Protein tagging at rare codons is caused by tmRNA action at the 3' end of nonstop mRNA generated in response to ribosome stalling

XIA LI, RIEKO HIRANO, HIDEAKI TAGAMI, and HIROJI AIBA

Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa, Nagoya 464-8602, Japan

ABSTRACT

It has been believed that protein tagging caused by consecutive rare codons involves tmRNA action at the internal mRNA site. We demonstrated previously that ribosome stalling either at sense or stop codons caused by certain arrest sequences could induce mRNA cleavage near the arrest site, resulting in nonstop mRNAs that are recognized by tmRNA. These findings prompted us to re-examine the mechanism of tmRNA tagging at a run of rare codons. We report here that either AGG or CGA but not AGA arginine rare-codon clusters inserted into a model *crp* mRNA encoding cAMP receptor protein (CRP) could cause an efficient protein tagging. We demonstrate that more than three consecutive AGG codons are needed to induce an efficient ribosome stalling therefore tmRNA tagging in our system. The tmRNA tagging was eliminated by overproduction of tRNAs corresponding to rare codons, indicating that a scarcity of the corresponding tRNA caused by the rare-codon cluster is an important factor for tmRNA tagging. Mass spectrometry analyses of proteins generated in cells lacking or possessing tmRNA encoding a protease-resistant tag sequence indicated that the truncation and tmRNA tagging occur within the cluster of rare codons. Northern and S1 analyses demonstrated that nonstop mRNAs truncated within the rare-codon clusters are detected in cells lacking tmRNA but not in cells expressing tmRNA. We conclude that a ribosome stalled by the rare codon induces mRNA cleavage, resulting in nonstop mRNAs that are recognized by tmRNA.

Keywords: tmRNA; rare codon; ribosome stalling; mRNA cleavage; nonstop mRNA

INTRODUCTION

Bacterial tmRNA (SsrA RNA) possessing properties of both a tRNA and an mRNA is a central player in the unusual translation process called *trans*-translation (Keiler et al. 1996; Karzai et al. 2000; Withey and Friedman 2002). When a ribosome translates to the 3' end of an mRNA lacking in-frame stop codons (nonstop mRNA), tmRNA charged with alanine in complex with SmpB and EF-Tu enters the A-site of the ribosome to act first as an alanyl-tRNA and then as an mRNA to direct the addition of a short peptide tag to the growing polypeptide. As a result, the stalled ribosome is released and the incomplete polypeptide is marked for degradation by ATP dependent proteases. The rescue of the stalled ribosome and the degra-

dation of aberrant polypeptides are well-established biological functions of the tmRNA quality-control system (Karzai et al. 2000; Withey and Friedman 2002). In addition, we found that the tmRNA-mediated *trans*-translation facilitates degradation of truncated mRNAs by removing stalled ribosomes and thus allowing 3'-to-5' exonucleases to access the free mRNA 3' end (Yamamoto et al. 2003). Thus, the tmRNA quality-control system not only degrades aberrant polypeptides once produced but also prevents production of aberrant polypeptides through a rapid elimination of damaged mRNAs.

Protein tagging mediated by tmRNA was originally demonstrated to occur at the 3' end of an artificial nonstop mRNA (Keiler et al. 1996). Subsequent studies revealed that the 3' ends of nonstop mRNAs derived from native genes under certain conditions are indeed recognized by the tmRNA system. For example, truncated mRNAs produced by an incomplete transcription due to transcriptional roadblock leads to the tmRNA tagging of LacI (Abo et al. 2000). Proteins translated from truncated nonstop mRNAs generated by 3' exonuclease digestion are also efficiently tagged

Reprint requests to: Hiroji Aiba, Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa, Nagoya 464-8602, Japan; e-mail: i45346a@nucc.cc.nagoya-u.ac.jp; fax: 81-52-789-3001.

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by the tmRNA system (Yamamoto et al. 2003). In addition, a ribosome could reach the 3' end of an mRNA and become a target for the tmRNA system when a normal stop codon is erroneously translated either in the presence of nonsense suppressor tRNAs (Ueda et al. 2002) or in the presence of misreading drugs (Abo et al. 2002).

