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RESEARCH PAPER

Introduction of an AU-rich Element into the 5' UTR of mRNAs Enhances Protein Expression in *Escherichia coli* by S1 Protein and Hfq Protein

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Abstract AU-rich elements in 5' untranslated region (UTR) are known to increase translation efficiency by recruiting S1 protein that facilitates the assembly of ribosomes. However, AU-rich elements also serve a binding site for Hfq protein, RNase E, *etc*. To investigate their roles in translation, mRNAs containing either an AU-rich element, originated from *sodB* 5'-UTR or a non-AU-rich element were constructed. The non-AU-rich elements were designed to retain the thermodynamics of the AU-rich element-containing mRNAs to reduce structural effect on translation. The AU-rich element increased mRNA translation and knock-down of S1 protein decreased the translation of AU-rich element-containing mRNAs, confirming the essential role of S1 protein in translation. When their mRNA levels were measured in *hfq*-deleted cells, those containing a non-AU-rich element and a high AU-content N-terminal coding sequence decreased, representing an auxiliary role of Hfq in translation, specifically in mRNA protection. Interestingly, despite of decreased mRNA level in *hfq*-deleted cells, protein production was increased, implying the involvement of unknown factors in translation. Consequently, these results suggest that actively translating ribosomes recruited by S1 protein at an AU-rich element stabilize mRNAs from degradation. In the absence of S1 protein, Hfq protein protects mRNAs from degradation. Moreover, AU-rich elements can be used for improved protein production.

Keywords: protein expression, AU-rich element, secondary structure, Hfq protein, S1 protein

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1. Introduction

Protein overexpression is one of challenges in bioindustry, since proteins have been widely used as therapeutics and biological research tools. For decades, many technological achievements have been made, including the use of fusion partners to solubilize and improve yields of the proteins of interest in bacteria. For better production and solubility, a number of fusion partners have been developed including glutathione S-transferase (GST), maltose-binding protein (MBP), thioredoxin A, and small ubiquitin related modifier (SUMO) [1,2]. On the contrary, certain partners, such as ketosteroid isomerase, TrpLE, PurF, PagP, and GFIL8 [3,4], have been used to form inclusion bodies for easy purification. In addition, protein tags including c-myc, FLAG, intein, and chitin-binding domain have been developed for better purification as well as easy detection [1].

On the other hand, computational models have been developed to design mRNAs to achieve desired protein expression levels in bacteria [5-10]. Heterologous genes are usually poorly expressed, since the hairpin structure around Shine-Dalgarno (SD) sequence in the untranslated region (UTR) prevents the access of ribosomes to the mRNA. To predict the translation yield and design mRNA sequences, several computational models such as RBSDesigner [11], RBSCalculator [5], UTRDesigner [8], *etc*. have been

developed based on the thermodynamics of mRNA structures.

Besides mRNA structures, UTR may contain functional elements that regulate protein expression. For example, 5' UTR of mRNA contains several sites for post-transcriptional regulation including RNase binding sites for rapid degradation of mRNAs, riboswitches for expression regulation, *etc.* [12,13]. In addition, the AU-rich element in the 5' UTR of *fepB* mRNA increases the accessibility of ribosomes [14].

There are RNA binding proteins (RBPs) as well. These regulatory RBPs bind to the ribosome binding site (RBS) to directly regulate the accessibility of ribosomes, or bind to the nearby sites to open/close the SD sequence or to open RNase binding site for rapid degradation [13,15-17]. Therefore, translation efficiency is regulated by the sequence features embedded in 5' UTR of mRNAs and corresponding RBPs that bind to the sequences.

In this study, we found that the introduction of an AU-rich element (AAATTAATAATAAA), originated from *sodB* gene, into the 5' UTR of mRNAs enhances protein expression. To investigate whether the enhancement was due to the binding of the conserved bacterial RBPs, such as Hfq and ribosomal protein S1 (S1 protein), to the AU-rich element [13,18], we replaced the AU-rich element with other nucleotides that have thermodynamically similar secondary structures but non-AU-rich in sequence. We then evaluated the expression levels of mRNAs containing AU-rich or non-AU-rich elements in *hfq*-deleted *Escherichia coli* and *rpsA*-knocked down *E. coli*. Consequently, the AU-rich element increases protein expression by providing a ribosome standby site by S1 protein. Moreover, actively translating ribosomes and Hfq proteins are able to protect mRNAs from degradation, probably masking putative RNase E binding sites in coding sequences.

2. Materials and Methods

2.1. Bacterial strain and growth conditions

E. coli DH5α was utilized for the DNA cloning for plasmid construction and the experiments for protein expressions of GFP, RFP, and ZsYellow mRNAs containing either an AU-rich element or non-AU-rich element. LB medium, containing 10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per liter, was used for the cultivation of *E. coli* DH5α, with appropriate antibiotics (25 μg/mL of chloramphenicol and/or 100 μg/mL of ampicillin).

2.2. Molecular cloning

For the construction of plasmids expressing reporter proteins (GFP, RFP, or ZsYellow), the respective genes were cloned into a vector with a p15A replication origin and a chloramphenicol resistance gene, and were transcribed under the control of a *lac* promoter. The AU-rich element (AAATTAATAATAAA) originated from the *sodB* gene or three different non-AU-rich elements designed by using RBSDesigner [11] were introduced into the 5' UTR of *gfp*, *rfp*, and *zsYellow* genes. For comparison, another shorter AU-rich element (AAATAA) derived from the *malT* gene was cloned in 5'-UTR of *rfp* gene.

