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PROLYL-tRNA^{Pro} IN THE A SITE OF SecM-ARRESTED RIBOSOMES INHIBITS THE RECRUITMENT OF tmRNA*

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Abstract

Translational pausing can lead to cleavage of the A-site codon and facilitate recruitment of the tmRNA (SsrA) quality control system to distressed ribosomes. We asked whether A-site mRNA cleavage occurs during regulatory translational pausing using the *Escherichia coli* SecM-mediated ribosome arrest as a model. We find the 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 $tRNA_3^{Gly}$ in the P site, and prolyl-$tRNA_2^{Pro}$ 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-$tRNA_2^{Pro}$ on over-expressed SecM-arrested ribosomes, which produces a second population of stalled ribosomes with unoccupied A sites. Indeed, compensatory over-expression of $tRNA_2^{Pro}$ 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[1] mRNA cleavage is a novel RNase activity that acts on A-site codons within paused ribosomes. Ehrenberg, Gerdes and their colleagues first demonstrated that *Escherichia coli* RelE protein causes cleavage of A-site mRNA *in vitro* (1). 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).

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[1]The abbreviations used are: A site, aminoacyl-tRNA binding site; P site, peptidyl-tRNA binding site; tmRNA, transfer-messenger RNA; IPTG, isopropyl-β-D-thiogalactopyranoside; OD_{600}, optical density at 600 nm; PNPase, polynucleotide phosphorylase; NTA, nitrilotetraacetic acid; rRNA, ribosomal RNA; Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Kan^r, kanamycin resistance

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 such 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 shows that ~40% of SecM-arrested ribosomes contain a fully accommodated A-site tRNA (15). Ito and colleagues have recently analyzed SecM-arrested ribosomes prepared by *in vitro* translation and concluded that the A-site tRNA is a prolyl-tRNA^{Pro} (16). 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 I 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) λ lysogen kit according to manufacturer’s instructions. Strain CH2198 [X90 *ssrA(his6)* (DE3)] was obtained by introducing the *ssrA(His6)* allele (18) of tmRNA into the *ssrA* 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 *ssrA::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 *Nco*I and *Nde*I restriction sites. Plasmid pFG21b allows the production of N-terminal FLAG-tagged proteins, provided the initiation Met codon is engineered into an *Nde*I 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 (Pharmacia). 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´- GGA TGG CAA TCA TAT GAG TGG AAT ACT GAC GCG CTG G), and **secA-Bam** (5´-CCG GGA TCC GAT TTT CCA GCA CTT CGC C) . The *(Δ ss)secMA´* fragment was generated with the **secA-Bam** primer and **secM-A38** (5´-CCT GCG CTC AGC CAT ATG GCC GAA CCA AAC GCG CCC GC). The PCR products were digested with *Nde*I and *Bam*HI and ligated to plasmid pFG21b. The mutation changing SecM residue proline-166 to alanine was made using primer **secM-P166A** (5´ -CCG TGC TGG CGC TCA ACG CCT CAC C) in combination with primers **secA-Bam** and **secM-A38** by the PCR megaprimer method (21). LacZ translational fusions were made in two steps: i) ligation of the various *secMA´ Nde*I/*Bam*HI fragments into plasmid pTrc3 followed by, ii) ligation of a *Bam*HI/*Hind*III fragment containing the *E. coli lacZ* gene. The *lacZ* fragment was produced by PCR using primers, **lacZ-Bam** (5´- AGG GAT CCA AAT GAT TAC GGA TTC ACT GGC CGT CG) and **lacZ-Hind** (5´- GGA T*AA GCT T*AC GCG AAA TAC GGG CAG ACA TGG C).

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´- GTG GAG TTA CAT ATG AGC GAT AAA ATT ATT CAC C) and **trxA-Bam** (5´- AAA TGG ATC CCC GCC AGG TTA GCG TCG AGG AAC TC); and the second from primers **trxA-Eco** (5´- ATA GAA TTC CGA TAA AAT TCA CCT GAC TGA CGA C) and **trxA-Xho** (5´- GAA CTC GAG ATT CCC TTA CGC CAG GTT AGC GTC G). The two *trxA* PCR fragments were sequentially ligated into pFG21b using *Nde*I/*Bam*HI and *Eco*RI/*Xho*I digestions to generate plasmid pTrx-Trx. The oligonucleotides **ts´t-top** (5´- GAT CCA ATT CAG CAC GCC CGT CTG GAT AAG CCA GGC GCA AGG CAT CCG TGC TGG CCC TCA G) and **ts´t-bottom** (5´- AAT TCT GAG GGC CAG CAC GGA TGC CTT GCG CCT GGC TTA TCC AGA CGG GCG TGC TGA ATT G) were annealed to one another and ligated to *Bam*HI/*Eco*RI-digested plasmid pTrx-Trx to generate pTrx-SecM´-Trx. Similarly, pSecM´ was generated by annealing **secM´-top** (5´- TAT GCA ATT CAG CAC GCC GGT CTG GAT AAG CCA GGC GCA AGG AAT CCG TGC TGG CCC TCA AAA G) and **secM´-bottom** (5´- AAT TCT TTT GAG GGC CAG CAC GGA TTC CTT GCG CCT GGC TTA TCC AGA CCG GCG TGC TGA ATT GCA) followed by ligation to *Nde*I/*Eco*RI-digested pFG21b. The $ptRNA_2^{Pro}$ over-expression plasmid was constructed by amplifying the *proL* gene and its promoter with primers **proL-Sac** (5´- CTG GAG CTC AAC AAT AAC GGT AAA TAC C), and **proL-Kpn** (5´- AGC GGT ACC TTG TCA GTC AGC TAT GG) followed by ligation of the resulting PCR product into *Sac*I/*Kpn*I-digested plasmid pCH405Δ (2,17).

mRNA Expression and RNA Analysis

E. coli strains were grown overnight at 37°C in LB medium supplemented with the appropriate antibiotics (150 μg/ml of ampicillin, 25 μg/ml of tetracycline, or 50 μg/ml of kanamycin). The next day, cells were resuspended at an optical density at 600 nm (OD_{600}) of 0.05 in 15 ml of

fresh medium and grown at 37°C with aeration. Once cultures reached an OD_{600} of ~0.3, mRNA expression was induced with IPTG (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 1.0 mL of a solution containing 0.6 M ammonium isothiocyanate – 2 M guanidinium isothiocyanate – 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 isopropanol to precipitate total RNA. RNA pellets were washed once with ice-cold 75% ethanol and dissolved in either 10 mM 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 electroblotting (250 mA) to positively charged nylon membrane in 0.5 × TBE for 1 hour at 4 °C. All subsequent Northern hybridization conditions were as described (2). The following radiolabeled DNA oligonucleotide probes were used in hybridizations: ***proL*** for $tRNA_2^{Pro}$ (5′-CAC CCC ATG ACG GTG CG); ***proK*** for $tRNA_1^{Pro}$ (5′-CTT CGT CCC GAA CGA AGT G); ***glyV*** for $tRNA_3^{Gly}$ (5′- CTT GGC AAG GTC GTG CT); ***argQ*** for RNA_2^{Arg} (5′- CCT CCG ACC GCT CGG TTC G); and **RBS** for pET-derived ribosome binding site (5′- GTA TAT CTC CTT CTT AAA GTT AAA C). The following radiolabeled DNA oligonucleotides were used as probes for S1 nuclease protection experiments: ***secM*** **S1 probe** (5′- TTA ATA AAA TGA AGT AAA GGT TTA TTG TTG TTA GGT GAG GCG TTG AGG GCC AGC ACG GAT GCC TTG CGC CTG GCT TAT CC); and ***secM′-trxA*** **S1 probe** (5′- CTG TCG TCA GTC AGG TGA ATT TTA TCG GAA TTC TGA GGG CCA GCA CGG ATG CCT TGC GCC TGG CTT ATC CAG).

