RNA Technology

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Small-Molecule-Dependent Regulation of Transfer RNA in Bacteria**

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Ribonucleic acids such as messenger, transfer, and ribosomal RNAs play pivotal roles in gene expression. In addition to naturally occurring, RNA-based switches of gene expression,[1] ligand-binding sequences (aptamers) have been incorporated artificially into mRNAs in order to control the expression of these messages.[2,3] Recently, Ogawa and Maeda as well as our own group have introduced a different strategy by utilizing ligand-dependent ribozymes in order to switch on or off the translation of a given mRNA in bacteria.[4–7] Apart from these hammerhead-based systems for controlling mRNA translation in bacteria, ligand-dependent ribozymes have been utilized in yeast to program RNA-based Boolean logic gates.[8] In our opinion, the use of ligand-dependent, self-cleaving ribozymes is advantageous since it can be generalized for controlling RNA classes other than mRNAs. Here we show that the concept can be extended to switching the utilization of transfer RNAs (tRNAs). In the present example, we use a theophylline-dependent ribozyme in order to activate a tRNA for its use in translation.

To our knowledge, this is the first example of an engineered device for the small-molecule-based control of a tRNA in a living cell. Nevertheless, Westhof and co-workers have demonstrated that yeast $tRNA^{Asp}$ is specifically recognized by the antibiotic tobramycin, resulting in the inhibition of the aspartylation reaction.[9] Furthermore, incorporation of self-cleaving hammerhead ribozymes (HHRs) into yeast tRNA and rRNA has been used to study polymerase II-independent polyadenylation mechanisms in yeast.[10] Recently, an in vitro translation system for the label-free detection of theophylline based on minimal-motif HHR–tRNA conjugates has been introduced.[11]

We reasoned that it should be possible to utilize self-cleaving ribozymes in vivo for a gain of function of a tRNA upon triggering the catalytic activity. In order to engineer a tRNA analogue that can be controlled in *Escherichia coli* by ribozyme activity, we attached a fast-cleaving hammerhead motif comprising stem I/stem II contacts[12,13] to the 5′ end of a tRNA (Figure 1a). As a reporter system for in vivo activity of a specific tRNA in translation we used a $tRNA^{Ser}$ with the anticodon loop mutated to recognize the amber stop codon ($tRNA^{Ser}_{CUA}$) in combination with an eGFP reporter mRNA carrying a mutation from serine to an amber stop codon (Ser50UAG).[11,14] In case of a nonfunctional amber suppressor $tRNA^{Ser}_{CUA}$ premature termination of translation takes place, whereas activation of the $tRNA^{Ser}_{CUA}$ results in suppression of termination by incorporation of serine (Figure 1c). The introduction of the amber stop codon results in complete inhibition of eGFP expression, whereas utilization of the amber suppressor tRNA leads to full restoration of eGFP expression by means of amber suppression.

