not only increases ΔG from −9.4 to −12.6 kcal mol^{-1}, but also leads to a jump in inducing activity (see the arrow in Figure 3C). Further increase of the stem's stability shows an almost linear dependence between activity and stability. This dependence is further supported by the temperature experiments. Lower temperatures, which lead to more stable RNA structures, further increase the activity of the aptamer. In addition, a clear correlation exists between induction efficiency and the amount of expressed RNA (adjustable by different IPTG amounts) and the amount of TetR within the cell (compare pWH527 and pWH1413), respectively.

Figure 4. Dependence of Aptamer-Mediated TetR Induction on Incubation Temperature and TetR Steady-State Levels

β-Galactosidase activity was determined in *E. coli* WH207(λ*tet*50) as shown in Figure 1B. pWH527 expresses TetR at a low level (black bars), whereas pWH1413 leads to medium levels of TetR expression (gray bars). The left two bar doublets represent the controls with the β-galactosidase activity repressed by TetR (−) and induced with 0.2 μg tc (tc). The following bars doublets represent β-galactosidase activity induced by the RNA aptamer 12-1. RNA expression is induced with increasing amounts of IPTG as indicated on the *x* axis). Three independent clones were assayed and experiments were repeated twice.

(A) The measurements were performed at 37°C.

(B) The measurements were performed at 28°C.

This shows that the induction efficiency of TetR by the aptamer can be adjusted and fine-tuned depending on the requirements of the respective regulatory system by varying (i) the amount of TetR in the cell, (ii) the amount of the aptamer, and (iii) the stability of the aptamer. Thereby, favorable expression windows can be targeted with respect to the regulatory situation.

SIGNIFICANCE

Aptamers are small oligonucleic acid molecules that can be selected in vitro against nearly any target of choice. Aptamers often show remarkable binding affinity and speci-

ficity, and consequently have a huge potential for application. However, in vitro selected RNA aptamers often lose their activity in vivo. To overcome this limitation, we coupled in vitro selection and in vivo screening to identify aptamers that retain their activity in vivo. Using this approach, we identified a small noncoding RNA that is able to activate transcription by modulating the DNA-binding activity of the bacterial transcriptional regulator TetR. Thereby the aptamer displays similar activity in vivo as the natural inducer tc. Its in vivo activity as an inducer directly correlates with its expression level and its stability. The aptamer folds into a stem-loop structure with an internal loop that interrupts the stem. Mutational analysis and inline probing reveal this internal asymmetrical loop as the protein binding site.

Because of its small size, high induction efficiency, and stability under in vivo conditions, the TetR aptamer is well suited as an intracellularly expressed alternative inducer for TetR controlled gene expression. A highly promising application, therefore, is its use as an expression tag to determine RNA levels in living bacterial cells. The aptamer can easily be inserted into the nontranslated regions of mRNAs and sRNAs to monitor their expression. The classical way is to create a transcriptional fusion of a reporter gene to a promoter to monitor its expression strength, which completely disregards the influence of the coding region and the 3′ untranslated region on its expression level. In contrast, the insertion of a small RNA aptamer into the untranslated region of an RNA of interest more likely retains the integrity of the natural context and conserves intrinsic elements like stability determinants. Therefore, an easy and accurate determination of the intracellular level of a certain RNA is possible.

EXPERIMENTAL PROCEDURES

Pool Construction

We used a DNA pool of 120 bp length (5′-AATTC<u>TAATACGACTCACTATA</u>*GGGA GAGGAGG GAGATAGATATCAA*–**(N)₅₀**-*GAGTTTCGTGGATGCCACAGGAC*-3′) as template for the initial step of the SELEX. The sequence includes a 50-bp-long randomized region (**bold**), constant regions (*italics*) (which were necessary) for amplification, and the T7-promoter (underlined). In vitro transcription via the DNA-dependent T7 RNA polymerase led to an RNA-library of 98 nucleotides in length (5′-GGGAGAGGAGGGAGAUAGAUAUCAA-**(N)₅₀**-GA GUUUCGUGGAU GCCACAGGAC-3′). The enriched RNA-pool was reverse transcribed and the cDNA-pool was amplified using the following oligonucleotides: forward, 5′-AATTC<u>TAATACGACTCACTATA</u>GGGAGAGGAGGGAGATAG ATATCAA-3′ (T7 promoter is underlined); reverse, 3′-AAAGCACCTACGGTGTC CTG-5′.

