

Generation of synthetic RNA-based thermosensors

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Abstract

Structured RNAs with fundamental sensory and regulatory potential have been discovered in all kingdoms of life. Bacterial RNA thermometers are located in the 5'-untranslated region of certain heat shock and virulence genes. They regulate translation by masking the Shine-Dalgarno sequence in a temperature-dependent manner. To engineer RNA-based thermosensors, we used a combination of computer-based rational design and *in vivo* screening. After only two rounds of selection, several RNA thermometers that are at least as efficient as natural thermometers were obtained. Structure probing experiments revealed temperature-dependent conformational changes in these translational control elements. Our study demonstrates that temperature-controlled RNA elements can be designed by a simple combined computational and experimental approach.

Keywords: regulatory RNA; riboswitch; RNA thermometer; synthetic biology; translational control.

Introduction

Examples of naturally occurring RNA sensors are accumulating in the last years (Winkler and Breaker, 2005; Narberhaus et al., 2006). Usually, they are located in the 5'-untranslated region (5'-UTR) of mRNAs, fold into complex structures and control the expression of downstream genes by signal-induced conformational changes. While RNA thermometers sense temperature as a physical stimulus, riboswitches recognize chemical signals. Target molecules are bound by riboswitches with high specificity and affinity. Gene expression is regulated either at the level of translation initiation, transcription termination or RNA processing (Mandal and Breaker, 2004; Nudler and Mironov, 2004).

RNA sensors are comparatively simple regulatory devices, as they do not require accessory proteins. It has been speculated that they represent an ancient mode of gene regulation (Vitreschak et al., 2004). Great effort has been made to engineer different types of riboregulators, which may act as antisense RNAs, ribozymes or small molecule-binding aptamers (Isaacs et al., 2006; Gallivan, 2007; Suess and Weigand, 2008). Incorporation of a theophylline aptamer into a designed helix in the 5'-UTR of

a reporter gene by Suess and colleagues produced a synthetic riboswitch (Suess et al., 2004). Addition of theophylline caused a dose-dependent increase in gene expression when this riboswitch was assayed in the Gram-positive bacterium *Bacillus subtilis*. Theophylline also serves as inductor of a synthetic riboswitch that activates translation in *Escherichia coli* (Desai and Gallivan, 2004).

Construction of riboswitches includes several steps. The first step often is an *in vitro* selection procedure to search for binding of a desired ligand. In a second step, the regulatory potential of ligand binding is then analyzed in an *in vitro* activity assay or an *in vivo* system. In most cases, time-consuming optimization steps are needed to yield the desired regulatory effect. The function of RNA-based regulators is to a great extent dependent on their structure. Computer-based prediction of RNA structures from the primary sequence usually is quite reliable. This motivated studies using computer predictions to engineer riboswitches. Penchovsky and Breaker used a series of programs to design RNA switches with different Boolean logical functions (Penchovsky and Breaker, 2005). Their system is based on allosteric ribozymes whose activity is triggered by oligonucleotide binding. The binding region is located in a loop whose ligand-dependent conformation modulates the catalytic core of a minimal hammerhead ribozyme.

Recently, RNA thermometers have been recognized as an attractive subject for engineering (Lee and Kotov, 2007; Wieland and Hartig, 2007). Naturally occurring RNA thermometers undergo temperature-induced structural changes (Narberhaus et al., 2006). All presently known RNA thermometers control translation initiation. In most cases, entry to the ribosome binding site is blocked by complementary base pairs at low temperatures. At increasing temperatures, melting of the structure permits ribosome access. This simple regulatory principle can be realized by quite different RNA structures. Only a few distinct families of RNA thermometers have been discovered so far. ROSE (repression of heat shock gene expression)-like thermometers consist of several stem-loop structures and control expression of small heat shock genes in many α - and γ -proteobacteria (Nocker et al., 2001; Waldminghaus et al., 2005). Thermal control is achieved by temperature-labile, non-canonical base-pairs in the SD region (Chowdhury et al., 2006). The fourU thermometer has a simpler architecture (Waldminghaus et al., 2007b). It regulates expression of the *Salmonella* heat shock gene *agsA* and is composed of only two hairpins spanning 57 nucleotides. While the 5'-proximal hairpin remains stable up to 50°C, the second hairpin melts with increasing temperature in the physiological temperature range. The temperature responsive element harbors the SD sequence base-paired with a stretch of fourU residues.

As the underlying mechanism of temperature sensing is a gradual melting of a weak stem-loop structure rather than a switch between two mutually exclusive conformations, RNA thermometers are often considered as molecular dimmers rather than ON/OFF switches. An RNA element regulating phase λ development is the only notable exception (Altuvia et al., 1989, 1991). The 5'-UTR of the gene coding for *cIII* appears to be in equilibrium between two alternative conformations. Variations in temperature or Mg^{2+} concentration shift the equilibrium between both structures and thereby alter accessibility of the SD region.