For the construction of a plasmid producing the synthetic sRNA based on MicC scaffold to repress S1 protein expression, the target binding sequence (GAGTTGACG CAGACATTCAGT) of anti-S1 protein sRNA was inserted between the scaffold sequence and its promoter sequence in the previously developed plasmid [19]. The target-binding sequence is a reverse complementary sequence to the coding region of S1 protein.

2.3. Sample preparation for fluorescence measurement

For the measurement of gene expression level, wild-type cells, *hfq*-deleted cells, or *rpsA*-knocked down cells harboring the constructed plasmids were grown overnight in a test tube at 37°C with shaking at 200 rpm. The following day, 1/100 (v/v) of the cells were transferred into fresh LB media with appropriated antibiotics. When the cells reached a log phase, the cells were then harvested by centrifugation at 13,000 rpm for 3 min and stored at -80°C until use.

2.4. Flow cytometry

Harvested cells were resuspended with 1 mL of 1 × phosphate buffered saline (PBS) buffer and the resuspended samples were diluted to 1/100 with PBS buffer again for fluorescence intensity measurement by using a Guava EasyCyte flow cytometer (Millipore, Darmstadt, Germany). GFP fluorescence intensities were measured with an excitation at 488 nm and an emission collected with a 525/30 nm bandpass filter. RFP fluorescence intensities were measured with an excitation at an excitation at 488 nm and emission collected with a 583/26 nm bandpass filter. ZsYellow fluorescence intensities were measured with an excitation at an excitation at 532 nm and emission collected with a 575/25 nm bandpass filter.

2.5. Gene expression determination by RT-qPCR

Expression levels of *gfp* and *rfp* genes were determined by reverse transcription-qPCR (RT-qPCR). Briefly, prepared cells were subjected to RNA isolation using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as indicated by the manufacturer. The RNA samples were retrotranscribed into cDNA using cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed using SYBR Premix Ex TaqTM (Takara) on an applied Biosystems 7300 Real-Time PCR system. The expression

levels of *gfp* and *rfp* genes were normalized to *gadA* gene. Relative gene expressions were determined with the comparative C_T method [20].

2.6. Data analysis

Data obtained from at least three independent experiments were analyzed using GraphPad Prism v7.0 (GraphPad Software, Inc.). Replicates were plotted using average and standard error of the mean (SEM).

3. Results and Discussion

3.1. AU-rich element from the *sodB* gene

In this study, we attempted to investigate the effects of AU-rich elements and RBPs on translation. Generally, AU-rich elements are known as a binding site for S1 protein that can enhance translation efficiency by facilitating the assembly of ribosomes [21,22]. They are also known as a binding site for Hfq protein and Hfq protein not only alters secondary structure of mRNAs to regulate translation [23] but also protects mRNAs from degradation [24]. Interestingly, AU-rich elements are also known as a binding site for RNase E [25]. Consequently, AU-rich elements are the place of interplay among the RBPs.

In this study, the AU-rich element (AAATTAATAATAAA) originated from the *sodB* gene in *E. coli* was used. We chose this element because it has been already discovered to recruit Hfq protein. Though it has not been discovered yet whether it can also recruit S1 protein or RNase E, the element is long enough to recruit other RBPs compared with other known AU-rich elements [26,27].

The *sodB* gene encodes a superoxide dismutase in response to iron limitation [28]. The *sodB* mRNA is post-transcriptionally regulated by RyhB, a small regulatory RNA, that represses the translation of the *sodB* mRNA [23,29]. The *sodB* mRNA contains an unstructured AU-rich element in the upstream of RyhB binding site. The AU-rich element is unstructured and the RyhB bindig site is within a hairpin structure. Once Hfq protein binds to the AU-rich element, Hfq changes the secondary structure of the RyhB binding site and thereby exposes the RyhB binding site. Consequently, Hfq protein regulates the expression level of *sodB* mRNA by altering the secondary structure of the 5' UTR of *sodB* mRNA [23].

3.2. AU-rich element in 5' UTR enhances protein expression

To investigate whether the AU-rich element in 5' UTR can enhance the expression of other genes by altering the secondary structure of mRNA like the *sodB* gene, we introduced the AU-rich sequence (AAATTAATAATAAA) originated from the *sodB* gene into the 5' UTRs of the GFP and RFP genes. For comparison, we also designed three non-AU-rich elements with similar structural thermodynamics by using RBSDesigner that predicts translation initiation efficiency based on the thermodynamics of the secondary structure of mRNA [11]. Thus, four 5' UTR sequences (one AU-rich and three non-AU-rich elements) were designed to have similar translation efficiencies to avoid structural effects on translation (Fig. 1A). The estimated translation efficiencies are listed in Table 1.

Table 1. Computationally predicted scores of the mRNAs*

GFP	Relative translation efficiency
AU-rich element	0.851
Non-AU-rich element #1	1
Non-AU-rich element #2	1
Non-AU-rich element #3	0.930
RFP	Relative translation efficiency
AU-rich element	0.971
Non-AU-rich element #1	0.980
Non-AU-rich element #2	0.838
Non-AU-rich element #3	1
ZsYellow	Relative translation efficiency
AU-rich element	1
Non-AU-rich element #1	0.973
Non-AU-rich element #2	0.972
Non-AU-rich element #3	0.973

*Prediction scores were normalized by the highest score.

