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Critical Regions of *secM* That Control Its Translation and Secretion and Promote Secretion-Specific *secA* Regulation

Shameema Sarker and Donald Oliver*

Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, Connecticut 06459

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SecA is an essential ATP-driven motor protein that binds to presecretory or membrane proteins and the translocon and promotes the translocation or membrane integration of these proteins. *secA* is subject to a protein secretion-specific form of regulation, whereby its translation is elevated during secretion-limiting conditions. A novel mechanism that promotes this regulation involves translational pausing within the gene upstream of *secA*, *secM*. The *secM* translational pause prevents formation of an RNA helix that normally blocks *secA* translational initiation. The duration of this pause is controlled by the rate of secretion of nascent SecM, which in turn depends on its signal peptide and a functional translocon. We characterized the atypical *secM* signal peptide and found that mutations within the amino-terminal region specifically affect the *secM* translational pause and *secA* regulation, while mutations in the hydrophobic core region affect SecM secretion as well as translational pausing and *secA* regulation. In addition, mutational analysis of the 3′ end of *secM* allowed us to identify a conserved region that is required to promote the translational pause that appears to be operative at the peptide level. Together, our results provide direct support for the *secM* translational pause model of *secA* regulation, and they pinpoint key sequences within *secM* that promote this important regulatory system.

In bacteria nascent or fully synthesized presecretory or membrane proteins are selectively targeted to the translocon by interactions with SecB and SecA or the signal recognition particle and its receptor (3, 24, 40, 47, 50). These pathways converge at the translocon, which consists of the integral membrane proteins SecYEG and SecDFyajC and the peripheral membrane protein SecA ATPase. SecYE forms the preprotein channel and SecA receptor (10, 20, 27, 30), while SecG and SecDFyajC greatly enhance the rate of protein translocation by regulating SecA membrane cycling (11, 28, 34). SecA is central to protein translocation since it binds to the signal peptides or transmembrane segments of presecretory and membrane proteins, interacts with the SecB chaperone to promote release of the bound preprotein, and acts as a motor protein to drive protein translocation at the translocon (for a review, see reference 26). Considerable evidence suggests that SecA undergoes ATP-driven cycles of insertion and retraction at SecYE, thereby promoting the stepwise translocation of proteins across the plasma membrane (12, 13, 52).

The selectivity of the translocon for its protein cargo is remarkable, since erroneous translocation of cytoplasmic proteins is essentially undetectable. Current evidence suggests that the translocon possesses a proofreading activity that is responsible for aborting the translocation of preproteins that lack a functional signal peptide (for a review, see reference 7). *prl* alleles of *secA*, *secY*, *secE*, and *secG* have been isolated that allow translocation of preproteins with a defective signal peptide (4, 15, 17, 23, 48). A recent study suggested that the control of the ATP-dependent, preprotein insertion reaction by the SecA-SecYE complex may be the critical biochemical step that controls this proofreading activity (51).

Because it catalyzes what appears to be the first committed step in protein translocation, ATP-dependent insertion of the preprotein into the translocon, SecA occupies a pivotal position in this pathway. *secA* appears to be the only *sec* gene that is under protein secretion-specific regulation; inhibition of protein secretion by either genetic or biochemical means leads to 10-fold induction of *secA* translation (36, 38, 44). Analysis of this system has revealed that *secA* is the second gene in the *secM secA* operon and that translation of *secA* is coupled to translation of *secM*, since ribosomes translating the distal portion of *secM* are needed to disrupt an RNA repressor helix (helix II) that normally blocks *secA* translational initiation (29, 42, 45). Based on the recent findings that (i) *secM* encodes a presecretory protein, (ii) *secM* signal sequence defects render *secA* expression constitutive even during rapid secretion of other proteins, (iii) such *secM* signal sequence defects are suppressible by *prlA* (*secY*) mutations, and (iv) the *secM* signal sequence alleles are *cis* acting, the *secM* translational pause-arrest model for *secA* regulation was recently proposed (37, 41). This model postulates that there is coupling and feedback between SecM (secretion monitor) translation and secretion, whereby the frequency of *secA* translational initiation depends on a translational pause within the distal portion of *secM* and the length of the *secM* translational pause is governed by the rate of secretion of nascent SecM protein, which in turn depends on its signal peptide and interaction with SecA and the translocon. Recent biochemical analysis of this system has confirmed many of the basic features of this model (33). In particular, the presence of a natural translational pause within the distal portion of *secM* was demonstrated, and the length of the *secM* translational pause was shown to depend on the secretion of SecM protein; defects in the *secM* signal peptide or trans-

* Corresponding author. Mailing address: Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, CT 06459. Phone: (860) 685-3556. Fax: (860) 685-2141. E-mail: doliver@wesleyan.edu.

TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Description[a]	Source or reference
Strains		
AF118	MC4100 *lamBΔ111*	18
AF128	MC4100 *lamBΔ111 prlG1 zja*::Tn*10*	18
AF130	MC4100 *lamBΔ111 prlG3 zja*::Tn*10*	18
CG155	MC1000 *recA*	Jon Beckwith
CG29	MC1000 *secD1*(*Cs*) *phoR recA1 srl*::Tn*10*	Jon Beckwith
CC118	MC1000 *phoAΔ20 rpsE rpoB argE*(Am) *recA1*	Jon Beckwith
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*argF-lac*)*U169 deoR* [ϕ*80dlac* Δ(*lacZ*)*M15*]	Laboratory stock
GN40	MC4100 *ompT*::*kan leu*::Tn*10*	33
JH101	MC4100 *lamB14D prlD22 leu*::Tn*10*	23
KB311	MC4100 *lamB14D prlD5 leu*::Tn*10*	23
MC4100	F^- Δ(*argF-lac*)*U169 araD136 relA1 rpsL150 flbB5301 ptsF25 deoC1 thi*	Jon Beckwith
MC4100.2	MC4100 *recA1 srl*::Tn*10*	Laboratory stock
SE6004	MC4100 *prlA4 lamBS60*	15
SE4014	MC4100 *prlA3 lamBS60 rpsE*	15
Plasmids		
pIF-A	Ap^r pBR322 derivative carrying *secM* ϕ(*secA-lacZ*)Hyb	29
pH^+	pACYC184 derivative, Cm^r $prlH^+$	4
pH5	pH^+ *prlH5*	4
pH6	pH^+ *prlH6*	4
pNH22	Ap^r pUC118 derivative carrying *secM*-Met6	33
pPhIF	pIF-A derivative carrying bacteriophage M13 replication origin	29
pSS1	pPhIF ϕ(*secM-phoA*)Hyb	43
pSS6	pPhIF *secM3*	This study
pSS7	pPhIF *secM4*	This study
pSS8	pPhIF *secM6*	This study
pSS9	pPhIF *secM7*	This study
pSS10	pPhIF *secM8*	This study
pSS11	pSS1 *secM3*	This study
pSS12	pSS1 *secM4*	This study
pSS13	pSS1 *secM6*	This study
pSS14	pSS1 *secM7*	This study
pSS15	pSS1 *secM8*	This study
pSTD343	Cm^r pACYC184 derivative carrying *lac1*	33

