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Canonical translation-modulating OFF-riboswitches with a single aptamer binding to a small molecule that function in a higher eukaryotic cell-free expression system

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ABSTRACT

We have found that OFF-riboswitches that ligand-dependently downregulate the canonical translation in a higher eukaryotic expression system (wheat germ extract) can be easily created by inserting a single aptamer into the 5′ untranslated region (UTR) of mRNA, even if its ligand is as small as theophylline. The key is the position of the inserted aptamer: the 5′ end (+0 position) is much better than other positions for inhibiting canonical translation with the aptamer-ligand complex. The data showed that ribosome loading is suppressed by a rigid structure in the 5′ end, and this suppression is dependent on the structure's stability but not on its size. Although this preference of aptamer insertion point contradicts the results in a lower eukaryote, it accords with the fact that the 5′-end structural hindrance is more effective for blocking the ribosome in higher eukaryotes. Therefore, the present type of OFF-riboswitch would function in various higher eukaryotic expression systems.

Riboswitches are ligand-responsive, *cis*-acting gene regulators composed of an aptamer (a sensory domain) and an expression platform (a regulatory domain).[1] When a specific ligand binds to the former domain, the ligand-aptamer complex formation induces conformational changes (or alternative folding) of the latter domain, which regulates expression of the downstream (or sometimes upstream) gene. While natural riboswitches responsive to endogenous ligands have been identified mainly in untranslated regions (UTRs) in bacterial mRNA, several types of artificial riboswitches (including those functioning in eukaryotic expression systems) have been constructed with *in vitro*-selected or engineered, non-natural aptamers to regulate gene expression (mostly, translation initiation) with user-defined ligands.[2–4] In particular, eukaryotic OFF-riboswitches that ligand-dependently downregulate the canonical translation have been created with relative ease merely by inserting an *in vitro*-selected aptamer into the 5′ UTR of mRNA.[3] Because the eukaryotic ribosome is loaded onto the 5′ terminus of mRNA to scan the mRNA for the initiation codon in a canonical translation system, a ligand-aptamer complex in the 5′ UTR suppresses the ribosome progression and thus downregulates the expression. In other words, the aptamer domain also plays a role as an expression platform in these eukaryotic OFF-riboswitches. Therefore, to design them, we do not have to consider complicated, ligand-dependent hybridization switches (i.e., strand displacement),[2h] which are generally required for changing the conformations of an expression platform in typical riboswitches.

Inconveniently, however, it was reported that a single aptamer in the 5′ UTR was insufficient to exert a riboswitch function when a small molecule, theophylline (MW = 180.16), was used as the ligand,[3c] despite the fact that the dissociation constant (K_D) between theophylline and its aptamer is relatively low (~0.3 μM).[5] In that report, three theophylline aptamers were eventually inserted in tandem into the 5′ UTR to fabricate an efficient, theophylline-responsive OFF-riboswitch (the ON/OFF reduction ratio was approximately 7 at 1 mM theophylline in wheat germ extract (WGE)).[3c] In general, however, it is difficult to add iterative sequences to a regulated gene at narrow intervals because of the difficulty of designing specific primers. This is one of the reasons why we recently created other types of eukaryotic OFF-riboswitches, which modulate noncanonical, internal ribosome entry site (IRES)-mediated translation in a different manner from that of the canonical translation-modulating ones.[4] These IRES-based OFF-riboswitches require only a single aptamer each to satisfactorily downregulate gene expression in response to a small molecule such as theophylline. Nonetheless, they require a relatively long, cumbersome IRES sequence (~200 nt)[6] as an expression platform to be ligated to the regulated gene. In the present study, we therefore sought to construct a much simpler, eukaryotic canonical translation-modulating OFF-riboswitch with a single aptamer, even if its ligand is small.

