RNA (1995), *1*:745–753. Cambridge University Press. Printed in the USA.
Copyright © 1995 RNA Society.

Competition between ribosome and SecA binding promotes *Escherichia coli secA* translational regulation

REZA SALAVATI and DONALD OLIVER
Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, Connecticut 06459, USA

ABSTRACT

SecA protein, the protein translocation ATPase of *Escherichia coli*, autogenously regulates its translation during normal protein secretion by binding to a secretion-responsive element located near the 5′ end of its gene on geneX-*secA* mRNA. In order to characterize this autoregulation further, RNA footprinting and primer-extension inhibition (toeprinting) studies were carried out with a segment of geneX-*secA* RNA, 30S ribosomal subunits and $tRNA_f^{Met}$ along with purified SecA protein. The results show that ribosome and SecA-binding sites overlap, indicating that a simple competition for binding of geneX-*secA* mRNA presumably governs the translation initiation step. Further analysis showed that SecA protein was able to specifically dissociate a preformed 30S-$tRNA_f^{Met}$-geneX-*secA* RNA ternary complex as indicated by the disappearance of its characteristic toeprint after SecA addition. These findings are consistent with *secA* autoregulation, and they suggest a novel mechanism for the autoregulatory behavior of this complex protein.

Keywords: autogenous repression; *secA* regulation; translation; ribosome

INTRODUCTION

Genetic and biochemical studies of *Escherichia coli* have revealed a complex system for catalyzing the secretion of proteins across the inner membrane of this bacterium (reviewed by Schatz & Beckwith, 1990; Wickner et al., 1991). This system consists of a number of soluble and membranous components. Chaperones such as SecB protein prevent premature folding of secretory preproteins that would otherwise prevent their secretion (Kumamoto, 1991). SecA protein is the translocation ATPase, which promotes functional binding of secretory preproteins to the inner membrane (Cabelli et al., 1988; Cunningham et al., 1989; Lill et al., 1989). The catalytic mechanism of SecA protein appears to involve ATP-dependent cycles of insertion and de-insertion from the inner membrane in order to "thread" successive segments of the secretory preprotein across the inner membrane (Economou & Wickner, 1994; Kim et al., 1994). The integral membrane protein SecY/SecE/SecG has been shown to be essential for efficient reconstitution of in vitro protein translocation activity (Douville et al., 1994; Hanada et al., 1994). Presumably one or more of these proteins constitutes the preprotein channel that has been detected previously by electrophysiological methods (Simon & Blobel, 1992). The function of the integral membrane proteins, SecD and SecF, remains ill-defined and controversial (Matsuyama et al., 1993; Arkowitz & Wickner, 1994; Kim et al., 1994; Pogliano & Beckwith, 1994).

Little is known about the regulation of this complex secretion machinery. Because the rate of protein synthesis of *E. coli* varies considerably depending on growth rate (Bremer & Dennis, 1987), the secretion machinery must accommodate itself to considerable differences in the rate of production of secretory preproteins. Thus far, only one component of the secretion machinery has been shown to be regulated: *secA* translation varies over a 10-fold range, depending of the status of protein secretion within the cell. *secA* is the second gene in a three-gene operon, and its expression is translationally coupled to that of the preceding gene, geneX (Schmidt et al., 1988). During protein export-proficient conditions, SecA protein autogenously represses its translation by binding near the 5′ end of its gene on geneX-*secA* mRNA (Schmidt & Oliver, 1989; Dolan & Oliver, 1991). However, during inhibition of protein export by genetic or biochemical means, the rate of *secA* translation rises abruptly (Oliver & Beckwith, 1982; Rollo & Oliver, 1988; Schmidt &

Reprint requests to: Donald Oliver, Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, Connecticut 06459, USA; e-mail: doliver@wesleyan.edu.

Oliver, 1989; Oliver et al., 1990). The mechanism that leads to derepression of SecA synthesis during protein secretion defects is unknown, as is the precise mechanism governing translational repression when protein export rates are normal. In order to elucidate this important regulatory paradigm further, we have defined the sites of ribosome and SecA binding on geneX-*secA* RNA. Our results show that the ribosome and SecA protein must compete for overlapping binding sites on geneX-*secA* RNA, consistent with the autoregulation of *secA* translation that has been established previously. Furthermore, they demonstrate that SecA protein can promote dissociation of a preformed 30S-tRNA$_f^{Met}$-geneX-*secA* RNA ternary complex.

RESULTS

Footprinting of SecA protein bound to geneX-*secA* RNA

In order to undertake studies to determine the ribosome and SecA-binding sites on geneX-*secA* RNA, a 162-nt-long segment of this RNA was prepared that contains the region shown previously to regulate *secA* translation, along with purified SecA protein (Fig. 1A) (Schmidt & Oliver, 1989; Schmidt et al., 1991). The SecA protein used in these studies (Fig. 2A) was free of nuclease activity as indicated by its inability to degrade radiolabeled RNA during incubation at 37 °C for 30 min (data not shown). To locate the binding site of SecA protein on geneX-*secA* RNA, we used a primer extension RNA footprinting technique described by Winter et al. (1987). This procedure is similar to the standard RNA footprinting method except that it uses a reverse transcription reaction of primer-hybridized RNA to visualize the RNA cleavage products. Presumably, RNA species originating from cleavage upstream of the protein-binding site are visualized by this method due to dissociation of the bound protein during the reverse transcription reaction. Partial digestion of geneX-*secA* RNA by RNase T_2 in the presence of purified SecA protein led to the production a distinctive region of protection on this RNA at the optimum concentration of SecA protein (Fig. 2B). A region of nuclease hypersensitivity at the 3′ end of the region of protection (C+46) was observed. Although the primer-extension RNA footprinting was useful for accurately determining the 3′ end of the SecA-binding site, determination of the 5′ end of the binding site was rather imprecise. In order to locate the 5′ end of the SecA-binding site more precisely, we used standard RNA footprinting methods on the two RNA species shown in Figure 1. RNase T_1, RNase U_2, or alkaline hydrolysis was used to fragment the 5′ end-labeled RNA in order to create a sequence ladder, whereas cobra venom nuclease treatment of RNA with or without bound SecA protein was used to generate a footprint. In a typical reaction, the RNA was first renatured and allowed to form a complex with a 2.5-fold molar excess of SecA protein, followed by treatment with cobra venom nuclease. The results of this analysis show that the 5′ end of the SecA-binding site extends to C−50, and that SecA protects a large region from C−50 to C+46 (Fig. 3A, and also Fig. 2). Furthermore, our re-