When the AU-rich element was replaced with a non-AU-rich element, the expression levels of GFP and RFP mRNAs were dramatically reduced (Fig. 1B and C). For GFP mRNA, the expression level was reduced by 96% when a non-AU-rich element was used. For RFP mRNA, the expression level was reduced by > 83%. Since the non-AU-rich elements were computationally designed to have a translation efficiency similar with that of the AU-rich element, the non-AU-rich elements did not alter the structural thermodynamics of translation initiation region (TIR). These results indicate that the AU-rich element and functionally related RBPs played a role in translation, which was not found in other mRNAs lacking the AU-rich element. In addition, it also demonstrated that introduction of the AU-rich element from the *sodB* gene can enhance protein production by 5.5-fold to 28-fold depending on coding sequences.

3.3. S1 protein effect on translation enhancement

A well-known AU-rich element binding protein is S1 protein [21,22]. S1 protein, a ribosomal protein, contributes to the interaction of the 30S ribosomal subunit and mRNA,

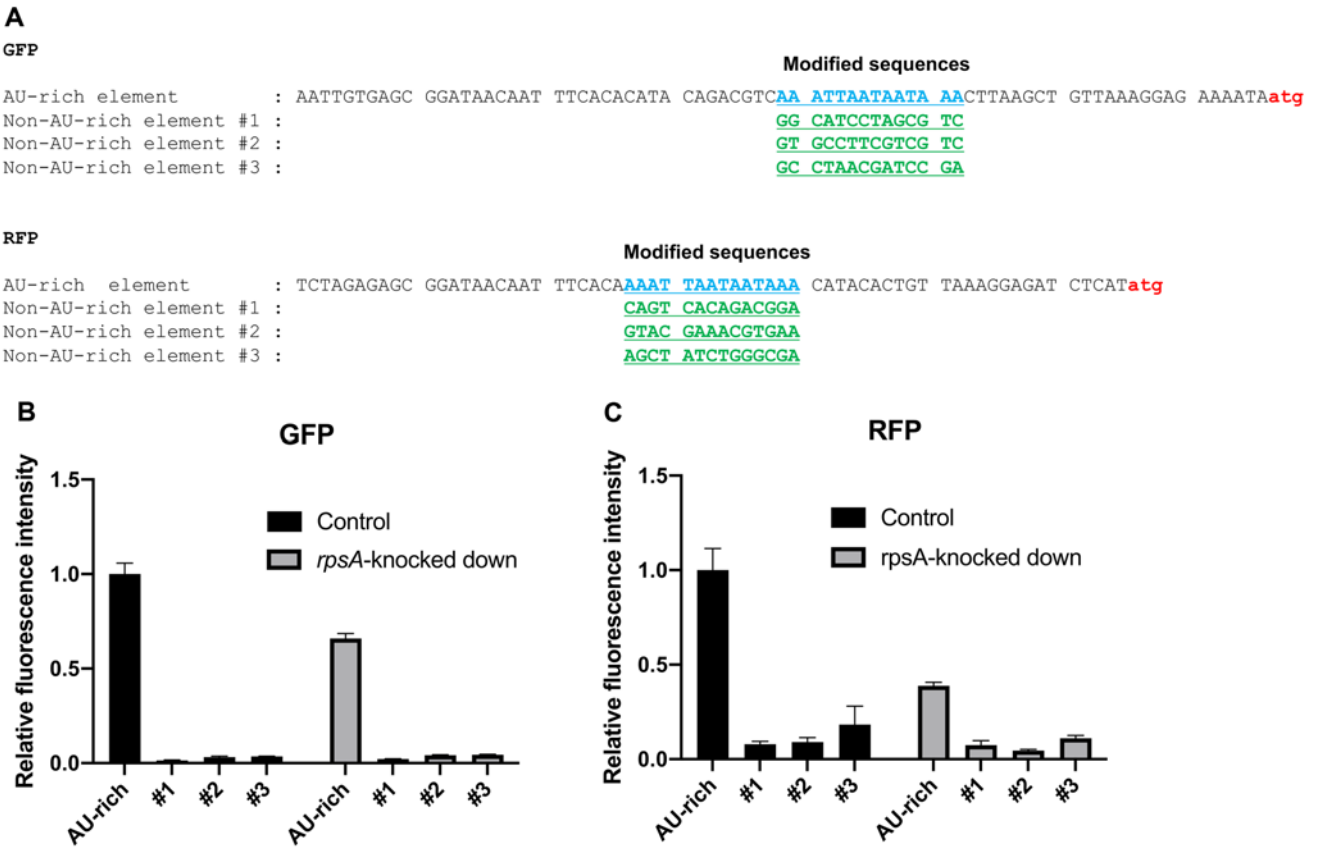

Fig. 1. Designed 5' UTR sequences of GFP and RFP mRNAs and their expression levels in wild type cells and *rpsA*-knocked down cells. (A) 5'-UTR sequences from the transcription start site to a start codon. Start codons are shown in red color, AU-rich elements are shown in blue, and the designed non-AU-rich sequences are shown in green. (B and C) The fluorescence intensities (protein levels) of the mRNAs. *AU-rich* denotes the *sodB* AU-rich element-containing mRNA, *#1*, *#2*, and *#3* denotes the mRNAs containing a designed non-AU-rich element. Respective designed nucleotides are shown in (A). Eight replicates for control experiments and three replicates for *rspA*-knockdown experiments. Mean ± SEM.

thus playing an important role in translation initiation [13,30]. The AU-rich element upstream of SD may provide a standby site for ribosomes, which enhances translation efficiency [22]. To investigate if S1 protein is implicated in the increased protein expression by the AU-rich element, the translation yields of the GFP and RFP mRNAs were measured in the *E. coli* in which *rpsA* (encoding S1 protein) was knocked down. Since the *rpsA* is an essential gene, it cannot be deleted from the *E. coli* genome. Thus, we used a synthetic sRNA based on MicC scaffold to repress the protein level of *rpsA*, S1 protein.

