Methods xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Identification of RNA aptamers with riboswitching properties

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A R T I C L E I N F O

Article history:
Received 8 September 2015
Received in revised form 7 November 2015
Accepted 1 December 2015
Available online xxxx

Keywords:
Aptamer
Riboswitch
Genetic engineering
SELEX
Screening system

A B S T R A C T

During the past years customized gene network design has become of tremendous interest among various disciplines in life science. The identification of artificial genetic elements sensitive to internal or external stimuli constitutes the foundation for the design and realization of conditional gene expression systems. Typically, strategies involving selection or screening steps are employed alongside approaches focusing on rational design to select for the desired functionality of a given element.

Here we present a fluorescence-based *in vivo* screening approach that combines an initial *in vitro* selection with subsequent extensive screening steps and a final rational design to identify RNA based regulators in baker's yeast. These artificial RNA regulators, termed synthetic riboswitches, are derived from RNA aptamers. Our method allows for the separation of aptamers featuring the potential to be transformed into a riboswitch from those inherently unable to confer control over gene expression. The system may be applied to virtually all existing aptamer-ligand pairs and as such presents a powerful means to enhance the setup of switchable genetic circuits.

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1. Introduction

During the past years, the ability to conditionally control gene expression has led to the discovery of novel functions of various cellular biomolecules such as proteins and nucleic acids. A plethora of genetic parts – where parts are defined as individually interchangeable genetic entities like promotors, terminators, reporter genes or regulatory elements – contributed to a better understanding, design and implementation of whole metabolic pathways in various cellular environments. However, design rules still remain elusive and the connection of all required parts is to be tested empirically rather than predicted computationally [1]. In addition, the majority of all available genetic elements is published for prokaryotic organisms but the integration of artificial gene regulation in lower eukaryotes as the yeast *Saccharomyces cerevisiae* still suffers from the lack of a greater pool of regulatory elements [2].

Regulatory building blocks are essentially protein- or nucleic acid-based and appear mainly as transcription factors or RNA-regulators. To render them sensitive to internal or external input a variety of adequate sensory elements has to be integrated. The availability of these elements is essential for the functionality of a genetic switch since they predefine the context in which it can be operated. As sensors for any given target cannot just be made by computational design due to the complexity of the sensor - target interaction, an *in vitro* selection strategy for nucleic acid sensors was developed [3,4]. This SELEX (Selective Evolution of Ligands by Exponential Enrichment) approach involves iterative interaction events between a large library of RNA sequences and a target molecule. After several rounds of *in vitro* selection only those RNA sequences are retained that form stable interactions with the target. These RNA sequences are called aptamers. Aptamers can be created against virtually any target molecule and therefore it is highly desirable to use them as sensory units for the conditional control of gene expression. However, aptamers are a priori not suited to regulate gene expression, because the applied *in vitro* selection strategy is designed to identify RNA sequences that bind strongly to their target and this does not imply a gene-regulatory activity as well. At this stage it is key to accomplish the transformation from an aptamer sequence to an RNA sequence capable of switching gene expression ON and/or OFF [5].

RNA-based regulatory elements, called riboswitches, were first discovered in 2002 by Breaker and co-workers. They were later confirmed to exist in all three kingdoms of life [6–8]. Naturally, they are highly structured RNAs globally arranged by secondary and tertiary interactions that are sensitive to metabolites or other small molecules and capable of controlling gene expression. They mostly reside in the 5′ untranslated regions (UTR) of bacterial mRNAs where they control both transcription or translation of several metabolic pathways. A few riboswitches have been discovered in eukaryotes, too, where they control splicing events and mRNA stability [8]. A prerequisite for functionality is the ability

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http://dx.doi.org/10.1016/j.ymeth.2015.12.001
1046-2023/© 2015 Published by Elsevier Inc.

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to transmit a conformational change of the aptamer domain to an expression platform upon selective binding of the ligand. The expression platform in turn undergoes a structural rearrangement that ultimately results in a change of gene product levels.