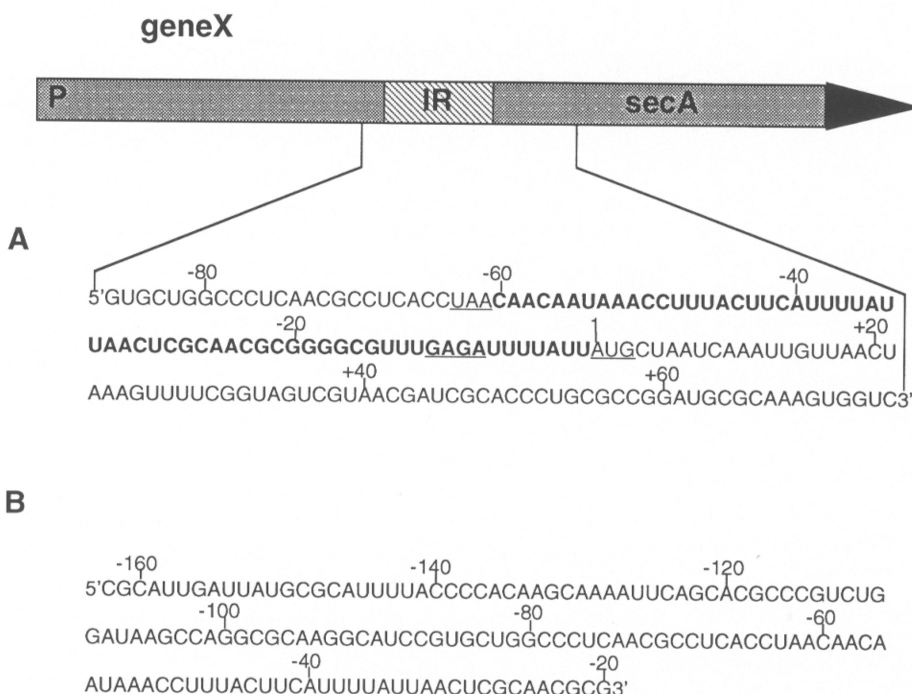

FIGURE 1. Graphic map of geneX-*secA* and the two RNAs used in these studies. The promoter (P), geneX, intergenic region (IR), and *secA* gene are indicated. **A:** 162-nt RNA. **B:** 143-nt RNA. The geneX termination codon, the Shine–Dalgarno sequence, and the *secA* initiation codon are underlined, and the intergenic region is shown in bold face. Coordinates are numbered with respect to the **A** of the initiation codon, which is taken as +1.

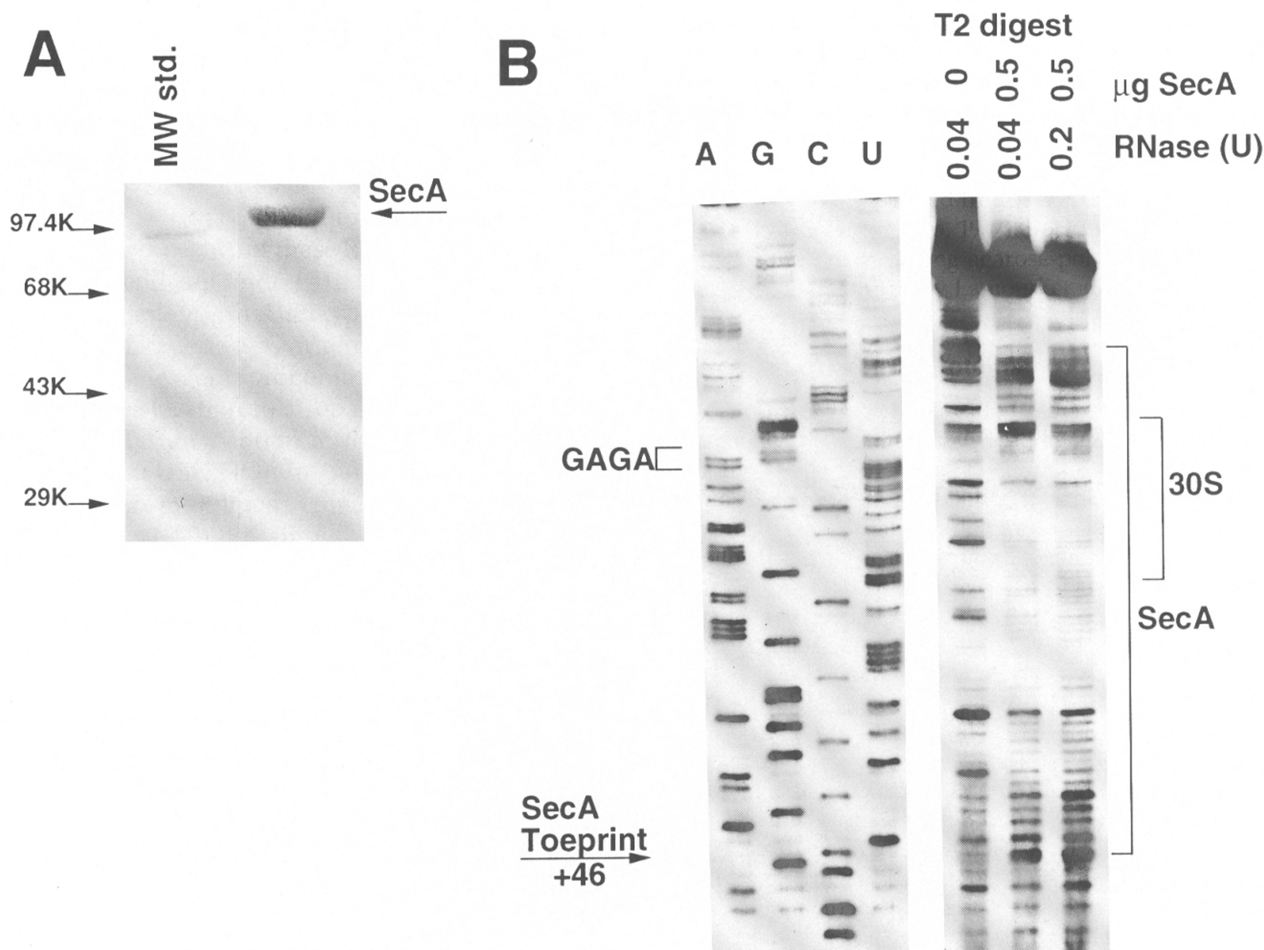

FIGURE 2. Primer-extension RNA footprint analysis of SecA protein bound to geneX-*secA* RNA. Three picomoles primer were hybridized to 1 pmol geneX-*secA* RNA, followed by incubation with the indicated amount of SecA protein or a comparable volume of storage buffer at 0 °C for 10 min. The indicated amount of RNase T_2 (T2 digest) was added and incubation was continued at 0 °C for an additional 5 min. The extension reaction was carried out by incubation at 37 °C for 15 min in the presence of 20 units of M-MLV reverse transcriptase and reactions were analyzed as described in the Materials and methods, along with the sequencing ladder (A G C U) shown. Positions of the Shine–Dalgarno sequence (GAGA), the putative SecA toeprint (SecA toeprint +46) shown in Figure 4B, and the overlapping binding sites of SecA protein (SecA) and the 30S subunit contained in the ternary complex (30S) are indicated. The binding site of the 30S subunit was determined from the primer-extension inhibition studies shown in Figure 4A and the assumption that this subunit protects 35 nt of RNA from RNase attack (Hartz et al., 1988).