Knockdown of S1 protein expression markedly decreased expression levels of mRNAs containing the AU-rich element (Fig. 1B and 1C). The expression levels of GFP mRNA and RFP mRNA were reduced by 34% and 61%, respectively, in *rpsA*-knocked down *E. coli*. However, for the GFP mRNAs containing non-AU-rich elements, the mRNA expression levels decreased slightly, whereas for the RFP mRNAs containing a non-AU-rich element, the mRNAs expression levels rather slightly increased in *rpsA*-knocked down *E. coli*. However, when compared with the decreases in AU-rich element-containing mRNAs, their changes were not significant. These results represent that the *sodB* AU-rich element introduced into the 5' UTR of mRNA increases translation initiation by providing a binding site for S1 protein for ribosomes assembly.

3.4. Effect of Hfq protein on translation

The AU-rich element obtained from the *sodB* gene is able to recruit Hfq protein [23]. Interestingly, AU-rich element is also a binding site of RNase [31]. Thus, Hfq protein can protect mRNAs from degradation [31] as well as unwind the secondary structure of TIR [23] for enhanced protein production. To investigate whether Hfq protein affects the translation of the mRNAs containing the AU-rich element, the GFP and RFP mRNAs were expressed in *hfq*-deleted *E. coli*. As shown in Fig. 2, the mRNAs containing the AU-rich element showed a similar expression level regardless of Hfq protein. In addition, the expression trend is identical: AU-rich element showed a high expression level

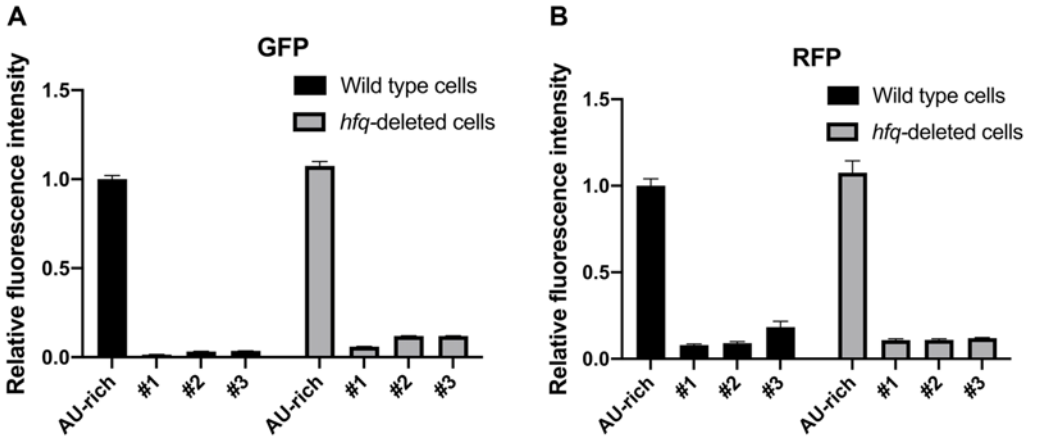

Fig. 2. Effect of AU-rich element on expression in wild type cells and *hfq*-deleted cells. The expression levels of GFP mRNAs (A) and RFP mRNAs (B) are shown. *AU-rich* denotes the *sodB* AU-rich element-containing mRNA, *#1*, *#2*, and *#3* denotes the mRNAs containing a designed non-AU-rich element. Eight replicates for control experiments and three replicates for *hfq*-deletion experiments. Mean ± SEM.

while non-AU-rich elements showed a very low expression level in both wild type cells and *hfq*-deleted cells. If Hfq protein plays an essential role in the mRNAs containing the AU-rich element, their expression level should decrease significantly like the case of *rpsA*-knockdown (Fig. 1B and 1C). However, there were no significant changes in gene expression in *hfq*-deleted cells. These results indicate that Hfq protein does not play an important role in the increase of protein expression associated with the AU-rich element.

To confirm the non-association of Hfq protein, mRNAs of ZsYellow were also designed to contain either the AU-rich element or a non-AU-rich element. The non-AU-elements were computationally designed like those for GFP and RFP mRNAs to retain similar thermodynamics with that of the AU-rich element-containing ZsYellow mRNA. The designed nucleotides are shown in Fig. 3A and their computationally predicted scores are shown in Table 1. As shown in Fig. 3B, a similar expression trend was also observed: the absence of Hfq protein did not affect protein expression significantly. This result confirms that Hfq protein does not play an essential role in translation of AU-rich element-containing mRNAs.

