**Prolyl-tRNA\textsuperscript{Pro} in the A-site of SecM-arrested Ribosomes Inhibits the Recruitment of Transfer-messenger RNA**

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Translational pausing can lead to cleavage of the A-site codon and facilitate recruitment of the transfer-messenger RNA (tmRNA) (SsrA) quality control system to distressed ribosomes. We asked whether aminoacyl-tRNA binding site (A-site) mRNA cleavage occurs during regulatory translational pausing using the *Escherichia coli* SecM-mediated ribosome arrest as a model. We find that SecM ribosome arrest does not elicit efficient A-site cleavage, but instead allows degradation of downstream mRNA to the 3'-edge of the arrested ribosome. Characterization of SecM-arrested ribosomes shows the nascent peptide is covalently linked via glycine 165 to tRNA\textsubscript{Gly} in the peptidyl-tRNA binding site, and prolyl-tRNA\textsubscript{Pro} is bound to the A-site. Although A-site-cleaved mRNAs were not detected, tmRNA-mediated ssrA tagging after SecM glycine 165 was observed. This tmRNA activity results from sequestration of prolyl-tRNA\textsubscript{Pro} on overexpressed SecM-arrested ribosomes, which produces a second population of stalled ribosomes with unoccupied A-sites. Indeed, compensatory overexpression of tRNA\textsubscript{Pro} readily inhibits ssrA tagging after glycine 165, but has no effect on the duration of SecM ribosome arrest. We conclude that, under physiological conditions, the architecture of SecM-arrested ribosomes allows regulated translational pausing without interference from A-site cleavage or tmRNA activities. Moreover, it seems likely that A-site mRNA cleavage is generally avoided or inhibited during regulated ribosome pauses.

A-site\textsuperscript{3} mRNA cleavage is a novel RNase activity that acts on A-site codons within paused ribosomes. Ehrenberg, Gerdes and their colleagues (1) first demonstrated that *Escherichia coli* RelE protein causes cleavage of A-site mRNA in vitro. Subsequently, A-site cleavage was also shown to occur at stop codons during inefficient translation termination in cells that lack RelE and related proteins (2, 3). The latter finding indicates that another unknown A-site nuclease also exists in *E. coli*. Indeed, it is possible the ribosome itself catalyzes A-site cleavage. The molecular requirements for A-site cleavage are incompletely understood, but an unoccupied ribosome A site appears to be important for both RelE-dependent and RelE-independent nuclease activity (1, 2).

A-site nuclease activity truncates mRNAs and produces stalled ribosomes that are unable to continue standard translation. In bacteria, ribosomes stalled at the 3' termini of truncated messages are "rescued" by the tmRNA quality control system. tmRNA is a specialized RNA that acts first as a tRNA to bind the A-site of stalled ribosomes, and then as an mRNA to direct the addition of the ssrA peptide degradation tag to the C terminus of the nascent polypeptide (4, 5). As a result of tmRNA activity, incompletely synthesized proteins are targeted for proteolysis and stalled ribosomes undergo normal translation termination and recycling (5). In this manner, A-site mRNA cleavage and tmRNA work together as a translational quality control system that responds to paused and stalled ribosomes.

Although a paused ribosome can be a manifestation of translational difficulty, translational pausing is also used to control and regulate protein synthesis. In many instances, the newly synthesized nascent peptide inhibits either translation elongation or termination (6, 7). A recently described example is the SecM-mediated ribosome arrest, which controls expression of SecA protein from the secM-secA mRNA in *E. coli* (8). The SecM nascent peptide interacts with the ribosome exit channel to elicit a site-specific ribosome arrest (9). The SecM-stalled ribosome is postulated to disrupt a downstream mRNA secondary structure that sequesters the secA ribosome binding site (9, 10). Thus, efficient initiation of secA translation depends upon ribosome pausing at the upstream secM open reading frame (11). SecM-mediated ribosome pausing is regulated in turn by the activity of SecA protein. SecM is secreted co-translationally by the general Sec machinery, which is powered in part by the SecA ATPase (12). It is thought that the mechanical pulling force exerted by SecA on the SecM nascent chain during secretion alleviates the ribosome arrest and allows translation to continue (13, 14). This intriguing regulatory circuit allows the cell to monitor protein secretion activity via ribosome pausing and adjust SecA synthesis accordingly.