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Cs, cold sensitive.

locon promoted a prolonged arrest of *secM* translation and resulted in *secA* derepression.

Despite these recent advances in our understanding of *secA* regulation, a number of features of this system remain poorly defined. For example, a recently revised translational start site for *secM* indicated the presence of a signal peptide consisting of 37 amino acid residues with an unusually long 19-amino-acid amino-terminal region that contains a number of atypical amino acids (43). The importance of this unique signal peptide in controlling the *secA* regulatory system remains poorly explored. In particular, the effect that the existing *secM* signal sequence mutations have on the rate of secretion of SecM protein was not investigated, and only one *secM* signal sequence mutant was studied with the translational pause assay (33, 37). In addition, the precise location of the *secM* translational pause site has not been defined (33). Thus, the proximity of this site to the RNA helix that normally blocks *secA* translational initiation remains unclear, as does the peptide or RNA sequence that is required to promote the translational pause itself. Understanding these features is critical for confirming and elucidating this important protein secretion-specific regulatory system.

In the present study we utilized a combined genetic and biochemical approach to further characterize the atypical *secM* signal peptide and the 3′ end of the gene where the translational pause site is localized. Our results indicate that the amino-terminal region (N-region) and the hydrophobic core region (H-region) of the *secM* signal peptide have different functions with respect to promoting SecM protein secretion, the *secM* translational pause, and *secA* regulation. In addition, analysis of mutations at the end of *secM* that affect *secA* regulation allowed us to identify a conserved region that is required to promote the translational pause and to demonstrate that pausing at this site allows the stalled ribosome to block formation of the *secA* repressor helix.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and chemicals. The bacterial strains and plasmids used in this study are listed in Table 1. M63 minimal medium and Luria-Bertani (LB) broth used for growth of bacteria have been described previously (31). 5-Bromo-4-chloro-3-indoylphosphate and isopropyl-1-thio-β-D-galactoside (IPTG) were obtained from Fisher Scientific, and cyclic AMP, *o*-nitrophenyl-β-D-galactoside, sodium azide, and protein A Sepharose were obtained from Sigma Chemical Co. DNA restriction enzymes were obtained from New England Biolabs, Inc., and were used as recommended by the supplier. Tran ^{35}S label (~1,100 Ci/mmol) was obtained from ICN Radiochemicals, and IgSorb was obtained from The Enzyme Center, Inc. The fluorographic reagent Amplify was obtained from Amersham Corp. (Piscataway, N.J.). 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside and XAR film were purchased from Eastman Kodak Co. Oligonucleotides were purchased from Integrated DNA Technologies.

DNA manipulation and oligonucleotide-directed mutagenesis. Mutations were made by using the QuikChange procedure as described by the manufacturer

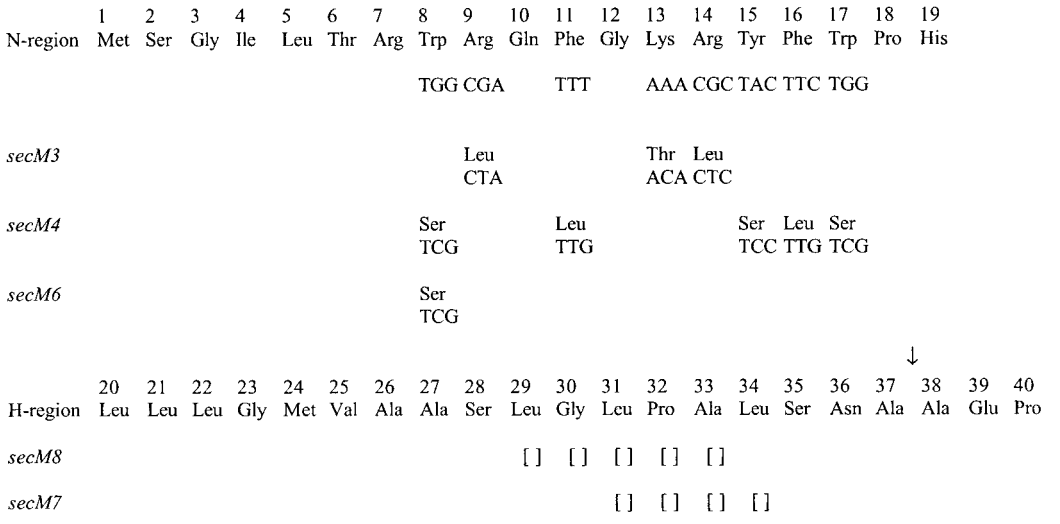

FIG. 1. *secM* signal sequence alleles used in this study. The N- and H-regions of the *secM* signal peptide are shown. The codon and amino acid substitutions for each allele are also shown. Empty brackets indicate deletions. An arrow indicates the presumed signal peptide processing site.

