Ribosome stalling during translation elongation induces cleavage of mRNA being translated in *Escherichia coli*

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Running title: mRNA cleavage induced by ribosome stalling

Key words: ribosome stalling /mRNA cleavage/SecM / tmRNA/ quality control

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SUMMARY

Recently, it has been found that ribosome pausing at stop codons caused by certain nascent peptides induces cleavage of mRNA in *Escherichia coli* cells (1,2). The question we addressed in the present study is whether mRNA cleavage occurs when translation elongation is prevented. We focused on a specific peptide sequence (AS17), derived from SecM, that is known to cause elongation arrest. When the *crp-crr* fusion gene encoding CRP-AS17-IIA\text{Glc} was expressed, CRP proteins truncated around the arrest sequence were efficiently produced and they were tagged by the tmRNA system. Northern blot analysis revealed that both truncated upstream *crp* and downstream *crr* mRNAs were generated along with reduced amounts of the full-length *crp-crr* mRNA. The truncated *crp* mRNA dramatically decreased in the presence of tmRNA due to rapid degradation. The 3’ ends of truncated *crp* mRNA correspond well to the C-termini of the truncated CRP proteins. We conclude that ribosome stalling by the arrest sequence induces mRNA cleavage near the arrest point resulting in nonstop mRNAs that are recognized by tmRNA. We propose that the mRNA cleavage induced by ribosome stalling acts in concert with the tmRNA system as a way to ensure quality control of protein synthesis and possibly to regulate the expression of certain genes.
INTRODUCTION

A special bacterial RNA called tmRNA or SsrA RNA is a central player in a unique quality control system during protein synthesis (3-5). When a ribosome translates to the 3' end of a broken or incomplete mRNA lacking a stop codon (nonstop mRNA), tmRNA charged with alanine enters the A-site of the ribosome to act first as an alanyl-tRNA and then is itself translated. This co-translation reaction (*trans*-translation) terminates at the stop codon that follows the tmRNA reading frame, releasing both the ribosome and the tagged polypeptide. The tagged polypeptide is recognized and degraded by several ATP dependent proteases. The rescue of the stalled ribosome and the degradation of aberrant polypeptides that are useless and/or potentially harmful to the cell are two well-established biological roles of the tmRNA quality control system (4,5). An additional important role of the tmRNA system is to facilitate the degradation of truncated mRNAs by removing stalled ribosomes and thus allowing 3'-to-5' exonucleases to access the free mRNA 3' end (6). 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.

Nonstop mRNAs could be generated either by nuclease cleavages of an mRNA or by incomplete transcription. For example, they are produced as degradation intermediates from mRNAs by 3'-to-5' exonucleolytic digestion (6). The tmRNA-mediated tagging of LacI typically represents the situation where truncated mRNAs are produced by an incomplete transcription due to transcriptional road block (7). A ribosome would reach also the 3' end of an mRNA when a normal stop codon is erroneously
translated either in the presence of nonsense suppressor tRNAs (8) or in the
presence of misreading drugs (9). Interestingly, the tmRNA system also
acts at stop codons to add the degradation tag to full-length proteins when
translation termination is prevented by certain nascent polypeptides (10-13).
During the study on the mechanism of nascent peptide dependent tagging
of the full-length protein at stop codons, we have found that ribosome
stalling caused by certain peptides leads to mRNA cleavage around the stop
codon resulting in nonstop mRNAs and therefore the mRNA cleavage is
the cause of trans-translation at stop codons (1). Cleavage of mRNA in
response to ribosome stalling at stop codons was also found in ybeL mRNA
by Hayes and Sauer (2).

The findings mentioned above have raised a question of whether the
cleavage of mRNA occurs at sense codons when translation elongation is
prevented. To answer this particular question, we focused on a specific
peptide sequence, derived from SecM, that is known to cause stalling of the
ribosome during translation elongation (14). In the present study, we
constructed the crp-crr fusion genes in which the coding regions of two
proteins (CRP and IIA^{Glc}) are connected by a short nucleotide sequence
corresponding to the SecM arrest sequence (14). By using this fusion
system, we found that the SecM arrest sequence strongly induces mRNA
cleavage resulting in nonstop mRNAs that are recognized by tmRNA. We
also found that the last 5 amino acid segment of the arrest sequence is
sufficient enough to induce the mRNA cleavage. Neither the bacterial toxin
RelE nor the known major endoribonucleases are involved in this cleavage,
suggesting that either other endoribonuclease(s) or ribosome itself would
be responsible for the mRNA cleavage in response to the ribosome stalling
caued by the particular nascent peptides. The mRNA cleavage induced by
ribosome stalling could act in concert with the tmRNA system as a way to ensure quality control of protein synthesis and possibly to regulate the expression of certain genes including the \textit{secM-secA} operon.

**EXPERIMENTAL PROCEDURES**

*Media and growth conditions*
Cells were grown aerobically at 37 °C in Luria-Bertani (LB) medium (15). Antibiotics were used at the following concentrations: ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), and tetracycline (5 μg/ml). Bacterial growth was monitored by determining the optical density at 600 nm.

*Strains and plasmids*
The \textit{E. coli} K-12 strains and plasmids used in this study are listed in Table 1. The \textit{rnc}14::Tn10 region of HT27 was transferred to W3110 by P1 transduction to construct ST201. All plasmids are derivatives of pHA7 carrying the \textit{crp} gene under the \textit{bla} promoter (16). Plasmid pHA7M expressing wild-type CRP is described previously (9). Plasmids expressing each of variant forms of CRP or CRP-IIA\text{Glc} were constructed from pHA7M and pIZ3A (9) by PCR mutagenesis using appropriate primers.