sults show that no additional SecA-binding sites can be detected within 100 nt upstream of this SecA-binding site (Fig. 3B). Finally, a parallel experiment in which a comparable mixture of SecA protein and bacteriophage T4 gene 32 RNA encompassing nt −92 to +80 was treated with cobra venom nuclease showed no indication of a region of protection on this latter RNA (Fig. 3C). These latter results confirm the RNA-binding specificity of SecA protein under these experimental conditions. We note that the region protected by SecA is unusually large, even taking into account the size of this protein (homodimeric molecular weight of 204 kDa [Schmidt et al., 1988]). This result may be due to the binding of more than one SecA molecule to this region, or alternatively, to the presence of a particular RNA secondary or tertiary structure that is bound by SecA protein and protected from nuclease attack. Additional studies will be required to determine more about the nature of this binding and the conformation of the bound RNA.

SecA protein prevents formation of the 30S-tRNA$_f^{Met}$-geneX-*secA* RNA ternary complex

The position of the SecA-binding site on geneX-*secA* RNA implies that such binding should exclude ribosome binding at the normal *secA* ribosome-binding site. In order to test this assumption, primer-extension inhibition analysis (toeprinting) (Hartz et al., 1988) was used to determine the binding site of the 30S ribosomal subunit on geneX-*secA* RNA, and the effect of SecA protein on this binding was assessed. For this purpose,

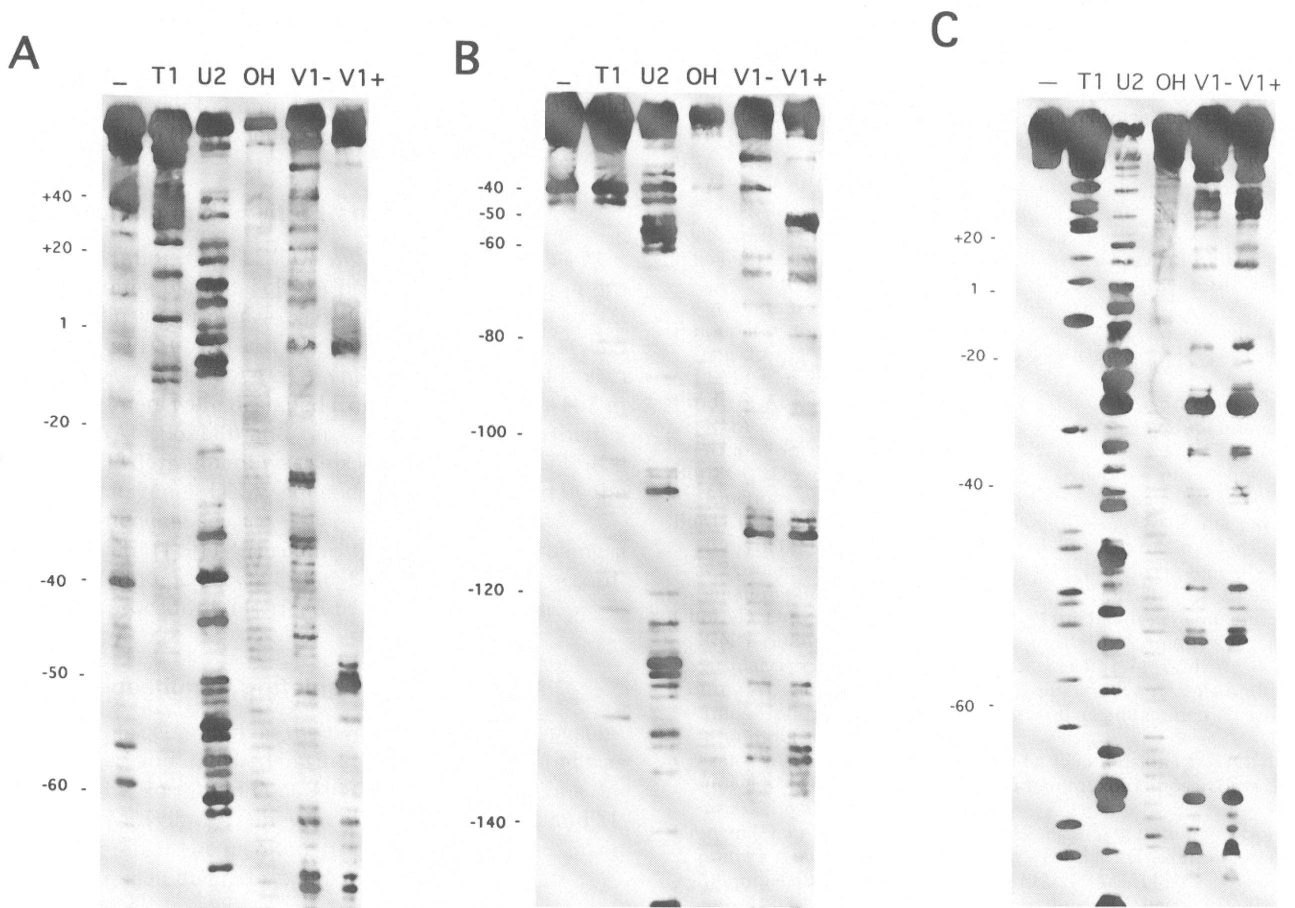

FIGURE 3. RNA footprint analysis of SecA protein bound to geneX-*secA* RNA. **A:** 162-nt geneX-*secA* RNA. **B:** 143-nt geneX-*secA* RNA. **C:** Bacteriophage T4 gene 32 RNA encompassing nt −92 to +80. All were analyzed by RNA footprinting as described in the Materials and methods. RNA treatment was as follows: untreated RNA (−), RNase T_1 (T1), RNase U_2 (U2), alkaline hydrolysis (OH), cobra venom nuclease in the absence (V1−) or the presence (V1+) of SecA protein, respectively. Coordinates are numbered with respect to **A** of the initiation codon, which is taken as +1.

