

# The Ribosomal Exit Tunnel Functions as a Discriminating Gate

Hitoshi Nakatogawa and Koreaki Ito<sup>1</sup>  
Institute for Virus Research and CREST  
Japan Science and Technology Corporation  
Kyoto University  
Kyoto 606-8507  
Japan

## Summary

Translation of SecM stalls unless its N-terminal part is “pulled” by the protein export machinery. Here we show that the sequence motif FXXXXWIXXXGIRAGP that includes a specific arrest point (Pro) causes elongation arrest within the ribosome. Mutations that bypass the elongation arrest were isolated in 23S rRNA and L22 r protein. Such suppressor mutations occurred at a few specific residues of these components, which all face the narrowest constriction of the ribosomal exit tunnel. Thus, we suggest that this region of the exit tunnel interacts with nascent translation products and functions as a discriminating gate.

## Introduction

The translational machinery, i.e., the ribosome, consists of both protein and RNA subunits. The atomic structure of the ribosomal large subunit (Ban et al., 2000; Nissen et al., 2000) indicates that its interior compartment, which functions in the peptidyl transfer reaction and subsequent movement of the polypeptide nascent chain, is composed largely of RNA. This feature could minimize aberrant interactions of the ribosomal constituents with the nascent product of translation, thus ensuring successful translation of proteins with unlimited sequence diversity. This was suggested specifically for the exit tunnel (Nissen et al., 2000). However, it is also known that some protein components contribute to the formation of the exit tunnel. More specifically, r proteins L4 and L22 protrude into the tunnel region, where their tips and RNA moieties form the narrowest constriction of the pathway. Why does the ribosome have such a constricted exit tunnel? Although either structural and/or functional roles are conceivable, this issue has not been addressed experimentally, except that there are some indications that erythromycin sensitivity of *Escherichia coli* is somehow related to this particular region of the ribosome (Chittum and Champney, 1994; Gregory and Dahlberg, 1999; Xiong et al., 1999; Gabashvili et al., 2001).

The *secM* (secretion monitor) gene of *E. coli* encodes a unique secretory protein that monitors cellular activity for protein export and accordingly regulates translation of the downstream *secA* gene (for the translocation ATPase) (Oliver et al., 1998). SecM is exported to the periplasm, where it is rapidly degraded by the tail-specific protease (Nakatogawa and Ito, 2001). Despite its translocation to the periplasm, the functional form of

the protein is the nascent polypeptide in the cytoplasm. The translation of SecM is subject to elongation arrest at a site close to the C terminus (Nakatogawa and Ito, 2001). This elongation arrest is transient under normal conditions, where the nascent SecM precursor interacts with the SRP-Sec translocation system. In contrast, the arrest is prolonged strikingly in the absence of active export of SecM, due either to a *cis* defect in its signal sequence or to a *trans* defect in the Sec machinery. The stalled ribosome may then disrupt the secondary structure of the *secM-secA* messenger RNA, leading to the exposure of the *secA* translation initiation sequence for the entry of new ribosomes that translate *secA* (McNicholas et al., 1997; Oliver et al., 1998; Nakatogawa and Ito, 2001).

Unlike the SRP-dependent translation arrest observed in mammalian cells (Walter and Johnson, 1994), neither the bacterial SRP nor the SecM signal sequence is required for the arrest (Nakatogawa and Ito, 2001). Instead, the nascent SecM product itself seems to be involved in the elongation arrest, since the elongation arrest was alleviated significantly in the presence of a proline analog, azetidine (Nakatogawa and Ito, 2001).

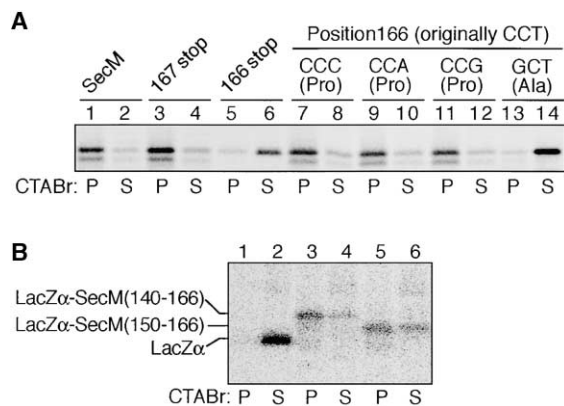
In this study, we identified Pro166 of SecM as the arrest point and a sequence motif including this residue FXXXXWIXXXGIRAGP as the element that causes arrest from within the ribosome during translation. This SecM segment acted as an obstacle to translation elongation even when it was present within unrelated sequences. The elongation arrest appeared to be brought about by specific interaction of these residues with ribosomal components, as we were able to identify mutations in both RNA (23S rRNA) and protein (L22) components of the ribosome that enabled completion of translation beyond Pro166. On the three-dimensional structure of the ribosomal large subunit, the mutated residues face the inner wall of the exit tunnel, clustering around the tunnel constriction. We propose that the constricted part of the tunnel acts as an exit gate by interacting with nascent chains. This interaction could regulate pausing or attenuate the rate of further translation.