*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 H\textsubscript{2}O. The cell suspensions were mixed with 50 μl of 2 x 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 indicated amount were subjected to a 0.1% SDS -12% or 15% PAGE and transferred to Immobilon membrane (Millipore). The membrane was probed with anti-CRP antibodies using ECL system (Amersham Life Science).

**Mass spectrometry**

The untagged and tagged CRP-GIRAGP proteins were immuno-purified from TA501 (W3110 Δcrp ΔssrA) and TA481(W3110 Δcrp ssrA<sup>DD</sup>) cells carrying pJK208. The cells were grown in 20 ml of LB medium containing 100 μg/ml of ampicillin to OD<sub>600</sub> = 0.6. The cell cultures were centrifuged and washed with 0.1 M NaCl, 10 mM Tris-HCl(pH8.0), 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 MgCl<sub>2</sub>, 0.1% Tween-20, 10 mM 2-mercaptoethanol, 10% glycerol, 0.2 mM phenylmethanesulfonyl fuluride). The cell suspensions were sonicated and centrifuged at 12,000 rpm for 5 min. 400 μl of the supernatants were mixed with 10 μl of anti-CRP antibodies conjugated to agarose beads. Immunoprecipitation was carried out for 2 hrs at 4°C with gentle agitation. After extensive washing with buffer A, bound proteins were eluted with 10 μl of 100 mM glycine-HCl (pH2.5). The polyclonal anti-CRP antibodies were crosslinked to Protein A-agarose (Roche) with dimethylpimelidate as described (17). For mass spectrometry (MS) analysis, the purified untagged and DD-tagged proteins were separated by 15% SDS-PAGE. The bands were cut out from the gel and a small piece of each band was treated with 0.1 μg of lysyl endopeptidase (Roche) in 20 μl of 25 mM Tris-HCl, pH 9.0 for 12 hr at 37 °C. The digested peptides were eluted with 300 μl of 50 % acetonitrile, 5 % formic acid and concentrated to 20 μl.
Then, the sample was desalted with zip-tip 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 RNAs were isolated from cells grown to mid-log phase as described (18). The total RNAs were resolved by 1.5 % agarose-gel electrophoresis in the presence of formaldehyde and blotted onto Hybond-N+ membrane (Amersham). The mRNAs were visualized using digoxigenin (DIG) reagents and kits for non-radioactive nucleic acid labeling and detection system (Roche) according to the procedure specified by the manufacturer. The DIG-labeled DNA probes used were 576 bp probe A corresponding to the crp coding region and 507 bp probe B corresponding to the crr coding region. The DIG-labeled RNA marker III (Roche) was used to estimate the size of RNA bands. The 3' end of crp mRNA and the 5' end of crr mRNA were determined by S1 nuclease assay as described (18). A DNA fragment corresponding to the junction region between the crp and crr was prepared by PCR from pJK208 encoding CRP-GIRAGP-IIA\textsuperscript{Glc} and digested with Sau3AI and HpaII. The 3' end of the resulting 102 bp fragment was labeled with $[^{\alpha-}\text{32P}]$ dGTP by Klenow enzyme and the strands were separated by electrophoresis on a 10 % polyacrylamide gel. The lower strand 32P-labeled at its Sau3AI 3' end was used as a DNA probe (probe C) to determine the 3' end of crp mRNA. Similarly, a DNA fragment (315 bp) corresponding to the junction region between the crp and crr was prepared by PCR from pJK208 encoding CRP-GIRAGP-IIA\textsuperscript{Glc} and the strands were separated by electrophoresis on a 10 % polyacrylamide gel. The lower strand 32P-labeled at its 5' end was used as a DNA probe (probe D) to
determine the 5' end of crr mRNA. The DNA probe and total RNAs were hybridized, and treated with increasing amounts of S1 nuclease for 15 min at 37 °C. The resulting products were analyzed on a 7% polyacrylamide-8 M urea gel. The ends of mRNAs were identified by using the Maxam-Gilbert A+G and C+T ladders of the DNA probe as references.

RESULTS

*The SecM translation arrest sequence induces tmRNA-mediated protein tagging at sense codons*