One outstanding question is how A-site cleavage and tmRNA activities affect regulatory translational pauses such as the SecM-mediated ribosome arrest. If all paused ribosomes are subject to A-site cleavage, then this nuclease activity would be expected to interfere with SecA regulation. The experiments presented in this paper demonstrate that A-site mRNA cleavage and the tmRNA quality control system do not significantly affect SecM-mediated ribosome arrest. Two recent reports...
have demonstrated that the A-site of SecM-arrested ribosomes is filled with tRNA (15, 16). The cryo-EM structure from Frank and colleagues (15) shows that ~40% of SecM-arrested ribosomes contain a fully accommodated A-site tRNA. Ito and colleagues (15) shows that the A-site of SecM-arrested ribosomes is filled with tRNA (15, 16). The cryo-EM structure from Frank and colleagues (15) shows that ~40% of SecM-arrested ribosomes contain a fully accommodated A-site tRNA. Ito and colleagues (15) shows that the A-site of SecM-arrested ribosomes is filled with tRNA (15, 16). The cryo-EM structure from Frank and colleagues (15) shows that ~40% of SecM-arrested ribosomes contain a fully accommodated A-site tRNA. In our analysis of SecM-arrested ribosomes in vivo, we also find that the P- and A-sites of the SecM-arrested ribosome are occupied with peptidyl- and aminoacyl-tRNAs, respectively. Additionally, we show that the occupied A-site prevents tmRNA recruitment during ribosome arrest and may also inhibit A-site mRNA cleavage. Thus, regulation by SecM ribosome arrest is able to operate efficiently in the presence of quality control systems that alleviate ribosome stalling.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—Table 1 lists the bacterial strains and plasmids used in this study. All bacterial strains were derivatives of *E. coli* strain X90 (17). Strain CH12 (X90 (DE3)) was generated using the Novagen (DE3) kit according to the manufacturer’s instructions. Strain CH2198 (X90 ssa::his6 (DE3)) was obtained by introducing the ssa::his6 allele (18) of tmRNA into the ssa chromosomal locus using the phage λ Red recombination method with minor modifications (17, 19). The same method was used to delete the rna (encoding RNase I), rnb (encoding RNase II), and pnp (encoding PNPase) genes. The rnr::kan disruption and the strain expressing truncated RNase E have been described previously (2, 20). All gene disruptions and deletions were introduced into strain CH113 by phage P1-mediated transduction. The kanamycin resistance cassette was removed from strain CH113 Δrnb::kan using FLP recombinase as described (19), allowing construction of the Δrnb rnr::kan double mutant. Lac− strains of X90 and X90 ssa::cat were obtained by curing the strain of the F’ episome as described (17). The details of all strain constructions are available upon request.

Plasmid pFG21b is a modified version of plasmid pET21d (Novagen), which encodes a FLAG peptide epitope between NcoI and NdeI restriction sites. Plasmid pFG21b allows the production of N-terminal NcoI restriction sites. Plasmid pFG21b allows the production of N-terminal FLAG-tagged proteins, provided the initiation Met codon is engineered into an NdeI restriction site. All expression plasmids were derived from plasmid pFG21b, with the exception of the LacZ translational fusions, which were constructed using a derivative of pTrc99a (GE Healthcare). Fragments containing secMA’ were obtained by PCR using oligonucleotide primers containing restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues). The secMA’ fragment was amplified from *E. coli* genomic DNA with oligonucleotides secM-Nde (5’-GGATGCGATCATATGATGGTGAATCC) in combination with restriction endonuclease sites (underlined residues).
Inhibition of tmRNA Activity during SecM Ribosome Arrest

Plasmid pTrxA-SecM'-TrxA was constructed from pFG21b in three steps. Two distinct trxA-containing PCR products were generated: the first from primers trxA-Nde (5'-GGTGAAGTTATTATGGGTATCAGC-NTCCGAGCTCCGCTTGAGGGAATTCC-3') and trxA-Bam (5'-GAACTCGAGTAATCCCTGACAGCTAGCGAATTTCC-3'); and the second from primers trxA-Eco (5'-ATAGATTACGCCGGAAATATTCACACTACC-3') and trxA-Xho (5'-GAATTACCGAGTAATCCCTGACAGCTAGCGAATTTCC-3'). The two trxA PCR fragments were sequentially ligated into pFG21b using NdeI and EcoRI/XhoI digestions to generate plasmid pTrx-Trx. The oligonucleotides ts-top (5'-GATCCATATTCAACGCGTCGCTGGTATCTGGGCGTGCTG-3') and ts-bottom (5'-AACATTGAGGGGCAAGCAGTCGATGCGTCCCGAACGACGG-3') were annealed to one another and ligated to BamHI/EcoRI-digested pTrx-Trx to generate pTrx-SecM'.

The two oligonucleotides were ligated to BamHI/EcoRI/XhoI-digested plasmid pTrx-Trx. After further incubation for 30 min, 15 ml of ice-cold methanol was added to the cultures, the cells collected by centrifugation, and the cell pellets frozen at 80 °C. Culture supernatants and pellets for analysis were resuspended at an optical density at 660 nm (OD660) of 0.3, mRNA expression was induced with isopropyl β-D-thiogalactopyranoside (1.5 mM). After further incubation for 30 min, 15 ml of ice-cold methanol was added to the cultures, the cells collected by centrifugation, and the cell pellets frozen at −80 °C. Total RNA was extracted from cell pellets using 1 ml of a solution containing 0.6 M ammonium isoiodcyanate, 2 M guanidinium thiocyanate, 0.1 M sodium acetate (pH 4.0), 5% glycerol, 40% phenol. The disrupted cell suspension was extracted with 0.2 ml of chloroform, the aqueous phase removed and added to an equal volume of isopropyl alcohol to precipitate total RNA. RNA pellets were washed once with ice-cold 75% ethanol and dissolved in 0.5× Tris-HCl (pH 7.5), 1 mM EDTA or 10 mM sodium acetate (pH 5.2), 1 mM EDTA.