(Stratagene) and were verified at the University of Pennsylvania DNA Sequencing Facility. In order to generate *secM-phoA* fusions containing the *secM3*, *secM4*, *secM6*, *secM7*, and *secM8* alleles, pSS6, pSS7, pSS8, pSS9, and pSS10 DNA were cut with *Hin*dIII and *Bst*BI, and the 0.57-kb *Hin*dIII-*Bst*BI DNA fragment containing the relevant *secM* allele was isolated and ligated to the 4.7-kb *Hin*dIII-*Bst*BI DNA fragment from pSS1 (43) to generate pSS11, pSS12, pSS13, pSS14, and pSS15, respectively. After transformation of CC118, blue colonies on LB plates containing 100 μg of ampicillin per ml and 20 μg of 5-bromo-4-chloro-3-indoylphosphate per ml were isolated and purified, and the fusions were verified by restriction enzyme mapping and DNA sequence analysis of the relevant plasmid DNA.

RESULTS

Effects of *secM* signal sequence mutations on *secA* regulation and SecM secretion. We have shown previously that *secM* possesses a signal peptide consisting of 37 amino acid residues with an unusually long N-region rich in basic and aromatic amino acids (Fig. 1) (43). In order to examine the importance of the N- and H-regions of the *secM* signal sequence in *secA* regulation, a number of mutations in these regions were constructed previously, and their effects on *secA* regulation were analyzed (43). The data indicated that both the N- and H-regions of the *secM* signal sequence are necessary for proper *secA* regulation. Truncation of the H-region by four residues (*secM7*) or five residues (*secM8*) resulted in constitutive *secA* expression during secretion-proficient conditions (Table 2). A mutation that reduced the positive charge within the N-region (*secM3*) while maintaining a single positively charged residue (Arg-7) that is often important for rapid protein secretion kinetics resulted in normal *secA* repression during secretion-proficient conditions but reduced induction during secretion-defective conditions. Mutations that reduced (*secM6*) or eliminated (*secM4*) the atypical aromatic amino acid residues within the N-region resulted in elevated *secA* expression during secretion-proficient conditions or an inability to fully induce *secA* expression during secretion-defective conditions (particularly for *secM4*).

In order to investigate the effects that these alleles had on the protein secretion function of the *secM* signal peptide, isogenic strains carrying a *secM-phoA* fusion with a relevant allele were constructed by utilizing a previous fusion in which the first 157 codons of *secM* were fused to Tn *phoA* (41). The joint in this fusion should have been prior to the translational pause site in *secM* (see below), thereby allowing us to study SecM-PhoA secretion unimpeded by effects on translation. This approach was necessary also because SecM is an unstable periplasmic protein that is rapidly degraded by the C-terminus-specific Tsp protease soon after its synthesis and secretion (33). As shown recently, addition of a hexamethionine tag to the C terminus of SecM (SecM-Met_6) stabilized it appreciably to proteolysis (33).

In order to assess the rate of secretion of the SecM-PhoA chimeras, the rate of processing of these chimeras was mea-

TABLE 2. Effects of *secM* signal sequence alleles on *secA* regulation

Mutation	Basal expression (%)[a]	Induction[b]
Wild type	100	6.42
secM3	119	3.21
secM4	445	0.77
secM6	306	2.79
secM7	513	1.21
secM8	635	1.10

[a] To determine basal expression, MC4100.2(pPhIF) (wild type) or an allelic derivative was grown in LB medium containing 100 μg of ampicillin per ml at 37°C to the mid-logarithmic phase, and then β-galactosidase assays were performed at 30°C in duplicate for each of two duplicate cultures as described previously (31). β-Galactosidase activities are expressed as percentages of the wild-type activity, which was defined as 100%.

[b] Induction was the ratio of the β-galactosidase activities present in secretion-defective CG29 [*secD1*(Cs)] and secretion-proficient CG155 (*sec*$^+$) strains carrying pPhIF (wild type) or on allelic derivative. Strains were grown in LB medium containing 100 μg of ampicillin per ml at 39°C to the mid-logarithmic phase, and then each culture was shifted to 23°C for 4 h. β-Galactosidase assays were performed as described above. Some data were taken from reference (43).

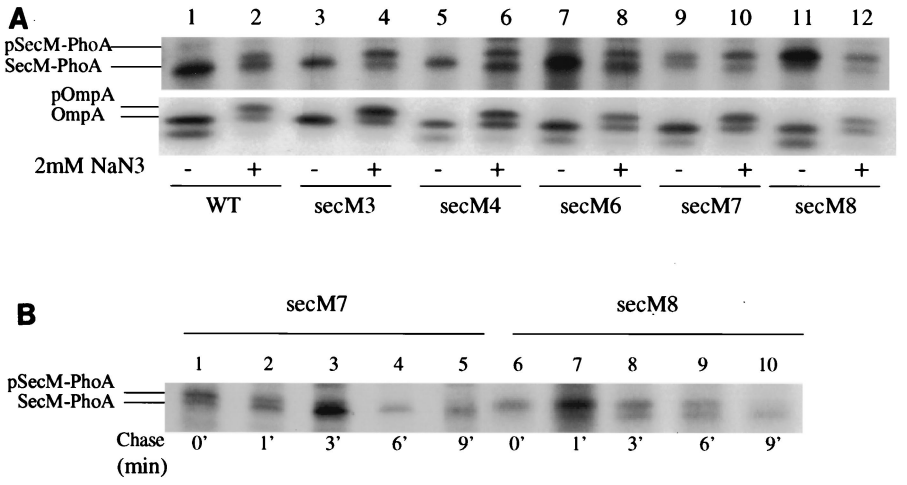

FIG. 2. Effects of *secM* signal sequence mutations on processing. (A) MC4100.2 containing pSS1 (WT) or an allelic derivative was grown in M63 minimal medium containing 0.4% glucose, 2 μg of thiamine per ml, 20 μg of each of 18 amino acids (not including methionine and cysteine) per ml, and 20 μg of ampicillin per ml at 37°C until the mid-logarithmic phase. Sodium azide (NaN3) was not added (−) or was added to a final concentration of 2 mM (+), and labeling was initiated after 5 min. A 0.5-ml aliquot of each culture was pulse-labeled with 10 μCi of Tran ^{35}S label (>1,000 Ci mmol^{-1}) for 1 min, and then an equal volume of ice-cold 10% trichloroacetic acid was added to terminate labeling. Samples were processed for immunoprecipitation with antisera to alkaline phosphatase and OmpA and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography as described previously (36). The positions of the precursor and mature forms of the SecM-PhoA fusion proteins (pSecM-PhoA and SecM-PhoA, respectively) and OmpA (pOmpA and OmpA, respectively) are indicated on the left. Both the precursor and mature forms of OmpA migrated as two bands, which were the heat-modifiable and non-heat-modifiable forms (22). (B) Similar to panel A, except that a mixture of methionine and cysteine (final concentration of each, 200 μg/ml) was added after 1 min of labeling to initiate the chase (0 min) and aliquots were removed at different times and mixed with an equal volume of ice-cold 10% trichloroacetic acid to terminate the chase.