Natively occurring riboswitches can serve as templates for the design of their artificial counterparts. In our studies we were pursuing a design that would combine the aptamer domain and the expression platform in a single compact structure (Fig. 1A). As mentioned earlier in this section it is not productive to rationally design a sensory unit and this goes as well for the combined functionality of sensing and switching. It is hardly possible to modify a given aptamer *in silico* to exhibit control over gene expression, because design rules are only poorly understood [5]. It is rather practical to apply a system that involves selection, library screening and in addition rational design to yield the desired structures.

Here, we present a method based on fluorescence readout which primarily focuses on screening RNA-aptamer libraries to identify sequences that control gene expression reversibly in a ligand-dependent fashion (see Fig. 1B and Section 3 for further information). Thereby an enriched RNA-aptamer pool obtained by SELEX is inserted into the 5′ UTR of a *gfp* reporter gene by homologous recombination in yeast and subsequently subjected to extensive screening steps performed in a 96-well format. We provides a detailed protocol for the method along with helpful tips and tricks to avoid pitfalls.

2. Material and methods

2.1. SELEX – pool design and selection strategy

A detailed protocol about *in vitro* selection (SELEX) is provided within this issue. It must be pointed out that for the design of the initial pool a rather long randomized region of 50–90 nt should be considered, which is optimal for the identification of small molecule-binding aptamers [9,10]. Long nucleotide stretches exhibit a greater structural flexibility and may probe the limited binding sites on a small molecule more efficiently [11]. To bias the evolution of RNA sequences towards a stable folding, structural elements as hairpins can be considered in the pool design [12,13]. This strategy however, as it compromises structural flexibility, has not proven to be beneficial to obtain small molecule binding aptamers that exhibit riboswitching properties (personal communication). The number of selection rounds depends on the target molecule, but typically 5–10 rounds of enrichment are performed yielding pools with moderate (μM) to high (nM) affinity binding profiles. A full length SELEX protocol for the selection of small molecule binding aptamers including buffers, temperatures, washing steps can be provided upon request.

2.2. RNA-library cloning for in vivo screening

The SELEX approach from 2.1 yields RNA aptamer pools from different rounds of selection differing in their enrichment of sequences that exhibit binding towards the target molecule. It is now recommended to use both pools that have a presumably strong aptamer target interaction and pools from earlier rounds with average binding properties as will be explained in Section 3. Once the user decides for two or more (cDNA) pools they are to be PCR-amplified with a forward (fwd) and reverse (rev) primer. These primers will attach 5′-overhangs that are homologous to the 5′ UTR upstream of the green fluorescent reporter gene (*gfp*) and 3′ overhangs to a 5′ part of the coding sequence of the reporter gene itself (for details see Fig. 2). The overhangs are proposed to have a length of 30–40 nt which is sufficient for efficient plasmid-borne homologous recombination. After PCR amplification of the selected pools with the correct primers the PCR product can be used without further purification, although the correct length of the product should be confirmed by gel electrophoresis.

The expression of GFP from the 2μ yeast shuttle vector pWHE601 is constitutively driven by a *pADH1* promotor with a 5′ UTR of 34 to 44 nt since two transcription start sites are described [14]. The template vector, termed pWHE601⁎, used in this study is