SecM Expression and Protein Analysis

Strains were cultured as described above for RNA analysis. Protein extraction and Western blot analyses were conducted as described (17). Anti-His6 polyclonal antibodies were obtained from Santa Cruz Biochemical. Monoclonal antibodies specific for *E. coli* β-galactosidase and the FLAG M2 epitope were obtained from Sigma-Aldrich. SsrA(His6)-tagged SecM proteins were purified by Ni^{2+}-NTA agarose (Qiagen) affinity chromatography as described (17,18). Ni^{2+}-NTA purified protein was further purified by reverse phase-HPLC. N-terminal gas-phase sequencing was performed on a Porton 2020 protein sequencer (Beckman-Coulter) with a dedicated in-line HPLC (model 2090) for separation of phenylthiohydantoin derivatives. Molecular masses were determined by liquid chromatography-mass spectrometry. Samples were applied to a Zorbax 300SB-C18 reverse phase column in aqueous 0.1% formic acid and proteins eluted using a linear gradient of acetonitrile using an Agilent 1100 LC nano-system. Eluted proteins were infused into a Waters Q-Tof II™ mass spectrometer for ionization.

β-Galactosidase assays were conducted essentially as described (23). Strains expressing *secA′::lacZ* translational fusions were inoculated at OD_{600} of 0.05 in LB medium and grown at 37° C with aeration to OD_{600} of 0.3 – 0.6. β-Galactosidase activity for each construct was measured from 5 to 8 independent cultures and reported as mean ± standard deviation.

Cell Extract Fractionation

Strains CH12 *Δ rna::kan* and CH113 *Δ rna::kan* containing plasmid pSecM′ or pSecM′ (P166A) were grown in 1 liter of LB media at 37°C with aeration in Fernbach flasks. At OD_{600} ~ 0.6, SecM′ expression was induced by the addition of IPTG to 1.5 mM and cultures

incubated for one hr at 37°C with aeration. Cultures were harvested over ice, the cells collected by centrifugation and washed once with cold, high-Mg^{2+} S30 buffer [60 mM potassium acetate – 30 mM magnesium acetate – 0.2 mM EDTA – 10 mM Tris-acetate (pH 7.0)]. Washed bacterial pellets were resuspended in 10 mL of cold high-Mg^{2+} S30 buffer and the cells broken by one passage through a French press at 12,000 psi. Cell lysates were cleared by centrifugation at 30,000 × g for 15 min at 4°C, and the supernatants layered onto cushions of cold high-Mg^{2+} S30 buffer containing 1.1 M sucrose in ultracentrifuge tubes (Beckman #344057). Samples were centrifuged in a Beckman-Coulter Optima™ ultracentrifuge at 45,000 rpm for one hr at 4°C using an MLS-50 rotor. Total RNA was extracted from the high-speed supernatants and pellets for analysis as described above.

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 (Figure 1A). Three SecM variants were used throughout this work: i) Flag-SecM, which is the wild-type protein fused to a n N-terminal FLAG epitope tag; ii) Flag- (Δss)SecM, which lacks the secretion signal sequence (Δss = deleted 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 using a probe specific for the ribosome binding site upstream of *secM*. In addition to the full-length mRNAs, truncated *flag-secMA´* and *flag-*(Δ*ss)secMA´* messages were also detected (Figure 1B). The truncated mRNAs did not hybridize to a probe specific for downstream *secA* sequence (data not shown). No truncated *flag-*(Δ*ss-P166A)secMA´* mRNA was apparent, suggesting that ribosome arrest was required for mRNA cleavage. Interestingly, steady state levels of truncated *flag-secMA ´* and *flag-(*Δ*ss)secMA´* mRNAs were similar in wild-type and ΔtmRNA cells (Figure 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 (Figures 1A & 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 (Figure 1C). 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 (Figure 1C, **truncated** lane). As suggested by the Northern analysis described above, mRNA cleavage occurred approximately 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 (2,3), 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 (F_{150}STPVWISQAQGIRAGP$_{166}$) sufficient for ribosome arrest (9). However, in contrast to the wild-type *secM* gene, the *flag-trxA-secM´-trxA* stop codon is 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) (Figure 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 (Figure 2A and Figure 1C). 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 exonuclease activity, then cleavage could be modulated by deletion of known 3´→5´ exoribonucleases. Figure 2B shows the effects of specific exoribonuclease deletions on mRNA cleavage using the *flag-trxA-secM´-trxA* message. Deletion of RNase R lead to an increase in the +19 cleavage product and a decrease in the +13 cleavage product compared to wild-type (Figure 2B). Similarly, removing polynucleotide phosphorylase (PNPase) activity also lead to increased levels of the +19 product (Figure 2B). In contrast, there was a slight decrease in the +19 product in ΔRNase II cells (Figure 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 (Figure 2B). Although RNase E is an endoribonuclease, the C-terminal domain is required for the organization of the degradosome, a multi-enzyme 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 $tRNA^{Gly}$ during ribosome arrest

The accumulation of truncated *secM* messages in $tmRNA^+$ 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 (Figure 1A). We sought to confirm the position of SecM-stalled ribosomes using a mini-gene that encodes SecM residues glutamine-149 to 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 (Figure 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 to a shift in the electrophoretic mobility of $tRNA_3^{Gly}$ but not that of $tRNA_2^{Pro}$ (Figure 3, ***glyV*** and ***proL*** probe blots). The $tRNA_3^{Gly}$ mobility shift was not observed when the Flag-(P166A)SecM´ variant was expressed (Figure 3, ***glyV*** probe blot). The $tRNA_3^{Gly}$ mobility shift was not seen when RNA samples were incubated at pH 8.9 for 1 hr at 37°C to deacylate tRNAs (data not shown) (22). The arrested ribosome could be positioned unambiguously because the recoded mini-gene contained only one codon (GGC of glycine-165) that is decoded by $tRNA_3^{Gly}$. Therefore, during SecM-mediated ribosome arrest, the nascent peptide is covalently linked to $tRNA_3^{Gly}$ via glycine-165 and the codon for proline-166 is positioned in the A site.