salt-washed 30S subunits and $tRNA_f^{Met}$ were mixed with the 162-nt geneX-*secA* RNA and the formation of a 30S-$tRNA_f^{Met}$-geneX-*secA* RNA ternary complex was monitored by formation of a reverse transcript that terminates at the 3′ edge of this complex. A reverse transcript of the expected size (stop at G+15 relative to the A of the *secA* initiation codon) was detected (Fig. 4A). In the absence of $tRNA_f^{Met}$, a 30S-geneX-*secA* RNA binary complex was not detected by this method (data not shown). Pre-incubation of geneX-*secA* RNA with increasing amounts of SecA protein led to an inhibition in the appearance of the characteristic toeprint for this ternary complex, consistent with the overlapping binding sites of the 30S subunit and SecA protein. SecA protein that was heated at 80 °C for 10 min was unable to inhibit formation of the ternary complex (data not shown). As little as 5 fmol of SecA protein (1 ng) inhibited substantially the appearance of this toeprint in a reaction where the concentrations of RNA, 30S subunits, and $tRNA_f^{Met}$ were 80 fmol, 10 pmol, and 20 pmol, respectively. Incubation of SecA protein alone with geneX-*secA* RNA lead to the appearance of a putative toeprint for SecA at high protein concentrations, along with the disappearance of the full-length transcript (Fig. 4B). SecA protein that was heated at 80 °C for 10 min was inactive for toeprinting (data not shown). The position of this putative toeprint (C+46) corresponded precisely to the 3′ end of the SecA-binding site as determined by RNA footprint analysis (Figs. 2, 3). The intensity of the putative toeprint, along with the disappearance of the full-length transcript, suggested that considerable nonspecific RNA binding of SecA protein may be occurring also at these high SecA concentrations. SecA gave a putative toeprint only in the absence of 30S subunits and $tRNA_f^{Met}$. Furthermore, the concentrations of SecA protein required to give a putative toeprint or footprint were substantially higher than those required to inhibit formation of a ternary complex. This discrepancy, along with the failure of SecA to toeprint in the presence of 30S subunits and $tRNA_f^{Met}$, is consistent with the interaction of SecA and the ribosome noted previously (Liebke, 1987;

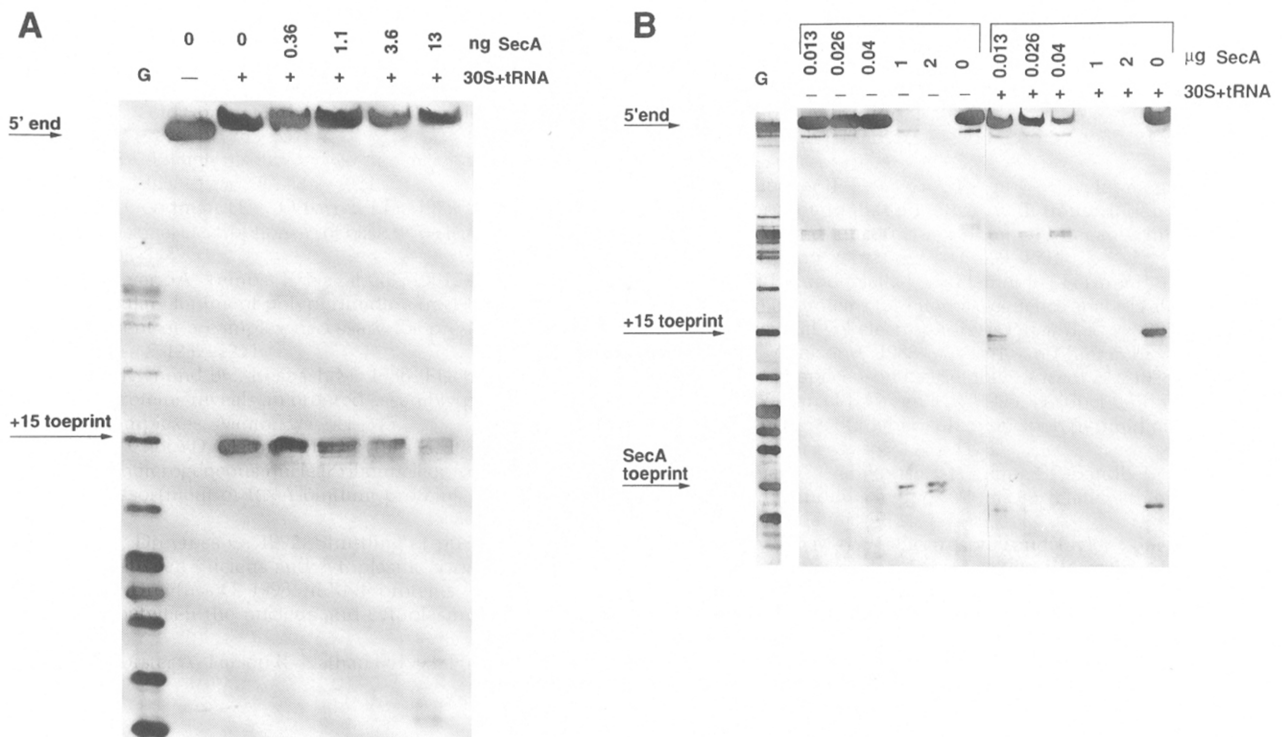

FIGURE 4. SecA prevents formation of the 30S-tRNA$_f^{Met}$-geneX-*secA* RNA ternary complex. **A:** Ten-microliter reactions containing VDG buffer, 80 fmol primer-hybridized geneX-*secA* RNA, and the indicated amount of SecA protein or a comparable volume of storage buffer were incubated at 0 °C for 5 min. Ten picomoles 30S subunits and 20 pmol tRNA$_f^{Met}$ were then added, followed by incubation at 37 °C for 15 min. The extension reaction was carried out by incubation at 37 °C for 15 min in the presence of 20 units of M-MLV reverse transcriptase, and the reactions were analyzed as described in the Materials and methods, along with the G sequencing ladder shown. The position of the full-length reverse transcript (5′ end), 30S-tRNA$_f^{Met}$ toeprint (+15 toeprint), and putative SecA toeprint (SecA toeprint) are indicated. **B:** Reactions were performed similarly to those described in A except for the absence or presence of 30S subunits and tRNA$_f^{Met}$.

Cabelli et al., 1991). Such interaction is supported further by an experiment demonstrating co-elution of SecA protein and 30S subunits during gel filtration through Superose 6 (R. Salavati & D. Oliver, unpubl. results). Examples of proteins that are capable of toeprinting include ribosomal protein S4 that autoregulates translation of the α operon (Spedding & Draper, 1993), SELB protein that is required for insertion of the unusual amino acid selenocysteine during translation (Ringquist et al., 1994), and Com protein, a positive regulator of mom translation of bacteriophage Mu (Wulczyn & Kahmann, 1991).