We first focused on the position of the aptamer inserted in the 5′

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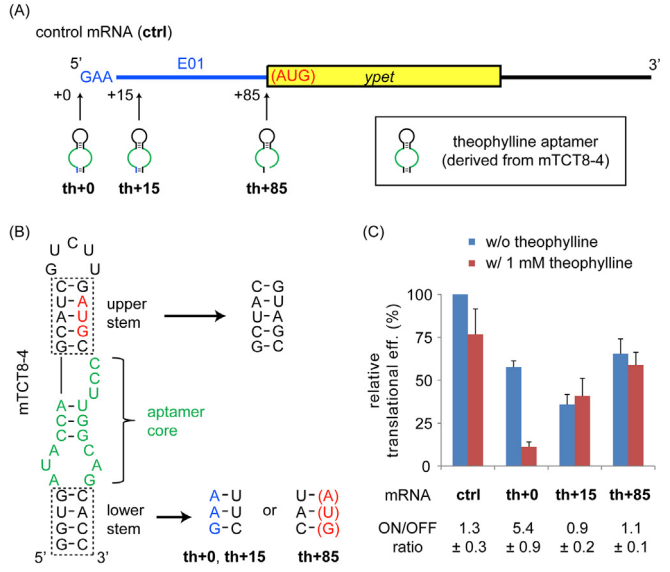

Fig. 1. Effect of the position of a theophylline aptamer in the 5′ UTR on riboswitch activity. (A) Schematic illustration of control mRNA (**ctrl**) and insertion of a theophylline aptamer into the 5′ UTR of **ctrl** to prepare three kinds of mRNAs with the aptamer at a different position (**th + 0**, **th + 15**, and **th + 85**). (B) Sequences of mTCT8-4 (the original theophylline aptamer, left) and its derivatives with some mutations in both upper and lower stems in **th + 0**, **th + 15**, and **th + 85** (right). The mutated upper stem was used for all mRNAs with the aptamer. The red letters in parentheses represent the start codon of the YPet gene. (C) The relative translation efficiencies of mRNAs shown in (A) in the absence (ON) or presence (OFF) of 1 mM theophylline in WGE.

UTR, because it has been reported that translational inhibition by structural hindrance in the 5′ UTR depends not only on the obstacle's size and stability but also on its position.[7] Although a theophylline aptamer was inserted into a +15 position in the above-mentioned previous report describing that one aptamer was insufficient,[3c] we expected that the 5′ end or the vicinity of the start codon was more appropriate, based on the results in other reports.[3d,7,8] We thus inserted a single theophylline aptamer derived from mTCT8-4[5] into a +0, +15, or +85 position of aptamer-free mRNA (**ctrl**), which has a 85-nt 5′ UTR (E01,[9] a translational enhancer with a 5′ terminal GAA sequence (*vide infra*)) and encodes yellow fluorescent protein (YPet),[10] to prepare three mRNAs (**th + 0**, **th + 15**, or **th + 85**, respectively, Fig. 1A). The length of the lower stem in the aptamer was set to 3 bp by reference to a theophylline-responsive, eukaryotic ON-riboswitch that we recently created.[2j] Specifically, 5′ GAA/UUC 3′ was used for **th + 0** (and **th + 15**) because the 5′ terminal GAA functions as a ribosome recruiter similarly to the 5′ cap in WGE,[11] and 5′ CAU/(AUG) 3′ was chosen for **th + 85** so that the lower stem could include the start codon (in parentheses, Fig. 1B). In addition, several bases in the upper stem, which are not involved in binding to theophylline,[12] were slightly mutated to remove the AUG sequence (Fig. 1B). We then checked the translation efficiencies of these mRNAs in WGE (Fig. 1C). In the absence of theophylline, all mRNAs with the aptamer showed moderate efficiencies (around half of that by **ctrl**), probably because a partial structure of the aptamer functioned somewhat as a hindrance and/or the aptamer insertion disturbed the translational enhancer sequence (E01), especially in **th + 15** with the aptamer in the middle of that sequence. When we added theophylline to the translation solution (f.c. = 1 mM), while **th + 15** and **th + 85** showed no responsiveness (ON/OFF = 0.9 ± 0.2 and 1.1 ± 0.1, respectively) along with **ctrl** (ON/OFF = 1.3 ± 0.3), **th + 0** exhibited surprisingly higher OFF-riboswitch activity (ON/OFF = 5.4 ± 0.9), which was comparable to that of the previously reported OFF-riboswitch with three tandem theophylline aptamers.[3c] The theophylline unresponsiveness of **th + 15** is in accordance with the results for the previously reported mRNA with a single theophylline aptamer at the same position.[3c] In contrast, the results of **th + 0** and **th + 85** contradict those of another report, in which an aptamer near the start codon was conversely more effective for ligand-dependent downregulation in yeast.[3d] However, it has been reported that higher eukaryotes such as wheat are more sensitive to ribosome loading than to ribosome scanning, in contrast to lower eukaryotes such as yeast.[7] In addition, it is expected to be kinetically and thermodynamically easier for the ligand to bind to the terminus than to the middle of the mRNA. Therefore, our results indicate that we successfully created a *higher eukaryotic* OFF-riboswitch by inserting a single aptamer at the +0 position (*i.e.*, the very 5′ end) to readily modulate ribosome loading, even though its ligand is very small.