In Vitro Selection

In vitro selection was performed on an automated platform as described elsewhere (Famulok and Huttenhofer, 1996; Mayer et al., 2008) with the following modifications. The incubation of the target protein with the RNA library was made in phosphate-buffered saline (PBS) (pH 7.4) supplemented with 0.8 μg/μl bovine serum albumin and 3 mM MgCl₂ at 37°C for 30 min. TetR (1 μg/μl) was biotinylated with a 5-fold molar excess of sulfo-NHS-LC biotin (Pierce) according to the manufacturer's protocol. The nonreacted biotin-esters were removed from the protein solution by gel filtration using Micro Bio-spin P6 columns (Bio-Rad). The biotinylated protein was subsequently coupled to 5 mg streptavidin beads (Invitrogen) and the resulting beads (267 μg/cycle) were directly used for the selection process. Twelve rounds of selection were performed. Specific binding of the RNA pool to TetR was verified by a filter retention analysis.
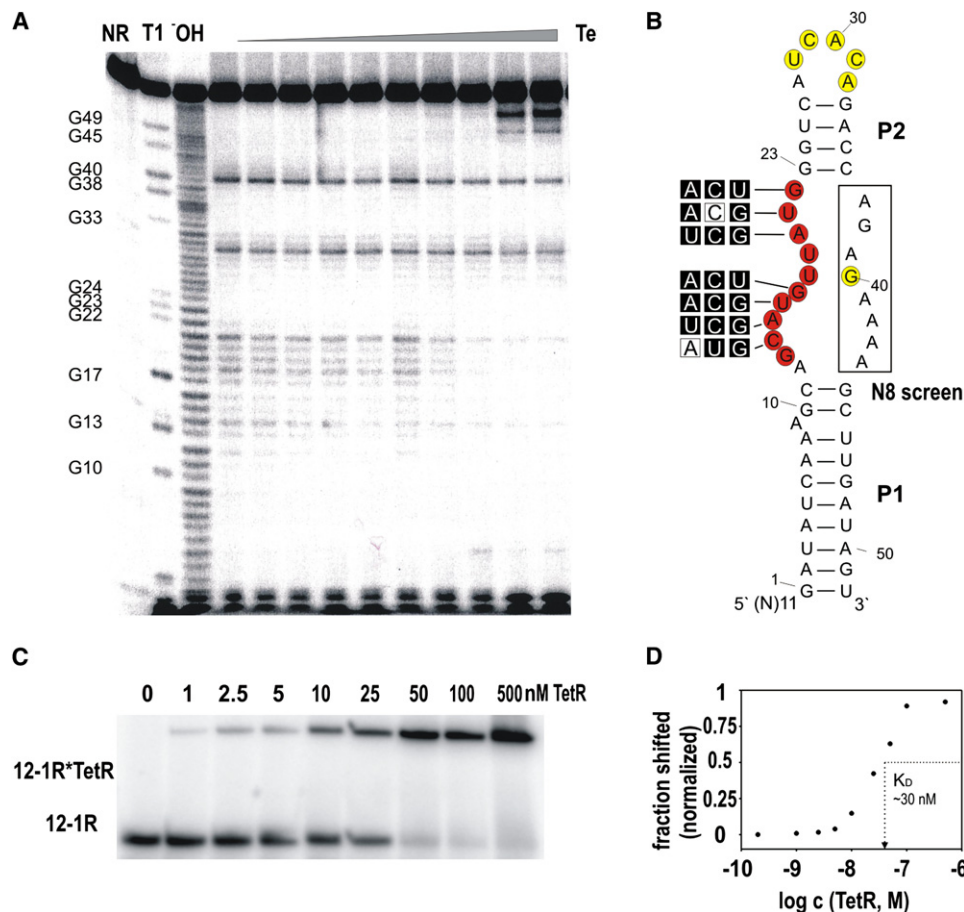
Figure 5. Identification of the Protein Binding Site

(A) Representative in-line probing of the TetR binding aptamer with increasing concentration of TetR. 5′-^{32}P-labeled RNA (500 pM) was examined after partial digestion with RNase T1 (T1), or alkali (OH), or after incubation for 40 hr with increasing concentrations of TetR (lanes 4–12 correspond to 0, 1, 10, 50, 100, 300, 600, 1000, and 5000 nM TetR). NR indicates no reaction. Selected bands in the T1 lane (G-specific cleavage) are identified by nucleotide positions.

(B) Secondary structure model of the aptamer used for in-line probing. TetR-induced structural modulations were identified by monitoring changes in spontaneous cleavage. These nucleotides are encircled in red and positions that showed no TetR dependency in their cleavage intensity are encircled yellow. The results of a saturating mutagenesis of nucleotides 14–17 and 20–22 are displayed on the left side of the structure. Loss-of-activity mutations are displayed with white capital letters on black squares. The nucleotides 37–44 (boxed, N8 screen) were subjected to a saturating mutagenesis screen.

(C) Gel retardation assay of the TetR binding aptamer 12-1R with increasing concentration of TetR. 5′-^{32}P-labeled RNA (500 pM) was incubated with increasing amounts of TetR and complex formation assayed on 10% native polyacrylamide gels.

(D) Plot of the normalized fraction of the RNA*TetR complex versus the logarithm of the molar concentration of TetR.