3.5. Measurement of mRNA levels

It has been reported that Hfq protein is able to protect RNAs from degradation by masking RNase E binding sites since AU-rich elements are also the binding sites of RNase E [31]. To investigate the association of Hfq protein and mRNA degradation, the level of mRNAs was measured by qPCR. As shown in Fig. 4, for GFP mRNAs, there were no statistically significant differences among the mRNA levels regardless of Hfq protein. For RFP mRNAs, the mRNAs in wild type cells also showed no significant differences in their levels except #3 mRNA. The #3 mRNA showed 40% lower mRNA level compared with that of mRNA containing the AU-rich element. However, this does not account for

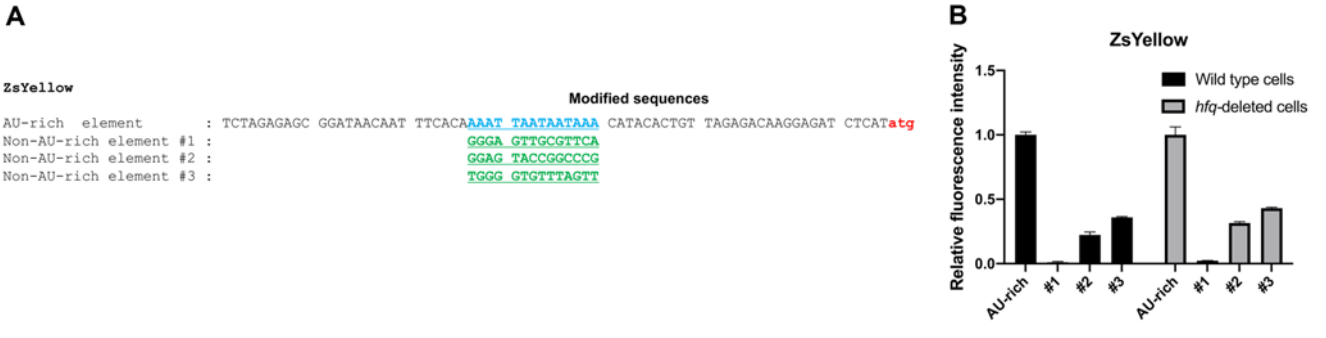

Fig. 3. Designed 5' UTR sequences of ZsYellow mRNA and their expression levels in wild type cells and *hfq*-deleted cells. (A) 5'-UTR sequences from the transcription start site to a start codon. Start codon is shown in red color, AU-rich element is shown in blue, and the designed non-AU-rich sequences are shown in green. (B) Fluorescence intensities (protein levels) of the constructed mRNAs. *AU-rich* denotes the *sodB* AU-rich element-containing mRNA, *#1*, *#2*, and *#3* denotes the mRNAs containing a designed non-AU-rich element. Respective designed nucleotides are shown in (A). Three replicates and mean ± SEM.

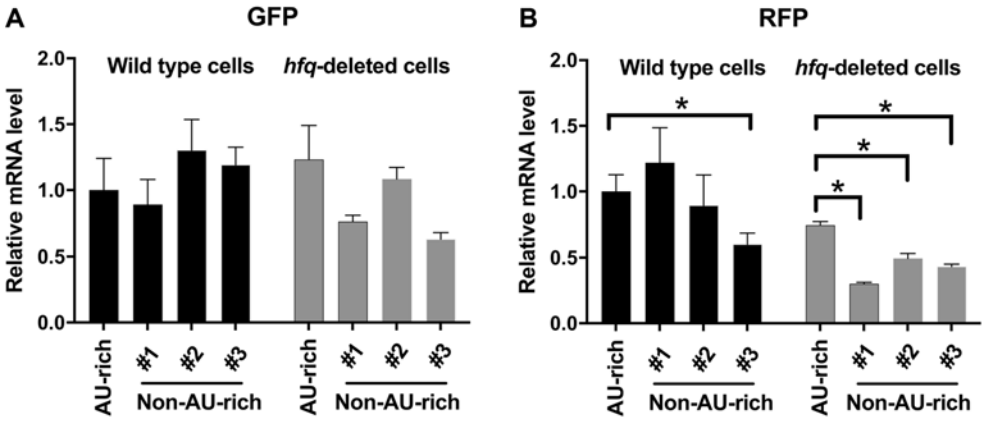

Fig. 4. mRNA levels measured by RT-qPCR. The levels of the constructed GFP (A) and RFP (B) mRNAs were measured by RT-qPCR in wild type cells or *hfq*-deleted cells. The cutoff of *p*-value obtained by *t*-test was 0.05. Three replicates and mean ± SEM.

the > 74% reduction in the protein expression of the mRNAs that did not contain the AU-rich element. In *hfq*-deleted cells, the mRNA levels were decreased significantly, which implies an auxiliary role of Hfq protein in the protection of mRNAs from degradation, probably from RNase E. The difference between GFP and RFP is a coding sequence: the AU contents of GFP and RFP N-terminal sequences (50 nt) are 35% and 58%, respectively. The relatively higher AU content of RFP than GFP may imply the existence of a putative RNase E binding site. We found a putative AU-rich sequence within N-terminal coding sequence of RFP, GUUAUCAAA, while no AU-rich sequences were found within N-terminal coding sequence of GFP [32]. This represents an interplay among the proteins of S1 protein, Hfq protein, and RNase E in translation.

Interestingly, even though the levels of RFP mRNAs were decreased (Fig. 4B), they showed similar expression levels in *hfq*-deleted cells to those obtained in wild type cells (Fig. 2B). This implies the implication of an unknown factor in translation, which should be elucidated further.