An important unsettled issue concerning tmRNA target is whether the tmRNA system could act within an mRNA when a translating ribosome stalls on an mRNA for any of several possible reasons. This question was first addressed by Roche and Sauer (1999), who found that tmRNA-mediated protein tagging occurs at a run of rare codons on an mRNA where the ribosome is expected to stall due to the deficiency of cognate aminoacyl-tRNAs. The tagging at rare codons could involve tmRNA recruitment to the ribosome stalled within an mRNA. Alternatively, it could be explained by the standard model if nonstop mRNAs are generated either by mRNA cleavage or by premature transcription termination. The failure to detect truncated mRNA species led Roche and Sauer to propose that the tagging at rare codons involves tmRNA action at an internal mRNA site rather than at the 3' end of a cleaved mRNA (Roche and Sauer 1999). More recently, protein tagging was shown to occur at a position corresponding to the normal termination codon in certain conditions depending on the presence of rare arginine codons near the adjacent inefficient UGA termination codon (Collier et al. 2002; Hayes et al. 2002b) or the amino acid sequence of nascent polypeptide prior to stop codons (Hayes et al. 2002a; Sunohara et al. 2002). The protein tagging at stop codons was thought to be an additional case for tmRNA action at an internal mRNA site where tmRNA may enter the A-site by competing with RFs or near-cognate aminoacyl-tRNAs. However, subsequent studies revealed that ribosome stalling at stop codons caused by certain peptides leads to mRNA cleavage resulting in nonstop mRNAs and, therefore, the generation of nonstop mRNAs through mRNA cleavage is the cause of *trans*-translation at stop codons (Hayes and Sauer 2003; Sunohara et al. 2004b). In addition, it has been established that ribosome stalling at sense codons during translation elongation caused by certain arrest sequences could also induce mRNA cleavage near the arrest point resulting in nonstop mRNAs that are recognized by tmRNA (Collier et al. 2004; Sunohara et al. 2004a).

These findings prompted us to re-examine the mechanism by which the tmRNA tagging occurs at a run of rare codons. In this paper, we report that either AGG or CGA but not AGA arginine rare-codon clusters inserted in a model *crp* mRNA encoding cAMP receptor protein (CRP) could cause an efficient protein tagging. Careful analyses of mRNAs demonstrate that truncated nonstop *crp* mRNAs are efficiently generated in the absence of tmRNA while they are rapidly degraded in the presence of tmRNA. We conclude that ribosome stalling caused by rare-codon clusters leads to endonucleolytic cleavages around the stalled

ribosome resulting in nonstop mRNAs, which, in turn, are recognized by the tmRNA system. Our study suggests that the 3' end of nonstop mRNA is the universal and probably only target for the tmRNA system.

RESULTS

Clusters of AGG and CGA codons induce tmRNA tagging

The work by Roche and Sauer (1999) demonstrated that tmRNA-mediated tagging of a model protein (N-terminal domain of λ repressor) occurs at positions encoded by the rare arginine codons AGA and CGA. To reinvestigate the effect of rare arginine codons on tmRNA tagging, we constructed a series of plasmid-borne *crp* gene variants in which five consecutive AGA, AGG, CGA, and CGC codons were placed either just after the last codon of CRP (Arg 209) or the codon for Val 205 (Fig. 1). The AGG is another rare codon for arginine, while the CGC is a control major codon. Each of the CRP variants was expressed in three isogenic *Escherichia coli* K12 strains with different *ssrA* allele (*ssrA*⁺, Δ *ssrA*, and *ssrA*^{DD}) and analyzed by Western blotting (Fig. 2). Anti-CRP-probed Western blots revealed that the full-length proteins corresponding to CRP variants were generated regardless of the *ssrA* allele. Additional bands of higher molecular weight were produced when AGG or CGA codons were placed at the position 209 or 205 of CRP in cells carrying the *ssrA*^{DD} allele (Fig. 2, lanes 9,13,17,20). The *ssrA*^{DD} allele encodes tmRNA-DD possessing a protease-resistant tag sequence (Roche and Sauer 1999; Abo et al.