Northern blot and S1 nuclease protection analyses of all mRNAs were performed as described (2). Northern blot analysis to identify the nascent peptidyl-tRNA species was performed using acid urea gels as described (22) with modifications. Total RNA (10 µg) was separated on 50% urea, 100 mM sodium acetate (pH 5.2), 1 mM EDTA, 6% polyacrylamide gels run at 4 °C. Gels were briefly soaked in 0.5× Tris borate-EDTA (TBE) buffer before electro blotting (250 mA) to positively charged nylon membrane in 0.5× TBE for 1 h at 4 °C. All subsequent Northern hybridization conditions were as described (2). The following radiolabeled DNA oligonucleotide probes were used in hybridizations: proL for tRNA3pro (5'-CACCCCATGACGGTGCG); proK for tRNA3pro (5'-CTCGTCCCAGCAAGAAGGTG); gyv for tRNA3gyv (5'-CTTGCGAACGTCGTGCT); argQ for RNA4P3 (5'-CCTCGGACGCTCGTGG); and RBS for pET-derived ribosome binding site (5'-GTATATCTCTTCTTATAAGTTAAAC). The following radiolabeled DNA oligonucleotides were used as probes for S1 nuclease protection experiments: secM1 probe (5'-TATATAAATGAAAGTGTTATTTGTTAAGTGAGGCGTGTAAGGGCCAGCAGTGCCCGGAATTTCC-3') and secM'-trxA S1 probe (5'-CTGTCGTAGTCTGAGATTTATTTAATCGACTTCGAACGATCGCCGCTGTGATCGGCGCTGTGATCGGCGCTGTG-3').
RESULTS

SecM Ribosome Arrest Leads to mRNA Cleavage—To determine whether SecM-mediated ribosome arrest leads to A-site cleavage, we generated plasmids to express mRNA encoding SecM and the first 62 residues of SecA (Fig. 1A). Three SecM variants were used throughout this work: (i) FLAG-SecM, which is the wild-type protein fused to an N-terminal FLAG epitope tag; (ii) FLAG-(Δss)SecM, which lacks the secretion signal sequence (residues 1–37); and (iii) FLAG-(Δss-P166A)SecM, which lacks the signal sequence and has alanine in place of proline 166. Deletion of the SecM signal sequence prevents its secretion and leads to a profound ribosome arrest, whereas the P166A variant completely abrogates arrest (9, 14). The FLAG sequence was added to facilitate analysis of SecM proteins by Western blot. However, secretion of FLAG-SecM protein resulted in the removal of the FLAG epitope along with the signal sequence (see below).

Each SecM protein was expressed in wild-type cells (tmRNA+), and cells that lack tmRNA (ΔtmRNA), and the corresponding messages examined by Northern blot analysis. In addition to the full-length mRNAs, truncated flag-secMA and flag-(Δss)secMA messages were also detected (Fig. 1B). The truncated mRNAs did not hybridize to a probe specific for the downstream secA sequence (data not shown). No truncated flag-(Δss-P166A)secMA mRNA was apparent, suggesting that ribosome arrest was required for mRNA cleavage. Interestingly, steady state levels of truncated flag-secMA and flag-(Δss)secMA mRNA were similar in wild-type and ΔtmRNA cells (Fig. 1B). This finding was noteworthy because tmRNA activity usually promotes rapid degradation of truncated mRNAs, including those produced by A-site mRNA cleavage (2, 3, 24). Moreover, the truncated mRNAs appeared to be somewhat larger than in vitro transcripts that terminate in the codon for glutamine 167, a position that is adjacent to the A-site of the arrested ribosome (Fig. 1A and B) (16).

The 3′ ends of the truncated messages were mapped more precisely using S1 nuclease protection analysis. Cleavages were detected in the secM stop codon and at positions 1–4 nucleotides downstream. No S1 protection was detected with RNA purified from a strain that had not been induced with isopropyl β-D-thiogalactopyranoside (IPTG). Truncated and full-length transcripts were produced by in vitro transcription and analyzed by S1 nuclease protection. The HphI and Sau96I oligonucleotide standards were generated by annealing the 3′-labeled S1 probe to a complementary DNA oligonucleotide followed by digestion with the appropriate endonucleases.

a probe specific for the ribosome binding site upstream of secM. In addition to the full-length mRNAs, truncated flag-secMA and flag-(Δss)secMA messages were also detected (Fig. 1B). The truncated mRNAs did not hybridize to a probe specific for the downstream secA sequence (data not shown). No truncated flag-(Δss-P166A)secMA mRNA was apparent, suggesting that ribosome arrest was required for mRNA cleavage. Interestingly, steady state levels of truncated flag-secMA and flag-(Δss)secMA mRNA were similar in wild-type and ΔtmRNA cells (Fig. 1B). This finding was noteworthy because tmRNA activity usually promotes rapid degradation of truncated mRNAs, including those produced by A-site mRNA cleavage (2, 3, 24). Moreover, the truncated mRNAs appeared to be somewhat larger than in vitro transcripts that terminate in the codon for glutamine 167, a position that is adjacent to the A-site of the arrested ribosome (Fig. 1, A and B) (16).