3.6. A proposed hypothesis on the interplay among S1 protein, Hfq protein, and RNase E

Taken together, we propose a trafficking hypothesis by which putative RNase E binding sites are protected by actively translating ribosomes recruited by S1 protein and

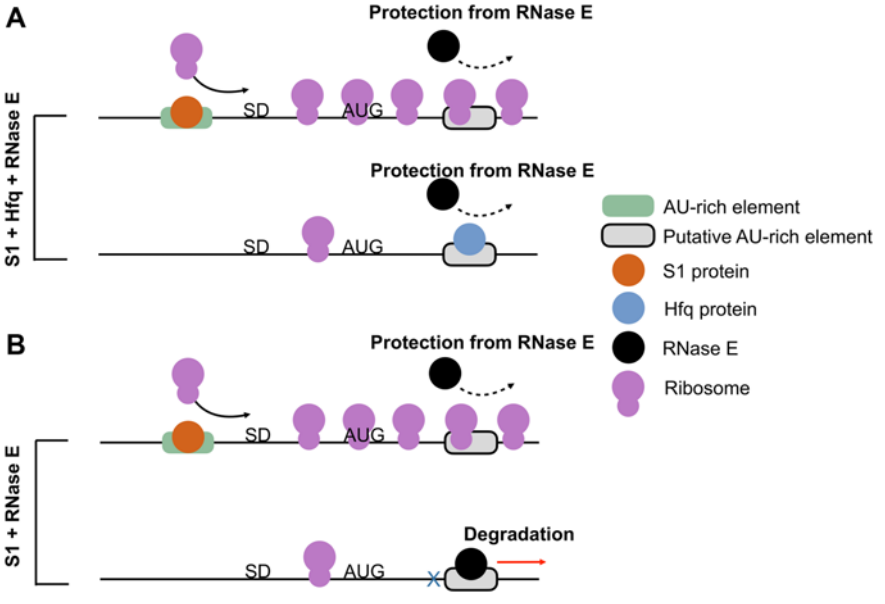

Fig. 5. Proposed hypothesis on the interplay of S1 protein, Hfq protein, and RNase E. (A) Competition of translating ribosomes and Hfq protein with RNase E in wild type cells. The translating ribosomes and Hfq protein protect the putative AU-rich element from RNase E. (B) Competition of translating ribosomes with RNase E in *hfq*-deleted cells. In the absence of Hfq protein and AU-rich element serving an S1 protein binding site, the putative RNase E binding site is likely to be exposed for degradation.

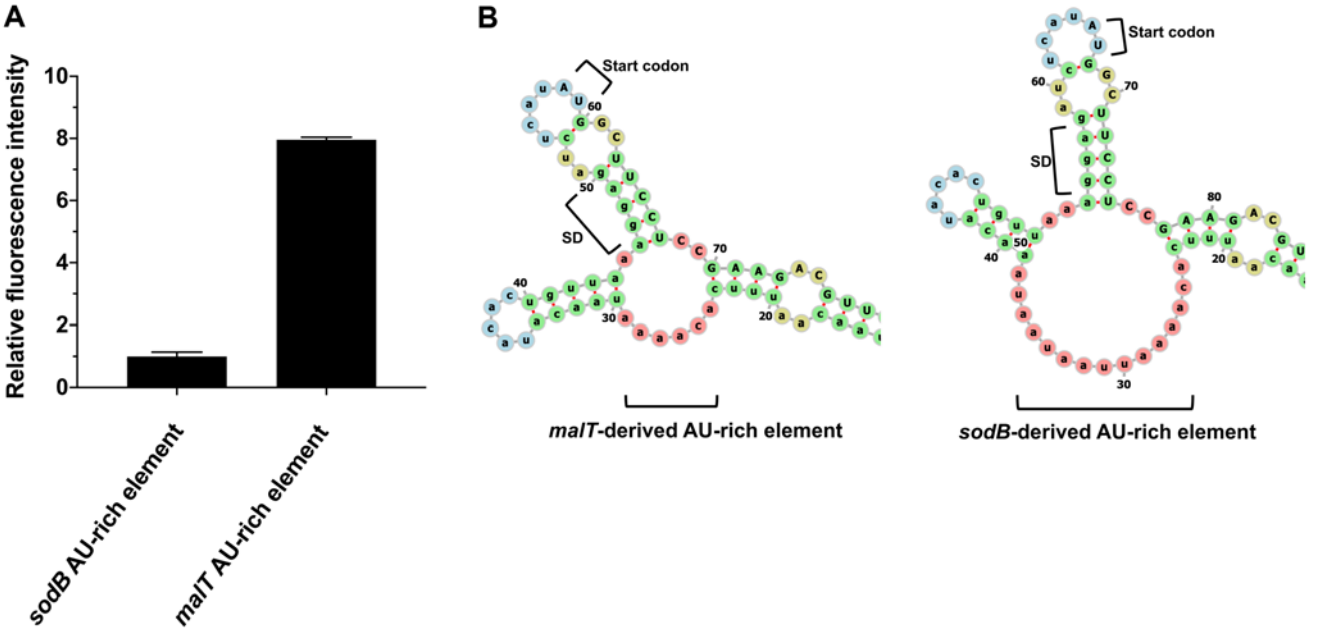

Fig. 6. The effect of *malT*-derived AU-rich element on protein production. (A) Protein expression levels of mRNAs harboring an AU-rich element derived from *sodB* or *malT*. Three replicates and mean ± SEM. (B) RNA secondary structures of the mRNAs. The structures were drawn by Forna [34].