sured by utilizing pulse-chase radiolabeling methods. Since the catalytic domain of the leader peptidase is periplasmically disposed, signal peptide processing is a good method for measuring the initial rate of secretion of a secretory protein (53). As controls, portions of the cultures were also treated with sodium azide prior to labeling in order to inhibit protein secretion (36), and the synthesis and processing of OmpA protein were assessed as well. The latter control allowed us to compare the rates of synthesis of the various SecM-PhoA chimeras to the rate of synthesis of OmpA as an internal standard. Given the proximity of the *secM* signal sequence mutations to the translational initiation region, these alleles could have effects on the rate of *secM* translational initiation (19). The *secM3*, *secM4*, and *secM6* mutants displayed rapid processing kinetics that were indistinguishable from those of the wild type (Fig. 2A). In contrast, the *secM7* and *secM8* mutants showed significant accumulation of the precursor form of SecM-PhoA during the 1-min pulse-labeling period. Additional pulse-chase analysis indicated that the *secM7* and *secM8* mutants had processing half-lives of the SecM-PhoA chimera of approximately 1 and 6 min, respectively (Fig. 2B). Of note were the relatively modest effects that the H-region truncations had on the secretion function of the *secM* signal peptide compared to the effects of similar mutations in other systems (2, 16). By contrast, the effects that these mutations had on *secA* regulation and *secM* translational pausing were more marked (Table 2) (see below). We also measured the levels of alkaline phosphatase activity of these strains, but our analysis was inconclusive due to the variable degrees of proteolysis of the SecM-PhoA chimeras (data not shown).

Effects of *secM* signal sequence mutations on translational pausing. The existence of a translational pause within the distal portion of *secM* was demonstrated recently, and the duration of this pause was shown to be dependent on the activity of the *secM* signal sequence and secretion machinery (33). We studied the effects that the *secM* signal sequence mutations had on translational pausing utilizing the system developed by Nakatogawa and Ito (33). The wild-type strain synthesized three species of SecM protein after a 1-min pulse-labeling period, corresponding to preSecM-Met$_6$, translationally paused SecM, and mature SecM-Met$_6$ (Fig. 3), as observed previously (33). The paused species of SecM presumably still contained the SecM signal peptide, since it was located in the cytoplasm in the cell (33). Relatively small amounts of the first two species were present initially, and these species disappeared rapidly during the chase period along with a modest amount of mature SecM-Met$_6$. In the case of the *secM8* mutant essentially all of SecM protein was in the translationally paused form even during the 8-min chase period, as noted previously (33). A similar result was obtained for the *secM4* mutant. The *secM4* and *secM8* mutants also gave similar results during an extended 20-min chase period in which only the translationally paused form of SecM was observed (data not shown). This result indicates that the *secM4* and *secM8* mutations have different effects on the secretion and translational pausing functions of the *secM* signal peptide and that the effects on the latter function are far more drastic than the effects on the former (compare Fig. 2 with Fig. 3). In contrast, although the *secM3* and *secM6* mutants displayed elevated levels of the translationally paused species compared to the wild type, this species did chase primarily into mature SecM-Met$_6$. Taken together, our results are consistent with the proposal that the

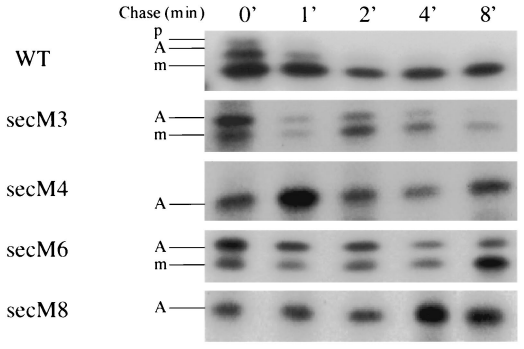

FIG. 3. Effects of *secM* signal sequence mutations on translational pausing. GN40(pSTD343) containing pNH22 (WT) or an allelic derivative was grown in M63 minimal medium supplemented with 0.4% glucose, 2 μg of thiamine per ml, 20 μg of each of 18 amino acids (not including methionine and cysteine) per ml, 20 μg of ampicillin per ml, and 10 μg of chloramphenicol per ml at 37°C until the mid-logarithmic phase, when IPTG and cyclic AMP were added at concentrations of 1 and 5 mM, respectively. Thirty minutes later an aliquot of each culture was pulse-labeled with 100 μCi of Tran ^{35}S label (>1,000 Ci mmol^{-1}) per ml for 1 min. Then a mixture of methionine and cysteine (final concentration of each, 200 μg/ml) was added to initiate the chase (0 min), and aliquots were removed at different times and mixed with an equal volume of ice-cold 10% trichloroacetic acid to terminate the chase. Samples were immunoprecipitated with a mixture of antisera against N- and C-terminal synthetic peptides of SecM (33) and analyzed on sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis gels by fluorography as described previously (35). p, preSecM-Met$_6$; A, translationally paused SecM; m, mature SecM-Met$_6$.