## Results

### SecM Translation Stalls at Pro166, a Residue that Is Essential for the Arrest

To determine the position of the elongation arrest, a TAA stop codon was inserted into various positions near the 3' end of the *secM* coding sequence. Insertions downstream of the arrest point will not affect the arrest. In contrast, the arrest will be abolished if the arrest point itself is converted to TAA or if this codon is inserted upstream of the arrest point. We assessed the elongation arrest using cetyltrimethylammonium bromide (CTABr), which precipitates nucleic acids. Thus, an elongation-arrested peptidyl-tRNA molecule is CTABr precipitable due to its RNA moiety (Gilmore et al., 1991). Both the wild-type SecM and a variant having a TAA

<sup>1</sup>Correspondence: kito@virus.kyoto-u.ac.jp



**Figure 1. C-Terminal SecM Segment within the Ribosome Causes Elongation Arrest**

(A) Elongation arrest occurs at Pro166. Plasmids encoding SecM and its derivatives were introduced into the SecY-defective strain, K1297/pST30 (Shimoike et al., 1995). Cells were pulse labeled with [<sup>35</sup>S]methionine, and samples were CTABr fractionated into pellet (P) and supernatant (S), followed by immunoprecipitation with anti-SecM, SDS-PAGE, and phosphor image visualization as described previously (Nakatogawa and Ito, 2001). Plasmids carried were: lanes 1 and 2, pNH21 (SecM); lanes 3 and 4, pNH52 (Gln167stop); lanes 5 and 6, pNH53 (Pro166stop); lanes 7 and 8, pNH57 (Pro166Pro(CCC)); lanes 9 and 10, pNH58 (Pro166Pro(CCA)); lanes 11 and 12, pNH59 (Pro166Pro(CCG)); and lanes 13 and 14, pNH61 (Pro166Ala). The multiple bands observed in the CTABr precipitates in this and other experiments were produced presumably by multiple ribosomes that had stalled on the messenger RNA.

(B) LacZ $\alpha$ -SecM fusion proteins. Cells of strain JM109 carrying pSTV28 (LacZ $\alpha$ ; lanes 1 and 2), pNH107 (LacZ $\alpha$ -SecM(140-166); lanes 3 and 4) or pNH108 (LacZ $\alpha$ -SecM(150-166); lanes 5 and 6) were pulse labeled and CTABr fractionated. After LacZ immunoprecipitation (antiserum from 5'→3', Inc.), samples were subjected to Tricine-SDS-PAGE (Schägger and von Jagow, 1987).

mutation introduced into the Gln167 position were mainly produced as the CTABr-precipitable forms (Figure 1A, lanes 1–4). In contrast, a TAA substitution for the Pro166 codon converted SecM to the CTABr-soluble form (Figure 1A, lane 6). Thus, the SecM translation is arrested at Pro166, the fifth position from the end of this open reading frame.

The Pro166 codon, CCT, was changed to the other proline codons, CCC, CCA, or CCG, without affecting the CTABr-precipitability of SecM (Figure 1A, lanes 7–12). In contrast, the translation arrest was completely abolished by an alanine substitution at this position (Figure 1A, lanes 13 and 14). These results suggest that the presence of proline at this position is particularly important for the elongation arrest, in agreement with the effect of a proline analog, azetidine (Nakatogawa and Ito, 2001).

### C-Terminal Region of SecM Functions within the Ribosome as an Independent Elongation-Arresting Element

We speculated previously that the nascent SecM might be folded outside the ribosome into a domain, which could somehow exert a feedback inhibition against the ribosome (Nakatogawa and Ito, 2001). However, we found that a SecM derivative lacking the N-terminal 122 residues remained CTABr precipitable (data not shown).

The elongation-arrested peptidyl-tRNA of this construct contains only 44 amino acids, which should be mostly inside the ribosome (Nissen et al., 2000; Blobel and Sabatini, 1970; Hardesty and Kramer, 2000). Thus, the SecM translation arrest seems to occur solely within the interior of the ribosome.

We fused the C-terminal regions of SecM to the C terminus of LacZ $\alpha$ . Whereas LacZ $\alpha$  itself was completely CTABr-soluble (Figure 1B, lane 2), a fusion protein having the Asp140-Pro166 segment of SecM was nearly quantitatively precipitated by CTABr (Figure 1B, lane 3). A shorter SecM segment, from Phe150 to Pro166, was also effective in converting LacZ $\alpha$  to the peptidyl-tRNA form, although slightly less efficiently than the Asp140-Pro166 segment (Figure 1B, lanes 5 and 6). The CTABr-precipitability of these fusion proteins was abolished after RNase treatment of the sample (data not shown). From these results, we have narrowed down the arrest-causing SecM sequence to the 17 residues of the Phe150-Pro166 interval.

When the C-terminal regions of SecM were attached to two unrelated proteins (MalE and RepA), the resulting fusion peptides were also subject to elongation arrest (data not shown). The SecM C-terminal segment also functioned at an N-terminal region of LacZ $\alpha$ , to abort enzyme production (see below). Thus, arrest of elongation can be conferred by the C-terminal region of SecM, independent of its sequence context.