Hfq proteins. As shown in Fig. 5A, given a putative AU-rich element serving an RNase E binding site, the putative RNase E site is protected by actively translating ribosomes that are facilitated by S1 protein and AU-rich element in 5' UTR. It has been discovered that actively translating ribosomes are able to stabilize mRNAs [22]. In the absence of AU-rich elements, though the density of translating ribosomes decreased, Hfq protein competes with RNase E to protect the mRNA. This explains the decreased protein production but invariable levels of GFP and RFP mRNAs containing no AU-rich elements in wild type cells.

In *hfq*-deleted cells, the existence of AU-rich element still induces in dense translating ribosomes, which in turn stabilize mRNA (Fig. 5B). However, in the absence of AU-rich element, the number of translating ribosomes decrease, and the putative RNase E binding site is likely to be exposed to RNase E because it is not protected any more by neither Hfq protein nor ribosomes.

In this hypothesis, S1 protein and an AU-rich element in 5' UTR play a critical role in translation enhancement and mRNA protection. On the other hand, Hfq protein has an auxiliary protective role, which is effective only when there are no AU-rich elements in 5' UTR for S1 protein recruitment. This hypothesis explains the decrease of mRNA levels in *hfq*-deleted cells (Fig. 4B). A greater decrease in mRNA level was observed in RFP mRNA than GFP mRNA. The AU content of N-terminal coding sequence of RFP is higher than that of GFP, which may provide more sites for RNase E and therefore RFP mRNA is more susceptible to RNase E-mediated degradation.

3.7. Enhancement of gene expression by the *malT*-derived AU-rich element

Employment of an AU-rich element in 5' UTR is a very effective strategy to increase protein production. To investigate if other AU-rich elements are also able to enhance protein production, an mRNA was constructed to harbor a *malT*-derived AU-rich element (AAATAA) instead of the *sodB*-derived AU-rich element. The *malT*-derived AU-rich element has been discovered to upregulate gene expression [26]. Interestingly, the new mRNA containing the *malT*-derived AU-rich element resulted in a higher protein production level than that containing the *sodB*-derived AU-rich element (Fig. 6A). When their mRNA secondary structures were predicted by UNAFold [33], there were no significant differences except the loop made by the AU-rich elements (Fig. 6B). The relatively long AU-rich element derived from the *sodB* gene could increase the accessibility of RNase E, which may reduce the stability of the mRNA. However, this should be elucidated further. Consequently, AU-rich elements enhance protein production, and shorter elements are more advantageous than longer ones.

4. Conclusion

In this study, we found that the AU-rich element originated

from the 5' UTR of the *sodB* and *malT* mRNA increased the expression of a downstream coding sequence. The increase in protein expression results from the protection of mRNAs by Hfq protein and actively translating ribosomes recruited by S1 protein. While AU-rich element and S1 protein play an essential role in the protection, Hfq protein plays an auxiliary role. The AU-rich element could be utilized to increase the production of recombinant proteins by stabilizing mRNAs, and the proposed hypothesis could give an insight into the strategy to enhance protein expression.