FIGURE 1. Schematic drawing and sequences of the *crp* variants containing clusters of arginine codons. The black box represents the CRP ORF. The nucleotide sequence and amino acid sequence (in one-letter code) of the 3' portion of the *crp* gene derived from pHA7M are shown below the diagram. The clusters of five arginine codons were inserted after the last codon of CRP (Arg 209) or the codon for Val 205. The sequence of five consecutive arginine codons are shown in italic along with plasmid and protein names.

FIGURE 2. SsrA tagging of CRP proteins expressed from *crp* variants. Lysates equivalent to $OD_{600} = 0.01$ prepared from TA341 (Δcrp *ssrA*⁺), TA501 (Δcrp $\Delta ssrA$), or TA481 (Δcrp *ssrA*^{DD}) cells harboring the indicated plasmids were analyzed by Western blotting using anti-CRP (lanes 1–9, 11–13, 15–20) and anti-DD-tag (lanes 10, 14) antibodies.

2000). These bands cross-reacted with anti-DD antibodies (Fig. 2, lanes 10, 14), indicating that the clusters of AGG and CGA codons efficiently induce tmRNA tagging presumably by reducing the ribosome movement. The position of the rare-codon clusters did not affect significantly the efficiency of tagging itself, although it somehow affected the tagging pattern. To our surprise, the AGA cluster, which was shown to induce tmRNA tagging previously by Roche and Sauer (1999), failed to cause any tagging in our system (Fig. 2, lanes 1–6). This suggests that the level of the cognate aminoacyl-tRNA corresponding to the AGA codon is relatively abundant in our strains. No tagging was observed when the five consecutive major CGC codons were placed in the *crp* gene (data not shown).

It should be noted that slightly shorter bands were also detected by anti-CRP antibodies just below the full-length CRP containing AGG or CGA codons in the absence of tmRNA (Fig. 2, lanes 7, 11, 15, 18). These shorter bands were markedly reduced in the presence of tmRNA, suggesting that they were eliminated by the tmRNA system. We assume that these bands are produced due to the rare-codon clusters. To confirm this, the five consecutive AGG codons were inserted just after the codon 145 of CRP (Fig. 3A). When the CRP145-5AGG protein was expressed in the absence of tmRNA, a band of the size expected for a protein truncated near the rare-codon cluster was generated in addition to the full-length CRP-5AGG protein (Fig. 3B, lane 1). This band was no longer detected in the presence of wild-type tmRNA (Fig. 3B, lane 2) while the DD-tagged truncated bands were produced in the presence of tmRNA-DD (Fig. 3B, lane 3). Taken together, we conclude that the 5AGG and 5CGA rare-codon clusters lead to the production of truncated CRPs that are efficiently tagged by the tmRNA system.

More than three consecutive AGG codons are required for an efficient tagging

It was reported that the tmRNA tagging occurs at the clusters of two or four consecutive AGA codons but not at the single AGA codon in their model system (Roche and Sauer 1999). We examined the effect of the number of consecutive AGG rare codons on tmRNA tagging. For this, each of the consecutive AGG codons in pRH32 was

replaced one by one with the major CGC codon. The resulting constructs encode the same CRP variant containing five consecutive arginine residues near the C terminus (Fig. 4A). These constructs were expressed in the presence of tmRNA-DD, and the lysates were analyzed by Western blotting using anti-CRP and anti-DD antibodies (Fig. 4B). The tmRNA tagging increased with an increasing number of AGG codons. Essentially no tagging was observed when only one AGG codon is present, while two AGG codons caused a very weak tagging. The extent of tagging significantly increased at three AGG codons. The tagging was further enhanced at four consecutive AGG codons, but five AGG codons caused only a little further increase in the tagging. We conclude that three or four consecutive AGG codons are needed to induce an efficient ribosome stalling therefore tmRNA tagging in our system.