To generate a temperature-responsive regulatory RNA element *de novo*, we set out to combine simple architectural characteristics of the fourU thermometer with a switch-like mechanism as described for the *cIII* thermometer. By computational design and *in vivo* screening, we were able to construct an RNA element capable of temperature-dependent induction of gene expression. The structural basis underlying this regulation was analyzed on the molecular level and revealed insights into thermosensing by a synthetic RNA thermometer.

Results

Architecture and design of RNA-based thermoswitches

The common principle of RNA-based thermosensors is the temperature-controlled access of the ribosome to the SD sequence (Narberhaus et al., 2006). On the basis of this principle, we simulated two temperature-dependent alternative structures (Figure 1A). Both structures contain the sequence of an optimal SD sequence (Curry and Tomich, 1988) and an AUG start codon in the appropriate

distance of 9 nucleotides. In the assumed OFF structure, both of these sequences are involved in base-pairing in an extended hairpin (Figure 1A). The predicted ON structure is composed of two smaller hairpins. The SD sequence is positioned in an exposed loop and the AUG start codon is single-stranded. To arrive at sequences potentially capable of folding into these two structures in response to temperature, we made use of software developed by Flamm and colleagues. The so-called switch program computes bistable RNA molecules with a predicted ability to switch between two given structures (Flamm et al., 2001).

Both alternative structures were converted into the dot-bracket annotation (Figure 1A) before being fed into the switch program. Temperatures of 30°C and 42°C were assigned as temperatures to the OFF and ON states, respectively. The expected output was a list of sequences that predominantly fold into the corresponding structures at the indicated temperature. As outlined in Figure 1B, candidate sequences retrieved by the switch program were processed through several *in silico* selection steps by making use of programs provided by the Vienna RNA folding package (Hofacker, 2003).

The switch program returned numerous sequences as basis for further analysis. A selection of candidates out of approximately 300 predicted sequences is shown in Figure 2A. Sequence 1 (Seq1) passed all of the following selection criteria (Figure 1B). First, the score was below 1.0 (Figure 2A). The score is an estimation of the switch potential. It summarizes multiple parameters. Lower values indicate better switch potential as sequences with higher values (Christoph Flamm, personal communication). Second, the calculated melting curve for the sequence shows a sharp peak in the temperature range between 30°C and 42°C (Figure 2A). Third, the RNAfold

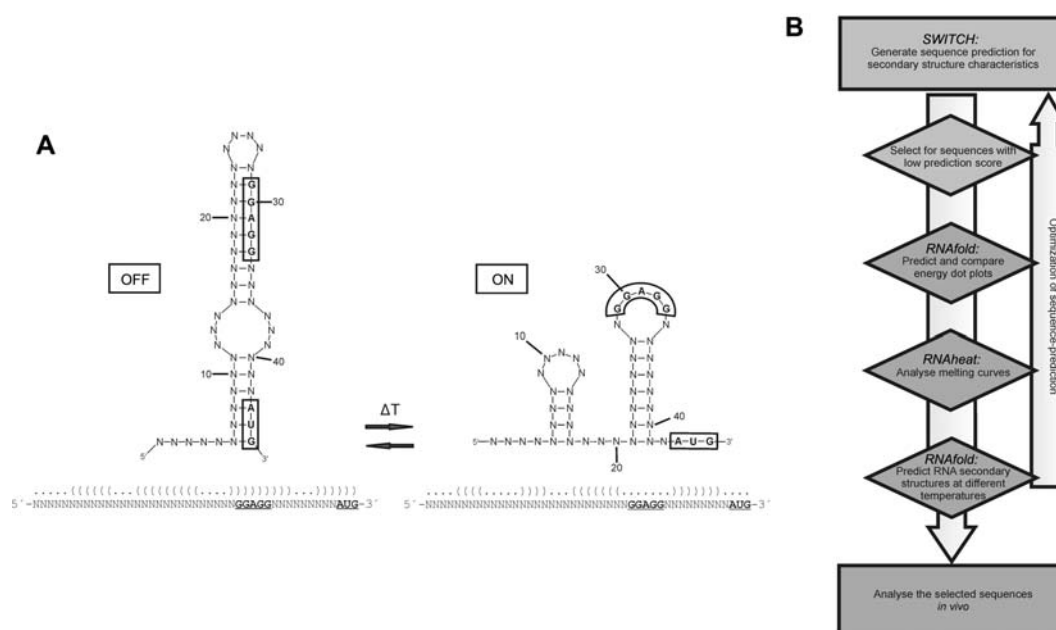


Figure 1 Bioinformatic prediction of temperature-controlled riboswitches.

(A) Schematic representation of the two alternative structures that the approach was based on. The dot-bracket annotations below the corresponding structures were used as input for the switch program. SD sequence (GGAGG) and the translation start sites (AUG) are framed or underlined, respectively. (B) Flow scheme of the individual steps used for *in silico* prediction. Computer programs are printed in bold font.