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, as well as our analysis, indicates that ribosome arrest occurs prior to proline-166 addition (16). 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 rRNA (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 $tRNA_2^{Arg}$ (Figure 4, ***argQ*** probe blot), which was not predicted to associate with SecM-arrested ribosomes. In contrast, a higher proportion of $tRNA_2^{Pro}$ was found in the pellet fractions from cells expressing Flag-(Δss)SecM, but not Flag-(Δss-P166A)SecM (Figure 4, ***proL*** probe blot). Enrichment of tRNA^{Pro} in pellet fractions was dependent upon cognate tRNA/codon interactions. $tRNA_2^{Pro}$, the cognate tRNA for CCU and CCC codons, was not enriched in high-speed pellets if SecM proline-166 was encoded by CCG (Figure 4, ***proL*** probe blot), even though the CCG codon fully supports ribosome arrest (9). Moreover, although $tRNA_1^{Pro}$ partitioned to the pellet fractions when the CCU construct was expressed, significantly more $tRNA_1^{Pro}$ was found in the pellet fraction when its cognate CCG codon was used to code for proline-166 (Figure 4, ***proK*** probe blot). The partitioning of $tRNA_1^{Pro}$ to the ribosome fraction with the CCU construct may be due to association with trailing ribosomes within the SecM-stalled polysome, because $tRNA_1^{Pro}$ is not known to decode CCU and is found in the high-speed supernatant in the absence of ribosome arrest (Figure 4, **(Δss) P166A** lanes). Finally, the association of tRNA^{Pro} with pellet fractions was not inhibited by the tmRNA quality control system (Figure 4, Δ**tmRNA** *vs.* **tmRNA**$^{+}$).

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 (Figure 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 (Figure 5A, **(Δss)P166A**). All ssrA(His6)-tagged species were also detected by Western analysis using antibody specific for the N-terminal FLAG epitope (Figure 5A, **anti-FLAG** panel).

To determine the sites of tagging, we purified ssrA(His6)-tagged Flag-SecM and Flag-(Δss) SecM by Ni^{2+}-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(His6) tags after glycine-165 (15,450 Da; calculated mass 15,451 Da) and threonine-170 (16,047 Da; calculated mass 16,047 Da) (Figure 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) (Figure 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) (Figure 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 (Figure 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 cells (Figure 5A, **anti-FLAG** panel, ΔtmRNA lane). The higher molecular weight protein represented full-length polypeptide and this species co-migrated with Flag-(Δss-P166A)SecM (which does not cause ribosome arrest) on SDS-polyacrylamide gels (Figure 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 (Figure 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 (Figure 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 (Figure 5A, **anti-FLAG** panel).

Prolyl-$tRNA^{Pro}$ 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 over-expression (27,28), which is predicted to deplete limiting $tRNA^{Pro}$ species. $tRNA_3^{Gly}$, which holds the SecM nascent chain during ribosome arrest, is found at ~4,400 molecules per *E. coli* cell, whereas $tRNA_2^{Pro}$ and $tRNA_3^{Pro}$, 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-$tRNA^{Pro}$ sequestration, potentially allowing for adventitious ssrA-tagging after glycine-165.

To test this model, we over-expressed $tRNA_2^{Pro}$ 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. Over-expression of $tRNA_2^{Pro}$ significantly suppressed ssrA(His6)-tagging after glycine-165, but increased tagging after threonine-170 (Figure 5A, **(Δss)SecM – ptRNA$_2^{Pro}$** lane). Although tmRNA activity was significantly altered, $tRNA_2^{Pro}$ over-expression had no effect on nascent peptidyl-$tRNA_3^{Gly}$ accumulation (Figure 3, ***glyV*** probe blot), and actually appeared to increase *flag-secM´* mRNA cleavage (Figure 3, **RBS** probe blot). Finally, $tRNA_2^{Pro}$ over-expression 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 (Figure 6). Over-expression of $tRNA_2^{Pro}$ had no significant effect on the ribosome arrest-dependent increase in β-galactosidase activity (Figure 6). Moreover, deletion of tmRNA had no effect on SecA´-LacZ expression, as determined by Western blot and β-galactosidase activity analyses (Figure 6). We also attempted to examine the effects $tRNA_1^{Pro}$ over-expression 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 $tRNA_1^{Pro}$-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 over-expression of $tRNA_1^{Pro}$ 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 $tRNA_2^{Pro}$ inhibits ssrA-tagging after SecM glycine-165. The surprising discovery of A site-bound prolyl-$tRNA^{Pro}$ 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 entirely 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 over-expression, 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 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 over-expressed protein saturated the secretion machinery. The (Δss)SecM-mediated ribosome arrest exhibits a $t_{1/2} > 4 – 5$ minutes *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 (Figure 7). Presumably, the 5´ portion of the message is protected by ribosomes queued behind the primary SecM-arrested ribosome. The influence of SecM ribosome arrest on mRNA degradation appears to differ from other reported ribosome pauses, which tend to stabilize mRNA downstream of the arrest site (32–34). Although specific endonuclease cleavage between cistrons has been observed in *E. coli* operons (35), we find the same cleavages in mRNAs that lack the *secM-secA* intergenic region. Moreover, the cleavage appears to require ribosome arrest, arguing against a sequence specific endonuclease activity. Our observations suggest that 3´→5´ exoribonucleases generate the 3´ termini of truncated *secM* messages. First, cleavage occurred downstream of the A-site codon, at sites consistent with the 3´ border of a stalled *E. coli* ribosome (36,37). Second, the proportion of +13 and +19 cleavage products was dependent upon exoribonuclease activities present in the cell. Longer cleavage products accumulated in the absence of either RNase R or PNPase, both of which

degrade secondary structure-containing RNAs more efficiently than RNase II (38,39). Although RNase R and PNPase are not known to work together, our data suggests that these enzymes may cooperate to convert the +19 cleavage product to the +13 product. Finally, RNase II can indirectly inhibit the degradation of structured mRNAs by removing 3′ single stranded regions required by PNPase to bind substrate (39,40). These biochemical properties are consistent with the accumulation of cleavage products in our exoribonuclease knock-out strains.

The details of mRNA cleavage notwithstanding, it is interesting 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 (Figure 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 (Figure 7, ribosome fate **II**). Because little full-length (Δss)SecM 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 over-expression. 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 (Figure 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 over-expressed. Ivanova *et al.* 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 (41). 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-tRNA^{Pro} (Figure 7, ribosome fate **III**). Alternatively, limited A-site mRNA cleavage may have occurred under SecM over-expression conditions (Figure 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 over-expression. Based on Northern blot analysis, approximately 60% of cellular $tRNA_3^{Gly}$ is sequestered as SecM nascent peptidyl-tRNA during SecM over-expression. This corresponds to roughly 2,600 SecM arrested ribosomes per cell, of which only ~1,300 can simultaneously contain A-site prolyl-tRNA^{Pro} (29). Therefore, we estimate ~50% of the SecM-arrested ribosomes have unoccupied A sites in the absence of compensatory $tRNA_2^{Pro}$ over-expression, 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] (2).