Overproduction of tRNAs corresponding to rare codons eliminates tmRNA tagging

Roche and Sauer (1999) demonstrated that overexpression of tRNA^{AGA} prevented tagging of their model proteins, arguing that a scarcity of free tRNA^{AGA} is a prerequisite for tmRNA-mediated tagging. To see whether the deficiency of cognate tRNAs is responsible for the tmRNA tagging induced by the rare-codon clusters, we examined the effect of the presence of a plasmid pRARE carrying

FIGURE 3. SsrA tagging of CRP proteins during translation elongation. (A) Schematic drawing and sequence of plasmid pRH36. The black box is the CRP ORF and the open box represents the region for rare codons inserted. The nucleotide sequence and amino acid sequence (in one-letter code) around this region are shown below the diagram. (B) Western blot analysis of CRP proteins expressed from plasmid pRH36. Lysates equivalent to $OD_{600} = 0.1$ prepared from TA341 (Δcrp), TA501 (Δcrp $\Delta ssrA$), or TA481 (Δcrp *ssrA*^{DD}) cells harboring pRH 36 plasmid were analyzed by Western blotting using anti-CRP antibodies.

FIGURE 4. Effect of the number of consecutive rare codons on SsrA tagging. (A) Schematic drawing and sequences of the *crp* variants. The black box is the CRP ORF and the open box represents the region of rare codon inserted. The nucleotide sequence and amino acid sequence (in one-letter code) around this region are shown below the diagram. The AGG codons are underlined. (B) Western blot analysis of variant CRP proteins. Lysates equivalent to OD₆₀₀ = 0.01 prepared from TA341 (Δ *crp*), TA501 (Δ *crp* Δ *ssrA*), or TA481 (Δ *crp* *ssrA*^{DD}) cells harboring the indicated plasmids were analyzed by Western blotting using anti-CRP and anti-DD-tag antibodies.

genes of a series rare-codon tRNAs including tRNA^{AGG} on the tagging. As shown in Figure 5, the tagging induced by the consecutive AGG rare codons was completely eliminated when pRARE was co-expressed with pRH31. This clearly indicates that scarcity of the corresponding tRNAs is an important factor for tmRNA tagging and that the rare-codon cluster causes the tmRNA tagging by reducing free tRNA levels.

Tagging occurs within rare codons

To determine the truncation and/or tagging sites in CRP containing the consecutive rare codons, proteins generated in Δ *ssrA*, *ssrA*⁺, and *ssrA*^{DD} cells carrying pRH31 were immuno-precipitated using anti-CRP antibodies. The purified proteins were subjected to SDS-polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining (Fig. 6A). As expected, only the full-length CRP209-5AGG protein (band I) was purified from the wild-type (*ssrA*⁺) cells. The full-length CRP209-5AGG protein was also obtained from Δ *ssrA* and *ssrA*^{DD} cells. In addition to band I, the truncated CRP (band II) and the DD-tagged CRP (band III) were recovered from lysates of Δ *ssrA* cells and *ssrA*^{DD} cells, respectively. Each of these bands was excised from the gel and digested in-gel with lysyl endopeptidase that specifically cleaves the peptide bond after lysine residues. The eluted peptides were analyzed by MALDI-

TOF mass spectrometry. The band I indeed produced a signal corresponding to the C-terminal fragment (TIVVYG TRRRRRR) of full-length CRP209-5AGG, while the band II corresponding to the truncated CRP gave three specific signals that correspond to those expected for the C-terminal fragments of TIVVGTR, TIVVGTRR, and TIVVGTRR (data not shown). The data for the mass spectrum of band III are shown in Figure 5B. The band III produced three specific signals that correspond to junction peptides containing the C-terminal fragments plus the tag. We conclude that the truncation and tmRNA tagging of CRP occur within the cluster of AGG rare codons.