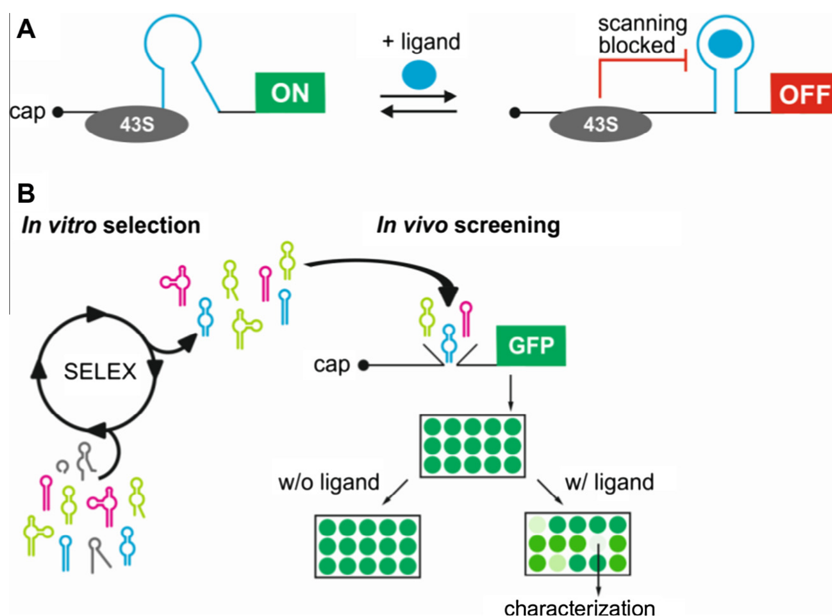

Fig. 1. Principles of the screening system to identify riboswitches. (A) Mechanism for conditional gene expression by synthetic riboswitches. In absence of the ligand the riboswitch adopts a loose conformation permitting scanning of the small ribosome complex for the start codon resulting in an ON state of gene expression. Addition of the ligand captures a highly structured riboswitch and scanning is blocked. As a result gene expression is reduced to a minimum. 43S (scanning-competent small ribosomal subunit), cap (5′-m^{7}GTP). (B) Scheme of the combined approach of *in vitro* selection and *in vivo* screening. Identification of target-binding sequences by SELEX (Systematic Evolution of Ligands by Exponential Enrichment) and subsequent fluorescence-based *in vivo* screening to yield RNA sequences with regulatory properties in baker's yeast. GFP (Green Fluorescent Protein), cap (5′-m^{7}GTP).

Please cite this article in press as: C. Schneider, B. Suess, Methods (2015), http://dx.doi.org/10.1016/j.ymeth.2015.12.001

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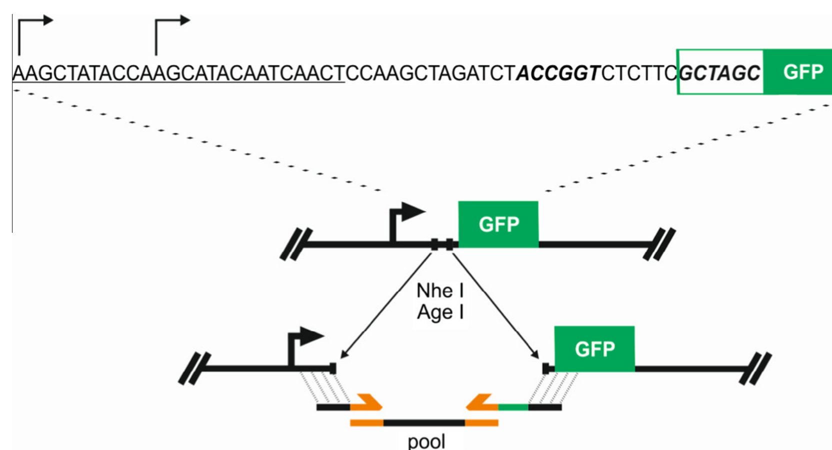

Fig. 2. Scheme of the cloning strategy. *Upper panel*: detailed display of the nucleotide sequence involved in homologous recombination. Arrows indicate the two transcription start sites of the adh1 promotor; the 3′-promotor sequence is underlined. Sequence of the *gfp* gene is artificially expanded for the Nhe I restriction site and translation incompetent due to a missing start codon (green square). Restriction sites are highlighted in bold letters and italicized. *Lower panel*: after a restriction digest with Nhe I and Age I the gel-purified and linearized vector serves as a template for homologous recombination. The inserts are prepared by PCR amplification of the RNA-aptamer pool with primer containing a 40 nt long 5′ overhang homologous to the upstream and downstream sequences of the restriction site (black lines connected by dashed lines) and binding on the constant parts flanking the randomized region of the pool (red lines). The Kozak sequence AAA-ATG-G including the start codon is introduced with the reverse primer (green line).

a derivative of the aforementioned pWHE601 where the start codon has been deleted. For homologous recombination it is best prepared by cutting with two restriction enzymes that produce incompatible ends. The restriction sites reside immediately upstream of the reporter gene to promote an efficient recombination event between promotor sequence and *gfp* gene [14]. The start codon including the yeast Kozak sequence 5′-AAA-start-G-3′ is introduced with the 5′ overhang of the rev primer [15]. This ensures that only yeast cells that have undergone an accurate homologous recombination will be selected for the *in vivo* screening step as incorrect recombined plasmids or survivors of the restriction digest cannot code for the fluorescent reporter and therefore remain colorless. That said, to minimize transformation of uncut plasmids, a gel extraction of the cut plasmids after the restriction digest is mandatory.