Filter Retention Analysis

Labeled RNA was obtained by in vitro transcription using α-[^{32}P]-GTP and purification of RNA was done using the nucleotide removal kit (QIAGEN) according to the manufacturer's protocol. For measuring protein-RNA interactions, radioactively labeled RNA was incubated with increasing concentrations of protein in selection buffer at 37°C for 30 min and then passed through nitrocellulose (0.45 μm, Schleicher & Schuell Bioscience). Subsequently, the membranes were washed with 1 ml wash buffer (PBS [pH 7.4]/3 mM MgCl$_2$) and dried. RNA retained on the membranes was quantified with a phosphorimager (FLA 5000, Fuji).

In Vivo System

The construction of the *E. coli* K12 strain WH207(λ*tet*50) (Wissmann et al., 1991) and the plasmids pWH527, pWH529, pWH1413, and pWH1415 has been described elsewhere (Klotzsche et al., 2005). For construction of pWH610L, an insert containing restriction sites for XhoI and NcoI was generated by hybridizing the following oligonucleotides: L_forward, 5′-TCGAGAAATCTAGAAA AAGATCTAAAC-3′; L_reverse, 3′-CTTTAGATCTTTTTCTAGATTTGGTAC-5′,

phosphorylated and ligated with the likewise-digested vector pWH610 (Schnappinger et al., 1999). The insert carries additional restriction sites for XbaI and BglII. Stabilizing stem-loop structures were introduced via cleavage with XhoI/XbaI, and BglII/NcoI, respectively. Inserts were prepared by hybridization and phosphorylation of the following oligonucleotides: F5_forward, 5′-T CGAGGGGAGACCACAACGGTTTCCCT-3′; F5_reverse, 3′-CCCTCTGGTGTTG CCAAAGGGAGATC-5′;F3_forward, 5′-GATCTTAGCATAACCCCTTGGGGCC TCTAAACGGGTCTTGAGGGGTTTTTTGCTC-3′; F3_reverse, 3′-AATCGTAT TGGGGAACCCCCGGAGATTTGCCCAGAACTCCCCAAAAAACGAGGTAC-5′. Aptamer-containing sequences were amplified using the primers 5′-AGTTC GCTCAGAGGGAGAGGAGGGAGATAGATATCAA-3′ and 3′-AAAGCACCTAC GGTGTCCTGTCTAGAGTCATC-5′ and ligated with pWH610L via XbaI and BglII.

β-Galactosidase Assay

Repression by and inducibility of TetR was assayed in *E. coli* WH207(λ*tet*50). This strain was transformed with pWH527 or pWH1413 derivatives constitutively expressing TetR at low or medium levels, respectively, and the

pWH610L plasmid series expressing the aptamers. Overnight cultures and log-phase cultures were grown at 37°C in Luria-Bertani medium supplemented with the appropriate antibiotics, and an additional 0.2 μg/ml tc for the positive control. Expression of the aptamers was induced by adding IPTG to a final concentration of 1 mM to the log-phase cultures in standard measurements. β-Galactosidase activities were determined as described elsewhere (Miller, 1972). Three independent clones were assayed for each construct combination, and experiments were repeated twice.

In-Line Probing

An RNA construct used for in-line probing was transcribed in vitro from PCR-amplified DNA, dephosphorylated, and 5′-^{32}P-labeled as previously described (Weigand et al., 2008). In-line probing was performed as described elsewhere (Winkler et al., 2002). In detail, 5′-^{32}P-labeled RNA (100 pM) was incubated for 40 hr at 25°C in 50 mM Tris-HCl (pH 8.3), 20 mM MgCl$_2$, and 100 mM KCl containing a defined tc concentration for each reaction. After incubation, 10 μl 10 M urea was added and the products separated by electrophoresis using denaturing 10% polyacrylamide gels. Gels were dried and analyzed using a Storm Phosphoimager (GE Healthcare). The ligand concentration needed for half-maximal modulation of spontaneous cleavage yields the K_D for the RNA-ligand interaction.

Gel Retardation Assay

5′-^{32}P-labeled 12-1 aptamer RNA was incubated with increasing amounts of TetR as indicated. Binding reactions were performed at room temperature for 30 min in 20 μl volumes in 1 x binding buffer containing 50 mM Tris-HCl (pH 8), 10 mM MgCl$_2$, 25 mM NaCl, and 5 μg/ml yeast tRNA. The mixture was loaded onto the gel after another 30 min incubation. All complexes were resolved on a 10% polyacrylamide gel in 1 x TBE buffer (0.89 M Tris, 0.89 M boric acid, 1 mM EDTA [pH 8.3]). To obtain K_D values in the low nanomolar range, the assay was conducted with RNA concentrations of 0.5 nM. The concentration of ligand needed to cause half-maximal complex formation yield reflects the K_D for the RNA–ligand interaction.

SUPPLEMENTAL DATA

Supplemental Data include two tables and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00006-4.

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