Truncation of mRNAs occurs at rare codons

Recently, it was found that endonucleolytic mRNA cleavage occurs in response to ribosome stalling at both stop and sense codons caused by certain nascent peptide sequences (Hayes and Sauer 2003; Collier et al. 2004; Sunohara et al. 2004a,b). This cleavage leads to the generation of nonstop mRNAs, which, in turn, are recognized by the tmRNA system. It is quite reasonable to assume that tmRNA tagging at the rare-codon clusters also occurs through the generation of a truncated mRNA lacking a stop codon. To test this possibility, total RNAs were prepared from cells carrying each of several plasmid-borne *crp* variants, both in the presence and absence of tmRNA and analyzed by Northern blotting using a DNA probe specific to the *crp* mRNA. Interestingly, a shorter band appeared along with the full-length *crp* mRNA when CRP209-5AGG and CRP205-5AGG were expressed in the absence of tmRNA (Fig. 7, lanes 3,5). The shorter *crp* mRNA was no longer observed in the presence of tmRNA (Fig. 7, lanes 4,6). This is because the truncated mRNA is

FIGURE 5. Effect of overproduction of tRNAs corresponding to rare codons on SsrA tagging. Lysates equivalent to OD₆₀₀ = 0.01 prepared from TA341 (Δ *crp*), TA501 (Δ *crp* Δ *ssrA*), or TA481 (Δ *crp* *ssrA*^{DD}) cells harboring the indicated plasmid(s) were analyzed by Western blotting using anti-CRP antibodies. Plasmid pRARE contains the tRNA genes for AGA, AGG, AUA, GGA, CUA, and CCC rare codons (Novagen).

FIGURE 6. Mass spectrometry of CRP proteins. (A) CRP proteins were purified from three isogenic strains carrying pRH31 by immuno-precipitation using anti-CRP agarose beads. Purified proteins were separated on a 15% SDS-polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining. The band I, II, and III corresponding to the full-length, truncated and DD-tagged CRP proteins were cut out from the gel, treated with lysyl endopeptidase, and subjected to mass spectrometry analysis. The expected peptide sequences and molecular weights of the C-terminal fragments of proteins are shown. The arginine rare codons are shown in bold, and the tag sequences derived from tmRNA-DD are underlined. (B) MALDI-TOF spectrum for the DD-tagged CRP protein purified from *ssrA^{DD}* cells. The specific signals corresponding to the C-terminal CRP fragments are shown by asterisks.

released from the stalled ribosome and rapidly degraded by *trans*-translation, as demonstrated previously (Yamamoto et al. 2003). Essentially the same results were obtained when RNAs from genes encoding CRP209-5CGA (Fig. 7, lanes 7,8) and CRP205-5CGA (data not shown) were analyzed. When the tagging-negative CRP205-5AGA was expressed, only the full-length *crp* mRNA was detected (Fig. 7, lanes 1,2). Thus, the tmRNA-mediated tagging of CRP at rare codons is tightly associated with the production of the shorter truncated *crp* mRNA. These results strongly suggest that the truncated *crp* mRNA is generated as a result of ribosome stalling at rare codons, which in turn would be recognized by tmRNA.

Determination of 3' ends of truncated mRNAs

To determine the sequence of the 3' end of the truncated *crp* mRNA, total RNAs prepared from tmRNA-deficient cells expressing CRP209-5AGG were hybridized with a DNA probe C ³²P-labeled at its 3' end. DNA probe C covers the 3' region of the *crp* gene including a part of the coding sequence and the terminator sequence. The hybrids were treated with S1 nuclease and the products were analyzed by

electrophoresis on a sequencing gel. As shown in Figure 8, three clusters of S1-resistant bands (referred to as I, II, and III) were detected. Cluster I represents the full-length *crp* mRNA and its major 3' ends were mapped just after the inverted repeat sequence of the *crp* terminator as previously shown (Abe et al. 1999). Cluster II presumably corresponds to the truncated *crp* mRNA, and their major 3' ends were mapped before the stop codon. The data are consistent with the view that the ribosome stalling caused by the consecutive arginine rare codons indeed generates nonstop mRNAs lacking a stop codon. When total RNAs prepared from the wild-type (*ssrA⁺*) cells expressing CRP209-5AGG were analyzed, the bands of cluster II were no longer observed, indicating they are removed through tmRNA-mediated *trans*-translation. Cluster III seems to be non-specific bands because these bands were also observed when RNAs were prepared from cells carrying a control plasmid pBR322 (data not shown).

