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Design of 5′-untranslated region variants for tunable expression in *Escherichia coli*

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Abstract

Redesign or modification of the cellular physiology requires a quantitatively well-controlled expression system known as the ''tunable expression.'' Although the modification of promoters demonstrates the great impact on the translation efficiency, it is difficult to detect the proper variants required for tunable expression. The 5′-untranslated region (UTR), however, can be an important target for tunable expressions because the ribosome binding affinity is directly modulated by the sequence variants of the Shine–Dalgarno (SD) sequence and the AU-rich sequence, which are the ribosome binding sites and a SD-sequence-independent translation enhancer, respectively. This study developed a simple method to obtain numerous 5′-UTR variants and analyze their translation efficiency based on the PCR-based site-directed mutagenesis and the expressional PCR using coupled *in vitro* transcription/translation system derived from *Escherichia coli* and eGFP gene as a template. SD sequence variants (18) and AU-rich sequence variants (36), which have a wide range of relative expression levels ranging from 0.1 to 2.0, were obtained. The translation efficiency was affected by the ribosome binding affinity and its accessibility that is dependent on the secondary structure around the 5′-UTR.

Keywords: 5′-UTR; Expressional PCR; Tunable expression; SD sequence; AU-rich sequence

Purposeful and effective redesigning the biological systems is a requisite for understanding the dynamic biological mechanism and the modulation of the expression level by the regulatory elements [1,2]. Intentional redesign of the biological systems requires dynamically controllable expressional regulation as well as a variety of quantitatively predictable regulatory elements. Recently, RNA-mediated control of gene expression based on the structural dynamics and the sequence-specific binding abilities has been actively investigated [2–4]. Issacs et al. developed riboregulators that facilitated the controlling gene expression via highly specific RNA–RNA interactions [5]. These techniques employ the dynamic regulation to introduce the sensitive response to environmental changes.

Numerous regulatory elements for various expression levels were obtained by several approaches such as the creation of promoter libraries and the introduction of secondary structures to control mRNA stability [6–9]. Although promoter libraries obtained by the error-prone PCR allow the generation of various promoters that show a wide range relative activities, ranging from 0.1 to 1000, additional elements are required for fine-tunable expression. Some works on the modulation of mRNA stability

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demonstrated that the secondary structure in the 5′-untranslated region (UTR) of the multi-cistronic mRNA molecules have the potential to function as an additional component for fine-tuning [3]. However, only few such structures are available and more elements are required for this technique.

5′-UTR plays a major role in the translation initiation that determines the translation rate, and it is composed of several regulatory elements, including the Shine–Dalgarno (SD) and the AU-rich sequences [10,11]. The SD sequence facilitates 16S rRNA-specific ribosome binding and the AU-rich sequence facilitates an auxiliary ribosome binding that is mediated by the ribosomal protein S1 [12–15]. Therefore, modulation of the 5′-UTR sequence is required for the sensitive expression control in addition to the transcriptional control by promoter libraries and the posttranscriptional control by altering the mRNA stabilities. Understanding the quantitative effect of the 5′-UTR structure on the translational efficiency will provide valuable information that can be used to design the additional regulatory elements required for the fine-tunable expression.

This study developed a simple method to investigate the translation efficiency in terms of the sequence variants in the 5′-UTR that contained the SD and the AU-rich sequences based on the expressional PCR coupled with the PCR-based site-directed mutagenesis [16,17]. The correlation between the expression level and each 5′-UTR variant with a point mutation was studied by using the eGFP as a model system, and its capability in fine tuning gene expression was illustrated.

Materials and methods

Reagents and primers. The *Pfu* polymerase and restriction endonucleases were purchased from Stratagene and New England Biolabs, respectively. The pET23b was purchased from Novagen. Oligonucleotides, which were used for the construction of pET23b-eGFP and linear expression templates, were synthesized by Bioneer Co., Ltd and are listed in Table 1 of Supplementary Material. All other reagents were obtained from Sigma.