Overall, one transformation mix for a homologous recombination contains around 100 fmol gel purified vector, a 10:1 M excess of the PCR-amplified inserts and competent yeast cells according to the protocol used. Transformation is carried out according to the protocol of the Frozen Yeast Transformation Kit II from Zymo Research or a lithium acetate-based method [16]. The transformation mix is plated on up to 40 petri dishes with a diameter of 9.2 cm. This assures a moderate colony density which simplifies the selection of clones for the *in vivo* screening. Agar plates are composed of minimal medium (0.2% [w/v] yeast nitrogen base, 0.55% [w/v] ammonium sulfate, 2% [w/v] glucose, 12 μg/mL adenine, MEM amino acid mix). The transformed yeast cells are cultured for 3–4 days at 28–30 °C.

2.3. Selection – fluorescence microscopy

The selection step is accomplished employing a standard stereo microscope (M50, Leica Microsystems) equipped with a setup to visualize the fluorescence of interest. Petri dishes are scanned by eye for fluorescent colonies. Controls have to be transformed alongside the pool to determine the minimal and maximal fluorescence. The negative control accords to the vector backbone pWHE601∗ unable to express GFP and the positive control, pWHE601, constitutively expresses GFP from the same backbone vector, but containing a start codon inclusive Kozak sequence. In addition it is suggested to have an already working riboswitch available that has been cloned according to Section 2.2 to have an idea which fluorescence levels are to be expected approximately (see Section 3 for further details). Fluorescent colonies are marked with a pen on the bottom of the petri dish and later picked into a 96-well plate as described in 2.4. We recommend to select over the whole spectrum of fluorescence intensities. Only those colonies displaying no or a very weak fluorescence can be omitted (see Sections 3 and 4).

2.4. In vivo screening

The screening step is carried out in transparent 96-well plates using the Tecan plate reader Infinite M200 Pro. Colonies chosen from plate in 2.3 are incubated as pre-culture in 200 μl minimal medium for 16–20 h at 30 °C using 96-well format, U-bottom, on the plate shaker Titramax 1000 (Heidolph *instruments*). The preculture is then transferred to fresh medium and grown for additional 30 h in the absence and presence of the target molecule. 20 μl are drawn each time to inoculate 2 triplicates for each independently grown colony. The first triplicate is cultured in absence of the ligand, the second triplicate in presence of the target molecule. These cultures are used to determine whether addition of the ligand reduces fluorescence levels and thereby indicates the presence of a riboswitch transcribed by the corresponding yeast cells.

Fluorescence is measured from the top at an emission wavelength of 510 nm after excitation at 482 nm at an optical density (OD_{600}) of 0.6–0.9 in 96-well flat-bottom plates. Cultures need to be diluted accordingly and have to be shaken up shortly prior to analysis. In addition to fluorescence the OD_{600} is recorded in parallel to normalize the fluorescence signal for cell growth. Yeast cells transformed with the positive and negative control as well as a working riboswitch are included in each plate and measured alongside the picked colonies. The determined fluorescence signal is normalized to an OD_{600} of 1, the fluorescence of the negative control is subtracted and the signal is displayed as relative fluorescence with the positive control set to 100% signal intensity. The riboswitch control is used to check whether a systematic error has occurred since the fluorescence values including the dynamic change of fluorescence of cells expressing this construct is known.

Cells in wells which display a dynamic change of the fluorescent signal are streaked out on agar plates and singularized. This

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separation step (singularization) after the first screening round is important, because sometimes we observed a partitioned fluorescence distribution within a given colony that is due to an uptake of multiple plasmids at once. Four individual colonies from one original candidate are incubated and subjected to a second round of screening as described above.