### Identification of the SecM Amino Acid Sequence that Causes Elongation Arrest

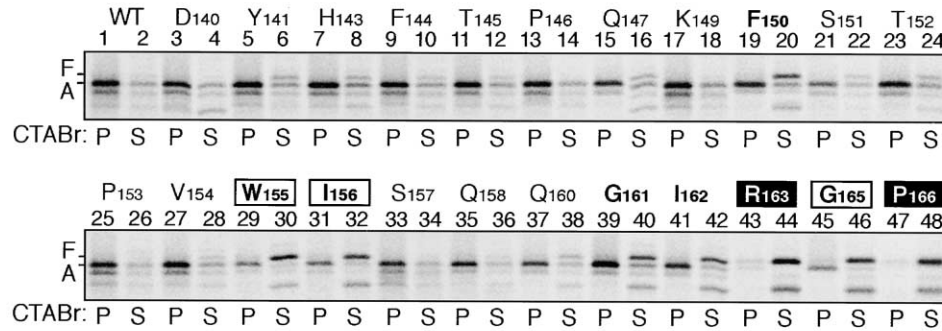
The Asp140-Pro166 segment of SecM (Figure 2A) was subjected to alanine-scanning mutagenesis (Figure 2B). The arrest was affected significantly by mutations at several residues in the Phe150-Pro166 segment. Mutations Pro166Ala and Arg163Ala completely abolished the CTABr-precipitability, allowing production of the full-length products (Figure 2B, lanes 44 and 48). Mutations at Trp155, Ile156, and Gly165 also resulted in the production of the full-length products (Figure 2B, lanes 30, 32, and 46), although small proportions of arrested fragments remained (Figure 2B, lanes 29, 31, and 45). Mutations at Phe150, Gly161, and Ile162 led to the production of small amounts of the full-length products (Figure 2B, lanes 20, 40, and 42). Ala substitutions for all the other residues were either completely or almost completely silent. The wild-type Ala residues at positions 142, 148, 159, and 164 were replaced by Ser; only the Ala164Ser mutation caused a significant defect in the translation arrest (Figure 2C, lane 10).

From these results, we conclude that Pro166 and Arg163 are essential; Trp155, Ile156, and Gly165 are important; and Phe150, Gly161, Ile162, and Ala164 are partially required for the ability of SecM to arrest translation elongation. The extent of the elongation arrest was examined by pulse-chase experiments (Figure 2E). The Phe150 and the Trp155 mutants both converted the arrested product into the full-length product more slowly than the Pro166 mutant protein, which produced full-length product from the start (Figure 2E, compare lanes 4, 7, and 10). It should be noted that not only the substitution mutations but also insertions and deletions of one residue significantly compromised the arrest activity

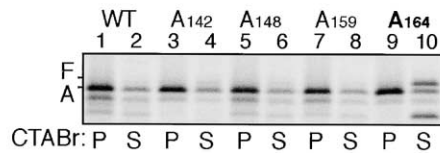
**A** Target sequence

140 145 150 155 160 165 170  
-DYAHFTPQAKFSTPVTWISQAQGGIRAGPQRLT

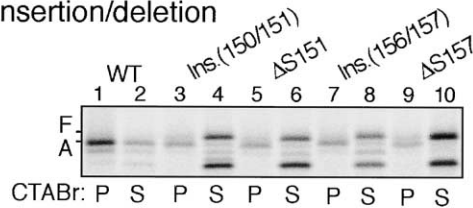
**B** Ala substitutions



**C** Ser substitutions



**D** Insertion/deletion



**E** Pulse chase

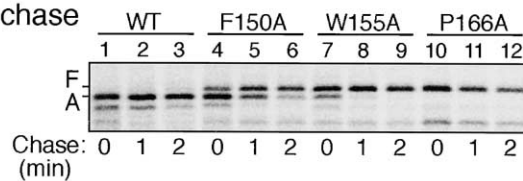


Figure 2. Mutational Analyses of the Asp140-Pro166 Segment of SecM

(A) SecM amino acid sequence for residues 140–170.

(B) Alanine-scanning mutagenesis. Each residue of this SecM segment, as indicated, was replaced by alanine.

(C) Serine substitutions. The wild-type alanine residues at the indicated positions were replaced by serine.

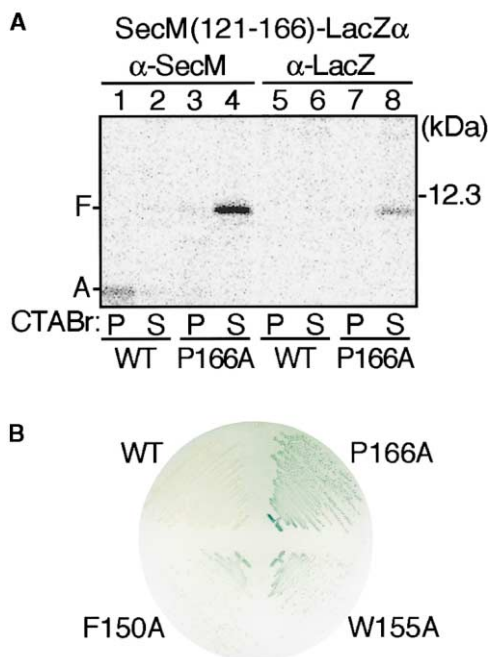
(D) One-residue insertions and deletions. Alanine was inserted between Phe150 and Ser151 as well as between Ile156 and Ser157, whereas Ser151 as well as Ser157 were deleted individually. All the SecM derivatives in (B)–(D) were expressed under the SecY-deficient conditions, pulse labeled, and CTABr fractionated.