PCR-based site-directed mutagenesis. The pET23b-eGFP plasmid was used and constructed by inserting the amplified eGFP gene in the *Nde*I and *Xho*I restriction sites of pET23b. It was used to construct the 5′-UTR variants as a template for the PCR-based site-directed mutagenesis. A PCR mixture for site-directed mutagenesis consisted of 100 ng of pET 23b-eGFP plasmid as a template, 10 pmole of each primer, 0.5 U *Pfu* DNA polymerase, 250 mM of each of the four dNTPs, 5 μl of the 10× buffer provided by the manufacturer, and H_2O up to 50 μl. PCR was carried out on a Bioneer Thermal Block (Bioneer) under the following conditions: 95 °C for 5 min, 12 cycles for point mutation or 18 cycles for multiple nucleotide deletion (30 s at 95 °C, 1 min at appropriate annealing temperatures depending on the T_m of primers, 5 min at 72 °C), and finally, 72 °C for 7 min. After the PCR cycles, PCR products were purified with a QIAquick PCR Purification Kit (Qiagen) and then template DNA was eliminated by treating with *Dpn*I at 37 °C for 30 min.

Expressional PCR. Reaction mixtures of the site-directed mutagenesis were used for the second-round PCR to produce the templates for the expressional PCR. Prior to the second-round PCR, further purification steps via gel extraction using a QIAquick Gel Extraction Kit (Qiagen) were carried out to eliminate *Dpn*I-digested fragments. The reaction mixture of the second-round PCR contained 5 μl of the first-round PCR mixture, 10 pmole of each primer for amplifying the region from the T7 promoter to the T7 terminator, and other reagents in the same amounts as that used in the first-round PCR. PCR conditions were as follows: 95 °C for 5 min, 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, and finally, 72 °C for 5 min. After amplification, the PCR products were isolated using a QIAquick PCR Purification Kit (Qiagen), before use in the *in vitro* eGFP synthesis.

The *Escherichia coli* S30 extract used for coupled *in vitro* transcription/translation system was prepared by the method reported by Ahn et al. [18,19]. T7 RNA polymerase was expressed endogenously during *E. coli* cultivation with IPTG at 0.6 OD_{600}. The cells were harvested 2 h after IPTG induction followed by preparation of the S30 extract. The reaction mixture (15 μl) used for the coupled *in vitro* transcription/translation of the PCR-amplified templates consisted of the following components: 57 mM Hepes–KOH (pH 8.2); 1.2 mM ATP; 0.85 mM each of GTP, UTP, and CTP; 1.7 mM dithiothreitol; 80 mM ammonium acetate; 0.17 mg/ml of the total tRNA mixture obtained from *E. coli* (strain MRE 600); 34 μg/ml 1-5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid); 2.0 mM of each amino acid; 0.3 U/ml creatine kinase; 67 mM creatine phosphate; 1.0 μg PCR-amplified DNA; and 4 μl of the S30 extract. The cell-free protein synthesis was carried out at 37 °C for 60 min, and the synthesized eGFP was quantified by measuring the fluorescence of the eGFP as described below.

In vivo expression of eGFP in E. coli BL21. After *Dpn*I-treatment for removing the template DNA in the first-round PCR, 1 μl of the reaction mixture was transformed to the *E. coli* BL21(DE3) strain. The *E. coli* BL21(DE3) culture containing the selected pET23b-eGFP mutants were grown in LB medium containing 50 μg/ml of ampicillin; 1 M IPTG was added to obtain a final concentration of 1 mM when the OD_{600} was 0.4, and the induced culture broths were collected every hour. The concentration of *E. coli* was measured by using the UV-1700 spectrophotometer (Shimadzu) at a wavelength of 600 nm.

Analysis of eGFP activity. The collected coupled *in vitro* transcription/translation mixture and BL21(DE3) cells containing the mutated pET23b-eGFP from the culture broth were chilled rapidly on ice, and the eGFP activities were detected by VICTOR$^{3\text{™}}$ 1420 multilabel counter (Perkin-Elmer). The detections were carried out using a 486-nm excitation filter and a 535-nm emission filter in a 96-well plate with a 1-s measurement time. In the case of the coupled *in vitro* transcription/translation, the reaction mixtures were diluted in 50 mM Hepes, whereas the eGFP activities of the cells were measured without carrying out dilution. Specific eGFP activity was defined as fluorescence in the arbitrary unit of the instrument per gram of cells or per microliter of cell-free translation mixture. All the quantitative analyses of the eGFP activities were carried out in triplicate.