Typically, only few colonies are identified to show a change in fluorescence signal intensity upon ligand addition. The dynamic range of the signal change is rather low and the user needs to set a threshold above which candidates are chosen. See Sections 3 and 4 for further information. Once candidates have been verified in the second screening round, the plasmids are isolated and transformed into *E. coli.* Isolated plasmids should then be retransformed into yeast and measured to confirm their switching activity. Then the plasmids are sequenced.

2.5. In silico rational design

The secondary structure of the identified sequences is simulated deploying online tools such as mfold [17] and the Vienna RNA package [18]. Based on these predictions it is now necessary to track down the minimal motif which is essential for ligand binding and *in vivo* activity. This is done by carefully evaluating the *in silico* projected structural data and a sequence alignment in parallel to structural probing experiments to identify mutual motifs that may constitute the active structure [19,20]. For the design of primers that will truncate the sequence step by step it is critical to start with short steps of about 5 nt, as it is completely unknown where the binding site and the structural information for *in vivo* activity are located. In case of an already characterized aptamer, the binding site may be known, but its involvement in the structural refolding leading to a change of gene expression is not. See Sections 3 and 4 for detailed information. Truncated sequences can be cloned using standard restriction and ligation, via homologous recombination, Gibson cloning or any other method the user feels appropriate. After cloning, the plasmids are sequenced and the regulatory effect is measured after transformation into yeast.

3. Theoretical considerations

The eukaryotic translation machinery is controlled by a variety of mechanisms and stimuli. Importantly for the *in vivo* screening system, local mRNA organization, structure and stability play key roles to facilitate ribosome assembly and translation initiation. The basic concept of the system is to interfere with ribosomal scanning and the initiation of translation at the start codon by inserting a road block in the 5′ UTR of the mRNA (see Fig. 1A) [14,21]. Thereby, mRNA levels are undisturbed [21]. The pre-selected aptamer pools are therefore cloned in front of a plasmid-borne *gfp* reporter gene expressed in baker's yeast.

Yeast colonies are in a first step selected for high gene expression in the absence of the ligand. This ensures that only cells harboring a correctly recombined plasmid with a functional 5′ UTR of the reporter gene are further processed. Functionality implies the lack of pre-mature start codons within the cloned aptamer sequence as these would abrogate protein expression, if they occurred not in frame with the actual translational start site. The occurrence of the triplet AUG is not linked to a potential riboswitching property, but the screening system cannot distinguish whether a functional riboswitch follows a pre-mature start codon and thus an *in vivo* active aptamer might be discarded.

In addition, only sequences with a moderately folded 5′ UTR that may be scanned by the 43S ribosomal pre-initiation complex, hence accessible for translation initiation, are subjected to the actual screening step.

The conformational stability depends strongly on the aptamer sequence and its capability to constitute the respective global architecture leading to translational repression even in absence of the ligand. At this point it becomes obvious why a pool of different aptamer sequences is needed and first to be screened for basal gene expression. Only from a rather broad spectrum of sequences candidates can be identified that satisfy the first selection criterion and do not interfere with gene expression in a way that abolishes gene expression right from the start.

Supplementation of the ligand is then supposed to reduce gene expression since the global fold of the aptamer is stabilized due to the binding event resulting in the inhibition of ribosome scanning. A prerequisite for a decrease of gene expression is, however, the ability of the aptamer to either undergo extensive structural refolding upon ligand binding or to display an ensemble of different ligand-free ground state conformations in thermodynamic equilibrium. One of these conformations is then captured by the ligand and stabilized. Several experiments in the past have shown that the ligand exclusively binds to an already pre-folded structure, typically energetically disfavored and short-lived, inducing only minor conformational rearrangements [22–26]. This conformational capture mechanism is mandatory for *in vivo* activity as it allows for the ligand-free ground states to be largely unstructured, hence to promote ribosome scanning, and on the other hand to spontaneously fluctuate into the folded conformation which is specifically bound and trapped by the ligand, hence to prevent ribosome scanning. The penalty to bind an entropically disfavored conformation is paid by the formation of enthalpically beneficial intermolecular interactions.