A 17 amino acid segment within SecM protein, FXXXXWIXXXXGIRAGP (AS17), was identified as an element to cause ribosome stalling (14). A striking feature of this specific segment is that it can inhibit translation elongation even when present within unrelated sequences. This provides us an opportunity to investigate a link between ribosome stalling during translation elongation and tmRNA tagging of the nascent peptide. We first constructed a plasmid (pJK217) carrying a *crp-crr* fusion gene encoding CRP-AS17-IIA\(^{\text{Glc}}\) protein in which CRP (cAMP receptor protein) and IIAGlc ORFs are connected by a nucleotide sequence corresponding to AS17 (Fig. 1). Another plasmid (pJK216) carrying a fusion gene encoding CRP-AS17 was also constructed, in which a stop codon was placed just after the AS17 sequence. We also used plasmids pST602 and pJK107 that express CRP-GP and CRP-GP-IIA\(^{\text{Glc}}\), respectively. An advantage of the *crp-crr* fusion system is that both tmRNA tagging and mRNA cleavage can be easily monitored by Western and Northern analyses, respectively. These plasmids were introduced into three isogenic strains regarding the *ssrA* allele and the expression of proteins was analyzed by
Western blotting using anti-CRP antibodies. It is established that a C-terminal GP sequence causes an efficient tmRNA tagging at a stop codon (12,13). Accordingly, CRP-AS17 protein is expected to be efficiently tagged by the tmRNA system because this CRP variant possesses a terminal GP sequence. In fact, CRP-AS17 was highly expressed in the absence of tmRNA while it is markedly reduced in the presence of the wild-type tmRNA due to an efficient tagging and proteolysis (Fig. 2, lanes 7 and 8). When a mutant tmRNA-DD encoding a protease-resistant tag sequence was co-expressed, the DD-tagged CRP-AS17 was produced (Fig. 2, lane 9). These results are essentially the same as those observed in the expression of CRP-GP (Fig. 2, lanes 1-3). We showed previously that the conversion of the stop codon after CRP-GP ORF to a sense codon completely eliminated the tmRNA tagging (1). Thus, CRP-GP-IIA\textsuperscript{Glc} fusion protein encoded by plasmid pJK107 is stably expressed both in the absence and the presence of tmRNA without tmRNA tagging (Fig. 2, lanes 4-6). Interestingly, the truncated CRP and its tagging occurred efficiently even when the stop codon of CRP-AS17 was converted to a sense codon (Fig. 2, lanes 10-12). Namely, the fusion gene encoding CRP-AS17-IIA\textsuperscript{Glc} produced a truncated protein corresponding to CRP-AS17 along with a small amount of the full-length CRP-AS17-IIA\textsuperscript{Glc} protein (Fig. 2, lane 10). In the presence of the wild-type tmRNA, the truncated protein was no longer observed while a significant amount of DD-tagged protein was detected in the presence of tmRNA-DD (Fig. 2, lanes 11 and 12). These results imply that the AS17 sequence causes ribosome stalling not only at a stop codon but also at a sense codon by inhibiting translation elongation.

The pentapeptide (IRAGP) is sufficient to cause tmRNA tagging
Among the 17 amino acid SecM arrest sequence, the last hexapeptide GIRAGP seems to be particularly important to inhibit translation elongation (14). To examine whether this hexapeptide alone could cause translation arrest and tmRNA tagging at a sense codon, we constructed a fusion gene encoding CRP-GIRAGP-IIA^{Glc} protein and analyzed the expression of proteins by Western blotting using anti-CRP antibodies. A significant amount of truncated CRP was generated in the absence of tmRNA and it was efficiently tagged by the tmRNA system, indicating that GIRAGP essentially retains the ability to cause the ribosome stalling during translation (Fig. 2, lanes 13-15). A slight increase in the abundance of the full-length CRP-GIRAGP-IIA^{Glc} protein compared to that of CRP-AS17-IIA^{Glc} is consistent with the results of Nakatogawa and Ito (14) who showed several amino acid residues upstream of GIRAGP affect the efficiency of the arrest. We also constructed fusion genes encoding CRP-IRAGP-IIA^{Glc} and CRP-RAGP-IIA^{Glc} proteins, respectively, and tested their expression in the presence and absence of tmRNA. An efficient production of truncated CRP and its tagging was still observed when CRP-IRAGP-IIA^{Glc} (Fig. 2, lanes 16-18) but not CRP-RAGP-IIA^{Glc} (Fig. 2, lanes 19-21) was expressed. No particular amino acid residues corresponding to the consensus arrest sequence originally identified (14) were found in the immediate N-terminal side of the IRAGP segment in CRP-IRAGP-IIA^{Glc} protein. We conclude that the last pentapeptide (IRAGP) of the SecM arrest sequence is sufficient to cause the ribosome stalling during translation elongation. We also observed that the identity of the amino acid residue following the arrest sequence does not affect the production of truncated CRP and its tagging (data not shown).
Identification of the C-terminus of truncated CRP and tagging site

To determine the C-terminus of truncated CRP and tagging site, CRP proteins were purified from cells carrying pJK208 by immuno-precipitation using anti-CRP antibodies conjugated to agarose beads. The purified proteins were subjected to SDS-polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining (Fig. 3A). The profile of the purified proteins was essentially the same as that of Western blot analysis shown in Fig. 2. Interestingly, both truncated untagged and DD-tagged CRP proteins consisted of two bands. 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. Representative data for the mass spectrum are shown in Fig. 3B. The upper band of untagged proteins gave two specific signals that correspond to those expected for the C-terminal fragments of CRP-GIRAG and CRP-GIRAGP. The last Pro residue was identified as the translation arrest point during translation of SecM (14). Thus, the truncation of the fusion protein occurs immediately before and after the arrest point. The lower untagged band produced two specific signals corresponding to the expected C-terminal fragments of CRP-G and CRP. This implies that the truncation occurred also before the arrest point. As expected, the DD-tagged bands gave specific signals that correspond to junction peptides containing the C-terminal fragments plus the tag.