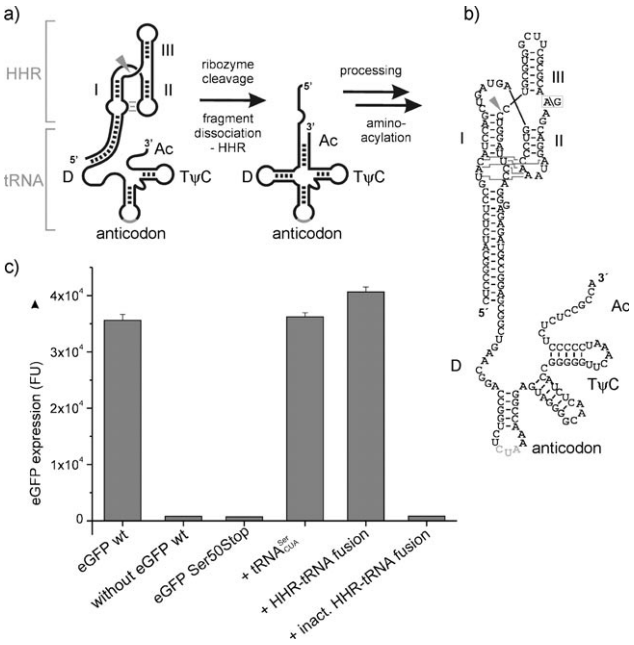

Figure 1. Ribozyme-mediated control of tRNA function in vivo. a) Connection of HHR to the tRNA sequence results in a nonfunctional tRNA since the formation of the acceptor and D arm is disrupted. The cleavage site is marked by a gray arrowhead. b) Nucleotide sequence of the HHR–tRNA fusion construct. The outlined nucleotides highlight the position of an A-to-G point mutation resulting in ribozyme inactivation.[15] The CUA anticodon matching the mRNA amber stop codon is highlighted in gray. c) Fluorescence analysis of eGFP expression in the *E. coli* strain BL21 (DE3) utilizing the following constructs: wt eGFP mRNA and wt serine tRNA (eGFP wt); a control clone lacking the eGFP gene (without eGFP); an eGFP mRNA containing an amber stop codon (eGFP Ser50Stop); amber eGFP mRNA and the wt serine tRNA (+ $tRNA^{Ser}_{CUA}$), the amber mRNA and the HHR–tRNA fusion construct (+ HHR–tRNA fusion); and the same constructs containing the ribozyme-inactivating A-to-G mutation (+ inact. HHR–tRNA fusion).

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In order to achieve ribozyme-mediated control of a tRNA in vivo, we aimed at a gain of function when the ribozyme cleavage reaction is activated. For this purpose, we connected the ribozyme such that the typical tRNA cloverleaf secondary structure is not able to fold in the case of an inactive ribozyme (Figure 1 a and b). We achieved this design utilizing the mfold secondary-structure prediction algorithm[16] by extending stem I of the HHR to pair with the 5′ end of the tRNA, disrupting the formation of the acceptor and D stem-loops. The resulting misfolding inhibits the processing of the tRNA and triggers premature degradation,[17–19] hence preventing the tRNA from functioning properly. We anticipated that upon cleavage of the ribozyme, the two RNA fragments should dissociate and enable the folding of the functional tRNA (Figure 1 a). We have successfully used this "gain of function" principle of cleavage fragment dissociation before in order to liberate the ribosome binding site for switching on mRNA translation.[4–6]

Indeed, when we connected an active HHR to the $tRNA^{Ser}_{CUA}$, strong eGFP expression occurred rivalling the level of wt-eGFP mRNA expression in combination with an unaltered tRNA (Figure 1 c, HHR–tRNA fusion). The fact that the combination of a suppressor tRNA with the corresponding amber mRNA results in expression levels of the reporter comparable to the natural system is intriguing since usually amber suppression is known to occur with maximum efficiencies of around 20–30 %.[20] Importantly, in this setup the tRNA function proved to be completely dependent on the ribozyme activity, as proven by an inactivating ribozyme point mutation (Figure 1 b). The A-to-G mutation in the catalytic core results in an inactivated ribozyme. Interestingly, gene expression in this construct drops to background levels comparable to the absence of the eGFP gene (Figure 1 c). Thus, the specific connection of a hammerhead ribozyme and a tRNA as shown in Figure 1 establishes a very powerful system for the self-cleavage-mediated control of tRNA function.

Next, we aimed at rendering the system to be dependent on a small-molecule stimulus. In order to do so, aptamers can be incorporated into the ribozyme to yield ligand-dependent aptazymes. Recently, we have shown that theophylline-dependent ribozymes can be used to cleave a given RNA in vivo upon addition of theophylline. We have now tested whether the same sequence used to cleavage an mRNA in the previous study (previously termed theoHHAz,[4] Figure 2) is also suited to mediate small-molecule-dependent control of tRNA utilization in the present setup. Surprisingly, the theophylline-controllable hammerhead theoHHAz for switching mRNA translation optimized by an in vivo screening experiment shows the same switching performance in the tRNA context (termed theoHHAz–tRNA fusion, Figure 2 c. Upon addition of theophylline to the growth medium, tRNA utilization and hence expression of eGFP is switched on. In order to verify the results generated by measuring GFP fluorescence from intact *E. coli* cells, we quantified the GFP levels by detection of the His tag from cell lysates by western blots using a conjugate composed of nickel nitrilotriacetate (Ni-NTA) and alkaline phosphatase (AP) (for experimental details see the Supporting Information). The results obtained