As with the selection for high gene expression in the absence of the ligand, aptamer pools are required to provide a sufficient sequence space to accommodate structural motifs that might confer *in vivo* activity. For the *in vivo* screening approach, enriched RNA-pools are chosen that exhibit moderate to good binding properties with respect to the target. It is necessary to work with pools of differential binding strength, because an aptamer with high affinity for its target is very likely strongly pre-structured and may interfere extensively with basal gene expression rendering it inactive for transformation into a riboswitch. The link between high affinity and extensive pre-structuring is given by the selection pressure inherent to the SELEX approach. Only those aptamers may be subjected to the next selection round that could withstand the stringent conditions under with selection is performed. This means the closer the actual folding resembles the perfect folded aptamer the higher is the affinity for the target. Extensive fluctuations between different conformations may therefore prevent efficient binding. On the other side, aptamer sequences with low affinity are not of interest as they do not efficiently bind the target at all and will not be of relevance in a biological matrix since they would require high concentrations of ligand, leading to unspecific binding and unwanted side effects. Aptamers that bind in the nanomolar range are ideally suited, because they efficiently accommodate the target, are moderately pre-structured and exhibit structural flexibility to be identified as a riboswitch candidate.

Once identified, the sequences in question are edited *in silico*. Sequence alignments are employed to reveal possible motifs that might be involved in ligand binding and/or switching activity. Uncovered motifs can guide the design of truncated derivatives. It is always desirable to work with minimal motifs, because these are accessible for further in depth structural studies and rational design. Besides, the introduction of a small artificial sequences in a native context of a given gene cassette reduces unnecessary sequence load and therefore increase the likelihood of a well-performing synthetic element. In general, truncations are performed in 5–10 nt steps to minimize the chance of preliminary abrogating ligand binding or switchability and target the closing

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stem of the folded aptamer. It has been shown that switching activity is closely related to the stability of the closing stem while the binding pocket is usually not constituted by this helix motif, making it an ideal target structure for modifications. Truncations, point mutations, swapping entire nucleotide stretches from opposite strands and the addition of bases are possible modifications. In total, a decrease of stability in the ligand-free state and an increase in stem stability in the ligand-bound conformation is pursued by rational design.

4. Results and discussion

This section describes and discusses the results of the *in vivo* screening for the neomycin-sensitive riboswitch generated in 2008 by Weigand et al. [19] and follow-up experiments investigating relevant biochemical properties [22–26]. Additionally, insights from further *in vivo* screening experiments on different aptamer pools currently performed in the lab (unpublished data) and the characterization of the tetracycline-binding riboswitch complement the data set wherever appropriate.

An aptamer pool enriched for binding neomycin B over six cycles of *in vitro* selection was chosen to start the *in vivo* screening [27]. The pool consisted of 111 nt-long RNA sequences with a 74 nt randomized region flanked by constant parts for amplification purposes during *in vitro* selection. The same initial pool design was also used to identify tetracycline-binding aptamers [28]. The neomycin-binding aptamer pool (after the sixth selection cycles) constituted an approximated starting diversity of about 10^3 sequences after insertion in front of a constitutively expressed *gfp* reporter gene. The estimated sequence space is a product of the pool diversity which is given by the length of the randomized region, but drastically diminished as the enrichment of binding aptamers proceeds [29], and the transformation efficiency in yeast.

During the first step of the screening only 5% of all colonies displayed green fluorescence, meaning that all other transformants harbored plasmids with (i) incorrectly recombined plasmids, (ii) aptamer sequences that incorporated premature start codons or (iii) whose global folding stopped the 43S complex from scanning for the original start codon (see Sections 2 and 3 for details). Other *in vivo* screening approaches revealed that interference with basal gene expression in absence of the ligand is strongly dependent on the actual aptamer size and its ability to form extensive secondary and tertiary interactions (unpublished data). Small aptamers (or pools of randomized sequences originating from an already characterized aptamers) generally produced > 80% bright fluorescent colonies after transformation. For longer sequences opposite observations were made. In accordance with this rule is the fact that aptamer pools enriched over many rounds are normally extensively pre-structured and lose their competence to fluctuate between loose and ridged conformations resulting eventually in low basal gene expression and no switching activity.

