論文内容の要旨

論文題目：Studies on rescue mechanism of stalled ribosome in *E. coli*（大腸菌における異常停止したリボソームのレスキューメカニズムの解析）

氏　名：　斉　浩

Introduction

Translation is an important process in cells that takes place on a ribosome, a huge complex comprised of RNAs and proteins. Troubles in the translation process would cause serious problems: Not only cells cannot synthesize proteins but also premature translation products are harmful and disturb other normal processes in cells. Therefore, regulatory systems are necessary to monitor and maintain the translation process in cells.

Generally, the untranslated region encoded in mRNA regulates the translation process. Hence, the lack of such regulatory information in mRNA makes troubles for the translation system. In a eukaryotic system, a regulatory process called nonsense-mediated mRNA decay degrades mRNA that has a stop codon at an incorrect position inside the open reading frame. Another process called no-go decay also degrades mRNA that is stalled on a ribosome by the strong secondary conformation. By these systems, stalled ribosomes are released from process error and returned to be in a normal process.

Defective mRNA also causes problems in a prokaryotic system. Transcriptional error or non-specific mRNA cleavage produces mRNA without a stop codon. If such mRNA is translated, the ribosome is arrested at a 3'-terminous of mRNA. In *Escherichia coli*, a trans-translation system is known as a regulatory process for rescuing such error. SsrA RNA is a central factor in the trans-translation system. The outline of the reaction mechanism of the trans-translation has been elucidated by several reports, but some questions about its detailed mechanisms still remain. In *E. coli*, SsrA RNA mediated trans-translation cannot deal with all kinds of the translation arrest. Previous researches have shown that the stalled ribosome complex arrested in the middle of mRNA cannot

proceed to the trans-translation and the mRNA cleavage is necessary prior to the trans-translation. In addition, it had been shown that the SsrA RNA is not essential to the cell viability. Therefore, it is likely that there are some other processes that can assist with trans-translation or are alternative to the trans-translation system.

Here, I investigated the rescue mechanism of stalled ribosome in *E. coli* by using the reconstituted cell-free protein synthesis system, the PURE system. The system is constructed with specific factors and enzymes necessary for the translation. Since the components are all identified in the PURE system, it is suitable for the analysis of the molecular mechanism of translation machinery. By using this system, I analyzed the detailed mechanism of trans-translation system and explored a novel pathway for rescuing the stalled ribosome.

1. Ribosome protein S1 function in the trans-translation system

In *Escherichia coli*, the trans-translation system is known as a unique process to release the translation stalling. SsrA RNA and SmpB protein are the two necessary components of the trans-translation system. SsrA RNA, also called as tmRNA, has a unique feature that it can act as both tRNA and mRNA. The complex comprised of aminoacylated tmRNA and SmpB enters into the A site of the stalled ribosome and then the mRNA on the ribosome is switched to the open reading frame (ORF) of tmRNA, directing ribosome to translate a specific peptide sequence encoded on the ORF. In addition to SmpB, some other factors are thought to play some roles in the trans-translation system. Ribosomal protein S1 is one of these factors. Ribosomal protein S1 is known as an RNA binding protein. Some studies had reported that S1 has significantly high affinity for the SsrA RNA. However, the detailed function S1 is unclear. I investigated the function of S1 by constructing the assay system utilizing the PURE system.

1. 1. Trans-translation in the PURE system

The trans-translation system was reconstructed in the PURE system using purified SsrA RNA and SmpB. SsrA RNA and SmpB were two essential factors in the trans-translation system. A stalled ribosome complex was formed using a template without a stop codon. By the addition of SsrA RNA and SmpB to this stalled ribosome complex, the tag-peptide that is encoded on SsrA RNA was found to be attached to the nascent polypeptide.

1. 2. Ribosomal protein S1 is not essential for the trans-translation machinery

An S1-free ribosome was prepared by using a poly (U) column and the S1-free trans-translation detection system was constructed by utilizing the PURE system. By using this system, the effect of the presence or the absence of S1 on the trans-translation was investigated. The results showed that the trans-translation reaction still proceeded even in the absence S1. Furthermore, the addition of the purified S1 did not affect the initial

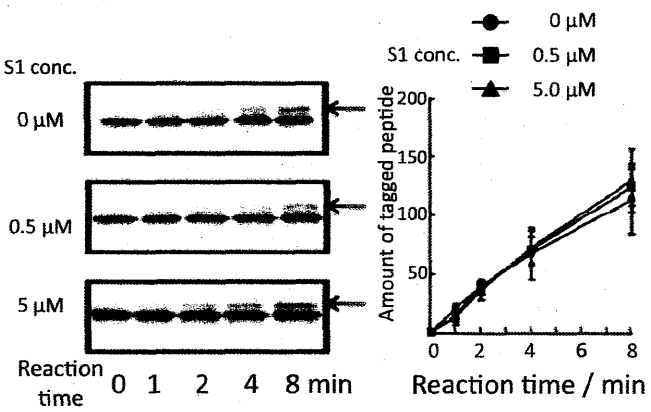

Figure 1: Time course of trans-translation with additon of S1

rate of the trans-translation reaction. Therefore, I concluded that the ribosomal protein S1 is not essential for the trans-translation machinery.