The arrest-causing sequence induces mRNA cleavage

We demonstrated previously that the ribosome pausing at stop codons caused by certain nascent peptides generates nonstop mRNAs through
mRNA cleavage within the stalled ribosome and that this cleavage is responsible for the trans-translation at stop codons (1). It is highly possible that the similar cleavage reaction occurs when translation elongation is inhibited by the SecM arrest sequence. To test this, total RNAs were prepared from cells expressing various fusion proteins both in the presence and absence of tmRNA and analyzed by Northern blotting. When a crp-crr fusion gene is normally expressed without mRNA cleavage, a full-length crp-crr transcript of about 1200 nt should be detected. On the other hand, when the SecM arrest sequence induces mRNA cleavage, both upstream crp mRNA and downstream crr mRNA would be produced. Northern blot analysis using a DNA probe specific to the crp mRNA clearly demonstrated that a significant amount of truncated crp mRNA was produced along with a lesser amount of the full-length crp-crr mRNA when the fusion gene encoding CRP-AS17-IIA\textsuperscript{Glc} is expressed in the absence of tmRNA (Fig. 4, lane 1). The short crp mRNA was dramatically reduced in the presence of tmRNA, indicating that tmRNA facilitates degradation of the truncated mRNA (Fig. 4, lane 2). Essentially the same results were obtained when RNAs from genes encoding CRP-GIRAGP-IIA\textsuperscript{Glc} or CRP-IRAGP-IIA\textsuperscript{Glc} were analyzed although the relative abundance of truncated crp mRNA to the full-length crp-crr mRNA was slightly reduced (Fig. 4, lanes 3-6). On the other hand, only the full-length crp-crr mRNA was produced both in the presence and absence of tmRNA when the fusion gene encoding CRP-RAGP-IIA\textsuperscript{Glc} or CRP-GP-IIA\textsuperscript{Glc} was expressed (Fig. 4, lanes 7-10). Thus, the production of the shorter truncated crp mRNA occurs only when the elongation arrest sequence was placed between CRP and II\textsuperscript{Glc} ORFs. We conclude that mRNA cleavage occurred as a result of ribosome stalling by the arrest sequence during translation elongation. The truncated mRNAs
would be recognized normally by tmRNA, released from the stalled ribosome and rapidly degraded by 3’ to 5’ exonucleases. It should be noted that the level of the full-length crp-crr mRNA is also markedly reduced in the presence of tmRNA (Fig. 4, lanes 1-6). This suggests that the mRNA cleavage is enhanced by tmRNA.

**Determination of the 3’ end of the upstream cleavage product**

To determine the 3' end of the truncated crp mRNA, an S1 analysis was performed by using a DNA probe that corresponds to the junction region of the crp-crr gene encoding CRP-GIRAGP-IIA\textsuperscript{Glc}. Total RNAs prepared from cells expressing CRP-GIRAGP-IIA\textsuperscript{Glc} were hybridized with the DNA probe C\textsuperscript{32}P-labeled at its 3’ end. The hybrids were treated with S1 nuclease and the products were analyzed by electrophoresis on a sequencing gel. As shown in Fig. 5, several clusters of S1-resistant bands (referred to as I to V) were detected when RNAs from cells without tmRNA were used (Fig. 5, lane 3). The abundance of these bands except the band V was markedly reduced when RNAs from cells with tmRNA were used (Fig. 5, lane 4). The cluster III is mapped around the Pro codon of GIRAGP sequence that corresponds to the A-site when ribosome stalls at this position while the cluster IV is located 12-15 nt upstream of the arrest point. The location of the cluster III and IV well correspond to the C-terminus of the truncated CRP demonstrated by the mass analysis. It is possible that the cluster IV-ended message would be produced by a second ribosome that was forced to stall by the SecM-arrested ribosome in front of it. The clusters I and II were mapped about 11-19 nt downstream from the arrest point. These results suggest that mRNA cleavage occurs at several points within/and near stalled ribosome. The cluster V appears to be non-specific because it was
detected in cells without the fusion plasmid (Fig. 5, lane 5).

Identification of the downstream cleavage product

To obtain additional evidence for mRNA cleavage, we performed Northern blot analysis by using a crr DNA probe. When CRP-AS17-IIA\textsuperscript{Glc}, CRP-GIRAGP-IIA\textsuperscript{Glc}, or CRP-IRAGP-IIA\textsuperscript{Glc} but not CRP-RAGP-IIA\textsuperscript{Glc} CRP-GP-IIA\textsuperscript{Glc} was expressed, an RNA band of about 550 nt corresponding to the downstream crr mRNA was detected along with the crp-crr mRNA (Fig. 6). These results are expected when endonucleolytic cleavage of the full-length crp-crr mRNA occurs in response to the ribosome stalling caused by the SecM arrest sequence during translation elongation. The abundance of the crr band increased while the level of the full-length crp-crr mRNA decreased in the presence of tmRNA when the cleavage positive mRNAs are expressed (Fig. 6, lanes 1-6). This again suggests that the mRNA cleavage occurs more efficiently in the presence of tmRNA.

Determination of the 5' end of the downstream cleavage product

An S1 analysis was performed to determine the 5' end of the truncated crr mRNA. Total RNAs prepared from cells expressing CRP-GIRAGP-IIA\textsuperscript{Glc} were hybridized with the probe D\textsuperscript{32}P-labeled at its 5' end. The hybrids were treated with S1 nuclease and the products were analyzed by electrophoresis on a sequencing gel. In contrast to the 3' end mapping, only one major S1-resistant band was detected and the abundance of this band increased in the presence of tmRNA as expected (Fig. 7, lanes 3 and 4). Interestingly, the 5' end of this band was mapped about 15 nt upstream of the arrest point while no signal corresponding to the Pro codon of GIRAGP.
sequence was detected. This suggests again that the mRNA cleavage occurs at several points within/and near stalled ribosome and some downstream cleavage products are very unstable.