DISCUSSION

This study was to re-examine the mechanism by which the protein tagging mediated by tmRNA occurs at a run of rare codons. We constructed a series of model variant *crp* genes containing different numbers of consecutive arginine rare codons (Fig. 1). We demonstrated that five consecutive AGG and CGA but not AGA rare codons could cause an efficient protein tagging (Figs. 2, 3). We also showed that more than three consecutive rare codons are needed for an efficient tmRNA tagging (Fig. 4). It should be noted that AGG, AGA, and CGA are all among the least frequently used in *E. coli* cells (0.16%, 0.27%, and 0.37%, respectively) (Nakamura et al. 1999). This means that infrequent codon usage itself is not directly correlated with the efficiency of tmRNA tagging. Instead, the scarcity of cognate tRNAs would be important for the tmRNA tagging at rare codons, as shown previously by Roche and Sauer (1999). In fact, we also demonstrated that the tagging at consecutive AGG rare codons was completely eliminated by introducing pRARE plasmid carrying genes for a series of tRNAs corresponding to rare codons. The tmRNA-mediated tagging was shown

FIGURE 7. Northern blot analysis of *crp* mRNA derived from the *crp* genes encoding CRP variants. Total RNAs prepared from TA341 (Δcrp) and TA501 ($\Delta crp \Delta ssrA$) cells harboring indicated plasmids were resolved by electrophoresis on a 2.0% agarose-formaldehyde gel. Northern blot analysis was performed using the DIG-labeled *crp* probe.

strain are reported to be 1.34% and 0.65%, respectively, out of total tRNA (Dong et al. 1996). In addition, our results are consistent with the previous observation by Varenne and colleagues that AGG codon clusters in the mRNA of chloramphenicol acetyltransferase from *E. coli* dramatically reduced the production of this enzyme when the mRNA transcription reached a certain level (Varenne et al. 1989). It is highly possible that ribosomal stalling time at AGG codon clusters increases dramatically, resulting in mRNA cleavage when the transcription increases above a critical limit. It was proposed that the nature of termination codon could affect the efficiency of protein tagging at rare codons (Collier et al. 2002; Hayes et al. 2002b). However, we observed that the replacing of a UAA termination codon with a less efficient UGA codon had no effect on the tagging efficiency (data not shown), suggesting that the scarcity of cognate tRNA is fully responsible for the tmRNA tagging at rare codons.

The depletion of the available pool of tRNA caused by the repeated rare codon is expected to lead to ribosome stalling resulting in tmRNA tagging. Indeed, mass-spectrometry analysis of the purified proteins indicated that truncation and tmRNA tagging of the model CRP occurs at the positions corresponding to the rare codons. Furthermore, we detected a truncated *crp* mRNA in cells lacking tmRNA by Northern blotting, and the 3' ends of the truncated mRNA were mapped within the cluster of rare codons. The truncated mRNA was no longer detected in wild-type (*ssrA*⁺) cells, indicating that it is released by *trans*-translation and rapidly degraded. All of these data are consistent with the view that generation of nonstop mRNA induced by ribosome stalling is responsible for the tmRNA tagging at consecutive rare codons.

It was established that ribosomes pausing either at stop or sense codons caused by certain nascent peptides generates nonstop mRNAs through mRNA cleavage, which, in turn, leads to the *trans*-translation (Hayes and Sauer 2003; Collier et al. 2004; Sunohara et al. 2004a,b). In addition, a recent in vitro study demonstrated that tmRNA targeting to ribosomes stalled at sense or stop codons is preceded by an mRNA truncation (Ivanova et al. 2004). In this study, we demonstrate that mRNA cleavage induced by ribosome stalling is responsible for the tmRNA tagging at the clusters of rare codons. Moreover, our preliminary study indicates that mRNA cleavage occurs in response to ribosome stalling caused by a depletion of release factors (data not shown). Thus, it is now quite clear that ribosome stalling is tightly associated with tmRNA tagging through mRNA cleavage in general. In other words, endonucleolytic cleavage of ribosome-bound mRNA may always occur prior to the tmRNA action when a ribosome stalls under various situations. We suggest that the 3' end of nonstop mRNA is the universal and probably only target for the tmRNA system.