2. Exploring a novel pathway for rescuing the stalled ribosome complex

In *E. coli*, it is known that some specific sequences encoded on mRNA arrest the translation process. In such a case, unlikely to the case of mRNA without a stop codon, the ribosome is stalled at the middle of mRNA and the 3'-terminus of mRNA is left on the ribosome. Several reports showed that such a stalled ribosome complex is resistant to the SsrA RNA mediated trans-translation and mRNA cleavage at the A site of the ribosome is necessary prior to the SsrA RNA entry into the ribosome. Furthermore, SsrA RNA is not an essential gene in *E. coli*. Cells without a gene for the SsrA RNA showed normal growth under the normal condition. Thus, these studies suggest that there are some other processes that can assist with trans-translation or are alternative to the trans-translation system. According to this aspect, I explored another pathway that rescues and releases the stalled ribosome complex. Particularly, several recent reports suggested the involvement of peptidyl-tRNA hydrolase (Pth) in rescuing such complex, although clear evidence is not proposed. Therefore, I investigated the effect of Pth on the stalled ribosome complex and found a novel activity of Pth on the stalled ribosome complex.

2. 1. In vitro analysis of the stalled ribosome complex

The nucleotide sequence that induces translation arrest was inserted into the 3'-terminous of GFP (green fluorescent protein) template for the PURE system. By using this template, the stalled ribosome complex comprised of ribosome, mRNA, and peptidyl-tRNA that carries GFP, was formed. The stalled ribosome complex formation was analyzed by sucrose density gradient centrifugation. The results showed that GFP, mRNA, and the ribosome were detected in the same fraction suggesting that the stalled ribosome complex was successfully formed. Same analysis was also examined by the gel filtration assay. The results showed that almost all the synthesized GFP was detected in the same fraction with the ribosome, suggesting that the complex formed in the PURE system is extremely stable.

2. 2. Identification of the novel activity of peptidyl-tRNA hydrolase on the stalled ribosome rescue process

An experiment was constructed to analyze Pth activity on the stalled ribosome complex using the complex formed in the PURE system. After the complex was treated with Pth, the complex was subjected to a gel filtration chromatography to analyze the peptide release from the stalled ribosome. The results showed that the treatment by Pth released specific amount of GFP from the ribosome complex, suggesting Pth promoted the release of translation product from the stalled ribosome.

2. 3. Mechanism analysis of Pth in the stalled ribosome rescue process

SDS-PAGE analysis of the stalled ribosome complex showed that the translation product arrested in the ribosome was kept as peptidyl-tRNA. This indicates that the stalled ribosome is in the elongation phase and the peptidyl-tRNA is protected from the hydrolysis attack stimulated by the interaction between the ribosome and the translation release factors. On the contrary, the treatment of the stalled ribosome by Pth released

the peptide from the ribosome. SDS-PAGE analysis showed that this released peptide is no longer attached to the tRNA. As described above, the stalled ribosome complex is extremely stable and therefore, the results suggest that Pth hydrolyzed the peptidyl-tRNA on the ribosome and this hydrolysis is not originated from the drop-off of the peptidyl-tRNA from the ribosome.

To elucidate how Pth accesses to the peptidyl-tRNA on the ribosome, the dependency of magnesium ion concentration on the Pth activity was examined. The results indicated that the lower concentration of magnesium showed higher activity of Pth on the stalled ribosome. Since the magnesium ion is known to affect the affinity between the large and small subunit of the ribosome, this suggests that the Pth cannot access to the peptidyl-tRNA on the 70S ribosome but can access to the one on the 50S subunit.

To verify this point more clearly, the stalled ribosome complex was analyzed by the sucrose density gradient centrifugation analysis. The results showed that under low concentration of magnesium ion, a specific proportion of the stalled ribosome is dissociated into two subunits. Furthermore, Pth treatment released almost all peptide from 50s ribosome subunit. These results suggest that Pth can promote hydrolysis of peptidyl-tRNA and release it from 50s ribosome subunit following a stalled complex dissociation into two subunits.

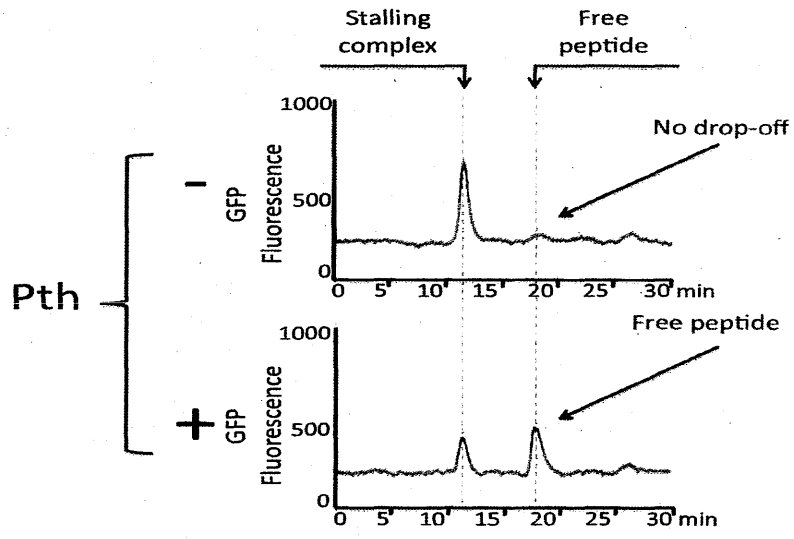

Figure 2: Translation product GFP detected in Gel filtration assay