The 3′ ends of the truncated messages were mapped more precisely using S1 nuclease protection analysis. The termini were somewhat heterogeneous but strong cleavage was detected inside and adjacent to the secM stop codon (Fig. 1C).
Inhibition of tmRNA Activity during SecM Ribosome Arrest

No cleavages were detected in the codon for glutamine 167, which would have produced an S1 protection pattern similar to that observed with the truncated control in vitro transcript (Fig. 1C, truncated lane). As suggested by the Northern analysis described above, mRNA cleavage occurred ~13 to 19 nucleotides downstream of the predicted A-site codon during SecM ribosome arrest.

3’ → 5’ Exonucleases Generate Truncated secM mRNA during Ribosome Arrest—Two models account for ribosome arrest-dependent cleavage at the secM stop codon: (i) A-site cleavage due to inefficient translation termination as originally described in Refs. 2 and 3, or (ii) exonucleolytic trimming of downstream mRNA to the 3’ margin of the arrested ribosome. To differentiate between these possibilities, we fused secM codons 150–166 in-frame between two thioredoxin genes (trxA). The encoded FLAG-TrxA-SecM’-TrxA fusion protein contained the minimal SecM peptide motif (150-FST-PVWISQAQGIRAGP166) sufficient for ribosome arrest (9). However, in contrast to the wild-type secM gene, the flag-trxA-secM’-trxA stop codon was positioned several hundred nucleotides downstream of the predicted ribosome arrest site (21).

Northern analysis of flag-trxA-secM’-trxA mRNA also showed ribosome arrest-dependent truncated messages (data not shown), and S1 nuclease protection analysis detected two prominent cleavage sites at 13 and 19 nucleotides downstream of the proline 166 codon (wild-type SecM numbering) (Fig. 2B, wild-type lane). The cleavages were the same distance from the proline 166 codon as was observed with flag-secMA’ and flag-(Δss)secMA’ mRNAs (Figs. 1C and 2A). Although the cleavage patterns were not strictly identical between truncated messages, the secM stop codon was clearly not required for mRNA cleavage.

We reasoned that if ribosome arrest-dependent mRNA cleavage was due to exonucleolytic activity, then cleavage could be modulated by deletion of known 3’ → 5’ exoribonucleases. Fig. 2B shows the effects of specific exoribonuclease deletions on mRNA cleavage using the flag-trxA-secM’-trxA message. Deletion of RNase R leads to an increase in the +19 cleavage product and a decrease in the +13 cleavage product compared with wild-type (Fig. 2B). Similarly, removing polynucleotide phosphorylase (PNPase) activity also led to increased levels of the +19 product (Fig. 2B). In contrast, there was a slight decrease in the +19 product in ΔRNase II cells (Fig. 2B). The RNase R/RNase II double deletion strain exhibited less cleavage at both sites, whereas deletion of the C-terminal domain of RNase E had little effect on cleavage (Fig. 2B). Although RNase E is an endoribonuclease, the C-terminal domain is required for the organization of the degradosome, a multienzyme complex that contains PNPase and is important for the degradation of many mRNAs in E. coli (25, 26). In general, the accumulation of specific cleavage products was dependent upon exoribonuclease activities.

The SecM Nascent Peptide Is Linked to tRNA<sup>gly</sup> during Ribosome Arrest—The accumulation of truncated secM messages in tmRNA<sup>−</sup> cells and the involvement of exoribonucleases in mRNA cleavage are inconsistent with what is known about A-site cleavage. Moreover, the SecM-induced ribosome arrest occurs at the codon for proline 166 (9, 16), a position that is 13–15 nucleotides upstream of the stop codon (Fig. 1A). We sought to confirm the position of SecM-stalled ribosomes using a mini-gene that encodes SecM residues glutamine 149–glutamine 167 directly downstream of the FLAG epitope. Additionally, the flag-secM’ mini-gene was synonymously recoded to change the codon for proline 153 from CCC to CCG, and the codon for glycine 161 from GGC to GGA.