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 50S exit channel and are propagated to the 30S subunit via inter-subunit bridges and ribosome-bound tRNAs (9,15). Although structural

[2]Garza-Sánchez and Hayes, unpublished results

changes are transmitted by tRNA, SecM-arrested ribosomes adopt the same conformation independent of A-site bound prolyl-$tRNA^{Pro}$ (15). Several elements of 16S rRNA are rearranged during ribosome arrest, including helix 44, which forms part of the 30S A site and makes contact with A-site mRNA (15). Clearly, alteration of A-site structure could significantly affect A-site nuclease activity, be it catalyzed by the ribosome or a *trans*-acting factor. Interestingly, Aiba and colleagues showed that expression of a fusion protein containing the SecM-derived residues G_{161}IRAGP$_{166}$ 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 (28). We also observed similar cleavages near the A-site codon when expressing a fusion protein containing the longer SecM-derived Q_{149}FSTPVWISQAQGIRAGP$_{166}$ sequence (Figure 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 twenty 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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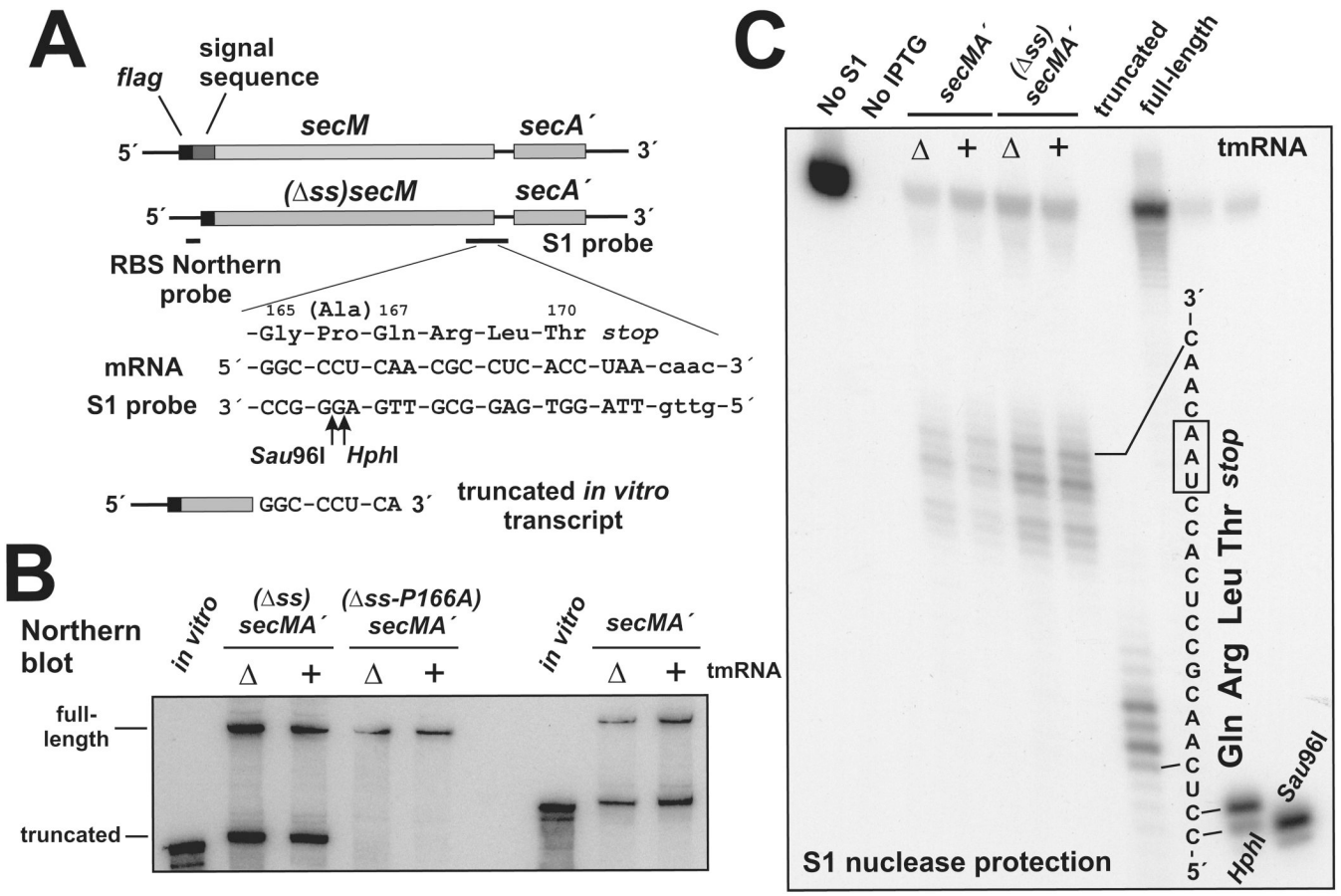

Figure 1. SecM ribosome arrest leads to mRNA cleavage

(A) *secMA´* mRNA variants are shown with the FLAG epitope, signal sequence and oligonucleotide probe binding sites indicated. SecM residues glycine-165 to threonine-170 and the encoding mRNA sequence are shown, as is the complementary sequence of the S1 nuclease probe and the 3´ terminus of the truncated *in vitro* transcripts used. The position of the P166A alteration is indicated by **(Ala)**. Arrows indicate the positions of *Hph*I and *Sau*96I restriction endonuclease cleavages in the S1 probe used to generate gel migration standards. **(B)** Northern blot of *secMA´* mRNAs purified from $tmRNA^+$ and ΔtmRNA cells. The positions of full-length and truncated *flag-(Δss)secMA´* mRNA are indicated. Both *in vitro* transcripts were truncated after the second nucleotide of the glutamine-167 codon. **(C)** S1 nuclease protection map of truncated *secMA´* mRNAs. Cleavages were detected in the *secM* stop codon and at positions one to four nucleotides downstream. No S1 protection was detected with RNA purified from a strain that had not been induced with IPTG. Truncated and full-length transcripts were produced by *in vitro* transcription and analyzed by S1 nuclease protection. The *Hph*I and *Sau*96I oligonucleotide standards were generated by annealing the 3´-labeled S1 probe to a complementary DNA oligonucleotide followed by digestion with the appropriate endonucleases.

