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Aptazyme-based biosensors using a eukaryotic cell-free translation system

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ABSTRACT

I have constructed a novel aptazyme-based biosensor system for detecting cofactors of the aptazymes using a cell-free luciferase synthesis in wheat germ extract. In this system, the activity of the aptazyme that is fused to a 5'-untranslated region of a luciferase gene can be detected as luciferase expression. In translating the aptazyme-fused mRNA as-is using a wheat germ cell-free translation system, the luciferase is almost not expressed because of the following triple suppression effects: (1) 5'-terminal three bases and (2) 5'-terminal duplex prevent the ribosome from binding to own mRNA; (3) if the ribosome binds, translation of a mimic gene in the aptazyme inhibits that of the downstream luciferase gene (OFF state). In contrast, in the presence of the aptazyme cofactor, the aptazyme in mRNA is self-cleaved to produce an aptazyme-free luciferase gene, which is translated efficiently (ON state). The ON/OFF efficiency and the detection limit of the aptazyme-based biosensor for theophylline are much higher and lower, respectively, compared to those of previously-reported one that utilizes a prokaryotic translation system.

INTRODUCTION

Aptazymes are one of functional RNAs that function as ribozymes only in the presence of specific molecules (i.e. their cofactors). They are expected to be versatile biosensors for various molecules because the aptazyme in response to an arbitrary molecule can be obtained by an in vitro selection method. Nonetheless, to detect cofactors with ease and high sensitivity using aptazymes, we need inventive approaches for how to detect activities of the aptazymes, in other words, how to convert those into more detectable signals. Although some detection methods have been reported thus far, attention is currently focused on "biofunction-assisted" methods, in which the aptazyme activities are converted into detectable protein expression using a biotic translation system, due to their simplicity and their potential for applications to development of molecule-dependent gene expression systems. Recently, I reported one of these methods using a prokaryotic cell-free translation system.[1,2] In this method, a hybridization switch of the ribosome binding site (RBS) is used. However, in a eukaryotic translation system, other strategies are required because there is no definite RBS in eukaryotic mRNA like a prokaryotic Shine-Dalgarno sequence. I report here a new conversion method from the aptazyme activity to protein expression using a eukaryotic (wheat germ) cell-free translation system.

RESULTS AND DISCUSSION

Generally, in a eukaryotic system, the ribosome is said to recognize 5'-capped mRNA in association with various initiation factors and to scan the mRNA from the 5'-terminus to start translation at the first start codon in a favorable strong context unless there is an internal ribosomal entry site (IRES) in the mRNA. However, according to the literatures by Endo et al.,[3] a wheat germ cell-free translation system that they have reported has an intriguing aspect: the 5'-cap of mRNA isn't necessarily required and the translation efficiency depends on three bases at the 5'-terminus without the 5'-cap although it is unclear whether the ribosome entries from 5'-terimus without the 5'-cap. On the basis of these features in wheat germ, I designed 5'-cap-free aptazyme-fused mRNA to convert the aptazyme activity into luciferase expression using its extract (Fig. 1).

In translating this uncleaved mRNA as-is in wheat germ extract, luciferase expression must be suppressed (OFF state). This can be achieved by three effects caused by "three bases at the 5'-terminus", "duplex formation of the 5'-terminus", and "a mimic gene" in the aptazyme. In terms of the first effect, because the translation efficiency depends on three bases at the 5'-terminus of mRNA without the 5'-cap as described above, it should decrease by optimizing 5'-terminal three bases to be less effective for translation. As far as the latter two effects are concerned, if the ribosome entries from the 5'-terminus even without the 5'-cap, the 5'-terminal duplex should inhibit the ribosome entry and the mimic gene (from mSTART and mSTOP) should be translated somewhat instead of the downstream "genuine" luciferase gene (from gSTART to

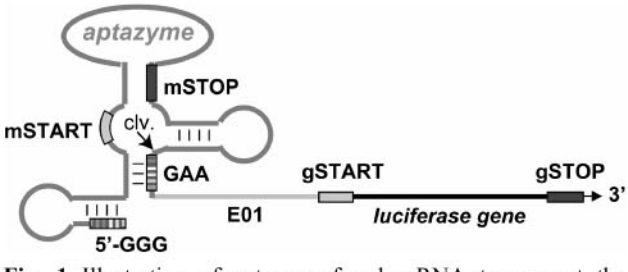

Fig. 1 Illustration of aptazyme-fused mRNA to convert the aptazyme activity into luciferase expression. The arrow shows the self-cleavage site.

gSTOP). In contrast, in the presence of the aptazyme cofactor, the aptazyme-fused mRNA should cleave itself to produce a non-fused (i.e. aptazyme-free) mRNA. This cleaved mRNA has GAA trinucleotides as a part of a translation enhancer sequence (E01) at 5'-terminus that are the most effective for translation, so that it is expected that its translation efficiency is relatively higher although it lacks a 5'-phosphoryl group (ON state).