(E) Arrest-release kinetics. SecM and its Ala substitution mutants, as indicated, were pulse labeled under the SecY-deficient conditions for 1 min and chased with unlabeled methionine for 0, 1, and 2 min, as indicated. A and F represent the translation-arrested and the full-length products, respectively.

(Figure 2D). Thus, the exact spacing of the relevant residues is important.

We constructed a genetic system to monitor the elongation-arresting function of SecM. SecM residues 121–166 were fused to the N terminus of the enzymatically active LacZ $\alpha$  domain at the DNA level [SecM(121–166)-LacZ $\alpha$ ]. This construct produced only a CTABr-precipitable fragment of about 5 kDa that reacted with anti-SecM (Figure 3A, lane 1) but not with anti-LacZ (Figure 3A, lane 5). When the Pro166Ala mutation was introduced into the fusion protein, the resulting mutant protein, SecM(121–P166A)-LacZ $\alpha$ , was synthesized as a CTABr-soluble product of about 12 kDa, which reacted with both antibodies (Figure 3A, lanes 4 and 8). The latter product seemed to be the full-length fusion protein

and thus should exhibit enzymatic activity. This was confirmed by expression in a LacZ $\omega$  strain. Whereas cells expressing SecM(121–166)-LacZ $\alpha$  formed white colonies, those expressing the Pro166Ala mutant version formed dark blue colonies on agar medium containing X-Gal, a chromogenic substrate of  $\beta$ -galactosidase (Figure 3B). Other arrest-compromising mutations also gave blue-colored colonies (Figure 3B). Taking advantage of this reporter system, we isolated a large number of arrest-deficient mutations in SecM(121–166)-LacZ $\alpha$  by site-directed random mutagenesis. Thus, we isolated all possible substitutions at positions 166 and 163 as arrest-impairing mutations. Also, most of the substitutions so far identified at the other important residues were destructive. From these results, we conclude that these



**Figure 3.** SecM Sequence 121–166 Blocks Translation of C-Terminally Fused LacZ $\alpha$

Strain JM109 was transformed either with pNH122 encoding SecM(121–166)-LacZ $\alpha$  or its variants containing a mutation in the SecM part.

(A) CTABr precipitability and antigenicity. Fusion proteins SecM(121–166)-LacZ $\alpha$  (lanes 1, 2, 5, and 6) and SecM(121–P166A)-LacZ $\alpha$  (lanes 3, 4, 7, and 8) were pulse labeled and CTABr fractionated into pellet (P) and supernatant (S). The samples were then immunoprecipitated with anti-SecM (lanes 1–4) and anti-LacZ (lanes 5–8).

(B)  $\beta$ -galactosidase activities as represented by colony color. The JM109 transformants expressing SecM(121–166)-LacZ $\alpha$  (wt) and its variants with a SecM amino acid substitution as indicated were grown at 37°C overnight on L agar plates supplemented with 40  $\mu$ g/ml X-Gal, 1 mM IPTG, 0.5 mM phenylethyl- $\beta$ -D-thiogalactopyranoside, and 20  $\mu$ g/ml chloramphenicol.

residues are uniquely required for the elongation arrest of SecM. Taken together, these results suggest that the amino acid alignment FXXXXWIXXXGIRAGP causes arrest from within the ribosome during translation.

### Ribosomal RNA Mutations that Circumvent Elongation Arrest

By what mechanism does the SecM sequence cause elongation arrest? We thought it possible that interaction of the SecM arrest motif with the interior of the ribosome, such as the exit tunnel components, could result in the stalled movement of the translation product. If so, it might be possible to isolate mutations in the ribosomal components that would alleviate the arrest. According to the crystal structure of the large subunit of the *Haloarcula marismortui* ribosome, the exit tunnel is composed mostly of RNA (Ban et al., 2000; Nissen et al., 2000). Thus, a plasmid carrying the *rrnB* operon (Xiong et al., 2000) was mutagenized and introduced into cells expressing SecM(121–166)-LacZ $\alpha$ . Ten independently mutagenized plasmid pools were screened

for clones that gave dark blue or pale blue colonies (Figure 4B, left). Strikingly, sequencing revealed only two single-base changes that had been isolated repeatedly by these screenings. The dark blue clones all possessed a single-base change, A2058 to G, in domain V of 23S rRNA, whereas the pale blue clones contained one adenine insertion within the five consecutive A residues (A749–A753) in hairpin 35 of domain II (Figure 4A). These base changes, named *rrlB2058* and *rrlB751*, respectively, were mapped on the published three-dimensional structure of the large subunit. A2058 (the *E. coli* coordinate) (Ban et al., 2000) was found to be located on the inner wall of the narrowest part of the exit tunnel (Figure 4D, orange). The A749–A753 region was shown to occupy a part of this wall on the opposite side (Figure 4D, yellow). The mutations *rrlB2058* and *rrlB751* indeed resulted in the production of significant amounts of the full-length SecM-LacZ $\alpha$  protein (Figure 4C, lanes 4 and 6). These results indicate that alterations at two specific regions of 23S rRNA lead to suppression of the elongation-arresting function of SecM. These regions coincide with the narrowest part of the exit tunnel.