We performed an order of addition experiment in order to demonstrate the competition between ribosome and SecA binding to geneX-*secA* RNA. Initial experiments in which 30S subunits, tRNA$_f^{Met}$, and SecA were added simultaneously to geneX-*secA* RNA revealed that SecA prevented the formation of the 30S-tRNA$_f^{Met}$-geneX-*secA* RNA ternary complex (Fig. 5A). However, to our surprise, we found that a previously assembled ternary complex was dissociated upon subsequent addition of SecA protein, as indicated by the disappearance of its characteristic toeprint (Fig. 5B). With dissociation of the ternary complex, we noticed the appearance of a doublet species that migrated more slowly than the reverse transcript, terminating at +15. This new species was due to stalling of reverse transcriptase at a sequence that is unusually GC-rich and presumably formed a stable helix (other examples of this species can be seen in Fig. 4). The specificity of this dissociation reaction was shown by a parallel experiment, where comparable concentrations of SecA protein had no effect on the stability of a ternary complex formed from ribosomes, tRNA$_f^{Met}$, and bacteriophage T4 gene 32 RNA (Fig. 5C). In such experiments with gene 32 RNA, even decreasing the 30S subunit concentration by 10-fold still resulted in the formation of a stable ternary complex in the presence of SecA protein (data not shown). To our knowledge, this represents the first example of a translational regulatory protein promoting the dissociation of a preformed ternary complex involved in its translational initiation.

DISCUSSION

We have undertaken these studies to understand further the nature of autoregulation of *secA* expression. Previous investigations of proteins that autoregulate

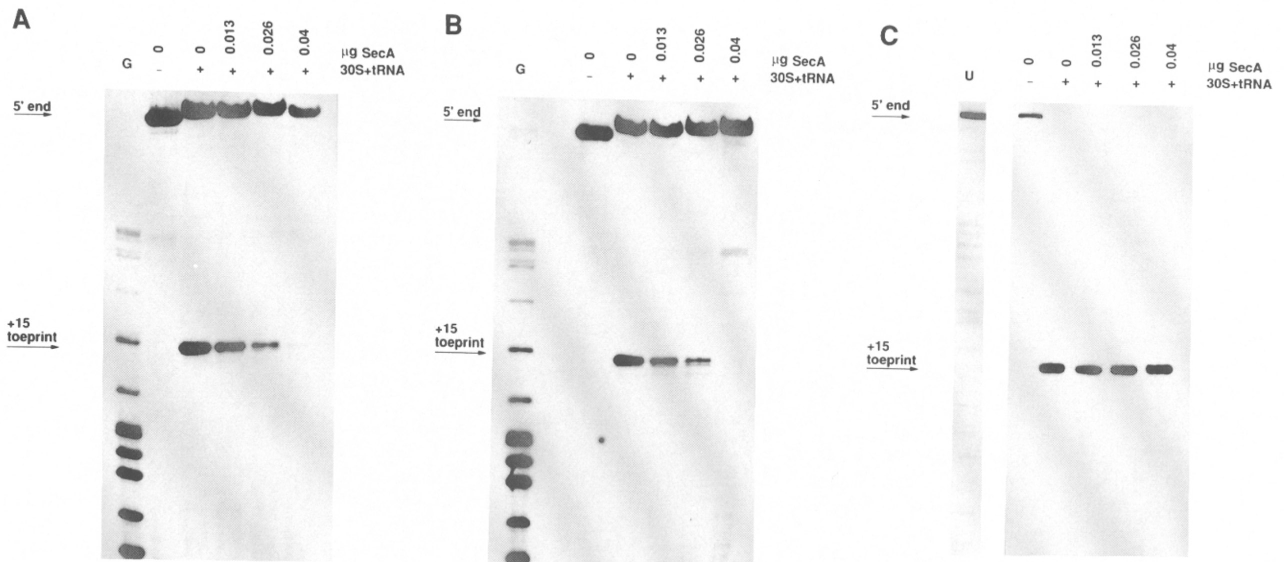

FIGURE 5. SecA protein can dissociate a pre-formed 30S-tRNA$_f^{Met}$-geneX-*secA* RNA ternary complex. Reactions were performed similarly to those described in the Figure 4 legend. **A:** To add SecA protein and the components of the ternary complex simultaneously (30S+tRNA+SecA pre-mix), 30S subunits, tRNA$_f^{Met}$, and the indicated amount of SecA protein or a comparable volume of storage buffer were mixed on ice and added to primer-hybridized geneX-*secA* RNA followed by incubation at 37 °C for 10 min. **B,C:** To pre-form the ternary complex (30S+tRNA pre-bound), 30S subunits and tRNA$_f^{Met}$ were pre-incubated with either primer-hybridized geneX-*secA* RNA or bacteriophage T4 gene 32 RNA at 37 °C for 10 min, at which time the indicated amount of SecA protein or a comparable volume of storage buffer was added and incubation continued at 4 °C for 5 min. Both sets of reactions were then analyzed using the extension reaction procedure described in the Figure 2 legend.

their translation suggest a variety of mechanisms, all of which require the protein of interest to recognize a target site on their mRNA. In certain cases, such as T4 gene 32 or RegA proteins, the regulatory protein appears to bind directly to single-stranded portions of the mRNA within the initiation region to directly block access of ribosomes to these elements (Winter et al., 1987; McPheeters et al., 1988). In other cases typified by bacteriophage R17 coat protein, bacteriophage T4 DNA polymerase, and many of the ribosomal proteins that autoregulate ribosomal operons, the regulatory protein binds to and stabilizes mRNA secondary structures that include the Shine–Dalgarno sequence and/or initiation codon, thereby cloistering these elements from recognition by ribosomes (Uhlenbeck et al., 1983; Lindahl & Zengel, 1986; Tuerk et al., 1990). The relatively few examples of positive translational regulation seem to work similarly to this latter mechanism except that the activator protein stabilizes an alternative mRNA secondary structure that exposes the ribosome-binding site rather than cloistering it (Altuvia et al., 1987; Wulczyn & Kahmann, 1991). Other more complex manifestations of this theme have been suggested. For example, within the *E. coli* α operon, it has been proposed that the regulatory protein, ribosomal protein S4, binds to a pseudoknot structure containing the ribosome-binding site, thus trapping its mRNA in a conformation able to bind 30S subunits, but unable to form an initiation complex with tRNA$_f^{Met}$ (Spedding & Draper, 1993). Action of autogenous repressors at sites other than those involved in translational initiation have been documented also. For example, within the *E. coli str* operon, where expression of the S7 gene is largely coupled to that of the upstream S12 gene, it has been proposed that S7 represses its translation by binding to its target within the S12–S7 intergenic region, thereby preventing formation of an RNA secondary structure needed to promote efficient translation coupling (Saito & Nomura, 1994; Saito et al., 1994).