Results

The effect of the variants in SD sequence on the translation efficiency

The pET23b-eGFP plasmid contains two major 5′-UTR elements that affect the translational efficiency: the SD sequence and the AU-rich sequence. The specific interaction between the SD sequence on mRNA and the 3′-end of the 16S rRNA (anti-SD sequence) is a key step in forming a translation initiation complex. Mutations in the SD sequence, therefore, significantly change the translation efficiency [20,21]. On the other hand, the AU-rich sequence is another 5′-UTR element enhancing the translational efficiency with the involvement of the S1 protein [22,23]. Since the ribosomal protein S1 can interact with a wide range of primary structures, it is difficult to modulate the translation efficiency by altering the specificity between the S1 protein

and the sequence variant with a single mutation. Instead, a change in the secondary structure around the AU-rich sequence, which is caused by single point mutation, influences the efficiency of translational initiation in the *E. coli* due to competition between the mRNA structures such as hairpin loops and the 30S subunit [24,25]. Among the two major elements in 5′-UTR, the SD sequence was modified to obtain the expressional variations of the eGFP.

A wild-type SD sequence of the pET23b-eGFP plasmid, AAGGAG, was mutated at every single nucleotide position by the site-directed mutagenesis. Then, the expression cassette from the T7 promoter to the T7 terminator containing the eGFP gene was directly amplified using PCR, and *in vitro* eGFP synthesis was carried out after PCR purification as described in Materials and methods.

As a result, 19 different 5′-UTR variants containing the single point mutations in the SD sequence were obtained (Fig. 1). The effect of mutation on the translation efficiency was highly dependent upon the nucleotide position. When mutagenesis resulted in the substitution of the first nucleotide from A to T or G, the eGFP expression was 1.2 or 0.9 times that of the wild-type SD sequence, respectively. The mutations in the second and the third nucleotides—A and G— resulted in an expression which was 0.4–0.6 times that of the wild-type, except when the mutation caused the substitution of G to C at the third nucleotide position. The relative expression level due to mutations from the forth to the sixth nucleotides decreased significantly to around 0.1–0.3 times that of the wild-type SD sequence.

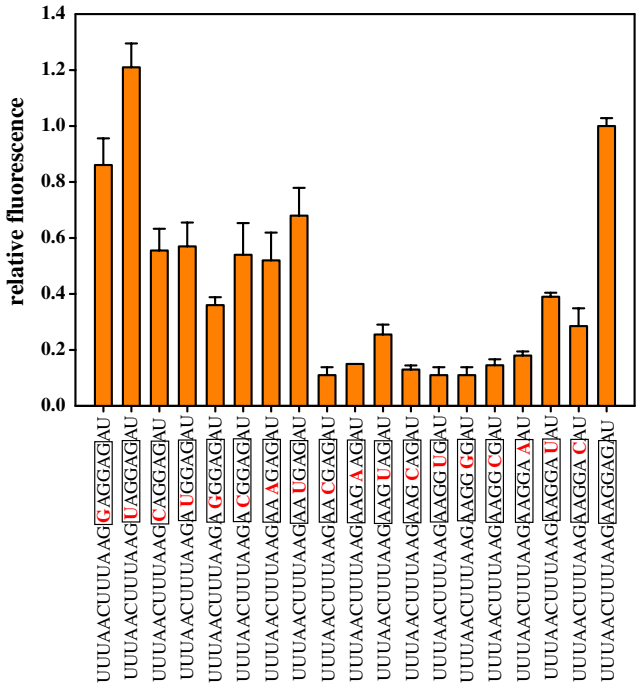

Fig. 1. Relative eGFP expression levels after *in vitro* translation using linear templates that were mutated at each position in the ribosome binding site. The *Y*-axis is the eGFP expression level normalized by the eGFP fluorescence level of the control RBS. The position of the mutated sequence is marked by a red letter.

These results show that from among the six nucleotides AAGGAG in the SD sequence, the last four nucleotides GGAG interact strongly with anti-SD sequence, whereas the first two nucleotides interact only weakly.

The effect of the mutations in the AU-rich sequence on the translation efficiency

The AU-rich sequences located upstream of the SD sequence are known to be other 5′-UTR elements that enhance the translation efficiency. Protein S1 interacts with the AU-rich single stranded region within the 5′-end of the mRNA during the formation of the translation initiation complex, and it has been found that S1-mRNA interaction contributes to the translation yield [24]. Therefore, the AU-rich sequence was investigated in terms of the effect of the mutations on the translation efficiency.

5′-UTR variants with the point mutations in the AU-rich sequence were obtained from a wild-type AU-rich sequence, AUUAACUUUAAG, by PCR-based site-directed mutagenesis, and expressional PCR for each mutant was carried out as described in Materials and methods.