### Ribosomal Protein Mutations that Circumvent Elongation Arrest

The above region of the ribosome is also characterized by the presence of domains of two proteins, L22 and L4, which surround the constricted part of the tunnel (Nissen et al., 2000). A  $\beta$  hairpin loop of L22 lies between the sites of the two 23S rRNA mutations (Figure 4D, green). We mutagenized a *rpIV* (L22) plasmid and screened for L22 mutations that gave blue colonies when expressed in the reporter strain. Three amino acid alterations, Gly91 to Ser, Ala93 to Thr, and Ala93 to Val, were isolated repeatedly in several independent experiments (Figure 4A). Thus, residues 91 and 93 were the hot spots for the arrest-suppressing mutations in L22. These residues were located on the segment of L22 that protrudes into the exit tunnel at the constricted region (Figure 4D, green spheres). There are several highly conserved amino acid residues of L22 around the hot spot (Unge et al., 1998; underlined in Figure 4A). We carried out alanine/serine-scanning mutagenesis of these residues. Among the Pro87Ala, Arg88Ala, Ala89Ser, Gly91Ala, Arg92Ala, and Ala93Ser mutations thus constructed, only the Gly91Ala and the Ala93Ser mutations resulted in the production of blue-colored colonies (Figure 4B, right) as well as in the increased production of the full-length SecM(121–166)-LacZ $\alpha$  protein (Figure 4C, lanes 10 and 12). Therefore, the two residues, Gly91 and Ala93, are specifically important for elongation arrest.

To further ascertain the importance of the specific residues of L22, we carried out nonbiased isolation of chromosomal mutations that affected SecM elongation arrest. The arrest reporter plasmid was introduced into bacterial cells that had been treated with a mutagen, ethyl methanesulfonate. Among about  $4 \times 10^6$  transformants screened, 15 formed blue colonies. Three of the mutations were linked to a transposon insertion at 73 min on the chromosome, a marker close to the ribosomal protein gene cluster. Remarkably, these mutants all contained a single base change within *rpIV*: two of them for