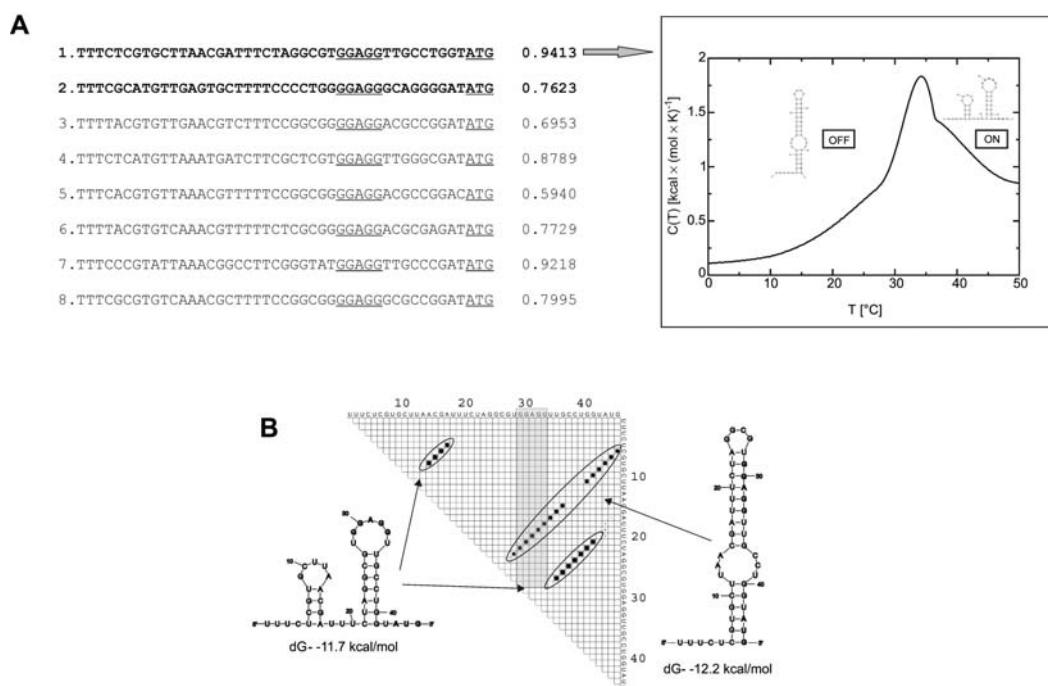


Figure 2 Output of the *in silico* prediction.

(A) Assorted sequences derived from the switch predictions are based on the secondary structures shown in Figure 1A. SD sequences and translation start sites are underlined. Sequence 1 (Seq1) and sequence 2 (Seq2) were selected for further analysis. The melting curve of Seq1, calculated with the RNAheat program of the Vienna RNA Package, is depicted to the right. According to this calculation, the OFF and ON states are separated by a narrow energy-maximum and both conformations are predicted to switch at around 35°C. (B) Energy dot plot of Seq1 calculated with RNAfold. The black squares describe the equilibrium base pairing probabilities. The sequence has two dominating conformations with energies of -11.7 (ON, left) and -12.2 kcal/mol (OFF, right). The SD sequence in the dot plot is shaded in gray.

program predicted a similar probability of both alternative structures. This is visualized by the comparable square sizes in the energy dot plot (Figure 2B). Finally, RNA secondary structure predictions at different temperatures supported a conformational switch between 37°C and 42°C (data not shown). Most predicted sequences failed to pass the first selection criterion and had a score above 1.0. Apart from Seq1, only Seq2 conformed to all criteria. The ability of both sequences to function as translational regulatory control elements was assessed in *E. coli*.

In vivo characterization of thermoswitch candidates

Translational reporter gene fusions to Seq1 and Seq 2 were placed downstream of the arabinose-inducible pBAD promoter (Guzman et al., 1995), which allows for temperature-independent control of transcription (Figure 3A). Upon annealing of appropriate oligonucleotides (Table 1), the AUG start codons of Seq1 and Seq2 were ligated to the *bgaB* sequence. *bgaB* codes for a heat stable β -galactosidase (Hirata et al., 1984). As positive control, we used the recently described *Salmonella* fourU thermometer (Waldminghaus et al., 2007b). In the presence of 0.01% (w/v) of the inducer L-arabinose, the expression in *E. coli* of both putative thermoswitch fusions was very low. This basal expression is clearly above background, which is zero since *E. coli* has no chromosomally encoded thermosensitive β -galactosidase. Repression was not relieved when cells were grown at 42°C (Figure 3B). In contrast, the positive control (fourU)

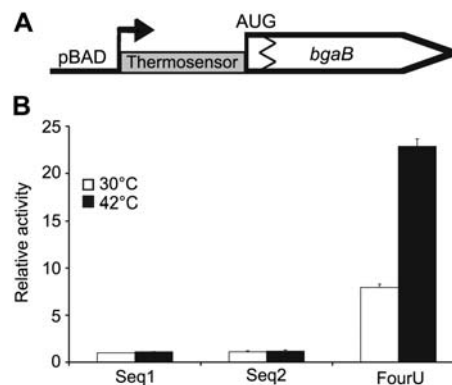


Figure 3 Expression of translational *bgaB* fusions to predicted RNA thermometers.