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Ethical Statements

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

References

1. Young, C. L., Z. T. Britton, and A. S. Robinson (2012) Recombinant protein expression and purification: A comprehensive review of affinity tags and microbial applications. *Biotech. J.* 7: 620-634.
2. Nallamsetty, S. and D. S. Waugh (2007) A generic protocol for the expression and purification of recombinant proteins in *Escherichia coli* using a combinatorial His6-maltose binding protein fusion tag. *Nat. Protoc*. 2: 383-391.
3. Wang, X., B. Zhou, W. Hu, Q. Zhao, and Z. Lin (2015) Formation of active inclusion bodies induced by hydrophobic self-assembling peptide GFIL8. *Microb. Cell Fact*. 14: 88.
4. Hwang, P. M., J. S. Pan, and B. D. Sykes (2014) Targeted expression, purification, and cleavage of fusion proteins from inclusion bodies in *Escherichia coli*. *FEBS Lett*. 588: 247-252.
5. Salis, H. M., E. A. Mirsky, and C. A. Voigt (2009) Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol*. 27: 946-950.
6. Na, D., S. Lee, and D. Lee (2010) Mathematical modeling of translation initiation for the estimation of its efficiency to computationally design mRNA sequences with desired expression levels in prokaryotes. *BMC Syst. Biol*. 4: 71.
7. Bonde, M. T., M. Pedersen, M. S. Klausen, S. I. Jensen, T. Wulff, S. Harrison, A. T. Nielsen, M. J. Herrgard, and M. O. A. Sommer (2016) Predictable tuning of protein expression in bacteria. *Nat. Methods*. 13: 233-236.
8. Seo, S. W., J. S. Yang, I. Kim, J. Yang, B. E. Min, S. Kim, and G. Y. Jung (2013) Predictive design of mRNA translation initiation region to control prokaryotic translation efficiency. *Metab. Eng*. 15: 67-74.
9. Barrick, D., K. Villanueba, J. Childs, R. Kalil, T. D. Schneider, C. E. Lawrence, L. Gold, and G. D. Stormo (1994) Quantitative analysis of ribosome binding sites in *E. coli*. *Nucleic Acids Res*. 22: 1287-1295.
10. Allert, M., J. C. Cox, and H. W. Hellinga (2010) Multifactorial determinants of protein expression in prokaryotic open reading frames. *J. Mol. Biol*. 402: 905-918.
11. Na, D. and D. Lee (2010) RBSDesigner: software for designing synthetic ribosome binding sites that yields a desired level of protein expression. *Bioinformatics*. 26: 2633-2634.
12. Lotz, T. S. and B. Suess (2018) Small-molecule-binding riboswitches. *Microbiol. Spectr*. 6: 6.4.26.
13. Kaberdin, V. R. and U. Bläsi (2006) Translation initiation and the fate of bacterial mRNAs. *FEMS Microbiol. Rev*. 30: 967-979.
14. Hook-Barnard, I. G., T. J. Brickman, and M. A. McIntosh (2007) Identification of an AU-rich translational enhancer within the *Escherichia coli fepB* leader RNA. *J. Bacteriol*. 189: 4028-4037.
15. Van Assche, E., S. Van Puyvelde, J. Vanderleyden, and H. P. Steenackers (2015) RNA-binding proteins involved in post-transcriptional regulation in bacteria. *Front. Microbiol.* 6: 141.
16. Nakagawa, S., Y. Niimura, K. Miura, and T. Gojobori (2010) Dynamic evolution of translation initiation mechanisms in prokaryotes. *Proc. Natl. Acad. Sci. USA*. 107: 6382-6387.
17. Valentin-Hansen, P., M. Eriksen, and C. Udesen (2004) The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol. Microbiol*. 51: 1525-1533.
18. Hoekzema, M., C. Romilly, E. Holmqvist, and E. G. H. Wagner (2019) Hfq-dependent mRNA unfolding promotes sRNA-based inhibition of translation. *EMBO J*. 38: e101199.
19. Na, D., S. M. Yoo, H. Chung, H. Park, J. H. Park, and S. Y. Lee (2013) Metabolic engineering of *Escherichia coli* using synthetic small regulatory RNAs. *Nat. Biotechnol.* 31: 170-174.
20. Schmittgen, T. D. and K. J. Livak (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc*. 3: 1101-1108.
21. Komarova, A. V., L. S. Tchufistova, E. V. Supina, and I. V. Boni (2002) Protein S1 counteracts the inhibitory effect of the extended Shine-Dalgarno sequence on translation. *RNA*. 8: 1137-1147.
22. Komarova, A. V., L. S. Tchufistova, M. Dreyfus, and I. V. Boni (2005) AU-rich sequences within 5' untranslated leaders enhance translation and stabilize mRNA in *Escherichia coli*. *J. Bacteriol*. 187: 1344-1349.
23. Geissmann, T. A. and D. Touati (2004) Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. *EMBO J*. 23: 396-405.
24. Folichon, M., V. Arluison, O. Pellegrini, E. Huntzinger, P. Régnier, and E. Hajnsdorf (2003) The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic Acids Res*. 31: 7302-7310.
25. Baek, Y. M., K. J. Jang, H. Lee, S. Yoon, A. Baek, K. Lee, and D. E. Kim (2019) The bacterial endoribonuclease RNase E can cleave RNA in the absence of the RNA chaperone Hfq. *J. Biol. Chem*. 294: 16465-16478.
26. Park, H. S., Y. Ostberg, J. Johansson, E. G. H. Wagner, and B. E. Uhlin (2010) Novel role for a bacterial nucleoid protein in translation of mRNAs with suboptimal ribosome-binding sites. *Genes Dev.* 24: 1345-1350.
27. Mitta, M., L. Fang, and M. Inouye (1997) Deletion analysis of *cspA* of *Escherichia coli*: requirement of the AT-rich UP element for *cspA* transcription and the downstream box in the coding region for its cold shock induction. *Mol. Microbiol*. 26: 321-335.
28. Massé, E. and S. Gottesman (2002) A small RNA regulates the

expression of genes involved in iron metabolism in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. 99: 4620-4625.
29. Večerek, B., I. Moll, T. Afonyushkin, V. Kaberdin, and U. Bläsi (2003) Interaction of the RNA chaperone Hfq with mRNAs: direct and indirect roles of Hfq in iron metabolism of *Escherichia coli*. *Mol. Microbiol*. 50: 897-909.
30. Gold, L., D. Pribnow, T. Schneider, S. Shinedling, B. S. Singer, and G. Stormo (1981) Translational initiation in prokaryotes. *Annu. Rev. Microbiol*. 35: 365-403.
31. Moll, I., T. Afonyushkin, O. Vytvytska, V. R. Kaberdin, and U. Bläsi (2003) Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. *RNA*. 9: 1308-1314.
32. McDowall, K. J., S. Lin-Chao, and S. N. Cohen (1994) A+U content rather than a particular nucleotide order determines the specificity of RNase E cleavage. *J. Biol. Chem*. 269: 10790-10796.
33. Markham, N. R. and M. Zuker (2008) UNAFold: software for nucleic acid folding and hybridization. *Methods Mol. Biol*. 453: 3-31.
34. Kerpedjiev, P., S. Hammer, and I. L. Hofacker (2015) Forna (force-directed RNA): Simple and effective online RNA secondary structure diagrams. *Bioinformatics*. 31: 3377-3379.