Northern analysis using a probe specific for the ribosome binding site of flag-secM’ detected truncated mRNA, and this cleavage appeared to depend upon ribosome stalling because truncated mRNA was not observed with the P166A variant (Fig. 3, RBS probe blot). The position of the arrested ribosome was determined by identifying the nascent peptidyl-tRNA by Northern blot analysis. Induction of FLAG-SecM’ synthesis led
tRNA2 Pro in the A-site. If this model is correct, tRNA Pro

SecM-arrested ribosomes. If this model is correct, tRNA Pro

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SecM-arrested Ribosomes Contain Prolyl-tRNA Pro in the A-site—Elegant studies have shown that SecM ribosome arrest is prevented if proline residues are replaced with the imino acid analog, azetidine-2-carboxylic acid (14). Based on this finding, it has been reasonably assumed that proline 166 is incorporated into the SecM nascent peptide during ribosome arrest (8, 9, 14). However, recent work from Ito and colleagues (16), as well as our analysis, indicates that ribosome arrest occurs prior to proline 166 addition. One model that is consistent with all available data postulates that prolyl-tRNA Pro occupies the A-site of the SecM-arrested ribosome. If this model is correct, tRNA Pro should be stably associated with arrested ribosomes.

Extracts from cells expressing FLAG-(Δss)SecM were separated into high-speed pellet and supernatant fractions by ultracentrifugation through sucrose cushions. Polyacrylamide gel analysis of RNA extracted from these fractions showed that the tRNA (i.e. ribosomes) was present in the pellet fraction, whereas the majority of tRNA was in the supernatant fraction (data not shown). Partitioning of tRNA to the supernatant fraction was confirmed by Northern analysis for tRNA Pro (Fig. 4, argQ probe blot), which was not predicted to associate with SecM-arrested ribosomes. In contrast, a higher proportion of tRNA Pro was found in the pellet fractions from cells expressing FLAG-(Δss)SecM, but not FLAG-(Δss-P166A)SecM (Fig. 4, proL probe blot). Enrichment of tRNA Pro in pellet fractions was dependent upon cognate tRNA/codon interactions. tRNA Pro is the cognate tRNA for CCU and CCC codons, was not enriched in high-speed pellets if SecM proline 166 was encoded by CCU (Fig. 4, proL probe blot), even though the CCG codon fully supports ribosome arrest (9). Moreover, although tRNA Pro partitioned to the pellet fractions when the CCU construct was expressed, significantly more tRNA Pro was found in the pellet fraction when its cognate CCG codon was used to code for proline 166 (Fig. 4, proK probe blot). The partitioning of tRNA Pro to the ribosome fraction with the CCU construct may be due to association with trailing ribosomes within the SecM-stalled polypeptide, because tRNA Pro is not known to decode CCU and is found in the high-speed supernatant in the absence of ribosome arrest (Fig. 4, Δss P166A lanes). Finally, the association of tRNA Pro with pellet fractions was not inhibited by the tmRNA quality control system (Fig. 4, ΔtmRNA versus tRNA Pro).

tmRNA Activity at SecM-arrested Ribosomes—The data presented thus far indicate that tmRNA does not play a significant role in rescuing SecM-arrested ribosomes. However, published reports show SecM and SecM variants are ssrA-tagged by tmRNA as a consequence of ribosome arrest (27, 28). We examined tmRNA-mediated peptide tagging of SecM proteins in cells that express tmRNA(His6), which encodes a hexahistidine-containing ssrA peptide that is resistant to proteolysis (18). Western blot analysis using antibodies specific for His6 detected two ssrA(His6)-tagged species of (Δss)SecM (Fig. 5A, Δss SecM His6 lane). A similar ssrA(His6)-tagged doublet was observed with signal sequence-containing FLAG-SecM (data not shown), but not with the FLAG-(Δss-P166A)SecM protein, which does not cause ribosome arrest (Fig. 5A, Δss P166A). All ssrA(His6)-tagged species were also detected by Western analysis using antibody specific for the N-terminal FLAG epitope (Fig. 5A, anti-FLAG panel).
To determine the sites of tagging, we purified ssrA(His<sub>6</sub>)-tagged FLAG-SecM and FLAG-(Δss)SecM by Ni<sup>2+</sup>-NTA affinity chromatography and subjected the purified proteins to mass spectrometry and N-terminal sequence analysis. Although FLAG-SecM was initially expressed as an N-terminal FLAG fusion, the N-terminal amino acid sequence (AEPNA) of the purified protein indicated that the epitope tag had been removed along with the signal sequence peptide during secretion (data not shown). The masses of tagged SecM species were consistent with the addition of ssrA-(His<sub>6</sub>) tags after glycine 165 (15,450 Da; calculated mass 15,451 Da) and threonine 170 (16,047 Da; calculated mass 16,047 Da) (Fig. 5B, SecM spectrum). Similarly, FLAG-(Δss)SecM was also tagged after residues corresponding to glycine 165 (16,845 Da; calculated mass 16,846 Da) and threonine 170 (17,439 Da; calculated mass 17,442 Da) (Fig. 5B, (Δss)SecM spectrum). We suspected that the tagged proteins detected by Western blot analysis corresponded to the two species observed by mass spectrometry. These assignments were confirmed through analysis of the FLAG-(Δss-Q167UAA)SecM protein, which was synthesized from a construct containing a mutation that changes glutamine 167 codon to a stop codon (UAA) (Fig. 1A). The FLAG-(Δss-Q167UAA)SecM protein lacks four C-terminal amino acid residues, but still causes ribosome arrest (9). FLAG-(Δss-Q167UAA)SecM protein was tagged after glycine 165, but not after threonine 170 (Fig. 5A, (Δss)Q167UAA, and data not shown). Presumably, the premature stop codon prevented ribosomes from translating to the 3′ end of truncated mRNA.