_The mRNA cleavage occurs in cells lacking either RelE, RNase E, RNase G, or RNase III_

A bacterial toxin RelE induces endonucleolytic cleavage of mRNAs bound to ribosomes at specific sites including stop codons in response to a stalled ribosome (19). To examine whether RelE is involved in the mRNA cleavage caused by the arrest sequence during elongation, total RNAs prepared from the ΔrelEB cells expressing CRP-GIRAGP-IIA Glc were analyzed by Northern blotting. The disruption of the relEB did not affect the generation of truncated _crp_ and _crr_ mRNAs (Fig. 8, lanes 1-3), suggesting that RelE is not responsible for the mRNA cleavage in response to ribosome stalling. We also tested the effects of mutations in genes encoding RNase E, RNase G, and RNase III on the cleavage reaction. None of these mutations affected the generation of truncated _crr_ mRNA (Fig. 8, lanes 4-6), indicating that these major endonucleases are also not responsible for the cleavage reaction.

**DISCUSSION**

The major finding of the present study is that inhibition of translation elongation by the SecM arrest sequence generates nonstop mRNAs through mRNA cleavage around the stalled ribosome. This conclusion is derived from the following observations: 1) the 17 amino acid SecM arrest
sequence placed between CRP and IIAGle ORFs leads to truncation of the fusion protein and their tagging by the tmRNA system; 2) the last 5 amino acid segment of the SecM arrest sequence is sufficient to cause the same effect; 3) the truncation and tagging occur around the arrest sequence; 4) truncated crp and crr mRNAs are generated when the protein truncation occurs; 5) the 3' ends of the truncated crp mRNA well correspond to the C-termini of the truncated CRP proteins.

A plausible model for mRNA cleavage induced by ribosome stalling at the SecM arrest sequence is shown in Fig. 9. How does ribosome stalling induce the mRNA cleavage? Nakatogawa and Ito provided convincing genetic evidence that the SecM segment causes elongation arrest by inhibiting movement of the translation product through the interaction with components of the exit tunnel of ribosome (14). Although how the interaction between the arrest sequence and the exit tunnel leads to elongation arrest is not worked out yet, it is possible that the A-site of ribosome becomes empty for a while, which in turn allows endonucleolytic attack around A-site when elongation arrest occurs. Once the mRNA cleavage occurs, the ribosome would be located at the 3' end of the truncated mRNA and accordingly is recognized by the tmRNA system. As a result, the stalled ribosome is rescued and the truncated peptide is tagged for degradation. In addition, the tmRNA-mediated trans-translation leads to a rapid degradation of truncated mRNAs by removing stalled ribosomes and thus allowing 3'-to-5' exonucleases to access the free mRNA 3' ends (6). The rapid degradation of truncated mRNAs prevents further the production of aberrant polypeptides. Although we can not exclude the possibility that tmRNA could also act without mRNA cleavage after the elongation arrest by the SecM arrest sequence, it is likely that tmRNA could enter the A-site
only after the mRNA cleavage. In other words, the primary event that occurs after the elongation arrest would be mRNA cleavage rather than tmRNA entry.

We observed that the endonucleolytic cleavage of mRNA also occurs when ribosomes stall at a run of AGG rare arginine codon (unpublished results). In the previous study, we demonstrated that ribosome pausing at stop codons caused by certain nascent peptides generates nonstop mRNAs through mRNA cleavage within the stalled ribosome and that this cleavage is responsible for the trans-translation at stop codons (1). Cleavage of mRNA in response to ribosome stalling at stop codons was also found in ybeL mRNA by Hayes and Sauer (2). In addition, several previous reports suggested that mRNA cleavage occurs in response to ribosome stalling. For example, it was reported that ribosome stalling at a stop codon in an artificial gene produced a short mRNA derived from the downstream portion (20). There are two reports proposing mRNA cleavage during translation elongation induced by ribosome stalling. They are the processing of fimbrial mRNA encoded by the daa operon in E. coli (21) and the cleavage of ermC mRNA coding for a ribosomal RNA methyltransferase in Bacillus subtilis (22). Although how the present finding is related to these previous observations remains to be studied, cleavage of mRNA appears to be a general event associated with ribosome pausing during translation under various situations.

We demonstrated that the mRNA cleavage in response to a stalled ribosome does not require tmRNA itself because it occurs in the absence of tmRNA. However, we also found that the mRNA cleavage is enhanced in the presence of tmRNA. This suggests that tmRNA interacts with the stalled ribosome prior to the cleavage and affect the cleavage reaction. In
fact, it is known that a significant fraction of tmRNA is associated with 70S ribosome in cells (23,24). In particular, it should be noted that tmRNA interacts strongly with 16S rRNA and several stretches in tmRNA are complementary to the sequences in 16S rRNA (23). Thus, it is quite possible that tmRNA modulates the conformation of the stalled ribosome through the interaction with 16S RNA to enhance the cleavage reaction. Further study is necessary to understand how tmRNA stimulates the mRNA cleavage reaction within the stalled ribosome.