It was demonstrated that bacterial toxins such as RelE and MazF could induce mRNA cleavages at the ribosomal

A-site in codon-specific manners (Christensen et al. 2003; Pedersen et al. 2003). However, we showed previously that neither RelE nor known endoribonucleases such as RNase E, RNase G, and RNase III are involved in the mRNA cleavage in response to ribosome stalling (Sunohara et al. 2004a). Hayes and Sauer (2003) also observed that the cleavage of a test mRNA does not require any bacterial toxins such as RelE, MazF, chpBK, YafQ, and YoeB. We speculate that the ribosome itself or some factors associated with the ribosome would be responsible for the mRNA cleavage in response to ribosome stalling. The recent in vitro experiment revealed that stalled ribosome complexes containing a test “full-length” mRNA did not cause any mRNA cleavage during extended incubations, suggesting that the stalled ribosome alone may be inactive regarding the cleavage reaction at least in vitro (Ivanova et al. 2004). Thus, how ribosome stalling induces the mRNA cleavage is still a challenging question for future study.

It is known that rare codons, particularly in clusters and when the cognate tRNA is limiting, slow translation and cause various translational errors (Bonekamp and Jensen 1988; Kane 1995). The analysis of the *E. coli* W3110 strain genome reveals that only five genes contain AGG-AGG and 28 genes contain CGA-CGA rare-codon clusters, and only one gene contains a CGA-CGA-CGA cluster. For example, the *fecC* gene encoding Iron (III) dicitrate transport system permease is an interesting case because it possesses an AGG-AGG-CGA-GGA sequence just before the termination codon. Another interesting example is *yeeO* because the sequence from codon 162 to codon 168 of this gene is CGG-GAT-CGA-CGA-CGA-GCG-AGG. It is quite possible that ribosome stalling, and therefore mRNA cleavage, may occur constitutively in these natural rare-codon clusters. Although there are no other genes in which more than three consecutive rare codons exist, ribosome stalling could occur at even one or two rare codons under certain conditions, such as amino acid starvation and other nutrient limitations. Therefore, the mRNA cleavage and tmRNA mediated *trans*-translation at rare codons could become significant under these situations. In addition, the mRNA cleavage and tmRNA mediated *trans*-translation could occur at other rare codons such as AUA (isoleucine), CUA (leucine), CCC (proline), and GGA (glycine). It will be interesting to investigate how the mRNA cleavage and tmRNA action at rare codons are involved in the regulation of gene expression and in quality control during protein synthesis in general.

MATERIALS AND METHODS

Media and growth condition

Cells were grown aerobically at 37°C in Luria-Bertani (LB) medium (Miller 1972). Antibiotics were used at the following concentrations: ampicillin (50 µg/mL), chloramphenicol (30 µg/mL).

Bacterial growth was monitored by determining the optical density at 600 nm.

Strains and plasmids

The *E. coli* K12 strains used—TA341 (W3110 Δ *crp*), TA501 (W3110 Δ *crp* Δ *ssrA*), and TA481 (W3110 Δ *crp* *ssrA*^{DD})—are described (Sunohara et al. 2004b). All plasmids that express wild-type and variant forms of CRP are derived from pHA7M carrying the *crp* gene under the *bla* promoter (Abo et al. 2000). Plasmids pRH11, pRH12, pRH31, pRH32, pRH51, pRH52, pRH61, pRH62, pRH36, pRH37, pRH34, pRH38, and pRH39 were constructed from pHA7M by PCR mutagenesis using appropriate primers. Plasmid pRARE is a derivative of pACYC184 carrying *argU*, *argW*, *ilex*, *glyT*, *leuW*, and *proL* encoding tRNAs for AGA, AGG, AUA, GGA, CUA, and CCC rare codons (Novagen).