The effect of tmRNA activity on total SecM protein production was examined by Western blot analysis using a monoclonal antibody specific for the N-terminal FLAG epitope present on all FLAG-(Δss)SecM variants. Two species of FLAG-(Δss)SecM accumulated in ΔtmRNA

FIGURE 5. tmRNA activity at SecM-arrested ribosomes. A, Western blot analyses of FLAG-(Δss)SecM variants expressed in tmRNA<sup>−</sup>, ΔtmRNA, and tmRNA(His<sub>6</sub>)<sup>−</sup> cells. Anti-His<sub>6</sub> antibodies recognized ssrA(His<sub>6</sub>) peptide tags added to the C termini of FLAG-(Δss)SecM proteins. Anti-FLAG antibody detected the N-terminal FLAG epitope present on all (Δss)SecM variants. Cells expressing the following proteins were analyzed: FLAG-(Δss-P166A)SecM ((Δss)P166A), FLAG-(Δss)SecM ((Δss)SecM), and FLAG-(Δss-Q167UAA)SecM ((Δss)Q167UAA). The positions of all untagged and ssrA(His<sub>6</sub>)-tagged proteins are indicated by labeled arrows. Plasmid ptRNA<sup>−</sup> overexpresses tRNA<sup>−</sup>. B, mass spectrometry of ssrA(His<sub>6</sub>)-tagged SecM and FLAG-(Δss)SecM proteins. Measured masses were consistent with proteins containing C-terminal ssrA(His<sub>6</sub>) tags added after SecM residues glycine 165 and threonine 170.
cells (Fig. 5A, anti-FLAG panel, ΔtmRNA lane). The higher molecular weight protein represented full-length polypeptide and this species co-migrated with FLAG-(Δss)SecM (which does not cause ribosome arrest) on SDS-polyacrylamide gels (Fig. 5A, anti-FLAG panel). The lower molecular weight species seen in ΔtmRNA cells corresponded to incompletely synthesized FLAG-(Δss)SecM protein (to residue glycine 165) produced during ribosome arrest (Fig. 5A, and data not shown). However, analyses of cetyl trimethylammonium bromide precipitates and isolated ribosomes indicated that most of the incompletely synthesized FLAG-(Δss)SecM protein was not covalently linked to tRNA and therefore did not represent ribosome-bound nascent chains (data not shown). Therefore, incompletely synthesized FLAG-(Δss)SecM polypeptide chains were released from the arrested ribosome in a tmRNA-independent manner. In contrast to ΔtmRNA cells, full-length FLAG-(Δss)SecM protein was not detected in tmRNA + cells (Fig. 5A, anti-FLAG panel, tmRNA + lane). Presumably, the full-length FLAG-(Δss)SecM protein was ssrA-tagged and degraded rapidly in wild-type cells. Similarly, full-length FLAG-(Δss)SecM did not accumulate to very high levels in tmRNA(His6) expressing cells, although the two ssrA(His6)-tagged species were readily detected (Fig. 5A, anti-FLAG panel). 

Prolyl-tRNAPro in the A-site Inhibits tmRNA Activity—SsrA tagging after glycine 165 appears to contradict the other data indicating that tmRNA plays no significant role in resolving SecM-arrested ribosomes. However, this work and previous studies relied upon SecM overexpression (27, 28), which is predicted to deplete limiting tRNAPro species. tRNAPro gly, which holds the SecM nascent chain during ribosome arrest, is found at ~4,400 molecules per E. coli cell, whereas tRNAPro and tRNAPro, which occupy the arrested ribosome A-site, are present at only ~1,300 copies per cell (29). Therefore, if the number of SecM-arrested ribosomes exceeds 1,300 per cell, a second population of stalled ribosomes with unoccupied A-sites will accumulate due to prolyl-tRNAPro sequestration, potentially allowing for adventitious ssrA tagging after glycine 165.