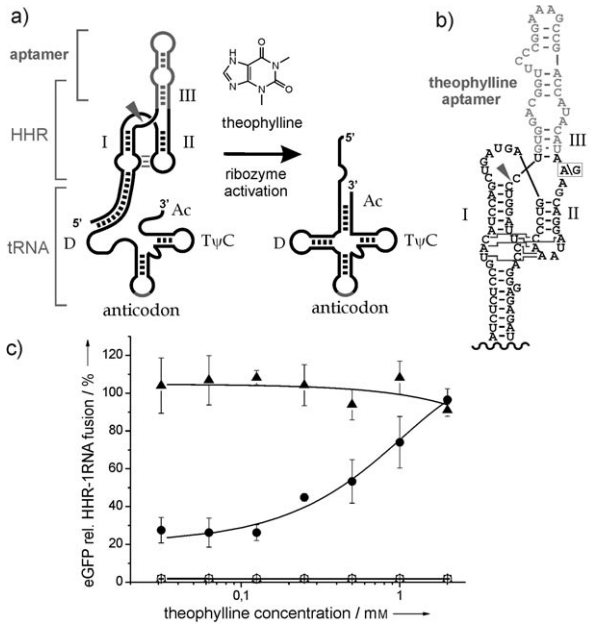

Figure 2. Small-molecule-dependent regulation of tRNA function in vivo. a) The theophylline-dependent aptazyme (theoHHAz, which contains an aptamer in stem III shown in gray) is connected to the tRNA to yield the theoHHAz–tRNA fusion construct. b) Nucleotide sequence of the theoHHAz–tRNA fusion construct (the tRNA part of the construct is identical to that shown in Figure 1 b). c) Theophylline-triggered utilization of tRNA: eGFP expression in vivo with the parental HHR–tRNA fusion construct (triangles) and the theophylline-dependent theoHHAz–tRNA fusion construct (circles). Open triangles and circles represent the respective constructs inactivated by the A-to-G point mutant.

by the western blots are in good accordance with the fluorescence measurements (Figure 3): The active HHR–tRNA fusion construct shows expression of GFP, whereas the ribozyme-inactivating point mutation results in suppressed expression. In the theophylline-responsive sequences, only the cleavage-competent sequence shows activation upon addition of theophylline (Figure 3 a and b). Figure 3 c and d show a concentration-dependent increase of GFP expression by western blot in accordance with the results observed when GFP fluorescence is measured.

By using ribozyme assays in vitro with isolated RNAs we have previously shown that the catalytic activity of the ligand-dependent ribozyme theoHHAz is indeed enhanced upon addition of theophylline.[4] We next studied the fate of the ribozyme-controlled RNA by northern blot analysis utilizing a hybridization probe detecting the 5′ end of the respective fusion constructs (see Figure S1 in the Supporting Information). In the active HHR–tRNA fusion construct, a short RNA species is detected corresponding to the ribozyme-cleaved 5′ product (66 nt, lanes 1 and 2). The band of the 5′-cleavage product of the ribozyme reaction is less intense than that of the inactivated variant (lanes 3 and 4). A possible reason could be the activity of nucleases reducing the stability of the cleavage fragment.[19,21] The inactivated HHR–tRNA fusion construct is found in high abundance at an increased length. Importantly, theophylline addition does not induce changes of the abundance of both constructs, indicating that