(A) Schematic representation of the reporter gene fusion on plasmid pBAD-*bgaB* and (B) expression analysis of *bgaB* fusions. The *Salmonella* fourU-*bgaB* fusion served as positive control. Cells were grown in LB medium to exponential phase at 30°C. Immediately after addition of 0.01% (w/v) arabinose, the cultures were heat-shocked to 42°C for 30 min. The relative β -galactosidase activity is shown as average of three parallel measurements with the indicated standard deviations.

showed approximately three-fold induction at the elevated temperature.

Thermosensor engineering

Since Seq1 was incapable of conferring temperature control to a reporter gene fusion, we established an *in*

Table 1 Strains, plasmids and oligonucleotides used in this study.

Strain, plasmid or oligonucleotide	Relevant characteristic(s) or sequence ^a	Source or reference
Strains		
<i>Escherichia coli</i> DH5 α	<i>F</i> F80 <i>dlacZDM15D(lacZYAargF)</i> <i>U169deoRrecA1endA1hsdR17</i> (<i>r_Km_K⁺</i>) <i>sup44thi-1 gyrA69</i>	(Hanahan, 1983)
Plasmids		
pBAD-bgaB	Translational <i>bgaB</i> fusion vector, <i>bgaB</i> : heat-stable β -galactosidase, Amp ^R	(Waldminghaus et al., 2007a)
pBO681	Seq1 fusion in pBAD-bgaB	This study
pBO684 ^b	Seq1-C37U fusion in pBAD-bgaB	This study
pBO685	Seq1-C10U fusion in pBAD-bgaB	This study
pBO686	Seq1-C26G fusion in pBAD-bgaB	This study
pBO690	Seq1-C38U fusion in pBAD-bgaB	This study
pBO692	Seq1-C26U-UG39/40AA fusion in pBAD-bgaB	This study
pBO693	Seq1-C10U-G36A fusion in pBAD-bgaB	This study
pBO694	Seq1-C26U-U39A fusion in pBAD-bgaB	This study
pBO695	Seq1-C10U-C26U fusion in pBAD-bgaB	This study
pBO696	Seq1-G24A-G36U fusion in pBAD-bgaB	This study
pBO699	Seq2 fusion in pBAD-bgaB	This study
pBO472	FourU fusion in pBAD-bgaB	(Waldminghaus et al., 2007b)
Oligonucleotides		
RBS1_fw	CTAGCTTTCTCGTGCTTAACGATTCTAGGCGTGGAGGTTGCCTGGTATGG (construction of pBO681)	
RBS1_rv	AATTCATACCAGGCAACCTCCACGCCTAGAAATCGTTAAGCACGAGAAAA (construction of pBO681)	
RBS3_fw	CTAGCTTTTCGCATGTTGAGTGCTTTTCCCCTGGGGAGGGCAGGGGATATGG (construction of pBO699)	
RBS3_rv	AATTCATATCCCTGCCCCTCCCAGGGGAAAAGCACTCAACATGCGAAA (construction of pBO699)	
mut <bgab< b="">fw</bgab<>	GTCTATAATCACGG (error-prone PCR)	
mut <bgab< b="">rv</bgab<>	TCTTGCTCCAACG (error-prone PCR)	
Fw-bgab-probe	AGAGCAATGGCCAGAGGAAA (generation of Northern blot probe)	
Rv-bgab-probe	TAATACGACTCACTATAGATCGGCAAAGAATCTGGAT (generation of Northern probe)	

^aIntroduced restriction sites are underlined.^bThe RNA sequence (U instead of T) is given.

vivo screening system to convert it into a functional thermosensor. For this, we amplified the Seq1 fragment by error-prone PCR. Different conditions, in which each one of the four nucleotides dGTP, dATP, dCTP and dTTP were used in four- or eight-fold excess, were used (see experimental procedures). The resulting PCR fragments were cloned into the pBAD-bgaB vector and transformed into *E. coli*. Transformants were plated on Luria-Bertani agar plates containing L-arabinose as inducer and X-gal to monitor β -galactosidase activity. Following overnight incubation at 30°C, plates were transferred to 42°C and the color of the colonies was visually inspected for several hours. Colonies that turned blue were selected, and the corresponding plasmids were isolated and sequenced. Four different single point mutations were obtained (Table 2). Strikingly, the C10U mutation and C37U mutation were both selected twice from different mutagenic conditions. This strongly suggests that the number of possible point mutations facilitating temperature-dependent regulation is limited.