To test this model, we overexpressed tRNAPro and examined the effects on ssrA peptide tagging and three other properties of SecM ribosome arrest: (i) nascent peptidyl-tRNA stability, (ii) cleavage of flag-secM mRNA, and (iii) regulation of secA translation. Overexpression of tRNAPro significantly suppressed ssrA(His6) tagging after glycine 165, but increased tagging after threonine 170 (Fig. 5A, (Δss)SecM-tRNAPro lane). Although tmRNA activity was significantly altered, tRNAPro overexpression had no effect on nascent peptidyl-tRNAPro gly accumulation (Fig. 3, glyV probe blot), and actually appeared to increase flag-secM mRNA cleavage (Fig. 3, RBS probe blot). Finally, tRNAPro overexpression had no effect on the regulation of secA translation. We made secA::lacZ translational fusions and confirmed that deletion of the SecM signal sequence increased SecA-LacZ expression, whereas further introduction of the P166A mutation reduced fusion protein synthesis (Fig. 6). Overexpression of tRNAPro had no significant effect on the ribosome arrest-dependent increase in β-galactosidase activity (Fig. 6). Moreover, deletion of tmRNA had no effect on SecA-LacZ expression, as determined by Western blot and β-galactosidase activity analyses (Fig. 6). We also attempted to examine the effects tRNAPro overexpression on ribosome arrest from constructs that encoded proline 166 as CCG. Unfortunately, all plasmid clones carrying the prok gene under its own promoter also contained mutations in the tRNAPro-encoding sequence (data not shown). Seven distinct mutations were found mapping to the D-arm, T-arm, anticodon loop, and the promoter (data not shown). These results suggest that high-level overexpression of tRNAPro is deleterious to the cell.

DISCUSSION

Several lines of evidence indicate that the primary SecM-mediated ribosome arrest is resistant to A-site mRNA cleavage and subsequent tmRNA recruitment. First, although the secM mRNA was truncated in a ribosome arrest-dependent manner, the cleavage sites were 13 to 19 nucleotides downstream of the A-site codon. Second, the steady-state number of SecM-arrested ribosomes (as determined by Northern analysis of nascent peptidyl-tRNA) was not significantly affected by tmRNA. Third, incompletely synthesized SecM protein (to residue glycine 165) accumulated in tmRNA + and tmRNA(His6)-expressing cells. Fourth, SecM-dependent regulation of secA translation was essentially identical in ΔtmRNA and tmRNA + cells (27). Finally, A-site-bound tRNAPro inhibits ssrA tagging after SecM glycine 165. The surprising discovery of A-site-bound prolyl-tRNAPro has also been recently reported by Ito and colleagues (16). That study used entirely different methods than ours to characterize arrested ribosomes produced in vitro (16), and is completely congruent with our analysis of the in vivo SecM ribosome arrest. Altogether, our data strongly suggest that tmRNA recruitment during the primary ribosome arrest is an artifact of SecM overexpression, and that A-site mRNA cleavage and ssrA tagging at this site do not occur under physiological conditions. We feel this conclusion makes biological sense because A-site cleavage is predicted to interfere with cis-acting SecM regulation of secA translation initiation. Moreover, co-translational
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secretion of the SecM nascent peptide ensures that SecA is synthesized in close proximity to the inner membrane (30), a phenomenon that presumably requires synthesis of SecM and SecA from the same mRNA molecule.

Deletion of the SecM signal sequence prevents co-translational secretion and thereby precludes the mechanism that normally alleviates ribosome arrest (14). Secreted SecM also elicited ribosome arrest in our study, presumably because the overexpressed protein saturated the secretion machinery. The \( t_{1/2} \) of SecM-mediated ribosome arrest is at least 4–5 min in vivo (14), which exceed the half-life of bulk \( E. coli \) mRNA turnover (~2.4 min at 37 °C) (31). Thus, prolonged translational pausing allows degradation of the downstream mRNA to the 3′-edge of the arrested ribosome by exoribonucleases. Resumption of translation on 3′-edge cleaved mRNA leads to secondary ribosome arrest at the 3′ end of the message, recruitment of tmRNA, and ssrA tagging of SecM after threonine 170. If the A-site is unoccupied, tmRNA may be directly recruited to ribosomes arrested on 3′-edge-cleaved mRNA, resulting in ssrA tagging after glycine 165. IV, ribosomes with unoccupied A-sites may undergo A-site mRNA cleavage at a low rate, also allowing ssrA tagging after glycine 165. Exoribonuclease cleavage to the 3′-edge of the arrested ribosome could also precede A-site mRNA cleavage. Protein release factor is labeled RF, and the peptidyl-tRNA and aminoacyl-tRNA binding sites are labeled P and A, respectively.