Thirty-six expression cassettes were expressed under the coupled *in vitro* transcription/translation system, showing a relatively wide range of translation efficiency (Fig. 2). The eGFP expression varied from 0.5 to 2.0 times that of the wild-type AU-rich sequence. An AU-rich sequence deleted mutant, used as a negative control, displayed 0.4 times the expression level of the wild-type (data not shown). Therefore, the mutations in the AU-rich sequence affected the translation efficiency to varying degrees, while allowing control of the expression level.

While the effect of the mutations in the SD sequence on the translation efficiency was highly position-dependent, the position carrying the mutation in the AU-rich sequence was not significant for the translation efficiency. This is because the primary access of the S1 protein to the AU-rich sequence is more important for the translation initiation, and this binding is dependent on the secondary structure around the AU-rich sequence rather than its primary structure [26,27].

In vivo expression of eGFP gene with 5′-UTR variants

The selected pET23b-eGFPs with 5′-UTR variants were transformed to the *E. coli* BL21(DE3) strain after DpnI treatment, and then the cells were cultivated under the IPTG induction to confirm the translation efficiency of 5′-UTR variants *in vivo*. Aliquots of 1.5 ml were collected at 3 h after IPTG induction, and the eGFP activities were measured as described in Materials and methods.

Fig. 3 shows that relative expression levels of eGFP from *in vivo* experiments were similar to the *in vitro* data. These results demonstrate that the effects of 5′-UTR variants on the translation *in vitro* can sufficiently reflect the effects of each variant *in vivo*. Hence, the expression levels can be intentionally modulated by selecting the correct

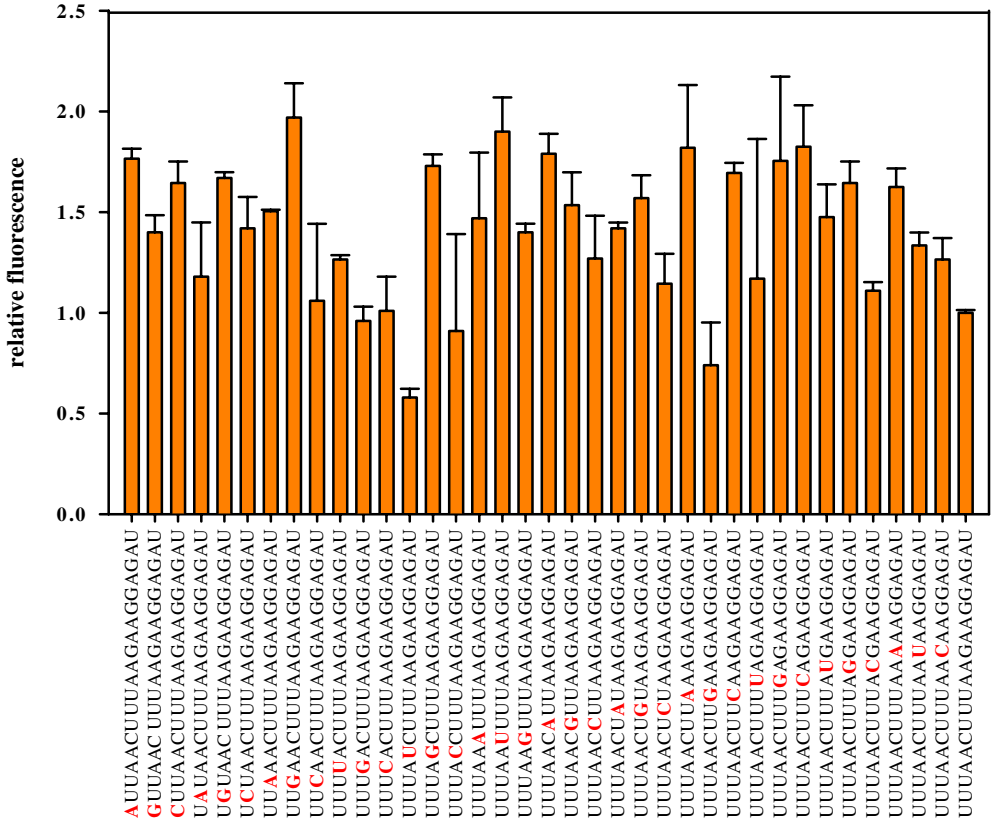

Fig. 2. Relative eGFP expression levels after *in vitro* translation using linear templates that were mutated at each position in the AU-rich sequence. The *Y*-axis value is the eGFP expression level normalized by the eGFP fluorescence level of the control AU-rich sequence. The position of the mutated sequence is marked by a red letter.