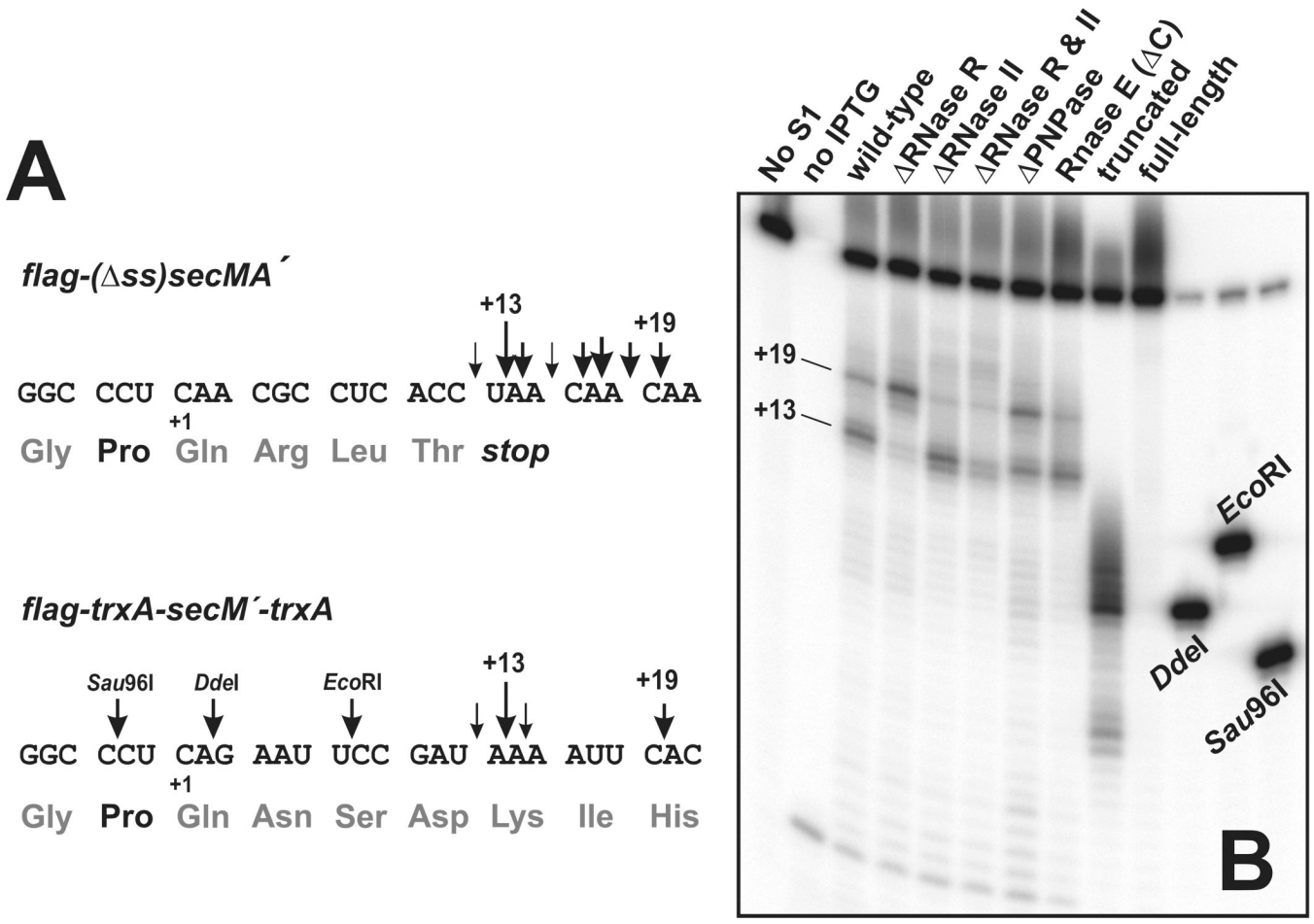

Figure 2. mRNA cleavage is modified in cells lacking 3´→5´ exoribonucleases

(A) RNA sequences of *flag-(Δss)secMA´* and *flag-trxA-secM´-trxA* messages near the observed cleavage sites are shown along with the encoded protein sequences. Downward arrows indicate the position and relative intensity of mRNA cleavage as determined by S1 nuclease protection analyses shown in panel B and Figure 1C. Numerical position is reported with respect to the codon corresponding to SecM proline-166, where position +1 is the first nucleotide of the codon corresponding to SecM glutamine-167. Downward arrows labeled ***Dde*I**, ***Eco*RI** and ***Sau*96I** indicate mRNA cleavage sites corresponding to the migration positions of S1 oligonucleotide probe standards. **(B)** S1 nuclease protection analysis of *flag-trxA-secM´-trxA* mRNA purified from cells lacking 3´→5´ exoribonucleases. Gene deletions and disruptions were constructed as described in Experimental Procedures. Positions +13 and +19 downstream of the codon corresponding to SecM proline-166 are indicated. The *Dde*I, *Eco*RI and *Sau*96I oligonucleotide standards were generated by annealing the labeled S1 probe to a complementary DNA oligonucleotide followed by digestion with the appropriate endonucleases.