Usually around 1300 colonies were subjected to the screening step in 96-well format. The calculation of the dynamic change of the fluorescent reporter signal yielded factors below 3-fold. Although these values are low, they are typical for the initial screening step since a raw, unmodified pool with a sequence length of around 100 nt and sequence space in the order of 10^4 cannot be expected to yield better regulation as was empirically seen in various experiments for different ligands. After two screening rounds and the singularization step (see Section 2) the original pool was narrowed down to 10 candidates conferring slight ligand-induced inhibition of reporter gene expression. Sequence analysis of these clones revealed a putative motif responsible for the *in vivo* activity regarding the neomycin-sensitive switches. Uncovering a common motif is not a prerequisite to identify riboswitches during the screening, but hints towards a successful experiment. Interestingly, the active sequences found in the neomycin screening were underrepresented in the pool cloned into the screening system. This exemplifies the experimental difficulty not to lose the few active sequences overshadowed by a vast majority of good binding but inactive aptamers.

Truncation analysis and structural probing of sequenced neomycin-binding aptamers lead to the identification of a minimal motif comprising the consensus sequence responsible for the regulatory phenotype and yielding an improved dynamic change (4-fold) of fluorescence values upon ligand supplementation (Fig. 3). Further modifications of the minimal motif increased the dynamic range up to 8-fold resulting in the successful transformation of an aptamer into a riboswitch.

Although *in silico* predictions of secondary structures of RNA sequences around 100 nt in length are more or less accurate, tertiary interactions cannot be mapped reliably. This inability of computational modelling is reflected in the fact that rational designs of aptamer mutants may not lead to an improvement at all or show impaired functionality as crucial motifs remain hidden in the primary and secondary structure. In case of the neomycin-binding as well as the tetracycline-binding riboswitches only approximations with regard to the actual motifs accounting for the switching

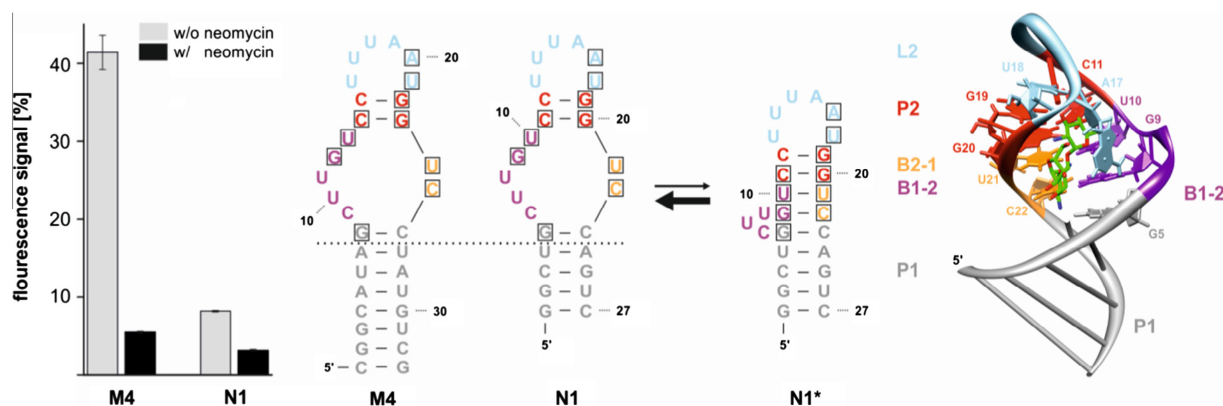

Fig. 3. The neomycin riboswitch. A comparison of the secondary structures of two individual sequences that regulate gene expression neomycin-dependent is shown. Sequence N1 was obtained after *in vitro* selection and *in vivo* screening, whereas M4 is the result of a rational modification of N1. As discussed in the text, the riboswitch fluctuates between an open ligand-free state (N1) and a folded ligand-free conformation (N1*) that is exclusively captured and stabilized by neomycin. The column plot illustrates gene expression levels based on the fluorescence readout of both sequences in absence and presence of neomycin. The 3D structure depicts the antibiotic ribostamycin bound to sequence N1. Ribostamycin was chosen due to superior spectral resolution but exhibits a similar repression and binding profile. Motifs involved in ligand binding are highlighted in color and nucleotides interacting with the ligand are boxed (2D structure) or specified in the 3D structure. Adopted from Wittmann et al. [31] and further modified.

