

Identification of SRP Particle in Escherichia coli

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1. Introduction

In gram-negative bacteria, up to 40% of cellular proteins are exported to the periplasm or are freely secreted through a conserved secretion system referred to as the Type II secretion or Sec system. This is a universal pathway first described by studying protein secretion in *E. coli* (Lee & Schneewind, 2001; Beckwith, 2013; Collinson et al., 2001; Tseng et al., 2009).

The polypeptides that translocate across the inner membrane usually contain signal peptides (20–30-residue) as part of their translated sequence. These signal sequences are often cleaved from the pre-protein during localization to generate the mature protein site (Kudva et al., 2013; Low et al., 2013; Papanikou et al., 2007; Peterson et al., 2006; Lee & Schneewind, 2001; Edman et al., 1999; Fekkes et al., 1999; Lammertyn et al., 1998; Nesmeyanova et al., 1997; Collier, 1994; Pugsley, 1993). These signal peptides play a decisive role in targeting the protein to the Sec machinery and direct the protein across the inner membrane in bacteria (Edman et al., 1999; Low et al., 2013).

It is well recognized that the pre-proteins destined for export through the inner membrane are recognized by factors such as the ribonucleoprotein signal recognition particle (SRP). This particle is a complex consisting of a 4.5S RNA and a 54-kDa Ffh protein (a homologue of eukaryotic SRP) (Beckwith, 2013; Mori & Ito, 2001). The SRP carries the nascent amino acid chain/ribosome complex to the translocon within the plasma membrane. Once the SRP pre-protein complex is bound to SRP receptors and the ribosome is docked on the SecYEG pore, the polypeptides are translocated across the membrane (Lee & Schneewind, 2001; Papanikou et al., 2007; Mori & Ito, 2001; Beckwith, 2013; Kudva et al., 2013; Pugsley, 1993). It has been well documented that SRP targets the highly hydrophobic signal peptides of nascent secretory proteins and membrane proteins rather than SecB chaperones to the Sec translocase (Low et al., 2013) (Papanikou et al., 2007).

There have been several studies that show that changing the structure of the signal peptide can eliminate or reduce the efficiency of protein secretion (Van der Wolk et al., 1998). However, other alterations in the signal peptide sequence can enhance protein sorting or have no effect (Petersen et al., 2006; Kaiser and Botstein, 1986; Koshl; Nevo-Dinur, et al., 2011).

Additionally, there are many mRNA studies proposed that the mRNA that codes for the secreted protein can actively participate in protein targeting rather than or in addition to the N-terminal

amino acid signal sequence. (Habyarimana et al., 2013; Sorg et al., 2005; Wilhelm, et al., 1993; Okita, et al., 2002; Nevo-Dinur, et al., 2011; Cuia, et al., 2012). The previous mRNA hypothesis was established because mRNA targeting and subcellular localization have been shown to exist in eukaryotes and prokaryotes (Wilhelm, et al., 1993; Okita, et al., 2002; Nevo-Dinur, et al., 2011; Cuia, et al., 2012). It has been further reported that fliC export by the flagellar system in *E. coli* requires 5' untranslated RNA (UTR) sequences signal (Habyarimana et al., 2013).

In this study, the periplasmic protein propyl isomerase chaperone SurA, which is involved in the biogenesis of outer membrane proteins (Lazar et al., 1996, 1998; Bitto et al., 2003; Sklar et al., 2007) was utilized to identify the novel RBP that involved in the secretory pathway in *E. coli*. As well as surA, 3- isopropylmalate dehydratase which involved in the biosynthesis of the amino acid leucine was used as a control for the non-secreted protein (Velíšek et al., 2006; He et al., 2010). Additionally, we also examined the 5'UTR (Berg et al., 2009) of alkaline phosphatase phoA and gMP reductase mRNAs that might achieve efficient isolation of such cytoplasmic factors. Biotinylated transcripts of SurA, phoA, IsoH and gMP were produced in vitro system. Thus used these as bait for pull down assays with streptavidin-magnetic beads in order to identify a cytoplasmic factor that associate with these mRNAs by SDS-PAGE and MALDI-MS identification.