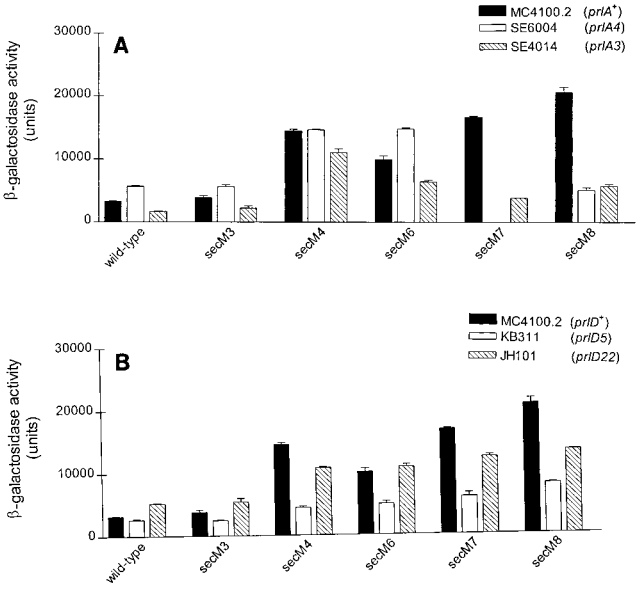

FIG. 4. Effects of *prl* suppressors on the phenotypes of *secM* signal sequence mutations. A strain containing pPhIF (wild type) or its allelic derivative was grown in LB broth containing 100 μg of ampicillin per ml and 5 μg of chloramphenicol per ml, when necessary, at 37°C to the mid-logarithmic phase. β-Galactosidase assays were performed as described in Table 2, footnote *a*. (A) *prlA* suppressor; (B) *prlD* suppressor.

duration of the *secM* translational pause controls the frequency of *secA* translational initiation (33, 37). In particular, the two *secM* alleles that arrested translation (*secM4* and *secM8*) exhibited strongly elevated levels of *secA* expression during secretion-proficient conditions (compare Fig. 3 with Table 2). By contrast, the two *secM* alleles that only delayed the release of the translational pause (*secM3* and *secM6*) exhibited lower levels of *secA* expression, with the longer delay (*secM6*) corresponding to a higher level of *secA* expression. The latter results also suggest that the release of the *secM* translational pause must be significantly delayed in order to result in appreciable *secA* derepression.

Effects of *prl* suppressors on the phenotype of *secM* signal sequence mutations. *prl* alleles of *secA* (*prlD*), *secY* (*prlA*), *secE* (*prlG*), and *secG* (*prlH*) that allow translocation of proteins with a defective signal peptide have been isolated (4, 15, 17, 23, 48). In order to genetically characterize the interaction of the *secM* signal peptide with the secretion machinery and its effect on *secA* regulation, we examined the effects that the *secM* signal sequence alleles had on *secA* regulation utilizing different *prl* suppressor strains. The most striking effects were observed for the *prlA* and *prlD* mutants. The H-region mutations were generally suppressed by the *prlA* alleles, as indicated by restoration of *secA* repression (Fig. 4A) (43). Interestingly, although *prlA4* has been found to be a stronger suppressor of signal sequence defects in the H-region than *prlA3* (14, 15), comparable suppression activities were observed for these two alleles with *secM8*. In addition, the *secM7* allele was found to be synthetically lethal with *prlA4*, further confirming the importance of this interaction. This result may have been due to an unproductive interaction between the SecM7 protein and the PrlA4-containing translocon that led to translocon jamming, although further studies are required to explore this hypothesis. The N-region mutations showed little or no suppression with the *prlA* alleles. The most notable effects were with *secM6*; the two *prlA* alleles had modest but opposite effects on *secA* regulation in this case. In contrast to the results described above, both the N- and H-region mutations were suppressed by *prlD* alleles, particularly *prlD5*, although the degree of suppression of the H-region mutations was less than that observed for *prlA* (compare Fig. 4A and B). It has been noted previously that *prlD* alleles are efficient suppressors of defects within the N-region of signal sequences (39). The *prlG* and *prlH* suppressors had little or no effect on this system despite the fact that the strongest *prlG* and *prlH* alleles available (4, 18) were used (data not shown). Although these genetic studies were indirect, they did support the notion that proper interaction of the *secM* signal peptide with SecA and SecY proteins is important for control of *secA* regulation.

TPE mutations lie within the *secM* translational pause site. In previous analyses of *secA* regulation, two different classes of mutations within the distal portion of *secM* and the *secM-secA* intergenic region were studied (29, 42). Class II mutations lie within the repressor helix (helix II) that normally cloisters the *secA* Shine-Dalgarno sequence. It was predicted that these mutations would disrupt this helix, and they were found to render *secA* expression constitutive. Class I mutations were constructed within a second predicted helix (helix I) immediately upstream of helix II in order to test its importance in *secA* regulation. Certain class I mutations rendered *secA* expression noninducible, although the mutational pattern suggested that

TABLE 3. *secA* regulation in class I mutants

Mutant	Codon	Type[a]	Basal expression (%)[b]	Induction[c]
Wild type			100	6.26
S151S[d]	AGC→UCG	HD	133	5.21
T152C[d]	ACG→UGC	AA + HD	141	4.06
T152M	ACG→AUG	AA	83	5.89
V154R[d]	GUC→CGG	AA + HD	75	6.26
I156K	AUA→AAA	AA	80	4.12
I156R	AUA→AGA	AA	94	3.57
I156T	AUA→ACA	AA	124	4.33
I156T	AUA→ACC	AA	86	3.36
I156I	AUA→AUC	AA	101	5.57
S157N	AGC→AAC	AA	87	4.74
S157I	AGC→AUC	AA	84	4.73
S157T	AGC→ACC	AA	106	4.42
Q158H-A159W[d]	GCG→CUG	AA + HD	90	5.52
Δ160[d]	CAA→Δ	AA	49	3.26
Q160P	CAA→CCA	AA	31	3.50
R163A[d]	CGU→AGC	AA + HD	30	1.26
R163R	CGU→AGA	HD	106	5.08
A164R[d]	GCU→CGA	AA + HD	90	1.00
A164V	GCU→GUU	AA	64	0.68

[a] AA, alteration of amino acid residue; HD, helix disruption.
[b] See Table 2, footnote *a*.
[c] See Table 2, footnote *b*.
[d] Data from reference 29, except for the data for T152C, which was reconstructed because a secondary mutation was found.

the RNA secondary structure may not be important for *secA* regulation (29). In particular, mutations on the 3′ side of helix I elicited the most defective phenotype. Below, we refer to the latter portion of helix I sequences as the TPE (three prime element) region.