The translational regulation of *secA* appears to be complex (Oliver, 1993). The region that controls the rate of initiation of *secA* translation in response to the protein secretion capability of the *E coli* cell lies at the end of geneX and within the geneX-*secA* intergenic region and has been termed the secretion-responsive element (SRE) (Schmidt & Oliver, 1989; Schmidt et al., 1991). We have shown previously that SecA protein binds around the SRE, where it is able to act as an autogenous repressor during protein export-proficient conditions (Dolan & Oliver, 1991). In this work, we have attempted to elucidate the mechanism of this repression further by defining the ribosome and SecA-binding sites on a 162-nt segment of geneX-*secA* RNA using primer-extension inhibition (toeprinting) and RNA footprinting analysis. Our results demonstrate that ribosome and SecA binding are mutually exclusive, suggesting that *secA* translation rate is governed by the concentrations of cytosolic SecA protein, free

30S ribosomal subunits, and conformationally correct geneX-*secA* mRNA. We have preliminary evidence that the *secA* ribosome-binding site has limited availability because a substantial fraction of this RNA possesses a secondary structure that occludes partially the Shine–Dalgarno sequence (P. McNicholas, R. Salavati, & D. Oliver, manuscript in prep.). In this regard, the function of translational coupling in this system could be analogous to other well-studied systems where terminating ribosomes of a preceding gene disrupt a secondary structure that blocks a ribosome-binding site, thereby allowing initiation of translation (Berkhout et al., 1987). Normally, cellular SecA protein is in equilibrium between at least three different forms: cytosolic; that which is peripherally bound to the inner membrane via interaction with anionic phospholipids; and that which is inserted into the inner membrane and is actively engaged in protein translocation (Lill et al., 1990; Cabelli et al., 1991; Breukink et al., 1992; Ulbrandt et al., 1992; Economou & Wickner, 1994; Kim et al., 1994). Thus, there is the expectation that as more SecA is recruited for protein translocation purposes, there will be a diminished pool of cytosolic SecA to repress its translation. Whether the mere titration of free SecA into protein translocation complexes is sufficient to account for the derepression of *secA* expression that is observed during protein export-deficient conditions is unknown. It is clear that such derepression can occur within one or two minutes after imposing a protein export block (Oliver et al., 1990), and models invoking posttranslational modification of SecA or other proteins in this system have not been ruled out.

Our results point to a novel activity of SecA protein, namely its ability to promote dissociation of a ternary complex of 30S-tRNA$_f^{Met}$-geneX-*secA* RNA. To our knowledge, this represents the first example of a translational repressor that can dissociate such a ternary complex. It remains an important goal to understand the mechanism of this novel activity and whether it plays an important role in *secA* regulation. It is tempting to speculate that SecA dissociates such ternary complexes in vivo, and therefore, there is a kinetic time window during which initiation of *secA* translation is sensitive to displacement by SecA protein that is not actively engaged in protein export. It is worth noting in this regard that a potential eucaryotic homologue of SecA protein, signal recognition particle (SRP) is found in association with ribosomes also, and that SRP plays a direct role in modulating the translation elongation step of preproteins (Siegel & Walter, 1988). It is conceivable that SecA may also play a role in regulating the initiation of translation of preproteins and that this biochemical activity has been utilized for autoregulatory purposes. For example, it has been reported that SecA contains sequence motifs predictive of RNA helicase activity; such an activity could stimulate the translation of preproteins by affecting the secondary structure of their mRNAs, while inhibiting its own translation in a similar manner (Koonin & Gorbalenya, 1992). It is known that there is separate global control of translation of periplasmic and outer membrane preproteins in *E. coli*, but the effectors of these regulatory systems remain undefined (Hengge-Aronis & Boos, 1986; Click et al., 1988). Clearly, additional work will be required to decipher the mechanisms employed by the *E. coli* cell for regulating protein secretion.

MATERIALS AND METHODS

Materials

RNase T_1 and tRNA$_f^{Met}$ were purchased from Boehringer. Cobra venom nuclease CV1 and RNase U_2 were purchased from Pharmacia. Calf intestinal phosphatase and T4 polynucleotide kinase were purchased from New England Biolabs. M-MLV reverse transcriptase and RNase T_2 were purchased from Bethesda Research Laboratories, and bacteriophage T3 and T7 RNA polymerase were purchased from Stratagene. High salt-washed *E. coli* 30S ribosomal subunits and bacteriophage T4 gene 32 RNA or pRS170 (Hartz et al., 1989) were generously provided by R. Traut and L. Gold, respectively.

Purification of SecA protein and preparation of RNA

SecA protein was purified from BL21.19 (pT7secA2) by affinity chromatography on Cibacron Blue Agarose 3GA (Sigma) as described previously (Mitchell & Oliver, 1993), and it was stored in 25 mM Tris-HCl, pH 7.5, 25 mM KCl, 0.5 mM Na_3EDTA, 5 mM β-mercaptoethanol, 10% (v/v) glycerol (storage buffer) at −80 °C until needed. Its purity was determined by SDS-PAGE and staining with Coomassie brilliant blue. General laboratory glassware was treated by baking at 180 °C for 8 h and, wherever possible, solutions were treated with 0.1% DEPC for at least 12 h to avoid any nuclease activity in the protein preparation steps. Prolonged incubation of RNA with purified SecA protein did not give any evidence of nuclease activity. The 162- and 143-nt geneX-*secA* RNAs were prepared by in vitro transcription of geneX-*secA* DNA fragments using T3 RNA polymerase. These DNA fragments were generated by PCR amplification using pGJ27 DNA (Jarosik & Oliver, 1991) and oligonucleotides 5′-CTATTAACCCTCACTAAAGGGAGTGCTGGCCCTCAA-3′ and 5′-TGACCACTTTGCGCATCC-3′ for the 162-nt RNA, and 5′-CTATTAACCCTCACTAAAGGGACGCATTGATTATGC-3′ and 5′-CGCGTTGCGGAGTTAATAAA-3′ for the 143-nt RNA, where the underlined portion represents a portable T3 promoter sequence. Bacteriophage T4 gene 32 RNA encompassing nt −92 to +80 was prepared similarly to geneX-*secA* RNA using

pRS170 (Hartz et al., 1989) and oligonucleotides 5′-ATCTTCAGAAGAAAAACCTTT-3′ and 5′-GGGTAATACGACTCACTATAGGGAAGACCCAGAGTATTGCG-3′, where the underlined portion represents a portable T7 promoter sequence. All RNAs were purified by electrophoresis through 8% sequencing gels as described by D'Alessio (1982).