2. Research Problem

- Why do alterations that disrupt the basic features of signal peptides have no impact on protein secretion in some cases while in other cases some alterations appear to significantly influence secretion without significantly altering signal peptides structure?
- Does the alterations of features of signal peptides leads to a possible alternative, mRNA hypothesis?
- What are the structures that recognized by secretory factors to secrete protein across the inner and outer membrane through the type III secretion pathway ?

3. Research Importance

In the ribosomes, one or more polypeptides will form after the translation of mRNA that carries genetic information for protein synthesis. Once translated, these peptides are translocated across the inner membrane via the Sec machinery to assemble in the outer membrane. Several studies have proved that mRNA binding proteins (RBP) regulate the translocation process in *E.coli* through association with a single peptide. Using *E.coli* as a model I will identify and characterize unknown RBPs. We will also utilize the periplasmic protein propyl isomerase chaperone SurA, which is involved in the biogenesis of outer membrane proteins to identify novel RBP involved in the secretory pathway in *E.coli*. As well as surA, 3-isopropylmalate dehydratase will be used as a control for the non-secreted protein.

Additionally, I will also examine the 5'UTR of alkaline phosphatase phoA and gMP reductase mRNAs that might achieve efficient isolation of such cytoplasmic factors.

4. Objectives

The objectives of the present study were to:

- I propose an alternate model where instead of the SRP only binding the signal sequence of polypeptides it also interacts with the 5' region of the mRNA.
- To use two *E. coli* proteins, one localized to the cytoplasm (3-isopropylmalate dehydrogenase IsodH and GMP reductase) and the other (periplasmic propyl isomerase chaperone SurA and alkaline phosphatase PhoA) secreted through the Sec-dependent pathway.
- Produce transcripts *in vitro* system and to use these as bait for pull down assays to identify a cytoplasmic factor that associate with these mRNAs.
- Look for factors in the cell that may interact with the mRNA of the secreted protein and not in the cytoplasmic protein. This factor could be involved in targeting that mRNA/ribosome complex to the membrane to effect secretion.

5. Hypothesis

The signal-recognition particle (SRP), which targets the ribosome to the membrane translocon for secretion of the protein, is hypothesized to recognize these features and therefore differentiate these proteins from non-secretory proteins. Recent work in other laboratories has suggested a role for the mRNA itself, rather than only the amino acid sequence of the N-terminus of the pre-protein as playing a role in targeting the pre-protein-ribosome complex to the translocon within the membrane.

6. Literature Review

6.1 Protein Translation and its Regulation in *Escherichia coli*

Within all cells, protein biosynthesis usually occurs on a specialized super molecular complex called the ribosome via a translation mechanism. In this event, the genetic information that encodes these proteins from the DNA is carried to the ribosome through mRNA (Laursen et al., 2005). Decades ago, translation factors were acknowledged as highly conserved factors that are required in both eukaryotes and prokaryotes. During translation these translation factors (Table 1) are involved in facilitating initiation, elongation, and termination of translation (Kaczanowska et al., 2007). In prokaryotes the initiation factors IF1, IF2, and IF3 bind to 30 S ribosomal subunit, tRNA, and mRNA to create a supra-molecular complex required for the creation of the translation machinery. The start of translation appears to involve an interacting between a sequence in the mRNA called the Shine-Dalgarno (SD) sequence and the 16S rRNA component of the 30S ribosome which results in expulsion of IF2 and the binding of the large 50S ribosome subunit to create the functional ribosome (Benelli et al., 2009; Kaczanowska et al., 2007; Malys et al., 2011; Marintchev et al., 2004). Once translation has been initiated a group of elongation factors, including EF-TU, EF-Ts and EF-G, promote recruitment and addition of aminoacylated tRNA to the complex and allows incorporation of the appropriate amino acid into the growing peptide chain depending on interactions between the mRNA codon and tRNA anticodon sequences. Lastly, the translation termination process is mostly carried out in the presence of RF1 and RF2 factors which bind appropriate mRNA stop codons and promote disassembly of the mRNA, 30S ribosome and 50S ribosome complex (Marintchev et al., 2004; Kaczanowska et al., 2007).