Given the existence of a translational pause site in the distal portion of *secM*, we decided to reinvestigate the importance of helix I sequences, particularly the TPE region, in translational pausing and *secA* regulation. In particular, both the location and the phenotype of the TPE mutations were consistent with their lying within the *secM* translational pause site. Accordingly, utilizing Watson-Crick base pairing rules (including allowance for G-U base pairs) and the ambiguity of the genetic code, we designed additional class I mutations that either would disrupt the predicted RNA secondary structure while conserving a particular amino acid residue or, alternatively, would minimally perturb the predicted RNA secondary structure while altering a given amino acid residue. We reasoned that this approach might allow us to locate the *secM* translational pause site and determine whether RNA or peptide sequences (or both) are operative in the translational pausing mechanism.

A summary of class I mutations and their effects on *secA* regulation is shown in Table 3. Mutations in the 5′ portions of helix I sequences (at codons 151, 152, and 154) (Fig. 5) that either disrupted the predicted RNA secondary structure or altered a particular amino acid residue or both had little effect on *secA* regulation (29). The minor differences between mutants with these mutations and the wild type may have been due to reduced stability of *secM-secA* mRNA or another nonspecific effect. Similar results were obtained for mutants with mutations in the loop region of helix I (at codons 156 and 157) or the upper portion of the 3′ side of the helix (at codons 158 and 159). The rare codon AUA at position 156 did not appear to be important for *secA* regulation, since synonymous or nonsynonymous substitutions that utilized more abundant tRNAs had little effect on *secA* regulation. Deletion or an amino acid substitution in a predicted bulge (at codon 160) on the 3′ side of helix I resulted in a modest decline in *secA* basal expression and induction, suggesting that this region may help enhance *secA* expression and regulation by some means. Most strikingly, however, mutations in the distal portion of helix I sequences within the TPE region (at codons 163 and 164) resulted in a decline in *secA* basal expression and elimination of *secA* induction. In such cases it appeared that the amino acid sequence of SecM rather than the predicted RNA secondary structure or sequence was the important factor. For example, a mutation that maintained the former but perturbed the latter resulted in correct *secA* regulation (compare R163R with R163A), whereas the converse resulted in a loss of *secA* regulation (A164V).

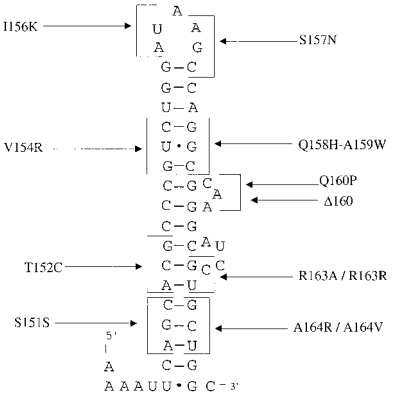

FIG. 5. Proposed RNA secondary structure for helix I. *secM* mutants are indicated by codons and single-letter codes for amino acids. The structure was taken from reference 29.

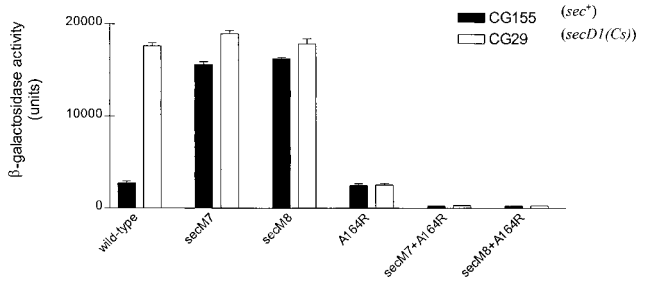

FIG. 6. Genetic interaction of *secM* signal sequence and TPE alleles. CG155 or CG29 carrying pPhIF (wild type) or an allelic derivative was grown and assayed for β-galactosidase activity as described in Table 2, footnote *a*.

We next turned our attention to genetic and biochemical experiments to prove that the TPE region is the *secM* translational pause site and that TPE mutants are defective in translational pausing. If TPE mutations are defective in the *secM* translational pause, then they should be epistatic to *secM* signal sequence mutations. This is logical because the *secM* signal sequence is not required for initiation of the translational pause itself but rather is required for release of the pause (33). Accordingly, we constructed *secM* double mutants that contained both H-region (*secM7* or *secM8*) and TPE (*secM*-A164R) mutations and analyzed their effects on *secA* regulation. While the H-region mutants were constitutive for *secA* expression and the TPE mutant was noninducible, the double mutants were also noninducible (Fig. 6). However, they displayed a lower level of *secA* expression than the TPE mutant, indicating that the *secM* signal sequence may have some effect on reducing TPE function.

The class I mutants were also directly examined to determine their effects on the *secM* translational pause. While the wild-type strain synthesized the three SecM species, including the translationally paused species, the strains carrying the TPE mutations that eliminated *secA* induction synthesized only pre-SecM-Met_6 and mature SecM-Met_6 and therefore were defective in translational pausing (Fig. 7). This pausing defect may also account for the lower level of SecM-Met_6 observed in this case, since it would have accelerated the kinetics of SecM-Met_6 secretion to the periplasm, where it would have been subjected to limited proteolysis. Even pretreatment of the latter cultures with sodium azide to induce a protein secretion block did not result in appearance of the translationally paused species, although it did result in a greater accumulation of preSecM-Met_6. Selected class I mutations in the 5′ region of helix I (T152C), the loop region (I156K), or the 3′ bulge (Q160P) gave patterns of translational pausing that were similar to that of the wild type (data not shown). These results demonstrate that codons 163 and 164 of *secM* are part of its translational pause site, and they agree with the observations of Nakatogawa and Ito that the *secM* translational pause site is located quite close to the 3′ end of *secM* (33). They also demonstrate that most of the helix I sequences are unimportant in the *secM* translational pause.