Despite the clearly visible phenotype on plates, a qualitative β -galactosidase assay of the four point-mutated Seq1 variants showed only a minor increase of expression at 42°C as compared to 30°C (Figure 4A). The fact that we were able to select for such subtle changes in temperature-dependent expression demonstrates the

Table 2 Single and multiple nucleotide exchanges in Seq1 obtained by error-prone PCR mutagenesis.

Mutation in Seq1	PCR conditions
C10U ^a	Seq1 as template; selected twice; (a) four-fold dGTP and (b) four-fold dATP (0.8 mM)
C37U	Seq1 as template; selected twice; (a) four-fold dGTP and (b) four-fold dCTP (0.8 mM)
C26G	Seq1 as template; four-fold dTTP (0.8 mM)
C38U	Seq1 as template; eight-fold dTTP (1.6 mM)
C10U-C26U	C10U as template; standard dNTPs (0.2 mM), MnCl ₂
C10U-G36A (HIM)	C10U as template; eight-fold dATP (1.6 mM)
C26U-U39A	Seq1 as template; standard dNTPs (0.2 mM), addition of 0.2 mM MnCl ₂
C26U-UG39/40AA	See above
G24A-G36U	See above

^aThe RNA sequence (U instead of T) is given.

high sensitivity of the X-gal based selection procedure and its suitability for an *in vivo* screening approach.

To further optimize thermal induction, we carried out a second round of error-prone mutagenesis. Conditions of

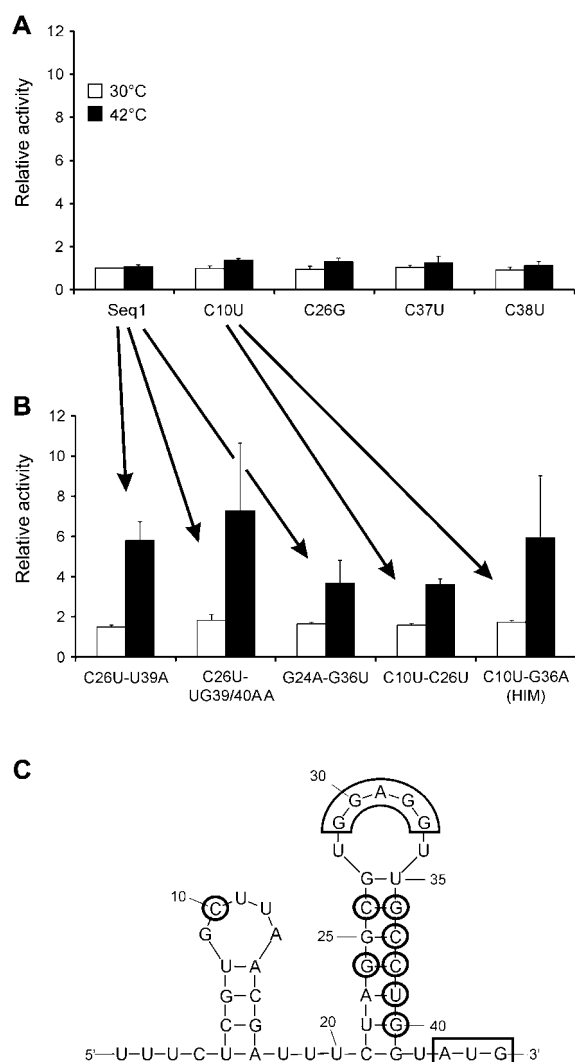


Figure 4 Effect of selected single (A) and multiple (B) mutations in Seq1 on temperature-dependent expression.

The experimental procedures are as described in Figure 3. Arrows indicate the template sequence from which the mutations in (B) originate. (C) Schematic representation of single and multiple mutations in the proposed ON structure. Point-mutated positions are encircled. SD sequence (GGAGG) and translation start site (AUG) are framed.

higher mutagenic strength were used, namely an eight-fold excess of one nucleotide and the addition of MnCl_2 (see the materials and methods section for details). Seq1 and the mutated variant C10U served as PCR templates. Cloning and selection were performed as above. Five new variants, four with two-point mutations and one with three mutations, were obtained (Table 2). β -Galactosidase expression of the new constructs was slightly higher at 30°C compared to Seq1 or C10U (Figure 4B). In each case, the performance as thermosensor was improved, since expression increased approximately two-fold (C10U-C26U and G24A-G36U) or approximately three-fold (C10U-G36A, C26U-U39A and C26U-UG39/40AA) at 42°C.

Except for C10U, all selected mutations are located in the second stem region of the ON structure (Figure 4C). Furthermore, all these mutations would destabilize the stem structure by either introducing a mismatch (G24A, C26G, G36U, G36A and U39A) or by changing a stable

GC base pair to a weaker GU pair (C26U, C37U, C38U). Taken together, these findings point to a critical role of hairpin stability in thermosensing.