Processes in Translation	Factor	Function
Initiation	IF1	Stabilizes 30S subunit.
	IF2	Binds fmet-tRNA to 30S–mRNA complex: bind to GTP and stimulates hydrolysis.
	IF3	Binds 30S subunit to mRNA
Elongation	EF-Tu	Binds GTP; brings aminoacyl-tRNA to the A site of ribosome
	EF-Ts	Generates active EF-Tu
	EF-G	Stimulates translation ; GTP-dependent
Termination	RF1	Catalyzes release of the polypeptide chain from tRNA and disassociation of the translocation complex; specific for UAA and UAG termination codons
	RF2	Behaves like RF1; specific for UGA and UAA codons
	RF3	Stimulates RF1 and RF2

Table 1: The table above shows the kind of factors involved in protein synthesis in prokaryotes (Benelli, et al 2009).

6.2 Sec-dependent Secretion and The Signal Peptide Hypothesis

In gram-negative bacteria, up to 40% of cellular proteins are exported to the periplasm, are present in the outer membrane (OM), or are freely secreted through a conserved secretion system referred to as the Type II secretion or Sec system. This is a universal pathway first described by studying protein secretion in *E. coli* (Lee & Schneewind, 2001; Beckwith, 2013; Collinson et al., 2001; Tseng et al., 2009). The core complex of the protein-conducting channel, which contains the SecY, SecE, and SecG proteins (Sec YEG complex or translocon), is necessary for mediating polypeptide movement through the inner membrane and/or into the periplasmic space during protein secretion (Collinson, 2001; Lee & Schneewind, 2001; Papanikou et al., 2007; Mori & Ito, 2001; Kudva et al., 2013).

The polypeptides that translocate across the inner membrane usually contain signal peptides (20–30-residue) as part of their translated sequence. These signal sequences are often cleaved from the pre-protein during localization to generate the mature protein. The signal sequences typically consist of three distinct domains including the N terminal hydrophilic domain (1-5 residues long) which is flanked by positively charged amino acids, followed by a large domain comprised of hydrophobic amino acids, named the H region (about 8-12), and finally a polar carboxy-terminal domain, the C-terminal region (3-7 residues) which contains the signal peptidase cleavage site (Kudva et al., 2013; Low et al., 2013; Papanikou et al., 2007; Peterson et al., 2006; Lee & Schneewind, 2001; Edman et al., 1999; Fekkes et al., 1999; Lammertyn et al., 1998; Nesmeyanova et al., 1997; Collier, 1994; Pugsley, 1993). These signal peptides play a decisive role in targeting the protein to the Sec machinery and direct the protein across the inner membrane in bacteria (Edman et al., 1999; Low et al., 2013).

It is well recognized that the pre-proteins destined for export through the inner membrane are recognized by factors such as the ribonucleoprotein signal recognition particle (SRP). The SRP carries the nascent amino acid chain/ribosome complex to the translocon within the plasma membrane. Once the SRP pre-protein complex is bound to SRP receptors and the ribosome is docked on the SecYEG pore, the polypeptides are translocated across the membrane (Lee &

Schneewind, 2001; Papanikou et al., 2007; Mori & Ito, 2001; Beckwith, 2013; Kudva et al., 2013; Pugsley, 1993). The SRP complex consists of a 4.5S RNA and a 54- kDa Ffh protein (a homologue of eukaryotic SRP). During translation the SRP-nascent polypeptide complex is formed and reacts with the FtsY SRP-receptor (docking protein) on the cytoplasmic side of the membrane (Beckwith, 2013; Mori & Ito, 2001). Once translocation has occurred and the mature protein is released into the periplasmic space, the signal peptides are subsequently removed by signal peptidase on the exterior side of the membrane (Lee & Schneewind, 2001; Mori & Ito, 2001; Kudva et al., 2013).