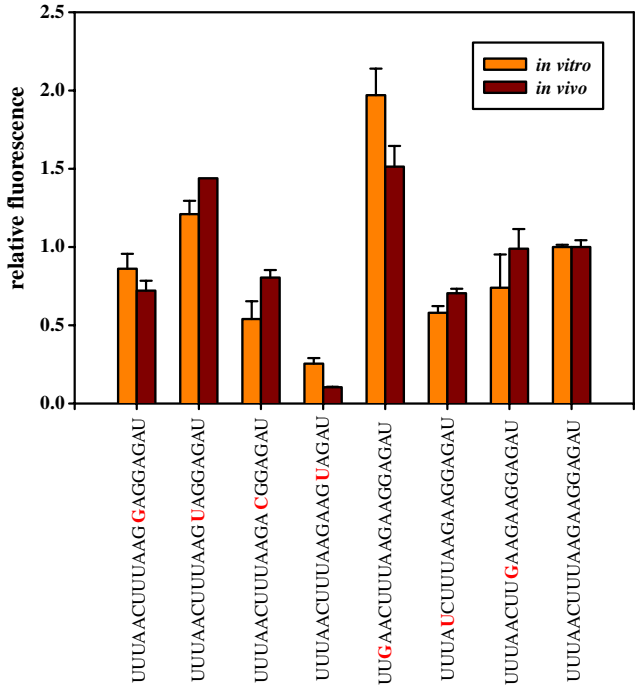

Fig. 3. Relative eGFP expression levels 3 h after IPTG induction. The *Y*-axis value is the eGFP expression level normalized by the eGFP fluorescence level of the control RBS and the AU-rich sequence per gram of cells. The position of the mutated sequence is marked by a red letter.

5′-UTR variant obtained in this study. Furthermore, a method based on the expressional PCR coupled with the PCR-based site-directed mutagenesis, which is developed in this study, can be a useful tool for predicting and searching the sequence variants in the 5′-UTR.

Discussion

Quantitatively controlled expression is critical for the regulation of cellular physiology and the effective redesign of the cellular functions. For several years, promoters have been modified for the quantitative gene expression [6]. The modification of promoters, however, causes significant changes in the gene expression level; hence, it is difficult to modulate the expression level due to promoter variation. On the other hand, 5′-UTR containing the SD sequence and the AU-rich sequence can influence the protein synthesis in a more direct manner, and the expression level can be accurately controlled by simple variation in their sequence.

The SD sequence was selected as a primary target for 5′-UTR variation to facilitate tunable expression because ribosome binds to the SD sequence directly and a single point mutation in the SD sequence significantly affects the translation efficiency. In particular, the last four nucleotides (GGAG) of the six-nucleotide SD sequence (AAG GAG) are known to be crucial for the translation efficiency

[14,28,29]. Mutations at the first and second nucleotide positions showed a higher expression level than mutations at the other positions (Fig. 1). On the other hand, mutations at last three nucleotides (GAG) resulted in significant reduction of the expression level, and these results reflect the high position-dependency of their translation efficiency. However, the relative expression levels for the 5′-UTR variants mutated at the third nucleotide position of the SD sequence varied from 0.1 to 0.7. Although the interaction between the SD sequence and the anti-SD sequence in the 3′-end of 16S rRNA primarily affects the translation efficiency, the secondary structure around the SD sequence also influences the expression level by controlling accessibility of the anti-SD sequence. Fig. 4A shows the secondary structures of the 5′-UTR variants mutated at the third nucleotide of the SD sequence estimated by M-fold [30]. A weaker secondary structure, i.e., fewer base pairs, can enhance the accessibility of the ribosome to the SD sequence, and consequently, increase the translation efficiency [5,31].

Mutations in the AU-rich sequence did not show strong correlation with the position where mutation occurred, unlike the SD sequence, because protein S1 interacts with a wide range of AU-rich sequences [23,26,32–34]. Therefore, point mutations in the AU-rich sequence mostly affect the accessibility of protein S1 by altering the secondary structure rather than the binding affinity of protein S1. Fig. 4B shows that a greater degree of base pairing in the AU-rich sequence reduced the translation efficiency. Furthermore, the mutation at the third position resulting in a G substitution in the AU-rich region resulted in an approximate 2-fold increase of the eGFP expression level due to its loose secondary structure.

It should be noted that all processes in this method, from mutation to detection of eGFP, were carried out *in vitro*. The direct translation of the PCR-amplified genes does not require time- and labor-intensive cloning procedures, and hence, it provides significant features for high-throughput test of many gene cassettes. Moreover, the various gene cassettes for the target protein expressions could be obtained by only single point mutations in the 5′-UTR region.