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The details of mRNA cleavage notwithstanding, it is interest-
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ing that the secM stop codon is in position to be cleaved during prolonged ribosome arrest. SecM-arrested ribosomes clearly resumed translation, and upon reaching the 3′ end of the truncated mRNA, they stalled for a second time (Fig. 7). However, tmRNA is readily recruited to ribosomes stalled at the extreme 3′ termini of mRNAs, and SecM was ssrA-tagged after the C-terminal residue threonine 170 (Fig. 7, ribosome fate II). Because little full-length (ΔssSecM) protein accumulated in tmRNA− or tmRNA(His6) cells, it appears that degradation of mRNA to the ribosome edge preceded the resumption of translation. It is unclear whether exoribonuclease cleavage also leads to ssrA-dependent degradation of SecM under physiological conditions. Secreted SecM was shown to be rapidly degraded in tmRNA− cells (14), and we find the non-degradable ssrA(His6) tag stabilizes SecM in the periplasm. However, both of these studies employed SecM overexpression. At lower expression levels, co-translational secretion of SecM is expected to prevent ribosome arrest, and thereby inhibit mRNA cleavage and subsequent tmRNA recruitment/ssrA-tagging (Fig. 7, ribosome fate I). In any event, prolonged ribosome arrest stimulates SecA expression, so significant protein synthesis must occur prior to degradation of the downstream secA cistron.

A-site mRNA cleavage and tmRNA activities were clearly not able to resolve the majority of primary SecM-arrested ribosomes. However, ssrA tagging after glycine 165 indicates limited tmRNA recruitment during primary ribosome arrest, at least when SecM is overexpressed. Ivanova et al. (41) showed tmRNA is recruited to ribosomes stalled on mRNAs where the 3′ terminus is 12 nucleotides downstream of the A-site codon, albeit at a ~20-fold lower rate than maximum. Therefore, cleavage of mRNA to the 3′-edge of the arrested ribosome could allow relatively inefficient tmRNA recruitment, provided the A-site is not occupied with prolyl-tRNAPro (Fig. 7, ribosome fate III). Alternatively, limited A-site mRNA cleavage may have occurred under SecM overexpression conditions (Fig. 7, ribosome fate IV). It appears that A-site nuclease activity is restricted to codons within unoccupied A-sites (1, 2), so presumably A-site cleavage in this instance would be an artifact of SecM overexpression. Based on Northern blot analysis, ~60% of cellular tRNA3Gly is sequestered as SecM nascent peptidyl-tRNA during SecM overexpression. This corresponds to roughly 2,600 SecM-arrested ribosomes per cell, of which only ~1,300 can simultaneously contain A-site prolyl-tRNAPro (29). Therefore, we estimate ~50% of the SecM-arrested ribosomes have unoccupied A-sites in the absence of compensatory tRNAPro overexpression, in accord with recent studies of SecM-arrested ribosomes (15, 16). Given incomplete A-site occupancy, perhaps the lack of A-site mRNA cleavage reflects sequence specificity of the A-site nuclease. The RelE protein shows marked preference for A-site codons, cleaving CAG and UAG at the highest rate (1). However, we have observed RelE-independent A-site mRNA cleavage at several different codons, suggesting that many sequences are potential substrates (2).4

Alternatively, the low rate of A-site mRNA cleavage may be due to the substantial structural rearrangements that occur in the ribosome during SecM-mediated arrest (15). SecM-induced structural rearrangements originate in the 50 S exit channel and are propagated to the 30 S subunit via inter-subunit bridges and ribosome-bound tRNAs (9, 15). Although structural changes are transmitted by tRNA, SecM-arrested ribosomes adopt the same conformation independent of A-site bound prolyl-tRNAPro (15). Several elements of 16 S rRNA are rearranged during ribosome arrest, including helix 44, which forms part of the 30 S A-site and makes contact with A-site mRNA (15). Clearly, alteration of A-site structure could significantly affect A-site nuclease activity, whether catalyzed by the ribosome or a trans-acting factor. Interestingly, Aiba and colleagues (28) showed that expression of a fusion protein containing the SecM-derived residues 161GIRAGP166 resulted in significant mRNA cleavage at sites immediately adjacent to the A-site codon, although the majority of cleavages still occurred at other positions corresponding to the 3′- and 5′-edges of the paused ribosome. We also observed similar cleavages near the A-site codon when expressing a fusion protein containing the longer SecM-derived 149QFSTPWIQAQGIRAGP166 sequence (Fig. 2B), but failed to detect these mRNA cleavages when expressing full-length SecM sequences. Perhaps the full-length SecM nascent peptide is required for complete structural rearrangement and inhibition of A-site mRNA cleavage.

Gene regulation by translational pausing has long been recognized in prokaryotes, although its importance is still often underestimated. Indeed, the SecM ribosome arrest is a newly characterized example of translational attenuation, which was shown to control inducible expression of erythromycin and chloramphenicol resistance genes over 20 years ago (42–45). The role of translational pausing in transcriptional attenuation of the E. coli trp operon was recognized even earlier (46, 47). In each case, A-site mRNA cleavage and tmRNA activities have the potential to interfere with regulation by “rescuing” paused ribosomes. However, in our view, it makes little sense to employ regulatory strategies that are undermined by translational quality control systems, and we predict that regulatory ribosome pauses are generally immune to A-site cleavage and tmRNA activities. The mechanisms involved are likely varied, and characterization of other ribosome pauses will hopefully increase our understanding of the molecular requirements for A-site mRNA cleavage.

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