6.3 Logistical Challenges for the Cell with the Present Signal Peptide

Hypothesis

The bacterial cell faces several challenges in the function of the protein secretion system based on a signal peptide. After a protein is synthesized on the ribosome and before it reaches its final destination, the process of proteins sorting, which involves a number of interactions with the Sec machinery must occur (Low et al., 2013). Specifically, SecB chaperones and the ribonucleoprotein signal-recognition particle (SRP) play a significant role in mediating protein translocation. These proteins are characterized as piloting factors that recognize the nascent pre- proteins at the first stage of protein sorting and targeting (Papanikou et al., 2007). It has been well documented that SRP targets the highly hydrophobic signal peptides of nascent secretory proteins and membrane proteins than SecB chaperones to the Sec translocase (Low et al., 2013) (Papanikou et al., 2007). The cytoplasmic SecB chaperone function by creating a transport- competent state when it is bound to the pre-protein and it interacts with membrane-bound SecA (Low et al., 2013) (Papanikou et al., 2007). Nevertheless, SecB is not available in all bacteria (Papanikou et al., 2007). SecB binds to the mature region of the pre-protein, and some signal peptides delay pre-protein folding. Finally the SecB-pre-protein and SRP-pre-protein complexes bind to the SecYEG channel at the membrane (Papanikouetal, 2007). The Sec machinery can distinguish secreted from non-secreted proteins by binding to the signal peptide. Different sequences of these signal sequences were identified in prokaryotes and eukaryotes cells (Low et al., 2013). Experimental studies comparing different signal peptides has shown that the sequence properties of the signal peptide, such as charge, length, and hydrophobicity, were highly variable and rapidly evolving in different groups of organisms (Edman et al., 1999). The diversity of the signal peptides can control the differences

in the ability of these signals to drive protein export (Low et al., 2013). For instance, blocking or impairing the secretion of *Escherichia coli* and *Bacillus subtilis* can result from disrupting the H region of the signal peptide (Collier, 1994). Recent findings also have shown that cytoplasmic proteins can be highly secreted when fused to signal peptides. For example, Manduca diuresin (MD) and the winter flounder antifreeze protein in *E.coli* can be secreted when fused to the OmpA signal peptide (Low et al., 2013).

There is a significant variety in the sequence of signal peptides, which makes it difficult to determine which alteration can enhance the performance of targeting secreted proteins to the Sec system.

6.4 The mRNA Hypothesis in Describing Membrane Targeting of the Pre-protein/Ribosome Complex

There have been several studies that show that changing the structure of the signal peptide can eliminate or reduce the efficiency of protein secretion (Van der Wolk et al., 1998). However, other alterations in the signal peptide sequence can enhance protein sorting or have no effect (Petersen et al., 2006; Kaiser and Botstein, 1986; Nevo-Dinur, et al., 2011). For example, deletions and two substitution mutations in the signal peptide sequence of the secreted *Saccharomyces cerevisiae* enzyme invertase still allowed normal secretion and expression of this enzyme which indicates this sequence can tolerate significant changes (Kaiser and Botstein, 1986). Although this protein secretion system is present in yeast, the structure of signal sequences in eukaryotes and prokaryotes are similar. Indeed, these findings suggest a central question: why do alterations that disrupt the basic features of signal peptides have no impact on protein secretion in some cases while in other cases some alterations appear to significantly influence secretion without significantly altering signal peptides structure? This leads to a possible alternative, mRNA hypothesis. The mRNA hypothesis proposes that the mRNA that codes for the secreted protein can actively participate in protein targeting rather than or in addition to the N- terminal amino acid signal sequence. (Habyarimana et al., 2013; Sorg et al., 2005; Wilhelm, et al., 1993; Okita, et al., 2002; Nevo-Dinur, et al., 2011; Cuia, et al., 2012). The mRNA hypothesis was established because mRNA targeting and subcellular localization have been shown to exist in eukaryotes and prokaryotes (Wilhelm, et al., 1993; Okita, et al., 2002; Nevo-Dinur, et al., 2011; Cuia, et al., 2012).