First, to decrease the translation efficiency in the OFF state, the 5'-terminal sequence was optimized using theophylline-dependent-aptazyme-fused mRNA, wherein mSTART that the theophylline-dependent aptazyme originally has with mSTOP was mutated to eliminate the mimic gene effect in this stage. With considering the in vitro transcription efficiency by T7 RNA polymerase for preparation of mRNA, the 5'-terminal three bases for less-effective translation were searched from 5'-GGX. As a result, 5'-GGG was found to be ~16% less effective than 5'-GAA. Moreover, duplex formation of 5'-terminal some bases resulted in another suppression effect (~14% less effective). These results strongly suggest that the ribosome binds to and entries not from E01 in an IRES-like manner but from the 5'-terminus without the 5'-cap. Next, to verify the mimic gene effect, the mutated mSTART in the aptazyme of 5'-terminus-optimized mRNA was turned back to the original one. As a result, translation of the downstream "genuine" luciferase gene was additionally suppressed (~39% less effective). This is clearly considered as evidence of the mimic gene effect and also supports that the ribosome entries from the 5'-teruminus.

Finally, using the optimized theophylline-dependent-aptazyme-fused mRNA that is the lowest effective for translation in the OFF state, self-cleavage reactions were performed in the presence of various concentrations of theophylline followed by translation in wheat germ extract. As a result, luciferase was expressed in response to theophylline concentrations in aptazyme reactions (Fig. 2). The ON/OFF efficiency, which is the activity ratio of the expressed luciferase in the ON state to the OFF state, was about 50 at 500 μM theophylline. The detection limit was estimated 5 μM (ON/OFF = 2.0+/-0.3). In comparison to a previously reported biosensor for theophylline using a prokaryotic cell-free translation system,[1] this ON/OFF efficiency at 500 μM theophylline and this detection limit are 10-fold higher and 20-fold lower, respectively. This higher sensitivity is mainly because whereas blocking the "effective" RBS suppresses translation in the OFF state in the previous prokaryotic method,[1] blocking the 5'-terminal "less-effective" RBS-like sequence (i.e. 5'-GGG) much suppresses translation in the OFF state in collaboration with the mimic gene effect in the present eukaryotic method. Because blocking the RBS is achieved by its duplex formation (hybridization) that is equilibrated reaction, it is not complete for inhibition of the ribosome-binding that is also hybridization of nucleic acids. In the present system, however, if the 5'-duplex unwinds, the translation efficiency is not almost restored due to the "less-effective"

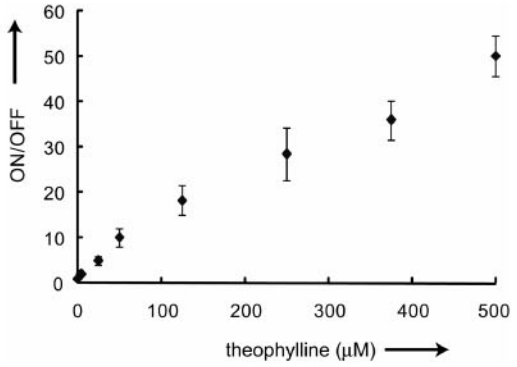

Fig. 2 ON/OFF efficiencies of the luciferase translated using as templates theophylline-dependent-aptazyme-fused mRNA self-cleaved at various concentrations of theophylline.

RBS-like sequence and the mimic gene. Moreover, the most effective RBS-like sequence (i.e. 5'-GAA) emerges only after self-cleavage in the ON state. These effects, which are attributed to the mRNA design utilizing the feature that the ribosome entries from 5'-terminus of mRNA without the 5'-cap in wheat germ extract, allow the gap in the translation efficiency between the ON and OFF state to be extended in the present method.

CONCLUSION

In summary, I developed a new method for converting the aptazyme activity into easy-detectable protein (luciferase) expression with high efficiency using a eukaryotic (wheat germ) cell-free translation system to use it as a biosensor for detecting the aptazyme cofactor. The ON/OFF efficiency and the detection limit of the aptazyme-based biosensor for theophylline were much higher and lower, respectively, compared to those of previously-reported one using a prokaryotic cell-free translation system.

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