Primer-extension inhibition analysis

Primer-extension inhibition studies were performed as described by Hartz et al. (1988). The 5′-^{32}P-end-labeled primer, 5′-TGACCACTTTGCGCATCC-3′, which is complementary to the 3′ end of the 162-nt geneX-*secA* RNA, was annealed as described by Hartz et al. (1988). Toeprint reactions were performed in VDG buffer (10 mM Tris-acetate, pH 7.4, 60 mM NH_4Cl, 10 mM Mg-acetate, 6 mM β-mercaptoethanol) and contained 80 fmol RNA in a 10-μL volume. SecA was pre-bound to RNA by a 5-min incubation on ice, and the 30S subunits and $tRNA_f^{Met}$ were bound to RNA by a 10-min incubation at 37 °C. M-MLV reverse transcriptase was added and incubation was continued at 37 °C for 15 min. After addition of 26 μL of sample buffer (94% formamide, 36 mM Tris-borate, pH 8.0, 36 mM boric acid, 0.8 mM EDTA), the samples were heated to 95 °C for 3 min and separated by electrophoresis on 8% sequencing gels, followed by autoradiography (Sambrook et al., 1989). Sequencing reactions were done as described above using dideoxynucleotide triphosphates at a final concentration of 200 μM, without preincubation or addition of the ribosomes and tRNA to the reactions.

Primer-extension RNA footprinting analysis

The annealing mixture of geneX-*secA* RNA (1 pmol) and 5′-^{32}P-end-labeled oligonucleotide 5′-TGACCACTTTGCGCATCC-3′ (3 pmol) was heated to 60 °C for 3 min, placed into dry ice–ethanol for 1 min, and allowed to thaw on ice. After addition of VDG buffer to 10 μL, SecA protein was added and the mixture was incubated at 0 °C for 10 min. Ribonuclease was then added, and the reactions were incubated at 0 °C for 5 min. Finally, M-MLV was added, and incubation was continued at 37 °C for 15 min. Reactions were terminated and analyzed as described above for primer-extension inhibition analysis.

RNA footprinting analysis

RNA was prepared by in vitro transcription and was purified as described above. The RNA was dephosphorylated with calf intestinal phosphatase, followed by phosphorylation with T4 polynucleotide kinase in the presence of [γ-32P]ATP. RNA (0.2 μM) in 40 mM Tris-HCl, pH 7.2, 17 mM $MgCl_2$, 222 mM NaCl, 1 mg/mL yeast tRNA in a 4-μL volume was preincubated at 42 °C for 20 min, and then chilled on ice for at least 10 min. SecA protein was added to 0.5 μM, and incubation was continued for 15 min at room temperature. One microliter of cobra venom nuclease CV1 (6×10^{-3} units) was added to the RNA or SecA-RNA mixture, followed by incubation at 37 °C for 12 min. To generate an RNA sequence ladder, the RNA was partially digested with RNase T_1, RNase U_2, or subjected to alkaline hydrolysis. For alkaline hydrolysis, 1 μL of labeled RNA (200,000 cpm), 8 μL of H_2O, and 1 μL of 10× CO_3/HCO_3 (0.5 M Na_2CO_3, pH 9.0, 10 mM EDTA, pH 8, and 5 mg/mL yeast tRNA) were combined, incubated at 90 °C for 7 min, and then chilled on ice. Five microliters of sample buffer were added to each reaction described above, followed by incubation at 90 °C for 30 s. Reactions were analyzed by electrophoresis on 8% sequencing gels followed by autoradiography (Sambrook et al., 1989).

ACKNOWLEDGMENTS

We thank Steven Ringquist for provision of bacteriophage T4 gene 32 RNA and pRS170, along with helpful discussions; Robert Traut for 30S ribosomal subunits; and Paul McNicholas for useful comments and encouragement during the course of these studies. This work was supported by grant GM42033 from the National Institutes of Health to D.O.

Received May 4, 1995; returned for revision June 27, 1995; revised manuscript received September 12, 1995

REFERENCES

Altuvia S, Locker-Giladi H, Koby S, Ben-Nun O, Oppenheim AB. 1987. RNase III stimulates the translation of the CIII gene of bacteriophage λ. *Proc Natl Acad Sci USA 84*:6511–6515.

Arkowitz R, Wickner W. 1994. SecD and SecF are required for the proton electrochemical gradient stimulation of preprotein translocation. *EMBO J 13*:954–963.

Berkhout B, Schmidt BF, van Strien A, van Boom J, van Westrenen J, van Duin J. 1987. Lysis gene of bacteriophage MS2 is activated by translational termination at the overlapping coat gene. *J Mol Biol 195*:517–524.

Bremer H, Dennis P. 1987. Modulation of chemical composition and other parameters of the cell by growth rate. In: Neidhardt FC, Ingraham JM, Low KB, Magasanik B, Schaechter M, Umbarger HE, eds. *Escherichia coli and Salmonella typhimurium: Molecular and cellular biology*. Washington, DC: American Society for Microbiology. pp 1527–1542.

Breukink E, Demel RA, de Korte-Kool G, de Kruijff B. 1992. SecA insertion into phospholipids is stimulated by negatively charged lipids and inhibited by ATP: A monolayer study. *Biochemistry 31*:1119–1124.

Cabelli RJ, Chen L, Tai PC, Oliver DB. 1988. SecA protein is required for secretory protein translocation into *E. coli* membrane vesicles. *Cell 55*:683–692.

Cabelli RJ, Dolan KM, Qian L, Oliver DB. 1991. Characterization of membrane-associated and soluble states of SecA protein from wild-type and *secA51(Ts)* mutant strains of *Escherichia coli*. *J Biol Chem 266*:24420–24427.

Click EM, McDonald GA, Schnaitman CA. 1988. Translational control of exported proteins that results from OmpC porin overexpression. *J Bacteriol 170*:2005–2011.

Cunningham K, Lill R, Crooke E, Rice M, Moore K, Wickner W, Oliver D. 1989. SecA protein, a peripheral protein of the *Escherichia*

coli plasma membrane, is essential for the functional binding and translocation of proOmpA. *EMBO J 8*:955–959.

D'Alessio JM. 1982. RNA sequencing. In: Rickwood D, Hames BD, eds. *Gel electrophoresis of nucleic acids: A practical approach*. Oxford, England: IRL Press. pp 173–197.

Dolan KM, Oliver DB. 1991. Characterization of *Escherichia coli* SecA protein binding to a site on its mRNA involved in autoregulation. *J Biol Chem 266*:23329–23333.

Douville K, Leonard M, Brundage L, Nishiyama K, Tokuda H, Mizushima S, Wickner W. 1994. Band 1 subunit of *Escherichia coli* preprotein translocase and integral membrane export factor P12 are the same protein. *J Biol Chem 269*:18705–18707.

Economou A, Wickner W. 1994. SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. *Cell 78*:835–843.