Western blotting

Bacterial cells were grown in LB medium containing appropriate antibiotic(s) to mid-log phase. Culture samples (1 mL) were centrifuged and the pellets were suspended in 50 µL of H₂O. The cell suspensions were mixed with 50 µL of 2× loading buffer (4% SDS, 10% 2-mercaptoethanol, 125 mM Tris-HCl, pH 6.8, 10% glycerol, 0.2% bromophenol blue) and heated for 5 min at 100°C. For Western blotting, the total extracts of the indicated amount were subjected to a 0.1% SDS-PAGE (–12% or 15%) and transferred to Immobilon membrane (Millipore). The membrane was probed with anti-CRP antibodies using the ECL system (Amersham Life Science).

Mass spectrometry

TA341 (W3110 Δ *crp*), TA501 (W3110 Δ *crp* Δ *ssrA*), and TA481 (W3110 Δ *crp* *ssrA*^{DD}) cells carrying pRH31 were grown in 20 mL of LB medium containing 50 µg/mL ampicillin to A₆₀₀ = 0.6. The cell cultures were centrifuged and washed with 0.1 M NaCl₂, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. The cell pellets were suspended in 600 µL of buffer A (20 mM Tris-HCl, pH 8.0, 0.1 M KCl, 5 mM MgCl₂, 0.1% Tween 20, 10 mM β-mercaptoethanol, 10% glycerol, 0.2 mM phenylmethanesulfonyl fluoride). The cell suspensions were sonicated and centrifuged at 12,000 rpm for 5 min. The supernatants were mixed with 10 µL of anti-CRP agarose beads (Sunohara et al. 2004a). Immuno-precipitation was carried out for 2 h at 4°C with gentle agitation. After extensive washing, with buffer A, 2× SDS-PAGE loading buffer (125 mM Tris-HCl at pH 6.8, 4% SDS, 10% glycerol, 10% β-mercaptoethanol, 0.2% bromophenol blue) was used to elute bound proteins instead of 100 mM glycine-HCl (pH 2.5) because it failed to elute CRP proteins inserted with consecutive arginine rare codons. For mass spectrometry (MS) analysis, the purified untagged and DD-tagged proteins were separated on 15% SDS-polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining. The bands were cut out from the gel and treated with 0.1 µg of lysyl endopeptidase (Roche Applied Science) in 20 µL of 25 mM Tris-HCl, pH 9.0 for 12 h at 37°C. The digested peptides were eluted with 50% acetonitrile, 5% formic acid, and then desalted with

ZipTip C18 (Millipore) reverse-phase column, mixed with 1% α -CHCA (α -cyano-4-hydroxycinnamic acid) in 70% acetonitrile, and subjected to MALDI/TOF-MS.

RNA analyses

Total RNA was isolated from cells grown to mid-log phase as described (Aiba et al. 1981). For Northern blot analysis, an indicated amount of total RNAs was resolved by either 2.0% or 1.5% agarose-gel electrophoresis in the presence of formaldehyde and blotted onto a Hybond- N^+ membrane (Amersham). The mRNAs were visualized using digoxigenin (DIG) reagents and kits for nonradioactive nucleic acid labeling and detection system (Roche) according to the procedure specified by the manufacturer. The DIG-labeled DNA probe used was a 576-bp probe corresponding to the *crp* coding region. The DIG-labeled RNA marker III (Roche) was used to estimate the size of RNA bands. The 3' end of *crp* mRNA was determined by S1 nuclease assay as described (Aiba et al. 1981). A DNA fragment corresponding to the 3' region of *crp* mRNA was prepared by PCR from pRH31 and digested with *Sau*3AI and *Eco*RV. The *Sau*3AI 3' end of the resulting 140-bp fragment was labeled with [α - 32 P]dGTP by Klenow enzyme. The 32 P-labeled fragment was used as a DNA probe for S1 assay. Total RNAs (50 μ g) prepared from TA341 (Δ *crp*) and TA501 (Δ *crp* Δ *ssrA*) cells harboring pRH31 were hybridized with the DNA probe, and treated with 200 U of S1 nuclease. The resulting products were analyzed on an 8% polyacrylamide-8M urea gel. The ends of mRNAs were identified by using the Maxam-Gilbert A+G ladder of the DNA probe as reference.

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