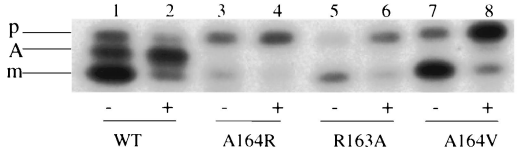

FIG. 7. Effects of *secM* TPE mutations on translational pausing. GN40(pSTD343) containing pNH22 (WT) or an allelic derivative was grown and pulse-labeled with 100 μCi of Tran ^{35}S label per ml for 1 min, and samples were processed and visualized as described in the legend to Fig. 3. Where indicated (+), sodium azide was added 5 min prior to labeling at a final concentration of 2 mM. p, preSecM-Met_6; A, translationally paused SecM; m, mature SecM-Met_6. The data are representative of the data obtained in three separate experiments.

DISCUSSION

In this work we investigated the role that the *secM* signal sequence plays in promoting SecM protein secretion, *secM* translational pausing, and *secA* regulation. A number of interesting and important conclusions were reached. It is clear that the N- and H-regions of the *secM* signal peptide have different functions with respect to these properties. Mutations in the H-region affected the rate of SecM protein secretion, the duration of the *secM* translational pause, and the fidelity of *secA* regulation, while mutations in the N-region affected only the latter two functions. This indicates that the N-region, which is unusually long and rich in basic and aromatic amino acids, plays a more exclusive role in promoting *secA* regulation by modulation of the *secM* pause-release cycle. While it is uncertain what this role is, analysis of the available SecM sequences showed that both the N-region and the early H-region of the *secM* signal peptide are highly conserved (Fig. 8). Recently, Nakatogawa and Ito showed that the *secM* signal peptide was not required for the *secM* translational pause but it was needed to promote the release through proper interaction with the secretion machinery (33). We speculate that the N-region makes important contact with one or more components of the Sec machinery in order to facilitate this event. While H-regions of signal peptides have been shown to interact with SecA and the translocon as well (32), it appears that in this specialized case H-region interaction is insufficient to promote proper signaling of the translation and secretion machinery and that an additional module (i.e., the atypical N-region) is required as well. Clearly, it will be of interest to investigate the detailed biochemical mechanisms that accomplish such coordination in order to facilitate the secretion-responsive regulation of *secA*.

Of note in our study were the relatively modest effects that the H-region truncations had on the secretion function of the *secM* signal sequence compared to the more dramatic effects observed for *secM* translational pausing and *secA* regulation. By comparison, similar mutations in other systems had more severe effects on secretion of the cognate protein. For example, truncation of the H-regions of the signal peptides of maltose-binding protein and lambda receptor led to strong secretion defects for these two proteins (2, 16). Genetic reversion analysis in the latter case, however, suggested that the proximity of α-helix-disruptive proline and glycine residues was responsible for the observed defect (16). It is important to note that the *secM* H-region is relatively rich in leucine residues and that four of six leucine residues remain in the *secM7* and *secM8* signal peptides. It also appears that conservation of the early portion of the *secM* H-region is more important than conser-

FIG. 8. Alignment of SecM proteins. SecM proteins from *Escherichia coli* (secMec) (43), *Salmonella enterica* serovar Typhi (secMst), *Klebsiella pneumoniae* (secMkp), and *Yersinia pestis* (secMyp) were multiply aligned by the Clustal X method (49). The *S. enterica* and *Y. pestis* sequences are available at ftp://ftp.sanger.ac.uk/pub/pathogens, and the *K. pneumoniae* sequence was produced by the Genome Sequencing Center at Washington University, St. Louis, Mo. (personal communication). Asterisks indicate completely conserved amino acid residues, while the arrow indicates the predicted signal peptide processing site.

vation of the later part, where the *secM7* and *secM8* alleles reside (Fig. 8). It has been shown that H-region function is highly dependent on net hydrophobicity, as well as mean hydrophobicity per residue, and that polyleucine-containing H-regions can therefore function efficiently when the regions are comparatively short (6, 8). This property should give the *secM* signal peptide a competitive advantage over other signal peptides for interacting with the Sec machinery. In this regard it has been found that relatively small differences in H-region hydrophobicity can have relatively large effects on preferential secretion of one protein over another (5). Preferential interaction of nascent preSecM with the Sec machinery could be important for the establishment of a low basal level of *secA* expression, since this association would promote release of the *secM* translational pause and resumption of *secA* translational repression.

In part of our genetic analysis we used *prl* suppressors in order to investigate the interaction of the *secM* signal peptide with the Sec machinery and its effect on *secA* regulation. While this type of study needs to be ultimately linked to biochemical investigations, some of our data are highly suggestive of specific interactions. In particular, the strong suppression and synthetic lethality of the H-region mutations with *prlA*, as well as the allele specificity noted for *prlD* and the N- and H-region mutations, suggest that SecY and SecA interact with these regions of the *secM* signal peptide. These inferences are consistent with the results of previous genetic and biochemical studies which indicated that SecYE and SecA interact with the H-region of signal peptides and with the N- and H-regions of signal peptides, respectively (1, 14, 23, 32, 39). By contrast, our data for the *prlG* and *prlH* suppressors were less striking, both from the standpoint of the strength of suppression and from the standpoint of allele specificity. The data suggest that the interaction of the *secM* signal peptide with SecA and SecY proteins is a key factor in controlling the steps that lead to correct *secA* regulation.

In part of our study we focused on the effects that mutations in the *secM* signal sequence and 3′ region had on the duration of the *secM* translational pause. This allowed us to directly correlate this property with the observed pattern of *secA* regulation and to obtain support for the *secM* translational pause-arrest model of *secA* regulation. We obtained evidence through examination of the different *secM* alleles that the duration of the *secM* translational pause was consistent with the observed *secA* expression levels. Our results may have been affected somewhat by the possibility that certain *secM* alleles could have other effects on *secA* expression, such as altering *secM-secA* mRNA folding or half-life. However, the consistency of our data, along with the observed *prl*-dependent suppression of these mutations, indicated that such considerations were minimal.