What is the biological relevance of the mRNA cleavage in response to ribosome stalling during translation? First, it could play a specific regulatory role in certain genes and operons. Second, it could act as an important step in a quality control during protein synthesis. The regulatory role of mRNA cleavage could be exemplified by the SecM translation arrest sequence on which we focused in this study. SecM is a unique secretory protein that monitors cellular activity for protein export to regulate translation of the downstream secA gene (25). The elongation block by the arrest sequence is transient when SecM is normally exported to periplasm. However, it is markedly enhanced when SecM export is impaired either by a defect in its signal sequence or by a defect in the Sec machinery (14). It is believed that the stalled ribosome rearranges the secondary structure of secM-secA mRNA resulting in the opening of the translation initiation region of SecA (25-27). The present study suggests that the ribosome stalling by the arrest sequence disrupts the mRNA secondary structure through mRNA cleavage. However, we have not shown yet whether the similar mRNA cleavage occurs in the native secM-secA mRNA and whether the mRNA cleavage around the arrest point could affect the secondary structure and/or the expression of the secA translation.
These questions remain to be addressed in future studies.

There are a number of systems in which specific regulatory nascent peptides may affect translation elongation and/or termination presumably by interacting with ribosome. In most of these cases, the peptides appear to exert a regulatory role by causing ribosome stalling at specific points. For example, it is known that the 24-residue product of the \textit{tnaC} gene prevents the release of the peptide at the stop codon depending on the availability of tryptophan to regulate the expression of downstream \textit{tna} operon (28). Another example is translation arrest in the chloramphenicol transacetylase gene (\textit{cat}) in gram-positive bacteria by the nascent pentapeptide (MVKTD) that interacts with the ribosome in the presence of chloramphenicol (29). Specific nascent peptides are also involved in the ribosome stalling in the \textit{daa} and \textit{ermC} systems mentioned above. It is likely that a specific mRNA cleavage is induced by ribosome stalling in all of these cases.

The mRNA cleavage in response to ribosome stalling could play a more general role in quality control during protein synthesis. Translation arrest is one of serious problems that cells encounter during protein synthesis. It may occur widely and frequently in cells depending on a variety of factors such as sequence of nascent peptide and amino acid and/or tRNA availability. The translation arrest would be further enhanced in the presence of some drugs. If the ribosome stalling occurs without mRNA cleavage, a large number of translating ribosomes would be trapped on mRNAs and many useless aberrant proteins would accumulate. The mRNA cleavage in response to ribosome stalling certainly allows the cells to avoid this situation because it provides a way by which tmRNA system acts. Namely, the mRNA cleavage preludes to the tmRNA-mediated \textit{trans}-translation reaction that rescues the stalled ribosome, targets the aberrant
polypeptide for degradation, and facilitates degradation of the cleaved mRNA. Thus, the mRNA cleavage in concert with the tmRNA system apparently plays a fundamental role in quality control of protein synthesis in *E. coli* cells. It is certainly interesting to investigate whether the mRNA cleavage in response to ribosome stalling occurs in other bacteria and in eukaryotic cells.

Recently, it was shown that several bacterial toxins induce or exhibit endonucleolytic cleavage of mRNAs with the codon or sequence specificity (19,30-32). For example, RelE induces endonucleolytic cleavage of mRNAs bound to ribosomes at UAA and other codons in response to a stalled ribosome (19). However, the cleavage of *ybeL* mRNA at a stop codon was observed in various toxin-deficient strains including Δ*relE*, Δ*yoeB*, Δ*yafQ*, Δ*mazF*, and Δ*chpBK* (2). We also observed that the disruption of *relEB* gene did not affect the mRNA cleavage in response to ribosome stalling at a stop codon (1) and at a sense codon (Fig. 8). In addition, we showed that the mRNA cleavage in response to ribosome stalling occurs in strains lacking either RNase E, RNase G, or RNase III (Fig. 8). Thus, neither bacterial toxins nor the major endoribonucleases seem to mediate the cleavage reaction. An attractive possibility would be that the RNA and/or protein components of the ribosome are directly involved in the cleavage reaction depending upon ribosome stalling. In any cases, the factors responsible for the mRNA cleavage in response to a stalled ribosome remains to be identified.

**ACKNOWLEDGMENTS**
This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

FIGURE LEGEND

Fig. 1. Schematic drawing of crp-crr fusion genes used in this study. The open and shaded rectangles represent the coding region for CRP and IIA$^{Glc}$, respectively. The black box represents the short variable region between two ORFs. Nucleotide sequences and amino acid sequences (in one-letter symbol) of the junction region are shown below the diagram. The amino acid sequences of the variable region are in bold and the sequences derived from the SecM segment are underlined. DNA probes used for Northern and S1 analyses are shown on the upper part.

Fig. 2. Western blot analysis of proteins expressed from the crp-crr fusion genes. Lysates equivalent to 0.005 OD$_{600}$ unit prepared from TA341 (Δcrp ssrA$^+$), TA501 (Δcrp ΔssrA), or TA481 (Δcrp ssrA$^{DD}$) cells harboring indicated plasmids were analyzed by Western blotting using anti-CRP antibodies. The “peptide” represents the amino acid sequence between CRP and IIA$^{Glc}$ ORFs.