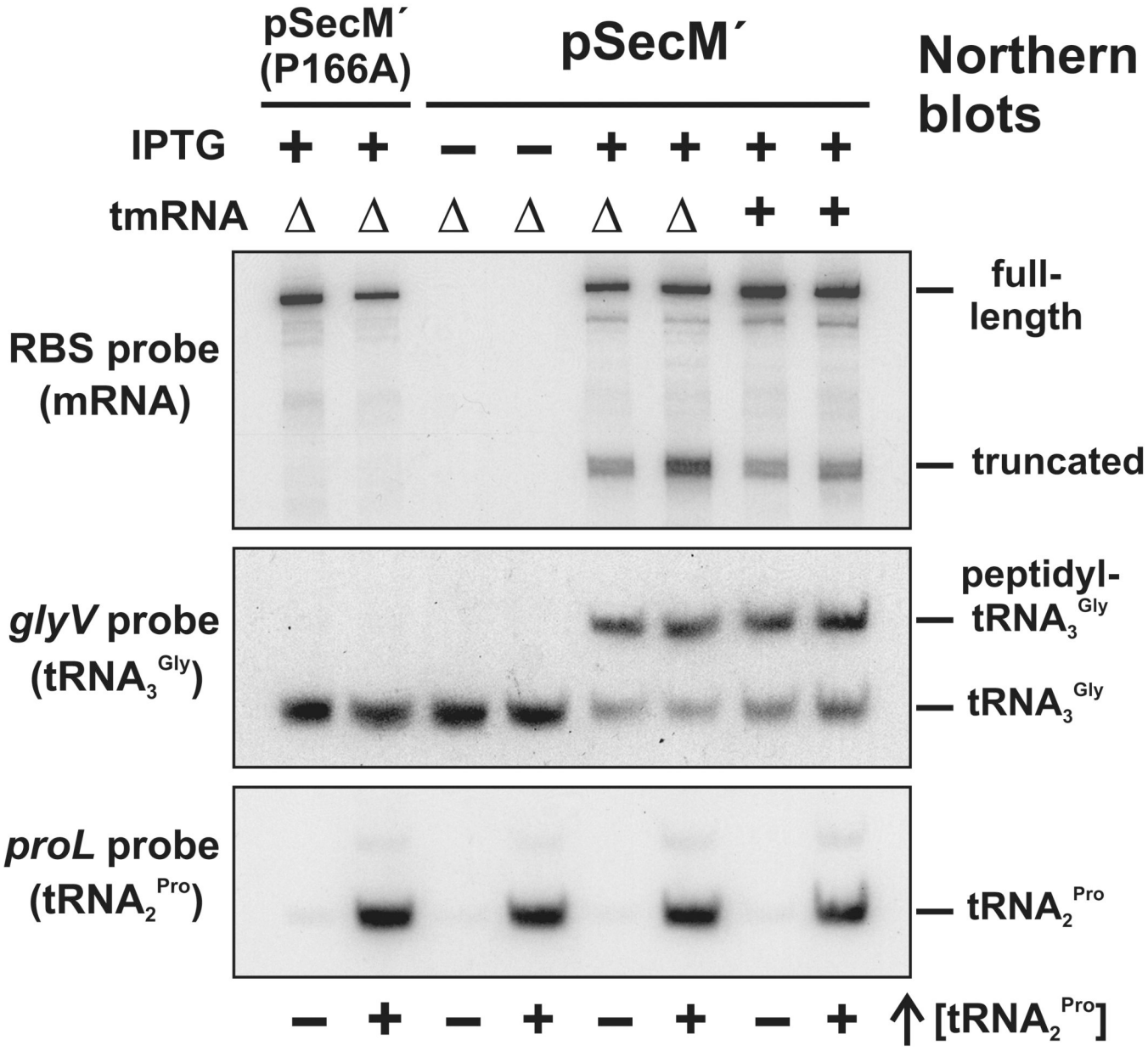

Figure 3. The SecM nascent peptide is covalently linked to $tRNA_3^{Gly}$

RNA from strains expressing *flag-secM´* [**pSecM´**] and *flag-(P166A)secM´* [**pSecM´ (P166A)**] mini-genes was analyzed by Northern blot to identify ribosome arrest-dependent peptidyl-tRNA. The **RBS**, ***glyV*** and ***proL*** oligonucleotide probes were specific for the ribosome binding site of mRNA, $tRNA_3^{Gly}$ and $tRNA_2^{Pro}$, respectively. The migration positions of full-length mRNA, truncated mRNA, $tRNA_3^{Gly}$, peptidyl-$tRNA_3^{Gly}$, and $tRNA_2^{Pro}$ are indicated. Samples containing over-expressed $tRNA_2^{Pro}$ are indicated by ↑[$tRNA_2^{Pro}$]. The slower migrating species detected in the ***proL*** probe blot is probably incompletely processed $tRNA_2^{Pro}$, as it is present in all over-expressed $tRNA_2^{Pro}$ samples, regardless of SecM expression.

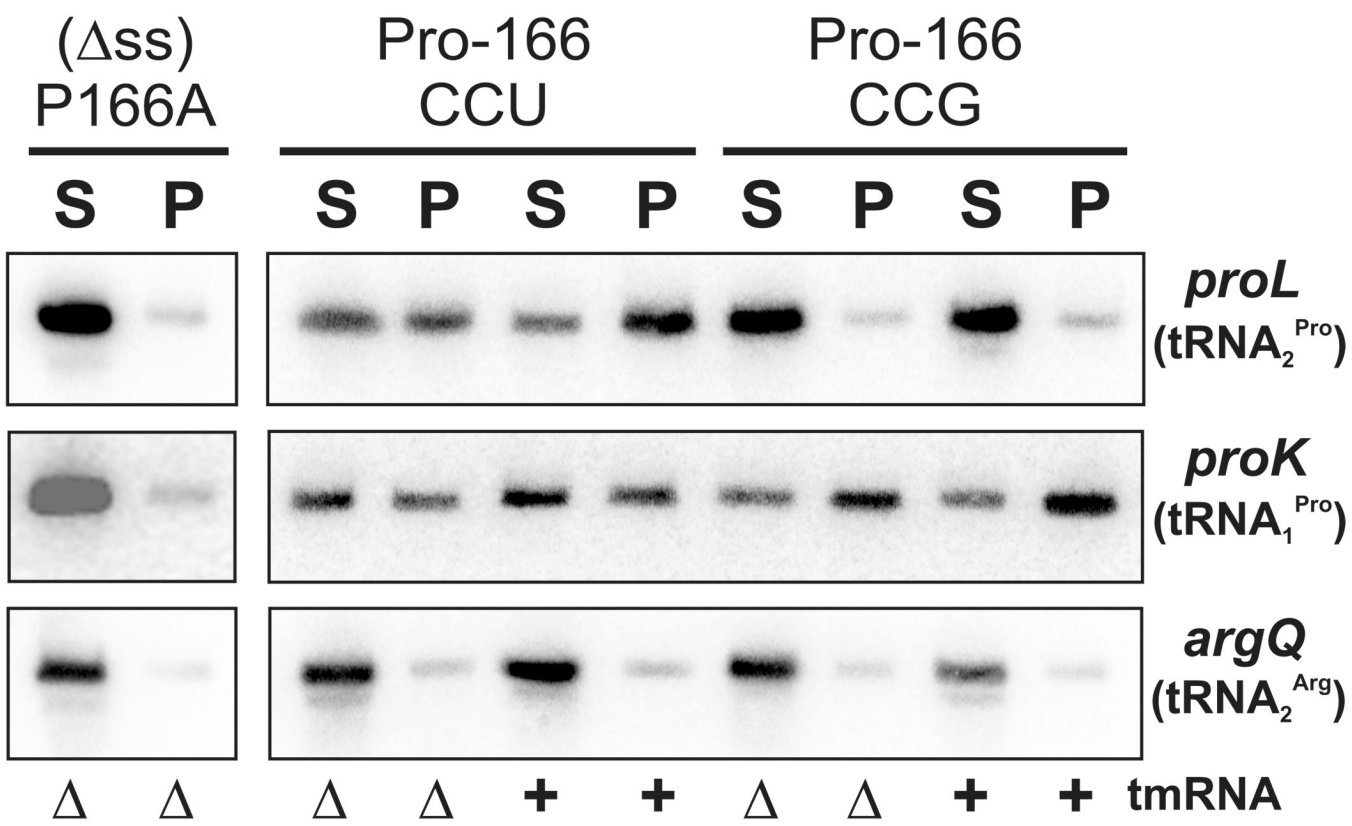

Figure 4. tRNA^{Pro} is associated with SecM-arrested ribosomes

Northern blot analysis of RNA purified from supernatant (**S**) and pellet (**P**) fractions obtained by ultracentrifugation of cell lysates through sucrose cushions. Cells expressed Flag-(Δss) SecM in which proline-166 was encoded by CCU, CCG, or changed to alanine (P166A). The ***proL***, ***proK*** and ***argQ*** oligonucleotide probes were specific for $tRNA_2^{Pro}$ (cognate for CCU and CCC), $tRNA_1^{Pro}$ (cognate for CCG), and $tRNA_2^{Arg}$ (cognate for CGU, CGC, and CGA), respectively. Samples from $tmRNA^+$ and ΔtmRNA strains are indicated.