Our analysis of helix I sequences allowed us to locate residues at the 3′ end of *secM* within the TPE region that promote translational pausing, as well as to conclude that the pausing mechanism appears to be operative at the peptide level rather than the RNA level. The latter inference is consistent with previous results obtained in our laboratory, in which double frameshift mutations revealed the importance of the translational reading frame in the later portion of *secM*, as well as the results of Nakatogawa and Ito, who found that the proline analog azetidine inhibited the translational pause (29, 33). While the pausing mechanism itself remains to be elucidated, we note that the region identified (codons 163 and 164) falls within a hexapeptide sequence (GIRAGP) that is completely conserved in the available *secM* homologues (Fig. 8). There are no rare codons in this six-codon region or the last four codons of *secM*, which argues against the importance of limiting charged tRNAs in the pausing mechanism. Furthermore, the

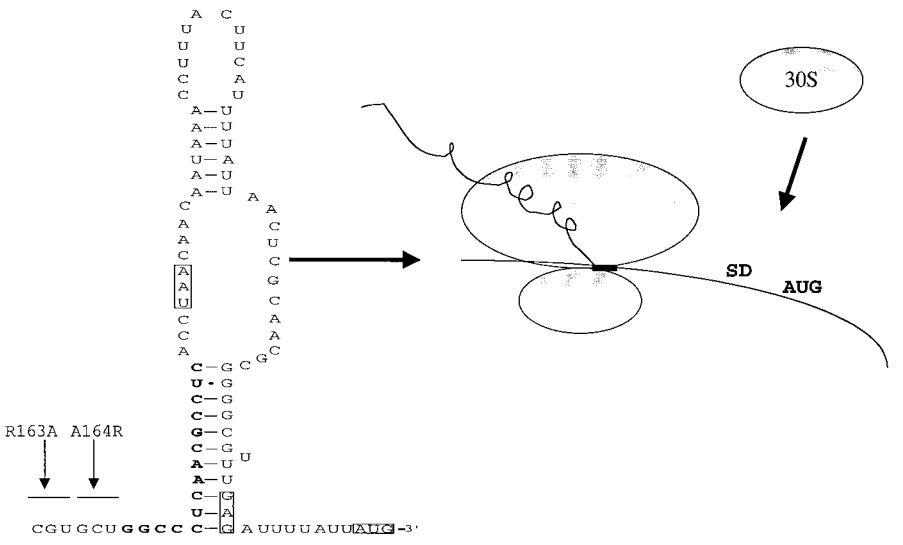

FIG. 9. Model for *secA* regulation. The proposed structure of the *secA* repressor helix (helix II) and its disruption by the *secM* translational pause are shown. The positions of the TPE mutations in codons 163 and 164 are indicated, along with the positions of 3′ nucleotides that would be sequestered by the translating ribosome (boldface type and box). The termination codon of *secM* (UAA) and the *secA* Shine-Dalgarno (SD) and the initiation codon (AUG) are enclosed in boxes. 30S, 30S ribosomal subunit. The structure of the *secA* repressor helix was taken from reference 29.

sequence in this region is more highly conserved at the amino acid level than at the RNA level, indicating the importance of a peptide in the translational pausing mechanism. Nascent peptide sequences that promote translational pausing have been found previously (for example, in control of chloramphenicol resistance in bacteria, which is referred to as translational attenuation [for a review, see reference 25). The precise location of the sequence is completely consistent with its ability to induce *secA* expression (Fig. 9). In particular, the translating ribosome, which should sequester at least 15 nucleotides 3′ of codon 164 of *secM* (21), should stall over mRNA sequences that normally comprise the 5′ portion of helix II, thereby activating the *secA* translational initiation region by exposure to the translational apparatus (29, 42).

It is too early to say what specific elements control the duration of the *secM* translational pause beyond the need for appropriate N- and H-regions of the *secM* signal sequence and a functional interaction with the Sec machinery (33, 37, 43). One attractive model is that the pause is released simply by the mechanical action of SecA as it threads nascent preSecM into the translocon and dislodges the stall peptide from the translational apparatus. In this scenario *secA* induction occurs when the stalled nascent preSecM translational complex is prevented from docking with SecA and the translocon due to blockage of the latter components by other presecretory and membrane proteins. While our kinetic analysis of certain *secM* signal sequence mutants may appear to be at odds with this model (given the temporal disparity between rapid signal peptide processing and slower translational pause release), there is no reason that the translational pause release cannot occur later in the translocation of SecM protein, particularly given the loop model in which the N-region of the SecM signal peptide remains cytoplasmically exposed during SecM translocation (for a review, see reference 9). Furthermore, our results with the *secM-phoA* fusions probably do not depict the correct sequence of events since these fusions lack the *secM* translational pause site. Indeed, in the wild-type system there was no evidence of two translationally paused species that differed in the presence and absence of the *secM* signal peptide, indicating that translocation and processing are probably coordinated with the translational pause event (33; this study). More complex regulatory models can be envisioned as well; for example, the buildup of translocation intermediates of other presecretory and membrane proteins could titrate away a factor that is needed to promote the translational pause release. A role for SecA RNA helicase activity in promoting *secA* autoregulation has been ruled out recently, since *secA* helicase-defective mutants showed normal *secA* regulation (46). This observation precludes models in which the translational pausing agent is an RNA secondary or tertiary structure that is unwound by SecA helicase activity in order to release the *secM* translational pause. Clearly, additional genetic and biochemical analyses that are under way will be required to reveal many of the subtleties of this complex and fascinating system.

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ADDENDUM IN PROOF

Nakatogawa and Ito have recently identified a similar SecM translational arrest peptide, FXXXXWIXXXXGIRAGP, that includes the specific arrest point (Pro), and they have also identified mutations in 23S rRNA and L22 protein near the

ribosomal exit tunnel that bypass the translational arrest (H. Nakatogawa and K. Ito, Cell, in press).

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