Hanada M, Nishiyama KI, Mizushima S, Tokuda H. 1994. Reconstitution of an efficient protein translocation machinery comprising SecA and the three membrane proteins, SecY, SecE and SecG (p12). *J Biol Chem 269*:23625–23631.

Hartz D, McPheeters DS, Gold L. 1989. Selection of the initiator tRNA by *Escherichia coli* initiation factors. *Genes & Dev 3*:1899–1912.

Hartz D, McPheeters DS, Traut R, Gold L. 1988. Extension inhibition analysis of translation initiation complexes. *Methods Enzymol 164*:419–425.

Hengge-Aronis R, Boos W. 1986. Translational control of exported proteins in *Escherichia coli*. *J Bacteriol 167*:462–466.

Jarosik G, Oliver D. 1991. Isolation and analysis of dominant *secA* mutations in *Escherichia coli*. *J Bacteriol 173*:860–868.

Kim YJ, Rajapandi T, Oliver D. 1994. SecA protein is exposed to the periplasmic surface of the *E. coli* inner membrane in its active state. *Cell 78*:845–853.

Koonin E, Gorbalenya A. 1992. Autogenous translation regulation by *Escherichia coli* ATPase SecA may be mediated by an intrinsic RNA helicase activity of this protein. *FEBS Lett 298*:6–8.

Kumamoto C. 1991. Molecular chaperones and protein translocation across the *Escherichia coli* inner membrane. *Mol Microbiol 5*:19–22.

Liebke H. 1987. Multiple SecA protein isoforms in *Escherichia coli*. *J Bacteriol 169*:1174–1181.

Lill R, Cunningham K, Brundage LA, Ito K, Oliver D, Wickner W. 1989. SecA protein hydrolyzes ATP and is an essential component of the protein translocation ATPase of *Escherichia coli*. *EMBO J 8*:961–966.

Lill R, Dowhan W, Wickner W. 1990. The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. *Cell 60*:271–280.

Lindahl L, Zengel JM. 1986. Ribosomal genes in *Escherichia coli*. *Annu Rev Genet 20*:297–326.

Matsuyama S, Fujita Y, Mizushima S. 1993. SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of *Escherichia coli*. *EMBO J 12*:265–260.

McPheeters DS, Stormo GD, Gold L. 1988. Autogenous regulatory site on the bacteriophage T4 gene 32 messenger RNA. *J Mol Biol 201*:517–535.

Mitchell C, Oliver DB. 1993. Two distinct ATP-binding domains are needed to promote protein export by *Escherichia coli* SecA ATPase. *Mol Microbiol 10*:483–497.

Oliver DB. 1993. SecA protein: Autoregulated ATPase catalyzing preprotein insertion and translocation across the *Escherichia coli* inner membrane. *Mol Microbiol 7*:159–165.

Oliver D, Beckwith J. 1982. Regulation of a membrane component required for protein secretion in *Escherichia coli*. *Cell 30*:311–319.

Oliver D, Cabelli R, Dolan K, Jarosik G. 1990. Azide-resistant mutants of *Escherichia coli* alter the SecA protein, an azide-resistant component of the protein export machinery. *Proc Natl Acad Sci USA 87*:8227–8231.

Pogliano JA, Beckwith J. 1994. SecD and SecF facilitate protein export in *Escherichia coli*. *EMBO J 13*:554–561.

Ringquist S, Schnider D, Gibson T, Baron C, Bock A, Gold L. 1994. Recognition of the mRNA selenocysteine insertion sequence by the specialized translational elongation factor SELB. *Genes & Dev 8*:376–385.

Rollo E, Oliver D. 1988. Regulation of the *Escherichia coli secA* gene by protein secretion defects: Analysis of *secA, secB, secD,* and *secY* mutants. *J Bacteriol 170*:3281–3282.

Saito K, Mattheakis LC, Nomura M. 1994. Post-transcriptional regulation of the *str* operon in *Escherichia coli*: Ribosomal protein S7 inhibits coupled translation of S7 but not its independent translation. *J Mol Biol 235*:111–124.

Saito K, Nomura M. 1994. Post-transcriptional regulation of the *str* operon in *Escherichia coli*: Structural and mutational analysis of the target site for translational repressor S7. *J Mol Biol 235*:125–139.

Sambrook J, Fritsch E, Maniatis T. 1989. *Molecular cloning: A laboratory manual, 2nd ed*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schatz P, Beckwith J. 1990. Genetic analysis of protein export in *Escherichia coli*. *Annu Rev Genet 24*:215–248.

Schmidt M, Dolan K, Oliver D. 1991. Regulation of *Escherichia coli secA* mRNA translation by a secretion-responsive element. *J Bacteriol 173*:6605–6611.

Schmidt M, Oliver D. 1989. SecA protein autogenously represses its own translation during normal protein secretion in *Escherichia coli*. *J Bacteriol 171*:643–649.

Schmidt M, Rollo E, Grodberg J, Oliver D. 1988. Nucleotide sequence of the *secA* gene and *secA(Ts)* mutations preventing protein export in *Escherichia coli*. *J Bacteriol 170*:3404–3414.

Siegel V, Walter P. 1988. Each of the activities of signal recognition particle (SRP) is contained within a distinct domain: Analysis of biochemical mutants of SRP. *Cell 52*:39–49.

Simon SM, Blobel G. 1992. Signal peptides open protein-conducting channels in *E. coli*. *Cell 69*:677–684.

Spedding G, Draper DE. 1993. Allosteric mechanism of translation repression in the *Escherichia coli* a operon. *Proc Natl Acad Sci USA 90*:4399–4403.

Tuerk C, Eddy S, Parma D, Gold L. 1990. Autogenous translational operator recognized by bacteriophage T4 DNA polymerase. *J Mol Biol 213*:749–761.

Uhlenbeck OC, Carey J, Romaniuk PJ, Lowary PT, Beckett D. 1983. Interaction of R17 coat protein with its RNA binding site for translational repression. *J Biomol Struct Dynam 1*:539–552.

Ulbrandt N, London E, Oliver D. 1992. Deep penetration of a portion of *Escherichia coli* SecA protein into model membranes is promoted by anionic phospholipids and by partial unfolding. *J Biol Chem 267*:15184–15192.

Wickner W, Driessen AJM, Hartl FU. 1991. The enzymology of protein translocation across the *Escherichia coli* plasma membrane. *Annu Rev Biochem 60*:101–124.

Winter RB, Morrissey L, Gauss P, Gold L, Hsu T, Karam J. 1987. Bacteriophage T4 regA protein binds to mRNAs and prevents translation initiation. *Proc Natl Acad Sci USA 84*:7822–7826.

Wulczyn FG, Kahmann R. 1991. Translational stimulation: RNA sequence and structure requirements for binding of Com protein. *Cell 65*:259–269.