Fig. 3. Mass spectrometry analysis of untagged and tagged CRP proteins generated from the crp-crr fusion gene encoding CRP-GIRAGP-IIA$^{Glc}$. (A) CRP proteins were purified from three isogenic strains carrying pJK208 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 bands corresponding to untagged and tagged CRP proteins, generated from ΔssrA (lane 1) and ssrADD (lane 3) cells, respectively, were cut out from the gel, treated with lysyl endopeptidase and subjected to mass spectrometry analysis. The deduced sequences and molecular weights of the C-terminal fragments, that were identified as specific signals, are shown. The amino acid residues derived from the SecM arrest sequence are shown in bold. The tag sequences derived from tmRNA-DD is underlined. The approximate proportions of the C-terminal fragments in each band are shown in parenthesis. (B) The MALDI/TOF spectra for the lower bands of CRP proteins purified from ΔssrA (top) and ssrADD (bottom) cells. The specific signals corresponding to the C-terminal fragments are shown by arrows. The signals matched to the other CRP fragments are shown by asterisks.

Fig. 4. **Northern blot analysis of crp mRNAs derived from the crp-crr fusion genes.** Total RNAs (0.5 μg) prepared from TA341 (Δcrp ssrA+) and TA501 (Δcrp ΔssrA) cells harboring indicated plasmids were resolved by electrophoresis on a 1.5 % agarose-formaldehyde gel. Northern blot analysis was performed using the DIG-labeled crp probe A. RNA bands corresponding to the full-length and truncated crp mRNAs are shown by arrows. Lane M represents RNA size markers.

Fig. 5. **Determination of 3' ends of the crp mRNAs.** Total RNAs (50 μg) prepared from TA341 (Δcrp ssrA+) and TA501 (Δcrp ΔssrA) cells harboring pJK208 were hybridized with the single stranded DNA probe C32P-labeled at its 3’ end, and the hybrids were treated with indicated
amounts of S1 nuclease. The products were dissolved in 20 µl of loading buffer (98% formamide, 0.025 % bromophenol blue, 0.025 % xylene cyanol, and 10 mM EDTA) and 5 µl of each sample was analyzed on a 7% polyacrylamide-8M urea gel along with products of A+G and C+T chemical sequencing reaction of the fragment. The lane 5 is the result when RNAs from TA501 (Δcrp ΔssrA) cells harboring a control plasmid pBR322 were analyzed. Both nucleotide and amino acid sequences around the GIRAGP sequence (bold letter and boxed) are shown on the right. The asterisks are the 3' ends identified by the S1 analysis.

Fig. 6. **Northern blot analysis of crr mRNAs derived from the crp-crr fusion genes.** Total RNAs (1.5 µg) prepared from TA341 (Δcrp ssrA+) and TA501 (Δcrp ΔssrA) cells harboring indicated plasmids were resolved by electrophoresis on a 1.5 % agarose-formaldehyde gel. Northern blot analysis was performed using the DIG-labeled crr probe B. RNA bands corresponding to the full-length crp-crr and truncated crr mRNAs are shown by arrows. Lane M represents RNA size markers.

Fig. 7. **Determination of 5' ends of the crr mRNAs.** Total RNAs (50 µg) prepared from TA341 (Δcrp ssrA+) and TA501 (Δcrp ΔssrA) cells harboring pJK208 were hybridized with the single stranded DNA probe D ³²P-labeled at its 5' end, and the hybrids were treated with indicated amounts of S1 nuclease. The products were dissolved in 20 µl of loading buffer (98% formamide, 0.025 % bromophenol blue, 0.025 % xylene cyanol, and 10 mM EDTA) and 5 µl of each sample was analyzed on a 7% polyacrylamide-8M urea gel along with products of A+G and C+T chemical sequencing reaction of the fragment. The lane 5 is the result when
RNAs from TA501 (Δcrp ΔssrA) cells harboring a control plasmid pBR322 were analyzed. Both nucleotide and amino acid sequences around the GIRAGP sequence (bold letter and boxed) are shown on the right. The asterisks are the 5' ends identified by the S1 analysis.

Fig. 8. Northern blot analyses of crp and crr mRNAs derived from cells lacking either RelE, RNase E, RNase G, or RNase III. Total RNAs were prepared from ST100 (ΔrelEB), ST101 (ΔssrA ΔrelEB), GW20 (rneEs), GW11 (rng), and ST201 (rnc) cells harboring pJK208 under the standard condition. Total RNAs were prepared from GW20 (rneEs) harboring pJK208 cells 10 min after the temperature shift (30 °C to 42°C). RNAs (0.5 μg: lanes 1 and 2 or 1.5 μg: lanes 3-6) were resolved by electrophoresis on a 1.5 % agarose-formaldehyde gel. Northern blot analysis was performed using the DIG-labeled crp probe A and crr probe B. RNA bands corresponding to the full-length crp-crr and truncated crp and crr mRNAs are shown by arrows.