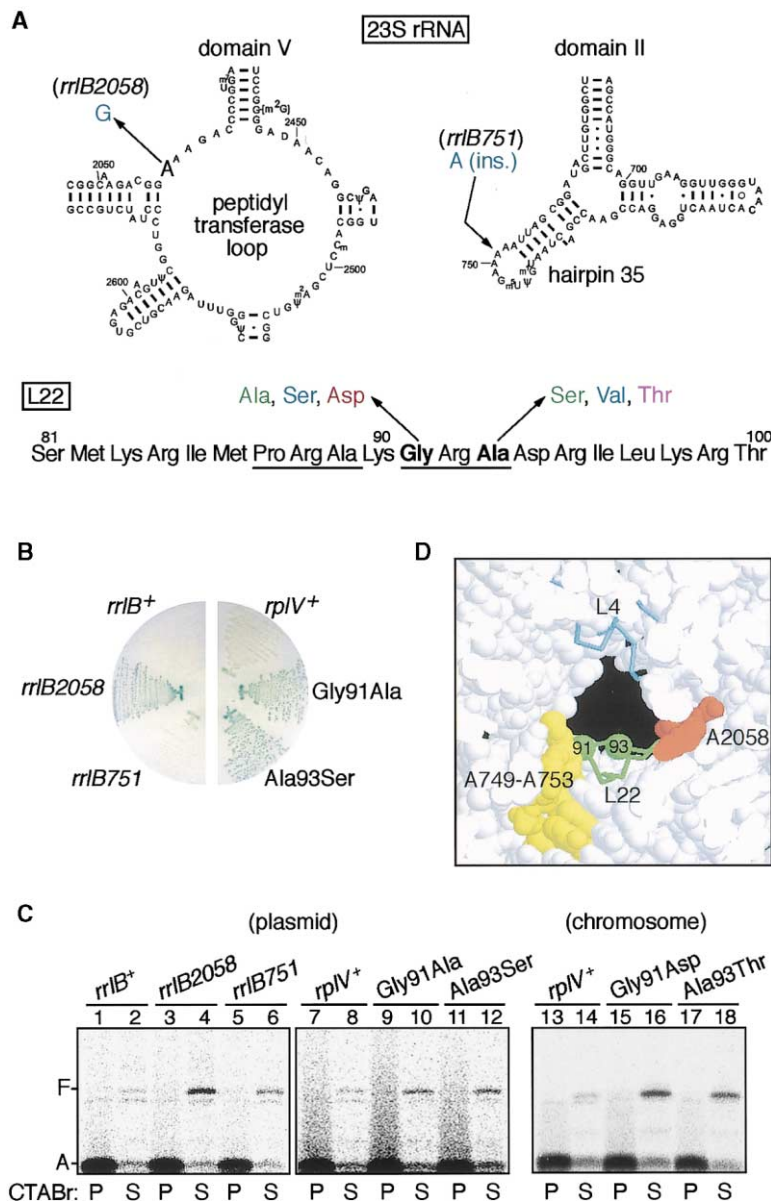


Figure 4. Ribosomal Mutations Alleviating the SecM Translation Arrest

(A) Ribosomal residues altered by the arrest-suppressing mutations. The 23S rRNA sequence is shown for domain V and domain II regions, with the secondary structure as downloaded from <http://www.rna.icmb.utexas.edu> (Ban et al., 2000). The L22 amino acid sequence is also shown for the interval of residues 81–100. The mutational alterations are shown by arrows. Those shown in blue were obtained on the genes carried on plasmids after random mutagenesis; those in red were obtained after random mutagenesis of the chromosome; and those in purple were obtained by both of the above procedures. Mutations shown in green were identified after alanine/serine-scanning mutagenesis of the underlined L22 residues.

(B) Colony color development. Plasmids pNK (*rrIB*<sup>+</sup>), pNH152 (*rrIB2058*), pNH153 (*rrIB751*), pNH106 (*rpIV*<sup>+</sup>), pNH146 (Gly91Ala), and pNH148 (Ala93Ser) were introduced into strain AD16 (Kihara et al., 1997) that carried pNH122 (SecM(121–166)-LacZ $\alpha$ ). Cells were grown on X-Gal-containing L agar plates additionally supplemented with 50  $\mu$ g/ml ampicillin, as described in Figure 3B.

(C) CTABr fractionation. Cells were pulse labeled, CTABr fractionated, and subjected to anti-SecM immunoprecipitation. In the experiments for lanes 1–12, the mutations on the plasmids were examined in the presence of chromosomal *rrIB*<sup>+</sup> and *rpIV*<sup>+</sup>, whereas lanes 13–18 were for experiments using the chromosomal *rpIV* mutants.

(D) Clustering of the mutation sites on the constricted region of the ribosomal exit tunnel. The nucleotide and amino acid residues, at which the arrest-circumventing mutations had been isolated, were allocated on the 3D structure of *Haloarcula marismortui* ribosomal large subunit, using the RasMol format file obtained from Protein Data Bank. The main exit tunnel is viewed from the peptidyl-transferase center side. The black part indicates the opening on the cytoplasmic end of the tunnel. Some of the front residues are omitted to allow visualization of the whole image of the tunnel. 23S rRNA, L22, and L4 are shown in white, green, and cyan, respectively.

tively. Note that L22 and L4 are protruding from the cytoplasmic side and their main bodies are invisible in this figure. Residues A749–A753 of 23S rRNA are shown in yellow, and A2058 is shown in orange. Gly91 and Ala93 of L22 are shown by green spheres. The residue numbers are those of the *E. coli* components (Ban et al., 2000).

a Gly91 to Asp amino acid change and another for an Ala93 to Thr change in L22 (Figure 4A). These chromosomal L22 mutations alleviated the elongation arrest of SecM(121–166)-LacZ $\alpha$  more effectively than the plasmid-carried mutations that were expressed in the presence of the chromosomal wild-type *rpIV* (Figure 4C, compare lanes 13–18 with lanes 7–12).

Colony color observations suggested that, of the remaining 12 mutants, 8 were complemented by the rRNA plasmid, although we have not determined which of the 7 rRNA operons had the arrest-suppressing mutation for each case. The remaining 4 mutations are currently under investigation. Thus, the results so far reinforce the conclusion that residues 91 and 93 of L22 are specifically involved in the elongation arrest of SecM.

## Discussion

We have shown here that the ribosome can discriminate among certain amino acid sequences as they emerge from the peptidyl transferase center, and this discrimination might occur through interaction between the nascent chain and the exit tunnel. Our mutational studies show that the motif FXXXXWIXXXGIRAGP is required for efficient elongation arrest of SecM. The fact that the arrest can be overcome by the Sec export implies that physical force can influence the process. If one assumes that this region of polypeptide cannot be folded inside the ribosome, the participation of multiple residues would suggest that the arrest is brought about by an accumulation of interactive forces generated between

participating residues and ribosomal components. In this scenario, the ribosomal constriction site mutations that alleviate the arrest might do so by decreasing one or several of the contributing interactions. It is conceivable that the arrest-essential residues near the C-terminal end of SecM interact with other ribosomal residues that are closer to the peptidyl transferase center and that we failed to isolate suppressor mutations affecting these residues because such alterations compromised essential translation functions.

On the other hand, if the nascent chain can fold to some extent inside the ribosome (Hardesty and Kramer, 2000), the SecM arrest segment could possibly form some higher order structure, and thus a more complex interaction between the arrest domain and the ribosomal components might be possible. For instance, arrest-essential C-terminal residues such as the tRNA-linked Pro166 might induce a special configuration of the SecM arrest segment within the ribosome such that it cannot move across the constricted region of the exit tunnel.