Temperature-dependent regulation is due to translational control

Our experimental setup was designed to select for thermosensors that act as translational control elements. However, in principle the selected mutations might also affect the mRNA level by altering RNA stability. To rule this out, we repeated the β -galactosidase assay for Seq1 and the two variants C10U and C10U-G36A (henceforth called HIM for High Induction Mutant). In parallel, we took samples for RNA isolation and subsequent Northern blot analysis. RNA levels were monitored using a probe directed against the coding region of the *bgaB* reporter gene (Figure 5). Ribosomal RNAs served as internal loading control. Although the recorded mRNA levels were equivalent at 30°C and 42°C for all three constructs, β -galactosidase activity was slightly induced in the C10U mutant and highly induced in the HIM strain. Translational repression at 30°C is illustrated by the finding that different C10U and HIM mRNA levels resulted in essentially the same basal β -galactosidase activity (Figure 5). Temperature-induced expression despite constant mRNA levels clearly supports a translational control mechanism.

Enzymatic probing experiments reveal structural basis for thermosensing

To address the structural requirements for thermosensing, we performed enzymatic probing experiments using

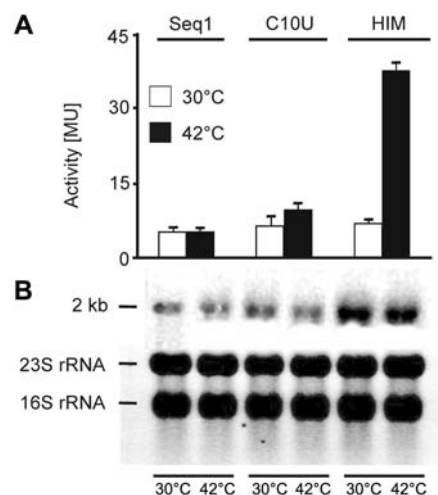


Figure 5 Translational control by selected RNA elements of temperature-dependent expression of *bgaB* fusions to Seq1, the C10U and HIM (C10U, G36A) RNAs.

(A) Temperature-dependent expression was monitored in *E. coli* DH5 α . Cells were grown in LB medium at 30°C and heat-shocked to 42°C for 30 min before β -galactosidase activity was measured. The results are the average of three independent measurements with the indicated standard deviations. (B) In parallel, total RNA was extracted from the same *E. coli* cultures. Equal amounts were separated on a 1.2% denaturing agarose gel, transferred to a positively charged nylon membrane and immobilized for subsequent hybridization. Northern blot experiments were carried out using digoxigenin-labeled RNA probes to detect *bgaB* transcripts.

RNase T1 which cuts selectively 3' of single-stranded guanine residues. We probed the structures of synthetic Seq1 and HIM RNAs at temperatures ranging from 20°C to 50°C in intervals of 5°C (Figure 6). The overall cleavage pattern of Seq1 at 20°C is in good agreement with the calculated ON structure (Figure 6C). G-residues in the terminal loop of hairpin II at positions 28–34, which contains the SD sequence, were cut by RNase T1. Conversely, protection of regions 23–26 and 34–40 against cleavage confirmed the predicted stem regions in hairpin II. Consistent with the low reporter gene activity, the RNA structure was not temperature-responsive, as it remained resistant against cleavage by RNases T1 at 40°C and was only moderately cleaved at 45°C and 50°C in the stem region at positions 24 and 25 (see box in Figure 6A). The structure probing results clearly show that the computer-predicted switch between alternative structures does not occur. Under our experimental conditions, the sequence does not fold into the OFF structure at all.

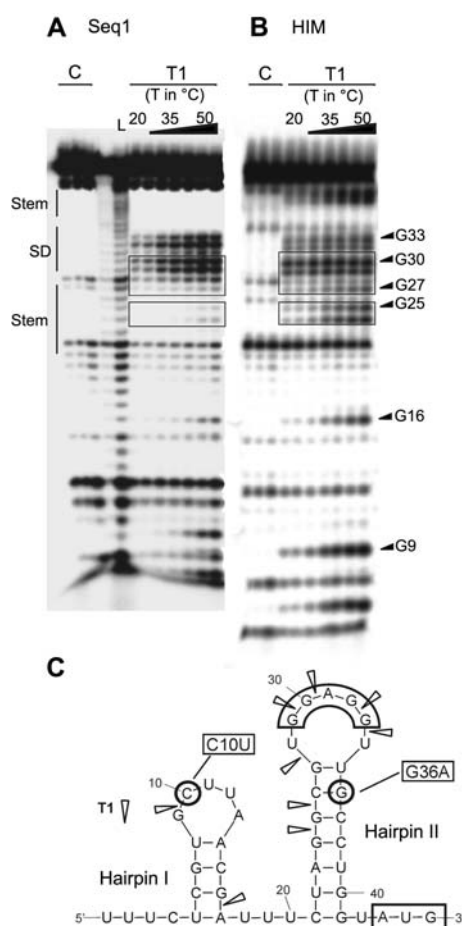


Figure 6 Structure probing of synthetic Seq1 (A) and HIM (B) RNAs.