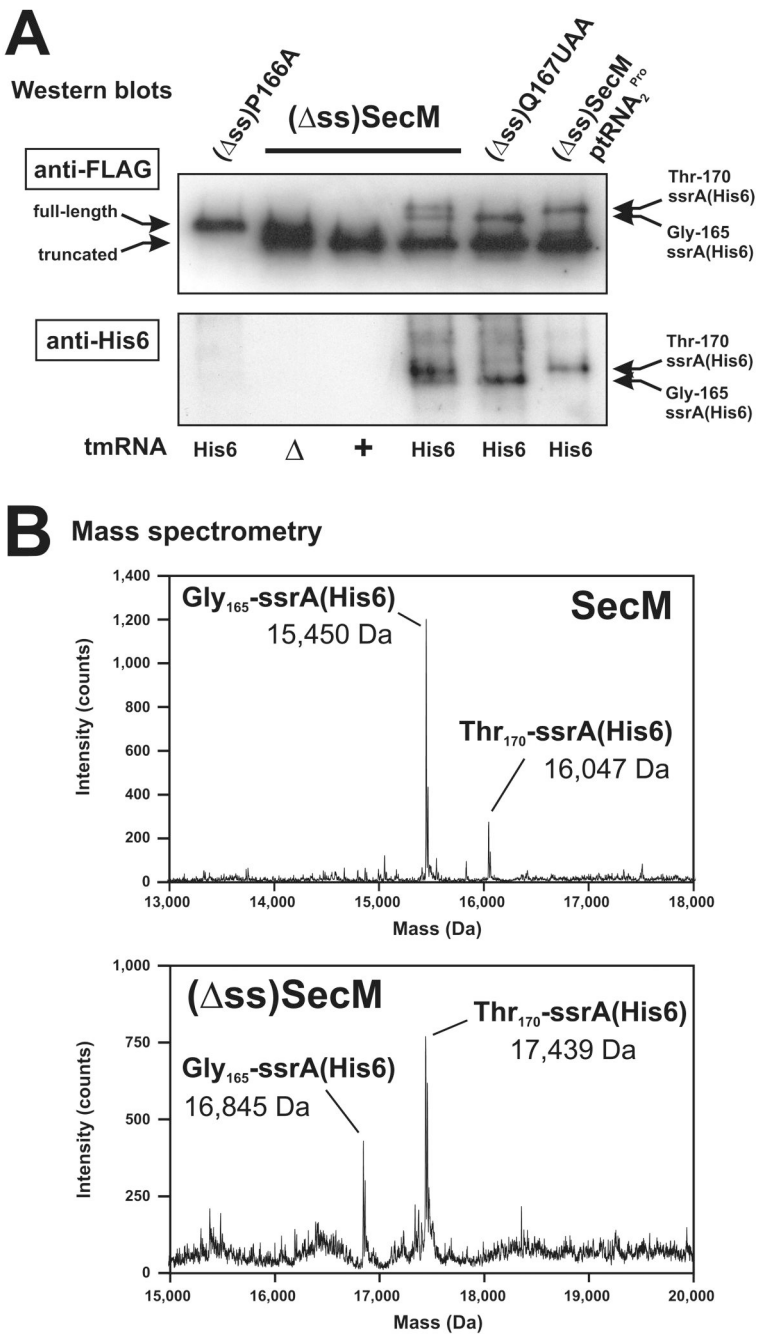

Figure 5. tmRNA activity at SecM-arrested ribosomes

(**A**) Western blot analyses of Flag-(Δss)SecM variants expressed in tmRNA^{+}, ΔtmRNA and tmRNA(His6) cells. Anti-His6 antibodies recognized ssrA(His6) 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(His6)-tagged proteins are indicated by labeled arrows. Plasmid ptRNA$_2^{Pro}$ over-expresses tRNA$_2^{Pro}$. (**B**) Mass spectrometry of ssrA(His6)-tagged SecM and Flag-(Δss)SecM proteins. Measured

masses were consistent with proteins containing C-terminal ssrA(His6) tags added after SecM residues glycine-165 and threonine-170.

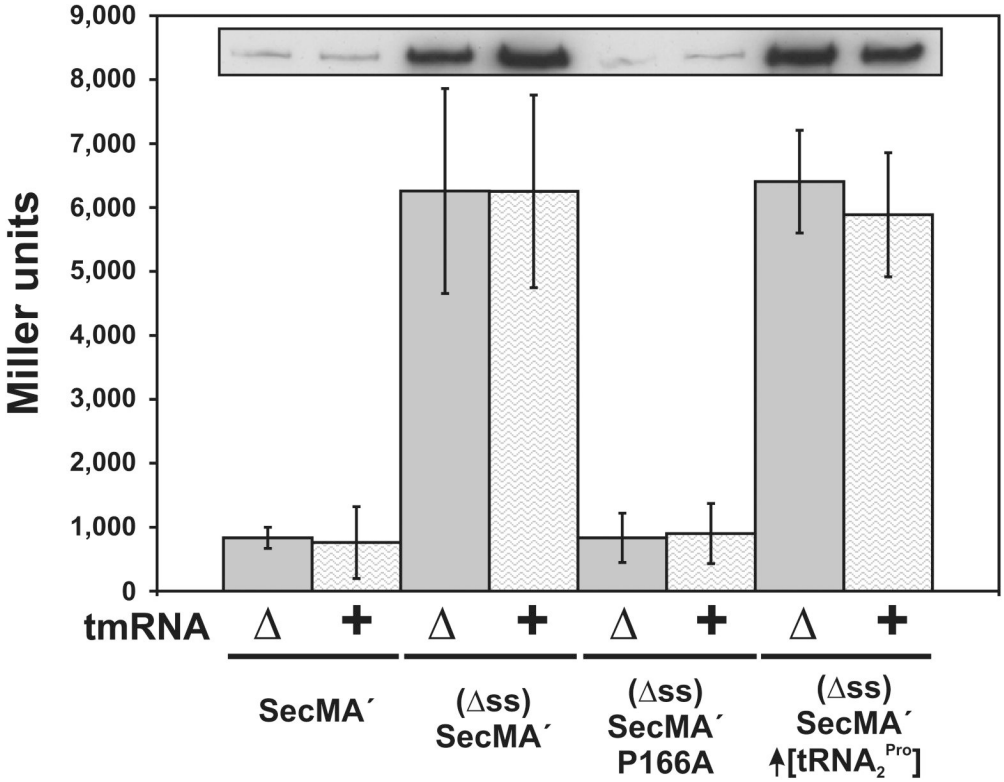

Figure 6. Over-expression of $tRNA_2^{Pro}$ does not affect translational regulation of SecA

SecM-dependent regulation of SecA-LacZ fusion protein expression was analyzed by β-galactosidase activity assay and Western blot analysis (inset). The SecA-LacZ translational fusion was expressed from constructs in which SecM was secreted [**SecMA´**], not secreted [**(Δss)SecM**], or failed to elicit ribosome arrest [**(Δss)SecM P166A**]. Western blot analysis was performed with polyclonal antibodies specific for *E. coli* β-galactosidase. $tRNA_2^{Pro}$ was over-expressed from plasmid $ptRNA_2^{Pro}$.