It is known that the type 3 effector proteins can be trafficked from the cytoplasm of the bacterium into the cytoplasm of the host cell. A particular signal sequence within the first 30 amino acids of the protein structure is sufficient for protein targeting to the type 3-secretion system (T3SS). However, the type 3 effectors are still secreted even when a frame shift mutation in the signal sequence is introduced indicating that the amino acid sequences are unnecessary for targeting. Surprisingly, this observation confirmed the hypothesis that the required signal might be in the nucleotide sequences (mRNA), rather than the amino acid sequences of the signal peptide. It has been further reported that *fliC* export by the flagellar system in *E. coli* requires 5' untranslated RNA (UTR) sequences. Moreover, it was observed that the C-terminal region of *Y. enterocolitica* protein *yopR*, requires a nucleotide sequence, as an essential element for its export confirming the importance of an mRNA secretion signal (Habyarimana et al., 2013). In the *Yersinia* species, 12 additional types of Yop proteins are exported into the eukaryotic cytosol, where they are required due to their toxic function. These polypeptides translocate across the inner and outer membrane through the type III secretion pathway even though they do not possess a common secretion signal within the amino acid sequence of the target polypeptides (Anderson et al., 1997).

6.5 Evidence of Specific mRNA Recognition and Targeting in Eukaryotic System

In the last decade, a number of mRNAs have been shown to be specifically localized to different cellular regions in animal, plant, and yeast cells using *in situ* hybridization techniques. The intracellular localization of mRNA can have distinct functions in regulating translation and targeting proteins to specific regions where they are required (Palacios & Johnston, 2001). Controlling gene expression is the primary advantage of mRNA transport (Glisovic et al., 2008; Martin & Ephrussi, 2009). It is also obviously a useful mechanism to localize copies of proteins in response to translations of localized mRNAs occur (Martin & Ephrussi, 2009).

The majority of mRNA targeting to the membrane requires becomes the presence of *cis*-acting elements or localization elements (Nevo-Dinur et al., 2011; Martin & Ephrussi, 2009). These *cis*-acting elements are mostly located in the 3'untranslated region (UTR). Subsequent work has shown that there are RNA-binding proteins (RBP) that recognize and bind to the *cis*-acting elements (Palacios & Johnston, 2001; Martin & Ephrussi, 2009; Slobodin, et al., 2010). These

RBP are considered to be the *trans*-acting elements and contribute significantly to the transcript localization and translational regulation mechanisms (Palacios & Johnston, 2001; Bashirullah et al., 1998; Martin & Ephrussi, 2009). The well-studied RBPs reveal how mRNAs can reach their destinations (Martin & Ephrussi, 2009; Palacios & Johnston, 2001; Bashirullah et al., 1998). For instance, RNA-RBP complexes, named ribonucleoproteins, can be transported along cytoskeletal elements by motor proteins (Martin & Ephrussi, 2009; Slobodin, et al., 2010; Kloc, et al., 2002). The ribonucleoprotein granule (RNP) also contributes to mitochondria, endoplasmic reticulum ER or peroxisomes to localization of mRNA toward distinct sites (Slobodin, et al., 2010).

Over 100 RNAs are known to localize to different intracellular regions have been characterized in eukaryotes. There are also 25 transcripts that are differentially localized in yeast (Palacios, 2007). For instance, cell division of yeast, the ASH1 mRNA is specifically transported to the bud tip of daughter cell in *Saccharomyces cerevisiae* as an assembly with myosin (Myo4) and actin (Glisovic et al., 2008; Martin & Ephrussi, 2009; Slobodin et al., 2010). The She2 and She3 proteins are also essentially in the localization of ASH1 mRNA. The process initiates when the She2 protein binds to the *cis*-acting elements in the 3' UTR of ASH1 mRNA. A high affinity binding between She2 and the C-terminus of She3 is also created. The She3-Myo4 complex further binds to the N-terminus of Myo4 and as a result, the ASH1 protein is localized (Glisovic, et al., 2008; Martin & Ephrussi, 2009; Palacios, 2007). The previous mechanism is needed for the repression of *HO* endonuclease gene transcription, which causes the repression of a mating type protein in the yeast bud (Glisovic et al., 2008).

A particularly clear example of transcript localization has been provided by the observation that β -actin mRNA targets to the lamella region in fibroblasts (Martin & Ephrussi, 2009). The interaction depends on the zipcode-binding protein (ZBP), a 68-kDa protein, which forms one RNA recognition motif (RRM) and four (hnRNP K homology) KH domains (Kindler et al., 2007). ZBP binds to the “RNA-zipcode,” a 54-nucleotide sequences, that exists on the 3' UTR site of the β -actin mRNA and forms a complex called ribonucleoproteins (RNP) (Glisovic et al., 2008; Martin & Ephrussi, 2009; Kloc et al., 2002). A KH domain facilitate the formation of an RNP- β -actin mRNA complex and also is involved in the association with actin microfilaments while the localization of the β -actin RNP complex is achieved by the RRM domains (Martin & Ephrussi, 2009).