In either case, our results suggest that the amino acid alignment of FXXXXWXXXXGIRAGP interacts with the exit tunnel of the ribosome, especially the narrowest region formed by the RNA and protein components. In our mutant isolation, only very specific alterations of the ribosomal components have been isolated as the arrest suppressors. Mutagenesis of the rRNA plasmid only yielded mutations at the two 23S rRNA sites that constitute the constricted part of the exit tunnel. Mutagenesis of either the L22 plasmid or even the entire chromosome yielded mutations at two specific residues of L22, residues 91 and 93. These results suggest that the arrest suppression was not caused by nonspecific ribosomal alterations, but only by alterations of specific residues of the ribosomal components.

The two L22 residues identified as crucial for the arrest are glycine and alanine which have the two smallest side chains. Thus, the suppressor mutations may contract the opening. This argues against a notion that the elongation arrest of SecM is essentially due to a jamming-like effect, although structural understanding of the mutational effects awaits actual determination of the altered structure. The results of our random mutagenesis also indicate that arrest-compromising alterations in SecM can result from either bulkier or smaller side chains. Thus, it is unlikely that a jamming effect primarily accounts for the elongation arrest. We propose that interaction between the nascent sequence and the ribosomal components restricts the movement of the translation product, leading to the inhibition of the peptidyl transfer and/or the ribosomal translocation reaction. This might involve some kind of signal transmission from the gate to the peptidyl transfer center. Alternatively, more steric mechanisms are conceivable. The transpeptidation reaction might require proper positioning of the amino acyl- and peptidyl-tRNA in each binding site (Polacek et al., 2001), and this feature might be disordered by the interaction between the arrest segment and the ribosomal components including the exit gate.

We noted some similarity between the action of SecM and that of erythromycin; they both cause elongation arrest by interacting primarily with the exit gate components (Gabashvili et al., 2001; Chittum and Champney, 1994; Gregory and Dahlberg, 1999; Xiong et al., 1999).

The A2058 to G alteration (*rrlB2058* in this work) was reported previously by Vester and Garrett (1987) as an erythromycin-resistance mutation. Another erythromycin-resistance mutation, *rplV281* (a deletion of Met82-Lys-Arg in L22), has been reported to open the exit tunnel, including the constricted region (Gabashvili et al., 2001). This mutation was found also to alleviate the elongation arrest of SecM(121–166)-LacZ $\alpha$  (H.N., unpublished results). However, our chromosomal L22 mutations at residues 91 and 93 did not confer erythromycin resistance (H.N., unpublished results). We also examined a published erythromycin-resistant mutant of L4, but it did not affect the elongation arrest. Incidentally, this L4 mutation has been reported to constrict the tunnel even further (Gabashvili et al., 2001). Thus, erythromycin resistance only partially correlates with the SecM elongation arrest.

The central dogma assumes that messages can direct the synthesis of proteins with any amino acids according to the proper arrangement of in-frame codons. It has been interpreted that the ribosomal tunnel does not interact strongly with translation products of diverse amino acid sequences (Nissen et al., 2000). However, our finding that the SecM arrest sequence is incompatible with exit through the tunnel gate challenges this notion. We suggest that the exit gate discriminates against certain sequences. In other words, there are certain limitations on the amino acid sequence in proteins—not every protein sequence exits the ribosome equally well.

SecM homologs are known to exist in at least three other bacterial species (Sarker et al., 2000). The FXXXXWXXXXGIRAGP motif is well conserved among them. Within *E. coli*, the arrest sequence is unique and only a few proteins contain a segment that significantly resembles the SecM arrest sequence (Y. Makita and K. Nakai, personal communication). It would be interesting to assay whether these proteins undergo elongation arrest. We found that overproduction of SecM without the signal sequence is highly toxic to the cell, perhaps because a large number of ribosomes are sequestered by the arrest mechanism. In spite of its potentially deleterious nature, the arrest sequence has been exploited by SecM to regulate translation of SecA. This type of regulatory mechanism may exist for other biological systems. The completion step of translation can be utilized for the expression of downstream genes in operons not only at the level of translation (McNicholas et al., 1997; Oliver et al., 1998; Nakatogawa and Ito, 2001) but also at the level of transcription (Gong et al., 2001). Translation arrest that involves the interactive nature of the nascent chain may provide a mechanism that guarantees a co-translational process, such as subcellular localization (Walter and Johnson, 1994) and subunit assembly (Young and Andrews, 1996). We observed that some mutant forms of the arrest sequence cause incomplete arrest, with the peptide released at different rates (Figure 2E). Thus, such a sequence could be utilized to modulate local rates of translation and to optimize protein-folding processes. The ribosomal subdomain that constitutes the exit gate might have active roles in these regulatory processes by scanning the sequences of the newly synthesized polypeptides. It is tempting to propose that the mRNA includes the information that determines the

optimal rate of translation elongation, which is decoded by the ribosomal exit gate.