Fig. 9. Model for mRNA cleavage induced in response to ribosome stalling caused by the SecM arrest sequence.
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Table 1. Bacterial strains and plasmids used in this study.

| Strain/plasmid | Relevant genotype and property | Source |
|----------------|--------------------------------|--------|
| Strain         |                                |        |
| W3110          | Wild-type                      | Laboratory stock |
| TA341          | W3110 Δcrp                     | Ref. 1 |
| TA501          | W3110 Δcrp ΔssrA               | Ref. 1 |
| TA481          | W3110 Δcrp ssrA<sup>OD</sup>   | Ref. 1 |
| ST100          | W3110 ΔrelEB                   | Ref. 1 |
| ST101          | W3110 ΔrelEB ΔssrA             | Ref. 1 |
| GW20           | W3110 am<sub>5</sub>-I<sub>zce</sub>-726::Tn10 | Ref. 33 |
| GW11           | W3110 rng::cat zce-726::Tn10   | Ref. 33 |
| ST201          | W3110 rnc-14::Tn10             | This work |
| HT27           | N4903 rnc-14::Tn10             | Ref. 34 |
| Plasmid        |                                |        |
| pHA7           | Derivative of pBR322 carrying the *crp* expressed from the *bla* promoter | Ref. 16 |
| pHA7M          | Derivative of pHA7 in which a *Mlu*I site is introduced near the last codon of *crp* | Ref. 7 |
| pIZ3A          | Derivative of pHA7 in which the *crr* gene is placed downstream of *crp* | Ref. 9 |
| pST602         | Derivative of pHA7 carrying the *crp-crr* fusion gene encoding CRP-GP | Ref. 1 |
| pJK107         | Derivative of pHA7 carrying the *crp-crr* fusion gene encoding CRP-GP-IIA<sup>Glc</sup> | Ref. 1 |
| pJK216         | Derivative of pHA7 carrying the *crp-crr* fusion gene encoding CRP-AS17 | This work |
| pJK217         | Derivative of pHA7 carrying the *crp-crr* fusion gene encoding CRP-AS17-IIA<sup>Glc</sup> | This work |
| pJK208         | Derivative of pHA7 carrying the *crp-crr* fusion gene encoding CRP-GIRAGP-IIA<sup>Glc</sup> | This work |
| pJK220         | Derivative of pHA7 carrying the *crp-crr* fusion gene encoding CRP-IRAGP-IIA<sup>Glc</sup> | This work |
| pJK219         | Derivative of pHA7 carrying the *crp-crr* fusion gene encoding CRP-RAGP-IIA<sup>Glc</sup> | This work |
plasmid protein

pST602  CRP-GP  GGCACGCGTGGCCCTTAAGGTACCCCT--
         GTRGP*GT

pJK107  CRP-GP-IIA^{Glc}  GGCACGCGTGGCCCTAAAAGGTACCCCT--
                     GTRGPKGT

pJK216  CRP-AS17  GGCACCGGTGTCAGCACGCCCTGCTGGATAAGCCAGGCGCAAGGCATCCGTGCTGGCCCTTGAGGTACCCCT--
        GTRFSTPVSQAQGIRAGPKGT

pJK217  CRP-AS17-IIA^{Glc}  GGCACCGGTGTCAGCACGCCCTGCTGGATAAGCCAGGCGCAAGGCATCCGTGCTGGCCCTAAAGGTACCCCT--
                     GTRFSTPVSQAQGIRAGPKGT

pJK208  CRP-GIRAGP-IIA^{Glc}  GGCACCGGTGTCATCCGTGCTGGCCCTAAAAGGTACCCCT--
                      GTRGIRAGPKGT

pJK220  CRP-IRAGP-IIA^{Glc}  GGCACCGGTATCCGTGCTGGCCCTAAAAGGTACCCCT--
                      GTRIRAGPKGT

pJK219  CRP-RAGP-IIA^{Glc}  GGCACCGGTGCTCCGTGGCCCTAAAAGGTACCCCT--
                      GTRRAGPKGT

Fig. 1. Sunohara et al.
| plasmid peptide | pST602 GP-stop | pJK107 GP | pJK216 AS17-stop | pJK217 AS17 | pJK208 GIRAGP | pJK220 IRAGP | pJK219 RAGP |
|-----------------|----------------|-----------|------------------|-------------|---------------|-------------|-------------|
| tmRNA            | - AA DD        | - AA DD   | - AA DD          | - AA DD     | - AA DD       | - AA DD     | - AA DD     |

CRP-IIAGlc

DD-tagged CRP

CRP

Fig. 2. Sunohara et al.
Fig. 3. Sunohara et al.
| plasmid | peptide | tmRNA | crp-crr mRNA | crp mRNA |
|---------|---------|-------|--------------|----------|
| pJK217  | AS17    | -     | -            | -        |
| pJK208  | GIRAGP  | -     | -            | -        |
| pJK220  | IRAGP   | -     | -            | -        |
| pJK219  | RAGP    | +     | -            | -        |
| pJK107  | GP      | +     | -            | -        |

Fig. 4. Sunohara et al.
| plasmid peptide | pJK217 | pJK208 | pJK220 | pJK219 | pJK107 |
|----------------|--------|--------|--------|--------|--------|
| tmRNA          | -      | +      | -      | +      | -      |
| crp-crr mRNA   |        |        |        |        |        |
| crr mRNA       |        |        |        |        |        |
|                | 1      | 2      | 3      | 4      | 5      |
| nt             | 1517   | 1049   | 575    | 483    | 310    |

Fig. 6. Sunohara et al.
Fig. 8. Sunohara et al
The SecM arrest sequence (red) interferes translation elongation. Degradation of nonstop mRNA, Tagging and degradation of aberrant polypeptide, Recycling of stalled ribosome.

Fig. 9. Sunohara et al.
Ribosome stalling during translation elongation induces cleavage of mRNA being translated in Escherichia coli
Takafumi Sunohara, Kaoru Jojima, Hideaki Tagami, Toshifumi Inada and Hiroji Aiba

J. Biol. Chem. published online January 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M312805200

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