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phenotypes could be made by structural probing experiments and *in silico* predictions [19,20]. Not till the employment of the more sophisticated analytical methods like EPR, NMR and fluorescence spectroscopy a detailed mechanistic insight could be gained [22–26]. Two distinct mechanisms can be drawn from these data sets that operate under the target-receptor model called conformational capture or selection, respectively. For the neomycin-dependent aptamer a switching element could be identified that destabilizes the ligand-free ground state, weakening the global fold of the riboswitch and resulting only in a moderate reduction of basal gene expression [25]. In turn, addition of the ligand shifts the thermodynamic equilibrium towards the entropically disfavored folded conformation which is captured by the ligand. The complex is stable due to the beneficial release of enthalpic energy compensating the entropic penalty. Therefore the neomycin-sensitive riboswitch gains its *in vivo* activity from the fact that the ligand-free conformations are loose and rather unstructured and above all highly populated in comparison to the folded conformation that can only stably exist when captured by the ligand. This ensures relatively high gene expression values without ligand and a decrease of gene expression upon ligand addition (compare Fig. 3).

The *in vivo* activity of the tetracycline-dependent riboswitch is governed by a transiently folded loose tertiary structure with high ligand affinity that is captured by the ligand as well. Since the ligand-free conformations fluctuate on biological relevant time-scales and do mostly not exhibit ligand affinity, basal gene expression values are relatively high, whilst the transient tertiary structure serves as a scaffold for ligand docking resulting in only minor conformational changes to impair expression from the controlled gene [22,24,26].

A feature of both mechanisms is found in the reduced gene expression already in the absence of the ligand that originates from trade-offs between the presence of a loosely pre-structured RNA and the ability to efficiently fold into the stable global architecture preventing the 43S complex scanning process. A very small aptamer (<30 nt) poses a weak roadblock whether ligand-stabilized or not. To observe gene product down regulation, the aptamer must increase in size and structural complexity. Exactly here an increased size – better switching behavior – is traded for a lower basal gene expression as the roadblock is already strengthened in absence of the ligand. Vice versa, an aptamer structure too large will reduce basal gene expression to a minimum and the system is not accessible for a further reduction. This "switching window" is ideally pre-selected by the screening system itself. Recently, such an *in vivo* selection system was established in baker's yeast by Hartig and co-workers [30]. Here, riboswitch identification was achieved by applying positive and negative selective pressure to generate a functional window in which the riboswitch is supposed to operate. To further improve our screening system an automatic unbiased pre-selection, rather than a manual and user-dependent microscope-assisted marking, will be established. In total, these improvements could greatly reduce the generation time of an *in vivo* active riboswitch from months to weeks once an aptamer has been successfully identified by SELEX which in turn takes up to several months. System upgrades could also aim at the *in vitro* selection and the randomization of already characterized aptamers.

In this article we have demonstrated the applicability of an *in vivo* screening system for the identification of novel riboswitches transformed from aptamers. Due to the small number of screens performed until now success rate is hard to predict. However, an improved system can more predictably yield sequences that bind to virtually any target of interest and interfere with gene expression in a user-defined manner. These regulatory active parts can be implemented in a variety of genetic circuits operating for instance in accordance with Boolean logic to gain a better control over in- and output signals as well as in simpler approaches that study the conditional impact of a gene product on its targets.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft Germany (SFB902 A2) and EU FP7-KBBE-2013-7 no.613745, PROMYS.

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Please cite this article in press as: C. Schneider, B. Suess, Methods (2015), http://dx.doi.org/10.1016/j.ymeth.2015.12.001