We next investigated the effect of stem length in the aptamer to optimize the obtained riboswitch in **th + 0** toward a higher switching efficiency (Fig. 2A and Supplementary Fig. S1A). The lower stem should form with theophylline (and the middle stem) to block the ribosome loading only in the OFF state (Fig. 2A, left). However, **th + 0-L4** with a 1-bp-longer lower stem (4 bp) inhibited translation (probably, ribosome loading) even in the absence of theophylline, as considerably as **th + 0** did in the presence of 1 mM theophylline (Supplementary Fig. S1B). This means a 4-bp-lower stem is too long to evade formation of a duplex without the ligand (at least at the 5′ end). Incidentally, the ON/OFF ratio of **th + 0-L4** was 2.2 ± 0.8 at 1 mM theophylline, which was much lower than that by **th + 0**. In contrast, **th + 0-L2** with a 1-bp-shorter lower stem (2 bp), which still had the 5′ GAA, allowed the ribosome to be slightly more easily loaded on the mRNA.[13] However, it showed only a weak theophylline responsiveness (ON/OFF = 1.6 ± 0.2 at 1 mM theophylline, Supplementary Fig. S1B), indicating that this length of lower stem is not sufficient for binding to the ligand. As far as

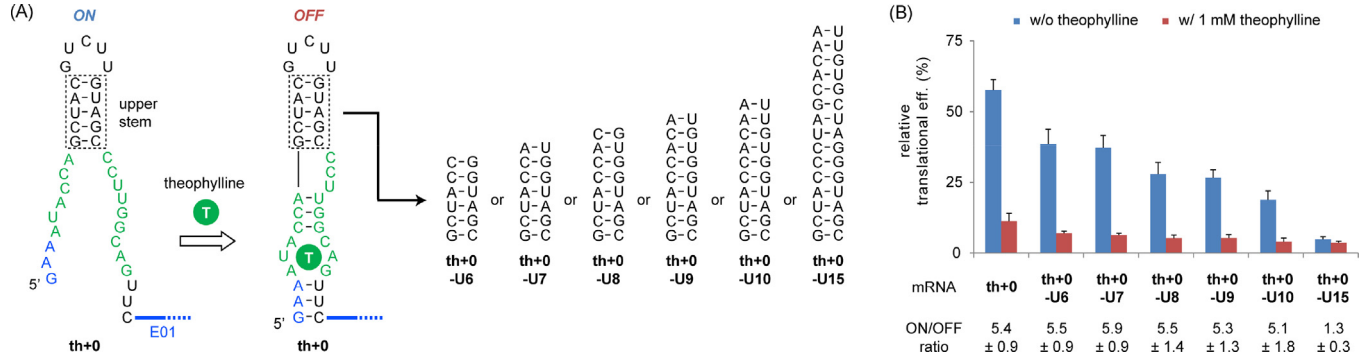

Fig. 2. Optimization of the upper-stem length of the theophylline aptamer in **th + 0**. (A) The proposed mechanism of theophylline-dependent riboswitching in the 5′ terminal region in **th + 0** (left) and alteration of the upper stem of the theophylline aptamer to construct various kinds of **th + 0** derivatives (a series of **th + 0-UX** (**X = 6–10** and **15**), right). (B) The relative translation efficiencies of mRNAs shown in (A) in the absence (ON) or presence (OFF) of 1 mM theophylline in WGE.