In conclusion, this study represents a rapid, easy, and simple method for synthesizing and analyzing gene expression that could lead to a much higher throughput approach to obtain 5′-UTR variants for expression control in *E. coli*.

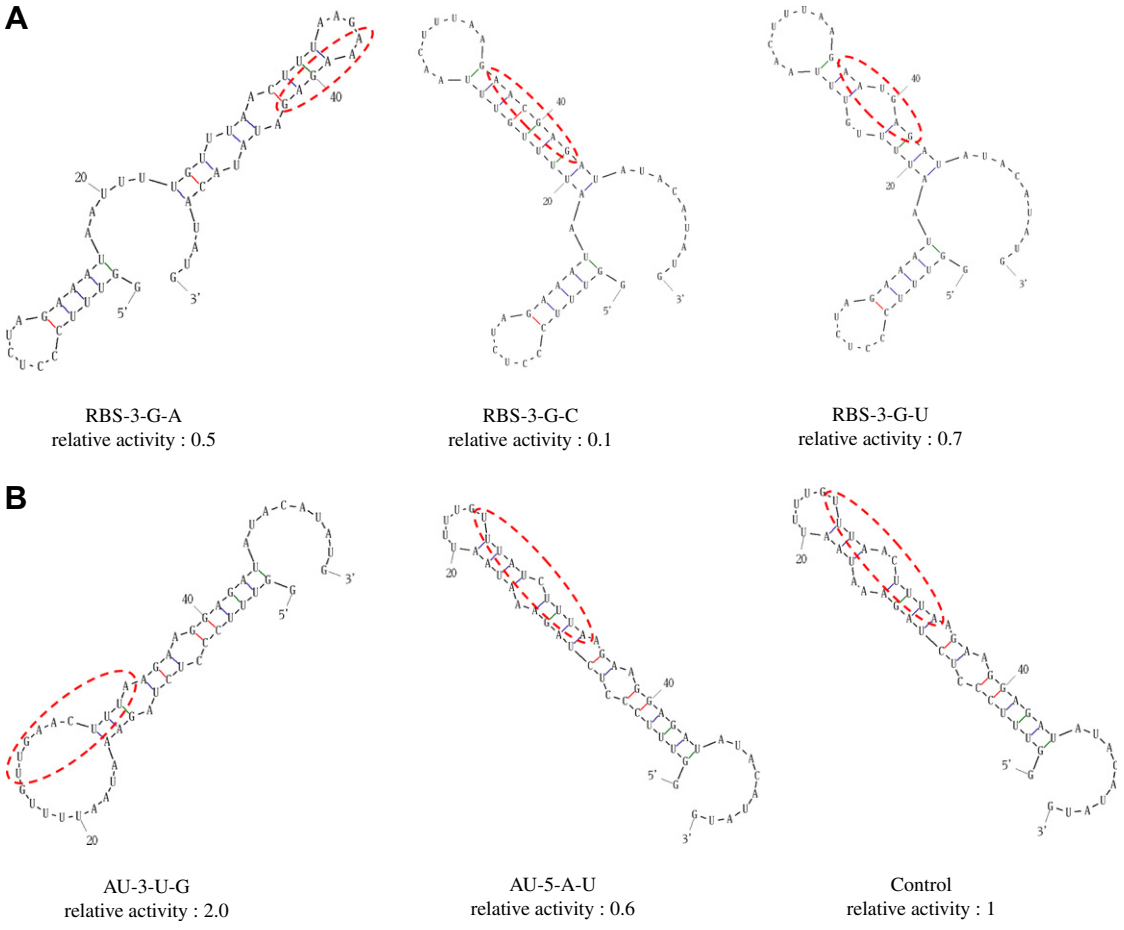

Fig. 4. mRNA secondary structures evaluated by the M-FOLD program [30]. (A) Mutations at the third position in RBS to A, C, and T. (B) Mutation at the third position in the AU-rich sequence to G, the fifth position to T, and the control 5′-UTR mRNA. Each relative eGFP expression level *in vitro* is indicated below the structure illustrations.

The results indicate that the expressional PCR after point mutations in the 5′-UTR region is a reliable method for the direct control of the target gene expression. Furthermore, 5′-UTR variants obtained in this study facilitate the tunable expression for redesigning the cellular physiology in *E. coli*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.127.

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