RNase T1 (0.01 U) cleavage of 5'-end-labeled RNAs was carried out from 20°C to 50°C in intervals of 5°C. RNA fragments were separated on 8% polyacrylamide gels. Lane C: incubation controls with water were taken at 20°C, 35°C and 50°C. Lane L: alkaline ladder. Relevant G-residues are marked with arrowheads to the right. The SD and stem sequences are labeled to the left. Selected parts of SD and stem region are highlighted with boxes. (C) Secondary structure model of Seq1 RNA consisting of hairpin I and II. RNase T1 cleavage sites are shown by arrows. Circled nucleotides mark exchanges that result in the thermoresponsive HIM RNA. SD sequence (GGAGG) and translation start site (AUG) are framed.

Also, the HIM structure does not seem to fold into the predicted OFF structure. There are essentially no differences between the structures of hairpin I in Seq1 and HIM (Figure 6). The predicted hairpin II of the HIM structure, however, is less stable as in Seq1. The G36A exchange results in an extended loop that is readily accessible to RNase T1, even at low temperature (Figure 6B). The flanking regions are more resistant to this enzyme at 20°C, indicating a double-stranded RNA at low temperature. Residual cleavage by T1 suggests that a minor population might be in an open conformation. In accordance with induced expression (Figures 4 and 5), the fraction of single-stranded residues substantially increased with increasing temperature as illustrated by the accumulation of T1-derived products at nucleotides 24 and 25 (see box in Figure 6B and arrows in Figure 6C).

Discussion

Heat-inducible systems have been shown to be suitable for controlled expression of recombinant genes and various industrial applications (Heitzer et al., 1992). Engineering of RNA-based modules to achieve temperature-regulated gene expression is attractive for several reasons. First, *cis*-active RNA thermometers are independent of additional (protein) factors and therefore transferable to various biological systems. Second, there is no need for chemical inducers as in the case of riboswitches. Such inducers often face technical drawbacks, such as poor membrane permeability or high costs in large scale applications. Third, various computational tools have recently been developed to support rational design of RNA elements. Using a two-step approach composed of computational design and *in vivo* screening, we successfully engineered RNA-based thermosensors.

Prediction of temperature-dependent RNA switches

RNA secondary structures are to a great extent composed of complementary base pairs. Algorithms to predict the structure of a given RNA molecule based on the maximal number of base pairs possible have been developed 30 years ago (Nussinov and Jacobson, 1980). Later, experimental data were included to further optimize prediction quality (Mathews et al., 1999). Matters are complicated by the fact that one RNA molecule can often fold into two or more different but energetically similar structures. On the other hand, a given structure might be formed by RNAs with various sequences. Flamm and colleagues developed a program to calculate sequences able to fold into two given structures (Flamm et al., 2001). Since RNA folding is strongly temperature-dependent, this parameter was implemented in the program. Our experimental study, however, revealed that such theoretical assumptions might not necessarily be transferable to an *in vitro* or *in vivo* situation. The computer-derived sequences Seq1 and Seq2 should have resulted in high reporter gene activities at elevated temperatures, which was not the case. Furthermore, determination of the RNA structure of Seq1 *in vitro* (Figure 6) shows that it falls into the predicted ON structure, regardless of the temperature. Although the G-residues in the SD sequence in loop

If were accessible to RNase T1, the small loop was not sufficient to allow entry of the 30S ribosomal subunit, since translation was inefficient. Apparently, it remains a challenge to reliably predict RNA sequences that switch between two alternative structures.

A promising approach for RNA regulator engineering

The power of *in vivo* screening systems has been widely used to improve enzyme function and to develop genetic control systems and RNA-based regulators (Buskirk et al., 2003; Haseltine and Arnold, 2007; Yuen and Liu, 2007). Also, our study demonstrates that a functional RNA regulator can be developed successfully by application of a suitable selection approach. Two findings are especially remarkable. First, two out of four selected single-point mutations were selected independently, despite different experimental conditions (Table 2). This might reflect the limited possibilities to evolve a functional regulator from the given input structures. Such a narrow window for *de novo* design of a functional RNA thermometer is conceivable, since most RNA structures would be either too tight or too loose to allow temperature control in the physiological temperature range.

The second interesting finding is that the combination of only two-point mutations turned the translation-incompetent structure Seq1 into a synthetic thermosensor that is as efficient as natural thermometers. Only minor changes in the nucleotide composition can have drastic effects on natural RNA thermometers. Deletion of a single G-residue that introduces a labile structure in ROSE elements results in a SD sequence that remains base-paired even at high temperatures (Chowdhury et al., 2003, 2006). On the other hand, single-point mutations can lead to derepression at low temperatures as described not only for the ROSE thermometer but also for the *prfA* thermometer of *Listeria monocytogenes* and the *rpoH* thermometer of *E. coli* (Morita et al., 1999; Johansson et al., 2002).