#### Experimental Procedures

##### Plasmid Constructions

Plasmid pNH21 carried *secM* (Nakatogawa and Ito, 2001). pNH52, containing a Gln167 stop mutation, was constructed by amplifying the *secM* region from pNH21, using primers 5'-GAATCCGAGCTC GGCAATAACGTGAGTGG-3' and 5'-AAGCTTGCATGCTTAAGGGC CAGCACGGATGCCTTGG-3' (TAA stop codon underlined) with the *SacI/SphI* recognition sequences, and cloning it into pUC118 after *SacI-SphI* digestions. Similarly, pNH53 had CCT (Pro166) to TAA alterations. pNH57, pNH58, pNH59, and pNH61 contained alterations of CCT (Pro166) to CCC (Pro), CCA (Pro), CCG (Pro), and GCT (Ala), respectively, in addition to the Gln167 stop mutation. Amino acid residue numbers of SecM are those starting from the initiator GTG codon of precursor SecM (Sarker et al., 2000). pNH107 (for LacZ $\alpha$ -SecM(140-166)) and pNH108 [for LacZ $\alpha$ -SecM(150-166)] encoded LacZ $\alpha$ , whose C terminus was followed by the indicated segments of SecM. A LacZ $\alpha$  fragment was amplified from pSTV28 (a pACYC184-based vector from Takara), using the M13-reverse primer as well as the downstream primers (containing the *SphI* recognition sequence) designed such that the SecM segments 140-166 and 150-166 were fused in-frame to the C terminus of LacZ $\alpha$ . The products were cloned into pSTV28 after *SacI-SphI* digestions.

For alanine- and serine-scanning mutagenesis, the codon for each of the indicated amino acid residues of SecM on pNH21 was changed to GCT (Ala) or TCT (Ser), using an appropriately designed mutagenic oligonucleotide (Sawano and Miyawaki, 2000). Deletion and insertion mutations were similarly introduced. pNH122 encoded SecM(121-166)-LacZ $\alpha$ , in which SecM residues 121-166 were inserted into an N-terminal region (between residues 9 and 10) of LacZ $\alpha$ . For its construction, the *secM* segment was amplified from pNH21 using primers having extensions of the *SacI* and the *SphI* recognition sequences and inserted into pSTV28 after *SacI-SphI* digestions. Plasmids encoding mutant forms of SecM(121-166)-LacZ $\alpha$  with Pro166Ala, Trp155Ala, and Phe150Ala mutations were similarly constructed with downstream primers containing a required alteration. pNH106 carrying *rpIV* (for L22 r protein) was constructed by amplifying a chromosomal *rpIV* region from *E. coli* strain MC4100 (Nakatogawa and Ito, 2001) using primers 5'-GAATTCGAG CTGCGACCTGATCGTTAAAC-3' and 5'-AAGCTTGCATGCTGCG GTGCACGCAGCACC-3' and cloning it into pTWV228 (a pBR322-based *lac* promoter vector) after *SacI-SphI* digestions. Alanine/serine-scanning mutagenesis of L22 was carried out essentially as described above for SecM. All the plasmid regions that experienced in vitro DNA synthesis were confirmed by nucleotide sequencing.

##### Isolation of 23S rRNA and L22 Mutations as Arrest Suppressors

pNK, a pBR322 derivative carrying the entire *rrnB* operon (Xiong et al., 2000), was mutagenized by passing it through the mutator strain KD1087 (Degnen and Cox, 1974) for two cycles of overnight growth in L medium containing 50  $\mu$ g/ml ampicillin and then introduced into strain AD16 (Kihara et al., 1997) that harbored pNH122 (SecM(121-166)-LacZ $\alpha$ ). Selection was on L agar plates containing isopropyl-thio- $\beta$ -D-galactoside (1 mM), 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal; 40  $\mu$ g/ml), phenylethyl- $\beta$ -D-thiogalactopyranoside (0.5 mM), ampicillin (50  $\mu$ g/ml), and chloramphenicol (20  $\mu$ g/ml) at 37°C for 2 days. Colonies that developed blue color were saved for extraction of plasmids and sequence determination. Finally, the *SacI-BIPI rriB* fragment was cloned back to the original pNK background to avoid any additional mutations outside *rriB*. Two nucleotide changes, A2058 to G and A insertion at A749-A753, were the only mutations obtained repeatedly from ten independent rounds of mutagenesis/screening, and they were named *rriB2058* and *rriB751*, respectively. Plasmids carrying them were pNH152 and pNH153, respectively.

Arrest-suppressing L22 mutations on plasmid were obtained by similar procedures, using pNH106 carrying *rpIV*. Three mutations for Gly91Ser, Ala93Thr, and Ala93Val alterations in L22 were obtained repeatedly. Chromosomal arrest suppressors were isolated as fol-

lows. Cells of AD16 (Kihara et al., 1997) were treated with ethyl methanesulfonate as described by Taura et al. (1994) and then transformed with the arrest reporter plasmid (pNH122). Blue-colored transformants were screened as described above. P1 transduction using strain IQ86 (MC4100, *zhd-33::Tn10*; Taura et al., 1994) as a donor revealed that 3 out of 15 mutants obtained carried mutations cotransducible, at frequencies of about 60%, with *zhd-33::Tn10* located within *bfr* (G. Matsumoto, personal communication) in the vicinity of the r protein gene cluster. The chromosomal *rpIV* gene was then sequenced, revealing two mutants with the identical Gly91Asp alteration and one with Ala93Thr alteration in L22.

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