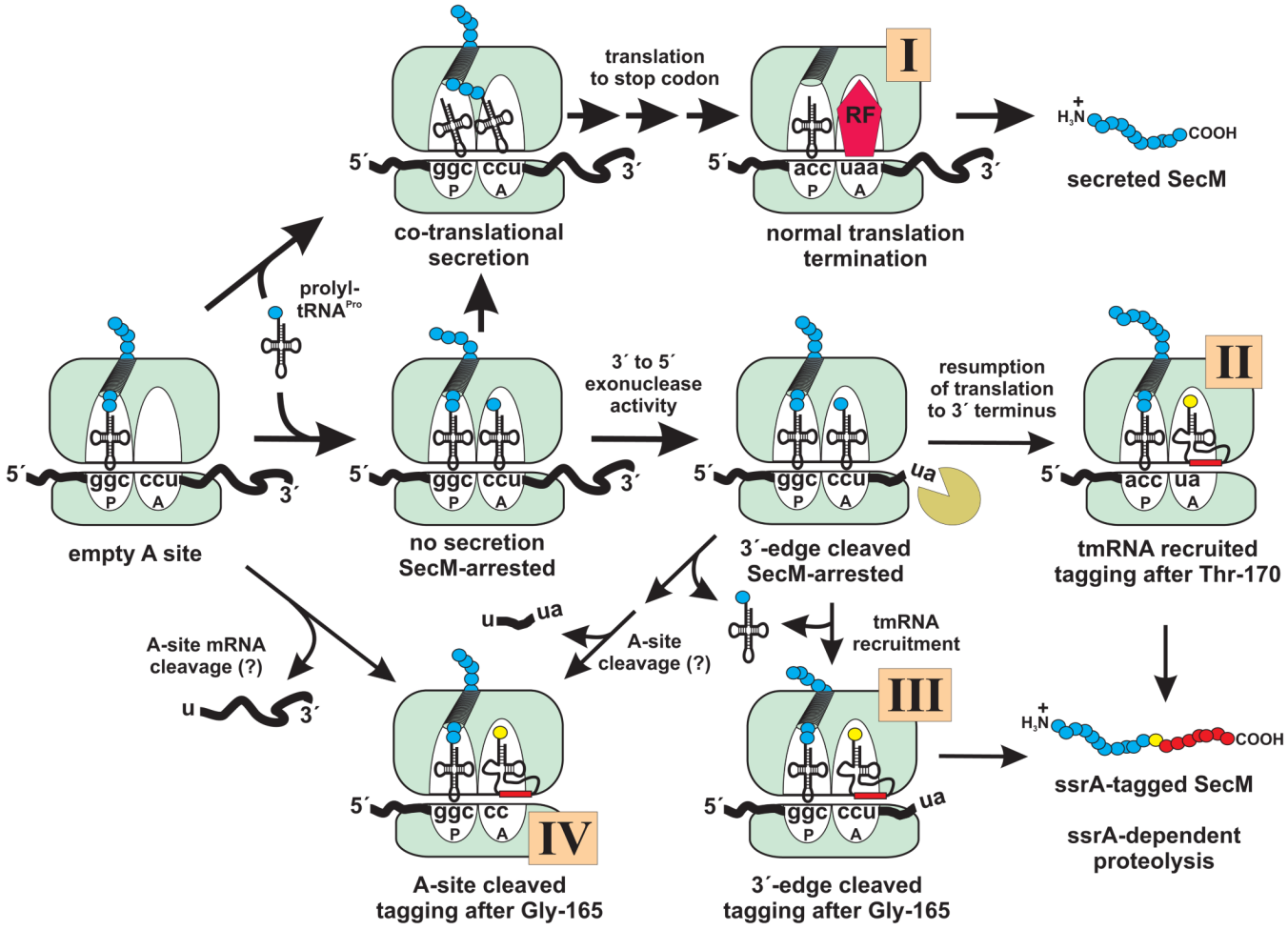

Figure 7. Fates of SecM-arrested ribosomes

Ribosomes that synthesize SecM have at least four distinct fates. (**I**) Ribosome arrest is prevented if prolyl-tRNA^{Pro} binds the A site during co-translational secretion of the SecM nascent chain. Such ribosomes continue to the stop codon and terminate translation normally. (**II**) Binding of prolyl-tRNA^{Pro} in the absence of co-translational secretion results in ribosome arrest and allows degradation of 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. (**III**) 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.

TABLE 1

Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Reference
Strains		
X90	F´ *lacI*^{q} *lac´ pro´/ara Δ[lac-pro] nalA argE[am] rif*^{r} *thi-1*	(17)
CH1924	X90 F^{-}	This study
CH1925	X90 F^{-} *ssrA::cat*, Cm^{r}	This study
CH113	X90 *ssrA::cat* (DE3), Cm^{r}	This study
CH12	X90 (DE3)	This study
CH2198	X90 *ssrA(his6)* (DE3), Kan^{r}	This study
CH988	CH113 *rnr::kan*, Cm^{r} , Kan^{r}	(20)
CH1002	CH113 *rne515::kan*, Cm^{r} , Kan^{r}	(2)
CH1207	CH113 *Δrnb::kan*, Cm^{r} , Kan^{r}	This study
CH1208	CH113 *Δpnp::kan*, Cm^{r} , Kan^{r}	This study
CH1227	CH113 *Δrnb rnr::kan*, Cm^{r},Kan^{r}	This study
CH1915	CH12 *Δrna::kan*,Kan^{r}	This study
CH1916	CH113 *Δrna::kan*, Cm^{r}, Kan^{r}	This study
Plasmids		
pFG21b	derivative of pET21b with FLAG epitope-encoding sequence, Amp^{r}	This study
pSecMA´	pFG21b*::secMA´*, Ampr^{r}	This study
p(Δss)SecMA´	pFG21b*::(Δss)secMA´*, Amp^{r}	This study
p(Δss-P166A)SecMA´	pFG21b*::(Δss-P166A)secMA´*, Amp^{r}	This study
pTrx-SecM´-Trx	pFG21b*::trxA-secM´-trxA*, Amp^{r}	This study
pSecM´	pFG21b*::secM´*, Amp^{r}	This study
pTrc3	derivative of pTrc99a containing 5´ *Nde*I restriction site, Amp^{r}	This study
pSecMA´-LacZ	pTrc3*::secMA´::lacZ*, Amp^{r}	This study
p(Δss)SecMA´-LacZ	pTrc3*::(Δss)secMA´::lacZ*, Amp^{r}	This study
p(Δss-P166A) SecMA´-LacZ	pTrc3*::(Δss-P166A)secMA´::lacZ*, Amp^{r}	This study
pCH405Δ	derivative of pACYC containing multi-cloning site, Tet^{r}	(2)
ptRNA$_2^{Pro}$	pCH405Δ*::proL*, over-expresses tRNA$_2^{Pro}$, Tet^{r}	This study