Another example of RNAs trafficking has been described within the growing oocyte or syncytial embryo in *Drosophila*. RNAs targeting is important in the definition of the oocyte and the specification of embryonic axes in the developing embryo (Lasko, 1999). *Staufen*, a double-stranded RNA-binding protein homologue, is important in mediating RNA localization and plays translation control in diverse cell types during embryogenesis (Kindler et al., 2007; Roegiers, 2000). In Oocytes, the localization of *Oskar*, *bicoid* and *nanos* mRNAs are fundamental for initiating protein gradients in the anterior and posterior domains required for proper development (Roegiers, 2000; Lasko, 1999; Du, 2007). Whereas *bicoid* is directed to move along the anterior end of the embryo, the *oskar* mRNA is directed to the pole plasma at the posterior of the embryo (Du, 2007; St Johnston, 1995).

6.6 Evidence of mRNA Targeting in Prokaryotes

A better understanding of the widespread phenomenon of RNA transport has also been demonstrated in prokaryotes. In a translation-independent manner, mRNAs have been characterized in *E. coli* to reach a specific destination of their encoded proteins, cytoplasm, inner membrane, and poles (Nevo-Dinur et al., 2011; Martin & Ephrussi, 2009; Nevo-Dinur, et al., 2012). Interestingly, RNA localization was first observed in foci near transcribed genes. Investigations into mRNA localization demonstrated that the mRNAs are located at the center of the *E. coli* cells and show a limited amount of movement. This was achieved using a fluorescent RNA binding protein, MS2. Thus far, most RNA moved to a specific area related to their attachment to RNA polymerase. This area is known as F plasmid localized to the quarter central region of the bacterial cell (Nevo-Dinur et al., 2012; Keiler, 2012).

Likewise in *E. coli*, a parallel approach of localization of RNA to the cytoplasm, membrane, and pole has been further detected by fluorescence protein complementation using the eIF4A RNA-binding protein (Nevo-Dinur et al., 2012; Broude, 2011). Significantly, through the previous approach, the distribution of the *lacZ* mRNA, 5S RNA, and a short artificial untranslated RNA (Nevo-Dinur et al., 2012) which encodes the same plasmid were observed. All of these types of RNAs displayed different patterns of localization (Nevo-Dinur et al., 2012; Broude, 2011). The 5S RNA was predominately localized to the pole or in foci in regions devoid of nucleoids, while

the *lacZ* mRNA was distributed in the cytoplasm (Broude, 2011). In addition to the 5S RNA, the short non-coding RNA was in fact revealed at the cell poles (Nevo-Dinur et al., 2012).

7. Research Methodology

- Bacterial Cultures
- Extraction and Quantitation of Genomic Nucleic Acids
- Genomic DNA Amplification and Transcription by T7 RNA Polymerase
- Agarose Gel Electrophoresis and Biotin Labeling of mRNA
- Chromogenic Detection of Biotinylated mRNA and Evaluation of Labeling Efficiency
- Protein Extraction and Protein Assay (UV Crosslinking)
- Pull-Down Assay
- RNA–Protein Interaction Assay
- SDS PAGE
- Protein Identification Using Matrix-Assisted Laser Desorption Time-of-Flight (MALDI- Mass Spectrometry).

8. Limitation

- The nature of the mRNAs used to detect RNA binding proteins.
- It is also important to point out that the periplasmic propyl isomerase chaperone SurA is exported to the periplasmic space but is not completely secreted.
- Potentially it is possible that other factors will not interact with the mRNA of periplasmic proteins in same way as secreted proteins.
- Other factual issues also the small size of mRNAs.
- The protein pattern from the blocking experiments may not support the idea of preventing a number of proteins that binds non-specifically to RNA.

9. Research Structure

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Appendix

10. Reference

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