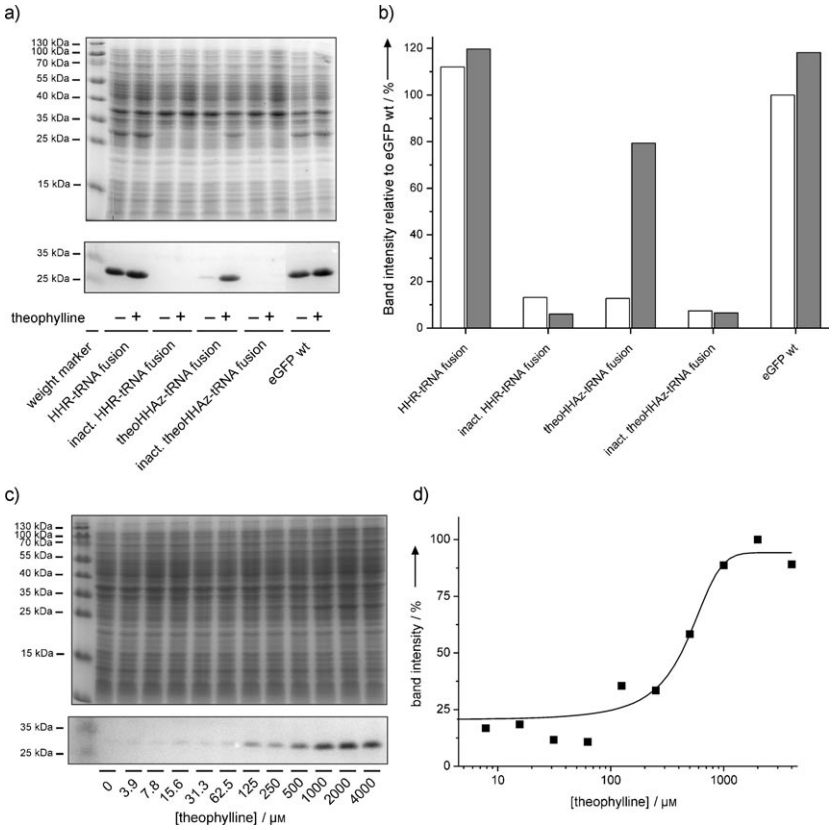

Figure 3. Western blot analysis of GFP expression in *Escherichia coli*: a) SDS-PAGE (top) and western blot (bottom) using a Ni-NTA–AP conjugate detecting the His-tagged GFP. Theophylline concentration was 2 mM in the lanes marked with +. b) Quantification of the western blot shown in (a). c) Western blot of theophylline-dependent eGFP expression utilizing theoHHAz–tRNA fusion construct. d) Quantification of the blot shown in (c).

tRNA expression and stability are not affected by theophylline when the aptamer is absent. With the theophylline-dependent theoHHAz–tRNA fusion construct (which is slightly longer than the HHR–tRNA fusion construct because of the inserted aptamer sequence), a pronounced decrease of the noncleaved fusion RNA is visible upon addition of theophylline in accordance with ribozyme cleavage activation by the small molecule (see Figure S1B in the Supporting Information). The 5′-cleavage fragment seems to be degraded rapidly in accordance with the literature.[19,21] The mature tRNA is not evident since it is not recognized by the hybridization probe (see the last lane in the northern blot analysis corresponding to RNA isolates from the parental strain lacking the introduced ribozyme).

In conclusion, we have developed a system for the external, small-molecule-mediated control of tRNA function in *E. coli*. It is interesting to note that high levels of the eGFP reporter were translated in our setup, although the activated amber suppressor $tRNA^{Ser}_{CUA}$ competes with the action of release factor 1.[22,23] The latter mechanism is the main reason why usually a maximum of only 20–30% efficacy of amber tRNA suppression is observed.[20] The presented technology of utilizing ligand-triggered ribozymes for activating tRNAs could prove useful in strategies for the site-specific incorporation of unnatural amino acids. The strategies developed so far allow for incorporation of a variety of unnatural building blocks utilizing native as well as orthogonal translational components.[24–26] Nevertheless, the huge complexity of the tRNA world still sets limitations on these approaches.[27] In this context, the possibility to specifically turn on or off a tRNA of interest in vivo could prove useful for such endeavors, allowing for enhanced control in the incorporation of unnatural amino acids.

In principle, the introduced system should make it possible to read a given message differently depending on the ribozyme-determined decoding of specific codons. For such applications, we will aim at a second, orthogonal tRNA system that is triggered by an alternate ligand. Since the general pathway of tRNA maturation based on structural features is conserved throughout all kingdoms of life,[17] the presented approach should be transferable to other organisms as well. In combination with other known ligand-dependent systems for regulating the genetic machinery, the presented approach should allow for the construction of combined pre-, co-, and post-transcriptional, hierarchical multilevel information processors with gene expression as output. In addition, the general approach of utilizing ribozymes for controlling key features of the genetic apparatus has the potential to be extended even beyond the regulation of mRNA and tRNA functions.

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