It remains a matter of speculation whether the short path to generate an RNA sensor in our study means that the starting structure was already close to being an RNA thermosensor or whether many different structures might be transformed into an RNA thermosensor by only a few screening steps. It is noteworthy that RNA thermometers with different architectures and riboswitches, which sense the same metabolite by different structures, have been described (Corbino et al., 2005; Narberhaus et al., 2006). Nevertheless, there might be a limitation in the number of possible RNA structures with the same functionality. *In vivo* screening approaches starting from random sequences could help to address how many options there might be in nature.

Materials and methods

Plasmid construction and *in vivo* screening

To generate plasmids pBO681 and pBO699, oligonucleotides corresponding to the predicted sequences were ordered (MWG-Biotech, Ebersberg, Germany) and cloned into the NheI and EcoRI restriction sites upstream of *bgaB* in pBAD-*bgaB* (Table

1). Plasmid pBO681 served as template for random mutagenesis by error-prone PCR using primers *mutbgaBfw* and *mutbgaBvr* to generate pBO684, 685, 686, 690, 692, 694 and 696 (Table 1). Plasmids pBO693 and 695 were generated with the same primers and pBO685 as template. The error-prone PCR was performed by using *Taq* DNA polymerase in PCR buffer [20 mM Tris HCl (pH 8), 10 mM KCl, 6 mM (NH₄)₂SO₄, 2 mM MgSO₄·6H₂O, 0.1% Triton X-100], 25 pmol of each primer, 3 mM MgCl₂ and a nucleotide concentration of 0.2 mM each in a total volume of 50 µl. To favor misincorporation of nucleotides, the following modifications to the standard protocol were used. One of the four nucleotides dGTP, dATP, dCTP and dTTP were used in four- (0.8 mM) or eight-fold (1.6 mM) excess. In some cases, 0.2 mM MnCl₂ were added in the presence of standard nucleotide concentration. The PCR program was as follows: initial DNA denaturation at 94°C for 2 min was followed by 35 cycles at 94°C for 30 s, primer annealing at 40°C for 30 s and elongation at 72°C for 30 s.

PCR products were digested with EcoRI and NheI, cloned into the corresponding sites upstream of *bgaB* in pBAD-*bgaB* and transformed into *E. coli* DH5α. Cells were grown on LB agar plates containing X-gal (40 µg/ml) in the presence of 0.01% (w/v) L-arabinose overnight at 30°C. Following a temperature shift to 42°C, candidates that turned blue earlier than others were collected and the assay was repeated before the β-galactosidase activity was measured at 30°C and 42°C. Plasmids from clones with elevated *bgaB* expression at 42°C were isolated and sequenced.

β-Galactosidase assay

E. coli cells carrying *bgaB* fusions were grown to exponential growth phase at 30°C. Samples of 10 ml were transferred to 42°C for 30–60 min, before β-galactosidase activity was measured as described previously (Miller, 1972), except that enzyme activity was measured at 55°C. The β-galactosidase activity in Figures 3B and 4A is denoted relative to the activity of Seq1-*bgaB* at 30°C and in Figure 4B relative to the parental construct (Seq1-*bgaB* for the first three and C10U-*bgaB* for the last two) at 30°C.

Isolation of RNA and Northern blot analysis

Total RNA was extracted from *E. coli* cells using the hot phenol method (Aiba et al., 1981). Equal amounts of total RNA samples (5 µg) were separated on a 1.2% formaldehyde-agarose gel, transferred to a positively charged nylon membrane (Hybond N1; Amersham Biosciences, Buckinghamshire, UK), and hybridized at 68°C using a DIG-HIGH prime labeling and detection kit as per the manufacturer's protocol (Roche Applied Science, Mannheim, Germany). The probe used was a 240-bp RNA fragment corresponding to the *bgaB* ORF. Detection was carried out by exposing the blot to a luminescence detector using chemiluminescence substrate (CSP-D-Star, Roche Molecular Biochemicals, Mannheim, Germany).

Structure probing experiments

RNA oligomers for structure probing experiments were purchased from Vbc biotech (Vbc-biotech GmbH, Vienna, Austria). Partial digestions of 5'-end-labeled RNAs with ribonuclease T1 were conducted as follows. 30 000 cpm of labeled RNA were mixed with 1 µl 5× TMN buffer (100 mM Tris acetate, pH 7.5, 10 mM MgCl₂, 500 mM NaCl) and 0.4 µg tRNA, and distilled water was added to a volume of 4 µl. Samples were pre-incubated for 5 min at the indicated temperature, before 1 µl of T1 RNase (0.01 U) was added. After 5 min of cleavage, 5 µl formamide loading dye was added and the samples were heated at 95°C for 5 min prior to separation on denaturing 8% poly-

acrylamide gels. Alkaline ladders were generated as described previously (Brantl and Wagner, 1994).

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